European Food Research and Technology (2019) 245:997–1010
https://doi.org/10.1007/s00217-018-3201-6

ORIGINAL PAPER

Characterization of catechins, theaflavins, and flavonols by leaf processing step in green and black teas (*Camellia sinensis*) using UPLC-DAD-QToF/MS

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Received: 3 September 2018 / Revised: 5 November 2018 / Accepted: 10 November 2018 / Published online: 23 November 2018

Abstract

Most previous studies have been focused on the variation of tea chemical composition by fermentative processes as well as different cultivars and regions. The detailed changes of flavonoid profiles were described for the first time by each processing step of green and black tea leaves in this study. A total of 24 flavonoid derivatives including catechins, theaflavins, and flavonols were separated and identified from the tea samples based on UPLC-DAD-QToF/MS data and constructed library. Among these, the fragmentation pathway of theaflavins was proposed specifically in positive ionization mode for structural interpretation. During leaf processing, the individual flavonols were changed as diverse patterns according to their aglycone types and glycosylated forms, but their total content showed a slight difference. EGCG and ECG were increased after roasting approximately twofold higher than that of fresh leaves (EGCG, 2709.5 →6085.6; ECG, 1548.0 →2318.2 mg/100 g dry weight, respectively) in green tea while considerably decreased their contents due to oxidation and conversion to theaflavins after fermentation during black tea processing. Especially, the drying steps also found to be factor to influence positively to increase the flavonoid contents in both tea processing. Therefore, this result indicated that detailed conditions of each processing step played important roles in changing the flavonoid profiles from tea leaves.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00217-018-3201-6) contains supplementary material, which is available to authorized users.

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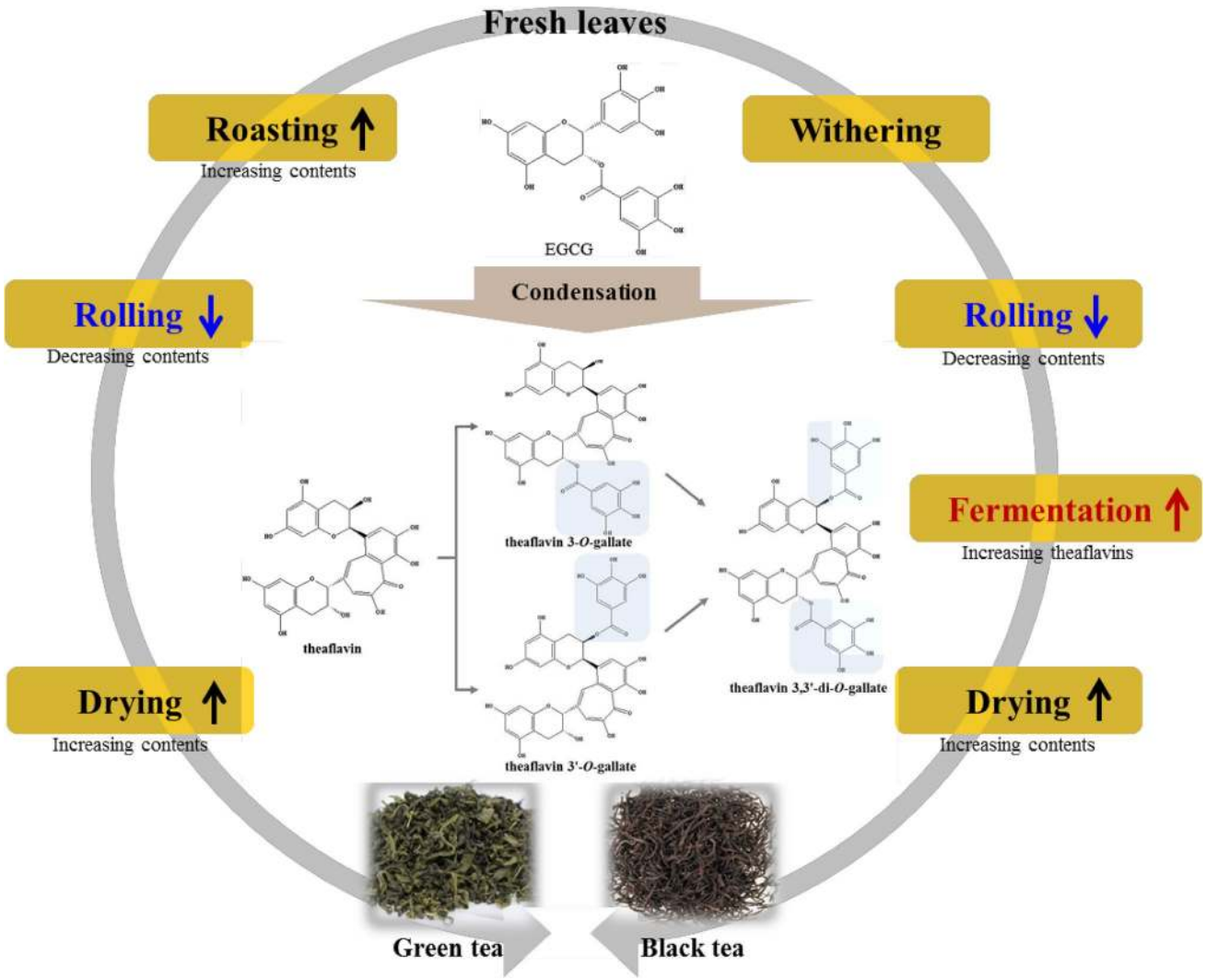
Graphical abstract



Keywords *Camellia sinensis* · Flavonoids · Processing steps · UPLC-DAD-QToF/MS

Introduction

Green tea (*Camellia sinensis*) is one of the most widely consumed beverages in mainly Asia and Europe. Health-promoting compounds such as catechins, theaflavins, thearubigins, flavonols, caffeine, and other phenolic compounds are predominantly distributed in the leaves of green and black (fermented) tea [1–4]. Catechin derivatives are belonging to flavan-3-ols and known to be abundant in tea products [5]. Especially, theaflavins are the responsible compounds to determine the color and taste of black tea infusions as conjugates of epicatechin (EC), epigallocatechin (EGC), and gallic acid by enzymatic oxidation reaction in fermentations [6].

Biological activities have been evaluated variously from tea products for potential health benefit. Epicatechin 3-*O*-gallate (ECG) and epigallocatechin 3-*O*-gallate (EGCG) were reported as the major compounds with strong radical-scavenging activities [7]. In addition, myricetin 3-*O*-galactoside and myricetin 3-*O*-glucoside showed potent antioxidant activities [8]. In tea consumption, dietary polyphenols played an important role in the preventive aspects of human diseases such as Alzheimer's, cardiovascular disease, diabetes mellitus, and obesity [9–12], and revealed remarkable activity as anti-carcinogenic agent [13–15].

Most polyphenolic studies have performed as qualitative and quantitative analysis using liquid chromatography–mass spectrometric (LC–MS) technologies. In addition,

polyphenolic composition was focused on observation at the level of fermentation from green, oolong, black, and white teas [4, 16]. A total of 40 flavonoids were tentatively identified including flavonol glycosides with glucose, galactose, rhamnose, and rutinose from green and black teas using a triple quadrupole MS [17]. Similarly, Lin et al. [18] determined a total of 63 flavonoids from various fermented tea samples, but other compounds except for two acylated flavonol glycosides (kaempferol 3-*O*-6″-*p*-coumaroylglucoside and kaempferol 3-*O*-2″,6″-di-*p*-coumaroylglucoside) were still unclear in acylated positions.

The phytochemical composition of tea leaves is affected by several factors such as cultivated regions, climatic conditions, varieties, brewing techniques, and processing conditions. The profiles of catechin derivatives including EC, EGC and EGCG were differentiated by geographical origins and climatic conditions in green teas [19, 20]. In brewing time of green teas, the content of EC, EGC and EGCG were no longer increased after 4 to 5 minutes [21]. Furthermore, Peterson et al. [22] suggested optimal brewing techniques (tea weight and brewing time) as well as varietal differences for determining the contents of catechins, theaflavins, and thearubigins in black teas. In leaf processing step, a slight increase of total catechins and polyphenols was observed during withering stage of Assam (*C. sinensis* var. *assamica*) green and black teas [23]. During black tea preparation, the conversion of phenolic compounds was derived by different fermentation and drying stages with much higher loss of catechins [24]. Far-infrared radiation (FIR) replacing the roasting step might also enhance total catechins and phenols [25]. These results indicated that processing methods might be responsible for tea quality related with contents of catechin derivatives.

Despite conducting qualitative and quantitative analysis of phenolic compounds on tea leaves and products, the accurate determination of flavonoid derivatives at each step of tea leaf processing remains unstudied. In this study, a comprehensive flavonoid library was constructed from the literature data, and was used for the identification of individual flavonoid component with UPLC-DAD-QToF/MS analysis. Therefore, a quantitative change was evaluated at each steps of leaf processing in green and black teas based on flavonoid profiles. Furthermore, it will be valuable to provide chemical information for optimal processing conditions to produce high quality teas.

Materials and methods

Chemical reagents

A galangin was used as an internal standard (Extrasynthese, Genay, France). Acetonitrile, methanol (MeOH), and purified water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid was obtained from the Junsei Chemical Co. Ltd. (Tokyo, Japan).

Plant materials

Fresh tea leaves (variety Chamnok) were harvested from same age of trees (fifteen years old) in May 2014 for green tea and September 2014 for black tea. These tea trees were cultivated from Tea Industry Institute at Boseong-gun, Jeollanamdo, Republic of Korea (latitude, 34°46′N; longitude, 127°46′E) by organic farming system.

Leaf processing methods of green and black teas

The scheme of leaf processing steps is presented in Fig. 1. For preparing green tea, the fresh leaves were taken and roasted at 250–300 °C for 10 min (roasting) to inactivate enzymatic activities and followed by rolling of roasted leaves meanwhile allowing it to cool for 10 min (rolling). To generate the flavor, the rolled leaves were further dried on oven: first at 150–200 °C for 10 min (first drying), then at 100–150 °C for 10 min (second drying), and finally at 90–100 °C for 10 min (third drying) under hot air. For preparing black tea, the fresh leaves were withered for 24 h at room temperature to reduce the moisture content of leaves below 60% (withering). The leaves were rolled for 30 min (rolling) and then allowed to ferment naturally at room temperature for 3 h (fermentation). After fermentation, two steps of drying were conducted; at 110 °C for 20 min

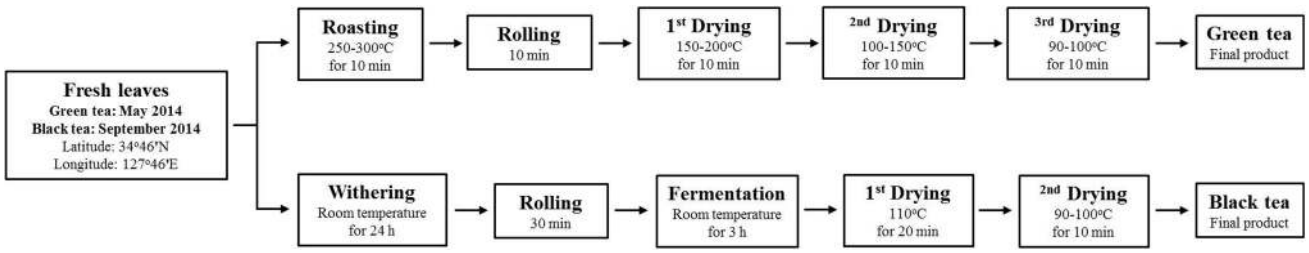


Fig. 1 Scheme for each processing condition in green and black tea (*Camellia sinensis*) leaves

and 90–100 °C for 10 min (first drying and second drying), respectively. After each processing stage completed, all samples were lyophilized (Programmable Freeze Dryer, Ilshin Lab Co. Ltd., Republic of Korea), pulverized, and stored below − 60 °C.

Extraction of catechins, theaflavins, and flavonols

Extraction procedures of flavonoids were conducted according to the method described by Kim et al. [26] with slight modification. The powdered samples (0.2 g) were shaken for 5 min with 40 mL of MeOH:water:formic acid (50:45:5, v/v/v) containing 20 µg/mL of internal standard (galangin). After centrifugation (3000 rpm, 4 °C, 15 min), the supernatant was immediately filtered using a syringe filter (PVDF 0.20 µm, Whatman, Kent, England), and then 0.5 mL of the filtrate was diluted with water to 5 mL of final volume. The extract was semi-purified using the Sep-pak C_{18} classic cartridge (Waters Co., Milford, MA, USA). The cartridge was activated by 2 mL of MeOH, followed by 2 mL of water for conditioning. The diluted extract was loaded into the cartridge, and impurities were removed by washing with 2 mL of water. The crude flavonoid extract was finally eluted by 3 mL of MeOH and concentrated using N_2 gas, and then redissolved with 0.5 mL of MeOH:water:formic acid (50:45:5, v/v/v) prior to UPLC-DAD-QToF/MS analysis.

UPLC-DAD-QToF/MS analysis

The individual flavonoid components were analyzed using an ultra-performance liquid chromatography-diode array detector (Waters Co., Milford, MA, USA) and quadrupole time of flight mass spectrometry (Waters Micromass, Manchester, UK) (UPLC-DAD-QToF/MS) equipped with Kinetex 1.7 µm XB C_{18} 100A column (150 × 2.1 mm i.d., Phenomenex, Torrance, CA, USA). According to our previous report [27], chromatographic condition was conducted: flow rate (0.3 mL/min), column oven temperature (30 °C), and representative wavelengths (280 nm for catechins and theaflavins; 350 nm for flavonols). The mobile phase was 0.5% formic acid in water (A) and 0.5% formic acid in acetonitrile (B). Elution gradient used as follows: initial 5% B; 20 min, 25% B; 25 min, 50% B; 30–32 min, 90%, and 35–40 min, 5% B. The mass spectrometric settings used was: capillary voltage 3.5 kV, sampling cone voltage 40 V, source temperature 120 °C, desolvation temperature 500 °C, and desolvation gas 1050 L/h. Mass analysis was run in positive ionization mode using an electrospray ionization (ESI) source, and their range measured at 200–1200 *m/z* in full-scan mode. The quantification was performed using an internal standard without considering relative response factor. All experimental analyses were conducted in triplicates.

Construction of LC–MS library for flavonoid identification

The flavonoid library was constructed from 21 literature sources related to green and black tea leaves based on structural evidences elucidated by NMR spectroscopy and mass spectrometry, and contained positive- and negative-ion fragmentations (Table S1).

Statistical analysis

To compare between treatments, a significant difference was verified by one-way ANOVA with Duncan's multiple range test ($p < 0.05$) in SPSS (version 24.0, SPSS Inc., Chicago, IL).

Results and discussion

Identification of catechins, theaflavins, and flavonols in green and black tea samples

From leaves of green and black teas, the mass fragmentations of flavonoid derivatives were summarized and presented in the constructed LC–MS library for identification based on the literatures (Table S1). The library contains a total 64 flavonoids including catechins, theaflavins, flavonols, and flavones, and provides their positive- and negative-ion fragmentations. The positive fragmentation was composed of both reported and proposed product ions.

On the previous studies, phenolic compounds such as flavonoids, hydroxycinnamic acid derivatives, and tannins have been characterized majorly by negative ionization mode [28, 29]. However, the present positive ionization study of flavonoids provides sodium (Na^+, *m/z* 23) and potassium (K^+, *m/z* 39) adduct ions that can help to distinct the parent ion easily, when peaks presented at low concentration as well as complexly, compared to negative-ion fragmentations (Table S1; Table 1). Actually, positive ions including $[M + Na]^+$, $[M + H]^+$, and glycosidic loss were fragmented clearly from flavonoid derivatives of green and black tea samples by Atoui et al. [30].

A total of 24 flavonoid derivatives including catechins (4), theaflavins (4), and flavonols (16) were separated and identified from the green and black tea samples using UPLC-DAD-QToF/MS (Table 1). Their chemical structures were described by R groups of each class in Fig. 2. The peak identification was completed by comparing retention time, UV spectra, and mass fragmentation presented in the literatures of constructed library.

(−)-epicatechin 3-*O*-gallate (ECG) and (−)-epigallocatechin 3-*O*-gallate (EGCG) were known as major compound in tea samples. Nevertheless, their positive-ion fragmentations

Table 1 Mass spectrometric data and contents (mg/100 g dry weight) of individual flavonoids in each processing step of green and black tea (*Camellia sinensis*) leaves

Peak No.[a]	RT	MW	Fragment ions (m/z)[b]	Green tea						
				Fresh leaves	Roasting	Rolling	First drying	Second drying	Third drying	Final product
4	10.65	788	811, 789, 627, 481, 319	4.3 ± 0.6b,A	4.8 ± 0.9ab	4.0 ± 0.6b	4.4 ± 0.5b	4.7 ± 1.0ab	4.4 ± 0.5b	5.8 ± 0.1a
5	10.83	788	811, 789, 627, 481, 319	6.9 ± 0.7d,A	8.7 ± 0.4ab	7.0 ± 0.2d	8.0 ± 0.3c	7.9 ± 0.2c	8.2 ± 0.3bc	9.2 ± 0.2a
6	11.44	480	503, 481, 319	70.3 ± 1.3e,A	99.6 ± 1.9b	85.4 ± 0.8d	93.9 ± 1.8c	102.7 ± 2.8ab	103.2 ± 2.5ab	105.5 ± 1.8a
7	11.7	480	503, 481, 319	36.0 ± 1.2d,B	57.6 ± 2.0b	49.3 ± 1.3c	57.6 ± 0.5b	63.0 ± 2.1a	62.7 ± 1.6a	60.7 ± 0.8a
8	12.25	772	795, 773, 611, 465, 303	123.8 ± 2.7d,A	142.5 ± 2.5bc	120.5 ± 0.8d	138.9 ± 2.1c	152.1 ± 5.0a	154.3 ± 2.9a	144.8 ± 2.2b
9	12.77	772	795, 773, 611, 465, 303	107.7 ± 0.7c,A	130.1 ± 2.5b	107.4 ± 1.9c	132.3 ± 2.8b	142.6 ± 4.9a	144.8 ± 3.4a	131.6 ± 3.1b
11	13.58	610	633, 611, 465, 303	59.7 ± 1.2c,C	71.9 ± 1.6ab	56.2 ± 0.2d	73.8 ± 2.3a	69.6 ± 2.6b	70.7 ± 1.8ab	62.2 ± 1.8c
12	13.58	756	779, 757, 595, 449, 287	220.8 ± 4.5 cd,A	234.9 ± 5.4b	205.0 ± 0.9e	210.9 ± 6.5de	226.2 ± 8.4bc	225.8 ± 5.9bc	246.1 ± 7.1a
13	13.68	464	487, 465, 303	65.5 ± 0.9e,A	80.5 ± 1.9c	68.6 ± 1.0d	79.4 ± 2.2c	87.7 ± 2.3a	88.9 ± 0.9a	84.0 ± 0.5b
14	14.08	464	487, 465, 303	34.1 ± 0.8b,AB	36.2 ± 2.7b	30.0 ± 1.5c	37.7 ± 0.6ab	40.9 ± 2.6a	41.1 ± 2.0a	37.5 ± 2.2ab
15	14.58	448	471, 449, 287	179.5 ± 2.2c,AB	189.0 ± 2.9b	160.5 ± 1.7e	170.4 ± 4.2d	182.2 ± 5.0c	180.8 ± 4.9c	197.3 ± 3.9a
16	15.28	756	779, 757, 595, 449, 287	61.9 ± 1.3d,A	69.3 ± 1.1b	60.3 ± 0.4d	64.9 ± 0.7c	67.4 ± 2.4bc	68.1 ± 2.0b	76.4 ± 2.0a
17	15.54	594	617, 595, 449, 287	30.6 ± 0.3b,B	32.9 ± 0.4ab	27.2 ± 2.4c	30.8 ± 2.6b	35.0 ± 1.6a	33.9 ± 1.0ab	33.3 ± 3.0ab
18	16.10	448	471, 449, 287	35.3 ± 4.5ab,A	35.9 ± 4.0ab	31.2 ± 3.0b	36.1 ± 2.7ab	37.1 ± 4.2ab	36.8 ± 3.3ab	40.8 ± 4.3a
19	21.73	302	341, 303	7.3 ± 0.9a,C	3.4 ± 0.4bc	ND	ND	3.9 ± 0.4b	3.0 ± 0.1c	ND
24	23.77	286	325, 287	10.7 ± 0.1a,CD	6.3 ± 0.2b	6.3 ± 0.2b	5.3 ± 0.6 cd	5.8 ± 0.8bc	4.8 ± 0.6d	3.7 ± 0.1e
Total flavonols				1054.3 ± 19.3c,A	1203.4 ± 27.3a	1019.1 ± 5.9c	1144.4 ± 17.9b	1228.8 ± 43.4a	1231.5 ± 28.8a	1238.8 ± 26.9a
1	5.75	306	345, 329, 307, 289	118.6 ± 1.4c,A	134.1 ± 11.7b	115.4 ± 11.0c	118.1 ± 7.9c	136.7 ± 6.4b	142.0 ± 6.2b	155.7 ± 4.1a
2	8.92	290	329, 313, 291, 273	133.3 ± 3.7e,B	276.6 ± 5.6b	237.7 ± 1.8d	252.0 ± 8.5c	282.8 ± 10.5ab	283.4 ± 13.3ab	297.6 ± 7.3a
3	9.21	458	481, 459, 307, 289	2709.5 ± 38.3f,B	5653.2 ± 54.3b	4741.0 ± 30.1e	5027.4 ± 76.4d	5409.6 ± 173.1c	5399.0 ± 149.9c	6085.6 ± 106.1a
10	13.34	442	465, 443, 291, 273	1548.0 ± 17.1d,A	2246.0 ± 58.5a	1830.5 ± 43.5c	2041.2 ± 76.7b	2087.6 ± 29.3b	2089.0 ± 41.5b	2318.2 ± 86.1a
Total catechins				4509.3 ± 42.7f,B	8310.0 ± 73.4b	6924.6 ± 72.8e	7438.7 ± 142.5d	7916.7 ± 206.4c	7913.4 ± 187.8c	8857.2 ± 172.0a
20	21.73	564	587, 565	22.7 ± 4.6C	ND	ND	ND	ND	ND	ND
21	22.47	716	739, 717	28.0 ± 6.8D	ND	ND	ND	ND	ND	ND
22	22.75	716	739, 717	30.3 ± 3.4D	ND	ND	ND	ND	ND	ND
23	22.75	868	891, 869	67.3 ± 7.5D	ND	ND	ND	ND	ND	ND
Total theaflavins				148.2 ± 19.5D	ND	ND	ND	ND	ND	ND
Total flavonoids				5711.8 ± 75.4f,A	9513.4 ± 96.3b	7943.7 ± 70.4e	8583.0 ± 142.6d	9145.6 ± 249.8c	9144.9 ± 214.5c	10096.0 ± 188.2a

Peak No.[a]	Black tea					
	Withering	Rolling	Fermentation	First drying	Second drying	Final product
4	3.9 ± 0.8A	2.5 ± 0.2B	ND[5)]	ND	ND	ND
5	6.1 ± 0.3B	4.8 ± 0.1C	ND	ND	ND	ND
6	73.6 ± 3.0A	46.8 ± 1.9B	12.8 ± 0.5D	23.5 ± 1.2D	24.3 ± 1.7D	30.6 ± 1.5C
7	38.8 ± 2.2A	25.6 ± 0.9C	6.4 ± 1.5F	13.2 ± 0.4E	12.8 ± 2.6E	17.1 ± 1.0D
8	119.8 ± 5.1A	120.7 ± 3.5A	96.9 ± 5.2C	104.8 ± 3.3B	103.2 ± 2.9 BC	118.3 ± 3.5A
9	108.6 ± 4.8A	111.6 ± 3.0A	86.5 ± 3.9C	98.8 ± 2.3B	97.4 ± 2.7B	111.2 ± 3.3A
11	60.1 ± 2.3C	74.8 ± 1.7A	61.0 ± 2.7 BC	71.6 ± 1.5A	59.6 ± 1.3C	63.8 ± 2.0B

Table 1 (continued)

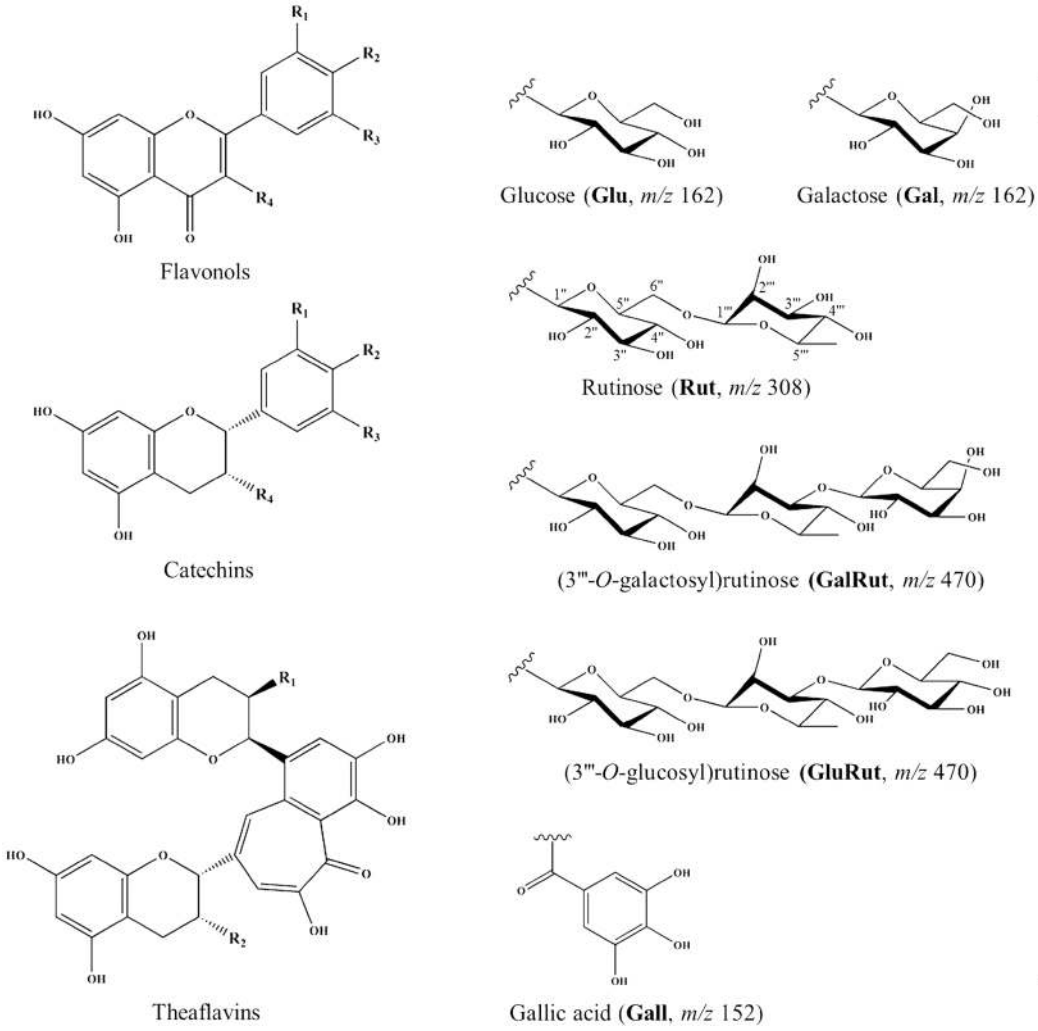
Peak No.[a]	Black tea					
	Withering	Rolling	Fermentation	First drying	Second drying	Final product
12	225.9 ± 8.7A	207.2 ± 4.8B	187.5 ± 8.3C	183.9 ± 4.0C	185.2 ± 4.2C	203.3 ± 7.4B
13	65.4 ± 1.5A	56.7 ± 1.1B	44.0 ± 0.6C	56.8 ± 1.0B	57.0 ± 2.1B	65.5 ± 2.2A
14	34.0 ± 3.5AB	34.3 ± 2.8A	27.4 ± 2.8D	29.6 ± 2.9BCD	28.3 ± 0.9CD	32.1 ± 2.0ABC
15	184.9 ± 7.3A	173.1 ± 3.6B	157.1 ± 7.3C	155.0 ± 2.7C	154.6 ± 3.6C	173.2 ± 4.1B
16	64.6 ± 3.1A	55.0 ± 1.1 BC	47.5 ± 2.6D	52.2 ± 1.8C	52.6 ± 0.9C	56.5 ± 1.6B
17	32.5 ± 1.2A	31.1 ± 1.5AB	28.1 ± 0.9C	29.6 ± 0.8 BC	28.6 ± 0.5C	32.4 ± 0.4A
18	35.1 ± 3.8A	34.2 ± 0.8AB	30.1 ± 1.8 BC	29.0 ± 0.4C	29.2 ± 0.6C	34.5 ± 0.9A
19	6.8 ± 0.2C	10.7 ± 0.5A	9.0 ± 0.6B	8.8 ± 0.4B	7.1 ± 0.5C	5.3 ± 0.3D
24	10.9 ± 0.1C	15.1 ± 0.1A	14.2 ± 0.5B	10.3 ± 0.2D	8.7 ± 0.1E	6.4 ± 0.2F
Total flavonols	1071.1 ± 43.3A	1004.3 ± 23.7B	804.4 ± 28.3E	859.2 ± 6.3D	840.6 ± 14.0DE	950.3 ± 25.5C
1	89.1 ± 6.3B	79.2 ± 16.0B	52.1 ± 12.6C	83.5 ± 2.7B	79.8 ± 4.1B	78.9 ± 4.9B
2	148.5 ± 5.8A	111.1 ± 2.2C	47.1 ± 2.3F	65.9 ± 1.8E	68.6 ± 1.5E	89.0 ± 1.0D
3	2869.9 ± 78.3A	1221.5 ± 5.2C	478.2 ± 19.6F	738.1 ± 9.8E	770.0 ± 15.9E	945.0 ± 18.2D
10	1584.6 ± 62.0A	1150.8 ± 19.5B	356.5 ± 13.5E	540.4 ± 2.0D	542.0 ± 42.0D	690.0 ± 12.8C
Total catechins	4692.1 ± 138.2A	2562.6 ± 24.3C	933.9 ± 37.9F	1428.0 ± 10.7E	1460.4 ± 55.0E	1803.0 ± 33.5D
20	17.0 ± 1.0C	21.4 ± 1.1C	22.2 ± 1.3C	43.0 ± 11.1B	43.5 ± 7.8B	65.0 ± 7.9A
21	21.9 ± 5.8D	36.2 ± 0.7D	71.1 ± 1.8C	122.4 ± 13.9B	121.7 ± 11.1B	168.4 ± 12.2A
22	21.3 ± 1.1D	28.9 ± 1.4D	43.4 ± 1.3C	70.1 ± 8.7B	69.7 ± 7.7B	97.8 ± 9.6A
23	45.6 ± 2.4D	78.2 ± 3.9D	163.8 ± 4.9C	251.0 ± 31.0B	245.8 ± 27.0B	305.6 ± 30.1A
Total theaflavins	105.7 ± 10.2D	164.7 ± 7.0D	300.5 ± 7.5C	486.4 ± 64.5B	480.6 ± 53.5B	636.8 ± 59.7A
Total flavonoids	5869.0 ± 183.1A	3731.5 ± 38.9B	2038.7 ± 58.5F	2773.7 ± 52.6D	2781.6 ± 120.0D	3390.1 ± 22.5C

Mean values ($n = 3$) in the same row with different letters were significantly different ($p < 0.05$) using Duncan's multiple range test

RT retention time, *MW* molecular weight, *ND* not detected

[a]Peak numbers: 1: (−)-epigallocatechin (EGC); 2: (−)-epicatechin (EC); 3: (−)-epigallocatechin 3-*O*-gallate (EGCG); 4: myricetin 3-*O*-(3″-*O*-galactosyl)rutinoside; 5: myricetin 3-*O*-(3″-*O*-glucosyl)rutinoside; 6: myricetin 3-*O*-galactoside; 7: myricetin 3-*O*-glucoside; 8: quercetin 3-*O*-(3″-*O*-galactosyl)rutinoside; 9: quercetin 3-*O*-(3″-*O*-glucosyl)rutinoside; 10: (−)-epicatechin 3-*O*-gallate (ECG); 11: quercetin 3-*O*-rutinoside (rutin); 12: kaempferol 3-*O*-(3″-*O*-galatosyl)rutinoside; 13: quercetin 3-*O*-galactoside (hyperoside); 14: quercetin 3-*O*-glucoside (isoquercitrin); 15: kaempferol 3-*O*-galactoside (trifolin); 16: kaempferol 3-*O*-(3‴-*O*-glucosyl)rutinoside; 17: kaempferol 3-*O*-rutinoside (nicotiflorin); 18: kaempferol 3-*O*-glucoside (astragalin); 19: quercetin; 20: theaflavin; 21: theaflavin 3-*O*-gallate; 22: theaflavin 3′-*O*-gallate; 23: theaflavin 3,3′-di-*O*-gallate; 24: kaempferol

[b]All samples analyzed in positive ionization mode using UPLC-DAD-QToF/MS



Peak No.	R_1	R_2	R_3	R_4
		Catechins		
1	OH	OH	OH	OH
2	OH	OH	H	OH
3	OH	OH	OH	OGall
10	OH	OH	H	OGall
		Flavonols		
4	OH	OH	OH	OGalRut
5	OH	OH	OH	OGluRut
6	OH	OH	OH	OGal
7	OH	OH	OH	OGlu
8	OH	OH	H	OGalRut
9	OH	OH	H	OGluRut
11	OH	OH	H	ORut
12	H	OH	H	OGalRut
13	OH	OH	H	OGal
14	OH	OH	H	OGlu
15	H	OH	H	OGal
16	H	OH	H	OGluRut
17	H	OH	H	ORut
18	H	OH	H	OGlu
19	OH	OH	H	OH
24	H	OH	H	OH
		Theaflavins		
20	OH	OH		
21	OH	OGall		
22	OGall	OH		
23	OGall	OGall		

Fig. 2 Chemical structures of catechins, theaflavins, and flavonols isolated from green and black teas (*Camellia sinensis*)

showed the limitation that reported only parent ions of *m/z* 443 and 459 $[M+H]^+$ from green and white tea samples, respectively [31]. EGCG (peak 3) was additionally observed for *m/z* 307 $[M+H\text{-Gall}]^+$ and 289 $[M+H\text{-Gall-}H_2O]^+$ in addition to *m/z* 481 and 459 corresponding to $[M+Na]^+$ and $[M+H]^+$. The fragments of ECG (peak 10) showed similar pattern to EGCG, and yielded regular ions at *m/z* 465, 443, 291, and 273. Unlike EGCG and ECG, potassium adduct ions were distinctively detected in aglycone types such as (−)-epigallocatechin (EGC, peak 1) and (−)-epicatechin (EC, peak 2). These compounds were fragmented with loss of H_2O as well as adduct ions (Na^+ and H^+) from parent ions (Table 1).

Only the positive ions corresponding to $[M+K]^+$, $[M+Na]^+$, and $[M+H]^+$ were presented in theaflavins isolated from fermented tea samples [3, 18]. However, in Fig. 3b,c and Table 1, the detailed fragmentations of theaflavins were proposed based on the structural pattern presented in negative ionization mode by Yassin et al. [32]. The fragments of theaflavins were produced primarily through Retro-Diels–Alder (RDA) fission ($C_7H_6O_3$, 138 Da) with the loss of H_2O and galloyl moiety (Gall, 152 Da). The main product ions of theaflavin 3-*O*-gallate (peak 21) and theaflavin 3′-*O*-gallate (peak 22) were largely divided into non-applied group (*m/z* 739 $[M+Na]^+$, 717 $[M+H]^+$, 699 $[M+H\text{-}H_2O]^+$, 547 $[M+H\text{-Gall-}H_2O]^+$, and 529 $[M+H\text{-Gall-}2H_2O]^+$), and applied group (*m/z* 579 $[M+H\text{-}C_7H_6O_3]^+$, 561 $[M+H\text{-}H_2O\text{-}C_7H_6O_3]^+$, 409 $[M+H\text{-Gall-}H_2O\text{-}C_7H_6O_3]^+$ and 391 $[M+H\text{-Gall-}2H_2O\text{-}C_7H_6O_3]^+$) relation to RDA fission (Fig. 3c). Especially, theaflavin 3,3′-di-*O*-gallate (peak 23) was determined as major theaflavin with fragmentation of *m/z* 891 $[M+Na]^+$, 869 $[M+H]^+$, 731 $[M+H\text{-}C_7H_6O_3]^+$, 717 $[M+H\text{-Gall}]^+$, 699 $[M+H\text{-Gall-}H_2O]^+$, 579 $[M+H\text{-Gall-}C_7H_6O_3]^+$, 561 $[M+H\text{-Gall-}H_2O\text{-}C_7H_6O_3]^+$, 547 $[M+H\text{-2Gall-}H_2O]^+$, 529 $[M+H\text{-2Gall-}2H_2O]^+$, 409 $[M+H\text{-2Gall-}H_2O\text{-}C_7H_6O_3]^+$ and 391 $[M+H\text{-2Gall-}2H_2O\text{-}C_7H_6O_3]^+$ (Fig. 3b). Thus, the fragmentations of theaflavin compounds were specifically described for the first time in positive ionization mode.

Flavonol-related derivatives were isolated from green and black tea samples, and found to be a class of kaempferol, quercetin, and myricetin conjugated with glycosides such as glucose, galactose, rutinose, galactosylrutinose, and glucosylrutinose (Fig. 2; Table 1). In the previous studies, de la Luz Cadiz-Gurrea et al. [33] who isolated four kaempferol glycosides from green tea extract did not provide the precise bonding position of glycosides. In addition, Scoparo et al. [17] could not confirm the glycosylated type and position as seen in myricetin 3-*O*-hexoside-rutinoside, quercetin 3-*O*-galactoside-rutinoside, quercetin 3-*O*-glucoside-rutinoside, kaempferol 3-*O*-galactoside-rutinoside, and kaempferol 3-*O*-glucoside-rutinoside isolated from green and black teas. On the current study, the isolated six flavonol tri-glycosides were characterized as galactosylrutinoside (peaks 4, 8, and 12) and glucosylrutinoside (peaks 5, 9, and 16) with the same molecular weight corresponding to kaempferol, quercetin, and myricetin, respectively (Fig. 2). Among them, it was confirmed that galactosyl form elutes faster than glucosyl form in the same aglycone (Fig. 4b) [34]. From the recent studies, these tri-glycosidic forms were elucidated as conjugates of galactose or glucose at 3‴- position of the rutinose moiety using NMR spectroscopy [35, 36]. Therefore, kaempferol 3-*O*-(3‴-*O*-galactosyl)rutinoside (peak 12) was identified as major flavonol observed at *m/z* 779 $[M+Na]^+$ and 757 $[M+H]^+$, and fragmented by losing of glycosidic bond clearly corresponding to 595 $[M+H\text{-Gal}]^+$, 449 $[M+H\text{-Gal-Rham}]^+$ and 287 $[M+H\text{-Gal-Rut}]^+$ (Fig. 3a). The other tri-glycosides were also completely confirmed as kaempferol 3-*O*-(3″-*O*-glucosyl)rutinoside (peak 16, *m/z* 757), quercetin 3-*O*-(3″-*O*-galactosyl)rutinoside (peak 8, *m/z* 773), quercetin 3-*O*-(3″-*O*-glucosyl)rutinoside (peak 9, *m/z* 773), myricetin 3-*O*-(3″-*O*-galactosyl)rutinoside (peak 4, *m/z* 789), and myricetin 3-*O*-(3‴-*O*-glucosyl) rutinoside (peak 5, *m/z* 789) in $[M+H]^+$ ions (Table 1).

Changes of flavonoid compositions during leaf processing steps

In each processing step of the green and black teas, the changes of composition and content were showed differently by catechins, theaflavins, and flavonols (Table 1). The final green and black teas contained the total catechins (mg/100 g DW) of 8857.2 and 1803.0, respectively, which are similar with data (7 green teas, range 5150–8430 → mean 6700; 12 black teas, range 560–4750 → mean 1540) provided by Khokhar and Magnusdottir [37] as well as a report (Meifoo and Shanghai green teas, 11,250 and 10,045; Fujian black, 1531) of Zuo et al. [4]. In green tea, total flavonoid content was dramatically increased after roasting step. Especially, as major catechins, EGCG and ECG were reaching to the highest level on the final product, and their contents (mg/100 g DW) increased approximately twofold higher than that of fresh leaves (EGCG, 2709.5 → 6085.6; ECG, 1548.0 → 2318.2). Theaflavins were present in the fresh leaves but completely disappeared after roasting. Heating processing (250–300 °C) was one of the important factors that affect the chemical composition of tea, and considered to cause the increase or decrease of certain components through drying or hydrolysis under water-free conditions, while Kim et al. [38] reported that the catechin contents were decreased with increasing infused temperature in green tea liquor. However, since the flavonol content was slightly increased after roasting (1054.3 → 1203.4 mg/100 g DW), it was hard to interpret that the great increase of catechin was entirely affected by water evaporation of the fresh leaf. Based on these points, the roasting step would be also expected to minimize the

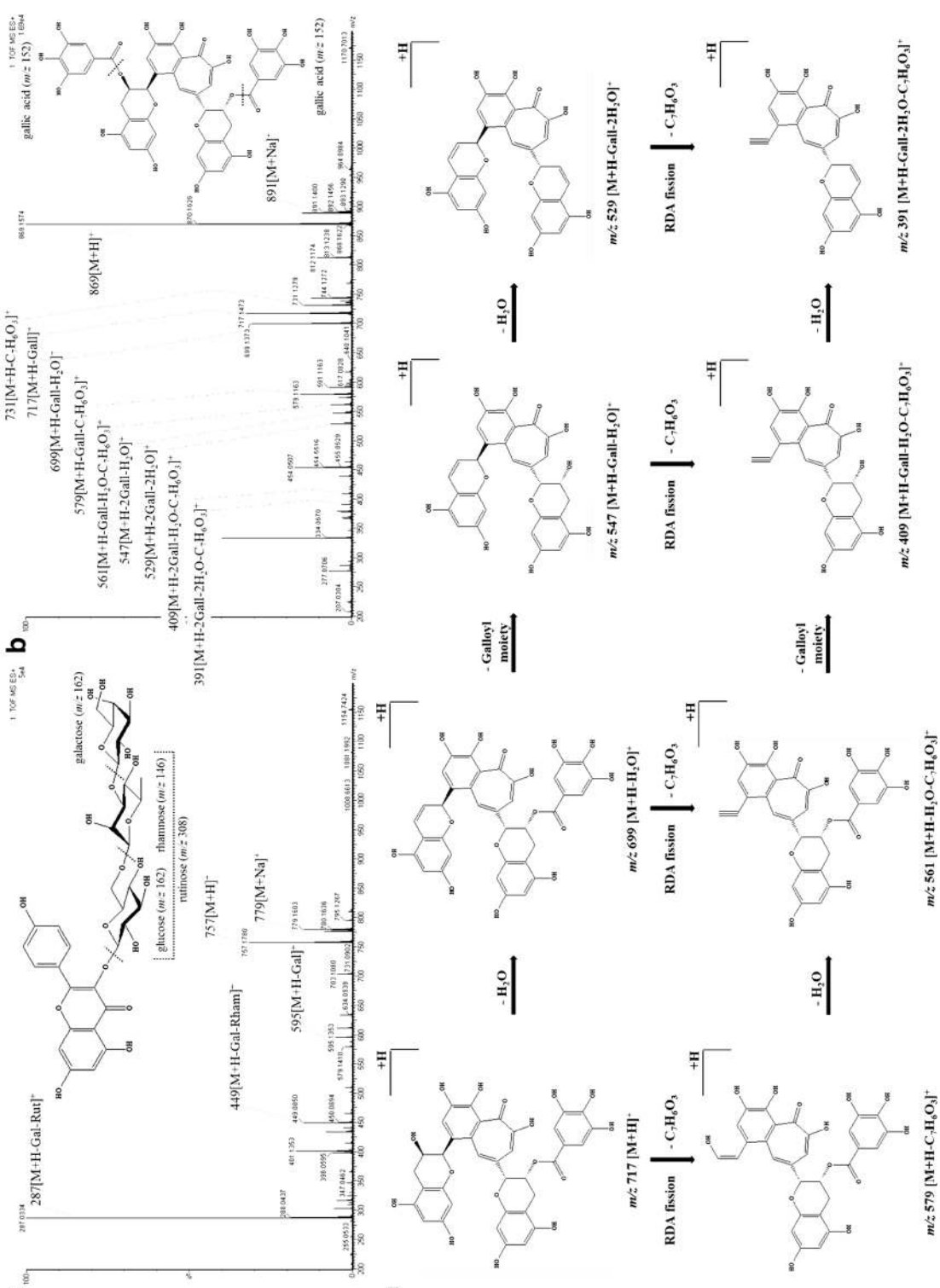
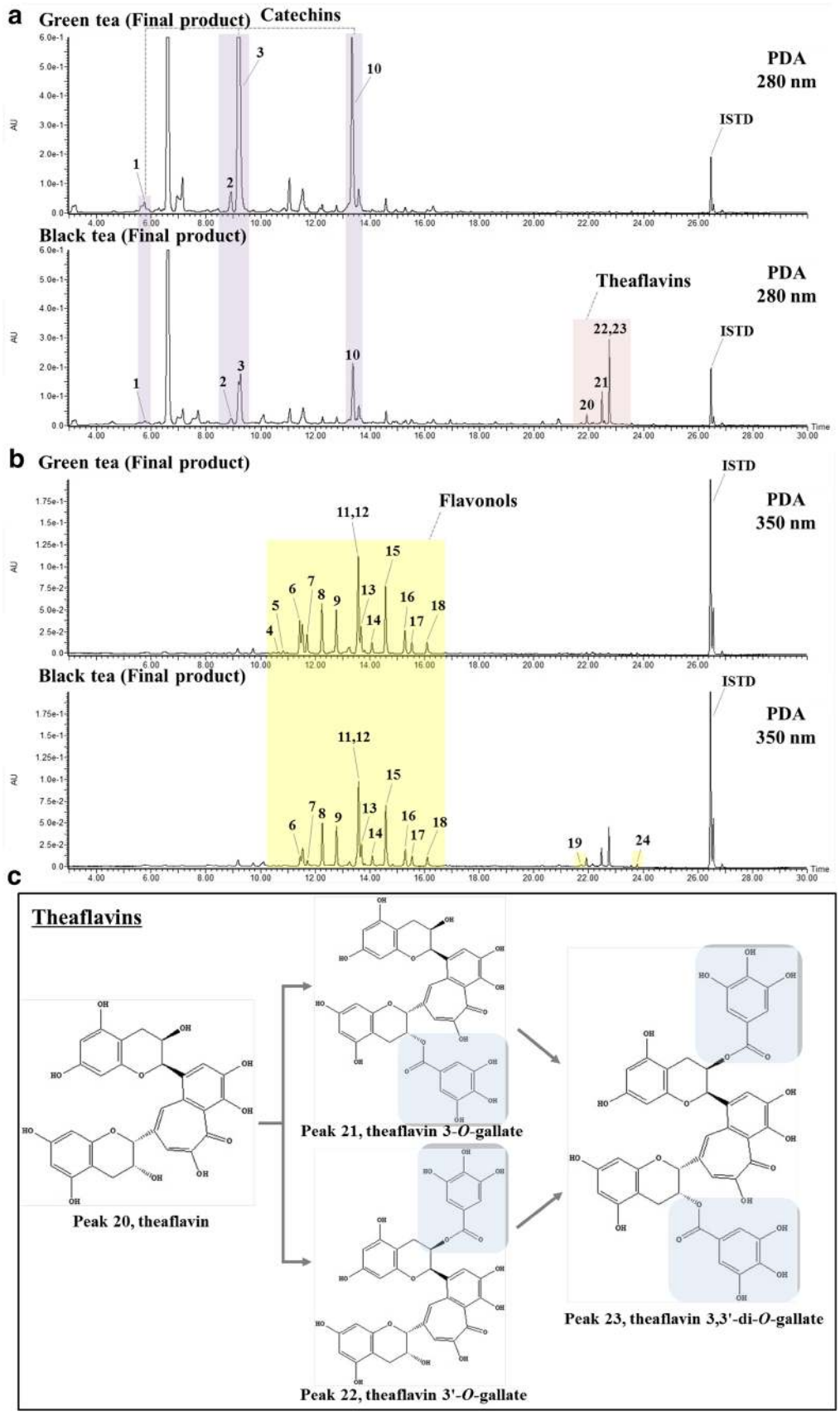


Fig. 3 Mass spectrometric characteristics of representative flavonoids isolated from green and black teas (*Camellia sinensis*). **a** Kaempferol 3-*O*-(3′′-*O*-galactosyl)rutinoside; **b** theaflavin 3,3′-di-*O*-gallate; **c** proposed fragmentation pathway of theaflavin 3-*O*-gallate. Abbreviations: *Gal* galactose; *Gall* gallic acid; *RDA* Retro-Diels–Alder; *Rham* rhamnose; Rut, rutinose


a
Green tea (Final product)
Catechins
PDA
280 nm
ISTD
Black tea (Final product)
Theaflavins
b
Green tea (Final product)
Flavonols
PDA
350 nm
Black tea (Final product)
c
Theaflavins
Peak 20, theaflavin
Peak 21, theaflavin 3-O-gallate
Peak 22, theaflavin 3'-O-gallate
Peak 23, theaflavin 3,3'-di-O-gallate

◂**Fig. 4** UPLC chromatograms of flavonoids in final green and black tea products. **a** Wavelengths at 280 nm for catechins and theaflavins; **b** 350 nm for flavonols; **c** biosynthetic pathway of theaflavin derivatives

loss of catechin content by inactivating the polyphenol oxidase (PPO) enzyme leading to catechin degradation in fresh leaf [23]. On the other hand, the contents of catechins and flavonols were significantly decreased by rolling step, and then increased again after drying steps in green tea. By Turkmen and Velioglu [39], as the rolling time was shortening, the loss of ECG and EGCG contents was reduced during leaf processing. The flavonols showed a tendency to reach maximum level through the three steps of drying, but there were no significant differences in mono- and tri-glycosides as well as total content until completion of the final product over second and third drying (Fig. 5b). In details, as major class, mono- and tri-glycosides of kaempferol (peak 12, 15, 16, and 18) were showed the highest contents, while mono- and tri-glycosides of quercetin (peak 8, 9, 13, and 14) were slightly lower than the previous step on the final product (Table 1). In green tea processing, roasting and drying were the most important factors in changing individual catechin and flavonol contents, and it was concluded that the flavonoid content could be optimized by controlling these steps in the future.

Fermentation during the production of black tea leaf is the important step that shows the greatest change in flavonoid composition and content. Most studies have been evaluated flavonoid changes in simple fermented differences by product types such as black, oolong, and Pu'er teas [18, 40]. Figure 4a chromatogram indicated that catechins were converted certainly to theaflavins through the fermentation step of black tea. In general, fermentation is one of the most affected methods to change the ingredients in agro-food processing. For example, as the major isoflavones of soybean, genistin and malonylgenistin tended to reproduce their hydrolyzed forms, namely genistein and acetylgenistin during fermentation [41]. In the contrary, catechins induces condensation reaction after oxidation by PPO enzyme to form theaflavins, thearubigins, and proanthocyanidins with large molecular weight during black tea processing, and the higher concentration of theaflavins might be caused by this chemical phenomenon (Fig. 4c) [6, 42]. In the present study, the catechin contents (mg/100 g DW) were slightly increased in the withering (4692.1) and then decreased significantly from rolling (2562.6) to fermentation (933.9) steps similar to the previous reports by Astill et al. [23] and Kim et al. [16]. The reduced content of individual catechins was increased approximately twofold again when it became the final product after drying (Table 1). The total theaflavin content (mg/100 g DW) increased remarkably throughout whole black tea processing following order: withering (105.7) → rolling (164.7) → fermentation (300.5) → first drying (486.4) → second drying (480.6) → final product (636.8). As shown in Figs. 4c and 5a, theaflavin 3,3′-di-*O*-gallate (305.6) and theaflavin 3-*O*-gallate (168.4) which were advanced with gallic acid in biosynthetic pathway, showed the greatest increases of 4.5- and sixfold as predominant compounds compared to fresh leaf, respectively. Unlike this proposed synthetic pattern, Liang et al. [43] reported that the concentration of theaflavin and theaflavin 3′-*O*-gallate was found to be approximately twofold higher than theaflavin 3-*O*-gallate and theaflavin 3,3′-di-*O*-gallate in black tea samples. Recently, it was provided that theaflavin-3,3′-di-*O*-gallate could be regulated inflammatory factors such as tumor necrosis factor-alpha (TNF-α), and interleukin (IL)-1β and -6 as potential therapeutic candidate [44].

On flavonol contents of black tea, significant differences were found between fresh leaf and final product, but only 23% of reduction was occurred in comparison with green tea. According to Del Rio et al. [45] study, an about 30% of reduction was discovered in total flavonols during black tea processing. Besides, Zhao et al. [31] investigated that the flavonol contents were not changed by different fermented degrees from green to Pu'er tea samples. Briefly, in Table 1, the change of kaempferol and quercetin glycosides had no significant difference between fresh leaf and final product. However, myricetin glycosides showed different patterns depending on the glycosidic forms. Interestingly, the tri-glycosides of myricetin were completely disappeared after fermentation as well as their mono-glycosides of final product have reduced more than 50% compared to fresh leaf. There is no report about focusing changes of flavonols during tea processing. It was considered that additional processing methods for increasing flavonol level are required to further optimize green and black tea qualities.

The composition of chemical compounds such as EGCG, ECG, and theaflavins could be evaluated as a potential quality indicator for grading of green and black tea infusion [46]. From our study, rolling step normally decreased catechins and flavonols both green and black tea. Thus, detailed rolling conditions are necessary to investigate minimum loss of catechins and flavonols. Theaflavins which were increased only in black tea processing might be considered to utilize as a health beneficial compounds for high qualities of black tea. However, the actual reaction mechanism of compounds on processing requires further studies. In the future, for the representative tea products by origins, it will be possible to evaluate the functional ingredients by their processing conditions to control the qualities more efficiently.

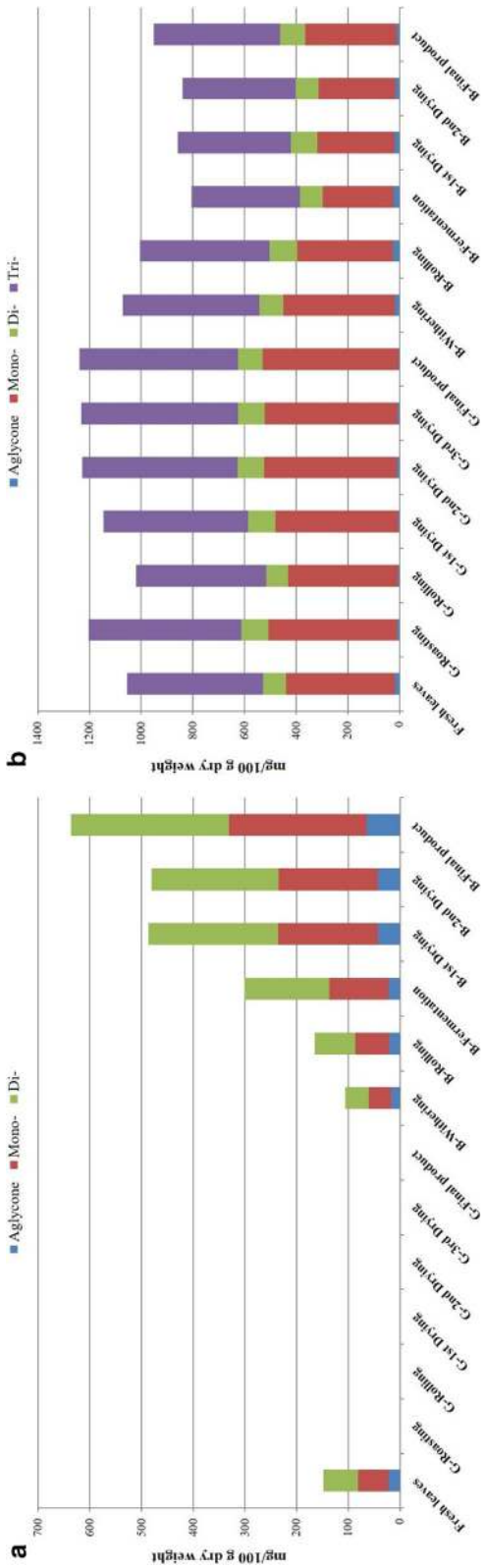


Fig. 5 Distribution of flavonoids by tea leaf processing. **a** Variation of theaflavin contents (mg/100 g dry weight, DW) by acylated forms (aglycone, mono- and di-); **b** variation of flavonol contents (mg/100 g DW) by glycosidic forms (aglycone, mono-, di-, and tri-)

Conclusions

The previous studies have been focused on the optimization of tea quality by comparing different varieties and cultivated regions. This study described specifically the changes of individual flavonoids according to each processing step of green and black tea leaves. A total of 24 flavonoid derivatives including catechins, theaflavins, and flavonols were separated and identified from the tea samples based on UPLC-DAD-QToF/MS data and constructed library. Among these, the fragmentations pathway of theaflavins was proposed for the first time in positive ionization mode. The contents of catechins, theaflavins, and flavonols showed a similar tendency to that of the previous studies on the final tea products (green and black), respectively. During leaf processing, the individual flavonol contents were changed diversely according to their aglycone types and glycosylated forms. In particular, roasting step generally enhanced catechin content including EGCG and ECG in green tea, but catechins were considerably reduced due to their oxidation and conversion to theaflavins after the fermentation step in black tea. The drying multi-steps also found to be factor to influence positively to increase the flavonoids in tea processing. Therefore, this result indicated that individual flavonoids might be optimized by controlling the detailed conditions of each processing step to obtain the excellent quality on final tea products.

Acknowledgements The authors would like to acknowledge the research funding from the 'Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ01178704)' provided by the Rural Development Administration, Republic of Korea.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subject.

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