

Development of RP-HPLC-DAD method for quantitative analysis of quercetin and piperine in botanical extracts

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Abstract: A simple, effective, and fast reversed-phase high-performance liquid chromatographic method was devised to separate and quantitatively determine quercetin and piperine in herbal extract. The analytes were eluted on the Intersil C18 ODS column (150 mm×4.6 mm, 5µm) as a stationary phase with a gradient elution technique. The mobile phase was composed of a mixture of water (with 0.1% formic acid) and methanol (with 0.1% formic acid) and the gradient program of 30 minutes was optimized. The mobile phase flow rate was adjusted to 1 mL min⁻¹ for 20 µL injection volume. The eluents were detected by the PDA detector at 342 nm wavelength. At the optimized chromatographic conditions, the developed method was validated for the parameters including system suitability, specificity, linearity, accuracy, precision, and robustness per the ICH Q2(R1) guidelines. The developed method was applied for the determination of quercetin and piperine in the prepared herbal extract.

Keywords: Herbal extract; piperine; quercetin; RP-HPLC; validation. © 2024 ACG Publications. All rights reserved.

1. Introduction

Quercetin is a flavonoid derived from several plants including but not limited to *Curcuma domestica valetton*, *Cuscuta reflexa*, *Daucus carota*, *Emblica officinalis*, *Foeniculum vulgare*, *Glycyrrhiza glabra*, *Mangifera indica*, *Momordica charantia*, *Ocimum sanctum*, *Psoralea corylifolia*, *Santalum album*, *Solanum nigrum*, *Swertia chirayita*, *Withania somnifera*, *Moringa oleifera* and many of others [1]. Structurally, it is 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one and its chemical structure is given in Figure 1 [2]. Quercetin shows therapeutic potential for diseases like cardiovascular disease, cancer, and neurological disorders through its antioxidant and anti-inflammatory properties. Along with this, it has shown potential for improving sexual behaviour [3, 4].

Piperine is a naturally occurring alkaloid found in fruits of plants belonging to the *Piperaceae* family- *Piper nigrum* and *Piper longum*. It is known for its anti-inflammatory, antihypertensive, hepatoprotective, anticancer, and bioavailability-enhancing actions [5, 6]. Structurally, piperine is (2E,4E)-5-(1,3-benzodioxol-5-yl)-1-piperidin-1-ylpenta-2,4-dien-1-one as shown in Figure 1 [7].

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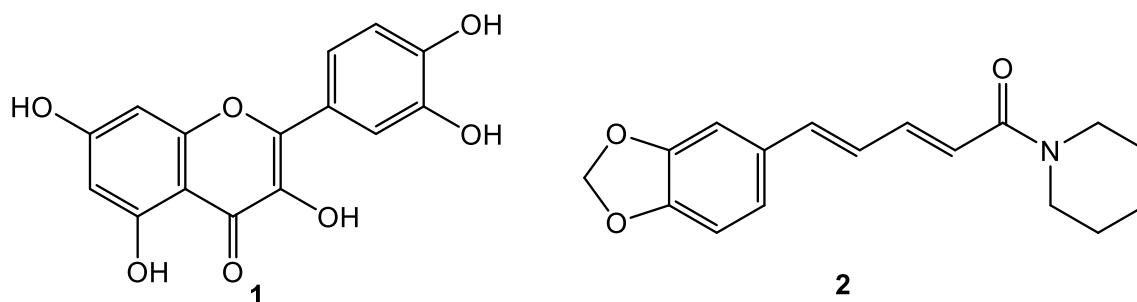


Figure 1. Chemical structure of (1) quercetin (2) piperine

High-performance liquid chromatography (HPLC) is a widely accepted and frequently employed chromatographic technique for the isolation and quantification of natural products. This method effectively separates compounds within complex mixtures and is extensively utilized in phytochemistry and analytical chemistry for the identification, purification, and quantification of specific constituents [8]. The primary objective of the developed analytical methods was to ascertain the purity and quantify the concentrations of quercetin and piperine within the extract. Validation of the analytical method delineated the performance parameters, thereby confirming its reliability for these measurements. Consequently, the developed method accurately determined the concentrations of quercetin and piperine present in the extract.

Numerous spectrophotometric [9-13] and chromatographic methodologies, including both normal and reverse-phase techniques [14-22], have been documented for the quantitative analysis of quercetin and piperine, either individually or in conjunction with other phytochemicals, within plant extracts or herbal formulations. A single validated analytical method has been described for the quantification of quercetin and piperine in nanostructured lipid carriers comprising both standard compounds [23]. When the same method was applied for the simultaneous determination of quercetin and piperine in the herbal extracts, the peak for quercetin merged with the solvent peak. No other technique has been reported for the simultaneous determination of quercetin and piperine in plant extracts, where the presence of other phytoconstituents may interfere with the analytical results. Consequently, the present study developed and validated a novel analytical method for the concurrent determination of quercetin and piperine in plant extracts. The validation process adhered to the guidelines set forth by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q2 (R1) [24].

2. Experimental

2.1. Chemicals and Reagents

Standard phytochemicals- quercetin (95%) and piperine (97%)- required for the experiments were purchased from Acros Organics, USA and Merck, India respectively. The remaining reagents, HPLC grade solvents and chemicals utilized for the development and validation of the present method were bought from Merck, India. High purity deionized water (Milli-Q) was obtained from Millipore (Prefil Kit Integral System, Merck) water purification system.

2.2. Chromatographic Conditions and Equipment

The analytical method for separation and quantitation of quercetin and piperine was developed on Shimadzu LC-20AD system (Shimadzu Corporation, Japan). The chromatographic instrument included a quaternary solvent pump, an auto-sampler, and a PDA detector (SPD-M40). The chromatographic signals were observed, analysed and recorded using LC solution (V 1.25) software. The chromatographic conditions such as solvent selection for solution preparation, stationary phase selection for separation, mobile phase selection for elution, and detection wavelength selection were based on the solubility and physicochemical properties of the standards. In brief, the initial trial for the mobile phase selection were carried out by isocratic elution method, trying different ration of acetonitrile: water and methanol: water. But, because of the vast difference in the polarity of two analytes, the experiments were shifted towards development of gradient elution method. The stock solutions of the standard phytochemicals and herbal

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extracts were prepared in methanol due to solubility and solution stability. The solutions were found to be stable even after 48 hours, though we preferred to prepare new solutions after two days. The prepared solutions were stored in the stoppered flasks at the refrigerated conditions throughout the studies. The final dilutions were made using the solvent mixture consisting of methanol (0.1% formic acid) and water (0.1% formic acid) (60:40 v/v) and were prepared from the stock solution for the daily use. Intersil C₁₈ ODS column (150 mm×4.6 mm, 5µm) was selected as a stationary phase. Gradient elution technique was selected for the analytical separation of two phytoconstituents using RP-HPLC method. The finalized gradient program is shown in Table 1. The flow rate of the mobile phase was adjusted to 1 mL min⁻¹ with 20 µL injection volume. The column oven temperature was fixed at 30 °C. the detection of the analytes was carried out at 342 nm wavelength.

Table 1. Optimized mobile phase (Gradient elution)

Time	0.1% FA in Water	0.1% FA in Methanol
0.01	50.00	50.00
9.00	50.00	50.00
11.00	30.00	70.00
20.00	30.00	70.00
25.00	50.00	50.00
30.00		Stop

2.3. Solution Preparation

Accurately weighed quercetin (50 mg) and piperine (5 mg) were transferred into two separate 50 mL volumetric flasks and dissolved in methanol. 50 mL of quercetin and 10 mL of piperine solutions were then transferred into a 100 mL volumetric flask and diluted to mark with the mobile phase to get the final concentration of 500 µg/mL and 10 µg/mL for quercetin and piperine, respectively. From the stock solutions, six serial dilutions were prepared in the concentration range of 50-300 µg/mL for quercetin and 0.5-3 µg/mL for piperine.

The test solution was prepared by transferring 20 mg *Moringa oleifera* extract and 10 mg *Piper nigrum* extract in a 10 mL volumetric flask. The content was mixed with methanol and sonicated for 20 minutes. The resulting solution was filtered using a 0.45 µm Millipore PVDF syringe filter.

The placebo solution used was a mobile phase consisting of a mixture of methanol (0.1% formic acid) and water (0.1% formic acid) (60:40 v/v) without any standard added.

2.4. Method Validation

The developed analytical method was validated in accordance with the International Council for Harmonisation (ICH) guidelines to prove its applicability for the denoted purpose. The studied validation parameters were specificity, detection limit, quantification limit, linearity, range, accuracy, precision, and robustness. The linearity solutions were prepared by mixing standard solutions of quercetin and piperine in the range of 50 - 300 µg/mL and 0.5 - 3 µg/mL respectively. The solution mixtures were injected six times and a linear regression equation was derived by plotting the mean peak area against concentration. The LOD and LOQ were calculated using the formulas specified in the ICH guidelines: $LOD = 3.3 \times \sigma/S$ and $LOQ = 10 \times \sigma/S$ where S is the slope of calibration curve and σ standard deviation of the response is the. The precision of the method was confirmed by carrying out repeatability studies and intermediate precision. The repeatability studies were performed by injecting six solutions of 150 µg/mL of quercetin and 1.5 µg/mL of piperine simultaneously on a single day. Intermediate precision was carried out as interday and intraday studies. Three different solutions containing 50, 150, and 300 µg/mL quercetin and 0.5, 1.5, and 3 µg/mL piperine respectively were injected. For interday precision, the solutions were injected in triplicate for three consecutive days. For intraday precision, the solutions were injected in triplicate on the same day. The mean peak area was calculated for both studies and the percentage relative standard deviation was determined. The accuracy solutions were prepared by adding 80%, 100%, and 120% level concentrations of standard quercetin and piperine solutions into the known, pre-analysed sample. The resulting solutions were injected in triplicate and percentage recovery was calculated for analytes. Small but deliberated

changes were made in some finalized chromatographic conditions including mobile phase composition ($\pm 0.02\%$ of formic acid in methanol and water) and flow rate ($\pm 0.1 \text{ mL min}^{-1}$), column oven temperature ($\pm 5^\circ\text{C}$) and detection wavelength ($\pm 2 \text{ nm}$) and %RSD was calculated. The results confirmed the robustness of the developed method [24].

3. Results and Discussion

3.1. Method Development

The present analytical method was developed for the effective separation and quantification of quercetin and piperine in prepared herbal extract. All the analytes were successfully separated with satisfactory peak parameters using a gradient elution technique in an Intersil C₁₈ ODS (150mm \times 4.6 mm, 5 μm) column. The gradient programming is shown in Table 1. All chromatographic conditions were finalized by considering the physicochemical properties and chemical structures of the analytes. Quercetin and piperine exhibit distinct polarity and solubility characteristics. Consequently, initial trials employing isocratic elution with a fixed polarity mobile phase mixture failed to elute the analytes adequately, resulting in suboptimal peak parameters. To address this issue, we transitioned to gradient method development. The gradual modification of the mobile phase composition in gradient methods facilitated the effective separation of both analytes, yielding satisfactory peak parameters.

3.2. Method Validation

3.2.1. System Suitability

The appropriateness of the chromatographic system for the intended analytical separation was assessed by performing system suitability studies. The system suitability was assessed by injecting six replicates of a solution mixture of quercetin and piperine at 50 $\mu\text{g/mL}$ and 0.5 $\mu\text{g/mL}$ respectively. Figure 2 represents the system suitability chromatogram in comparison with the placebo solution. The peak parameters corresponding to both analytes at system suitability conditions are presented in Table 2. All the parameters fall under the acceptance limits specified by ICH guidelines, suggesting the suitability and consistency of the developed method [24].

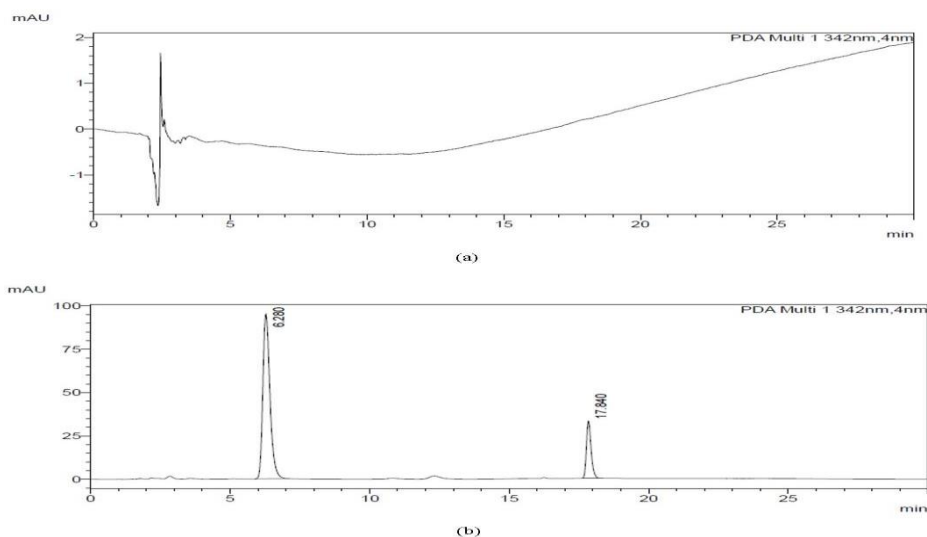


Figure 2. Chromatogram for (a) Placebo solution (mobile phase without analytes) (b) System suitability

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Table 2. System Suitability Data (mean \pm RSD%; n= 6)

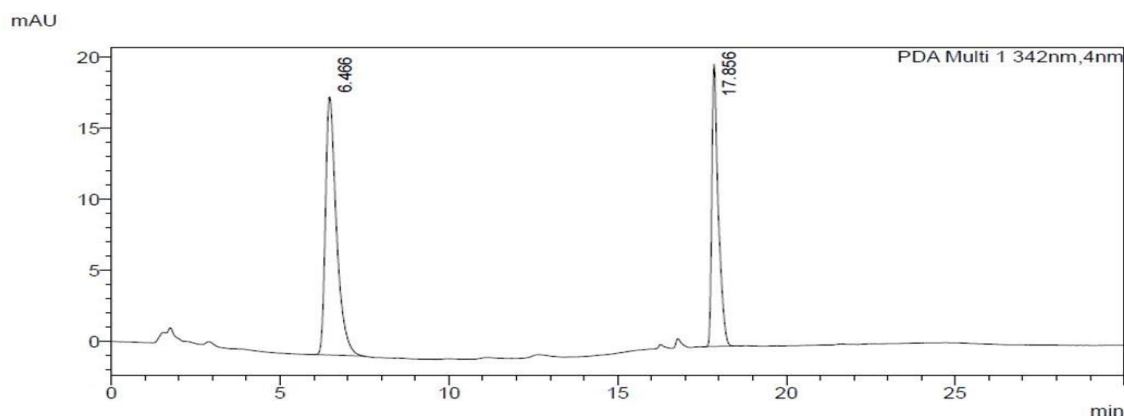
Parameter	Quercetin	Piperine
Relative retention (min)	6.393 \pm 0.42	17.867 \pm 0.32
Theoretical Plates (N > 2000)	21722 \pm 0.68	379328 \pm 0.82
Tailing factor (T \leq 2)	1.308 \pm 0.56	1.270 \pm 0.78
Resolution (R _s > 2)	--	30.602 \pm 0.46

3.2.2. Specificity

The peak purity of each analyte in the standard, sample and spiked solutions confirms that the developed method is specific for the analytes of interest only and their separation and quantification is not affected by the presence of other chemicals present in the solutions and solution mixtures [24]. A PDA detector was used to check the purity and uniformity of all peaks of quercetin and piperine. The peak purity index for both of the analytes found to be near 1, indicating unhindered separation of analytes in the herbal extract. The test solution containing *Moringa oleifera* and *Piper nigrum* extracts was injected into the HPLC system at the optimized chromatographic conditions and the amount of quercetin and piperine was calculated from the obtained peak area (Table 3). The chromatogram for the assay solution is shown in Figure 2.

Table 3. Results for Assay of Tablet Formulation (n=3)

	Quercetin	Piperine
<i>Moringa oleifera</i> extract	20 mg	-
<i>Piper nigrum</i> extract	-	10 mg
Amount	0.55 mg	0.01 mg
% Assay	2.75 % w/w	0.1 % w/w

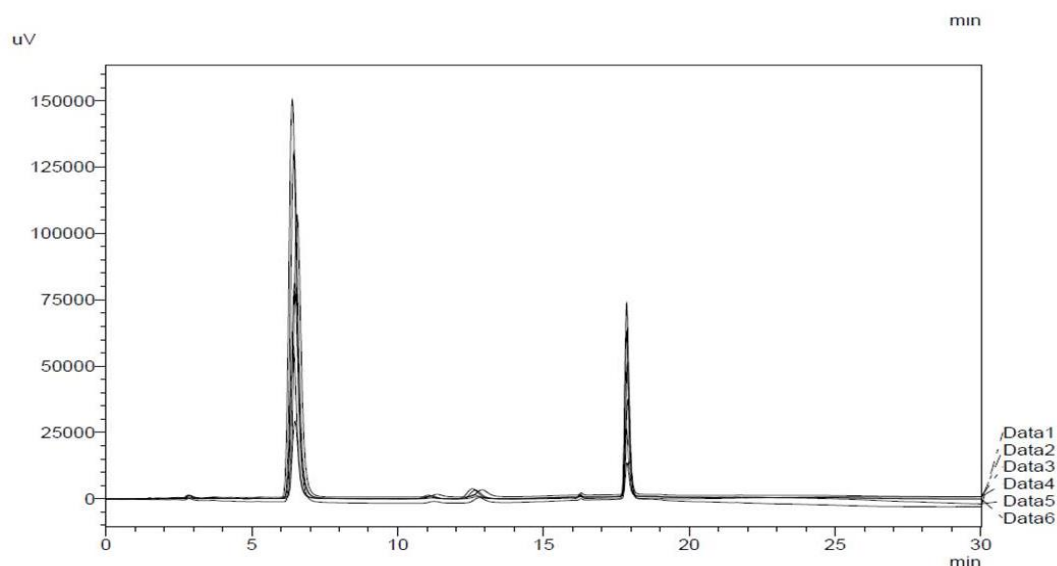
**Figure 2.** Assay chromatogram for the herbal extract

3.2.3. Linearity and Range

The linearity studies were performed to confirm that within the selected range of quercetin (50 $\mu\text{g/mL}$ to 300 $\mu\text{g/mL}$) and piperine (0.5 $\mu\text{g/mL}$ to 3 $\mu\text{g/mL}$), the responses obtained are in direct proportion to the concentration. The linear relation was confirmed with the plot of peak area vs. concentration. Figure 3 represents the overlay chromatogram for quercetin and piperine in the selected concentration range. The linearity equation is included in Table 4. For both analytes, the regression coefficient value is greater than 0.9, which confirms that the developed method is linear in the selected concentration range of analytes [24].

Table 4. Summary of Validation Parameters

Validation Parameter (Acceptable Limits)	Quercetin	Piperine
Peak Purity Index (>0.5)	1.00	0.99
Range (%)	25 – 150	25 – 150
Linearity ($\mu\text{g/mL}$)	50 – 300	0.5 – 3
Linearity Equation	$Y = 9216.5X + 1118.3$	$Y = 244213X + 3379.6$
r^2 (~1)	0.9999	0.9999
LOD ($\mu\text{g/mL}$)	3.09	0.03
LOQ ($\mu\text{g/mL}$)	9.37	0.10
Intraday Precision (%RSD)	0.86-1.01	0.63-0.95
Intermediate Precision (RSD% < 2)	1.21-1.96	1.08-1.37
Repeatability (%RSD)	0.95	1.21

**Figure 3.** Overlay chromatogram for Quercetin and Piperine

3.2.4. LOD and LOQ

The sensitivity of any analytical method is shown in terms of the lowest concentration, which the method can identify and quantify. They are known as limits of detection (LOD) and limits of quantification (LOQ) respectively [24]. For the present method, LOD and LOQ values are included in Table 4.

3.2.5 Accuracy

The suitability of the analytical method for the determination of samples in the matrix is confirmed by the accuracy studies to a great extent. The study confirms the optimum extraction of the analytes from the sample matrix. For the developed method, accuracy was assessed at 80%, 100% and 120% levels by spiking the known amount of analytes into the accuracy solution and calculating % recovery for each analyte, at each level [24]. The recovery data for the present method are presented in Table 5. The % recovery for both analytes were in the Ich acceptable range of 98 – 102 %, confirming the accuracy of the developed method for effective separation of quercetin and piperine in herbal formulations.

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Table 5. Results of Accuracy (n=3)

Recovery level	% Recovery (Mean \pm RSD%; n = 3)	
	Quercetin	Piperine
80 %	100.43 \pm 0.39	100.18 \pm 0.64
100 %	100.08 \pm 0.29	100.16 \pm 0.28
120 %	100.23 \pm 0.44	101.05 \pm 0.26

3.2.6. Precision

The results of repeatability and intermediate precision studies of the analytical method confirm the precision of the developed analytical method. The repeatability studies are performed by injecting sample solution at a medium concentration level in triplicates on the same day. The intermediate precision is performed at low, medium and high concentration levels in triplicates. When the samples are injected three times on the same day, the type of precision is intraday precision. When the samples are injected on three consecutive days in triplicates, the precision is inter-day precision. For each sample, the mean peak area and %RSD were calculated. %RSD below 2 confirms that the developed method is precise within the selected concentration range, as per ICH guidelines [24]. The %RSD for repeatability and intermediate precision are included in Table 4.

3.2.7. Robustness

The analytical methods' robustness demonstrates the reliability of the developed method when subjected to minor purposeful or unintentional alterations by the analyst. The devised method was validated by adjusting the column oven temperature, detecting wavelength, and mobile phase flow rate and mobile phase composition. The %RSD was computed for each chromatographic experiment, and the findings confirmed the suitability of the developed method [24]. The %recovery of analytes for each change is presented in Table 6. The %RSD value below 2 confirms the robustness of the developed method.

Table 6. Results of Robustness (n=3)

Parameter	Normal condition	Change in the condition	% Recovery (Mean \pm RSD%; n = 3)	
			Quercetin	Piperine
Formic acid in water	0.1 %	0.08 %	101.92 \pm 0.25	100.12 \pm 0.42
		0.12 %	100.67 \pm 0.45	100.69 \pm 0.62
Formic acid in methanol	0.1 %	0.08 %	99.62 \pm 0.45	101.74 \pm 0.45
		0.12 %	100.45 \pm 0.45	99.69 \pm 0.45
Flow Rate	1 mL min ⁻¹	0.9 mL min ⁻¹	101.68 \pm 0.17	99.04 \pm 0.52
		1.1 mL min ⁻¹	101.92 \pm 0.37	99.89 \pm 0.45
Column oven Temperature	30°C	25°C	101.60 \pm 0.15	99.33 \pm 1.01
		35°C	100.43 \pm 0.16	99.60 \pm 0.51
Wavelength	342 nm	340 nm	100.77 \pm 0.23	100.16 \pm 0.54
		344 nm	100.73 \pm 0.26	99.40 \pm 0.66

3.2.8. Assessment of Measurement Uncertainty

The analytical methods have been validated and an uncertainty assessment has been carried out to reduce errors from various experimental conditions. These conditions include instrumental calibration, chemical and solvent purity, sampling procedures, preparation of standard and sample solutions, and environmental factors such as temperature, humidity, and light exposure. The uncertainty evaluation for this method was performed in line with the EURACHEM/CITAC guide and relevant literature [25-26]. This report presents the combined uncertainty (U_{Combined}) and expanded uncertainty (U_{Expanded}) values. These

values are calculated from the uncertainties associated with standard preparation (U_{Standard}), the slope of the calibration curve ($U_{\text{Calibration}}$), recovery (U_{Recovery}), and repeatability ($U_{\text{Repeatability}}$) as given in equation 1.

$$U_{\text{Combined}} = \sqrt{(u_{\text{Standard}})^2 + (u_{\text{Calibration}})^2 + (u_{\text{Recovery}})^2 + (u_{\text{Repeatability}})^2} \quad (1)$$

The main factor contributing to uncertainty in the standard (U_{Standard}) preparation is the purity of the analyte. The purity percentage provided by the vendor was used to establish the U_{Standard} for each analyte, using Equation 2.

$$U_{\text{Standard}} = \frac{100 - \% \text{Purity}}{\sqrt{3}} \quad (2)$$

The uncertainty of calibration ($U_{\text{Calibration}}$) for each analyte was calculated using the slope of the calibration curve and the standard error of the slope, as outlined in equation 3.

$$U_{\text{Calibration}} = \frac{(\text{Standard Error of Slope} * 100)}{\text{Slope}} \quad (3)$$

The mean relative standard deviation (RSD) for the recovery tests is represented as U_{Recovery} , and for the repeatability studies, it is denoted as $U_{\text{Repeatability}}$. The expanded uncertainty at a 95% confidence level is calculated by multiplying the combined uncertainty by the coverage factor ($k = 2$). Table 7 provides the uncertainty profile for this method.

Table 7. Uncertainty assessment for the developed method

Uncertainty (U)	Quercetin	Piperine
u_{Standard}	2.88	1.73
$u_{\text{Calibration}}$	0.02	0.0003
u_{Recovery}	0.37	0.39
$u_{\text{Repeatability}}$	0.95	1.21
u_{Combined}	3.06	2.14
U_{Expanded}	6.12	4.29

4. Conclusions

A reverse-phase liquid chromatographic method with gradient elution was developed for the separation of quercetin and piperine in standard mixtures and herbal extracts. Under optimized chromatographic conditions, both analytes were separated with satisfactory peak parameters. The developed method was validated according to the ICH Q2(R1) guidelines. All system suitability parameters were within the acceptable range. The findings were within specified limits, confirming the method's suitability for its intended purpose. The optimized chromatographic conditions and validation analyses confirmed the method's selectivity, specificity, accuracy, linearity, precision, and robustness. Furthermore, the method was applied to the analysis of herbal extracts, where the amounts of quercetin and piperine were found to be 2.5% and 0.1% w/w, respectively.

Conflicts of Interest

Authors do not have any conflicts of interest to declare.

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Supporting Information

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