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Simple and rapid RP-HPLC method for simultaneous quantitation of gallic acid, catechin, and quercetin

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A R T I C L E I N F O	**A B S T R A C T**

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Several plants contain gallic acid (GA), catechin (CT), and quercetin (QT) as bioactive compounds which provide health benefits beyond basic nutrition. Previously reported high performance liquid chromatography ultraviolet (HPLC-UV) methods for the detection of aforementioned phytochemicals have several disadvantages including longer run time, unsatisfactory peak shape, poor resolution, or lack of method validation according to the standard guidelines of International Conference on Harmonisation (ICH). A simple and rapid HPLC-UV method with gradient elution was developed on a Thermo Scientific Syncronis C18 column (5 µ particle size, 250 × 4.6 mm) and detection was conducted at dual wavelengths of 273nm (for GA and CT) and 370nm (for QT). The effective separation was achieved within a run time of 20 minutes with a flow rate of 1.2 ml min^{-1} and column temperature of 25°C. The optimized method was validated and found to be precise, accurate, and robust. The developed method was applied for the quantitation of GA, CT, and QT from the methanolic extract of Banana Blossom Florets Powder (BBFP). The experimental results showed the presence of all three components. The developed HPLC-UV method can be a useful tool for the quantitative and qualitative evaluation of the selected phytochemical compounds.

Citation: Puranik, D., Kaur, G., & Purandare, K. 2024. Simple and rapid RP-HPLC method for simultaneous quantitation of gallic acid, catechin, and quercetin. *Journal of Postharvest Technology*, **12** (2): 126-137.

INTRODUCTION

The term "phytochemical" refers to an array of biologically active natural compounds with significant bioactive properties associated with human health benefits (Kahkeshani et al., 2019; Jaiswal et al., 2023). Simple phenols, phenolic acids,

flavonoids, coumarins, stilbenes, hydrolyzable and condensed tannins, lignans, and lignins are all included under the umbrella term "plant phenolics" (Nour et al., 2013). The leaves, flowers, roots, fruit, and seeds of plants are the sources of these secondary metabolites. Because they can be employed in the production of complex chemical molecules, the study of these plant chemicals is vital (Zaheer et al., 2021). With a basic chemical structure of C6-C1 (hydroxybenzoic acids) or C6-C3 (hydroxycinnamic acids), which consists of a phenolic ring and a carboxyl substituent, phenolic acids are a significant and prevalent subclass of phenolic compounds (Kahkeshani et al., 2019; Sun and Shahrajabian, 2023). Recently, there has been a growing interest in phenolic compounds especially those which exhibit significant antioxidant properties (Kumar and Goel, 2019). A naturally occurring phenolic substance called gallic acid (GA) is present in many fruits and medicinal plants. It is reported to have a number of health-enhancing properties including strong antioxidant activity (Kahkeshani et al., 2019). In addition, GA has a number of evident pharmacological effects, such as anti-bacterial, anti-diabetic, anti-obesity, anti-microbial, and anti-myocardial ischemia (Bai et al., 2021). As presented in (Figure 1A) chemically GA is a 3, 4, 5-trihydroxybenzoic acid with the molecular formula $C_7H_6O_5$ and it has increasingly gained a lot of attention as it is ubiquitous in fruits, vegetables, and herbal medications (Yang et al., 2020). Pharmacokinetic studies show that GA is quickly absorbed and eliminated following oral administration; however, GA's structural optimization or dosage form modification can help to boost its bioavailability (Bai et al., 2021).

Catechins are natural polyphenolic compounds- flavan-3-ols, belonging to the flavonoid family which can be obtained from various sources including green tea, black tea, wine, grapes, and other food crops such as fruits and cocoa (Bernatoniene and Kopustinskiene, 2018; Emilia et al., 2020). Catechins are weakly acidic, soluble in water, and easily undergo oxidation when exposed to air and at a pH close to neutral (pH 6.9). They are well-researched compounds with proven antioxidant properties (Bae et al., 2020). Catechin derivatives are considered to be promising drug candidates in the pharmaceutical and nutraceutical fields and thus a reliable, rapid, and simple analytical method is required for their characterization and pharmacokinetic studies (Li et al., 2012). Figure 1B shows the chemical structure of catechins. Quercetin belongs to the class of flavonoids and is a subclass of flavonol with promising bioactive effects and strong antioxidant activity (Salehi et al., 2020; Ang et al., 2014). The International Union of Pure and Applied Chemistry's (IUPAC's) name for quercetin is 3, 3', 4', 5, 7-pentahydroxyflavone and its chemical structure is shown in Figure 1C. An array of methods has been reported for quantitatively detecting quercetin, including TLC, HPTLC, HPLC, and many more (Ang et al., 2014).
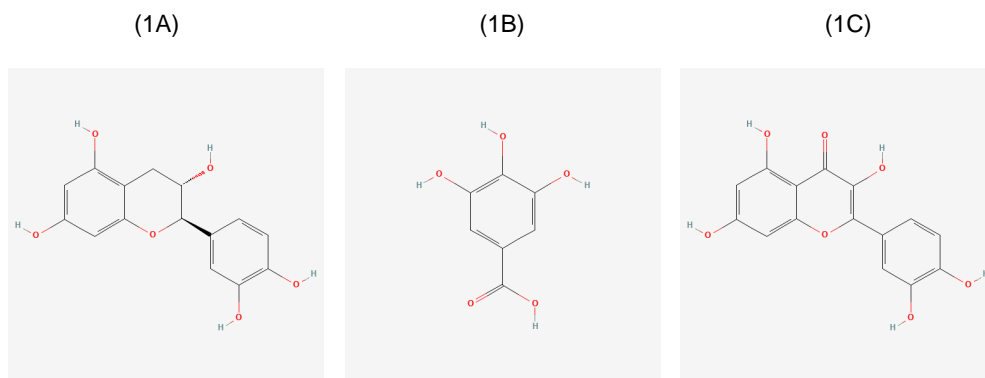
(1A)	(1B)	(1C)

Figure 1: Chemical structure of (1A) GA, (1B) CT, and (1C) QT

Determining the chemical marker, which consists of a constituent (or groups of constituents), is a crucial step in the quality control of herbal medicine products (Fernandes et al., 2015). Banana flower or banana blossom which is the edible by-product of banana cultivation is consumed in many Asian countries including Sri Lanka, Malaysia, Indonesia, Philippines, and India due to its good nutritional value. They are rich sources of bioactive compounds which include phenols and flavonoids possessing strong antioxidant activity (Soni and Saxena, 2021).

High-performance liquid chromatography (HPLC) is one of the most widely used analytical technique for the identification, and quantification of bioactive compounds (Kuppusamy et al., 2018). There are several reasons for this including applicability to diverse groups of analytes, sensitivity, precision and many more. Specifically for flavonoids, due to the presence of chromophores HPLC with ultraviolet (UV) detection has become the popular choice for routine analysis. Several HPLC methods have been reported for both individual and simultaneous detection, but they have certain disadvantages: longer run time or poor resolution or distorted peak shape or need a certain detector as an additional requirement, or lack of validation as per standard guidelines (Ang et al., 2014). Thus, this study aimed to develop and validate the RP-HPLC method, which is rapid, simple, robust, and can be easily employed for routine quality control analysis. The method was also applied to BBFP to check the presence of GA, CT, and QT.

MATERIALS AND METHODS

Chemicals and reagents

Banana blossoms used in this study were procured from a local market in Mumbai. The gallic acid standard was purchased from Molychem, the quercetin standard from Research Lab Fine Chem Industries, and the catechin standard from Yucca Enterprises. All chemicals and solvents used were of HPLC grade. Nylon syringe filters of 0.45 μm were purchased from PerkinElmer.

Instrumentation and chromatographic conditions

HPLC analysis was performed employing a Shimadzu-LC 2010A HT system (Shimadzu, Japan, serial no: C21244504995 LP) equipped with a pump, degasser, autosampler, detector, and column oven. Chromatographic separation was achieved using a Thermo Scientific Syncronis C18 column (250 mm × 4.6 mm I.D.: 5 μm). A reverse phase HPLC method was carried out using gradient elution with a flow rate of 1.2 mL/minute, a column temperature of 25°C, and at dual detection wavelength of 273 nm and 370 nm. The mobile phase consisted of 0.4% orthophosphoric acid & acetonitrile [90:10- solvent A] and methanol [solvent B]. The system was run with the gradient elution program given in Table 1. The injection volume was 10 μL for each solution. The total run time was 20.0 minutes for each injection. Data were acquired and processed with LabSolutions software. Solvents and distilled water were prior filtered through a 0.45μm nylon membrane by using a set of glass bottles with the aid of a vacuum pump.

Table 1: Mobile phase gradient program of the developed HPLC method

Time (minutes)	Composition	
	Solvent A	Solvent B
0.10	90	10
5.00	90	10
6.00	40	60

7.00	5	95
8.00	15	85
13.00	40	60
15.00	60	40
18.00	90	10
19.00	90	10
20.00	90	10

Preparation of standard solutions

Standard stock solutions of GA, CT, and QT were prepared in methanol, each at a concentration of 500ppm. A mixture of standards as working range solutions were prepared for the calibration standard curves. From stock, each working concentration was prepared by taking appropriate volume from each standard stock solution and diluting with methanol:water (60:40- solvent mixture) to get a concentration range of 125ppm, 187.5ppm, 250ppm, 312.5ppm, and 375ppm (50%, 75%, 100%, 125%, and 150% level respectively).

Preparation of sample solution

Freshly procured banana blossoms were thoroughly washed to remove dirt or any extraneous matter. To minimize darkening due to enzymatic browning, the uniformly sliced were pretreated with 0.2% citric acid solution for 30 minutes followed by drying at 50°C for 6 hours. Dehydrated florets were then ground to fine powder by use of a grinder and stored at 4°C till further use (Wickramarachchi and Ranamukhaarachchi, 2005). The extraction was carried out using 1g of Banana Blossom Powder (BBP) with 50mL of solvent mixture and was sonicated in an ultrasonic bath (ultrasound extraction) for 30 minutes followed by maceration for 24 hours. The obtained extract was then filtered through a 0.45µm nylon syringe filter.

Method development

Various combinations of buffer & organic solvents were employed for the development of a suitable HPLC method for the simultaneous separation of GA, CT & QT. Different parameters were considered while selecting the composition of solvents for the mobile phase such as resolution, run time, miscibility of solvents, economy, and peak parameters (Alsuwyeh et al., 2018). After taking into consideration above mentioned parameters, different solvents and combinations of hydro-organic solvents were tried in different ratios. Isocratic elution was carried out with a mobile phase composition of methanol or acetonitrile in combination with water or a mixture of water with varying concentrations of formic acid, orthophosphoric acid, and glacial acetic acid. After trial and error, the desired separation was not achieved with isocratic elution, where GA and CT peaks showed poor resolution thus gradient elution was used. Subsequent optimization of different method parameters and mobile phase solvents, lead to well-resolved peaks of GA, CT, and QT with gradient elution using mobile phase composition of 0.4% orthophosphoric acid and acetonitrile (90:10) as solvent A and methanol as solvent B.

Validation studies

The developed method was validated for various parameters including system suitability, specificity, precision, accuracy, linearity, sensitivity, and robustness following the ICH Q2 (R2) guidelines (ICH Harmonised Tripartite Guidleine, 2005).

System suitability: System suitability testing was performed to determine if the developed chromatographic system is appropriate for the analysis. It was assessed by different performance parameters including number of theoretical plates, tailing factor, and resolution. % RSD for retention time and peak area was calculated. Acceptance criteria for system suitability were followed as per standard guidelines that is %RSD not more than 2%, number of theoretical plates not less than 2000, tailing factor not more than 1.5, and the resolution between peaks not less than 2.0. After stabilizing the HPLC system, blank, standard, and sample solutions were injected and evaluated for system suitability parameters.

Specificity: The developed method needs to be specific, and the response of the mobile phase should not interfere with the retention time of the analyte. The specificity of the method was investigated by injecting blank (solvent mixture), mobile phase (0 µL), individual standards solutions, the mixture of standard solutions and methanolic BBFP extract to demonstrate the absence of interference with the elution of GA, CT, and QT in standard and sample solutions.

Precision: The precision of the assay method was evaluated in terms of repeatability by performing six independent assays (intra-day). The mixture of standards (GA, CT, and QT) was injected. Following the same experimental conditions, the intermediate precision of the method was checked by another person performing the same procedure on a different day (inter-day). It was assessed based on the %RSD of the peak area obtained, where not more than 2% was taken as the limit.

Accuracy: An accuracy study was performed by spiking the known concentration of the mixture of standard solution to the methanolic extract of BBFP (sample solution). The actual and measured concentrations were compared, and recovery was calculated. The recovery of the method was evaluated by spiking a known amount of standard solution at three different concentration levels (corresponding to 50%, 100%, and 150% of working concentration). An analysis of the as such sample solution (without spiking) was also conducted. The analysis was performed in triplicate for each concentration level.

Linearity: To study the linearity parameter, five different concentration levels of standard solutions were injected into HPLC. Five concentration levels prepared were from 50% to 150% of working concentration (125ppm - 375ppm)**.** Standard solutions were prepared in triplicates for each level. To determine the linear response of the instrument with analyte concentration, a calibration curve was constructed for each standard by plotting the concentration of that analyte on the X-axis and the average peak area on the Y-axis. The regression equation and value of the co-relation coefficient were obtained from the graph.

Sensitivity: The sensitivity of the analytical method was determined in terms of limit of detection (LOD) and limit of quantification (LOQ). LOD and LOQ were determined for each of the studied compounds. Their values were calculated using the expressions $3.3\sigma/s$ and $10\sigma/s$, respectively, in which σ is intercepted standard deviation and s is the slope of the calibration curve. LOQ was taken as the lowest concentration of analyte that could be quantitatively determined with acceptable accuracy and precision.

Robustness: The robustness of the developed HPLC method was investigated by making deliberate changes in the chromatographic conditions on the performance parameters of the developed method. The factors chosen for this study were change in flow rate (±0.2mL) and column temperature (±2 °C).

RESULTS AND DISCUSSION

Method development: During the method development stage, the isocratic mobile phase system was employed with an array of solvents that were unable to resolve GA and CT peaks. Different concentrations of orthophosphoric acid were tried ranging

from 0.1-0.5% for solvent A of the mobile phase. For solvent B, methanol and acetonitrile were tried in different ratios. Among these, 0.4% orthophosphoric acid (solvent A) and methanol (solvent B) showed the presence of each peak with good resolution of QT peak while GA and CT peak showed poor resolution. To resolve GA and CT peak, the flow rate was optimized to 1.2mL/min, a gradient time program (Table 1) was designed after trial and error and the addition of acetonitrile in solvent A (acetonitrile: 0.4% orthophosphoric acid in the ratio of 10:90 v/v) resulted in good resolution of peaks. Column oven temperature was kept at 25°C and detection wavelengths as 273nm (for GA and CT) and 370nm (for QT). The developed method resulted in a shorter run time of 20 minutes with acceptable performance parameters.

System suitability: The system suitability study showed results that comply with the standard guidelines. The average tailing factor for GA, CT and QT was 1.01, 0.97, and 1.24 respectively. A good number of theoretical plates was obtained for each peak (average 5097 for GA, 34122 for CT, and 88406 for QT). Resolution between GA and CT (at 273 nm) was found to be greater than 2.0. % RSD of retention time, peak area, tailing factor, and resolution was reported to be below 2% (Table 2).

Table 2: System suitability study

Component	GA	CT	QT
Average retention time (n=6)	3.59	8.39	9.93
SD	0.01	0.01	0.00
%RSD	0.15	0.06	0.04
Average peak area (n=6)	5721423	1981493	7014652
SD	23647.72	11776.63	40122.79
%RSD	0.41	0.59	0.57
Average asymmetry factor (n=6)	1.01	0.97	1.22
Average theoretical plates (n=6)	5097	34122	88780
Average resolution (between GA and CT)	-	25.04	-

Specificity: No interference was observed at the retention time of any of the standard peaks, confirming that each peak represents a single compound.. The method was proved to be specific.

Precision: Repeatability (intraday precision) showed %RSD of 0.11, 0.50, and 0.78 respectively for GA, CT, and QT. For intermediate precision, %RSD was found to be 0.28, 1.14, and 0.72 for GA, CT, and QT respectively (Tables 3 and 4).

Table 3: Precision study-repeatability (intra-day)

Component	GA	CT	QT
Average peak area	5703637	2001178	7029252
SD	6381.87	9910.52	54982.27
%RSD	0.11	0.50	0.78

Table 4: Precision study-intermediate (inter-day)

Component	GA	CT	QT
Average peak area	5720670	2011868	7035331
SD	15955.21	22977.58	50879.23
%RSD	0.28	1.14	0.72

Accuracy: The developed method was employed to quantify GA, CT, and QT from methanolic extract of BBFP and the concentration was found to be 4160.29ppm, 2321.61ppm, and 7413.21ppm respectively (Figure 1, 2, 3 & 4). %RSD was calculated for each level that was below 2% indicating the accuracy of the method. The value of mean recoveries was found to be 97.06%, 99.92%, and 98.84% respectively (Table 5).

Table 5: Accuracy study

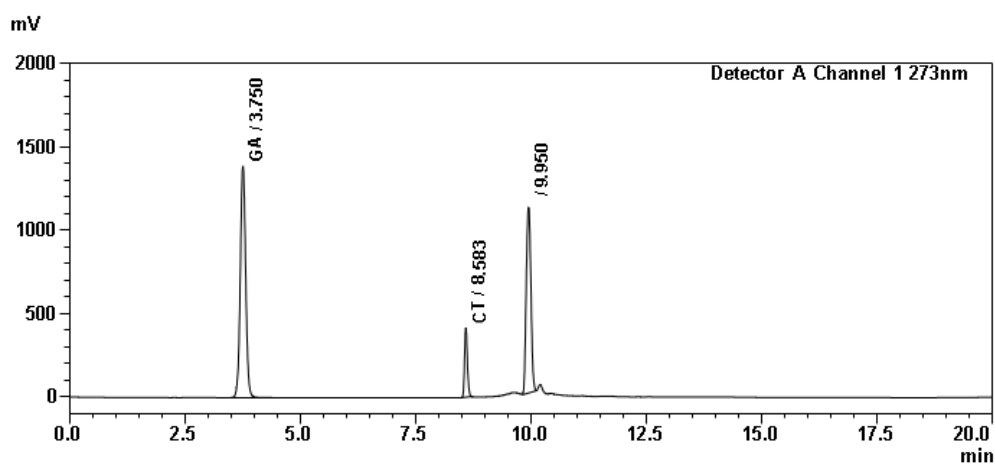| Level	% Recovery	Mean % recovery	%RSD
	97.41		
50%	95.67	97.06	1.29
	98.10		
	101.80		
100%	98.61	99.92	1.67
	99.35		
	97.68		
150%	100.31	98.84	1.36
	98.53		

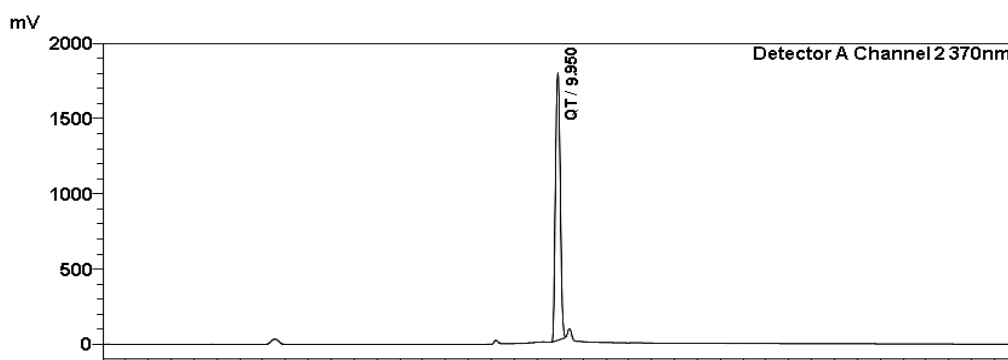**Figure 2: Chromatogram of standard (GA and CT) at 273nm**

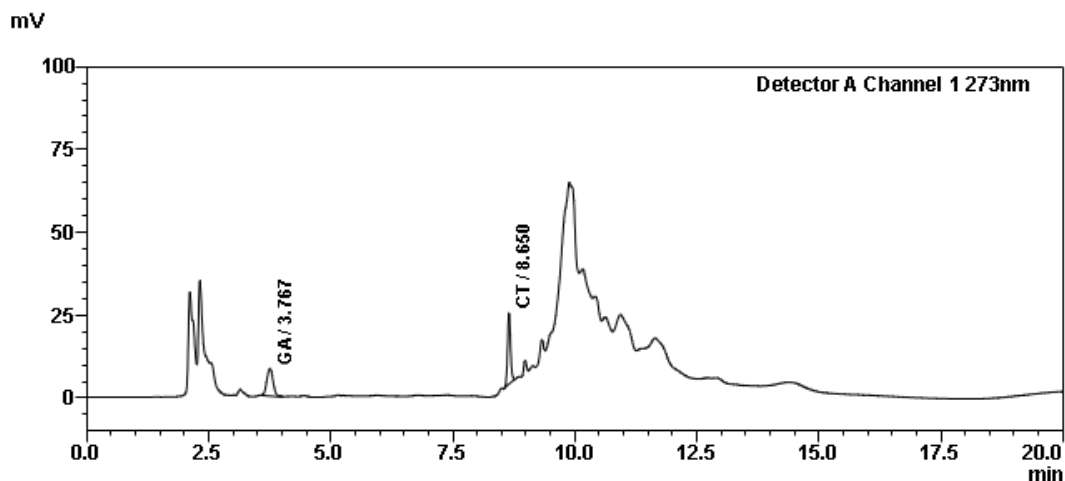**Figure 3: Chromatogram of standard (QT) at 370nm**

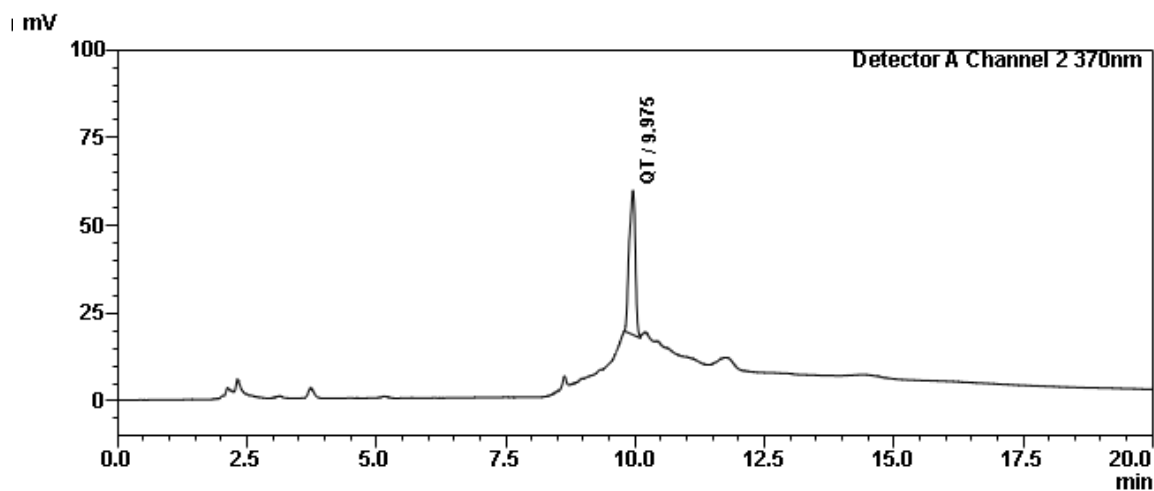**Figure 4: Chromatogram of sample (GA and CT) at 370nm**

Figure 5: Chromatogram of sample (QT) at 370nm

Figure 6: Standard calibration curve of GA
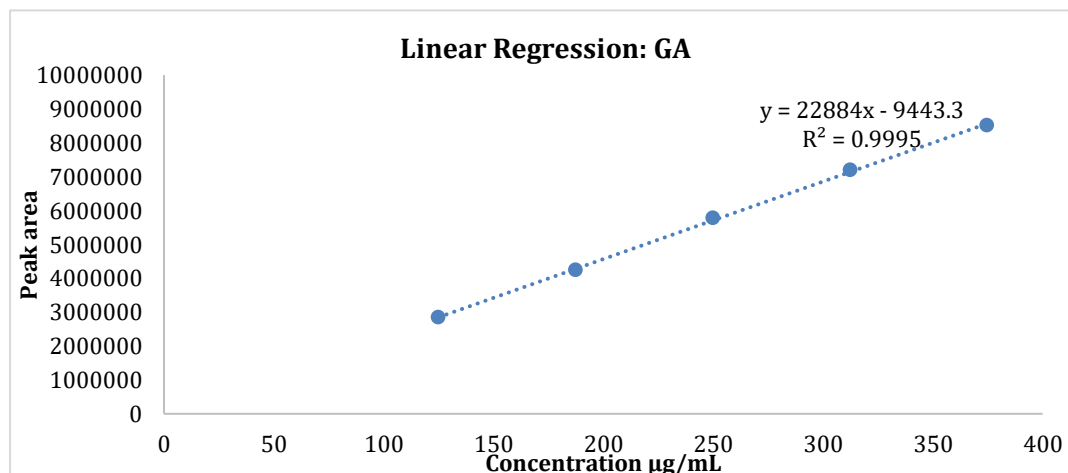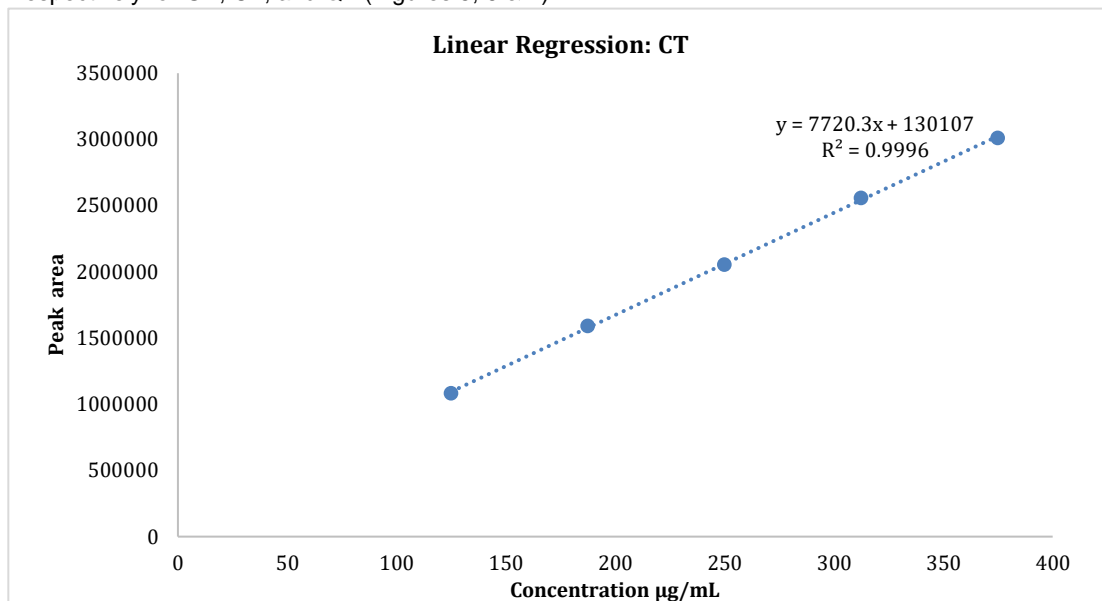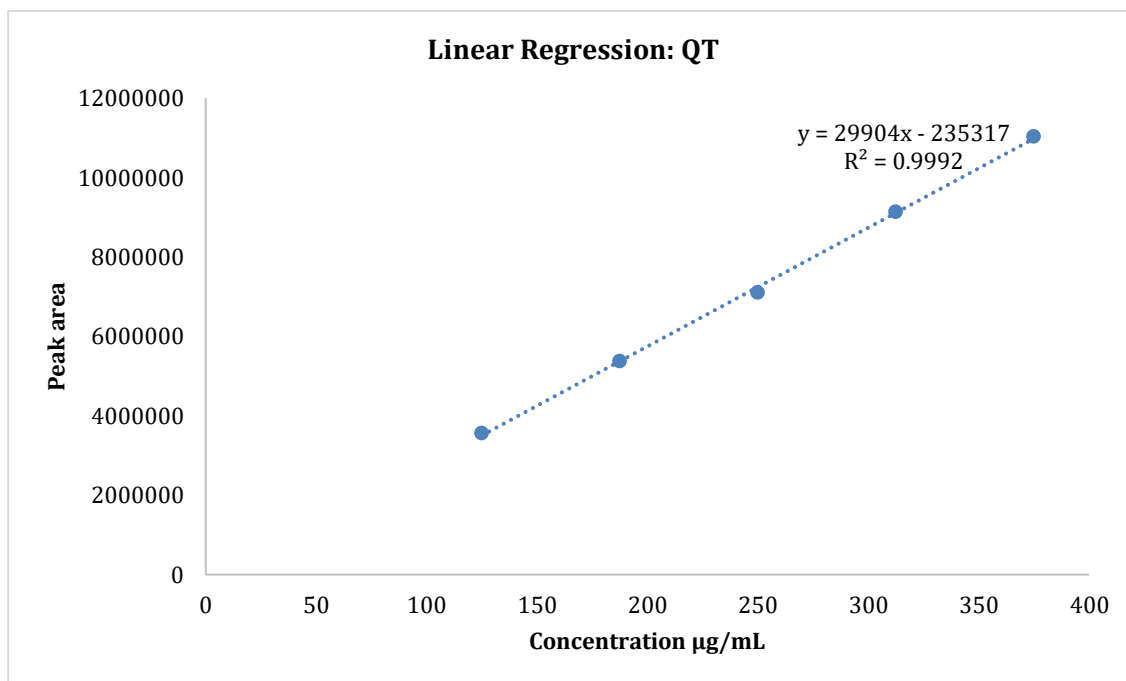
Linearity: Linearity was studied by analyzing mixed standard solution at 5 different levels (n=3 for each level) in the range of 125ppm-375ppm (50%-150% levels). Calibration curves with concentration versus peak area were plotted for each standard and the obtained data was assessed through regression analysis. The correlation coefficient (r^2) value was graphically calculated to be 0.9941, 0.9948, & 0.9962 respectively for GA, CT, and QT (Figures 5, 6 & 7).

Linear Regression: CT

$y = 7720.3x + 130107$
$R^2 = 0.9996$

Figure 7: Standard calibration of CT

Linear Regression: QT

$y = 29904x - 235317$
$R^2 = 0.9992$

Figure 8: Standard calibration curve of QT

Sensitivity: Regression analysis of obtained data showed LOD and LOQ values as 86.67ppm-262.65 ppm for GA, 81.46ppm-246.85 ppm for CT, and 69.80ppm- 211.50 ppm for QT (Table 6).

Table 6: Sensitivity of the proposed method

Component	SE of intercept	SD of intercept	LOD (ppm)	LOQ (ppm)
GA	295868.29	661561.50	86.67	262.65
CT	87703.17	196104.29	81.46	246.85
QT	276356.07	617932.18	69.80	211.50

Robustness: Change in flow rate and column temperature resulted in peak area values with not more than 2% of relative standard deviation (Table 7).

Table 7: Robustness study

Performance parameter (n=2)	GA	CT	QT
Flow rate (+0.2mL/min)			
Average retention time	3.50	8.25	9.83
%RSD	**1.79**	**1.19**	**0.71**
Average peak area	5642186	1945608	6982562
%RSD	**0.99**	**1.29**	**0.32**
Flow rate (-0.2mL/min)			
Average retention time	3.62	8.51	10.02
%RSD	**0.59**	**1.00**	**0.64**
Average peak area	5821482	2032576	7132580
%RSD	**1.22**	**1.80**	**1.18**
Column temperature (+2°C)			
Average retention time	3.51	8.55	9.91
%RSD	**1.59**	**1.33**	**0.14**
Average peak area	5674921	2004886	6974214
%RSD	**0.58**	**0.83**	**0.40**
Column temperature (-2°C)			
Average retention time	3.65	8.56	10.03
%RSD	**1.17**	**1.42**	**0.71**
Average peak area	5652389	1930304	6987284
%RSD	**0.86**	**1.85**	**0.28**

CONCLUSION

A simple, easy, and rapid RP-HPLC method with UV detection was developed for the simultaneous detection of GA, CT, and QT. The analytes were well resolved and separated within 20 minutes. The method was validated for specificity, precision, accuracy, linearity, sensitivity, and robustness following ICH guidelines. The results showed good sensitivity, high precision and accuracy, and a wide linear range. The method showed sensitivity towards changes in chromatographic conditions; that is, change in flow rate and column temperature. Both parameters affected retention time and area response but did not affect the resolution of the analytes. The developed HPLC method was applied to the BBFP sample and showed good resolution between desired analytes. The developed HPLC method is simple, easy, rapid, utilizes a UV detector, and can be conveniently employed for routine quality control analysis of GA, CT, and QT based formulations or products.

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