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Simultaneous Estimation of Quercetin and Hesperidin in Herbal Formulations Using a Validated RP-HPLC Method

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Abstract

Background: A green, rapid, and reliable analytical method is required for simultaneous estimation of flavonoids in herbal formulations used for the management of Polycystic Ovary Syndrome (PCOS). **Aim:** To develop and validate an eco-friendly Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) method for simultaneous estimation of quercetin and hesperidin in a polyherbal formulation following Green Analytical Chemistry (GAC) principles. **Methods:** Chromatographic separation was achieved on a C18 column (250 × 4.6mm, 5µm) using a low-toxicity methanol–water mobile phase (55:45, v/v). Isocratic elution was performed at a flow rate of 0.6 mL/min with a total run time of 8 minutes to minimise solvent consumption. The developed method was validated according to the International Council for Harmonisation (ICH) Q2(R1) guidelines. We assessed greenness using the Complex Green Analytical Project Index (GAPI), Appraisal of Guidelines for Research and Evaluation (AGREE), Eco-Scale, and Blue Applicability Grade Index (BAGI) tools.**Results:** The method showed good linearity in the concentration range of 20–120 µg/mL with correlation coefficients of 0.9994 for quercetin and 0.9999 for hesperidin. The limits of detection and quantification were 3.19 and 6.90 µg/mL for quercetin, and 0.066 and 1.74 µg/mL for hesperidin, respectively. Precision studies showed %RSD values below 2%. Accuracy studies demonstrated recovery between 98.5% and 101.8%. Greenness evaluation indicated reduced environmental impact with an AGREE score of 0.76 and an Eco-Scale score of 85. **Conclusion:** The proposed RP-HPLC method is rapid, simple, validated, and environmentally sustainable. It is suitable for routine quality control analysis of quercetin and hesperidin in herbal formulations intended for PCOS management.

Major Findings: A simple, green RP-HPLC method was developed for simultaneous estimation of quercetin and hesperidin that required less than 8 min separation time with a low-toxicity mobile phase (methanol–water), which showed high linearity ($R^2 \geq 0.999$), precision (%RSD < 2%), and recovery (98–102%). It has been confirmed by greenness assessment (ComplexGAPI, AGREE, Eco-Scale, BAGI) to have less environmental impact and good analytical performance. The method is simple, ecological and reliable for standard quality control of herbal formulations containing flavonoids.

Keywords: AGREE, Eco-Scale, Green Analytical Chemistry, Hesperidin, Quercetin, RP-HPLC

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1. Introduction

Sustainable chemistry, commonly referred to as Green Chemistry (GC), focuses on designing chemical processes and products that have minimal environmental footprint through reduced waste generation and safer chemical use. damage through the reduction of waste, conserving energy and the use of materials that are safer and friendlier to the environment[1]. Its history dates back to the increased awareness of the environment in the mid-20th century, especially after the publication of the book titled 'Silent Spring' by Rachel Carson, bringing forth the harmful environmental characteristics of industrial chemicals such as pesticides[2]. GC as a discipline officially developed during the 1990s, as a concept of 12 green chemistry principles was proposed by Paul Anastas and John C. Warner as a set of principles intended to minimise

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the environmental and health effects of chemical manufacturing[3–7]. utilised in the pharmaceutical sector as the most efficient and adaptable method for the quantitative analysis of mixture components. Nonetheless, most HPLC techniques do not adopt environmentally friendly practices. The concept of "green analytical chemistry" emerged in the 2000s [8,9]. and is concerned with coming up with methods that have little or no impact on the environment and yet ensure the safety of the analysts[10]. Preferably, green chemistry champions the absence of vile solvents and reagents or restricting them to the bare minimum in duration of analysis, and energy-saving analysis instruments[11]. Some useful instruments include a more recent index called Analytical Greenness Metric (AGREE)[12]. Such techniques provide detailed data on the process that the sampling to the analysis process entails. These techniques will give in-depth details about the methodology, including sampling to analysis, and it is suggested to employ these tools in order to have a clear report on the greenness of the method. The key aim is to design and justify an HPLC procedure for the quantitative analysis of Quercetin and hesperidin through a green method (Figure 1). Quercetin, a bioflavonoid prevalent in foods such as grapefruit, onions, and berries, has been shown to have strong therapeutic potential, especially for Type 2 diabetes and its associated complications in cell line models[12-14]. Quercetin has been found to have a comparable effect to a widely prescribed antidiabetic drug, metformin, in cell line models. Also, *in vivo* experiments demonstrate that quercetin is a promising drug to treat diabetes and its pathophysiological complications[15,16]. Quercetin has effects on various pathways encompassed by diabetes, so it is able to regulate some of the most important signalling processes that would improve the symptoms and pathophysiological complications of Type 2 diabetes[17-19].

Figure 2, (7-O -rutinoside) and its aglycone hesperidin (4 -methoxy3,5,7- trihydroxy flavanone) are found in citrus fruits with a high concentration of flavonoids (grapefruits, lemons, and oranges). Hesperidin has anti-inflammatory, antiviral, anticancer, and antidepressant-like effects in animal models and was found to be useful in the treatment of numerous different forms of central nervous system diseases in humans[20]. Hesperidin is used in numerous Ayurvedic preparations sold to health-care providers[21]. Monitoring of the quality of hesperidin-containing formulations is of vital importance in Ayurveda formulations. In the literature, there are analytical methods used to estimate the amount of hesperidin in different plant extracts, such as spectrophotometric[22], HPLC[23] and HPTLC [24] methods. The mentioned methods have their limitations, which include lengthy time consumption and the need to use expensive and complicated chemicals. The literature review revealed that no Food and Drug Administration (FDA)-approved ultraviolet (UV) spectrophotometric technique exists that can be used to determine the presence of hesperidin in pure form in Ayurvedic preparations and novel topical nano preparations. Consequently, the present paper also sets out to design and prove a simple, fast, sensitive, accurate and precise UV-spectrophotometric procedure to measure Hesperidin.

2. Materials and Methods

2.1 Reagents and Chemicals

Hesperidin and quercetin were acquired from TCI Chemicals (Mumbai, India). Ethanol (≥99.5%), acetic acid and Milli-Q water were purchased from Parul

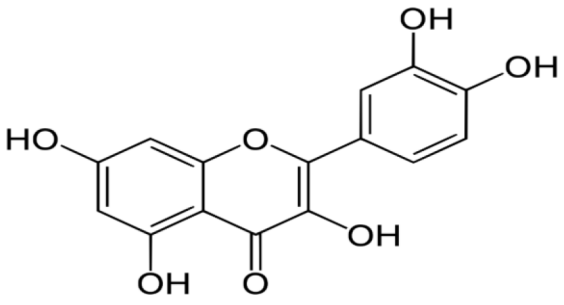


Figure 1. Structure of Quercetin.

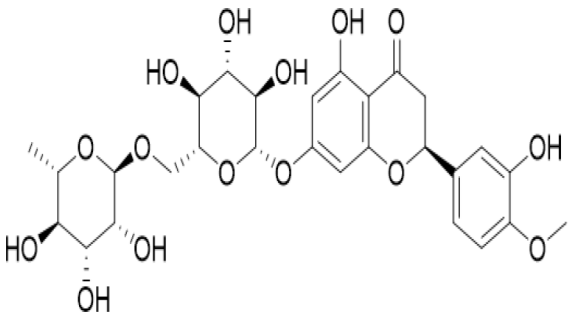


Figure 2. Structure of Hesperidin.

Institutes of Pharmacy (Vadodara, Gujarat, India. HPLC-grade methanol was purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). All the reagents were environmentally friendly and followed the GAC recommendations.

2.2 Instruments and Chromatographic Conditions

Separation was chromatographically performed using a Shimadzu LC-2010CHT HPLC system fitted with a Photodiode Array Detector (PDA). The C18 column (250 × 4.6mm, 5µm) was used. The mobile phase comprised methanol: Water (55:45 v/v); detection wavelength: 227nm; column temperature: 30°C; run time: less than 8 min.

2.3 Diluent

The diluent methanol-water (55:45 v/v) was used to ensure it was eco-friendly and reduced toxicity.

2.4 Equipment and Software

A Shimadzu HPLC system, model LC-2050C, fitted with a PDA, was used for chromatographic analysis. Shim-pack Gist C18 (250 × 4.6mm, 5µm) column was used for the separation, along with an autosampler injector (10µL injection volume). Mobile phase was made of methanol: water mobile phase. The flow rate was maintained at 0.6mL/min and identification was done at 227nm wavelength. The samples were weighed using a Sartorius sensitive electronic balance, and degreasing of the mobile phase was done using a sonicator ultrasonic liquid processor. The pH of the buffer solution was measured with the help of a pH meter (µ Controller based pH System 361) and a glass electrode (Systronics, India). The filtration of the mobile phase was carried out on a Millipore 220-220 glass filter and Thermo Scientific™ Nylon Syringe Filters with 0.20µm and 0.45µm pore sizes.

2.5 Preparation of Diluent

In order to enhance the stability of the product, the diluent was prepared by adding methanol and water in a 5:95 v/v ratio.

2.6 Preparation of Standard Solutions

The accurate measurement of 10mg of Quercetin and Hesperidin was performed, and then a 10mL volumetric flask was filled to the mark with a mobile phase consisting of methanol and water (55: 45 v/v). The sample was sonicated for 5 minutes in order to make the sample fully dissolve, and a 0.22 µm nylon syringe filter was used to filter the solution before analytical analysis.

2.7 Method Validation

The validated chromatographic procedure was authenticated well following the procedures implemented in the ICH standards Q2 (R1) of analytical method validation[25-27].

2.8 Specificity

The accuracy of the developed HPLC method was confirmed by verifying that the target analytes were effectively separated from potential interfering substances, including contaminants, degradation products, and excipients. Chromatographic analysis was performed after injecting 10µL of individual standard

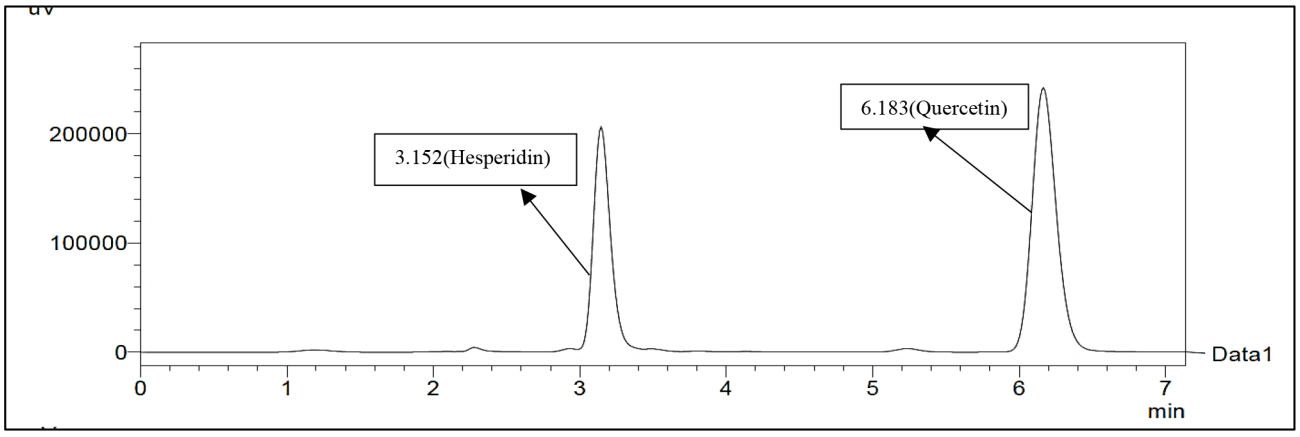


Figure 3. Specificity chromatogram of Hesperidin and Quercetin.

solutions or excipient mixtures. No interfering peaks were observed at the retention times of Hesperidin (3.152min) or Quercetin (6.183min) (Figures 3 and 4). These results confirm that the method is specific and reliable for the precise quantification of Quercetin and Hesperidin. No interference from other peaks in the blank as well as the mix was observed.

2.9 System Suitability

The parameters to verify the system's applicability were: A 10µL volume of a standard solution combination comprising Hesperidin and Quercetin at an amount of 20 µg/mL was injected under optimised chromatography conditions of use (Table 1 and Figure 5)

2.10 Linearity and Range

In order to determine the linearity and range of the developed approach, different concentrations of Quercetin (QUE) and Hesperidin (HESP) stock solutions, 20 µg/mL to 120 µg/mL, were obtained using the mobile phase (Methanol and water 55:45 v/v). Each test concentration was determined six times (n=6). The peak areas were plotted against their sample concentrations. The data were evaluated using the least-squares method. Linearity was observed by looking at the Regression coefficient (R^2) and the y-intercept from the calibration curve, which shows the response in relation to concentration. (Figure 6 and Table 2)

2.11 LOD and LOQ

Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated from the standard deviation of the response (σ) and slope of the calibration curve using LOD = 3.3 × (σ/slope) and LOQ = 10 × (σ/slope) (Table 3).

2.12 Precision

Intra-day precision was assessed by analysing three different concentrations of QUE and HESP (60, 80 and

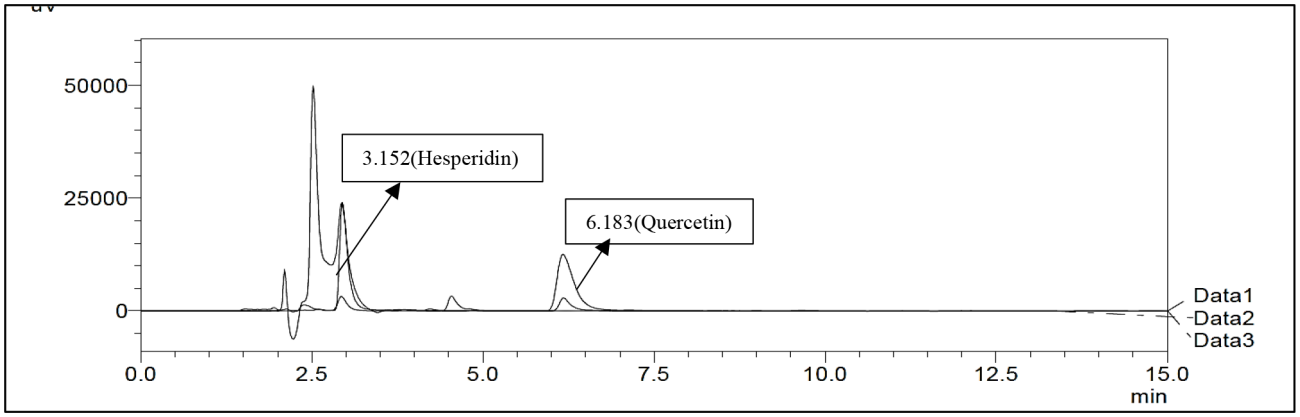


Figure 4. Sample chromatogram of the herbal formulation.

Table 1. System suitability parameters for Quercetin and Hesperidin

Sr. No	Quercetin			Hesperidin		
	RT	TP	Tailing	RT	TP	Tailing
1	3.16	3174	1.225	6.222	6270	1.164
2	3.152	3155	1.234	6.183	6197	1.168
3	3.142	3156	1.229	6.16	6193	1.167
4	3.139	3147	1.229	6.15	6169	1.168
5	3.144	3153	1.224	6.164	6190	1.168
6	3.144	3155	1.223	6.166	6166	1.167
Mean	3.146833	3156.667	1.227333	6.174167	6197.5	1.167
SD	0.007	9.09	0.004	0.02	37.78	0.001
%RSD	0.24	0.28	0.33	0.41	0.609	0.132

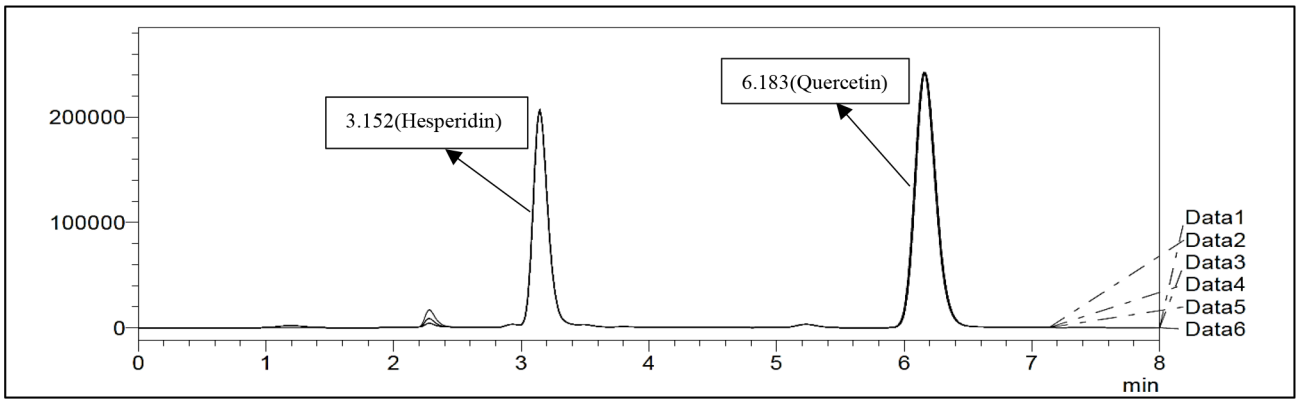


Figure 5. System suitability chromatogram.

Table 2. Linearity table for Quercetin and Hesperidin

Quercetin		Hesperidin	
Conc (µg/mL)	**Peak Area**	**Conc (µg/mL)**	**Peak Area**
20	74179	20	94607
40	99887	40	125450
60	122652	60	155190
80	145180	80	184260
100	168690	100	214494
120	192206	120	243439

Table 3. Sensitivity table for Quercetin and Hesperidin

Drug	LOD((µg/mL)	LOQ(µg/mL)
Quercetin	3.190941	6.905293
Hesperidin	0.066496	1.745749

100 µg/mL) at multiple time measurements on the same day. Inter-day precision was further assessed by repeating the analysis the next day to ensure the method's accuracy; both the Standard Deviation (SD) and % Relative Standard Deviation (RSD) values were evaluated (Tables 4 and 5).

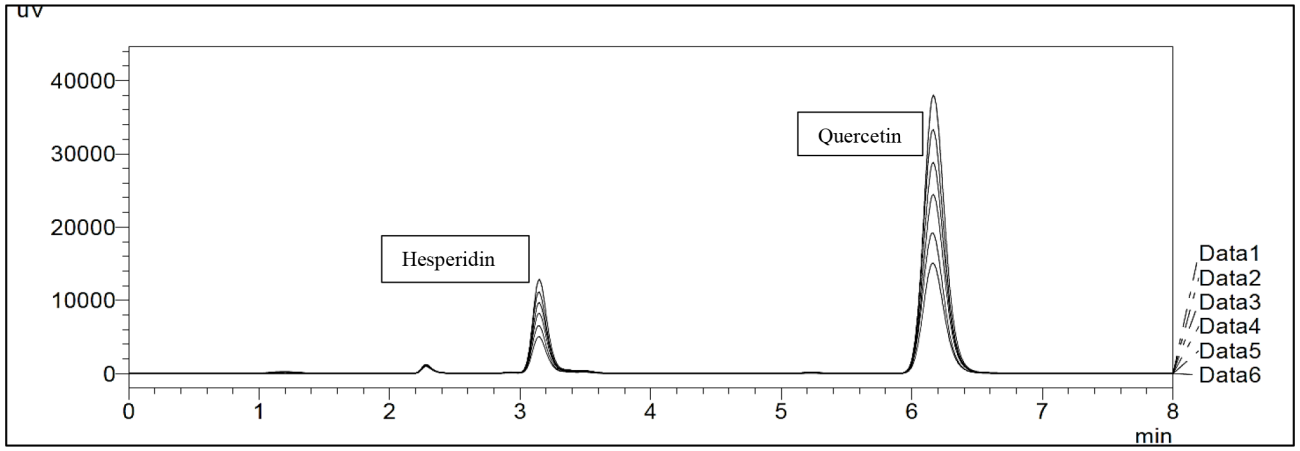


Figure 6. Linearity chromatogram of Quercetin and Hesperidin.

Table 4. Intraday table for Quercetin and Hesperidin

Drug	Conc. (µg/mL)	Area 10 am	Area 12 pm	Area 2 pm	Mean	SD	%RSD
Hesperidin	60	122652	122452	122276	122460	188.12	0.15
	80	145239	145432	145580	145417	170.99	0.11
	100	168690	168790	168980	168820	147.30	0.08
Quercetin	60	155190	155240	155430	155286.7	126.62	0.08
	80	184260	184980	184298	184512.7	405.16	0.21
	100	214387	214494	214594	214491.7	103.51	0.04

Table 5. Interday table for Quercetin and Hesperidin

Drug	Conc. (µg/mL)	Area Day1	Area Day2	Area Day3	Mean	SD	%RSD
Hesperidin	60	122652	124532	125276	124153.3	1352.36	1.08
	80	145239	145632	145765	145545.3	273.50	0.18
	100	168690	168987	168989	168888.7	172.05	0.10
Quercetin	60	155190	154540	154654	154794.7	347.08	0.22
	80	184260	185765	186298	185441	1056.92	0.56
	100	214387	214494	214594	214491.7	103.51	0.04

Table 6. Accuracy table for Quercetin and Hesperidin

Drug	Level	Amount of sample µg/mL	Amount of Std. Spike µg/mL	Total Amount µg/mL	Amount Found µg/mL	%Recovery
Hesperidin	50%	2.25	1.13	3.38	3.35	99.11
	100%	2.25	2.25	4.5	4.55	101.11
	150%	2.25	3.38	5.63	5.55	98.58
Quercetin	50%	3.5	1.75	5.25	5.20	99.05
	100%	3.5	3.5	7	7.1	101.43
	150%	3.5	5.25	8.75	8.63	98.63

Table 7. Robustness data for Quercetin and Hesperidin at different wavelengths

Wavelength	Quercetin			Hesperidin		
	60 µg/mL	80 µg/mL	100 µg/mL	60 µg/mL	80 µg/mL	100 µg/mL
226 nm	125987	145180	167602	155190	184260	214494
227 nm	126138	145138	167931	155436	185776	218339
228 nm	126208	145208	167991	155575	185835	218497
Mean	126111	145175.3	167841.3	155400.3	185290.3	217110
SD	112.94	35.23	209.42	194.96	892.78	2266.89
%RSD	0.089	0.024	0.124	0.125	0.481	1.044

2.13 Accuracy

To check the uniformity of the method, recovery evaluation was conducted to assess the suitability of the designed procedure. To determine accuracy, placebos with known concentrations of QUE and HESP were subjected to the proposed method to measure the accuracy over the nominal concentrations of 50,100 and 150 of the expected concentration levels. Accuracy was assessed through the percentage of recovery obtained from the recovered quantity of QUE and HESP (Table 6).

2.14 Robustness

The designed method was tested by changing the chromatographic conditions including the method was tested at flow rates of 0.5 and 0.7 mL/min and wavelengths of 226 and 228nm. Changes in Retention Time (RT) and peak area were assessed by computing the per cent RSD for each parameter (Tables 7 and 8).

2.15 Method of Greenness and Whiteness Assessment

Various green analytical assessment methods, including ComplexGAPI, AGREE, the Eco-Scale, and BAGI, were used to assess the method's environmental sustainability.

2.16 GAPI Software

The latest instrument created for environmental friendliness used in analytical techniques is the

Table 8. Robustness data for Quercetin and Hesperidin at different flow rates

Flow Rate	Quercetin			Hesperidin		
	60 µg/mL	80 µg/mL	100 µg/mL	60 µg/mL	80 µg/mL	100 µg/mL
0.5 mL/min	124987	145180	166602	155436	185776	218339
0.6 mL/min	126138	145234	167931	155436	185776	218339
0.7 mL/min	127208	145432	169091	155875	186435	218997
Mean	126111	145282	167874.7	155582.3	185995.7	218558.3
SD	1110.74	132.68	1245.45	253.45	380.47	379.89
%RSD	0.88	0.091	0.74	0.162	0.204	0.173

ComplexGAPI software, which is a sophisticated variation of the popular GAPI indicator. This software is exclusively aimed at analysing environmental performance through the selection of the following critical parameters, including collecting and preparing samples, the nature of used reagents, energy expenditure, waste, and environmental footprint[28-30]. The use of scores awarded on the basis of these factors allows ranking the methods and using the most eco-friendly one as the result of the ComplexGAPI[31-33].

2.17 AGREE Software

Analytical GREEnness metric is quite well-known as one of the crucial instruments to assess the environmental footprint of analytical procedures. It is a self-assessment instrument that can be found in a downloadable format. The AGREE tool is a software-based evaluation system (accessible at https://mostwiedzy.pl/AGREE) designed around the 12 principles of GAC[34-35]. It assesses factors such as reduced energy and solvent consumption, lower waste production, and improved safety, assigning a score between 0 and 1, where a higher value indicates better compliance with green analytical practices. These individual scores are displayed in the form of a radar diagram, providing a visual summary of the method's overall level of greenness. This holistic assessment not only reflects the environmental impact of an analytical procedure but also highlights specific areas where laboratory sustainability can be improved[36,37].

2.18 ESA Tool

The Eco Scale Assessment (ESA) aims to assess the environmental effect of analytical processes. This scale determines different variables such as the usage of resources, emission levels, and safety, among others, giving a rating that reflects the general friendliness of the approach. All the principles, including reducing consumption of toxic chemicals, minimising energy, and greener solvents, are calculated from the assessment. The ESA score obtained, and which is 0 to 100 in number, is a clear indication of how efficient the method in question is in terms of the environment. The Eco-Scale provides a continuous development of analytical practice as it emphasises the areas of improvement, promoting the appearance of greener approaches[38-40].

2.19 Blue Applicability Grade Index (BAGI)

BAGI is a key parameter in analytical chemistry that evaluates non-toxic and practical analytical methods by assessing multiple performance criteria. A higher BAGI score indicates greater practicality and reliability of the proposed method. Based on ten evaluation criteria, BAGI provides both a numerical score and a graphical representation, offering a comprehensive assessment of environmental acceptability and practical applicability of the analytical approach[41,42].

3. Result and Discussion

3.1 Checking the Method of Analysis

The new method was tested to determine whether it works well in everyday lab work. As the process had to meet the ICH Q2(R1) standards, aspects such as how straight the response is, the useful measurement span, the lowest detectable levels, the correctness, the repeatability, and the resistance to small changes were checked. The chromatographic conditions for this method for simultaneous estimation of HESP and QUE were systematically developed using an RP-HPLC system with a PDA detector and an autosampler injector (Model-SIL-10ADvp). A C18 column

(Phenomenex Luna, 250 × 4.6 mm, 5 µm) was used at a temperature of 30 °C, and the mobile phase was composed of methanol and water (55:45)v/v mobile phase, wavelength of detection: 227nm; temperature of the column: 30°C; and run time: less than 8 min. The flow rate was set at 0.6mL/min, and the injection volume for all analyses was 10µL. System suitability tests were run to ensure that the HPLC setup was effective. All the key parameters are listed in Table 1 and Figure 5. The tailing factor was less than 2, so the peaks looked good and symmetrical. The number of theoretical plates was over 2000, which means that the column worked efficiently. The resolution topped 1.5—so the system separated the peaks well, with no overlap or interference. All of these results show that the method produced sharp, clean peaks and effectively separated the analytes. Moreover, the %RSD for every peak remained under 2%. This tells us that the system performed reliably, with repeatable results across several injections, all under the same conditions. For both QUE and HESP, results were very consistent from 20 to 120 µg/mL, as shown in Table 2, with near-perfect linearity, with values of 0.9994 and 0.9999. This calibration helps determine the smallest amounts detectable and quantifiable. For quercetin, the detection limit was 3.19 µg/mL, whereas the quantification limit was 6.90 µg/mL; Meanwhile, HESP had a detection limit of 0.066 µg/mL, with a concentration of 1.74 µg/mL, as shown in Table 3. In addition, setup reliability was checked through RSD analysis across several run traits, such as how long the peaks remained, their size, shape symmetry, and column efficiency, and every value remained within the normal range. These parameters had RSD values that were less than 2%, which shows that the method is acceptable. Precision measurements, which were performed via intraday and interday measurements, indicated an RSD of less than 2% for both drugs, shown in Tables 4 and 5. An RSD of less than 2 was also observed by performing robustness studies where minor changes to the chromatographic conditions, including the solvent ratio, flow rate, and wavelength detection, were made, and the results are described in Tables 7 and 8. The level of accuracy based on the percentage of recovery was used to indicate the level of closeness of the measured values to the actual values. The results obtained through the experiment at concentrations of 50, 100 and 150% demonstrated a high degree of accuracy of the new method developed. Table 6 describes the results of the accuracy studies. Therefore, the proposed RP-HPLC method is suitable for routine quality control and stability testing of herbal formulations containing QUE and HESP.

3.2 Greenness and Whiteness Evaluation of the Suggested Approach

Using ComplexGAPI, the new method turned out to be safe for nature since risks stay low. Findings show up as colored symbols - five pentagrams stand for steps like prep work, chemicals used, tools involved, and technique type, while a hexagram covers early testing stages. Green hints at minimal harm, yellow points to some concern, red warns of danger needing immediate action. The E number hit 2 because eight greens popped up, just one red appeared, alongside six yellows - all

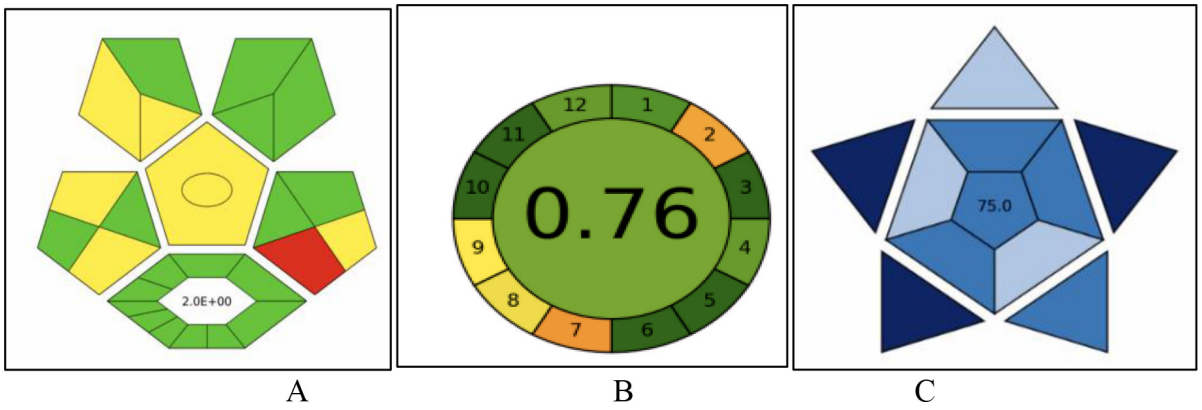


Figure 7. Greenness and whiteness profiling of the proposed analytical method using **(A).** ComplexGAPI, **(B).** AGREE, and **(C).** BAGI.

from the suggested HPLC way (Figure 7A). Using the greenness meter, twelve guidelines shaped a clock-style AGREE image - score sits in the middle, colors shift from dark green to bright red based on eco-impact. The suggested HPLC approach got 0.76 on AGREE, leaning toward greener tones (Figure 7). Eco-Scale works as a handy rough estimate tool - it starts at 100, then subtracts points for harm like waste, heavy power draw, or risky chemicals. When factoring in danger symbols during this scoring, the method hit 85, marking it solidly green. Results from BAGI testing revealed a mark of 75 (Figure 7C), backing its real-world usability and smooth setup.

4. Conclusion

A green, rapid, and reliable RP-HPLC method was successfully developed and validated for the simultaneous determination of quercetin and hesperidin in a novel herbal formulation intended for the management of PCOS. The method was designed in accordance with the principles of GAC, employing a low-toxicity methanol–water mobile phase, reduced solvent consumption, mild operating conditions, and a short analysis time, while maintaining excellent chromatographic performance. The method demonstrated satisfactory linearity, sensitivity, precision, accuracy, robustness, and system suitability, fully complying with the ICH Q2(R1) validation requirements. Comprehensive greenness and applicability evaluations using ComplexGAPI, AGREE, Eco-Scale, and BAGI confirmed the low environmental impact and high practical utility of the proposed method. Overall, this approach provides a sustainable and analytically sound alternative for routine quality control and standardisation of flavonoids in herbal pharmaceutical formulations, supporting the broader adoption of green practices in analytical laboratories. Assay results for the commercial herbal formulation demonstrated satisfactory content uniformity, supporting the applicability of the method for routine quality control. Overall, the proposed RP-HPLC method is robust, reproducible, and well suited for routine analysis and stability testing of herbal formulations containing quercetin and hesperidin, addressing a key analytical requirement for complex phytochemicals in herbal formulations.

5. Acknowledgement

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Author Contribution

Riya Rai: Conceptualisation, methodology, investigation, data interpretation, formal analysis, data curation, and manuscript writing. Dr Avani Khristi: Supervision, methodology, manuscript review and editing. All authors have read and agreed to the published version of the manuscript.