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Massive Accumulation of Gallic Acid and Unique Occurrence of Myricetin, Quercetin, and Kaempferol in Preparing Old Oolong Tea

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Old oolong tea, tasting superior and empirically considered beneficial for human health, is prepared by long-term storage accompanied with periodic drying for refinement. Analyzing infusions of three old and one newly prepared oolong teas showed that significant lower (−)-epigallocatechin gallate (EGCG) but higher gallic acid contents were detected in the old teas compared to the new one. The possibility of releasing gallic acid from EGCG in old tea preparation was supported by an in vitro observation of gallic acid degraded from EGCG under heating conditions mimicking the drying process. Moreover, three minor flavonols, myricetin, quercetin, and kaempferol, that were undetectable in the new tea occurred in all of the three old teas. Converting the new oolong tea into an old one by periodic drying revealed the same characteristic observation, i.e., massive accumulation of gallic acid presumably released from EGCG and unique occurrence of flavonols putatively decomposed from flavonol glycosides.

KEYWORDS: EGCG; flavonol; flavonol glycosides; gallic acid; old oolong tea

INTRODUCTION

Oolong tea, possessing a taste and color somewhere between green and black teas, is the most popular tea in Taiwan, and versatile process conditions have been practiced to generate variable products of this tea by local manufacturers. Some unique compounds have been identified in different preparations of oolong tea (*1*−*6*). In the production of oolong tea, young green shoots are freshly harvested and allowed to undergoing a semi-fermentation process, where the term "fermentation" refers to natural browning reactions induced by oxidative enzymes in the cells of tea leaves (*7*). The final fermentation degree of oolong tea is empirically controlled by experts during the semi-fermentation process and usually ranges from 20 to 80%, depending upon the demand of customers.

After long-term storage, oolong tea tends to absorb substantial moisture from the air and thus needs to be refined by periodic drying. Traditionally, old oolong tea is named for those oolong teas that have been stored for more than 5 years and refined annually by a professional drying process using a specialized oven, batch after batch of approximately 5 kg of tea each time, at various desired temperatures. Experientially, the longer oolong tea is stored and further oxidized gradually, the better it is in terms of taste and beneficial effects to human health. In general, semi-fermentation and periodic drying are regarded as two key steps for quality control of old oolong tea during its tedious preparation. The time-consuming and labor-intensive preparation of old oolong tea considerably raises its production cost and thus limits its commercialization at a large scale. Typically, the price of old oolong tea is 3−10 times higher than that of its original oolong tea.

Different preparations of old oolong tea may vary noticeably because of their divergent semi-fermentation and drying process, and therefore, there is no clear definition for old oolong tea thus far, not to mention its quality control. However, almost all preparations of old oolong tea possess some common characteristics, such as their infusions look dark red or black and taste slightly sour in company with the special umami sensation known for oolong tea. Whether the common characteristic of slightly sour taste is resulted from a unique chemical reaction during the preparation of old oolong tea has not been addressed.

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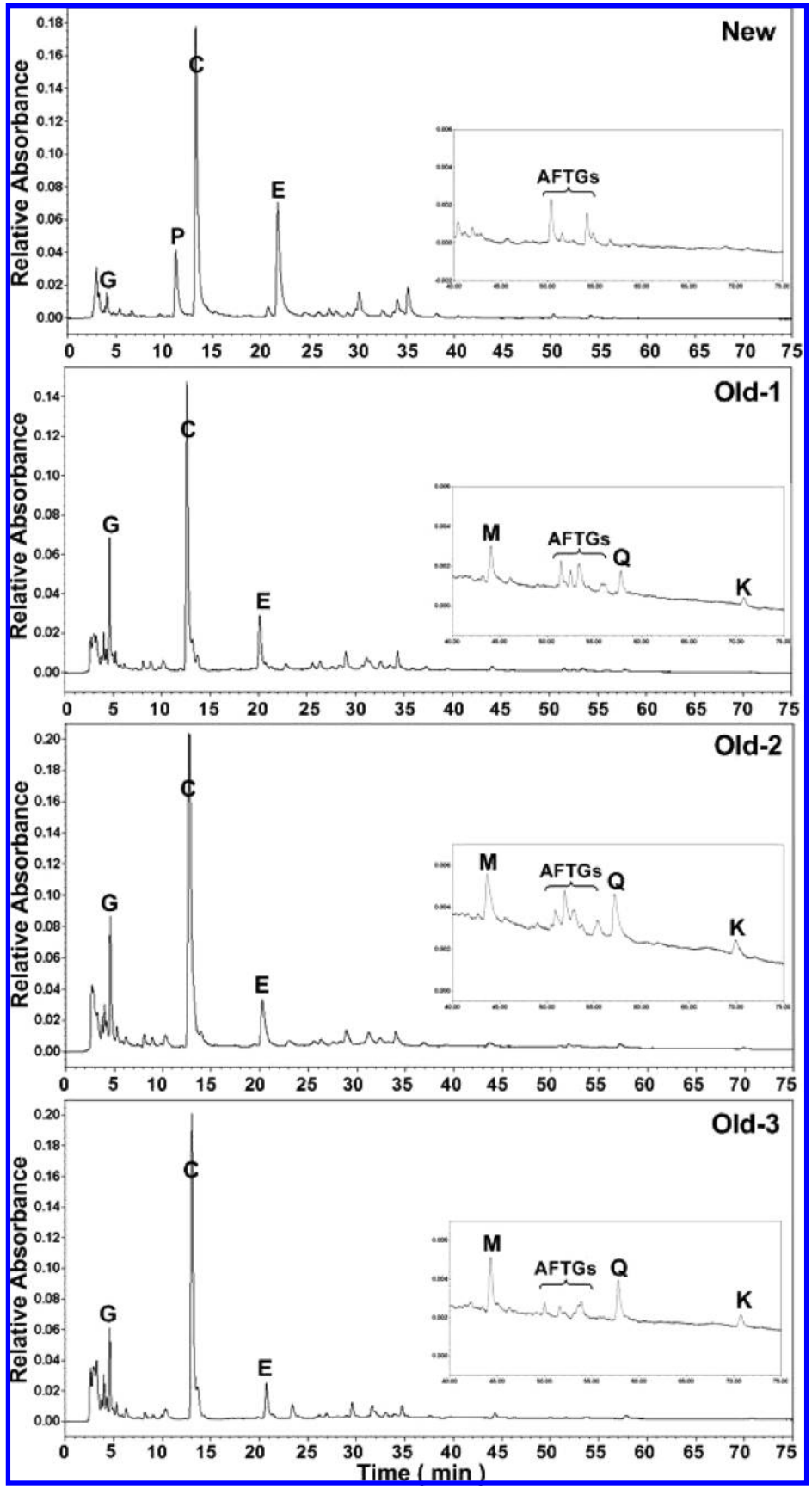

Figure 1. HPLC profiles (0−75 min) of infusions of three old and one new oolong teas at 254 nm. Amplification of each HPLC profile from 39 to 74 min is shown in an inserted panel within the diagram. G, P, C, E, M, Q, and K represent gallic acid, prodelphinidin A-2 3′-*O*-gallate, caffeine, EGCG, myricetin, quercetin, and kaempferol, respectively. AFTGs represent acylated flavonol tetraglycosides identified previously (*5*).

In this study, we aimed to search for unique characteristics of old oolong tea. First, infusions of three old and one newly prepared oolong teas were analyzed and compared. According to the difference observed between the old and new oolong teas,

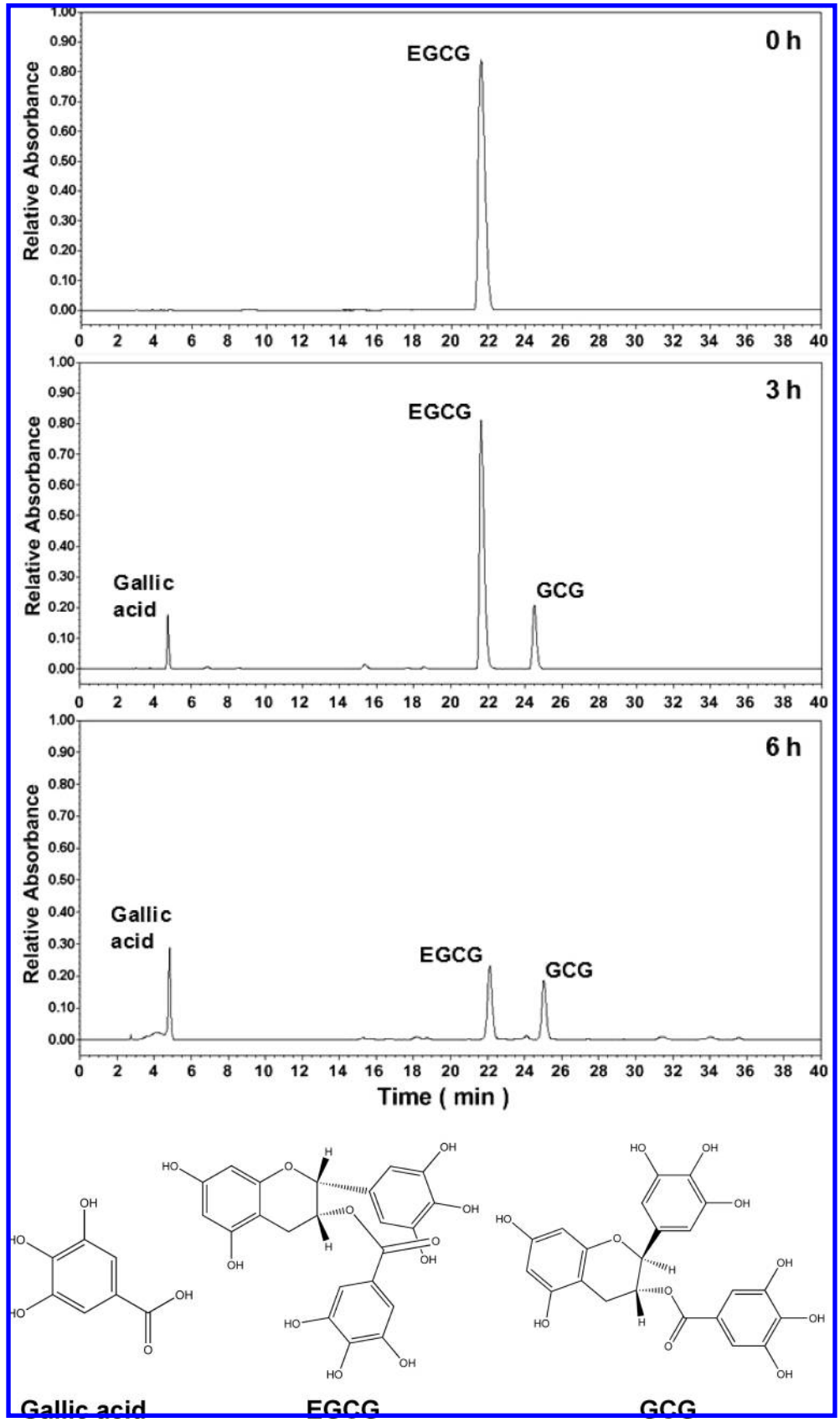

Figure 2. Chemical conversion of EGCG by heating. Chemical changes of EGCG at 120 °C for 3 and 6 h were detected by HPLC. EGCG was partly epimerized into GCG and degraded to release gallic acid after heating. Chemical structures of EGCG, GCG, and gallic acid are shown at the bottom.

an in vitro observation of releasing gallic acid from (−)-epigallocatechin gallate (EGCG) by heating was executed to simulate the chemical conversion that might occur in the drying process for preparing old oolong tea and led to the characteristic of slightly sour taste. Finally, the new oolong tea was converted into an old one by periodic drying, and relative contents of

Figure 3. Tea leaves and infusions of new and old oolong teas. The new oolong tea (right) was converted into an old one (left) by periodic drying. New and old oolong tea leaves are shown before and after tea preparation.

EGCG, gallic acid, flavonols, and flavonol glycosides in the tea were monitored during this conversion process to verify the characteristics observed in the three old oolong teas.

MATERIALS AND METHODS

Chemicals and Materials. Gallic acid, (-)-epigallocatechin-3-*O*-gallate (EGCG), and caffeine were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Acetonitrile (HPLC grade) and phosphoric acid (85%) were purchased from Merck KGaA (Darmstadt, Germany). Acetic acid (99.7%) was obtained from J. T. Baker (Mallinckrodt Baker, Inc., Phillipsburg, NJ). Water was purified by a Millipore clear water purification system (Millipore Direct-Q, Billerica, MA). Three preparations of old oolong tea were obtained from local suppliers in Taiwan; they were prepared using the same tea plant cultivar, *Camellia sinensis* L., Chin-shin oolong, grown at different locations. Fresh oolong tea was prepared with young green shoots of tea plant (the same cultivar as the three old teas) harvested from Lugu village, Nantou County, Taiwan, following the traditional semi-fermentation process, with a final fermentation degree of approximately 80%. To convert the newly prepared oolong tea into an old one, the tea was refined by a drying process at 120 °C for 10 h every 4 months in the following 2 years. Samples of the newly prepared oolong tea and its converted teas refined by periodic drying for 3 times (1 year old) and 6 times (2 year old) were collected for further analysis and comparison.

Preparation of Tea Infusions. Tea infusions were prepared by adding 18 mL of boiling water to 1 g of various oolong tea preparations. After 3 min, the brew was filtered through a 0.45 μm polyvinylidene difluoride (PVDF) membrane filter (Pall Corporation, Glen Cove, NY) and used for the following analysis.

LC/UV and LC/MS/MS Analysis. Tea infusions were analyzed on a liquid chromatography system coupled to a Model 600E photodiode array detector (Waters Corporation, Milford, MA) and performed using a 250 × 4.6 mm i.d., 5 μm, C18 reversed-phase column (Waters, Milford, MA). The mobile phase consisted of (A) water containing 0.026% phosphoric acid and (B) acetonitrile. The gradient was as follows: 0−60 min, linearly gradient from 10 to 30% B; 61−70 min, 30% B; and 70−100 min, linear gradient from 30 to 10% B. In all experiments, the injection volume was 10 μL and the flow rate was 1 mL/min at room temperature. The UV absorbance detection wavelength was set at 254 nm. For mass analysis, the liquid chromatograph system was coupled to a Finnigan LCQ ion-trap mass spectrometer (Thermo Finnigan Corporation, San Jose, CA) as described previously (*4*).

In Vitro Observation of Releasing Gallic Acid from EGCG by Heating. To examine if gallic acid might be released from EGCG during the drying process for the preparation of old oolong tea, EGCG of 2 mg was dissolved in an acetate buffer (pH 5) of 200 μL and kept in an oven at 120 °C. After heating for 3 or 6 h, the sample volume was adjusted with the acetate buffer to a final concentration of 10 mM EGCG. The samples were filtered through a 0.45 μm PVDF membrane filter, and 20 μL of each sample was subjected to LC/UV and LC/MS/MS analysis. Gallic acid was identified by comparing its retention time and mass fragmentation patterns with those of an authentic standard. (−)-Gallocatechin-3-*O*-gallate (GCG) epimerized from EGCG was identified according to the analysis described by Wang et al. (*8*) and confirmed by its mass fragmentation patterns.

RESULTS AND DISCUSSION

Analysis and Comparison of Infusions of Three Old and One New Oolong Teas. To identify unique characteristics of old oolong tea, infusions of three old oolong teas were analyzed and compared to that of a newly prepared oolong tea. The analysis was performed under the same condition as described in our previous work that thoroughly screened and identified 57 peaks in oolong tea infusion by comparing with available authentic standards and analyzing their mass fragmentation patterns (*4*). The results showed that the relative contents of EGCG were significantly lower, while those of gallic acid were notably higher in the infusions of the three old oolong teas compared to those contents in the infusion of the new tea (**Figure 1**). Moreover, the relatively abundant content of prodelphinidin A-2 3′-*O*-gallate in the infusion of the new tea nearly vanished in those of old teas.

In Vitro Observation of Releasing Gallic Acid from EGCG. To examine the possibility of releasing gallic acid from EGCG during the preparation of old oolong tea, an in vitro observation of releasing gallic acid from EGCG by heating at 120 °C was executed to simulate the chemical conversion that might occur in the drying process. The results showed that EGCG, partly converted into its isomer GCG as reported previously (*8*), was degraded to release gallic acid under the heating condition (**Figure 2**). The chemical isomerization and degradation were not observed when EGCG was kept at room temperature for long-term storage, e.g., 6 months (data not shown).

Identification of Three Minor Flavonols in Old Oolong Teas. In addition to the drastic alteration of EGCG and gallic acid, three minor peaks that were undetectable in the new tea occurred in all of the three old teas (**Figure 1**). They were identified as myricetin, quercetin, and kaempferol by their mass fragmentation patterns. The deprotonated ion at *m*/*z* 317 (myricetin) generated the MS^2 fragment ions at 289, 179, and 151 in keeping with the loss of one CO, $C_7H_6O_3$, and $C_8H_6O_4$, respectively. The loss of $C_7H_6O_3$ was through retro-Diels−Alder (RDA) fission. The deprotonated ion at *m*/*z* 301 (quercetin) generated the MS^2 fragment ions at 273, 257, 179, and 151 corresponding to the loss of one CO, CO_2, $C_7H_6O_2$, and $C_8H_6O_3$, respectively. The *m*/*z* 285 (kaempferol) ion produced MS^2 fragment ions at 257, 241, and 151 corresponding to the loss of one CO, CO_2, and $C_8H_6O_2$, respectively. These three flavonols were found to be fundamental structures of many flavonol glycosides detected in the infusion of oolong tea as reported previously (*4*).

Converting the New Oolong Tea into an Old One. To examine fidelity and reproducibility of the characteristics observed in the three old oolong teas, the new oolong tea was

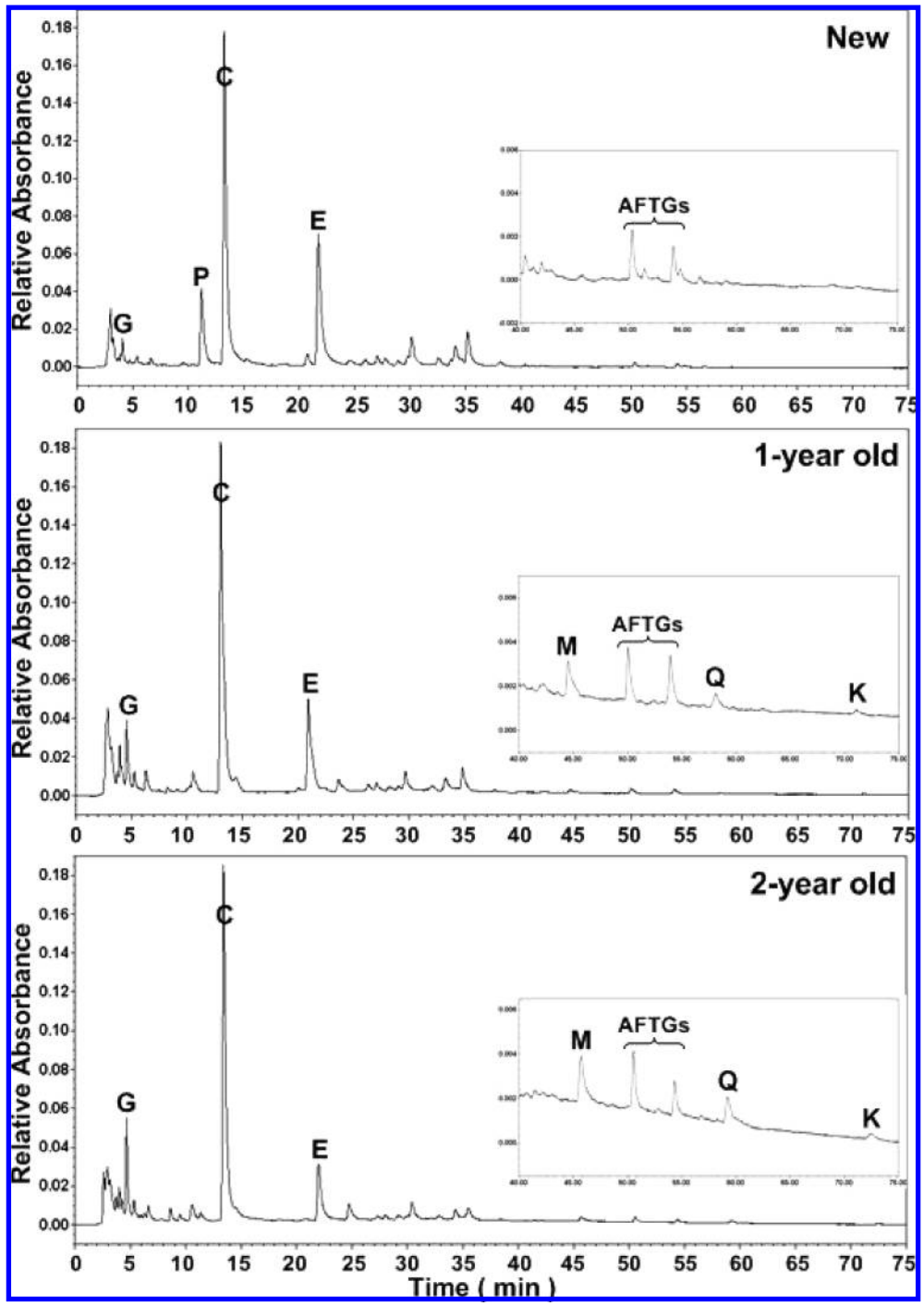

Figure 4. HPLC profiles (0−75 min) of infusions of the new oolong tea and its converted old teas (1 and 2 year old) at 254 nm. Amplification of each HPLC profile from 39 to 74 min is shown in an inserted panel within the diagram. G, P, C, E, M, Q, and K represent gallic acid, prodelphinidin A-2 3′-*O*-gallate, caffeine, EGCG, myricetin, quercetin, and kaempferol, respectively. AFTGs represent acylated flavonol tetraglycosides identified previously (*5*).

converted into an old one by periodic drying. After 2 year conversion, the tea transformed from yellow−green to nearly black, its infusion color changed from light yellow to dark red, and the tea leaves could no longer fully expand to their original sizes when they absorbed hot water in a regular tea preparation (**Figure 3**). Similar to the results observed in infusions of the three old oolong teas (**Figure 1**), the content of EGCG significantly decreased, that of gallic acid drastically increased, and those of myricetin, quercetin, and kaempferol gradually appeared in tea infusions during the conversion processes (**Figure 4**). In accordance with the accumulation of gallic acid, the pH value of tea infusion shifted from 5.5 to 5.0 after conversion.

Quantitative estimation indicated that the content of EGCG was reduced by half and that of gallic acid was approximately doubled after the 2 year conversion processes (**Table 1**). In contrast with the occurrence of three flavonols, myricetin, quercetin, and kaempferol, during the tea conversion, relative contents of almost all flavonol glycosides in oolong tea was subsequently reduced during the conversion processes. Quantita-

Table 1. Peak Area Ratios of Gallic Acid, EGCG, Flavonols, and Flavonol Glycosides to Caffeine in Converting the New Oolong Tea into an Old One

tea constituents	new (%)	1 year old (%)	2 year old (%)
gallic acid	7.51	13.34	16.73
EGCG	53.44	32.43	25.54
Flavonol			
myricetin	ND[a]	1.53	1.70
quercetin	ND[a]	0.46	0.76
kaempferol	ND[a]	0.01	0.23
Flavonol Glycosides			
myricetin-3-*O*-Gal	3.83	2.17	2.59
myricetin-3-*O*-Glu	3.06	2.65	2.57
quercetin-3-*O*-Glu-Rham-Glu	3.90	3.20	2.29
quercetin-3-*O*-Glu-Rham-(p-coumaroyl-Hex)Hex	1.57	1.32	1.30
quercetin-3-*O*-Glu-Rham-(p-coumaroyl)Hex	0.26	ND[a]	ND[a]
kaempferol-3-*O*-Gal-Rham-Glu	1.37	ND[a]	ND[a]
kaempferol-3-*O*-Glu-Rham-Glu	13.44	9.54	4.86
kaempferol-3-*O*-rutinoside	1.71	0.60	0.27
kaempferol-3-*O*-Glu	0.37	0.38	ND[a]
kaempferol-3-*O*-Glu-Rham-(p-coumaroyl-Hex)Hex	1.30	1.34	0.90
kaempferol-3-*O*-Glu-Rham-(p-coumaroyl)Hex	0.34	ND[a]	ND[a]
total flavonol glycosides	31.15	21.2	14.78

[a] Nondetectable.

tive calculation showed that total flavonol glycosides in the oolong tea after 1 and 2 year drying processes dropped by 32 and 52.5%, respectively.

Two distinct characteristics were observed between old and new oolong teas and confirmed by monitoring the constituent alteration in tea during the conversion of a new tea into an old one in this study. One was the massive accumulation of gallic acid in old oolong tea presumably released from EGCG during the drying process, and therefore, significantly lower content of EGCG was observed in old oolong tea compared to the new one. Of course, the accumulation of gallic acid was also possibly released from other gallate-containing compounds, such as gallocatechin-3-*O*-gallate, epicatechin-3-gallate, and prodelphinidin A-2 3′-*O*-gallate. The massive accumulation of gallic acid was in agreement with the decrease of the pH value of tea infusion from 5.5 to 5.0 after conversion by periodic drying and might, at least, partly lead to the slightly sour taste commonly experienced for old oolong tea. The other characteristic was the unique occurrence of three flavonols, myricetin, quercetin, and kaempferol, in old oolong tea. Instead of free forms, these three flavonols were mainly present as their glycoside derivatives in fresh tea leaves and newly prepared oolong tea. These flavonol glycosides seemed to be deglycosylated under the heating condition of periodic drying, and thus their elemental flavonols occurred in old oolong tea.

Gallic acid, a naturally occurring polyphenol antioxidant identified as an excellent free-radical scavenger, is regarded as an important constituent responsible for the health benefits in many food sources, such as fruit, vegetable, red wine, coffee, and tea (*9*). Plant extracts rich in gallic acid were found to possess antidiabetic, antiangiogenic, and antimelanogenic effects and reduced heart infarction incidence and oxidative liver and kidney damage (*10*−*14*). Moreover, gallic acid has also been reported to inhibit growth and induce apoptosis in various cancer cell lines, e.g., chemopreventive efficacy of oral gallic acid against prostate cancer was recently demonstrated by evaluating its inhibition on prostate tumor growth and progression in transgenic adenocarcinoma of the mouse prostate model (*15*). As observed in this study, gallic acid was significantly increased during the conversion of oolong tea into an old one. The empirical consideration of beneficial effects to human health in old oolong tea may be partially attributed to its massive accumulation of gallic acid.

Myricetin, quercetin, and kaempferol are flavonol compounds that belong to a large group of low-molecular-weight polyphenolic compounds termed flavonoids (*16*). Fruits, vegetables, red wine, and tea are especially rich in flavonols and assumed to attribute to certain beneficial health effects of these food sources; flavonols have been demonstrated to exhibit numerous biological and pharmacological effects, including antioxidant, chelation, anticarcinogenic, cardioprotective, bacteriostatic, and secretory properties (*17*−*19*). Comparably, many plant food sources that are also rich in flavonol glycosides were shown to possess certain similar beneficial health effects observed for flavonols, although distinct biological activities were also found between flavonol glycosides and flavonols (*20*). As observed in this study, more than half of flavonol glycosides were degraded and resulted in the occurrence of their fundamental flavonols when a new oolong tea was converted into an old one. It remains to be evaluated for the biological and pharmacological effects of old and new oolong teas in terms of their different contents resulting from the conversion of flavonol glycosides into flavonols during the drying process.

ABBREVIATIONS USED

EGCG, (−)-epigallocatechin gallate; GCG, (−)-gallocatechin-3-*O*-gallate.

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