JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

*J. Agric. Food Chem.* **2008,** *56,* 11187–11191 **11187**

# Identification and Quantification of Free Radical Scavengers in Pu-erh Tea by HPLC-DAD-MS Coupled Online with 2,2′-Azinobis(3-ethylbenzthiazolinesulfonic acid) Diammonium Salt Assay

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Pu-erh tea, a well-known traditional beverage in China, has attracted more attention because of its beneficial health effects and special flavor and taste. Generally, it is believed that Pu-erh tea with a longer preservation period has better quality and taste. Antioxidant activity is one of the major beneficial activities of tea. In this study, a HPLC-DAD-MS coupled with 2,2′-azinobis (3-ethylbenzthiazoline-sulfonic acid) diammonium salt (ABTS) assay was employed for identification and quantification of free radical scavengers in different samples of Pu-erh tea. Among 12 main peaks detected in Pu-erh raw tea, 11 compounds were identified as gallic acid, (−)-gallocatechin, (−)-epigallocatechin, (+)-catechin, caffeine, (−)-epicatechin, (−)-epigallocatechingallate, rutin, (−)-epicatechingallate, quercetin-3-glucoside, and kaempferol-3-glucoside by comparison of their UV and MS data with standard compounds or literature data, respectively. The contents of 12 investigated compounds were also determined or estimated using caffeine, (−)-epicatechin, or rutin as standard. ABTS assay showed that 10 out of 12 compounds were free radical scavengers. Their total amount was used as the marker for evaluation of free radical scavenging activities of different Pu-erh teas, which indicated that the activity of different Pu-erh teas varied; Pu-erh raw tea was stronger than the ripe one, and the activity decreased with the increase of preservation period.

**KEYWORDS: Pu-erh tea; free radical scavenging activity; HPLC; ABTS; online**

## INTRODUCTION

Oxygen free radicals or reactive oxygen species (ROS) at high concentration can induce many human diseases such as cancer, atherosclerosis, stroke, rheumatoid arthritis, neurodegeneration, and diabetes (*1, 2*). Consequently, free radical scavengers, which can alleviate the oxidative stress, are beneficial for improving human health. However, some currently used synthetic free radical scavengers have various side effects such as cytotoxicity and genotoxicity (*3, 4*). Therefore, searching for novel free radical scavengers from natural sources becomes a focus (*5*−*7*).

Pu-erh tea, a well-known traditional beverage mainly produced in Yunnan Province of China, is derived from dry leaves of *Camellia sinensis* var. *assamica* (*8*). In recent years, Pu-erh tea has attracted more attention because of its beneficial health effects and special flavor and taste. Modern pharmacological studies have also demonstrated that Pu-erh tea has antimutagenic (*9*), antimicrobial (*9*), anticancer (*10*), antioxidant (*11, 12*), antiobesity (*13, 14*), and antiarteriosclerosis (*15*) activities. Traditionally, it is believed that Pu-erh tea with a longer preservation period has better quality and taste. Therefore, the prices of Pu-erh tea with different preservation periods are greatly variant, and the difference can be up to tens to hundred of times in the market (*16, 17*). Generally, teas represent a rich source of dietary antioxidants (*18, 19*), which are attributed to their phenolic compounds (*20, 21*). Several studies have reported the antioxidant activity of Pu-erh tea (*11, 22*). But its components with free radical scavenging capacity were not elucidated. In addition, the effect of aging on free radical scavenging activity of Pu-erh tea has not been investigated.

Many methods, such as 2,2′-azinobis(3-ethylbenzthiazoline-sulfonic acid) diammonium salt (ABTS) assay, diphenylpicrylhydrazyl (DPPH) assay, and electron spin resonance spectrometry, have been used for evaluation of free radical scavenging capacity of compounds (*23*). However, these methods could not determine the active components in a mixture. Generally, a conventional procedure for screening and identification of free radical scavengers in herbal extracts is separating and purifying chemical compounds with the guidance of bioassay, which is tedious and time-consuming work. Recently, a technique was developed to measure the radical scavenging activity of individual compounds online when they elute from an HPLC column (*24*−*26*).

In the present study, free radical scavengers in Pu-erh tea were identified and quantitatively determined or estimated using

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10.1021/jf8025716 CCC: $40.75 
Published on Web 11/06/2008

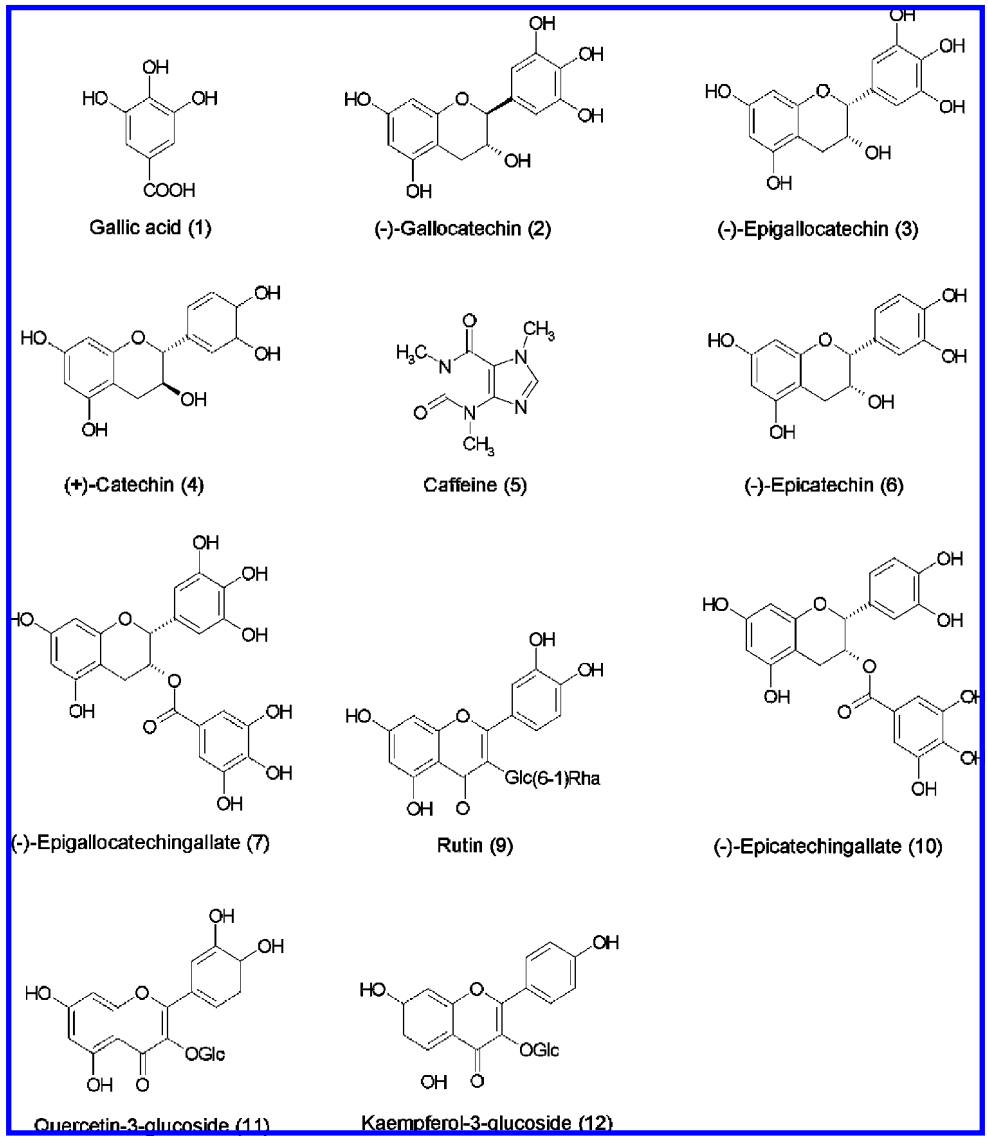


**Figure 1.** Chemical structures of the investigated compounds in Pu-erh tea.

HPLC-DAD-MS coupled online with ABTS-based analysis. The free radical scavenging capacities of Pu-erh teas with different preservation periods have also been investigated and compared.

## MATERIALS AND METHODS

**Materials.** Caffeine, (−)-epicatechin, and rutin (**Figure 1**) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). ABTS was purchased from International Laboratory (San Bruno, CA). Potassium persulfate was from Fluka (Seelze, Germany). HPLC grade formic acid, acetonitrile, and ethanol were purchased from Merck (Darmstadt, Germany). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Billerica, MA).

Six samples of Pu-erh tea were supplied by Macau International Food Safety Association (**Table 1**). Lipton green tea (Lipton Company, China) was purchased in the local supermarket.

**Preparation of Sample Solutions and Standard Solutions.** Sample preparation was performed using an ultrasonic cleaning bath (Branson Ultrasonics Corporation, Danbury). In brief, the dried samples were ground using a Knifetec 1095 Sample Mill (Foss Tector, Sweden). Ground powder (0.5 g, less than 0.45 mm) was transferred into a 100 mL conical flask with stopper. Ultrasonication (43 kHz, 320 W) was performed at room temperature for 30 min with 50 mL of 50% methanol. After centrifugation (3000 revolutions/min, 15 min), the supernatant was filtered through a 0.45 $\mu$m filter (Agilent Technologies) before injection into the HPLC system for analysis. Standard stock solution was prepared by dissolving the accurately weighted analytes [caffeine, (−)-epicatechin, and rutin] in an appropriate volume of 50% methanol, which was stored in the refrigerator at 4 °C before analysis.

**Table 1.** The List of Tea Samples

| sample no. | name | type | production date | source |
|---|---|---|---|---|
| A | *Ren Wu Yi Wu Quing Bing* | raw tea | 2002 | Macao |
| B | *Geng Chen Jing Mai Gong Zhuan* | unknown | 2000 | Yunnan |
| C | *Yi You Yi Wu Quing Bing* | raw tea | 2005 | Macao |
| D | *Gu Fa Qi Zi Bing* | unknown | unknown | Yunnan |
| E | *Ren Wu Gong Ting* | unknown | 2002 | Macao |
| F | *Ba Shi Hong Zhi* | unknown | unknown | Yunnan |
| G | *Lipton Green Tea* | green tea | 2007 | Guangzhou |

**Preparation of $ABTS^{\bullet+}$ Solution.** ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS solution (7 mM in water) with 2.5 mM potassium persulfate (final concentration) for 12 h at 4 °C in dark (stock solution). Then the $ABTS^{\bullet+}$ stock solution was diluted with ethanol to an absorbance of approximately 1.0 at 750 nm, which was stable for at least 2 days in the dark (*26*).

**HPLC-DAD-MS and ABTS-Based Assay.** HPLC-DAD-MS and ABTS-based assay was performed according to our previous report (*26*). HPLC-DAD-MS analysis was performed on an Agilent series 1100 (Agilent Technologies) liquid chromatograph, equipped with a vacuum

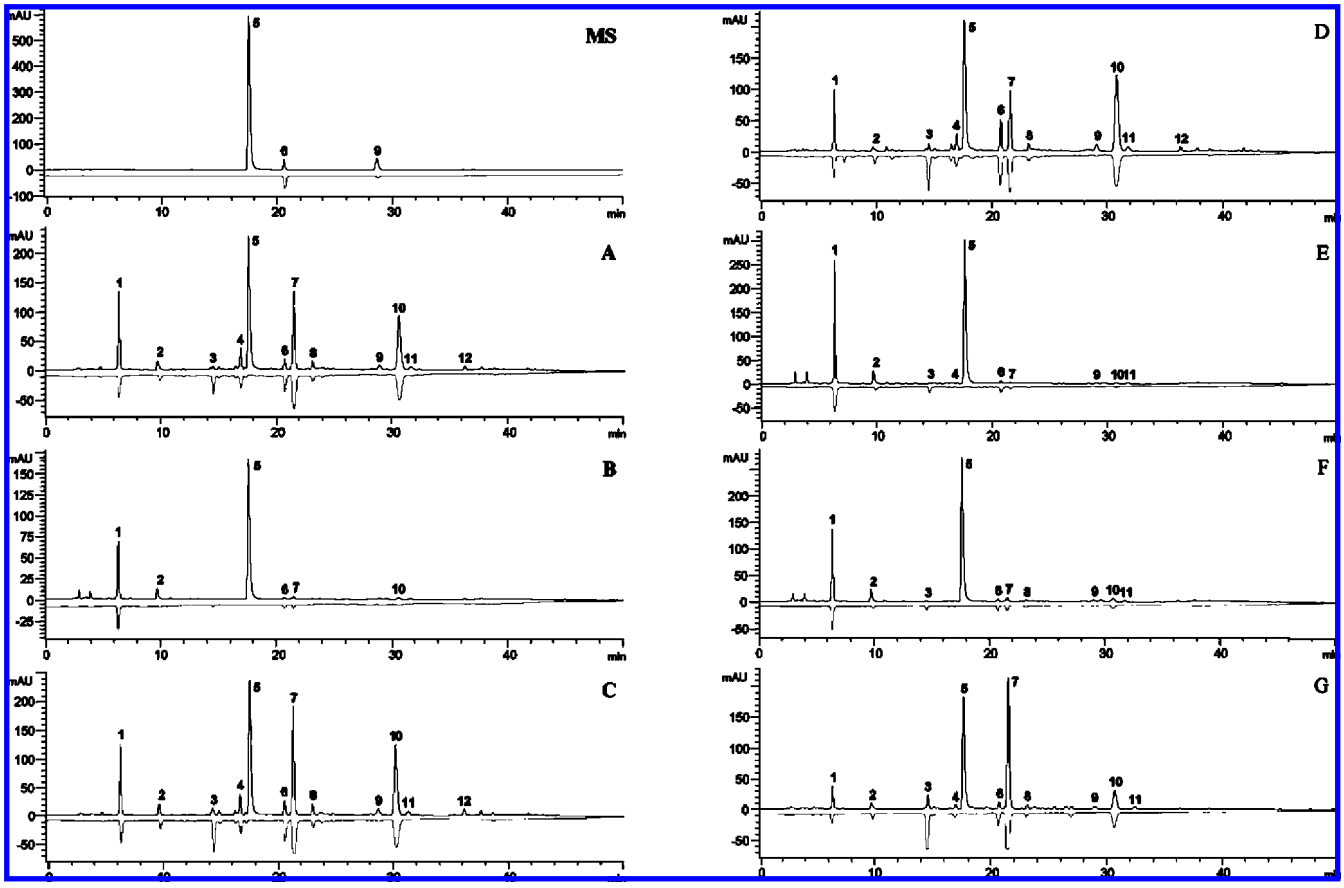


**Figure 2.** The typical UV chromatograms and ABTS assay profiles (free radicals scavenger detected as negative) of (**MS**) mixed standards, (**A**−**F**) samples of Pu-erh tea, and (**G**) Lipton green tea: (**A**) *Ren Wu Yi Wu Quing Bing*; (**B**) *Geng Chen Jing Mai Gong Zhuan*; (**C**) *Yi You Yi Wu Quing Bing*; (**D**) *Gu Fa Qi Zi Bing*; (**E**) *Ren Wu Gong Ting*; (**F**) *Ba Shi Hong Zhi*.

degasser, a quaternary pump, an autosampler, and a diode array detector (DAD) and an ion-trap mass spectrometer with electrospray ionization interface, controlled by Agilent LC/MSD Trap software. A Zorbax SB C18 column (250 mm × 4.6 mm i.d., 5 $\mu$m) with a Zorbax SB C18 guard column (12.5 mm × 4.6 mm i.d., 5 $\mu$m) was used. The column temperature was set at 30 °C. The separation was achieved by a gradient elution of 0.5% formic acid solution (solvent A) and acetonitrile (solvent B) at a flow rate of 1 mL/min: 0−20 min, 5%−17% B; 30−45 min, 17%−40% B; 45−50 min, 40%−80% B; 50−55 min, 80%−100% B. The injection volume was 5 $\mu$L, and the detection wavelength was set at 270 nm. Mass spectrometry was carried out in positive scan mode from *m*/*z* 50 to 1400. ESI-MS conditions were as follows: drying gas, $N_2$, 8.5 L/min; temperature, 350 °C; nebulizer pressure, 35 psi. ESI-MS/MS conditions were as follows: smart mode with compound stability 50%, isolation width 4, fragment amplification 1.5. The flow rate of the diluted $ABTS^{\bullet+}$ solution was set to 0.5 mL/min, and any bleaching of the initial color was detected as a negative peak at 750 nm.

**Calibration Curves.** . Stock solutions containing caffeine, (−)-epicatechin, and rutin were prepared and diluted to appropriate concentrations for the construction of calibration curves. At least six concentrations of the three analyte solutions were analyzed in triplicate, and then the calibration curves were constructed by plotting the peak areas versus the concentration of each analyte.

**Limits of Detection and Quantification.** The stock solution was diluted to a series of appropriate concentrations with the same solvent, and an aliquot of the diluted solution was injected into the HPLC apparatus for analysis. The limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively.

**Precision and Accuracy.** Intra- and interday variations were chosen to determine the precision of the developed assay. For intraday variability test, the mixed standards solution was analyzed for six replicates within one day, while for interday variability test, the solution was examined in duplicate for three consecutive days. The relative standard deviation (RSD) was taken as a measure of precision. The recovery was performed by adding a known amount of individual standards into a certain amount (0.25 g) of Pu-erh tea (sample A). Three replicates were performed for the test. The mixture was extracted and analyzed using the method mentioned above. The recovery was calculated as recovery (%) = 100 × (amount found − original amount)/amount spiked.

**Statistical Data Analysis.** Hierarchical clustering analysis was performed by SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA). A method named as between-group linkage was applied, and Pearson correlation, which is a pattern similarity measure, was selected as measurement.

## RESULTS AND DISCUSSION

**Method Validation.** The regression equations, correlation coefficients, and linear ranges (LR) of the three marker compounds were as follows: caffeine, $y = 4.87x + 153.92$ with $r^2 = 1.000$ and LR = 33.0−1320 $\mu$g/mL; (−)-epicatechin, $y = 1.21x + 7.29$ with $r^2 = 0.9999$ and LR = 8.3−2491.0 $\mu$g/mL; and rutin, $y = 2.78x + 12.20$ with $r^2 = 0.9999$ and LR = 12.8−512.0 $\mu$g/mL. The overall LODs and LOQs of caffeine, (−)-epicatechin, and rutin were less than 1.6 $\mu$g/mL and 5.6 $\mu$g/mL, and their intra- and interday precisions were less than 1.33% ($n = 6$) and 2.45% ($n = 6$), respectively. The recoveries of (−)-epicatechin, caffeine, and rutin were between 96.5% and 107.8% ($n = 3$).

**Identification of Free Radical Scavengers in Pu-erh Tea.** The extracts of Pu-erh and Lipton green teas were applied onto the system. Twelve major compounds can be detected in their HPLC-DAD chromatograms (**Figure 2**) Among them, 10 peaks

**Table 2.** The MS and UV Data of 12 Components in Pu-erh Tea

| peak no. | retention time (min) | MS (*m*/*z*) | UV $\lambda_{max}$ (nm) | identification | ref |
|---|---|---|---|---|---|
| 1 | 6.5 | 171 $[M + H]^+$, 153 $[M + H - H_2O]^+$ | 270 | gallic acid | *27,28* |
| 2 | 9.7 | 329 $[M + Na]^+$, 307 $[M + H]^+$, 289 $[M + H - H_2O]^+$, 139 | 275 | (−)-gallocatechin | *28,29* |
| 3 | 14.5 | 329 $[M + Na]^+$, 307 $[M + H]^+$, 289 $[M + H - H_2O]^+$, 139 | 275 | (−)-epigallocatechin | *28,29* |
| 4 | 16.7 | 313 $[M + Na]^+$, 291 $[M + H]^+$, 273 $[M + H - H_2O]^+$, 139 | 270 | (+)-catechin | *29,30* |
| 5 | 17.6 | 217 $[M + Na]^+$, 195 $[M + H]^+$, 138 | 280 | caffeine | *28,31* |
| 6 | 20.8 | 313 $[M + Na]^+$, 291 $[M + H]^+$, 273 $[M + H - H_2O]^+$, 139 | 270 | (−)-epicatechin | *28,29* |
| 7 | 21.5 | 481$[M + Na]^+$, 459 $[M + H]^+$, 289, 139 | 275 | (−)-epigallocatechingallate | *29,30* |
| 8 | 23.2 | 565, 547, 427 | 280 | unknown | |
| 9 | 28.9 | 633 $[M + Na]^+$, 611$[M + H]^+$, 465 $[M + H - rha]^+$, 303 $[M + H - rha - glc]^+$ | 265, 355 | rutin | *27,30* |
| 10 | 30.6 | 465 $[M + Na]^+$, 443 $[M + H]^+$, 273, 139 | 280 | (−)-epicatechingallate | *29,30* |
| 11 | 31.7 | 487 $[M + Na]^+$, 465 $[M + H]^+$, 303 $[M + H - glc]^+$ | 265, 355 | quercetin-3-glucoside | *27,30* |
| 12 | 36.3 | 471 $[M + Na]^+$, 449 $[M + H]^+$, 287 $[M + H - glc]^+$ | 265, 355 | kaempferol-3-glucoside | *27,30* |

**Table 3.** The Contents of 12 Investigated Compounds in Pu-erh Teas

| | content (mg/g)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| analytes | A | B | C | D | E | F | G |
| gallic acid | 37.1 | 18.27 | 34.58 | 27.27 | 68.25 | 36.25 | 9.81 |
| (−)-gallocatechin | 5.83 | 4.35 | 7.75 | 2.98 | 9.77 | 8.43 | 3.61 |
| (−)-epigallocatechin | 11.76 | *c* | 5.43 | 3.52 | 0.37 | 0.32 | 6.92 |
| (+)-catechin | 13.47 | *c* | 13.38 | 9.63 | 0.60 | *c* | 2.32 |
| caffeine[b] | 24.7 | 17.63 | 25.29 | 22.38 | 32.79 | 29.48 | 19.4 |
| (−)-epicatechin | 6.07 | 0.40 | 8.72 | 17.38 | 1.66 | 1.66 | 3.85 |
| (−)-epigallocatechingallate | 52.74 | 0.75 | 74.43 | 37.32 | 0.68 | 2.43 | 83.57 |
| unknown | 5.56 | *c* | 7.28 | 4.89 | *c* | 0.7 | 2.06 |
| rutin | 1.77 | *c* | 3.14 | 2.71 | 0.36 | 0.51 | 0.95 |
| (−)-epicatechingallate | 61.78 | 1.33 | 80.26 | 83.75 | 1.26 | 4.41 | 20.58 |
| quercetin-3-glucoside | 1.55 | *c* | 2.18 | 1.98 | 0.41 | 0.52 | 0.68 |
| kaempferol-3-glucoside[b] | 0.93 | *c* | 1.14 | 0.78 | *c* | *c* | *c* |
| amount of antioxidants | 197.63 | 25.10 | 237.15 | 191.43 | 83.36 | 55.23 | 134.35 |

[a] The contents of gallic acid, (−)-gallocatechin, (−)-epigallocatechin, (+)-catechin, (−)-epigallocatechingallate, (−)-epicatechingallate, and peak 8 were estimated using (−)-epicatechin as standard, while contents of quercetin-3-glucoside and kaempferol-3-glucoside were determined using rutin as standard. [b] Non-free-radical scavengers. [c] Under the limited of quantification.

(1−4, 6−11) were detected as negative using the ABTS assay, which indicated that these components were free radical scavengers in these tea samples.

Peaks 5, 6, and 9 were identified as caffeine, (−)-epicatechin, and rutin, respectively, by comparing their retention time, UV, and MS data with those of standards. The peaks 1−4, 7, and 10−12 were identified as gallic acid (1), (−)-gallocatechin (2), (−)-epigallocatechin (3), (+)-catechin (4), (−)-epigallocatechingallate (7), (−)-epicatechingallate (10), quercetin-3-glucoside (11), and kaempferol-3-glucoside (12) by comparing their MS data with references (*27*−*31*). Peak 8 was still unknown based on its MS and UV data (**Table 2**).

**Determination or Estimation of 12 Major Compounds in Pu-erh Tea Samples.** The contents of the 12 investigated compounds in tea samples were determined or estimated. The contents of (−)-epicatechin, rutin, and caffeine were determined by their individual calibration curves, while the contents of gallic acid, (−)-gallocatechin, (−)-epigallocatechin, (+)-catechin, (−)-epigallocatechingallate, (−)-epicatechingallate, and peak 8 were estimated using (−)-epicatechin as standard, and those of quercetin-3-glucoside and kaempferol-3-glucoside were estimated using rutin as standard. The results were summarized in **Table 3**, which showed that the contents of investigated compounds varied greatly in different tea samples.

**Comparison of Different Pu-erh Teas.** In order to compare free radical scavenging capacities of different Pu-erh teas, hierarchical clustering analysis of the seven tested samples was performed based on the contents of the 10 main free radical scavengers. As a result, seven tested samples were divided into two main clusters, cluster 1 (samples B, E, and F) and cluster 2 (samples A, C, D, and G) (**Figure 3**). The total amount of free radical scavengers in the two clusters were significantly different, 25.10−83.36 mg/g for the samples of cluster 1 and 134.35−237.15 mg/g for those of cluster 2 (**Table 3**). Generally, there are two types of process methods for production of Pu-erh tea; consequently raw and ripe ones are obtained. The ripe Pu-erh tea usually has less antioxidants because of a fermentative process (*15*). Furthermore, samples A and C are known as Pu-erh raw tea. Therefore, sample D and the samples in cluster 1 may be Pu-erh raw and ripe teas, respectively. In addition, the three Pu-erh raw tea samples and Lipton green tea were further divided into two subclusters. The differences might derive from their different origins and cultivation locations (*8, 32*).

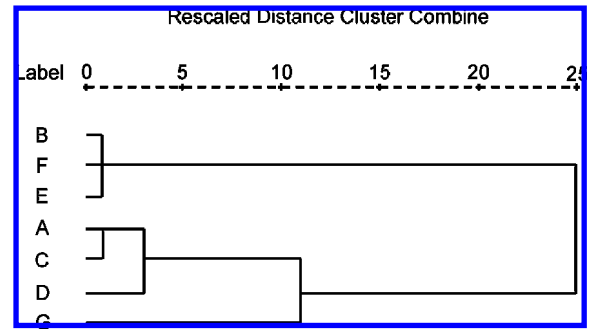


**Figure 3.** Dendrograms resulting from hierarchical clustering analysis base on 10 free radical scavengers.

Pu-erh tea that has been preserved for longer time generally has good odor and taste, and it is usually considered that the older is more valuable than younger one. Therefore, the price of older Pu-erh tea is higher than that of younger ones. Among the samples with different ages in the same type of Pu-erh tea, the younger teas have higher amounts of free radical scavengers than older ones, such as sample C (237.15 mg/g) > sample A (197.63 mg/g) and sample E (83.36 mg/g) > sample B (25.10 mg/g). The results are in accordance with the previous report (*18*). Therefore, the value of Pu-erh tea should be evaluated based on their taste, odor, and beneficial component amounts.

In summary, ten major free radical scavengers were found in Pu-erh tea, and nine of them were identified as gallic acid, (−)-gallocatechin, (−)-epigallocatechin, (+)-catechin, (−)-epicatechin, (−)-epigallocatechingallate, rutin, (−)-epicatechingallate, and quercetin-3-glucoside by HPLC-DAD-MS coupled online with ABTS-based analysis. The amount of free radical scavengers in Pu-erh raw tea was higher than that in ripe ones, while older Pu-erh tea had less free radical scavenging capacity in the same type.

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**Received for review August 20, 2008. Revised manuscript received October 7, 2008. Accepted October 10, 2008.**

JF8025716