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Development and Validation of a HPLC based analytical method, towards the determination of sugar concentration in processed black tea

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Abstract A rapid and sensitive reversed phase High Performance Liquid Chromatographic (HPLC) method was developed and validated for the quantitative estimation of soluble sugars viz fructose, glucose and sucrose in black tea brew. The method includes an isocratic elution of mobile phase comprising 83% aqueous acetonitrile for 30 minutes at a flow rate of 1.5 mL/min over Zorbax NH_2 (150 x 4.6 mm, 5µm) column at column oven temperature of 33 ºC. The detection was done using Reflective Index Detector (RID) at the temperature of 33 ºC. Validation was conducted with respect to selectivity, linearity and linear range, sensitivity, accuracy and precision. The calibration plots were linear in response with correlation coefficient (r^2) value of 0.99 over the concentration range of 50 ppm – 1000 ppm for all three standard solutions. More than 98% recovery was observed during the recovery study of sugar spiked samples. The precision of the developed method in terms of Co-efficient of Variance (CV %) were in the range of 1.07-1.98, 0.85-1.15 and 0.9-1.92 at the spike levels of 0.25%, 0.375% and 0.5% respectively. Therefore, the methodology was found to be accurate, reproducible and sensitive, which could be successfully applied for the determination of sugar compounds in black tea brew.

Key words: Liquid chromatography; Sugar; Fructose; Glucose; Sucrose; Black tea

1. Introduction

Tea is a universally accepted natural beverage which is produced from young succulent harvestable shoots of the plant *Camellia sinensis (L.)* O. Kuntze (Tea Plant). Thus, the bio-chemical composition of tea leaves is the basis for the transformation and synthesis of bio organic compounds under different manufacturing conditions. This ultimately leads to the quality aspects of made tea [15].

Tea is a complex matrix which consists diverse biochemical constituents. A range of biochemical parameters in tea, such as polyphenols and tannins, catechins, amino acids,

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antioxidants, volatile compounds etc. have been studied extensively throughout the world [6, 8, 9, 10, 15]. However availability of studies on the impact of naturally existing sugar content on quality of tea is very scarce [2]. The unavailability of a standard, efficient, reliable and validated quantitative methodology for the determination of sugar components in tea, could be one of the reasons for scarcity of studies on tea sugar components.

As in all plants, tea plant also synthesizes glucose, a monosaccharide as a primary product of photosynthesis which provides energy and input materials for the biosynthesis of various other structural components. In terms of plant physiological aspects, glucose is converted to sucrose, a disaccharide for translocation, and when energy is needed, sucrose is converted back to glucose and fructose (Figure 1). Accordingly the presence of inherent sugar in made tea and tea brew is un-preventable as tea is produced directly from the harvestable shoots of tea plant, which necessarily contains photosynthetically derived carbohydrates.

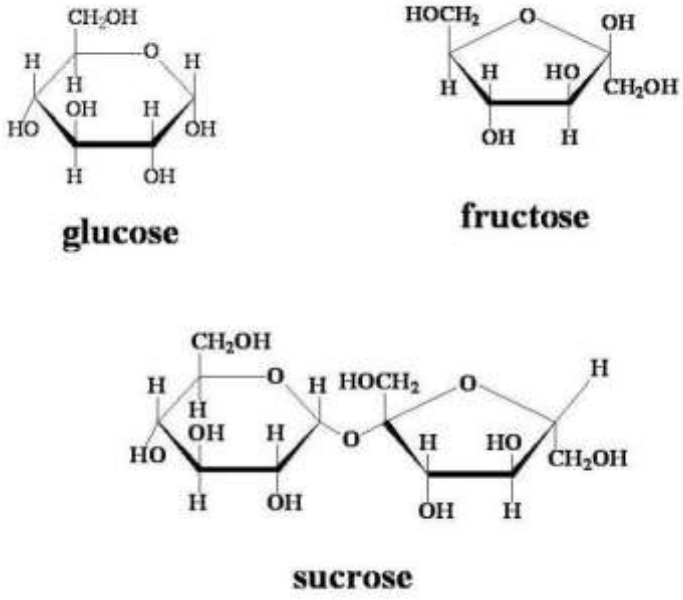

Figure 1: Chemical structures of glucose, fructose and sucrose

The level of sugar content present in made tea could vary due to a range of factors which affect the metabolic pathways in tea plant. This includes climatic factors, elevation of tea growing regions, types of clones, harvesting practices as well as the time of the harvest etc. Thus, the establishment of a validated analytical method is necessarily important towards the establishment of such information.

Though several colorimetric methods including, Anthrone, Phenol Sulphuric and Modified Lane and Eynone etc., with the basis of oxidation – reduction process, have been tried out for this purpose, [7,5], the information generated through these methods is less accurate. This is because the oxidation-reduction processes can also be taken place in other compounds such as polyphenolics in tea brew [5]. Further, those colorimetric methods could determine only the total sugar content, but not the concentrations of each of individual components [7]. Literature survey revealed only one chromatography method for the quantitative determination of sugar components in processed tea, which is based on Amperometric Detector [2].

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Thus, the development and validation of a reliable chromatographic methodology for the detection and quantification of sugar concentration in black tea is imperative. Therefore during this study, a reliable Reflective Index Detector (RID) based High Performance Liquid Chromatography (HPLC) analytical methodology was developed for the determination of glucose; fructose and sucrose concentration in black tea brew and the validation study clearly revealed that the methodology is applicable for the intended purposes.

2. Materials and Methodology

2.1. Instrumentation

Agilent Technologies 1260 HPLC system with auto-sampler (model G7129A), quaternary pump (model G7111B), Agilent Technologies- Zorbax NH_2 column (150 x 4.6 mm (5µm)) (Part No: 883952-708) equipped with RID (model G 7162A) was used for method development and validation.

2.2. Chemicals and reagents

Standard glucose (Purity - 99.5%), fructose, sucrose (Purity - 99.99%) were purchased from Chem Service chemicals and analytical grade Acetonitrile (Purity - 99.99%) was purchased from Sigma-Aldrich.

2.3. Chromatographic conditions

The chromatographic conditions used to develop and validate the methodology using Zorbax NH_2 column (150 x 4.6 mm (5µm)) was as follows:

Isocratic system with a mobile phase comprised of 83% Acetonitrile: 17% water (Filtered through 0.45 µM nylon membrane filters) - (Agilent Technologies); at the flow rate of 1.5 mL min^{-1}

Column oven temperature is 33 ºC; Detector (RID) Temperature is 33 ºC.

Injection volume is 10 µL for all the standards and the samples.

2.4. Preparation of Standard Solutions

The standard solutions of fructose, glucose and sucrose were prepared by dissolving an accurately weighted 0.2000 g of each of the compound in 100 mL of mobile phase to make 2000 ppm of stock solution. Through the serial dilution of the stock solution with the mobile phase, the standard series were prepared for each compound in the range of 50 ppm – 1000 ppm.

2.5. Establishment of the calibration curves

Three calibration curves were prepared separately for each of the standard compound, in the range of 50 ppm – 1000 ppm.

For each of the concentration level, three replicates were prepared separately and the average response value of triplicates was used for the establishment of the calibration curve.

2.6. Extraction of the tea sample

The black tea samples were ground through 0.5 mm mesh to homogenize the samples and a weight of 4.00 g of homogenized sample was extracted with 50 mL of distilled water at room temperature. Samples were brewed for 30 mins, in a magnetic shaker and filtered through cotton wool. A volume of 1 mL of the extraction was diluted with 4 mL of 100% Acetonitrile. Diluted brew was again filtered through 0.45 µM Nylon filters (Agilent Technologies) prior to the HPLC injection.

2.7. Analytical Method Development and Optimization

The optimizations for analytical method development was conducted with mobile phases using different solvent strengths at different flow rates and at different column temperatures, considering one variable at a time, while keeping other variables constant.

Mobile phases, 75%, 80% and 83% Acetonitrile: MilliQ water and 0.5 ml/min^{-1}, 1 ml/min^{-1}, 1.5 ml/min^{-1} flow rates, whereas 30 ºC, 33 ºC and 35 ºC column oven temperatures were assessed for the determination of optimal chromatographic conditions for the best resolution.

The injection volume was 10 µL for all the standards and samples.

2.8. Method Validation

Validation of an analytical procedure demonstrates its suitability for intended purposes. [14, 1]. Thus for the process of defining the performance characteristics of developed chromatographic system, validation parameters including selectivity, linearity and liner range, sensitivity in terms of Limit Of Detection (LOD) and Limit Of Quantification (LOQ), accuracy and precision in terms of Co-efficient of Variance (CV%) were determined.

2.8.1. Selectivity

Selectivity of an analytical method is defined as the degree to which a method can quantify the analyte in the presence of inerferents [4].

It was evaluated by injecting 10 µL solutions of standards, acetonitrile blank and the extracted tea brew sample separately and was identified by the eluted well defined peaks at respective retention times.

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2.8.2. Linearity and the Linear range

The ability of an analytical method to provide results directly proportional to the concentration of the substance is denoted by the linearity [11].

For its determination, the standard solutions in a concentration range of 50 ppm-1000 ppm were prepared and analyzed under optimized chromatographic conditions. Three injections from each concentration were analyzed under the same conditions. The linear regression analysis was used to evaluate the linearity of calibration curve, by using least square linear regression method.

2.8.3. Sensitivity

The sensitivity was measured in terms of LOD and LOQ, whereas the LOD is the concentration of the tea brew which gives a signal to-noise ratio of approximately 3:1, while the LOQ is the concentration which gives a signal to-noise ratio of approximately 10:1. [3]. This was conducted by the repeated dilution of sample extract, until a reliable detection and quantification is achieved for each of the three components.

2.8.4. Accuracy

The accuracy of the methodology was determined by recovery studies at three spiked concentration levels. The extend of ability to recover each target component after extraction and chromatographic separation was determined by recovery studies, and was calculated using the following equation.

Recovery % = (C1 – C2)/ C3 × 100, where;

C1 = Concentration determined in the spiked sample

C2 = Concentration determined in unspiked sample

C3 = Concentration of total spiked

2.8.5. Precision

Precision of an analytical method expresses the closeness of measurements in a series of analytical results obtained from multiple sampling of the same homogeneous sample under the prescribed conditions [12].

This was determined in terms of Relative Standard Deviation (RSD) or the Co-efficient of variance (CV), through successive separate injections of different extracts from the same sample and was calculated using the following equation.
CV % = S/$\bar{x}$ × 100
$\bar{x}$ = Mean value of five repeated determinations
S = Standard deviation of five repeated determinations

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3. Results and Discussion

3.1. Analytical method Development and Optimization

During the optimization of chromatographic conditions and solvent strength programming of method development, the isocratic elution of mobile phase comprising 83 % aqueous acetonitrile at flow rate of 1.5 mL/min, along with column oven temperature of 33 ºC were identified as the optimal conditions for the determination of target sugar compounds in black tea brew. Under these optimal conditions, target compounds were eluted at desirable retention times (RT), with symmetrical peak shapes along with good resolution within a total running time of 30 minutes. (Figure 2).

The results of the method optimizations are summarized in Table 1.

Table 1: Results of the optimization of chromatographic conditions

Mobile Phase Composition		**Mobile Phase Flow rate (mL/ min)**	**Column Oven Temperature (ºC)**	**Detector Temperature (ºC)**	**Resolution**	**Method Accepted/ Rejected**
Acetonitrile	**MilliQ Water**					
75%	25%	0.5	30	30	Poor	Rejected
		1	30	30	Poor	Rejected
		1.5	30	30	Poor	Rejected
80%	20%	0.5	30	30	Poor	Rejected
		1	30	30	Poor	Rejected
		1.5	30	30	Poor	Rejected
83%	17%	0.5	30	30	Poor	Rejected
		1	30	30	Poor	Rejected
		1.5	30	30	Good	Accepted
83%	**17%**	**1.5**	**33**	**33**	**Best**	**Accepted & Used for validation**
		1.5	35	35	Good	Accepted

3.2 Method validation

3.2.1 Selectivity

The absence of co-eluting peaks at the RT of the desired compounds, in black tea sample confirmed the high degree of selectivity under the optimal operating conditions.

At the total running time of 30 minutes, the corresponding RT values in standard solutions were 3.655, 4.207 and 6.731 min (Figure 2). Whereas, the RT values for those compounds

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in tea sample were 3.661, 4.211 and 6.739 min for fructose, glucose and sucrose respectively (Figure 3)

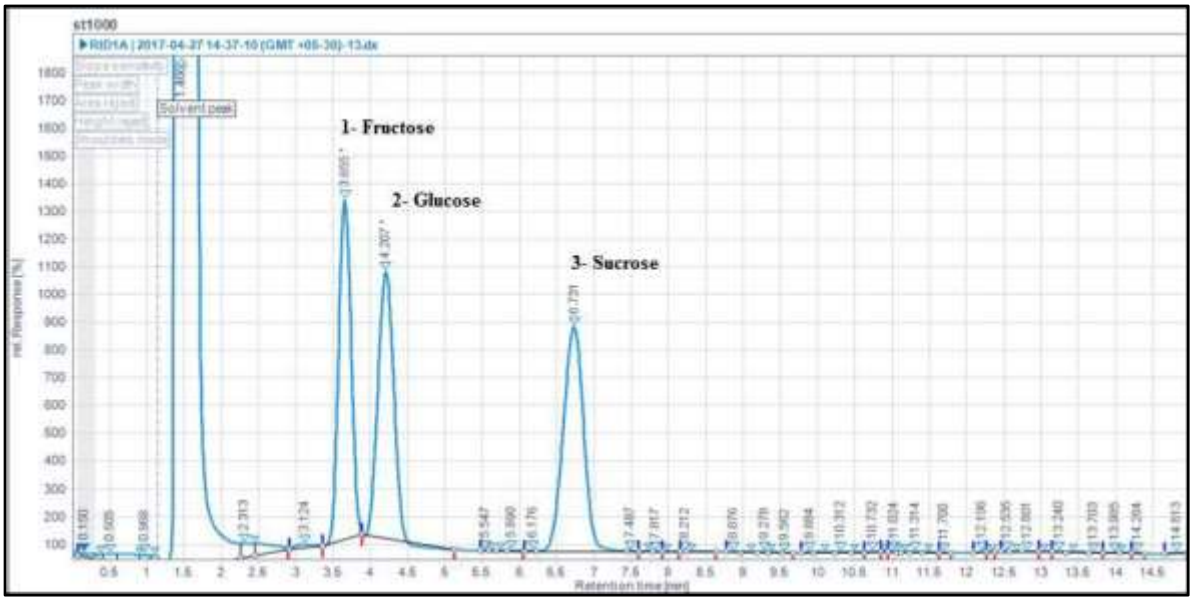

Figure 2: Chromatogram for standard solutions of fructose, glucose and sucrose at optimal operating conditions using Zorbax NH_2 column (150 x 4.6 mm (5µm))
1. Fructose (RT 3.665 min) 2. Glucose (RT 4.207 min) 3. Sucrose (RT 6.731 min)

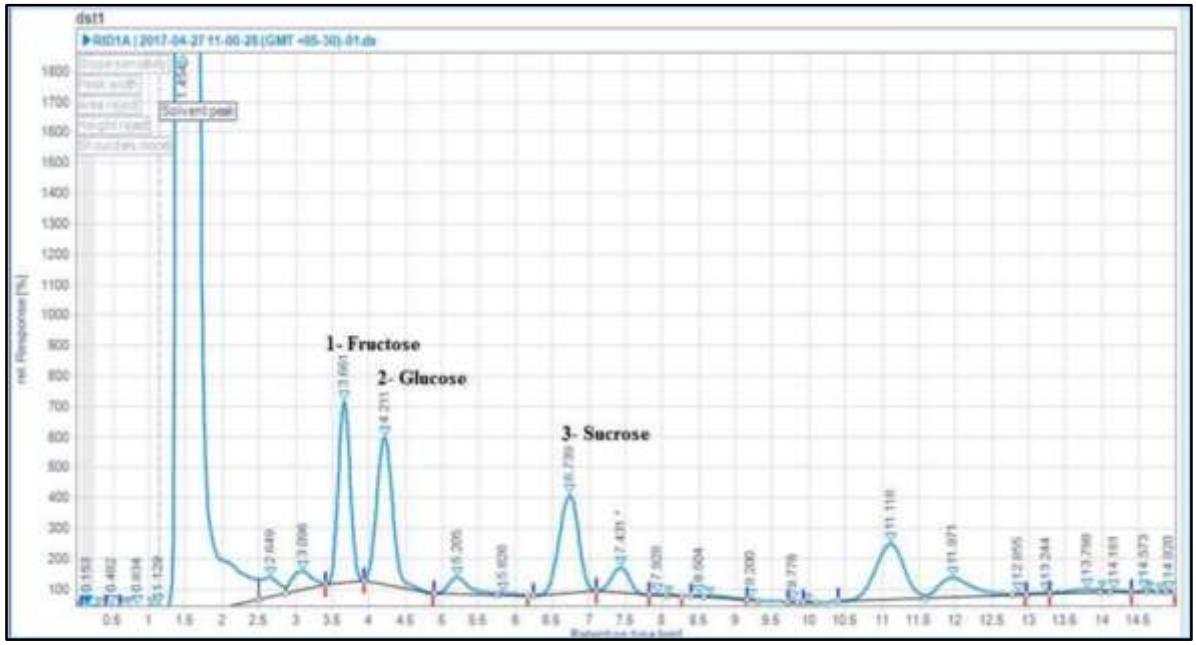

Figure 3: Chromatogram for black tea brew (4.00 g of tea sample extracted with 50 mL of distilled water at Room Temperature), at optimal operating conditions using Zorbax NH_2 column (150 x 4.6 mm (5µm))
1. Fructose (RT 3.661 min) 2. Glucose (RT 4.211 min) 3. Sucrose (RT 6.739 min)

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3.2.2. Linearity and Linear Range

Linearity was assessed for each of the sugar components, by linear regression analysis for the of three authentic calibration curves in the concentration range of 50 ppm - 1000 ppm of standard solutions. (Figure 4)

Table 2 denotes the linear regression equations obtained using three authentic calibration curves established in three different dates. "Y" represents the average integrated peak area in the chromatogram and "X" represents the concentration (ppm) of each of the sugar compound. Correlation coefficient is indicated by r^2.

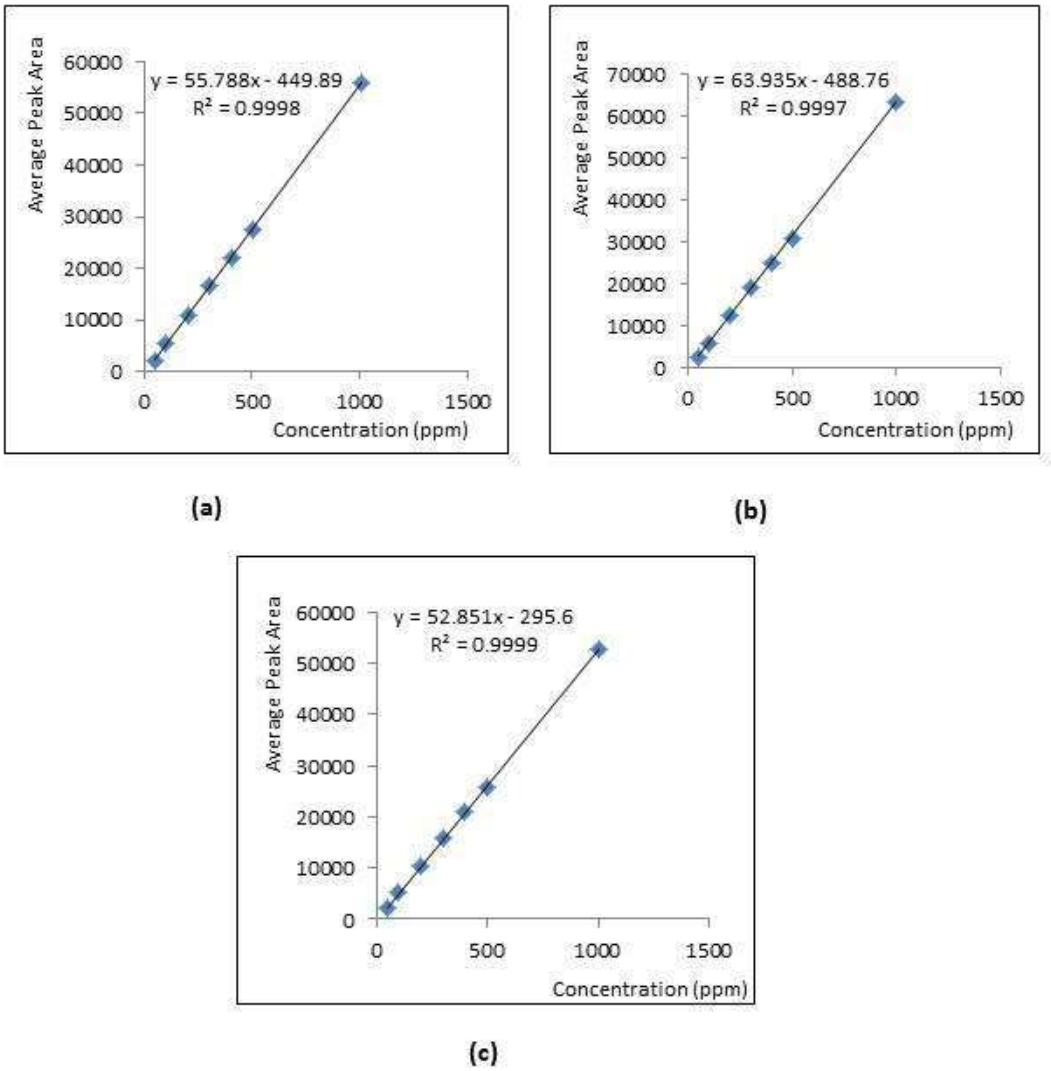

Figure 4: External calibration curves, established using standard solutions in the range of 50 ppm – 1000 ppm; (a) Fructose (b) Glucose (c) Sucrose

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Table 2: Linear regression equations obtained for calibration curves, Sucrose (Suc), Glucose (Gluc) and Fructose (Fruc)

Suc		**Gluc**		**Fruc**	
Regression Equation	r^2	Regression Equation	r^2	Regression Equation	r^2
Y=52.851x-295.60	0.9999	Y=63.935x-488.76	0.9997	Y=55.788x-449.89	0.9998

Correlation coefficient, r^2>0.99, in regression analysis indicates a linear relationship between concentration of the analyte and area under peak over concentration range of 50 to 1000 µg/mL^{-1}, for all three analyzed sugar compounds .

3.2.3. Sensitivity

3.2.3.1 Limit Of Detection (LOD) and Limit Of Quantification (LOQ)

The lowest amount of the analyte in a sample which can be detected, but not necessarily be quantified is termed as the LOD. Whereas, the lowest amount of the analyte in sample that can be quantitatively determined with acceptable precision is termed as the LOQ [13]. According to the observed results, the concentration values of 35 ppm and 50 ppm were identified as the LOD and LOQ values respectively for all three sugar components.

3.2.4. Accuracy %

The degree of closeness of the results obtained by an analytical method, to the true value is termed as accuracy and was determined by the recovery % [12]. This was conducted at three different spiked levels including 2500 ppm, 3750 ppm and 5000 ppm (0.25%, 0.375% and 0.5%) in three successive analysis (n=3) of the proposed method. At all three spiked levels, the average recovery of fructose, glucose and sucrose were in the range of 98.80%- 106.27% (Table 3).

3.2.5. Precision

The precision of the proposed method was determined by the repeatability of responses after replicate injections (n = 3). The precision was expressed in terms of CV % and it was found to be in the range of 1.07-1.98, 0.85-1.15 and 0.9-1.92 at the spike levels of 0.25%, 0.375% and 0.5% respectively. (Table 3)

Table 3: Average detection (mg/g), SD, CV% and Recovery%, at three different spike levels of standards, Fructose (Fruc), Glucose (Gluc) and Sucrose (Suc)

	Fruc			**Gluc**			**Suc**		
Spiked amount (mg/g)	**2.50**	**3.75**	**5.00**	**2.50**	**3.75**	**5.00**	**2.50**	**3.75**	**5.00**
%Spiked level	**0.25**	**0.375**	**0.50**	**0.25**	**0.375**	**0.50**	**0.25**	**0.375**	**0.50**
Av. Detection (mg/g)	2.66	3.79	5.13	2.54	3.94	5.11	2.47	3.80	5.22
SD	0.04	0.03	0.10	0.05	0.05	0.09	0.03	0.04	0.05
CV%	1.52	0.85	1.92	1.98	1.15	1.66	1.07	0.95	0.9
Recovery%	106.27	100.98	102.60	101.47	104.98	102.27	98.80	101.33	104.47

4. Conclusion

During this study, a reverse phase High Performance Liquid Chromatographic method was developed and validated with respect to linearity and linear range, selectivity, sensitivity, accuracy and precision. All performance parameters of the developed method are in acceptable levels for a validation study. The developed method is simple, accurate and an efficient methodology with sufficient repeatability. Thus the methodology can be successfully applied for the efficient determination of fructose, glucose and sucrose concentration in tea brew.

Conflicts of Interest

The authors declare no conflicts of interest in publication of this research

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