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Original Research Article

Simultaneous Analysis Method for Rutin, Diosmin, Hesperidin, and Quercetin in Solid Food Supplements by HPLC-PDA

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ABSTRACT

Flavonoids are important component of dietary supplements, playing major roles as antioxidants, anti-aging agents, and numerous health benefits. The aim of this study was to develop a method for the simultaneous analysis of four flavonoids - rutin, diosmin, hesperidin, and quercetin in dietary supplements. A high-performance liquid chromatography (HPLC) method with reversed-phase separation and photodiode array detector was developed to simultaneously quantify rutin, diosmin, hesperidin, and quercetin in solid dietary supplements. The method used a Phenomenex Gemini C18 column (250 mm × 4.6 mm, 5 µm), controlled at 60°C. Separation was performed using a gradient mobile phase of acetonitrile and phosphoric acid (0.1%) at a 1.0 mL/min flow rate. Detection was executed at 284 nm for hesperidin and 254 nm for rutin, diosmin, and quercetin. Under the optimized chromatographic conditions, the method linearity was verified over the following concentration ranges: 6.2 – 794.0, 3.4 – 434.0, 3.3 – 420.0, and 3.2 – 414.0 µg/mL for rutin, diosmin, hesperidin, and quercetin, respectively. The method was validated for accuracy, linearity, and precision. The method exhibited high accuracy, with recoveries of 99.95±0.43, 100.08±0.42, 100.12±0.32, and 100.23±0.44% for rutin, diosmin, hesperidin, and quercetin, respectively. The relative standard deviation (RSD, %) of all four flavonoids did not exceed 1%. The method's robustness and simplicity make it well-suited for routine quality control of herbal products containing these flavonoids. The results showed that the method is a valuable tool for ensuring the quality consistency of dietary supplements containing rutin, diosmin, hesperidin, and quercetin, frequently used for human health benefits.

Keywords: Rutin, Hesperidin, Diosmin, Quercetin, Food supplement.

Introduction

In recent years, individuals have struggled to accept the fact that their quality of life is deteriorating, as evidenced by the rapid increase in the prevalence of diseases, especially cancer-related diseases and cardiovascular problems. The health crisis has progressively impacted the younger population, prompting them to seek medical intervention and rely on dietary supplements for health promotion.[1,2] Dietary additives, which contain a variety of flavonoids, play a pivotal role in complementing conventional drug therapies.[3]
Flavonoids, a group of secondary plant metabolites, are divided into five subgroups: anthocyanins, catechins, flavonols, flavones, and flavanones.[4,5] Flavonoids are commonly present in medicinal herbs, fruit juices, teas, and beverages.[6] They have become known for their beneficial properties, such as antioxidant,[7,8] anti-inflammatory, anti-carcinogenic, hypoglycaemic,[9] cholesterol lowering, and antihypertension effects,[6] they also help in the management of chronic venous insufficiency, and hemorrhoids.[10] A broad range of biological properties are responsible for the health benefits of flavonoids found in dietary supplements.[11]

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Numerous multi-ingredient supplements and nutraceuticals distributed globally contain vitamins and flavonoids, of which rutin, diosmin, hesperidin, and quercetin are the most common active components.[12] The rapid growth in the food industry, particularly in the development of modern dietary supplements, which are designed to meet a variety of human health needs, including health promotion, anti-aging, and disease prevention, has led to the proposition of legal, regulatory framework for the control of functional food products, and flavonoids-containing products are no exception. The control of functional foods is challenging due to the ambiguity between pharmaceuticals and foods.[10] In general, flavonoids are a safe group of compounds with little or no side effects; however, at very high doses, they may cause undesired or adverse effects, which may be due to the presence of impurities.[13] Consequently, it is imperative to implement quality control measures for this product category. Accordingly, many studies have been conducted to analyze flavonoid compounds from dietary supplements such as: single-component analysis (rutin,[14] hesperidin,[15] etc.), multi-component analysis (Rutin, diosmin, hesperidin and troxerutin,[10] diosmin, diosmetin, hesperidin, and hesperitin).[13]
Various analytical methods have been employed for flavonoid analysis, with the primary focus being on high-performance liquid chromatography (HPLC). Reverse phase-HPLC is a widely used method for the detection and identification of compounds, including quercetin and curcuminoid,[16] diosmin and hesperidin,[17] rutin, quercetin, and naringenin.[18] Till date, no method has been proposed for the simultaneous quantification of four flavonoids: rutin, diosmin, hesperidin, and quercetin in multi-flavonoid dietary supplements. For the first time, this study proposed an HPLC-PDA method for the simultaneous quantification of rutin, diosmin, hesperidin, and quercetin in dietary supplements. The method was successfully validated for the following validation parameters: specificity, linearity, limit of detection (LOD), and limit of quantification (LOQ) in compliance with the OAOC[19] and ICH guidelines.[20]

Materials and Methods

Dietary supplements

The dietary supplements (tablets and capsules) were sourced from Vietnam local markets. These supplements include NC1 - Royal Care Bye Tree from Vesta Pharma (10.7909° N, 106.6882° E), NC2 - Diosmin Chiba from Duc Hung Pharmaceuticals (10.7563° N, 106.7091° E), NC3 - Giap Ca Plus Tat Thanh from Tat Thanh Pharma (10.7700° N, 106.6884° E), NC4 - Viet A Diosmin Rutin Tablets from Viet A Pharmaceuticals (10.7600° N, 106.6992° E), NC5 - Tan Tri Vuong Nano Tablets from Goldpha (10.7601° N, 106.6992° E), and NC6 - Q Plus from BV Pharma (10.7812° N, 106.6671° E). A placebo, without analytes, was used as a control sample in this study.

Chemicals and reagents

Acetonitrile and methanol (for HPLC) were obtained from J.T. Baker Chemical Company (Switzerland). Phosphoric acid and dimethyl sulfoxide were obtained from Merck (Germany).

Equipment

The HPLC system was a Shimadzu UFLC (Japan) comprising the CBM-20Alite as the HPLC system controller, SIL-20AC HT thermostatted autosampler, CTO-20AC column oven, SPD-M20A photodiode array detector (PDA), and LC solution software version 1.14. Separations were conducted using a Gemini C18 column with a dimension of 250 mm x 4.6 mm and a particle size of 5 µm. The column was kept at a temperature of 60ºC, and the flow rate was at 1 mL/min. UV wavelengths were recorded at 284 nm for hesperidin and 254 nm for rutin, diosmin and quercetin. Effective separations and quantification were achieved within a 70-min run, utilizing a 10 µL injection volume. The autosampler temperature was fixed at 20ºC, and the elution was accomplished using a gradient technique, as detailed in Table 1.

Preparation of mobile phase solvents

Mobile phase A was prepared by dissolving 1 mL of phosphoric acid in 1 L of distilled water, and mobile phase B was acetonitrile. They were filtered through a 0.45 µm membrane and sonicated for 15 minutes to remove gas.

Table 1: Elution programme of the mobile phase

Time (min)	Solvent A **(%)**	Solvent B **(%)**
0.00	90	10
15.00	84	16
50.00	30	70
60.00	0	100
65.00	90	10
70.00	90	10

Reference standards

Standard substances were supplied by the Institute of Drug Quality Control in Ho Chi Minh City (Vietnam) and were used without further purification. Rutin, diosmin, hesperidin, and quercetin working standards' purities were 88.5% $C_{27}H_{30}O_{16}$ (as internal standard), 88.0% $C_{28}H_{34}O_{15}$ (as internal standard), 88.4% $C_{28}H_{32}O_{15}$ (as internal standard) and 95.8% $C_{15}H_{10}O_7$ (as internal standard), respectively.

Preparation of standard solutions

The standard substances were prepared in methanol to form homogeneous solutions, which were subsequently diluted to obtain working standard solutions at progressively lower dilutions. Diluted solutions of the four flavonoids were in the concentration ranges of 6.20 – 794.02 µg/mL for rutin, 3.39 – 434.04 µg/mL for diosmin, 3.28 – 420.02 µg/mL for hesperidin, and 3.23 – 413.95 µg/mL for quercetin. The calibration curves were prepared from the nine-point dilutions shown in Table 2.

Table 2: The concentration ranges of rutin, diosmin, hesperidin and quercetin in methanol

	Concentration (µg/mL)			
Solution	Rutin	Diosmin	Hesperidin	Quercetin
1	6.20	3.39	3.28	3.23
2	12.41	6.78	6.56	6.47
3	24.81	13.56	13.13	12.94
4	49.63	27.13	26.25	25.87
5	99.25	54.26	52.50	51.74
6	198.51	108.51	105.01	103.49
7	397.01	217.02	210.01	206.98
8	595.52	325.53	315.02	310.46
9	794.02	434.04	420.02	413.95

Preparation of sample solutions

Solid dietary supplements, comprising no fewer than 20 tablets or capsules, were precisely weighed before being finely powdered. The powder was then dissolved in methanol in an appropriate volumetric flask. The solutions were freshly prepared and kept in the dark just before the experiments. Sample solutions were prepared in the concentration ranges of 6.20 – 794.02 µg/mL for rutin, 3.39 – 434.04 µg/mL for diosmin, 3.28 – 420.02 µg/mL for hesperidin, and 3.23 – 413.95 µg/mL for quercetin including stepwise dilution with 50% dimethyl sulfoxide (v/v).[14,21-25] The solutions were sonicated for 5 minutes, followed by the addition of 25% methanol (v/v), sonicated again for another 5 minutes, and finally, the solutions were filled to the mark with methanol. The solutions were filtered through a 0.45 µm Polytetrafluoroethylene (PTFE) membrane.

Preparation of placebo solution

A 100 mg placebo devoid of rutin, hesperidin, diosmin, and quercetin was weighed and placed into a 100 mL volumetric flask. Subsequently, 50 mL of dimethyl sulfoxide was added and sonicated for 5 minutes. The volumetric flask was filled up to volume with methanol, and the solution was then filtered through a 0.45 µm PTFE membrane.

Method validation

Following the International Conference on Harmonization (ICH) guidelines, the analytical method was validated, explicitly following "Validation of Analytical Procedure: Text and Methodology." [19,20] The validation assessed critical parameters such as specificity, linearity, limit of detection (LOD), and limit of quantification (LOQ). Accuracy was evaluated at three different concentration levels for each flavonoid: 6.78, 54.25, and 325.51 µg/mL for diosmin; 6.57, 52.54, and 315.22 µg/mL for hesperidin; 6.27, 50.12, and 300.74 µg/mL for quercetin; and 12.57, 100.57, and 603.43 µg/mL for rutin. Precision (repeatability and reproducibility) was determined by analyzing samples on the same day and different days. At the same time, recovery was assessed by spiking placebo samples with known amounts of flavonoids and calculating the recovery percentage (%).

The HPLC system suitability was rigorously tested, including parameters such as retention time, peak area, theoretical plates, resolution, and tailing factor, based on six injections of the standard solution. Linearity was established by analyzing nine standard solutions at varying concentrations and creating calibration curves for each

flavonoid by plotting peak areas against concentrations. The LOD and LOQ values were determined using the signal-to-noise ratio, and precision was evaluated through relative standard deviation (RSD). Recovery tests involved spiking approximately 100 mg of placebo with known amounts of flavonoids. The percentage recovery was calculated using the formula (1):

$$\text{Recovery (\%)} = \frac{\text{Amount found}}{\text{Amount spiked}} \times 100 \quad (1)$$

Statistical analysis
The concentrations of each flavonoid in the test solutions were determined by comparing the peak areas from the test chromatograms with the regression lines from standard solutions. Analytical results were processed using Microsoft Excel 2016 software and expressed as mean ± standard deviation.

Results and Discussion

The present approach enables the concurrent determination of multiple flavonoids-rutin, hesperidin, diosmin, and quercetin-in solid dietary supplements. The study comprehensively evaluated and selected the optimal chromatographic conditions, including mobile phase composition, flow rate, and temperature for the simultaneous determination of rutin, hesperidin, diosmin, and quercetin. Optimal separation and quantification were achieved using a mobile phase mixture of acetonitrile and 0.1% phosphoric acid with a gradient programme, a column temperature of 60°C, and a 1.0 mL/min flow rate. The autosampler was set to 20°C.

Specificity
The chromatograms of blank, placebo, standard, sample, and QC solutions were injected into the HPLC system at the wavelength of 254 nm (rutin, diosmin and quercetin) and 284 nm (hesperidin). The chromatograms of standard, sample, and QC solutions based on the spectrums and retention time of the peaks were used to identify the four flavonoids. The retention times of rutin, hesperidin, diosmin, and quercetin were about 18 min, 23 min, 24 min and 27 min, respectively. The peak purity value of the sample solution was greater than the threshold value of 0,99. Besides, the chromatograms of blank and placebo did not appear as corresponding peaks. Thus, the method has th+-e specificity and selectivity for the analysis of the four flavonoids mentioned above. The results */are shown in Figure 1.

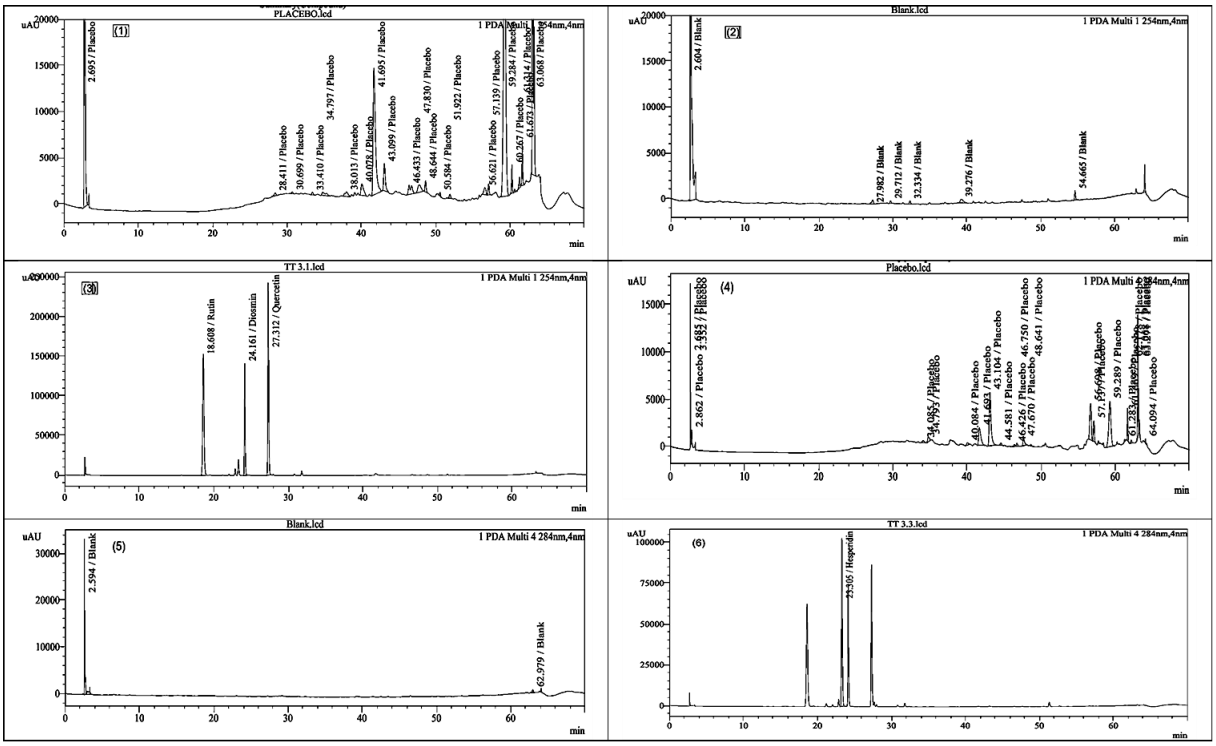

Figure 1: Chromatograms under optimal conditions: **(1)** Placebo (λ = 254 nm), **(2)** Blank (λ = 254 nm), **(3)** HPLC chromatogram of rutin, diosmin and quercetin (λ = 254 nm), **(4)** Placebo (λ = 284 nm), **(5)** Blank (λ = 284 nm), **(6)** HPLC chromatogram of hesperidin (λ = 284 nm)

System suitability
The method and the validation procedure showed the system suitability parameters. The values for the system suitability parameters including retention time, resolution, peak area, number of theoretical plates, and peak tailing factor, are presented in Table 3.

Linearity
Linearity was assessed by analyzing nine distinct solutions at varying concentrations for each flavonoid: 6.20 to 794.02 µg/mL for rutin, 3.39 to 434.04 µg/mL for diosmin, 3.28 to 420.02 µg/mL for hesperidin, and 3.23 to 413.95 µg/mL for quercetin. The signal was recorded at 284 nm for hesperidin and 254 nm for rutin, diosmin, and quercetin. The correlation coefficients (r^2) were more significant than 0.9996 for all flavonoids, indicating a robust linear correlation between concentration and peak area for rutin, diosmin, hesperidin, and quercetin. The results of the linearity verification are presented in Table 4 and Figure 2.

Limit of detection and limit of quantification
LOD and LOQ for the flavonoids were determined using visual assessments of signal-to-noise ratios and calculations based on the standard deviation and the slope of the calibration curves. To establish these limits, reference solutions were diluted with methanol to achieve the appropriate concentrations.

Table 3: System suitability of the developed method

Rutin

Solution	Statistical analysis	Retention time (min)	Area	Number of theoretical plates	Tailing factor
Standard (n = 6)	Average	18.59	962787	47269	1.12
	RSD %	0.02	0.15	0.31	0.17
Sample (n = 3)	Average	18.59	952491	46080	1.12
	RSD %	0.02	0.22	0.23	0.18

Diosmin

Solution	Statistical analysis	Retention time (min))	Area	Number of theoretical plates	Tailing factor
Standard	Average	24.16	447096	235790	1.28
(n = 6)	RSD %	0.01	0.20	0.34	0.61
Sample	Average	24.16	445434	233849	1.28
(n = 3)	RSD %	0.00	0.05	0.10	0.30

Hesperidin

Solution	Statistical analysis	Retention time (min)	Area	Number of theoretical plates	Tailing factor	Resolution between diosmin and hesperidin
Standard	Average	23.32	417940	150180	1.22	3.20
(n = 6)	RSD %	0.02	0.04	0.08	0.17	0.48
Sample	Average	23.30	416581	149655	1.21	3.19
(n = 3)	RSD %	0.01	0.10	0.15	0.21	0.51

Quercetin

Solution	Statistical analysis	Retention time (min)	Area	Number of theoretical plates	Tailing factor
Standard	Average	27.33	901624	217420	1.21
(n = 6)	RSD %	0.04	0.09	0.16	0.53
Sample	Average	27.32	890952	216361	1.22
(n = 3)	RSD %	0.04	0.10	0.16	0.13

The LOD was defined as the lowest concentration that produced a peak area three times higher than background noise, resulting in values of 0.04 µg/mL for rutin and 0.02 µg/mL for diosmin, hesperidin, and quercetin. The necessary LOQ to obtain a signal-to-noise ratio (S/N) of 10 was 0.12 µg/mL for rutin and 0.06 µg/mL for diosmin, hesperidin, and quercetin. The results are provided in Table 5, highlighting the sensitivity of the analytical method for each flavonoid.

Accuracy

The recovery rates of rutin, hesperidin, diosmin, and quercetin were recorded at three distinct concentration levels for each flavonoid to assess the analytical method accuracy. Placebo samples were spiked with known quantities of each flavonoid standard, and the experiments were run in triplicate under consistent conditions. Concentrations were deduced from calibration curves and peak areas on the chromatogram. Accuracy was estimated based on the percentage recovery of the added reference compounds. Average recovery ranged from 99.80 – 100.24%, 99.55 – 100.39%, 100.03 – 100.20%, and 99.72 – 100.10% for rutin, diosmin, hesperidin, and quercetin, respectively with RSD values between 0.07 – 0.44%. The detailed results are presented in Table 6.

Repeatability and reproducibility

The repeatability and reproducibility of the analytical method were conducted to compare the analytical results of seven samples from the same batch. These samples were subjected to two analyses utilizing two distinct HPLC chromatographic columns on two separate days. The results of the repeatability and reproducibility measurements for the flavonoids are shown in Table 7.

Table 4: The linearity between the concentration (Conc) of four flavonoids and the corresponding peak areas (Area)

N.o	Rutin		Diosmin		Hesperidin		Quercetin	
	Conc. (µg/mL)	Area	Conc. (µg/mL)	Area	Conc. (µg/mL)	Area	Conc. (µg/mL)	Area
1	6.20	107446	3.39	55408	3.28	49844	3.23	107756
2	12.41	214085	6.78	108525	6.56	98510	6.47	215216
3	24.81	439248	13.56	216493	13.13	187597	12.94	438893
4	49.63	860575	27.13	446254	26.25	397992	25.87	900230
5	99.25	1734702	54.26	886011	52.50	786505	51.74	1843432
6	198.51	3467866	108.51	1765618	105.01	1589030	103.49	3659360
7	397.01	6866963	217.02	3515157	210.01	3146315	206.98	7294286
8	595.52	10393092	325.53	5308153	315.02	4734771	310.46	11137913
9	794.02	13710178	434.04	7035052	420.02	6249524	413.95	15221123

Table 5: The LOD and LOQ of the developed method

	Rutin		Diosmin		Hesperidin		Quercetin	
	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ
Mean	0.04	0.12	0.02	0.06	0.02	0.06	0.02	0.06
RSD	1.52	1.61	1.97	1.62	1.97	1.54	1.89	1.79

LOD: Limit of Detection, LOQ: Limit of Quantification, RSD: Relative standard deviation.

Table 6: Accuracy of the analytical method

Rutin		Diosmin		Hesperidin		Quercetin	
Amount found (µg/mL)	Average recovery (%) %RSD (found)	Amount found (µg/mL)	Average recovery (%) %RSD (found)	Amount found (µg/mL)	Average recovery (%) %RSD (found)	Amount found (µg/mL)	Average recovery (%) %RSD (found)
12.57	100.24 (0.15)	6.78	99.55 (0.13)	6.57	100.20 (0.07)	6.27	99.75 (0.44)
100.57	99.92 (0.29)	54.25	100.39 (0.18)	52.54	100.12 (0.15)	50.12	100.10 (0.31)
603.43	99.80 (0.42)	325.51	100.33 (0.19)	315.22	100.03 (0.13)	300.74	99.72 (0.42)

Table 7: Precision **(r**epeatability and reproducibility of the analytical method

Compound	Measurement Type	Content (g/100 g)	RSD (%)	n (number of samples)	Acceptance Criteria for AOAC RSD (%)
Rutin	Repeatability	100.59	0.39	7	≤ 1.3
	Reproducibility	100.25	0.42	14	≤ 2.0
Diosmin	Repeatability	54.38	0.34	7	≤ 1.3
	Reproducibility	54.31	0.31	14	≤ 2.0
Hesperidin	Repeatability	52.61	0.27	7	≤ 1.3
	Reproducibility	52.78	0.28	14	≤ 2.0
Quercetin	Repeatability	50.35	0.53	7	≤ 1.3
	Reproducibility	50.31	0.64	14	≤ 2.0

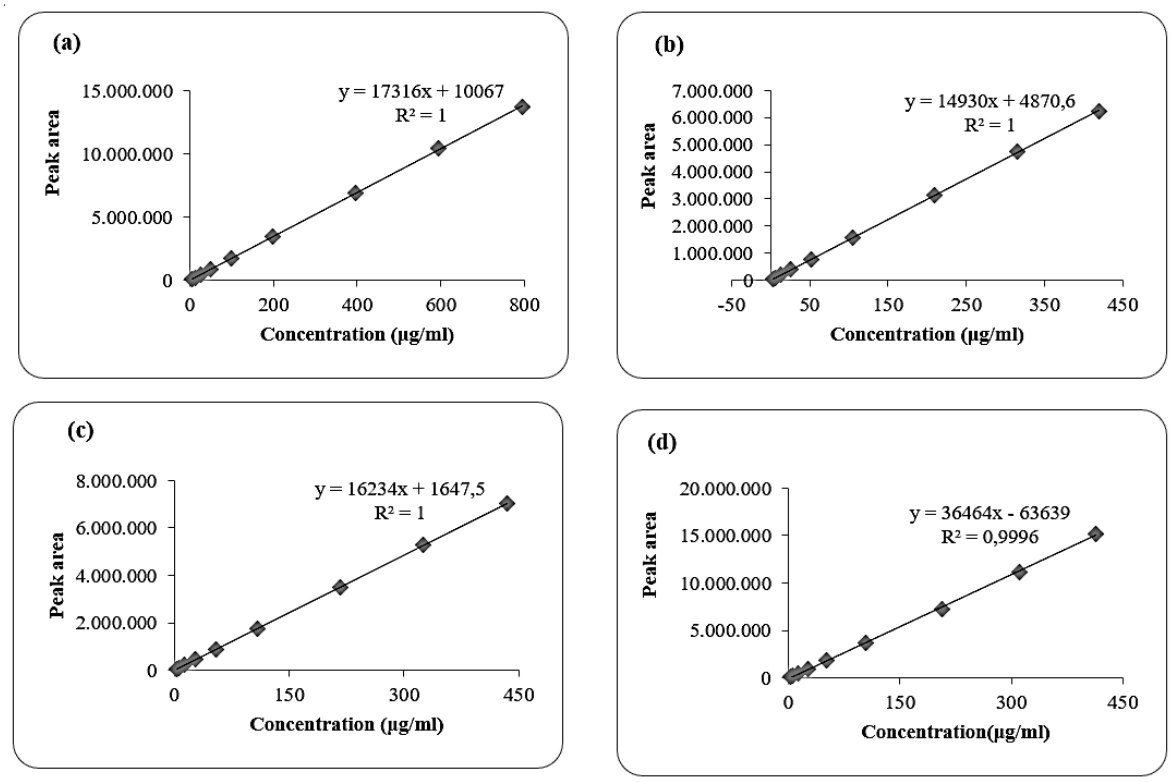

Figure 2: Linearity of: **(a)** rutin, **(b)** hesperidin, **(c)** Diosmin, and **(d)** quercetin

Analysis of variance (ANOVA) was performed to evaluate the results between the two analytical days. No significant differences were observed for the analytes, including rutin, diosmin, hesperidin and quercetin at 95% confidence interval. The intermediate precision and accuracy tests showed that the RSD of the flavonoids was not greater than 2.0%. Therefore, it can be concluded that the values were confirmed to meet the exact requirements of Annex K of the AOAC for the simultaneous evaluation of flavonoids in dietary supplements.

Table 8: Contents of flavonoids in dietary supplements

Sample	Rutin		Diosmin		Hesperidin		Quercetin	
	Label content	Found content	Label content	Found content	Label content	Found content	Label content	Found content
NC 1	30 mg	30.02 mg	150 mg	149.95 mg.	50 mg	49.99 mg		
NC 2			200 mg	199.55 mg				
NC 3	50 mg	49.92 mg						
NC 4	50 mg	50.08 mg	100 mg	99.69 mg				
NC 5	20 mg	19.99 mg	125 mg	124.81 mg				
NC 6							250 mg	250.10 mg

Contents of flavonoids in dietary supplements

Six dietary supplement products (tablets and capsules) with single or blended rutin, hesperidin, diosmin, and quercetin in varied concentrations were supplied by the local pharmacies. Table 8 displays the results of the quantification of the four flavonoids in the formulation.

All examined formulations exhibited a flavonoid content, with a recovery rate falling within the specified range of 90 – 110% compared to the labelled content. The RSD (%) values obtained when measuring the precision of three independent analyses were below 2%. The validation and analysis results demonstrated adherence of the dietary supplements to AOAC and ICH Q2 general standards through a liquid chromatography analysis procedure.

Conclusion

In the present investigation, an HPLC method was successfully validated and certified to conform with AOAC 2016 and ICH Q2 (R1) guidelines for the concurrent determination of rutin, diosmin, hesperidin, and quercetin in dietary supplements. The developed method is characterized by its simplicity, speed, sensitivity, accuracy and precision, rendering it suitable for flavonoid quantification. The study successfully demonstrated the method's applicability for confirming these four flavonoids in tablets and capsules. The HPLC analysis achieved baseline separation with excellent resolution for all flavonoids in an individual run, with strong concurrence with the stated values. Based on this validated method, future investigations will explore the simultaneous separation of different flavonoids in dietary supplements.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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