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# DoE-assisted RP-HPLC Method for Simultaneous Estimation of Caffeine, Eugenol, and Zingerone in *Ashwagandha* Green Tea

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## Abstract

**Background:** The objective of this research was to develop a reliable RP-HPLC analytical technique for the concurrent determination of Caffeine, Eugenol, and Zingerone in a marketed formulation. **Aim:** This research aimed to develop a novel High-Performance Liquid Chromatography (HPLC) method that is simple and accurate for standardising the three Phytoconstituents, Caffeine, Eugenol, and Zingerone, in TATA TE-A-ME *Ashwagandha* Green Tea. Green tea is used because it has anti–oxidant, anti-inflammatory, and anti-bacterial properties. It includes dried variants of the Tulsi, Ginger, Turmeric, and Liquorice plant species. **Method:** A C-18 column was used as the stationary phase, while Methanol and water (35: 65 v/v) were used as the mobile phase in the method development and validation. For Caffeine, Eugenol, and Zingerone, detection and quantification were carried out at 254 nm. **Result:** The Retention time was 4.76, 8.64 and 10.48 for Caffeine, Eugenol and Zingerone, respectively. In terms of linearity, precision, specificity, Limit of Detection (LOD), Limit of Quantification (LOQ), accuracy, and robustness, the method was validated in accordance with ICH guidelines. The calibration plots for Caffeine, Eugenol and Zingerone in the range showed a satisfactory linear relationship according to the regression analysis data. **Conclusion:** The study integrates the Experiment Planning (DOE) methodologies with High-Performance Liquid Chromatography in the Reverse Phase (RP-HPLC) to develop an analytical method for estimating phytoconstituents. Design of Experiments is applied to Robustness.

**Major Findings:** The developed and validated HPLC method is simple, precise, specific and robust, making it suitable for the simultaneous estimation of eugenol, zingerone, and caffeine in commercial formulations.

**Keywords:** Caffeine, Eugenol, HPLC, Validation, Zingerone

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## 1. Introduction

Around the world, green tea is a popular beverage, especially in Asian nations. Green tea is said to offer more health benefits than almost any other food or drink. Green tea helps prevent hypertension and reduce blood pressure. Tea causes the body to produce more nitric oxide, which widens arteries and lowers blood pressure[1-3]. TATA-TE-A-ME *Ashwagandha* green tea keeps you active and focused while promoting relaxation, regulating nerves. It lessens the symptoms of anxiety and depression, making it the ideal infusion for a busy workday. An excellent detox tea that may promote better digestion is made with liquorice root, tulsi, ginger, and turmeric[4].

*Ashwagandha* green tea is a polyherbal beverage formulation that contains several bioactive ingredients derived from various plant sources, such as zingerone

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(*Zingiber officinale*), eugenol (*Ocimum tenuiflorum*), and caffeine (*Camellia sinensis*). Together, these ingredients give the anti-inflammatory, stimulant, and antioxidant qualities. It takes a lot of time, solvents, and money to develop specific analytical techniques for every marker ingredient, especially when it comes to routine quality evaluation of herbal formulations. Therefore, simultaneous estimation is crucial for thorough quality control, batch-to-batch homogeneity, and regulatory compliance[5,6].

Traditional RP-HPLC techniques are often developed using the One-Factor-at-a-Time (OFAT) paradigm, which often ignores interaction effects between important method factors such as mobile phase composition, pH, flow rate, and detection wavelength. This limitation becomes particularly crucial when analysing chemically diverse analytes with unique chromatographic properties, such as caffeine (polar), eugenol (moderately lipophilic), and zingerone (phenolic ketone). Applying Design of Experiments (DoE) enables systematic evaluation of multiple components simultaneously, identifies Critical Method Parameters (CMPs) and their interactions and creates a reliable and effective analytical technique requiring few experimental runs[7]. By following the principles of Analytical Quality by Design (AQbD), as recommended by ICH Q8–Q14 recommendations, DoE-based method development guarantees enhanced method robustness, reproducibility, and regulatory acceptance[8].

## 2. Materials and Methods

### 2.1 Standards and Chemicals

The standard phytoconstituents Caffeine, Eugenol and Zingerone were purchased from Yucca Enterprises, Mumbai, India. All analytical grade (HPLC-grade) solvents for HPLC were purchased from Merck India Pvt. Ltd, and HPLC-grade water is purchased from the Research and Development Centre, Parul University.

### 2.2 Marketed Formulation

TATA TE-A-ME *Ashwagandha* Green Tea was procured from a local market of Vadodara, Gujarat.

### 2.3 Instruments and Software

The Shimadzu LC 2030 C auto sampler series system was used for analysis. The stationary phase was a C-18 column (4.6 mm × 250) (5 µm). Design Expert version 13 was used for optimisation and experimental design.

### 2.4 Preparation of Standard Reference Solution

Accurately weigh about 1 mg of standard caffeine and zingerone and dissolve in 10 mL of methanol in a volumetric flask to make a 100 µg/mL solution, respectively. For eugenol, dissolve 0.1 mL of eugenol in 10 mL of a volumetric flask to make a 1000 µg/mL solution. From a stock solution, a 100 µg/mL solution was prepared with methanol.

Working standard solution caffeine (100 µg/mL), eugenol (400 µg/mL) and zingerone (100 µg/mL) and standard mixtures were prepared, and chromatograms were recoded according to the final optimised chromatographic conditions.

### 2.5 Preparation of Sample Solution

The sample solution of TATA- TE-A-ME *Ashwagandha* green tea is prepared by refluxing 5 gm of the sample with 50 ml methanol for 1 hour. Then the filtered extract is transferred in to China dish and gently heated on waterbath at 50°C until the methanol evaporates completely, and kept in a dry and dark place.

Measure 1 mg of this sample in a 10 mL volumetric flask (100µg/mL), and volume was made up to the mark with methanol. Then filter this solution using a Whatman no. 42 filter. From the filtrate, 10 µL of the solution was subjected to HPLC analysis for the simultaneous estimation of caffeine, eugenol and zingerone.

### 2.6 Chromatographic Conditions for Optimisation

The stationary phase in the HPLC was a C-18 column, while the mobile phase was a 35:65, v/v mixture of methanol and water. Set to 1.2 mL/min as the flow rate. The detection was done at 254 nm at 40°C - 80°C. Run time for optimisation is 12 min.

### 2.7 Method Validation[9-12]

The International Council for Harmonisation's (ICH) Q2 (R2) guidelines were followed in the systematic establishment of the validation of the HPLC method. Critical parameters such as linearity, precision, accuracy, specificity, LOD, LOQ, and robustness were all included in the thorough validation procedure.

#### 2.7.1 System Suitability

System Suitability is primarily used to determine the column efficiency, resolution, and repeatability of a particular chromatographic system, verifying its ability to perform a defined analysis. The most important parameters that are investigated for different HPLC analyses are Resolution (R), Height, Area, retention time, Theoretical Plate and Tailing Factor (TF).

#### 2.7.2 Specificity

The specificity is examined by injecting blank samples of the mobile phase to show that there is no interference in the standard samples. In this investigation, the Retention time ($R_t$) values for both test samples and reference standards were thoroughly analysed in order to evaluate the method specificity.

#### 2.7.3 Linearity and Range

An analytical procedure is claimed to be linear if it can yield test results that are precisely proportional to the analyte concentration in the sample (within a given range). A linear relationship must be assessed at each step of the analysis. The recommended technique involves diluting a typical stock solution of the drug product's constituents to display them directly on the drug material. The standard way to express linearity is to utilise the confidence interval around the slope of the regression line. Following ICH requirements, linearity must be established at least at five concentrations. The distance between the higher and lower levels that can be estimated exactly, accurately, and linearly is known as the analytical approach's range.

#### 2.7.4 Precision

The precision of an analytical technique is the degree of agreement (or scatter) between a set of measurements made by continuously sampling the same consistent sample under particular conditions. Three levels of accuracy can be distinguished: reproducibility, moderate precision, and repeatability. The standard deviation or relative standard deviation of a data series is usually used to express the precision of an analytical technique.

#### 2.7.5 Accuracy

The method's accuracy was proven by a recovery study at three levels (80%, 100%, and 120% standard addition). The % recovery of all three phytoconstituents was determined as per the ICH guideline.

#### 2.7.6 LOD and LOQ

The calibration curve approach was used to determine the LOD. A sample containing an analyte within the Detection Limit (DL) should be used to study a calibration curve. The y-intercepts of regression lines are used to compute the standard deviation. It was calculated using the equation mentioned below.

$$DL = 3.3 \times \sigma / S$$

The calibration curve approach was used for the LOQ. When examining a calibration curve, a sample that contains an analyte that falls within the Quantification Limits (QL) range should be used. Through the use of regression line y-intercepts, the standard deviation is computed. The following equation was used to calculate it.

$$QL = 10 \times \sigma / S$$

Where σ stands for the standard deviation of the y-intercepts of regression lines, and S represents the slope of the calibration curve.

#### 2.7.7 Robustness[13,14]

Robustness is the ability of an analytical technique to tolerate small but deliberate variations in method parameters (*e.g.*, pH, mobile phase composition, temperature, wavelength, flow rate, *etc.*). It represents the method's dependability under typical operating conditions. The systematic method of changing a parameter and assessing the impact on the approach through sample analysis and/or system appropriateness monitoring is known as robustness determination.

#### 2.7.8 Quantification of the Formulation

Adequate quantification of standards in formulation was crucial, determined using formulation areas and regression equations.

## 3. Results and Discussion

### 3.1 Selection of UV Wavelength

For the selection of a good wavelength, multiple concentrations of standard solutions of caffeine, eugenol and zingerone were prepared and scanned using a UV

Spectrophotometer. The detection range was kept from 200-400 nm, and the overlay spectra of caffeine, eugenol and zingerone were obtained at 254 nm.

## 3.2 Development of Chromatogram

The chromatogram was developed where the retention times of caffeine, eugenol and zingerone were found to be 4.76 min., 8.64 min and 10.48 min. respectively. The chromatograms of caffeine, eugenol and zingerone are shown in Figure 1. The optimised chromatographic conditions for the developed HPLC method are described in Table 1.

## 3.3 System Suitability

After every validation run, a system suitability analysis was conducted. Theoretical plates, retention time, height, area, and tailing factor were calculated and mentioned in Table 2. The results of system suitability are shown in Figure 2.

## 3.4 Specificity

The specificity was done for the confirmation of the separation potential of the method. It was tested by injecting a blank solution of methanol. At the Rt 2.85, the peak of methanol is seen. There were no additional peaks eluting at the Rt of caffeine, eugenol and zingerone. Hence, the method was specific. The chromatogram is shown in Figure 3.

## 3.5 Linearity

The calibration curves of caffeine, eugenol and zingerone are shown in Figures 4, 5 and 6, respectively. All three phytoconstituents showed a correlation coefficient of 0.9996 (caffeine), 0.9994 (eugenol) and 0.9999 (zingerone). The linear chromatogram of caffeine, eugenol and zingerone is presented in Figure 7. The results of linearity are mentioned in Table 3.

## 3.6 Limit of Detection and Limit of Quantification

The limit of detection and limit of quantification were determined by the standard calibration curve method

**Table 1.** Optimised chromatographic conditions

| Sr. No. | Parameter | Condition |
|---|---|---|
| 1 | **Column** | Hypersil BDS Premium (250 ×4.6 mm) (5µm) |
| 2 | **Mobile phase** | Methanol: Water (35: 65; v/v) |
| 3 | **Flow rate** | 1.2 mL/min |
| 4 | **Injection Volume** | 10 µL |
| 5 | **Wavelength** | 254 nm |
| 6 | **Column temperature** | 40 °C - 80 °C |
| 7 | **Mode of separation** | Isocratic |
| 8 | **Run time** | 12 min |
| 9 | **Retention Time** | **Caffeine:** 4.46 min, **Eugenol:** 8.64 min, **Zingerone:** 10.48 min |

**Table 2.** System suitability parameters

| Phyto constituents | $R_t$ | Height | Area | TF | TP | Resolution |
|---|---|---|---|---|---|---|
| **Caffeine** | 4.78 | 92577 | 1185 | 1.686 | 3997 | 0.221 |
| **Eugenol** | 8.62 | 4075 | 6809 | 1.935 | 7130 | 20.541 |
| **Zingerone** | 10.44 | 10082 | 2117 | 1.823 | 6742 | 22.27 |

$R_t$ = Retention time, TF =Tailing Factor, TP= Theoretical Plate

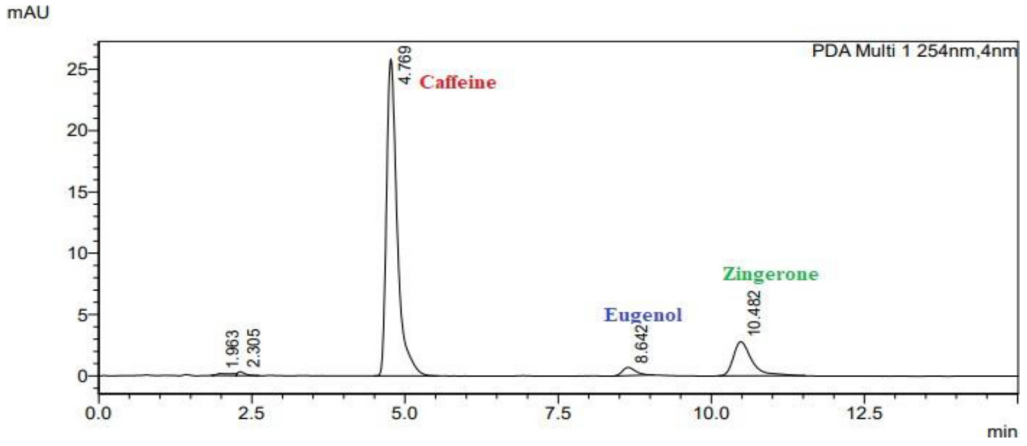



**Figure 1.** Chromatogram of caffeine, eugenol and zingerone.

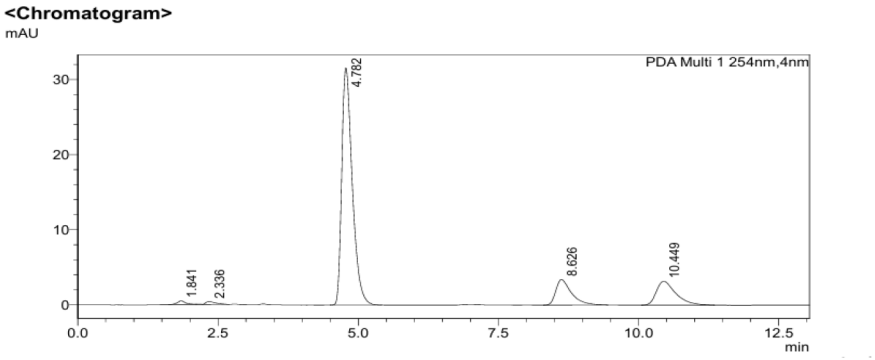



**Figure 2.** Chromatogram for system suitability of caffeine, eugenol and zingerone.

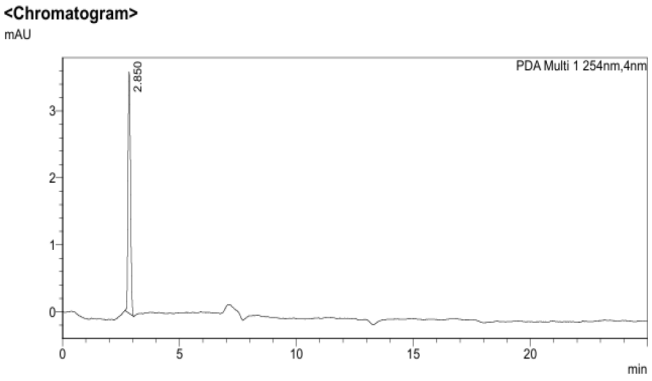



**Figure 3.** Blank chromatogram.

as per the ICH guidelines, and the results are shown in Table 4.

## 3.7 Accuracy

The accuracy was determined by the standard recovery method. The % Recovery of Caffeine, Eugenol and Zingerone are depicted in Tables 5, 6, and 7. The results of accuracy were found to be in the range of 98-102%. According to ICH guidelines, the method was found to be accurate as it is within the acceptable limits.

**Table 3.** Linear regression for calibration of caffeine, eugenol and zingerone

| Parameter | Caffeine | Eugenol | Zingerone |
|---|---|---|---|
| **Linearity Range (µg/mL)** | 33.33 - 133.32 µg/mL | 133.32 - 266.64 µg/mL | 33.33- 166.65 µg/mL |
| **Regression Equation** | y= 2376.8x + 2214.4 | y= 381.44x – 38832 | y= 440.28x – 259.47 |
| **Correlation Coefficient ($R^2$)** | 0.9996 | 0.9994 | 0.9999 |
| **Slope** | 2376.8 | 381.44 | 440.28 |
| **Intercept** | 2214.4 | 38832 | 259.47 |

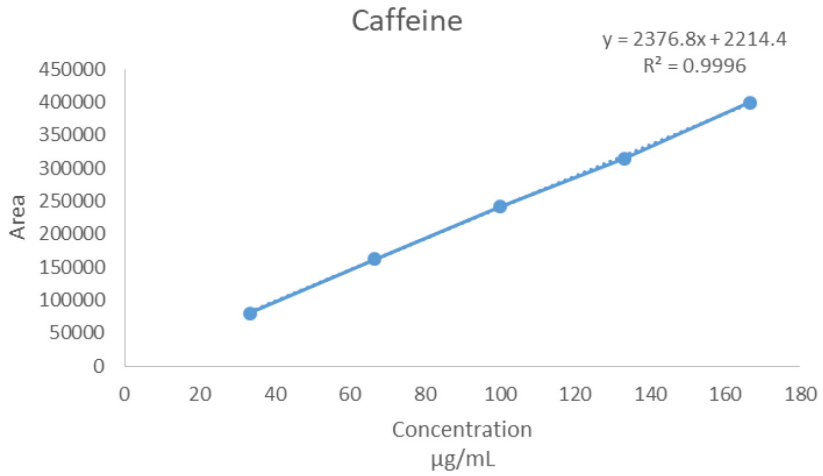



**Figure 4.** Calibration curve for caffeine.

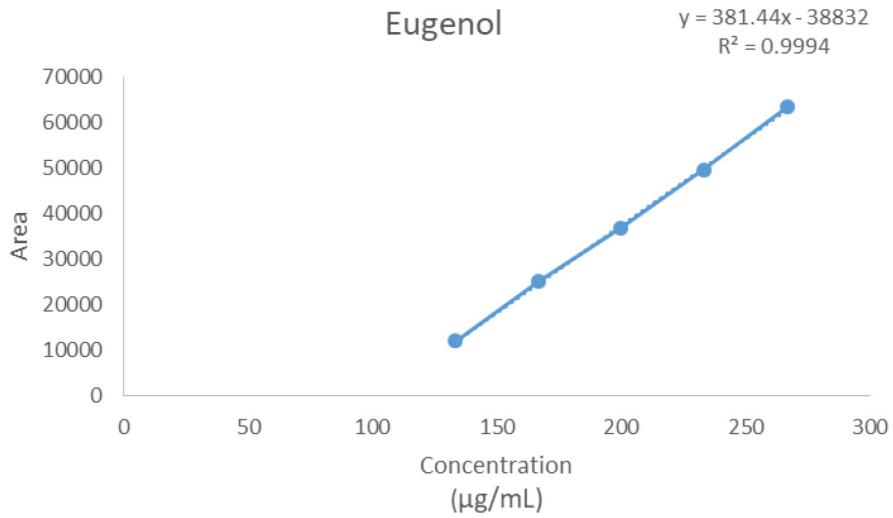



**Figure 5.** Calibration curve for eugenol.

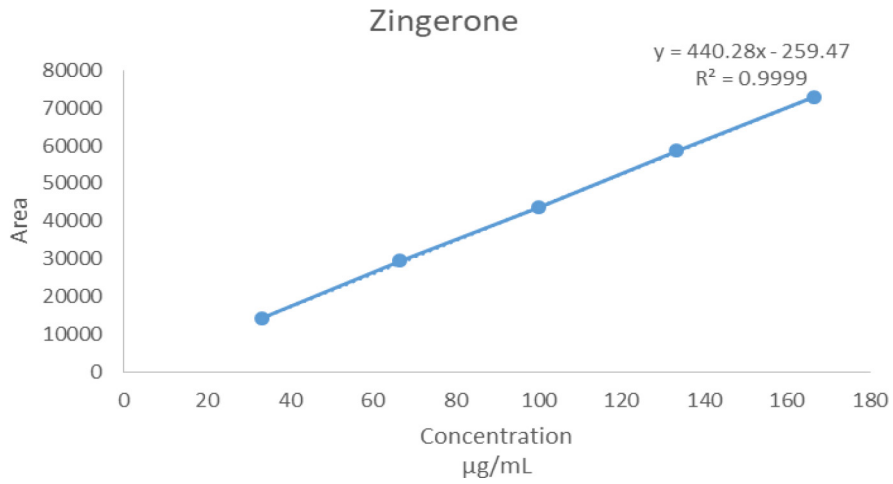



**Figure 6.** Calibration curve for zingerone.

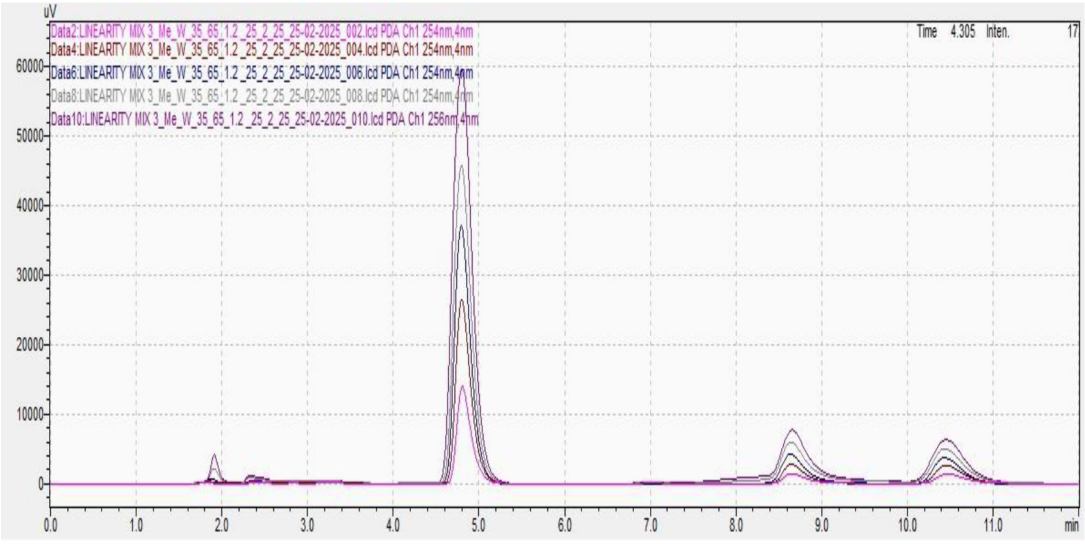



**Figure 7.** Linearity chromatogram of caffeine, eugenol and zingerone.

## 3.8 Precision

### *3.8.1 Repeatability*

The % RSD for repeatability of caffeine, eugenol and zingerone were found to be < 2. So, the proposed method for the estimation of caffeine, eugenol and zingerone is precise (Table 8).

### *3.8.2 Intraday and Interday Precision*

Intraday and Interday precision assess method performance by evaluating result variations from repeated assays across different days (interday) and within the same day (intraday), indicating the repeatability of a testing method. Interday and Intraday precision are crucial measures in assessing the consistency and repeatability of the HPLC method for caffeine, eugenol and zingerone. The results are shown in Tables 9 and 10, respectively. The proposed method for the estimation of caffeine, eugenol and zingerone is precise.

Experimental results were computed by Design Expert Trial Version 15. A multivariate approach was employed, in which simultaneous changes were made to method factors using a design matrix. Robustness was performed using Full Factorial Design (FFD) (2+1). All the runs were independent, and each run was carried out according to the design domain. Design identifies complications that may develop during implementation and emphasizes the aspects of the approach that significantly impact its outcomes. Based on the analytical process, the elements under investigation were believed to have an impact on the separation of caffeine, eugenol, and zingerone. When the procedure is transferred, the interval between the levels is the limit between which factors are anticipated to change. The responses studied were the retention time of Caffeine, Eugenol and Zingerone. The domain constructed to show the effects produced on the response is shown in Table 11.

**Table 4.** Limit of detection and limit of quantification of caffeine, eugenol and zingerone

| Phytoconstituents | LOD(µg/mL) | LOQ(µg/mL) |
|---|---|---|
| **Caffeine** | 0.1060 | 0.32135 |
| **Eugenol** | 0.0094 | 0.02855 |
| **Zingerone** | 0.0165 | 0.05112 |

**Table 5.** % Recovery of caffeine

| % Level | Area Found | Conc. Found | % Re covery | Mean Recovery± SD | % RSD |
|---|---|---|---|---|---|
| 80 | 189846 | 79.63 | 99.53 | 99.42± 0.09539392 | 0.09595 |
| 80 | 189515 | 79.49 | 99.37 | | |
| 80 | 189672 | 79.56 | 99.36 | | |
| 100 | 238556 | 100.26 | 100.26 | 100.03± 0.21656408 | 0.216499 |
| 100 | 237942 | 100 | 100 | | |
| 100 | 237530 | 99.83 | 99.83 | | |
| 120 | 283958 | 119.49 | 99.47 | 99.5266667± 0.05131601 | 0.05156 |
| 120 | 283851 | 119.45 | 99.54 | | |
| 120 | 284748 | 119.83 | 99.57 | | |

**Table 6.** % Recovery of eugenol

| % Level | Area found | Conc. found | % Re covery | Mean Recovery ± SD | % RSD |
|---|---|---|---|---|---|
| 80 | 28876 | 318.05 | 99.39 | 99.63± 0.210 | 0.21156791 |
| 80 | 28982 | 319.21 | 99.75 | | |
| 80 | 28985 | 319.25 | 99.76 | | |
| 100 | 36272 | 398.89 | 99.72 | 99.75±0.026 | 0.02652382 |
| 100 | 36289 | 399.08 | 99.77 | | |
| 100 | 36287 | 399.06 | 99.76 | | |
| 120 | 43457 | 477.00 | 99.37 | 99.52±0.13 | 0.13861747 |
| 120 | 43527 | 478.19 | 99.58 | | |
| 120 | 43533 | 478.26 | 99.63 | | |

**SD** = Standard Deviation, **% RSD** = Relative Standard Deviation.

**Table 7.** % Recovery of zingerone

| % Level | Area Found | Conc. Found | % Re covery | Mean Recovery ± SD | % RSD |
|---|---|---|---|---|---|
| 80 | 34899 | 79.85 | 98.33 | 98.29 ± 0.032 | 0.032703645 |
| 80 | 34887 | 79.82 | 98.27 | | |
| 80 | 34879 | 79.8 | 98.28 | | |
| 100 | 43551 | 99.5 | 98.32 | 98.36 ± 0.037 | 0.038489331 |
| 100 | 43577 | 99.56 | 98.38 | | |
| 100 | 43580 | 99.57 | 98.39 | | |
| 120 | 52678 | 120.23 | 100.19 | 100.08 ± 0.095 | 0.095317666 |
| 120 | 52589 | 120.03 | 100.02 | | |
| 120 | 52593 | 120.04 | 100.03 | | |

**Table 8.** Repeatability data of caffeine, eugenol and zingerone

| Sr. no. | Area of Caffeine (133.3 µg/mL) | Area of Eugenol (133.3 µg/mL) | Area of Zingerone (133.3 µg/mL) |
|---|---|---|---|
| 1 | 415818 | 66014 | 76421 |
| 2 | 412894 | 64972 | 75769 |
| 3 | 412746 | 67564 | 76347 |
| 4 | 415693 | 65823 | 77020 |
| 5 | 415024 | 65529 | 76552 |
| 6 | 419912 | 66080 | 77406 |
| **Mean± SD** | 415347 ±2610269 | 65997 ±867.4782 | 76585.83 ±568.1103 |
| **% RSD** | 0.628454 | 1.314421 | 0.741796 |

n* =6, **SD** = Standard Deviation, **% RSD** = Relative Standard Deviation

### 3.8.3 ANOVA Analysis of Each Response

The model was validated by ANOVA (Analysis of Variance). Where no single factor had been significantly affecting the $R_t$ of caffeine, eugenol and zingerone as evident from the model p-value. The model p-value was not significant for the retention time of caffeine, eugenol and zingerone. This indicated the $R_t$ of Caffeine, Eugenol and Zingerone was not affected by changes in factor, conforming to the robustness of the method. The 3D Response plot of Caffeine, Eugenol and Zingerone is illustrated in Figure 8.

### 3.9.2 Polynomial Equation for Each Response

It is possible to predict the reaction for certain levels of each element by using the equation in terms of coded factors. The factors' low values are coded as -1

**Table 9.** Intraday precision data of caffeine, eugenol and zingerone

| Phytoconstituents | Concentration (µg/mL) | Intraday Precision | | | Mean Peak Area | SD | % RSD |
|---|---|---|---|---|---|---|---|
| | | Peak Area | | | | | |
| | | Interval 1 | Interval 2 | Interval 3 | | | |
| **Caffeine** | 66.66 | 169130 | 166899 | 166899 | 16763 | 1290 | 0.77 |
| | 99.99 | 250566 | 250426 | 254512 | 25183 | 2319 | 0.92 |
| | 133.29 | 334216 | 332680 | 335305 | 33406 | 1318 | 0.39 |
| **Eugenol** | 133.29 | 24725 | 24739 | 24592 | 24685 | 81.13 | 0.32 |
| | 166.62 | 39053 | 38720 | 38802 | 38858 | 173.5 | 0.44 |
| | 199.95 | 52328 | 52712 | 52531 | 52523 | 192.1 | 0.39 |
| **Zingerone** | 66.66 | 30668 | 30373 | 30552 | 30531 | 148.6 | 0.48 |
| | 99.99 | 42419 | 42578 | 42212 | 42403 | 183.5 | 0.43 |
| | 133.29 | 61363 | 61237 | 61348 | 61316 | 68.82 | 0.11 |

n*=3, SD = Standard Deviation, % RSD = Relative Standard Deviation

**Table 10.** Interday precision data of caffeine, eugenol and zingerone

| Phytoconstituents | Conc. (µg/mL) | Interday Precision | | | Mean Peak Area | SD | % RSD |
|---|---|---|---|---|---|---|---|
| | | Peak Area | | | | | |
| | | Day 1 | Day 2 | Day 3 | | | |
| **Caffeine** | 66.66 | 168076 | 167484 | 168052 | 16787 | 335.0 | 0.19 |
| | 99.99 | 250366 | 250416 | 254012 | 25159 | 2090 | 0.83 |
| | 133.29 | 332216 | 332380 | 335325 | 33330 | 1749 | 0.52 |
| **Eugenol** | 133.29 | 24469 | 24579 | 2472 | 24573 | 101.6 | 0.41 |
| | 166.62 | 38453 | 38550 | 38802 | 38601 | 180.1 | 0.46 |
| | 199.95 | 52228 | 52512 | 52621 | 52453 | 202.8 | 0.38 |
| **Zingerone** | 66.66 | 30638 | 30727 | 30565 | 30643 | 81.13 | 0.26 |
| | 99.99 | 42519 | 42575 | 422282 | 42458 | 155.5 | 0.36 |
| | 133.29 | 61453 | 661307 | 61348 | 61369 | 75.30 | 0.12 |

n*=3, SD = Standard Deviation, % RSD = Relative Standard Deviation

**Table 11.** Effect of various factors on the retention time of caffeine, eugenol and zingerone

| | | Factor 1 | Factor 2 | Factor 3 | Response 1 | Response 2 | Response 3 |
|---|---|---|---|---|---|---|---|
| Std | Run | A: Flow rate (mL/min) | B: Temperature (°C) | C: Wavelength (nm) | $R_t$ Caffeine (min) | $R_t$ Eugenol (min) | $R_t$ Zingerone (min) |
| 2 | 1 | 1.3 | 38 | 252 | 4.676 | 7.49 | 10.32 |
| 5 | 2 | 1.1 | 38 | 256 | 5.504 | 9.803 | 12.89 |
| 7 | 3 | 1.1 | 42 | 256 | 5.155 | 9.753 | 11.92 |
| 3 | 4 | 1.1 | 42 | 252 | 5.256 | 9.332 | 12.67 |
| 6 | 5 | 1.3 | 38 | 256 | 4.785 | 7.72 | 10.319 |
| 8 | 6 | 1.3 | 42 | 256 | 4.663 | 7.985 | 9.606 |
| 4 | 7 | 1.3 | 42 | 252 | 4.563 | 7.885 | 9.706 |
| 1 | 8 | 1.1 | 38 | 252 | 5.404 | 9.921 | 12.79 |

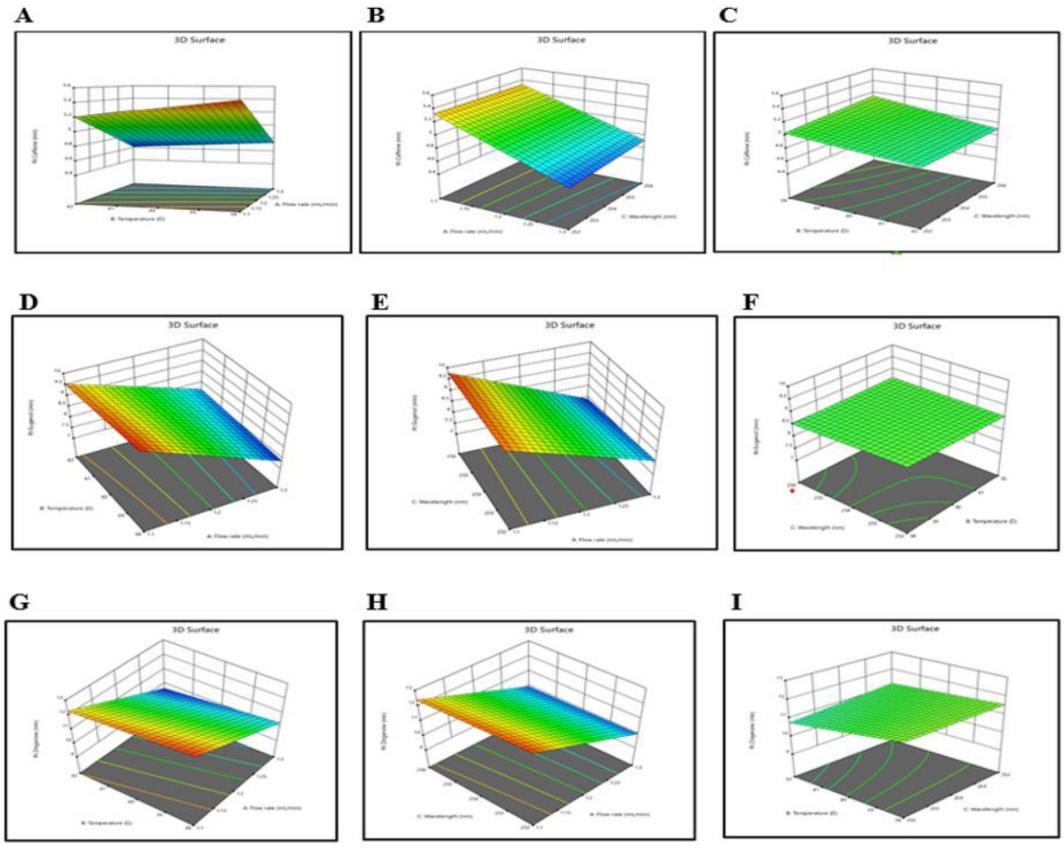



**Figure 8.** **(A,B,C).** 3D Response plot of caffeine; **(D,E,F)**. Eugenol; **(G,H,I)**. Zingerone.

**Table 12.** Polynomial equation for responses

| Sr. No. | Response | Polynomial Equation |
|---|---|---|
| 1 | Caffeine Rt | +5.00-0.3290A- 0.0915B+0.0260C +0.0328AB+0.0263AC-0.0262 |
| 2 | Eugenol Rt | +8.62-1.08A-0.0024B-0.0296C+ 0.1574AB- 0.1054AC+0.0549 |
| 3 | Zingerone Rt | +11.28-1.29A-0.3021B-0.0939C-0, 0296AB+0.0686AC- 0.1186 |

by default, and their high levels are coded as +1. By comparing the factor coefficients, one can determine the relative importance of the elements using the coded equation mentioned in Table 12.

### *3.9.3 Pareto Charts of Caffeine, Eugenol and Zingerone*

The Pareto chart is best utilised after effects have been chosen on the half-normal plot and helps determine

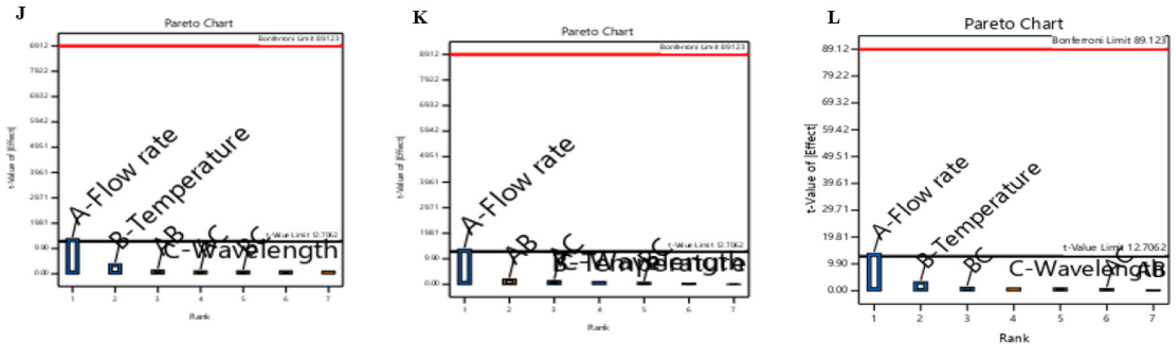



**Figure 9.** Pareto charts of caffeine, eugenol and zingerone.

**Table13.** Quantification data of caffeine, eugenol and zingerone

| Sr. No. | Phytoconstituents | Amount Present in Formulation (mg) |
|---|---|---|
| 1 | **Caffeine** | 2.04 |
| 2 | **Eugenol** | 0.27 |
| 3 | **Zingerone** | 0.16 |

the significance of the next greatest unselected effect. Above the Bonferroni Limit, effects are most likely to be significant. Effects that exceed the t-value limit may be significant. Effects below the t-value limit are not likely to be significant. Pareto chart shows the effect of factors on $R_t$ of drugs in decreasing order for Caffeine A>B>AB>AC>BC, for Eugenol A>AB>AC>AB>C and for Zingerone A>B>BC>C>AC. The Pareto charts of Caffeine, Eugenol and Zingerone is presented in Figure 9.

J, K and L represent the Pareto chart for caffeine, eugenol and zingerone. Pareto chart showed that factor are significant impact on their flow rate and not a significant impact on the wavelength.

## 3.10 Quantification of Formulation

All three phytoconstituents are quantified by using a standard calibration curve equation, and the outcomes are displayed in Table 13.

## 4. Conclusion

The present study describes the applicability of the chromatographic method to develop a new, sensitive, HPLC method for the determination of Caffeine, Eugenol and Zingerone in *Ashwagandha* green tea formulation. A simple, selective and precise HPLC method has been developed and validated for the analysis of Caffeine, Eugenol and Zingerone. The chromatographic separation was achieved on a C-18 column with Methanol: Water (35: 65 v/v), as mobile phase at a flow rate of 1.2 mL/min. Detection was performed at 254 nm, and a sharp peak was obtained for Caffeine, Eugenol and Zingerone at a retention time of 4.76 min. 8.64 min and 10.48 min. Caffeine, Eugenol and Zingerone were in the range of 33.33-166.65 µg/mL, 133.32- 266.64 µg/mL, 33.33- 166.65 µg/ml. The recoveries for Caffeine, Eugenol and Zingerone were found to be in the range of 99.48-110.03%, 98.63-99.75 % and 99.29-100.08%, respectively, using the proposed RP-HPLC method. The high values convey that the proposed HPLC method was found to be accurate. The limit of detection and limit of quantification of Caffeine, Eugenol and Zingerone were 0.106 µg/mL and 0.0094 µg/mL, 0.0165 µg/mL and 0.321 µg/mL and 0.028 µg/mL and 0.051 µg/mL, respectively. The intra-day precision and inter-day precision showed relative standard deviation (%RSD) values less than 2.00%, which were adequate to elucidate that the developed HPLC method was precise and reproducible. Deliberate changes to the analytical parameters, such as the mobile phase ratio and detector wavelength, were used to examine the method's robustness. The percentage change was found to be less than 2% in all the changed conditions of the robustness study. Hence, small variations in the method conditions did not influence the results. The developed and validated HPLC method is simple, precise, specific and robust, making it suitable for the simultaneous estimation of eugenol, zingerone, and caffeine in commercial formulations.

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## Author Contribution

Vrutika Patel: Conceptualisation, design, literature search, experimental studies, data acquisition, data analysis, and manuscript preparation. Dr Tanvi Dodiya: Contributed to the concept, Visualisation supervision, manuscript editing, and manuscript review. Dr. Disha Prajapati: manuscript editing and manuscript review. All authors have read and agreed to the published version of the manuscript.