RESEARCH PAPER

Development and Validation of a Green RP-HPLC Method for Determination of Chloramphenicol in Pharmaceutical Formulations

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ABSTRACT

Objective: To develop and validate an RP-HPLC method for the determination of chloramphenicol (CHL) in bulk drug and marketed formulations with assessment of analytical greenness based on Green Analytical Chemistry (GAC) principles.

Methods: Chromatographic separation was achieved using a Shimpack GIST C18 column (250 × 4.6 mm, 5 µm) with a mobile phase consisting of methanol and water (70:30, v/v) delivered at a flow rate of 1.0 mL/min. Detection was performed at 275 nm with an injection volume of 20 µL. The method was validated as per ICH Q2(R1) guidelines for system suitability, specificity, linearity, precision, accuracy, robustness, LOD, LOQ, and greenness assessment using AGREE.

Results: The method showed linearity over the concentration range of 30–200 µg/mL (R^2 = 0.999), with recovery values ranging from 99.4% to 101.9%.LOD and LOQ were 2.53 µg/mL and 7.68 µg/mL, respectively. Precision studies showed %RSD < 0.5% for intra- and inter-day analyses. Robustness testing confirmed minimal influence of deliberate variations. The AGREE score of 0.79 suggested acceptable analytical greenness of the developed method. Application to marketed formulations (capsule, eye drops, ointment) yielded assay values within 95–105%.

Conclusion: The developed green HPLC method is precise, accurate, robust, and environmentally sustainable. It offers a reliable tool for routine pharmaceutical analysis of chloramphenicol while minimizing environmental impact.

Keywords: *Chloramphenicol; RP-HPLC; Green analytical chemistry; Method validation; AGREE assessment*
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1. INTRODUCTION:

In recent years, there has been growing interest in the use of green solvents for the development of sustainable analytical techniques. Green Analytical Chemistry (GAC) focuses on reducing or eliminating hazardous substances in analytical workflows while simultaneously lowering energy consumption and minimizing waste generation without compromising method reliability or performance [1–3].

Ocular infections such as conjunctivitis and keratitis are common ophthalmic disorders associated with symptoms including eye pain, blurred vision, and photosensitivity [4]. Chloramphenicol (CHL) is a broad-spectrum bacteriostatic antibiotic widely used in the treatment of ocular bacterial infections due to its activity against both Gram-positive and Gram-negative microorganisms [5,6]. Owing to its therapeutic effectiveness and widespread availability, CHL continues to be extensively used in pharmaceutical formulations [7].

Despite its therapeutic advantages, CHL is prone to degradation through hydrolysis of its amide group, producing 2-amino-1-(4-nitrophenyl) propane-1,3-diol. This hydrolysis product is frequently encountered in

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pharmaceutical formulations and can affect the drug's stability and efficacy [8].

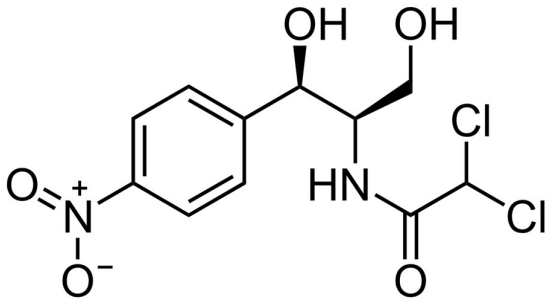

Figure 1: Chemical Structure of Chloramphenicol

The analysis of chloramphenicol in pharmaceutical formulations, biological fluids, and food products (such as milk, honey, and meat) is crucial to ensure compliance with regulatory limits and to monitor its presence due to potential health risks. Over the years, various analytical methods have been developed for chloramphenicol detection, including spectrophotometry, high-performance liquid chromatography (HPLC), gas chromatography (GC), and liquid chromatography-mass spectrometry (LC-MS). However, most of these methods rely heavily on volatile organic compounds (VOCs) as solvents, which pose significant environmental and health risks [9–13].

Growing awareness regarding environmental sustainability has encouraged the adoption of Green Analytical Chemistry principles in pharmaceutical analysis. Nevertheless, only limited studies have focused on the development of comparatively greener chromatographic methods for chloramphenicol determination.

Previously reported analytical methods for chloramphenicol mainly focused on sensitivity and chromatographic performance; however, many of them employed toxic organic solvents, involved complex extraction procedures, required longer analysis times, or lacked systematic greenness evaluation. In addition, limited attention has been given to the development of comparatively eco-friendly RP-HPLC methods applicable to routine pharmaceutical quality control. The present study attempts to address these limitations by developing a simple RP-HPLC method using a comparatively less hazardous mobile phase system with reduced environmental impact, satisfactory chromatographic performance, and AGREE-based greenness assessment. The proposed method also offers acceptable precision, accuracy, and robustness suitable for routine analysis of chloramphenicol in different pharmaceutical dosage forms.

This research aims to develop a green, environmentally friendly analytical method for the detection and quantification of chloramphenicol. The proposed method will minimize the use of hazardous solvents, reduce environmental impact, and ensure accurate, reliable quantification of chloramphenicol in various matrices. Additionally, the method was validated in accordance with ICH guidelines.

2. MATERIALS AND METHODS

2.1 Materials and reagents

Chloramphenicol working standard was obtained as a gift sample from a reputed pharmaceutical industry. Methanol and acetonitrile (HPLC grade) were procured from Merck Life Sciences Pvt. Ltd., Mumbai, India. HPLC-grade water was prepared using an in-house Milli-Q water purification system. All weighing procedures were carried out using a NABL-calibrated analytical balance, and Type A grade glassware was used throughout the study. Marketed chloramphenicol formulations, including capsules (500 mg), eye ointment (1% w/w), and eye drops (1% w/v), were purchased from a local pharmacy.

2.2 Instruments

Chromatographic analysis was performed using a Waters HPLC system equipped with a quaternary pump and diode array detector (DAD). Data acquisition and processing were carried out using Waters Empower software. A Mettler Toledo analytical balance and ultrasonicator were used during sample preparation.

2.3 Preparation of standard (std) stock solution

An accurately weighed quantity of chloramphenicol standard (50 mg) was transferred into a 50 mL volumetric flask and dissolved in approximately 25 mL of diluent (water, 50:50 v/v). The solution was sonicated to ensure complete dissolution and the volume was adjusted to the mark using the same diluent to obtain a final concentration of 1000 µg/mL.

2.4. Preparation of mobile phase

The mobile phase consisted of methanol and water in the ratio of 70:30 (v/v). Methanol was selected as the organic modifier considering its comparatively lower cost and acceptable chromatographic performance for chloramphenicol analysis. The mobile phase was filtered through a 0.45 µm membrane filter and degassed prior to use.

2.5 Chromatographic conditions

The chromatographic analysis was performed using a Shimpack GIST C18 column (250 × 4.6 mm, 5 µm). The mobile phase consisted of a 70:30 ratio of methanol to water. A flow rate of 1.0 mL/min was maintained, with an injection volume of 20 µL. Detection was carried out at a wavelength of 275 nm, and the column temperature was set at 30°C.

2.6 Method validation

The developed method was validated based on the ICH Q2(R1) guideline (International Conference on Harmonization of Technical Requirements for registration of pharmaceuticals for human use, 2005).

2.7 System suitability testing (SST)

The standard stock solution (treated as 100% level) was used for the evaluation of SST. The results were considered acceptable if the percent Relative Standard Deviation (%RSD) of 6 replicate injections for peak area is within 2%, the mean theoretical plate was Not Less Than (NLT) 2000, tailing factor (Tf) Not More Than (NMT) 2.0. A blank sample at the beginning of the injection sequence was analysed to check interference due to solutions. The sequence also contained a blank sample at the end to ascertain any carryover effect.

2.8 Specificity and Selectivity

Specificity of the developed method was evaluated by analyzing blank, standard, and sample solutions to assess any potential interference at the retention time of chloramphenicol. No interfering peaks from diluent or formulation excipients were observed at the analyte retention time, demonstrating the specificity and selectivity of the method for chloramphenicol analysis.

2.9 Linearity and range

Linearity of the developed method was evaluated over the concentration range of 30–200 µg/mL for chloramphenicol. Calibration solutions corresponding to 30%, 50%, 80%, 100%, 120%, 150%, and 200% of the target concentration were prepared and analyzed in triplicate. Calibration curves were constructed by plotting peak area versus concentration, and linear regression analysis was performed. The method was considered linear when the correlation coefficient (R^2) was not less than 0.99 and %RSD values for replicate measurements were within acceptable limits.

2.10 Precision

Precision of the method was evaluated in terms of repeatability (system precision), intraday precision, and interday precision. System precision was assessed using six replicate injections of the standard solution at the target concentration level. Intraday precision was evaluated by analyzing three different concentration levels (50, 100, and 150 µg/mL) within the same day, whereas interday precision was determined on different days under similar experimental conditions. The method was considered precise when the %RSD values were less than 2%.

2.11 Accuracy/recovery studies

Accuracy of the method was evaluated by recovery studies using the standard addition method at four concentration levels (30%, 50%, 100%, and 150%). Known amounts of chloramphenicol standard were added to pre-analyzed sample solutions and analyzed in triplicate. The percentage recovery was calculated by comparing the obtained concentration with the added concentration. The method was considered accurate when recovery values were within the acceptable range of 98–102%.

2.12 Detection and quantification limits

The limit of detection (LOD) and limit of quantification (LOQ) were determined based on the standard deviation of the response and slope of the calibration curve according to the following equations:

$$LOD = 3.3\sigma/S$$

$$LOQ = 10\sigma/S$$

where σ represents the standard deviation of the response and S represents the slope of the calibration curve

2.13 Robustness

Robustness of the developed method was evaluated by introducing deliberate variations in chromatographic conditions, including flow rate (1.0 ± 0.1 mL/min) and column temperature (30 ± 2°C). The effect of these variations on chromatographic performance was assessed using peak area and %RSD values. The method was considered robust when no significant variation in analytical performance was observed.

2.14 Greenness of the developed method

The analytical greenness of the developed RP-HPLC method was evaluated using AGREE (Analytical GREEnness Metric Approach), GAPI (Green Analytical Procedure Index), and NEMI (National Environmental Methods Index) assessment tools based on the principles of Green Analytical Chemistry. The AGREE tool was used to determine the overall greenness score of the analytical method, whereas GAPI and NEMI assessments were employed to evaluate different environmental aspects of the analytical procedure, including reagent hazards, waste generation, instrumentation, and sample preparation steps. Comparative greenness evaluation of the developed method with previously reported HPLC methods for chloramphenicol analysis was also performed using these assessment approaches (see Section 3.9 and Table 8).

3. RESULT AND DISCUSSION

3.1 Method development and method optimization

Different chromatographic conditions were investigated to obtain acceptable peak symmetry, retention behavior, and analytical response for chloramphenicol. Various mobile phase compositions consisting of methanol and water were evaluated during method optimization. Among the tested conditions, methanol (70:30, v/v) provided satisfactory chromatographic performance with appropriate retention time, symmetrical peak shape, and acceptable theoretical plate count.

The detection wavelength was selected at 275 nm based on the UV absorption spectrum of chloramphenicol, which showed adequate analytical response at this wavelength. Chromatographic separation was achieved using a Shimpack GIST C18 column maintained at 30°C with a flow rate of 1.0 mL/min. Under the optimized chromatographic conditions, chloramphenicol was eluted with satisfactory peak characteristics and reproducible analytical response suitable for routine pharmaceutical analysis.

METHOD VALIDATION

3.2 System suitability testing (SST)

System suitability parameters demonstrated satisfactory chromatographic performance for chloramphenicol

analysis. The retention time was found to be 3.60 min with acceptable peak symmetry and theoretical plate count. The %RSD values obtained for replicate injections were within acceptable limits, indicating adequate system precision and reproducibility of the developed method (Table 1).

Table 1: System suitability parameters of HPLC method

Parameters	Results	
	Mean ± SD (n=6)	**% RSD**
Retention time (min)	3.603 ± 0.005	0.16
Tailing factor (T_f)	1.166 ± 0.005	0.49
Theoretical plate (N)	4546 ± 7.93	0.17

1.3 Specificity

Specificity of the developed RP-HPLC method was confirmed by analyzing blank, standard, and sample solutions. No interfering peaks from diluent or formulation excipients were observed at the retention time of chloramphenicol, indicating adequate specificity of the method for pharmaceutical analysis. Representative chromatograms of blank and standard solutions are shown in Figures 2 and 3.

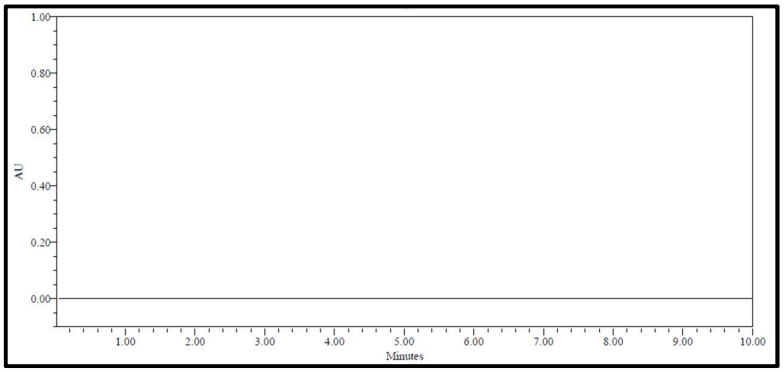

Figure 2: Chromatogram of blank in mobile phase

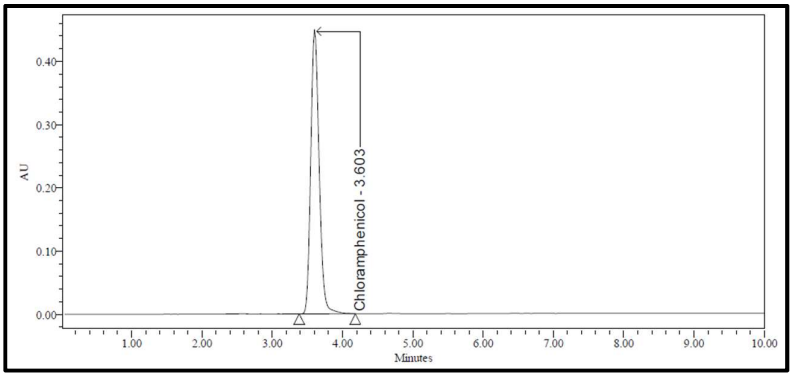

Figure 3: Chromatogram of CHL standard solution (100 µg/mL)

3.4 Linearity and range

The developed method demonstrated linear response over the concentration range of 30–200 µg/mL for chloramphenicol. Calibration data showed a correlation coefficient (R^2) of 0.999, indicating satisfactory linearity between concentration and peak area response (Table 2 and Figure 4). The low %RSD values obtained for replicate measurements further confirmed the reliability of the calibration model for quantitative analysis.

Table 2: Calibration curve data for CHL

Conc. (µg/mL)	Mean peak area ± SD (n=3)	%RSD
30	1116636 ± 2482	0.22
50	1895916 ± 3659	0.19
80	2997380 ± 1679	0.05
100	3725424 ± 7477	0.20
120	4522377 ± 3985	0.08
150	5612234 ± 5644	0.10

200	7423121 ± 2771	0.03

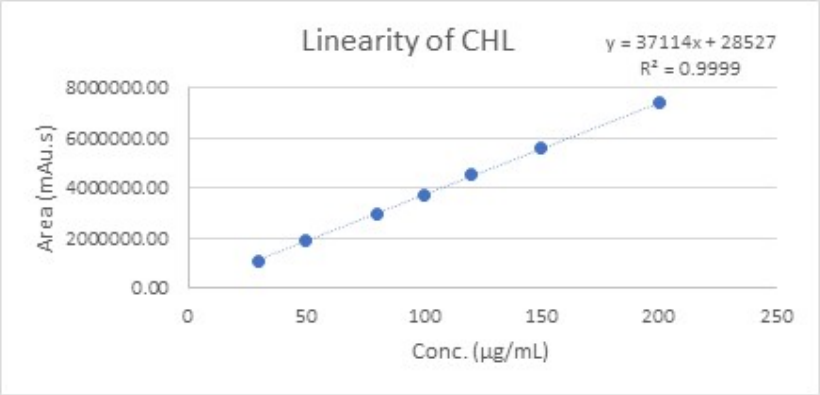

Figure 4: Calibration plot of CHL

1.4 Precision

The precision of the developed RP-HPLC method was evaluated in terms of system precision, intraday precision, and interday precision. The %RSD values obtained for all evaluated concentration levels were within acceptable limits (<2%), indicating satisfactory repeatability and reproducibility of the analytical method. The obtained results demonstrated adequate precision of the developed method for routine quantitative analysis of chloramphenicol (Tables 3 and 4).

Table 3: Repeatability data for CHL

Sr. No.	CHL	
	Conc. (µg/mL)	Peak area (mAU.s)
1	100 (µg/mL)	3716804
2		3729313
3		3730157
4		3726035
5		3710179
6		3724105
Mean	3722765.5	
SD	7796.76	
%RSD	0.20	

Table 4: Intraday precision data for CHL

API	Conc. (µg/mL)	Mean peak area (mAU.s) ± SD (n=3)	% RSD
CHL	50	1894346 ± 1477	0.07
	100	3720106 ± 8651	0.23
	150	5622868 ± 4871	0.08

Table 5: Interday precision data for CHL

API	Conc. (µg/mL)	Mean peak area (mAU.s) ± SD (n=3)	% RSD
CHL	50	1895916 ± 3659	0.19
	100	3725425 ± 7477	0.20
	150	5612234 ± 5644	0.10

1.5 Accuracy/Recovery studies

Accuracy of the developed method was evaluated by recovery studies using the standard addition technique at different concentration levels. The percentage recovery values ranged from 99.4% to 101.9%, indicating satisfactory accuracy of the analytical method for chloramphenicol quantification. The low %RSD values

obtained for recovery studies further confirmed the reliability of the developed method (Table 5).

Table 6: Recovery data for CHL

Sample	Spiking level	Percentage amount added	Amount added (mg/mL)	Amount recovered Mean ± SD (n = 3)	Percent recovery Mean ± SD (n = 3)	% RSD
CHL	30 %	30	0.3	0.303 ± 0.0011	101.05 ± 0.391	0.38
	50 %	50	0.5	0.509 ± 0.0002	101.90 ± 0.052	0.05
	100 %	100	1.0	0.994 ± 0.0026	99.43 ± 0.268	0.27
	150 %	150	1.5	1.528 ± 0.0018	101.88 ± 0.121	0.11

3.7 Detection and quantification limits

The limit of detection (LOD) and limit of quantification (LOQ) of the developed RP-HPLC method were determined based on the standard deviation of the response and slope of the calibration curve. The obtained LOD and LOQ values indicated adequate sensitivity of the method for chloramphenicol quantification at low concentration levels.

3.8 Robustness

Robustness of the developed RP-HPLC method was evaluated by introducing deliberate variations in chromatographic conditions, including changes in flow rate and column temperature. No significant variation in retention time, peak area, or chromatographic performance was observed under the modified conditions. The %RSD values remained within acceptable limits, indicating that the developed method was robust and reliable for routine pharmaceutical analysis (Table 7).

Table 7: Robustness data for CHL

Factor	Change level	Mean peak area (mAU*sec) ± SD (n=3)	% RSD
Flow rate (mL/min)	0.9	4137883 ± 3131	0.07
	1.0	3725425 ± 7477	0.20
	1.1	3384069 ± 5846	0.17
Column oven temp. (˚C)	28	3726903 ± 4339	0.11
	30	3720106 ± 8651	0.23
	32	3725076 ± 2651	0.07

3.9 Greenness of analytical procedure

The greenness profile of the developed RP-HPLC method was evaluated using AGREE, GAPI, and NEMI assessment tools. The AGREE assessment generated a greenness score of 0.79, indicating acceptable environmental performance of the developed analytical procedure according to the principles of Green Analytical Chemistry. The AGREE pictogram is presented in Figure 5.

The GAPI assessment demonstrated a favorable environmental profile for the developed method with predominantly green and yellow zones in the pictogram, reflecting simplified sample preparation, absence of derivatization steps, and comparatively reduced analytical waste generation. However, the use of methanol as the organic modifier contributed to moderate environmental impact in certain assessment zones (Figure 6).

The NEMI evaluation showed green quadrants for non-persistent/non-bioaccumulative chemicals, non-corrosive conditions, and comparatively low waste generation, while one non-green quadrant was observed due to the use of methanol in the mobile phase (Figure 7).

Comparative evaluation with previously reported HPLC methods demonstrated that the developed RP-HPLC method offered shorter analysis time, lower flow rate, reduced solvent consumption, and simplified chromatographic conditions with systematic greenness assessment using AGREE, GAPI, and NEMI tools (Table 8). These findings indicate that the proposed method possesses an improved greenness profile suitable for routine pharmaceutical quality control analysis.

Figure 5: Pictogram for Greenness assessment of the developed HPLC method using AGREE tool

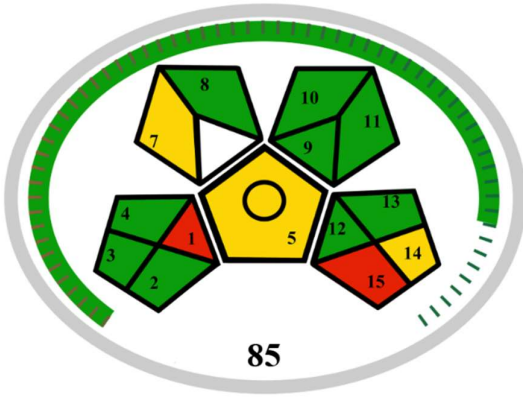

Figure 6: GAPI pictogram representing the greenness profile of the developed RP-HPLC method for chloramphenicol analysis

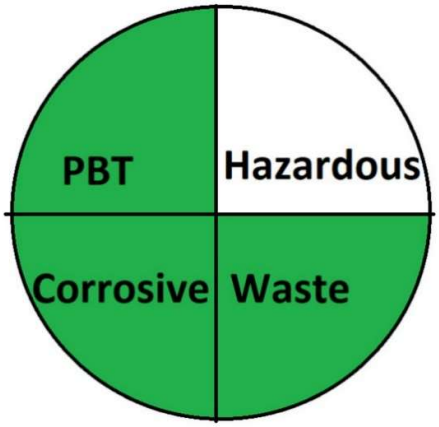

Figure 7: NEMI pictogram representing the environmental profile of the developed RP-HPLC method.

Table 8: Comparative evaluation of reported HPLC methods and the developed RP-HPLC method for chloramphenicol analysis

Parameter	Reported Method[10]	Developed Method
Mobile phase	Sodium pentanesulfonate: Acetonitrile: Acetic acid (85:15:1)	Methanol: Water (70:30, v/v)
Buffer/Ion-pair reagent	Present	Absent
Flow rate	2.0 mL/min	1.0 mL/min
Run time	10 min	~3.6 min

Sample preparation	Multiple dilution steps	Simple dissolution and dilution
Solvent consumption	Higher	Comparatively reduced
Greenness assessment	Not reported	AGREE, GAPI, and NEMI evaluated
Environmental profile	Conventional HPLC method	Comparatively improved greenness profile
Applicability	Pharmaceutical formulations	Pharmaceutical formulations

3.10 Application of developed method

The validated method was applied to marketed CHL dosage forms, including capsules, eye drops, and ointments. The assay results were 98.59% for capsules, 95.94% for eye ointments, and 98.80% for eye drops, all within the acceptable pharmaceutical limits of 95–105%. These findings confirm the suitability of the method for routine quality control, consistent with earlier reports of CHL quantification in dosage forms [7,8]. The assay results showed low variability (%RSD < 2), confirming suitability for routine quality control.

2. CONCLUSION

A simple, reliable, and comparatively greener RP-HPLC method was developed and validated for the determination of chloramphenicol in pharmaceutical formulations. The developed method demonstrated satisfactory specificity, linearity, precision, accuracy, robustness, and sensitivity in accordance with ICH Q2(R1) guidelines. Greenness assessment using AGREE, GAPI, and NEMI tools indicated acceptable environmental performance of the analytical procedure with comparatively reduced solvent consumption and analytical waste generation. Comparative evaluation with previously reported HPLC methods further demonstrated the improved greenness profile and simplified chromatographic conditions of the developed method. The proposed RP-HPLC method was successfully applied for routine quantitative analysis of chloramphenicol in marketed pharmaceutical formulations and may be considered suitable for pharmaceutical quality control applications.

Abbreviations:

- CHL – Chloramphenicol
- HPLC – High-Performance Liquid Chromatography
- RP-HPLC – Reverse Phase High-Performance Liquid Chromatography
- GAC – Green Analytical Chemistry
- ICH – International Council for Harmonisation
- UV – Ultraviolet
- LOD – Limit of Detection
- LOQ – Limit of Quantification
- R^2 – Correlation Coefficient
- SD – Standard Deviation
- %RSD – Percentage Relative Standard Deviation
- SST – System Suitability Testing
- AGREE – Analytical GREEnness Metric Approach
- DAD – Diode Array Detector
- VOCs – Volatile Organic Compounds

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AUTHORS' CONTRIBUTIONS
All authors contributed equally to the research work and manuscript preparation.

CONFLICT OF INTERESTS
Declared none.

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