Changes in major polyphenolic compounds of tea (*Camellia sinensis*) leaves during the production of black tea

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Abstract The objective of this study was to quantify the polyphenolic compounds present in tea samples during black tea processing, and to determine the correlation between the contents of individual catechins and theaflavins. Nine monomeric and four dimeric compounds were identified and quantified by HPLC. During black tea processing, the catechins content decreased, whereas the gallic acid content increased. The decrease in the catechins-in particular, the *cis*-catechins-was due to the formation of dimeric theaflavins. In the present study, we found a significant negative correlation between the changes in the catechins and theaflavins contents during black tea processing. Theaflavin-3-gallate showed the strongest correlations with the *cis*-catechins ((-)-epigallocatechin: $r=0.713$; (-)-epicatechin: $r=0.755$; (-)-epigallocatechin gallate: $r=0.681$; and (-)-epicatechin gallate: $r=0.771$).

Keywords: black tea, fermentation, gallic acid, catechins, theaflavins

Introduction

Tea, the most widely consumed beverage in the world after water, is made from the leaves of *Camellia sinensis*, and can be divided into three main categories based on the degree of fermentation: unfermented green tea, partially fermented Oolong tea, and fermented black tea. Fermentation is a very important process in fermented tea manufacture, where the term “tea fermentation” refers to natural browning reactions induced by oxidative enzymes in the cells of the tea leaves (1). Of the tea produced worldwide, black tea is the most commonly consumed (approximately 78%), followed by green (approximately 20%) and oolong tea (approximately 2%) (2).

The general process of manufacturing black tea from fresh tea leaves consists of five steps, including withering, rolling, fermentation, drying and sorting. Black tea is manufactured by different processes depending on geographical region; the main difference arises from the applied rolling method (3). The rolling of tea leaves may be accomplished by orthodox rollers or crush-tear-curl machines; the former method is more widely used (3). During black tea manufacture, the monomeric catechins are converted to more complex polyphenols, the theaflavins and thearubigins. Theaflavins contribute to the characteristic bright orange-red color of black tea. Four major theaflavins are found: theaflavin (TF), theaflavin-3-gallate (TF-3-G), theaflavin-3'-gallate (TF-3'-G), and theaflavin-3,3'-digallate (TF-3,3'-diG) (4).

Currently, Korean tea manufacturers produce mainly green tea. Unfortunately, because production is outstripping demand, the

industry needs to diversify its tea products, such as by producing black teas. To produce high quality black tea using domestic tea leaves, a better understanding of the major polyphenol changes in the tea leaves during processing is required. Polyphenol compounds, especially theaflavins, have become a critical parameter in estimating the quality of black tea, since they contribute to its color and are responsible for its taste and brightness (5).

Many studies have investigated the chemical changes that occur during particular black tea processing steps. For example, the changes in black tea components as a function of withering or fermentation times or different rolling methods have been examined (3,6–9). However, there is a lack of information about the chemical changes that occur over the entire process of black tea manufacturing. Therefore, this study aimed to investigate the effect of the processing procedure on the polyphenol compounds in tea leaves, to provide a foundation for the newly developing black tea manufacturing industry in Korea. Monomeric catechins and dimeric theaflavins were identified and quantified by HPLC, and Pearson's correlation analysis was applied to determine the relationship between the contents of the individual catechins and theaflavins.

Materials and Methods

Tea materials and chemicals Fresh tea leaves (*Camellia sinensis*) were harvested in 2015 from a tea garden in the Korean province of Boseong (34°42'N, 127°04'E). Black tea was prepared by the orthodox

method, which is commonly used as a commercial method for loose black tea in Korea. The fresh tea leaves were withered indoors at ambient temperature for about 20 h. The withered leaves were rolled in a pilot scale (kg/batch) orthodox rolling machine (6CRM-25; Fuyang Wealth Imp. & Exp. Co., Ltd., Zhejiang, China) for 30 min. The rolled leaves were fermented for 180 min at 30°C using a temperature-controlled incubator (Jeio-Tech, Daejeon, Korea) and dried for 120 min at 90°C to obtain black tea containing approximately 3% moisture. Samples were collected at each stage of the process, including a fresh tea leaf. During all stages of processing, tea samples were obtained and frozen immediately using dry ice, except the dried teas. The frozen samples were freeze-dried (FDCF-12012; Operon Co., Gimpoo, Korea) and ground before further analysis. (+)-Catechin (C), (–)-epicatechin (EC), (+)-catechin gallate (Cg), (–)-epicatechin gallate (ECg), (+)-gallo catechin (GC), (–)-epigallocatechin (EGC), (+)-gallo catechin gallate (GCg), (–)-epigallocatechin gallate (EGCg), and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Theaflavin (TF), theaflavin-3-gallate (TF-3-G), theaflavin-3'-gallate (TF-3'-G), and theaflavin-3,3'-digallate (TF-3,3'-diG) were purchased from Nacalai (San Diego, CA, USA). HPLC grade methanol and water were obtained from Fisher Scientific (Pittsburgh, PA, USA).

Extraction and standard preparation Sample extracts were prepared by soaking ground leaf powder (1 g) in boiling deionized distilled water (100 mL) for 10 min. The mixtures were filtered with 0.45 µm Stericup filter units (Millipore, Billerica, MA, USA). Stock solutions of reference standards were prepared by dissolving each standard individually in 20% aqueous methanol to a final concentration of 1 mg/mL. Working standards for calibration curve construction were prepared with concentrations ranging from 6.25 to 62.5 µg/mL.

Measurement of total polyphenol content The total polyphenol content was measured using the modified Folin-Ciocalteu assay, as described by Singleton and Rossi (10). Briefly, each test sample solution (20 µL) was mixed with Folin-Ciocalteu reagent (100 µL) in a 96-well plate. The mixtures were kept at room temperature for 5 min, after which time 7.5% sodium carbonate solution (80 µL) was added to each sample. The reaction mixtures were left to stand at room temperature for 120 min, and their absorbance was measured at 735 nm using a microplate reader. The total polyphenolic content for each sample was expressed in terms of mg of gallic acid equivalents (GAE) per 100 mL of infusion.

Quantification of phenolic compounds by HPLC The major components of the tea extracts were quantified using the modified method described by Lee and Ong (11). Extracts were analyzed at 35°C by HPLC (JASCO Co., Tokyo, Japan), using an XTerra RP18 column (3.5 µm, 4.6×150 mm, Waters, Milford, MA, USA) and a multi-wavelength detector set at 205 nm (MD-2010 Plus). The mobile phase was composed of solvent A (0.035% trifluoroacetic acid in 5% acetonitrile aqueous solution, v/v) and solvent B (0.035%

trifluoroacetic acid in 50% acetonitrile aqueous solution, v/v). The elution gradient employed was 10% B to 20% B over 10 min, 20–40% B over 6 min, 40–50% B over 4 min, 50–60% B over 5 min, holding at 60% B for 5 min, and re-equilibration to the initial conditions for 10 min. The caffeine, theanine, and phenolic contents of the samples were calculated by comparison with external standards. For quantitative analysis of the tea samples, a six-level calibration curve was generated by the analysis of known concentrations (6.25–62.5 µg/mL) of each standard. The equations for the standards are as follows: gallic acid ($y=55567.65x-44992.34$; $R^2=0.9987$); EGC ($y=110134.71x-171176.18$; $R^2=0.9995$); EC ($y=129634.93x-122477.37$; $R^2=0.9995$); EGCg ($y=71372.78x-776549.86$; $R^2=0.9958$); ECg ($y=61467.01x-275449.28$; $R^2=0.9950$); GC ($y=105116.07x-304959.82$; $R^2=0.9930$); C ($y=124489.74x-163144.95$; $R^2=0.9992$); GCg ($y=59692.46x-677375.86$; $R^2=0.9943$); Cg ($y=55400.19x-617980.28$; $R^2=0.9945$); TF ($y=43156.77x-238008.50$; $R^2=0.9942$); TF-3-G ($y=44487.67x-248482.25$; $R^2=0.9932$); TF-3-G ($y=32797.95x-207932.74$; $R^2=0.9906$); TF-3,3'-diG ($y=29923.75x-206763.11$; $R^2=0.9908$). The *cis*-catechins content was defined as the sum of the EGC, EC, EGCg, and ECg. The *trans*-catechins content was defined as the sum of the GC, catechin, GCg, and Cg. The concentrations of all the compounds in the extracts were quantified using standard curves and expressed as milligrams per 100 mL of infusion.

Statistical analysis Data are expressed as the mean and standard deviation and were analyzed by one-way ANOVA, followed by Tukey's test. Analyses were carried out to a significance level of $p<0.05$, using the software program SPSS (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Changes in total polyphenol content during black tea processing of fresh tea leaves The changes in the total polyphenol content during the black tea processing of fresh tea leaves are shown in Fig. 1. The maximum total polyphenol content was observed in the fresh leaves. As the leaves were withered, rolled, fermented, and dried during processing, the total polyphenol content decreased by approximately 53%. The total polyphenol content declined more quickly after fermentation, which suggested the oxidation of the polyphenols during this stage. The decrease in the total polyphenol content was associated with the changes in the chemical characteristics of the polyphenols resulting from their oxidation, hydrolysis, polymerization, and transformation during tea processing (12).

The withering process causes physicochemical changes in the tea leaves, including a decrease in their moisture content and increase in cell membrane permeability, as well as the formation of the tea aroma. Moreover, during withering, the leaves become more flaccid, facilitating rolling. The rolling process disrupts the cell structure in the withered leaves, which accelerates the enzymatic oxidation of

Fig. 1. Change in total polyphenol content during black tea manufacturing. FL, fresh leaf; WL, withered leaf; RL, rolled leaf; EFL, enzyme-fermented leaf; BT, black tea.

the polyphenols. The chemical changes proceed at a rapid rate during and/or after rolling. During fermentation, monomeric catechins are oxidized by the endogenous enzymes, polyphenol oxidase and peroxidase, producing more complex polyphenols such as the theaflavins and thearubigins (1,13,14).

Changes in monomeric polyphenol content during black tea processing of fresh tea leaves Fresh tea leaves are abundant in monomeric polyphenols, especially catechins. During black tea processing, the contents of all the catechins except for Cg decreased compared to the fresh leaves, while the gallic acid content increased (Table 1). Moreover, the depletion of the *cis*-catechins, EGC, EC, EGCg, and ECg, was higher than that of the corresponding epimers, GC, C, GCg, and Cg. At the end of tea processing, among the assayed monomeric polyphenols, gallic acid was the most abundant, followed by EGCg, Cg, and GCg. This was probably due to the chemical changes in the catechins caused by oxidation, epimerization,

and hydrolysis reactions. Most of the catechins were nearly completely degraded during black tea processing, particularly after fermentation. At the same time, the gallic acid content increased, because it is produced through the degradation of the galloyl moieties of catechins by oxidation (15). These results were consistent with those of previous studies, which showed that gallic acid increased by the enzymatic oxidation and thermal hydrolysis of catechins (12,16).

The present study also provides key information for the role of the stereochemistry of the catechins in tea processing. Catechins can be classified as catechol-catechins (B-ring dihydroxylated catechins, EC, ECg, C, and Cg) or gallo-catechins (B-ring trihydroxylated catechins, EGC, EGCg, GC, and GCg), depending on the number of hydroxyl groups on the B-ring. These are further classified as *cis*-catechins (EGC, EC, EGCg, and ECg) or *trans*-catechins (GC, C, GCg, and Cg) according to the relative configuration of the C2 and C3 positions of the C-ring (17). As shown in Fig. 2A, the sum of the *cis*-catechins content dramatically decreased to approximately 6% of the initial amount after final processing, whereas the *trans*-catechins only decreased by about 50%. Figure 2A also presents the degree of change in the catechin composition by expressing the *cis*-catechins as a percentage of the total catechins. The percentage of *cis*-catechins in black tea was much lower than that in fresh leaves. The *cis*-catechins were responsible for the formation of the components of black tea, which was consistent with previous results (4). Additionally, as shown in Fig. 2B, the content of the gallo-catechins was much higher than that of the catechol-catechins in fresh leaves. However, the percentage of gallo-catechins in the total catechins decreased from 65.1% in fresh leaves to 54.4% in black tea. This was probably related to the higher oxidation level of the gallo-catechins, with three B-ring hydroxyl groups, than the catechol-catechins, resulting in a lower redox potential (15). This result was consistent with previous studies where the concentration of gallo-catechins was much lower than that of the catechol-catechins after enzymatic oxidation of a mixture standard of catechins (18–20).

Table 1. Change in monomeric polyphenol content during black tea manufacturing

	Content (mg/100 mL infusion)				
	FL ²⁾	WL	RL	EFL	BT
Epigallocatechin	15.40±0.23 ^{aB1)}	13.21±0.22 ^{bB}	9.31±0.15 ^{cB}	1.79±0.20 ^{dC}	0.77±0.10 ^{eE}
Epicatechin	9.77±0.10 ^{aD}	6.85±0.23 ^{bC}	6.00±0.02 ^{cC}	1.34±0.02 ^{dD}	0.87±0.01 ^{eDE}
Epigallocatechin gallate	25.08±0.83 ^{aA}	21.13±0.60 ^{bA}	16.90±1.64 ^{cA}	2.51±0.27 ^{dB}	1.47±0.09 ^{dB}
Epicatechin gallate	13.04±1.06 ^{aC}	7.77±0.73 ^{bC}	7.62±0.68 ^{bBC}	1.19±0.05 ^{cDE}	0.71±0.04 ^{cE}
Gallocatechin	2.50±0.04 ^{aE}	1.60±0.10 ^{bD}	1.02±0.09 ^{cE}	0.34±0.02 ^{dF}	0.26±0.01 ^{dF}
Catechin	1.09±0.01 ^{aF}	0.42±0.00 ^{bE}	0.53±0.01 ^{cE}	0.22±0.01 ^{dF}	0.18±0.01 ^{eF}
Gallocatechin gallate	1.53±0.04 ^{aEF}	1.27±0.03 ^{bDE}	1.20±0.04 ^{bE}	0.97±0.01 ^{cE}	1.00±0.02 ^{cD}
Catechin gallate	ND	1.27±0.03 ^{bDE}	1.38±0.03 ^{aE}	1.14±0.02 ^{cDE}	1.15±0.01 ^{cC}
Gallic acid	1.13±0.05 ^{cEF}	1.73±0.09 ^{bD}	3.58±0.07 ^{aD}	3.38±0.10 ^{aA}	3.51±0.10 ^{aA}

¹⁾All values are means±SD (n=3). Different superscript small letters indicate significant differences within a row ($p < 0.05$) and different capital letters indicate significant differences within a column ($p < 0.05$).

²⁾ND, not detected; FL, fresh leaf; WL, withered leaf; RL, rolled leaf; EFL, enzyme-fermented leaf; BT, black tea.

Fig. 2. Change in catechins content during black tea manufacturing: (A) *cis*-catechins and *trans*-catechins; (B) gallo-catechins and catechol-catechins. FL, fresh leaf; WL, withered leaf; RL, rolled leaf; EFL, enzyme-fermented leaf; BT, black tea.

Changes in dimeric polyphenol content during black tea processing of fresh tea leaves During the manufacturing of black tea, the monomeric catechins in the fresh tea leaves are oxidized and polymerized to dimeric theaflavins and polymeric thearubigins, which contribute to the characteristic color of black tea. Theaflavins are formed from two different *cis*-catechins, catechol-catechin and gallo-catechin. The four major theaflavins found in black tea are TF, TF-3-G, TF-3'-G, and TF-3,3'-diG (4).

As shown in Table 2, theaflavins appeared after withering, and during the manufacturing processes of rolling and fermentation, the theaflavin contents reached a peak and began to decline. This decrease was probably due to either the degradation of the theaflavins or their conversion into other compounds, such as thearubigins (21,22). In fact, according to the literature, the considerable theaflavins synthesized during black tea fermentation are further oxidized by epicatechin quinone, which contributes to the production of thearubigins (23-25).

At the end of tea processing, among the dimeric theaflavins tested, TF was the most abundant, followed by TF-3-G, TF-3'-G, and TF-3,3'-diG. During rolling and throughout fermentation, in particular, the TF and TF-3-G contents significantly decreased from 1.85 and 0.74 to 1.37 and 0.64 mg/100 mL infusion, respectively, but the TF-3-G and TF-3,3'-diG contents showed minimal change. Therefore, the contents of the individual theaflavins are influenced by the stereo-

chemical configuration and the amount of their catechin precursors. These results were supported by a previous study, which showed that the oxidative degradation of theaflavins becomes apparent during fermentation after the gallo-catechins have been utilized (20). The results are also consistent with data from the model fermentation of black tea, which showed that the combination of EGCg and ECg gives a lower yield compared to the combination of EGCg with EC (22).

Pearson's correlation analysis was used to find out significant correlations between the catechins and theaflavins obtained at each tea processing step. As shown in Table 3, most of the theaflavins negatively and significantly correlated with catechins. Of these, the TF-3-G levels had the strongest correlations with all the catechins (EGC: $r=0.713$; EC: $r=0.755$; EGCg: $r=0.681$; and ECg: $r=0.771$). Only TF showed no correlation with any catechins. Moreover, all theaflavins except for TF were more negatively and significantly correlated with the gallo-type catechins (TF-3-G: $r=0.744$; TF-3'-G: $r=0.593$; and TF-3,3'-diG: $r=0.722$) than with the catechol-type catechins (TF-3-G: $r=0.708$; TF-3'-G: $r=0.505$; and TF-3,3'-diG: $r=0.646$). One explanation for these results might be that theaflavins formation is dependent on the catechins, which act as the precursors. As mentioned earlier, the major theaflavins formed from *cis*-catechins are TF (EGC+EC), TF-3-G (EGCg+EC), TF-3'-G (EGC+ECg), and TF-3,3'-diG (EGCg+ECg) (4).

In conclusion, although further studies on the influence of

Table 2. Change in dimeric theaflavins content during black tea manufacturing

	Content (mg/100 mL infusion)				
	FL ²⁾	WL	RL	EFL	BT
Theaflavin	ND	1.13±0.05 ^{ca1)}	1.85±0.06 ^{aA}	1.37±0.01 ^{bA}	0.95±0.02 ^{dA}
Theaflavin-3-gallate	ND	0.58±0.01 ^{cc}	0.89±0.02 ^{aB}	0.90±0.01 ^{aB}	0.73±0.01 ^{bB}
Theaflavin-3'-gallate	ND	0.69±0.02 ^{bb}	0.74±0.02 ^{aC}	0.64±0.02 ^{bcC}	0.60±0.01 ^{cc}
Theaflavin-3,3'-digallate	ND	0.56±0.01 ^{bc}	0.62±0.02 ^{aD}	0.61±0.00 ^{aD}	0.59±0.00 ^{aC}

¹⁾All values are means±SD ($n=3$). Different superscript small letters indicate significant differences within a row ($p<0.05$) and different capital letters indicate significant differences within a column ($p<0.05$).

²⁾ND, not detected; FL, fresh leaf; WL, withered leaf; RL, rolled leaf; EFL, enzyme-fermented leaf; BT, black tea.

Table 3. Pearson's correlation between catechins and theaflavins obtained at each tea processing step

	Pearson's correlation coefficient (<i>r</i>)									
	EGC ²⁾	EC	EGCg	ECg	Catechol-type	Gallo-type	TF	TF-3-G	TF-3'-G	TF-3,3'-diG
EGC	1	0.986** ¹⁾	0.995**	0.961**	0.998**	0.976**	-0.420	-0.713**	-0.504	-0.645**
EC		1	0.987**	0.990**	0.990**	0.997**	-0.465	-0.755**	-0.593*	-0.723**
EGCg			1	0.969**	0.999**	0.983**	-0.374	-0.681**	-0.478	-0.621*
ECg				1	0.970**	0.997**	-0.489	-0.771**	-0.638*	-0.760**
Catechol-type					1	0.984**	-0.409	-0.708**	-0.505	-0.646**
Gallo-type						1	-0.450	-0.744**	-0.593*	-0.722**
TF							1	0.931**	0.925**	0.894**
TF-3-G								1	0.932**	0.961**
TF-3'-G									1	0.984**
TF-3,3'-diG										1

¹⁾The direction and magnitude of the correlation between variables was quantified by the correlation coefficient *r*, **p*<0.05; ***p*<0.01.

²⁾EGC, epigallocatechin; EC, epicatechin; EGCg, epigallocatechin gallate; ECg, epicatechin gallate; TF, theaflavin; TF-3-G, theaflavin-3-gallate; TF-3'-G, theaflavin-3'-gallate; TF-3,3'-diG, theaflavin-3,3'-digallate.

parameters such as time and temperature on each processing step are needed, this study will provide important information that will help to improve the manufacturing process for high-quality black tea produced in Korea.

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