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Regular Paper

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

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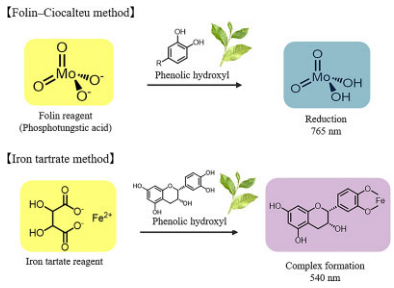
Abstract

Two colorimetric methods are used to determine the total polyphenol contents of tea, namely, "the Folin-Ciocalteu method," defined by the International Organization for Standardization, and the "iron tartrate method," specified in the Standard Tables of Food Composition in Japan. In this study, we compared the Folin-Ciocalteu and iron tartrate methods using green tea extracts. When comparing the 2 methods, the sum of the 4 major catechins measured using high-performance liquid chromatography (HPLC) was regarded as the standard value. The total polyphenol contents obtained using the Folin-Ciocalteu method were closer to the HPLC value than those obtained using the iron tartrate method. However, the iron tartrate method is adequate if the current official method is improved, that is, our results suggest that the coefficients appropriate for common green tea varieties, as well as the degree and duration of cover cultivation, in the official iron tartrate method must be considered.

Keywords: Folin-Ciocalteu method, iron tartrate method, colorimetric method, total polyphenol contents, green tea

Graphical abstract



The values of total polyphenol contents by the Folin-Ciocalteu method were closer to high-performance liquid chromatography values than those by the iron tartrate method.

Tea is a commonly consumed beverage worldwide. Differences in the chemical composition of green, oolong, black tea, and postfermented tea are mainly based on manufacturing methods (Abudureheman *et al.* 2022). Green tea is a popular beverage in Japan and is mainly divided into sencha, gyokuro, and matcha. Sencha is manufactured by plucking, twisting, and drying the tea leaves grown under sunlight. Conversely, tea leaves shaded from sunlight for a certain period are used for gyokuro and matcha. Gyokuro is prepared by rolling and drying plucked tea leaves (Omori 1983; Krahe and Krahe 2022), whereas matcha is made by drying plucked tea leaves without rubbing and grinding them into a powder using a stone mortar (Murakami 2020; Devkota *et al.* 2021).

Green tea is rich in polyphenols, which have high functionality and are believed to promote human health (Chacko *et al.* 2010; Liu, Li and Shen 2020). Polyphenols are compounds that contain numerous phenolic hydroxyl groups in their molecules and are classified into flavonoids, tannins, stilbenoids, and lignans according to their diverse chemical structure (Wisnuwardani *et al.* 2019). Tannins are compounds that possess specific properties to bind to proteins, alkaloids, and metal ions, among which tea catechins are widely known (Tong *et al.* 2022). Catechins are the most important constituents of tea (Almatrood *et al.* 2020). Green tea contains 4 main catechins: (–)-epigallocatechin (EGC), (–)-epicatechin (EC), (–)-epicatechin gallate (ECg), and (–)-epigallocatechin gallate (EGCg) (Sano *et al.* 2001) (Figure S1). Many polyphenols, which include catechins that are abundant in green tea, possess a variety of functional properties (Li *et al.* 2022).

Currently, 2 colorimetric methods are utilized for the analysis of total polyphenol contents of green tea. The first is "the

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Folin-Ciocalteu method," which is defined by the International Organization for Standardization (ISO) as the method for analyzing total polyphenol contents of green and black tea (ISO 14502-1:2005). The colorimetric method is based on reducing phosphotungstic acid in reagents by the phenolic hydroxyl groups of polyphenols. On the other hand, there is the Folin-Denis method that uses the same Folin reagent (Folin and Denis 1915). However, the Folin-Denis method is known to produce turbidity during the operation depending on the type of food, which may interfere with the measurement results. Therefore, the Folin-Ciocalteu method is currently used for the analysis of total polyphenol contents of tea (Nikolaeva, Lapshin and Zagoskina 2022). Another method is the "iron tartrate method," which is specified in the Standard Tables of Food Composition in Japan as the method for determining tannins in green tea (https://www.mext.go.jp/a_menu/syokuhinseibun/index.htm). The iron tartrate method was established for tea in Japan in 1962 (Iwasa and Torii 1962) and was subsequently improved and defined in the Standard Tables of Food Composition in Japan 2020 (8th Revision) Analysis Manual. The iron tartrate method utilizes the quantitative reaction of phenolic hydroxyl groups with iron ions to form complexes. Although it is a tannin determination method, the iron tartrate method is considered for determining the amounts of total polyphenols (Nakabayashi 1988).

Both colorimetric methods (Folin-Ciocalteu and iron tartrate methods) have long been used, particularly the iron tartrate method, which has been applied mainly in Japan. However, the total polyphenol content analysis of green tea has not been standardized internationally; in particular, scientific data comparing both colorimetric methods under the same conditions are not available. Therefore, this study aims to compare the total polyphenol contents of green tea obtained using the 2 colorimetric methods and clarify their characteristics to propose an appropriate method for the analysis of total polyphenol contents of green tea. These experimental results are expected to provide rapid, accurate, and simplified analytical data for green tea based on scientific evidence.

Materials and methods

General experimental requirements

Absorption data were obtained using a FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices, Tokyo, Japan). High-performance liquid chromatography (HPLC) 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). Data were analyzed using ChromNAV ver. 2 software (Jasco).

Green tea samples

Details of the green tea samples used in this study are presented in Table 1. However, the identification of the variety of some gyokuro and matcha samples was difficult as they are a mixture of different varieties (Table 1). Sencha and matcha samples were provided by Y.N., one of the authors, University of Shizuoka (Japan). Gyokuro was purchased online. Matcha was stored in a freezer at −20 °C until use. Sencha and gyokuro were stored at room temperature and in the dark until use. The extraction was performed according to the method described by ISO 14502-1. First, 50 mL of 70% methanol was added to 0.2 g of the ground green 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 3500 1644 ×g (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.

Table 1. Information of green tea samples used in this study

Sample	Collection site	Variety[a]	Year of manufacture
Sencha-1	Shizuoka	Yamakai	2022
Sencha-2	Shizuoka	Yumesuruga	2022
Sencha-3	Shizuoka	Ooiwase	2022
Sencha-4	Shizuoka	Tsuyuhikari	2022
Sencha-5	Shizuoka	Shizukaori	2022
Sencha-6	Shizuoka	Koushun	2022
Gyokuro-1	Shizuoka	–	2022
Gyokuro-2	Kyoto	–	2022
Gyokuro-3	Fukuoka	–	2022
Gyokuro-4	Shizuoka	–	2022
Matcha-1	Kyoto	–	2015
Matcha-2	Mie	–	2015
Matcha-3	Shizuoka	Samidori	2015
Matcha-4	Shizuoka	Gokou	2015
Matcha-5	Shizuoka	–	2015
Matcha-6	Shizuoka	–	2015

[a]Not specified.

Folin-Ciocalteu method

The method described in ISO (ISO 14502-1:2005) was used in this study. Green tea extracts were prepared in water at a final concentration of 100 µg/mL. For the calibration curve, 0.022 g of gallic acid monohydrate was prepared at a concentration of 1 mg/mL by constant volume in 20 mL of water. Subsequently, 1 mg/mL gallic acid solution was diluted to final concentrations of 10, 20, 30, 40, and 50 µg/mL. The green tea extract solution, gallic acid standard solution, and blank (water) were dispensed in 50 µL portions into a 96-well microplate. Next, 50 µL of 10% of the Folin-Ciocalteu reagent (Kanto Chemical, Tokyo, Japan) was added to microplate wells. After 3 min, 50 µL of 7.5% Na_2CO_3 solution was added and stirred. The solutions were placed in the dark at 25 °C for 60 min, and the absorbance was measured at 765 nm. The total polyphenol contents were calculated as gallic acid equivalents.

Iron tartrate method

The method described in the Standard Tables of Food Composition in Japan 2020 (8th Revision) Analysis Manual (https://www.mext.go.jp/a_menu/syokuhinseibun/index.htm) was used in this study. Green tea extracts were prepared in water at a final concentration of 200 µg/mL. For a calibration curve, 0.020 g of ethyl gallate (Tokyo Chemical Industry, Tokyo, Japan) was prepared at a concentration of 1 mg/mL by constant volume in 20 mL of water. Subsequently, 1 mg/mL ethyl gallate solution was diluted to final concentrations of 10, 20, 30, 40, and 50 µg/mL. Iron tartrate reagent was prepared by mixing 100 mg of ferrous sulfate (Kokusan Chemical, Tokyo, Japan) and 500 mg of potassium sodium (+)-tartrate 4-hydrate (Kishida Chemistry, Osaka, Japan) in 100 mL of water. The green tea extract solution, ethyl gallate standard solution, and blank (water) were dispensed in 50 µL portions into a 96-well microplate. Next, 50 µL of iron tartrate reagent was added, followed by 50 µL of 1/15 M phosphate buffer (pH 7.5), and stirred. The absorbance was measured at 540 nm. The total polyphenol

content was calculated by multiplying the ethyl gallate equivalent by 1.5.

Quantitative analysis of catechins in green tea extracts using HPLC

Green 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). Quantitative analysis of catechins in green tea extracts was performed using HPLC under the following conditions: column, Capcell Pak UG 120 C18 (5 μm, 4.6 × 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); and detection wavelength, 280 nm. The temperature of the column oven was set to 40 °C, and the injection volume was 10 μL. EGC, EC, ECg, and EGCg (Tokyo Chemical Industry) were used as standards to obtain calibration curves for each compound. The limits of detection (LOD) and quantification (LOQ) of each compound were 0.1 and 0.3 μg/mL, respectively (defined as signal-to-noise ratios of 3 and 10, respectively). Green tea extracts were independently analyzed 3 times and the SD was calculated. The purity of all compounds separated using HPLC at a wavelength of 280 nm was more than 99.6%.

Quantitative analysis of ascorbic acid in green tea extracts using HPLC

Green tea extracts were diluted with water at a concentration of 5 mg/mL and passed through a 0.22-μm membrane filter (AS ONE). Quantitative analysis of ascorbic acid in green tea extracts was performed using HPLC under the following conditions: column, Capcell Pak UG 120 C18 (5 μm, 4.6 × 250 mm; Osaka Soda); flow rate, 1.0 mL/min; eluent, 0.1% phosphoric acid in H_2O; and detection wavelength, 254 nm. The temperature of the column oven was set to 40 °C, and the injection volume was 10 μL. (+)-Ascorbic acid (Fujifilm Wako Pure Chemical, Osaka, Japan) was used as a standard to obtain a calibration curve. LOD and LOQ of the compound were 0.3 and 1.0 μg/mL, respectively (defined as signal-to-noise ratios of 3 and 10, respectively). Green tea extracts were independently analyzed 3 times, and the SD was calculated.

Statistical analysis

The data were expressed as mean ± SD ($n = 3$). Statistical differences between the data were assessed by one-way analysis of variance with Tukey's test for quantitative analysis of total polyphenol contents ($P < .05$).

Results

Total polyphenol contents of green tea extracts using the Folin-Ciocalteu and iron tartrate methods

Total polyphenol contents in the extracts of (1) sencha, (2) gyokuro, and (3) matcha obtained using the Folin-Ciocalteu and iron tartrate methods and HPLC are shown in Figure 1. The detailed data for each value are listed in Table S1. EGC, EC, EGCg, and ECg accounted for more than 95% of the total catechin content of green tea (Saijo and Takeda 1999). Therefore, the sum of the 4 catechins was regarded as the total polyphenol contents of green tea extracts determined using HPLC. The total polyphenol contents determined by the Folin-Ciocalteu method were equal to or lower than those obtained using HPLC for (1), (2), and (3). In contrast, the total polyphenol contents determined using the iron tartrate method tended to be higher than those obtained using the Folin-Ciocalteu method and HPLC for all samples. In addition, a significant difference in total polyphenol contents was observed between the iron tartrate method and HPLC, particularly in (2) and (3).

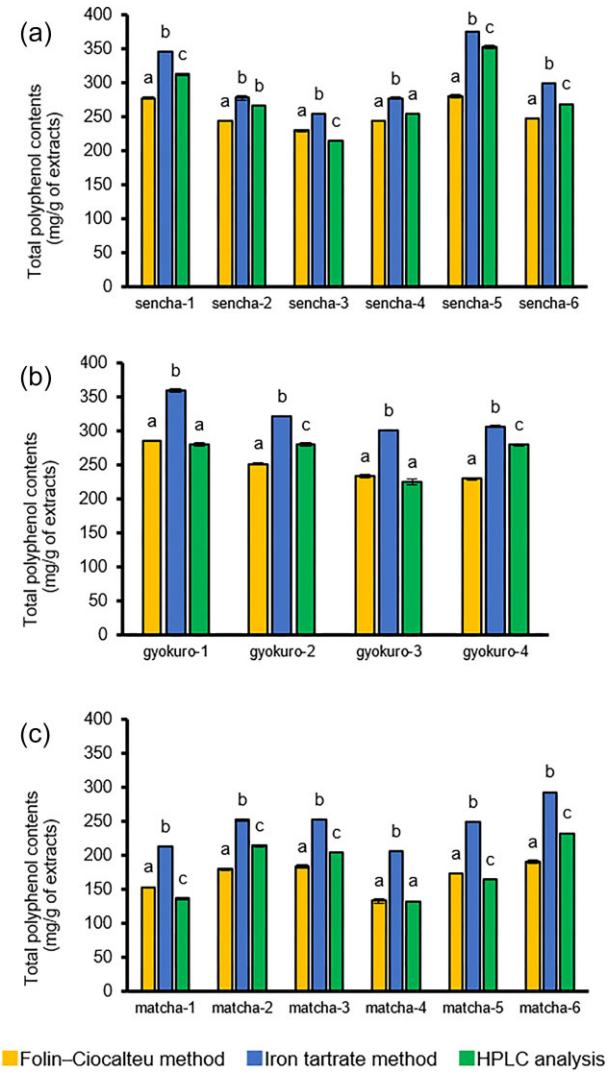

Figure 1. Total polyphenol contents in green tea extracts determined by various methods: (a) sencha; (b) gyokuro; and (c) matcha. Values followed by different letters within each sample are significantly different ($P < .05$).

Composition rates of catechins in green tea extracts

The composition rates of EGC, EC, EGCg, and ECg in the green tea extracts determined using HPLC are shown in Figure 2. The percentages of EGC and EC in sencha were higher than those in gyokuro and matcha. In contrast, the percentages of EGCg and ECg in gyokuro and matcha were higher than those in sencha. The percentages of these 4 catechins vary depending on the type of green tea used.

Influence of ascorbic acid on the Folin-Ciocalteu method

Because the Folin-Ciocalteu method is based on a reduction reaction, the oxidation of substances other than polyphenols has been

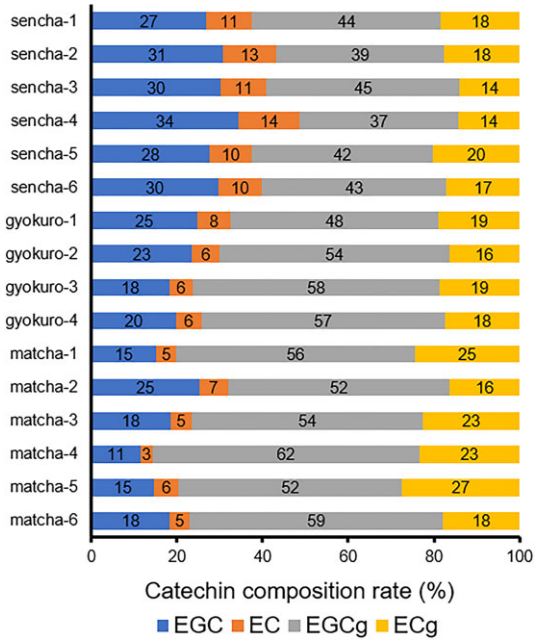

Figure 2. Composition rate of 4 main catechins (EGC, EC, EGCg, and ECg) in green tea extracts determined by HPLC analysis.

Table 2. Ascorbic acid contents in green tea extracts[a]

Sample	HPLC analysis[b] (mg/g of extracts)	Folin-Ciocalteu method (mg/g of extracts)
Sencha-1	7.5 ± 0.00	6.5 ± 0.00
Sencha-2	6.5 ± 0.02	5.9 ± 0.01
Sencha-3	4.5 ± 0.02	4.5 ± 0.02
Sencha-4	6.5 ± 0.04	5.9 ± 0.02
Sencha-5	7.3 ± 0.12	6.4 ± 0.07
Sencha-6	7.1 ± 0.07	6.3 ± 0.04
Gyokuro-1	7.2 ± 0.09	6.3 ± 0.09
Gyokuro-2	2.5 ± 0.04	2.2 ± 0.04
Gyokuro-3	3.3 ± 0.01	3.2 ± 0.01
Gyokuro-4	1.1 ± 0.07	ND
Matcha-1	ND	ND
Matcha-2	ND	ND
Matcha-3	1.4 ± 0.06	ND
Matcha-4	0.5 ± 0.01	ND
Matcha-5	ND	ND
Matcha-6	ND	ND

[a]Each value is the mean ± SD ($n = 3$).
[b]r (correlation coefficient) = 0.99; LOD = 0.1 μg/mL; LOQ = 0.3 μg/mL.
Abbreviation: ND, not determined.

reported to affect the results (Lester *et al.* 2012). Therefore, the effect of ascorbic acid in green tea extracts on the Folin-Ciocalteu method was investigated. First, the ascorbic acid content of green tea extracts was quantified using HPLC (Table 2). Next, the values obtained using the Folin-Ciocalteu method based on the ascorbic acid content of each tea sample were determined (Table 2). The results showed that the ascorbic acid content measured using HPLC and the values obtained using the Folin-Ciocalteu method were similar and low. Sencha's higher value compared to matcha and gyokuro is attributed to the increased production of ascorbic acid in the tea leaves, which results from longer hours of sunlight during the growing process (Nomura, Monobe and Matsuo 2016).

Discussion

In this study, the total polyphenol contents of green tea measured using HPLC were considered the standard value and compared with those obtained using the Folin-Ciocalteu and iron tartrate methods. The total polyphenol contents determined using the Folin-Ciocalteu method was equal to or lower than that determined using HPLC. In contrast, the total polyphenol contents determined using the iron tartrate method were higher than those determined using HPLC.

First, we examined the cause of the high value of total polyphenol contents determined using the iron tartrate method. The current official iron tartrate method is defined as the measured value times 1.5 as the total polyphenol contents of the ethyl gallate equivalent. The value of 1.5 is considered because the absorbance of whole green tea extracts is equal to that of ECg and the absorbance of ethyl gallate multiplied by 1.5 is equal to that of ECg. However, the green tea used when the method was defined was an Assam variety called Benihomare, which has a different catechin composition than the Chinese variety used in this study (Nakagawa and Torii 1964). The Assam variety is generally used as black tea, whereas the Chinese variety is currently consumed as green tea in Japan. Additionally, the absorbance values of EGC, EC, EGCg, and ECg are different (Iwasa, Ota and Torii 1970). The percentage of ECg in total catechins is tended to be higher in the green tea variety than in the black tea variety (Table S2). Therefore, the factor 1.5 determined in the 1970s was appropriate, while it is not suitable for Chinese variety currently consumed for green tea in Japan. In this study, we calculated the total polyphenol contents using the iron tartrate method with various factors (Figure 3). As shown in Figure 3a, the total polyphenol contents determined using the iron tartrate method with a factor of 1.35, not 1.5, were closer to those obtained using HPLC. In addition, the coefficient of 1.5 was not applied to green teas under cover cultivation, such as gyokuro and matcha. The catechin content decreased under cover cultivation. In particular, under cover cultivation, the relative ratio of ECg increases because the EGC content decreases, although the ECg content does not show significant changes (Matsunaga *et al.* 2016). Hence, in the determination of total polyphenol contents using the iron tartrate method, the application of a coefficient value of 1.35 (b-1) or 1.15 (b-2) for gyokuro and 1.2 (c-1) or 1.0 (c-2) for matcha would be close to the values obtained using HPLC (Figure 3). Therefore, in the official iron tartrate method, appropriate coefficients for common green tea varieties, as well as the degree and duration of cover cultivation, must be considered. In addition, when using gallic acid as the standard solution for both colorimetric methods, the total polyphenol contents determined by the iron tartrate method were lower than those obtained using ethyl gallate (Figure S4). The reason for this is considered as the difference in absorbance between gallic acid and ethyl gallate. Therefore, the appropriate standard solution should also be selected according to the polyphenol in the sample.

Next, the effect of ascorbic acid on the Folin-Ciocalteu method was examined. The ascorbic acid content of green tea extracts was low and did not affect the total polyphenol content, as shown in Table 2. Therefore, in green tea extracts, ascorbic acid had no influence on the Folin-Ciocalteu method.

In conclusion, when the 2 official methods are compared, the Folin-Ciocalteu method is considered a more appropriate assay to obtain total polyphenol contents of green tea. Samples other than green tea should use the Folin-Ciocalteu method, considering the ascorbic acid content and the type of polyphenols.

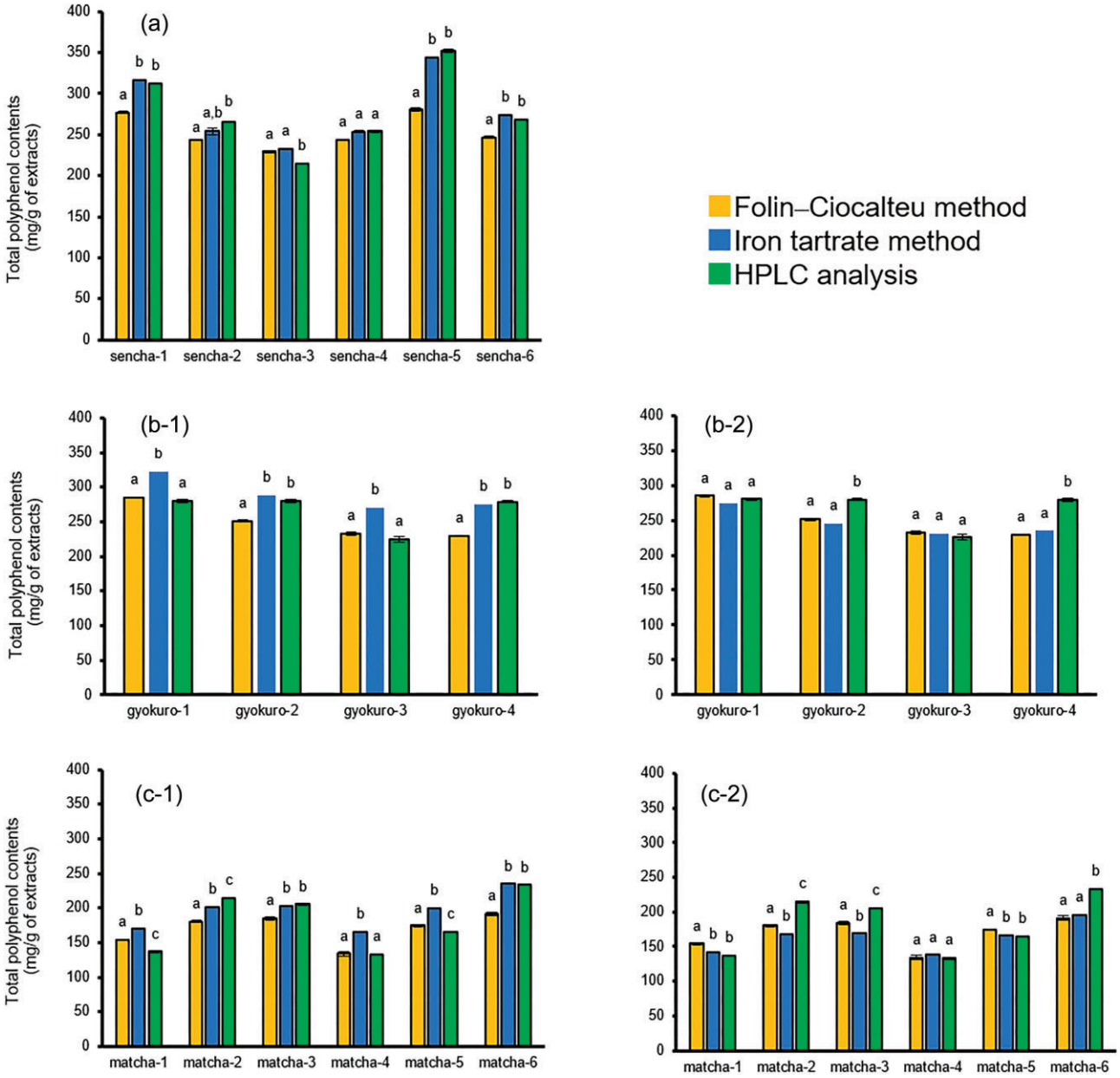

Figure 3. Total polyphenol contents of green tea extracts by various factors applied to ethyl gallate equivalents in the iron tartrate method. The factor multiplied by ethyl gallate equivalents is (a) 1.35 for sencha, (b-1) 1.35 for gyokuro, (b-2) 1.15 for gyokuro, (c-1) 1.2 for matcha, and (c-2) 1.0 for matcha. Values followed by different letters within each sample are significantly different ($P < .05$).

Moreover, our results suggest that in the official iron tartrate method, appropriate coefficients for current common green tea varieties must be considered and fixed according to the degree and duration of cover to adapt them to green tea under cover cultivation. However, the cost and safety of the reagents used in the experiments and the experimental time required to obtain the results are also important factors. Therefore, the use of 2 different colorimetric methods may be necessary to determine the total polyphenol contents of green tea according to the priorities of the measurers.

Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Author contribution

K.M. performed the experiments and wrote the manuscript; C.H, Y.N, and S.K. supervised all the processes in the experiments and the manuscript preparation. All the authors have read and approved the submitted manuscript.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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