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Development of a validated efficient HPLC-DAD analysis for assessing polyphenol transformation during black tea processing

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ABSTRACT

Tea (*Camellia sinensis*) is valued for its polyphenolic compounds, which define its sensory and health attributes. Accurate quantification across processing stages is hindered by analytical and extraction challenges. We developed and validated a rapid high-performance liquid chromatography with diode array detection (HPLC-DAD) method for simultaneous analysis of 12 key constituents - gallic acid, theobromine, caffeine, (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate (ECG), theaflavin (TF), theaflavin-3-gallate (TF3G), theaflavin-3′-gallate (TF3'G), theaflavin-3,3′-digallate (TF3–3'G), in green and black tea. The method achieved superior linearity ($r^2 > 0.9995$), high sensitivity (LOD: 0.03–1.68 μg/mL), strong precision (RSD < 4.68 %), and high recovery, while also resolving co-elution with a 40-min runtime. Extraction was optimized using ultrasonication with 70 % methanol, which outperformed hot water and ISO-standard methods. Applied to black tea processing, the method revealed a 79.1 % reduction in catechins, post-rolling theaflavin peaks, and dynamic fluctuations in gallic acid, caffeine, and theobromine. These changes were associated with enzymatic oxidation, leaching, and cultivar effects. The validated HPLC-DAD method provides a robust tool for tea polyphenol profiling and enables improved understanding of processing-induced transformations. It holds potential for use in quality control, nutritional labeling, and functional food research in tea and other polyphenol-rich systems.

1. Introduction

Tea (*Camellia sinensis*) is a globally consumed beverage renowned for its intricate flavour nuances, deep-seated cultural heritage, and health-promoting properties. These attributes are largely conferred by a diverse spectrum of polyphenolic compounds and alkaloids, including catechins, theaflavins, gallic acid, caffeine, and theobromine (Chaudhary et al., 2023; Wang et al., 2025). Green tea is characterized by high levels of catechins, such as epigallocatechin gallate (EGCG) and epicatechin gallate (ECG), which contribute to its antioxidant capacity. Conversely, black tea undergoes enzymatic oxidation during processing, resulting in the formation of theaflavins via catechin condensation. This process yields unique sensory characteristics, including colour, briskness, and astringency (Deka et al., 2021). The production of black tea involves a series of stages, including plucking, withering, rolling, cutting, oxidation, and firing. Throughout the oxidation phase, polyphenol oxidases (PPOs) catalyze biochemical modifications that shape the tea's bioactivity and quality (Takeo, 2014; Tanaka and Matsuo, 2020). The variability observed across tea cultivars, terroirs, and processing parameters underscores the necessity for precise analytical techniques capable of monitoring polyphenol transformations in diverse tea types (Li et al., 2021).

The precise quantification of tea's constituents is paramount for ensuring quality, providing accurate nutritional labelling, and functional food development, as polyphenols are the key to the tea's health benefits, such as anti-inflammatory and cardioprotective effects (Sun

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Table 1
Final concentrations (μg/mL) of each compound in the six-level mixed calibration standards.

Compound	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6
Gallic acid	1.8750	3.75	7.5	15	30	60
Theobromine	0.9375	1.875	3.75	7.5	15	30
EGC	0.9375	1.875	3.75	7.5	15	30
Caffeine	4.6875	9.375	18.75	37.5	75	150
Catechin	0.9375	1.875	3.75	7.5	15	30
EC	0.9375	1.875	3.75	7.5	15	30
EGCG	4.6875	9.375	18.75	37.5	75	150
ECG	0.9375	1.875	3.75	7.5	15	30
TF	2.8125	5.625	11.25	22.5	45	90
TF3G	2.8125	5.625	11.25	22.5	45	90
TF3′G	2.8125	5.625	11.25	22.5	45	90
TF3–3′G	2.8125	5.625	11.25	22.5	45	90

Abbreviations: EC = (-)-epicatechin, EGC = (-)-epigallocatechin, EGCG = (-)-epigallocatechin gallate, ECG = (-)-epicatechin gallate, TF = theaflavin, TF3G = theaflavin-3-gallate, TF3′G = theaflavin-3′-gallate, TF3–3′G = theaflavin-3,3′-digallate.

et al., 2022). Although high-performance liquid chromatography with diode array detection (HPLC-DAD) is a well-known analytical technique for quantifying polyphenols because of its sensitivity and specificity, current methods have limitations. These limitations include the co-elution of structurally similar compounds, like catechins and theaflavins, which complicates separation. Additionally, method optimization is needed for specific tea types, reducing the techniques' versatility. Traditional C_{18} columns, commonly used in HPLC-DAD, often result in long run times, reducing sample throughput (Dimcheva et al., 2019; Rha et al., 2021). Ultra-high-performance liquid chromatography offers faster analyses, but its high costs can limit accessibility, emphasizing the need for optimized and cost-effective HPLC-DAD methods (Wang et al., 2025).

Extraction efficiency is another pivotal factor, as the chemically complex matrix of tea, comprising cellulosic structures and diverse metabolites, challenges consistent recovery. Traditional techniques, such as hot water extraction or ISO-standard infusions, often fail to solubilize all bioactives, particularly less-polar compounds like theaflavins (Shaukat et al., 2023). Ultrasonication, combined with solvents like methanol or ethanol, enhances extraction through cavitation, improving solvent penetration and yield (Demesa et al., 2024). However, optimal solvent polarity, temperature, and extraction duration vary by tea type and compound, necessitating systematic optimization to ensure reproducibility across green and black tea matrices (Alshammari et al., 2021; Shaukat et al., 2023).

To fill such gaps, the current study aimed to develop and validate a robust HPLC-DAD method for the simultaneous quantification of 12 key tea constituents of tea (both green and black), including gallic acid, theobromine, caffeine, catechins (C, EC, EGC, EGCG, ECG), and theaflavins (TF, TF3G, TF3′G, TF3–3′G). We also aimed to optimize the extraction process by comparing hot water, ISO-standard, and ultrasonication methods with graded methanol and ethanol solutions. By integrating method validation with extraction optimization and evaluating polyphenol dynamics during black tea processing, this study provides a comprehensive framework for polyphenol profiling, supporting industrial quality control, nutritional research, and academic exploration of processing-induced metabolic transformations.

2. Materials and methods

2.1. Chemicals and instrumentation

High-purity reagents were used for accurate quantification of tea polyphenols and alkaloids. Reference standards, including (+)-catechin, (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG), caffeine, theobromine, gallic acid, theaflavin (TF), theaflavin-3-gallate (TF3G), theaflavin-3′-gallate (TF3′G), and theaflavin-3,3′-digallate (TF3–3′G), were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Phytolab (Vestenbergsgreuth, Germany), meeting analytical or HPLC-grade standards. All standards were of ≥ 98 % purity. HPLC-grade solvents, methanol, ethanol, and acetonitrile, as well as orthophosphoric acid (≥85 %) for mobile phase preparation, were sourced from Fisher Scientific (Fair Lawn, NJ, USA). Ultrapure water was produced using a Milli-Q system (Millipore, Billerica, MA, USA). Polypropylene 50 mL volumetric flasks and 0.22 μm Stericup filter units (Millipore, Billerica, MA, USA) were employed for solution preparation and filtration. Ceylon green and black tea powders purchased from local supermarkets, finely ground with a coffee grinder (Brevelle, New Zealand), were stored in sealed, desiccated containers until analysis. A Fisherbrand ultrasonic water bath (Model 1123, Fisher Scientific, Hampton, USA) was used for ultrasound-assisted extractions.

Chromatographic analyses were conducted using a Vanquish HPLC system (Thermo Fisher Scientific, Waltham, MA, USA), equipped with a binary solvent pump, autosampler, column oven, and diode array detector (DAD). Separation was performed on a Kinetex XB-C_{18} column (100 × 4.6 mm, 2.6 μm particle size, 100 Å pore size; Phenomenex, Torrance, CA, USA), chosen for its high-resolution capabilities and low backpressure (Lee et al., 2018). UV absorbance was monitored simultaneously at 210 nm and 280 nm to capture phenolic acid and flavonoid spectra, respectively (Lee et al., 2018; Wang et al., 2003).

2.2. Preparation of standard solutions

Standard solutions were prepared for HPLC calibration following a modified method based on ISO (2021), with stabilizing agents to prevent oxidation of polyphenols. Accurately weighed amounts (3.000 ± 0.001 mg) of each reference compound—gallic acid, theobromine, EGC, caffeine, EC, EGCG, ECG, TF, TF3G, TF3′G, and TF3–3′G — dissolved in 2 mL acetonitrile with 1 min of ultrasonication. A stabilization solution consisting of 12.5 % (w/v) EDTA and 12.5 % (w/v) ascorbic acid was freshly prepared and added at 10 % (v/v) to all stock standard solutions to prevent oxidative degradation of polyphenols (ISO, 2021). Each was subsequently diluted to 10 mL with HPLC-grade water to yield individual stock solutions at 300 μg/mL. Six levels of mixed calibration standards were prepared by combining appropriate volumes of the individual stock solutions (300 μg/mL) to achieve target concentrations ranging from 0.9375 to 150 μg/mL, as listed in Table 1. Preparation of six-level calibration standards is given in detail in Table S1. Calibration curves were constructed by plotting concentration against peak areas, which are also given in Figure S1.

2.3. HPLC analysis

The HPLC method was designed for the simultaneous separation of twelve tea compounds within a 40-min run time. The mobile phase comprised solvent A (0.1 % v/v orthophosphoric acid in ultrapure water) and solvent B (0.1 % v/v orthophosphoric acid in acetonitrile). The HPLC elution gradient was programmed as follows: starting at 10 % solvent B, increasing to 11 % B over 5 min, then rising to 20 % B over the next 5 min. This was followed by a 6-min hold at 20 % B, an increase to 30 % B over 8 min, and a further rise to 45 % B over 8 min. The gradient then ascended to 90 % B over 2 min, held at 90 % B for 2 min, and concluded with a 4-min re-equilibration to the initial 10 % B condition. The total run time was 40 min. The flow rate was 0.4 mL/min, with a column temperature of 25 °C and an injection volume of 10 μL. Analytes were identified by comparing retention times and UV absorption profiles with standards, ensuring no co-elution, as verified by chromatograms.

2.4. Calibration and validation

The HPLC method was validated to ensure reliability. Calibration curves were evaluated using least-squares regression for each compound

Table 2
Calibration parameters for standard compounds analyzed by HPLC, including retention times, linearity ranges, regression equations, correlation coefficients (r^2), limits of detection (LOD), and limits of quantification (LOQ).

Compound	Retention Time (min)	Range (μg/mL)	Regression Equation	Correlation Coefficient (r^2)	LOD (μg/mL)	LOQ (μg/mL)
Gallic acid	3.75	1.875–60	y = 1.593x + 0.353	0.9997	0.21	0.07
Theobromine	4.75	0.937–30	y = 0.493x + 0.030	0.9998	0.12	0.04
EGC	10.22	0.937–30	y = 1.279x - 0.160	0.9997	0.46	0.15
Caffeine	11.35	4.6875–150	y = 0.576x + 0.129	1.0000	1.68	0.56
Catechin	11.83	0.937–30	y = 0.673x + 0.085	0.9997	0.04	0.01
EC	14.08	0.937–30	y = 0.744x + 0.144	0.9997	0.03	0.01
EGCG	14.86	4.6875–150	y = 0.840x - 0.668	0.9999	0.07	0.02
ECG	19.12	0.937–30	y = 1.005x - 0.006	0.9999	0.05	0.02
Theaflavin	29.69	2.8125–90	y = 0.584x - 0.305	0.9995	0.85	0.28
TF3G	31.05	2.8125–90	y = 0.642x - 0.542	0.9998	0.10	0.03
TF3′G	31.72	2.8125–90	y = 0.412x - 0.159	0.9996	1.67	0.55
TF3–3′G	32.5	2.8125–90	y = 0.398x - 0.612	0.9998	0.89	0.30

Abbreviations: EC = (-)-epicatechin, EGC = (-)-epigallocatechin, EGCG = (-)-epigallocatechin gallate, ECG = (-)-epicatechin gallate, TF = theaflavin, TF3G = theaflavin-3-gallate, TF3′G = theaflavin-3′-gallate, TF3–3′G = theaflavin-3,3′-digallate.

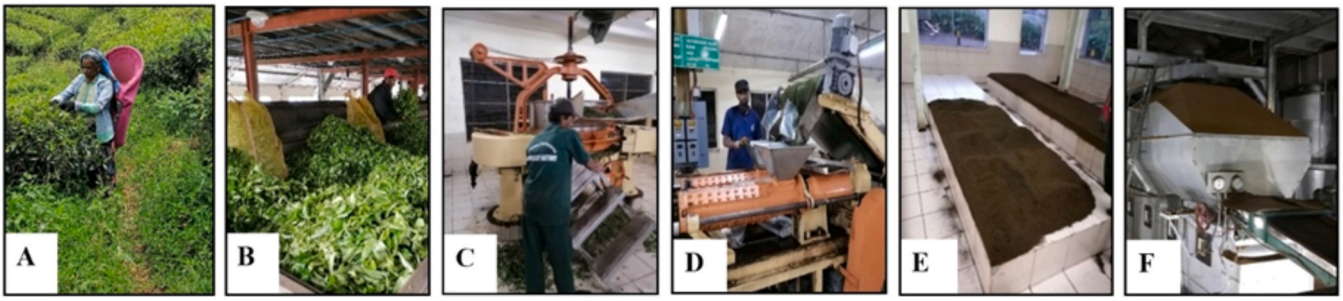

Fig. 1. Black tea processing stages, including plucking (**A**), withering (**B**), Rolling (**C**), Rotovaning (**D**), Oxidation (**E**), and Firing (**F**).

(Table 2). Limits of detection (LOD) and quantification (LOQ) were determined by injecting serial dilutions of standards, based on signal-to-noise ratios of 3:1 and 10:1, respectively (Saadati et al., 2013). Precision was assessed by analyzing six replicate injections of a 150 μg/mL standard mixture within a single day (intra-day) and across three consecutive days (inter-day). Recovery tests were performed by spiking ultrapure water and black tea extract with known analyte concentrations, followed by identical processing to evaluate matrix effects (Xu et al., 2012).

2.5. Extraction procedure and optimization

The dry matter content of both green and black tea samples was determined by measuring the loss in mass at 103 °C on a portion of the test sample, following the ISO 1573 method (ISO, 1980). Extraction methods were optimized to maximize polyphenol yields from both tea types. For ultrasound-assisted extraction, a solid-to-solvent ratio of 1:20 was employed. Specifically, 2.5 g of freeze-dried and finely powdered tea was mixed with 20 mL of solvent, either water or aqueous ethanol/methanol at 20 %, 50 %, 70 %, or 90 % (v/v), and subjected to ultrasonication in an ultrasonic water bath operating at 20 kHz and 60 W for 30 min (Athirojthanakij and Rashidinejad, 2024). The extracts were centrifuged (3500 rpm) for 10 min., and the supernatant was collected into 50 mL tubes. The remaining residue was then re-extracted with an additional 25 mL of the same solvent under the same ultrasonic conditions. The supernatant from the second extraction was combined with the first, filtered through a 0.22 μm Stericup filter, and transferred to a 50 mL volumetric flask. The flask was rinsed with the respective solvent and brought to volume. A 1 mL aliquot of the resulting extract was analyzed by HPLC. For comparison, polyphenols were also extracted using the ISO 14502–1:2005 standard method and traditional hot water extraction (80 °C for 20 min.), with all extractions performed in triplicate with two repeats (n = 6) (Athirojthanakij and Rashidinejad, 2024; ISO, 2005b).

2.6. Dynamics of the major tea polyphenols during black tea processing

Tea leaves (*C. sinensis* (L.) Kuntze) were collected from Somerset Tea Estate, Talawakelle Tea Estates, Sri Lanka (Latitude 6.92403° N, Longitude 80.68446° E). Trained workers, wearing facemasks and gloves, plucked leaves at the "two leaves and a bud" stage, adhering to the standard grade for black tea production as outlined by the Tea Research Institute (Wijeratne, 2003). Freshly plucked leaves were transported in aerated nylon bags to the processing facility and manufactured into black tea using a modified orthodox method, consisting of withering, rolling, rotovane cutting, oxidation (sampled thrice in 45-min intervals), and firing (see Fig. 1). To minimize contamination risks, all handlers wore gloves and masks throughout processing.

Samples were collected from three independent batches at two-month intervals between January and August 2023. Each sample was wrapped in aluminum foil, sealed in sterile polyethylene zip lock bags, transported on dry ice, and stored at −80°C for 24 h. Subsequently, samples were freeze-dried, ground, and stored in aluminum foil bags at −20 °C until analysis (Punyasiri et al., 2015). Each batch contained duplicate samples for each processing stage. With three independent experiments conducted, this resulted in a total of 18 replicates per stage. The dry matter content was determined by loss in mass at 103 °C on a portion of the test sample following the ISO1573 (ISO, 1980) method for all tea samples. Polyphenol extraction was performed using 70 % methanol in an ultrasonic water bath operating at 20 kHz frequency and 60 W power for 30 min (as described in Section 2.5), which was determined to be optimal for quantitative analysis. The total phenolic content (TPC) of each extract was determined using Folin-Ciocalteu's phenol reagent according to ISO 14502–1 (ISO, 2005a) method using a spectrophotometer set at 765 nm. A series of gallic acid standard solutions was prepared with concentrations ranging from 10 to 50 μg/mL.

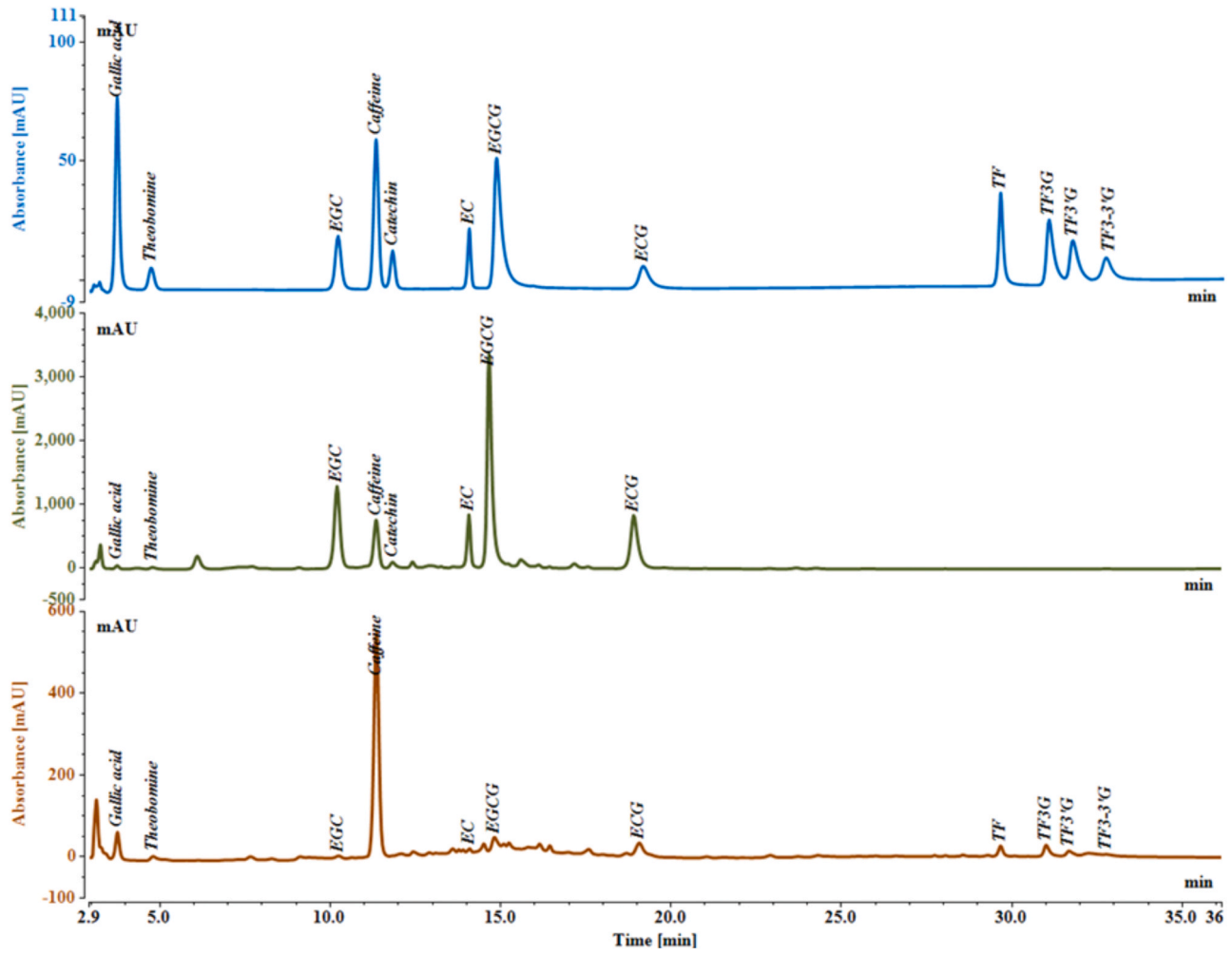

Fig. 2. HPLC-DAD chromatograms of a mixed standard of 12 compounds (A), Green tea extract using 70 % methanol by ultrasonication (B), and Black tea extract using 70 % methanol by ultrasonication (C) at 210 nm. Peaks shown are gallic acid (3.75 min), theobromine (4.75 min), EGC (10.22 min), caffeine (11.35 min), catechin (11.83 min), EC (14.08 min), EGCG (14.86 min), ECG (19.12 min), TF (29.69 min), TF3G (31.05 min), TF3'G (31.72 min), and TF3–3'G (32.50 min). Abbreviations: EC = (-)-epicatechin, EGC = (-)-epigallocatechin, EGCG = (-)-epigallocatechin gallate, ECG = (-)-epicatechin gallate, TF = theaflavin, TF3G = theaflavin-3-gallate, TF3′G = theaflavin-3′-gallate, TF3–3′G = theaflavin-3,3′-digallate.

Table 3
Intra-day and inter-day precision (relative standard deviations; RSD) of the HPLC method in terms of the retention time and peak areas of the 12 standards.

Standards	Precision in retention time		Precision in peak area	
	Intra-day (n = 6) % RSD	**Inter-day (n = 6) % RSD**	**Intra-day (n = 6) % RSD**	**Inter-day (n = 6) % RSD**
Gallic acid	0.12	0.21	1.70	2.75
Theobromine	0.14	0.10	1.94	0.57
EGC	0.05	0.31	1.97	1.37
Caffeine	0.02	0.32	1.84	2.45
Catechin	0.04	0.24	1.14	2.71
EC	0.02	0.08	1.84	2.00
EGCG	0.26	0.38	1.16	2.81
ECG	0.15	0.15	1.96	2.27
Theaflavin	0.08	0.22	3.59	2.51
TF3G	0.10	0.52	2.94	2.86
TF3′G	0.12	0.75	3.33	3.68
TF3–3′G	0.51	0.86	1.70	4.20

Abbreviations: EC = (-)-epicatechin, EGC = (-)-epigallocatechin, EGCG = (-)-epigallocatechin gallate, ECG = (-)-epicatechin gallate, TF = theaflavin, TF3G = theaflavin-3-gallate, TF3′G = theaflavin-3′-gallate, TF3–3′G = theaflavin-3,3′-digallate.

2.7. Statistical and data analysis

Bioactive compound concentrations (gallic acid, theobromine, caffeine, catechins (C, EC, EGC, EGCG, ECG), theaflavins (TF, TF3G, TF3′G, TF3–3G) in green tea (GT) and black tea (BT) were quantified using a validated HPLC-DAD method (Vanquish Core HPLC System) and reported as mean concentrations (mg/g DW ± SD, n = 18). Concentrations across 22 extraction methods (hot water [HW], ultrasonication with water [U_W], ethanol [U_E20–U_E90], methanol [U_M20–U_M90], ISO-standard) and black tea processing stages (FTL, WTL, ROL, RTL, OTL 1–3, FBT) were analyzed in Google Colab using Python 3.10. Data were standardized to z-scores, and principal component analysis (PCA) was performed, generating a biplot visualizing GT (green) and BT (black) samples on PC1 and PC2, with compound loading arrows (orange). A scree plot displayed explained variance ratios. A clustered heatmap (Seaborn's clustermap) visualized z-scores with a blue-white-red colormap, applying hierarchical clustering (Ward's linkage). Pearson correlation coefficients were calculated to assess compound relationships (Section 3.3.1). One-way ANOVA with Tukey's HSD post-hoc test evaluated significant differences across stages and methods ($p < 0.05$). Values below the limit of detection (LOD: 0.03–1.68 μg/mL), denoted as Not Detected (ND), were treated as 0 mg/g for ANOVA, justified by the low LOD and, for theaflavins in FTL, their biological

Table 4
Recovery analysis of key analytes of tea using blank (ultrapure water) and black tea extract (n = 3).

	Ultrapure water			Black tea extract			
Standards	**Spiked amount (ug/mL)**	**Found amount**	**Recovery %**	**Content in the sample**	**Spiked amount**	**Found amount**	**Recovery %**
Gallic acid	50	50.00	100	4.77	50	52.32	95.10
Theobromine	50	50.00	100	3.02	50	50.18	94.32
EGC	50	49.22	98.44	0.46	50	50.04	99.16
Caffeine	50	47.78	99.56	74.85	50	120.95	92.20
Catechin	50	48.80	97.6	1.34	50	50.07	97.46
EC	50	49.92	99.84	3.01	50	51.23	96.44
EGCG	50	45.54	95.08	3.22	50	52.22	98.00
ECG	50	50.00	100	6.01	50	54.32	96.62
Theaflavin	50	44.55	95.10	2.66	50	49.44	93.56
TF3G	50	47.77	95.54	3.49	50	51.67	96.36
TF3′G	50	48.90	97.8	2.32	50	48.76	92.88
TF3–3′G	50	46.32	92.64	2.37	50	44.30	85.86

Abbreviations: EC = (-)-epicatechin, EGC = (-)-epigallocatechin, EGCG = (-)-epigallocatechin gallate, ECG = (-)-epicatechin gallate, TF = theaflavin, TF3G = theaflavin-3-gallate, TF3′G = theaflavin-3′-gallate, TF3–3′G = theaflavin-3,3′-digallate.

Table 5
Comparison of analytical performance parameters between the present HPLC method and previously reported methods.

Parameter	Present Study	Ai et al. (2024)	Peng et al. (2008)	Wang et al. (2010)
No. of Compounds	12	14	14	18
Run time (min)	40	55	45	86
Limit of Detection (LOD)	0.03–1.68 (0.855*) (μg/mL)	0.19–5.14 (2.665*) (μg/mL)	0.2–2.8 (1.5*) ng/μL	0.2–3.21(1.705*) ng/μL
Relative standard deviation (RSD Inter-day, %)	≤ 3.68	≤ 3.48	< 2.81	0.09–4.03
Linearity (r^2)	> 0.9995	> 0.9993	> 0.9994	> 0.9991
Recovery (%)	85.86–100 (91.93)	98.02–102.57 (100.295)	85.56–103.86 (94.71)	85.67–103.47 (94.57)
Column used	Kinetex XB-C_{18}, 100 × 4.6 mm, 2.6 μm	Agilent SB-C_{18}, 250 × 4.6 mm, 5 μm	RP-Amide C_{16}, 150 × 4.6 mm, 5 μm	RP-Amide C_{16}, 250 × 4.6 mm, 5 μm
Detection wavelength	210 & 280 nm	280 nm	210 & 280 nm	210 nm

* Mid-point in parentheses.

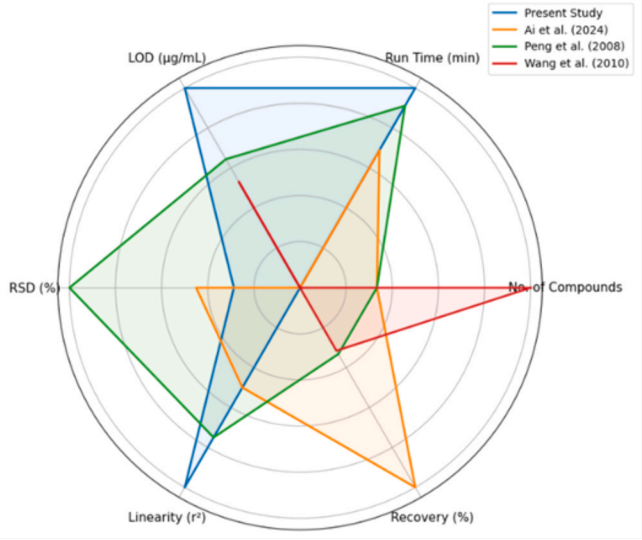

Fig. 3. Radar plot comparing normalized analytical performance of the present HPLC method with previously reported methods.

absence (Li et al., 2012). Statistical analyses ensured robust interpretation of polyphenol dynamics, supporting the method's superiority over hot water and ISO-standard extractions in yield and precision (Section 2.4).

3. Results and discussion

3.1. Separation of tea compounds

The developed HPLC method successfully separated and quantified 12 key compounds in *C. sinensis* leaves across black tea processing stages at Somerset Tea Factory. These compounds included gallic acid, theobromine, EGC, caffeine, catechin, EC, EGCG, ECG, TF, TF3G, TF3′G, and TF3–3′G.

Fig. 2 presents a representative chromatogram of mixed standard, green tea extract, and black tea extract at 210 nm, showcasing well-resolved peaks for all analytes, with retention times ranging from 3.75 min (gallic acid) to 32.50 min (TF3–3′G) (Table 2).

3.1.1. Method validation and performance

Validation confirmed the method's robustness for routine tea analysis. Calibration curves showed excellent linearity (correlation coefficients, $r > 0.9995$) across a 0.9375–150 μg/mL range for all standards (Table 2). Figure S1 illustrates the calibration curves for all tea compounds, exemplifying the method's reliability. Limit of detection (LOD) and limit of quantification (LOQ) values ranged from 0.03 to 1.68 μg/mL and 0.01–0.56 μg/mL, respectively, surpassing those reported by Lee et al. (2018), particularly for EC (LOD: 0.03 μg/mL) and catechin (LOQ: 0.01 μg/mL).

Precision was assessed through intra-day and inter-day variability (Table 3). Relative standard deviations (RSD) for peak areas were below 3.59 % (intra-day) and 4.68 % (inter-day), and retention time RSDs were below 0.51 % (intra-day) and 0.86 % (inter-day), as shown in Table 3, improving on Lee et al. (2018) (RSD < 5.2 %). Recovery rates, as shown in Table 4, ranged from 92.64 % to 100 % in ultrapure water and 85.86–99.16 % in black tea extract, comparable to Wang et al. (2010) (85–98 %) but covering more compounds. TF3–3′G showed the

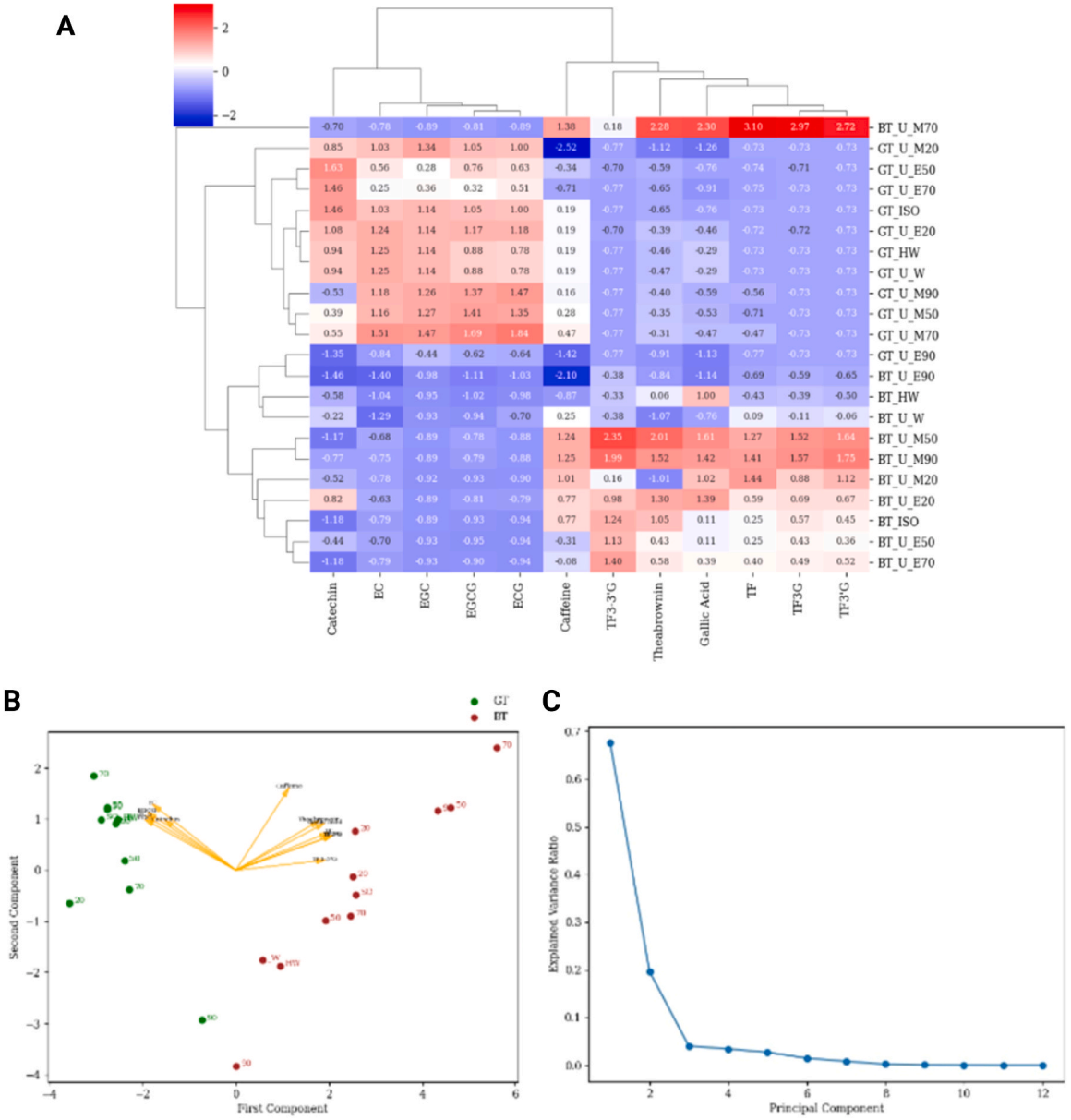

Fig. 4. Analytical visualizations of bioactive compound concentrations in green tea (GT) and black tea (BT) across 22 extraction methods. (A) Clustered heatmap of standardized concentrations (z-scores). Rows represent extraction methods: GT (green tea), BT (black tea); HW (hot water); U_W (ultrasonication with water); U_E20–U_E90 (ultrasonication with ethanol, 20–90 %); U_M20–U_M90 (ultrasonication with methanol, 20–90 %); ISO (International Organization for Standardization method). Columns represent compounds: Gallic Acid, Theobromine, EGC, Caffeine, Catechin, EC, EGCG, ECG, TF, TF3G, TF3'G, TF3–3′G. Color scale: blue = below-average, white = average, red = above-average concentrations. Hierarchical clustering groups similar methods and compounds. (B) PCA biplot of standardized concentrations. Points represent samples (GT: green; BT: black) projected onto the first two principal components. Orange arrows indicate compound loadings. (C) Scree plot showing the proportion of variance explained by each principal component. The elbow point indicates components of statistical significance[1] . [1]Statistical significance determined using the elbow criterion or eigenvalue threshold.

lowest recovery (85.86 %) in black tea, likely due to matrix effects, a common issue noted by Peng et al. (2008).

3.1.2. *Method comparison with previously published methods*

The comparison of analytical methods from the present HPLC method and those reported in similar previous publications, including Ai et al. (2024), Peng et al. (2008), and Wang et al. (2010), is summarized in Table 5 and visualized in Fig. 3. Six parameters were evaluated: number of compounds, run time, LOD, inter-day RSD, linearity (r^2), and recovery. LOD values were converted to μg/mL for consistency (1 ng/mL = 0.001 μg/mL), and parameters were normalized (0−1) for the radar chart, with run time, LOD, and RSD inverted so higher values reflect better performance.

Based on the data presented in Table 5, our present new HPLC method analyzed the fewest compounds (12) but achieved the shortest run time (40 min), demonstrating high efficiency. This performance is primarily attributed to the use of a Kinetex XB-C_{18} column (2.6 μm), which enhances chromatographic resolution by minimizing band broadening and improving mass transfer. Additionally, its eXtra Base (XB) deactivation chemistry reduces peak tailing, especially for structurally diverse tea polyphenols and alkaloids, while maintaining compatibility with acidic mobile phases such as 0.1 % orthophosphoric acid. These characteristics collectively enabled rapid, high-resolution separation of multiple analytes within a short runtime.

In contrast, Wang et al. (2010) analyzed most compounds (18) but required the longest run time (86 min), reflecting complex and

Table 6
Major polyphenolic content during black tea processing (mg/g DW ± SD, n = 18).

Processing Stage	FTL	WTL	ROL	RTL	OTL 1	OTL 2	OTL 3	FBT
Gallic acid	15.10 ± 0.06[a]	6.89 ± 0.04[d]	9.50 ± 0.06[b]	9.18 ± 0.12[b]	4.14 ± 0.07 [f]	5.41 ± 0.08[e]	4.47 ± 0.08 [f]	4.88 ± 0.08[e]
Theobromine	4.42 ± 0.04[a]	4.26 ± 0.03[a]	4.79 ± 0.03[c]	3.50 ± 0.04[d]	3.60 ± 0.06[d]	3.40 ± 0.05[d]	3.67 ± 0.06[d]	3.08 ± 0.05[e]
EGC	32.77 ± 0.14[a]	17.33 ± 0.11[b]	18.66 ± 0.12[b]	1.07 ± 0.01[c]	0.27 ± 0.00[d]	2.00 ± 0.03[c]	0.90 ± 0.02[c]	0.47 ± 0.01[d]
Caffeine	101.70 ± 0.54[a]	101.62 ± 0.69[a]	98.51 ± 0.68[c]	95.89 ± 1.20[d]	94.77 ± 1.49[e]	96.89 ± 1.28 [f]	95.70 ± 1.45 [g]	95.67 ± 1.47 [g]
Catechin	16.02 ± 0.07[a]	6.77 ± 0.04[b]	6.99 ± 0.04[b]	3.26 ± 0.04[c]	2.03 ± 0.03[d]	1.37 ± 0.02[e]	1.01 ± 0.02 [f]	1.37 ± 0.02[e]
EC	42.65 ± 0.18[a]	23.92 ± 0.14[b]	31.34 ± 0.20[c]	13.00 ± 0.16[d]	4.12 ± 0.07[e]	4.00 ± 0.06[e]	2.80 ± 0.05 [f]	3.07 ± 0.05 [f]
EGCG	186.66 ± 0.79[a]	111.68 ± 0.69[b]	88.57 ± 0.57[c]	9.31 ± 0.12[d]	7.65 ± 0.03[e]	5.80 ± 0.20[d]	4.70 ± 0.04[e]	3.29 ± 0.06[e]
ECG	50.26 ± 0.21[a]	31.25 ± 0.19[b]	34.70 ± 0.22[b]	14.25 ± 0.18[c]	8.80 ± 0.14[d]	9.99 ± 0.15[d]	7.35 ± 0.13[e]	6.13 ± 0.10 [f]
Theaflavin	ND[e]	4.29 ± 0.03[b]	6.21 ± 0.04[a]	2.99 ± 0.04[c]	1.68 ± 0.03[d]	3.46 ± 0.05[c]	2.70 ± 0.05[c]	2.72 ± 0.05[c]
TF3G	ND[e]	2.94 ± 0.02[a]	3.67 ± 0.02[a]	3.43 ± 0.04[a]	2.71 ± 0.04[b]	3.36 ± 0.05[a]	3.06 ± 0.05[a]	3.57 ± 0.06[a]
TF3′G	ND[e]	2.26 ± 0.01[b]	4.25 ± 0.03[a]	2.79 ± 0.03[b]	1.62 ± 0.03[c]	2.73 ± 0.04[b]	2.34 ± 0.04[b]	2.32 ± 0.04[b]
TF3–3′G	ND[e]	2.91 ± 0.01[b]	1.84 ± 0.03[c]	3.25 ± 0.03[b]	2.71 ± 0.03[c]	1.45 ± 0.04[d]	1.42 ± 0.04[d]	2.37 ± 0.04[b]
TPC (GAE mg/g DW)	831.73 ± 23.32[a]	436.22 ± I7.54[c]	554.08 ± 18.65[b]	517.25 ± 20.05[b]	497.96 ± 10.61[c]	391.74 ± 9.37[d]	505.61 ± 19.77[b]	374.18 ± 8.44[d]

Data show concentrations (mg/g DW ± SD, n = 18). ND = Not Detected (below LOD, treated as 0 mg/g for ANOVA and Tukey's HSD). Superscripts ([a–g]) denote significant differences within rows ($p < 0.05$). LODs for theaflavins range from 0.03–1.68 μg/mL.

comprehensive separations. Peng et al. (2008) achieved the lowest midpoint LOD (1.5 ng/μL), leveraging an RP-Amide C_{16} column and 210 nm detection, ideal for trace analysis. Meanwhile, Ai et al. (2024) reported the highest LOD (2.665 μg/mL), possibly due to 280 nm detection, which may be less sensitive for certain analytes.

In terms of precision, Peng et al. (2008) showed the best inter-day reproducibility (RSD < 2.81 %), while Wang et al. (2010) displayed the widest RSD range (0.85–4.03 %), suggesting less consistent repeatability across compounds. All studies exhibited excellent linearity (> 0.999), with the present study outperforming others (> 0.9995), indicating robust quantification over a wide concentration range. Recovery rates varied, with Ai et al. (2024) reporting the highest (100.295 %), while the present study had the lowest (91.93 %, mid-point), suggesting potential matrix interference.

The radar chart (Fig. 3) visualizes and compares the normalized analytical performance of each method across key parameters. The data was normalized on a 0–1 scale, with lower values inverted where appropriate (e.g., run time, LOD, RSD) to ensure the higher values consistently represent better performance. The chart illustrates that the present study demonstrated superior performance in terms of run time, linearity, speed, and precision, making it well-suited for high-throughput, targeted polyphenol profiling. Peng et al. (2008) led in sensitivity and reproducibility, ideal for trace analysis. Wang et al. (2010) demonstrated strengths in compound coverage, while Ai et al. (2024) prioritized high recovery but at the cost of sensitivity. These trade-offs primarily stem from the choice of column type and detection wavelength. The use of a modern column in the present study enhanced efficiency, while Peng et al.'s selection of a 210 nm detection wavelength improved sensitivity.

Overall, the present method is optimal for rapid and reproducible screening of tea polyphenols. Methods like Peng et al. (2008) are better suited for trace-level quantification, while Wang et al. (2010) offer breadth in analyte coverage. Ai et al. (2024) may be preferred in applications emphasizing analyte recovery. Future analytical approaches could benefit from integrating these strengths to develop a more versatile and balanced method.

3.2. Optimization of extraction methods

The analysis of the standardized dataset, comprising 12 bioactive compounds – gallic acid, theobromine, EGC, caffeine, catechin, EC, EGCG, ECG, TF, TF3G, TF3′G, TF3–3′G–across 22 extraction methods for green tea (GT) and black tea (BT), offers comprehensive insights into compound concentrations and interrelationships. The methods include hot water (HW), ultrasonication with water (U_W), ethanol (20–90 %) (U_E20–U_E90), methanol (20–90 %) (U_M20–U_M90), and the ISO method (ISO, 2005b). Table S2 describes the values of total catechins, total theaflavins, caffeine, and gallic acid yield values for different extraction methods and conditions. Fig. 4 integrates three visualizations: a clustered heatmap (Fig. 4A), a scree plot (Fig. 4B), and a PCA biplot (Fig. 4C), offering a consolidated view of data trends collected in this experiment.

The heatmap (Fig. 4A), using a blue-white-red colormap (blue for low z-scores, white for near-mean, red for high z-scores), reveals distinct concentration patterns. GT methods show high catechin levels (red), particularly with methanol-based ultrasonication (e.g., GT_U_M70: 1.99 for EGCG, 1.65 for ECG), indicating effective extraction of polyphenols, aligning with findings that polar solvents enhance catechin yield (Wang et al., 2000). Theaflavins (TF, TF3G) are negligible (blue) in GT (e.g., 0 for TF3G in GT_U_M70), consistent with GT's unfermented nature, which limits theaflavin formation (Harbowy et al., 1997). BT methods exhibit elevated theaflavins (e.g., BT_U_M70: 2.31 for TF, 2.07 for TF3G) with methanol (U_M50–U_M70), reflecting oxidation-driven catechin conversion (Liang et al., 2003).

The PCA biplot (Fig. 4B) complements these findings, with GT methods (green) clustering on the left, driven by catechins (EGCG, EGC), and BT methods (black) on the right, influenced by theaflavins (TF, TF3G) and caffeine. Orange arrows indicate compound contributions, with EGCG and TF showing strong influence on GT and BT, respectively. BT methods are more dispersed, suggesting variability due to oxidation, while GT methods cluster tightly, indicating consistency. Outliers like GT_U_E90 and BT_U_E90, near the origin, reflect poor extraction at 90 % ethanol. The scree plot (Fig. 4C) supports this, showing the first two principal components explain most variance (likely ~80 %), with a sharp eigenvalue drop after PC2, justifying the 2D biplot representation.

Methanol and ethanol ultrasonication (50–70 %) outperform HW and ISO methods, likely due to enhanced solvent penetration and cavitation, disrupting tea matrices (Pan et al., 2003). The decline at 90 % solvent (e.g., GT_U_E90) may indicate degradation, a known issue in high-polarity extractions (Xu et al., 2012). The GT-BT contrast underscores processing impacts: GT retains catechins, while BT favors theaflavins, affecting antioxidant properties (Cabrera et al., 2006). Optimizing extraction (e.g., 70 % methanol for BT theaflavins) could enhance yields.

3.3. Dynamics of the major tea polyphenols during black tea processing

Black tea processing transforms *Camellia sinensis* leaves through enzymatic and oxidative reactions, reshaping polyphenol profiles critical to sensory and health attributes (Tanaka and Matsuo, 2020). This section quantifies the dynamics of 12 key compounds, catechins (C, EC, EGC, EGCG, ECG), theaflavins (TF, TF3G, TF3′G, TF3–3′G), gallic acid, theobromine, and caffeine—across processing stages: fresh tea leaves (FTL), withered tea leaves (WTL), rolled leaves (ROL), rotovaned (cut)

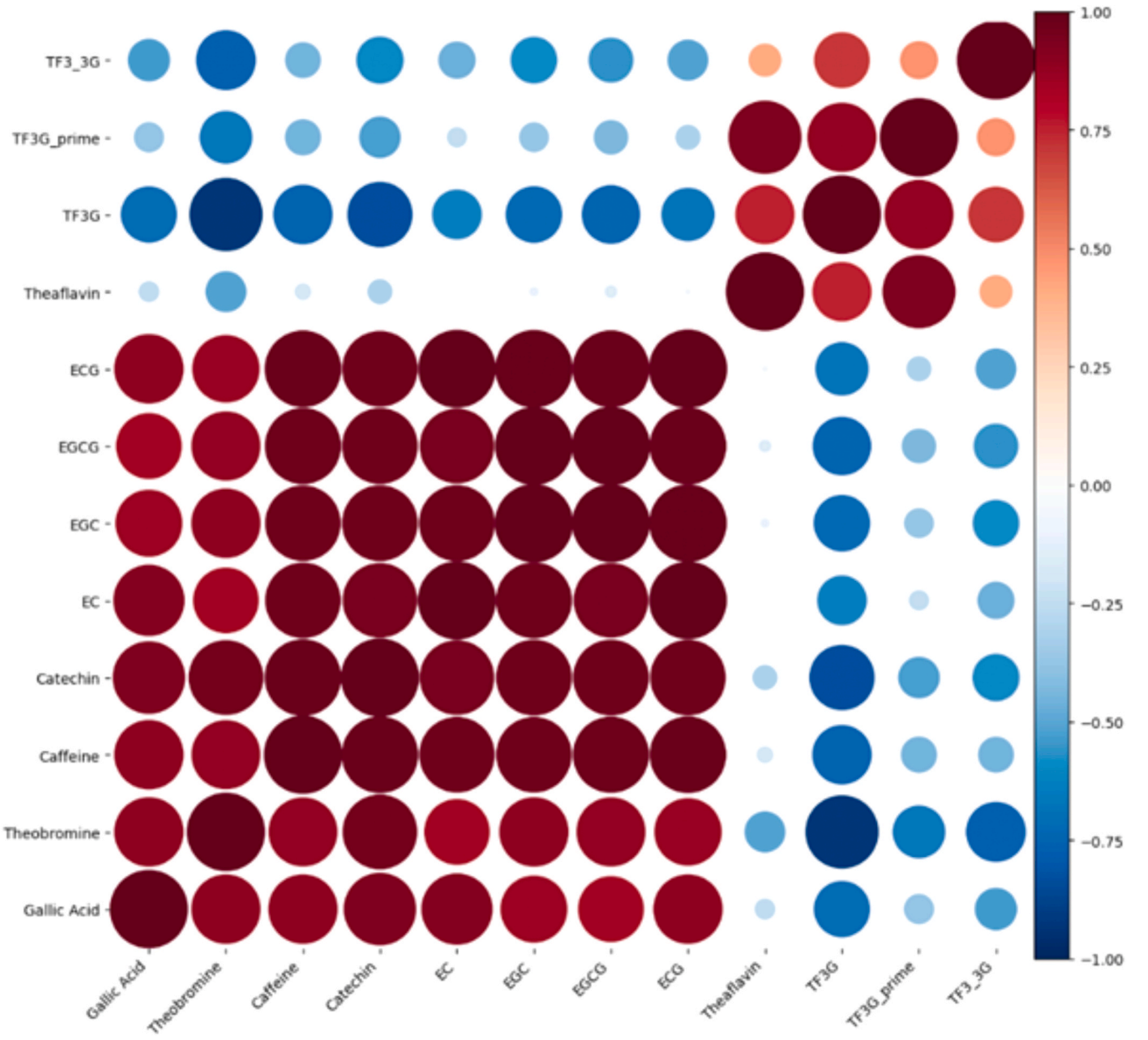

Fig. 5. Correlogram of Pearson correlation coefficients among bioactive compounds during black tea processing. Compounds include catechins (Catechin, EC, EGC, EGCG, ECG), theaflavins (Theaflavin, TF3G, TF3'G, TF3–3'G), and others (Gallic acid, Theobromine, Caffeine), assessed across processing stages: FTL, WTL, ROL, RTL, OTL 1, OTL 2, OTL 3, and FBT. Circle size represents the absolute value of the correlation coefficient. Color scale: blue = positive correlation, red = negative correlation, ranging from −1–1.

leaves (RTL), orthodox tea leaves (OTL 1–3), and fired black tea (FBT). Data, obtained via a validated high-performance liquid chromatography with diode array detection (HPLC-DAD) method using a Vanquish Core HPLC System (Section 2.4), reveal significant shifts driven by PPO activity, microbial interactions, and processing conditions (Table 6, Figures S2, S5, and S6). Figure S2 illustrates the concentration trends of catechins, theaflavins, and other compounds (gallic acid, theobromine, caffeine) across processing stages, with column charts displaying error bars and pairwise comparison ($p < 0.05$, Tukey's HSD) for clarity.

3.3.1. *Polyphenol dynamics*

Total catechins declined 78.5 % from 328.36 mg/g in FTL to 70.33 mg/g in FBT (ANOVA, $p < 0.05$), with EGCG exhibiting the steepest drop (98.2 %, 186.66–3.29 mg/g; Tukey's HSD, $p < 0.01$), reflecting PPO-catalyzed oxidation into theaflavins and thearubigins during rolling and oxidation (Deka et al., 2021). Withering reduced catechins by 44.6 % to 190.95 mg/g, driven by cell disruption and moisture loss, enhancing PPO activity, while rolling further decreased them by 27.8 % to 180.26 mg/g (Pou et al., 2019).

Theaflavins, not detected in FTL (below LOD, treated as 0 mg/g for ANOVA, Section 2.7), emerged during withering (12.40 mg/g), peaked post-rolling (15.97 mg/g, +28.8 %), and declined 24.2 % to 12.11 mg/g during extended oxidation due to microbial conversion to thearubigins (Pou et al., 2019). Firing (90–100°C) stabilized theaflavins at 10.98 mg/g by inactivating PPO.

Gallic acid decreased 67.7 % from 15.10 to 4.88 mg/g ($p < 0.01$), with a transient 37.7 % rise during rolling (9.50 mg/g) from esterase-mediated hydrolysis of galloylated catechins (Karas et al., 2017). Theobromine dropped 30.3 % from 4.42 to 3.08 mg/g ($p < 0.01$), possibly due to oxidative coupling with polyphenols (Ye et al., 2018). Caffeine decreased 5.93 % from 101.70 to 95.67 mg/g ($p < 0.05$), reflecting physical losses, such as leaching during withering

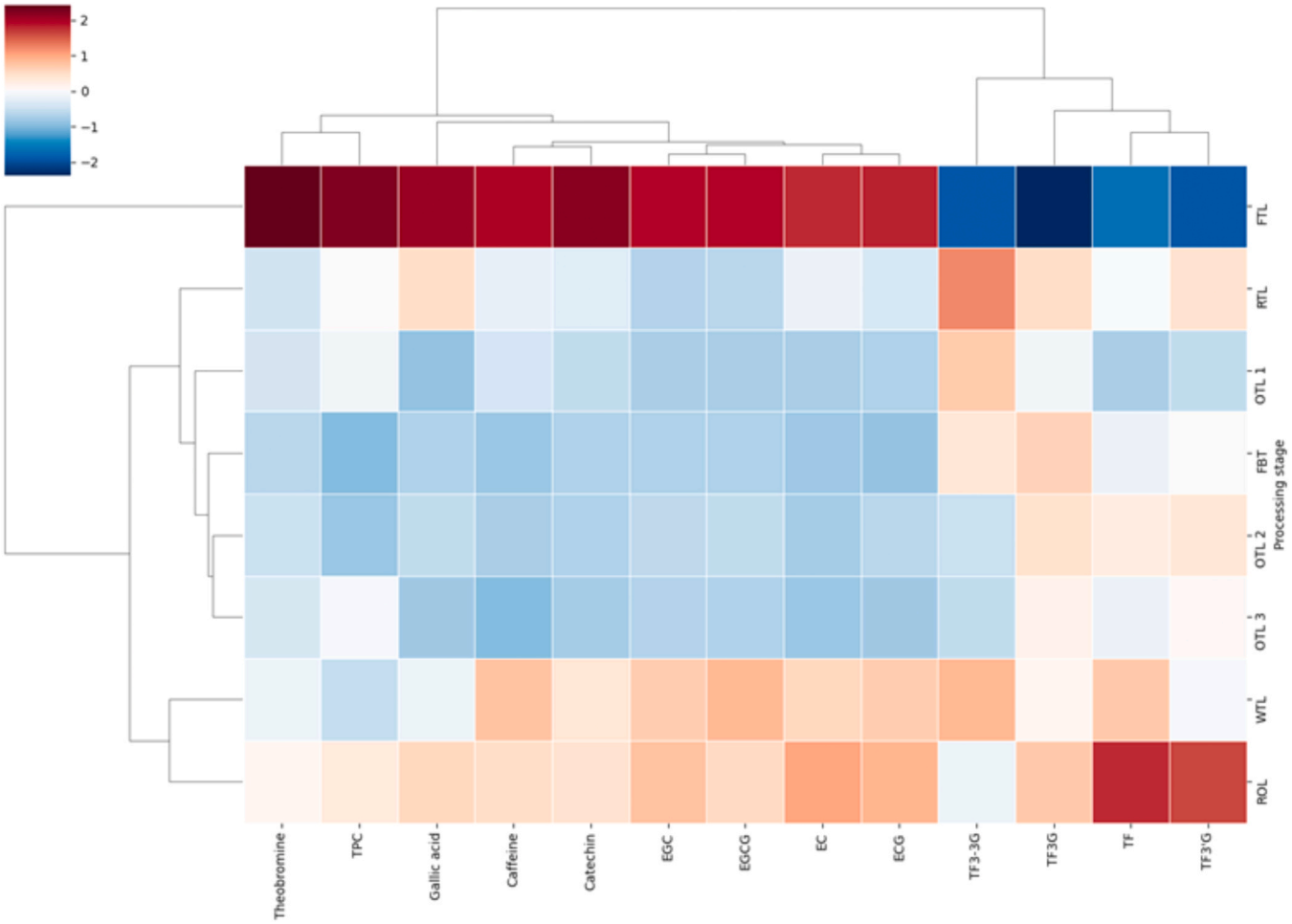

Fig. 6. Clustered heatmap of normalized z-scores for 12 bioactive compounds across black tea processing stages. Compounds are grouped into catechins, theaflavins, and others. Processing stages include FTL, WTL, ROL, RTL, OTL 1, OTL 2, OTL 3, and FBT. Z-scores are normalized per compound to highlight relative concentration trends. Color scale: blue = low z-scores (low concentrations), red = high z-scores (high concentrations). Dashed lines separate compound categories.

(101.62 mg/g) and rolling (98.51 mg/g), facilitated by mechanical disruption and high humidity (65–70 %) (Lee et al., 2016). High initial caffeine levels, characteristic of high-altitude Somerset Tea Estate cultivars, may amplify absolute losses (Hazarika et al., 2024; Zhang et al., 2018), contrasting with reported increases due to moisture-related concentration effects (Sanaeifar et al., 2020).

Minor thermal degradation during firing or reduced extractability in the fired leaf matrix using 70 % methanol could contribute (Theppakorn, 2016). Total polyphenol content (TPC) fell 55.0 % from 831.73 to 374.18 GAE mg/g DW, mirroring catechin declines but with a rebound at OTL 3 (505.61 mg/g), possibly due to variable oxidation rates or microbial activity (Leonard et al., 2021). One-way ANOVA confirmed significant differences ($F(7,16) = 124.6$ for catechins, 89.3 for theaflavins, $p < 0.001$), with Tukey's HSD identifying rolling and oxidation as critical stages ($p < 0.01$, Section 2.7).

3.3.2. *Correlation and clustering analysis*

Pearson correlation analysis (Fig. 5) revealed strong within-group associations: catechins (0.81–0.98, e.g., Catechin-EC: 0.98), theaflavins (0.58–0.89, e.g., TF-TF3G: 0.82), and others (0.79–0.92, e.g., Gallic Acid-Theobromine: 0.92). Cross-group correlations showed positive links between catechins and others (0.62–0.87, e.g., EGCG-Caffeine: 0.76) but negative ties with theaflavins (-0.47–0.15, e.g., EGCG-TF: −0.42), consistent with oxidative conversion (Ito and Yanase, 2022). Theaflavins exhibited weak correlations with others (0.05–0.41), reflecting distinct biosynthetic pathways, while caffeine, gallic acid, and theobromine patterns aligned with stability trends (Lee et al., 2016). These insights suggest monitoring EGCG or theaflavins for quality control, with oxidation duration adjustments optimizing flavor and antioxidants.

Hierarchical cluster analysis (Fig. 6) identified two metabolite clusters. The first, including EGCG, EGC, EC, catechin, ECG, gallic acid, and theobromine, dominated early stages (FTL, WTL) but declined through rolling (ROL) and rotovane cutting (RTL), becoming minimal in oxidized (OTL 1–3) and fired (FBT) stages, consistent with PPO and peroxidase-driven oxidation (Lee et al., 2016; Liang et al., 2003; Liu et al., 2022). The second cluster, comprising theaflavins (TF, TF3G, TF3′G, TF3–3G), emerged post-rolling, peaking in OTL stages, reflecting catechin condensation (Roberts and Smith, 1961; Samanta et al., 2015). TPC mirrored catechin trends, declining post-RTL as monomers degraded or polymerized. Stage clustering grouped FTL with WTL, ROL with RTL, and OTL 1–3 with FBT, providing a metabolite fingerprint for quality monitoring (Chandini et al., 2011; Liang et al., 2003). These findings underscore the pivotal role of processing stages in modulating polyphenol profiles, offering a foundation for quality optimization.

The heatmap revealed two principal metabolite clusters. The first cluster, comprising EGCG, EGC, EC, catechin, ECG, gallic acid, and theobromine, was abundant in the early stages, particularly in fresh tea leaves (FTL) and withered leaves (WTL). These compounds exhibited a progressive decline through rolling (ROL) and rotovane cutting (RTL) stages and were largely diminished in the oxidized (OTL1–3) and fired black tea (FBT) stages. This pattern aligns with established findings that

catechins are oxidized during fermentation by polyphenol oxidase and peroxidase (Lee et al., 2016; Liang et al., 2003; Liu et al., 2022) In contrast, the second cluster, dominated by theaflavins (TF, TF3G, TF3'G, TF3,3 G), was nearly undetectable in the early stages but significantly increased during ROL, RTL, and peaked in OTL stages. This rise reflects the oxidative condensation of catechins to form theaflavins, a hallmark of black tea quality (Roberts and Smith, 1961; Samanta et al., 2015).

The total phenolic content (TPC) pattern mirrored catechin dynamics, declining after RTL as monomeric phenolics were degraded or polymerized. Cluster analysis of processing stages showed FTL and WTL closely grouped, ROL and RTL as a second group, and OTL1–3 with FBT forming the final cluster. Overall, the heatmap provides a metabolite-level fingerprint of black tea processing, validating the biochemical conversions reported in classical tea chemistry and offering a visual tool to monitor quality-determining compounds throughout production (Chandini et al., 2011; Liang et al., 2003).

4. Conclusions

This study successfully developed and validated an HPLC-DAD method for the simultaneous quantification of 12 key polyphenolic compounds in *Camellia sinensis*, including catechins, theaflavins, gallic acid, caffeine, and theobromine. Utilizing a Kinetex XB-C_{18} column and dual-wavelength detection (210 and 280 nm), the method demonstrated excellent performance: linearity ($r^2 > 0.9995$), sensitivity (LOD: 0.03–1.68 μg/mL; LOQ: 0.01–0.56 μg/mL), precision (RSD < 4.68 %), and recovery (85.86–99.16 %). It also outperformed many existing methods in terms of run time (40 min) and robustness.

Among extraction techniques, ultrasonication with 70 % methanol (U_M70) yielded the highest polyphenol concentrations. Green tea showed elevated catechin levels (214.30 ± 12.86 mg/g), while black tea was richer in theaflavins (19.73 ± 1.33 mg/g), both surpassing hot water and ISO-standard extractions. Analysis of black tea processing stages (FTL to FBT) revealed significant polyphenol transformations ($p < 0.001$, ANOVA). Catechins declined by 79.1 % (from 321.02 to 66.04 mg/g), primarily due to PPO-mediated oxidation, while theaflavins peaked post-rolling (15.98 mg/g) before partial conversion to thearubigins. Gallic acid and theobromine decreased by 67.9 % and 70.4 %, respectively, whereas caffeine remained relatively stable, with only a 5.93 % reduction (101.70–95.67 mg/g). These trends, supported by Table 6 and Figs. 5–6, provide a detailed biochemical framework for understanding tea quality and bioactivity.

The method's high throughput, sensitivity, and applicability across diverse tea matrices make it well-suited for industrial quality control, nutritional labeling, and functional food research. However, limitations include matrix effects for TF3–3'G (recovery: 85.86 %) and the study's focus on green and black teas, highlighting the need for validation in other tea types (e.g., oolong, white). Future research should explore solid-phase extraction to reduce matrix effects, enzyme-assisted extraction for greener alternatives, and LC-MS for profiling minor metabolites such as thearubigins. Additionally, investigating cultivar-specific and terroir-related influences on polyphenol profiles could enhance generalizability. Integrating sensory evaluations with chemical data would further align this method with consumer preferences and industry standards, reinforcing its value in advancing tea science and quality assurance.

CRediT authorship contribution statement

Ali Rashidinejad: Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Carl H. Mesarich:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Conceptualization. **Chandrika M. Nanayakkara:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Conceptualization. **Punyasiri P. A Nimal:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Conceptualization. **David G. Popovich:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Conceptualization. **Prishanthini Muthulingam:** Writing – original draft, Visualization, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2025.108330.

Data availability

Data will be made available on request.

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