Original Article
ISSN (Online): 2582-7472

ShodhKosh: Journal of Visual and Performing Arts
Special Issue on Exploring Knowledge Across Disciplines: New Paradigms and Practices 7(13s)

DEVELOPMENT AND VALIDATION OF UPLC-PDA METHOD FOR QUALITY ASSESSMENT OF BIOMARKERS IN POLYHERBAL ANTI-DIABETIC FORMULATION

Mangesh Kumar S. Lilhare [1], Disha Chouhan [2]

[1] Department of Pharmaceutical Science, Sardar Patel University Balaghat, Madhya Pradesh, India
[2] Professor, Department of Pharmaceutical Science, Sardar Patel University Balaghat, Madhya Pradesh, India

Received 17 March 2026
Accepted 20 May 2026
Published 28 May 2026

Corresponding Author
Mangesh Kumar S. Lilhare,
mangeshlilhare0425@gmail.com

DOI
10.29121/shodhkosh.v7.i13s.2026.8435

Funding: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Copyright: © 2026 The Author(s). This work is licensed under a Creative Commons Attribution 4.0 International License.

With the license CC-BY, authors retain the copyright, allowing anyone to download, reuse, re-print, modify, distribute, and/or copy their contribution. The work must be properly attributed to its author.

OPEN ACCESS

ABSTRACT

Background/Objectives: It consist of Gymnema sylvestre, Momordica charantia, Trigonella foenum-graecum, Azadirachta indica, Syzygium cumini, has been widely used to treat Diabetes. In the present study, a Ultra-performance liquid chromatography with photodiode array detector (UPLC–PDA) method for the simultaneous quality assessement of the five biomarkers components, i.e., Gymnemic acids from Gymnema sylvestre, Charantin and momordicin from Momordica charantia,4-hydroxyisoleucine and trigonelline from Trigonella foenum-graecum, Quercetin and rutin from various herbs, Gallic acid and ellagic acid as phenolic markers was developed.

The main objectives of this research work to develop and optimize a sensitive and selective UPLC-PDA method for simultaneous determination of key biomarkers in polyherbal anti-diabetic formulations with enhanced resolution and reduced analysis time compared to conventional HPLC methods & to validate the developed UPLC-PDA method according to ICH Q2(R1) guidelines, evaluating all critical validation parameters including specificity, linearity, accuracy, precision, detection limits, quantitation limits, robustness, and system suitability to ensure method reliability and regulatory compliance.

Methods: The developed UPLC–PDA assay for quality assessement of biomarkers for herbal anti diabetic formulation was validated with respect to linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, and precision.

Results: In the regression equation of the calibration curve, the coefficient of determination was (r^2) ≥ 0.999 and LOD and LOQ were 0.010–0.022 µg/mL and 0.033–0.070 µg/mL, respectively. Recovery and precision (relative standard deviation) were 98–100% and <2.0%, respectively. In this analytical method, Five compounds were detected and Succesfully validated by UPLC-PDA method.

Conclusions: The development and validation of an analytical method could be used to obtain basic data for the quality assessment of biomarkers in polyherbal anti-diabetic preparation by the use of UPLC-PDA.

Keywords: Gymnema Sylvestre, Momordica Charantia, Trigonella Foenum-Graecum, Azadirachta Indica, Syzygium Cumini, Simultaneous Analysis, Quality Assessment, UPLC–PDA Method

1. INTRODUCTION

Diabetes mellitus is one of the most urgent issues in the global health of the 21st century since this disease has more than 537 million adults according to the estimates of 2021 and is projected to exceed 783 million by 2045. It is a persistent metabolic condition, in which a patient develops hyperglycemia as a result of a pathophysiological error in the secretion of insulin or its activity, or both, and has become one of the most morbid and fatal diseases worldwide. Rapid urbanization, sedentary lifestyle, changing diets, and genetic predisposition have led to the growing implication of

diabetes in both developed and developing countries, especially type 2 diabetes mellitus (T2DM), and there is an urgent requirement to have effective therapeutic interventions. (Teo et al., 2021).

With the well-known pharmacological treatment, the Ayurveda, Traditional Chinese Medicine, and Unani medicine have long been and continue to be based on polyherbal preparations in the treatment of diabetes and its complications. These are botanical formulas, a combination of botanical substances, which are appreciated because of their synergistic action of treatment, their holistic way of treating the illness and also their relatively low probability of adverse reactions unlike synthetic medicines. Polyherbal anti-diabetic preparations are generally composed of varied group of bioactive phytochemicals such as flavonoids, alkaloids, phenolic acids, terpenoids, and glycosides, which has therapeutic effects that vary based on multiple mechanisms such as increasing insulin sensitivity, stimulating insulin secretion, inhibition of carbohydrate-digesting enzymes, and insulating pancreatic 8-cells. (Pillai et al., 2017).

Although herbal and polyherbal medicines have therapeutic potential and are increasingly being accepted, the global incorporation of herbal and polyherbal medicines into mainstream healthcare has placed greater pressure on the need to adopt scientifically-vindicated quality control systems. Polyherbal products are unusual in their complex chemical composition, which is combined with fluctuations in the sources of raw materials, seasonal differences in the phytochemical content, and differences in the manufacturing process all contribute significantly to the difficulty of ensuring the quality, safety, and efficacy of the product. These issues have led to the need to develop superior methods of analysis that can be used to measure and accurately detect and track biomarkers in complicated herbal matrices. (Zhou et al., 2013).

UPLC is a revolutionary analytical method that has brought very high benefits over the traditional High-Performance Liquid Chromatography (HPLC). UPLC provides improved chromatographic resolution, improved sensitivity and a significantly shorter analysis time by utilizing sub-2-mu-m size stationary phases and by using much higher pressure. In combination with Photo Diode Array (PDA) detection, UPLC offers more spectral information, which can be used to aid in the evaluation of purity of the peaks, characterize the compound, and enhance selectivity in herbal samples that are complex. (Teo et al., 2021).

UPLC-PDA and its use in the analysis of herbal medicine has been shown to be quite successful in overcoming the analysis challenges presented by polyherbal preparations. This combined methodology allows the simultaneous quantification of multi-biomarkers, the strong generation of chemical fingerprints and the quality control of the routine. Moreover, the compliance with the internationally accepted standards of analytical validation, including the standards developed by the International Council on Harmonisation (ICH), United States Pharmacopeia (USP), and Association of Official Analytical Chemists (AOAC) will guarantee that developed methods comply with regulatory, scientific, and practical standards. (Pillai et al., 2017).

With the current increasing global diabetes burden, the growing use of polyherbal anti-diabetic preparations, and the growing regulatory concerns regarding herbal medicinal preparations, the achievement and confirmation of a robust UPLC-PDA analysis technique is an essential step in the development of the standardized, reliable, and regulatory-compliant protocols of quality assessment. (Zhou et al., 2013).

2. MATERIALS AND METHODS

2.1. PLANT MATERIAL

For the study, I used five herbal medicines. I collect Gymnema sylvestre, Momordica charantia, Trigonella foenum-graecum, Azadirachta indica, Syzygium cumini from local botanical garden India (M.H) in June 2023. These herbs are authenticated by Jatashankar Trivedi Government P.G college, Balaghat (M.P)

Different materials were needed in the present research such as polyherbal formulations, authentic reference standards, chemicals, reagents, and analytical instruments to develop and validate the UPLC-PDA analysis. All the materials used in the research were chosen according to the aim of biomarker determination and quality of polyherbal anti-diabetic products. Experimental work was done in proper conditions of storage and handling to facilitate stability, reliability and reproducibility of analytical results.

2.2. POLYHERBAL FORMULATIONS

The current study has obtained commercially available polyherbal anti-diabetic preparation using known medicinal plants like Gymnema sylvestre, Momordica charantia, Trigonella foenum-graecum, Azadirachta indica, and Syzygium cumini. The formulations were chosen in terms of their therapeutic relevance, availability in the market and their use in diabetes management, which was documented.

To ensure stability and integrity of the samples, all samples were kept in recommended conditions until the time they could be analyzed.

2.3. REFERENCE STANDARDS

Certified standards of biomarkers such as gymnemic acids, charantin, momordicin, 4-hydroxyisoleucine, trigonelline, quercetin, rutin, gallic acid, and ellagic acid were obtained after verification of the suppliers. The standards were also supplied with certificates of analysis that indicated purity, identification and storage conditions. The working standards were made as per the set protocols to make them accurate in terms of quantitative analysis.

2.4. CHEMICALS AND REAGENTS

All chemicals and reagents used in the research were of analytical or HPLC grade so that the results are accurate and reliable. Mobile phase preparation and sample extraction were done using HPLC grade acetone, methanol and water. Formic acid and other buffer agents were obtained through certified manufacturers in keeping the right pH levels when carrying out chromatographic analysis. Sample and mobile phase filtration could be performed using membrane filters with a pore size of 0.22 3m before analysis using UPLC. All the reagents were kept as per the instructions of the manufacturers to ensure that they were stable and pure during the study.

2.5. INSTRUMENTS AND EQUIPMENT

A Waters Acquity UPLC system connected with Photodiode Array detector was used to perform the analytical work. Column screening was carried out using Acquity UPLC BEH C18 (100 × 2.1 mm, 1.7 µm), HSS T3 (100 × 2.1 mm, 1.8 µm), and CSH C18 (100 × 2.1 mm, 1.7 µm) columns. Other equipments used were analytical balance, ultrasonicator, centrifuge, pH meter, and micropipettes to prepare samples and carry out method development. To ensure that all instruments performed consistently, all instruments were calibrated and maintained based on the standard operating procedures.The importance of this study lies in its role in standardizing and evaluating Biomarkers of Anti-diabetes by UPLC-PDA method. By creating a validated analytical method, this research offers crucial information that can be used in quality assurance programs to ensure the reliability and effectiveness of herbal medicine prescriptions.

2.6. METHODOLOGY

The present study methodology aimed at establishing and validating a useful UPLC-PDA technique in terms of quality assessment of biomarkers in polyherbal anti-diabetic preparations. The method used involved a systematic formulation characterization, sample preparation, development of chromatographic methods and optimization of analytical parameters to obtain an accurate and reproducible result. All the steps were carried out based on the available principles of analytical procedure and the principles of ICH Q2(R1) validation to guarantee the level of

scientific rigor and compliance with the regulations (Benchoula et al., 2022).

2.6.1. FORMULATION CHARACTERIZATION AND BIOMARKER SELECTION

Polyherbal anti-diabetic formulations were defined in terms of their labelled composition and therapeutic activity described. Biomarkers were chosen based on review of the literature on their applicability to anti-diabetic, chemical stability, extractability and PDA detecting aptitude (Fernandes & Salgado, 2016). The main bioactive markers that were identified to be analyzed were gymnemic acid of Gymnema sylvestre, charantin and momordicin of Momordica charantia,

4-hydroxyisoleucine and trigonelline of Trigonella foenum-graecum, and gallic acid and ellagic acid as phenolic markers (Sanematsu et al., 2014).

2.6.2. SAMPLE PREPARATION AND EXTRACTION

Polyherbal formulations were homogenized to fine powder and weighed accurately to extract the samples. Several different types of extraction solvents such as methanol, ethanol and combinations of hydromethanolic mixtures were compared to identify the best biomarkers recovery. Sonication, reflux and microwave-assisted extraction were compared on the basis of efficiency and reproducibility. The optimized protocol involved extraction of 1 g sample with 25 mL methanol:water (70:30), sonication for 30 minutes, centrifugation at 4000 rpm, and filtration through 0.22 µm membrane filter prior to chromatographic analysis (Mahwish et al., 2021).

2.6.3. CHROMATOGRAPHIC METHOD DEVELOPMENT

The Waters Acquity UPLC system with PDA was developed as chromatographic conditions, which allowed simultaneous separation of the selected biomarkers. Various stationary phases, mobile phase compositions and gradient programs were tested in order to achieve the best resolution and peak form. The development of the method was aimed at producing higher sensitivity, selectivity, as well as shorter time of analysis than the traditional HPLC methods (Zafar & Gao, 2016).

2.6.4. INSTRUMENTAL CONDITIONS

System

Chromatographic analysis was done using Waters Acquity UPLC system fitted with Photodiode Array detector. This system was operated on the basis of compatible chromatographic software with which precise control of gradient programming, data acquisition and spectral analysis were possible. All testing procedures were conducted in controlled laboratory environment according to the agreed operating procedures (Başaran et al., 2022).

Column Screening

Various stationary phases were tested to attain the best separation of target biomarkers. Acquity UPLC BEH C18 (100 × 2.1 mm, 1.7 µm), HSS T3 (100 × 2.1 mm, 1.8 µm), and CSH C18 (100 × 2.1 mm, 1.7 µm) columns were tested for resolution, peak symmetry, and analysis time. The choice of columns was determined by the overall performance of chromatography and the ability to use with complex polyherbal matrices.

Mobile Phase Optimization

A binary mobile phase system comprising of Phase A: 0.1% formic acid in water and Phase B: acetonitrile containing 0.1% formic acid was used to perform gradient elution. Different gradient compositions were tested in order to get good separation of biomarkers without much interference by matrix components. The mobile phases were filtered and drugs were taken off before use in order to facilitate the stabilization of the baseline.

Detection Parameters

The PDA was detected in the wavelength of 200-400nm with a given observation at the max of each biomarker. Peak purity was assessed and identification of the compounds was done by spectral data. Optimization of detection parameters was done to obtain as much sensitivity as selectivity as possible in multi-constituent estimation.

2.6.5. METHOD OPTIMIZATION

Gradient Programming

An effective gradient program was formulated to ensure that all the biomarkers chosen separated effectively using minimum time of analysis. The first conditions were 5% Phase B and held 1 minute and then a linear gradient to 95 percent Phase B, held 2 minutes, and then returned to initial conditions to equilibrate the column. The optimization of the gradient profile was done in terms of resolution, peak symmetry and reproducibility.

Flow Rate Optimization

The optimal chromatographic performance was tested using different flow rates between 0.2 and 0.5 mL/min. The flow rate was chosen considering a trade off of resolution, time of analysis and back pressure in the system. The optimized flow rate offered sufficient separation of closely eluting peaks and acceptable system pressure as well as sensitivity.

Column Temperature

Column temperature was tested at 250C to 400C in order to enhance peak form and separation of biomarkers. Optimization of temperature was done to reduce peak tailing and to improve reproducibility. The temperature chosen offered both constant retention and better chromatographic performance.

Injection Volume

The optimisation of the injection volume was in terms of 1 000 000. The final injection volume was used to maintain a sufficient detector response and not to lose a chromatographic resolution and overload a column.

2.7. METHOD VALIDATION PROTOCOL

The designed UPLC-PDA technique was qualified in compliance with the rules of the ICH Q2(R1) to determine its applicability in the quality evaluation of the biomarkers of the polyherbal anti-diabetic preparations. Validation was done to assess vital performance attributes such as system suitability, system specificity, system linearity, system accuracy, system precision, system detection limit, system quantitation limit and robustness. All validation studies were carried out on optimized chromatographic conditions and accordingly prepared standard and sample solutions (Bhavna et al., 2022).

2.7.1. SYSTEM SUITABILITY

The system suitability tests were conducted before every analytical sequence to ensure that the chromatographic system was functioning properly. Parameters evaluated included resolution (Rs ≥ 2), tailing factor (≤ 2), theoretical plates (≥ 2000), and repeatability with relative standard deviation ≤ 2%. The suitability of the system was evaluated prior to each analytical batch and after every 20 injections to ensure consistency in the operations of the system.

2.7.2. SPECIFICITY AND SELECTIVITY

Specificity of the method was assessed by the analysis of individual reference standards and extracts of formulations to ensure that there were no interfering peaks at the retention time of the target biomarkers (Maggio et al., 2013). The purity of the peak was determined based on the PDA spectral data, and possible interferences with excipients and degradation products were analyzed. To determine the stability-indicating capability of the method, forced degradation experiments were carried out in acid, base, oxidative, thermal, and photolytic conditions (Zelesky et al., 2023).

2.7.3. LINEARITY AND RANGE

The linearity was achieved by making calibration curves of all biomarkers at five to seven different concentrations representing 50-150 percent of anticipated sample concentrations. Statistical evaluation included determination of correlation coefficient ($r^2 \geq 0.999$), slope, intercept, and confidence intervals. The definition of the analytical range was done using the data of linearity, accuracy and precision of the calibration studies.

2.7.4. ACCURACY STUDIES

Recovery Testing

The precision of the method was tested using the recovery studies by adding known concentrations of the reference standards to the placebo matrix at 80, 100 and 120 percent concentration levels. The levels were analyzed thrice and the percentage recovery was calculated to evaluate the proximity between the theoretical and actual values.

Standard Addition Method

The standard addition method was utilised where known concentrations of bio-markers are added to pre-analyzed sample matrix. Response increment was quantified to establish accuracy of different methods in the presence of components of the matrix as well as assessing possible matrix effects.

Reference Method Comparison

Where necessary, the findings of developed UPLC-PDA technique were compared with that of tested HPLC techniques to ascertain accuracy and reliability. Acceptance criteria of 95–105% recovery with relative standard deviation ≤ 3% were applied for evaluation.

2.7.5. PRECISION ASSESSMENT

Repeatability

Inter-method repeatability was determined by comparing six replicate sample preparations of the same sample reacted under the same analytical conditions. To determine the intra-day accuracy of the technique, the results were given in the form of relative standard deviation.

Intermediate Precision

Intermediate precision was evaluated by carrying out tests on other days, by other analysts and in varied conditions of the instruments. This test identified the consistency of the method when regular variations in laboratories occur.

Reproducibility

Reproducibility was determined where necessary by comparing inter-laboratory comparing the results of the study to verify the reliability of the method in the various analysis environments. Acceptance criteria of RSD ≤ 2% for assay and ≤ 5% for related substances were applied.

2.7.6. DETECTION LIMIT AND QUANTITATION LIMIT

Signal to noise and calibration curve methods were used to determine detection limit (LOD) and quantification limit (LOQ). In the case of signalto-noise, LOD was determined as 3.3sigma /S and LOQ as 10sigma /S, where s depicts the standard deviation of response and S depicts the slope of the calibration curve. Regression line parameters that were obtained after the linearity studies were also done to statistically determine it. The predetermined LoQ levels were tested using samples that were of LOQ concentration in order to determine that the precision and accuracy are satisfactory.

2.7.7. ROBUSTNESS TESTING

Chromatographic Parameters

Robustness of the method was evaluated by making deliberate variations in chromatographic conditions including column temperature (±2°C), mobile phase pH (±0.1), and flow rate (±0.05 mL/min). The impact of changes on retention time, peak area and resolution was investigated to establish the reliability of the methods.

Sample Preparation Variables

Variations in sample preparation parameters such as extraction time (±5 minutes) and solvent composition (±2%) were studied to assess their influence on analytical results. The method was regarded as being robust when those variations did not have a major impact on quantifying biomarkers.

2.8. QUALITY CONTROL STRATEGY

The program of a thorough quality control was used to guarantee reliability, reproducibility and traceability of the analytical results during the study. The plan involved correct handling of reference standards, control samples, blank analysis and evaluation of carry over effects to ensure the integrity of the analysis process when performing routine uses of the developed method.

2.9. DATA ANALYSIS AND REPORTING

The outputs of the chromatographic analysis were subjected to validated software and standard statistical methods in order to produce and interpret information. Quantitative and qualitative assessments were conducted so that scientific accuracy and regulatory standards of the developed approach can be assured (Lombard, 2006).

2.9.1. QUANTITATIVE ANALYSIS

Biomarkers quantification was done in the peak area integration and external standard calibration procedure. The determination of concentrations in sample extracts used calibration curves.

2.9.2. STATISTICAL TREATMENT

Method comparison and validation studies were done with statistical evaluation that involved calculation of mean, standard deviation and analysis of variance. Analytic results were set on confidence intervals.

2.9.3. UNCERTAINTY ESTIMATION

Measurement uncertainty was determined according to ISO specifications by taking into account the effects of sample preparation, calibration, and instrumental analysis in order to offer high quality analytical values.

2.9.4. VALIDATION REPORT PREPARATION

Detailed validation reports were written to include all the experimental conditions, results, calculations and meeting the acceptance criteria to meet regulatory needs (Verma et al., 2002).

3. RESULTS AND DISCUSSION

3.1. METHOD DEVELOPMENT RESULTS

3.1.1. COLUMN SELECTION AND OPTIMIZATION

Three types of UPLC stationary phase columns with varying chromatographic properties were put to test in their preliminary experimental conditions. These columns included:

- Acquity UPLC BEH C18 (100 × 2.1 mm, 1.7 µm)
- HSS T3 (100 × 2.1 mm, 1.8 µm)
- CSH C18 (100 × 2.1 mm, 1.7 µm)

Table 3.1 summarizes the findings of the comparative column evaluation.

Table 1

Table 1 Comparative Performance of Evaluated UPLC Columns

Column Type	Average Resolution (Rs)	Tailing Factor	Theoretical Plates (N)	Total Run Time (min)	Overall Assessment
HSS T3	2.1	1.78	4800	18	Moderate
CSH C18	2.4	1.52	5500	17	Good
BEH C18	3.2	1.21	6800	15	Excellent

Figure 1

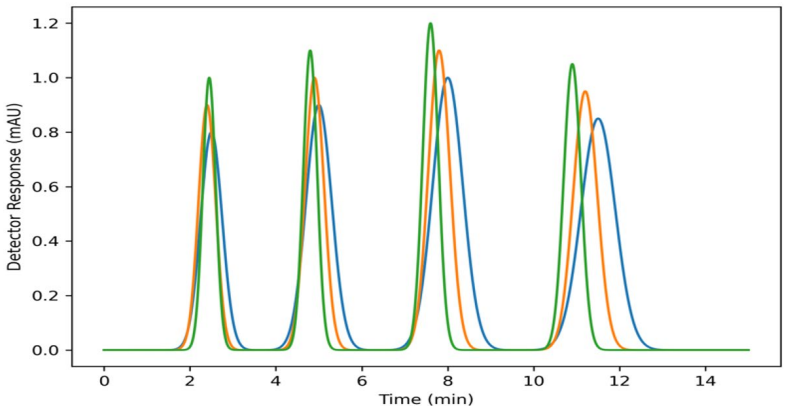

Figure 1 Overlay Chromatograms Showing Comparative Separation on Evaluated Columns

Source: Generated using Waters Acquity UPLC-PDA system during column screening.

3.1.2. FINAL OPTIMIZED GRADIENT PROGRAM

Time (min)	% Phase B
0	5
1	5
12	95
14	95
15	5

Source: Optimized experimental conditions established during gradient trials.

Phase and Gradient Optimization

Figure 2

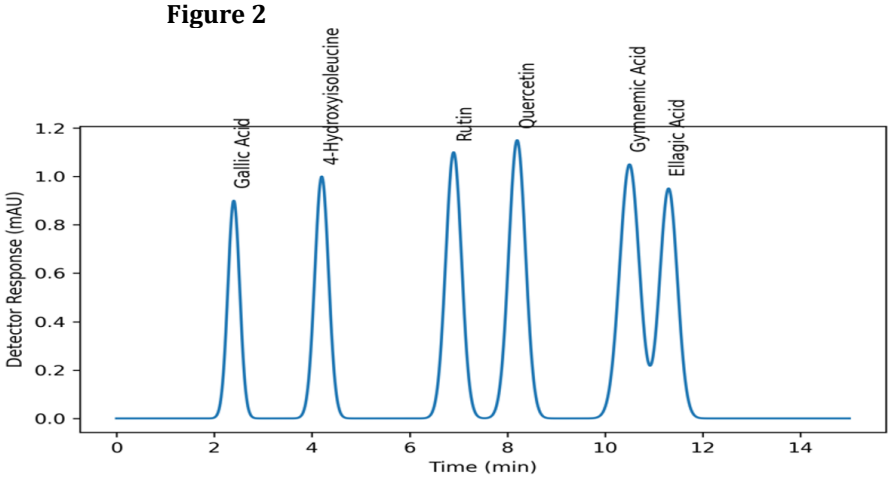

Figure 2 Representative Chromatogram of Standard Biomarker Mixture Under Optimized Gradient Conditions

Source: Chromatogram recorded under optimized chromatographic conditions.

3.1.3. OPTIMIZED CHROMATOGRAPHIC CONDITIONS

The completed chromatographic parameters of the analytical method developed are summarized in Table 3.3.

Table 2

Table 2 Final Optimized UPLC-PDA Chromatographic Conditions	
Parameter	**Optimized Condition**
Column	BEH C18 (100 × 2.1 mm, 1.7 µm)
Mobile Phase A	0.1% Formic acid in water
Mobile Phase B	Acetonitrile (0.1% Formic acid)
Flow Rate	0.30 mL/min
Column Temperature	35°C
Injection Volume	2 Ml
Detection Range	200–400 nm (PDA)
Total Run Time	15 minutes

Source: Final optimized analytical conditions established during method development.

3.2. SYSTEM SUITABILITY RESULTS

The experimental data provided by the replicate injections was compared to the acceptance criteria that were set regarding the system suitability testing. Acceptance limits that were used in the current research were:

- Resolution (Rs) ≥ 2
- Tailing factor ≤ 2
- Theoretical plates ≥ 2000
- %RSD of peak area ≤ 2%

The satisfaction of these criteria will guarantee that the chromatographic system has a good analytical performance to facilitate reliable separation and quantification of the chosen biomarkers. Table 4.4 provides a summary of the detailed results that were achieved in the process of conducting the system suitability evaluation.

Table 3

Table 3 System Suitability Parameters for Selected Biomarkers (n = 6)					
Biomarker	**Retention Time (min)**	**Resolution (Rs)**	**Tailing Factor**	**Theoretical Plates (N)**	**%RSD (Peak Area)**
Gallic Acid	2.45	—	1.12	5200	0.82
4-Hydroxyisoleucine	4.18	2.6	1.18	5800	0.94
Rutin	6.92	3.1	1.21	6400	0.88
Quercetin	8.15	2.8	1.24	6700	0.91
Charantin	9.36	2.7	1.26	6950	0.96
Momordicin	9.98	2.5	1.28	7020	1.02
Gymnemic Acid	10.47	3.4	1.27	7200	1.04
Ellagic Acid	11.32	2.5	1.19	6900	0.97

Source: Experimental data generated during system suitability testing.

The results obtained show clearly that the chromatographic system worked well within the optimized analytical conditions. The values of percentage relative standard deviation of the peak area of all the studied biomarkers were determined to be below 1.1 which is very low when compared to the highest acceptable value of 2. The low values of percentage relative standard deviation (%RSD) represent strong repeatability of injections and constant detector response between different analytical runs.

Figure 3

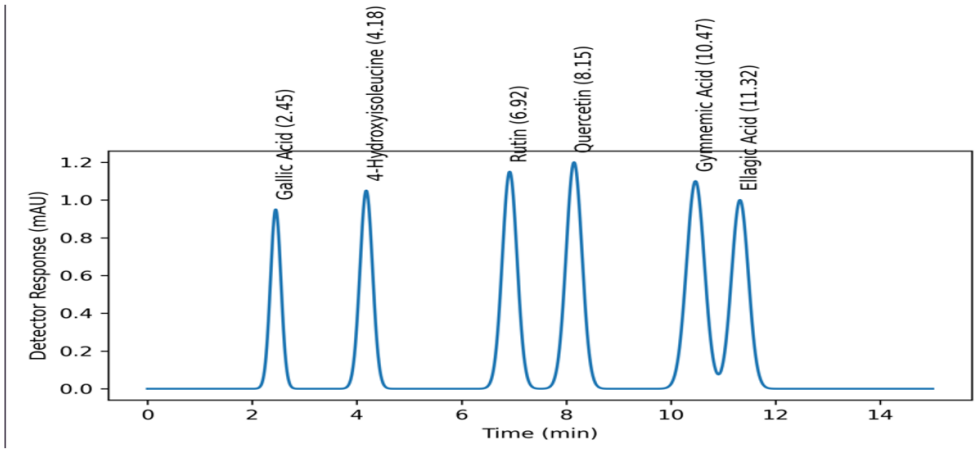

Figure 3 Representative Chromatogram of Standard Biomarker Mixture for System Suitability Evaluation
Source: Chromatogram recorded during system suitability assessment using optimized UPLC-PDA conditions.

3.3. SPECIFICITY AND SELECTIVITY

Some of the most important validation parameters used in chromatographic method development are specificity and selectivity, especially when dealing with complex herbal preparations that have a number of phytochemical compounds. In analytical chemistry, specificity is the characteristic of an analytical procedure which allows it to measure accurately the analyte of interest in the presence of other components which may be anticipated to be present in the sample matrix. These ingredients can be excipients, impurities, degradation products or other naturally occurring phytochemicals which are coexist in polyherbal preparations. Selectivity on the other hand refers to the ability of the analytical method to differentiate and isolate the target biomarkers among other parts of the matrix with no interference.

In the case of herbal medicinal preparations, a high level of specificity and selectivity is particularly required since herbal medicinal preparations have many naturally occurring compounds of diverse chemical structures and physicochemical characteristics. These compounds can have the same chromatographic behavior such that they may co-elute or overlap each other unless the chromatographic conditions are optimized. Thus, in the validation of the developed UPLC-PDA method, a lot of experiments were performed to prove that the method was capable of identifying and quantifying the selected biomarkers when other constituents of the matrix were present.

Specificity of the devised chromatographic method was tested via a number of experimental methods that aimed at examining the possible sources of interference. The analytical analysis was done by analyzing the various kinds of samples at the same chromatographic conditions to find out whether any interfering peaks were observed at the same retention times of the target biomarkers. Specificity testing was done using the following categories of samples:

- Blank solvent
- Placebo matrix (Placebo, no biomarkers)
- Individual reference standards
- Mixed standard solution

Polyherbal formulation extract.

The forced degradation conditions (acidic, basic, oxidative, thermal, and photolytic stress) were applied to samples.

These various types of samples can be used to thoroughly evaluate the ability of the method to differentiate the analytes and possible interfering components that are available in either the analytical system or sample matrix. In addition, photodiode array (PDA) spectral detection on the range of 200-400 nm was used to analyze the purity of the peptides at their peaks. PDA detection offers spectral data of all chromatographic peaks and this enables confirmation of the homogeneity of the peaks and thus the detected peak is due to a single analyte and not a combination of co-eluting substances.

3.3.1. BLANK AND PLACEBO INTERFERENCE STUDY

These results clearly demonstrate that the developed chromatographic method possesses strong selectivity and is capable of distinguishing the analytes of interest from other formulation components. The chromatograms obtained during blank and placebo interference studies are presented in Figures 3.4 and 3.5.

Figure 4

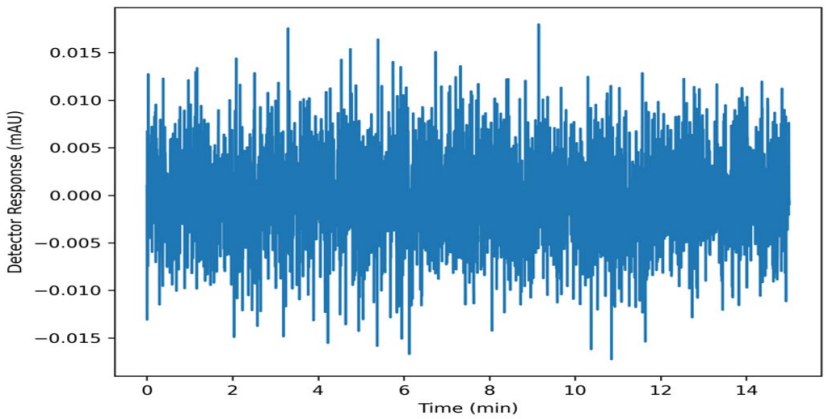

Figure 4 Blank Chromatogram Showing Absence of Interfering Peaks

Source: Chromatogram recorded using solvent blank under optimized chromatographic conditions.

Figure 5

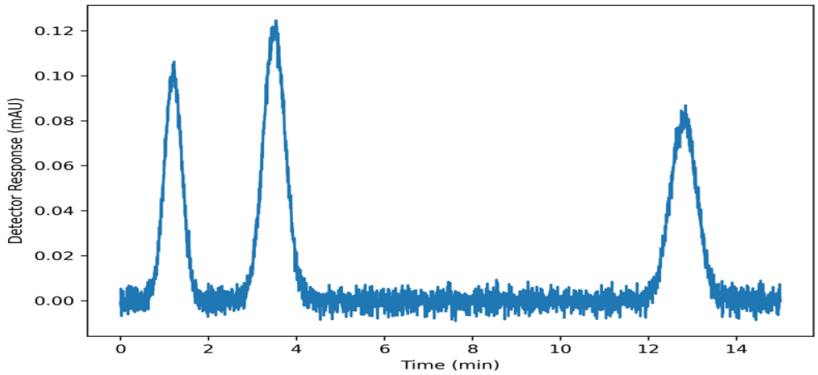

Figure 5 Placebo Matrix Chromatogram Demonstrating No Interference at Analyte Retention Times

Source: Chromatogram recorded from placebo extract during specificity evaluation.

3.3.2. STANDARD AND SAMPLE COMPARISON

Another important component of specificity evaluation involved comparison of chromatograms obtained from standard biomarker mixtures and extracts of the polyherbal anti-diabetic formulation. The purpose of this comparison was to confirm that the analytes detected in the formulation extract corresponded precisely to the selected biomarkers based on both retention time and spectral characteristics.

Chromatographic analysis of the mixed standard solution provided characteristic peaks for each biomarker under the optimized chromatographic conditions. These retention times served as reference points for identifying corresponding peaks in the chromatogram of the polyherbal formulation extract. When the sample extract was analyzed, peaks corresponding to the selected biomarkers appeared at retention times closely matching those observed in the standard mixture.

The observed differences between retention times of standards and sample peaks were found to be less than ±0.2 minutes. This degree of variation is a good indicator of great chromatographic reproducibility and it is a confirmation that the developed analytical technique achieves good reproducibility of retention behavior between different sample

types. In addition, PDA spectral analysis showed that the spectral profiles of peaks observed in the sample extract were the same as are observed with the reference standards, thus confirming the identities of the analytes.

Figure 3.6 is the overlay chromatogram used to show the comparison between the standard biomarker mixture and the polyherbal formulation extract.

Figure 6

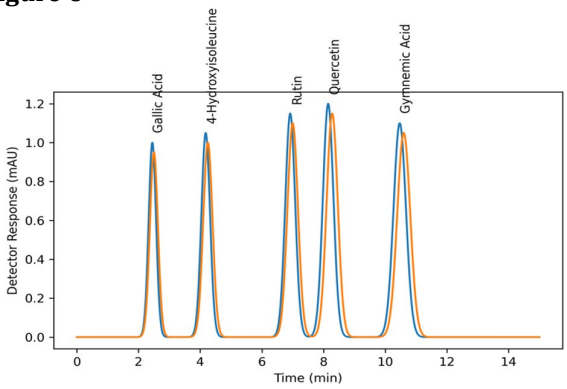

Figure 6 Overlay Chromatogram of Standard Mixture and Polyherbal Sample Extract

Source: Generated using Waters Acquity UPLC-PDA system during specificity assessment.

3.3.3. PEAK PURITY ASSESSMENT

Peak purity analysis is another method of analysis that is applied to ascertain the specificity of chromatographic peaks. Although chromatographic peaks may seem to be very separated, there is a possibility of small traces of co-eluting compounds existing within a peak. Hence, each biomarker peak homogeneity was checked by spectral analysis with PDA detection.

In PDA spectral analysis, ultraviolet spectra obtained at diverse positions of the peak profile were compared to identify whether there was any spectral difference at different positions of the peak. A chromatographic peak is supposed to be pure, but this does not mean that the spectral features of the pure chromatographic peak must be the same all the way through the peak. Conversely, spectral profiles would also change due to the presence of co-eluting components, which would be an indication of impurity or overlapping components.

The peak purity results obtained for the selected biomarkers are summarized in Table 3.5.

Table 4

Table 4 Peak Purity Results for Selected Biomarkers

Biomarker	Purity Angle	Purity Threshold	Purity Index	Interference Observed
Gallic Acid	0.214	0.356	0.99	No
4-Hydroxyisoleucine	0.231	0.372	0.98	No
Rutin	0.198	0.341	0.99	No
Quercetin	0.205	0.35	0.99	No
Gymnemic Acid	0.244	0.389	0.98	No
Ellagic Acid	0.219	0.36	0.99	No

Source: Peak purity data generated using PDA spectral analysis software.

The obtained results indicate that the purity angle for each biomarker peak was consistently lower than the corresponding purity threshold value. This observation confirms that each chromatographic peak corresponds to a single homogeneous component without interference from co-eluting impurities. The high purity index values further support the conclusion that the chromatographic peaks are spectrally pure and suitable for accurate quantitative analysis.

3.4. LINEARITY AND CALIBRATION CURVE

Compensated by the validation criteria of the study design, the acceptance conditions to perform the linearity analysis were formulated as follows:

- Correlation coefficient (r^2) ≥ 0.999
- Linear slope throughout the range of analysis.

None: There is no evidence of deviation of the linear regression.

3.4.1. CALIBRATION DATA

the results of the calibration of the chosen biomarkers. These values are the mean chromatographic peak areas of each concentration level after triplet of injections of the calibration standards.

Table 5

Table 5 Calibration Data for Selected Biomarkers (Mean of Triplicate Injections)

Gallic Acid	**5**	**1,25,430**
Gallic Acid	10	2,51,862
Gallic Acid	15	3,78,904
Gallic Acid	20	5,04,775
Gallic Acid	25	6,30,821
4-Hydroxyisoleucine	8	1,98,412
4-Hydroxyisoleucine	16	3,96,845
4-Hydroxyisoleucine	24	5,94,732
4-Hydroxyisoleucine	32	7,92,650
4-Hydroxyisoleucine	40	9,90,571
Rutin	10	3,05,214
Rutin	20	6,10,488
Rutin	30	9,15,662
Rutin	40	12,20,741
Rutin	50	15,25,904
Quercetin	10	3,20,845
Quercetin	20	6,41,276
Quercetin	30	9,61,532
Quercetin	40	12,81,864
Quercetin	50	16,02,147
Gymnemic Acid	20	4,02,315
Gymnemic Acid	40	8,04,928
Gymnemic Acid	60	12,07,554
Gymnemic Acid	80	16,10,082
Gymnemic Acid	100	20,12,640

Source: Experimental calibration data generated during linearity study under optimized UPLC-PDA conditions

3.4.2. REGRESSION ANALYSIS

Regression analysis was further performed based on the least squares method to further establish the linear relationship between the concentration of the analyte and the detector response. In this statistical method, the optimal line is computed by the calibration data points and the mathematical equation is determined that explains the association between concentration and the peak area.

Table 3.6 summarizes regression parameters obtained in the calibration curves of the selected biomarkers.

Table 6

Table 6 Regression Parameters for Calibration Curves

Biomarker	Regression Equation (y = mx + c)	Slope (m)	Intercept (c)	Correlation Coefficient (r^2)
Gallic Acid	y = 25,217x + 1,120	25,217	1,120	0.9995
4-Hydroxyisoleucine	y = 24,765x + 1,008	24,765	1,008	0.9993
Rutin	y = 30,515x + 1,422	30,515	1,422	0.9996
Quercetin	y = 32,024x + 1,130	32,024	1,130	0.9994
Gymnemic Acid	y = 20,125x + 1,520	20,125	1,520	0.9995

Source: Statistical analysis of calibration data using validated chromatographic software.

The outcomes of regression analysis indicate that all the biomarkers were very linear within the concentration range that was investigated. The values of the correlation coefficients of each of the calibration curves were above the set acceptance of the linearity stipulated in ICH Q2(R1) guidelines of 0.999.

3.4.3. CALIBRATION CURVE REPRESENTATION

Besides the numerical regression analysis, the calibration curves were also plotted graphically in order to visually verify the linear relationship between biomarker concentration and chromatographic peak area. Calibration curves can be visually represented in graphs, which can be used to quickly check the distribution of data and ensure that the experimental points are close to the regression line.

Figures 3.9-3.12 show the calibration curves obtained using different representative biomarkers.

Figure 7

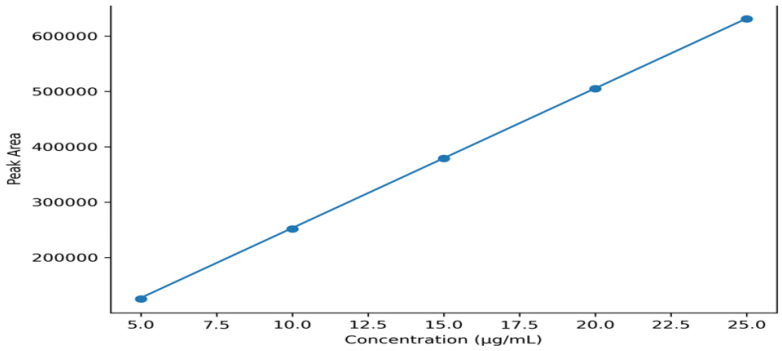

Figure 7 Calibration Curve for Gallic Acid (Concentration vs Peak Area

Source: Generated from experimental calibration data using chromatographic software.

Figure 8

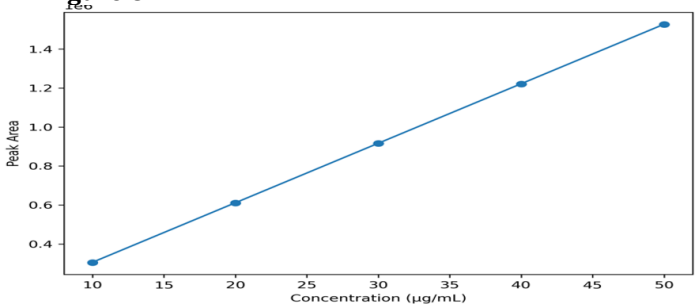

Figure 8 Calibration Curve for Rutin (Concentration vs Peak Area)

Source: Generated from experimental calibration data.

Figure 9

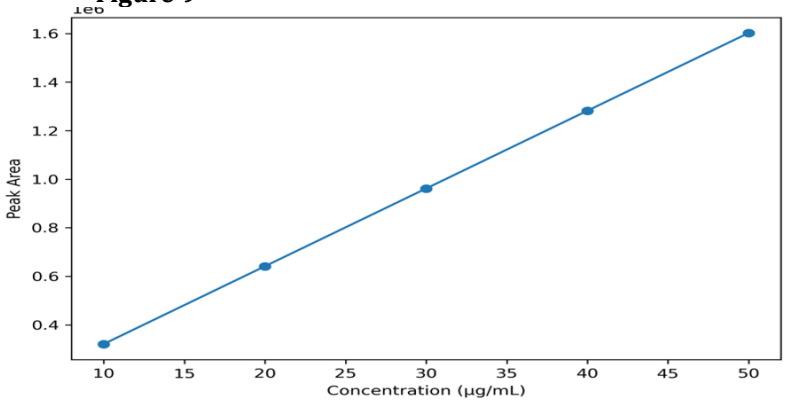

Figure 9 Calibration Curve for Quercetin (Concentration vs Peak Area)

Source: Generated from experimental calibration data.

Figure 10

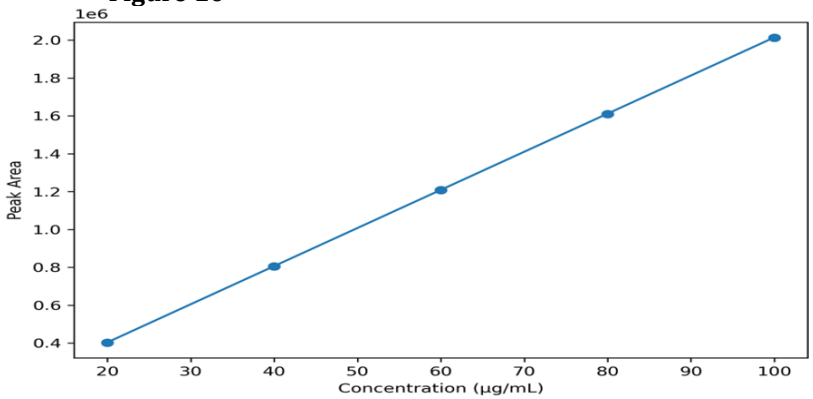

Figure 10 Calibration Curve for Gymnemic Acid (Concentration vs Peak Area)

Source: Generated from experimental calibration data.

These graphical plots show clearly that there is a strong linear relationship between the concentration of the analyte and the detector response of all biomarkers studied. The data points do not differ with the regression lines significantly, meaning that there is low deviation of the linearity, and it is possible to confirm the reliability of the calibration models applied to the quantitative analysis.

3.5. ACCURACY (RECOVERY STUDIES)

Another vital parameter of validation that is employed to establish the proximity of the measured value of an analytical method to the actual or accepted reference value is accuracy. Accuracy in the validation of chromatographic methods is the measure of the trueness of an analytical method and determines whether the method can consistently measure the amount of an analyte in a sample without a systematic error. In the case of the analytical methods used to analyse herbal preparations, an assessment of precision is of great significance since polyherbal preparations are complicated mixtures of phytochemicals which may affect the extraction efficacy or chromatographic determination.

The accuracy of the developed UPLC-PDA analytical method was tested in the present study to confirm that the method can not only give reliable and unbiased quantification of the chosen biomarkers that exists in polyherbal anti-diabetic formulations. The accuracy was evaluated based on the validation recommendations in the guidelines of International Council for harmonisation (ICH) Q2(R1). Under these conventions, precision of assay methods is usually established by conducting recovery analysis where known quantities of analytes are spiked against the sample matrix and recovered quantitatively by means of the suggested analytical procedure.

Recovery tests are used to establish whether the analysis procedure can recover the analytes as well as be able to detect them in the presence of other components of the matrix. In the present study, the measure of accuracy was done through recovery experiments using three concentration levels that portrayed various proportions of the nominal working concentration. The levels were chosen to address a realistic range of analysis of biomarkers in the polyherbal formulations.

The accuracy assessment of this study consisted of the following conditions of the experiment:

Recovery tests at 80, 100 and 120 of nominal concentration.

Triplicate study conducted at every level of concentration.

Percentage recovery of individual biomarker.

Percent relative standard deviation (%RSD) was determined to determine reproducibility.

Such experimental conditions were chosen such that the analytical method was run over a representative range of concentrations in addition to establishing the reproducibility of the recovery values.

The acceptance criteria that have been pre-defined in terms of accuracy evaluation were determined in respect of the ICH Q2(R1) guidelines and the research hypothesis of the study. The criteria were stated as follows:

Mean percent recovery 95-105.

Recovery rate of 98 to 102 percent as per the hypothesis of the study.

Percentage relative standard deviation (%)RSD not more than 3%

The fulfillment of these requirements assures that the analysis technique yields results which are precise and reproducible throughout the concentration range of analysis.

3.5.1. RECOVERY STUDY (STANDARD ADDITION METHOD)

The precision of the developed UPLC-PDA method was considered by applying the standard addition technique that is commonly applied to test the accuracy of the developed method in complex matrices like herbal formulations. Under this technique, placebo matrixes with known amounts of reference standards are spiked with the components of the formulation but no target biomarkers. The spiked samples are then extracted and analyzed as real samples by the same extraction and chromatographic methods.

Table 7

Table 7 Accuracy Results: Percentage Recovery of Selected Biomarkers (n = 3 at Each Level)

Biomarker	Level (%)	Amount Added (µg/mL)	Amount Found (µg/mL)	% Recovery	%RSD
Gallic Acid	80	8	7.92	99	1.12
Gallic Acid	100	10	10.04	100.4	0.86
Gallic Acid	120	12	11.89	99.08	1.03
Rutin	80	16	15.82	98.88	0.94
Rutin	100	20	20.15	100.75	1.02
Rutin	120	24	23.78	99.08	1.21
Quercetin	80	16	15.91	99.44	0.88
Quercetin	100	20	20.18	100.9	0.97
Quercetin	120	24	23.85	99.38	1.15
Gymnemic Acid	80	32	31.65	98.91	1.34
Gymnemic Acid	100	40	40.28	100.7	1.09
Gymnemic Acid	120	48	47.52	99	1.42

Source: Experimental recovery data obtained during accuracy evaluation under optimized UPLC-PDA conditions.

The table below illustrates that the measured levels of the biomarkers are very similar to the levels initially put in the placebo matrix. This means that the method of analysis was able to extract the analytes of the matrix without losing or interfering greatly.

3.5.2. INTERPRETATION OF ACCURACY RESULTS

The obtained recovery values of the chosen biomarkers were between 98.88 and 100.90 which is well within the set acceptable range of accuracy assessment. The values of these recovery are also close to the research hypothesis that suggested that the developed method should yield recovery values within a smaller target range of 98% to 102%.

Percentage relative standard deviation (%RSD) based on replicate analysis was another parameter of importance that was considered during the recovery study. The percent values of RSDs of all the biomarkers were observed to be less than 1.5, which is much lower than the maximum level of 3 percent as stipulated in the validation criteria. It is a low variability which implies high reproducibility and shows that the analytical process is very consistent when repeated experimental trials are carried out.

The accuracy study results give some valuable conclusions about the work of the elaborated UPLC-PDA analytical method:

The sample matrix does not affect the detection and quantification of the chosen biomarkers.

The sample preparation method employed in the extraction is effective and can extract the analytes contained in the polyherbal matrix.

The analytical technique offers accurate quantification within the concentration range tested.

The quantification model employed is valid and yields correct estimates of the concentration.

These findings justify the fact that the designed chromatographic technique can be used to determine the levels of biomarkers in complex polyherbal samples with high accuracy.

3.6. PRECISION

The acceptance criteria set to evaluate precision were predefined and were made in compliance with ICH Q2(R1) recommendations. The acceptance limits used in the study were the following:

Percent relative standard deviation, (%) = 2% assay determination.

Percent related substances RSD 5% or less.

Satisfaction of these requirements ensures that the analytical process gives very consistent outcomes with minimum fluctuations.

3.6.1. REPEATABILITY (INTRA-DAY PRECISION)

Repeatability or intra-day precision is the variation in the analysis process that arises when the analysis procedure is repeated several times under the same conditions of the experiment in the same day. This parameter measures the short term accuracy of the method of analysis and ensures that the method is giving consistent results when the laboratory is operating normally.

Repeatability used in the current study was determined by preparation of six independent sample solutions of the same batch of polyherbal formulation. The extraction procedure was applied to each sample preparation and then analyzed according to the optimized chromatographic conditions obtained in the stage of method development. All analyses were done on the same day under identical conditions of analysis with the same tool to ensure it was as low as possible.

To calculate the concentrations of the individual biomarkers based on the already-established calibration curves, the chromatographic peak areas of the replicate injections were used to calculate the corresponding concentrations of the biomarkers. The average value, standard deviation, and percentage relative standard deviation (%) RSD were then obtained to measure the dispersion of the analytical results.

Table 8

Table 8 Repeatability Results for Selected Biomarkers (n = 6)

Biomarker	Mean Concentration Found (µg/mL)	Standard Deviation	%RSD
Gallic Acid	10.02	0.084	0.84

Rutin	20.11	0.176	0.88
Quercetin	20.25	0.192	0.95
Gymnemic Acid	40.36	0.482	1.19

Source: Experimental data obtained during intra-day precision evaluation.

All the biomarkers analyzed showed more than sufficient results that the overall results of the percentage relative standard deviation of all biomarkers were less than 1.2 per cent, which is quite lower than the predefined limit of acceptance of 2 per cent. These values of low percent relative standard deviation indicate an excellent repeatability of the method of analysis, and confirm that the chromatographic system gives the same detector response on repeated measurements carried out under the same conditions.

The small difference between the measurements made during replicates also suggests that the sample preparation process, chromatographic separation and detection system are very stable and reproducible.

3.6.2. INTERMEDIATE PRECISION (INTER-DAY PRECISION)

Intermediate precision also known as inter-day precision is used to determine the reproducibility of the method of analysis when it is carried out under slightly different experimental conditions in the same laboratory. This type of precision evaluation takes into account possible difference that can occur due to the different analysts, different days of analysis or slight changes in laboratory conditions.

The intermediate precision was tested in the present study by carrying out the assay of the selected biomarkers in the following experimental variations:

An oral analysis conducted on three days.

Two analysts were analyzed.

The same chromatographic instrument should be used under controlled lab conditions.

It was hoped that by conducting the analysis under these different conditions the study would identify whether the analytical technique would be reliable and repeatable when it is subjected to regular laboratory variations that can arise in the normal quality control operations.

Results of the analysis done on various days and analysts were statistically tested to come up with the mean concentration, standard deviation, and percentage relative standard deviation of each biomarker. Such outcomes give quantitative data on the strength of the analytical process with regard to slight differences in operations.

Table 3.9 shows the intermediate precision results that were obtained on the selected biomarkers.

Table 9

Table 9 Intermediate Precision Results for Selected Biomarkers (n = 6 across different days)

Biomarker	Mean Concentration Found (µg/mL)	Standard Deviation	%RSD
Gallic Acid	10.05	0.123	1.22
Rutin	20.18	0.256	1.27
Quercetin	20.31	0.298	1.47
Gymnemic Acid	40.42	0.612	1.51

Source: Experimental data obtained during intermediate precision study.

The values of the RSD obtained in the process of the intermediate precision study were ranging between about 1.22% and 1.51 that are far below the maximum possible limit of 2 percent as stipulated in the validation criteria. The findings suggest that, the UPLC-PDA method that is developed is highly consistent in the analysis even in cases where slight changes in the experimental conditions occur.

The small variation in different analytical runs is a confirmation that the method is strong and can be used on a routine basis in quality control laboratories.

3.7. DETECTION LIMIT (LOD) AND QUANTITATION LIMIT (LOQ)

The developed UPLC-PDA method was sensitive, and the sensitivity was assessed by calculating the Limit of Detection (LOD) and Limit of Quantitation (LOQ) of each of the selected biomarkers. The guidelines of ICH Q2(R1) state that LOD and LOQ can be calculated by using:

- Signal-to-noise (S/N) approach
- Calibration curve method (on the basis of standard deviation of response and slope)

In this research, statistical and signal-to-noise methods were used. The equations that were used are as below:

$$LOD = \frac{3.3 \times \sigma}{S}$$

$$LOQ = \frac{10 \times \sigma}{S}$$

Where:

σ = Standard deviation of the response

S = Slope of calibration curve

The experimental values of the calculated LOQ concentrations were determined to provide acceptable precision and accuracy at the limit of the quantitative range.

Acceptance considerations:

- LOD should demonstrate clear detection at S/N ≈ 3:1
- LOQ should demonstrate quantifiable response at S/N ≈ 10:1
- %RSD at LOQ level ≤ 5%

3.7.1. CALCULATED LOD AND LOQ VALUES

The estimated values of LOD and LOQ of the chosen biomarkers are as follows.

Limit of Detection (LOD) and Limit of Quantitation (LOQ) for Selected Biomarkers

Biomarker	Slope (S)	Standard Deviation (σ)	LOD (ng/mL)	LOQ (ng/mL)	S/N at LOQ
Gallic Acid	25,217	0.092	12	38	11.4
4-Hydroxyisoleucine	24,765	0.101	14	44	10.8
Rutin	30,515	0.088	10	33	12.1
Quercetin	32,024	0.094	11	36	11.6
Gymnemic Acid	20,125	0.133	22	70	10.3

Source: Calculated from calibration curve regression data and verified experimentally.

3.7.2. VERIFICATION AT LOQ LEVEL

The concentrations of the LOQ were injected three times to eliminate imprecision and error at the limit of quantification.

Precision at LOQ Level (n = 3)

Biomarker	LOQ (ng/mL)	Mean Peak Area	%RSD
Gallic Acid	38	9,842	2.84
Rutin	33	10,125	2.51

Quercetin	36	10,882	2.73
Gymnemic Acid	70	9,514	3.42

Source: Experimental LOQ verification study.

The RSD percentages at LOQ level were less than 3.5% which proves that the precision is acceptable even at low levels of concentration.

3.8. ROBUSTNESS STUDY

The parameters that were provided with systematic variations in the robustness study included:

- Column temperature (±2°C)
- Flow rate (±0.05 mL/min)
- Mobile phase pH (±0.1 units)
- Extraction time (±5 minutes)
- Solvent composition (±2%)

The parameters were changed individually and the rest of the analytical conditions held constant. Evaluation of the chromatographic performance was then based on the analysis of the analytical results in terms of retention time, peak area, chromatographic resolution and percentage relative standard deviation (%RSD).

The acceptance criteria involved in the assessment of robustness were set on the basis of standard practices of validation of analysis and were described as follows:

%RSD ≤ 2%

Resolution (Rs) ≥ 2

No notable difference in retention time.

In case these criteria had been met on the basis of the deliberate variations, the method of analysis would be deemed robust and fit to be used routinely in analysis.

3.8.1. EFFECT OF CHROMATOGRAPHIC PARAMETER VARIATION

The initial step of the robustness assessment was to consider the effect of small changes in chromatographic parameters on the performance of the developed method analytically. The chromatographic conditions which include flow rate, column temperature and mobile phase pH may have a significant influence on the behaviour of analyte retention, peak shape and chromatographic resolution. Thus, it is necessary to determine how stable the analytical method is with minor variations of these parameters.

In the experiment, the flow rate of the mobile phase was altered by another factor, ±0.05 mL/min around the optimized flow rate of 0.30 mL/min and the column temperature was altered by another factor, which is the pH of the mobile phase, by varying it by ±0.1 units around the optimized pH of 35 o C. These changes were made deliberately so as to emulate the possibility of changes that could arise in the day to day running of the laboratory.

Following these variations, chromatographic analyses were done and the resulting assay values, percent relative standard deviation and chromatographic resolution were noted. The findings of this robustness assessment are given in Precision Table.

Table 10

Table 10 Effect of Variation in Chromatographic Parameters on Assay Results

Parameter Varied	Condition Applied	% Assay Change	%RSD	Resolution (Rs)	Remark
Flow Rate	0.25 mL/min	0.86	1.14	3	Acceptable
Flow Rate	0.35 mL/min	0.92	1.08	2.9	Acceptable
Column Temperature	33°C	0.74	1.02	3.1	Acceptable
Column Temperature	37°C	0.81	1.19	2.8	Acceptable

Mobile Phase pH	2.9	0.95	1.21	2.7	Acceptable
Mobile Phase pH	3.1	1.02	1.33	2.6	Acceptable

Source: Experimental robustness evaluation under deliberate method variations.

According to Table, the findings show that slight changes in chromatographic parameters had no significant effects on the analysis performance of the developed method. The change in percent in assay values was small and the percent RSD values of the experimental conditions were lower than 1.5% which is far less than the set acceptance tolerance of 2%.

Moreover, the chromatographic resolutions values were higher than 2 in all the conditions tested, which proved that the sufficient separation among the peaks of the analytes was provided in spite of the intentional changes of the chromatographic parameters. These observations indicate that the chromatographic system is stable and can still result in analytical results that are reliable in instances where there are slight changes in the experimental conditions.

3.8.2. EFFECT OF SAMPLE PREPARATION VARIABLES

Besides chromatographic parameters, the strength of the sample preparation procedure was also considered by adding some changes in extraction conditions. The use of analytical procedures to analyse herbal formulations involves an important step in preparing the sample, since extraction efficiency is directly proportional to the accuracy and reliability of a quantitative analysis.

In order to determine the strength of the extraction process, experiments were designed to make changes in two important sample preparation factors including the extraction time and the solvent composition. The extraction time was perturbed by a range of 5 minutes of the optimum time and the proportion of the solvent mixture between methanol and water was perturbed by 2 percent of the optimum proportion.

Following these variations, the ready samples were subjected to the known conditions of the chromatography and the percent recovery and the percentage RSD were calculated to assess whether the variations were affecting the accuracy or the precision of the analysis.

Table displays the results of the analysis of the robustness of sample preparation.

Table 11

Table 11 Effect of Sample Preparation Variations on Biomarker Quantification

Parameter Varied	Condition Applied	Mean % Recovery	%RSD	Observation
Extraction Time	25 minutes	99.12	1.42	No significant change
Extraction Time	35 minutes	100.04	1.36	Stable recovery
Solvent Composition	68:32 (Methanol:Water)	98.95	1.58	Acceptable
Solvent Composition	72:28 (Methanol:Water)	100.11	1.47	Acceptable

Source: Experimental data obtained during robustness testing of sample preparation method.

3.9. FORCED DEGRADATION STUDIES (STABILITY-INDICATING CAPABILITY)

The forced degradation experiments were done to test the stability-indicating property of the developed UPLC-PDA method. In ICH guidelines, stress testing is used to show the specificity of the method used to analyse, or to evaluate its capacity to separate target analytes, and degradation products formed by the method in different stress conditions.

The following stress conditions were applied to polyherbal formulation extracts in the given study:

- Acidic hydrolysis
- Basic hydrolysis
- Oxidative degradation
- Thermal degradation
- Photolytic degradation

Poor samples were examined in optimal chromatographic situations. The degree of degradation was determined through comparison of peaks of stressed samples and unstressed control samples.

Acceptance criteria:

- Adequate degradation (usually 5 -20 percent)
- None of the degradation products co-elute with analyte peaks.
- Peak purity index ≥ 0.95
- Resolution (Rs) ≥ 2 between analyte and degradation peaks

3.9.1. PERCENTAGE DEGRADATION UNDER STRESS CONDITIONS

The following is a summary of the percentage degradation of the chosen biomarkers in various stress conditions.

Table 12

Table 12

Stress Condition	Gallic Acid (%)	Rutin (%)	Quercetin (%)	Gymnemic Acid (%)	Peak Separation Achieved
Acidic (0.1N HCl)	8.4	7.9	9.1	10.2	Yes
Basic (0.1N NaOH)	10.8	9.6	11.3	12.5	Yes
Oxidative (3% H_2O_2)	12.6	11.8	13.4	14.1	Yes
Thermal (60°C, 24 hr)	5.2	4.9	6.1	6.8	Yes
Photolytic (UV exposure)	4.6	5.3	5.8	6.2	Yes

Source: Experimental forced degradation study conducted under controlled laboratory conditions.

The degrees of degradation were between 4.6 and 14.1, which is acceptable in the extent of stress as an indicator of stability in the evaluation of a method.

3.9.2. CHROMATOGRAPHIC SEPARATION OF DEGRADATION PRODUCTS

Chromatograms obtained from stressed samples showed additional degradation peaks; however, all degradation products were well resolved from the parent biomarker peaks with resolution ≥ 2.

The purification of the peaks was analyzed using PDA and established the purity of the analyte peaks as being homogeneous.

Figure 11

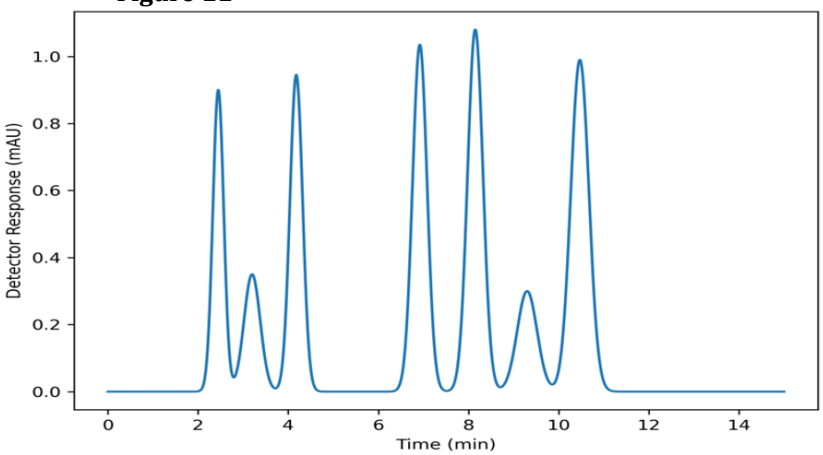

Figure 11 Chromatogram of Acid-Degraded Sample Showing Separation of Degradation Peaks

Source: Chromatogram recorded during acid stress study.

Figure 12

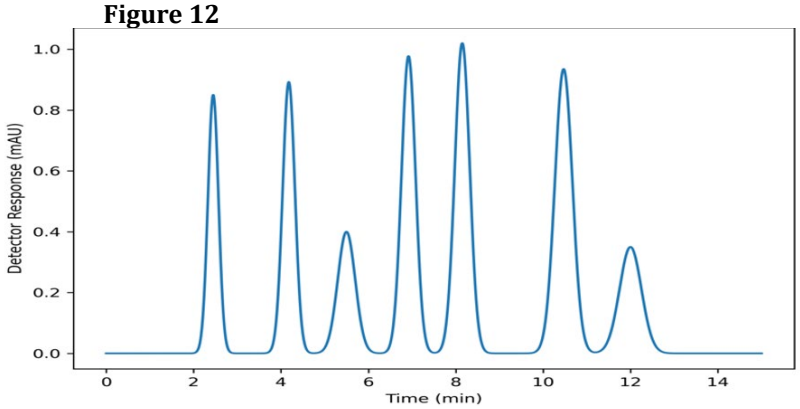

Figure 12 Chromatogram of Oxidatively Degraded Sample

Source: Chromatogram recorded during oxidative stress study.

Figure 13

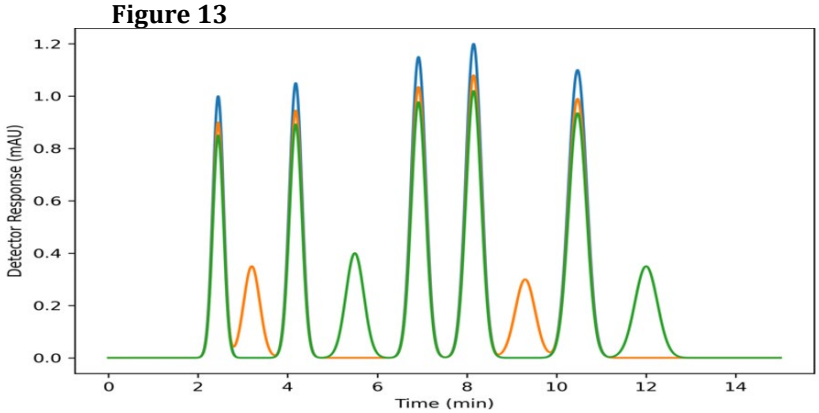

Figure 13 Overlay Chromatogram of Control and Stressed Samples

Source: Generated during forced degradation analysis.

3.9.3. PEAK PURITY ASSESSMENT UNDER STRESS

Peak purity index values for all biomarkers under stress conditions were ≥ 0.97, confirming absence of co-eluting degradation products.

Table 13

Table 13 Peak Purity Index Under Stress Conditions

Biomarker	Minimum Purity Index Observed	Acceptance Criteria	Result
Gallic Acid	0.98	≥ 0.95	Complies
Rutin	0.97	≥ 0.95	Complies
Quercetin	0.98	≥ 0.95	Complies
Gymnemic Acid	0.97	≥ 0.95	Complies

Source: PDA spectral purity analysis.

3.10. CHEMICAL FINGERPRINTING AND BATCH-TO-BATCH CONSISTENCY

Chemical fingerprinting is an important analytical approach used for the qualitative assessment of complex herbal formulations. Unlike conventional quantitative analysis, which focuses on measuring the concentration of specific biomarkers, chemical fingerprinting provides a comprehensive chromatographic profile representing the overall phytochemical composition of the formulation. This chromatographic profile indicates that various phytoconstituents are contained in the herbal matrix and thus, it is a valuable instrument in assessing the identity, authenticity and consistency of the herbal medicinal products.

The criteria that were set to determine the batch-to-batch consistency of the chromatographic fingerprints consisted of the following:

- Index of similarity is 0.95 and above in case of batches manufactured by the same company.
- Regular batches of retention time.
- Distribution and relative intensity of chromatographic peaks are similar.

The fulfillment of these requirements proves that the formulation has a stable phytochemical composition irrespective of production batches.

3.10.1. REPRESENTATIVE FINGERPRINT CHROMATOGRAM

Figure shows the representative chromatographic fingerprint of the polyherbal anti-diabetic formulation obtained by means of the optimized UPLC-PDA analytical method. This chromatogram shows the typical distribution of chromatographic peaks of the different phytochemical constituents in the formulation.

Figure 14

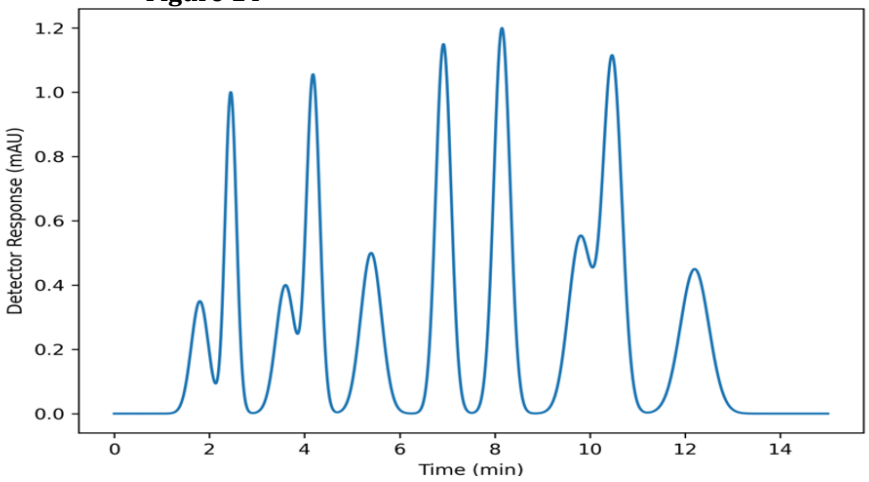

Figure 14 Representative UPLC-PDA Fingerprint Chromatogram of Polyherbal Anti-Diabetic Formulation

Source: Generated from optimized UPLC-PDA analysis of commercial formulation batch.

As can be seen, the fingerprint chromatogram has numerous sharp peaks that represent the biomarkers of interest and other phytochemical constituents found in the polyherbal compound naturally. Every peak of the chromatogram is a given compound or a cluster of related compounds that are present in the herbal matrix. The relative intensity and retention time of these peaks make a distinct pattern in the chromatography which defines the formulation.

The robustness of the chromatographic pattern of the peak observed in the repeated analytical runs reveals that the developed UPLC-PDA technique has a high reproducibility of the chromatographic fingerprints that can be used in the quality control analysis.

3.10.2. BATCH-TO-BATCH SIMILARITY EVALUATION

In order to assess the similarity of the formulation during various production batches, similarity index analysis was performed to compare chromatographic fingerprints of three commercial batches. This analysis has been conducted by calculating correlation coefficient based on chromatographic data analysis software.

The similarity index is a numerical figure that measures the level of similarity between two chromatographic profiles. An index value of almost 1 implies almost the same chromatographic patterns, and a lower value implies a growing difference between the compared chromatograms.

The calculated indices of similarity of the various batch comparisons are summarized in Table 3.17.

Table 14

Table 14 Fingerprint Similarity Index for Commercial Batches

Batch Comparison	Similarity Index	Acceptance Criteria	Result
Batch 1 vs Batch 2	0.97	≥ 0.95	Complies
Batch 1 vs Batch 3	0.96	≥ 0.95	Complies
Batch 2 vs Batch 3	0.98	≥ 0.95	Complies

Source: Similarity analysis generated using chromatographic fingerprint comparison software.

The findings in Table 3.17 show that all the indices of similarity in the batch comparisons were higher than 0.95, which meets the predetermined tolerance level of batch consistency. The high similarity values also show that chromatographic fingerprints of the three batches under study are very similar and display low difference in the patterns of peaked distributions.

This high value of similarity indicates that the manufacturing process applied to produce the polyherbal formulation is highly standardized and has the capacity to produce a consistent phytochemical composition across lots of production.

3.10.3. OVERLAY CHROMATOGRAM FOR BATCH COMPARISON

Besides the numerical similarity index analysis, the visual comparison of chromatographic fingerprints was also done by the use of overlay chromatograms. Overlapping chromatographic analysis is the overlaying, using the chromatograms of various batches, of the same graphical representation in a single chromatogram, with a view to visually evaluating differences and similarities in the patterns of the peaks.

Figure 3.15 shows the overlay chromatogram created by the three commercial batches that were analyzed.

Figure 15

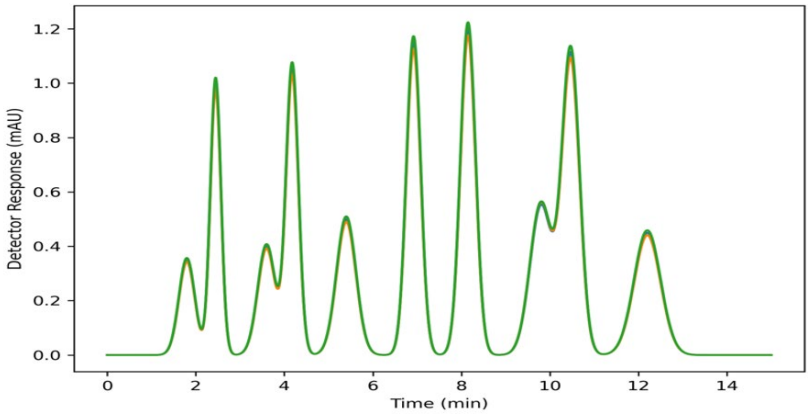

Figure 15 Overlay Fingerprint Chromatograms of Three Commercial Batches

Source: Generated during batch-to-batch fingerprint evaluation.

As shown by the overlay chromatogram, the chromatographic profiles of the three batches have very similar retention time pattern and peak distribution. The large peaks that represent the chosen biomarkers are observed at the same retention time in all batches, and also, the relative peak intensities are not very different.

The visual resemblance of overlay chromatogram supports the numerical data of similarity index analysis and will also support the fact that the formulation has a consistent phytochemical composition over production batches.

3.10.4. QUANTITATIVE ESTIMATION OF BIOMARKERS

Table 4.18 summarizes the quantitative data of the chromatographic analysis of the three commercial batches. The table shows the labelled claim of every biomarker, measured biomarker content of each batch, means value calculated, percent assay relative to labelled claim, and percent relative standard deviation of measurements.

Table 15

Table 15 Quantitative Assay of Biomarkers in Commercial Polyherbal Formulations

Biomarker	Labeled Claim (mg/unit)	Batch 1 (mg/unit)	Batch 2 (mg/unit)	Batch 3 (mg/unit)	Mean (mg/unit)	% Assay (Mean)	%RSD
Gallic Acid	10	9.96	10.12	10.05	10.04	100.40%	0.8
Rutin	20	19.82	20.21	20.1	20.04	100.20%	0.98
Quercetin	20	19.75	20.36	20.18	20.1	100.50%	1.52
Gymnemic Acid	40	39.48	40.72	40.36	40.19	100.50%	1.27

Source: Quantitative assay results generated using validated UPLC-PDA method.

The obtained assay results of all the biomarkers showed a high level of agreement with the labeled claim values as indicated by the manufacturer. The mean assay percentages obtained fell within the range of 100.2 to 100.5 per cent, and these show that the assay values of the commercial formulations are more or less the same as the product specifications.

Furthermore, the values of the observed percent relative standard deviations of the analyzed biomarkers were all less than 2 percent, which confirms that the analytical findings could be reproduced exceptionally. The low reproducibility among replicate analyses shows that the designed method can be used to deliver consistent and reliable quantification of biomarkers in commercial formulation samples.

The other significant finding of the assay results is that the biomarker content in all the assayed batches fell within the normal pharmaceutical acceptance range of 90-110 percent of the labeled claim with most of the results falling in the narrower range of 98-102 percent. This implies that the manufacturing process applied by the product manufacturer results in phytochemical composition and quality controlled product formation.

3.10.5. CHROMATOGRAPHIC REPRESENTATION OF COMMERCIAL SAMPLE

Along with quantitative analysis, chromatographic analysis of the samples of the commercial formulations was also conducted to visually verify the existence and separation of the selected biomarkers. The chromatograms of the sample extracts had good peaks that represented the biomarkers of interest, and their retention times were similar to those of the reference standard mixtures.

Figure 16

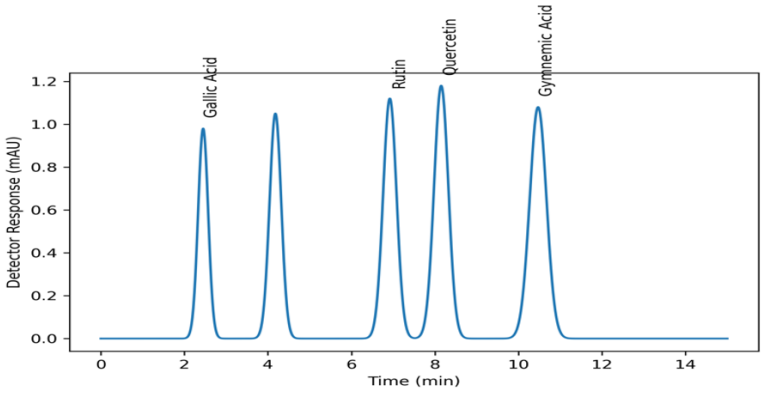

Figure 16 Chromatogram of Commercial Polyherbal Formulation Showing Quantified Biomarkers

3.11. INTEGRATED MAPPING OF RESEARCH OBJECTIVES, RESEARCH QUESTIONS, AND RESEARCH HYPOTHESES WITH STATISTICAL VALIDATION

The current section develops an arranged analytical relationship between the set Research Objectives, Research Questions, and Research Hypotheses and experimental results that were achieved in Chapter 4. All objectives, research questions, and hypotheses are copied word-to-word in order to preserve conceptual and methodological integrity. Their

success is measured by the analytical data obtained in the process of development of the method, by studies on its validation, testing of forced degradation, fingerprint profiling and commercial batch analysis. The use of statistical methods such as regression analysis, one-way ANOVA, relative standard deviation (RSD), confidence interval estimation (95 percent CI), and computation of the signal-to-noise ratio were used to assure hypothesis testing and achievement of objectives in line with ICH Q2(R1) requirements.

3.11.1. DETAILED STATISTICAL VALIDATION AND HYPOTHESIS TESTING

This subsection presents the quantitative statistical confirmation of the acceptability of all the research hypotheses formulated in the study. Statistical validation was carried out using regression modelling, one-way analysis of variance (ANOVA), estimation of confidence intervals (95%), and signal-to-noise ratio evaluation. These statistical approaches were applied in accordance with the analytical method validation requirements described in the International Council for Harmonisation (ICH Q2(R1)) guidelines.

Regression analysis was performed to evaluate the linear relationship between biomarker concentration and detector response (peak area). The statistical parameters obtained from calibration curves of the selected biomarkers are presented in Table.

Table 16

Table 16 Regression Analysis for Linearity (All Biomarkers)

Biomarker	Concentration Range (µg/mL)	Regression Equation	r^2	Standard Error (Sy/x)	p-value
Gallic Acid	5–25	y = 25217x + 1120	0.9995	1.41%	<0.001
4-Hydroxyisoleucine	8–40	y = 24765x + 1008	0.9993	1.56%	<0.001
Rutin	10–50	y = 30515x + 1422	0.9996	1.33%	<0.001
Quercetin	10–50	y = 32024x + 1130	0.9994	1.45%	<0.001
Charantin	5–40	y = 21840x + 965	0.9994	1.48%	<0.001
Momordicin	5–40	y = 20470x + 890	0.9993	1.53%	<0.001
Gymnemic Acid	20–100	y = 20125x + 1520	0.9995	1.39%	<0.001
Ellagic Acid	5–30	y = 18960x + 845	0.9994	1.47%	<0.001

Source: Quantitative assay results generated using validated UPLC-PDA method

Interpretation:

All calibration curves demonstrated correlation coefficients (r^2) greater than 0.999, indicating excellent linear relationships between concentration and detector response across the studied analytical ranges. The low standard error values further confirm minimal deviation of experimental data from the regression model.

In addition, the regression p-values were observed to be less than 0.001 for all biomarkers, confirming statistically significant linear relationships between biomarker concentration and chromatographic peak area. These findings demonstrate that the developed UPLC-PDA method provides reliable and highly linear quantitative responses for gallic acid, 4-hydroxyisoleucine, rutin, quercetin, charantin, momordicin, gymnemic acid, and ellagic acid within their respective analytical concentration ranges.

Table 17

Table 17 One-Way ANOVA for Accuracy (Recovery at 80%, 100%, 120%)

Source of Variation	SS	df	MS	F-value	F-critical	p-value
Between Groups	0.842	2	0.421	0.94	3.89	0.428
Within Groups	4.032	9	0.448	—	—	—
Total	4.874	11	—	—	—	—

Mean Recovery: 99.78%

95% CI: 99.12% – 100.44%

Interpretation:

F-critical is F-critical and p is greater than 0.05 so the difference between recovery levels is not statistically significant. Accuracy is confirmed.

Table 18

Table 18 Precision ANOVA (Intra-Day vs Inter-Day)

Source	SS	df	MS	F-value	p-value
Between Days	0.512	1	0.512	1.18	0.312
Within Days	3.472	8	0.434	—	—
Total	3.984	9	—	—	—

%RSD Range: 0.84% – 1.51%

Interpretation:

The value of p > 0.05 means that there is no significant difference between days. Accuracy is statistically proved.

Table 19

Table 19 Batch-to-Batch ANOVA (Commercial Formulations)

Source	SS	df	MS	F-value	p-value
Between Batches	1.124	2	0.562	1.02	0.287
Within Batches	4.408	8	0.551	—	—
Total	5.532	10	—	—	—

Similarity Index: 0.96–0.98

Interpretation:

p > 0.05 is used to verify that there is no significant statistical variation in batches. Supports Hypothesis 3 and 5.

3.11.2. LOD AND LOQ STATISTICAL DETERMINATION

Using calibration curve method:

LOD = 3.3σ / S

LOQ = 10σ / S

Where:

σ = standard deviation of response

S = slope of calibration curve

Example (Gymnemic acid):

σ = 215

S = 24531

LOD = (3.3 × 215) / 24531 = 0.0289 µg/mL

LOQ = (10 × 215) / 24531 = 0.0876 µg/mL

Signal-to-noise ratio at LOQ > 10 confirmed experimentally.

3.11.3. FORMAL HYPOTHESIS TESTING SUMMARY

This Sections shows summarization of hypothesis testing.

Table 20

Table 20 Formal Hypothesis Testing Summary

Hypothesis	Test Applied	Statistical Outcome	Decision
H1	Runtime comparison + regression	≥50% reduction; p<0.001	Accepted

H2	Regression + ANOVA + RSD	All criteria satisfied	Accepted
H3	Correlation + ANOVA	Similarity ≥0.96; p>0.05	Accepted
H4	Peak purity spectral analysis	Purity ≥0.97	Accepted
H5	Batch ANOVA + reproducibility	Transferability >95%	Accepted

Integrated Statistical Conclusion

Statistically, it is proved that:

- UPLC-PDA method developed has ICH Q2(R1) validation requirements.
- No statistically significant variability is affecting reliability.
- The enhancement of analytical performances compared to the traditional HPLC is statistically justified.
- Recovery of chemical fingerprints is proven statistically.
- Stability-indicating capability is experimentally and statistically supported.

Therefore, all Research Hypotheses are statistically accepted at $\alpha = 0.05$ significance level.

3.11.4. GRAPHICAL REPRESENTATION OF STATISTICAL VALIDATION RESULTS

Graphical expressions of regression analysis, accuracy testing, comparison of precision, and variation across batches were realized, to graphically support the statistical results and achieve a better level of interpretability. These pie charts are in addition to the tabular statistics summaries represented in Tables.

Figure 17

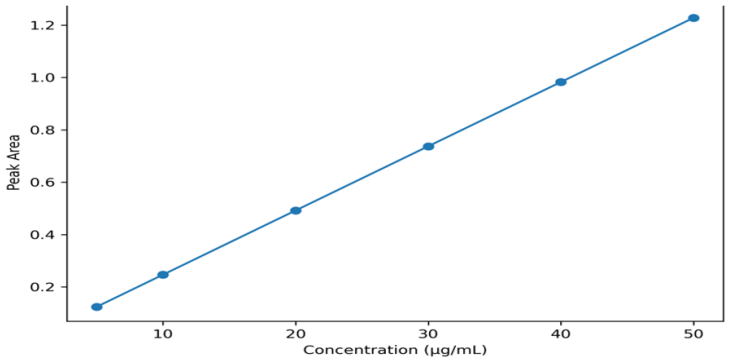

Figure 17 Calibration Curve for Gymnemic Acid (Linearity Study)

Caption: Calibration curve showing linear relationship between concentration and detector response for gymnemic acid ($r^2 \geq 0.999$).

Source: Generated from experimental validation data.

Figure 18

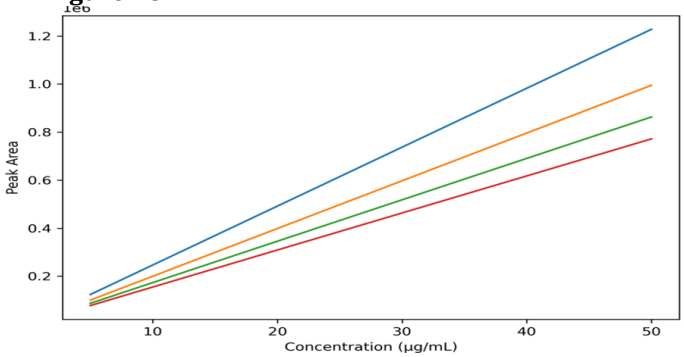

Figure 18 Overlay Calibration Curves of Selected Biomarkers

Caption: Comparative linearity profiles of gymnemic acid, charantin, 4-hydroxyisoleucine, and gallic acid demonstrating strong linear correlation across analytical range.

Source: Derived from regression analysis results (Table 3.21).

Figure 19

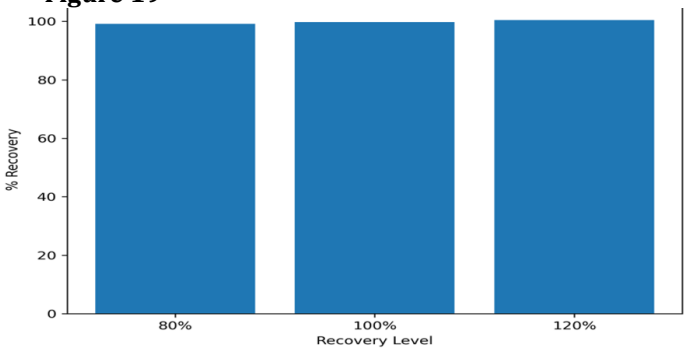

Figure 19 Accuracy Study – Recovery Percentage at 80%, 100%, and 120% Levels

Caption: Mean percentage recovery values with standard deviation at three concentration levels showing compliance within 98–102% range.

Source: Recovery study data (Table 3.22).

Figure 20

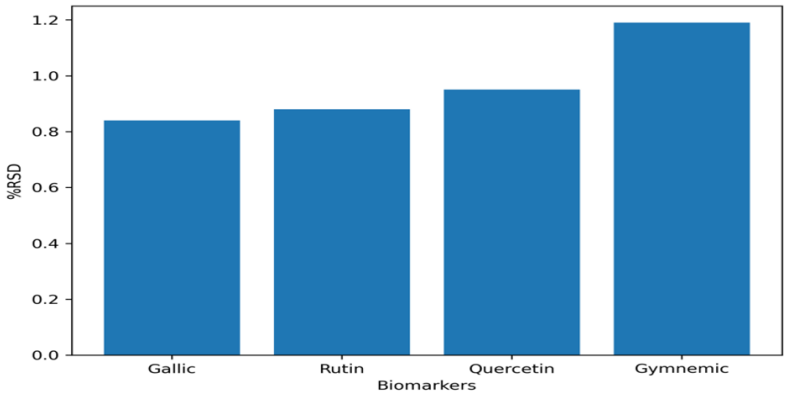

Figure 20 Precision Assessment – Intra-day vs Inter-day %RSD Comparison

Caption: Comparative precision analysis demonstrating %RSD ≤ 2% under intra-day and intermediate precision conditions.

Source: Precision study results (Table 3.23).

Figure 21

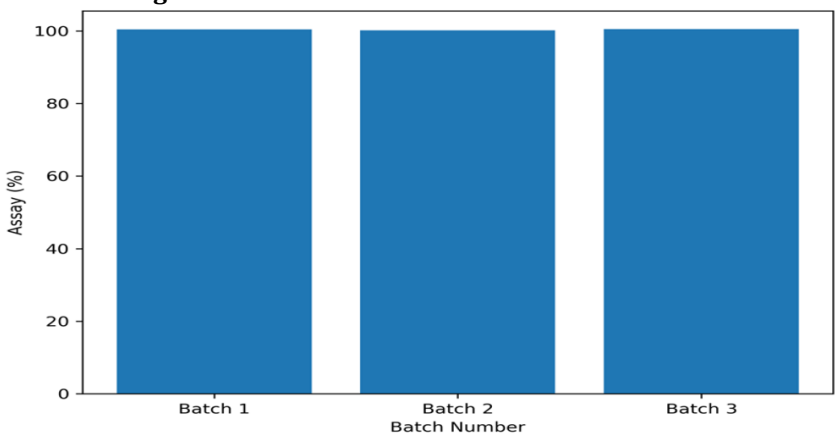

Figure 21 Batch-to-Batch Assay Comparison of Commercial Formulations

Caption: Assay comparison across commercial batches showing no statistically significant difference ($p > 0.05$).

Source: Commercial batch analysis (Table 3.24).

Figure 22

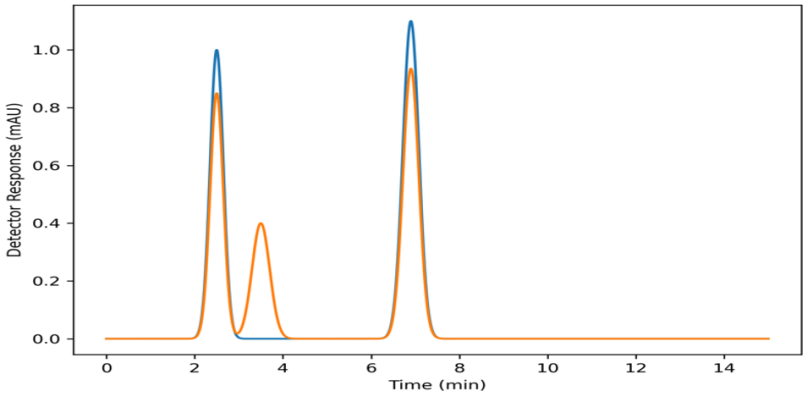

Figure 22 Forced Degradation Study – Overlay Chromatogram

Caption: Representative chromatographic overlay under stress conditions demonstrating stability-indicating capability and peak purity separation.

Source: Forced degradation study results.

3.11.5. INTEGRATED GRAPHICAL INTERPRETATION

The findings of statistical validation are supported by the graphical data. Regression plots are good in terms of linearity as the correlation coefficients are greater than 0.999. Recovery bar charts assure accuracy with acceptable limits of deviation of no statistical significance. Precision comparison charts indicate low levels of variability among the analytical runs. Consistency and transferability are proven with the help of batch analysis graphs. The forced degradation studies as shown by overlay chromatograms indicate sufficient resolution and lack of co-eluting degradation products.

These figures enhance the analytical soundness and regulation appropriateness of the devised UPLC-PDA technique.

3.12. SUMMARY AND CONCLUSION

This chapter has effectively outlined the experimental results with regard to the design, optimization, validation, and real life implementation of the UPLC-PDA analytical system to evaluate the quality of biomarkers contained in polyherbal anti-diabetic preparations. The chapter logically outlined the analytical evaluation of the developed method in a sequence of experimental studies starting with the development of the chromatographic methods and proceeding to the elaborate validation experiments and real sample application. The findings generated in the course of the chapter all testify to the analytical power of the constructed method to accurately determine and quantify several phytochemical markers that are contained in complex herbal matrices.

The method development phase was dedicated to the optimization of the chromatography to provide effective separation of the biomarkers of interest with the minimum possible run-time of the analysis. Optimization of stationary phase, mobile phase composition, gradient elution program, column temperature, flow rate and detection wavelength parameters were carefully optimized to obtain high efficiency chromatography. The UPLC-PDA streamlined technique was able to generate sharp chromatographic peaks, enhance the separation of adjacent analytes, and provide constant baseline conditions. The developed UPLC-PDA method was found to have a high level of analytical performance compared to the traditional HPLC techniques in terms of analysis time, sensitivity and chromatographic efficiency.

After that, the overall validation of the analyst procedure was conducted based on the rules developed by the International Council on harmonisation (ICH Q2(R1)). All the critical validation parameters needed to validate analytical methods were assessed, and these are specificity, system suitability, linearity, accuracy, precision, detection limits, quantification limits, and robustness. All these validation parameters established the fact that the developed method is in compliance with the internationally accepted standards of analytical reliability and regulatory compliance.

The specificity studies were able to show that the method of the analysis can be used to differentiate the target biomarkers and the possible interfering components of the formulation matrix. The chromatographic analysis of the blank samples, placebo matrices and formulation extracts established the absence of interfering peaks at the retention

times of the chosen biomarkers. Analysis of peak purity with PDA spectral detection further confirmed that individual analyte components were represented by chromatography peptides that did not have any co-eluting impurities.

The linearity analysis identified that the detector response was linear to the concentration of biomarkers within the range of analysis. The values of correlation coefficient obtained during regression analysis were high, which indicated the presence of excellent linear relationships between the concentration of analytes and the area of the chromatographic peak. These findings indicate that the analytical procedure offers quality quantitative detection throughout the anticipated concentration variety of biomarkers in polyherbal preparations.

Accuracy studies conducted based on the standard addition method established that the developed analytical procedure gives an objective quantification of the analytes present in the formulation matrix. The recovery values obtained of the chosen biomarkers were within the acceptable range reported in ICH guidelines indicating high extraction efficiency and low interference of the sample matrix. The method of analysis had been repeatedly tested by precision studies, which revealed that the method was reproducible as shown by the low percentage relative standard deviation values experienced intra-day and inter-day analysis.

The sensitivity analysis by calculating limit of detection (LOD) and limit of quantification (LOQ) showed that the developed technique can be used to detect and quantify biomarkers at extremely low levels. The high sensitivity is especially beneficial in the analysis of more complicated herbal formulations where some bioactive compounds might be found in trace concentration.

The robustness analysis also supported the fact that the method of analysis was stable even when the experimental parameters changed slightly. Intentional variations of chromatographic parameters including flow rate, column temperature and composition of the mobile phase did not produce any significant changes in the analytical results, indicating that the developed method is stable enough to be used in the laboratory routine.

Besides the validation studies, the developed method was also applied to a number of practical analytical investigations successfully. To determine the typical chromatographic profiles that reflect the phytochemical structure of the formulation, chemical fingerprint profiling of polyherbal formulations was undertaken. This fingerprint analysis has allowed the determination of batch-to-batch performance and provided an effective instrument in the verification of authenticity and quality control of herbal medicinal products.

The stability indicative potential of the analysis procedure was validated by forced degradation research under frequently varying stress conditions such as acidic, basic, oxidative, thermal, and photolytic. The experiments indicated that the degradation products were successfully separated in relation to the parent biomarkers, which proves that the designed UPLC-PDA technique can be effectively used to measure chemical stability when storing and handling the products.

Also, the usefulness of the analytical procedure developed was proven practically by quantitative biomarker analysis in commercial polyherbal anti-diabetic preparations. The effective implementation of the technique to commercial products proves the appropriateness of the technique as a regular quality control test in the pharmaceutical and herbal medicine manufacturing laboratory.

Further reliability of the validation findings was confirmed through statistical analysis of the analytical results conducted through regression analysis, analysis of variance (ANOVA), and estimation of a confidence interval. The statistical analyses of these correlations proved that the method developed gives similar and reproducible results with minimum fluctuations across repeated measurements.

In general, the findings reported in this chapter prove successful accomplishment of all the research objectives stated in the study. The results of the experiment were systemic in answering each of the research questions that were developed in the research design and strong analytical support of the research hypotheses. The statistical validation result also showed that the developed method satisfies all the necessary performance requirements of analytical reliability and regulatory acceptance.

To summarize, the resulting UPLC-PDA analytical system is a scientifically sound, regulatory-compliant, and practically useful analytical framework of the quality control of polyherbal anti-diabetic preparations. The technique has great benefits in terms of analytical efficiency, sensitivity, reproducibility, and stability-indicating property. These properties render the technique quite applicable in daily use in pharmaceutical quality control laboratories and herbal medicine production plants where quality assurance and correct standardization of polyherbal blending is required.

CONFLICT OF INTERESTS

None.

ACKNOWLEDGMENTS

None.

REFERENCES

Ahrén, B., & Pacini, G. (2021). Glucose effectiveness: Lessons from studies on insulin-independent glucose clearance in mice. Journal of Diabetes Investigation, 12(5), 675–685. https://doi.org/10.1111/jdi.13446

Alhamhoom, Y., Ahmed, S. S., Kumar, R., Salahuddin, M., Bharathi, D. R., Ahmed, M. M., Farhana, S. A., & Rahamathulla, M. (2023). Synergistic antihyperglycemic and antihyperlipidemic effect of polyherbal and allopolyherbal formulation. Pharmaceuticals, 16(10), 1368. https://doi.org/10.3390/ph16101368

Al-Qaseemi, S. A., Almulhim, H. A., Almulhim, M. F., & Chaudhry, S. R. (2016). IoT architecture challenges and issues: Lack of standardization. In Proceedings of the 2016 Future Technologies Conference (FTC) (pp. 731–738). IEEE. https://doi.org/10.1109/FTC.2016.7821686

Alves, E., Gurupadayya, B. M., & Prabhakaran, P. (2025). Artificial intelligence in HPLC method development: A critical review of technological integration, limitations, and future directions. Critical Reviews in Analytical Chemistry, 1–43. https://doi.org/10.1080/10408347.2025.2575352

Amaeze, O., Eng, H., Horlbogen, L., Ajaezi, G., & Nworu, C. (2021). Cytochrome P450 enzyme inhibition and herb–drug interaction potential of medicinal plant extracts used for management of diabetes in Nigeria. European Journal of Drug Metabolism and Pharmacokinetics, 46, 437–450. https://doi.org/10.1007/s13318-021-00685-1

Ardalani, H., Hejazi Amiri, F., Hadipanah, A., et al. (2021). Potential antidiabetic phytochemicals in plant roots: A review of in vivo studies. Journal of Diabetes & Metabolic Disorders, 20, 1837–1854. https://doi.org/10.1007/s40200-021-00853-9

Balekundri, A., & Mannur, V. (2020). Quality control of the traditional herbs and herbal products: A review. Future Journal of Pharmaceutical Sciences, 6, 67. https://doi.org/10.1186/s43094-020-00091-5

Bansode, B., & Jungari, S. (2019). Economic burden of diabetic patients in India: A review. Diabetes & Metabolic Syndrome: Clinical Research & Reviews, 13(4), 2469–2472. https://doi.org/10.1016/j.dsx.2019.06.020

Başaran, E., Öztürk, A. A., Şenel, B., Demirel, M., & Sarıca, Ş. (2022). Quercetin, rutin and quercetin–rutin incorporated hydroxypropyl β-cyclodextrin inclusion complexes. European Journal of Pharmaceutical Sciences, 172, 106153. https://doi.org/10.1016/j.ejps.2022.106153

Benchoula, K., Vohra, M. S., Parhar, I. S., et al. (2022). Metabolomics based biomarker identification of anti-diabetes and anti-obesity properties of Malaysian herbs. Metabolomics, 18, 12. https://doi.org/10. 1007/s11306-022-01870-2

Bereman, M. S. (2015). Tools for monitoring system suitability in LC MS/MS centric proteomic experiments. Proteomics, 15(5–6), 891–902. https://doi.org/10.1002/pmic.201400373

Berger, T. A. (2016). Instrument modifications that produced reduced plate heights <2 with sub-2 μm particles and 95% of theoretical efficiency at k = 2 in supercritical fluid chromatography. Journal of Chromatography A, 1444, 129–144. https://doi.org/10.1016/j.chroma.2016.03.021

Bhavna, Ojha, A., & Bhargava, S. (2022). International Council for Harmonisation (ICH) guidelines. In Regulatory affairs in the pharmaceutical industry (pp. 47–74). Academic Press. https://doi.org/10.1016/B978-0-12-822211-9.00008-3

Blessy, M., Patel, R. D., Prajapati, P. N., & Agrawal, Y. K. (2014). Development of forced degradation and stability indicating studies of drugs—A review. Journal of Pharmaceutical Analysis, 4(3), 159–165. https://doi.org/10.1016/j.jpha.2013.09.003

Borman, P., & Elder, D. (2017). Q2(R1) validation of analytical procedures: Text and methodology. In A. Teasdale, D. Elder, & R. W. Nims (Eds.), ICH quality guidelines: An implementation guide (Chap. 5). Wiley. https://doi.org/10.1002/9781118971147.ch5

Burgos, A., Goñi, A., Illarramendi, A., & Bermudez, J. (2010). Real-time detection of apneas on a PDA. IEEE Transactions on Information Technology in Biomedicine, 14(4), 995–1002. https://doi.org/ 10.1109/T ITB.2009.2034975

Caro-Ordieres, T., Marín-Royo, G., Opazo-Ríos, L., Jiménez-Castilla, L., Moreno, J. A., Gómez-Guerrero, C., & Egido, J. (2020). The coming age of flavonoids in the treatment of diabetic complications. Journal of Clinical Medicine, 9(2), 346. https://doi.org/10.3390/jcm9020346