Research Article

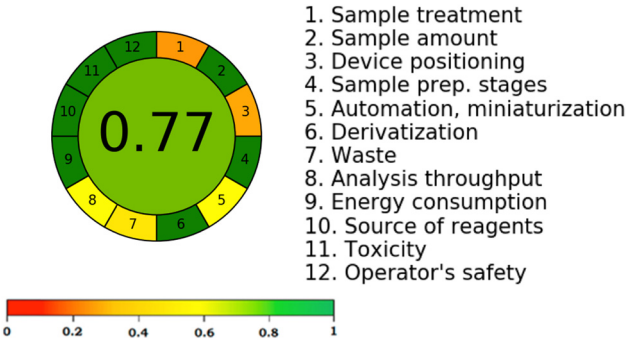
Norah Alanazi, Nazrul Haq, Ibrahim A. Alsarra, Faiyaz Shakeel*

A fast, sensitive, greener, and stability-indicating HPLC method for the standardization and quantitative determination of chlorhexidine acetate in commercial products

https://doi.org/10.1515/chem-2024-0079
received April 20, 2024; accepted August 4, 2024

Abstract: The goal of the proposed work was to create and verify a fast, sensitive, greener, and stability-indicating high-performance liquid chromatography (HPLC) method to quantify chlorhexidine acetate (CHDA) in commercial products. The developed method was validated for numerous validation metrics and greenness. The greener mobile phase was made up of a ternary mixture of ethanol, water, and glacial acetic acid (50:49:1 v/v/v). CHDA was detected at a wavelength of 265 nm. The developed HPLC method showed a coefficient of determination of 0.9981 and was linear in the 1–100 µg/mL range. In addition, the developed method for CHDA analysis was rapid, accurate, precise, robust, and sensitive. The outstanding greenness profile was indicated by the derived values of the Analytical Eco-Scale, ChlorTox, and AGREE scales for the current approach, which are 89, 0.74 g, and 0.77, respectively. With its breakdown products present, the proposed analytical approach was still able to identify CHDA, demonstrating its selectivity- and stability-indicating qualities. Two distinct commercial products, A and B, were found to contain 1.96 and 2.05% w/v of CHDA, respectively. These results revealed that CHDA in commercially accessible products can be routinely standardized and quantified using the proposed HPLC approach.

Keywords: chlorhexidine acetate, greener HPLC, greenness tools, quantitative determination, standardization, validation



Graphical abstract

1 Introduction

Chemical compounds known as antiseptics are employed to either eradicate or impede the growth and reproduction of germs [1]. Belonging to the biguanide class, chlorhexidine (CHD) is a highly effective cationic antimicrobial [2,3]. Because it dissolves poorly in water, it is employed in commercial formulations in a variety of salt forms, including acetate, gluconate, digluconate, and hydrochloride [3,4]. In topical commercial products like hand and mouth wash, it is the most commonly used cationic antiseptic [3–7]. The molecular structure of chlorhexidine acetate (CHDA) is presented in Figure 1. The pharmaceutical industries place great importance on the development and validation of analytical procedures to standardize and manage the quality of the active components in commercial products [8]. The presence of CHDA in a number of commercially available mouthwashes and hand washes makes both its qualitative and quantitative standardization crucial.

Numerous analytical approaches have been used in the literature to standardize and quantify CHD and its many salts in biological samples and commercially accessible products. To evaluate CHD sorption in soft contact lenses, high-performance liquid chromatography (HPLC) was used [9]. There have also been reports of stability-indicating HPLC techniques for the detection of CHD and its breakdown

* **Corresponding author: Faiyaz Shakeel**, Department of Pharmaceutics, College of Pharmacy, King Saud University, P. O. Box 2457, Riyadh 11451, Saudi Arabia, e-mail: fsahmad@ksu.edu.sa
Norah Alanazi: Quality Control in Pharmacy Unit, Department of Pharmaceutics, College of Pharmacy, King Saud University, P. O. Box 2457, Riyadh 11451, Saudi Arabia
Nazrul Haq, Ibrahim A. Alsarra: Department of Pharmaceutics, College of Pharmacy, King Saud University, P. O. Box 2457, Riyadh 11451, Saudi Arabia

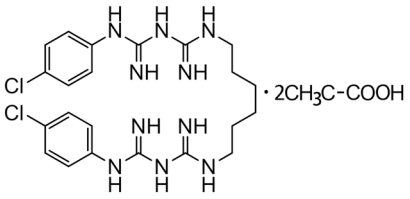


Figure 1: Molecular structure of CHDA.

products [10–12]. The most popular approach for analyzing CHD and its many salts in distinct medicinal products is the HPLC method [10,13–18]. Numerous physiological fluids, including human urine, serum, and saliva, have been reported to be tested for the presence of CHD and its salts using a variety of HPLC techniques [19–27]. A method for measuring CHD in human hemolyzed blood utilizing liquid chromatography–electrospray ionization-tandem mass spectrometry has also been disclosed [28]. There have also been reports of the greener high-performance thin-layer chromatography (HPTLC) technology being used to quantify CHDA in four distinct commercial products [29]. Several other techniques, including spectrometry [30], fluorescence spectroscopy [31], and solid-phase microextraction [32], have also been proven to be effective in identifying CHD in human saliva samples. There have also been reports of other techniques for determining CHD in its commercial products, including gas chromatography [33], solid-phase extraction [34], capillary electrophoresis [35], flow-injection spectrometry [36,37], voltammetry [38], and agar diffusion [7].

Most of the documented methods have a number of shortcomings, like limited sensitivity, long analysis times, difficult extraction and sample preparation procedures, and a greater use of hazardous and toxic eluents such as acetonitrile or methanol. Despite this, the aforementioned techniques are capable of identifying and quantifying CHD and its salts. These solvents were subjected to several treatments before being released into the environment. The use of substitute green or environmentally friendly solvents to reduce the harmful effects of hazardous solvents on the environment is emphasized in one of the 12 principles of green analytical chemistry [39]. The usage of greener and more ecologically friendly solvents has skyrocketed in the last 10 years, according to a review of the literature [40–42]. Analytical assays' environmental friendliness or greenness profile has been evaluated using a variety of methods [43–51]. This study evaluated the greenness profile of the proposed HPLC methodology utilizing three different tools: the Analytical Eco-Scale (AES) [46], ChlorTox [50], and the Analytical GREEnness (AGREE) [51] methods. According to published HPLC procedures, CHD and its salts cannot be measured to produce a greenness profile or index. Several analytical methods have been reported for the analysis of CHDA as a single analyte. However, greener HPLC methods have not been reported for the determination of CHDA as a single analyte in pharmaceutical products or biological materials. In view of this, the goal of this study was to use an isocratic stability-indicating HPLC coupled with a UV–Vis detector to create and validate a fast, sensitive, greener, and stability-indicating methodology to standardize and quantify CHDA in its commercial mouthwashes. The AES, ChlorTox, and AGREE methodologies were also used to determine the greenness profile of the developed method. Using the "International Council for Harmonization (ICH)-Q2-R2" recommendations, the proposed method for CHDA detection was validated [52].

2 Materials and methods

2.1 Chemicals and reagents

Santa Cruz Biotechnology (Dallas, TX, USA) provided the CHDA (purity: 99.2%) working standard. Fischer Scientific UK Ltd. (Loughborough, UK) provided HPLC-grade solvents, including ethanol (EtOH), ethyl acetate (EA), and acetone (Ace). Using the Milli-Q® device (Millipore, Lyon, France), highly pure deionized water (H_2O) was produced. The source of glacial acetic acid (GAA) was E-Merck (Darmstadt, Germany). The other reagents and compounds were AR graded. The commercial CHDA mouthwashes A and B were bought at Lulu Hypermarket in Riyadh, Saudi Arabia, and each contained 2% w/v of CHDA.

2.2 Chromatography and analysis

The CHDA was quantified at an ambient temperature (25°C) using the "Waters HPLC system (Waters, Milford, MA, USA)," which included a column oven, an SCL 10AVP system controller, an inline vacuum degasser, a UV–visible wavelength detector, a 1515 isocratic pump, and 717 autosampler. To handle and interpret the data, "Millennium programmer (version 32, Milford, MA, USA)" was used. CHDA was measured using a Nucleodur (150 mm × 4.6 mm, 5 µm particle size) RP C18 analytical column. An environmentally friendly mobile phase comprising 50:49:1% v/v/v of EtOH, H_2O, and GAA was used. The pumping rate for the greener mobile phase was 1 mL/min. It was found that CHDA had a wavelength of 265 nm. Each sample was injected with a 20-µL injection volume with the help of a Waters autosampler.

2.3 CHDA calibration curve

The necessary amount of CHDA was dissolved using a greener eluent system (EtOH–H_2O–GAA, 50:49:1% v/v/v) to create a CHDA stock solution with a concentration of 100 µg/mL. The required aliquots from the CHDA stock solution (100 µg/mL) were diluted using the greener eluent systems to produce the serial dilutions in the needed range of 1–100 µg/mL. The peak area of each CHDA solution was recorded utilizing the present HPLC technique. Ten distinct CHDA concentrations (1, 10, 20, 30, 40, 50, 60, 70, 80, and 100 µg/mL) were plotted against the observed peak area to generate the CHDA calibration curve. Every sample preparation and experiment were repeated three times (n = 3).

2.4 Samples preparation for CHDA analysis in commercial products

Each commercial mouthwash (25 mL), A and B, containing 2% w/v of CHDA, was transferred individually to a 100-mL volumetric flask and diluted with 25 mL of the greener mobile phase to standardize and quantify the amount of CHDA in each. The solutions of both products were sonicated for 15 min and shaken vigorously for another 15 min. The volume of both products was adjusted again using the greener mobile phase. Both products underwent a 10-min centrifugation at 4,000 rpm, after which the supernatants were removed and filtered through a 0.45 µm Millipore membrane filter. The CHDA concentrations were then quantified using the suggested HPLC method [15].

2.5 Development of HPLC method

A variety of blends of green solvents were investigated as the greener mobile phase solutions to develop an accurate stability-indicating greener HPLC method for the standardization and quantitative analysis of CHDA in commercially available products. Some of the greener solvent compositions that were investigated included EtOH–H_2O, EtOH–EA, EtOH–Ace, EA–Ace, EtOH–GAA, EA–GAA, EtOH–H_2O–GAA, EtOH–EA–GAA, EtOH–Ace–GAA, and EA–Ace–GAA. Numerous considerations had to be made to determine the greenest solvent system: the cost of the solvents, their toxicity and greenness, their compatibility, the length of the analysis, the chromatographic parameters, and their sensitivity. Many green solvent combinations were therefore considered to be used as the mobile phases. Ultimately, it was decided that the ideal eluent system for further research would be a ternary mixture of EtOH, H_2O, and GAA (50:49:1% v/v/v).

2.6 Validation parameters

Through the use of ICH-Q2-R2 standards, many parameters for the proposed approach for measuring CHDA were validated [52]. By graphing the linearity, the proposed method's linearity was assessed between 1 and 100 µg/mL. Following the administration of three copies (n = 3) of freshly made CHDA solutions to the system, the peak area was assessed. Peak area vs CHDA concentration was used to create the CHDA calibration curve.

At the target concentration of 10 µg/mL for CHDA, the system suitability criteria for the environmentally friendly HPLC technique were computed. As the "capacity factor (k), retention time (R_t), tailing factor (S), and theoretical plate number (N)" [53,54], these parameters were established.

The accuracy of the suggested CHDA analytical procedure was evaluated using the spiking/standard addition methodology in terms of percentage recoveries [52]. To establish low-quality control (LQC) levels of CHDA of 15 µg/mL, middle-quality control (MQC) levels of 20 µg/mL, and high-quality control (HQC) levels of 25 µg/mL, the previously measured CHDA solution (10 µg/mL) was spiked with additional 50, 100, and 150% CHDA concentrations. Three replicates (n = 3) were used to calculate the % recovery at each QC level.

Through the computation of intra- (repeatability) and inter-day (reproducibility) variances as a percentage of relative standard deviation (% RSD), the precision of the suggested CHDA analysis method was assessed. To evaluate the intra-day precision (repeatability), three replicates of the CHDA solutions – LQC (15 µg/mL), MQC (20 µg/mL), and HQC (25 µg/mL) – were utilized on the same day. The inter-day precision (reproducibility) was evaluated using the same CHDA QC levels for 3 days in a row. Both precisions were evaluated in triplicate (n = 3) and expressed as % RSD [52].

The robustness of the suggested HPLC technique was examined to determine how deliberate adjustments affected the CHDA chromatographic responses. To gauge robustness, the MQC level of CHDA (20 µg/mL) was chosen. Robustness was evaluated by adjusting the detection wavelength, flow rate, and composition of the greener mobile phase. When the original EtOH−H_2O−GAA (50:49:1 v/v/v) eluent system was substituted with EtOH−H_2O−GAA (52:47:1 v/v/v) and EtOH−H_2O−GAA (48:51:1 v/v/v), the variations in peak area (quantitative parameter) and R_t (separation parameter)

were recorded. The initial flow rate of 1 mL/min was changed to 1.10 and 0.90 mL/min to evaluate robustness. Changes in R_t and peak area were observed. When detection wavelengths of 270 and 260 nm were used in place of the original (265 nm) detection wavelength, changes in peak area and R_t were observed to assess robustness [52].

The proposed HPLC technology's sensitivity was evaluated utilizing the standard deviation methodology, and the results were reported as "limit of detection (LOD) and limit of quantitation (LOQ)." After injecting the blank sample, the peak area's standard deviation was computed in terms of triplicates (n = 3). CHDA "LOD and LOQ" were calculated with the help of the following equations (n = 3) [52]:

$$\text{LOD} = \frac{3.3 \times \sigma}{\text{slope}}, \tag{1}$$

$$\text{LOQ} = \frac{10 \times \sigma}{\text{slope}}, \tag{2}$$

where σ is the standard deviation of the intercept and slope is for the CHDA calibration curve.

2.7 Evaluation of stability-indicating properties and selectivity using forced degradation investigations

To evaluate the selectivity and stability-indicating qualities of the proposed HPLC method, forced-degradation studies under various stress settings, such as acidic (HCl), base (NaOH), oxidative (H_2O_2), and thermal stress conditions, were carried out [55,56]. In these studies, the eluent system was fed freshly produced CHDA at the MQC level (20 µg/mL). An aliquot (1 mL) of this solution was added to 4 mL of 1 M HCl or 4 mL of 1 M NaOH to execute acid or base hydrolysis. Before being evaluated using the recommended HPLC method, these combinations were refluxed for 48 h at 60°C to identify CHDA in the presence of its acid- and base-degradation products, respectively [55].

For oxidative degradation tests, freshly produced CHDA at the MQC level (20 µg/mL) was added to the eluent system. This solution underwent oxidative degradation when 1 mL of it was combined with 4 mL of 30% H_2O_2. Prior to being subjected to a greener HPLC method of analysis to detect CHDA in the presence of its oxidative-degradation products, this combination was refluxed for 48 h at 60°C [55].

An eluent system was used to dilute the MQC level of CHDA (20 µg/mL) from 1 to 5 mL to do thermal degradation studies. It was next assessed for CHDA detection using the proposed HPLC approach when its thermal-degradation products were found [55,56].

2.8 Greenness evaluation

The greener HPLC method to measure CHDA was evaluated for its greenness profile using three distinct approaches: AES [46], ChlorTox [50], and AGREE [51]. AES is a semi-quantitative method that takes into account all of the analytical steps, waste, and tools. A perfect analysis with 100 points is anticipated for the solvents/reagents that need little to no reagent use, low energy, and no waste. If any of these requirements are broken, penalty points are given and subtracted from the total of 100 [46]. The ChlorTox scale is determined using equation (3) [50] in accordance with the ChlorTox scale approach.

$$\text{ChlorTox} = \frac{\text{CH}_{\text{sub}}}{\text{CH}_{\text{CHCl3}}} \times m_{\text{sub}}. \tag{3}$$

The chemical hazards of standard chloroform are represented by CH_{CHCl3}, the mass of the substance of interest required for a single analysis is indicated by m_{sub}, and the chemical risks of the substance of interest required for a single analysis are indicated by CH_{sub}. The safety data sheet provided by "Sigma Aldrich (St. Louis, MO, USA)" was used to help in the computation of the values of CH_{sub} and CH_{CHCl3} using the "weighted hazards number" model [50]. The AGREE-metric approach was used to evaluate the AGREE scale for the greener HPLC method for the analysis of CHDA [51]. The AGREE scales for the greener HPLC method were determined using the "AGREE: The Analytical Greenness Calculator (version 0.5, Gdansk University of Technology, Gdansk, Poland, 2020)". The values, which were determined by 12 distinct green analytical chemistry principles, varied from 0.0 to 1.0.

2.9 Application of greener HPLC method in analysis of CHDA in commercial products

Following the processing of samples for commercial products A and B and their injection into the apparatus in three repetitions (n = 3), the chromatographic responses were recorded. Using the CHDA calibration curve, the amount of CHDA in commercial products A and B was calculated. Additionally, investigated was the possibility of influence by commercial product excipients.

3 Results and discussion

3.1 Development of HPLC method

The combination of several greener mobile phases and the recorded chromatographic parameters are mentioned in Table 1.

Table 1: The optimization of greener solvent systems and recorded chromatographic parameters for CHDA (mean ± SD, n = 3)[a]

Greener eluent system	*S*	*N*	R_t
EtOH–H_2O (50:50 v/v)	1.21 ± 0.05	2,781 ± 3.34	2.97 ± 0.03
EtOH–EA (50:50 v/v)	1.41 ± 0.07	1,279 ± 2.23	3.69 ± 0.06
EtOH–Ace (50:50 v/v)	1.67 ± 0.12	1,152 ± 1.91	5.16 ± 0.16
EA–Ace (50:50 v/v)	1.83 ± 0.17	1,024 ± 1.84	5.68 ± 0.19
EtOH–GAA (99:1 v/v)	1.28 ± 0.07	2,456 ± 2.13	5.34 ± 0.13
EA–GAA (99:1 v/v)	1.53 ± 0.13	1,078 ± 1.62	5.92 ± 0.18
EtOH–H_2O–GAA (50:49:1 v/v/v)	1.10 ± 0.04	4,876 ± 5.41	2.81 ± 0.02
EtOH–EA–GAA (50:49:1 v/v/v)	1.34 ± 0.06	2,574 ± 3.16	3.46 ± 0.05
EtOH–Ace–GAA (50:49:1 v/v/v)	1.47 ± 0.08	1,722 ± 2.58	3.89 ± 0.07
EA–Ace–GAA (50:49:1 v/v/v)	1.72 ± 0.11	1,257 ± 1.97	4.32 ± 0.09

[a]R_t: retention time, *S*: peak asymmetry factor, *N*: number of theoretical plates, EtOH: ethanol, H_2O: water, EA: ethyl acetate, Ace: acetone, GAA: glacial acetic acid.

The chromatographic response of CHDA was below par because EtOH–H_2O (50:50 v/v), EtOH–EA (50:50 v/v), EtOH–Ace (50:50 v/v), and EA–Ace (50:50 v/v) were utilized during the HPLC method development. Low *N* values (*N* = 1,024–2,781) and higher *S* values (*S* = 1.21–1.83) were observed by CHDA. Furthermore, when EtOH–GAA (99:1 v/v) and EA–GAA (99:1 v/v) were used, CHDA's chromatographic response was subpar, exhibiting higher *S* values (*S* = 1.28–1.53) and lower *N* values (*N* = 1,078–2,456). The chromatographic parameters were improved when the ternary combination of EtOH–H_2O–GAA (50:49:1 v/v/v), EtOH–EA–GAA (50:49:1 v/v/v), EtOH–Ace–GAA (50:49:1 v/v/v), and EA–Ace–GAA (50:49:1 v/v/v) was studied as the greener mobile phases compared to their binary combinations. Nevertheless, a well-resolved and intact CHDA chromatographic peak with a decent *S* value and higher *N* values was shown by the ternary combination of EtOH, H_2O, and GAA (50:49:1 v/v/v), which also produced a respectable R_t and *S* and *N* values (Figure 2). It was consequently determined that a ternary mixture of EtOH, H_2O, and GAA (50:49:1 v/v/v) would be the final greener mobile phase for the standardization and quantitative analysis of CHDA with an appropriate *S* (1.10) and *N* (4,876), rapid analysis (R_t = 2.81 min), and a fast analysis duration (6 min).

3.2 Validation assessment

Using the ICH-Q2-R2 recommendations, a number of parameters were determined for the validation of the greener HPLC method [52]. The linearity curves were created using the freshly produced CHDA solution, which ranged from 01 to 100 µg/mL. Table 2 displays the outcomes of a linear regression analysis of the CHDA calibration plot. The

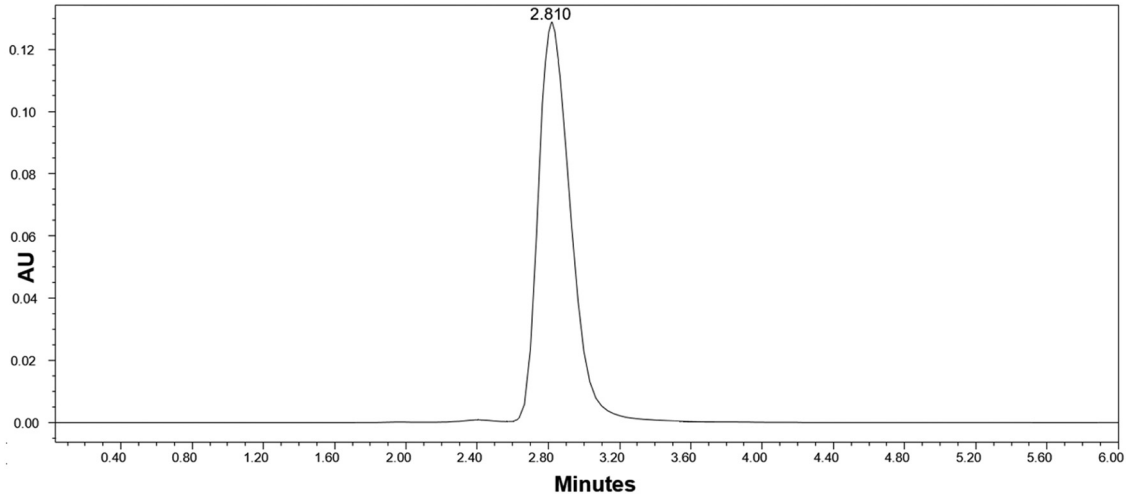


Figure 2: A representative chromatogram of CHDA (10 µg/mL concentration) derived by EtOH:H_2O:GAA (50:49:1 v/v/v) greener solvent system.

Table 2: Findings of linear regression analysis of the CHDA calibration curve for the developed HPLC method (mean ± SD, n = 3)

Parameters	Values
Linearity range (μg/mL)	1–100
Regression equation	y = 24,204x − 51,219
R^2	0.9981
R	0.9990
Standard error of slope	37.14
Standard error of intercept	79.92
95% confidence interval of slope	24,044–24,363
95% confidence interval of intercept	50,875–51,562
LOD (ng/mL)	18.87 ± 0.84
LOQ (ng/mL)	56.62 ± 2.52

Table 3: Recorded system suitability values of CHDA for the developed HPLC method (mean ± SD, n = 3)

Parameter	Recorded value	Reference value	Ref.
R_t (minutes)	2.81 ± 0.02	>1	[52]
S	1.10 ± 0.04	0.80–1.15	[52]
k	2.64 ± 0.09	>2	[52]
N	4,876 ± 5.41	>2,000	[52]

CHDA calibration plot was linear and ranged from 1 to 100 μg/mL. The findings demonstrate a strong relationship between the concentrations of CHDA and the observed responses, with correlation coefficient (R) and coefficient of determination (R^2) values on the calibration curve of 0.9990 and 0.9981, respectively. These results demonstrated the linear nature of the currently employed CHDA assessment approach.

The system appropriateness criteria for the current technique were ascertained by utilizing the R_t, S, k, and N. The values of R_t, S, k, and N along with their reference values are presented in Table 3. R_t, S, k, and N using the current approach were determined to be 2.81 min, 1.10, 2.64, and 4,876, respectively. For the purpose of measuring CHDA, the values were appropriate and satisfying as mentioned in Table 3.

Table 4: Accuracy results of CHDA for the developed HPLC method (mean ± SD; n = 3)

Conc. (μg/mL)	Conc. found (μg/mL) ± SD	Recovery (%)	RSD (%)
15	14.76 ± 0.16	98.40	1.08
20	20.34 ± 0.18	101.70	0.88
25	24.79 ± 0.20	99.16	0.80

To gauge the accuracy of the greener HPLC process, the extra spiked recovered samples at three different concentrations were calculated. Table 4 contains the findings. It was shown that the percentage recoveries of CHDA at three distinct QC levels were 98.40–101.70%. The accuracy of the present CHDA measurement approach is demonstrated by the high recovery rates.

The intra-day and inter-day precision results, reported as % RSD, are shown in Table 5. It was shown that the intraday precision % RSDs for CHDA vary from 0.67 to 0.85%. Conversely, the inter-day precision percentage RSDs ranged from 0.77 to 0.97%. The current CHDA measurement process demonstrated its precision with low percentage RSDs.

Table 6 displays the findings of the robustness study at the CHDA MQC level (20 μg/mL). To assess robustness, the composition of the greener mobile phase was changed. The resulting percentage RSD and R_t were determined to be 1.07–1.29% and 2.79–2.83 min, respectively. The results of a robustness investigation involving a change in flow speed indicated that the percentage RSD and R_t were, respectively, 0.94–1.29% and 2.61–3.14 min. The percentage RSD and R_t in the case of a robustness examination by changing the detecting wavelength were found to be 1.11–1.30% and 2.80–2.82 min, respectively. The robustness of the current methodology for assessing CHDA is indicated by low RSDs and limited R_t value variations.

The findings of assessing the sensitivity of the current method in terms of “LOD and LOQ” are shown in Table 2. Based on the findings, the current method’s “LOD and LOQ” are 18.87 ± 0.84 and 56.62 ± 2.52 ng/mL, respectively.

Table 5: Intra-day and inter-day precision results of CHDA for the developed HPLC method (mean ± SD; n = 3)

Conc. (μg/mL)	Intra-day precision			Inter-day precision		
	Conc. found (μg/mL) ± SD	SE	RSD (%)	Conc. found (μg/mL) ± SD	SE	RSD (%)
15	15.26 ± 0.13	0.07	0.85	15.32 ± 0.15	0.08	0.97
20	19.51 ± 0.15	0.08	0.76	20.23 ± 0.17	0.09	0.84
25	25.13 ± 0.17	0.09	0.67	24.62 ± 0.19	0.10	0.77

Table 6: Robustness results of CHDA for the developed HPLC method (mean ± SD; n = 3)

Parameters	Conc. found (µg/mL) ± SD	RSD (%)	R_t ± SD	RSD (%)
Eluent system (EtOH–H_2O–GAA, % v/v/v)				
(52:47:1)	19.56 ± 0.21	1.07	2.79 ± 0.04	1.43
(48:51:1)	20.13 ± 0.26	1.29	2.83 ± 0.05	1.76
Flow speed (mL/min)				
(1.10)	21.64 ± 0.28	1.29	2.61 ± 0.03	1.14
(0.90)	18.97 ± 0.18	0.94	3.14 ± 0.06	1.91
Measurement wavelength (nm)				
260	19.71 ± 0.22	1.11	2.80 ± 0.04	1.42
270	20.76 ± 0.27	1.30	2.82 ± 0.05	1.77

These results showed that CHDA could be identified and quantified in a range of concentrations with greater sensitivity utilizing the current method.

3.3 Evaluation of stability-indicating properties and selectivity using forced degradation investigations

The current methodology's selectivity and stability-indicating capabilities were examined through the exposure of CHDA at its MQC level (20 µg/mL) to a range of stress scenarios. An overview of the selectivity results obtained under different settings using the current approaches is presented in Figure 3 and Table 7. In addition to well-separated CHDA peaks, the chromatograms of the forced degradation studies revealed multiple additional peaks of degradation products (Figure 3). Under conditions of acid stress, 49.30% of CHDA was recovered and 50.70% degraded (Table 7). Consequently, it was determined that CHDA was unstable in the presence of acidic degradations. A minor shift in the R_t value (R_t = 2.84 min) was observed for CHDA under acid stress (Figure 3a). Table 7 shows that only 33.70% of CHDA was recovered during alkali-stress degradations, whereas 67.30% was broken down. As a result, it was shown that CHDA was extremely unstable in alkali degradation conditions. Following alkali-stress degradation, the R_t value of CHDA changed little (R_t = 2.83 min) (Figure 3b). It was discovered that 51.30% of CHDA was recovered and 48.70% was destroyed under oxidative hydrolysis. It was proven as a result that CHDA was sufficiently unstable during the degradation caused by oxidative stress. Additionally, following oxidative hydrolysis, there was a minor change in

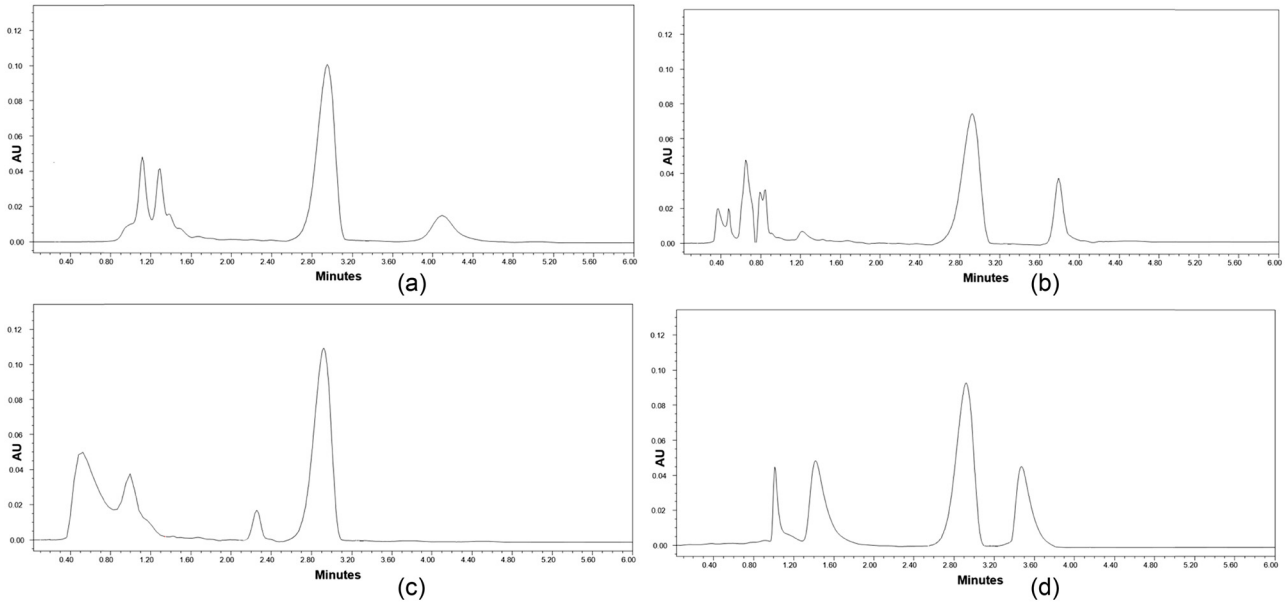


Figure 3: The representative chromatograms of CHDA recorded under (a) acid (b) base, (c) oxidative, and (d) thermal degradation of CHDA.

Table 7: Results of the current method's forced-degradation study of CHDA at MQC (20 µg/mL) under various stress scenarios (mean ± SD; n = 3)

Stress condition	CHDA R_t (min)	CHDA remained (µg/mL)	CHDA recovered (%)
1 M HCl	2.84	9.86	49.30 ± 1.41
1 M NaOH	2.83	6.74	33.70 ± 1.38
30% H_2O_2	2.84	10.26	51.30 ± 1.52
Thermal	2.84	8.05	40.25 ± 1.40

CHDA R_t value (R_t = 2.84 min) (Figure 3c). Of the CHDA, 59.75% degraded and 40.25% remained after heat degradation. Consequently, it was found that in the event of heat degradation, CHDA was likewise highly unstable. Furthermore, under thermal deterioration, there was a minor change in CHDA R_t value (R_t = 2.84 min) (Figure 3d). Alkali degradation was shown to exhibit the highest rate of CHDA degradation overall. It was discovered that the CHDA degradation patterns agreed with those previously published in the literature [13]. Since the current methodology can detect CHDA when its degradation products are available, it facilitates stability-indicating features. Overall, our findings supported the existing technique's selectivity and stability-signaling capacities.

3.4 Greenness evaluation

The established analytical approaches' eco-friendliness is assessed using a variety of greenness tools. The current investigation evaluated the greenness of the current HPLC method using three different methods: AES [46], ChlorTox [50], and AGREE [51]. Table 8 shows the outcomes of AES scales with penalty points for the current HPLC approach. AES scores above 75 indicated good greenness, below 75 but above 50 indicated sufficient greenness, and below 50 showed inadequate greenness [46]. The AES scale for the current HPLC procedure was found to be 89. Additionally, we calculated the AES scales of two HPLC methods found in the literature and compared them to the current HPLC method used for CHDA assessment (Table 8). AES scales for two literature HPLC methods were derived to be 67 and 71, respectively [14,18]. Both of the previously reported HPLC methods were shown to be much less effective than the HPLC approach currently in use for CHDA assessment, based on AES scales [14,18].

Table 8: Evaluation of the greenness of the current method using penalty points and the AES, and its comparison to published HPLC methods

Reagents/instruments/ waste	Penalty points		
	HPLC [14]	HPLC [18]	Present HPLC
EtOH		4	4
H_2O		0	0
GAA			2
Acetonitrile	12		
Formic acid (1%)	12		
Phosphate buffer	0	0	
Methanol		18	
Triethylamine		6	
Instruments	0	0	0
Waste	5	5	5
Total penalty points	29	33	11
AES scale	71	67	89

The overall ChlorTox and solvent-specific ChlorTox scale results for the current HPLC approach are shown in Table 9 in comparison to previously published HPLC

Table 9: Results of the ChlorTox scales for the present method compared to reported HPLC methods in terms of the relative dangers concerning chloroform (CH_{sub}/CH_{CHCl3}) calculated using the weighted hazards number model

Stage	Solvent/reagent	Relative hazard (CH_{sub}/CH_{CHCl3})	m_{sub} (mg)	ChlorTox (g)	Total ChlorTox (g)	Ref.
Sample preparation	EtOH	0.26	394	0.10	0.74	Present HPLC
	GAA	0.43	10	0.00		
HPTLC analysis	EtOH	0.26	2,367	0.61		
	GAA	0.43	63	0.03		
Sample preparation	Acetonitrile	0.39	157	0.06	1.16	[14]
	Formic acid (1%)	0.56	10	0.01		
HPLC analysis	Acetonitrile	0.39	2,751	1.07		
	Triethylamine (0.5%)	0.78	24	0.02		
Sample preparation	EtOH	0.26	394	0.10	5.44	[18]
HPLC analysis	Methanol	0.56	9,504	5.32		
	Triethylamine (0.4%)	0.78	23	0.02		

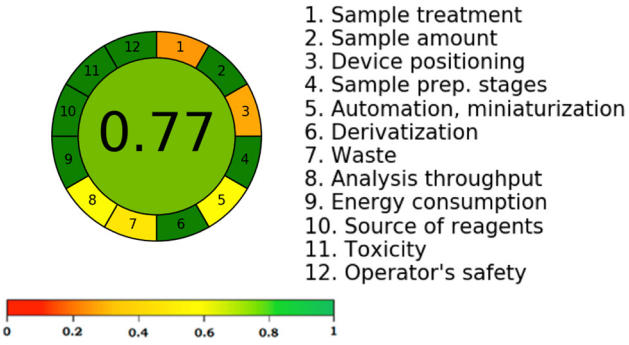


Figure 4: AGREE scale for the present HPLC method of CHDA determination obtained by AGREE calculator.

methods. The current HPLC method's estimated total ChlorTox scale was 0.74 g, indicating that it was environmentally benign and safe [50]. Additionally, we calculated the ChlorTox scales for two literature HPLC methods and compared them to the current HPLC method used currently for CHDA assessment (Table 9). It was found that the ChlorTox scales for two literature HPLC methods were 1.16 and 5.44 g, respectively [14,18]. Based on ChlorTox scales, it was inferred that both literature HPLC methods were significantly less effective than the current HPLC method [14,18].

The most popular quantitative approach for assessing greenness is the AGREE methodology, which considers all 12 green analytical chemistry criteria [51]. Figure 4 displays the overall AGREE scale for the current HPLC technique. The present HPLC method measured the total AGREE scale of 0.77. The outstanding greenness profile for the present HPLC method of CHDA analysis was once again demonstrated by the AGREE results. Overall greenness approaches compared to literature HPLC methods show that the current HPLC method for CHDA assessment in commercial products has an excellent greenness profile.

3.5 Standardization and quantitative determination of CHDA in commercial products

It has been demonstrated that the current CHDA HPLC method is sensitive, fast, and efficient. To ascertain CHDA in its commercial products A and B, this approach was used. The CHDA calibration curve for the greener stability-indicating HPLC method was used to determine the CHDA contents of two distinct commercial products. The representative chromatograms of CHDA in commercial products A and B are shown in Figure 5. Figure 5 shows a single chromatographic peak of CHDA in commercial product A (Figure 5a) and product B (Figure 5b), indicating that there was no interaction between CHDA and formulation excipients. Using the greener stability-indicating HPLC technique, the CHDA concentrations of two distinct commercial products, A and B, were found to be 1.96% w/v (label claim 2.00% w/v) and 2.05% w/v (label claim 2.00% w/v), respectively. These findings suggested that the greener stability-indicating HPLC approach might be used to determine and standardize the CHDA in commercially available CHDA products.

4 Conclusion

There are no documented greener HPLC techniques available for the standardization and quantitative determination of the CHDA content of its commercial products. Hence, to standardize and determine CHDA in its commercial formulations, this work established and validated an HPLC methodology that is fast, sensitive, environmentally friendly, and stability-indicating. The new HPLC technique was validated using the ICH-Q2-R2 recommendations. The

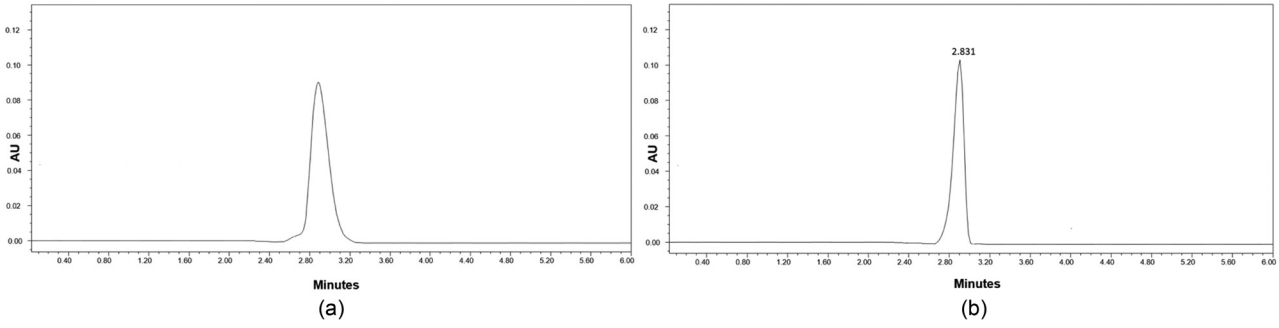


Figure 5: The representative chromatograms of CHDA in (a) commercial product A and (b) commercial product B.

greener HPLC methodology is fast, linear, accurate, precise, robust, sensitive, stability-indicating, selective, and environmentally friendly for the standardization and quantitative determination of CHDA in its commercial formulations. The CHDA contents of commercial products were successfully analyzed using the proposed methodology. The findings of the AES, ChlorTox, and AGREE assessments confirmed the outstanding greenness of the proposed methodology for determining CHDA. The selectivity- and stability-indicating qualities of CHDA are indicated by the fact that the suggested methodology was able to identify it under a variety of stress situations, even in the presence of its degradation products. These findings all pointed to the possibility of routinely determining the CHDA level in commercial products using the existing HPLC approach. Further research can be performed to detect CHDA in biological samples like plasma, blood, and urine to exploit its biological/biochemical potential.

Acknowledgments: The authors are thankful to the Researchers Supporting Project number (RSPD2024R1040), King Saud University, Riyadh, Saudi Arabia, for supporting this work.

Funding information: This work was funded by the Researchers Supporting Project number (RSPD2024R1040), King Saud University, Riyadh, Saudi Arabia.

Author contributions: N.A.: conceptualization, methodology, investigation, software, validation; writing, review, and editing; N.H.: supervision, methodology, investigation, writing, review, and editing; I.A.A.: formal analysis, data curation, validation, writing, review, and editing; F.S.: conceptualization, supervision, project administration, investigation, funding acquisition; software; visualization, resources; writing original draft.

Conflict of interest: The authors declare no competing financial interest.

Ethical approval: The conducted research is not related to either human or animal use.

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

References

[1] Mazzola PG, Penna TCV, Martins AMS. Determination of decimal reduction time (D value) of chemical agents used in hospitals for disinfection purposes. BMC Infec Dis. 2003;3:1–10.

[2] Risso NH, Stopiglia CDO, Oliveira MT, Haas SE, Maciel TR, Lazzari NR, et al. Chlorhexidine nanoemulsion: a new antiseptic formulation. Int J Nanomed. 2020;15:6935–44.

[3] Hoang TPA, Ghori MU, Conway BR. Topical antiseptic formulation for skin and soft tissue infections. Pharmaceutics. 2021;13:E558.

[4] Thomas L, Maillard JY, Lambert RJW, Russell AD. Development of resistance to chlorhexidine diacetate in *Pseudomonas aeruginosa* and the effect of a residual concentration. J Hosp Infec. 2000;46:297–303.

[5] Cone RA, Hoen T, Wong X, Abusuwwa R, Anderson DJ, Moench TR. Vaginal microbicides: detecting toxicities *in vivo* that paradoxically increase pathogen transmission. BMC Infect Dis. 2006;6:E90.

[6] Haq N, Shahid M, Alaofi AL, Ahmad ZH, Alrayyes YF, Alsarra IA, et al. Evaluation of the physicochemical and antimicrobial properties nanoemulsion-based polyherbal mouthwash. ACS Omega. 2023;8:41755–64.

[7] Fiorentino FAM, Correa MA, Salgado HRN. Development and validation of a microbiological assay for determination of chlorhexidine digluconate in aqueous solution. Braz J Pharm Sci. 2013;49:351–8.

[8] Fiorentino FAM, Correa MA, Salgado HRN. Analytical methods for the determination of chlorhexidine: a review. Crit Rev Anal Chem. 2010;40:89–101.

[9] Stevens LE, Durrwachter JR, Helton DO. Analysis of chlorhexidine sorption in soft contact lenses by catalytic oxidation of (14C) chlorhexidine and by liquid chromatography. J Pharm Sci. 1986;75:83–6.

[10] Hu OYP, Wang SY, Fang YJ, Chen YH, King ML. Simultaneous determination of thimerosal and chlorhexidine in solutions for soft contact lenses and its applications in stability studies. J Chromatogr. 1991;523:321–6.

[11] Doub WH, Ruhi DD, Hart B, Mehelic PR, Revelle LK. Gradient liquid chromatographic method for determination of chlorhexidine and its degradation products in bulk material. J AOAC Int. 1996;79:636–9.

[12] Gavlick WK. High-performance liquid chromatographic analysis of chlorhexidine and *p*-chloroaniline using a specialty column and a photodiode-array detector. J Chromatogr. 1992;623:375–80.

[13] Ha Y, Cheung AP. New stability-indicating high performance liquid chromatography assay and proposed hydrolytic pathways of chlorhexidine. J Pharm Biomed Anal. 1996;14:1327–34.

[14] Havlıkova L, Matysova L, Novakova L, Hajkova R, Solich P. HPLC determination of chlorhexidine gluconate and *p*chloroaniline in topical ointment. J Pharm Biomed Anal. 2007;43:1169–73.

[15] Cardoso MA, Favero MLD, Gasparetto JC, Hess BS, Stremel DP, Pontarolo R. Development and validation of an RP-HPLC method for the determination of chlorhexidine and p-chloroaniline in various pharmaceutical formulations. J Liq Chromatogr Relat Technol. 2011;34:1556–67.

[16] Sobaih AE, Magdy N, Hussein LA. HPLC method development for determination of chlorhexidine in pharmaceutical formulations alone and in presence of hexamidine and p-chlorocresol, and in spiked human saliva. Arch Pharm Sci Ain Shams Uni. 2022;6:45–59.

[17] Vrachas A, Gkountanas K, Boutsikaris H, Dotsikas Y. Development and validation of a novel RP-HPLC method for the determination of cetrimide and chlorhexidine gluconate in antiseptic solution. Analytica. 2022;3:79–91.

[18] Muniz VM, Junior JVC, Aragao CFS, de Souza FS, Sampaio FCA. HPLC method for simultaneous quantification of chlorhexidine and

thymol using Box-Behnken design for robustness of the method assessment. J Liq Chromatogr Relat Technol. 2023;46:168–9.
[19] Houston CE, Wainwright P, Cooke M, Simpson R. High performance liquid chromatographic method for the determination of chlorhexidine. J Chromatogr. 1982;237:457–64.
[20] Gaffney MH, Cooke M, Simpson R. Improved method for the determination of chlorhexidine in urine. J Chromatogr. 1984;306:303–13.
[21] Brougham LR, Cheng H, Pittman KA. Sensitive high performance liquid chromatographic method for the determination of chlorhexidine in human serum and urine. J Chromatogr. 1986;383:365–73.
[22] Wainwright P, Cooke M. Direct determination of chlorhexidine in urine by high-performance liquid chromatography. Analyst. 1986;111:1343–4.
[23] Lam YWF, Chan DCN, Rodriguez SY, Lintakoon JH, Lam TH. Sensitive high-performance liquid chromatographic assay for the determination of chlorhexidine in saliva. J Chromatogr. 1993;612:166–71.
[24] Medlicott NJ, Ferry DG, Tucker IG, Rathbone MJ, Holborow DW, Jones DS. High performance liquid chromatographic (HPLC) assay for the determination of chlorhexidine in saliva film. J Liq Chromatogr. 1994;17:1605–20.
[25] Pesonem J, Holmalahti J, Pohjola J. Determination of chlorhexidine in saliva using high-performance liquid chromatography. J Chromatogr B. 1995;665:222–5.
[26] Kudo K, Ikeda N, Kiyoshima A, Hino Y, Nishida N, Inoue N. Toxicological analysis of chlorhexidine in human serum using HPLC on a polymer-coated ODS column. J Anal Toxicol. 2002;26:119–22.
[27] Below H, Lehan N, Kramer A. HPLC determination of the antiseptic agent chlorhexidine and its degradation products 4-chloroaniline and 1-chloro-4-nitrobenzene in serum and urine. Microchim Acta. 2004;146:129–35.
[28] Usui K, Hishinuma T, Yamamguchi H, Tachiiri N, Goto J. Determination of chlorhexidine (CHD) and nonylphenolethoxylates (NPEO*n*) using LC-ESI-MS method and application to hemolyzed blood. J Chromatogr B. 2006;831:105–9.
[29] Alam P, Shakeel F, Alqarni MH, Foudah AI, Alshehri S. A rapid, sensitive, and greener stability-indicating normal-phase HPTLC method with univariate calibration for the estimation of chlorhexidine acetate in its commercial formulations. Sus Chem Pharm. 2021;24:E100552.
[30] Jensen JE, Christensen F. A study of the elimination of chlorhexidine from the oral cavity using a new spectrophotometric method. J Periodon Res. 1971;6:306–11.
[31] de Vries J, Ruben J, Arends J. Determination of chlorhexidine in saliva and in aqueous solutions. Caries Res. 1991;25:410–4.
[32] Musteata FM, Pawliszyn J. Assay of stability, free and total concentration of chlorhexidine in saliva by solid phase microextraction. J Pharm Biomed Anal. 2005;37:1015–24.
[33] Miribel L, Brazier JL, Comet F, Lecompte D. Gas-liquid chromatographic determination of chlorhexidine in pharmaceutical formulations. J Chromatogr. 1983;268:321–8.
[34] Bonazzi D, Andrisano V, Gatti R, Cavrini V. Analysis of pharmaceutical creams: A useful approach based on solid-phase extraction (SPE) and UV spectrophotometry. J Pharm Biomed Anal. 1995;13:1321–9.
[35] Abad-Villar EM, Etter SF, Thiel MA, Hauser PC. Determination of chlorhexidine digluconate and polyhexamethylene biguanide in eye drops by capillary electrophoresis with contactless conductivity detection. Anal Chim Acta. 2006;561:33–137.
[36] Calatayud JM, Falco PC, Sanpedro AS. Turbidimetric determination of chlorhexidine using flow injection analysis. Analyst. 1987;112:87–90.
[37] Perez-Ruiz T, Matınez-Lozano C, Sanz A, Sanchez A. Flow injection extraction-spectrophotometric method for the determination of chlorhexidine in pharmaceutical preparations. J Pharm Biomed Anal. 1999;21:709–14.
[38] Wang LH, Tsai SJ. Voltammetric behavior of chlorhexidine at a film mercury electrodes and its determination in cosmetic and oral hygiene products. Anal Chim Acta. 2001;441:107–16.
[39] Galuszka A, Migaszewski Z, Namiesnik J. The 12 principles of green analytical chemistry and the significance mnemonic of green analytical practices. Trends Anal Chem. 2013;50:78–84.
[40] Alam P, Ezzeldin E, Iqbal M, Anwer MK, Mostafa GAE, Alqarni MH, et al. Ecofriendly densitometric RP-HPTLC method for determination of rivaroxaban in nanoparticle formulations using green solvents. RSC Adv. 2020;10:2133–40.
[41] Foudah AI, Shakeel F, Alqarni MH, Alam P. A rapid and sensitive stability-indicating green RP-HPTLC method for the quantitation of flibanserin compared to green NP-HPTLC method: Validation studies and greenness assessment. Microchem J. 2021;164:E105960.
[42] Alam P, Shakeel F, Alshehri S, Iqbal M, Foudah AI, Aljarba TM, et al. Simultaneous etimation of lesinurad and allopurinol in the new FDA-approved products using a greener normal-phase HPTLC method: Greenness assessment using the NEMI, analytical eco-score, ChlorTox, and AGREE methods. ACS Sustain Chem Eng. 2024;12:1526–35.
[43] Keith LH, Brass HJ, Sullivan DJ, Boiani JA, Alben KT. An introduction to the national environmental methods index. Env Sci Technol. 2005;39:173A–6A.
[44] Gaber Y, Tornvall U, Kumar MA, Ali Amin M, Hatti-Kaul R. HPLC-EAT (Environmental Assessment Tool): a tool for profiling safety, health and environmental impacts of liquid chromatography methods. Green Chem. 2011;13:2021–5.
[45] Hartman R, Helmy R, Al-Sayah M, Welch CJ. Analytical method volume intensity (AMVI): a green chemistry metric for HPLC methodology in the pharmaceutical industry. Green Chem. 2011;13:934–9.
[46] Galuszka A, Konieczka P, Migaszewski ZM, Namiesnik J. Analytical eco-scale for assessing the greenness of analytical procedures. Trends Anal Chem. 2012;37:61–72.
[47] Plotka-Wasylka J. A new tool for the evaluation of the analytical procedure: Green analytical procedure index. Talanta. 2018;181:204–9.
[48] Hicks MB, Farrell W, Aurigemma C, Lehmann L, Weisel L, Nadeau K, et al. Making the move towards modernized greener separations: introduction of the analytical method GREEnness score (AMGS) calculator. Green Chem. 2019;21:1816–26.
[49] Nowak PM, Koscielnaik P. What color is your method? Adaptation of the RGB additive color model to analytical method evaluation. Anal Chem. 2019;91:10343–52.
[50] Nowak PM, Wietecha-Posluszny R, Plotka-Wasylka J, Tobiszewski M. How to evaluate methods used in chemical laboratories in terms of the total chemical risk? a ChlorTox Scale. Green Anal Chem. 2023;5:E100056.
[51] Pena-Pereira F, Wojnowski W, Tobiszewski M. AGREE-Analytical GREEnness metric approach and software. Anal Chem. 2020;92:10076–82.

[52] International Conference on Harmonization (ICH), Q2 (R2). Guideline on validation of analytical procedures–text and methodology. Geneva, Switzerland; 2023.
[53] Haq N, Alanazi FK, Salem-Bekhit MM, Rabea S, Alam P, Alsarra IA, et al. Greenness estimation of chromatographic assay for the determination of anthracycline-based antitumor drug in bacterial ghost matrix of *Salmonella typhimurium*. Sus Chem Pharm. 2022;26:E100642.
[54] Haq N, Alshehri S, Alam P, Ghoneim MM, Hasan Z, Shakeel F. Green analytical chemistry approach for the determination of emtricitabine in human plasma, formulations, and solubility study samples. Sus Chem Pharm. 2022;26:E100648.
[55] Haq N, Iqbal M, Alanazi FK, Alsarra IA, Shakeel F. Applying green analytical chemistry for rapid analysis of drugs: Adding health to pharmaceutical industry. Arab J Chem. 2017;10:S777–85.
[56] Haq N, Alshehri S, Alsarra IA, Alenazi M, Alwhaibi A, Shakeel F. Environmentally friendly stability-indicating HPLC method for the determination of isotretinoin in commercial products and solubility samples. Heliyon. 2023;9:E18405.