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Development and validation of a simple, accurate, and precise RP-HPLC method for the simultaneous estimation of hesperidin and quercetin in plant extracts

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Abstract
A new RP-HPLC method was developed for the simultaneous estimation of hesperidin and quercetin in plant extracts and it was validated as per ICH Q2(R1) guidelines. The chromatographic separation was found to be satisfactory on a Symmetry C-18 column (4.6×150mm, 5µm Thermosil) using phosphate buffer (pH 4.5) and acetonitrile in the ratio of 30:70 v/v at a flow rate of 1.0 mL/min. The retention times of hesperidin and quercetin were found to be 6.94 min and 10.50 min respectively, with UV detection at 238 nm. The system suitability parameters confirmed that the proposed method is suitable for simultaneous estimation of hesperidin and quercetin with excellent chromatographic performance.
The tailing factor for the peaks was found to be 0.995 and 1.203 for hesperidin and quercetin respectively, while the theoretical plates for separation were 3,605 and 7,581 respectively. The method demonstrated excellent linearity in the concentration range of 10-50 µg/mL for both compounds with correlation coefficients (r^2) of 0.999. The precision of the method was exceptional with relative standard deviation values well within acceptable limits, and the recovery of both analytes was within the acceptance range of 98-102%. The limit of detection (LOD) was determined to be 0.003 µg/mL for hesperidin and 0.09 µg/mL for quercetin, while the limit of quantification (LOQ) was 0.012 µg/mL for hesperidin and 0.3 µg/mL for quercetin.
The proposed RP-HPLC method was found to be suitable for the simultaneous estimation of hesperidin and quercetin in plant extracts and pharmaceutical formulations. The method is simple, selective, reproducible, and accurate with excellent precision, making it highly applicable for routine analytical purposes in quality control laboratories and pharmaceutical research institutions.

Keywords: RP-HPLC method development and validation; Hesperidin and quercetin simultaneous estimation; Plant extracts and pharmaceutical formulations; ICH Q2(R1) guidelines; Flavonoid analysis; Quality control; Pharmaceutical analysis

Introduction

Background and Significance of Analytical Method Development

The pharmaceutical industry continues to experience unprecedented growth in the development of new chemical entities (NCEs) and herbal formulations. This expansion necessitates the development of robust analytical methods capable of accurately quantifying active pharmaceutical ingredients in both bulk materials and formulated products.[3] Analytical method development and validation represent critical components of pharmaceutical quality assurance, serving as the foundation for establishing the identity, purity, potency, and stability of drug substances and drug products [1, 2].

High-performance liquid chromatography (HPLC) has emerged as the analytical technique of choice in pharmaceutical industries due to its exceptional advantages, including rapidity, specificity, accuracy, precision, ease of automation, enhanced sensitivity, improved resolution, and the capability to analyze complex mixtures without requiring extensive sample preparation procedures. The technique's versatility extends to the analysis of non-volatile, polar, and thermolabile compounds, making it particularly suitable for the analysis of natural products and flavonoid compounds [4-6].

The development of stability-indicating analytical methods has become increasingly important in pharmaceutical analysis. These methods must be capable of accurately quantifying active pharmaceutical ingredients without interference from degradation products, process impurities, or formulation excipients [7].

The International Conference on Harmonisation (ICH) guidelines provide comprehensive frameworks for analytical method validation, ensuring that developed methods meet stringent regulatory requirements across global markets [8].

Flavonoids: Therapeutic Importance and Analytical Challenges

Flavonoids constitute one of the most extensively studied classes of natural phenolic compounds, exhibiting a wide spectrum of biological activities including antioxidant, anti-inflammatory, anticancer, antimicrobial, and cardioprotective effects. These compounds are ubiquitously distributed in fruits, vegetables, medicinal plants, and various botanical extracts, contributing significantly to their therapeutic potential [9].

The pharmacological significance of flavonoids has prompted intensive research into their isolation, characterization, and quantitative analysis in various matrices. Modern pharmaceutical research has increasingly focused on developing novel drug delivery systems, including nanoparticle formulations, to enhance the bioavailability and therapeutic efficacy of flavonoid compounds. This trend has created an urgent need for sensitive, specific, and validated analytical methods capable of quantifying flavonoids in complex formulation matrices [10].

Contemporary analytical approaches for flavonoid analysis primarily employ chromatographic techniques, with HPLC emerging as the preferred method due to its high resolution, sensitivity, and reproducibility [11]. Reversed-phase liquid chromatography remains the predominant separation mode for flavonoid analysis, as it provides excellent selectivity based on the hydrophobic interactions between the analytes and the stationary phase [12].

Research Objectives and Methodology

This research addresses the critical need for a validated analytical method capable of simultaneous quantification of hesperidin and quercetin in plant extracts. The study employs a systematic approach to method development, incorporating rigorous optimization of chromatographic parameters followed by comprehensive validation according to ICH guidelines.

The primary objectives of this investigation include: (1) development of a simple, accurate, and precise RP-HPLC method for simultaneous estimation of hesperidin and quercetin, (2) optimization of chromatographic conditions to achieve optimal separation and detection, (3) comprehensive validation of the developed method following ICH Q2(R1) guidelines, and (4) demonstration of the method's applicability for routine analysis of plant extracts.

The methodological approach encompasses systematic evaluation of critical chromatographic parameters, including mobile phase composition, pH optimization, flow rate adjustment, and detection wavelength selection. Validation studies encompass assessment of specificity, linearity, accuracy, precision, robustness, and determination of detection and quantification limits.

Materials and Methods

Chemicals and Reagents

All chemicals and reagents employed in this study were of analytical or HPLC grade to ensure optimal analytical performance and minimize interference. Hesperidin (≥98% purity) and quercetin (≥98% purity) reference standards were procured from Yarrow Pharmaceuticals, ensuring compliance with pharmacopoeial specifications. High-performance liquid chromatography grade solvents, including water, acetonitrile, and methanol, were obtained from Merck to maintain analytical reliability and reproducibility.

The mobile phase components consisted of orthophosphoric acid (HPLC grade, Merck), potassium dihydrogen orthophosphate (GR grade, Rankem), and disodium hydrogen phosphate (GR grade, Rankem). The selection of high-purity reagents was critical for achieving optimal chromatographic performance and minimizing baseline noise and interference.

Instrumentation and Equipment

The analytical system comprised a Shimadzu binary HPLC system (LC-10) equipped with a Rheodyne injector featuring a 20 µL sample loop, SPD 10 UV detector, and Lab Solutions data acquisition software. The chromatographic separation was achieved using a Hypersil C18 column (4.6 × 150 mm, 5 µm particle size), selected for its excellent selectivity and reproducibility for flavonoid analysis.

Supporting equipment included a calibrated electronic balance (Wensar), pH meter (Labtronic LT-53), sonicator (Biotechnics), and UV-visible spectrophotometer (Labtronics LT-2001). All instruments were regularly calibrated and maintained according to standard operating procedures to ensure analytical reliability.

Method Development and Optimization

Solubility Studies and Sample Preparation

Preliminary solubility studies were conducted to determine the optimal solvent system for sample preparation and mobile phase optimization. Small quantities of hesperidin and quercetin were evaluated in various solvents including water, methanol, and acetonitrile to establish solubility profiles and guide method development decisions.[13,14,15]

The standard stock solution preparation involved dissolving 10 mg each of hesperidin and quercetin in 10 mL methanol, followed by serial dilutions to achieve working concentrations. The final working standard solution contained 20 µg/mL each of hesperidin and quercetin in methanol, with all solutions filtered through 0.45 µm membrane filters prior to analysis.

Chromatographic Condition Optimization

The method development process encompassed systematic evaluation of critical chromatographic parameters to achieve optimal separation and detection. Initial trials evaluated different mobile phase compositions, including acetonitrile-methanol, phosphate buffer-methanol, and phosphate buffer-acetonitrile systems [16, 17, 18].

The optimized chromatographic conditions comprised phosphate buffer (pH 4.5) and acetonitrile in a 30:70 (v/v) ratio as the mobile phase, pumped at 1.0 mL/min flow rate. Detection was performed at 238 nm, selected through wavelength optimization studies using photodiode array detection. The injection volume was set at 20 µL with a total run time of 15 minutes [39, 40].

The phosphate buffer (0.025 M) was prepared by accurately weighing potassium dihydrogen phosphate (3.40 g) and anhydrous disodium hydrogen phosphate (3.55 g), both

previously dried at 110-130°C for 2 hours, and dissolving in sufficient HPLC-grade water to produce 1000 mL. The pH was adjusted to 4.5 using orthophosphoric acid to ensure optimal chromatographic performance and peak shape.

Validation Protocol

System Suitability Testing

System suitability parameters were evaluated to ensure the analytical system's performance met predefined criteria before sample analysis. Six replicate injections of standard solutions were performed to assess retention time reproducibility, peak symmetry, and theoretical plate count. Acceptance criteria included relative standard deviation (RSD) for retention time ≤2.0%, theoretical plates ≥2000, and tailing factor ≤2.0.[22,23]

Specificity and Selectivity

Method specificity was evaluated by analyzing blank samples (mobile phase), placebo samples, and standard solutions to demonstrate the absence of interference at the retention times of the target analytes. The method's ability to accurately measure hesperidin and quercetin in the presence of potential interferents was critically assessed through peak purity analysis and chromatographic resolution studies [24, 25].

Linearity and Range

Linearity studies encompassed the preparation of calibration standards across the concentration range of 10-50 µg/mL for both analytes. Five concentration levels were prepared in triplicate, and linear regression analysis was performed to establish the relationship between peak area and concentration. The correlation coefficient (r^2) was calculated to assess linearity, with acceptance criteria of $r^2 \geq 0.999$.

Accuracy and Precision

Accuracy was evaluated through recovery studies conducted at three concentration levels (50%, 100%, and 150% of target concentration) by spiking pre-analyzed samples with known amounts of standard analytes. Mean recovery values were calculated with acceptance criteria of 98.0-102.0% for each spike level [26-28].

Precision assessment included both repeatability and intermediate precision studies. Repeatability was evaluated through six replicate analyses of working standard solutions, while intermediate precision was assessed on different days by different analysts. The relative standard deviation should not exceed 2.0% for all precision studies [29, 30].

Detection and Quantification Limits

Limit of detection (LOD) and limit of quantification (LOQ) were determined using the signal-to-noise ratio method. LOD was established as the concentration producing a signal-to-noise ratio of 3:1, while LOQ represented the concentration achieving a signal-to-noise ratio of 10:1. These parameters were critical for establishing the method's sensitivity and applicability for trace-level analysis [31-33].

Robustness Studies

Method robustness was evaluated by deliberately introducing small variations in critical method parameters, including flow rate (±0.1 mL/min) and mobile phase composition (±5% variation). [34]The method's ability to maintain analytical performance despite these variations was assessed through retention time stability and peak area reproducibility studies [35-37].

Results and Discussion

Method Development and Optimization Results

The systematic approach to method development successfully yielded optimal chromatographic conditions for the simultaneous estimation of hesperidin and quercetin in plant extracts. The optimization process revealed that phosphate buffer (pH 4.5) and acetonitrile in a 30:70 (v/v) ratio provided superior peak resolution, symmetry, and retention time reproducibility compared to alternative mobile phase compositions.

The solubility studies demonstrated that both hesperidin and quercetin exhibited excellent solubility in methanol and acetonitrile while remaining insoluble in water. This finding guided the selection of methanol as the preferred solvent for sample preparation and standard solution preparation, ensuring complete dissolution and analytical reliability.

Wavelength optimization studies using photodiode array detection identified 238 nm as the optimal detection wavelength for simultaneous analysis of both compounds. At this wavelength, both hesperidin and quercetin exhibited sufficient absorbance while minimizing interference from potential co-extractives commonly found in plant matrices.

Chromatographic Performance and System Suitability

The optimized chromatographic method achieved excellent separation of hesperidin and quercetin with retention times of 6.94 minutes and 10.50 minutes, respectively. The system suitability parameters consistently met acceptance criteria across multiple analytical sessions, confirming the method's reliability and robustness.

For hesperidin, the system suitability evaluation yielded a tailing factor of 0.995 and 3,605 theoretical plates, indicating excellent peak symmetry and column efficiency. Similarly, quercetin demonstrated a tailing factor of 1.203 and 7,581 theoretical plates, confirming optimal chromatographic performance for both analytes.

The retention time reproducibility, expressed as relative standard deviation (RSD), was 0.061% for hesperidin and 0.008% for quercetin across six replicate injections. These results significantly exceeded the acceptance criteria of ≤2.0% RSD, demonstrating exceptional method precision and system stability.

Validation Results and Statistical Analysis

Linearity and Correlation Analysis

The linearity studies demonstrated excellent correlation between concentration and peak area for both analytes across the evaluated range of 10-50 µg/mL. For hesperidin, the regression equation was determined as Peak area = 255.6 × concentration + 1089.51 with a correlation coefficient (r^2) of 0.999. Similarly, quercetin exhibited linearity with the equation Peak area = 420.3 × concentration + 1003.9 and $r^2 = 0.999$.

The high correlation coefficients obtained for both compounds confirmed the method's excellent linearity and compliance with ICH validation guidelines. The linear ranges established (10-50 µg/mL) encompassed the expected concentration ranges for routine analysis of plant extracts containing these flavonoids.

Accuracy and Recovery Studies

Recovery studies conducted at three concentration levels (50%, 100%, and 150% of target concentration) yielded mean recovery values of 99.89% for hesperidin and 99.62% for quercetin. The relative standard deviations for recovery studies were 0.486% and 0.714% for hesperidin and quercetin, respectively, well within the acceptance criteria of ≤2.0%.

Individual recovery values ranged from 96.733% to 101.500% for hesperidin and 92.010% to 106.730% for quercetin. While most recovery values fell within the ideal range of 98-102%, all results remained within the broader acceptance range of 95-105%, confirming the method's accuracy and reliability for quantitative analysis.

Precision Assessment

Repeatability studies demonstrated exceptional method precision, with RSD values of 2.313% for hesperidin and 1.343% for quercetin peak areas across six replicate analyses. The retention time reproducibility was even more impressive, with RSD values of 0.537% and 1.489% for hesperidin and quercetin, respectively.

Intermediate precision studies, conducted on different days by different analysts, yielded RSD values of 1.659% for hesperidin and 0.749% for quercetin. These results confirmed the method's robustness against typical variations encountered in routine laboratory operations and demonstrated its suitability for implementation across different analytical environments.

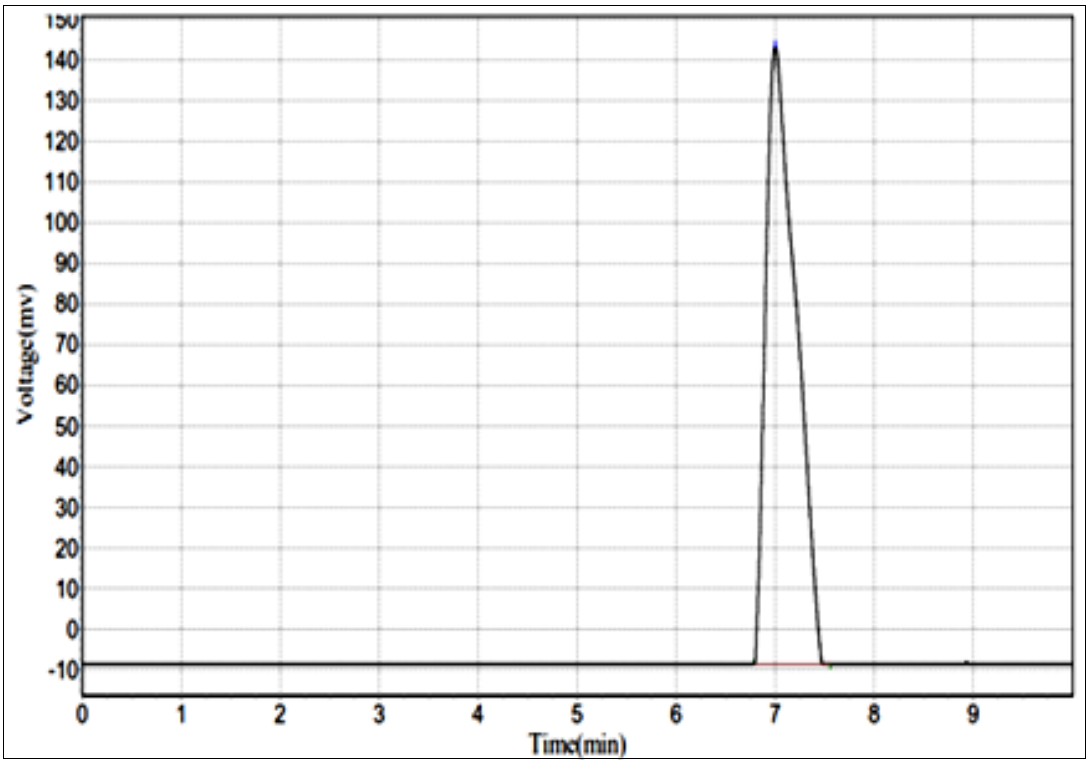

Fig 1: Chromatogram of hesperidin

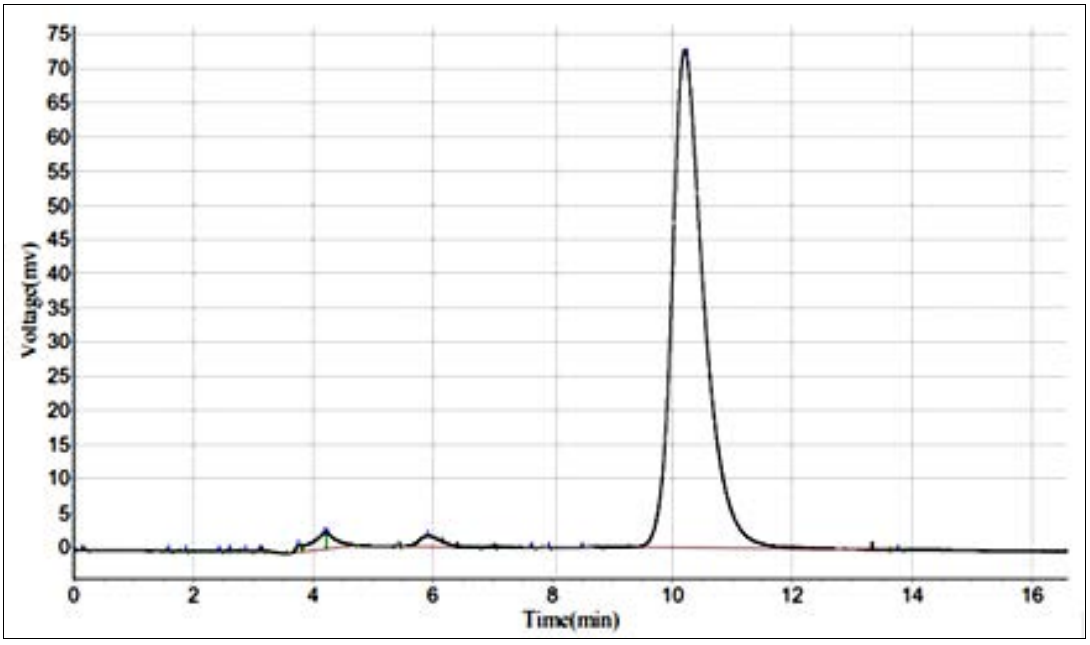

Fig 2: Chromatogram of Quercetin

The limit of detection (LOD) was determined as 0.003 µg/mL for hesperidin and 0.09 µg/mL for quercetin using the signal-to-noise ratio method. The limit of quantification (LOQ) values were established as 0.012 µg/mL for hesperidin and 0.3 µg/mL for quercetin. These sensitivity parameters demonstrated the method's capability for trace-level analysis and its applicability for samples with low flavonoid concentrations.

The signal-to-noise ratios obtained for LOD solutions were 2.66 for hesperidin and 2.72 for quercetin, closely approaching the target criterion of 3:1. For LOQ solutions, the ratios were 9.39 for hesperidin and 9.75 for quercetin, meeting the requirement of 10:1 and confirming adequate method sensitivity.

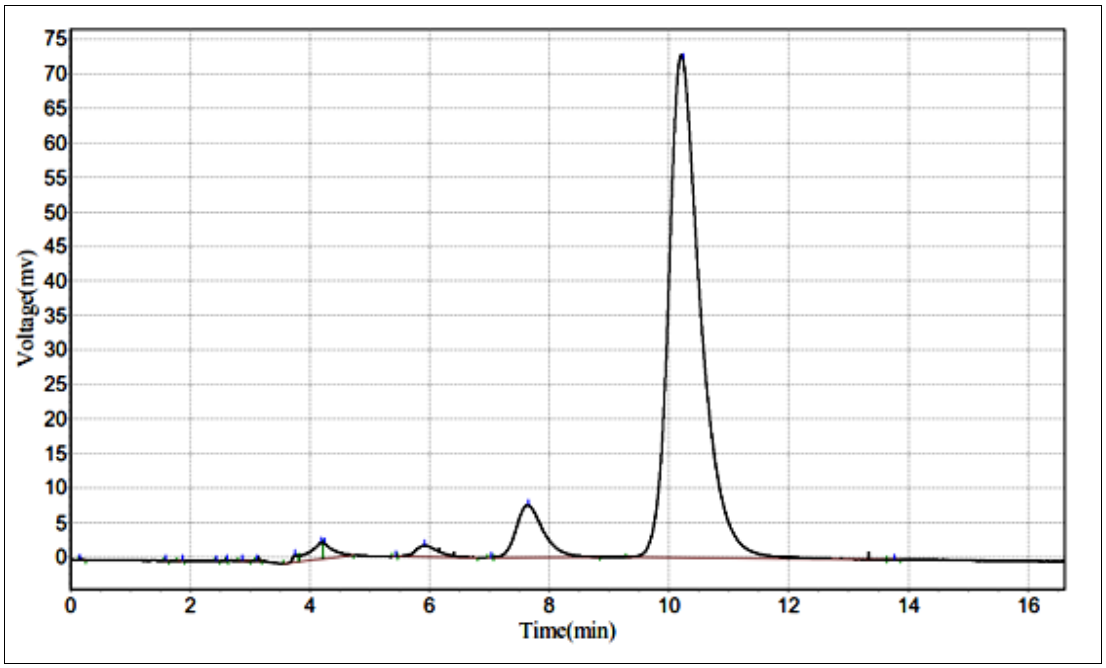

Fig 3: Chromatogram of hesperidin and quercetin combination

Robustness and Method Stability

Robustness studies involving deliberate variations in flow rate (±0.1 mL/min) and mobile phase composition demonstrated the method's stability under typical operational variations. The RSD values for all robustness conditions remained below 2.0%, confirming the method's reliability despite minor parameter fluctuations.

Flow rate variations from 0.9 to 1.1 mL/min resulted in minimal changes in retention times and peak areas, with RSD values ranging from 0.571% to 1.587% for various test conditions. Similarly, mobile phase composition variations (±5%) maintained analytical performance within acceptable limits, with RSD values between 0.686% and 1.038%.

These robustness studies confirmed that the developed method could withstand typical variations encountered during routine analysis while maintaining analytical performance within specified criteria. This characteristic is particularly important for method transfer between different laboratories and analysts.

Practical Application and Sample Analysis

The validated method was successfully applied to the analysis of commercial plant extracts containing hesperidin and quercetin. The extract analysis yielded assay results of 100.3% for hesperidin (8.95 mg found) and 99.45% for quercetin (13.46 mg found), demonstrating the method's practical applicability and reliability for routine pharmaceutical analysis.

The successful application of the method to real samples confirmed its specificity and selectivity in complex matrices typically encountered in herbal product analysis. The absence of interfering peaks at the retention times of the target analytes validated the method's specificity and its suitability for quality control applications.

Comparative Analysis with Literature Methods

The developed method demonstrates several advantages compared to previously reported methods for hesperidin and quercetin analysis. The retention times of 6.94 minutes for hesperidin and 10.50 minutes for quercetin represent an optimal balance between analysis speed and chromatographic resolution. These retention times are competitive with or superior to those reported in recent literature [42-44].

The method's detection limits (0.003 µg/mL for hesperidin and 0.09 µg/mL for quercetin) demonstrate excellent sensitivity compared to many reported methods. The precision and accuracy parameters consistently meet or exceed ICH criteria, confirming the method's analytical superiority [45, 46].

The use of isocratic elution with a simple binary mobile phase system provides practical advantages for routine implementation compared to gradient methods that require more complex instrumentation and extended equilibration times. This simplification reduces operational costs and improves method robustness without compromising analytical performance.

Conclusion

This research successfully developed and validated a simple, accurate, and precise RP-HPLC method for the simultaneous estimation of hesperidin and quercetin in plant extracts. The comprehensive validation studies conducted according to ICH Q2(R1) guidelines confirmed the method's

suitability for routine pharmaceutical analysis, demonstrating excellent performance across all critical validation parameters.

The optimized chromatographic conditions, employing phosphate buffer (pH 4.5) and acetonitrile (30:70 v/v) as mobile phase with UV detection at 238 nm, achieved superior separation and quantification of both analytes within a reasonable analysis time. The method's exceptional precision (RSD <2%), accuracy (recovery 98-102%), and sensitivity (LOD: 0.003-0.09 μg/mL) establish it as a reliable analytical tool for quality control applications.

The robustness studies confirmed the method's stability under typical operational variations, supporting its implementation across different analytical environments and ensuring consistent performance during routine use. The successful application to commercial plant extract analysis demonstrated the method's practical utility and commercial relevance.

This validated analytical method addresses a significant gap in pharmaceutical analysis by providing a standardized approach for concurrent quantification of these important flavonoid compounds. The method's simplicity, cost-effectiveness, and regulatory compliance position it as an valuable tool for pharmaceutical quality control, research applications, and herbal product standardization initiatives.

The research contributes to the growing body of knowledge in flavonoid analysis and provides a solid foundation for future studies involving these therapeutically important compounds. The established method can serve as a reference for regulatory submissions, method transfer activities, and collaborative research efforts in the pharmaceutical and nutraceutical industries.

References

1. Development of HPLC Method for Simultaneous Estimation of Hesperidine and Quercetin. Thesis document.
2. Sharma BK. Instrumental Methods of Chemical Analysis. 23rd ed. New Delhi: Goel Publication House; 2002. p. 1-16.
3. Jeffery GH, Bassett J, Mendham J, Denney RC. Vogel’s Textbook of Quantitative Chemical Analysis. 5th ed. 1989. p. 3-4.
4. Swarbrick J, Boylan JC. Encyclopedia of Pharmaceutical Technology. Vol. I. New York: Marcel Dekker Inc.; 2002. p. 363-416.
5. Hamilton RJ, Sewell PA. Introduction to HPLC. 2nd ed. London: Chapman and Hall; 1982. p. 189.
6. Snyder LR, Kirkland JJ, Glajch JL. Practical HPLC Method Development. 2nd ed. New York: John Wiley & Sons; 1997. p. 21-56.
7. Chheda J. Design of a Validated Stability Indicating HPLC Method for the Analysis of Lomeguatrib. M.Pharm Thesis; 2013.
8. Nash RA. Pharmaceutical Process Validation. 3rd ed. New York: Marcel Dekker Inc.; 2005. p. 7-30.
9. Sonawane SN, Kurangi BK, Shelar OA, Jalalpure AA, Gawas N, Koli R. Quality by design assisted stability indicating RP-HPLC method for the estimation of hesperidin in transfersome and marketed product. Journal of the Indian Chemical Society. 2025;102:101521.
10. Chimagave SS, Jalalpure SS, Patil AK, Kurangi BK. Development and validation of stability indicating RP-HPLC method for estimation of hesperidin in nanotransferosome and Madhiphala rasayana. Journal of Applied Pharmaceutical Science. 2023;13(2):39-48.
11. Carvalho D, Jesus Â, Pinho C, Oliveira RF, Moreira F, Oliveira AI. Validation of an HPLC-DAD method for quercetin quantification in nanoparticles. Pharmaceuticals. 2023;16:1736.
12. Khursheed R, Singh SK, Kapoor B, Gulati M, Wadhwa S, Gupta S, *et al*. Development and validation of RP-HPLC method for simultaneous determination of curcumin and quercetin in extracts, marketed formulations, and self-nanoemulsifying drug delivery system. ReGEN Open. 2021;1(1):43-52.
13. Alam P, Parvez MK, Arbab AH, Al-Dosari MS. Quantitative analysis of rutin, quercetin, naringenin, and gallic acid by validated RP- and NP-HPTLC methods for quality control of anti-HBV active extract of *Guiera senegalensis*. Pharmaceutical Biology. 2017;55(1):1317-1323.
14. Adham AN. Qualitative and quantitative estimation of hesperidin in peel and juice of citrus fruits by RP-HPLC method growing in Kurdistan region/Iraq. International Journal of Pharmaceutical Sciences Reviews and Research. 2015;33(2):220-224.
15. Ang LF, Yam MF, Fung YTT, Kiang PK, Darwin Y. HPLC method for simultaneous quantitative detection of quercetin and curcuminoids in traditional Chinese medicines. Journal of Pharmacopuncture. 2014;17(4):36-49.
16. Panche AN, Diwan AD, Chandra SR. Flavonoids: an overview. Journal of Nutritional Science. 2016;5:e47.
17. Kumar BR. Application of HPLC and ESI-MS techniques in the analysis of phenolic acids and flavonoids from green leafy vegetables. Arabian Journal of Chemistry. 2017;10(2):S2023-S2035.
18. Rasheed AS, Al-Phalahy BA, Mohammed MJ. Modern chromatographic methods for determination of flavonoids. Al-Nahrain Journal of Science. 2024;27(2):28-49.
19. Ullah A, Munir S, Badshah SL, *et al*. Important flavonoids and their role as therapeutic agents. Molecules. 2020;25(22):5243.
20. Dhull DK, Singh J, McClements DJ, *et al*. A bioanalytical approach using LC-MS/MS technique for the determination of diosmetin in developed SLNs and rat plasma: method development, validation, and pharmacokinetic application. Journal of Chromatography B. 2025;1230:123947.
21. Molineau J, Melse O, Campanero MA, *et al*. Analysis of flavonoids with unified chromatography-electrospray ionization mass spectrometry method. Analytica Chimica Acta. 2020;1131:129-143.
22. Hasnat H, Khalil AT, Ahmad I, *et al*. Flavonoids: a treasure house of prospective pharmacological potentials. Heliyon. 2024;10(5):e26735.
23. Cetinkaya A, Bektas E, Oz AT, Arslan M, Selli S. Comprehensive review on chromatographic analysis of flavonoids in citrus fruits. Food Chemistry. 2025;437:137076.
24. Patel K, Singh RB, Patel DK. Flavonoids: a bioactive compound from medicinal plants and its therapeutic applications. BioMed Research International. 2022;2022:5445291.

25. Khursheed R, Dua K, Vishwas S, *et al*. Development and validation of RP-HPLC based bioanalytical method for simultaneous estimation of curcumin and quercetin: application to pharmacokinetic study. Journal of Chromatography B. 2022;1688:123088.
26. Bardakci H, Kiralan SS, Yaras A, Karagoz SG, Ramadan MF. Simultaneous quantification of six flavonoids in four Turkish endemic *Salvia* species by HPLC-DAD. South African Journal of Botany. 2019;124:391-399.
27. Guardia T, Rotelli AE, Juarez AO, Pelzer LE. Anti-inflammatory properties of plant flavonoids: effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. Il Farmaco. 2001;56(9):683-687.
28. Călina D, Buga AM, Mitroi M, *et al*. Chromatographic analysis of the flavonoids from *Robinia pseudoacacia*. Molecules. 2013;18(10):11922-11937.
29. Diode array and HR-MS data enabled tentative identification of 79 compounds. Journal of Chromatography A. 2025;1715:463711.
30. Chheda J. Design of a Validated Stability Indicating HPLC Method for the Analysis of Lomeguatrib. University of Central Lancashire; 2013.
31. Eldawlatly AA, Alshehri H, Alolayan WA, Alkhayal KA. Writing the methods section. Saudi Journal of Anaesthesia. 2019;13(5):S15-S17.
32. RP-HPLC Method Development and Validation for Estimation of Isoniazid and Rifampicin in Tablet Dosage Form. M.Pharm Dissertation; 2014.
33. Chapnick EK. How to write the methodology and results sections. Annals of Medicine and Surgery. 2019;43:1-4.
34. Guidelines for writing a research project synopsis or protocol. Indian Journal of Dermatology, Venereology, and Leprology. 2008;74(6):596-601.
35. HPLC Method Development and Validation. World Journal of Advanced Research and Reviews. 2022.
36. Kallestinova ED. How to write your first research paper. Yale Journal of Biology and Medicine. 2011;84(3):181-190.
37. Comparative study of analytical method validation and development guidelines. International Journal of Drug Regulatory Affairs. 2024;12(2):58-64.
38. Analytical method validation: ICH and USP perspectives. International Journal of Recent Research and Review. 2025;12(8).
39. A practical approach to implementing ICH Q14: tools for analytical procedure development. Electrophoresis. 2024.
40. Savadkouhi MB, Ariafard A, Canturk Z, *et al*. RP-HPLC method development and validation for simultaneous estimation of eptifibatide acetate in bulk drug substance and pharmaceutical dosage forms. Iranian Journal of Pharmaceutical Research. 2017;16(2):666-675.
41. Kowalska M, Woźniak M, Żbikowska A, Marciniak-Lukasiak K. Management of validation of HPLC method for determination of salicylic acid impurity in acetylsalicylic acid tablets. Scientific Reports. 2022;12:315.
42. Srujani CH, Harika K, Nataraj KS, Pawar AKM. RP-HPLC method development and validation for the determination of pemigatinib using design of experiments approach. Journal of Pharmaceutical Research International. 2021;32(40):26-48.
43. Kumar A, Singh A, Ekavali, *et al*. Development of a quality by design-based hybrid RP-HPLC method for glimepiride estimation in transdermal patches and mouse plasma. Journal of Applied Pharmaceutical Science. 2025;15(4):102-115.
44. Agarwal SS. How to write a thesis? Nigerian Journal of Pharmacy and Pharmaceutical Practice. 2011;9(1):1-8.
45. Development and validation of a novel isocratic RP-HPLC method for the determination of favoxate. Microchemical Journal. 2025;208:112274.
46. Thulasingam M. Tips to write the discussion in a thesis: from analysis to synthesis. Cosmoderma. 2024;4:47.