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HPLC METHOD FOR SIMULTANEOUS QUANTIFICATION OF URSOLIC ACID AND SCOPOLETIN IN NONI FRUIT (*MORINDA CITRIFOLIA* L.)

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Summary

Noni fruit (*Morinda citrifolia* L.) is used widely in traditional Vietnamese medicine and in other countries around the world. The Vietnamese Pharmacopoeia has a monograph on Noni fruit, but there is no specific quantitative criterion for the main chemical components to control the quality of this herbal material rigorously. This study developed an HPLC-PDA method for simultaneous quantification of ursolic acid and scopoletin, two main components of Noni fruit. The method was evaluated according to the guidelines of ICH (2007) and AOAC (2023), fulfilling criteria for system compatibility, specificity, linear range, repeatability, and intermediate accuracy. The method had been applied to quantify 6 samples of Noni fruit collected from various regions in Vietnam. The results showed that ursolic acid and scopoletin are the main chemical constituents of Noni fruit in Vietnam with a range of content (7.360 - 13.570 mg/g) and (0.225 - 1.360 mg/g), respectively.

Keywords: *Noni fruit; Morinda citrifolia; Ursolic acid; Scopoletin; HPLC.*

1. Background

Noni fruit (*Morinda citrifolia* L.) is a popular herbal remedy in Vietnam and many countries worldwide for managing hypertension, alleviating inflammation, assisting diabetes treatment, promoting digestion, and preventing cancer [1],[2]. The chemical constituents of Noni fruit include volatile components, triterpenes, coumarins, iridoids, flavonoids, anthranoids, etc. [2]. Although the Vietnamese Pharmacopoeia has a monograph on Noni fruit, there is no quantitative criterion of the main chemical in this herbal material [3]. Several studies have been conducted to improve the quality standards of Noni fruit, including the development of an HPLC quantification method for scopoletin (SPL) [4] and an HPLC method for simultaneous quantification of deacetylasperulosidic acid and rutin in Noni fruit samples in Vietnam [5]. Investigations into the chemical constituents of Noni fruit indicated that the important main compounds were scopoletin, which contributed to antioxidant and anti-inflammatory activities [2], and ursolic acid (UA), which is associated with the anti-cancer activity of Noni fruit [6]. To enhance the quality standards of Noni fruit, an HPLC method for simultaneous quantification of ursolic acid and scopoletin was developed and evaluated according to the guidelines of ICH (2005) [7] and AOAC International (2023) [8] and then applied to quantify some Noni fruit samples collected from various regions in Vietnam.

2. Material and methods

2.1 Material

The Noni fruit samples were collected when they turned green-yellow. The codes of samples are presented in Table 1:

Table 1: List of samples

Number	Code	Location	Time of collection
1	HCM-1	Ho Chi Minh City	4-2023
2	QT-2	Quang Tri province	1-2024
3	LD-3	Lam Dong province	3-2024
4	BT-4	Binh Thuan province	1-2024
5	BD-5	Binh Duong province	3-2024
6	LA-6	Long An province	3-2024

The samples were dried and ground to a powder capable of passing through a 0.22 mm sieve. Store in a dry place away from sunlight. The powder was assessed for humidity prior to quantification application.

The sample collected in HCM city was utilized for the investigation of extraction and chromatographic conditions as well as validation of the established method. The residual samples were used for the application of the quantitative method.

2.2. Chemicals, apparatus

Chemicals: Ursolic acid (Lot No. 0224, retest

date: 28th Feb 2027, content: 99.8%, calculated on an as-is basis) and scopoletin (Lot No. 0224, retest date: 28th Feb 2027, content: 100.0%, calculated on an as-is basis) were supplied by the Institute of Drug Quality Control, Ho Chi Minh City.

Solvents: Methanol, acetonitrile, ethyl acetate (Fisher, HPLC grade) ethanol, formic acid, trichloroacetic acid, and acetic acid (Prolabo).

Apparatus: Ultrasonic bath (Sonorex), infrared moisture balance (Ohaus), analytical balance (Sartorius), centrifuge (EBA 20 Hettich), pH meter (pH 7110-WTW), UPLC-PDA system (Waters ACQUITY Arc QDA), and chromatographic column Purospher ®STAR RP-18 endcapped (150 x 4.6 mm, 3 µm).

2.3. Method

2.3.1. The analytical procedure:

Sample preparation

- Stock standard solution (AU, SPL): approximately 1 mg/mL

- UA standard solution: Accurately take 2 mL of the stock solution in a 10 mL volumetric flask, add MeOH to the mark, and mix well. The resulting solution has a concentration of 200 µg/mL. Filter through a 0.22 µm membrane filter.

- SPL standard solution: Accurately take 0.15 ml of the stock solution in a 10 mL volumetric flask, add MeOH to the mark, and mix well. The resulting solution has a concentration of 15 µg/mL. Filter through a 0.22 µm membrane filter.

- Test sample: Take approximately 0.500 g of the powdered herbal sample into a centrifuge tube, add 8 mL of MeOH, vortex for 1 minute and sonicate for 30 minutes, centrifuge at 4000 rpm for 5 minutes, realize extracting 4 times, collect all supernatants, evaporate the solvent in 50 °C to around 8 mL of MeOH, transfer to a 10 mL volumetric flask, rinse thoroughly by MeOH and add MeOH to the mark, and filter through a 0.22 µm membrane filter before UHPLC analysis under the selected conditions.

- Blank sample: MeOH.

- Sample under high temperature conditions: Take approximately 0.500 g of the powdered herbal sample into a centrifuge tube and store it at 80°C for 8 hours. Then, continue the extraction procedure similar to that of the test sample.

- Sample exposed to light: Take approximately 0.500 g of the powdered herbal sample into a petri dish and expose it to 254 nm UV light for 8 hours. Then, continue the extraction procedure similar to that of the test sample.

- Oxidated sample: Take approximately 0.500 g of the powdered herbal sample into a centrifuge tube, add 1 mL of 30% H_2O_2, vortex for 15 minutes, then stand at room temperature for 8 hours. Afterward, continue the extraction procedure similar to that of the test sample.

Chromatographic condition: Stationary phase: Column Puropher ®STAR RP-18 endcapped (150 x 4.6 mm, 3µm). Column temperature: 35°C. Mobile phase: (A) acetonitrile and (B) 0.05% trichloroacetic acid. Elution program: 0 - 19 min: A (18%), 20 - 37 min: A (90%), 38 - 48 min: A (98%). Injection volume: 5 µL. Flow rate: 0.5 mL/min.

The percentage content of UA, SPL in 1g of Noni fruit sample is calculated using the formula:

$$X\ (mg) = \frac{S_{Sample}}{S_{Ref}} \times C_{Ref} \times P\% \times \frac{D_F}{1000} \times \frac{1000}{m_{sample} \times (1 - H)}$$

Where: S_{sample}: Peak area of the test sample (µV*sec); S_{Ref}: Peak area of the standard sample (µV*sec); C_{Ref}: Concentration of the standard sample (µg/mL); P%: Purity of the corresponding standard substance; D_F: Dilution factor of the test sample; m_{sample}: Weighed mass of the test sample (mg); H: Moisture content of the herbal powder sample.

2.3.2. Optimization of the sample preparation process

Sample preparation factors investigated included extraction solvent (acetonitrile, ethanol, ethyl acetate, methanol, methanol-water mixtures at various ratios), extraction method (ultrasonic, heat reflux, and magnetic stir), extraction time (20, 30, and 40 minutes), sample-to-solvent ratio (g/mL), and the time of extraction (1 - 4 times). The sample preparation method was selected to get the highest contents of ursolic acid and scopoletin.

2.3.3. Validation of method

Analytical method validation was performed in accordance with the guidelines of ICH (2005) [7] and AOAC International (2023) [8], including assessment of the specificity, system suitability, calibration curves, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy.

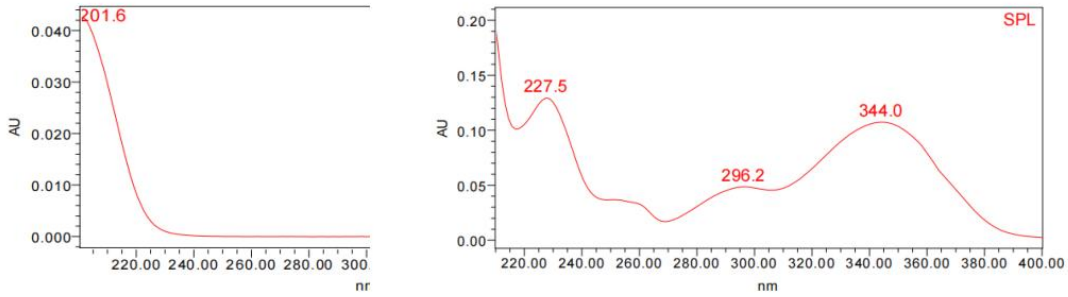
2.3.4. Application of the validated method for sample analysis

The proposed method was applied to six samples of Noni fruit collected in different cultivation regions (Table 1).

3. Results and discussion

3.1. Optimization of extraction conditions

After investigating the mobile phase and elution program, the chromatographic conditions were established as 2.3.1. The analytical time: 48 min (Fig. 2).



UV spectrum of ursolic acid

UV spectrum of scopoletin

Fig 1. UV spectrum of ursolic acid and scopoletin

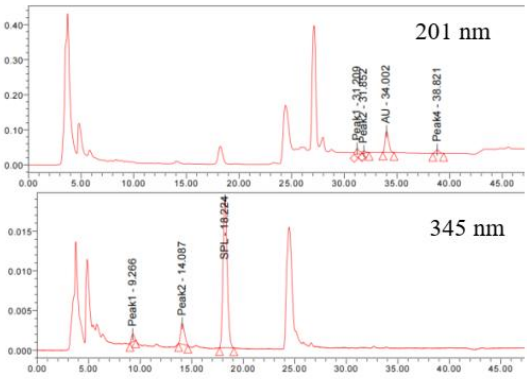


Fig. 2. Chromatograms for analysis of ursolic acid and scopoletin in Noni fruit

Extraction factors were investigated including extraction solvent (methanol, ethanol, and acetonitrile), extraction method (ultrasonic, reflux, and magnetic stirrer), extraction time (20, 30, and 40 minutes), and times of extraction (1-4 times). Each factor was changed sequentially and the parameter for the largest peak area value was selected. The chosen parameter was fixed, and then the next factor was changed.

From the investigation results (Table 2) the sample preparation procedure was established as 2.3.1. (the analytical procedure of the test sample).

Table 2. The results of extraction conditions optimization

Extraction factors		Peak area/sample weight (µV*s/mg)	
		Ursolic acid	Scopoletin
Extraction solvent	Methanol	2624.9	1052.3
	Ethanol	2029.5	576.5
	Acetonitrile	2643.3	1085.2
Extraction method	Ultrasonic	2640.2	1052.3
	Reflux	1804.0	845.9
	Magnetic stirrer	2587.1	378.9
Extraction time (min)	20	2554.3	909.4
	30	2640.3	1052.3
	40	2778.2	1054.0
Time of extraction (n)	1	2640.2	1052.3
	2	5773.3	1059.8
	3	8865.5	1117.3
	4	9533.5	1128.3

3.2. Method validation

3.2.1 System suitability

Analyze the mixture of ursolic acid and scopoletin by HPLC method six times, assessing the retention time, peak area, peak asymmetry, and theoretical plates. The findings showed that RSD % of retention time and peak area for ursolic acid and scopoletin was less than 0.22 (Table 3). These indicated that the method satisfied the system suitability criteria.

Table 3. The results of system suitability testing (*n = 6)*

Injection	Retention time (min)		Peak area (µV*s)		As		N	
	UA	SPL	UA	SPL	UA	SPL	UA	SPL
1	34.176	18.017	2265083	328216	0.94	1.15	69551	9300
2	34.173	18.017	2225430	326801	0.95	1.17	68609	9051
3	34.173	18.018	2231945	326398	0.92	1.18	68921	9074
4	34.148	17.923	2228272	327378	0.93	1.17	68549	9136
5	34.148	17.988	2262001	327203	0.93	1.17	68729	8971
6	34.155	17.958	2218922	328871	0.93	1.16	70112	9232
Mean (n=6)	**34.162**	**17.987**	**2238608**	**328543**				
RSD (n=6) %	**0.04**	**0.22**	**0.88**	**0.14**				

3.2.2. Selectivity

The chromatograms of the reference sample, test sample, and spiked sample indicated that the retention time and UV spectrum of the ursolic acid (UA) and scopoletin (SPL) peaks were identical (Fig. 3). The blank sample exhibited no signals from these peaks. The height and peak area of these peaks in the spiked sample significantly exceeded those in the test sample. The samples which were subjected to harsh conditions (temperature, light, oxidation), demonstrated that any disintegrating contaminants did not compromise the purity of the analytical peak (Rs ≥ 1.5, peak asymmetry 0.8 ≤ As ≤1.8, the purity angle PA < the purity threshold PT of the analytical peak). The process was determined to fulfill the specificity of the method (Table 4).

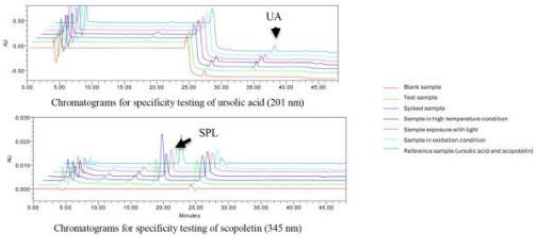


Fig 3. Chromatograms for the testing of specificity

Table 4. The results of specificity testing

Compounds	Samples	t_R (min)	PA	PT	Spic (µV.s)	Rs	As
UA	Blank	-	-	-	-	-	-
	UA & SPL mixing standard solution	34.155	1.328	1.974	2218922		0.93
	Sample	34.788	5.722	7.852	2273544	-	1.25
	Spike sample	33.943	4.607	4.643	4217528	-	1.29
	Sample in high temperature condition	33.920	6.229	8.652	2144769	-	1.28
	Sample exposed to light	33.933	5.079	5.174	2284161	-	1.29
	Oxidated sample	33.914	5.676	6.846	2057855	-	1.21
SPL	Blank	-	-	-	-	-	-
	UA & SPL mixing standard solution	18.052	0.762	1.804	287962	5.8	1.16
	Sample	18.214	0.153	0.532	600796	5.4	1.15
	Spike sample	18.064	0.630	1.820	264187	5.9	1.15
	Sample exposed to high temperature	18.056	0.746	2.173	213905	5.8	1.16
	Sample exposed to light	18.050	0.602	1.864	255047	6.0	1.18
	Oxidated sample	18.018	0.408	1.182	326398	-	1.18

3.2.3. Calibration curve

The calibration curve was constructed by plotting the peak areas versus the concentrations of the analyte. The results showed satisfactory linearity in the concentration ranges of 28-900 µg/mL for ursolic acid and 1.875-60 µg/mL for scopoletin. The regression equation with R^2 was more than 0.998 (Table 5). The limit of quantification (LOQ) and limit of detection (LOD) were established according to the results of experiments (Table 6). The sample was diluted by methanol until the signal/noise (s/n) value (calculated by Empower 4.0 software) reached around 3 and 10 for LOD and LOQ, respectively.

Table 5. The result of the calibration curve of ursolic acid (UA) and scopoletin (SPL)

Substance	Parameter	Result	Conclusion
UA	The regression equation	y = 8761.9x + 137.9	y = 8761.9x
	Range (µg/mL)	28.131 - 450.1	
	R < 0.998	1.0000	Comply
	Significance F < F0,05 (95%)	0,00 < 6,61	Compatibility
	P - value a < 0,05	0,00 < 0,05	The coefficient a has statistical significance
	P - value b < 0,05	0,98 > 0,05	The coefficient b has no statistical significance
	Bias %	1.81 - 3.23	
SPL	The regression equation	y = 27519x + 6835.9	y = 27519x
	Range (µg/mL)	1.625 - 26	
	R < 0.998	0,9991	Comply
	Significance F < F0,05 (95%)	0,00 < 6,61	compatibility
	P - value a < 0,05	0,00 < 0,05	The coefficient a has statistical significance
	P - value b < 0,05	0,54 > 0,05	The coefficient b has no statistical significance
	Bias %	0.18 - 3.43	

Table 6. The result of LOD and LOQ of ursolic acid and scopoletin in Noni fruit

	Compound	t_R (min)	Spic (µV.s)	s/n (USP)	Concentration (µg/mL)
LOD	UA	34.119	9371	2.41	0.87
	SPL	18.369	3411	3.22	0.23
LOQ	UA	34.162	17668	10.63	1.75
	SPL	18.344	5177	10.02	0.47

3.2.4. Precisions

The intra-and inter-day precisions were summarized in Table 7. The results showed that the method had good precision.

Table 7. Intra and inter-day precision of HPLC analysis for ursolic acid and scopoletin

Inter-day precision			Intra-day precision		
Samples	Concentration (mg/g)		Samples	Concentration (mg/g)	
	UA	SPL		UA	SPL
1	9.92	0.57	7	9.94	0.57
2	9.93	0.57	8	10.84	0.57
3	9.87	0.57	9	9.82	0.57
4	9.72	0.56	10	9.88	0.57
5	9.61	0.57	11	10.06	0.57
6	9.84	0.58	12	10.21	0.56
Mean (n=6)	9.82	0.57	**Mean** (n=6)	10.12	0.57
RSD (n=6) %	1.28	0.92	RSD (n=6) %	3.73	0.91
			TB (n=12)	9.87	0.57
			RSD (n=12) %	2.74	0.90

3.2.4. Accuracy

The accuracy was evaluated by adding a known amount of reference sample at three different levels (low - medium - high levels compared to 9.82 mg/g and 0.57 mg/g of UA and SPL, respectively) to the known sample. Take approximately 0.500 g of the powdered herbal sample into a centrifuge tube, add 2.50 - 3.50 - 4.50 ml Stock standard solution of UA (1030 µg/ml) and 0.20 - 0.25 - 0.30 mL Stock standard solution of SPL (970 µg/mL) and fill up to 8 ml of MeOH, continuing extraction as the analytical procedure. Each value was analyzed with three independent samples. The results in Table 8 showed that recovery rates of ursolic acid and scopoletin were in the range of 96.42 - 107.89%.

Table 8. Recovery studies of ursolic acid and scopoletin by the proposed procedure.

Samples	Ursolic acid			Scopoletin		
	Amount spiked (mg)	Amount found (mg)	Recovery (%)	Amount spiked (mg)	Amount found (mg)	Recovery (%)
1	2.570	2.579	100.35	0.194	0.188	96.81
2	2.570	2.537	98.70	0.194	0.193	99.68
3	2.570	2.553	99.34	0.194	0.193	99.37
4	3.598	3.507	97.49	0.243	0.261	107.42
5	3.598	3.469	96.42	0.243	0.259	106.94
6	3.598	3.542	98.46	0.243	0.262	107.89
7	4.626	4.533	97.99	0.291	0.295	101.51
8	4.626	4.534	98.01	0.291	0.296	101.74
9	4.626	4.570	98.79	0.291	0.295	101.34
Mean			98.40			102.52

3.2.5. Application of the validated method for sample analysis

The proposed method was applied to six samples of Noni fruit collected in different cultivation regions. The quantitative results are presented in Table 9:

Table 9. Content of ursolic acid and scopoletin in Noni fruit samples collected in Vietnam.

Samples	Mean ± SD		
	Humidity content (n=3, %)	UA (n=3, mg/g)	SPL (n=3, mg/g)
HCM-1	8.56 ± 0.615	9.815 ± 0.125	0.571 ± 0.006
QT-2	9.46 ± 0.229	12.215 ± 0.885	1.360 ± 0.091
BT-3	12.39 ± 0.501	13.575 ± 0.015	0.225 ± 0.005
LD-4	14.12 ± 0.587	7.360 ± 0.070	0.545 ± 0.005
BD-5	9.10 ± 0.694	7.950 ± 0.020	0.651 ± 0.001
LA-6	10.57 ± 0.872	10.375 ± 0.005	0.643 ± 0.005

Quantitative results indicated that the content of ursolic acid and scopoletin in Noni fruit samples varied, with ursolic acid exhibiting a high content (7.360 - 13.575 mg/g) while scopoletin had a lower content (0.225 - 1.360 mg/g) and had a wide variation. The difference in the content of these two substances in different Noni samples indicated that soil and climate significantly affect the chemical composition of Noni fruit.

4. Discussions

Noni fruit is widely utilized in Vietnam and has numerous products on the market; however, the monograph on Noni fruit in the Vietnamese Pharmacopoeia V does not have specific quantitative criteria for the main components. The chemical constituents of Noni fruit are diverse; therefore, numerous studies have quantified coumarins, flavonoids, iridoids, and fatty acids using HPLC-MS methods for quality control [9],[10]. The quantification methods with MS detectors may encounter several challenges due to the high cost of equipment and factors influencing sensitivity and precision. In Vietnam, there was a report on developing quantification procedures for the main component, scopoletin [4], as well as another study quantifying both deacetylasperulosidic acid and rutin compounds in Noni fruit [5]. In this study, the simultaneous quantification method for two biologically active compounds, ursolic acid and scopoletin [2],[8] was developed and validated, which will be suitable for use in the quality control of this herbal material. When selecting markers to test and control the quality of herbal medicines, the basic principle is to choose substances with proven therapeutic or pharmacological effects (ursolic acid and scopoletin), high specificity (scopoletin), or high content (ursolic acid) in the Noni fruit. Quality control of Noni fruit based on ursolic acid and scopoletin can provide a more accurate assessment compared to other publications that use only scopoletin [4], or simultaneously both deacetylasperulosidic acid and rutin [5]. It can be recognized that scopoletin (which has a relatively low content), deacetylasperulosidic acid (an iridoid that is easily degraded during extraction and storage), and rutin (which is common in herbal medicines but has a low content in Noni fruit) are not the most ideal markers when used alone.

Both ursolic acid and scopoletin are non-polar compounds that dissolve well in organic solvents such as MeOH and ACN. Survey results show that ACN's extraction efficiency is 0.07% better than MeOH. However, MeOH also minimizes the risk of introducing non-polar impurities into the chromatography column, extending column life,

and is more biodegradable than ACN, resulting in less environmental pollution. The ultrasonic extraction method generates high-frequency mechanical waves that break down plant cell structures and release substances, making extraction more efficient than reflux and magnetic stirring methods. Due to the extraction volume per time being 8 mL, depending on the laboratory equipment, four times extraction in 30 minutes ensures complete extraction of ursolic acid and scopoletin.

Ursolic acid, being a triterpene structure, exhibited poor UV absorption; although it had a high content, this compound displayed significantly higher LOD and LOQ values compared to scopoletin. However, the established quantification procedure satisfied the criteria for system compatibility, specificity, linearity, accuracy, and repeatability in accordance with ICH (2007) and AOAC (2023) guidelines, which demonstrated the method could be applied to analyzing ursolic acid and scopoletin levels in Noni fruit. Published literature indicates that the scopoletin concentration in Noni fruit from Costa Rica is 0.0132 mg/g [11], whereas in Mexico, it varies from 0.024 to 0.065 mg/g [12]. Prior studies on Noni fruit in Vietnam established that the concentration of scopoletin was 0.265 mg/g [4], consistent with the research results. The finding showed that scopoletin concentration in Noni fruit in Vietnam is markedly elevated. Furthermore, there is no available literature reporting the concentration of ursolic acid in Noni fruit, even though this compound was responsible for numerous biological effects of Noni. The analytical data in this study indicated that ursolic acid is present in significant quantities in Vietnamese Noni fruit (7.360 - 13.575 mg/g).

5. Conclusion

This study has established a method for the simultaneous quantification of two main constituents in Noni fruit: ursolic acid and scopoletin. The quantification procedure has been validated in accordance with international guidelines, meeting the criteria of specificity, linearity, accuracy, and precision. The approach has been utilized to quantify various Noni fruit samples collected in different provinces in Vietnam. The obtained results provide useful information for the quality control of Noni fruit in Vietnam.

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