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# Development and Validation of High-Performance Liquid Chromatography Method for Apigenin from Parsley Leaves Extract

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***Abstract*: A new, simple, rapid, accurate and precise high-performance thin layer chromatography (HPLC) method has been developed and validated according to the guidelines of the International Conference on Harmonization (ICH Q2(R1) for the estimation of Apigenin in *parsley leaves* extract. The chromatographic analysis were performed by an Shimadzu HPLC instrument using a was Kromasil C18 column (250mm x 4.6mm x 5µm).and mobile phase comprising Acetonitrile and water were mixed in the ratio of 75:25 v/v at flow rate of 1.0 ml/min. The eluent was monitored at 268 nm for determination of Apigenin. The total run time was 11 min and the average retention time of Apigenin was found to be 7.102 min. The calibration curves were linear over the range of 10-100 ng/mL ($R^2$ = 0.999). The intra- and inter-day accuracy and precision values for all the analytes were within the acceptable range. The LOD and LOQ were 0.1723and 0.9748 ng/mL. The developed method was found to be robust. A simple, precise, accurate, linear and rapid RP-HPLC method was developed and validated as per ICH guidelines. The results suggest that the developed method was found to be robust and it can be applicable in routine analysis and efficiently used for the estimation of Apigenin in bulk as well as in plant extract.**

***Keywords*: Apigenin, HPLC method development, Validation.**

## 1. Introduction

Apigenin, is a natural product belonging to the flavone class. It is chemically known as 4′, 5, 7,-trihydroxyflavone is found in many plants. Apigenin (Fig. 1) is the aglycone of several naturally occurring g lycosides, Apigenin is one of the predominant monomeric favonoids found in a daily diet. The molecular formula and molecular weight of Apigenin is $C_{15}H_{10}O_5$ and 270.24 respectively. Flavones and some of their synthetic derivatives, have been shown four biological activities, including antioxidant, anti-inflammatory, antitumor, ant-genotoxic, antiallergic, neuroprotective, cardioprotective and antimicrobial. Apigenin has large effects on various cancers. Interesting aspect of the effects of Apigenin on bacteria is its interactions with gut microbes. Apigenin is synthesized in a number of plants as secondary metabolite. A variety of plants such as parsley, celery, onions, oranges, chamomile, maize, rice, tea, wheat sprouts, some grasses etc., are known to synthesis Apigenin and its derivatives. Therefore, considering the therapeutic importance of the Apigenin and the need of simple yet precise and robust analytical methodology for the same, it was envisaged that development of UV-Visible spectrophotometric method for the determination of Apigenin in bulk and the formulation as well as in plant extract by using co-solvent system consisting economic percentage of organic solvent will be worth.

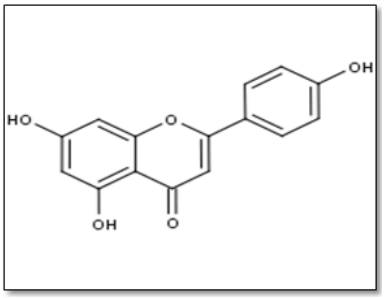

Fig. 1. Chemical structure of Apigenin

## 2. Materials and Methods

Apigenin (> 95 % by HPLC) was purchased from TCI Chemicals (India) Pvt. Ltd. All other chemicals of analytical grade were used for study.

### *A. Chemicals*

Apigenin (> 95 % by HPLC) was purchased from TCI Chemicals (India) Pvt. Ltd. All other chemicals of analytical grade were used for study.

### *B. Instrumentation and Condition*

The chromatographic separation was performed on a Shimadzu prominence HPLC system provide with LC-10AT binary pump, SIL-20AHT auto sampler, and was employed for this work. The detector consists of a SPD-10A UV detector and operated at 268 nm. Data acquisition was carried out using LC solutions software. The column used was Kromasil C18 column (250mm x 4.6mm x5µm particle size). Chromatographic analysis was carried out at 400C temperature. Ultrasonicator (PCi Analyticals) is use for mobile phase degasing. Vibra HT (Essae) analytical balance was used for weighing of chemicals.

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### *C. Preparation of Mobile Phase*

The mobile phase Acetonitrile (ACN) and water were mixed in the ratio of 75:25 v/v and filtered through membrane filter (Millipore Nylon disc filter of 0.45 μ). This filtered mobile phase was sonicated for 15 min in ultrasonic bath.

### *D. Stock Solution Preparation*

Stock solutions (1 mg/mL) of morin prepared in HPLC grade CAN and filtered through 0.45-m nylon membrane syringe filter.

### *E. Preparation of Standard Calibration Curve*

Calibration curve was prepared by diluting the stock-I solution to achieve the seven different calibration standards representing 10, 20, 30, 40, 60, 80, 100 ng/ml strength of Apigenin. All these solutions were injected into HPLC column and the peak area of each solution was measured. The standard calibration curves of peak area Vs concentration (ng) were plotted.

### *F. Method Validation*

The validation of pre-optimized chromatographic method was performed according to the Q2 (R1) guidelines of International Conference on Harmonization (ICH). Various analytical method validation parameters like system suitability, linearity, range, LOD, LOQ, accuracy, precision and stability were assessed.

### *G. System Suitability*

Before performing the main analysis, the system suitability test was carried out using freshly prepared standard working solutions of 15 ng/mL of Apigenin. Standard working solution was repeatedly analyzed by using proposed HPLC conditions. During analysis, various parameters viz. retention time, peak area, and the number of theoretical plate were measured. Acceptable upper limit of % RSD for peak area and retention time was set at 2 whereas acceptable lower limit of number of theoretical plates was set at 2000. System was considered to be suitable only when obtained values were within the set limits.

### *H. Linearity & Range*

Linearity of the proposed method was calculated by using seven different calibration standards of Apigenin. The calibration curves were constructed using the Calibration Standards representing 10, 20, 30, 40, 60, 80, 100 ng/ml strength of Apigenin. Concentration vs. peak areas were plotted, subjected to linear regression analysis and linearity in terms of R-squared values and respective range were reported.

### *I. Accuracy (% Recovery)*

Accuracy of pre-optimized HPLC method was assessed using recovery studies by standard addition method. To the solutions with predefined amount of Apigenin (15, 50 and 95 ng/mL), its 80, 100 and 120 % amount was added externally and the % recovery of the drugs was calculated.

### *J. Precision*

The precision of the developed method was evaluated by performing Intra-day and Inter-day studies. Intra-day precision study was carried out by analyzing five replicates of three different concentrations (15, 50 and 95 ng/ml of Apigenin) at morning, afternoon and evening time of the same day. Similarly, inter-day precision study was carried out by analyzing the samples on three consecutive days. Intra- and inter-day precision results were expressed in terms of % RSD.

### *K. Robustness*

Robustness of the proposed HPLC method was evaluated by making slight, deliberate change in chromatographic parameters viz. column temperature, flow rate of mobile phase and the mobile phase composition. Modified chromatographic conditions for the assessment of robustness were ±1ºC deviation in column temperature, ±0.5 ml/min deviation in flow rate of mobile phase and ±1 unit deviation in volume of methanol. For the robustness study, a solution (50 ng/ml) was repeatedly (n=5) analyzed for retention time and peak area of Apigenin using above mentioned modified chromatographic conditions. Results of the robustness study were expressed in terms of % RSD. Proposed method was considered to be robust only when the % RSD values for both retention time and peak areas were below 2.

### *L. Limit of Detection (LOD) and Limit of Quantification (LOQ)*

LOD is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. LOQ is the lowest concentration of analyte that can be determined with acceptable accuracy and precision. LOD and LOQ were calculated using following formula

$LOD = 3.3 \times SD/S$

$LOQ = 10 \times SD/S$

where SD = standard deviation of response (peak area) and S = slope of the calibration curve.

### *M. Estimation of Apigenin from Parsley Leaves*

Dried Parsely leaves were taken and powdered using twin blade mixer (Bajaj electrical ltd., Mumbai, India). To select uniform particle size, powder was sifted in a sieve shaker (CIP Machineries, Ahmedabad, India) with sieves of different sizes (12, 24, 45, 85 and 120 mesh, Swastika electric and scientific works, Ambala, India) for a period of 10 min. Powder passed through 120 mesh sieve was collected and used for further extraction.

Soxhlet assisted extraction (SAE) technique was used for the extraction of Apigenin from Parsely leaves. Ten gm of powdered Parsely leaves was placed in a thimble (Borosil, Mumbai, India) which was inserted into a Soxhlet apparatus. The material was exhaustively extracted with 95% ethanol. SAE was performed for 1 h. After predefined extraction period, solvent was distilled off under reduced pressure using rotary vaccum evaporator (Heidolph instruments GmbH & co. Germany) to obtain the dry extract.

Accurately weighed 5 mg of dry extract of parsley leaves was transferred in to the calibrated volumetric flask and dissolved using 5 ml of ethanol to achieve a stock solution of 1000 μg/ml (Stock-III). Stock- III solution was suitably diluted with co-

solvent system and analyzed for the Apigenin content using proposed HPLC method.

## 3. Results and Discussion

### *A. Optimization of RP-HPLC Method*

While developing HPLC method for estimation of Apigenin, various mobile phase combinations and the stationary phases were tried. Selection of mobile phase composition and stationary phases was based on the solubility behavior, pKa values and the relative retention of Apigenin. Apigenin was optimally resolved (Figure 2) over C-18 HPLC column using combination of ACN and water (75:25 v/v) as a mobile phase. The details of optimized chromatographic conditions are shown in Table 1.

Table 1
The optimized chromatographic conditions

| Separation variable | Optimized conditions |
|---|---|
| **Chromatography** | Shimadzu HPLC system |
| **Column** | Kromasil (C18 -250mm x 4.6mm x5µm particle size) |
| **Mobile phase** | ACN and water (75:25v/v) |
| **Flow rate** | 1 mL/min |
| **Total Run Time** | 11 Min |
| **Temperature** | $40^0$C |
| **Detection wavelength** | 268 nm |
| **Retention time** | 7.102 min |

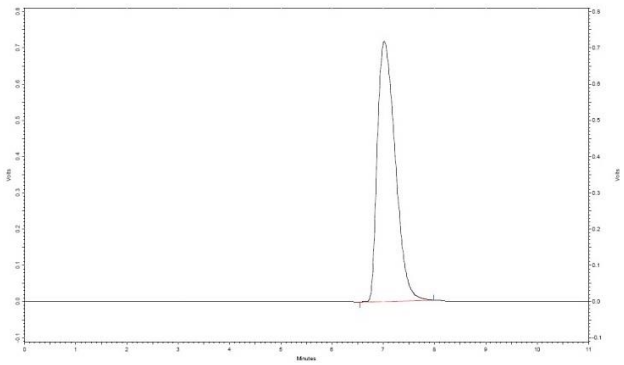

Fig. 2. A typical RP-HPLC chromatogram of Apigenin system suitability

During system suitability test, RSD of all parameter were calculated to evaluate the suitability of the developed method. From the results, it was found that %RSD for retention time and peak area was less than 2 and the number of theoretical plates were more than 2000 (Table 2). On the basis of obtained results, it was found that system is suitable for the analysis. The details of system suitability results are summarized in Table 2.

Table 2
System suitability parameters for Apigenin

| S.No. | Parameter | Acceptance criteria | Results | | |
|---|---|---|---|---|---|
| | | | Apigenin | %RSD | Status |
| 1 | Retention Time | %RSD ≤ 2% | 7.108 | 0.6598 | Passed |
| 2 | Area | %RSD ≤ 2% | 27597 | 0.5239 | Passed |
| 3 | Theoretical plates | ≥ 2000 | 5894 | 0.6124 | Passed |

### *B. Method Validation*

#### *1) Linearity and Range*

Linearity and range are the important parameters of analytical method that demonstrates the limit within which the intended method is to be used for its optimum performance. Considering the prime importance of linearity and the range, seven-point calibration curve of Apigenin (10-200 ng/ ml) were constructed. Different concentrations and peak area values are depicted in Table 3. Calibration curve when subjected to least square regression analysis yielded an equation; y = 1841.6x + 288.59 with correlation coefficient 0.9998 respectively (Fig. 3). From the linearity study, it was revealed that, there is a linear relationship between response and amount of drug within the range 10-100 ng/ml.

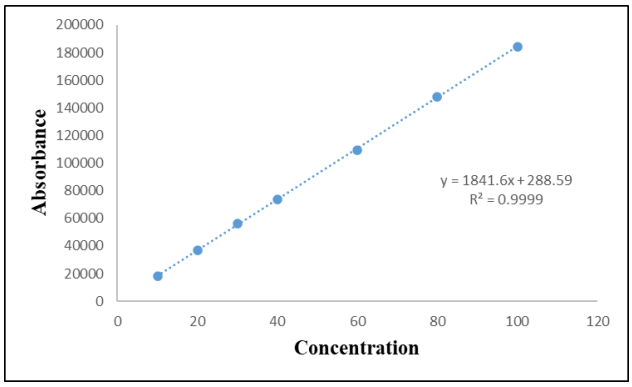


Fig. 3. Calibration curve for Apigenin

Table 3
Calibration standard data for Apigenin

| S. No. | Conc. (ng/mL) | Peak Area |
|---|---|---|
| 1 | 10 | 18462±0.58 |
| 2 | 20 | 37120±0.64 |
| 3 | 30 | 56394±0.80 |
| 4 | 40 | 73954±0.87 |
| 5 | 60 | 109567±0.68 |
| 6 | 80 | 148121±0.19 |
| 7 | 100 | 184534±0.38 |

#### *2) Accuracy (Percentage recovery)*

Accuracy is the closeness of test results to the true value obtained by proposed method. The accuracy of an analytical method should be established over its calibration range so that at any point of determination, results obtained would be accurate. For Apigenin, accuracy was determined using

Table 4
Recovery studies of Apigenin

| S. No. | Sample | Spiked level | Theoretical Concentration (ng/mL) | Practical Concentration (ng/mL) | % Recovery | Mean % Recovery | % RSD |
|---|---|---|---|---|---|---|---|
| 1 | Apigenin | 80% | 12 | 11.98 | 99.83 | 99.97 | 0.995 |
| | | 100% | 50 | 49.99 | 99.98 | | |
| | | 120% | 114 | 114.12 | 100.10 | | |

Table 5
Intra-day precision data for Apigenin

| | Apigenin | | | |
|---|---|---|---|---|
| **S. No.** | **Amount present (ng/ml)** | **Amount recovered (ng/ml)** | **% Assay** | **% RSD** |
| 1 | 15 | 14.972 | 99.81 | 0.9581 |
| 2 | 50 | 49.998 | 99.99 | 0.6581 |
| 3 | 95 | 95.12 | 100.12 | 0.9482 |

Table 6
Inter-day precision data for Apigenin

| | Apigenin | | | |
|---|---|---|---|---|
| **S.No.** | **Amount present (ng/ml)** | **Amount recovered (ng/ml)** | **% Assay** | **% RSD** |
| 1 | 15 | 14.968 | 99.78 | 0.8941 |
| 2 | 50 | 49.9855 | 99.99 | 0.4426 |
| 3 | 95 | 95.18 | 100.18 | 0.9861 |

Table 7
Robustness study for Apigenin

| **S.No.** | **Parameter** | **Setting** | **Apigenin** | | | |
|---|---|---|---|---|---|---|
| | | | **RT** | **% RSD** | **Peak Area** | **% RSD** |
| 1 | Column temperature (°C) | 39 | 7.118 | 0.51 | 92157 | 0.4984 |
| | | 40 | 7.106 | 0.25 | 92284 | 0.4383 |
| | | 41 | 7.111 | 0.41 | 92216 | 1.0425 |
| 2 | Mobile phase flow rate (ml/min) | 9 | 7.112 | 0.34 | 92315 | 0.4869 |
| | | 1 | 7.108 | 0.38 | 92456 | 0.5964 |
| | | 1.1 | 7.105 | 0.56 | 92368 | 0.9965 |
| 3 | Mobile phase composition (%, v/v) | 74.5:25.5 | 7.113 | 0.94 | 92128 | 0.5589 |
| | | 75:25 | 7.109 | 0.55 | 92358 | 0.6528 |
| | | 75.5:24.5 | 7.102 | 0.34 | 92452 | 1.0435 |

recovery studies. At 80, 100 and 120 % standard addition, mean recovery of Apigenin was found to be in between 99.83 to 100.10 %. The relative standard deviation (% RSD) was found to be less than 2 (Table 4). From the results of accuracy studies, it was concluded that the proposed method is accurate.

*3) Precision*

Precision was studied by analysis LQC, MQC and HQC STDs containing the drugs at concentrations covering the entire calibration range**.** The results expressed in terms of % RSD for the intra- and inter-day precision study (Table 5 and 6). Percent RSD values of intra-day precision study were found to be in between 0.6581 to 0.9581, whereas inter-day precision was found to be in between 0.4426 to 9861. It was concluded that the analytical technique showed good repeatability.

*4) Robustness*

An analytical method is considered to be robust when the small, internal changes in method parameters did not alter the final results significantly. Robustness of the proposed method was established by slightly changing the column temperature, mobile phase flow rate and mobile phase composition. It was found that, slight change in internal method parameters did not alter the final result (retention time and peak area) significantly. The % RSD values were found to be less than 2 (Table No.7). Thus, proposed method was found to be robust.

*5) LOD and LOQ*

LOD and LOQ of proposed HPLC method was found to be 0.1723and 0.9748 ng/ml. Lower LOQ value indicated that proposed method would be sensitive enough to quantify the Apigenin content of samples at its lower level.

*6) Estimation of Apigenin content in marketed formulation*

Developed HPLC method was successfully applied for estimation of Apigenin content in dried parsley leaves extract. By proposed HPLC method, Apigenin content in dried parsley leaves was found to be 37.23 ± 0.41 mg/g feed.

## 4. Conclusion

An accurate, precise, sensitive yet robust RP-HPLC method was developed and validated for the determination of Apigenin from parsley leaves. Proposed HPLC method was found to be specific for Apigenin and was free from any interference of excipients. Proposed HPLC method can be used for routine analysis of Apigenin in bulk as well as formulation.

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