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RESEARCH ARTICLE

Development and Validation of HPLC and UV Spectrophotometric Method for the Quantification of Cinnamaldehyde in Cinnamon Bark Extract

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Abstract

Cinnamaldehyde is the prime constituent of cinnamon bark and cassia oil. It is used as a flavoring agent. Numerous methods described the determination of cinnamaldehyde based on volumetric analysis, ultraviolet spectrometry, fluorimetry, thin layer chromatography, liquid chromatography and gas chromatography. The natural absorbance of cinnamaldehyde is at 286 nm which is used as a base for its determination through spectrophotometry or a suitable derivatizing reagent is used for its estimation. Most of these methods were simultaneous estimation methods and if non-simultaneous than not sensitive. Therefore, in the present study, sensitive HPLC and UV Spectrophotometric procedures have been established for the estimation of cinnamaldehyde in Cinnamon extract. The retention time of cinnamaldehyde was 7.21 minutes and absorption maxima come out to be 282 nm. 2.55 ± 0.003 mg/ml quantity of cinnamaldehyde was present in the cinnamon extract which is detected by UV Spectrophotometric method. Accuracy information appeared in the range that gives decent recovery figures for both processes. Sensitivity data furnished LOD 0.062 µg/ml and LOQ 0.19 µg/ml for HPLC and LOD 0.104 µg/ml and LOQ 0.312 µg/ml for UV Spectrophotometric method. The developed methods were found to be rugged and robust. The repeatability, Inter-day,and Intra-day precision of cinnamaldehyde provided RSD below 2% presenting the planned process to be extremely specific. Various factors to validate HPLC and UV Spectrophotometric methods of cinnamaldehyde were estimated and both methods show no significant difference. Developed procedures were statistically checked as per ICH guidelines.

Keywords: Cinnamaldehyde, Cinnamon Extract, HPLC Method, UV Spectrophotometric Method, Validation

1. Introduction

Cinnamon is acquired from the interior bark of the various trees of the genus Cinnamomum which is used in the making of both sweet and spicy foods. The word cinnamon came from the Greek kinnamomon[1]. It is a small classic tree, 11-16 meters in height. It is a member of the Lauraceae family, indigenous to various regions of Sri Lanka and India. The flowers are green in color and have a specific odour. The fruit contains one single seed which is a purple one-centimeter berry. Aromatic essential oil is responsible for its flavor which makes up 0.5 to 1% of its composition. Cinnamon shows good antioxidant and antibacterial property and it is also beneficial in suppressing cold and cough, tooth pain, loose motions and various other problems of the digestive system and prevents foul breath, and have significant pharmacological effects in curing type II diabetes. Cinnamon oil helps in the preservation of several food items through its antimicrobial properties[2–4].

Cinnamaldehyde is the key component of cinnamon bark and cassia oil and is used as a flavoring agent. It has effective antimicrobial activity, reduces fever, sedative and holds insecticidal properties[5]. Numerous procedures

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described the determination of cinnamaldehyde based on titrimetry[6], second derivative ultraviolet spectrometry[7], fluorimetry,[8,9] thin layer-chromatography[10,11], liquid chromatography[12–19] and gas chromatography[20–26]. Most of these analytical methods are a simultaneous estimation of cinnamaldehyde along with other phytoconstituents. UV spectrophotometric method was a derivative method so to have a single evaluation of cinnamaldehyde from the extract the authors developed and validated sensitive HPLC and UV Spectrophotometric analytical procedures.

2. Materials and Methods

2.1 Reagents and Chemicals

The diluents and compounds of High-Performance Liquid Chromatography grade were used. Each needed solution was made in the water of HPLC grade. Unless otherwise mentioned, solutions were strained across a 0.2 µm Ultipor® N66® Nylon 6, 6 membrane filter (Pall Life Sciences, USA) before starting the experiment. The crude powder of cinnamon bark was procured from Kshipra Biotech Private Limited. Standard of cinnamaldehyde was provided as a present by the Tokyo Chemical Industry (TCI), Japan. Methanol solvent was sourced from Thermo Fisher Scientific, India. Acetonitrile was bought from Merk Ltd., Mumbai.

2.2 Instruments and Apparatus

- Shimadzu's HPLC system has an auto-sampler and SPD-10AVP UV-VIS detector alongside column Phenomenex Luna, C_{18} bonded with 5 µm (4.6 x 250 mm) particle size, attached with LC-solution software to record and process chromatographic data.
- Spectrophotometric quantifications were done on a Shimadzu 1700 double beam UV Visible spectrophotometer having a fix slit width of 1 nm attached with a computer loaded with Shimadzu UV Probe software of version 2.31.
- Analytical balance (Shimadzu, AUX220), Digital pH meter (Lab India, OHPL-OP_024) and Bath Sonicator (PCI, Mumbai, NSW-133) were used.

2.3 Chromatographic Conditions

Solvent constitutes of ethanol and 0.5% v/v solution of acetonitrile was made, filtration was done by passing across 0.45 µm screens and gasses were removed by an ultrasonic bath every time prior to use. Good sensitivity and resolution of the process were found at 280 nm with a rate of flow of 1 ml min^{-1} at 37 °C with isocratic elution (50:50 v/v). The solvent phase was made to run continuously in the system for at least 10 mins to equilibrate the column[27]. All solutions were formed with diluent (ethanol:water) in the ratio of 8:2 (v/v) and were inserted thrice.

2.3.1 Standard Stock Solution for HPLCs

A fresh solution of cinnamaldehyde standard of reference (10 mg/ml) was made by adding solvent and sonication for a period of 10 mins was done and kept at 2–9°C till further use. The solution for the standard was made by diluting the standard stock solution along with ethanol:water (8:2) (v/v) to get 10 µg/ml. The calibration curve was achieved while using this solution which helps in the optimization of the method.

2.3.2 Cinnamon Bark Extract Sample for HPLC Method

A precisely measured amount of 10 mg of cinnamon extract was shifted to 10 ml of the vessel. Enough solvent was put to mix the extract and sonication was done for thirty minutes after which the volume was altered to get 100 µg/ml. This mixture was strained in a 0.2 µm Ultipor® N66® membrane filter of Nylon 6,6 and introduced into the HPLC system.

2.4 Standard Stock Solution for Spectrophotometric Method

10 mg cinnamaldehyde was measured correctly and 10 ml ethanol was put into a graduated flask, 5 ml from the above stock solution was taken and added to a 50 ml graduated flask and volume was modified by adding ethanol up to the mark and sonication was done for 5 mins. This solution was then mixed with ethanol, to get several dilutions from 0.5-4.5 µg/ml. The absorbance of these solutions was checked at 282 nm against ethanol as blank in a UV-visible spectrophotometer and the standard curve was designed in contrast to concentration. Equations of straight lines and coefficient of correlation were obtained from the calibration curve.

2.4.1 Cinnamon Bark Extract Sample for Spectrophotometric Method

Dried extract (1 g) of cinnamon was weighed precisely and then diluted with 10 ml of ethanol in a cork tube. The contents of the tube were transferred to a beaker

made up of glass and mixed using an electric mixer for 15 mins at 25°C. The solution was strained and the filtrate solution was suitably diluted and determined spectrophotometrically. Cinnamaldehyde level was estimated using a calibration curve[28].

2.4.2 UV Spectrophotometric Method

Each one of the samples turned out to be balanced in all experimental measurements (spectra were constant for about 24 hrs). All measurements were recorded at room temperature. A range of 200-400 nm was used to record spectra of different samples using a 1.0 cm quartz cell in UV-A 4.5 µg/ml solution of cinnamaldehyde in ethanol was checked on the scale of 200-400 nm. Absorption maxima of cinnamaldehyde in ethanol come at 282 nm[29].

2.5 HPLC Method Optimization

After several checks of various chromatographic properties, ethanol:acetonitrile (0.5% v/v) with isocratic elution at a rate of flow of 1 ml/min comes out to be the optimum mobile phase. Good sensitivity and resolution of the process were attained for cinnamaldehyde at 282 nm. A distinctive chromatogram with advanced conditions gives a symmetric and sharp peak having a retention time of 7.21 min.

2.6 Validation Parameters for Developed Methods

Both methods essentially are validated. The suggested methods were authenticated according to ICH terms for linearity, robustness, accuracy, range, precision, ruggedness, sensitivity and limit of quantification and limit of detection.

2.6.1 Linearity

The linearity of the process defines its capability to get test results which commensurate to the analyte concentration in the sample within a given range. The range of the analytical process gives the interval in the center of the advanced and subordinate levels of analyte that has to be determined within an acceptable precision level, accuracy and linearity. The selected linearity range for cinnamaldehyde was 1-10 µg/ml.

2.6.2 Accuracy and Precision

Accuracy was estimated using recovery experiments, % and the mean recovery of the sample at 3 different levels (80-120%) of the sample solution (8 mg, 10 mg, and 12 mg) of the cinnamon extract was calculated. Three resolutions were carried out at all stages respectively. The average recovery was determined. The suitable limits of recovery are 80% - 120%.

Parameters like repeatability and intermediate precision were used to establish the precision of the method. It tells about the arrangement's proximity present in asequence of arrangements attained from frequent samplings of the alike similar sample. The in-between precision was affirmed by intraday and inter-day examination which was performed thrice in a day and also consecutively for 3 days. Repeatability was the similarity between several estimations of a single quantity when the experiments are performed in identical circumstances (apparatus, instruments, analyst and day) in a continuous flow. In the HPLC method, a sample solution of cinnamaldehyde (10 mg of cinnamon extract in 10 ml of ethanol) was formed and analysis was performed six times as per the planned method. In UV Spectrophotometric method, a cinnamon extract standard solution containing cinnamaldehyde equivalent to 3 μg/ml was formed and analyzed six times at 282 nm as per the planned method. The absorbance was checked and % RSD was also calculated.

2.6.3 Specificity

HPLC specificity was checked by estimating the peak purity of the specimen that was estimated by evaluation of the cinnamon bark extract peak at 3 levels, the start of the peak (S), apex of the peak (M) and end of the peak (E) positions in the spectrum.

2.6.4 Sensitivity

The sensitivity of UV and HPLC was checked by estimating the Limit of Determination (LOD) and Limit of Quantitation (LOQ):

$$LOD = 3.3\ \sigma/S$$

$$LOQ = 10\ \sigma/S$$

Where σ = Standard deviation of the response; S = Slope of the calibration curve.

2.6.5 Robustness

Robustness is the capability to give precise and accurate results under different conditions. The robustness of the process was determined by interchanging the most crucial factors while the other factors were kept unchanged and simultaneously the chromatographic characteristics

were recorded. The focused parameter was a change in wavelength and change in flow rates.

2.6.6 Ruggedness

Ruggedness was calculated by analysis of a similar specimen of cinnamaldehyde by two dissimilar analysts and the corresponding output of the percentage was estimated and the results were indicated as % RSD.

2.7 Statistical Comparison of the Results of the Developed Methods

For statistical comparison of both methods, recovery experiment data was used. The standard sample of cinnamaldehyde was added to the cinnamon extract at three different levels and recovery % was calculated. The mean % recovery in both methods was compared statistically by Student's t-test.

3. Results and Discussion

At first, a chromatogram of the blank sample was obtained using HPLC analysis, as shown in Figures 1 and 2. The figures represents the chromatogram of the cinnamaldehyde standard (10 µg/ml) with a retention time of 6.98 min. Cinnamaldehyde in ethanol (4.5 µg/ml) was scanned on the scale of 200-400 nm. Absorption maxima of cinnamaldehyde in ethanol at 282 nm come out to be similar to the literature as given in Figure 3.

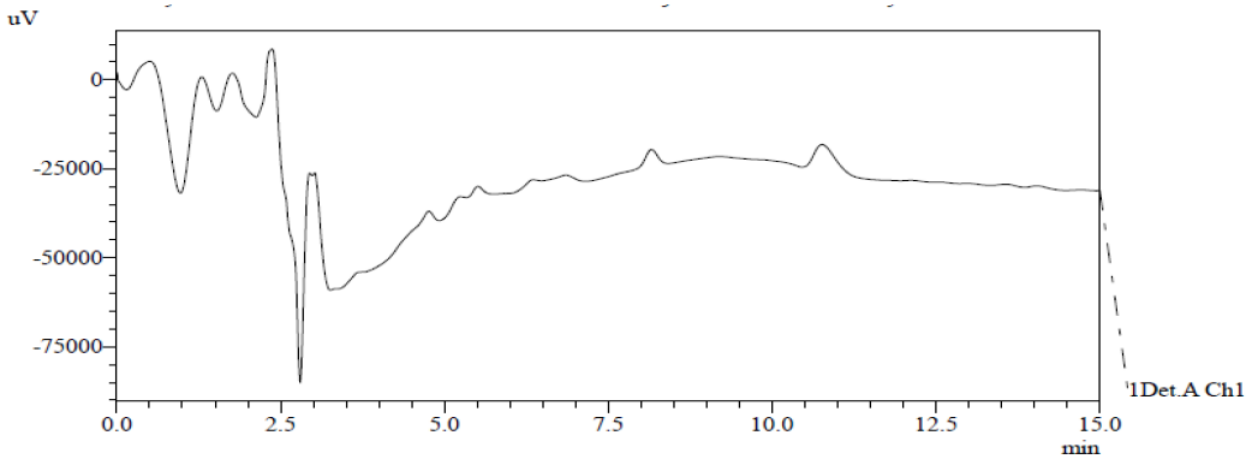

Figure 1. Chromatogram of blank sample.

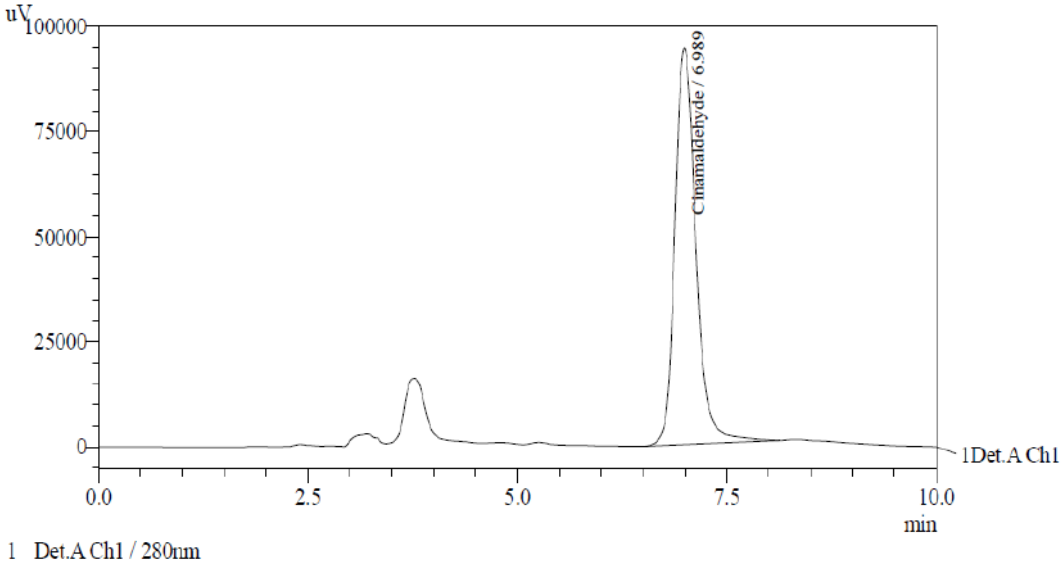

Figure 2. Chromatogram of cinnamaldehyde Standard (10 µg/ml).

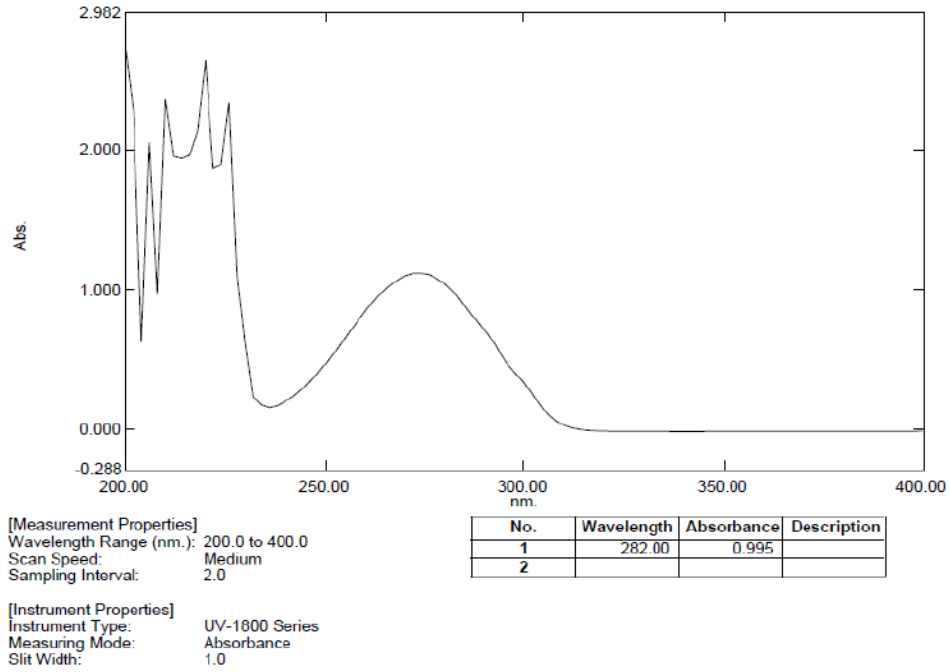

Figure 3. Absorption maxima of cinnamaldehyde in ethanol.

3.1 Validation Parameters

3.1.1 Linearity

At an analytical scale of 1–10 µg/ml, the chromatographic result was straight for HPLC and for the UV Spectrophotometric method, the absorbance was linear over 0.5 to 4.5 µg/ml for the determination of cinnamaldehyde. The least-squares method was used to calculate the linear regression equation using GraphPad Prism 9.0.0 program. Table 1 represents the slope variance and intercepts for HPLC and Table 2 for UV Spectrophotometry. The intercept is not profoundly different from zero; so, there was no interference in the calculation of the cinnamaldehyde by both methods. Furthermore, the slope and intercept lie in the confidence interval. The overlay spectrum of cinnamaldehyde is given in Figure 4.

Table 1. Validation parameters for RP-HPLC method of vinnamaldehyde

Parameters	Cinnamaldehyde
Absorption maxima (nm)	282
Linearity range (µg/ml)	1-10
Coefficient of determination (r^2)	0.998
Correlation coefficient (r)	0.999
Regression equation (Y^a)	Y=39002x + 298.49
Slope (m)	158715
Intercept (c)	54762
LOD (µg/ml)	0.062
LOQ (µg/ml)	0.19
Precision Repeatability (%RSD) Intra-day (%RSD) Inter-day (%RSD)	 1.79 1.36 1.26

Table 2. Validation parameters for UV Spectrophotometric method of cinnamaldehyde

Parameters	Cinnamaldehyde
Absorption maxima (nm)	282
Linearity range (µg/ml)	0.5-4.5
Coefficient of determination (r^2)	0.9990
Correlation coefficient (r)	0.9994
Regression equation (Y^a)	Y= 0.212x +0.019
Slope (m)	0.212
Intercept (c)	0.019
LOD (µg/ml)	0.104
LOQ (µg/ml)	0.312
Precision Repeatability (%RSD) Intra-day (%RSD) Inter-day (%RSD)	 0.941 0.570 0.563
% Cinnamaldehyde in Cinnamon extract (mg/ml) (Mean ± SD)	2.55±0.003

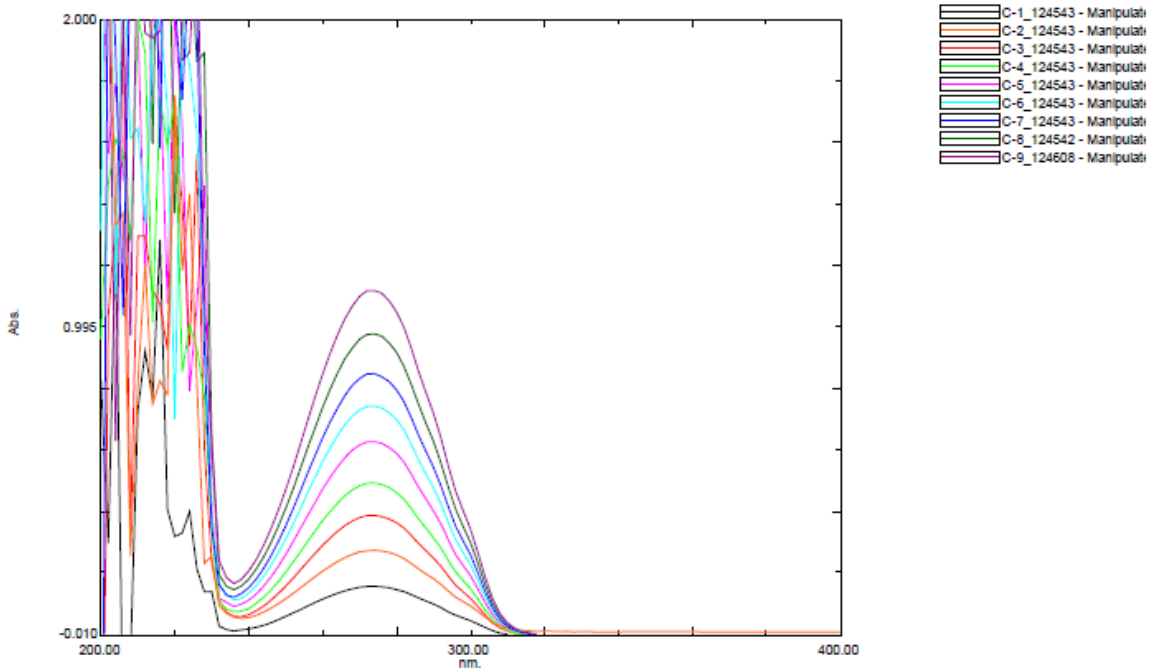

Figure 4. Overlay spectrum of cinnamaldehyde.

3.1.2 Accuracy

Accuracy was estimated by using recovery tests, by the estimation of % mean recovery of the sample at three distinct levels (80-120%) of the sample solution (8 mg, 10 mg and 12 mg) of cinnamon extract. Individually at all levels, three determinations were carried out. The average percentage recovery was estimated as given in Table 3 for HPLC and Table 4 for UV Spectrophotometry. The passed recovery values are 80%-120%. Recovery study data shows there were good recovery values for both methods indicating that cinnamaldehyde is present within the acceptable range.

Table 3. Results of recovery study of cinnamaldehyde by HPLC method

Level	The percentage recovery of cinnamon extract (Mean)	SD	% RSD
80%	103.97	0.42	0.40
100%	102.38	0.33	0.32
120%	100.53	0.39	0.39

Table 4. Results of recovery study of cinnamaldehyde by UV Spectrophotometric method

Level	The percentage recovery of Cinnamon extract (Mean)	SD	% RSD
80%	101.86	0.22	0.20
100%	100.63	0.63	0.63
120%	102.40	0.21	0.19

3.1.3 Precision

Precision was determined by performing Intra and inter-day variation and repeatability experiments. Tables 1 and 2 present the relative standard deviation in percentage. RSD value less than 2.0% was obtained for cinnamaldehyde, hence proving its precision.

3.1.3.1 Repeatability

A similar pattern of calculations is used again and again for the alike component in tests under optimum situations like an instrument, analyst, day and apparatus. For the HPLC method, the %RSD was calculated was found to be 1.79% as described in Table 1. For UV Spectrophotometric method, the % RSD was estimated and found to be 0.941 and presented in Table 2.

3.1.3.2 Intra-day and Inter-day Precision

The intra-day and inter-day variation for the estimation of cinnamaldehyde was performed six times in one day and three consecutive days using a sample solution of concentration of 10 µg/ml of cinnamaldehyde (10 mg of cinnamon extract in 10 ml of ethanol) for the HPLC method and 3 μg/ml for UV method. Percent RSD was estimated and found to be within 2% and is given in Table 1 for HPLC and Table 2 for UV Spectrophotometry.

3.1.4 Robustness

Robustness for HPLC and UV Spectrophotometry is given in Table 5 and Table 6 suggested that the little changes in the circumstances did not remarkably affect the estimation of cinnamaldehyde. Change in wavelength and change in flow rates was studied for HPLC and change in wavelength for UV Spectrophotometry. It was perceived that no remarkable changes were found in both methods' parameters proving that the established methods are robust.

Table 5. Robustness parameter of cinnamaldehyde studied for HPLC method

Parameters	Alterations	%RSD for Area (n = 6)
Change in flow rate	-2%	0.46
	Normal	0.40
	+2%	0.10
Detection wavelength	-2 unit	0.12
	Normal	0.31
	+2 unit	0.24

Table 6. Robustness parameter of Cinnamaldehyde studied for UV Spectrophotometric method

Parameters	Alterations	%RSD for Area (n = 6)
Detection wavelength (of 3 **µg/ml)**	-4 unit (278)	0.88
	Normal (282)	0.97
	+4 unit (286)	0.94

3.1.5 Ruggedness

For the HPLC method, percent RSD comes out to be 1.06 and 1.35 for the first and second analysts respectively and UV Spectrophotometric method shows 0.78 and 0.79 for the first and second analysts. No differences were found in the HPLC conditions proving the developed HPLC as rugged.

3.1.6 Sensitivity

The LOQ and LOD of the process were estimated depending on the slope from the calibration curve and the standard deviation of the response at estimated levels of the limit of detection and the limit of quantification. The LOD comes out to be 0.062 µg/ml and LOQ 0.19 µg/ml for HPLC and LOD comes out to be 0.104 µg/ml and LOQ 0.312 µg/ml respectively for UV, proving the high sensitivity of the process.

3.1.7 Specificity

Specificity is the degree to which the process focuses on the main analyte of interest and is assessed by probing the cinnamon extract samples for the detection of any

interfering peaks. The specificity of the process was estimated in comparison to the interference due to the presence of any other constituents of the extract. None of the constituents interfere with the cinnamaldehyde peak and therefore the method is specific.

3.2 Determination of Cinnamaldehyde Content in Cinnamon Extract

It was found that a solution of 1 gm of cinnamon extract contains 2.55 ± 0.003 mg/gm of cinnamaldehyde by UV Spectrophotometric method and 1.42 ± 0.002 mg/gm by HPLC method. This value is the mean of three independent determinations. These values suggest that the other constituents of the extract may be interfering when analyzed using UV Spectrophotometric method. Therefore, it is suggested that extracts must be subjected to extraction before analyzing in UV Spectrophotometric method.

3.3 Statistical Comparison of the Results of the Developed Methods

Validated methods were successfully applied to the analysis of cinnamaldehyde amount in cinnamon extract without any interference from other constituents. Recovery experiment results as shown in Table 7 are correlated statistically. No remarkable variations were found in the outcomes of these established procedures when checked at a 95% confidence level (two-tailed p-value – 0.0913) using Student's *t*-test.

4. Conclusion

The developed new RP-HPLC method in the present study was established to be simple, distinct, sharp, pinpoint, robust and authentic that can be successfully used in research organizations. This process can be adopted as a quality assurance tool for estimating total cinnamaldehyde, which makes it a useful tool for the quality control of cinnamon extract.

Table 7. Statistical comparison of the results obtained by the developed methods

	HPLC method	UV Spectrophotometric method
Cinnamaldehyde content Mean ± SD	102.29 ± 0.38	101.86 ± 0.35
	$t_{calculated}$= 0.298 $t_{theoretical}$= 2.92	$t_{calculated}$= 0.298 $t_{theoretical}$= 2.92

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