Research Article

Evidence based seasonal variances in catechin and caffeine content of tea

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Abstract
Tea is a popular refreshing beverage and its popularity is also due to its multidimensional health benefits. The health effects of tea are due to its polyphenolic contents, viz. catechins, and methyl xanthenes like caffeine etc. The variance in concentration of these secondary metabolites both in fresh tea leaves and processed black tea owing to seasonal variances was studied by a chemometrically optimized and validated quantitative HPLC method. As per quantitative HPLC results, the fresh tea leaves plucked during spring and also the processed black tea made from it showed the highest yield of catechins and caffeine. Catechin and caffeine content were found to decrease quantitatively in monsoon and further decrease in autumn as observed from HPLC analysis of fresh tea leaves and processed black tea made from it.

Keywords Black tea · Secondary metabolites · Poly phenols · Caffeine · Catechins · Seasonal variances · Chemometrics

1 Introduction

Tea (*Camellia sinensis*) of family theaceae, is not only a refreshing beverage from time unknown but has attracted global research attraction owing to its much explored multidimensional health potentials [1]. Tea can be considered as a store house of several pharmacoactive compounds, i.e. plant secondary metabolites (PSM). The PSMs exhibit a wide array of pharmacologic actions [2, 3]. The tender leaves and buds of *Camellia sinensis* are used for manufacturing of tea. Tea plant being perennial in nature, tea leaves can be harvested almost all the year round [4]. The world agro-eco system is affected by climatic variance and abiotic stress factors thus affecting both the yield and crop quality. Concentrations of PSMs are influenced by seasonal variations and the same fact is revealed in case of tea plantations [5]. In tea, fluctuations in phenolic content, amounts of methyl xanthenes etc. affects both the pharmacologic profile and sensory attributes of tea. This study reports a quantitative reverse phase high performance liquid chromatographic (RP-HPLC) method, the chromatographic conditions being optimized by D-optimal combined design approach of chemometrics and validated as per the guidelines of International Conference on Harmonization. The purpose is to study the quantitative variances in catechin (major phenolics) and caffeine content (major methyl xanthenes compound) both in fresh tea leaves of Tocklai Vegetative clone 25 variety in spring, monsoon and autumn and the processed black tea prepared thereof.

2 Materials and methods

2.1 Plant material

Fresh tea leaves (TV 25 variety, *Voucher specimen*: IIT-KGP/HB/2018/T1) were plucked from the tea garden of Kharagpur, India. Tea leaves were collected during spring,

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monsoon and autumn. Leaves of first flush were plucked in mid February during spring, during monsoon the leaves of second flush were plucked in mid July and leaves of third flush at November onset during autumn. From the harvested tea leaves of three different seasons processed black tea was also prepared.

2.2 Instrument used

Electronic balance; HPLC (Model Waters 2998).

2.3 Software

Design Expert version 7.0 for chemometrics work.

2.4 Sample preparation of fresh tea leaf and processed black tea of different seasons

Sample preparation was done as per the literature methodology [6–9]. About 20 g of fresh tea leaves were dipped in liquid nitrogen, crushed and grinded well in mixer. Next 1 g of tea leaf powder and 20 mL methanol were mixed well in mortar for 15 min and filtered through Whatmann filter paper. The final volume was adjusted to 25 mL with methanol.

For sample preparation of black tea, infusions were prepared by adding 1 g of black tea to 25 mL of boiling milli-Q™ water and brewed for 5 min. The brewed, aqueous infusions were centrifuged at 8000 × *g* for 10 min. The supernatant were filtered through 0.45-µm membrane filter (Pall Gelman Laboratory, South Wagner road, Ann arbor, USA) before analysis on HPLC [7].

2.5 Chemometrics assisted optimization of HPLC chromatographic conditions

D-optimal combined design approach of chemometrics was used with mobile phase acetic acid: acetonitrile compositional ratio and flow rate as the inputs and retention time of caffeine, catechin and epigallocatechin gallate (EGCG) as the dependent variables. The optimized chromatographic conditions consisted of 92% of 0.2% acetic acid and 8% of acetonitrile as the mobile phase, with a flow rate of 1 mL/min, injection volume of 20 µl, PDA detector was set at 200–600 nm and chromatograms were recorded at 274 nm. Separations were achieved at room temperature on RP-C18 column 5 µm (250 mm × 4.6 mm i.d.). Authenticated standards were used for identifying peaks and calculate the concentration of tea components. Each peak was confirmed by comparing the retention times and absorption spectra of unknown to that of standard compounds.

2.6 HPLC method Validation

Chemometrics assisted optimized HPLC chromatographic conditions have been validated as per ICH guidelines in terms of precision, specificity, robustness. Precision was studied in terms of repeatability (system precision), method precision and ruggedness. For system precision, standard solution (20 µg/mL) was injected six times into the HPLC system as per test procedure. For method precision, six replicates of standard and sample of 20 µg/mL were prepared and injected into the HPLC system and % RSD was calculated. Intermediate precision study or ruggedness of experimentation was carried out by different analyst, on different instrument and on different days. From the sample and stock solutions, six replicates of 20 µg/mL were prepared and injected into the HPLC system and % RSD was calculated. As a measure of robustness of the method, deliberate alterations in the flow rate (0.5 mL/min and 1.5 mL/min) was made to evaluate the impact of the method. The tailing factor, % RSD of asymmetry and retention time of standard should not be more than 2% due to the intentional alterations in the flow rate.

2.7 Abiotic factors and secondary metabolites

Kharagpur, India is a non traditional tea growing zone. TV 25 variety of tea used as the research material has been grown in the tea garden of IIT Kharagpur. Located at latitude 22°01′ N and longitude 87°07′ E in South-western Midnapore, Kharagpur covers an area of about 127 km^2 with an average elevation of 29 meters (95 ft). This region lies in the alluvial tract of Midnapore district. Kharagpur has a tropical wet and dry climate. Summers start in March; are mostly hot and humid, with average temperatures close to 30 °C (86 °F). The average estimated rainfall is about 1140 mm (45 inches). Winters are brief but chilly with average temperatures around 22 °C (72 °F) and last from December to mid February. Total annual rainfall is around 1400 mm (55 in). Considering temperature and rainfall as abiotic stress factors, variances in concentration of secondary metabolites have been studied.

3 Results

3.1 Response surface methodology (RSM)

The Design Expert software generated Analysis of variance (ANOVA) tables with three dependable outputs

Table 1 Software generated ANOVA table considering retention time of caffeine, catechin, EGCG as responses

Source	Sum of squares			df			Mean square			F value			p value Prob > F					
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Model	96.62	783.02	403.15	3	3	3	32.21	261.01	134.38	385.33	365.78	476.81	< 0.0001	< 0.0001	< 0.0001	Significant	Significant	Significant
Linear mixture	86.13	699.27	383.89	1	1	1	86.13	699.27	383.89	1030.54	979.96	1362.11	< 0.0001	< 0.0001	< 0.0001			
AB	5.13	72.58	14.53	1	1	1	5.13	72.58	14.53	61.39	101.71	51.55	< 0.0001	< 0.0001	< 0.0001			
AB (A-B)	5.53	12.15	5.01	1	1	1	5.53	12.15	5.01	66.17	17.03	17.78	< 0.0001	< 0.0009	< 0.0007			
Residual	1.25	10.70	4.23	15	15	15	0.084	0.71	0.28									
Lack of fit	0.45	8.79	2.79	10	10	10	0.045	0.88	0.28	0.28	2.29	0.97	0.9574	0.1860	0.5515	Not significant	Not significant	Not significant
Pure error	0.80	1.91	1.44	5	5	5	0.16	0.38	0.29		365.78							
Cor total	97.87	793.73	407.38	18	18	18		261.01			979.96							

EGCG Epigallocatechingallate; A, B, C are the three different responses where 'A' is response 1-the retention time of caffeine, 'B' is response 2-the retention time of catechin, 'C' is response 3-the retention time of EGCG

(the retention times of caffeine, catechin and epigallocatechin gallate or EGCG) as responses are presented in Table 1.

Considering the retention time of caffeine as a response, The Model F-value of 385.33 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case Linear Mixture Components, AB, AB(A-B) are significant model terms. The "Lack of Fit F-value" of 0.28 implies the Lack of Fit is not significant relative to the pure error. There is a 95.74% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good and is required to fit the model. The "Pred R-Squared" of 0.9798 is in reasonable agreement with the "Adj R-Squared" of 0.9846. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Here the ratio of 43.581 indicates an adequate signal. This model can be used to navigate the design space.

Considering the retention time of catechin as a response, the Model F-value of 365.78 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case Linear Mixture Components, AB, AB(A-B) are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

The "Lack of Fit F-value" of 2.29 implies the Lack of Fit is not significant relative to the pure error. There is a 18.60% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good and we require the model to fit. The "Pred R-Squared" of 0.9761 is in reasonable agreement with the "Adj R-Squared" of 0.9838.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Here the ratio obtained is 41.213 indicating an adequate signal. This model can be used to navigate the design space.The Model F-value of 476.81 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case Linear Mixture Components, AB, AB(A-B) are significant model terms. The "Lack of Fit F-value" of 0.97 implies the Lack of Fit is not significant relative to the pure error. There is a 55.10% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good for the model to fit. The "Pred R-Squared" of 0.9834 is in reasonable agreement with the "Adj R-Squared" of 0.9875.

"Adeq Precision" that measures the signal to noise ratio and a ratio greater than 4 is desirable. The ratio of 44.430 indicates an adequate signal and this model can be used to navigate the design space [10, 11].

The software generated final equations for the response (retention time of caffeine) is + 0.10830*Acetic acid + 1.45602*Acetonitrile − 0.027532*Acetic acid*Acetonitrile + 2.04763E − 004* Acetic acid*Acetonitrile*(Acetic acid–Acetonitrile). The software generated final equations for the response (retention time of catechin) is + 0.26885*Acetic acid + 2.35678*Acetonitrile − 0.047552*Acetic acid*Acetonitrile + 3.03477E − 004* Acetic acid*Acetonitrile* (Acetic acid–Acetonitrile). The software generated final equations for the response (retention time of EGCG) is + 0.27737*Acetic acid + 1.49237*Acetonitrile − 0.028369*Acetic acid*Acetonitrile + 1.94892E − 004* Acetic acid*Acetonitrile*(Acetic acid–Acetonitrile).

3.2 HPLC analysis and method validation

The quantitative yield of secondary metabolites in fresh tea leaves (Fig. 1) procured in three different flushes in spring, monsoon and autumn and also the processed black tea prepared from them (Fig. 2) have shown significant quantitative variance in tea catechins and caffeine content. The amount of the phenolic compounds and methyl xanthenes were found to be highest in leaves plucked during spring (Fig. 1); decrease in phenolic content were observed amongst leaves plucked during monsoon (Fig. 1) and the content was found to be minimum in autumn season (Fig. 1). The same fact was revealed in evidence based quantitative HPLC analysis where black tea processed from tea leaves of three different flushes also exhibited significant variations in phenolic compounds and methyl xanthenes. Processed black tea obtained from the fresh tea leaves of first flush exhibited higher phenolic content (Fig. 2) however a diminution in pharmacologically active components were observed with processed black

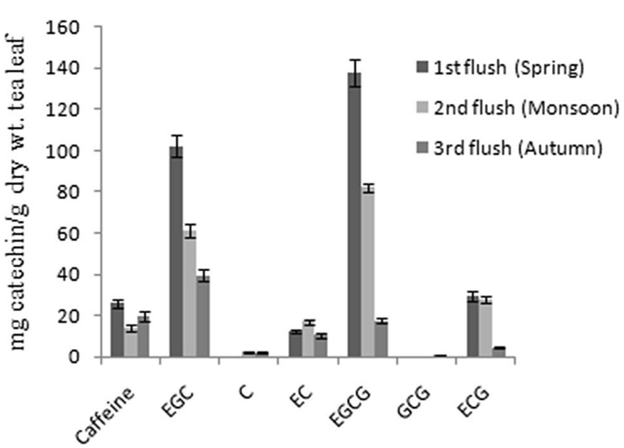

Fig. 1 Comparative histograms showing the variations in secondary metabolites in fresh tea leaves due to seasonal variation. EGC–epigallo catechin; C–catechin; EC–epicatechin; EGCG–epigallocatechin gallate; GCG–gallo catechin gallate; ECG–epicatechin gallate

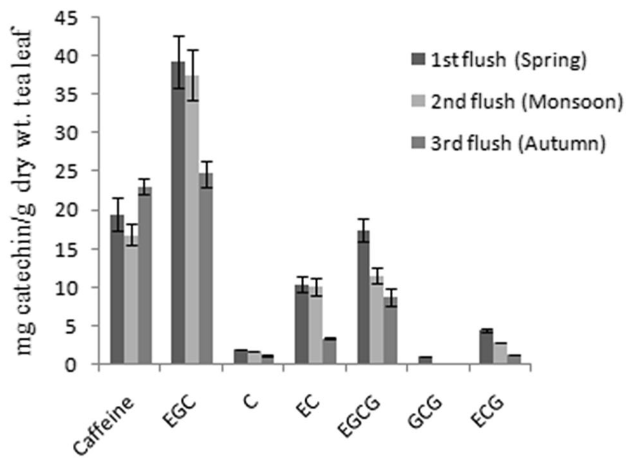

Fig. 2 Comparative histograms showing the variations in secondary metabolites in processed black tea due to seasonal variation. EGC–epigallo catechin; C–catechin; EC–epicatechin; EGCG–epigallocatechin gallate; GCG–gallo catechin gallate; ECG–epicatechin gallate

tea obtained from the fresh tea leaves of second flush of monsoon season (Fig. 2) and still more lower content were observed in processed black tea as obtained from the fresh tea leaves of third flush of autumn season (Fig. 2). Amongst different tea compounds, the main interest of research was the tea catechins and caffeine. Authenticity of the eluted compounds were checked by running the corresponding standards and checking the absorption spectra (λmax) of the eluted compounds. In aqueous media the λmax of caffeine was found to be 273 nm, the λmax values of oter tea catechins include: epigallocatechin—274 nm, epigallocatechin gallate—273.6 nm, gallocatechins—271 nm, gallocatechin gallate—274 nm, epicatechin gallate—278 nm. Considering the method validation parameters for the developed RP-HPLC method, no interferences of peaks from other metabolites was observed as regards specificity of the method thus considering the specific nature of the method. The percentage relative standard deviation for inter and intra day precision are 0.3 and 0.2 respectively, for repeatability of the method it is 0.31, for ruggedness it is 0.33 and for robustness (with deliberate alterations in flow rate) its 0.2. In all cases the values lied within the recommended limits i.e. not more than 2.00. Thus all parameters of method validation studies were found to lie within the specified limits.

4 Discussion

Plants in order to combat and adapt to environmental stress factors viz. drought, extremes of temperature fluctuations, high salinity are equipped with Plant secondary metabolites (PSMs). PSMs not only provide protection and increase the adaptive capacity of plants but themselves are of great pharmacological significance and of nutrotherapeutic and industrial applications [12]. Under stressful environmental conditions, metabolic responses are triggered; there will be excess presence of reduction equivalents (NADPH + H^+) due to decrease in CO_2 uptake owing to stomatal closure. Excess reduction equivalents will generate huge oxidative stress; this stressful condition will shift the metabolic processes towards biosynthetic activities. PSMs viz. phenolics, flavonoids, alkaloids, triterpenoids will be synthesized, so as to quench the reduction equivalents [13]. Biosynthesis of PSMs through biosynthetic pathways like phenylpropanoid pathway, naringenin-chalcone via naringenin to dihydrokaempferol pathway lead to the synthesis of secondary metabolites viz. catechins. These biosynthetic reactions are catalyzed by different enzymes viz. phenylalanine ammonia lyase, chalcone synthase, chalcone isomerase, flavanone-3-hydroxylase. Activities of these enzymes are also influenced by environmental variables. Secondary metabolites in tea, the polyphenols and catechins affect the sensory attributes of black tea brew and are influenced by climatic variations, quality of fresh tea leaves, time of harvest, processing conditions adopted etc. [14, 15]. Catechin biosynthesis is influenced by the expression of genes viz. phenylalanine ammonia lyase, flavanone-3-hydroxylase (F3H), dihydroflavonol-4-reductase (DFR), anthocyanidin synthase (ANS). Expression level of F3H and ANS are reduced in autumn, again DFR is over expressed in bright sunlight [14]. The concentration of the secondary metabolites gets diluted in monsoon owing to level of precipitation [5]. The fact is revealed in processed commercial teas where the polyphenolic content also varies with the quality of tea leaves used, harvesting time, manufacturing process and variables [16–19]. The astringent taste and flavor of black tea, its multi dimensional health potentials and wide range of pharmacological effects are due to tea catechins, methyl xanthenes like caffeine, benzotropolone compounds the theaflavins, astringency due to thearubigins [1–3, 9]. Thus climatic variations affect the concentrations of secondary metabolites of black tea which in turn affect the pharmacologic and sensory attributes of black tea.

5 Conclusion

Black tea being a rich source of catechins, benzotropolone compounds, methyl xanthenes, amino acids, volatile components is found to exhibit versatile pharmacological effects. Not only as a refreshing beverage, black tea has gained research attention owing to its health effects. Experimental results have shown highest concentration of secondary metabolites in fresh tea leaves harvested during spring, concentration declined in monsoon and further in autumn. The same fact is reflected in the processed black tea made thereof. Thus the seasonal effect is of vital importance in assessing the nutrotherapeutic potentials and sensory attributes of black tea.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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