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Comparison of colorimetric methods for the analysis of total polyphenols in black tea extracts

Kokoro MATSUSHITA¹, Chihiro HONDA¹, Yoriyuki NAKAMURA², and Shigenori KUMAZAWA^{1*}

¹ Graduate School of Integrated Pharmaceutical and Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan

² Tea Science Center, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan

Received November 18, 2024 ; Accepted January 10, 2025

Because polyphenols are bioactive components in foods, an accurate and simple method for their determination is required. Currently, the Folin-Ciocalteu and iron tartrate methods are utilized for analyzing the total polyphenol content (TPC) in black tea. However, there are no scientifically based comparative data for these colorimetric methods. Therefore, in this study, an appropriate method for analyzing the TPC in black tea was proposed. Comparison of the efficacy of these colorimetric methods for the analysis of fresh and fermented black tea leaf samples, including quantitative HPLC data, showed that both colorimetric methods provided characteristic data depending on the degree of tea-leaf fermentation. The iron tartrate method was affected by the type of polyphenols in tea leaves, whereas in the Folin-Ciocalteu method, the reducing power of the polyphenols directly affected the total polyphenol equivalents. Both colorimetric methods were effective for analysis of the TPC in black tea leaves.

Keywords: Folin-Ciocalteu method, iron tartrate method, black tea, polyphenols, total polyphenol analysis, fermentation

Introduction

Plant-based foods contain polyphenols with a wide range of complex structures. The molecular structure of polyphenols contains numerous hydroxyl groups (Abbas *et al.*, 2017). Polyphenols can be divided into flavonoids, phenolic acids, lignans, tannins, and stilbenes based on their structural differences (Lang *et al.*, 2024).

Polyphenols are the most common dietary antioxidants. Their main dietary sources are fruits and plant-derived beverages such as fruit juices, tea, coffee, and red wine. Vegetables, cereals, chocolate, and dry legumes also contribute to the total polyphenol intake. Research on the antioxidant properties and disease-preventive effects of flavonoids and other polyphenols began in 1995 (Scalbert *et al.*, 2005). Flavonoids, including catechins, are the most studied polyphenols found in tea. Condensed tannins are among the theaflavins present in black tea. Many epidemiological studies have demonstrated that the daily intake of black tea is inversely associated with many diseases,

such as cardiovascular disease, hypertension and cancer (Gardner *et al.*, 2007; Chiang *et al.*, 2020).

The quantitative determination of polyphenols is affected by their structural complexity and diversity. However, because polyphenols are bioactive substances, accurate, rapid, and simple methods of total polyphenol content (TPC) determination are required. Currently, two simple colorimetric methods are used to analyze the TPC in tea. The first is the Folin-Ciocalteu method, which is defined by the International Organization for Standardization (ISO) as a method for analyzing the TPC in green and black tea (ISO 14502-1, 2005). Polyphenols in tea react with specific redox reagents (Folin-Ciocalteu reagent) to form a blue chromophore comprising a phosphotungstic-phosphomolybdenum complex (Fig. 1). The maximum absorption of these chromophores depends on the alkaline solution and concentration of phenolic compounds (Schofield *et al.*, 2001; Gülçin *et al.*, 2004). Another method is the iron tartrate method, which is specified in the Standard Tables of Food Composition in Japan as a method for

*To whom correspondence should be addressed.

E-mail address: kumazawa@u-shizuoka-ken.ac.jp

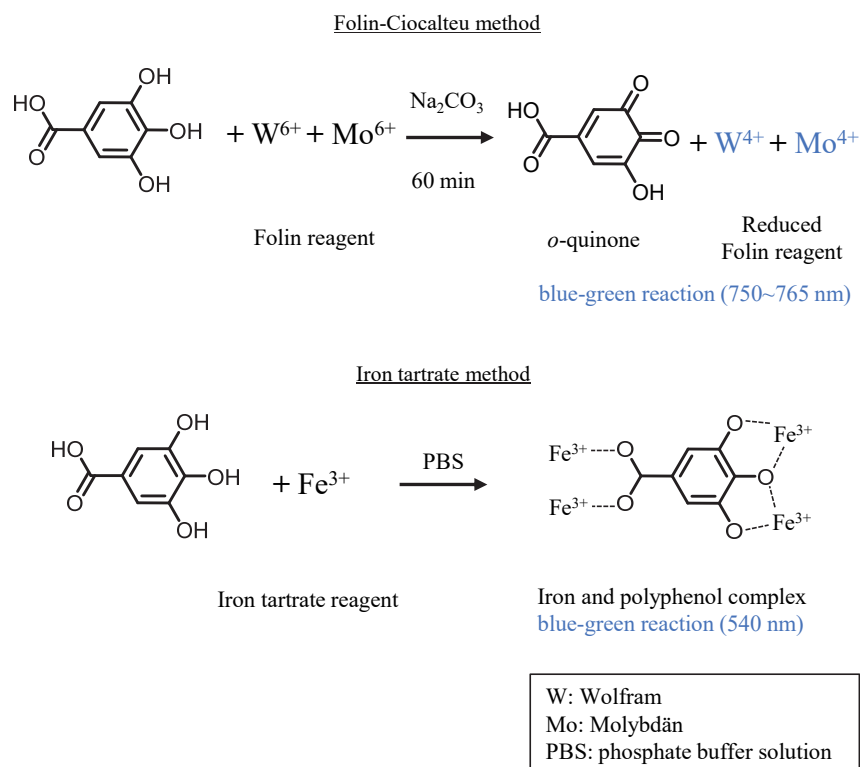


Fig. 1. Reaction mechanisms for the Folin-Ciocalteu and iron tartrate methods.

determining tannins in green tea¹⁾. The iron tartrate method involves the quantitative reaction of phenolic hydroxyl groups with iron ions to form complexes (Fig. 1). Although it is a tannin determination method, the iron tartrate method can potentially be used to determine the amounts of total polyphenols (Nakabayashi, 1988). The iron tartrate method is the first excellent analytical method for TPC analysis developed in Japan, but compliance with international standards such as ISO has been delayed. Therefore, the iron tartrate method is rarely employed overseas. Colorimetric reactions are widely used in UV/vis spectrophotometric analyses, which are easy to perform, rapid, applicable for routine laboratory use, and inexpensive (Blainski *et al.*, 2013). The Folin-Ciocalteu and iron tartrate colorimetric methods are both enable simple analysis of the TPC in tea. However, no studies have compared the colorimetric methods under the same conditions. Therefore, herein, these two colorimetric methods are compared in the analysis of tea leaves and are further compared to analytical methods used internationally.

Recently, we compared the Folin-Ciocalteu and iron tartrate methods for analyzing the TPC in green tea (Matsushita *et al.*, 2024). In that report, the TPC determined by the Folin-Ciocalteu method was comparable to that obtained by HPLC analysis. However, the TPC obtained using the iron tartrate method was higher than that detected using the Folin-Ciocalteu method. Thus, the Folin-Ciocalteu method is recommended for analysis of the TPC in green tea leaves. In

addition, we conclude that for the iron tartrate method, the factor for multiplication by the ethyl gallate concentration must be determined, depending on the green tea variety.

After the above study, we compared the effectiveness of the colorimetric methods for the analysis of oolong tea, black tea, and hojicha. Tea is typically classified into three fundamental categories based on the various processing methods, particularly the level of fermentation: green tea (non-fermented), oolong tea (semi-fermented), and black tea (fully fermented) (Deo *et al.*, 2024). In contrast to those three types of tea, hojicha is processed by roasting autumn flush tea or stem leftover from the production of green tea (Tan *et al.*, 2019). Following our previous study on green tea, we compared the two colorimetric methods using four types of tea leaves (green tea, oolong tea, black tea, and hojicha).

Black tea is the second most-consumed beverage worldwide after water (Abudurehman *et al.*, 2022). Green tea is prepared by the immediate heat treatment of plucked leaves to stop fermentation (oxidation). On the other hand, black tea is made by withering and twisting the picked leaves to enable full fermentation. Leaf fermentation affects the taste, aroma, and appearance of black teas. For this reason, appropriate types of leaves for black tea are produced worldwide, and there are many varieties and blends of black tea (Ono, 2014). Therefore, compared to green tea, it is more difficult to apply colorimetric methods to black tea. To conduct comparative study of black tea, tea leaves were prepared at each stage of

Table 1. Information for green tea, oolong tea, black tea, and hojicha samples used in this study.

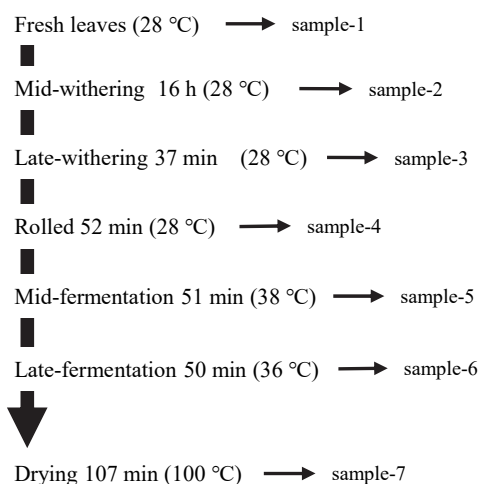
Sample	Collection site
green tea-1	Japan (Kagoshima)
green tea-2	Japan (Kyoto)
green tea-3	Japan (Kyoto)
green tea-4	Japan (Miyazaki)
oolong tea-1	Taiwan
oolong tea-2	Taiwan
oolong tea-3	Taiwan
black tea-1	Japan (Kagoshima)
black tea-2	Japan (Shizuoka)
black tea-3	Japan (Shizuoka)
black tea-4	Japan (Shizuoka)
black tea-5	Sri Lanka
black tea-6	Sri Lanka
black tea-7	Türkiye
hojicha-1	Japan (Nara)
hojicha-2	Japan (Shizuoka)
hojicha-3	Japan (Shizuoka)
hojicha-4	Japan

processing and, the same leaves were followed from the raw material until the final processing into black tea. Using these samples, the influence of the type of black tea leaves can be eliminated, and the effect of black tea processing procedures on the results of both colorimetric methods can be evaluated.

To compare the colorimetric methods, the TPC in the tea samples is analyzed herein using HPLC. Black tea contains theaflavins and thearubigins, which are catechins polymerized by oxidative fermentation. However, the determination of thearubigins by HPLC is not currently possible. Therefore, the sum of the quantified amounts of catechins and theaflavins from HPLC analysis was considered the TPC in the tea samples. In this study, the TPC in black tea leaves determined using both colorimetric methods is compared. Thus, the aim of this study is to clarify the efficacy of both analytical methods for the determination of green and black teas and to propose a universal colorimetric method for determining the TPC in tea leaves.

Materials and Methods

General experimental procedures Absorption data were obtained using a FlexStation 3 Multi-mode Microplate Reader (Molecular Device, Tokyo, Japan). High-performance liquid chromatography (HPLC) analysis was conducted using a PU-2080 Plus intelligent HPLC Pump (Jasco, Tokyo, Japan), a UV-1570 intelligent UV/Vis detector (Jasco), an LG-2080-02 HPLC ternary gradient unit (Jasco), and a Gastorr AG-40 degasser (FLOM, Tokyo, Japan). The data were analyzed using the Chromato-PRO software (LabLab Company, Tokyo, Japan).

**Fig. 2.** Sampling of tea leaves at various stages during preparation of black tea leaves.

Tea samples Collection sites of the tea samples using in this study (green tea, oolong tea, black tea, and hojicha) are shown in Table 1. However, the identification of cultivated species of these teas is difficult because each sample comprises a mixture of different varieties. Tea samples were provided by Y. N., one of the authors, and were stored at room temperature (25–28 °C) and in the dark until use. Figure 2 shows the tea leaf samples at each stage of black tea production using the same fresh leaves (*sei 7132*) sample-1: fresh leaves, sample-2: mid-withering leaves, sample-3: late-withering leaves, sample-4: rolled leaves, sample-5: mid-fermentation leaves, sample-6: late-fermentation leaves, and sample-7: dried leaves. These tea leaf samples (*sei 7132*) were collected from Ikumi Tea Garden in Shimada City, Shizuoka Prefecture, Japan. The leaves were stored in a freezer at −20 °C until use. All samples were extracted using procedures consistent with those described in ISO 14502-1 (Matsushita *et al.*, 2024). First, 50 mL of 70 % methanol was added to 0.2 g of the ground tea samples. The mixture was then stirred and extracted in a thermostatic incubator (Taitec, Tokyo, Japan) at 70 °C for 10 min. The extracted solution was subsequently cooled at 25 °C and centrifuged at 3 500 rpm (Hitachi Industrial Equipment Systems, Tokyo, Japan) for 10 min; the supernatant was carefully decanted. The extraction procedure was repeated twice. The supernatant was concentrated by evaporation and completely dried under nitrogen gas. To eliminate errors due to extraction conditions, all tea extracts used in the Folin-Ciocalteu, iron tartrate, and HPLC analysis methods were samples extracted under the above conditions.

Folin-Ciocalteu method The method described by the ISO (ISO 14502-1, 2005) was used in this study. Tea extracts were prepared in water at a final concentration of 100 µg/mL. For the calibration curve, 1 mg/mL gallic acid (Fujifilm Wako

Pure Chemical, Osaka, Japan) solution was diluted to final concentrations of 10, 20, 30, 40, and 50 $\mu\text{g/mL}$. The absorbance was measured at 765 nm. The TPC was calculated as gallic acid equivalents.

Iron tartrate method The method described in the Standard Tables of Food Composition in the Japan 2020 (8th revision) Analysis Manual¹⁾ were used in this study. Tea extracts were prepared in water at a final concentration of 200 $\mu\text{g/mL}$. To construct the calibration curve, 1 mg/mL ethyl gallate (Tokyo Chemical Industry, Tokyo, Japan) solution was diluted to final concentrations of 10, 20, 30, 40, and 50 $\mu\text{g/mL}$. The absorbance was measured at 540 nm. The TPC was calculated by multiplying the ethyl gallate equivalent by 1.5.

Quantitative analysis of catechins and theaflavins in tea extracts at each stage of black tea production using HPLC Tea extracts were diluted with water at a concentration of 5 mg/mL and passed through a 0.22 μm membrane filter (AS ONE, Osaka, Japan). The catechins and theaflavins in the tea extracts were quantitatively analyzed using HPLC under the following conditions column: Capcell Pak UG 120 C18 (5 μm , 4.6 $\times$ 250 mm, Osaka Soda, Osaka, Japan); flow rate: 1.0 mL/min; solvent A: 0.1 % phosphoric acid in H_2O ; solvent B: acetonitrile and 0.1 % phosphoric acid in H_2O (2:3); gradient conditions: linear gradient from B% (time) = 30 % (0 min) to 75 (25 min) to 100 (35–45 min); and detection wavelength: 280 nm. The temperature of the column oven was set to 40 $^{\circ}\text{C}$ and the injection volume was 10 μL . (–)-epigallocatechin (EGC), (–)-epicatechin (EC), (–)-epigallocatechin gallate (EGCg), and (–)-epicatechin gallate (ECg) (Tokyo Chemical Industry), gallic acid, theaflavin (TF1), theaflavin-3-gallate (TF2a), and theaflavin-3,3'-digallate (TF3) were used as standards for constructing the calibration curves of each compound. The limits of detection (LOD) and quantification (LOQ) of each compound were 0.1 and 0.3 $\mu\text{g/mL}$, respectively (defined as signal-to-noise ratios of 3 and 10, respectively). The tea extracts were independently analyzed three times, and the standard deviation (SD) was calculated. The purity of all compounds separated by HPLC at a wavelength of 280 nm was > 99.6 %.

Statistical analysis The data were expressed as the mean $\pm$ SD ($n = 3$). Statistical differences between the data were assessed by one-way analysis of variance with Tukey's test for quantitative analysis of the TPC ($p < 0.05$).

Results and Discussion

TPCs in green tea, oolong tea, and hojicha from different collection sites were analyzed. Then, the results of the respective samples obtained via the Folin-Ciocalteu method and the iron tartrate method were compared. Some tea samples showed significant differences in TPC values with asterisks by the two colorimetric methods, and some did not (Fig. 3).

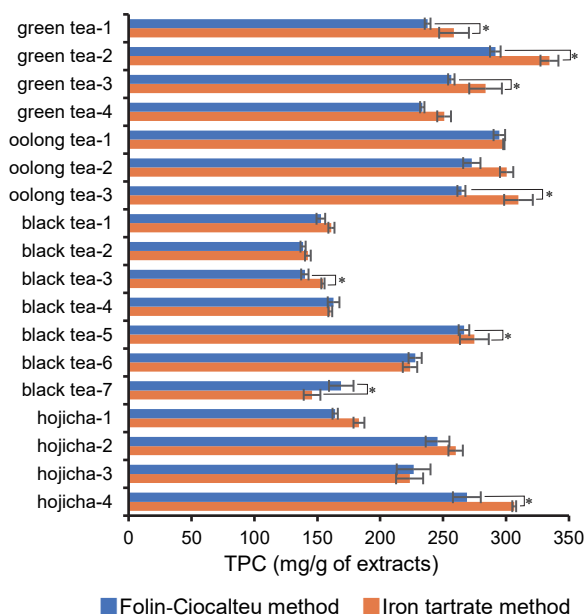


Fig 3. TPC in green tea, oolong tea, black tea, and hojicha extracts by Folin-Ciocalteu and iron tartrate methods. Asterisks (*) indicate significant differences in TPC determined by the Folin-Ciocalteu and tartrate methods for each sample ($p < 0.05$).

Trends indicating that there was not enough difference in the TPC determined by the two colorimetric methods were shown, except for the green tea samples.

Next, the two colorimetric methods were applied to analyze the extracts of the leaves with the respective steps from the fresh leaves to the final products of black tea leaves. Among the three methods (Folin-Ciocalteu, iron tartrate, and HPLC), the iron tartrate method gave the highest TPC values for the respective tea samples with relatively low levels of oxidative fermentation (sample-1 to -3) (Fig. 4). Black tea with low levels of oxidative fermentation indicated a condition similar to that of unfermented green teas. Consequently, this trend was similar to that observed in our previous study on green teas (Matsushita, 2024) and was due to the similarity of the components in the tea samples with low fermentation (sample-1 to -3) to those of green teas. However, in tea samples with advanced fermentation (sample-4 to -7), the total polyphenol values determined by both colorimetric methods were comparable.

As described above, the procedure for tea leaf fermentation affected the TPC determined by both colorimetric methods. Through oxidative fermentation, the catechins in fresh tea leaves are converted into low- and high-molecular-weight polyphenols. Therefore, the polyphenol components in tea leaves plausibly affected the colorimetric measurements. Theaflavins, low-molecular-weight catechin

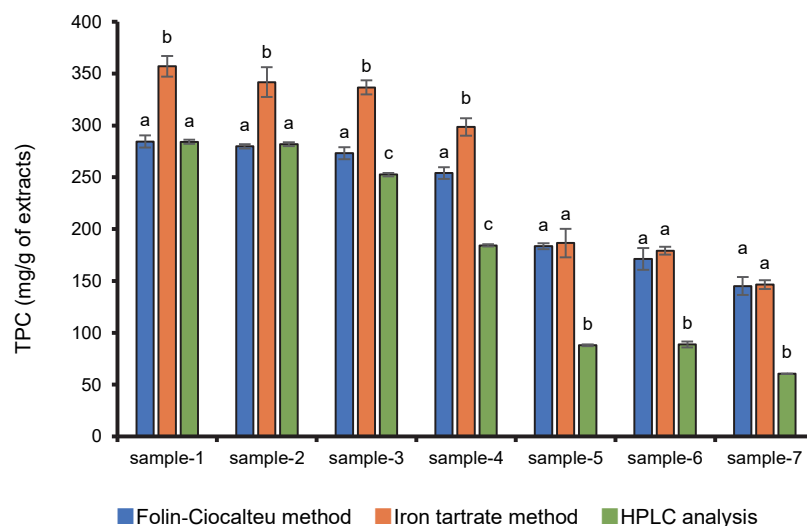


Fig 4. TPC in tea extracts from sample-1 to -7 determined by Folin-Ciocalteu method, tartrate method, and HPLC analysis. Values followed by different letters indicate significant differences in TPC determined by the three methods for each sample ($p < 0.05$).

polymers, are coupled components of o-quinones formed by the oxidation of catechol and pyrogallol (Kusano *et al.*, 2015). On the other hand, the high-molecular-weight polyphenols, thearubigins, are most likely formed from catechins, not theaflavins, as intermediates (Yanase, 2022). The theaflavins and thearubigins are formed competitively, and tea leaves that undergo advanced oxidative fermentation contain more of these components. The Folin-Ciocalteu method involves an indirect color reaction of polyphenols through the reduction of phosphotungstic acid, whereas the iron tartrate method involves a direct color reaction of iron ions and polyphenols. The absorbance of polyphenols complexed with iron ion varies depending on the type of polyphenol (Iwasa and Torii, 1962). In tea samples rich in catechins, the TPC determined using the iron tartrate method was higher than that determined using the Folin-Ciocalteu method, indicating the need to revise the standard substance used as the total polyphenol equivalent.

In contrast, in tea samples with high theaflavin and thearubigin contents, the TPC determined by both colorimetric methods was equivalent. This also indicates the validity of ethyl gallate as a standard substance for analyzing samples rich in theaflavin and thearubigin. The color reaction in the Folin-Ciocalteu method depends on the reducing power of the polyphenols, such as the number of hydroxyl groups and conjugated double bonds. Because the Folin-Ciocalteu method is not affected by anything other than the reducing power of polyphenols in the color reaction, it proposed that the reducing power of the polyphenols was equivalent to the amount of total polyphenols.

The TPC determined by HPLC in sample-5 to -7 was lower than that determined by the Folin-Ciocalteu and iron tartrate methods. This is expected because sample-5 to -7

contained more complex catechin polymers, such as thearubigin, which could not be quantified in this HPLC analysis. Detailed data on thearubigins are still lacking. Further research on thearubigins, which are abundant in fermented teas, such as black tea, is desirable. In this study, two colorimetric methods were used to determine the TPC in tea leaves. The TPC is the sum of individual components, such as catechins, theaflavins, and thearubigins. Polyphenols have different functionalities and activity levels depending on their type. Therefore, the TPC is not directly related to functionality. Notably, although the TPC of sample-1 was the highest in this experiment, this does not mean that the tea leaves of sample-1 have the highest functionality.

In summary, in the determination of TPC in tea leaves, the iron tartrate method was greatly affected by the type of polyphenol in the tea leaves, whereas with the Folin-Ciocalteu method, the reducing power of the polyphenols properly reflected the value of the total polyphenol equivalent. Both colorimetric methods were effective for analyzing the TPC in black tea, which contains high amounts of theaflavin and thearubigin. However, the cost and safety of the reagents used in experiment and the experimental time are also important factors. Therefore, the experimenter must decide which colorimetric method is most appropriate.

Acknowledgements We thank Yukihiro Hara (Tea Solutions, Hara Office Inc., Japan) for valuable suggestions. We also thank Yasuhiko Saito (owner of a tea plantation on a farm in Ikumi Village, Shimada City, Shizuoka Prefecture, Japan) for providing tea samples.

Conflict of interest There are no conflicts of interest to declare.

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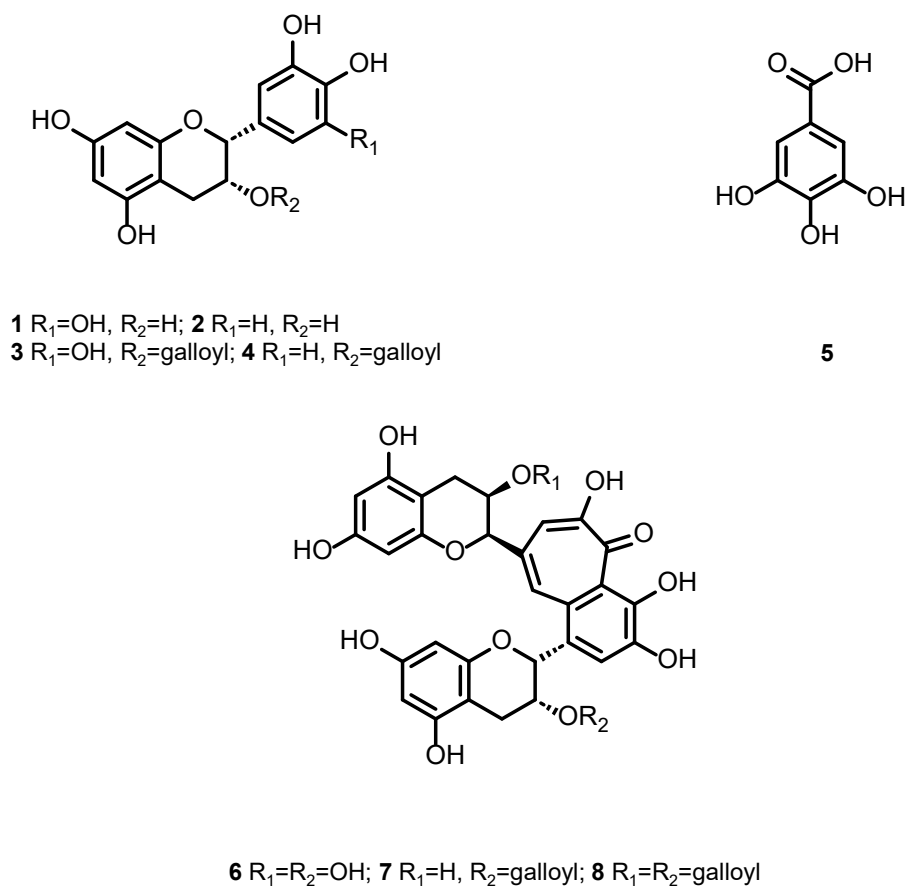


Fig. S1. Structures of catechins, gallic acid, and theaflavins **1**: (–)-epigallocatechin (EGC), **2**: (–)-epicatechin (EC), **3**: (–)-epigallocatechin gallate (EGCg), **4**: (–)-epicatechin gallate (ECg), **5**: gallic acid, **6**: theaflavin (TF1), **7**: theaflavin-3-gallate (TF2a), **8**: theaflavin-3,3'-digallate (TF3).

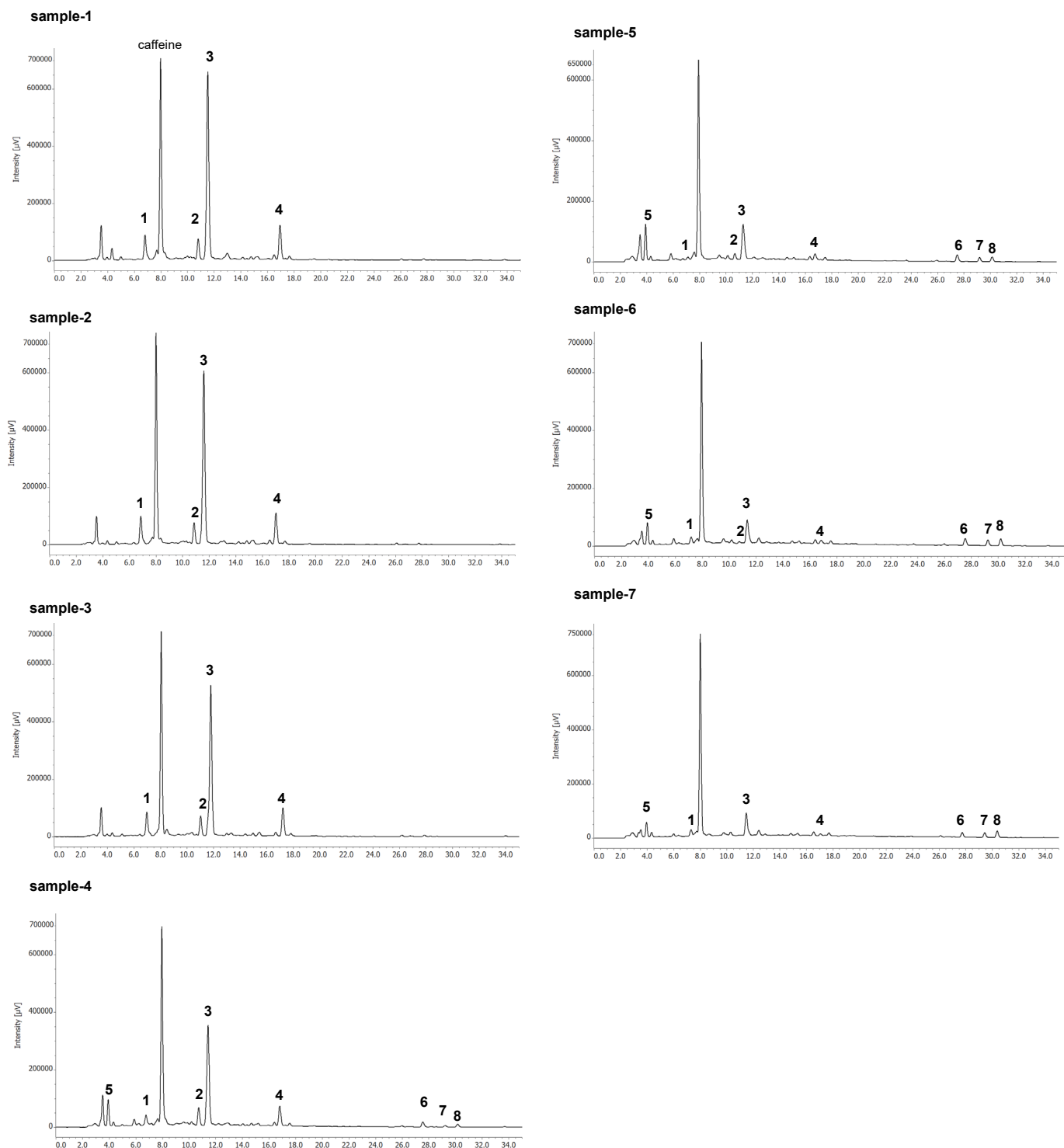


Fig. S2. HPLC chromatograms of the methanol extracts of black tea sample-1 to -7. Peak identification **1**: (–)-epigallocatechin (EGC), **2**: (–)-epicatechin (EC), **3**: (–)-epigallocatechin gallate (EGCg), **4**: (–)-epicatechin gallate (ECg), **5**: gallic acid, **6**: theaflavin (TF1), **7**: theaflavin-3-gallate (TF2a), **8**: theaflavin-3,3'-digallate (TF3).