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A Simple, Cost-Effective, and Green HPTLC Method for the Estimation of Ascorbic Acid in Solvent and Ultrasound-Assisted Extracts of *Phyllanthus emblica*, *Capsicum annuum*, and *Psidium guajava*

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Abstract: Greener analytical methodologies for the estimation of ascorbic acid (AA) are poorly reported in the literature. Furthermore, the green indexes of the literature's analytical assays of AA estimation have not been assessed. As a consequence, the aim of this research is to invent and validate a simple, cost-effective, and green reverse-phase "high-performance thin-layer chromatography (HPTLC)" method for the estimating AA in the solvent extracts (SE) and ultrasound-assisted extracts (UAE) of *Phyllanthus emblica*, *Psidium guajava*, and *Capsicum annuum*. The greener mobile phase for AA estimation was a binary mixture of water and ethanol (70:30, *v*/*v*). At a wavelength of 265 nm, the detection of AA was carried out. The greener HPTLC technique was linear in the 25–1200 ng/band range. In addition, the method was simple, cost-effective, accurate, precise, robust, sensitive, and green. The amount of AA was highest in the SE and UAE of *P. emblica* compared to the SE and UAE of *P. guajava* and *C. annuum*. The amount of AA in the SE of *P. emblica*, *P. guajava*, and *C. annuum* was found to be 491.16, 168.91, and 144.30 mg/100 g, respectively. However, the amount of AA in the UAE of *P. emblica*, *P. guajava*, and *C. annuum* was found to be 673.02, 218.71, and 199.30 mg/100 g, respectively. Using the "analytical GREEnness (AGREE)" methodology, the greenness index for the developed method was calculated to be 0.88, showing that the developed method has an excellent green profile. When it came to extracting AA, the UAE method outperformed the SE method. These findings suggested that the developed method might be used to estimate the AA in a variety of vegetable crops, plant-based extracts, and commercial formulations. Furthermore, because of the use of greener solvent systems against the commonly utilized hazardous solvent systems for AA determination, this technique is also safe and sustainable.

Keywords: AGREE; ascorbic acid; greener HPTLC; *Phyllanthus emblica*; *Psidium guajava*; *Capsicum annuum*; ultrasound extraction; validation; vegetable crops

1. Introduction

Ascorbic acid (AA), also known as vitamin C or ascorbate, is a water-soluble vitamin [1]. It is involved in several biological processes and is mainly synthesized by plants [2]. It is found in several fruits and vegetable crops [3]. It is abundantly found in fresh fruits

and leafy vegetable crops such as *Phyllanthus emblica* or *Emblica officinalis* (family: Phyllanthaceae, common name: amla), *Psidium guajava* (family: Myrtaceae, common name: guava), *Capsicum annuum* (family: Solanaceae, common name: capsicum), *Mangifera indica* (family: Anacardiaceae, common name: mango), *Carica papaya* (family: Caricaceae, common name: papaya), *Brassica oleracea* (family: Brassicaceae, common name: cabbage), *Brassica juncea* leaves (family: Brassicaceae, common name: mustard), and *Spinacea oleracea* leaves (family: Amaranthaceae, common name: spinach) [2–4]. The content of AA in different fruits and vegetable crops varies depending on the species, variety, and climate conditions [1]. AA has been reported as a potent antioxidant and is also present in various commercial formulations [2,3]. As a result, the qualitative and quantitative standardization of AA is necessary for commercial formulations and plant extracts.

Several analytical methods have been proposed to estimate AA either alone or in combination with other compounds in commercial formulations, plant extracts, and physiological fluids. Some titration-based assays have been reported for AA estimation in plant extracts [5,6]. The wide range of "high-performance liquid chromatography (HPLC)" methods are reported to estimate AA in the variety of fruits and vegetable crops [6–12]. An HPLC method has also been reported to estimate AA in human milk samples and infant milk formulas [13]. Various "high-performance thin-layer chromatography (HPTLC)" methods are also reported to estimate AA in various fruits, vegetable crops, and commercial formulations [14–18]. A variety of electrochemical methods have been reported for the simultaneous determination of AA, dopamine, and uric acid in different samples [19–27]. Biosensor, electro-oxidation, and electrochemical methods have also been used to estimate AA in plant extracts and pharmaceutical formulations [28–31]. Some other methods such as Folin–Ciocalteu assay, voltammetry, and ultra-performance liquid chromatography methods have also been used to estimate AA in plant extracts and pharmaceutical formulations [32–34].

A single green HPTLC methodology has been used to determine AA in fruit juices and pharmaceutical formulations [15]. However, its greenness index was not estimated. After conducting a literature review, we discovered that the safety and environmental profiles of reported analytical methods for estimating AA were not evaluated. Greener HPTLC technologies have a number of benefits over traditional liquid chromatography—such as HPLC and TLC—methods [35,36]. These benefits include simplicity, economy, low operation costs, fast analysis, the parallel detection of multiple samples, sharp detection, and reduced environmental pollution [35–38]. As a result, the greener reverse-phase HPTLC method for measuring AA was chosen in this investigation. There have been several methodologies for determining the greenness of various analytical procedures [37–42]. Only the "analytical GREEnness (AGREE)" approach for greenness assessment employs all twelve principles of "green analytical chemistry (GAC)". As a result, the greenness index of the greener HPTLC method was determined using the "AGREE methodology" [41]. The present research aims to invent and validate a simple, cost-effective, accurate, precise, robust, sensitive, and green reverse-phase HPTLC method to estimate AA in its bulk form, solvent extract (SE), and ultrasound-assisted extract (UAE) of *P. emblica*, *P. guajava*, and *C. annuum*, based on these ideas. The greener HPTLC approach for estimating AA was validated using the "International Council for Harmonization (ICH)" Q2-(R1) requirements [43].

2. Materials and Methods

2.1. Materials

The standard AA was procured from "Sigma Aldrich (New Delhi, India)". The HPLC grade ethanol (EOH) and water (WTR) were obtained from "E-Merck (Darmstadt, Germany)". Other solvents and reagents used were of analytical grade and obtained from "E-Merck (Darmstadt, Germany)". The fresh fruits of *P. emblica* (amla), *P. guajava* (guava), and *C. annuum* (capsicum) were procured from a supermarket in "Al-Kharj, Saudi Arabia".

2.2. Chromatography and Instrumentation

The estimation of AA in its bulk form (pure drug or standard), SE, and UAE of *P. emblica*, *P. guajava*, and *C. annuum* was performed using the "HPTLC CAMAG TLC system (CAMAG, Muttenz, Switzerland)". The HPTLC estimation of AA in reverse-phase conditions was conducted on "10 cm × 20 cm glass plates pre-coated with RP silica gel 60 F254S plates (E-Merck, Darmstadt, Germany)". The solutions were spotted as the 6 mm bands with the help of a "CAMAG Automatic TLC Sampler 4 (ATS4) Sample Applicator (CAMAG, Geneva, Switzerland)". The "CAMAG microliter Syringe (Hamilton, Bonaduz, Switzerland)" was linked with the sample applicator. The application rate for estimating AA was fixed to 150 nL/s. Under linear ascending mode, the TLC plates were developed in a "CAMAG automated developing chamber 2 (ADC2) (CAMAG, Muttenz, Switzerland)" at a distance of 80 mm. The greener solvent system/mobile phase was a binary mixture of WTR-EOH (70:30, *v*/*v*). For 30 min at 22 °C, the development chamber was saturated with vapors of WTR-EOH (70:30, *v*/*v*). The bands in TLC plates were visualized under a UV cabinet at different wavelengths. Finally, the detection of AA was carried out at a wavelength of 265 nm. The slit size (band length × width) and scanning rate were set to 4 mm × 0.45 mm and 20 mm/s, respectively. Each estimation was performed in three or six replicates. The software used was "WinCAT's (v1.4.3.6336, CAMAG, Muttenz, Switzerland)".

2.3. AA Calibration Curve and Quality Control (QC) Samples

AA is freely soluble in WTR. Hence, the needed quantity of AA (10 mg) was dispensed in 5 mL of WTR and the volume was increased to 100 mL by diluting with methanol (95 mL) to obtain a stock solution of 100 µg/mL. Variable quantities of this stock solution were diluted again with methanol to achieve AA concentrations in the 25–1200 ng/band range. The resultant AA solutions were spotted in various concentrations on reverse-phase HPTLC plates. The HPTLC response for AA was measured using the greener HPTLC method at each AA concentration. Graphing the AA concentrations vs. the measured TLC response produced the AA calibration curve. Furthermore, low QC (LQC; 100 ng/band), middle QC (MQC; 400 ng/band), and high QC (HQC; 1200 ng/band) samples were created individually for the validation evaluation of the developed method.

2.4. Sample Preparation for the Estimation of AA in SE of P. emblica, P. guajava, and C. annuum

The fresh fruits of *P. emblica, P. guajava*, and *C. annuum* were obtained from the market. The fruits were crushed and 1.0 g of each fruit was dispersed in 20 mL of WTR. The obtained dispersions were dried via a lyophilization process using a "Lyophilizer (Freezone® 2.5 model 76530, Labconco Corp., Kansas, MO, USA)" for 40 h and then stored at 20 °C until further use. The lyophilized samples of crushed fruits were extracted by maceration with WTR (3 × 100 mL) at room temperature. Each sample was filtered using Whatman filter paper (No. 41). The filtered samples were dried again via the lyophilization process. The extract of lyophilized samples was initially dissolved in 5 mL of WTR and the volume was maintained at 100 mL using methanol. This solution was used to determine the AA content using the developed analytical method.

2.5. Sample Preparation for the Estimation of AA in the UAE of P. emblica, P. guajava, and C. annuum

The fresh fruits of *P. emblica, P. guajava*, and *C. annuum* were obtained from the market. The fruits were crushed and 1.0 g of each fruit was dispersed in 20 mL of WTR. The obtained dispersions were dried via a lyophilization process using a "Lyophilizer (Freezone® 2.5 model 76530, Labconco Corp., Kansas, MO, USA)" for 40 h and then stored at 20 °C until further use. The UAE was performed using ultrasonic vibrations with the help of an ultrasonic probe type (Ultrasonic processor-200 Ht, power 200 W, Darmstadt, Germany) at low frequency (26 kHz), power (30%), duration (20 min), and temperature (25 °C). The probe used for extraction has a vibrating horn diameter of 7 mm which was directly immersed in the solvent-containing sample, which was then irradiated with the ultrasonic waves

generated from the tip of the probe. Approximately 1 g of each crushed fruit was separately extracted with 20 mL of WTR using the above apparatus. The residue obtained was dissolved in 5 mL of WTR and volume was maintained at 50 mL using methanol. The obtained samples were used to determine the AA content using the developed analytical method.

2.6. Validation Parameters

The developed method for determining AA content was tested for multiple validation settings using the ICH-Q2-R1 criteria [43]. The linearity of AA was tested by graphing its concentrations vs. its recorded TLC response. The AA linearity was determined in the 25–1200 ng/band range for the developed method. To evaluate the system suitability parameters for the developed method, the "retardation factor (R_f), tailing factor (As), and theoretical plates number per meter (N/m)" were employed. The "R_f, As, and N/m" were calculated using the formulae provided [44].

The accuracy of the developed method was determined using % recovery. The % recovery was assessed at LQC, MQC, and HQC for the greener HPTLC method.

The intra/interday precision of the developed method was estimated. The intraday fluctuation was determined by measuring AA at LQC, MQC, and HQC on the same day. The interday precision was determined by measuring AA at LQC, MQC, and HQC on three consecutive days [43].

The method's robustness was evaluated by introducing some planned alterations in the greener solvent systems. The original WTR-EOH (70:30, v/v) greener solvent system was altered to WTR-EOH (72:28, v/v) and WTR-EOH (68:32, v/v) systems, and the necessary changes in chromatographic response and R_f values were recorded [43].

Using the standard deviation method of blank, the sensitivity of the proposed method was determined as "limit of detection (LOD) and limit of quantification (LOQ)". The "LOD and LOQ" for AA were calculated using regular formulae previously reported [43,44].

The method specificity was determined by comparing the R_f values and overlaid UV-absorption spectra of AA in the TE and UAE of *P. emblica, P. guajava,* and *C. annuum* with those of standard AA.

2.7. Estimation of AA in the SE and UAE of P. emblica, P. guajava, and C. annuum

On reverse-phase TLC plates, the TLC responses of the obtained SE and UAE solutions of *P. emblica*, *P. guajava*, and *C. annuum* were recorded. The AA content of all of these samples was determined using the developed method's AA calibration curve.

2.8. Greenness Assessment

The developed method's greenness index was assessed using the "AGREE metric approach" [41]. The AGREE index (0.0–1.0) of the developed method was calculated using the "AGREE: The Analytical Greenness Calculator (v0.5, Gdansk University of Technology, Gdansk, Poland, 2020)".

3. Results and Discussion

3.1. Method Development

In the literature, the greener analytical methodologies for determining AA are poorly reported. Accordingly, the aim of this research was to invent and validate a simple, cost-effective, sensitive, and greener reverse-phase HPTLC method for estimating AA in the SE and UAE of *P. emblica, P. guajava*, and *C. annuum*.

For the AA determination using the greener HPTLC method, different proportions of WTR and EOH, including WTR-EOH (30:70, v/v), WTR-EOH (40:60, v/v), WTR-EOH (50:50, v/v), WTR-EOH (60:40, v/v), WTR-EOH (70:30, v/v), WTR-EOH (80:20, v/v), and WTR-EOH (90:10, v/v) were investigated as the greener solvent systems for the development of a suitable band for the estimation of AA in SE and UAE of *P. emblica*, *P. guajava*, and *C. annuum*. The greener solvent system, which was developed under chamber saturation conditions, is depicted in Figure 1.

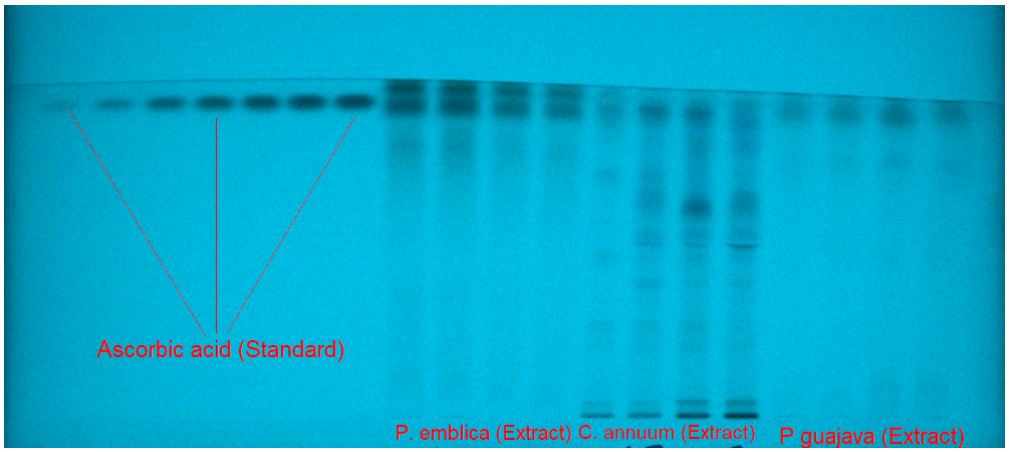

Figure 1. Developed thin-layer chromatography (TLC) plate for standard ascorbic acid (AA), *P. emblica* extract, *C. annuum* extract, and *P. guajava* extract developed using water-ethanol (70:30 v/v) as the mobile phase for the greener "high-performance thin-layer chromatography (HPTLC)" method.

The greener solvent compositions and different chromatographic parameters are included in Table 1.

Table 1. The optimization of the greener solvent systems and chromatographic conditions of ascorbic acid (AA) estimation for the greener high-performance thin-layer chromatography (HPTLC) method [a].

Mobile Phase	As	N/m	R_f
WTR-EOH (30:70, v/v)	1.290 ± 0.0200	1653 ± 1.912	0.7800 ± 0.0300
WTR-EOH (40:60, v/v)	1.280 ± 0.0200	1871 ± 1.942	0.8000 ± 0.0200
WTR-EOH (50:50, v/v)	1.270 ± 0.0200	1945 ± 2.021	0.8200 ± 0.0300
WTR-EOH (60:40, v/v)	1.260 ± 0.0300	2067 ± 2.123	0.8400 ± 0.0200
WTR-EOH (70:30, v/v)	1.070 ± 0.0300	5378 ± 2.980	0.8600 ± 0.0200
WTR-EOH (80:20, v/v)	1.120 ± 0.0300	4143 ± 2.432	0.8800 ± 0.0200
WTR-EOH (90:10, v/v)	1.160 ± 0.0300	3243 ± 2.121	0.9000 ± 0.0200

[a] Mean ± SD; n = 3; WTR: water; EOH: ethanol; R_f: retardation factor; As: asymmetry factor; N/m: theoretical plates number per meter.

The findings indicated that the WTR-EOH (30:70, v/v), WTR-EOH (40:60, v/v), WTR-EOH (50:50, v/v), WTR-EOH (60:40, v/v), WTR-EOH (80:20, v/v), and WTR-EOH (90:10, v/v) greener solvent systems offered a poor densitogram of AA with a high value of As (As = 1.120–1.290) (Table 1). However, the WTR-EOH (70:30, v/v) greener solvent system showed an intact and well-resolved peak of AA at R_f = 0.8600 ± 0.0200 with an acceptable As value (As = 1.070 ± 0.0300), as illustrated in Figure 2. As a consequence, for the estimation of AA in the SE and UAE of *P. emblica, P. guajava,* and *C. annuum*, the WTR-EOH (70:30, v/v) greener solvent system was selected as the final solvent system. The chromatogram for the suggested analytical method was densitometrically determined, and the highest chromatographic response for the proposed analytical method was observed at 265 nm. Accordingly, the entire estimation of AA was performed at 265 nm.

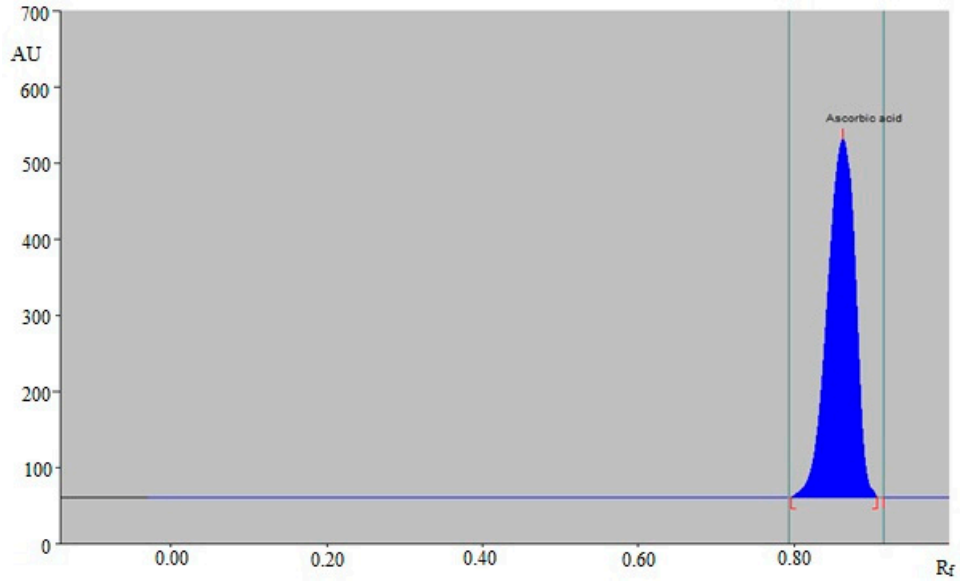

Figure 2. Representative HPTLC densitogram of standard AA for the greener HPTLC method.

3.2. Validation Parameters

The developed method for estimating AA was validated for different parameters using the ICH-Q2-R1 criteria [43]. The results for the linear regression analysis of the calibration curve of AA are included in Table 2. The AA calibration curve was linear in the 25–1200 ng/band range for the developed method. The values of the "determination coefficient (R^2)" and "regression coefficient (R)" for AA were determined to be 0.9956 and 0.9977, respectively, for the developed method. These results revealed a solid linear relationship between AA concentrations and TLC response.

Table 2. Results of the regression analysis for the estimation of AA using the greener HPTLC method [a].

Parameters	Values
Linearity range (ng/band)	25–1200
Regression equation	y = 31.78x + 1974
R^2	0.9956
R	0.9977
SE of slope	0.3919
SE of intercept	9.959
95% CI of slope	30.10–33.47
95% CI of intercept	1931–2017
LOD ± SD (ng/band)	8.630 ± 0.1600
LOQ ± SD (ng/band)	25.89 ± 0.3900

[a] Mean ± SD; n = 6; R^2: determination coefficient; R: regression coefficient; CI: confidence interval; LOD: limit of detection; LOQ: limit of quantification.

Table 1 lists the results of the system suitability parameters for the developed method. For the developed method, the "R_f, As, and N/m" were determined to be 0.8600 ± 0.0200, 1.070 ± 0.0300, and 5378 ± 2.980, respectively. These results revealed that the developed method was suitable for estimating AA in the SE and UAE of *P. emblica*, *P. guajava*, and *C. annuum*.

Table 3 lists the findings of the accuracy assessment for the developed method. The % recovery of AA for the developed method was determined to be 100.26%, 99.05%, and 101.28%, respectively, at LQC, MQC, and HQC. These % recovery results illustrate the accuracy of the developed method for estimating AA in the SE and UAE of *P. emblica*, *P. guajava*, and *C. annuum*.

Table 3. Determination of the accuracy of AA for the greener HPTLC method [a].

Conc. (ng/Band)	Conc. Found (ng/Band) ± SD	Recovery (%)	CV (%)
50	50.13 ± 0.61	100.26	1.21
400	396.21 ± 3.71	99.05	0.93
1200	1215.45 ± 10.58	101.28	0.87

[a] Mean ± SD; $n = 6$.

The precision assessment results for the developed method were calculated in the percent of the coefficient of variation (% CV), and the results are shown in Table 4. The % CVs of AA for the developed method were 0.68%, 0.56%, and 0.53% at LQC, MQC, and HQC, respectively, for intraday precision. The % CVs of AA for the developed method at LQC, MQC, and HQC, respectively, were estimated to be 0.81%, 0.61%, and 0.54% for interday precision. These findings suggested the precision of the developed method for estimating AA in the SE and UAE of *P. emblica*, *P. guajava*, and *C. annuum*.

Table 4. Determination of the intra/inter-day precision of AA for the greener HPTLC method [a].

Conc. (ng/Band)	Intraday Precision			Interday Precision		
	Conc. (ng/Band) ± SD	SE	CV (%)	Conc. (ng/Band) ± SD	SE	CV (%)
50	49.89 ± 0.34	0.13	0.68	50.24 ± 0.41	0.16	0.81
400	407.41 ± 2.32	0.94	0.56	409.12 ± 2.52	1.02	0.61
1200	1187.43 ± 6.41	2.61	0.53	1184.74 ± 6.48	2.64	0.54

[a] Mean ± SD; $n = 6$.

The findings of the robustness assessment for the developed method are listed in Table 5. The % CVs for the robustness assessment were evaluated to be 0.73–0.77% for the developed method. The R_f values of AA were determined to be 0.85–0.87 using the developed method. The robustness of the developed method for estimating AA in the SE and UAE of *P. emblica*, *P. guajava*, and *C. annuum* was demonstrated by minor fluctuations in R_f values of AA and low CVs.

Table 5. Robustness analysis for AA for the greener HPTLC method [a].

Conc. (ng/Band)	Mobile Phase Composition (Water-Ethanol, *v*/*v*)			Results		
	Original	Used	Level	Conc. (ng/Band) ± SD	CV (%)	R_f
400	70:30	72:28	+2.0	393.54 ± 2.89	0.73	0.85
		70:30	0.0	398.71 ± 2.98	0.74	0.86
		68:32	−2.0	404.61 ± 3.12	0.77	0.87

[a] Mean ± SD; $n = 6$.

The developed method's "LOD and LOQ" values were determined, and the results are shown in Table 1. For estimating AA, the developed method's "LOD and LOQ" were calculated to be 8.630 ± 0.1600 and 25.89 ± 0.3900 ng/band, respectively. The sensitivity of the developed method for estimating AA in the SE and UAE of *P. emblica*, *P. guajava*, and *C. annuum* was revealed by these "LOD and LOQ" values.

By comparing the overlaid UV-absorption spectra of AA in the SE and UAE of *P. emblica*, *P. guajava*, and *C. annuum* with those of standard AA, the method specificity was determined. Figure 3 represents the overlaid UV spectra of standard AA and AA in the SE and UAE of *P. emblica*, *P. guajava*, and *C. annuum*. For the developed method, the maximum chromatographic response for AA in standard AA and AA in the SE and UAE of *P. emblica*, *P. guajava*, and *C. annuum* was measured at 265 nm. The specificity of the developed method was revealed by the identical UV spectra, R_f values, and wavelengths of AA in standard AA and AA in the SE and UAE of *P. emblica*, *P. guajava*, and *C. annuum*.

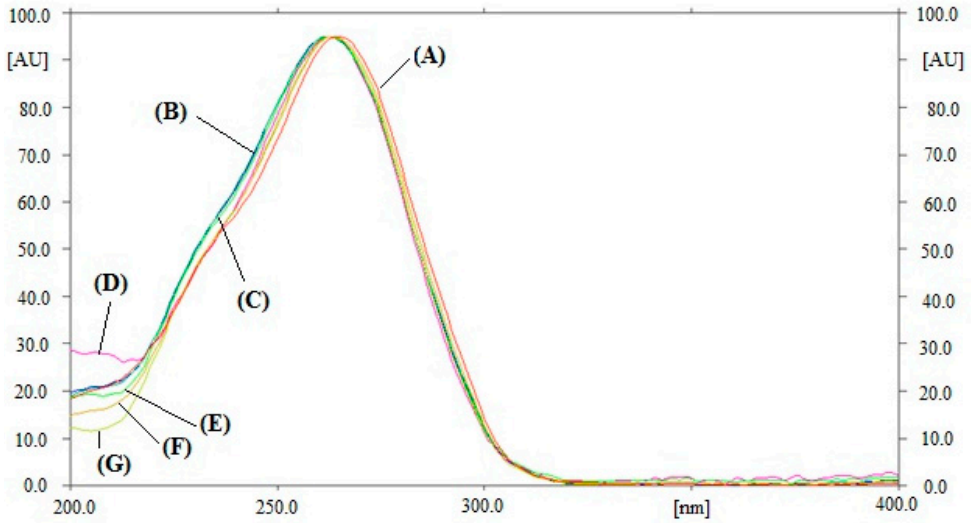

Figure 3. Overlaid UV-absorption spectra of (**A**) standard AA, (**B**) the UAE of *P. emblica*, (**C**) the SE of *P. emblica*, (**D**) the UAE of *P. guajava*, (**E**) the SE of *P. guajava*, (**F**) the UAE of *C. annuum*, and (**G**) the SE of *C. annuum*.

3.3. Estimation of AA in the SE and UAE of P. emblica, P. guajava, and C. annuum

The developed method was used for the estimation of AA contents in the SE and UAE of *P. emblica, P. guajava*, and *C. annuum*. The HPTLC chromatogram of AA from the SE and UAE of *P. emblica*, *C. annuum*, and *P. guajava* were identified by comparing their single TLC band at $R_f = 0.86 \pm 0.02$ with that of a standard AA for the developed method. The representative HPTLC densitogram of AA in the UAE of *P. emblica*, *C. annuum*, and *P. guajava* is presented in Figure 4, which showed an identical peak of AA with that of standard AA. In addition, four, six, and ten additional peaks were also detected in the UAE of *P. emblica* (Figure 4A), *P. guajava* (Figure 4B), and *C. annuum* (Figure 4C), respectively. The detection of additional peaks in the UAE of *P. emblica*, *C. annuum*, and *P. guajava* suggested that the developed method can be efficiently used in the estimation of AA in the presence of impurities/other phytoconstituents.

The calibration curve of AA was used to determine the amount (mg/100 g) of AA in all samples, and the findings are shown in Table 6. The amount of AA in the SE of *P. emblica, P. guajava*, and *C. annuum* was determined to be 491.16 ± 2.34 mg/100 g, 168.91 ± 1.41 mg/100 g, and 144.30 ± 1.17 mg/100 g, respectively. However, the amount of AA in the UAE of *P. emblica, P. guajava*, and *C. annuum* was determined to be 673.02 ± 3.16 mg/100 g, 218.71 ± 1.67 mg/100 g, and 199.30 ± 1.32 mg/100 g, respectively. The amount of AA was computed as higher in the SE and UAE of *P. emblica* compared to the SE and UAE of *P. guajava*, and *C. annuum*. Furthermore, the amount of AA in all UAE samples was considerably greater than the respective SE ($p < 0.05$). Based on these observations and results, the UAE method for extracting the AA in *P. emblica*, *P. guajava*, and *C. annuum* is superior to the SE method of extraction. Overall, our results showed that the developed method may be utilized to assess AA in a variety of food and pharmaceutical samples with AA as one of the constituents.

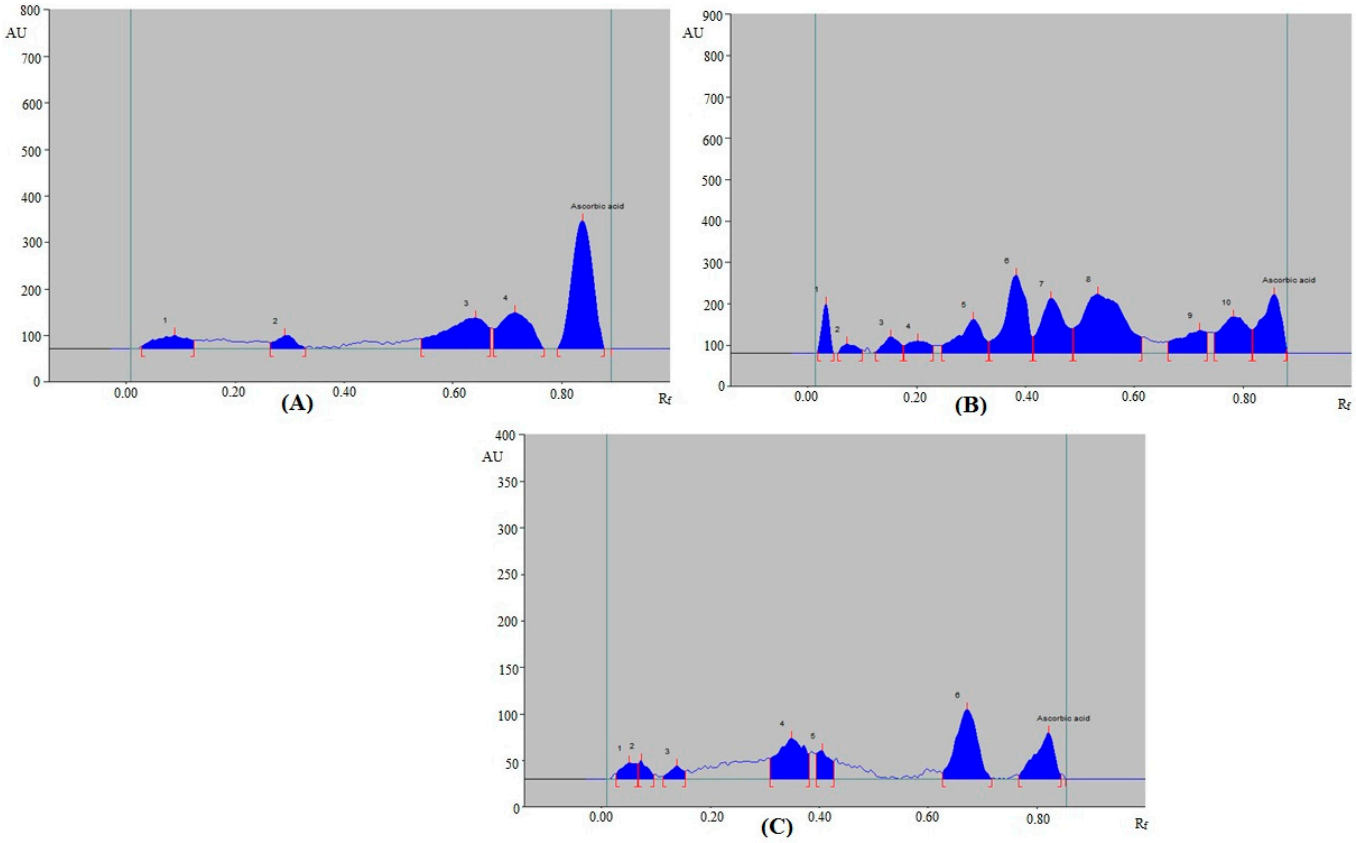

Figure 4. Representative HPTLC densitograms of AA in the UAE of (**A**) *P. emblica*, (**B**) *P. guajava*, and (**C**) *C. annuum*.

Table 6. Application of the greener HPTLC method for the estimation of AA in the SE and UAE of *P. emblica*, *P. guajava*, and *C. annuum* [a].

Samples	SE	UAE
	Amount of AA (mg/100 g)	
P. emblica (Amla)	491.16 ± 2.34	673.02 ± 3.16
P. guajava (Guava)	168.91 ± 1.41	218.71 ± 1.67
C. annuum (Capsicum)	144.30 ± 1.17	199.30 ± 1.32

[a] Mean ± SD; $n = 3$.

3.4. Greenness Assessment

For the quantitative estimation of the greenness of analytical methods, various methodologies have been used [37–42]. Only the "AGREE methodology", on the other hand, applies all twelve GAC principles [41]. As a consequence, the developed analytical method's greenness index was determined using the "AGREE methodology". Figure 5 depicts the calculated AGREE index for the developed analytical method using the twelve GAC principles. The overall AGREE index for the developed analytical method was 0.88, demonstrating that the developed analytical method was extremely green for determining the AA in studied samples. A single green HPTLC technique has been applied in the determination of AA in fruit juices and pharmaceutical formulations. The quaternary combination of ethyl acetate–acetone–WTR–formic acid was used as the green solvent system in this study [15]. However, the greenness index of this method has not been reported. As a consequence, the greenness index of the present HPTLC methodology could not compare with the reported HPTLC technique of AA estimation.

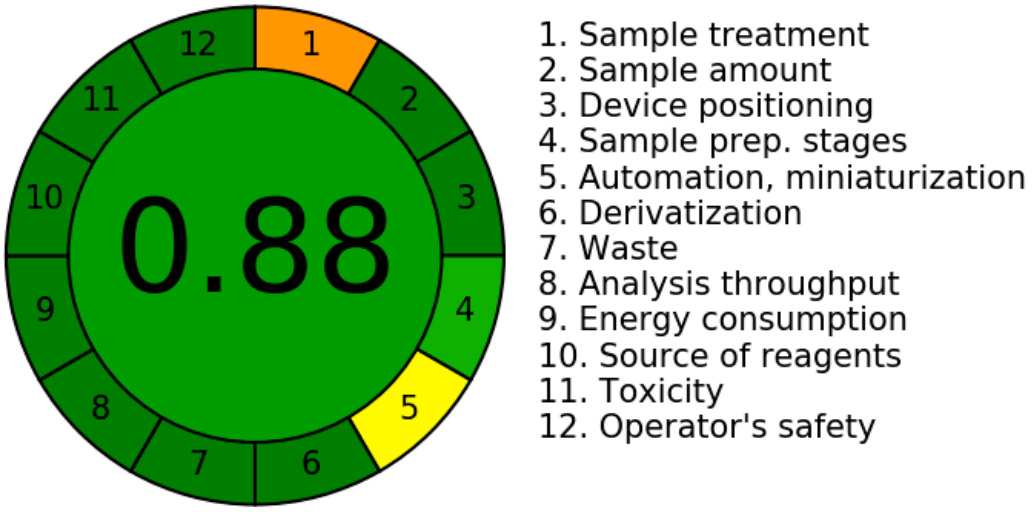

Figure 5. "Analytical GREEnness (AGREE)" index for the developed method.

3.5. Comparison with Reported HPTLC Methods

The developed HPTLC method of AA estimation was compared with reported HPTLC methods of AA estimation. The findings of the comparative analysis are included in Table 7. Three different validation parameters, such as "linearity, accuracy, and precision", in addition to the solvent system and environmental toxicity of the developed HPTLC method, were compared with reported HPTLC methods. The linearity range, accuracy, and precision of a reported HPLC method have been reported as 200–800 ng/band, 98.25–99.12%, and 0.60–1.30%, respectively [15]. The linearity range of this method was much inferior to the present HPTLC method. The solvent system used in this study was green (ethyl acetate–acetone–WTR–formic acid), but the greener index of this method was not estimated using any of the reported quantitative approaches [15].

Table 7. Comparative assessment of the greener HPTLC method with reported HPTLC methods of AA analysis.

Analytical Method	Linearity Range	Accuracy (% Recovery)	Precision (% CV)	Solvent System	Environmental Toxicity	Ref.
HPTLC	200–800 (ng/band)	98.25–99.12	0.60–1.30	Ethyl acetate–acetone–water–formic acid	Non-toxic/green	[15]
HPTLC	400–2400 (ng/band)	100.41	0.98–1.11	Chloroform–acetone–trifluoroacetic acid	Toxic	[16]
HPTLC	400–1400 (ng/band)	99.25–99.97	0.18–0.23	Ethyl acetate–methanol–formic acid	Toxic	[17]
HPTLC	1000–15,000 (ng/band)	100.10–101.58	0.48–1.77	Toluene–ethyl acetate–methanol–acetic acid	Toxic	[18]
HPTLC	25–1200 (ng/band)	99.05–101.28	0.53–0.81	Water–ethanol	Non-toxic/greener	Present work

The accuracy and precision of other literature HPTLC methods were within the limit of ICH guidelines and hence similar to the present HPTLC method [16–18]. However, the linearity range of all these methods was much inferior to the present HPTLC method. In addition, the solvent systems of all these methods, such as chloroform–acetone–trifluoroacetic acid, ethyl acetate–methanol–formic acid, and toluene–ethyl acetate–methanol–acetic acid were environmentally toxic compared with the greener solvent systems (WTR–EOH) of the present HPTLC method [16–18]. Overall, the present HPTLC method to estimate AA was found to be superior to all reported HPTLC methods of AA analysis.

4. Conclusions

The goal of this study was to invent and validate a simple, cost-effective, sensitive, and green reverse-phase HPTLC method for measuring AA in the SE and UAE of *P. em-blica*, *P. guajava*, and *C. annuum*, due to the scarcity of greener analytical methodologies for determining AA in the literature. The developed analytical method for estimating AA is simple, cost-effective, sensitive, accurate, precise, robust, and green. The UAE of *P. emblica*, *P. guajava*, and *C. annuum* had much more AA than their corresponding SE. As a result, it is recommended that the UAE process be used to extract AA from *P. emblica*, *P. guajava*, and *C. annuum*. The calculated overall AGREE index indicated the excellent green profile of the developed method for AA analysis. The present HPTLC method was found to be superior to other reported HPTLC methods of AA analysis. These findings indicate that the developed HPTLC method can be used to estimate AA in a variety of food, plants, and pharmaceutical products containing AA as one of the ingredients.

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