Heliyon 8 (2022) e09177

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

A stability-indicating HPLC-UV method for the quantification of anthocyanin in Roselle (*Hibiscus Sabdariffa L.*) spray-dried extract, oral powder, and lozenges☆

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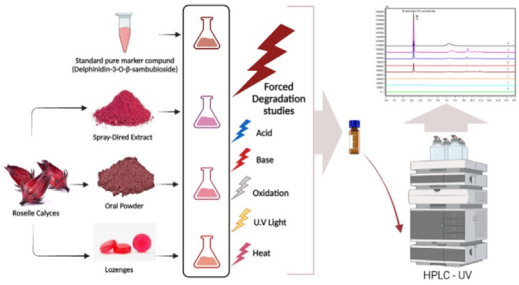
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Hibiscus Sabdariffa L.
Roselle
Anthocyanin
Delphinidin-3-O-Sambubioside
Stability indicating HPLC method
Forced degradation studies

ABSTRACT

Hibiscus sabdariffa L. (H.S.) plant and its calyces have received much attention from researchers because of their potential medicinal and nutritional values. Calyces are the major source of anthocyanin in this plant. Therefore, a well-developed, efficient, and accurate analytical method is needed to assure proper standardization and control the quality of H.S. plant herbal and nutraceutical products. The objective of this work is to develop a simple, rapid, stability-indicating HPLC-UV method for the quantitative determination of anthocyanin in spray-dried aqueous extract (SDE), oral powder, and compressible lozenges formulations using Delphinidin-3-O-sambubioside (Dp3S) as a marker compound. The chromatographic conditions were optimized using Eclipse plus® C18 column. The mobile phase comprised water acidified with 0.2% formic acid (FA) and acetonitrile (ACN) (90:10, v/v) using a gradient system at a flow rate of 0.8 mL/min. The detection wavelength was 525 nm. The column was maintained at 45 °C, and the injection volume was 15 μL. The developed method was validated according to the international conference of harmonization (ICH) guidelines for linearity, detection and quantitation limits, accuracy, precision, specificity, and robustness. Forced degradation studies under acid, base, oxidation, heat, and U.V light, were performed on the pure compound, extract, and the H.S. developed formulations. Significant degradation of the compound was observed under all tested conditions except U.V. light,

☆ This article is a part of "Epigenetic Mechanisms" Special issue.

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https://doi.org/10.1016/j.heliyon.2022.e09177
Received 1 October 2021; Received in revised form 17 December 2021; Accepted 17 March 2022

where degradation was minimum. There was no interference from impurities, degradation products, or excipients at the retention time of Dp3S 3.2 min indicating the specificity of the method. The developed method was statistically confirmed to be accurate, precise, and reproducible. This simple, rapid, and specific method can be employed efficiently to determine anthocyanin in H.S. plant extract and nutraceutical products.

1. Introduction

Roselle (*Hibiscus sabdariffa L.*) is a tropical plant that belongs to the Malvaceae family. The plant is cultivated in tropical and subtropical regions around the globe. The annual or perennial plant is grown for its edible calyx, leaves, stem, fibers, and seeds. The calyces and the pods of the flowers are used in many countries such as China, Malaysia, India, Indonesia, Egypt, and Mexico to prepare hot and cold beverages, jams, cakes, and ice cream. In addition, the leaves are consumed as fresh or cooked vegetables, while the seeds are utilized to prepare oils that could be used in the cosmetic industry to produce scrubs and soaps [1]. Moreover, the H.S. plant is used in folk or complementary medicine due to its cardioprotective, antilipidemic, antihypertensive, hepatoprotective, antidiabetic immunomodulatory, and antioxidant effects as remedies for chronic diseases like high blood pressure, liver diseases, fever, inflammation, mutagenicity [2, 3, 4].

Such pharmacological activities are mainly due to the chemical constituents of this plant that include organic acids [5], phenolic compounds [6], and anthocyanins [7]. Different types of anthocyanins have been previously detected. Among them, delphinidin-3-O sambubioside (Dp3S) and cyanidin-3-O sambubioside (C3S) are considered the major components in the aqueous extract of the H.S. plant [7, 8]. They are potent bioactive agents having significant beneficial effects on human health [9, 10]. Dp3S and C3S are believed to be the responsible compounds for the antihypertensive and hypocholesterolemic activities of H.S. plant extracts [11]. The mechanism of blood pressure-lowering was suggested to be due to their ability to competitively inhibit the angiotensin-converting enzyme (ACE) [12, 13, 14]. In addition, anthocyanins demonstrated potential hepatoprotective activities. They are reported to restore the altered hepatic architecture and reduce the hepatic enzymes, inflammatory and oxidative stress markers following thioacetamide-induced hepatotoxicity in rats [15, 16]. Furthermore, Dp3S was also connected to the anticarcinogenic properties of the plant extracts since it has been demonstrated to trigger apoptosis in neoplastic leukemia cells via the p38-FasL and Bid pathways [17]. Anthocyanins were also suppressed melanoma cancer metastasis in vitro and in vivo studies [18].

Among the different analytical techniques, the U.V. Spectrophotometry [19, 20, 21, 22, 23], and high-performance liquid chromatography with U.V. detection (HPLC-UV) are the most prominent methods used to determine anthocyanins in H.S. plant extract and nutraceutical products for human consumption. However, using a U.V. spectrophotometer only may not detect or quantify the anthocyanins at low concentrations where more advanced and precise techniques such as the HPLC are required. The use of HPLC-UV can provide the needed accuracy and specificity in detecting and quantifying anthocyanins in the H.S. plant extracts or formulations. However, many of the developed HPLC methods for the detection and quantification of anthocyanins are either not thoroughly validated or uneconomical with very long run times or high limits of detections (LOD) or quantification (LOQ) [24, 25, 26, 27, 28].

Besides, to the best of our knowledge, until now, no stability-indicating HPLC-UV method for the determination of anthocyanin was developed. The implementation of forced degradation studies is of paramount importance. They demonstrate the likely degradation pathways and chemical behavior of the tested molecule, which assist in the pre-formulation studies, development of the suitable formulations, packages, and the selection of proper storage requirements. They also help in the identification of the possible degradation products [29, 30, 31].

The aim of this contribution is to develop a simple, rapid, stability-indicating HPLC-UV method for the quantitative determination of anthocyanin in spray-dried extract (SDE), oral powder, and compressible lozenges formulations using Dp3S as a marker compound. It involved the implementation of forced degradation studies on anthocyanin under various stressing conditions where new findings regarding the stability of anthocyanin of the H.S. plant under acidic conditions were included. Besides, the article includes details for proper HPLC method development and validation procedures for medicinal plants. The method is developed and validated according to the international conference on harmonization of technical requirements for registration of pharmaceuticals for human use (ICH) guidelines [32]. The method is suitable for detecting and quantifying anthocyanin in H.S. plant extract and nutraceutical products.

2. Materials and methods

2.1. Chemicals and reagents

Delphinidin-3-O-β-sambubioside chloride, product number 2407-1 (purity >97 %), was purchased from Polyphenols Laboratories AS, Hanaveien Sandnes, Norway. HPLC grade acetonitrile, methanol, and formic acid (99%) were sourced from J.T. Baker (NJ, USA), Fisher Scientific (U.K.), and Acros Organics (Morris Plains, NJ, USA), respectively. Hydrochloric acid (HCl) and A.R. grade hydrogen peroxide (H_2O_2) were obtained from QRëC® Asia (Darul Ehsan, Selangor, Malaysia). Anhydrous citric acid and Sodium hydroxide (NaOH) pellets were purchased from R & M (Essex, England). Distilled water was produced in-house using Favorit® water system (Model-W4L) (Genristo Ltd., England). Other organic solvents and chemicals used were either of analytical grade or HPLC grade. Polytetrafluoroethylene (PTFE) syringe membrane filters of 0.45 μm were supplied by (Acrodisc, Pall Corporation, USA). All materials required for oral powder drink and lozenges formulations were of food or pharmaceutical grade. Herbagus Sdn. Bhd., Malaysia supplied dried Roselle calyces. Beneo-Palatinit GmbH, Germany gifted Isomalt Galen IQ™ 721. L-Leucine was a kind gift from Ajinomoto Private Limited (Singapore). Ascorbic acid, citric acid, polyethylene glycol (PEG-4000) powder, Talcum powder, sodium benzoate, sodium citrate, and stevia were supplied by Prima Inter-Chem Sdn. Bhd, Malaysia as a gift. Colloidal silicon dioxide was provided by Evonik (SEA) Pte. Ltd., Singapore. Konjac natural gum was purchased from Hubei Yizhi konjac biotechnology co. Ltd, China. Pharmaceutical-grade aluminum foil was kindly provided by Nutratix Biotech Sdn. Bhd. Malaysia. Thermal impulse sealer was purchased from the local market.

2.2. Instrumentation

HPLC analyses were performed on a Shimadzu Prominence series HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a solvent reservoir, a prominence degasser (DGU-20A3), a prominence pump (LC-20AD), prominence auto-sampler (SIL-20A HT), a column oven (CTO-10AS VP), a prominence UV/VIS detector (SPD-20A), and computer software LabSolutions® Version 5.30 SP1, 2010.

2.3. Optimization of the chromatographic conditions

During the initial method development, different mobile phase compositions, columns, flow rates, detection wavelength, and oven temperatures were tested to achieve the best separation of Dp3S peak

from the other peaks of the excipients, degradants, and impurities with good resolution, sharpness, and symmetry. Moreover, to get the separation with shortest sample run and retention times with cost-effective and easy mobile phase preparation.

In the preliminary studies, several mobile phase systems used different combinations of methanol or ACN as organic phase and pure or acidified water with acetic, FA, or phosphoric acids as the aqueous phase at various levels ratios were evaluated. Three different C18 columns including: (Waters Symmetry® W, 5 µm, 4.6 × 150 mm, Agilent Zorbax® SB, 5 µm, 4.6 × 250 mm and Agilent Eclipse plus®, 5 µm, 4.6 × 250 mm), were put into trials as stationary phases for proper separation and determination of Dp3S peak. Furthermore, different levels of the flow rate (0.6–1 mL/min), the injection volume (5–20 µl), the detection wavelengths (520–530 nm), and oven temperatures (30–45 °C) were also investigated.

2.4. Preparations

2.4.1. Preparation of stock solution, calibration standards and quality control samples

Dp3S reference standard of 1 mg was weighed using Sartorius microbalance (Sartorius Weighing Technology GmbH, Gottingen, Germany) and transferred to a 5 mL volumetric flask and dissolved in 3 mL of methanol. The volumetric flask was shaken using an ultrasonic bath for 5 min. The solution was diluted to volume with methanol. The stock standard solution had a concentration of 200 µg/mL of Dp3S. This stock solution was further diluted with methanol to achieve a standard working solution of 100 µg/mL. From the stock standard solution, serial dilutions for calibration curve were prepared by diluting with the methanol to give the concentration in the range of 4–40 µg/mL. Three quality control (QC) solutions were prepared at low (LQC), medium (MQC), and high (HQC) concentrations by diluting the working standard to 8, 16, and 36 µg/mL, respectively.

2.4.2. Preparation of spray dried extract

Briefly, the H.S. extract was prepared by soaking dried ground calyx in water at a sample-to-solvent ratio of 1:15 for 2 h at room temperature and then sonicated at 24 ± 1 °C and 40 kHz for another 0.5 h (ultrasonic bath, Branson 5510, USA) for exhaustive extraction. Then the liquid extract was filtered with muslin cloth followed by 0.45 µm pore size Whatman filter paper. The filtrate was spray dried using a Büchi Mini Spray Dryer B-29 (Büchi Labortechnik AG, Switzerland), with a feed flow rate of (2.5 mL/min), inlet temperature of 150 °C, while the aspirator rate was fixed at maximum (100%).

2.4.3. Preparation of oral powder formulation

A geometric mixing method was used to prepare the oral powder formulation. First, all the required materials were carefully dispensed in the controlled environment of 25 ± 2 °C temperature and <33% relative humidity (Relative humidity range was identified experimentally, unpublished data). Next, the SDE, Isomalt, stevia, konjac natural gum, sodium citrate, citric acid, ascorbic acid, sodium benzoate, and PEG powder were passed through sieve # 40 to break any lumps. This was then transferred into the mixing jar of the Turbula (Type T2F, Willy A. Bachofen AG Switzerland) laboratory-scale mixer. The mixer was run for ~45 min at the speed of 30 rpm to ensure uniform mixing of the blend. Finally, a powder blend of 7 g per sachet, containing 0.5 g of SDE (equivalent to 10 ± 2 mg of Anthocyanin), was manually filled and sealed, using thermal impulse sealer, in pharmaceutical-grade moisture-resistant aluminum foil.

2.4.4. Preparation of direct compressible lozenges formulation

The lozenges were prepared by the direct compression method. First, all the required materials were carefully dispensed in a controlled environment of 25 ± 2 °C temperature and <33% relative humidity. The SDE, Isomalt, stevia, natural gum, sodium citrate, ascorbic acid, sodium benzoate, and talcum powder were passed through sieve # 40 to break any lumps. This was then transferred into the mixing jar and mixed. After mixing for ~35 min, the pharmaceutical lubricants (Talcum and silicon dioxide) were added and again mixed for 5–7 min. Finally, the powder blend was compressed into lozenges with hardness in the range of 18–20 kg using a rotary compression machine (Model CDM-49, Clit Press, India) equipped with an 18 mm bevel edge, bi-planer punches, and dies set. The weight of the compressed Lozenges was 2 g having SDE of 0.5 g (equivalent to 10 ± 2 mg of Anthocyanin).

2.5. System suitability studies

To verify reproducibility and the proper performance of the chromatographic system the system suitability test was performed [33]. At the three QC levels, chromatographic parameters, including tailing factor (T), theoretical plates (N), and height equivalent of a theoretical plate (HETP), were investigated and quantified according to United States Pharmacopoeia specifications [34]. This study employed six replicates for each concentration.

2.6. Specificity

Interferences' possibilities from the compounds present in the extract, formulation excipients, or the mobile phase at the retention time of Dp3S was evaluated by comparing the chromatograms obtained from the reference standard Dp3S solution, SDE solution, formulations, mobile phase, and blank formulations.

2.7. Forced degradation studies

Forced degradation studies were performed as per the ICH guidelines [26] under stress conditions of acid, base, oxidation, heat, and light. The tests were conducted on seven solutions: Dp3S standard solution, SD extract solution, the powder formulation, and lozenges formulation solutions, the mobile phase, and the blank formulations of the oral powder and lozenges. A fresh Dp3S standard solution of 250 µg/mL was prepared and used for this study. Oral powder formulation and lozenges formulation samples were calculated for the equivalent weight of 2 mg/mL of the SDE (previously standardized as 1 mg SDE contains 17 µg of Dp3S) and carefully weighed. The samples were dispersed in methanol and sonicated for 15 min and then centrifuged at 3000x g for 10 min. Then the required volume was taken and diluted with methanol to get the final concentration of 25 µg/mL of Dp3S. The same method was used to prepare the blank formulations sample.

2.8. Selection of stress-inducing and neutralizing agents for acid-base degradation studies

2.8.1. Study for the selection of molarity of acid and its neutralizing agent

The stress degradation studies were initially performed on the pure standard Dp3S to identify the suitable strengths of the stress-inducing agents and the corresponding neutralizing agents. For acid stress at 0.5 mol/L strength, two pairs A and B of 10 mL volumetric flasks were filled with 1 mL of the sample solution. Pair-A was stressed with acid and Pair-B with alkali. Out of the pair A, one sample was neutralized with 1 mL of 0.5 mol/L NaOH, and subsequently, the volume was made up to 10 mL with methanol. The second sample of the Pair-A was counterbalanced with only methanol. Contrary, the Pair-B (which was stressed with alkali) was neutralized by using HCl and methanol for one sample and only methanol for the other sample. In both cases, the neutralization was done after 30 min of inducing the stress. The same procedure was repeated with 1 mol/L acids and alkali as stressing agents. All the prepared samples were run on HPLC in triplicates. As a result of this study, the selected strength of stress-inducing and neutralizing agents would be used for further studies.

2.8.2. Study for the selection of molarity of alkali and its neutralizing agent

For the alkali stress degradation, the same procedure described above was adopted. In this case, the only change was that NaOH was used as a stress-inducing agent, and HCl and methanol were used as neutralizing solutions.

2.8.3. Acid and alkali degradation studies

In the acid degradation study, eighteen volumetric flasks of 10 mL capacity were divided into three sets nominated as A, B, and C. Each set consisted of six volumetric flasks. Each flask was filled with 1 mL of sample solution of Dp3S standard, SDE, oral powder formulation, lozenges formulation, and blank formulations. Then, 1 mL of 0.5 mol/L HCl was added to each flask of all three sets as the acid stress-inducing agent. Set A was immediately neutralized by adding methanol and make-up the volume up to 10 mL; this served as 0 h samples. Solutions of set B and C flasks were kept without neutralization for 1 h (as an intermediate sample) and 24 h (as the final sample) respectively on the benchtop at the room temperature of (25 ± 2 °C/65% relative humidity). After a specified time, these samples were neutralized and top-up with methanol up to 10 mL. All the prepared samples were run by HPLC in triplicates.

The same approach was employed for alkali stress degradation. The sole difference was that the alkali stress-inducing agent was NaOH, and the neutralising agent was HCl, with methanol as the volume-making solution.

2.8.4. Oxidative degradation studies

In the oxidative degradation study, a total of eighteen volumetric flasks of 10 mL capacity were divided into three sets nominated as A, B, and C. Each set had six volumetric flasks. Each flask was filled with 1 mL of sample solution of Dp3S standard, SDE, oral powder formulation, lozenges formulation, and blank formulations. Then to each flask of all the three sets, 1 mL of 1% H_2O_2 was added as the oxidative stress-inducing agent. Set A was immediately neutralized by adding methanol and make-up the volume up to 10 mL. These served as 0 h samples. Solutions of the set B and set C flasks were kept for 1h (as an intermediate sample) and 24 h (as the final sample) on the benchtop at the room temperature of (25 ± 2 °C/65 % relative humidity). After a specified time, these samples were also neutralized and top-up with methanol up to 10 mL. HPLC was used to analyse all of the produced samples in triplicate.

2.8.5. UV light degradation

In the photodegradation study, a total of eighteen volumetric flasks of 10 mL capacity were divided into three sets nominated as A, B, and C. Each set had six volumetric flasks. Each flask was filled with 1 mL of sample solution of Dp3S standard, SDE, oral powder formulation, lozenges formulation, and blank formulations, respectively. Set A was immediately neutralized by adding methanol and make-up the volume up to 10 mL. These served as 0 h samples. The flasks of the other two sets, A and B, were stored for 1 h and 24 h in a UV cabinet (365 nm), respectively. After 1 h the flasks of the set B were taken out from the UV cabinet and neutralized by adding methanol and make-up the volume of each flask up to 10 mL. These served as intermediate stress samples. After 24 h, the third set of flasks were taken out, neutralized with methanol, and make-up the volume of each flask up to 10 mL. HPLC was used to analyse all of the produced samples in triplicate.

2.8.6. Heat degradation study

For thermal degradation study, in two sets of 10 mL volumetric flasks, 1 mL of each solution, namely, Dp3S standard, SDE, oral powder formulation, lozenges formulation, and blank formulations solutions, filled respectively. To one set of the flasks, methanol was added and make-up the volume up to 10 mL. This set serves as 0 h samples. The second set of flasks were heated for 2 h in a water bath at 80 °C temperature. After 24 h, methanol was added to these samples and make-up the volume of each flask up to 10 mL. HPLC was used to analyse all of the produced samples in triplicate.

2.9. Method validation

The developed method validation was conducted as per the ICH, the association of official agricultural chemists (AOAC) guidelines and Unites States Pharmacopoeia (USP). The linearity, specificity, the limit of detection (LOD), the limit of quantification (LOQ), accuracy and precision, ruggedness, and robustness of the method were evaluated.

2.9.1. Linearity

The calibration curve was prepared using six solutions of Dp3S at concentrations of 4, 8, 16, 20, 30, and 40 μg/mL. The standard calibration curve was constructed using peak area versus known concentrations of Dp3S. Triplicate injections of each solution were performed. The linear regression line was used to determine the linearity and concentration of the samples.

2.9.2. Accuracy and precision

Accuracy and precision are the two critical factors of a chromatographic test method. The aim of analyzing herbals and other botanical natural products is the quantitative analysis of marker compounds in the matrix. In natural products analysis techniques, the most popular procedure for determining accuracy is the spike recovery method, in which a known amount of the marker compound is added to the matrix system. Then through analysis, the added compound is identified as a percentage of the theoretical quantity present in the system. The quantity found compared to the amount added provides the recovery of the marker compound. Analyte spiking is a frequently practiced method, and other techniques can also be used to validate the accuracy of the method, for example, the exhaustive extraction method. However, in the case of certified reference standard availability, it could be used in place of the spiking method. In our study, we used reference standard Dp3S with a known purity of >98%.

Precision refers to the percentage of the relative standard deviation (% RSD) and is determined by dividing the estimated concentration's standard deviation by the mean value. On the other hand, the accuracy which is represented by the percent relative error (%RE), is calculated by dividing the difference between the computed and nominal concentrations of the standard solution by the known concentration of the standard solution.

To determine the precision and accuracy of the procedure, three quality control (QC) samples of Dp3S were made from the stock solution, namely LQC, MQC, and HQC of 6, 18, and 36 g/mL.Six sets of the standard solution were injected on the first day for intra-day precision and accuracy, and six sets of the standard solution were injected on the following six consecutive days for inter-day precision and accuracy, with one standard curve injected each day.

2.9.3. Limit of detection (LOD) and lower limit of quantification (LLOQ)

The limit of detection (LOD) is defined as the lowest detectable amount of an analyte in a sample. In contrast, the lower limit of quantification (LLOQ) is the minimal amount of the analyte that could be quantified in a particular sample with acceptable accuracy and precision. Serial dilutions of Dp3S stock solutions determined these parameters to obtain a signal-to-noise (S/N) ratio of at least 3:1 for LOD and 10:1 for LLOQ.

2.9.4. Robustness studies

The developed method was assessed for robustness by intentionally making minor changes in the optimized values of the chromatographic conditions. Following variables were evaluated for this purpose: wavelength (±2 nm), pH of mobile phase (±0.2), the flow rate of the mobile phase (±0.1 mL/min), and oven temperature (±2 °C). The standard DP3S

Table 1. System suitability studies of Dp3S at three quality control concentrations.

Conc. (μg/mL)	Rt*	%RSD	TF*	%RSD	N*	%RSD	HETP*	%RSD
6	3.20 ± 0.002	0.073	1.47 ± 0.02	1.45	13881.0 ± 179.28	1.29	10.79 ± 0.16	1.48
18	3.21 ± 0.004	0.124	1.67 ± 0.03	1.90	8901.8 ± 117.19	1.32	16.96 ± 0.25	1.49
36	3.21 ± 0.003	0.083	1.63 ± 0.03	1.92	9708.0 ± 185.71	1.91	15.99 ± 0.31	1.93

* mean ± SD, n = 6; TF = Tailing factor; N = number of theoretical plates; HETP = Height equivalent to the theoretical plate; RSD: Relative standard deviation.

concentration of 18 μg/mL was used in this study, and the peak areas and %RSD obtained under these conditions were calculated and statistically analyzed.

2.9.5. Stock solution stability

Due to the known sensitivity of anthocyanin to temperature, the stability of the standard stock solution was evaluated by keeping the

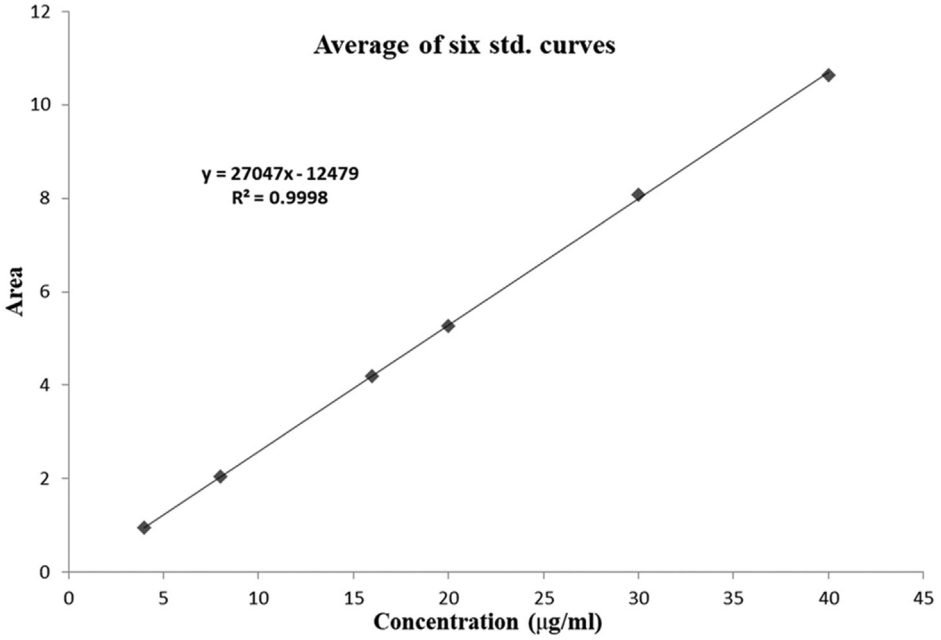

Figure 1. Least square line for the 6 concentrations (4, 8, 16.20, 30 and 40 μg/mL) of Dp3S, with y = 27047x – 12479 (Average of six std. curves).

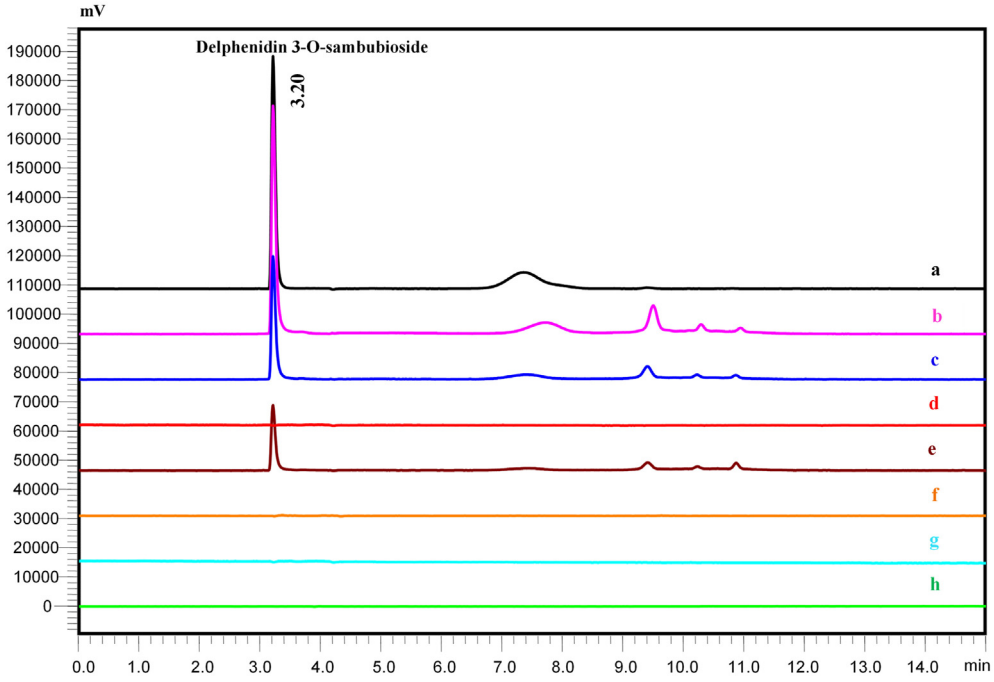

Figure 2. Chromatograms of the reference standard Dp3S [a], SDE [b], oral powder formulation [c] and blank powder formulation [d], lozenges formulation, [e] and blank lozenges formulation [f], methanol [g] and mobile phase [h], indicating specificity of the method.

Table 2. Results of intra-day and inter-day precision, recovery, and accuracy for Dp3S at three quality control concentrations.

Theoretical Conc. (μg/mL)	Intra-day					Inter-day				
	Found conc. (μg/mL)*	Precision (%RSD)	Accuracy (%RE)	Recovery n = 3	RSD (%)	Found conc. (μg/mL)*	Precision (%RSD)	Accuracy (%RE)	Recovery n = 3	RSD (%)
6	5.97 ± 0.05	0.86	-0.45	99.71 ± 0.670	0.672	6.00 ± 0.05	0.81	0.00	99.82 ± 0.255	0.255
18	17.77 ± 0.17	0.93	-0.38	99.97 ± 1.004	1.004	18.07 ± 0.32	1.80	0.41	100.21 ± 1.324	1.321
36	36.28 ± 0.16	0.46	1.55	101.79 ± 0.363	0.357	36.64 ± 0.06	0.16	1.78	101.75 ± 0.174	0.171

* mean ± SD, n = 6.

Table 3. Analysis of method robustness using Dp3S at a concentration of 18 μg/mL.

Parameters	Changes	Mean area* ± SD	%RSD	Mean retention time (min)* ±SD	%RSD
Detector wavelength (nm)	528	451922 ± 2445.2[a]	0.54	3.208 ± 0.001[a]	0.03
	525	448639 ± 108.1[a]	0.02	3.209 ± 0.001[a]	0.03
	522	449298 ± 490.8[a]	0.11	3.208 ± 0.000[a]	0.01
pH of the mobile phase	2.48	444044 ± 1573.9[a]	0.35	3.212 ± 0.003[a]	0.06
	2.46	445972 ± 488.9[a]	0.11	3.209 ± 0.001[a]	0.03
	2.44	446138 ± 282.9[a]	0.06	3.210 ± 0.002[a]	0.07
Flow rate (mL)	0.9	409287 ± 1238.7[c]	0.30	2.867 ± 0.000[c]	0.00
	0.8	441489 ± 1296.8[b]	0.29	3.221 ± 0.000[b]	0.01
	0.7	520498 ± 1272.9[a]	0.24	3.679 ± 0.000[a]	0.00
Temperature (°C)	47	462320 ± 2575.3[a]	0.56	3.208 ± 0.002[a]	0.06
	45	450440 ± 1296.6[b]	0.28	3.209 ± 0.001[a]	0.02
	43	449321 ± 1424.5[b]	0.32	3.210 ± 0.001[a]	0.04

* mean ± SD, n = 3; Values that do not share a letter are significantly different ($p < 0.05$).

stock solution at two different temperatures. The stock solution of Dp3S (60 μg/mL) was kept for 24 h in two flasks, one flask was left on the benchtop at room temperature (25 ± 2 °C), and the second one was kept in the refrigerator (2–6 °C). The next day the samples were diluted to bring them within the standard calibration curve range of 18 μg/mL. A comparison was made between the peak area of the zero h samples and the peak areas obtained from the samples stored at the two different temperatures for 24 h.

2.10. Quantification of delphinidin-3-O-sambubioside in different samples

2.10.1. Assay of delphinidin-3-O-sambubioside in Hibiscus sabdariffa L. SDE

Freshly prepared H.S. SDE (10 mg) was dissolved in 10 mL of methanol and vortexed for 0.5 min and then sonicated in an ultrasonic bath (Branson 5510, USA) for 15 min. The sample was then centrifuged for 10 min at 3000x g to separate any suspended particle. The supernatant was filtered through a 0.45 μm PTFE syringe filter into an amber-colored HPLC vial and subjected to chromatographic analysis. The sample was injected into HPLC in triplicate to determine the potency of Dp3S in H.S. SDE.

2.10.2. Assay of delphinidin-3-O-sambubioside in oral powder formulation

From the powder formulation, 140 mg of the powder, which is equivalent to 10 mg of SDE, was carefully weighed and dispersed in 10 mL of methanol, vortexed for 3–5 min, and then sonicated in an ultrasonic bath (Branson 5510, USA) for 15 min. The sample was then centrifuged for 10 min at 3000 rpm to separate any suspended excipient particle. The supernatant was filtered through a 0.45 μm PTFE syringe filter into an amber-colored HPLC vial and subjected to chromatographic analysis.

2.10.3. Assay of delphinidin-3-O-sambubioside in lozenges formulation

Five compressed lozenges were taken and crushed in mortar and pestle; out of it, 40 mg of the powder equivalent to 10 mg of SDE was carefully weighed and dispersed in 10 mL of methanol, vortexed for 3–5 min, and then sonicated in an ultrasonic bath (Branson 5510, USA) for 15 min. The sample was then centrifuged for 10 min at 3000x g. The supernatant was filtered through a 0.45 μm PTFE syringe filter into an amber-colored HPLC vial and subjected to chromatographic analysis.

Table 4. Results of the initial acid-base stress degradation studies of Dp3S standard solution treated for a short duration and immediately neutralized.

Column # 1	Dp3S[a] +HCl[b] + NaOH[c]		Dp3S + HCl[b] + MeOH[d]		Dp3S + NaOH[e] + HCl[f]		Dp3S + NaOH[e] + MeOH[d]	
	# 2	# 3	# 4	# 5	# 6	# 7	# 8	# 9
Strength of the Solution	Drug[a] % Remaining*	% Loss	Drug[a] % Remaining*	% Loss	Drug[a] % Remaining*	% Loss	Drug[a] % Remaining*	% Loss
0.5 mol/L	86.61 ± 0.39	13.4	96.99 ± 1.11	3.01	10.71 ± 0.25	89.3	4.02 ± 0.05	95.9
1 mol/L	70.39 ± 1.14	29.6	88.89 ± 1.06	11.1	10.81 ± 0.19	89.2	3.65 ± 0.02	96.4

* mean ± SD, n = 3.
[a] Drug = Marker compound "Dp3S".
[b] HCL used as Stress inducing agent.
[c] NaOH used as Neutralizing agent.
[d] MeOH (Methanol) used as Neutralizing solvent.
[e] NaOH used as Stress inducing agent.
[f] HCL used as Neutralizing agent.

2.11. Statistical analysis

The results (the peak area and or the retention time) of the stock solution stability and method robustness were statistically analyzed using Minitab® statistical software, version 17.2.1.0 (Minitab, Inc., State College, PA, USA). One-way analysis of variance (ANOVA) and a post hoc Tukey's HSD (honest significant difference) test were performed to analyze the data. The difference was considered statistically significant when $p < 0.05$. All values were expressed as mean ± SD. Student t-test was employed wherever required.

3. Results and discussion

3.1. The optimized chromatographic conditions

In this method, using methanol and pure water at various ratios did not lead to proper analyte separation. Likewise, when combinations of methanol: acidified water with acetic acid 0.25%, FA, or phosphoric acid at 0.1% are used, no desirable peak parameters are obtained and thus were not selected. However, upon using a mobile phase composed of a combination of ACN–acidified water (0.2% FA), Dp3S was optimally fractionated at a ratio of 10:90 v/v under gradient conditions. The optimized used linear gradient for the analysis was as follows: mobile phase: A: water (0.2% FA) 90% and B: ACN 10%. The linear gradient elution program was run as: 0.5 min, 10% (B); 4 min 20% (B); 5 min 10% (B); 15 min, isocratic of B 10%. Among the tested columns, Agilent Eclipse® plus displayed better separation parameters at short-run and retention time and was preferred for this method due to its high endurance in the 90% water phase. The other selected optimized conditions were injection volume was 15 μL, flow rate of 0.8 mL/min, oven temperature of 45 °C, and detection wavelength of 525 nm.

Table 5. Results of acid, base, oxidation, light and heat stress degradation studies.

Stress condition	Remaining drug (%) at 0 h*	Remaining drug (%) after 1 h or 2 h*	Remaining drug (%) after 24 h*
Stress degradation studies results for the standard Dp3S solution at 0, 1 and 24hr			
Acid (0.5 mol/L HCl)	95.42 ± 2.13	83.18 ± 0.43	66.64 ± 1.37
Base (0.5 mol/L NaOH)	10.77 ± 0.08	5.74 ± 0.14	ND
Oxidation (1% H_2O_2)	99.11 ± 1.81	59.16 ± 0.23	ND
Light (365 nm)	100.26 ± 0.11	95.27 ± 0.18	85.73 ± 0.29
Heat (at 80 °C)	100.22 ± 0.37	66.01 ± 0.15	-
Stress degradation studies results for the SDE at 0, 1 or 2 and 24 h			
Acid (0.5 mol/L HCl)	96.60 ± 0.56	85.75 ± 0.21	72.16 ± 1.82
Base (0.5 mol/L NaOH)	11.02 ± 0.18	7.16 ± 0.19	ND
Oxidation (1% H_2O_2)	96.69 ± 0.73	54.15 ± 0.59	ND
Light (365 nm)	99.77 ± 0.41	91.18 ± 0.35	83.25 ± 0.20
Heat (at 80 °C)	100.05 ± 0.28	67.73 ± 0.29	-
Stress degradation studies results for the oral powder formulation at 0, 1 or 2 and 24 h			
Acid (0.5 mol/L HCl)	93.30 ± 1.61	84.91 ± 2.12	70.00 ± 1.50
Base (0.5 mol/L NaOH)	14.69 ± 0.28	12.51 ± 0.61	ND
Oxidation (1% H_2O_2)	97.23 ± 0.063	54.55 ± 0.210	ND
Light (365 nm)	99.71 ± 0.28	92.87 ± 0.31	83.90 ± 0.36
Heat (at 80 °C)	100.13 ± 0.53	57.83 ± 0.15	-
Stress degradation studies results for the lozenges formulation at 0, 1 or2 and 24 h			
Acid (0.5 mol/L HCl)	96.53 ± 1.97	86.18 ± 0.43	65.61 ± 3.27
Base (0.5 mol/L NaOH)	12.97 ± 0.06	7.82 ± 0.02	ND
Oxidation (1% H_2O_2)	97.86 ± 0.932	57.57 ± 1.74	ND
Light (365 nm)	99.79 ± 0.29	92.26 ± 0.28	81.48 ± 0.33
Heat (at 80 °C)	99.86 ± 0.81	67.02 ± 0.37	-

* mean ± SD, n = 3; Drug = Marker compound "Dp3S"; ND = Not detected.

3.2. Method validation

3.2.1. System suitability studies

The system suitability studies' results of the method at three QC concentrations of 6, 18 and 36 μg/mL are summarized in Table 1. The peak retention time of the Dp3S was about 3.20 min with %RSD <1%. The obtained average theoretical plate was >10,000 with an acceptable tailing factor of <2, indicating the suitability of the developed method for the quantification of Dp3S and the quality of the method for correct measurements. Such critical parameters were not reported in other HPLC methods [24, 25, 26, 28] to quantify anthocyanins.

3.2.2. Linearity

The linearity of Dp3S was ascertained through six sets of calibration standards. The linear regression equation was y = 27047x (±636.80) – 12478.9 (±4068.69) with a correlation coefficient of 0.9998 (±0.0001) and a percentage of relative error (%RE) of ˂ 2 %. This indicated the good linearity of the method. The linearity graph is shown in Figure 1. The correlation coefficient of the developed method is higher than the previously reported coefficients of HPLC methods for the quantification of anthocyanins [25, 26, 27, 28].

3.2.3. Specificity

The chromatograms of different sample solutions are shown in Figure 2. The chromatogram of Dp3S standard (a) shows a peak at a retention time (3.20 min) followed by chromatograms of the SDE (b), oral powder formulation (c), blank powder formulation (d), lozenges formulation (e), blank lozenges formulation (f), mobile phase (g), and the solvent used for sample preparation, methanol (h). All chromatograms that do not contain Dp3S showed no peaks at the specific retention time of 3.20 min. From the results, it is clear that there is no interference from the excipients and the mobile phase used, as shown by the absence of the peak at the retention time of Dp3S reflecting method specificity.

3.2.4. Precision and accuracy

The limits of ±2% for precision and accuracy are generally recommended for an ideal HPLC method of a pharmacopeial pharmaceutical drug [35]. The results for precision and accuracy at three QC concentrations of Dp3S are tabulated in Table 2. The intra-day precision lies between 0.46% - 0.93%, while that of inter-day, was between 0.16% - 1.80%. On the other side, the accuracy for both intra-day and inter-day ranged between -0.38% to 1.55% and 0.00%–1.78%, respectively. The results revealed that the procedure was precise and accurate. The acquired % RSD values were less than 2%, indicating that the devised HPLC method had good inter- and intra-day variation precision.

The recovery range of the marker compound (99.71%–101.79%) also reveals that the method is accurate with %RSD less than 2%. The obtained values fall within the 95–105% accepted range, which is generally accepted for method accuracy. In quantitative analysis, there is no official criterion available for the recovery rate. AOAC, 2002, guideline proposes that the compound recovery rates depend on the concentration of an analyte within the sample; as much as the concentration is low, that much bigger is the tolerance limit. The limits, as per AOAC recovery ranges for analyte concentration of 10%, 1%, 0.1% and 0.01 % ranges from 95-102%, 92–105%, 90–108% and 85–110%, respectively. Similarly, these guidelines also state that recovery can be obtained as a byproduct of the precision determinations [36]. In this study, the recovery results of approx. 97–102% meet the criterion outlined in AOAC guidelines and were taken from precision data acquired.

3.2.5. LOD and LLOQ

The limit of detection (LOD) and lower limit of quantification (LLOQ) of Dp3S of the proposed method were 0.01 μg/mL and 0.04 μg/mL, respectively.

3.2.6. Robustness

A suitable HPLC method must have an adequate capacity to remain unaffected by minor variations in the method parameters. Thus, robustness studies are essential because the analyst may need to change one or more of the method's chemicals and/or instrumental parameters, and this can only be done if the changes do not affect the method's accuracy when compared to the previously optimized results [37, 38]. Unfortunately, robustness studies were not performed in many of the developed HPLC methods for anthocyanin quantification [24, 25, 26, 27, 28].

The results of the robustness studies are shown in Table 3. Variations in the detection wavelength and pH of the mobile phase had no statistically significant ($p > 0.05$) impact on the peak area or retention time. While the alteration of oven temperature was associated with some changes in Dp3S peak area but showed no effect on retention time. On the other hand, The flow rate modification caused significant (p 0.05) changes in the peak area along with significant shifts in the retention time.

3.3. Stock solution stability

The obtained results indicated that the percentage of Dp3S remained 100.5 ± 0.2% after 24 h at refrigeration temperature (2–6 °C). Furthermore, there was no statistically significant difference ($p > 0.05$) in peak area between the samples measured at zero time and after 24 h at (2–6 °C), reflecting that the stock solution was stable for at least 24 h at refrigeration temperature. On the other hand, samples stored at room temperature (25 ± 2 °C), after 24 h (98.85 ± 0.09%) showed a slight degradation in the standard solution leading to the conclusion that storage at room temperature is not favorable and stock solution should preferably be kept in the refrigerator.

3.4. Selection of stress-inducing and neutralizing agents for acid-base degradation studies

Based on the evidence from the literature that alkaline medium is highly detrimental to anthocyanins, therefore it was mandatory to

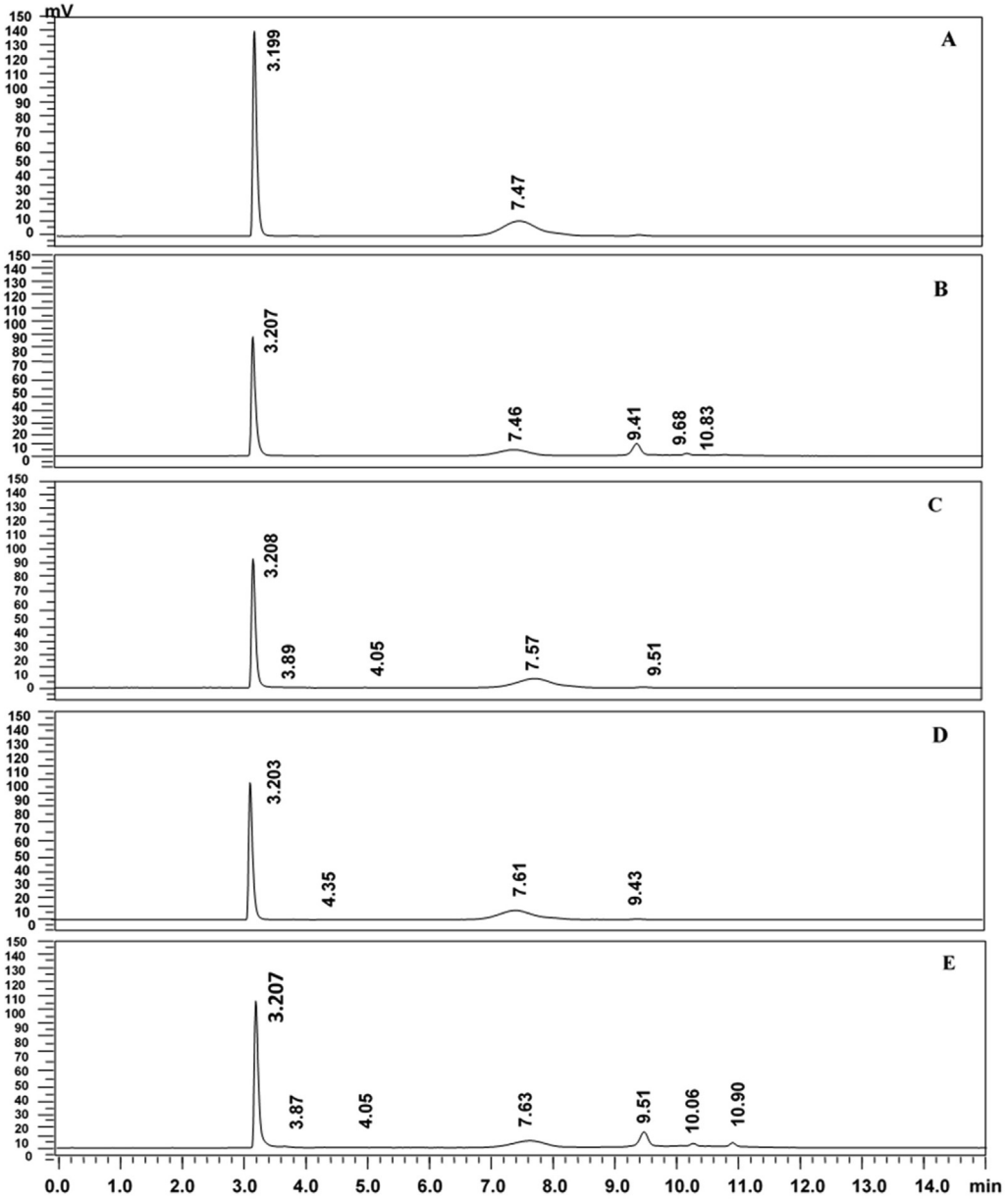

Figure 3. Chromatograms of Dp3S standard solution at 0 h (A) and after thermal stress for 2 h, Dp3S standard sol. (B), SDE (C), oral powder formulation (D) and lozenges formulation (E).

carefully select the stress-inducing and neutralizing agents before commencing the stress degradation studies [39, 40, 41]. The results of the performed initial acid-base stress studies to select suitable strength of stress-inducing and neutralizing agents are compiled in Table 4. It is evident from the results that in the case of HCl stress, the use of sodium hydroxide (NaOH) to neutralize the HCl interestingly fails to do so; instead, it further increased the degradation process, where within 30 min 13.4% of the compound was lost as compared to only 3.01% when only methanol was used as neutralizing solvent. The reason for this increment of degradation is most likely that with the addition of 0.5 mol/L NaOH, the pH of the solution is raised to 7 and above, and hence immediate degradation occurs where anthocyanins are highly sensitive to pH $\geq$ 7 [33].

The use of methanol alone was able to efficiently neutralize the stress induced by the acidic solution, as shown in Table 2, column no. 2–5. Contrary, during alkali stress, the use of HCl as the neutralizing agent was proved to be adequate to counter the stress of NaOH solution, whereas the use of methanol alone was not able to neutralize the stress induced by NaOH solution.

Generally, three concentrations of 0.1 mol/L, 0.5 mol/L, and 1 mol/L of acid (HCl) and Alkali (NaOH) are commonly used for stress degradation purposes. However, as it is known that at a low concentration of 0.1 mol/L HCl (pH = 1), anthocyanins are pretty stable; therefore, in the present study, two strengths (i.e., 0.5 mol/L and 1 mol/L) of solutions for both acid and base, were employed to know the appropriate strength of stress-inducing agents. Based on the obtained results shown in Table 4, the concentration of 0.5 mol/L for both HCl and NaOH were selected for the forced degradation studies because they are less harsh than 1 mol/L. The selection was made on the basis that during the stress degradation study, the compound should be stressed in a manner to degrade the drug from 5 to 20% of the claimed potency, and more harsh stress conditions should be avoided [29, 43].

3.5. Stress degradation studies

Stress degradation studies helps to identify the degradation products, the stability of the molecule and specificity of the analytical procedure [33]. The forced degradation study results of acid, alkali, oxidation, light (UV), and thermal stresses are tabulated in Table 5. The chromatographic presentations of the stressed Dp3S standard, SDE, and the oral powder and lozenges formulations after 24 h under various stress conditions are presented in Figures 3, 4, 5, and 6. It was revealed that Dp3S in pure form, SDE, and the developed formulations exhibited substantial degradation under highly acidic, alkali, heat, and oxidation conditions.

In acidic stress (at pH 0.3), the remaining compound percentage for different samples ranged between 65 – 72% after 24 h — this loss in potency contradicted the established hypothesis that anthocyanins are stable at acidic pH < 2. The results for alkali stress were devastating for all samples, and only less than 14% compound remained in the 0 h sample, less than 12.5% after 1 h and entirely vanished after 24 h stress.

The results of hydrogen peroxide stress (Figures 4, 5, and 6) show a complete loss after 24 h; however, unlike alkali stress, its degradation was gradual but fast (the compound remained at 0 h was ranged from 96 – 99% and 54–59% after 1 h stress). The heat stress results after 2 h also showed a significant decay of the compound (Figure 3), and the remained quantity was quantified as 58–67%. Contrary, all the samples appeared to be least affected by UV light stress as the least amount of compound degraded over

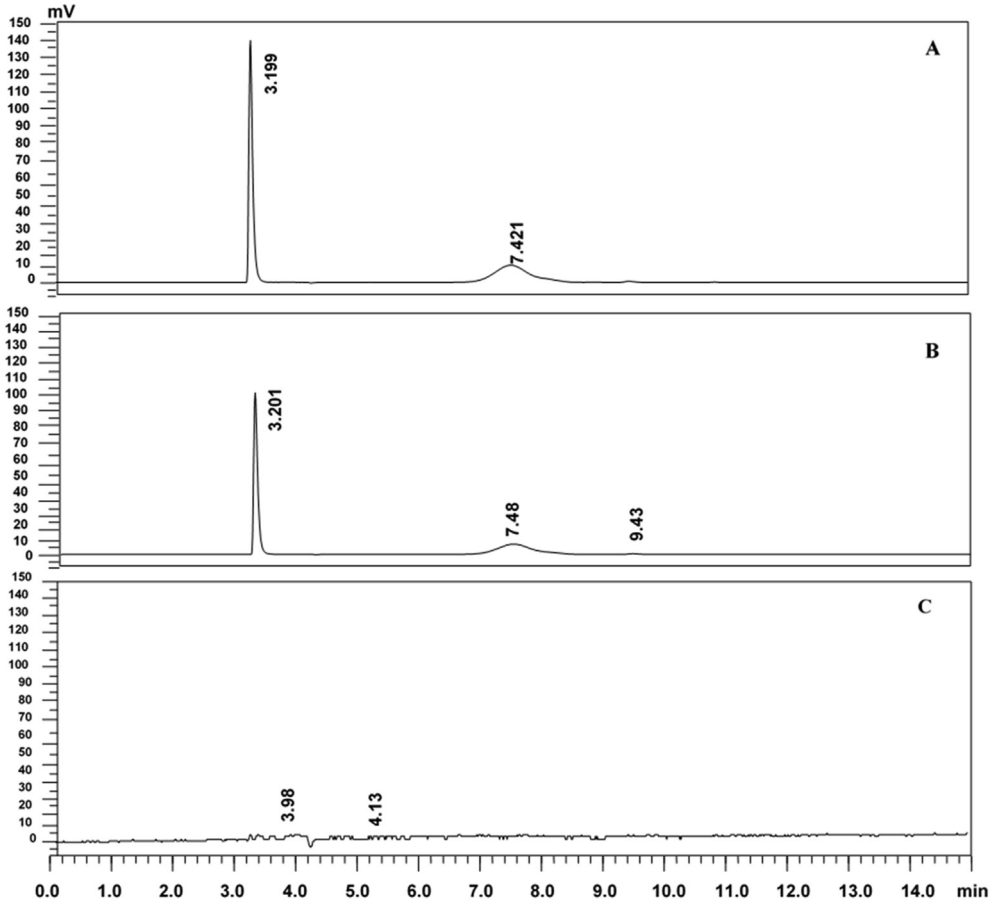

Figure 4. Chromatograms of Dp3S standard at 0 h (A), after stressed with 1% Hydrogen peroxide for 1 h (B) and for 24 h (C).

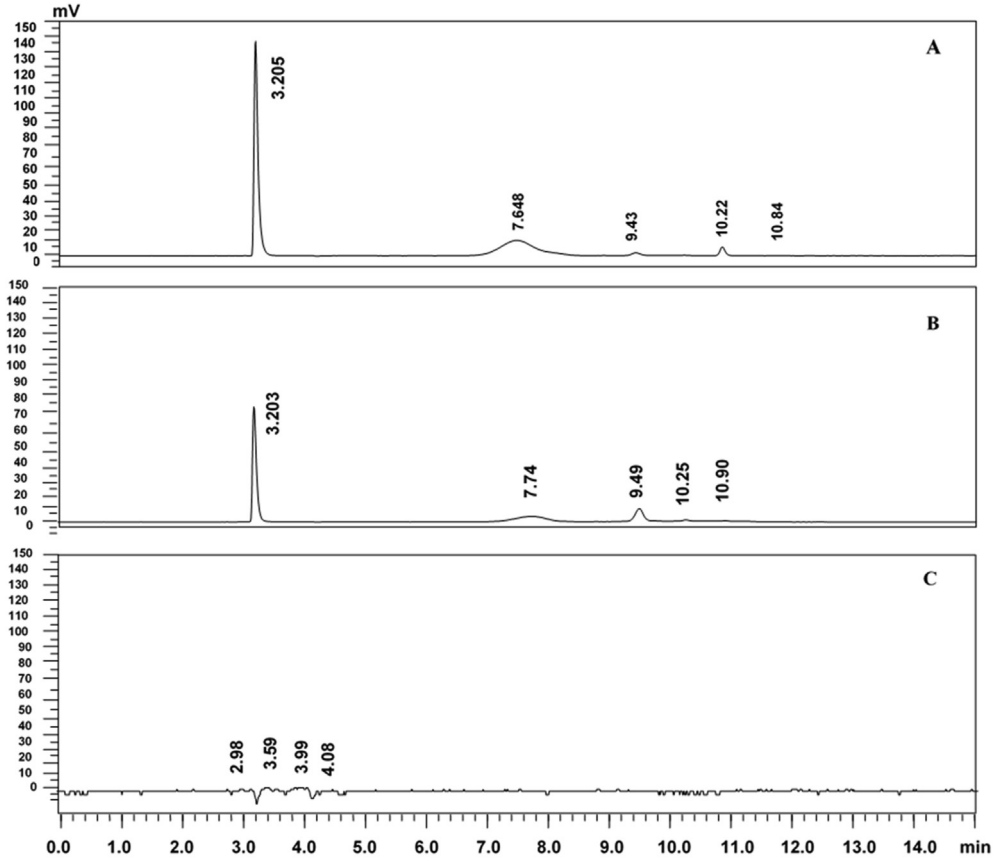

Figure 5. Chromatograms of spray dried extract at 0 h (A), after stressed with 1% Hydrogen peroxide for 1 h (B) and for 24 h (C).

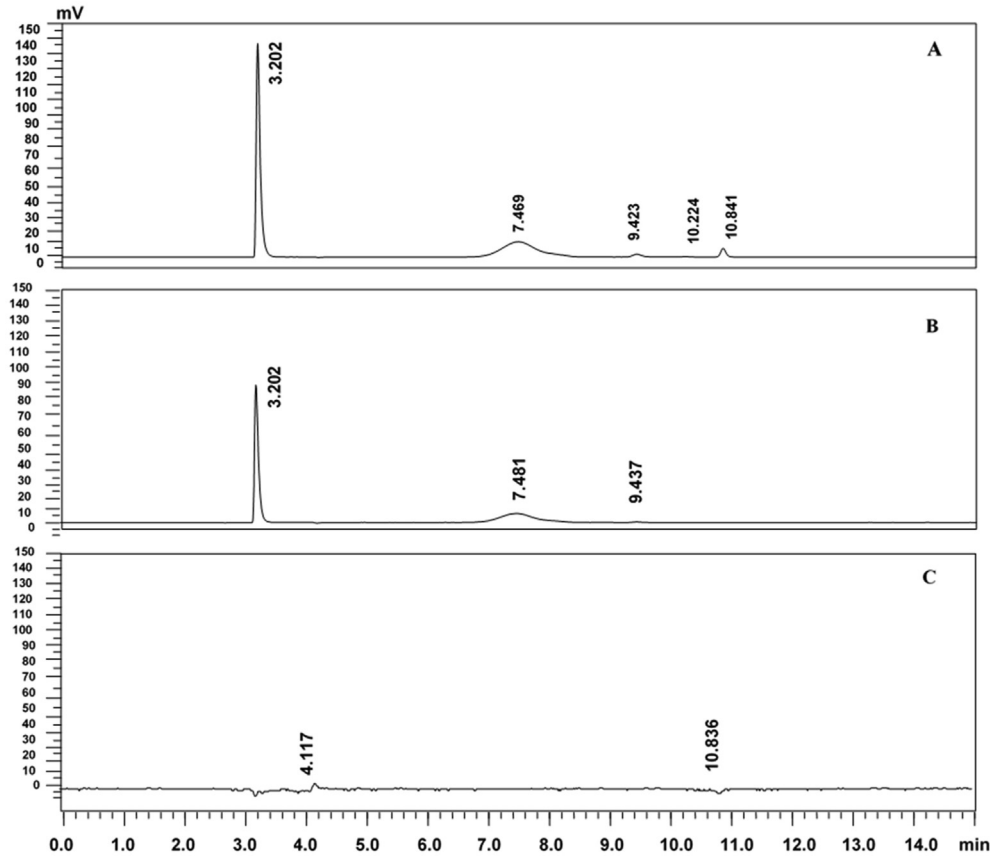

Figure 6. Chromatograms of oral powder formulation at 0 h (A), after stressed with 1% Hydrogen peroxide for 1 h (B) and for 24 h (C).

time, and the remaining compound concentration ranged from 81.48 – 85.73% after 24 h, which is in good agreement with the requirements of ICH guidelines for forced degradation studies [44]. However, photosensitivity is there, and chances of product instability via light cannot be rolled out entirely; therefore, proper attention must be paid during processing and storage to protect the product from direct light for product integrity during shelf life.

An excellent stability-indicating method can quantify the drug and resolve the degradation products [45]. It is a fact that anthocyanins are extremely sensitive to pH change [41]. The inherent instability of anthocyanins is an essential factor that must also be considered in their analysis. During the extraction process, the extracting solution should be somewhat acidic to maintain the red flavylium cation form, which is stable in a highly acidic medium, but it should not be extremely acidic to cause partial hydrolysis of the acyl moieties in acylated anthocyanins [40]. According to Du and Francis (1975), stronger acid solutions have proven detrimental to anthocyanins; therefore, the use of only 0.05% of HCl in methanol was recommended as the extraction solvent to protect the decomposition of acylated anthocyanins [39]. As mentioned, few researchers indicated the damaging effects of acidic pH, but until now based on authors' knowledge, no research was conducted on the degradation of anthocyanins under extremely acidic (pH 0.3) stress conditions as performed in this work. Most of the researchers worked on the pH stability of anthocyanins, starting from the pH range of 1–14 [41,42,46]. Only Nielsen et al. (2003) investigated the pH stability of pure anthocyanins in aqueous solutions starting from the lowest pH of 0.6 up to 13 (pH) and concluded that for stability purposes, the pH should be kept below 2 when handling with the aqueous solution of anthocyanins [38]. The findings obtained from this study showed instability of anthocyanins at extremely high acidic (pH 0.3) conditions contradicting the conclusions from previous reports that considered anthocyanins are stable at pH < 2 [7,47]. However, this may be specific to some of the anthocyanins where highly acidic conditions may lead to partial hydrolysis of the glycosidic moiety of some of the anthocyanin. Therefore, we suggest that anthocyanins are stable under pH-2 up to a certain extent, such as pH-0.6 as stated above, but more astringent acidic conditions at pH < 0.6 is proven to be risky for anthocyanins stability. Therefore, care should be observed during acid degradation studies whenever a stability-indicating method is desired.

3.6. Assay of Dp3S in SDE and formulations

The assay of Dp3S in standardized SDE, oral powder, and compressed lozenges formulations were 105.20 %, 98.15%, and 96.65 %, respectively.

4. Conclusion

A rapid, simple, specific, sensitive, and stability-indicating HPLC–UV method was developed and validated to determine Dp3S in the spray-dried extract and various dosage forms. Method validation was carried out as per ICH guidelines and was statistically confirmed to be accurate, precise, and reproducible with superior values of correlation coefficient (0.9998), LOD, and LOQ (0.01 μg/mL and 0.04 μg/mL respectively) in comparison to the previously reported methods. There were no peaks of formulation excipients, diluents, or degradation products that interfered with the peak of Dp3S. The forced degradation studies showed that Dp3S undergo moderate degradation against highly acidic conditions while it is extremely vulnerable to alkali, heat, and oxidation degradation. The current method was confirmed statistically to be accurate, precise, and reproducible $P < 0.05$. The method is robust in terms of the detection wavelength and pH of the mobile phase ($p < 0.05$). The developed method could be used for the routine quality control assay, content uniformity, standardization of plant materials, verification of raw material origins, verification of commercial Hibiscus products, and nutraceuticals quality control.

Declarations

Author contribution statement

Nasir Hayat Khan, Ibrahim M Abdulbaqi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Yusrida Darwis, Siok-Yee Chan: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Nafiu Aminu: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Funding statement

This work was supported by Malaysia Toray Science Foundation (210608STRG0319).

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgments

Nasir Hayat Khan and Ibrahim M. Abdulbaqi gratefully acknowledge Universiti Sains Malaysia (USM), the Institute of Postgraduate Studies (IPS), Penang, Malaysia for awarding of USM Fellowship.

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