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DEVELOPMENT AND VALIDATION OF HESPERIDIN FROM ORANGE PEEL *CITRUS AURANTIUM* BY RP-HPLC METHOD

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ABSTRACT

The present study was investigated to develop and validate a reversed phase high performance liquid chromatography method for the determination of Hesperidin from orange peel of *Citrus aurantium* belonging to rutaceae family. Hesperidin was isolated from orange peel. A new method was developed and validated for Hesperidin using methanol and water as mobile phase. The separation was achieved on a reversed-phase C18 column (Chromatopak (250mm×4.6×5micron) using a mobile phase composed of water (0.1% orthophosphoric acid): methanol (65:35) at a flow rate of 1.0 ml min^{-1}. The peak response time for hesperidin was observed at 5.669 minutes the detection was carried out on a UV detector at 280 nm. The developed method was validated according to the requirements for International Conference on Harmonisation (ICH) guidelines, which includes specificity, linearity, precision, accuracy, limit of detection and limit of quantitation. The developed method validates good linearity with excellent correlation coefficient (R2 > 0.999). In repeatability and intermediate precision, the percentage relative standard deviation (% RSD) of peak area was less than 1% shows high precision of the method. The developed HPLC method is a simple, rapid, precise, accurate and widely accepted and it is recommended for efficient assays in routine work.

INTRODUCTION

Hesperidin is a flavanone glycoside (flavonoid) ($C_{28}H_{34}O_{15}$) found abundantly in citrus fruits. Its aglycone form is called Hesperidin. Hesperidin, an abundant and inexpensive bioflavonoid in (*Citrus aurantium*) peel, has been reported to possess a wide range of pharmacological properties like antioxidant, antiinflammatory, hypolipidemic, vasoprotective and anticarcinogenic and cholesterol lowering actions. Hesperidin is also an enzyme inhibitor and inhibits phospholipase A2, lipoxygenase, HMG-CoA reductase and cyclo-oxygenase8. Hesperidin improves the health of capillaries by reducing the capillary permeability. Hesperidin is used to reduce hay fever and other allergic conditions by inhibiting the release of histamine From mast cells. The possible anti-cancer activity of hesperidin could be explained by the inhibition of polyamine synthesis. Sources of hesperidin include citrus fruits, berries, onions, parsley and green tea. Hesperidin is a flavanone glycoside found abundantly in citrus fruits, vegetables and medicinal plants possess antioxidant activity [1, 2]. It has breast cancer activity [3]. Anti-allergic activity. [4] Hesperidin has anti-inflammatory and analgesic effects [5–7]. In addition, the results revealed that hesperidin exhibited pronounced anticancer activity against the selected cell lines [8]. In literature, several analytical methods for the determination of hesperidin have been reported. Several high performance liquid

chromatography (HPLC) methods were developed for the estimation of hesperidin either alone or in mixture with other flavonoids in plant juices and pharmaceutical formulations [9–15]. Literature survey revealed that only few analytical methods were reported for hesperidin by RP-HPLC method. [16-22] Some RP-HPLC methods were not economical in terms of mobile phase composition, column dimensions and run times. Therefore the aim of present investigation was to develop a simple, precise and accurate high performance liquid chromatography (HPLC) method for the identification and determination quantity of hesperidin in citrus fruits.

MATERIALS AND METHODS

Chemicals and Reagents: Hesperidin 80% pure was procured from Sigma-Aldrich, ortho-phosphoric acid from Merck GR Grade. Deionised water was obtained by means of a Milli-Q Water Purification system supplied by Millipore HPLC Grade. All the other chemicals were procured from SDFCL s d fine-chem limited.

Instrumentation and Chromatographic Conditions: HPLC was performed on a system equipped with a quaternary low-pressure gradient solvent delivery P3000A HPLC pump, a high-sensitivity UV3000 ultraviolet (UV) detector. The system controlled and data analyzed by HPLC workstation software.

Sonicator Model: 1.5L 50 degasser units, Double Beam UV Spectrophotometer-Analytical technologies Limited, Model 212R RI, LVDVE, Superfit Rotavap, Model-PBU-6. Servewell Instruments Pvt. Ltd.

Collection of Material: The fruits of *Citrus aurantium* (Orange) were collected from local area of Tirupati, Andhrapradesh and they were peeled off and peels were dried under shade.

Optimized Chromatographic Conditions: A separation was carried out in C_{18} Chromatopak (250mm×4.6×5micron). The mobile phase consists of isocratic elution with a low-pressure gradient using double-distilled HPLC grade Water: HPLC grade methanol (A: B): (65:35) with a flow rate of 1.0 ml/min and the injection volume of 20 µl. All solutions were degassed and filtered through 0.2 µm pore size filter. The column was maintained at 40°C throughout analysis, and the UV detector was set at 280 nm. The total LC run time was 30 min.

Extraction of Crude Hesperidin:

800 mL petroleum ether (40 – 60°C) was filled in a 250 mL round bottom flask with magnetic stir bar. 250g dried and powdered dried orange peel was placed in the extraction sleeve of a Soxhlet extractor and covered with a little glass wool. A reflux condenser is put on the Soxhlet extraction unit, and then the reaction mixture is stirred and heated for 4 hours under strong reflux. The petroleum ether extract was discarded. In order to remove the adherent petroleum ether, the content of the extraction sleeve was laid out in an extensive crystallisation dish. Afterwards the substance was placed again in an extraction sleeve and, like before, but with 800 mL methanol, extracted unless the solvent leaving the extraction sleeve was colour less (1 to 2 hours). After complete Soxhlet extraction and maceration the filtrate was then acidifying (pH 3-4) with 6% acetic acid, Keep the concentrated residual liquid in refrigerator (4-6 ◦C) over night when a solid crystalline substance appears. It was again filtered and the crude hesperidin was separated out on buchner funnel as amorphous powder. The hesperidin was further characterized and identified according to various Physical and analytical test.

Preparation of standard solution (25ppm):

50 mg of Hesperidin was weighed accurately into a 100 ml volumetric flask dissolved and diluted to the volume with mobile phase. Further 5 ml of the above solution was diluted to 100 ml with mobile phase.

Preparation of sample solution (25ppm):

50 mg of Hesperidin (sample) was weighed accurately into a 100 ml volumetric flask dissolved and diluted to the volume with mobile phase. Further 5 ml of the above solution was diluted to 100 ml with mobile phase.

Preparation of spiked sample solution:

The accuracy for the method was determined by spiking the blank samples at standard concentrations (10, 50 and 100 ppm) and analyzing their recoveries.

Validation of the method: The validation of the developed analytical method was done according to the International Conference on Harmonisation (ICH) guidelines. The method is validated for specificity, linearity, recovery, precision, and the limits of detection (LOD) and limits of quantification (LOQ).

Linearity: Linearity was determined by different known concentrations of hesperidin standard solution in triplicate by diluting the standard stock solution. For the determination of linearity, five aliquots were pipetted out from standard stock solution (i.e., 60 ppm). 2-10 ml of standard stock solution was pipetted out in to a series of 10 ml volumetric flasks and volume was made up with the solvent to obtain concentration ranging from 10 ppm to 60 ppm of hesperidin. The calibration curve was constructed by plotting the peak areas against concentration, and the linear regression equations were calculated. The correlation coefficient was also computed. Shown in Table 1 and Figure 6

Precision: Precision was determined by studying the repeatability (intraday) and intermediate (interday) precision. The repeatability and intermediate precision of the method was determined by calculating the percentage relative standard deviation (% RSD). The repeatability was examined on the same day, whereas intermediate precision examined on different days by the different analyst. Shown in Table 2

Accuracy: The accuracy for the method was determined by spiking the blank samples at standard concentrations (10, 50 and 100 ppm) and analyzing their recoveries. Shown in Table 3

The LOD and the LOQ for caffeine were found to be 2.81 ppm and 8.90 ppm, respectively. Low LOD and LOQ of Hesperidin enable the detection and quantitation of this alkaloid in *C. aurantium* at low concentrations.

RESULTS AND DISCUSSION

Optimized Chromatogram

Column: A separation was carried out in C_{18} Chromatopak (250mm×4.6×5micron).

Mobile phase: The mobile phase consists of isocratic elution with a low-pressure gradient using double-distilled HPLC grade Water: HPLC grade methanol (A: B): (65:35)

Flow rate: flow rate of 1.0 ml/min and the injection volume of 20 µl.

Degassing: All solutions were degassed and filtered through 0.2 µm pore size filter.

Column Temperature: The column was maintained at 40°C throughout analysis,

Detector nm: UV detector was set at 280 nm.

Run time: The total LC run time was 30 min.

Accuracy: The accuracy for the method was determined by spiking the blank samples at standard concentrations (10, 50 and 100 ppm) and analyzing their recoveries. Shown in Table 3

Robustness: The Robustness of the method was determined by making slight changes in the experimental conditions such as change in the flow rate, mobile phase and wave length.

Ruggedness: Ruggedness is the degree or measure of reproducibility under different situations such as in different laboratories, different analyst, different machines, environmental conditions, operators etc.

DISCUSSION

Validation of analytical procedure is the process for proving that an analytical procedure is suitable for its intended purpose. Results obtained from method validation study can be used to judge the quality, reliability, and consistency of analytical results. An RP-HPLC method was developed and validated for the determination of hesperidin in *C. aurantium*. Several mobile phase compositions were tried, and a satisfactory separation was obtained using the mobile phase composition water: methanol (A: B): (65:35 v/v). The RT of standard and sample peak was found to be at 5.669 min, respectively. The ICH defines specificity as "the ability to assess the analyte for the presence of various components which may be expected to be present". The 100% test (sample) chromatograms confirm the presence of hesperidin RT at 5.669 without any interference. The RT of sample solution is overlay with the standard solution, so the method was specific. To check the linearity of the analytical method, calibration curves were plotted by peak area versus concentration of hesperidin standard in the range 10–60 ppm. This indicated that good fitting of the curve. The correlation coefficient (R^2) of hesperidin standard solution was 0.999.

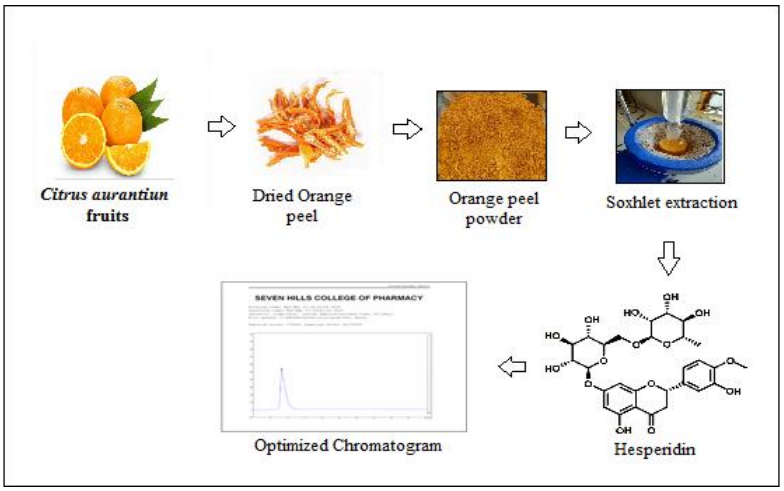

Figure 1: Graphical Abstract

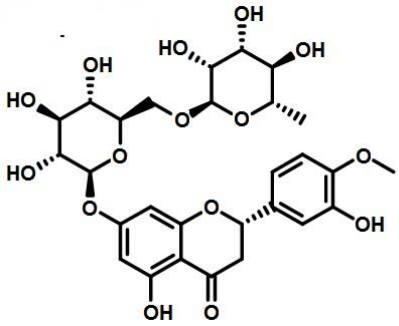

Figure 2: Structure of Hesperidin

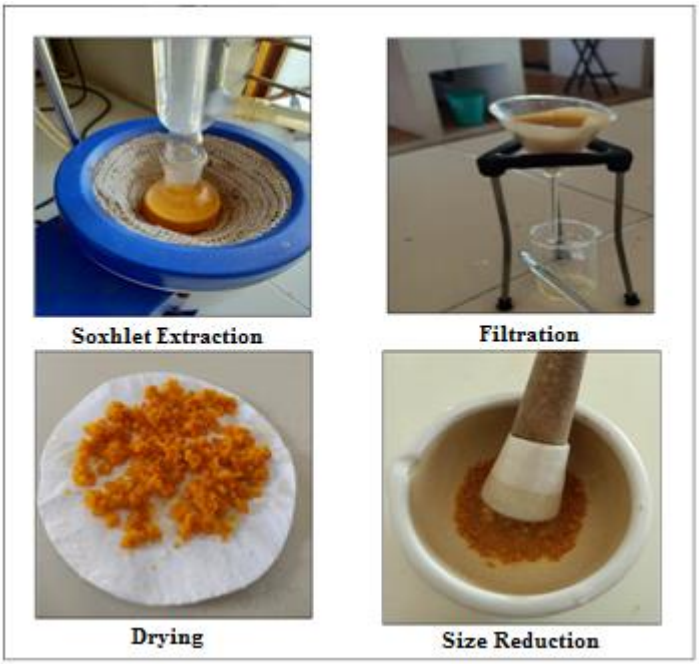

Figure 3: Isolation of hesperidin from orange

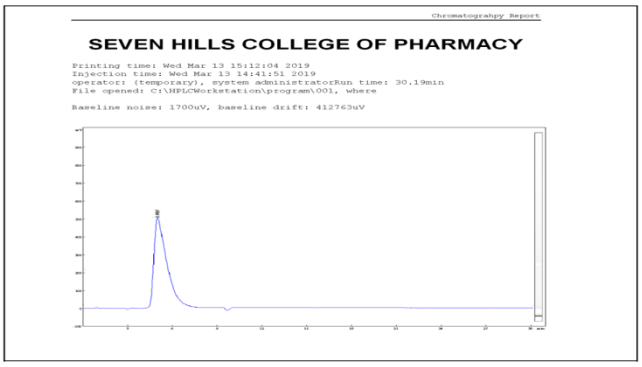

Figure 4: Optimized Chromatogram

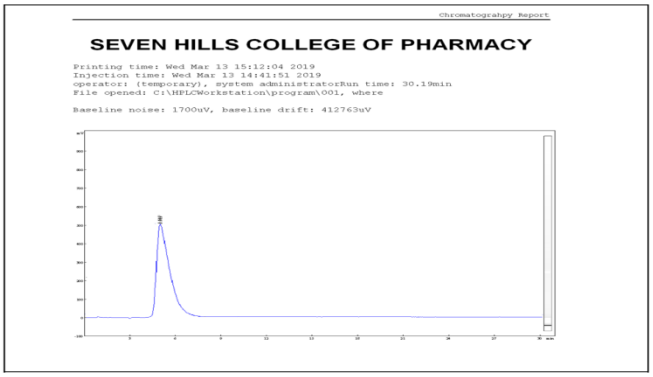

Figure 5: HPLC Chromatogram of Hesperidin Standard

Table 1: Linearity Data for Hesperidin

Concentration	Peak Area
10	1220001
20	2433802
30	3506773
40	4417784
50	5505535
60	6490823

Acceptance criteria: Correlation coefficient should not be less than 0.998.

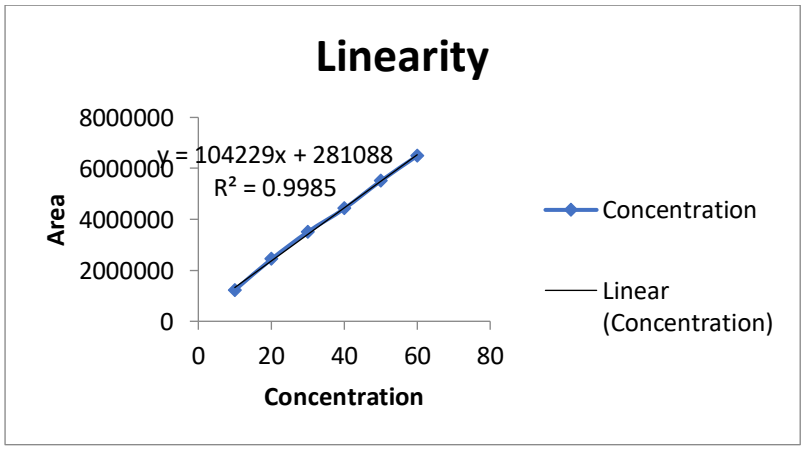

Figure 6: Linearity of Hesperidin

Table 2: Inter-day repeatability for 25 ppm

Number	Concentration of Sample (ppm)	Retention time (min)	Area	Result %
1	25	5.669	2453804	99.2
2	25	5.665	2433801	99.4
3	25	5.667	2453101	99.3
4	25	5.660	2401202	99.7
5	25	5.663	2471105	99.8
6	25	5.666	2441504	99.9
	Average	5.665	2442419.5	99.55
	Std. Dev.	0.0031	23835.09	1.87
	% RSD	0.06%	0.98%	0.19%

Table 3: Recovery study of Hesperidin

Concentration in the blank (ppm)	**Amount of spiked hesperidin (mg)**	**Amount of hesperidin found (mg)**	**Recovery %**
10	20 mg	19.1	98.7
50	100 mg	99.5	97.7
100	200 mg	199.3	98.5

Table 4: Summary of Robustness

Parameters	Condition	R_t
Flow rate	0.9ml/min	5.669
Actual flow rate	1ml/min	5.665
Flow rate	1.1ml/min	5.667
Wavelength	281nm	5.660
Wavelength	283nm	5.663
Wavelength	285nm	5.666

Table 5: Ruggedness study on hesperidin with various conditions

Various Conditions	Day		Analyst	
	1	2	1	2
No. of injections	6	6	6	6
Retention time	5.669	5.668	5.666	5.667
S.D	0.0026	0.0025	0.0045	0.0047
% R.S.D	0.19%	0.21%	0.37%	0.35%

The acceptance criteria for linearity are that the correlation coefficient (R^2) should not be less than 0.990. This indicates that the method showing good linearity. The repeatability of the method was investigated by performing 6 repeated analysis of 1 standard solution (25 ppm) on the same day (for intra-day repeatability) and different day for inter day precision. The results showed that the % RSD for retention time and area were satisfactory for further analysis. The results of repeatability are shown in Table 2. The accuracy for the method was determined by spiking the blank samples at standard concentrations (10, 50 and 100 ppm) and analyzing their recoveries. The LOD and the LOQ for hesperidin were found to be 2.81 ppm

and 8.90 ppm, respectively. Low LOD and LOQ of hesperidin enable the detection and quantitation of this flavonoid in *C. aurantium* at low concentrations.

CONCLUSION

The developed HPLC method is a simple, rapid, precise, accurate and widely accepted and it is recommended for efficient assays in routine work. Therefore, the method was proved to be suitable for hesperidin, flavonoid determination in *C. aurantium*. Further explorations are needed to investigate the standardization of individual phytoconstituents of *C. aurantium*. A simple reverse phase HPLC method was developed for the determination of hesperidin present in orange peel. The proposed method was also validated. The proposed study describes a new RP-HPLC method for the estimation of hesperidin. The method gave good results within a short analysis time. The developed method was validated in accordance with ICH guidelines and all of the results were within the limits. The HPLC method for the estimation of hesperidin in orange peel was also found to be simple, rapid, precise, accurate and sensitive. A good agreement was observed with HPLC method. The validated HPLC method can be used for the routine analysis of quality control samples. Since the developed method has been applied only to a single brand (hesperidin), the same method is applicable to different brands.

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