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Development and validation of stability indicating RP-HPLC method for estimation of hesperidin in nanotransferosome and Madhiphala rasayana—An Ayurvedic marketed product

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ABSTRACT

Hesperidin (4′-methoxy3′,5,7-trihydroxy flavanone) is a bioflavonoid molecule with remarkable wound healing properties as well as anticancer, antibacterial, antidiabetic, hepatoprotective, anti-inflammatory, and chemoprevention action. As a result, the current study used a stability-indicated reverse phase-high performance liquid chromatography (RP-HPLC) approach to synthesize and analyze hesperidin nanotransferosome. The important elements of the RP-HPLC method for hesperidin-based nanotransferosome stability highlighted were the retention time of 5.45 minutes, the limit of detection (LOD) of 0.008 µg/ml, the limit of quantification (LOQ) of 0.024 µg/ml, and the % relative standard deviation (RSD) of less than 2%. Hesperidin-loaded nanotransferosome were successfully demonstrated using the current RP-HPLC method, which has been proven to be stable. All vital factors, such as accuracy, linearity, LOD, LOQ, precision, and % RSD, were within the appropriate limits. Hesperidin was successfully quantified and estimated using the specified RP-HPLC method, and hesperidin-based nano transferosome confirmed hesperidin stability. This approach will be vital in evaluating the presence of hesperidin-containing therapeutic formulations.

INTRODUCTION

Hesperidin (C28H34O15) (Fig. 1) is a flavanone glycoside found in abundance in citrus fruits such as lemons, sweet oranges (*Citrus sinensis*), and grape fruits, as well as to a lesser level in vegetables such as tomatoes, mint, and other fresh herbs (Pandey and Khan, 2021; Sulaiman *et al.*, 2020). Flavonoids are the most common type of dietary polyphenol. Its name comes from the Latin word "hesperidium," which refers to the fruit of citrus trees. Recent studies have reported that hesperidin in experimental animals has a lower bioavailability and absorption due to the rutinoside moiety's binding to the dietary flavonoid (Rekha *et al.*, 2019). Hesperidin, on the other hand, has been identified as a promising phytocompound with antibacterial, anticancer, antioxidant, anti-inflammatory (Balakrishnan *et al.*, 2021; Rao *et al.*, 2018), antidiabetic, and cardiovascular protective properties. Hesperidin, in particular, has been shown to have chemotherapeutic and chemopreventive properties. A number of chromatographic methods, including high performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC/MS), have been used to quantify hesperidin in orange, lime peel, grapes, and plant extracts (Lee *et al.*, 2017). Spectrophotometric, HPLC, and HPTLC techniques have all been used to estimate hesperidin in various species extracts and have been published in the literature. The procedures outlined have their own drawbacks, such as the fact that they take longer and demand the use of expensive and complex chemicals. There is no Food and drug administration (FDA)-approved reverse phase (RP)-HPLC method for evaluating hesperidin in its Ayurvedic formulation and nanotransferosome, according to the literature study. As a result, to estimate hesperidin, an RP-HPLC method must be designed and validated utilizing a

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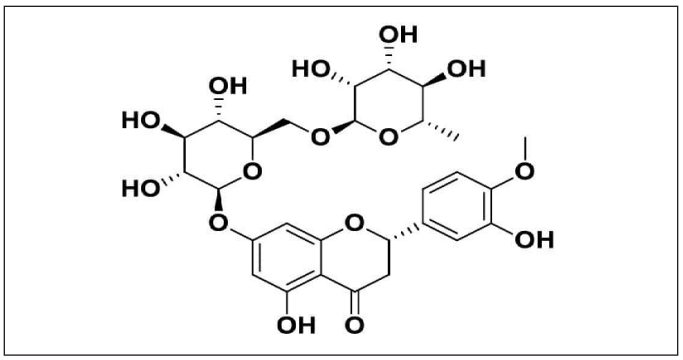

Figure 1. Chemical structure of hesperidin.

suitable solvent system. The purpose of this research is to develop and design a new RP-HPLC technique for assessing hesperidin in plant extract, Ayurvedic formulation, and nanotransferosome. Many assays have been described for diosmin (Šatínský *et al*., 2013), hesperetin, rutin, and quercetin in combination with hesperidin, including HPLC, HPTLC (Lobo *et al*., 2010), gas chromatography-MS, and UV-Spectroscopy. There are currently no reports of a hesperidin RP-HPLC method. The goal of the current investigation was to design and establish a simple, delicate, exact, and reliable RP-HPLC technique for assessing hesperidin. Hesperidin is now offered in a range of pharmaceutical formulations as nutritional health supplements (powders and capsules). These dose formulations, however, fall short of delivering therapeutic benefits due to their limited bioavailability (Majumdar and Srirangam, 2009), low aqueous solubility, rapid decomposition at alkaline pH, and rapid elimination of the system (Li *et al*., 2008). Hesperidin stability during formulation and analysis is also an issue related to its sensitivity that can harm its therapeutic potential. Several nanocarrier techniques, like liposomes, solid lipid nanoparticles (Ferrari *et al*., 2019), nanoemulsions (Somasundaram *et al*., 2018), and nanoparticles have been studied to overcome these obstacles. The nanotransferosome (Yuan *et al*., 2022) is the most useful vesicular system among these nanocarriers. On the other hand, particle size has an impact on the effectiveness of drug delivery systems based on nanoformulation. In this regard, the current research focuses on the formulation of hesperidin-loaded nanotransferosome as well as the assessment of the utility of the created approach. The goal of this work was to provide a clear, simple, and accurate RP-HPLC method in compliance with the International Council for Harmonization guideline (ICH, Q2 [R1] (2005)) to assess hesperidin in commercial Ayurvedic formulation (Kumar and Devanna, 2013), plant extract, and hesperidin loaded nanotransferosome. As far as we are aware, there is no one approach that has been documented that can analyze the quantity of hesperidin in a range of systems, including Ayurvedic formulations, plant extracts, and lipid-based nanoformulations. The established strategy was applied to investigate the behavior of hesperidin's degradation under diverse circumstances (acidic, basic, oxidation, and photochemical degradation). The stated approach can benefit the acceptable selection of experiments and its effects on design, formulation, and storage.

MATERIALS AND METHODS

Chemicals and reagents

Shaanxi Yi An Biological Technology Co., Lt., China, and Himalaya wellness Bangalore provided the hesperidin as a gift sample. HPLC grade methanol and Ortho phosphoric acid used from Merck Limited (Mumbai, India), Marketed Ayurvedic formulation Madhiphala rasayana from well-established Ayurveda local dispensing store Belagavi.

Instrumentation

The Agilent 1220 Infinity II LC System is a high-performance liquid chromatography system by Agilent Technologies (Agilent technologies, Germany). The Sorvall MX 150 Ultracentrifuge from Thermo Scientific was utilized to extract unentrapped hesperidin from Ayurvedic formulation, as well as nanotransferosome.

Chromatographic conditions

The analytical conditions were calibrated at room temperature using a Phenomenex Luna C18 Column (250 × 4.6 mm, 5 µm) with an Agilent 1220 infinity II LC System (Agilent technologies, Germany). Various combinations of methanol and 0.1% orthophosporic acid were tested on a C18 column in preliminary investigations in order to obtain a strong, isolated, and stable hesperidin peak. Methanol: 0.1% orthophosporic acid in a 50:50 (% v/v) ratio was shown to be the best combination. The mobile phase was filtered through a 0.45 mm membrane and ultrasonically degassed for 15 minutes (Chaudhary *et al*., 2013). With a run time of 7.0 minutes and a sample injection volume of 20 µl, the flow rate was maintained at 1.0 ml/minute. The system was equilibrated with the mobile phase for 1 hour prior to sample injections. Hesperidin absorbance was measured at 284 nm. All analysis tests were performed at room temperature.

Standard solution preparation

In the mobile phase, a stock solution was made (100 µg/ml). Dilutions from 0.5 to 16 µg/ml were used to develop the calibration curve (Kempwade *et al*., 2015; Kurangi *et al*.,2019).

Commercial orange peel and lime extracts samples

Precisely weighed 10 g and then mixed with 5 ml of methanol and 5 ml of dimethyl sulfoxide in 10 ml amber-colored volumetric flasks (Ahmed *et al*., 2021; Kurangi and Jalalpure, 2018; Rodrigues *et al*., 2022). The methanolic solution was vortex mixed and sonicated for 10 minutes to make sure that all of the hesperidin was extracted. For volume make-up, methanol was used. A 0.25 m membrane filter was used to filter the solution. After filtering, samples of orange and lime peel extract (filtrate) were used in the HPLC analysis (Jagwani *et al*, 2019; Kudatarkar *et al*.,2022; Kurangi and Jalalpure, 2020; Kurangi *et al*., 2021; Sharma *et al*.,2022).

Analysis of Madhiphala rasayana—a marketed Ayurvedic formulation

2 ml of the selected formulation was carefully measured and placed into a 10 ml amber-colored volumetric flask, where it was subjected to the identical method as the commercial formulation analysis and the Ayurveda formulation analysis. The HPLC analysis was used to quantify the parameter.

Preparation of hesperidin-loaded nanotransferosome

In the current investigation, a thin film hydration technique was used in the preparation process to develop the hesperidin-loaded nanotransferosome. Hesperidin was accurately

weighed and dissolved in 1 ml of dimethyl sulfoxide. Phospholipon 90G and tween 80 were dissolved in a mixture of chloroform and methanol (2:1, v/v) and hesperidin. The solvent mixture was evaporated in a rotary evaporator under pressure at 60°C and 60 rpm, forming a thin layer on the flask walls. To ensure that almost all organic solvents were completely removed, the flask was placed under a vacuum for an entire night after the solvents must have completely evaporated (Asensio-Regalado *et al*., 2022).

The lipid film was hydrated to either phosphate buffer (pH 7.4) or by rotating at 60 rpm for an hour at 60°C. The resulting suspension was allowed to stand at room temperature for 1 hour to achieve complete hydration in order to produce large multilamellar vesicles (LMLVs). Using an ultrasonic sonicator, LMLV was probe sonicated for 2 minutes (nanotransferosome) at 4°C to create smaller vesicles. To get uniformly sized vesicles, the sonicated vesicles were extruded through 0.45 and 0.2 m sterile syringe filters (Minisart, Sartorius, AG, Germany), and they were then stored at 4°C for further research (Hsieh *et al*., 2021).

Method development

The development of method for analysis of hesperidin was done by using different mobile phase ratios, concentration, and pH values of ortho phosphoric acid (OPA) and flow rates. Whenever a robust baseline was obtained, the chromatographic variables were modified. Injecting a standard hesperidin solution assessed the chromatogram, and the study was carried out six times with the same method (Parab *et al*., 2021).

Approach evaluation

Linearity

The linear calibration curve is plotted from values of 0.5 to 16 µg/ml, according to studies on linearity. Using common calibration curves, concentrations versus peak area were calibrated. A chromatogram was created following a sample run of each standard solution in triplicate (Parab *et al*., 2022).

Precision and accuracy

Three different quality control methods were employed on the hesperidin samples to evaluate their precision and accuracy. These approaches used low, medium, and high levels of 2, 4, and 16 µg/ml. In order to assure intraday precision and accuracy, triplicates of standard solutions (50%, 100%, and 150%) were injected on the same day in triplicates of 50%, 100%, and 150% including over the course of 3 days. Analysis was performed to determine relative standard deviation (RSD) and percent error.

Limit of detection (LOD) and limit of quantification (LOQ)

The signal-to-noise level was set with a minimum of 3.3:1 for LOD and 10:1 for LOQ to estimate the LOD using a series of hesperidin stock solutions. Both variables restrict the amount of analyte that may be examined (Shetti and Jalalpure, 2021).

System suitability

The tailing factor, theoretical plates, peak area, and resolution values are one of the variables that are considered when conducting suitability tests to evaluate the chromatographic process.

Robustness

By performing a simple adjustment to the optimal value, the robustness of the approach was examined. The parameters like flow rate (±0.1 ml/min), wavelength (±2 nm), and data were used to estimate the hesperidin peak areas and the % RSD of robustness testing (Chimagave *et al.*, 2020; Joshi *et al*, 2018).

Specificity

By analyzing the chromatograms from the standard hesperidin solution and phosphate buffer at the retention time of hesperidin, the specificity study chances of interferences from phosphate buffer and hesperidin based nano transferosome were investigated.

Force degradation studies

The devised HPLC method stability-indicating property was tested according to the ICH criteria. Hesperidin was subjected to acidic, basic, oxidative, and photochemical conditions to study its forced degradation (Anwer *et al.*, 2014; Bhalerao *et al.*, 2019; Kudatarkar *et al*; 2021; Peram *et al.*, 2019; Patil and Mall, 2012; Tavade *et al.*, 2022).

RESULTS

Conditions for sample preparation and chromatographic analysis

The composition of the mobile phase was modified to carry out various studies. Finally, a methanol solution comprising 50:50 v/v and 0.1% orthophosphoric acid resulted in the best separation. The injection volume of 20 µl and the flow rate of 1 ml/minutes were constant throughout this study.

Analytical method validation suitability of system

Table 1 provides an overview of the system suitability results. The theoretical plate average was higher than 6,000, however, the tailing factor was less than 2. The maximum retention time was 5.45 minutes. Figure 2 shows that the tailing factor was less than 2, and the average theoretical plate was > 6,000. A more efficient process has higher theoretical plates and a lower tailing number. The system was also well-equipped, showing percent RSD values of less than 1% for every parameter including retention time, peak area, theoretical plates, and tailing factor.

Linearity and range

Table 2 and Figure 3 present the linearity and regression results. The hesperidin peaks were not affected by any of the nanotransferosome ingredients or stress treatment derivatives.

Table 1. Results of system suitability studies of quality control samples of hesperidin.

Parameter	Mean ± SD	% RSD
Retention time (minute)	5.45	0.58
Peak area	7,073,707	0.63
Theoretical plates	6,681	1.09
Tailing factor	1.14	1.01

RSD: relative standard deviation.
$n = 6$.

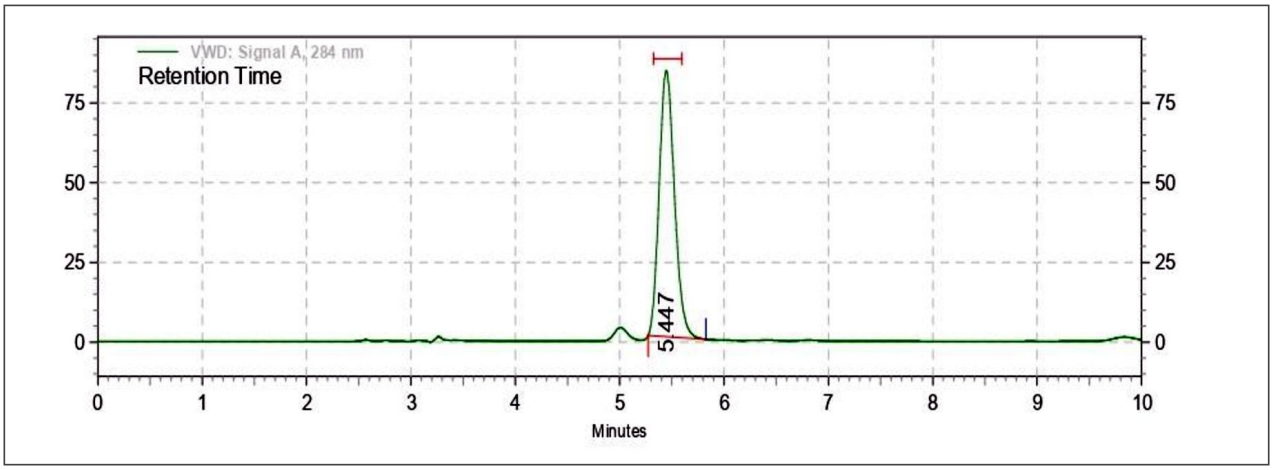

Figure 2. Chromatogram of standard hesperidin solution (10 µgml).

Table 2. Results of linearity and regression analysis of hesperidin.

Analyte	Hesperidin at 284 nm
Concentration range (µg/ml)	0.5–16
Slope	216,488
Intercept	21,910
R^2	0.999
LOD(µg/ml)	0.008
LOQ (µg/ml)	0.024

The concentration of hesperidin (×) was plotted against the peak area to produce a calibration curve. It was shown that there was linearity in the concentration range of 0.5–16 µg/ml. (R2 = 0.999; equation y = 216,488 ×–21,910). Hesperidin was detected with an accuracy and recovery of 100.85 to 101.43 using a C18 column with methanol to 0.1% orthophosphoric acid as the mobile phase.

LOD and LOQ

According to studies, hesperidin LOD and LOQ are 0.008 and 0.024 µg/ml, respectively.

Accuracy and precision

The findings are shown in Tables 3, 4, and 5. The intra-day precision of hesperidin was 1.16% to 1.54%, and its accuracy was 0.74% to 1.72%. The intraday precision was 1.04%, and the interday precision was 1.72%. The best recovery of three injected samples was 100.85%–103.69%, demonstrating that the approach is more accurate.

Robustness and ruggedness

Table 6 displays the findings of the robustness analysis. Mobile phase, flow rate, wavelength, and column type still have no influence on resolution in terms of the same retention time and minimum RSD values.

Stability-indicating study

Table 7 displays the outcomes of the experiments performed to identify how acid, base, oxidation, and photolysis affected the standard hesperidin solution. Figures 4 and 5 illustrate the chromatograms of the standard stressed hesperidin solution after two hours under different stress levels. In the present investigation, the percentage of hesperidin degradation varies from 9.99% for oxidative degradation to 53% for base degradation. In this study, samples were subjected to photochemical stress, which caused 10.84%. Additionally, 18.5%, 9.99%, and 53.14% of the samples were affected by acid, oxidation and bases analysis (Jagwani *et al*., 2019).

Preparation and characterization of nanotransferosome

The thin-film hydration method was successfully used to prepare the hesperidin-loaded nanotransferosome, which reveals results like, particle size as 160 nm and the blank nanotransferosome showed 182 nm. According to Table 8, the polydispersity index for drug-free and drug-based nano-transferosome was 0.22 and 0.27, correspondingly. These observations highlighted that the polydispersity index (PDI) value is less than 0.3 and suggested narrow homogeneous distribution of particle size dispersion. On the chromatogram of the nanotransferosome, as shown in Figure 5 and Table 8, hesperidin displays a visible, recognizable peak. The average particle size of hesperidin-based nanotransferosome was in the range from 177 ± 4.1 to 270.2 nm. The PDI ranges from 0.205 ± 0.02 to 0.220 ± 0.050 and being highly capable of being formulated into nutraceuticals. Additional research, hesperidin-loaded polymer-based nanoparticles (PLGAs) showed a particle size of 204.4 nm, PDI of 0.253. Hesperidin-based PLGA nanoparticles may be used as a good source of anti-cancer agent (Balakrishnan *et al*., 2021; Chimagave *et al*., 2022; Patil *et al*., 2022).

Comparison with previously published HPLC methods

On the basis of mobile phase ratios, mobile phase flow rate, wavelength, column, stability study, limits, and application of the HPLC methods, a comparison of previously published methods and the recently created HPLC method was carried out. The comparison details were shown in Table 9. There is currently no single HPLC method that can be used for a variety of analyses, such as the estimation of hesperidin in nanoformulation, plant extract, and Ayurvedic-marketed products like Madhiphala rasayana, as well as the evaluation of hesperdin's degradation behavior using the same parameters of developed HPLC method. When compared to previously developed methods and other hesperidin-related published literature, the current method, which

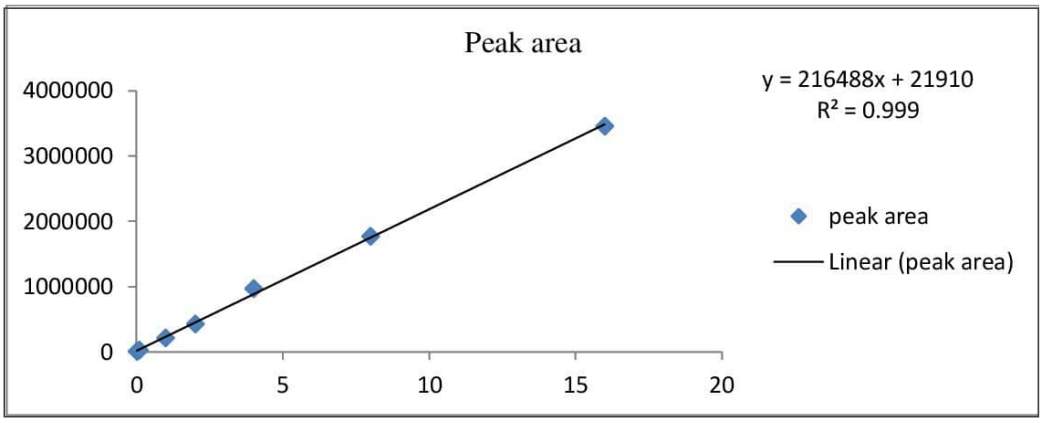

Figure 3. Calibration curve for hesperidin 284 nm.

Table 3. Results of accuracy study of hesperidin.

Sl. No.	Conc. (µg/ml)	Level in (%)	Added qty (µg/ml)	Recovery (%)	RSD (%)
1	2	50	1	100.85	1.72
2	2	100	2	103.69	0.74
3	2	150	3	101.43	1.50

Table 4. Results of intra-day precision of hesperidin solution.

Concentration (µg/ml)	Peak area	Retention time (minute) (%)	RSD of peak area
2	425,302.7	5.41	1.35
4	965,188.7	5.45	1.16
16	3,467,871	5.46	1.38
2	418,235.7	5.45	1.37
4	965,033.3	5.42	1.30
16	3,455,740	5.47	1.54
2	424,159.7	5.46	1.34
4	964,442.3	5.41	1.22
16	3,453,928	5.45	1.48

Table 5. Results of interday precision data of hesperidin samples.

Day	Concentration (µg/ml)	Peak area	Retention time (minute)	(%) RSD of peak area
Day 1	2	424,904.7	5.45	1.04
	4	958,065.7	5.43	1.00
	16	357,203.3	5.45	1.18
Day 2	2	428,222	5.41	1.49
	4	9,572,478	5.47	1.45
	16	3,512,377	5.45	1.66
Day 3	2	417,928	5.44	1.61
	4	951,381	5.45	1.26
	16	3,505,105	5.41	1.72

Table 6. Analysis of robustness and ruggedness using hesperidin solution.

Parameters	Variations	Time (minute)	%RSD
Mobile phase(± 2 v/v)	50:50	5.45	0.60
	48:52	5.41	0.71
	52:48	5.43	0.76
Flow rate(± 0.1 ml)	1 ml/minute	5.44	0.79
	0.9 ml/minute	5.46	1.77
	1.1 ml/minute	5.47	0.90
Detection wavelength (± 2 nm	282	5.45	0.81
	286	5.40	0.73
Column	c-18 column (5 µm, 4.6 mm × 250 mm, ZORBAX)	5.47	0.58
	Luna c-18 (5 µm, 4.6 mm × 250 mm) Phenomenex Inc.	5.46	0.48

Table 7. Results of the stress degradation studies of hesperidin solution at 2 hours.

Stress degradation studies	% Degradation
Acid hydrolysis	18.5
Base	53.1
Oxidation	9.9
Photolytic	10.8

uses methanol as the mobile phase and 0.1% OPA (50:50 v/v), 1 ml/minute flow rate, and 284 nm as the detection wavelength, is shown to be more accurate, affordable, and stable. In accordance with ICH rules that were within allowable bounds, the established procedure was validated. The developed approach shows a precise and simple assessment of hesperidin in nanotransferosomes,

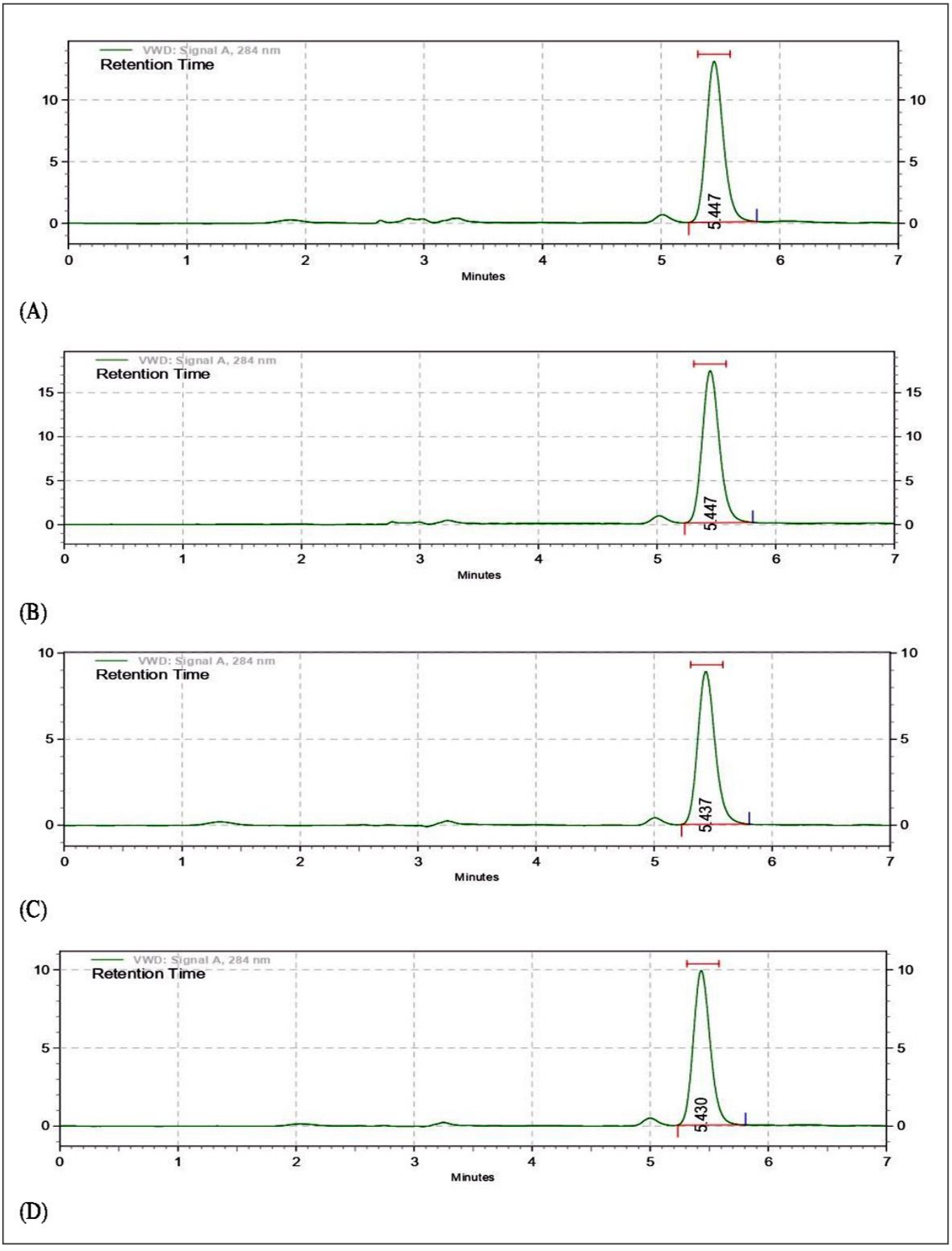

Figure 4. Chromatograms of stressed hesperidin samples (10 µgml) under a) acid hydrolysis (0.1 N HCI), b) base hydrolysis (0.1 N NaOH), c) oxidative degradation (3% H2O2), and d) photolytic.

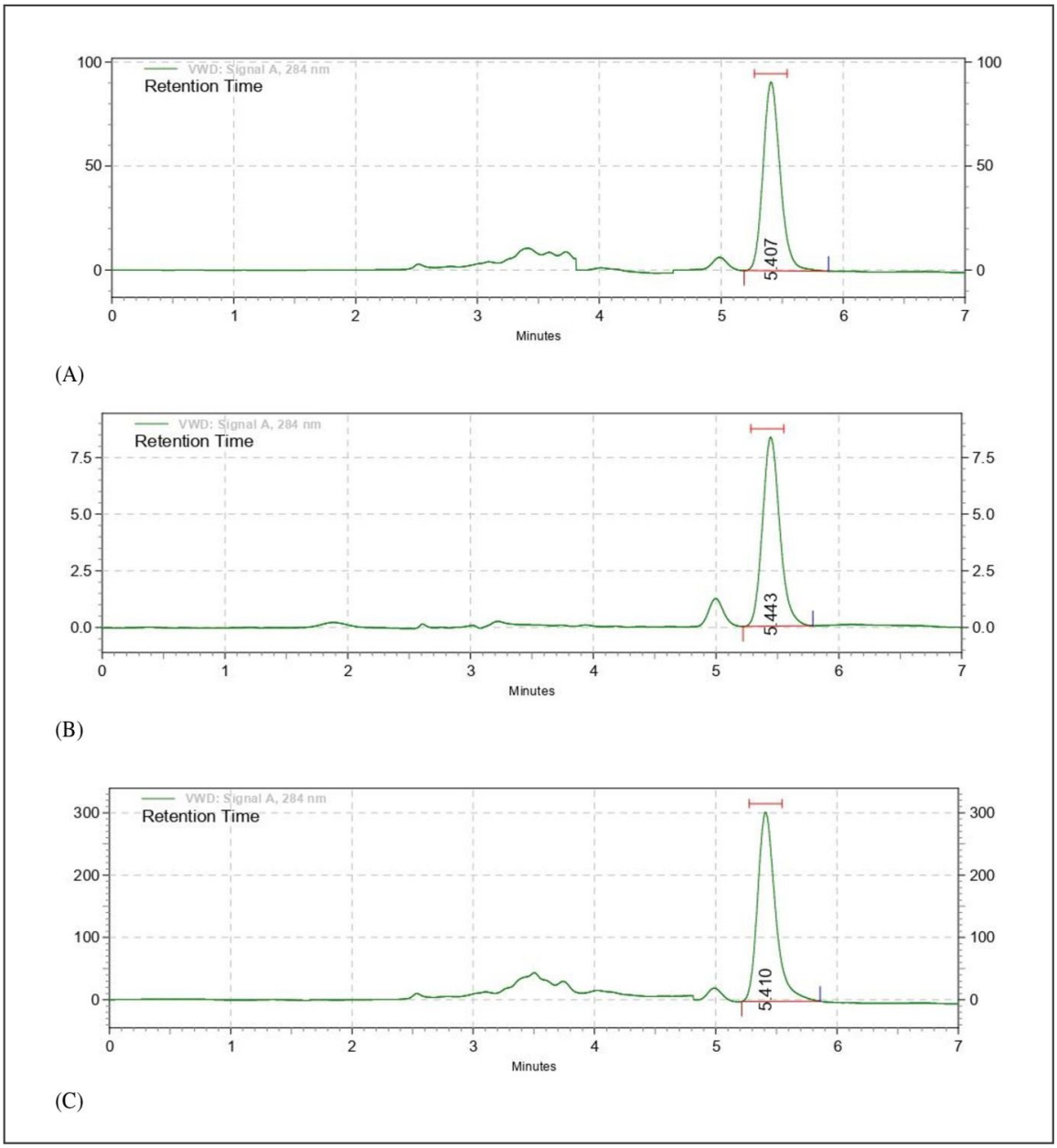

Figure 5. RP-HPLC chromatogram of a) plant extract (orange peel), b) hesperidin-loaded nano transferosome, and c) marketed Ayurveda formulation.

Table 8. Results of characterization of hesperidin loaded nanotransferosome.

Nanotransferosome formulation	Particle size diameter(nm)	PDI
Blank nanotransferosome	160 ± 0.02	0.22 ± 0.01
Hesperidin loaded nanotransferosome	182 ± 0.04	0.27 ± 0.02

RSD: relative standard deviation.
$n = 3$.

plant extracts, and Ayurvedic-marketed products, demonstrating the method is sensitive and reliable. Hesperidin appeared to be relatively resistant to acidic, alkaline, and high heat conditions, according to the results of a study on stress degradation. However, piperine was vulnerable to breakdown under conditions of oxidative and photolytic stress. This easy, stability-indicating RP-HPLC approach is therefore useful for further routine quality control analysis. Thus, this technique might be applied to regularly estimate hesperidin *in vitro* and *in vivo*.

DISCUSSION

The presence of higher theoretical plates with a lower tailing number indicates the system's efficiency. The percent RSD values were also less than 1% for all parameters, including retention interval, peak area, theoretical plates, and tailing number, indicating that the system was more efficient. Hesperidin peaks were not affected by any of the nano transferosome components or stress treatment. A calibration curve was generated by plotting the quantity of hesperidin (×) against the peak area.

Table 9. Comparison between previously published HPLC methods.

Sr. No	Mobile phase and flow rate	Wavelength (nm)	Column	Limitations	Application	Reference
Hesperidin with other drugs						
1	Formic acid (ph 4.1 and 0.05%, V/V) and methanol (58:42, V/V) Flow rate: 1.2ml	280	Lichrospher RP Select B 75 mm × 4 mm, 5 µm	High flow rate and less sensitive	Simultaneous determination of two flavonoids, hesperidin and diosmin, in combined tablets	Marjan *et al.* (2018)
2	Acetonitrile/water solution of acetic acid ph3 (30:70, v/v) Flow rate: 1.0 ml minutes−1	283	CTO 10 AC	Lack of stability study, expensive	Separation of rutin, troxerutin, diosmin, and hesperidin was developed and used for determination of these flavonoids in food supplements	Saeidi *et al.* (2011)
3	Acetonitrile (A) and de-ionized water (B) Flow rate: 1.0 ml/minute	280	C18 reverse-phase	Expensive	Clarify some inconsistent reports on hesperidin content in the literature and provide valuable information for the measurement of hesperidin in orange juice	Šatínský *et al.* (2013)
4	Water/acetonitrile/acetic acid (78:19:3, v/v) Flow rate: 0.8 ml/minute	280	C8 (25 × 4.6, 5 µm)	More than 2 mobile phases used, expensive	Study involved in specific areas	
5	A (1.0% v/v aqueous acetic acid) and solvent B (acetonitrile with 1.0% v/v acetic acid) Flow rate: gradient	280	C18 analytical column (250 × 4.6 mm; particle size 5 µm)	Lack of stability study, expensive	Method was developed and validated to determine liquiritin, hesperidin, and glycyrrhizin levels in a traditional Korean medicine, Pyungwi-san	Seo *et al.* (2011)

For the concentration range of 0.5–16 µg/ml, linearity of r = 0.999 was obtained using the equation y = 216,488 ×–21,910. Hesperidin was found using a C18 column with methanol and 0.1% orthophosphoric acid as the mobile phase. The best recovery of three injected samples was 100.85%–103.69%, demonstrating that the approach is more accurate. The method's RSD values for intra- and interday precision were less than 2%. In terms of similar retention time and lower RSD values, parameters such as mobile phase, flow rate, detection wavelength, and column type had no effect on resolution. Triplicate analysis of the samples revealed that the established procedure was more robust than those previously described. Hesperidin exhibited a degree of degradation ranging from 9.99% for oxidative degradation to 53% for base damage in the current investigation. The developed approach accurately predicted the degradation of hesperidin.

CONCLUSION

The present approach was effectively established and validated, using the same mobile phase to estimate hesperidin in plant extract, Ayurvedic formulation, and hesperidin-loaded nanotransferosome, as well as to determine degradation behavior. The established RP-HPLC method was reliable, accurate, and easy, and it met the FDA's criterion for hesperidin quantitative determination and its validation. Hesperidin may withstand acidic, oxidative, and photolytic environments, but is more likely to get damaged in base degradation conditions, according to forced degradation experiments. The results of the degradation experiments are used to determine the best conditions for formulation, development, and storage. The lack of excipient peaks in hesperidin analysis in plant extract, Ayurvedic formulation, and nanotransferosome further proved that the established method may be used for routine hesperidin analysis in various dosage forms.

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LIST OFABBREVIATIONS

ICH: International Council for Harmonisation; LOD: Limit of detection; LOQ: Limit of quantification; RP-HPLC: Reverse phase high performance liquid chromatography; RSD: Relative standard deviation.

CONFLICT OF INTEREST

The authors declare no conflict of interests for this manuscript.

ETHICAL APPROVAL

This study does not involve any animals or human subjects.

AUTHOR'S CONTRIBUTION

All the authors have equally contributed to the method development and its validation as well as design and characterization of hesperidin loaded nanoformulation. Supriya S. Chimagave contributed in the conceptualization and development & validation of RP-HPLC method for Hesperidin. Dr. Sunil S. Jalalpure guided for this project and given a technical support. Akshay K. Patil involved in design of nanotransferosome. Bhaskar K. Kurangi guided for the HPLC validation work and manuscript writing.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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