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Effect of brewing conditions on polyphenols in the dark tea (*Camellia sinensis L.*) infusions: content, composition and antioxidant activities

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Abstract

The study was the first to investigate the content and composition along with the antioxidant activities of polyphenols in the dark tea infusions. The effect of brewing conditions on total polyphenolic contents (TPC) in dark tea infusions was conducted by response surface methodology (RSM), and the composition of polyphenolic compounds was investigated using ultraperformance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS), and DPPH and ABTS assay were used to evaluate the antioxidant activity of dark tea. Results showed that brewing at water/tea radio 50 : 1 mL/g, temperature 92 °C and time 27 min was the best condition to obtain the highest TPC (3.90 mg/mL). The composition of polyphenolic compounds in the infusions included 11 catechins and derivatives, 19 flavones and flavone glycosides, and 1 phenolic acid, and the concentrations of epigallocatechin gallate (91.32 mg/L) and epicatechin-3-*O*-gallate (23.10 mg/L) was the highest among the quantitative compounds. Moreover, the dark tea had good scavenging activities on DPPH (IC_{50} = 9.94 μg/mL) and ABTS (IC_{50} = 17.26 μg/mL) free radicals.

Keywords: dark tea; polyphenols; composition; antioxidants; UPLC-Q-TOF/MS.

Practical Application: Guiding the brewing conditions of dark tea and lay a foundation for analyzing its health benefits.

1 Introduction

Tea (*Camellia sinensis L.*) is one of the most popular functional beverages globally and rich source of polyphenols (Pang et al., 2022; Zhou et al., 2022). According to the fermentation degree, tea is traditionally categorized into green, white, yellow, oolong, black, and dark tea (Gong et al., 2020). Unlike other types of tea, dark tea is a post-fermented tea with unique sensory and health beneficial characteristics, which is produced by a special piling fermentation involving microorganisms (Zheng et al., 2015). It has become the largest tea variety only after green tea in China, with the total output of more than 387 thousand tons in 2019 (Cheng et al., 2021). Previous studies have pointed out that dark tea has anti-obesity (Liu et al., 2022), anti-tumor (Zheng et al., 2019), anti-diabetic (Wu et al., 2017), anti-inflammatory (Yeh et al., 2022) and other health effects (Lin et al., 2021). Indeed, the antioxidant activities of polyphenols are strongly associated with above health benefits (Yildiz et al., 2021; Zhang et al., 2021).

Polyphenolic compounds proven to have significant contribution to organoleptic and health-promoting properties of food matrices (Pena et al., 2021; Pimpley et al., 2022). There are two groups of interesting polyphenolic compounds present in tea: catechins and flavonols. (-)-epigallocatechin gallate (EGCG) is generally regarded as the major catechins in tea, other ubiquitous catechins are (-)-epicatechin (EC), (-)-Epigallocatechin (EGC), (-)-epicatechin-3-*O*-gallate (ECG) (Wang & Ho, 2009). However, the fermentation process of dark tea converts simple catechins into complex theaflavins or thearubigins, which is responsible for its dark brown color and astringent properties, but they also possess strong antioxidant activity (Kayisoglu & Coskun, 2021). The major flavonols in tea are kaempferol, quercetin and myricetin conjugates, which are normally bound to sugar (Souza et al., 2020). Compared to catechins, the flavonol glucosides induce a mouth-drying, mouth-coating and silkiness sensation at a very low threshold concentration (Scharbert et al., 2004; Wu et al., 2012). Other polyphenolic compounds found in tea are anthocyanidins, leucoanthocyanidin, phenolic acid, and depside.

There is a positive dose-dependent relationship between healthy activities and polyphenol content. The extraction of polyphenols from tea depends on water/tea radio, temperature and time (Yu & He, 2018). Therefore, the preparation of tea is important, as the increase of polyphenol contents in the tea infusions may allow for enhancing scavenging of oxidative radical (Liu et al., 2018). Although some studies have reported that testing for the presence of polyphenols in tea under different infusion conditions, they mainly focused on the conditions

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of temperature and time, or used green tea or black tea as the research object (Liu et al., 2018; Liu et al., 2022). Studying the effect of infusion water/tea radio, temperature and time on polyphenols content and antioxidant properties of dark tea can provide information on how to prepare dark tea most efficiently.

To our knowledge, there is no detailed information on the influence of different infusion conditions on polyphenols and antioxidant capacity of dark tea. Also, there are very few studies on the composition and quantification of dark tea polyphenol compounds. As a result, the objectives of this study were to compare polyphenol contents of dark tea under different infusion conditions and evaluate antioxidant capacities. In addition, the polyphenol compounds in dark tea infusions were identified and quantified to confirm the reason for health benefits.

2 Materials and methods

2.1 Materials and chemicals

The dark tea was provided by Jiangnanchun An-tea Co., Ltd (Huangshan, Anhui province, China) and was crushed into fine powder and kept at -20 °C for later use.

2,2-diphenyl-1-picryl hydrazyl (DPPH), 2,2'-azino-bis-3-ethyl-benzothiazoline- 6-sulfonic acid (ABTS), potassium persulfate, absolute ethanol, gallic acid, Folin-Ciocalteu reagent, and sodium carbonate were obtained from Sinopharm Group Co., Ltd. (Shanghai, China). The standard chemicals, including kaempferol, (-)-epigallocatechin (EGC), (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin gallate (EGCG), and (-)-epicatechin-3-O-gallate (ECG), were purchased from Victory Biological Technology Co., Ltd. (Sichuan, China). Reagents used in this study were of analytical grade.

2.2 Tea infusion preparation

One gram of tea powder was brewed by pure water. The single factor trials for hot water brewing were conducted as follows: Firstly, we studied the effect of water/tea ratio on the extraction yield. The brewing performed for 15 min at 70 °C after different volumes of water (20, 30, 40, 50, 60, 70 mL) were added into a 100 mL-conical flask containing one gram of tea powder. Secondly, the impact of temperature was investigated. 20 mL water was added in tea powder (1 g) and the extraction was conducted for 15 min at different temperature (60, 70, 80, 90, 100 °C). Lastly, the influence of time on the extraction efficiency was investigated. 20 mL water was added in tea powder (1 g) and the extraction was conducted for different times (10, 15, 20, 25, 30 min) at 70 °C. After the brewing was completed, infusions were filtered immediately and analysis. Each sample was repeated three times for analysis.

2.3 Response surface experimental design and analysis

Design-Expert 12 software (State-Ease, Minneapolis, MN, USA) was used for the experimental design, statistical analysis, and regression model. The Box-Behnken design was applied to study the effect of brewing conditions on polyphenols. The coded factors in this work were shown in Table 1. Variables, including water/tea ratio (X_1), temperature (X_2) and time (X_3), were assessed at three proper levels based on above single factor experimental results. A second-order polynomial equation for predicted responses used a previous method (Bezerra et al., 2008).

Table 1. Design and results of Box-Behnken experiments.

Run	A (mL/g)	B (°C)	C (min)	TPC (mg/mL)
1	50 (0)	100 (1)	30 (1)	3.75
2	50 (0)	90 (0)	25 (0)	3.88
3	40 (-1)	90 (0)	20 (-1)	2.95
4	40 (-1)	100 (1)	25 (0)	3.32
5	50 (0)	90 (0)	25 (0)	3.95
6	50 (0)	100 (1)	20 (-1)	3.67
7	50 (0)	80 (-1)	20 (-1)	3.03
8	50 (0)	90 (0)	25 (0)	4.01
9	60 (1)	90 (0)	20 (-1)	3.22
10	40 (-1)	80 (-1)	25 (0)	2.76
11	50 (0)	80 (-1)	30 (1)	3.55
12	60 (1)	80 (-1)	25 (0)	3.34
13	50 (0)	90 (0)	25 (0)	3.91
14	60 (1)	90 (0)	30 (1)	3.65
15	50 (0)	90 (0)	25 (0)	3.82
16	60 (1)	100 (1)	25 (0)	3.47
17	40(-1)	90 (0)	30 (1)	3.49

2.4 Determination of Total Polyphenol Content (TPC)

TPC of tea were determined using modified Folin-Ciocalteu method (Turkmen et al., 2006). In brief, 1.0 mL of test solution was diluted 100 times with deionized water, then 1.0 mL of diluted solution was mixed with 5.0 mL of 10% Folin-Ciocalteu, and then 4.0 mL of 7.5% Na_2CO_3 was added at room temperature in the dark for 1 h. The absorbance of samples was measured at 765 nm using a spectrophotometer (UV-2700, Shimadzu Instrument Co., Ltd, Japan). The concentrations of TPC were calculated from the standard curve ($y = 0.0038 x - 0.0060$, $R^2 = 0.997$) of gallic acid with mg of gallic acid equivalent (GAE) per mL of tea infusions.

2.5 UPLC-Q-TOF/MS analysis of polyphenol compounds

The chromatographic separations were carried using a Waters Acquity UPLC H-Class system (Waters Corp., MA, and the U.S.A.) and a Waters HSS T3 column (1.8 μm, 2.1 × 100 mm) at 25 °C. The UV detection wavelength and the sampling rate were fixed at 231 nm and 20 points s^{-1}, respectively. 0.1% formic acid in water and acetonitrile were used as mobile phases A and B, respectively. The gradient program is shown in Table S1. Prior to the next injection, the mobile phase was reset to 95% A over 1 min and held for 3 min to re-equilibrate the system. 1 μL of samples or standard solutions were injected.

A Waters Xevo G2-XS Q-TOF/MS with electrospray ionization was used for Triple-quadrupole tandem MS under optimized parameters (i.e., capillary and cone voltage of 3.5 kV and 30 V, respectively, source and desolvation temperature of 120 and 500 °C, respectively, desolvation gas flow of 900 L/h). The acquisition was performed in MSE mode at 6 eV and ramp

collision energy from 10 to 25 eV under the optimized ESI+ conditions. Mass spectra were recorded across the range m/z 50 ~ 2000 in modes of positive and negative ion.

Individual compounds were quantified using a calibration curve of the corresponding standard compounds. The precision test was assessed by continuously analyzing five repetitions of tea infusions samples. The stability test was determined by analyzing the same sample solution at 0, 2, 6, 12, 24 h, respectively. The repeatability of the method was determined by analyzing five independently prepared solutions.

2.6 Antioxidant activities

DPPH assay

DPPH radical scavenging activity of tea at 517 nm was determined by (Molyneux, 2004). Briefly, tea was diluted into final volume of 2 mL using absolute ethanol solution (20 ~ 120 μg/mL, and 2 mL of DPPH ethanol solution (0.1 mM) was added and incubated at room temperature in the dark for 30 min. DPPH radical scavenging activity (%) was calculated using Equation 1:

$$DPPH\ \ (\%) = \left(1 - \frac{A_s - A_c}{A_b}\right) \times 100 \quad (1)$$

Where A_s and A_b refers to the absorbance of DPPH ethanol solution + diluted extract and DPPH ethanol solution + absolute ethanol solution, respectively; A_c and V_C are the absorbance of diluted extract + absolute ethanol solution and ascorbic acid (as a reference for comparison), respectively.

ABTS assay

ABTS assay was conducted by previous study (Re et al., 1999) with slight modification. Firstly, ABTS+ solution was obtained by adding the same volume of ABTS (7.4 mM) into potassium persulfate (2.45 mM) at room temperature in the dark for 24 h, and then solution was diluted with absolute ethanol to achieve an absorbance of 0.70 ± 0.05 at 734 nm. Similarly, the extract was diluted with absolute ethanol under various concentrations (20 ~ 120 μg/mL). The ABTS radical scavenging activity (%) was calculated using Equation 2:

$$ABTS\ (\%) = \left(\frac{A_b - A_s}{A_b}\right) \times 100 \quad (2)$$

Where A_s and A_b are absorbance for 0.4 mL of diluted extract and absolute ethanol solution + 3.6 mL ABTS+ solution, respectively.

2.7 Statistical analysis

All experiments were repeated three times, the results were demonstrated by means ± standard deviation. A second-order polynomial equation was used to fit the experimental data. Statistical differences of the results were measured by ANOVA, with the multiple range significant differences (Duncan) test ($p < 0.05$).

3 Results and discussion

3.1 Brewing conditions effects on TPC

In this present study, the dark tea brewing condition of water/tea radio, temperature and time effects on TPC were determined (Figure 1). In the tea infusions, the values of TPC

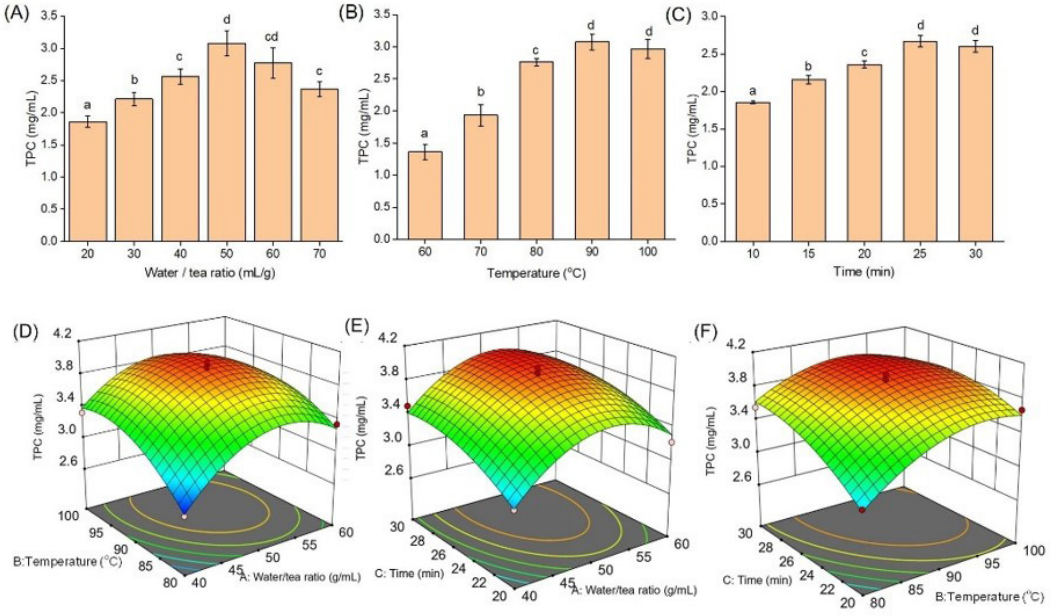

Figure 1. Effect of independent variables (A: water/tea ratio; B: temperature; C: time) on total polyphenol content (TPC) in the dark tea infusions. Response surface (D-F) for the effect of different variables on TPC in the dark tea infusions. The values represent the mean ± SD. The different letters above the bars indicate significant differences test ($p < 0.05$).

increased from 1.86 to 3.08 mg/mL with increasing water/tea radio from 20 to 50 mL/g. However, the decrease of TPC with further increasing water/tea radio. The enhancement of TPC (from 1.36 to 3.07 mg/mL) was observed as the increase of temperature from 60 to 90 °C. Then as temperature continued to rise, and the TPC did not change significantly. In addition, TPC increased with extending time from 10 to 25 min, whereas TPC did not change significantly exceeded 25 min.

Overall, higher brewing temperature, longer brewing time, and proper water/tea radio increased the contents of polyphenol in tea infusions, and consistent with the results of previous researches (Magammana et al., 2019; Vuong et al., 2011).

3.2 Fitting the models

According to the experimental results of single factor test (Figure 1), the best conditions of brewing tea used as central points for the experimental Box-Behnken design were shown in Table 1. The regression model generated the following equation of TPC (Y) as a function of water/tea radio (A), temperature (B), and time (C). $Y = 3.91 + 0.1450A + 0.1913B + 0.1963C - 0.1075AB - 0.0275AC - 0.1100BC - 0.4320A^2 - 0.2595B^2 - 0.1545C^2$

ANOVA analysis showed the significance of quadratic polynomial model. As shown in Table S2, the analysis showed that this model was significant ($p < 0.0001$) with high F-value of 30.70. The "Lack of Fit F-value" of not significant verified the validity of the model. Furthermore, R^2 for all of the responses were 0.9765 and adjusted R^2 were close to R^2, which indicated that this model was fitted well to the test. Moreover, a low value of the coefficient of variation (C.V. = 2.46%) and a high value of the adequately precision (Adeq = 16.9508) indicated the experimental values with a high reliable degree of precision.

The three-dimensional response surfaces and the two-dimensional contour plots illustrated the interaction between independent variables. Generally, the steep of the surface diagram showed that the interactive influence between related variables was significant (Chen et al., 2018). As shown in Figure 1D-1F, the surface diagrams of Figure 1D and Figure 1E were steep, and there was significant interaction between A and C, B and C, and consistent with the results in Table 1.

The optimal conditions for extracting polyphenols were water/tea ratio of 51.22 mL/g, temperature of 92.28 °C, and time of 27.71 min, with the corresponding $Y = 3.99$ mg/mL. To confirm the result, the actual yield of TPC was 3.90 mg/mL. To sum up, this model could be used to predict the release of polyphenols in dark tea infusions.

3.3 Identification of polyphenolic compounds in dark tea infusions

Total ions chromatograms of compounds in dark tea infusions were showed in Figure 2. Identification of polyphenol compounds was listed in Table 2. A total 31 compounds belonging to the polyphenols were identified, namely, 11 catechins and derivatives, 19 flavones and flavone glycoside, and 1 phenolic acid.

Catechins and derivatives

Catechins in tea can be divided into ester type catechins and nonester type catechins. As shown in Table 2, Peak 6, the major ester type catechins in tea infusions, was identified as EGCG (t_R 9.092 min, m/z 457) by comparison with standard, which exhibited characteristic fragmentations ion at m/z 331 $[M\text{-}H\text{-}Bring]^-$, m/z 305 $[M\text{-}H\text{-}C_7H_4O_4]^-$, m/z 287 $[M\text{-}H\text{-}C_7H_4O_4\text{-}H_2O]^-$, m/z 152 ($C_7H_4O_4$), and m/z 125 (Bring). Similarly, the other ester type catechins Peak 7, 8, and 17 were identified as EGCG isomer,

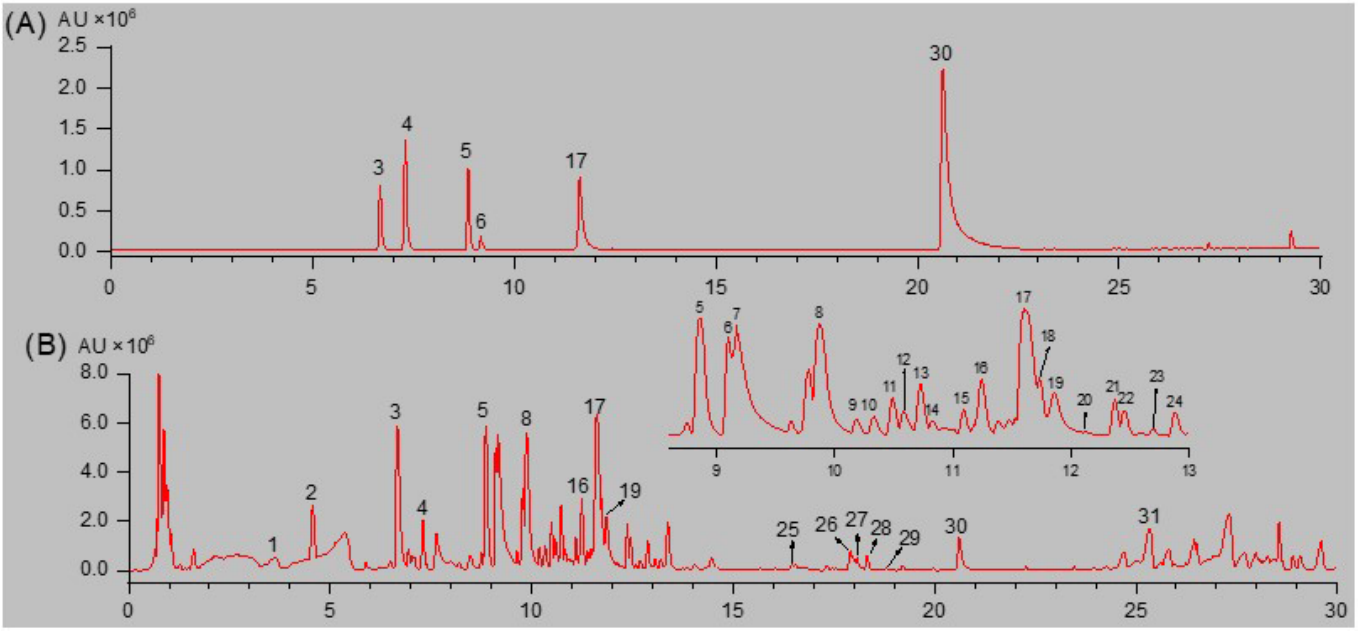

Figure 2. Total ion chromatogram of compounds in dark tea infusions. (A: standards; B: samples). Peak numbers correspond to Table 2.

Table 2. Identification of polyphenol compounds in dark tea infusions by UPLC-Q-TOF/MS.

Peak	Compounds	t_R/min	Cal. m/z $[M-H]^-$	Det. m/z $[M-H]^-$	Major fragments ions m/z	Formula
1	3-*p*-Coumaroylquinic acid	3.508	337.0981	337.0911	295;191;134;93	$C_{16}H_{18}O_8$
2	(+)-Gallocatechin(GC)	4.567	305.0661	305.0662	261; 237; 219; 191; 173	$C_{15}H_{14}O_7$
3	(-)-Epigallocatechin (EGC)*	6.620	305.0661	305.0645	261; 219; 179; 137; 125	$C_{15}H_{14}O_7$
4	(+)-Catechin (C)*	7.2600	289.0715	289.0708	245; 179; 151;137; 108	$C_{15}H_{14}O_6$
5	(-)-Epicatechin (EC)*	8.856	289.0699	289.0697	245; 151;137	$C_{15}H_{14}O_6$
6	(-)-epigallocatechin gallate (EGCG)*	9.092	457.0771	457.0762	331; 305; 287;152;125	$C_{22}H_{18}O_{11}$
7	EGCG isomer	9.156	457.0771	457.0762	331; 305; 287;152;125	$C_{22}H_{18}O_{11}$
8	(-)-Gallocatechin gallate(GCG)	9.786	457.0771	457.0762	331; 305; 287;152;125	$C_{22}H_{18}O_{11}$
9	myricetin 3-*O*-galactoside	10.19	479.0833	479.0826	448;317;282;197	$C_{21}H_{20}O_{13}$
10	myricetin 3-*O*-glucoside (isomyricitrin)	10.34	479.0833	479.0826	448;317; 282;197	$C_{21}H_{20}O_{13}$
11	Quercetin 3-*O*-galactosyl-rhamnosyl-glucoside	10.49	771.2043	771.2014	720; 589; 400;301	$C_{33}H_{40}O_{21}$
12	Quercetin 3-*O*-glucosy-rhamnosyl-glucoside	10.583	771.2043	771.2014	605; 593; 301	$C_{33}H_{40}O_{21}$
13	Quercetin	10.716	301.2397	301.2362	283; 257; 109	$C_{15}H_{10}O_7$
14	Epigallocatechin 3-*O*-(3-*O*-methyl) gallate	10.830	471.0927	471.0904	457; 287; 183	$C_{23}H_{20}O_{11}$
15	2-*O*-Rhamnosylvitexin	11.102	577.1557	577.155	568; 465; 431	$C_{27}H_{30}O_{14}$
16	Kaempferol 3-*O*-galactosyl-rhamnosyl-glucoside	11.249	755.2093	755.2069	726; 609; 568;285	$C_{33}H_{40}O_{20}$
17	(-)-Epicatechin-3-*O*-gallate(ECG)*	11.563	441.0822	441.0797	331; 289; 271; 169	$C_{22}H_{18}O_{10}$
18	Kaempferol-3-*O*-glucosyl-rhamnosyl-glucoside	11.73	755.2093	755.2069	739; 599; 411;285	$C_{33}H_{40}O_{20}$
19	(+)-catechin gallate (CG)	11.846	441.0822	441.0797	415; 289; 169	$C_{22}H_{18}O_{10}$
20	Kaempferol-rhamnosyl-robinoside (robinin)	12.111	739.2144	739.2099	685; 609; 457; 285	$C_{33}H_{40}O_{19}$
21	4'-*O*-Glucosylvitexin	12.372	593.1497	593.1506	525; 441; 431	$C_{27}H_{30}O_{15}$
22	Kaempferol 3-*O*-galactoside (trifolin)	12.451	447.0927	447.0915	401; 349; 285	$C_{21}H_{20}O_{11}$
23	Kaempferol 7-(6″-galloylglucoside)	12.608	599.1037	599.1034	313; 285	$C_{28}H_{24}O_{15}$
24	Kaempferol 3-*O*-glucoside (astragalin)	12.891	447.0927	447.0915	401; 400; 285	$C_{21}H_{20}O_{11}$
25	Theasinensin C	16.493	609.1244	609.1248	493; 467; 457	$C_{30}H_{26}O_{14}$
26	Kaempferol 3-*O*-rutinoside	17.899	593.1497	593.1506	516; 483; 447;301; 285	$C_{27}H_{30}O_{15}$
27	Kaempferol 3-*O*-rutinoside isomer	18.07	593.1497	593.1506	531;483; 447; 301;285	$C_{27}H_{30}O_{15}$
28	Kaempferol 3-*O*-rutinoside isomer	18.299	593.1497	593.1506	549; 497; 301;285	$C_{27}H_{30}O_{15}$
29	Kaempferol 3-*O*-rutinoside isomer	18.781	593.1497	593.1506	551; 457; 447;301;285	$C_{27}H_{30}O_{15}$
30	Kaempferol*	20.606	285.0399	285.0391	255; 151	$C_{15}H_{10}O_6$
31	2-*O*-Rhamnosylvitexin isomer	25.352	577.1557	577.155	505; 476; 431; 309	$C_{27}H_{30}O_{14}$

*Identified by standard substances.

GCG, and ECG in the tea infusions. Furthermore, nonester type catechins included GC (Peak 2), EGC (Peak 3), C (Peak 4), EC (Peak 5) and CG (Peak 19). For example, Peak 3 was identified as EGC (t_R 6.620 min, m/z 305) by comparison with standard, which had $[M-H]^-$ at m/z 261 $[M-H-CO_2]^-$, m/z 219 $[M-H-CO_2-C_2H_2O]^-$, m/z 179 [M-H- Bring]$^-$, and m/z 125 (Bring). Peak 14 and 25 were identified as catechin derivatives according to databases and references. The catechin derivatives identified in this manner were epigallocatechin 3-*O*-(3-*O*-methyl) gallate (Peak 14) and theasinensin C (Peak 25).

Flavones and flavone glycosides

The main flavonoids in tea were kaempferol, quercetin, myricetin and their flavonoid glycosides formed by combining with sugar. Due to the different binding sugars (glucose, rhamnose, galactose, rutose, etc.) and different connecting positions (mostly binding with sugar at the C3 position), various flavonol glycosides were formed (Lakenbrink et al., 2000).

As can be seen in Table 2, Peak 30 was identified as kaempferol (t_R 20.606 min, m/z 285) by comparison with standard, displayed typical fragment ions at m/z 255 $[M-H-CO]^-$, and m/z 151 $[M-H-C_8O_2H_6]^-$. Then, 10 kinds of kaempferol glycoside were identified on the basis of their retention time, MS fragmentation pattern and literature report (Kelebek, 2016; Zhong et al., 2020). Universal kaempferol glycoside deprotonated molecule $[M-H]^-$ showed the typical loss of kaempferol residue $[C_{15}H_{10}O_6-H]^-$, producing an ion at m/z 285. For example, the $[M-H]^-$ ion of kaempferol-3-*O*-galactoside (Peak 22, t_R 12.451 min) produced MS^2 fragment ions at m/z 401 $[M-H-H_2O-CO]^-$ and m/z 285 $[M-H-C_6H_{10}O_6]^-$.

Furthermore, quercetin and quercetin glycosides also were found in tea infusions, Peak 11, 12, and 13, were identified as quercetin-3-*O*-galactosyl-rhamnosyl-glucoside (t_R 10.490 min, m/z 771), quercetin-3-*O*-glucosy-rhamnosyl-glucoside (t_R 10.583 min, m/z 771) and, quercetin (t_R 10.716 min, m/z 301) according to references (Kelebek, 2016; Zhong et al., 2020) and retention time. And two glycosides both had fragment ion at m/z 301 [quercetin-H]$^-$. Quercetin (Peak 13) generated the deprotonated ion at m/z 109, corresponding to the loss of a C-bring residue $[C_6H_6O_5-H]^-$.

Besides, two myricetin glycosides and three vitexin glycosides were also detected in the dark tea based on literature (Kelebek,

2016; Zhong et al., 2020). Myricetin 3-*O*-galactoside (Peak 9), and myricetin 3-*O*-glucoside (Peak 10) had the same fragment ion at m/z 317 $[myricetin-H]^-$, and m/z 282 $[M-H-glu/gal-H_2O]^-$. 2-*O*-Rhamnosylvitexin (Peak 15), 4'-*O*-Glucosylvitexin (Peak 21), and 2-*O*-Rhamnosylvitexin isomer (Peak 31) had typical fragment ion at m/z 431 $[vitexin-H]^-$.

Phenolic acids

Only one phenolic acid was identified in tea infusions, it was Peak 1: 3-*p*-Coumaroylquinic acid (t_R 3.508 min, m/z 337). Peak 1 produced the characteristic fragment ions at m/z 191 $[quinic\ acid-H]^-$, and m/z 93 $[phenol-H]^-$.

3.4 Quantitative analysis of main polyphenols in dark tea infusions

As we all know, ECGC, EGC, ECG and EC are the four most important polyphenol compounds in various teas, and their content in tea infusions determines the quality and biological activity of tea. In this study, the contents of these four substances as well as C and kaempferol were determined, and the quantitative analysis method was validated.

As show in Table 3, regression equation analysis for six compound was performed by taking the peak aera (y) against the concentrations (x, mg/L) of the mixture standard solutions. Good linearity was discovered in the investigated ranges for all the analytes. The relative standard deviation (RSD) values of precision, stability, and repeatability test of the six markers were found in the range of 0.84 ~ 0.91%, 1.45 ~ 3.82%, and 6.10 ~ 10.55%, respectively. Furthermore, the recovery rates of six compound were 97.25 ~ 111.13%. The above results showed that the method was precise, accurate, reproducible and sensitive enough for simultaneously quantitative analyses of the six compounds in the dark tea infusions.

The contents of the six polyphenols in dark tea infusions were summarized in Table 4. The concentration of those were in order of EGCG (91.32 mg/L) > ECG (23.10 mg/L) > EC (17.19 mg/L) > EGC (9.14 mg/L) > C (1.14 mg/L) > Kaempferol (0.27 mg/L). However, He et al. (2022) reported that the main polyphenols concentrations in green tea brewed with boiling water were EGCG (502 mg/L) > EGC (315 mg/L) > ECG (44.3 mg/L) > EC (33.6 mg/L) > Kaempferol (24.3 mg/L) > C (5.93 mg/L). The concentrations of EGCG and ECG in green tea were significantly higher than those in dark tea in this study. The catechins in tea is related to the degree of fermentation, because the fermentation process would convert catechins into theaflavins, thearubigins, etc. Rigling et al. (2021) reported that all the green tea catechins reduced during the fermentation, and the degree of reduction was different from individual catechin and ranged between 9.7 ~ 52.9%. Therefore, green tea, as unfermented tea, may have higher catechin content than fermented tea, including dark tea and black tea.

3.5 The antioxidant activities of dark tea infusions

Most of the health benefits of tea are based on its antioxidant activities. According to the DPPH and ABTS scavenging ability assays, the antioxidant activity was increased with the concentration of dark tea (Figure 3A-3B). When the concentration of dark tea

Table 3. Linearity, recovery, precision, stability, and repeatability for used chemical standards.

Components	Regression equation	R^2	Linear range (mg/L)	RSD (%)			Recovery/% (n = 5)
				Precision (n = 5)	Stability (n = 5)	Repeatability (n = 5)	
EGC	y = 720.40x + 3508.2	0.999	1.032 ~ 103.2	0.84	3.82	7.33	111.13
C	y = 2526.8x + 5991.3	0.999	1.060 ~ 106.0	0.91	2.01	6.10	108.03
EC	y = 2587.1x - 43.966	0.999	1.039 ~ 103.9	0.90	2.40	10.55	102.98
EGCG	y = 4542.0x - 14467	0.999	1.077 ~ 107.7	0.86	1.45	7.55	98.25
ECG	y = 6138.6x + 45546	0.999	1.028 ~ 102.8	0.88	2.15	9.15	97.88
Kaempferol	y = 5489.3x + 1234.5	0.999	0.1041 ~ 104.1	0.89	3.28	8.83	106.43

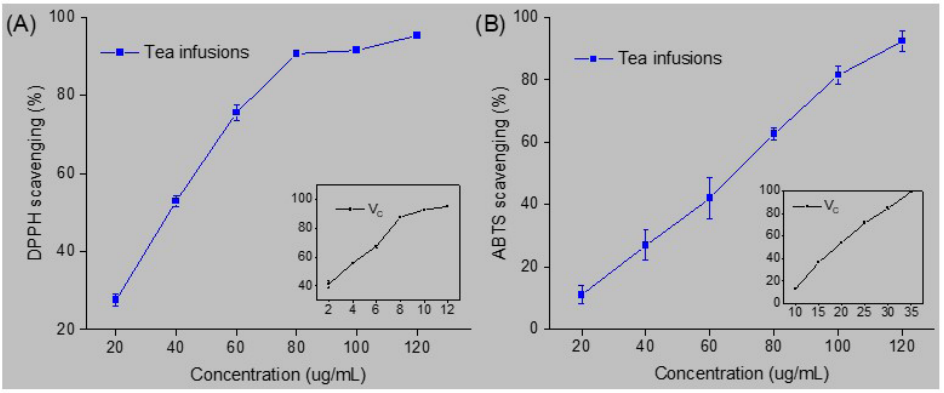

Figure 3. Antioxidant activity evaluated by DPPH (A) and ABTS (B) assays in dark tea infusions.

Table 4. Quantifications of six main polyphenols in dark tea infusions.

Components	Concentration (mg/L)
EGC	9.14 ± 0.28
C	1.14 ± 0.05
EC	17.19 ± 0.55
EGCG	91.32 ± 0.70
ECG	23.10 ± 0.39
K	0.27 ± 0.02

was 120 μg/mL, the DPPH and ABTS scavenging rate both reached the maximum of 95.40% and 92.44%, respectively. In addition, IC_{50}, an important value of the extract concentration required for 50% inhibition of the free radical scavenging, was also used to evaluate the antioxidant activity. The IC_{50} values of DPPH and ABTS scavenging ability assays of dark tea infusions were 38.94 and 66.65 μg/mL, but higher than V_C (DPPH: IC_{50} = 9.94 μg/mL; ABTS: IC_{50} = 17.26 μg/mL). Researchers have verified that polyphenols had the strong antioxidant activity (Anesini et al., 2008; Tagkouli et al., 2022). This result indicated that a positive dose-dependent relationship may was observed between the antioxidant activity with polyphenols of dark tea infusions.

4 Conclusions

After single factor experiment and RSM, optimal infusion conditions for dark tea were set at a water/tea radio 50 : 1 mL/g, temperature 92 °C and time 27 min. Under such conditions, the highest contents of polyphenols (3.90 mg/L) can be extracted into the dark tea brew. Moreover, thirty-one polyphenolic compounds were identified in dark tea infusions, among which 11 catechins and 10 kaempferol glycosides were the most abundant, and the concentration of EGCG (91.32 mg/L), ECG (23.10 mg/L), and EC (17.19 mg/L) were the highest in catechins. In addition, regardless of the antioxidant methods, dark tea showed the most effective scavenging ability, and the antioxidant activity has a positive dose-dependent relationship with the concentration of tea infusions. This study provides experimental evidence for guiding the brewing conditions of dark tea and it is a good source of dietary polyphenolic compounds.

Conflict of interest

The authors declare no conflict of interest.

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References

Anesini, C., Ferraro, G. E., & Filip, R. (2008). Total polyphenol content and antioxidant capacity of commercially available tea (*Camellia sinensis*) in Argentina. *Journal of Agricultural and Food Chemistry*, 56(19), 9225-9229. http://dx.doi.org/10.1021/jf8022782. PMid:18778031.

Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S., & Escaleira, L. A. (2008). Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta*, 76(5), 965-977. http://dx.doi.org/10.1016/j.talanta.2008.05.019. PMid:18761143.

Chen, S., Zeng, Z., Hu, N., Bai, B., Wang, H., & Suo, Y. (2018). Simultaneous optimization of the ultrasound-assisted extraction for phenolic compounds content and antioxidant activity of Lycium ruthenicum Murr. fruit using response surface methodology. *Food Chemistry*, 242, 1-8. http://dx.doi.org/10.1016/j.foodchem.2017.08.105. PMid:29037664.

Cheng, L., Wang, Y., Zhang, J., Xu, L., Zhou, H., Wei, K., Wei, X., Peng, L., Zhang, J., Liu, Z., & Wei, X. (2021). Integration of non-targeted metabolomics and E-tongue evaluation reveals the chemical variation and taste characteristics of five typical dark teas. *LWT*, 150, 111875. http://dx.doi.org/10.1016/j.lwt.2021.111875.

Gong, Z.-P., Ouyang, J., Wu, X.-L., Zhou, F., Lu, D.-M., Zhao, C.-J., Liu, N.-X., Zhu, W., Zhang, J.-C., Li, N.-X., Miao, F., Song, Y.-X., Li, Y.-L., Wang, Q.-Y., Lin, H.-Y., Zeng, X., Cai, S.-X., Huang, J.-A., Liu, Z.-H., & Zhu, M.-Z. (2020). Dark tea extracts: chemical constituents and modulatory effect on gastrointestinal function. *Biomedicine and Pharmacotherapy*, 130, 110514. http://dx.doi.org/10.1016/j.biopha.2020.110514. PMid:32707438.

He, G., Hou, X., Han, M., Qiu, S., Li, Y., Qin, S., & Chen, X. (2022). Discrimination and polyphenol compositions of green teas with seasonal variations based on UPLC-QTOF/MS combined with chemometrics. *Journal of Food Composition and Analysis*, 105, 104267. http://dx.doi.org/10.1016/j.jfca.2021.104267.

Kayisoglu, S., & Coskun, F. (2021). Determination of physical and chemical properties of kombucha teas prepared with different herbal teas. *Food Science and Technology*, 41(Suppl. 1), 393-397. http://dx.doi.org/10.1590/fst.12720.

Kelebek, H. (2016). LC-DAD–ESI-MS/MS characterization of phenolic constituents in Turkish black tea: effect of infusion time and temperature. *Food Chemistry*, 204, 227-238. http://dx.doi.org/10.1016/j.foodchem.2016.02.132. PMid:26988497.

Lakenbrink, C., Lapczynski, S., Maiwald, B., & Engelhardt, U. H. (2000). Flavonoids and other polyphenols in consumer brews of tea and other caffeinated beverages. *Journal of Agricultural and Food Chemistry*, 48(7), 2848-2852. http://dx.doi.org/10.1021/jf9908042. PMid:10898634.

Lin, F.-J., Wei, X.-L., Liu, H.-Y., Li, H., Xia, Y., Wu, D.-T., Zhang, P.-Z., Gandhi, G. R., Li, H.-B., & Gan, R.-Y. (2021). State-of-the-art review of dark tea: from chemistry to health benefits. *Trends in Food Science & Technology*, 109, 126-138. http://dx.doi.org/10.1016/j.tifs.2021.01.030.

Liu, J., Lv, Y., Pan, J., Jiang, Y., Zhu, Y., & Zhang, S. (2022). Effects of tea polyphenols and EGCG on glucose metabolism and intestinal flora in diabetic mice fed a cornstarch-based functional diet. *Food Science and Technology*, 42, e50821. http://dx.doi.org/10.1590/fst.50821.

Liu, Y., Luo, L., Liao, C., Chen, L., Wang, J., & Zeng, L. (2018). Effects of brewing conditions on the phytochemical composition, sensory qualities and antioxidant activity of green tea infusion: a study using response surface methodology. *Food Chemistry*, 269, 24-34. http://dx.doi.org/10.1016/j.foodchem.2018.06.130. PMid:30100430.

Magammana, C. M., Rock, C. R., Wang, L., & Gray, V. (2019). A comparison of the polyphenolic and free radical scavenging activity of cold brew versus hot brew black tea (Camellia Sinensis, Theaceae). *Journal of Food Research*, 8(3), 35-41. http://dx.doi.org/10.5539/jfr.v8n3p35.

Molyneux, P. (2004). The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. *Songklanakarin Journal of Science and Technology*, 26(2), 211-219.

Pang, X., Chen, F., Liu, G., Zhang, Q., Ye, J., Lei, W., Jia, X., & He, H. (2022). Comparative analysis on the quality of Wuyi Rougui (Camellia sinensis) tea with different grades. *Food Science and Technology*, 42, e115321. http://dx.doi.org/10.1590/fst.115321.

Pena, F. L., Souza, M. C., Valle, M. C. P., Bezerra, R. M., Rostagno, M. A., & Antunes, A. E. (2021). Probiotic fermented milk with high content of polyphenols: study of viability and bioaccessibility after simulated digestion. *International Journal of Dairy Technology*, 74(1), 170-180. http://dx.doi.org/10.1111/1471-0307.12735.

Pimpley, V. A., Maity, S., & Murthy, P. S. (2022). Green coffee polyphenols in formulations of functional yoghurt and their quality attributes. *International Journal of Dairy Technology*, 75(1), 159-170. http://dx.doi.org/10.1111/1471-0307.12813.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine*, 26(9-10), 1231-1237. http://dx.doi.org/10.1016/S0891-5849(98)00315-3. PMid:10381194.

Rigling, M., Liu, Z., Hofele, M., Prozmann, J., Zhang, C., Ni, L., Fan, R., & Zhang, Y. (2021). Aroma and catechin profile and in vitro antioxidant activity of green tea infusion as affected by submerged fermentation with Wolfiporia cocos (Fu Ling). *Food Chemistry*, 361, 130065. http://dx.doi.org/10.1016/j.foodchem.2021.130065. PMid:34023683.

Scharbert, S., Holzmann, N., & Hofmann, T. (2004). Identification of the astringent taste compounds in black tea infusions by combining instrumental analysis and human bioresponse. *Journal of Agricultural and Food Chemistry*, 52(11), 3498-3508. http://dx.doi.org/10.1021/jf049802u. PMid:15161222.

Souza, C. C., Oliveira, C. A., Pires, J. F., Pimentel, T. C., Raices, R. S. L., & Nogueira, L. C. (2020). Physicochemical characteristics and sensory acceptance of a mixed beverage based on organic apple juice and cardamom tea (Elettaria cardamomum) with allegation of functional properties. *Food Science and Technology*, 40(Suppl. 2), 669-676. http://dx.doi.org/10.1590/fst.35419.

Tagkouli, D., Tsiaka, T., Kritsi, E., Soković, M., Sinanoglou, V. J., Lantzouraki, D. Z., & Zoumpoulakis, P. (2022). Towards the optimization of microwave-assisted extraction and the assessment of chemical profile, antioxidant and antimicrobial activity of wine lees extracts. *Molecules*, 27(7), 2189. http://dx.doi.org/10.3390/molecules27072189. PMid:35408586.

Turkmen, N., Sari, F., & Velioglu, Y. S. (2006). Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin–Ciocalteu methods. *Food Chemistry*, 99(4), 835-841. http://dx.doi.org/10.1016/j.foodchem.2005.08.034.

Vuong, Q. V., Golding, J. B., Stathopoulos, C. E., Nguyen, M. H., & Roach, P. D. (2011). Optimizing conditions for the extraction of catechins from green tea using hot water. *Journal of Separation Science*, 34(21), 3099-3106. http://dx.doi.org/10.1002/jssc.201000863. PMid:21905216.

Wang, Y., & Ho, C. (2009). Polyphenolic chemistry of tea and coffee: a century of progress. *Journal of Agricultural and Food Chemistry*, 57(18), 8109-8114. http://dx.doi.org/10.1021/jf804025c. PMid:19719133.

Wu, C., Xu, H., Héritier, J., & Andlauer, W. (2012). Determination of catechins and flavonol glycosides in Chinese tea varieties. *Food Chemistry*, 132(1), 144-149. http://dx.doi.org/10.1016/j.foodchem.2011.10.045. PMid:26434273.

Wu, Y.-T., Du, W.-H., Shi, L., Liang, Q., & Zou, X.-Q. (2017). Vasculoprotective effects of water extracts of black, green and dark tea in vitro. *Natural Product Communications*, 12(3), 387-390. http://dx.doi.org/10.1177/1934578X1701200320. PMid:30549892.

Yeh, T.-M., Chang, C.-D., Liu, S.-S., Chang, C.-I., & Shih, W.-L. (2022). Tea seed kaempferol triglycoside attenuates LPS-induced systemic inflammation and ameliorates cognitive impairments in a mouse model. *Molecules*, 27(7), 2055. http://dx.doi.org/10.3390/molecules27072055. PMid:35408453.

Yildiz, E., Guldas, M., & Gurbuz, O. (2021). Determination of in-vitro phenolics, antioxidant capacity and bio-accessibility of kombucha tea produced from black carrot varieties grown in Turkey. *Food Science and Technology*, 41(1), 180-187. http://dx.doi.org/10.1590/fst.00320.

Yu, X. L., & He, Y. (2018). Optimization of tea-leaf saponins water extraction and relationships between their contents and tea (*Camellia sinensis*) tree varieties. *Food Science & Nutrition*, 6(6), 1734-1740. http://dx.doi.org/10.1002/fsn3.724. PMid:30258618.

Zhang, X., Zhu, K., Xie, J., Chen, Y., Tan, L., Liu, S., Dong, R., Zheng, Y., & Yu, Q. (2021). Optimization and identification of non-extractable polyphenols in the dietary fiber of jackfruit (*Artocarpus heterophyllus Lam.*) pulp released by alkaline, acid and enzymatic hydrolysis: content, composition and antioxidant activities. *LWT*, 138, 110400. http://dx.doi.org/10.1016/j.lwt.2020.110400.

Zheng, K., Zhao, Q., Chen, Q., Xiao, W., Jiang, Y., & Jiang, Y. (2019). The synergic inhibitory effects of dark tea (*Camellia sinensis*) extract and p38 inhibition on the growth of pancreatic cancer cells. *Journal of Cancer*, 10(26), 6557-6569. http://dx.doi.org/10.7150/jca.34637. PMid:31777585.

Zheng, W., Wan, X., & Bao, G. (2015). Brick dark tea: a review of the manufacture, chemical constituents and bioconversion of the major chemical components during fermentation. *Phytochemistry Reviews*, 14(3), 499-523. http://dx.doi.org/10.1007/s11101-015-9402-8.

Zhong, J., Chen, N., Huang, S., Fan, X., Zhang, Y., Ren, D., & Yi, L. (2020). Chemical profiling and discrimination of green tea and Pu-erh raw tea based on UPLC–Q–Orbitrap–MS/MS and chemometrics. *Food Chemistry*, 326, 126760. http://dx.doi.org/10.1016/j.foodchem.2020.126760. PMid:32447157.

Zhou, H., Wang, S., Wang, Z., Xie, W., Wang, C., & Zheng, M. (2022). Prepparation, characterization and antioxidant activity of polysaccharides selenides from Qingzhuan dark tea. *Food Science and Technology*, 42, e108421. http://dx.doi.org/10.1590/fst.108421.

Supplementary Material

Supplementary material accompanies this paper.

Table S1. Solvent gradient program of UPLC analysis.

Table S2. Analysis of variances for the developed regression equation.

This material is available as part of the online article from https://www.scielo.br/j/cta