A gradient based facile HPLC method for simultaneous estimation of antioxidants extracted from tea powder

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Abstract A new simple, rapid and precise RP-HPLC method was developed for the extraction and quantitative estimation of caffeine (C), (–)-epigallocatechin gallate (EGCG), (+)-catechin(Ct), (–)-epicatechin(EC), and (–)-epicatechin gallate (ECG) (collectively named as Tea Powder Bioactives **TPBAs**) extracted from tea powder using different ratios of ethanol: water. The simultaneous determination of TPBAs was performed using the UV spectrophotometric method which employs the absorbance at 205 nm ($\lambda_{\max}$ of caffeine and polyphenols). This method is a gradient based HPLC method with a flow rate

of 0.8 mL/min using Inertsil ODS 100 × 4.6 mm, 3 μ m column with methanol and ammonium dihydrogen phosphate (pH-2.8) as mobile phase. The method was validated in terms of specificity, precision, linearity, accuracy, limit of quantification (LOQ), and limit of detection (LOD). The linearity of the proposed method was investigated for concentration ranging between 0.5–60 μ g/mL with regression co-efficient, $R^2 = 0.999$ –1.0. This method estimates all the TPBAs simultaneously with enhanced precision and linearity as per the ICH guidelines. Also, to confirm the individual TPBA, the antioxidant property of the each TPBA was analyzed which was commensurate with that of the previous reports.

Highlights of the manuscript

- Based on LOQ, the method is sensitive for the tea bioactives ranging from ~0.4 to 0.7 ppm.
- The method has a detection limit of 0.13 to 0.23 ppm.
- The method is linear for 80, 100 and 120 % assay concentration.
- System suitability parameters lies within the limit of % RSD i.e., less than 2 % (~0.7 %)
- Accuracy of the method is 98–102 %.

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Introduction

Tea is the most popularly used refreshment beverage throughout the world. Along with its aroma, this anti-oxidant-rich drink has vast medicinal applications, because of which several research investigations have been attempted to explore the potential of its chemical constituents in therapeutics. The fact that these constituents possess various biological properties, such as antiallergic, antibacterial, anticarcinogenic, antimutagenic (Otsuka et al. 1998) as well as the antioxidant properties (Tanaka et al. 1998; Vinson and Dabbagh 1998) has magnified the attention of researchers. In general, fresh green tea leaves contain 36 % polyphenols predominated by the catechins. Catechins constitute four primary compounds Table 1 epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallo catechin (EGC) and

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Table 1. Chemical structures and molecular weight of TPBAs

<p>Structure of Caffeine (M.W. 194.19)</p>
<p>Common structure of the tea polyphenols</p>				
Name of the polyphenol	R ₁	R ₂	Molecular Formula	Formula weight
(-) Epicatechin (EC)	H	H	C ₁₅ H ₁₄ O ₆	290.27
(-) Epicatechin gallate (ECG)	H		C ₂₂ H ₁₈ O ₁₀	442.37
(-) Epigallo catechin (EGC)	OH	H	C ₁₅ H ₁₄ O ₇	306.27
(-) Epigallocatechin gallate (EGCG)	OH		C ₂₂ H ₁₈ O ₁₁	458.37

epicatechin (EC) (Perva-Uzunalić et al. 2006). Among these, EGCG is the most prime component in tea extract (48–55 % of total polyphenols) (Ho et al. 1997). The therapeutic effects of tea components mainly of the green tea and exclusively its catechins have been extensively examined in microbial and mammalian cell systems (Nugala et al. 2012). A study suggests that tea phenolics may suppress formation of mutagenic and carcinogenic heterocyclic amines in cooked foods (Oguri et al. 1998). The most dominant constituents in tea are polyphenols and they have been considered to be the major dietary bioactive responsible for its health benefits. The anticarcinogenic and antimutagenic activities

of tea polyphenols are attributed to their antioxidant properties which acts by inactivating the carcinogens directly and also inhibiting the activation of indirect carcinogens extracellularly (Kuroda and Hara 1999; Perva-Uzunalić et al. 2006; Zhao et al. 1989). The total polyphenol amounts determined from the same plant and their corresponding antioxidant and antimicrobial activities may vary widely, depending on extraction conditions applied. The exact measurement of TPBAs in Tea powder is extremely important as they possess considerable dietary importance which helps in also assessing the quality of the tea powder with respect to its health benefits. The aim of the present work was to develop

simple and precise RP-HPLC method which was optimized and validated in accordance with international conference on Harmonization (Willard 2012; Pharmacopeia 2009; Q2 (R1) Validation of Analytical Procedures: Text and Methodology 1995). This method is applicable for the isolation of TPBAs in the tea powder extracted by conventional way by varying the solvent ratio, temperature and soxhlet extraction time. Previously, acetonitrile gradient has been used (Goto et al. 1996) but, the use of a class 2 methanol and class 3 ethanol as per FDA guidelines is more recommended than acetonitrile which is a class 2 solvent (FDA 2012).

Materials and methods

Compounds chosen for extraction and estimation

Chemical structure, molecular formula and formula weight of the bioactives are as shown in Table 1.

Plant material and chemicals

Dry green tea leaves (*Thea sinensis* L) were collected from the local tea powdering industry of Assam. Phosphoric acid (85.0 %), Diammonium hydrogen phosphate AR grade, Caffeine (99.0 %), (+)-Catechin (98.0), (–)-Epigallocatechin gallate (100 %), (–) Epicatechin (98.0 %) and (–)-Epicatechin gallate (100 %) were purchased from Alfa Aesar (USA). HPLC grade methanol (Merck, USA) was used as mobile phase solvent. Ethanol used was of analytical reagent grade (Beijing Chemical Reagents). Deionized water was obtained from reverse osmosis and ion exchange purified water system (Milli-Q system, USA).

Preparation of extracts

The green tea leaves were shade-dried for three days, pulverized and sieved. Green tea leaves powder (50 g) was placed into a lintel free cloth and the bag containing tea powder was placed into a soxhlet apparatus containing extraction solvent of different combination of water and ethanol (ratio of tea powder and extraction solvent 1 g: 20 mL) was taken for the extraction (Corrales et al. 2009; Jun et al. 2011) at constant time (2 h) and temperature. The extracted sample was centrifuged at 4000 rpm for 10 min and the supernatant was subjected to vacuum filtration using 0.45 µm filter paper. The sample was evaporated by rotary evaporator under reduced pressure at 40 °C followed by freeze drying at –35 °C. The samples were stored at –20 °C until further use.

Extraction using different ratio of ethanol: water at 100 °C

Extraction was carried out as described in the section 2.3 at boiling temperature on an oil bath using different ratios of water and ethanol viz., 75:25, 50:50 and 100 % water.

Preparation of standards

TPBAs of 0.15 mg were dissolved in 1 mL of Water-Methanol (50:50 v/v).

HPLC sample preparation

The extract (40 mg) was dissolved in 20 mL of water-methanol (50:50 v/v) and further sonicated to dissolve and then diluted to 100 mL.

Chromatographic system and conditions

Two HPLC systems were employed during validation experiments viz., Agilent (1200series) quaternary HPLC with DAD detector and Shimadzu binary system with SP-20 Detector using HPLC column Inertsil ODS (100 mm × 4.6 mm, 5 µm, USA) at 25 °C. To adjust the pH of the mobile phase, pH-meter (EUTECH instruments, USA) was used.

The mobile phase consists of A- 100 mM monobasic ammonium phosphate buffer of pH 2.8 ± 0.2 and B-methanol. The ODS column (100 mm × 4.6. I.D., 3 µm, Inertsil, USA) and the flow rate was set to 0.8 mL/min. The column temperature was set to 25 °C. The PDA detector was kept at 205 nm. Water and methanol of ratio 1:1 v/v was used as a diluents. A 5 µL injection volume of each sample was injected on to the column, separated and eluted using the following gradient, 0 min, 15 % B; 6 min, 30 % B; 12 min, 30 % B; 15 min, 80 % B; 23 min, 80 % B; and allowed for 5 min for column stabilization with initial conditions.

HPLC method validation

Specificity and method precision

Specificity was demonstrated by the resolution of the two components which elute closest to each other. The ability of the procedure to discriminate between compounds of close structurally related polyphenols which are likely to be present was confirmed by obtaining positive results by comparing with a known reference material from samples containing the analyte.

Separately, 0.2 mg/mL of all standard polyphenol solution were injected to the column separately and 1 mL solution of each standard were mixed and injected as a spiked standard solution. The relative retention time (RRT) of

Table 2 Effect of extraction solvent ratio and extraction time on the yield of TPBAs

Name of the TPBA	Yield (g/Kg) based on Extraction solvent ratio of Water: Ethanol (v/v)			Yield (g/Kg) based on Extraction time using Water: Ethanol (75:25 v/v)			
	50 %	75 %	100 %	30 min	60 min	90 min	120 min
(+)-Catechin	0.9	2.9	0.7	2.4	3	3.4	3.5
Caffeine	41.1	101.3	46.8	63.5	69.2	69.5	75.4
(-)-Epigallocatechin gallate	1.5	3.9	0.6	2.2	2.4	3.3	3.2
(-)-Epicatechin	0.2	0.6	0.8	0.5	0.3	0.6	0.4
(-)-Epicatechin gallate	0.8	1.2	0.3	1.4	1.8	2.2	2.5

each of Caffeine, (+)-Catechin, (-)-Epigallocatechin gallate, (-)-Epicatechin, and (-)-Epicatechin gallate were 1, 0.85, 1.08, 1.17, and 1.64 respectively.

Limit of quantification and limit of detection (LOQ and LOD)

Signal-to-noise ratio was determined by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the

minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio was verified which suggests that the ratio of 10 S/N was obtained for quantification limit and 3 S/N for detection limit.

Procedure for the solution: preparation for LOQ and LOD

Exactly 5.03, 5.01, 5.0, 5.03, and 5.01 mg of (+)-Catechin, Caffeine, (-)-Epigallocatechin gallate, (-) Epicatechin, and

Fig. 1 Linearity plot for TPBAs **a** (+)-Catechin; **b** Caffeine; **c** (-)-Epicatechin; **d** (-)-Epicatechin gallate; and **e** (-)-Epigallocatechin gallate

Fig. 2 Separation of TPBAs using Inertsil ODS column. **a** Mixed Standards, **b**. TPBAs from Tea powder

(-)-Epicatechin gallate respectively were weighed and dissolved separately in 10 mL of Water-Methanol (50:50 v/v). From these solutions, 0.2, 0.35, 0.35, 0.25, and 0.25 mL were pipetted out into respective 250 mL volumetric flasks and diluted up to the mark. The resulting solution was used for LOQ. This concentration was established in the method developed for signal to noise ratio of 10 units (Q2 (R1) Validation of Analytical Procedures: Text and Methodology 1995). 5 μ L of the LOQ solution was injected into HPLC in six replicates and was calculated for S/N ratio 10. LOQ solution of 33.3 mL was pipetted out and made up to 100 mL using diluent and the resulting solution was used for LOD.

Linearity determination of TPBAs

Linearity of the method was tested for all the five polyphenols using the concentration range 80–120 %. The stock solution for linearity was prepared by weighing 5 mg of each polyphenol in 10 mL of Water-Methanol (50:50 v/v) and the stock solution was further diluted to 10 mL using 0.8 mL for 80 %, 1.0 mL for 100 %, and 1.2 mL for 120 % linearity solution.

Fig. 3 Dose dependent DPPH scavenging activity of the extracted TPBAs

Procedure for the accuracy solution preparation

Exactly 5.16, 5.24, 5.27, 5.34, and 5.32 mg of (+)-Catechin, Caffeine, (-)-Epigallocatechin gallate, (-) Epicatechin, and (-)-Epicatechin gallate were weighed and separately dissolved in 10 mL of Water-Methanol (50:50 v/v). From the stock, 6 mL was diluted to 10 mL to get the working standard and 1 mL of working standard was spiked with 50 mg of sample and diluted to 10 mL. Spiking samples were prepared in triplicate to calculate the recovery.

DPPH scavenging activity of the TPBAs

DPPH-free radical scavenging activity of (+)-catechin, caffeine, (-)-epigallocatechin gallate, (-)epicatechin, and (-)-epicatechin gallate was evaluated according to the method of Xu and Chang (Xu and Chang 2007). Briefly, 0.2 mL of the isolated tea components were separately taken and made up to 4 mL using ethanol solution of DPPH radical (final concentration was 0.1 mM). The mixture was vigorously vortexed for 1 min and left to stand in dark at room temperature for 30 min. Thereafter, the absorbance for the samples was measured at 517 nm against ethanol blank. A negative control (A_{sample}) was taken after adding DPPH solution to 0.2 mL of the respective compound (A_{control}). The percent of DPPH discoloration of the sample was calculated according to the equation:

$$\% \text{ discoloration} = \left[1 - \frac{A_{\text{sample}}}{A_{\text{control}}} \right] \times 100$$

The free radical scavenging capacity of the bioactives is expressed as an equivalent of that of Trolox standard curve.

Results and discussion

The extraction of TPBAs with different ratio of water and ethanol (75:25; 50:50; 100:0 v/v) was carried out at

the boiling temperature using soxhlet apparatus. It is observed from the data (Table. 2) that the ethanol: water mixture of the ratio 75:25 v/v was more efficient than other compositions and maximum yield was obtained using 90 mins of extraction time. There was no significant increase in the yield of any compound other than caffeine even after continuing the extraction for 120 min. Based on this data, 90 min extraction was found to be optimum.

With increase in concentration, linearity ($R^2 = 0.99$) was seen for all the TPBAs from the HPLC studies (Fig. 1). This method was precise in extracting and estimating the TPBAs with a run time of less than 20mins which was also observed with previously established methods (Dionex 2011).

From the results being comparable with the previous studies (Nanjo et al. 1996; Senba et al. 1999), it is clear from Fig. 3 that (+)-catechin showed the highest DPPH scavenging activity followed by caffeine, (–)-epicatechin, (–)-epicatechin gallate, and (–)-epigallocatechin gallate.

Conclusion

The developed RP-HPLC method can be successfully used for the isolation and quantification of five bioactives present in tea powder. Based on the results of the LOQ, the method was observed to be sensitive for measuring all TPBAs at concentrations ranging between 0.4 to 0.7 ppm. The method has a detection limit of 0.13 to 0.23 ppm. It is linear for 80 %, 90 %, 110 %, 100 %, and 120 % assay concentration. With a % RSD of 0.7 and the method has precision as the system suitability parameters lie within the limit of % RSD less than 2 % and the accuracy of the method was found to be 98–102 %. The use of ethanol instead of acetonitrile made the method safer as per the guidelines of Food and Drug Administration (FDA 2012). The methanol-ammonium dihydrogen phosphate used as mobile phase which gives higher resolution to the peaks by providing detection at shorter wavelength (205 nm). The same mobile phase is advised for preparative and semi-preparative separations (Meurant 2011). The DPPH scavenging activity of the bioactives (Fig. 2) confirms their antioxidant properties which are relevant with that of the results obtained in the previous studies (Nanjo et al. 1996; Senba et al. 1999). Furthermore, this method which is simple, precise, and accurate can be employed by industries for quality control and quality assurance.

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References

- Corrales M, García AF, Butz P, Tauscher B (2009) Extraction of anthocyanins from grape skins assisted by high hydrostatic pressure. *J Food Eng* 90:415–421. doi:10.1016/j.jfoodeng.2008.07.003
- Dionex (2011) Sensitive Determination of Catechins in Tea by HPLC. www.dionex.com/en-us/webdocs/110783-AN275-HPLC-CatechinsTea-19May2011-LPN2799.pdf. 2015
- FDA (2012) Guidance for Industry, <http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm073395.pdf>. 2015
- Goto T, Yoshida Y, Kiso M, Nagashima H (1996) Simultaneous analysis of individual catechins and caffeine in green tea. *J Chromatogr A* 749:295–299. doi:10.1016/0021-9673(96)00456-6
- Ho CT CC, Wanasundara UN, Shahidi F (1997) Natural Antioxidants from Tea in Natural Antioxidants AOCS Press, Champaign: 213–223
- Jun X, Deji S, Ye L, Rui Z (2011) Comparison of in vitro antioxidant activities and bioactive components of green tea extracts by different extraction methods. *Int J Pharm* 408:97–101. doi:10.1016/j.ijpharm.2011.02.002
- Kuroda Y, Hara Y (1999) Antimutagenic and anticarcinogenic activity of tea polyphenols. *Mutat Res* 436:69–97
- Meurant G (2011) Chromatography of alkaloids, part B: gas-liquid chromatography and high-performance liquid chromatography. Elsevier Science
- Nanjo F, Goto K, Seto R, Suzuki M, Sakai M, Hara Y (1996) Scavenging effects of tea catechins and their derivatives on 1,1-diphenyl-2-picrylhydrazyl radical. *Free Radic Biol Med* 21:895–902. doi:10.1016/0891-5849(96)00237-7
- Nugala B, Namasi A, Emmadi P, Krishna PM (2012) Role of green tea as an antioxidant in periodontal disease: the Asian paradox. *J Indian Soc Periodontol* 16:313–316. doi:10.4103/0972-124X.100902
- Oguri A, Suda M, Totsuka Y, Sugimura T, Wakabayashi K (1998) Inhibitory effects of antioxidants on formation of heterocyclic amines. *Mutat Res* 402:237–245
- Otsuka T, Ogo T, Eto T, Asano Y, Suganuma M, Niho Y (1998) Growth inhibition of leukemic cells by (–)-epigallocatechin gallate, the main constituent of green tea. *Life Sci* 63:1397–1403. doi:10.1016/S0024-3205(98)00406-8
- Perva-Uzunalić A, Škerget M, Knez Ž, Weinreich B, Otto F, Grüner S (2006) Extraction of active ingredients from green tea (*Camellia sinensis*): extraction efficiency of major catechins and caffeine. *Food Chem* 96:597–605. doi:10.1016/j.foodchem.2005.03.015
- Pharmacopeia US (2009). http://www.pharmacopeia.cn/v29240/usp29nf24s0_c621s12.html. Accessed 10 Jan 2015
- Q2 (R1) Validation of Analytical Procedures: Text and Methodology (1995) International conference of harmonisation
- Senba Y, Nishishita T, Saito K, Yoshioka H, Yoshioka H (1999) Stopped-flow and spectrophotometric study on radical scavenging by tea catechins and the model compounds. *Chem Pharm Bull* 47:1369–1374. doi:10.1248/cpb.47.1369
- Tanaka T, Kusano R, Kouno I (1998) Synthesis and antioxidant activity of novel amphipathic derivatives of tea polyphenol. *Bioorg Med Chem Lett* 8:1801–1806. doi:10.1016/S0960-894X(98)00311-4

- Vinson JA, Dabbagh YA (1998) Tea phenols: antioxidant effectiveness of teas, tea components, tea fractions and their binding with lipoproteins. *Nutr Res* 18:1067–1075. doi:[10.1016/S0271-5317\(98\)00089-X](https://doi.org/10.1016/S0271-5317(98)00089-X)
- Hobart H. Willard (2012) Instrumental methods of analysis. CBS Publishers & Distributors, New Delhi
- Xu BJ, Chang SK (2007) A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. *J Food Sci* 72:S159–S166. doi:[10.1111/j.1750-3841.2006.00260.x](https://doi.org/10.1111/j.1750-3841.2006.00260.x)
- Zhao BL, Li XJ, He RG, Cheng SJ, Xin WJ (1989) Scavenging effect of extracts of green tea and natural antioxidants on active oxygen radicals. *Cell Biophys* 14:175–185