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HPLC-MS^n Analysis of Phenolic Compounds and Purine Alkaloids in Green and Black Tea

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Tea is a complex mixture containing a range of compounds from simple phenolics to complex thearubigins, many of which have well-recognized antioxidant properties. This paper describes the application of high-performance liquid chromatography−mass spectrometry (HPLC-MS^n) methods for the rapid and routine analysis of more than 30 phenolics in tea. Green and black tea infusions were injected directly onto a reversed phase HPLC column, and the phenolics eluted using two different mobile phase gradients, one optimized to resolve catechin derivatives and the other, flavonols and theaflavins. Compounds, identified on the basis of their retention time, absorbance spectrum, and MS fragmentation pattern, included (+)-catechin, (−)-epicatechin, theaflavin and their various gallate derivatives, quercetin and kaempferol mono-, di-, and triglycosides, quinic acid esters of gallic acid and hydroxycinnamates, and the purine alkaloids, caffeine and theobromine.

KEYWORDS: ***Camellia sinensis*; green tea; black tea; HPLC-MS^n analysis; catechins; theaflavins; flavonols; gallates; quinic acid derivatives of hydroxycinnamates; purine alkaloids**

1. INTRODUCTION

Tea is one of the most widely consumed beverages in the world. Grown in about 30 countries worldwide, the tea plant (*Camellia sinensis* L.) originated in SE China. Tea is generally consumed in one of three forms: green, oolong, or black. Approximately 3.0 million metric tons of dried tea is produced annually, 20% of which is green tea, 2% is oolong, and the remainder is black tea (*1*). Although produced principally from *C. sinensis* and smaller amounts of *Camellia assamica*, each of the teas is processed differently and has a unique character, taste, and chemical profile. To produce green tea, after the leaves are picked, the young leaves are rolled and steamed to minimize oxidation. In the production of black tea, after the leaves are rolled, which disrupts cellular compartmentation and brings phenolic compounds into contact with polyphenol oxidases, the young *C. sinensis* leaves undergo oxidation for 90−120 min before drying. During this period, which is referred to as fermentation (although it is clearly distinct from yeast-mediated alcoholic fermentation), flavan-3-ols are converted to complex condensation products, the theaflavins and their polymers, thearubigins. Oolong tea, which is manufactured mainly in Taiwan and exported to Japan and Germany, is produced with a shorter fermentation period than black tea and is said to have a taste and color somewhere between green and black teas (*2*).

The major phenolics present in teas are the flavan-3-ols and flavonols. The main flavan-3-ols in green tea are (−)-epicatechin and its gallate derivatives. These compounds are present in lower amounts in black tea being replaced by theaflavins and thearubigins (*3*, *4*). The main flavonols in tea are conjugates of quercetin and kaempferol with lower levels of myricetin and the conjugating moiety varying from mono- to di- and triglycosides (*5*−*8*). Other related compounds found in tea are gallic acid, quinic esters of gallic, coumaric, and caffeic acids together with the purine alkaloids, theobromine and caffeine (**Scheme 1**) (*9*, *10*), proanthocyanidins (*11*, *12*), and trace levels of flavones (*13*).

Analysis of these compounds has typically involved the use of a variety of high-performance liquid chromatography (HPLC) systems with absorbance or diode array detection with each system tailored for separation of only a limited number of the many phenolic components in tea (*3*, *14*−*19*). There are few studies in which both the flavan-3-ols and the flavonol profiles in teas have been examined simultaneously and in depth (*20*). The current paper describes two high resolution, gradient elution reverse-phased HPLC systems for the separation of over 30 flavonols, flavan-3-ols, and related compounds in green and

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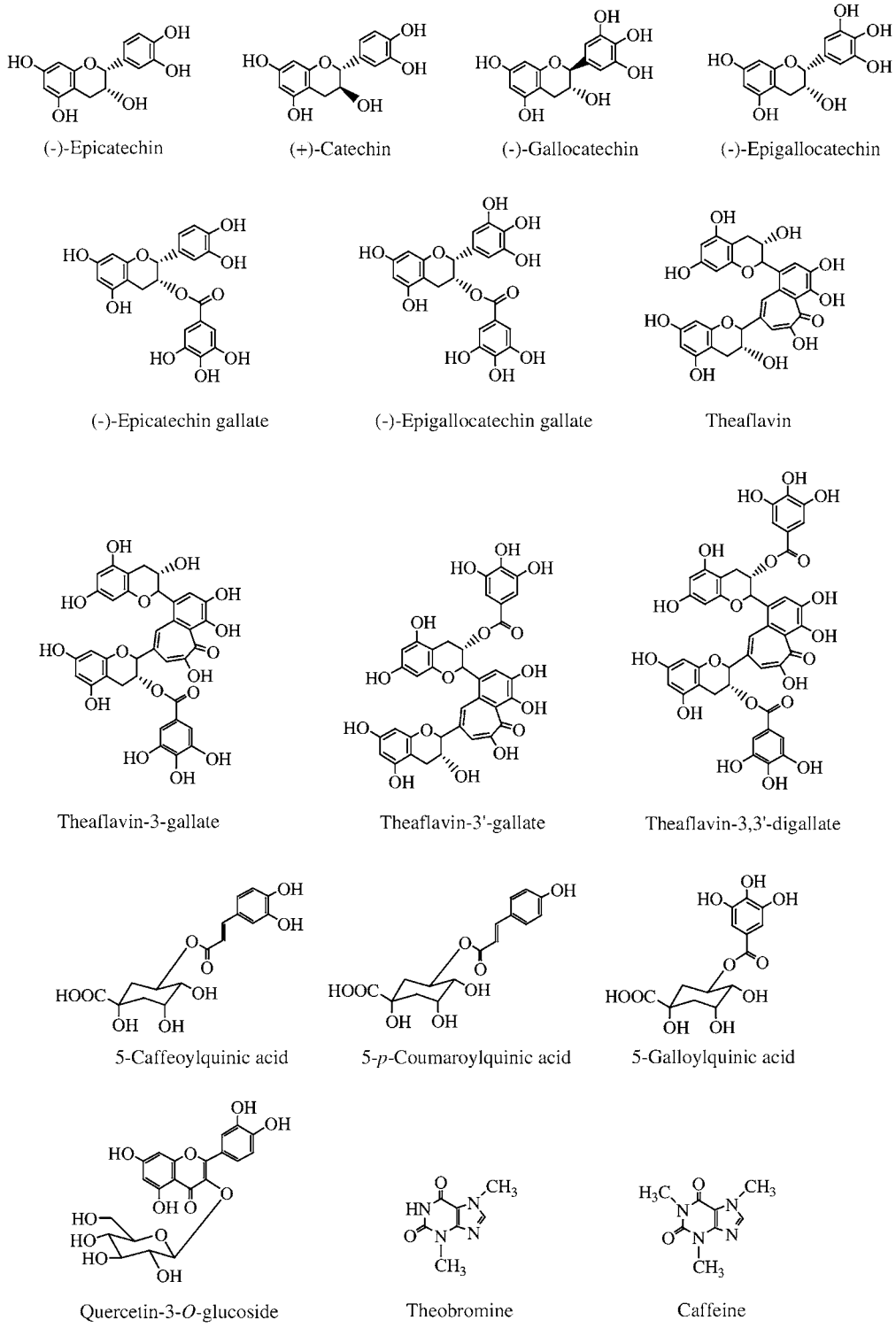
Scheme 1. Structures of the Major Phenolics and Purine Alkaloids Found in Teas



black teas, and their identification by diode array detection and electrospray mass spectrometry (MS) using an ion trap instrument with a MS^n facility.

2. MATERIALS AND METHODS

2.1. Materials. Tetley GB Ltd. (Greenford, Middlesex, U.K.) supplied samples of Indonesian green and black tea. (+)-Catechin, (−)-gallocatechin, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin, (−)-epigallocatechin gallate, gallic acid, caffeine, theobromine, quercetin-3-glucoside, quercetin-3-rutinoside, and Folin−Ciocalteu phenol reagent were supplied by Sigma (Poole, Dorset, U.K.). A black tea extract containing a mixture of theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate was also obtained from Sigma. Quercetin-3-arabinoside, quercetin-3-galactoside, kaempferol-3-glucoside, kaempferol-3-rutinoside, and 5-caffeoylquinic acid (chlorogenic acid) were supplied by AABB Chemicals (Southampton, U.K.). HPLC solvents were obtained from Rathburn Chemicals (Walkerburn, Scotland).

2.2. Preparation of Teas. Stock solutions of the tea infusions were prepared by adding 18 mL of boiling water to 1 g of leaves. After 3 min, the brew was filtered to remove particulate matter prior to analysis of the filtrate.

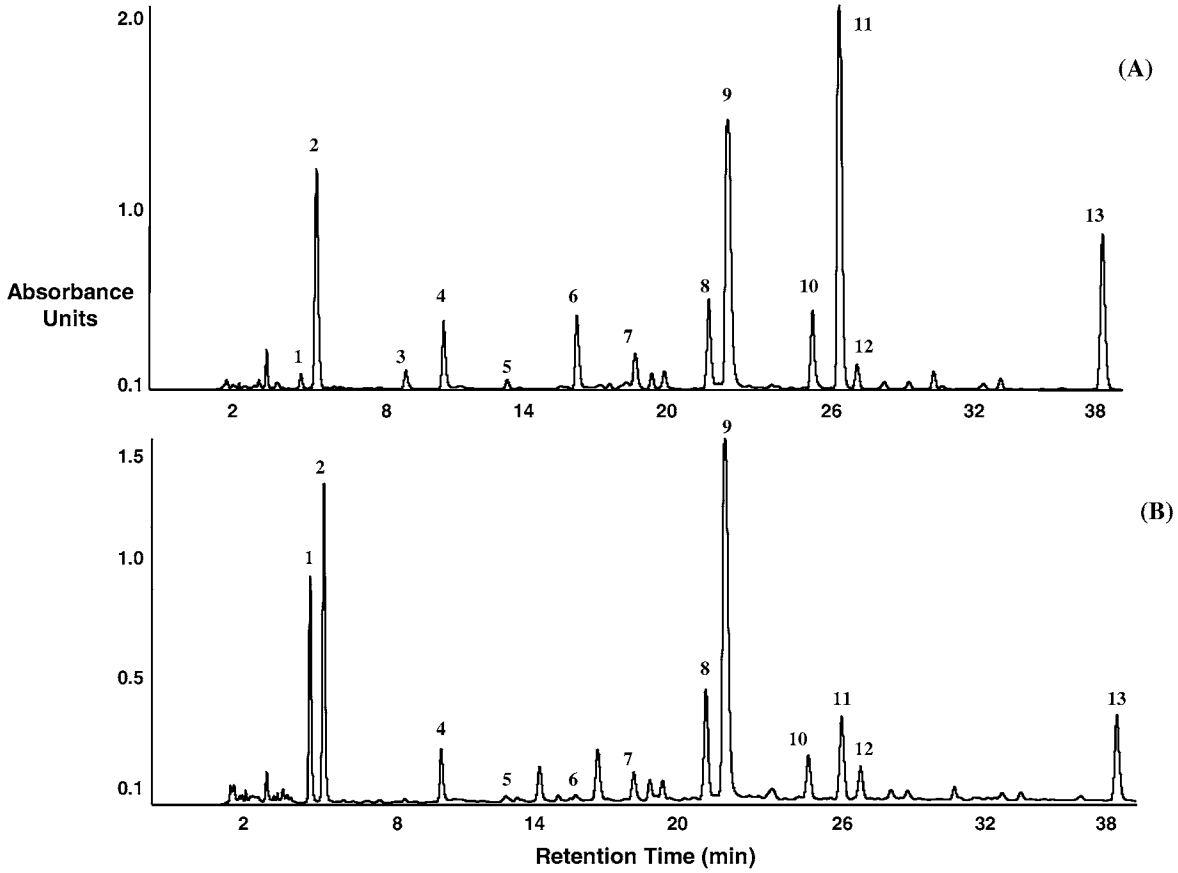


Figure 1. Major phenolic compounds and purine alkaloids in aliquots of (**A**) green tea (5 μL) and (**B**) black tea (10 μL) analyzed on a 250 mm × 4.6 mm i.d. Synergie RP Max 80 column eluted with a 60 min gradient of 4–25% acetonitrile in 1% aqueous formic acid at a flow rate of 1 mL min^{-1} and monitored at 280 nm. Peak identification: 1, gallic acid; 2, 5-galloylquinic acid; 3, (–)-gallocatechin; 4, theobromine; 5, 3-caffeoylquinic acid; 6, (–)-epigallocatechin; 7, (+)-catechin; 8, 5-caffeoylquinic acid; 9, caffeine; 10, (–)-epicatechin; 11, (–)-epigallocatechin gallate; 12, 4-*p*-coumaroylquinic acid; and 13, (–)-epicatechin gallate.

2.3. LC-Diode Array and MS^n Analysis. Initial analysis of tea samples highlighted the need to use two separate HPLC methods to separate out first the catechin-derived compounds and second the flavonols and theaflavins. Although this increased the analysis time, it greatly improved the chromatographic resolution of the compounds under study.

Tea infusions were analyzed using a Surveyor gradient HPLC system comprised of an HPLC pump, diode array absorbance detector, scanning from 250 to 700 nm, and an autosampler cooled to 4 °C (Thermo Finnigan, San Jose, CA). Separations were carried out using a Phenomenex RP-MAX 4 μm 250 mm × 4.6 mm i.d. C_{12} reverse phase column (Torrance, CA) 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. After the mixture was 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 analyzed using both negative and positive ionization 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, (+)-catechin, (−)-epicatechin, theaflavins, 5-caffeoylquinic acid, quercetin-3-glucoside, rutin, 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 3-galloylquinic acid was quantified in gallic acid equivalents, 4-*p*-coumaroylquinic acid and 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-glucoside equivalents, and all kaempferol-based compounds were quantified by reference to kaempferol-3-glucoside.

2.4. Measurement of Total Polyphenols and Thearubigins. To quantify the total polyphenols in black tea infusions, the Folin−Ciocalteu method was applied and the data were expressed in gallic acid equivalents (*21*). Thearubigin levels were estimated by adding up the overall level of individual phenolics determined by HPLC and subtracting this figure from the estimate of total polyphenols obtained with the Folin−Ciocalteu assay (*9*).

3. RESULTS AND DISCUSSION

3.1. Phenolic Profile of Teas. Two gradient elution systems were used for reverse phase HPLC analysis of phenolics in tea infusions. Although most studies reported in the literature make use of a single long gradient, this approach can result in the incomplete resolution of some of the phenolic components with minor peaks often being masked by more prominent components that elute nearby. To solve these problems, two 60 min gradients were used as follows: a 4−25% acetonitrile gradient optimized to separate catechins, gallocatechins, and gallate esters, and a 10−30% acetonitrile gradient devised to resolve flavonol conjugates and theaflavins. The HPLC absorbance traces obtained when these gradients were used to analyze green and black teas are illustrated in **Figures 1** and **2**.

3.2. Identification of the Major Phenolics and Purine Alkaloids in Green and Black Tea. The phenolic compounds

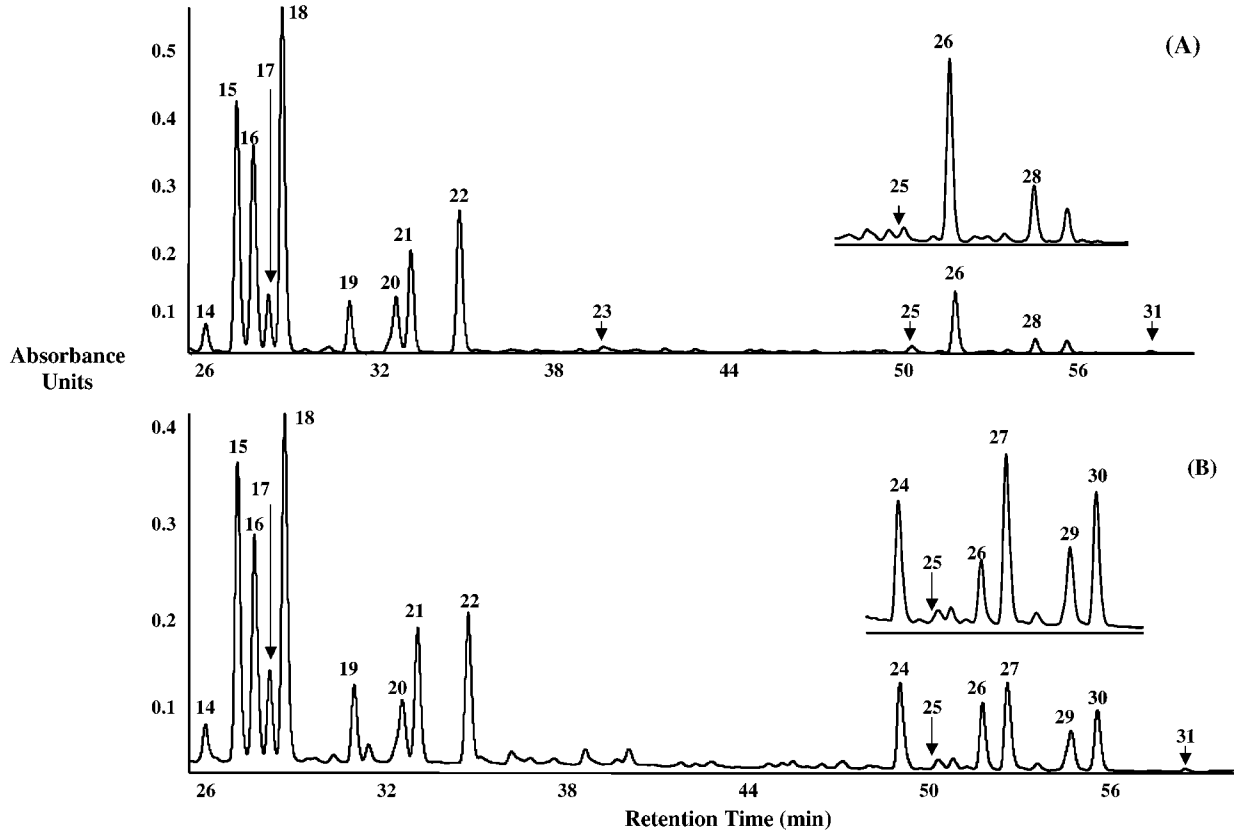


Figure 2. Major flavonol conjugates and theaflavins in aliquots of (**A**) green tea (10 μL) and (**B**) black tea (10 μL) analyzed on a 250 mm × 4.6 mm i.d. Synergie RP Max 80 column eluted with a 60 min gradient of 10–30% acetonitrile in 1% aqueous formic acid at a flow rate of 1 mL min^{-1} and monitored at 365 nm. The insert illustrates absorbance at 280 nm. Peak identification: 14, quercetin-rhamnosylgalactoside; 15, quercetin-3-rutinoside; 16, quercetin-3-galactoside; 17, quercetin-rhamnose-hexose-rhamnose; 18, quercetin-3-glucoside; 19, kaempferol-rhamnose-hexose-rhamnose; 20, kaempferol galactoside; 21, kaempferol-3-rutinoside; 22, kaempferol-3-glucoside; 23, kaempferol pentose conjugate; 24, theaflavin; 25 and 26, unknown quercetin conjugates; 27, theaflavin-3-gallate; 28, unknown kaempferol conjugate; 29, theaflavin-3′-gallate; 30, theaflavin-3,3′-digallate; and 31, unknown kaempferol conjugate.

Table 1. Retention Time, Mass Spectral Characteristics, and Identity of Flavan-3-ols, Hydroxycinammates, and Purine Alkaloids Present in Tea[a]

peak	t_R (min)	compound	[M – H]$^-$ (*m*/*z*)	MS^2 (*m*/*z*)
1	5.8	gallic acid	169	125
2	6.2	5-galloylquinic acid	343	191 (quinic acid; [M – H]$^-$-galloyl), 169
3	9.4	(–)-gallocatechin	305	261, 221, 219, 179
4	10.7	theobromine	181[b]	
5	13.2	3-caffeoylquinic acid	353	191 (quinic acid; [M – H]$^-$-caffeoyl), 179
6	15.9	(–)-epigallocatechin	305	261, 221, 219, 179
7	18.4	(+)-catechin	289	245, 205
8	21.3	5-caffeoylquinic acid	353	191 (quinic acid; [M – H]$^-$-caffeoyl)
9	22.1	caffeine	195[b]	
10	25.5	(–)-epicatechin	289	245, 205, 179
11	26.8	(–)-epigallocatechin-3-gallate	457	331, 305, 169
12	27.6	4-*p*-coumaroylquinic acid	337	173 (quinic acid; [M – H]$^-$-coumaroyl)
13	38.8	(–)-epicatechin-3-gallate	441	331, 289, 169

[a] Peak numbers and retention times refer to **Figure 1**. [b] [M + H]$^+$.

in green and black teas were identified on the basis of their retention time, absorbance spectrum, and MS fragmentation pattern and, where possible, by cochromatography with an authentic standard. It should be noted that a number of the phenolics found in teas are not available in a purified form. However, in most instances, compounds could be identified on the basis of MS fragmentation data coupled with characterizations in previously published studies (*3*, *6*, *7*, *22*).

Peaks 1−13 separated on the 4−25% acetonitrile gradient (see **Figure 1A**,**B**). Mass spectrometry utilized negative ionization, as indicated below, with the exception of peaks 4 and 9 where better spectra were obtained with positive ionization. The MS data obtained with individual compounds are summarized in **Table 1**.

Peak 1 (t_R, 5.8 min; λ_{max}, 277 nm) cochromatographed with gallic acid and had the same absorbance spectrum. This identification was confirmed by MS-MS analysis that revealed the presence of a negatively charged molecular ion ([M − H]$^-$) at *m*/*z* 169, which fragmented to produce a secondary fragment ion (MS^2) at *m*/*z* 125 (see **Table 1**).

Peak 2 (t_R, 6.2 min; λ_{max}, 271 nm) was identified as 5-galloylquinic acid (theogallin), which has previously been

Table 2. Retention Times, Mass Spectral Characteristics, and Identity of Flavonols and Theaflavins Present in Tea[a]

peak	t_R (min)	compound	$[M-H]^-$ (*m*/*z*)	MS^n (*m*/*z*)
14	25.9	quercetin-rhamnosylgalactoside	609	301 (Q; $[M-H]^-$-Gal-Rham)
15	27.0	quercetin-3-rutinoside	609	301 (Q; $[M-H]^-$-Glc-Rham)
16	27.6	quercetin-3-galactoside	463	301 (Q; $[M-H]^-$-Gal)
17	28.1	quercetin-rhamnose-hexose-rhamnose	755	609 ($[M-H]^-$-Rham), 301 (Q; $[M-H]^-$-Rham-Hex-Rham)
18	28.6	quercetin-3-glucoside	463	301 (Q; $[M-H]^-$-Glc)
19	30.9	kaempferol-rhamnose-hexose-rhamnose	739	593 ($[M-H]^-$-Rham), 431 ($[M-H]^-$-Rham-Hex), 285 (K; $[M-H]^-$-Rham-Hex-Rham)
20	32.5	kaempferol-galactoside	447	285 (K; $[M-H]^-$-Gal)
21	33.0	kaempferol-3-rutinoside	593	285 (K; $[M-H]^-$-Glc-Rham)
22	34.7	kaempferol-3-glucoside	447	285 (K; $[M-H]^-$-Glc)
23	39.6	kaempferol pentose conjugate	417	285 (K; $[M-H]^-$-Pen)
24	49.0	theaflavin	563	
25	50.3	unknown quercetin conjugate	901	755 ($[M-H]^- - 146$), 609 ($[M-H]^- - 146 - 146$), 301 (Q; $[M-H]^- - 146 - 146 - 308$)
26	51.7	unknown quercetin conjugate	901	755 ($[M-H]^- - 146$), 609 ($[M-H]^- - 146 - 146$), 463 ($[M-H]^- - 146 - 146 - 146$), 301 (Q; $[M-H]^- - 146 - 146 - 308$)
27	52.5	theaflavin-3-gallate	715	
28	54.5	unknown kaempferol conjugate	885	739 ($[M-H]^- - 146$), 593 ($[M-H]^- - 146 - 146$), 285 (K; $[M-H]^- - 146 - 146 - 308$)
29	54.7	theaflavin-3′-gallate	715	
30	55.5	theaflavin-3,3′-gallate	867	
31	58.4	unknown kaempferol conjugate	885	739 ($[M-H]^- - 146$), 593 ($[M-H]^- - 146 - 146$), 285 (K; $[M-H]^- - 146 - 146 - 308$)

[a] Peak numbers and retention times refer to **Figure 2**. Q, quercetin; K, kaempferol; Ara, arabinosyl; Gal, galactosyl; Glc, glucosyl; Hex, hexosyl; Pen, pentosyl; and Rham, rhamnosyl.

characterized and detected in significant quantities in green tea (*15*, *23*). An authentic standard was not available; however, MS analysis of the peak revealed a $[M-H]^-$ ion at *m*/*z* 343 that fragmented to yield a MS^2 spectrum with ions at *m*/*z* 191, the amu of a quinic acid, and *m*/*z* 169, which corresponds with gallic acid.

Peak 3 (t_R, 9.4 min; λ_{max}, 270 nm) was identified as (−)-gallocatechin on the basis of retention time, comparison with an authentic standard, and a $[M-H]^-$ at *m*/*z* 305, which yielded a major MS^2 fragment at *m*/*z* 261 and further minor fragments at *m*/*z* 221, 219, and 179.

Peak 4 (t_R, 10.7 min; λ_{max}, 272 nm) had a $[M+H]^+$ at *m*/*z* 181 and on the basis of the mass spectrum, absorbance spectrum, and cochromatography was identified as the purine alkaloid, theobromine.

Peak 5 (t_R, 13.2 min; λ_{max}, 325 nm) produced a $[M-H]^-$ at *m*/*z* 353 and MS^2 ions at *m*/*z* 191 and 179, which is in keeping with the presence of 3-caffeoylquinic acid as described by Clifford et al. (*22*).

Peak 6 (t_R, 15.9 min; λ_{max}, 270 nm) was identified as (−)-epigallocatechin on the basis of cochromatography with an authentic standard and a mass spectrum with a $[M-H]^-$ at *m*/*z* 305 and MS^2 fragments at *m*/*z* 261, 221, 219, and 179.

Peak 7 (t_R, 18.4 min; λ_{max}, 279 nm) was identified as (+)-catechin by comparison of its absorbance spectrum and retention time with those of an authentic standard. This was confirmed by the MS-MS, which yielded a $[M-H]^-$ at *m*/*z* 289 and a prominent MS^2 ion at *m*/*z* 245 and a minor fragment at *m*/*z* 205.

Peak 8 (t_R, 21.3 min; λ_{max}, 325 nm) cochromatographed with 5-caffeoylquinic acid. It also had the same absorbance and mass spectra ($[M-H]^-$ at *m*/*z* 353 and a single major MS^2 fragment at *m*/*z* 191) as 5-caffeoylquinic acid, which has previously been detected in black tea (*9*). In the HPLC system used, 4-caffeoylquinic acid elutes ca. 45 s after 5-caffeoylquinic acid, typically with baseline resolution. The two isomers can, therefore, be distinguished chromatographically as well as by MS as the predominant MS^2 ion derived from 4-caffeoylquinic acid is at *m*/*z* 179 while the corresponding fragment from 5-caffeoylquinic acid is at *m*/*z* 191 (*22*).

Peak 9 (t_R, 22.1 min; λ_{max}, 268 nm) was caffeine. It cochromatographed with the purine alkaloid and had a similar absorbance spectrum and an identical positive ion mass spectrum ($[M+H]^+$ at *m*/*z* 195).

Peak 10 (t_R, 25.5 min; λ_{max}, 278 nm) was identified as (−)-epicatechin on the basis of absorbance spectrum, retention time, and MS-MS fragmentation pattern ($[M-H]^-$ at *m*/*z* 289 and the main MS^2 fragment at *m*/*z* 245 with a further ion at *m*/*z* 205).

Peak 11 (t_R, 26.8 min; λ_{max}, 273 nm) has the same absorbance spectrum and cochromatographed with (−)-epigallocatechin-3-gallate. The presence of the flavan-3-ol was confirmed by MS-MS, which produced a $[M-H]^-$ at *m*/*z* 457 and MS^2 fragments at *m*/*z* 331, 305, and 169.

Peak 12 (t_R, 27.6 min; λ_{max}, 311 nm) had a similar absorbance spectrum but different HPLC retention time to *p*-coumaric acid. MS analysis yielded a $[M-H]^-$ at *m*/*z* 337 and a prominent MS^2 ion at *m*/*z* 173, which, in accordance with Clifford et al. (*22*), matches the mass spectrum of 4-*p*-coumaroylquinic acid, a known component of black tea (*9*).

Peak 13 (t_R, 38.8 min; λ_{max}, 275 nm) was identified as (−)-epicatechin-3-gallate on the basis of comparison of absorbance spectrum, retention time, and MS-MS fragmentation ($[M-H]^-$ at *m*/*z* 441 and the main MS^2 fragment at *m*/*z* 331, 289, and 169) with those of an authentic standard.

Using a 60 min, 10−30% acetonitrile gradient, reverse phase HPLC separated peaks 14−28 as illustrated in **Figure 2A**,**B**. A summary of the HPLC retention times, λ_{max}, and MS-MS spectra is presented in **Table 2**. The basis of the identifications is as follows.

Peaks 14 and 15 (t_R, 25.9 and 27.0 min; λ_{max}, 353 nm) were identified as quercetin-hexose-rhamnose on the basis of MS-MS and retention times. The $[M-H]^-$ in each case was *m*/*z* 609, which yielded a MS^2 fragment at *m*/*z* 301, the $[M-H]^-$ of the aglycone quercetin. This loss of *m*/*z* 308 corresponds to cleavage of rhamnose-hexose sugar. The lack of intermediate quercetin-hexose or quercetin-rhamnose ions suggests that the cleaved sugar is a rutinose type fragment as a rhamnose-glucose moiety does not fragment into its constitutive sugars. The two peaks have identical MS spectra, and it is probable from the chromatographic behaviors that the earlier eluting peak 14 is quercetin-rhamnosylgalactoside and the latter peak 15 is quercetin-rhamnosylglucoside. The identity of peak 15 was subse-

quently confirmed as quercetin-3-rhamnosylglucoside (quercetin-3-rutinoside, alias rutin) by reference to an authentic standard.

Peaks 16 and 18 (t_R, 27.6 and 28.6 min; λ_{max}, 353 nm) were identified as quercetin-hexose conjugates on the basis of elution order and MS-MS. Both peaks yielded an $[M - H]^-$ at *m*/*z* 463, which yielded a major MS^2 ion at *m*/*z* 301. This corresponds to quercetin with the loss of a hexose group. As with peaks 14 and 15, peaks 16 and 18 have identical mass spectra and are likely to be a quercetin galactoside and a quercetin glucoside, respectively. On the basis of cochromatography with reference compounds and identical MS-MS spectra, peak 18 was identified as quercetin-3-glucoside and peak 16, which is the minor component, is quercetin-3-galactoside. Both of these flavonols are known components of black tea (*6*, *8*).

Peak 17 (t_R, 28.1 min; λ_{max}, 343 nm) was identified as quercetin-rhamnose-hexose-rhamnose on the basis of MS-MS. Analysis revealed an $[M - H]^-$ at *m*/*z* 755, which fragmented to produce a major MS^2 ion at *m*/*z* 609. This 146 amu loss equates with the cleavage of a rhamnosyl unit. As discussed above for peaks 14 and 15, a fragment ion at *m*/*z* 609 corresponds to a quercetin-rhamnose-hexose conjugate. Further MS^3 analysis of the MS^2 *m*/*z* 609 ion produced an ion at *m*/*z* 301, corresponding to quercetin. Generally, in reversed phase HPLC, the more sugar moieties attached to the flavonoid aglycone, the earlier the retention time. However, the elution order of anthocyanins shows that the addition of a rhamnose to a glucosyl moiety can cause the anthocyanin to elute later rather than earlier (*24*). The position of the conjugating sugar can also have a marked effect as in the reversed phase system used in the present study quercetin-4′-glucoside is much more retained than quercetin-3-glucoside.

It should be noted that peak 17 is not the quercetin-3-glucosylrhamnosylglucoside previously identified in tea by Finger et al. (*8*) as this quercetin trisaccharide would yield a $[M - H]^-$ at *m*/*z* 787 not *m*/*z* 755. Nor is this peak the quercetin-rhamnose-rhamnose-glucoside identified by Englehardt et al. (*25*) as indicated by the initial *m*/*z* 146 loss of rhamnose leaving a fragment ion at *m*/*z* 609 (quercetin-rhamnose-glucose).

Peak 19 (t_R, 30.9; λ_{max}, 342 nm) was identified as a kaempferol-rhamnose-hexose-rhamnose conjugate on the basis of the MS-MS spectrum. The $[M - H]^-$ at *m*/*z* 739 ionized to produce the major MS^2 fragment at *m*/*z* 593. This loss of 146 amu corresponds with cleavage of a rhamnosyl unit. Further fragmentation of the *m*/*z* 593 ion yielded major MS^3 ions at *m*/*z* 431 and 285, which corresponds to a kaempferol rhamnoside (loss of 162 amu) and kaempferol (loss of 308 amu corresponding to cleavage of a rhamnose-hexose moiety), respectively. This kaempferol trisaccharide has a molecular weight 32 amu lower than kaempferol-3-glucosylrhamnosylglucoside, which has been detected in black tea by Finger et al. (*8*). Similarly, although the $[M - H]^-$ at *m*/*z* 739 is the same as would be obtained with the kaempferol-rhamnose-rhamnose-galactoside and kaempferol-rhamnose-rhamnose-glucoside identified in tea by Engelhardt et al. (*25*), the initial loss of a rhamnosyl group during fragmentation reveals the order of attachment of the conjugating sugar moieties to be different.

Peak 20 (t_R, 32.5 min; λ_{max}, 345 nm) was identified as a kaempferol galactoside. The ion at *m*/*z* 447 corresponds to the $[M - H]^-$ of a kaempferol-hexose conjugate, and the resultant MS^2 fragment at *m*/*z* 285 ($[M - H]^- - 162$) is kaempferol. Peak 22, which is a kaempferol-3-glucoside, has the same MS spectra so it is likely that peak 20 contains a kaempferol-galactoside. Galactosides elute earlier than the corresponding glucosides, and a kaempferol galactoside has been detected in black tea infusions by Price et al. (*6*).

Peak 21 (t_R, 33.0 min; λ_{max}, 345 nm) was identified as kaempferol rutinoside. The $[M - H]^-$ *m*/*z* 593 yielded a MS^2 fragment at *m*/*z* 285 (kaempferol; M − 308, loss of hexose-rhamnose with no intermediate ion at *m*/*z* 447). In keeping with this assignment, cochromatography revealed that the compound involved is kaempferol-3-rutinoside.

Peak 22 (t_R, 34.7 min; λ_{max}, 343 nm) is kaempferol-3-glucoside on the basis of its MS-MS spectrum ($[M - H]^-$ at *m*/*z* 447 and the major MS^2 fragment at *m*/*z* 285), absorbance spectrum, and cochromatography with a reference compound.

Peak 23 (t_R, 39.6 min; λ_{max}, 343 nm) is a kaempferol pentose conjugate, on the basis of negative ion MS-MS. The $[M - H]^-$ at *m*/*z* 417 produced a major MS^2 ion at *m*/*z* 285 (kaempferol) with the M − 132 equating with the loss of a pentosyl moiety.

Peaks 24, 27, 29, and 30 (t_R, 49.0, 52.5, 54.7, and 55.5 min; all with λ_{max}, 270, 374, and 453 nm) were identified as theaflavins on the basis of MS-MS, elution order, and cochromatography with a standard mixture containing theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate. The four major theaflavins in the test mixture elute after the main flavonol conjugates in the following order: theaflavin > theaflavin-3-gallate > theaflavin-3′-gallate > theaflavin-3,3′-digallate (*9*, *18*, *26*). These four compounds all had a similar absorbance spectrum, which was unlike those of catechin or flavonol spectra. Peak 24 yielded a $[M - H]^-$ at *m*/*z* 563, which corresponds to theaflavin. The $[M - H]^-$ of peaks 27 and 29 appeared at *m*/*z* 715, 152 amu higher than that of peak 24. This increase corresponds to the addition of a galloyl unit. On the basis of the mass spectrum and the elution order, it is concluded that peak 27 is theaflavin-3-gallate and peak 29 is theaflavin-3′-gallate. Both compounds are known to be present in black tea (*3*, *9*). Peak 30 is identified as theaflavin-3,3′-digallate on the basis of an $[M - H]^-$ at *m*/*z* 867, 152 amu higher than the $[M - H]^-$ of peaks 27 and 29, and 304 amu higher than that of peak 24.

Peaks 25, 26, 28, and 31 (t_R, 50.3, 51.7, 54.5, and 58.4 min) were identified. Peaks 25 and 26 had a λ_{max} of 314 nm while peaks 28 and 31 had a λ_{max} of 306 and 310 nm, respectively. Peaks 25 and 26 had an $[M - H]^-$ at *m*/*z* 901, which yielded varying combinations of MS^2 ions at *m*/*z* 755, 609, and 301 corresponding to the loss of two successive 146 amu fragments and one 308 amu fragment. A loss of 146 and 308 amu is in keeping with cleavage of a rhamnosyl and rutinosyl moiety, respectively. The fragment ion at *m*/*z* 301 corresponds to the aglycone quercetin. However, the long retention time of these compounds is not in keeping with them being quercetin tetraglycosides conjugated with one disaccharide and two monosaccharides. To confirm the aglycone was in fact quercetin, the tea was infused into the mass spectrometer and MS^4 was carried out on the ion at *m*/*z* 901. The resultant fragmentation of the *m*/*z* 301 ion confirmed that the aglycone was quercetin.

Peaks 28 and 31 had $[M - H]^-$ at *m*/*z* 885 and yielded similar fragmentation patterns to peaks 25 and 26 (loss of 146−146−308 amu) giving rise to a base ion at *m*/*z* 285, corresponding to kaempferol. However, as with the quercetin-based compounds, peaks 25 and 26, the long HPLC retention time of peaks 28 and 31 would appear to preclude the possibility them being flavonol tetraglycosides, which because of their high polarity, would elute at a much earlier point on the reverse phase gradient. An alternative possibility is that at least one of the losses of 146 amu is due to cleavage of a *p*-coumaroyl rather than a rhamnosyl unit (*27*). The addition of a *p*-coumaroyl moiety to

Table 3. Concentration of the Major Phenolics in Black and Green Tea Infusions[a]

compound (peak number)	green tea	black tea	black tea content as a % of green tea content
gallic acid (1)	6.0 ± 0.1	125 ± 7.5	2083
5-galloylquinic acid (2)	122 ± 1.4	148 ± 0.8	121
total gallic acid derivatives	**128**	**273**	**213**
(–)-gallocatechin (3)	383 ± 3.1	ND	0
(–)-epigallocatechin (6)	1565 ± 18	33 ± 0.8	2.1
(+)-catechin (7)	270 ± 9.5	12 ± 0.1	4.4
(–)-epicatechin (10)	738 ± 17	11 ± 0.2	1.5
(–)-epigallocatechin gallate (11)	1255 ± 63	19 ± 0.0	1.5
(–)-epicatechin gallate (13)	361 ± 12	26 ± 0.1	7.2
total flavan-3-ols	**4572**	**101**	**2.2**
3-caffeoylquinic acid (5)	60 ± 0.2	10 ± 0.2	17
5-caffeoylquinic acid (8)	231 ± 1.0	62 ± 0.2	27
4-*p*-coumaroylquinic acid (12)	160 ± 3.4	143 ± 0.2	89
total hydroxycinammate quinic esters	**451**	**215**	**48**
quercetin-rhamnosylgalactoside (14)	15 ± 0.6	12 ± 0.2	80
quercetin-3-rutinoside (15)	131 ± 1.9	98 ± 1.4	75
quercetin-3-galactoside (16)	119 ± 0.9	75 ± 1.1	63
quercetin-rhamnose-hexose-rhamnose (17)	30 ± 0.4	25 ± 0.1	83
quercetin-3-glucoside (18)	185 ± 1.6	119 ± 0.1	64
kaempferol-rhamnose-hexose-rhamnose (19)	32 ± 0.2	30 ± 0.3	94
kaempferol-galactoside (20)	42 ± 0.6	29 ± 0.1	69
kaempferol-rutinoside (21)	69 ± 1.4	60 ± 0.4	87
kaempferol-3-glucoside (22)	102 ± 0.4	69 ± 0.9	68
kaempferol-arabinoside (23)	4.4 ± 0.3	ND	0
unknown quercetin conjugate (25)	4.0 ± 0.1	4.3 ± 0.5	108
unknown quercetin conjugate (26)	33 ± 0.1	24 ± 0.9	73
unknown kaempferol conjugate (28)	9.5 ± 0.2	ND	0
unknown kaempferol conjugate (31)	1.9 ± 0.0	1.4 ± 0.0	74
total flavonols	**778**	**570**	**73**
theaflavin (24)	ND	64 ± 0.2	
theaflavin-3-gallate (27)	ND	63 ± 0.6	
theaflavin-3′-gallate (29)	ND	35 ± 0.8	
theaflavin-3,3′-digallate (30)	ND	62 ± 0.1	
total theaflavins	**ND**	**224**	
theobromine (4)	57 ± 0.1	25 ± 0.6	44
caffeine (9)	866 ± 16	541 ± 2.9	62
total purine alkaloids	**923**	**566**	**61**

[a] Data expressed as mg L^{-1} ± standard error ($n = 3$); ND, not detected.

anthocyanin glucosides markedly increases reverse phase HPLC retentions (*24*, *28*). It has been reported that addition of hydroxy-aromatic organic acids results in a characteristic shift in absorbance spectra toward 310 nm (*29*). This is in keeping with the λ_{max} of peaks 28 and 31, the spectra of which also retain a shoulder at ~370 nm, characteristic of the flavonol aglycone. However, more detailed analysis is required to establish the identity of these quercetin- and kaempferol-based putative *p*-coumaroyl-sugar conjugates.

Although 31 compounds were detected in total, it is noticeable that neither tea contained detectable levels of myricetin conjugates. This in is keeping with the findings of Price et al. (*6*) that myricetin conjugates are found in relatively low concentrations in some but not all teas. Proanthocyanidins (*11*, *12*) and flavones (*13*) were also not detected in the present study. These components are relatively minor in tea, and their detection is usually only feasible after sample enrichment.

3.3. Differences in the Levels of Phenolics and Purine Alkaloids in Green and Black Tea Infusions. During HPLC analyses, quantitative estimates of the individual phenolics and purine alkaloids in the green and black tea infusions were obtained from the response of the diode array detector while peak identity and purity were determined by MS^n analysis. The information obtained is presented in **Table 3**.

Despite the teas being produced from a single plucking, the caffeine and theobromine contents of the black tea were 62 and 44%, respectively, of the concentration in green tea. Gallic acid was present in black tea in ca. 20-fold higher amount than found in green tea while the 5-galloylquinic acid content was only marginally higher. The combined level of the six flavan-3-ols in green tea was 4572 mg L^{-1} while only 101 mg L^{-1} was present in the black tea.

The green tea contained 60 ± 0.2 and 231 ± 1.0 mg L^{-1}, respectively, of 3-caffeoylquinic acid and 5-caffeoylquinic acid with the black tea infusion containing ca. 4−5-fold lower quantities. In contrast, at 160 ± 3.4 and 143 ± 0.2 mg L^{-1}, respectively, for the green and black teas, there was little difference in the concentration of 4-*p*-coumaroylquinic acid.

A total of 14 flavonol glycosides were detected in varying concentrations in the two teas (**Table 3**). The parent flavonols were quercetin and kaempferol, which were conjugated to a range of sugars including glucose, galactose, pentose, and rhamnose, as mono-, di-, and trisaccharides. The overall level of flavonols in the black tea was 73% of that found in the green tea infusion, indicating that flavonols survive fermentation far better than flavan-3-ols.

Green tea contained no theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, or theaflavin-3,3′-digallate, but all four compounds were detected in black tea in concentrations ranging from 35 to 64 mg L^{-1} (**Table 3**). The albeit approximate estimate of the thearubigin content of black tea was 1681 mg L^{-1} of gallic acid equivalents indicating that during fermentation

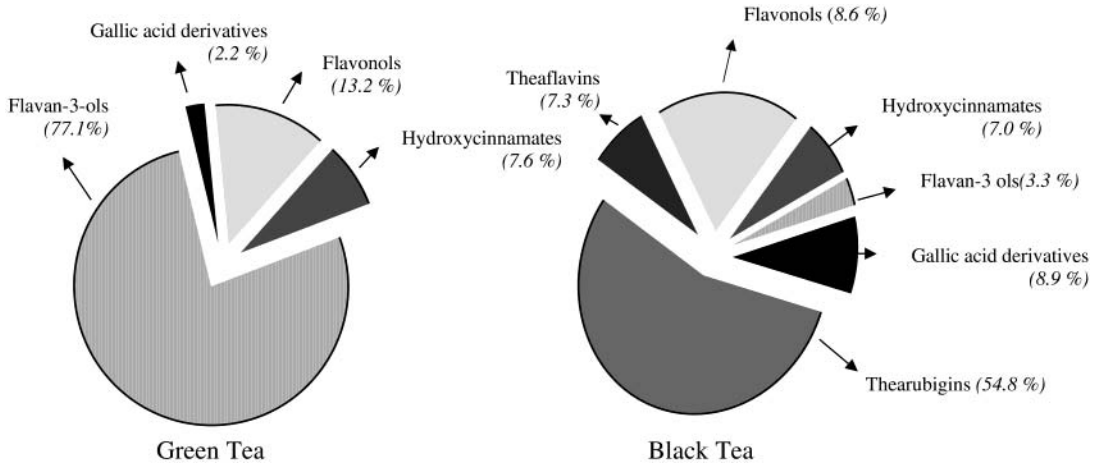


Figure 3. Percentile composition of the different classes of phenolics in green and black tea.

there is substantial condensation of flavan-3-ols yielding these high molecular weight derivatives. The overall levels of the different flavonoid and phenolic groups in the green and black tea are summarized in **Figure 3**.

The data obtained in the present study merit comparison with the results of Rechner et al. (*20*) who quantified 20 phenolics in seven black teas using a single gradient HPLC method with diode array detection. The illustrated HPLC trace in **Figure 1** of Rechner et al. (*20*) shows a large unresolved peak eluting over 60% of the chromatogram. This was attributed to "chromatographically irresolvable" thearubigins. However, this explanation cannot be correct as the traces in **Figures 1B** and **2B** in the present paper demonstrate. A more likely cause of the rise and fall in baseline is the incomplete separation of the phenolic compounds in the teas. This would explain the lack of detection of (+)-catechin and (−)-gallocatechin, despite the availability of reference compounds, and the ability to separate and quantify only five flavonols. In addition, Rechner et al. (*20*) reported that 4-caffeoylquinic acid was the main chlorogenic acid in black teas whereas in the present study 5-caffeoylquinic acid was found to be the main component and 4-caffeoylquinic acid was not present in detectable amounts. These compounds can be readily identified based on their MS^n fragmentation patterns cited by Clifford et al. (*22*). The importance of accurately quantifying the major phenolics in tea has additional compounding factors when an estimation of total thearubigins content is made. Underestimation of phenolics will result in an overestimate in the levels of thearubigins.

In summary, (+)-catechin, (−)-epicatechin, (−)-gallocatechin, (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-epigallocatechin gallate have previously been identified in both green and black tea by many investigators. One of the two gradient HPLC systems, used in the current study, with diode array and MS^n detection, facilitated the reliable identification and quantification, not only of these six flavan-3-ols but also of gallic acid, 5-galloylquinic acid, 3- and 5-caffeoylquinic acid, 4-*p*-coumaroylquinic acid, theobromine, and caffeine in green and black tea (**Figure 1**). The second gradient HPLC system was optimized for a similarly rapid analysis of 14 flavonols and four theaflavins found in teas. This study demonstrates the value of HPLC with MS^n rather than single stage MS detection for the reliable identification of trace levels of flavan-3-ols and hydroxycinnamates. It has been demonstrated in the current study and other investigations (*22*, *27*, *28*, *30*−*33*) that MS^n and MS^2 are especially useful in providing partial identifications of trace quantities of flavonols and other natural products when reference compounds are not available. Such structural information cannot be obtained when HPLC is used with diode array, fluorescence, or electrochemical detectors. Further information on the position of substituent groups and the positive identification of some of the flavonols detected in teas would require the use of NMR as exemplified by the elegant studies of Engelhardt and colleagues (*5*, *8*). However, this would involve not only extensive sample purification but also a requirement for several orders of magnitude more analyte than the low nanogram quantities needed for HPLC-MS^n and, as a consequence, would be extremely time consuming and not an approach that lends itself to routine quantitative analysis.

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