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Identification and Comparison of Phenolic Compounds in the Preparation of Oolong Tea Manufactured by Semifermentation and Drying Processes

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Oolong tea manufactured via a semifermentation process possesses a taste and color somewhere between green and black teas. Alteration of constituents, particularly phenolic compounds, in the infusion of oolong tea resulting from its manufacture, was analyzed by high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry. The identified constituents contained 2 alkaloids, 11 flavan-3-ols, 8 organic acids and esters, 11 proanthocyanidin dimers, 3 theaflavins, and 22 flavonoid glycosides, including 6 novel acylated flavonol glycosides. The tentative structures of these 6 novel compounds were depicted according to their mass fragmentation patterns in MS^n ($n = 1-4$). In comparison with caffeine as an internal standard, relative contents of the constituents in the infusions of fresh tea shoot and different oolong tea preparations were examined. Approximately, 30% catechins and 20% proanthocyanidins were oxidized during the manufacture of oolong tea from fresh tea shoots, and 20% of total flavonoids were decomposed in a follow-up drying process. Gallocatechin-3-*O*-gallate and theaflavins putatively produced in the semifermentation process of oolong tea were not detected in fresh tea shoots, and the majority of theaflavins were presumably transformed into thearubigins after drying.

KEYWORDS: Catechins; flavonoid; mass spectrometry; oolong tea; theaflavin

INTRODUCTION

Tea is a widely popular beverage consumed in the world for over several thousand years. Various teas as well as their active ingredients, such as catechins and polyphenols, have been demonstrated to possess antioxidant and biological activities (*1*). They are mainly classified into green tea (unfermented), oolong tea (partially fermented), and black tea (fully fermented) according to the degree of fermentation during their preparations, where the term "fermentation" refers to natural browning reactions induced by oxidative enzymes in the cells of tea leaves (*2*). Oolong tea, possessing a taste and color somewhere between green and black teas, is manufactured predominantly in Fujian and Guangdong provinces of China as well as in Taiwan.

In the production of oolong tea, young green shoots (usually the top three leaves of each branch) are freshly harvested in the early morning and allowed to wither under the sunlight for a few hours prior to undergoing the semifermentation process, in which tea leaves are oxidized, pan fired at ~200 °C, rolled to form a ball shape, and then dried in a specialized oven at various desired temperatures. The reaction time for the contact between phenolic compounds and oxidative enzymes is empirically controlled by experts during this semifermentation process, and the final fermentation degree of oolong tea 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 periodical drying. In general, 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. 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. Hence, fermentation (oxidation) and drying are two key steps for the manufacture of oolong tea.

Many kinds of compounds have been identified in various teas, such as flavan-3-ols, flavonoids, gallic acid, quinic esters of caffeic, coumaric acid, theaflavins, thearubigins, and alkaloids (*3*−*12*). Caffeine, an abundant alkaloid found in tea, is resistant to oxidation during the tedious processes of tea production. Therefore, the relative contents of other compounds to caffeine can be regarded as indices of chemical changes in these

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processes for tea preparation. Analyses of these compounds were mainly performed by high-performance liquid chromatography (HPLC) combined with ultraviolet (UV) absorbance or a diode array detector (DAD) (*3*−*5*, *13*−*16*). Recently, mass spectrometry (MS) was also employed to determine tea constituents resolved by HPLC (*8*, *17*−*20*). For example, HPLC coupled to electrospray ionization tandem mass spectrometry (LC/MS/MS) was performed to determine phenolic compounds and purine alkaloids in green and black teas (*8*).

Although oolong tea is getting more and more popular in the world, there is much less investigation on the infusions of different oolong tea preparations in comparison with the vigorous studies on the active ingredients found in green and black teas. In this study, a gradient elution reversed-phase HPLC program was developed for simultaneous separation of 57 constituents in the infusions of fresh tea shoot and oolong tea. The chemical structures of these constituents were identified on the basis of their retention times and mass fragmentation patterns. In comparison with caffeine, used as an internal standard, relative contents of these constituents were calculated to monitor the chemical changes during the semifermentation and drying processes of oolong tea.

MATERIALS AND METHODS

Chemicals and Materials. (+)-Catechin (C), (−)-gallocatechin (GC), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-gallocatechin-3-*O*-gallate (GCG), (−)-epigallocatechin-3-*O*-gallate (EGCG), (−)-epicatechin-3-*O*-gallate (ECG), quinic acid, gallic acid, quercetin-3-*O*-galactoside, 2″-*O*-rhamnosylvitexin, theobromine, and caffeine were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Acetonitrile (HPLC grade) were purchased from Merck KGaA (Darmstadt, Germany). Acetic acid (99.7%) was obtained from TEDIA (Fairfield, OH). Water was purified by a SG-Ultra clear water purification system (SG Water Company, Barsbüttel, Germany). Young green shoots of the tea plant (*Camellia sinensis* L.) and preparations of oolong tea were obtained from Lugu village, Nantou County, Taiwan. To attenuate the enzymatic oxidation of polyphenols in tea leaves, fresh tea shoots were packed in an ice bucket right after harvest. The fresh tea shoot containing approximately 75% water was stored in a freezer at −30 °C prior to analysis. Four oolong tea samples were prepared by the semifermentation process, and three of them were subjected to a follow-up drying process.

Preparation of Tea Infusions. Tea infusions were prepared by adding 18 mL of boiling water to 4 g of fresh tea shoot or 1 g of oolong tea. The weight difference between fresh tea shoot and oolong tea used for tea infusion was a consequence of the fresh tea shoot containing 75% water. After 3 min, the brew was filtered through a 0.22 μm polyvinylidene difluoride (PVDF) membrane filter (Millipore Corporation, Billerica, MA) and used for the following analysis.

LC/UV and LC/MS/MS Analysis. Tea infusions were analyzed on a Surveyor liquid chromatograph system coupled to a Finnigan LCQ ion-trap mass spectrometer (Thermo Finnigan Corporation, San Jose, CA). Separations were performed using a 250 × 4.6 mm i.d., 5 μm, BDS Hypersil C18 column from Thermo Electron Corporation (Waltham, MA). The mobile phase consisted of (A) water containing 0.5% acetic acid and (B) acetonitrile. The program for gradient elution started at 95% solvent A and 5% solvent B, increased linearly to 70% solvent A and 30% solvent B in 100 min. In all experiments, the column was kept at room temperature, the flow rate was 0.5 mL/min, and the injection volume was 5 μL. The UV absorbance detection wavelength was set at 280 nm. The mass spectra were obtained at a mass-to-charge ratio (*m*/*z*) scan range from 120 to 2000. Samples were analyzed in both negative and positive modes for all experiments. The following MS parameters were used for the analysis in full-scan mode: spray voltage, 4.0 kV; capillary temperature, 270 °C; sheath gas flow rate, 80 arbitrary units; auxiliary gas flow rate, 20 arbitrary units. The relative collision energy was 20−30% in collision-induced dissociation (CID) mode except for the instrumental parameters used in the full-scan mode.

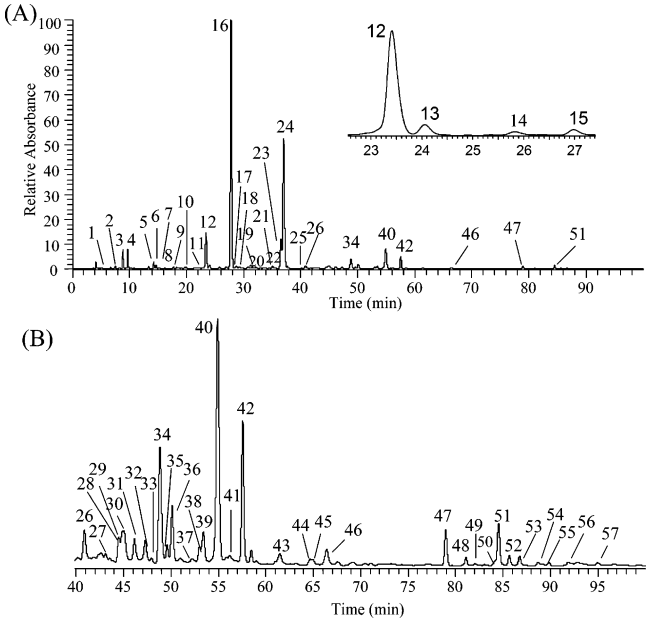


Figure 1. (**A**) HPLC profile of oolong tea infusion. (**B**) Enlargement of the 40–100 min profile at 280 nm.

RESULTS AND DISCUSSION

Profile of Constituents in Tea Infusion. In the LC/MS/MS analyses of constituents in the infusions of fresh tea shoot and oolong tea, the peak area ratio of each compound to caffeine was recorded by the UV detector. The chemical structures of these tea constituents were identified on the basis of their retention times and mass fragmentation patterns. The chromatogram of the tea infusion is shown in **Figure 1**, and the retention times and mass fragmentation data of 57 compounds in the tea infusion are listed in **Table 1**.

Alkaloids. Possessing the same $[M + H]^+$ ions, MS^2 fragment ions and retention times with authentic standards, peaks 5 and 16, were identified as theobromine and caffeine, respectively. The protonated ion at *m*/*z* 195 (caffeine) lost C_2H_3NO to yield the MS^2 fragment ion at *m*/*z* 138.

Flavan-3-ols. Peaks 6, 12, 15, 23, 24, 26, 32, 34, 40, 45, and 46 were determined as catechins belonging to the flavan-3-ol class of flavonoids. Peaks 6, 12, 15, 23, 24, 26, and 40 were identified as gallocatechin, epigallocatechin, catechin, epicatechin, epigallocatechin-3-*O*-gallate, gallocatechin-3-*O*-gallate, and epicatechin-3-*O*-gallate based on the same retention times and mass fragmentation comparion of $[M - H]^-$ ions with authentic standards. The deprotonated ion at *m*/*z* 305 (gallocatechin and epigallocatechin) generated the MS^2 fragment ions at *m*/*z* 261, 221, 219, 179, 167, and 165 in keeping with the loss of one CO_2, $C_4H_4O_2$, $C_4H_6O_2$, $C_6H_6O_3$, $C_7H_6O_3$, and $C_7H_8O_3$, respectively. The loss of $C_4H_4O_2$ and $C_4H_6O_2$ was due to the cleavage of the A ring of flavan-3-ol. The loss of $C_6H_6O_3$ was due to heterocyclic ring fission (HRF) (*21*). The loss of $C_7H_6O_3$ and $C_7H_8O_3$ were through retro-Diels−Alder (RDA) fission. The deprotonated ion at *m*/*z* 289 produced the MS^2 fragment ions at *m*/*z* 245, 205, 203, and 137 corresponding to the loss of one CO_2, $C_4H_4O_2$, $C_4H_6O_2$, and $C_8H_8O_3$, respectively. The loss of $C_4H_4O_2$ and $C_4H_6O_2$ was also due to the cleavage of the A ring of flavan-3-ol. The loss of $C_8H_8O_3$ was through RDA fission. The deprotonated ion at *m*/*z* 457 (epigallocatechin-3-*O*-gallate and gallocatechin-3-*O*-gallate) produced the MS^2 fragment ions at *m*/*z* 305 and 169 corresponding to the deprotonated ion of gallocatechin (or epigallocatechin) and gallic acid, respectively. The deprotonated ion at *m*/*z* 441 (epicatechin-

Table 1. Retention Time and Mass Spectrometric Data of 57 Active Compounds in Oolong Tea Determined by LC/MS/MS

peak	Rt (min)	$[M-H]^-$ (*m*/*z*)	MS/MS (*m*/*z*)	compounds
1	5.19	191		quinic acid
2	7.56	609	471, 333	theasinensin C
3	8.88	343	191, 169	5-galloylquinic acid
4	9.73	169	125	gallic acid
5	14.28	181[a]		theobromine
6	14.66	305	261, 221, 219, 179, 167, 165	gallocatechin
7	15.21	759	607, 589, 451	quinone dimmer
8	16.26	609	483, 441, 423, 305	prodelphinidin B-4
9	17.71	761	609, 591, 453	theasinensin B (or E)
10	19.96	759	741, 607, 589, 427	prodelphinidin A-2 3′-*O*-gallate
11	23.07	761	609, 591, 423, 305	prodelphinidin B-2 (or 4) 3′-*O*-gallate
12	23.41	305	261, 221, 219, 179, 167, 165	epigallocatechin
13	24.06	337	163	3-*p*-coumaroylquinic acid
14	25.82	353	191	5-caffeoylquinic acid
15	26.98	289	245, 205, 203, 137	catechin
16	27.81	195[a]	138	caffeine
17	28.75	633	463, 301	strictinin
18	29.29	577	451, 425, 407, 289	(epi)catechin(epi)catechin
19	31.56	913	743, 573	theasinensin A or D
20	32.15	337	173	4-*p*-coumaroylquinic acid
21	35.11	337	191	5-*p*-coumaroylquinic acid
22	35.57	745	619, 593, 577, 559, 289	epicatechin-epigallocatechin 3-*O*-gallate
23	36.60	289	245, 205, 203, 137	epicatechin
24	37.04	457	305, 169	epigallocatechin-3-*O*-gallate
25	40.16	865	739, 695	epiafzelechin-3-*O*-gallate-epicatechin-3-*O*-gallate
26	40.87	457	305, 169	gallocatechin-3-*O*-gallate
27	43.10	729	577, 559, 441, 407	procyanidin B-2 (or 4) 3′-*O*-gallate
28	44.50	563	545, 503, 473, 443, 383, 353	6-*C*-arabinosyl-8-*C*-glucosyl apigenin
29	44.87	563	545, 503, 473, 443, 383, 353	6-*C*-glucosyl-8-*C*-arabinosyl apigenin
30	45.06	479	317, 316	myricitin-3-*O*-galactoside
31	46.14	479	317, 316	myricitin-3-*O*-glucoside
32	47.28	471	305, 183	epigallocatechin-3-*O*-(4-*O*-methyl) gallate
33	47.93	771	609, 463, 301	quercetin-3-*O*-galactosyl-rhamnosyl-glucoside
34	48.85	471	305, 183	epigallocatechin-3-*O*-(3-*O*-methyl) gallate
35	49.60	593	413, 293	4″-*O*-glucosylvitexin
36	50.13	771	609, 463, 301	quercetin-3-*O*-glucosyl-rhamnosyl-glucoside
37	52.24	577	413, 293	rhamnosylvitexin
38	52.98	577	457, 413, 293	2″-*O*-rhamnosylvitexin
39	53.43	755	593, 447, 285	kaempferol-3-*O*-galactosyl-rhamnosyl-glucoside
40	54.92	441	289, 169	epicatechin-3-*O*-gallate
41	56.21	463	301	quercetin-3-*O*-galactoside
42	57.56	755	593, 447, 285	kaempferol-3-*O*-glucosyl-rhamnosyl-glucoside
43	61.50	593	447, 285	kaempferol-3-*O*-rutinoside
44	64.69	447	285	kaempferol-3-*O*-glucoside
45	64.94	455	289, 183	epicatechin-3-*O*-(4-*O*-methyl) gallate
46	66.42	455	289, 183	epicatechin-3-*O*-(3-*O*-methyl) gallate
47	78.98	1079	933, 915, 301	quercetin-3-*O*-glucosyl-rhamnosyl-(*p*-coumaroyl-hexosyl)hexoside
48	81.10	1079	933, 915, 301	
49	82.06	917	771, 753, 301	quercetin-3-*O*-glucosyl-rhamnosyl-(*p*-coumaroyl)hexoside
50	83.90	917	771, 753, 301	
51	84.55	1063	917, 899, 777, 285	kaempferol-3-*O*-glucosyl-rhamnosyl-(*p*-coumaroyl-hexosyl)hexoside
52	85.69	1063	917, 899, 777, 285	
53	86.76	563	545, 527, 519, 501, 407, 379	theaflavin
54	88.68	901	755, 737, 285	kaempferol-3-*O*-glucosyl-rhamnosyl-(*p*-coumaroyl)hexoside
55	89.84	901	755, 737, 285	
56	91.88	715	563, 545, 527, 501, 407	theaflavin-3-*O*-gallate
57	94.95	715	563, 545, 527, 501, 407	theaflavin-3′-*O*-gallate

[a] $[M+H]^+$.

3-*O*-gallate) produced the MS^2 fragment ions at *m*/*z* 289 and 169 corresponding to the deprotonated ion of catechin (or epicatechin) and gallic acid, respectively.

Peaks 32 and 34 had the same $[M-H]^-$ at *m*/*z* 471. The product ions of *m*/*z* 471 ion were *m*/*z* 305 and 183, in keeping with the cleavage of epigallocatechin (or gallocatechin) and methylgalloyl moiety. Epigallocatechin-3-*O*-methylgallates have been found in oolong tea (*22*). The content of epigallocatechin-3-*O*-(3-*O*-methyl) gallate was higher than that of epigallocatechin-3-*O*-(4-*O*-methyl) gallate in oolong tea. Putatively, compounds **32** and **34** were epigallocatechin-3-*O*-(4-*O*-methyl) gallate and epigallocatechin-3-*O*-(3-*O*-methyl) gallate, respectively.

Peaks 45 and 46 had the same $[M-H]^-$ at *m*/*z* 455. The product ions of the *m*/*z* 455 ion were *m*/*z* 289 and 183, in keeping with the cleavage of epicatechin (or catechin) and methylgalloyl moiety. In accordance with molecular weight and mass fragmentation characterization, the *m*/*z* 455 ion was identified as epicatechin-3-*O*-methylgallate, a known component of oolong tea (*10*). The ion at *m*/*z* 471 (compounds **32** and **34**)

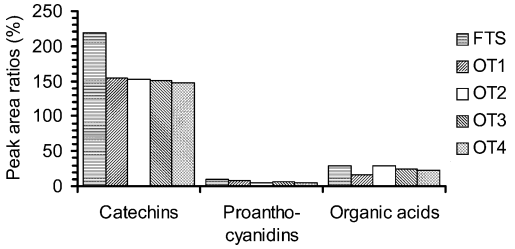
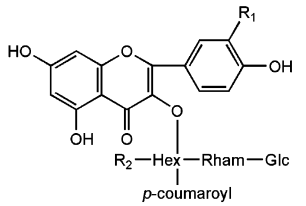


Figure 2. Peak area ratios of catechins, proanthocyanidins, and organic acids to caffeine in tea infusions. FTS, fresh tea shoot; OT1, oolong tea without drying; OT2–OT4, oolong tea with drying.



47, 48 R_1=OH, R_2=Hex; MS^2 ion: 301
49, 50 R_1=OH, R_2=H; MS^2 ion: 301
51, 52 R_1=H, R_2=Hex; MS^2 ion: 285
54, 55 R_1=H, R_2=H; MS^2 ion: 285

Figure 3. Structures of eight acylated flavonol glycosides.

was 16 Da more than the ion at *m*/*z* 455, and the fragment ion at *m*/*z* 305 corresponding to epigallocatechin was 16 Da more than *m*/*z* 289. The ions at *m*/*z* 471 and 455 had the same fragment at *m*/*z* 183. Consequently, compounds **45** and **46** were speculated as epicatechin-3-*O*-(4-*O*-methyl) gallate, a known component in oolong tea (*10*), and epicatechin-3-*O*-(3-*O*-methyl) gallate, an unknown component in tea, in accordance with structures and retention times of compounds **32** and **34**.

The combined ratio of the 11 flavan-3-ols to caffeine in the infusion of fresh tea shoot was 220% but around 150% in that of oolong teas; roughly, 30% of the catechins were oxidized in the semifermentation process of oolong tea preparation (**Figure 2**). Gallocatechin-3-*O*-gallate was not found in fresh tea shoot, but the peak area ratio of gallocatechin-3-*O*-gallate to caffeine was 1.4−1.8% in oolong teas. Possibly, gallocatechin-3-*O*-gallate was originated from the degradation of proanthocyanidins during the tea preparation.

Flavonoids. Peaks 28, 29, 30, 31, 33, 35−39, 41−44, 47−52, 54, and 55 were determined as flavonoids. According to the fragmentation characterization of $[M - H]^-$ ions and the retention times (*8*, *16*), peaks 41, 43, and 44 were identified as quercetin-3-*O*-galactoside, kaempferol-3-*O*-rutinoside, and kaempferol-3-*O*-glucoside, while peaks 54 and 55 were isomers with different linking sites among sugars and speculated as kaempferol-3-*O*-glucosyl-rhamnosyl-(*p*-coumaroyl)hexosides (tentative) (**Figure 3**).

Peaks 28 and 29 had the same $[M - H]^-$ at *m*/*z* 563 and MS^2 ions at *m*/*z* 545, 503, 473, 443, 383, and 353. In accordance with the deprotonated ions of the two compounds as well as mass fragmentation characterization and retention times, they were assigned as 6-*C*-arabinosyl-8-*C*-glucosyl apigenin and 6-*C*-glucosyl-8-*C*-arabinosyl apigenin (*23*), which have not been reported in tea.

Peaks 30 and 31 had the same $[M - H]^-$ at *m*/*z* 479 and MS^2 ions at *m*/*z* 317 and 316 corresponding to the loss of 162 and 163 Da. The loss of 162 and 163 Da was in keeping with the cleavage of a hexosyl group. Hence, they were assigned as myricitin-3-*O*-galactoside and myricitrin-3-*O*-glucoside (isomyricitrin), two known components in black tea (*6*). Myricitin-3-galactoside was eluted earlier than myricitin-3-*O*-glucoside in the reversed-phase HPLC system.

Peaks 33 and 36 had the same $[M - H]^-$ at *m*/*z* 771. The *m*/*z* 771 ion generated MS^2 fragment ions at *m*/*z* 609, 463, and 301 corresponding to the loss of a 162 Da fragment, a 308 Da fragment, and a 470 Da fragment. The loss of 162, 308, and 470 Da was in keeping with the cleavage of a hexosyl residue, a hexosyl-rhamnosyl group, and a hexosyl-rhamnosyl-hexosyl group, respectively. The fragment ion at *m*/*z* 301 corresponded to the aglycone quercetin. Quercetin and its glycosides were universally present in tea. The two compounds were identified as quercetin-3-*O*-galactosyl-rhamnosylglucoside and quercetin-3-*O*-glucosyl-rhamnosylglucoside based on the description of Price et al. (*6*).

Peaks 35, 37, and 38 were identified as a group that produced the same MS^2 fragment ions at *m*/*z* 413 and 293 in the negative-ion mode. Peaks 37 and 38 also had the deprotonated ion at *m*/*z* 577 in mass spectrum; hence, peaks 37 and 38 were isomers. However, peak 38 had a fragment ion at *m*/*z* 457 besides the same fragment ions at *m*/*z* 413 and 293 as peak 37 in the MS^2 spectrum. The mass fragmentation data and retention time of peak 38 was in accordance with 2″-*O*-rhamnosylvitexin, namely, 2″-*O*-rhamnosyl-8-*C*-glucosylapigenin (*24*). The ion at *m*/*z* 457 corresponded to the neutral loss of 120 Da, which was generated by cross-ring cleavage at the 0,2 two bonds of a glucosyl group. The analysis suggested that a (1−2) linking between rhamnosyl and glucosyl groups was present in the structure of peak 38. The ions at *m*/*z* 413 and 293 were in keeping with the cleavage of a rhamnose (164 Da) and a rhamnose-glucosyl residue (164 plus 120 Da) from the precursor ion, respectively. The ion at *m*/*z* 293 produced MS^3 fragment ions at *m*/*z* 275, 265, 249, and 175, in keeping with the cleavage of a H_2O, CO, CO_2, and C_8H_6O, respectively. Peak 37 was identified as rhamnosylvitexin. A rhamnosyl group linked with vitexin (8-*C*-glucosylapigenin) at the C3, C4, or C6 position of the glucosyl group. Peak 35 generated the deprotonated ion at *m*/*z* 593 and MS^2 fragment ions at *m*/*z* 413 and 293 corresponding to the loss of a 180 Da fragment (a glucose) and a 300 Da fragment (a glucose-glucosyl residue, 180 plus 120 Da). Peak 35 had two glucosyl groups but no 120 Da of neutral loss in the MS^2 spectrum, suggesting no (1−2) linking. Although the linking between two glucosyl groups could be (1−3), (1−4), or (1−6), peak 35 was speculated to be 4″-*O*-glucosylvitexin, which is widely present in plants (*25*). Compounds **35**, **37**, and **38** have never been reported in tea.

Peaks 39 and 42 generated the same $[M - H]^-$ at *m*/*z* 755. The *m*/*z* 755 ion produced MS^2 fragment ions at *m*/*z* 593, 447, and 285 corresponding to the loss of a 162 Da fragment, a 308 Da fragment, and a 470 Da fragment. The loss of 162, 308, and 470 Da was in keeping with the cleavage of a hexosyl residue, a hexosyl-rhamnosyl group, and a hexosyl-rhamnosyl-hexosyl group, respectively. The fragment ion at *m*/*z* 285 corresponded to aglycone kaempferol. Kaempferol and its glycosides were universally present in tea. The two compounds were identified as kaempferol-3-*O*-galactosyl-rhamnosylglucoside and kaempferol-3-*O*-glucosyl-rhamnosylglucoside based on the description of Price et al. (*6*).

Peaks 47 and 48 produced $[M - H]^-$ at *m*/*z* 1079 in the negative-ion mode and MS^2 fragment ions at *m*/*z* 933, 915, and 301 corresponding to the loss of a 146 Da fragment, a 164 Da fragment, and a 778 Da fragment. The fragment ion at *m*/*z* 301 corresponded to the aglycone quercetin. According to its molecular weight, the neutral loss of 778 Da corresponded to a pentosaccharide with two rhamnosyl and three hexosyl moieties

and one quercetin sugar conjugate with a pentosaccharide should shorten the retention time of its conjugated compound in the reversed-phase HPLC system because of the increase of its polarity. The product-ion fragment of the loss of 164 Da was also in the MS^2 spectrum. The loss of 146 and 164 Da was in keeping with the cleavage of a *p*-coumaroyl group and a *p*-coumaric acid (*26*−*29*). In the MS^3 spectrum of *m*/*z* 1079, the ion at *m*/*z* 933 had three main fragment ions at *m*/*z* 771, 753, and 301 corresponding to the loss of a 162 Da fragment, a 180 Da fragment, and a 632 Da fragment. The loss of 162 Da was in keeping with the cleavage of a hexosyl group, and the 180 Da fragment was speculated to result from the cleavage of one hexose. The fragment ion at *m*/*z* 771 was similar to the deprotonated ion of compound **33** (*6*), which produced fragment ions at *m*/*z* 609 and 301 in the MS^4 spectrum. Therefore, the two unknown compounds **47** and **48** were isomers with different linking sites among sugars and speculated as quercetin-3-*O*-glucosyl-rhamnosyl-(*p*-coumaroyl-hexosyl)hexosides (tentative) (**Figure 3**), according to the structure of a known acylated flavonol tetraglycoside (*26*).

Peaks 49 and 50 generated $[M - H]^-$ at *m*/*z* 917 in the negative-ion mode and MS^2 fragment ions at *m*/*z* 771, 753, and 301 corresponding to the loss of a 146 Da fragment, a 164 Da fragment, and a 616 Da fragment. The fragment ion at *m*/*z* 301 corresponded to the aglycone quercetin. Compounds **49** and **50** produced the same neutral loss of 146 and 164 Da as compound **47** and had longer retention times. Therefore, they also had a *p*-coumaroyl group. The fragment ion at *m*/*z* 771 was similar to the deprotonated ion of compound **33** (*6*), which produced fragment ions at *m*/*z* 609 and 301 in the MS^4 spectrum. Thus, the two unknown compounds **49** and **50** were isomers with different linking sites among sugars and speculated as quercetin-3-*O*-glucosyl-rhamnosyl-(*p*-coumaroyl)hexosides (tentative) (**Figure 3**).

Peaks 51 and 52 produced $[M - H]^-$ at *m*/*z* 1063 in the negative-ion mode, and the MS^2 spectrum of *m*/*z* 1063 displayed fragment ions at *m*/*z* 917, 899, 777, and 285 corresponding to the loss of a 146 Da fragment, a 164 Da fragment, a 286 Da fragment, and a 778 Da fragment. The fragment ion at *m*/*z* 285 corresponded to aglycone kaempferol. Compounds **51** and **52** produced the same neutral loss of 146, 164, and 778 Da as compound **47** and might have analogous stuctures. In the MS^3 spectrum of *m*/*z* 1063, the ion at *m*/*z* 917 had four main fragment ions at *m*/*z* 755, 737, 631, and 285. The fragment ion at *m*/*z* 755 was similar to the deprotonated ion of compound **39** (*6*), which produced fragment ions at *m*/*z* 593 and 285 in the MS^4 spectrum. Therefore, the two unknown compounds **51** and **52** were isomers with different linking sites among sugars and speculated as kaempferol-3-*O*-glucosyl-rhamnosyl-(*p*-coumaroyl-hexosyl)hexosides (tentative) (**Figure 3**).

A total of 22 flavonoid glycosides were detected in the five tea samples. The parent flavonoids were apigenin, myricetin, quercetin, and kaempferol, which were conjugated to a range of sugars, including glucose, galactose, and rhamnose, as mono-, di-, tri, and tetrasaccharides. Using caffeine as an internal standard, relative contents of these four major flavonoid classes as well as their sum in the tea samples were shown in **Figure 4**. Apigenin and myricetin glycosides were detected in all samples, and their combined proportion varied from 2 to 3% of caffeine. Quercetin and kaempferol glycosides were significant components possessing 3−5 and 7−9% of caffeine in these tea samples, respectively. Analyzing the proportions of these four flavonoid glycosides in tea samples indicated that no significant conversion occurred among these flavonoid glycosides in the semifermentation process, while the total flavonoid glycosides decreased by 20−25% after drying. Consequently, the drying process might lead to the decomposition of these four flavonoid glycosides.

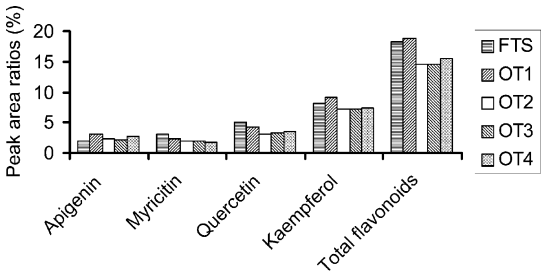


Figure 4. Peak area ratios of apigenin, myricetin, quercetin, and kaempferol glycosides to caffeine in tea infusions. FTS, fresh tea shoot; OT1, oolong tea without drying; OT2–OT4, oolong tea with drying.

Table 2. Peak Area Ratios of Organic Acids and Their Esters to Caffeine in Tea Samples Determined by LC/UV

peak	compounds	young tea shoots	oolong teas [a]			
			OT1	OT2	OT3	OT4
1	quinic acid	1.5	0.5	0.6	0.6	0.4
3	5-galloylquinic acid	17.5	5.5	16.2	14.3	12.3
4	gallic acid	3.9	4.7	5.1	3.4	4.7
13	3-*p*-coumaroylquinic acid	1.2	1.6	2.0	1.9	1.8
14	5-caffeoylquinic acid	0.9	0.5	0.5	0.4	0.4
17	strictinin	1.0	1.5	2.2	2.0	1.9
20	4-*p*-coumaroylquinic acid	1.2	0.8	0.2	0.3	0.2
21	5-*p*-coumaroylquinic acid	1.9	1.0	1.8	1.5	1.5

[a] OT1, oolong tea sample without drying; OT2–OT4, oolong tea with drying.

Organic Acids and Their Esters. Peaks 1, 3, 4, 13, 14, 17, 20, and 21 were determined to be organic acids and their esters. According to their fragmentation characterization of $[M - H]^-$ ions and retention times (*8*, *16*), peaks 1, 3, and 4 were identified as quinic acid, 5-galloylquinic acid (theogallin), and gallic acid, respectively. The $[M - H]^-$ ion of 5-galloylquinic acid produced MS^2 fragment ions at *m*/*z* 191 and 169 corresponding to the deprotonated ions of quinic acid and gallic acid. The $[M - H]^-$ ion of gallic acid produced a MS^2 fragment ion at *m*/*z* 125 corresponding to the loss of one CO_2. Peaks 13, 14, 20, and 21 were identified as 3-*p*-coumaroylquinic acid, 5-caffeoylquinic acid, 4-*p*-coumaroylquinic acid, and 5-*p*-coumaroylquinic acid in accordance with the description of Clifford et al. (*30*).

Peak 17 had a $[M - H]^-$ at *m*/*z* 633 and MS^2 ions at *m*/*z* 463 and 301 corresponding to the cleavage of gallic acid (170 Da) and gallic acid plus glucosyl residue (170 plus 162 Da). Hence, the compound was identified as strictinin, a known component in green tea (*31*).

5-Galloylquinic acid in oolong tea without drying (OT1) was about 3-fold less than that in oolong teas with drying (OT2−OT4) or fresh tea shoots (**Table 2**). The proportion of gallic acid varied subtly before and after fermentation. The proportion of 5-caffeoylquinic acid was reduced to ∼50% after fermentation. The proportions of 3-, 4-, and 5-*p*-coumaroylquinic acid were similar in fresh tea shoots (1.2, 1.2, and 1.9%, respectively) but changed variably after fermentation. The proportion of 3-*p*-coumaroylquinic acid increased, but the proportion of 4-*p*-coumaroylquinic acid decreased after fermentation. Interestingly, the proportion of 5-*p*-coumaroylquinic acid decreased after fermentation but increased after the drying process. The relative content of strictinin to caffeine increased after fermentation. The proportion of total organic acids and their esters in the five tea

samples is shown in **Figure 2**; no significant alteration was observed before and after semifermentation if the fluctuation of 5-galloylquinic acid was excluded.

Proanthocyanidin Dimers. The heterocyclic ring fragment pathways of the flavan-3-ols are through quinone-methide (QM), RDA, and HRF (*21*). Usually, flavan-3-ol gallates would also lose gallic acid (170 Da) or a galloyl group (152 Da) in mass spectra. For example, the protonated ion at *m*/*z* 609 (peak 2) produced MS^2 fragment ions at *m*/*z* 471 through RDA and *m*/*z* 333 through two RDAs. The protonated ion at *m*/*z* 609 (peak 8) produced MS^2 fragment ions at *m*/*z* 483 through HRF, *m*/*z* 441 through RDA, *m*/*z* 423 through RDA following the loss of one H_2O, and *m*/*z* 305 through QM. The protonated ion at *m*/*z* 761 (peak 9) produced MS^2 fragment ions at *m*/*z* 609 through the loss of one galloyl group, *m*/*z* 591 through the loss of one gallic acid, and *m*/*z* 453 through the loss of one gallic acid following RDA cleavage. The protonated ion at *m*/*z* 761 (peak 11) produced MS^2 fragment ions at *m*/*z* 609 through the loss of one galloyl group, *m*/*z* 591 through the loss of one gallic acid, *m*/*z* 423 through the loss of one gallic acid following RDA cleavage, and *m*/*z* 423 through the loss of one galloyl group following QM. Hence, peaks 2, 8−11, 19, 22, 25, and 27 were assigned as proanthocyanidin dimers, known components in tea (*9*, *11*, *12*, *32*).

Peak 7 generated the $[M - H]^-$ ion at *m*/*z* 759, which was 2 Da less than peak 9 (theasinensin B). Its product ions were also 2 Da less than those of theasinensin B, respectively. Hence, the structure of compound **7** was similar to theasinensin B, and it was probably a quinone dimer, an intermediate during tea oxidation (*33*).

Peak 18 generated the $[M - H]^-$ ion at *m*/*z* 577, which yielded product ions at *m*/*z* 451 through HRF, *m*/*z* 425 through RDA, *m*/*z* 407 through RDA following the loss of one H_2O, and *m*/*z* 289 through QM, in keeping with the description of Gu et al. (*21*). Hence, it was identified as (epi)catechin → (epi)-catechin.

In total, 11 proanthocyanidin dimers were confirmed. The total proanthocyanidin content was highest in fresh tea shoot, and it decreased by ∼20% after fermentation and diminished further by ∼15% after the drying process (**Figure 2**).

Theaflavins. Peaks 53, 56, and 57 were identified as theaflavins. Peak 53 produced $[M - H]^-$ at *m*/*z* 563 and was identified as theaflavin, a known component in oolong tea (*12*). The MS^2 fragment ions of *m*/*z* 563 were at *m*/*z* 545, 527, 519, 501, 407, and 379 corresponding to the loss of a 18 Da fragment, a 36 Da fragment, a 44 Da fragment, a 62 Da fragment, a 156 Da fragment, and a 184 Da fragment. The loss of 18, 36, 44, and 62 Da was in keeping with the cleavage of one H_2O, two H_2O units, one CO_2, and one CH_2O_3 (CO_2 plus H_2O). The fragment of 156 Da was the loss of a H_2O and a 138 Da fragment (RDA cleavage). The fragment of 184 Da was the loss of a H_2O, a CO, and a 138 Da fragment (RDA cleavage). Peaks 56 and 57 produced $[M - H]^-$ at *m*/*z* 715 and were identified as theaflavin-3-*O*-gallate and theaflavin-3′-*O*-gallate based on their relative retention times similar to two known components in oolong tea (*8*, *12*). The MS^2 fragment ions of *m*/*z* 715 were at *m*/*z* 563, 545, 527, 501, and 407 corresponding to the loss of a 152 Da fragment, a 170 Da fragment, a 188 Da fragment, a 214 Da fragment, and a 308 Da fragment. The loss of 152 Da corresponded to the cleavage of a galloyl group. The loss of 170 and 188 Da was in keeping with the cleavage of one H_2O and two H_2O groups from *m*/*z* 563. The fragment ions of *m*/*z* 715 at *m*/*z* 545, 527, 501, and 407 were the same as product ions of *m*/*z* 563.

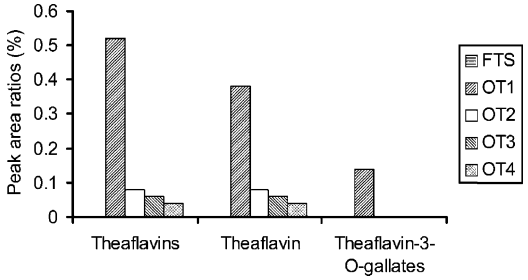


Figure 5. Peak area ratios of theaflavins to caffeine in tea infusions. The amount of theaflavins represents the sum of theaflavin and theaflavin-3-*O*-gallates. FTS, fresh tea shoot; OT1, oolong tea without drying; OT2–OT4, oolong tea with drying.

Fresh tea shoot and oolong tea contained theaflavin, theaflavin-3-*O*-gallate, and theaflavin-3′-*O*-gallate, but the ratios of these three theaflavins to caffeine were very low, ranging from 0 to 0.5% (**Figure 5**). In the semifermentation process, part of catechins might be oxidized and polymerized to form theaflavins. However, theaflavins decreased gradually by ∼90% during the drying process of oolong tea; particularly, theaflavin-3-*O*-gallates were not detected in oolong teas after drying (OT2−OT4). It was known that theaflavins contributed importantly to the properties of black tea, such as color and taste, but the properties of theaflavins in oolong tea were dissimilar according to the ratios of theaflavins to caffeine. While the amount of theaflavins was very low, flavan-3-ols and proanthocyanidins decreased by 30 and 20% after fermentation, respectively. Therefore, thearubigins might be transformed from flavonol-3-ols by enzymatic browning in addition to the theaflavin pathway described previously (*2*).

In conclusion, HPLC combined with UV and mass detection was suitable for the reliable identification and quantification of constituents in the infusion of tea. In this study, alteration of constituents in the infusion of oolong tea that resulted from its manufacture was analyzed, including 2 alkaloids, 11 flavan-3-ols, 8 organic acids and esters, 11 proanthocyanidin dimers, 3 theaflavins, and 22 flavonoid glycosides. As demonstrated by the structural determination of 6 novel acylated flavonol glycosides, LC/MS/MS seemed to be especially useful in speculating structures of trace amounts of natural compounds when standard compounds were not available. In addition, 5 apigenin glycosides were found in tea for the first time. The amount of theaflavins in oolong tea was very low and mostly transformed into thearubigins after drying. Our analyses showed that both the semifermentation and drying processes altered relative contents of many constituents in oolong tea.

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