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CHEMOMETRICS ASSISTED VALIDATED HPLC METHOD DETECTING SEASONAL VARIANCES OF SECONDARY METABOLITES OF *Camellia sinensis* CONSIDERING THE ABIOTIC STRESSES

[a] Koushik Bhandari, [b] Baishakhi De, [c] Tridib K. Goswami*
[a] Advanced Technology Development Centre, IIT Kharagpur, 721302, India
[b] Indira college of Pharmacy, Vishnupuri, Nanded, Maharashtra, India,
[c] Department of Agriculture and Food Engineering, Indian Institute of Technology, Kharagpur, 721302, India.

ABSTRACT

Climatic changes have great impact on the crops and agro-eco systems and such changes influences the concentration of secondary metabolites. IIT Kharagpur, India is a non-traditional tea growing zone where Tocklai Vegetative 25 variety was used as the research material. This study reports the development of a chemometrics assisted HPLC method validated as per ICH guidelines to explore the effect of seasonal variations in polyphenolics viz. catechins and methyl xanthenes like caffeine in fresh tea leaves and processed CTC black tea prepared from them. Further study was done on the variances amongst the concentration of secondary metabolites and abiotic stress factors. Good resolutions of secondary metabolites were obtained using 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. Results of quantitative HPLC analysis have clearly shown that highest yield of catechins and caffeine were observed in fresh tea leaves plucked during spring (24.3ºC temperature and average rainfall of 34 mm) and also the processed black tea made from it, followed by tea leaves plucked during monsoon (28.8 ºC temperature and 282 mm rainfall) and processed black tea prepared from it. The lowest concentrations of secondary metabolites were found in leaves plucked during autumn (26.2 °C temperature and 132 mm rainfall) and the processed tea prepared from it. The developed quantitative HPLC method showed an inter day precision of 0.3, intraday precision of 0.2, repeatability value of 0.31, ruggedness value of 0.33 and robustness value of 0.2. Considering temperature and rainfall as abiotic stress factors, highest total polyphenolic content was obtained during spring and lowest in autumn. From our experimental findings, the fresh tea leaves of spring season and also the processed black tea prepared from it showed higher yield of catechins.

Key word: Climatic changes, secondary metabolites, black tea, poly phenols, catechins, spring, autumn, monsoon.

*Corresponding email address: tkg@agfe.iitkgp.ac.in **Received: 22 May 2019** **Accepted: 02 July 2019**

INTRODUCTION

Tea (*Camellia sinensis*) of family theaceae, is the most popular beverage just after water. It has achieved global popularity from time unknown and currently its multifaceted health potentials are being largely explored (Sen and Bera, 2013). Apart from being a refreshing nonalcoholic beverage, tea can be considered as a combinatorial library of several pharmacologically active compounds that attributes to the wide range of its pharmacologic actions (Skotnicka *et al.*, 2011; Sharangi *et al.*, 2014). Tea is manufactured from the tender leaves and buds of *Camellia sinensis* and tea plant being perennial in nature leaves are harvested almost all the year round (Muthumani *et al.*, 2013). Climatic variance and abiotic stress factors has a worldwide impact on the agro-eco systems affecting both the yield and crop quality. Concentrations in plant secondary metabolites (PSMs) are influenced by seasonal variations and the same fact is revealed in case of tea plantations (Ahmed *et al.*, 2014). Variance in phenolic content, amounts of methyl xanthenes etc affects not only the quality of tea but also its pharmacologic profile. This study reports the development of chemometrics assisted HPLC method that has been validated as per ICH guidelines (International Conference on Harmonization) to show the quantitative variances of secondary metabolites both in fresh tea leaves of TV 25 variety (Tocklai Vegetative) grown in the non-traditional tea growing zone of IIT Kharagpur, India harvested in spring, monsoon and autumn and the CTC (cut, tear and curl). Black tea processed from them with a correlation amongst tea secondary metabolites concentrations and abiotic stress factors.

MATERIALS AND METHODS

Plant material: Fresh tea leaves (TV 25 variety, *Voucher specimen*: IITKGP/HB/2018/T1) grown in the non-traditional tea growing zone of IIT Kharagpur, India were collected from three different flushes in three different time periods of the year. Leaves of first flush were procured around mid-February during spring, leaves of second flush in mid-July during monsoon and leaves of third flush in the first month of November during autumn. From the harvested tea leaves of three different seasons processed black tea was also prepared.

Instrument used: Electronic balance; HPLC (Model Waters 2998)

Software: Design Expert version 7.0 for chemometrics work

Sample preparation of fresh tea leaf and processed black tea of different seasons: Sample preparation was done as per the literature methodology (Yao *et al.*, 2004; Shivaprasad and Khanam, 2006; Mandal *et al.*, 2013; Bhandari *et al.*, 2015). About 20g 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 of 60% methanol (very suitable for the extraction of phenolics) 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 1g of black tea to 25 mL of boiling milli-Q™ water and brewed for 5 min. The brewed, aqueous infusions were centrifuged at 8,000 × 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 (Mandal *et al.*, 2013).

For the purpose of human consumption, only aqueous media is used to prepare the tea brew. So secondary metabolites have been determined in aqueous media.

Chemometrics assisted optimization of HPLC chromatographic conditions: D-optimal combined design approach of chemometrics (Bhandari *et al.*, 2015) 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. Chromatographic separations were achieved on RP-C18 column 5 µm (250mm × 4.6 mm i.d.). 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. 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.

HPLC method Validation: Chemometrics assisted optimized HPLC chromatographic conditions have been validated as per ICH guidelines. Method validation was done in terms of precision, specificity, robustness. The proposed RP–HPLC method was validated as per ICH guidelines. Precision was studied in terms of repeatability (system precision), where 20 µg/mL of standard solution (catechin) was injected for six times into the HPLC system as per test procedure. For method precision, from sample and stock solution, 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 (Katakam *et al.*, 2014).

Abiotic factors and secondary metabolites: TV 25 variety of tea used as the research material has been grown in the tea garden of IIT Kharagpur. Geographically, Kharagpur is located at latitude 22°01'N and longitude 87°07'E in South-western Midnapore and covers an area of about 127 km^2, average elevation of 29 meters (95 ft), formed in the alluvial tract of Midnapore and intersected by numerous waterways. Kharagpur has a tropical wet and dry climate. Summers are hot and humid, start in March with average temperatures close to 30 °C (86 °F). It is followed by the monsoon season that estimates about 1140 mm (45 inches) of rain. Winters are brief but chilly, lasting from December to mid-February, with average temperatures around 22 °C (72 °F). Total annual rainfall is around 1400mm (55 in). Considering temperature and rainfall as abiotic stress factors, variances in concentration of secondary metabolites have been studied (recorded as per the meteorology lab in department of Physics, IIT Kharagpur, India),.

RESULTS

The software generated ANOVA tables with three dependable outputs (the retention times of caffeine, catechin and EGCG) are presented in Table 1-3 and the software generated final equations in terms of coded and actual factors for the responses are shown in Table 4.

The quantitative yield of secondary metabolites in fresh tea leaves procured at three different flushes and also the processed black tea prepared from them (Table 5-6) and Fig. 1-2 have shown significant quantitative variance in tea catechins and caffeine content have been observed in fresh tea leaves plucked at three different flushes during spring, monsoon and autumn. The amount of the phenolic compounds and methyl xanthenes were found to be highest in leaves plucked during spring (Table 5); decrease in phenolic content were observed in fresh tea leaves plucked during monsoon (Table 5) and the catechin content was found to be minimum in autumn season (Table 5). 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 (Table 6), however a diminution in pharmacologically active components were observed with processed black tea obtained from the fresh tea leaves of second flush of monsoon season (Table 6) and still more lower content were observed in processed black tea as obtained from the fresh tea leaves of third flush of autumn season (Table 6). The relative abundance of tea catechins, caffeine and other secondary metabolites in the fresh leaves of 1st flush and the processed black tea prepared from them are shown in figure 3. The average climatic condition of IIT Kharagpur, India as recorded a temperature of 24.3ºC and average rainfall of 34 mm during Spring, 28.8ºC temperature and 282 mm rainfall during monsoon and 26.2ºC temperature and 132 mm rainfall in autumn showed the highest yield of total phenolic content (TPC) in spring that was found to decrease in monsoon with further diminution in the autumn season (Figure 4). All parameters of method validation studies were found to lie within the specified limits (Table 7).

HPLC analysis and method validation: The quantitative yield of secondary metabolites in fresh tea leaves procured at three different flushes and also the processed black tea prepared from them (Table 5-6) and Fig. 1-2 have shown significant quantitative variance in tea catechins and caffeine content have been observed in fresh tea leaves plucked at three different flushes during spring, monsoon and autumn. The amount of the phenolic compounds and methyl xanthenes were found to be highest in leaves plucked during spring (Table 5); decrease in phenolic content were observed in fresh tea leaves plucked during monsoon (Table 5) and the catechin content was found to be minimum in autumn season (Table 5). 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 (Table 6), however a diminution in pharmacologically active components were observed with processed black tea obtained from the fresh tea leaves of second flush of monsoon season (Table 6) and still more lower content were observed in processed black tea as obtained from the fresh tea leaves of third flush of autumn season (Table 6). The relative abundance of tea catechins, caffeine and other secondary metabolites in the fresh leaves of 1st flush and the processed black tea prepared from them are shown in Fig. 3. The average climatic condition of IIT Kharagpur, India as recorded a temperature of 24.3ºC and average rainfall of 34 mm during Spring, 28.8 ºC temperature and 282 mm rainfall during monsoon and 26.2 ºC temperature and 132 mm rainfall in autumn showed the highest yield of total phenolic content (TPC) in spring that was found to decrease in monsoon with further diminution in the autumn season (Figure 4). All parameters of method validation studies were found to lie within the specified limits (Table 7).

Source	Sum of squares	df	Mean square	F value	p-value Prob>F	
Model	96.62	3	32.21	385.33	<0.0001	Significant
Linear mixture	86.13	1	86.13	1030.54	<0.0001	
AB	5.13	1	5.13	61.39	<0.0001	
AB (A-B)	5.53	1	5.53	66.17	<0.0001	
Residual	1.25	15	0.084			
Lack of fit	0.45	10	0.045	0.28	0.9574	Not significant
Pure error	0.80	5	0.16			
Cor Total	97.87	18				

Table 1: Software generated ANOVA table considering retention time of caffeine as response 1.

Considering the retention time of caffeine as a response, the Model F-value of 385.33 implies that 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.

Source	Sum of squares	df	Mean square	F value	p-value Prob>F	
Model	783.02	3	261.01	365.78	<0.0001	Significant
Linear mixture	699.27	1	699.27	979.96	<0.0001	
AB	72.58	1	72.58	101.71	<0.0001	
AB (A-B)	12.15	1	12.15	17.03	<0.0009	
Residual	10.70	15	0.71			
Lack of fit	8.79	10	0.88	2.29	0.1860	notsignificant
Pure error	1.91	5	0.38			
Cor Total	793.73	18				

Table 2: Software generated ANOVA table considering retention time of catechin as response 2

Considering the retention time of catechin as a response, the Model F-value of 365.78 implies that 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.

Source	Sum of squares	df	Mean square	F value	p-value Prob>F	
Model	403.15	3	134.38	476.81	<0.0001	Significant
Linear mixture	383.89	1	383.89	1362.11	<0.0001	
AB	14.53	1	14.53	51.55	<0.0001	
AB (A-B)	5.01	1	5.01	17.78	<0.0007	
Residual	4.23	15	0.28			
Lack of fit	2.79	10	0.28	0.97	0.5515	Not significant
Pure error	1.44	5	0.29			
Cor Total	407.38	18				

Table 3: Software generated ANOVA table considering retention time of EGCG as response 3.

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.

Variables	**Final equation in terms of coded factors**	**Final equation in terms of actual factors**
Retention time of caffeine	+14.27*A	+0.10830*Acetic acid
	+9.39*B	+1.45602*Acetonitrile
	-4.74*A*B	-0.027532*Acetic and acid*Acetonitrile
	+13.10*A*B*(A-B)	+2.04763E-004* Acetic acid * Acetonitrile * (Acetic acid-Acetonitrile)
Retention time of catechin	+26.82*A	+0.26885*Acetic acid
	+12.40*B	+2.35678*Acetonitrile
	+12.40*B	+2.35678*Acetonitrile
	-17.82*A*B	-0.047552*Acetic acid*Acetonitrile
	+19.42*A*B*(A-B)	+3.03477E-004* Acetic acid * Acetonitrile * (Acetic acid-Acetonitrile)
Retention time of EGCG	+28.39*A	+0.27737*Acetic acid
	+17.56*B	+1.49237*Acetonitrile
	-7.97*A*B	-0.028369*Acetic acid*Acetonitrile
	+12.47*A*B*(A-B)	+1.94892E-004* Acetic acid * Acetonitrile * (Acetic acid-Acetonitrile)

Table 4: Software generated final equations in terms of coded and actual factors for the responses

Sl. No.	**Compound Name**	**Retention Time (RT)**	**mg catechin/g dry weight tea leaf**		
			1st flush (Spring) fresh tea leaves (281.53 mg)	**2nd flush (Monsoon) fresh tea leaves (190.26 mg)**	**3rd flush (Autumn) fresh tea leaves (74.76 mg)**
1	Caffeine	8.3	26.02	13.93	19.55
2	EGC	9.9	102.14	61.2	39.39
3	C	11.9	-	2.34	2.03
4	EC	14.3	12.18	16.93	10.46
5	EGCG	17.8	137.61	82.26	17.39
6	GCG	19.9	-	-	1.02
7	ECG	22.2	29.60	27.52	4.47

Table 5: Quantitative yield of secondary metabolites in fresh tea leaves

*EGC-epigallocatechin; C-catechin; EC-epicatechin; EGCG-epigallocatechingallate; GCG-gallocatechingallate; ECG-epicatechingallate

Sl. No.	**Compound Name**	**Retention Time (RT)**	**mg catechin/g dry weight tea leaf**		
			Processed black tea of 1st flush (74.76 mg)	**Processed black tea of 2nd flush (63.88 mg)**	**Processed black tea of 3rd flush (74.76 mg)**
1	Caffeine	8.3	19.55	16.83	23.13
2	EGC	9.9	39.39	37.57	24.79
3	C	11.9	2.03	1.77	1.17
4	EC	14.3	10.46	10.07	3.48
5	EGCG	17.8	17.39	11.59	8.71
6	GCG	19.9	1.02	-	-
7	ECG	22.2	4.47	2.88	1.33

Table 6: Quantitative yield of secondary metabolites in processed black tea.

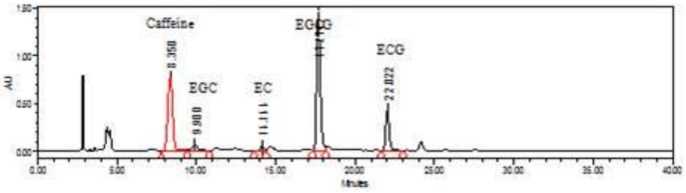
*EGC-epigallocatechin; C-catechin; EC-epicatechin; EGCG-epigallocatechingallate; GCG-gallocatechingallate; ECG-epicatechingallate.

Parameters	**Recommended limits**	**RP-HPLC method**
Specificity	No interferences	a
Precision*	NMT# 2.00	
Inter day		0.3*
Intraday		0.2*
Repeatability*	NMT#2.00	0.31 *
Ruggedness		0.33 *
Robustness *, (Flow rate, mL/min)	NMT# 2.00	0.2 * (1.4) 0.2 * (1.6)

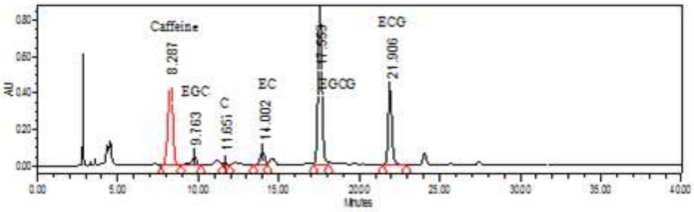
Table 7: Validation parameters of the developed RP-HPLC method (n=6)

*Percentage relative standard deviation, # Not more than, a= Specific nature of method

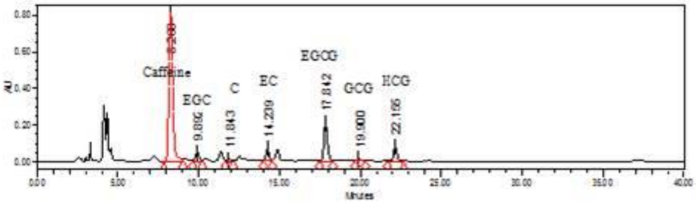
Figure 1: HPLC chromatograms of fresh tea leaves in three different flushes.



A: Compounds in tea leaves of spring season (281.53 mg catechin/gm dry weight)

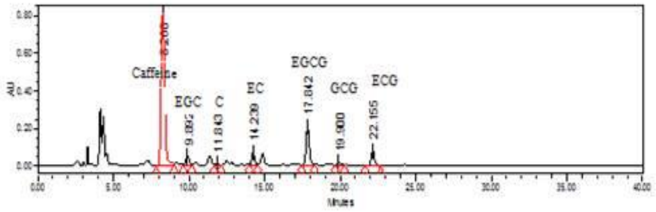


B: Compounds in tea leaves of Monsoon season (190.26 mg catechin/g dry weight)

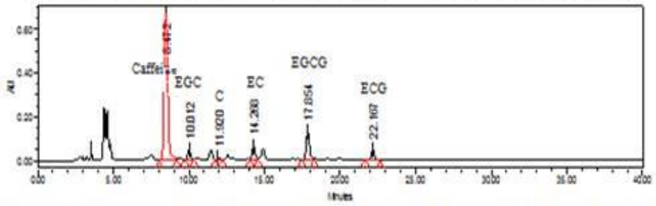


C: Compounds in fresh tea leaves of autumn season (74.76 mg catechin/g dry weight)

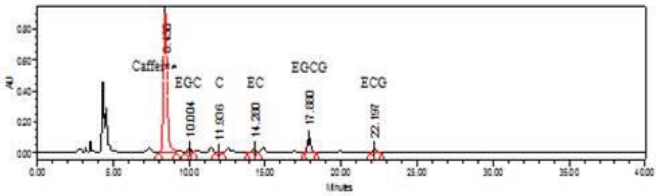
Figure 2: HPLC chromatograms of processed black tea prepared in three different flushes



A: Compounds in processed Black tea (spring) (74.76 mg catechin/gm dry weight)



B: Compounds in processed Black tea of Monsoon season (63.88 mg catechin/gm dry weight)



C: Compounds in processed Black tea of autumn season (39.48 mg catechin/gm dry weight)

Figure 3: Relative abundance of secondary metabolites in fresh tea leaves (top) procured during 1st flush (spring) and processed black tea (below) prepared from it

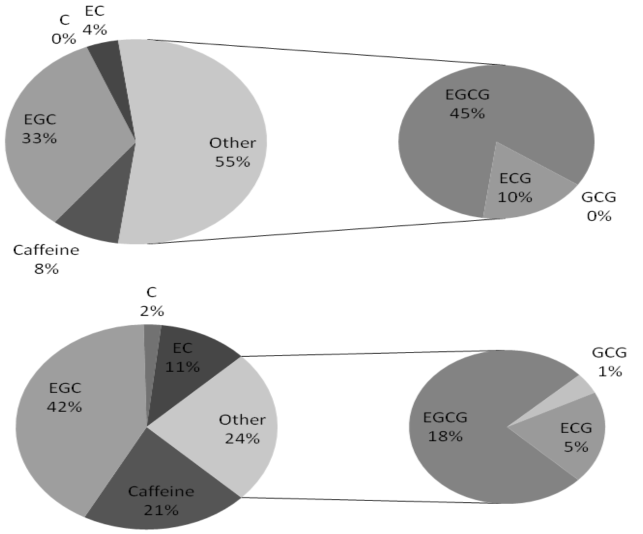
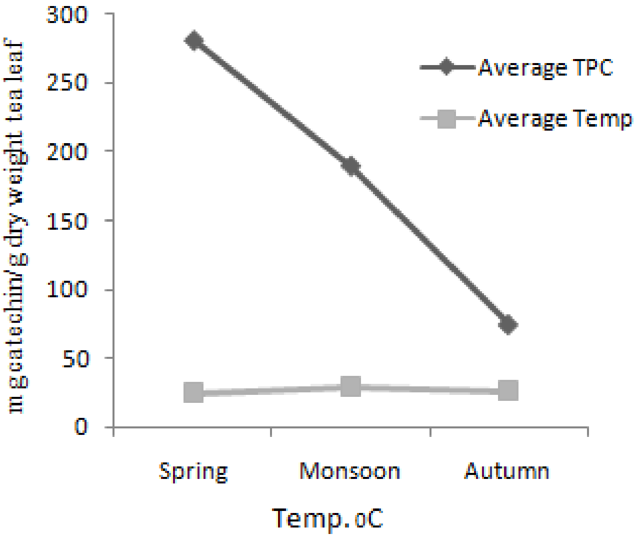


Figure 4: Variance in phenolic content (TPC) with the variance in temperature



Discussion

Plant secondary metabolites (PSMs) are not only important sources of phytopharmaceuticals, nutraceuticals or bio chemicals of industrial applications but also help plants in adaptation to environment and combating the stress factors viz. drought, high salinity, freezing temperatures (Akula and Ravishankar, 2011). Drought triggers stress induced metabolic responses, there will be stomatal closure, significant decrease in CO$_2$ uptake thus reducing the consumption of reduction equivalents (NADPH+H$^+$) for CO$_2$ fixation via the Calvin cycle; excess presence of reduction equivalents will generate huge oxidative stress and metabolic processes get shifted towards biosynthetic activities enhancing the synthesis of isoprenoids, phenolics, alkaloids so as to quench the reduction equivalents (Selmar and Kleinwächter, 2013). Catechins are the main astringent substances in tea synthesized by the phenylpropanoid pathway. Tea polyphenols which are closely associated with the sensory attributes and quality of tea brew are greatly influenced by leaf quality, harvesting season, climate, processing method etc (Turkmen *et al.*, 2009; Liu *et al.*, 2015). The fresh tea leaves of TV 25 variety grown in the non-traditional tea growing zone of IIT Kharagpur showed significant concentration of phenolics (mostly catechins estimated) as evidenced by our current study. The processed black tea also showed significant yield of the catechins. However, fresh tea leaves plucked during spring or processed tea made from leaves of spring time showed higher catechin content in comparison to monsoon and autumn Catechin biosynthesis which follows the pathway of naringenin-chalcone via naringenin to dihydrokaempferol pathway are catalyzed by a number of enzymes viz. phenylalanine ammonia lyase, chalcone synthase, chalcone isomerase, flavanone-3-hydroxylase. The activity of these enzymes are influenced by environmental and climatic variables; the expression of the genes phenylalanine ammonia lyase, flavanone-3-hydroxylase (F3H), dihydroflavonol-4-reductase (DFR), anthocyanidin synthase (ANS) are associated with catechin biosynthesis. Expression level of F3H and ANS are reduced in autumn, again DFR is over expressed in bright sunlight (Liu *et al.*, 2015). Again high level of precipitation in monsoon leads to dilution of secondary metabolites (Ahmed *et al.*, 2014). Thus the poly phenolic content in commercial teas greatly varies with the species, seasonal effect, degree of oxidation or fermentation during the manufacturing process, horticultural conditions etc (Yao *et al.*, 2005; Boehm *et al.*, 2016; Ghabru and Sud, 2017; Gogoi, 2017). Considering the abiotic stress factors (here temperature and rainfall at IIT Kharagpur), the climatic conditions of spring time was found to be suitable for the increased concentration of tea catechins in TV 25 variety of tea and was found to decrease in monsoon climatic conditions and further reduce in autumn months. The concentrations of secondary metabolites in tea viz. catechins, methyl xanthenes like caffeine, benzotropolone compounds the theaflavins are responsible for the multi-dimensional pharmacological effects of tea like antioxidant, anti-diabetic, cardio protective, neuroprotective, immunostimulatory, anti-cancer and other effects (Skotnicka *et al.*, 2011; Sen and Bera, 2013; Sharangi *et al.*, 2014; Bhandari *et al.*, 2015). Thus the climatic impacts on the variance in concentrations of pharmacologically active compounds of tea have been studied in an evidence based manner that also influences the sensory attributes and health potentialities of tea.

CONCLUSION

Food and nutritional scientists are considering tea as an important research material not only as a popular beverage after water but tea is a store house of wide range of pharmacologically active molecules. However as per experimental evidences processed tea from the leaves plucked during first flush of spring season exhibited highest concentration of secondary metabolites followed by tea processed during monsoon and the lowest concentration was observed in autumn. Thus the organoleptic qualities of the processed tea as well as its nutrotherapeutic potentials will greatly depend on the time of flush of fresh tea leaves.

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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