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3B QUANG TRUNG - HOAN KIEM - HANOI - VIETNAM
TEL: (+84-24) 38247058 FAX: (+84-24) 39348740
Email: tapchiduoclieu@nimm.org.vn; tapchiduoclieu@gmail.com

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METHOD DEVELOPMENT FOR SIMULTANEOUS QUANTIFICATION OF NARINGIN AND MELITIDINE IN YOUNG POMELO FRUITS BY HPLC-PDA

Nguyen Thi Anh Nguyet[1,*], Nguyen Kieu Quynh Trang[1], Nguyen Tan Phat[1], Nguyen Hoai Sang[1], Nguyen Le Hoang Son[2], Hoang Ngoc Cuong[3], Huynh Loi[2], Tran Thi Van Anh[1], Le Van Huan[2]

[1]*University of Medicine and Pharmacy at Ho Chi Minh City, Ho Chi Minh City, Vietnam;*

[2]*Institute of Pharmaceutical Education and Research, Binh Duong University, Vietnam;*

[3]*Faculty of Food Technology, Binh Duong University, Vietnam.*

*Corresponding author: ntanguyet@ump.edu.vn

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Summary

In this study, an HPLC method was developed for quantitative analysis of melitidine and naringin, which were found in young pomelo fruits. This procedure was performed on the Waters Acquity UPLC system, using a GL Sciences InertSustain C18 chromatographic column (4.6 × 250 mm; 5 µm). Mobile phase (acetonitrile - acetic acid 0.1%), flow rate of 1 mL/min, and injection volume of 10 µL, UV detection at 283 nm. The developed method met all validation requirements of ICH guidelines as follows: System suitability, specificity, and linear range of naringin (50 - 1000 µg/mL) and melitidine (25 - 500 µg/mL) with R^2 = 0.9999. The limits of detection (LODs) and limits of quantification (LOQs) of naringin and melitidine at 0.17 µg/mL and 0.56 µg/mL, respectively; the intraday precision (RSD = 0.45 - 1.68%) and interday precision (RSD = 0.61-1.92%); the accuracy from 95.08% to 102.94% (RSD = 0.55 - 1.82%). This method was applied for the analysis of naringin and melitidine in some samples of young pomelo fruit collected in Binh Duong.

Keywords: *Citrus maxima; Young pomelo; Naringin; Melitidin: HPLC.*

1. Introduction

Pomelo (*Citrus maxima* (Burm.) Merr., Rutaceae) is widely cultivated in Southeast Asia. Vietnam has the second-largest cultivation area for *Citrus* species globally, with a planted area of 87,921 hectares and a production volume of 1,142,581 tons [1]. Binh Duong province is one of the major contributors to national pomelo production. To enhance the yield of mature pomelos, farmers often thin out young fruits, resulting in a large number of underutilized by-products that may cause environmental issues. Research indicates that pomelo albedo provides important health benefits, notably its lipid-lowering properties, which have been clinically demonstrated for obesity treatment [2]. This is mostly due to naringin, which inhibits HMG-CoA reductase, hence reducing cholesterol and mevalonate levels [3]. Young pomelos exhibit greater amounts of naringin than mature ones [4] and display antioxidant, hepatoprotective, and anticancer properties [5],[6]. Thus, young pomelos yield a beneficial by-product with considerable potential for health supplements.

The existing genetic variety among pomelo types results in variations in flavonoid composition. The majority of pomelo cultivars predominantly contain naringin, although the occurrence of other flavonoids depends on the species or cultivar. Certain pomelo types in Thailand and China contain minimal or no hesperidin and neohesperidin [7]. Some common pomelo types cultivated in Binh Duong exhibited no presence of hesperidin or neohesperidin when preliminarily analyzed by High Performance Liquid Chromatography (HPLC). Nonetheless, they exhibited a significant concentration of melitidin, a flavonoid recognized for its vital biological properties, including hepatoprotection, cholesterol reduction, and antioxidant activity [8]. The study aimed to develop a method for quantifying naringin and melitidine (**Fig. 1**) concentrations in young pomelo cultivated in Binh Duong.

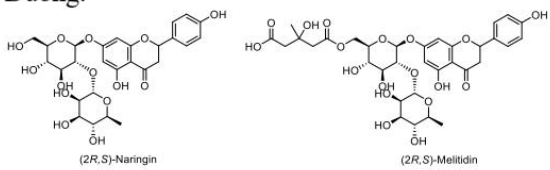


Fig. 1. Structure of naringin and melitidine

2. Materials and methods

2.1. Plant materials

The immature pomelo fruits were obtained in November 2022, Tan Uyen District, Binh Duong Province at an age of 1 - 3 months, measuring approximately 4 - 12 cm in diameter, from the pomelo tree (*Citrus grandis/Citrus maxima*). The specimen was authenticated by Dr. Le Van Ut

from the Institute of Pharmaceutical Education and Research at Binh Duong University. The voucher specimen (NP-23.004) was deposited at the Department of Biochemistry, Institute of Pharmaceutical Training and Research - Binh Duong University.

Young pomelo fruits from various cultivars - Duong la cam (DLC), Duong da lang (DDL), Da xanh (DX), and Buoi oi (O) - were collected (**Fig. 2**) from Dau Tieng, Tan Uyen, and Phu Giao districts at 1, 2, and 3 months ages of, with diameters varying from 4 to 12 cm. At every age, six pomelo trees were selected, and six fruits were collected from each tree. Immature pomelo fruits were sliced, dried at 50°C, pulverized, and sieved through two meshes (0.125 - 0.3 mm) to produce the herbal powder. Pomelo samples were collected at different growth stages, comprising 6-10 fruits from each of the six trees within the same study area. The samples' moisture content was determined using an infrared moisture analyzer (105°C, 10 min). All samples were stored at 4°C and deposited at the Department of Pharmacognosy and Traditional Pharmacy, University of Medicine and Pharmacy in Ho Chi Minh City.

Fig. 2. Some immature pomelo samples collected from Binh Duong

2.2. Chemicals

The reference substances (2*R,S*)-naringin (98.55%; HPLC-PDA) and (2*R,S*)-melitidine (98.07%; HPLC-PDA) were isolated and established as reference standards. The solvents used for HPLC analysis include methanol (Fisher, USA), acetic acid (Merck, Germany), acetonitrile (Merck, Germany), and distilled water.

2.3. Methods

2.3.1. HPLC method development and optimization

Optimization of HPLC chromatographic conditions: The detection wavelengths, mobile phase, gradient elution program, and flow rate were investigated in this study to meet chromatographic parameters for naringin and melitidine peaks, including %RSD (relative standard deviation) of retention time (Rt) and peak area (S), capacity factor (k'), asymmetry factor (As), resolution (Rs), and number of theoretical plates (N).

Optimization of sample preparation: To optimize extraction conditions for two major flavonoids in young pomelo for high yield, 200 mg of herbal powder was examined using reflux with 50% ethanol as the initial solvent. Methanol (100%, 80%, 70%, 50%, 20%, and 10%), ethanol (96%, 70%, 50%, 20%, and 10%), and water were studied in subsequent tests. We examined extraction times of 15, 30, 45, 60, 90, and 120 minutes. The number of extraction cycles was also assessed using three sequences (20, 10, and 10 mL); (30, 5, and 5 mL); and (35, 5, and 5 mL). All survey samples were conducted using a uniform sample mass of 200 mg of herbal powder. To establish the optimal extraction conditions, compare the peak areas of naringin and melitidine on HPLC-PDA chromatograms for all test sample solutions at 40 mg/mL.

2.3.1. The procedure for HPLC quantitative analysis

Reference solutions: A stock solution (1000 µg/mL) was diluted in 70% ethanol to obtain six working standard solutions with a concentration range of 50 - 1000 µg/mL and 25 - 500 µg/mL for naringin and melitidin, respectively. Serial dilutions were employed to construct calibration curves for each component.

Sample preparation: Accurately weigh about 200.0 mg of herbal powder and initially extract by reflux with 35 mL of 70% ethanol for 90 minutes at 95°C. Allow to cool and filter. Add 15 ml of 70% ethanol, reflux it a second time for 30 minutes, then filter and rinse the residue. Add 70% ethanol to get a total volume of 50 mL in a volumetric flask. Shake well and filter through a 0.45 µm membrane filter.

HPLC chromatographic conditions: The Acquity UPLC system with PDA detector and Empower3 software, using a GL Sciences InertSustain C_{18} column (250 × 4.6 mm; 5 µm). The HPLC chromatographic conditions were optimized using the mobile phase consisting of 0.1% acetic acid (pump A) and acetonitrile (pump B). The gradient elution program was as follows: 17 - 20% B (3 min); 20% B (5 min); 20 - 25% B (4 min); 25 - 30% B (3 min); 30% B (5 min); 30 - 95% B (1 min); 95% B (4 min); 95 - 17% B (1 min); 17% B (4 min), the flow rate was 1.0 mL/min, the total run time was 30 min, the inject volume was 10 µL and the UV detection wavelengths were set at 283 nm for the 2 flavonoids.

The determination of naringin and melitidine content: The percentage content (%, dry weight)

of each flavonoid in the sample was calculated using the following formula:

$$X\%\ (w/w) = \frac{St}{1000 \times a} \times \frac{50}{m\ (1-h)} \times p \times 100$$

In this equation, *X%* (yield, %); *St* (peak area of the analytical component in the sample solution); *a* (the regression coefficient of the linear calibration curve); *p* (the purity of reference standard, %); m (sample weight, mg); and *h* (loss on drying, %).

2.4. Method validation

The UPLC method was validated according to the International Conference on Harmonization (ICH) guidelines [9] for the validation of analytical procedures. The validation included assessing linearity, limit of detection (LOD), limit of quantification (LOQ), system suitability, intra-day and inter-day precision, and accuracy.

3. Results and Discussions

3.1. Investigation of the HPLC chromatographic conditions

Naringin and melitidine show similar UV spectra with three maximum absorption wavelengths at 213-214 nm, 282.8 nm, and 327-329 nm. Absorbance at 282.8 nm was best and had less interference. Therefore, a detection wavelength of 283 nm was optimal for the quantification of naringin and melitidin.

The solvent system initially employed acetonitrile and 0.1% acetic acid as the mobile phase. The gradient elution analysis provided better separation of naringin and melitidine peaks compared to isocratic methods. Peak resolution improved with ACN ratio modification.

Among the evaluated flow rates (0.5, 0.7, 1.0, and 1.2 mL/min), 1.0 mL/min exhibited the best results (**Fig. 3**), providing well-defined and symmetrical peaks and meeting the system suitability parameters (see **Table 2**). The test sample and reference mixture HPLC chromatograms are shown in **Fig. 4**.

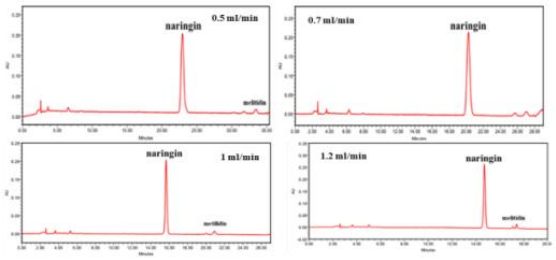


Fig. 3. Survey results of the flow rate for the HPLC chromatographic program

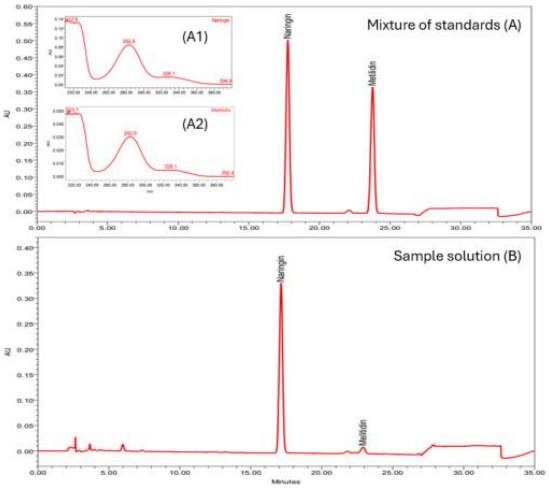


Fig. 4. HPLC chromatography of the mixture of standards (A) and the sample solution (B) with UV spectra of naringin (A1) and melitidine (A2) at 283 nm

3.2. Investigation of the sample extraction procedure

The investigation results are shown in **Fig.5** and **Table 1**, which found 96% EtOH was the least effective as an extractive solvent, while 70% EtOH and 80% MeOH demonstrated greater efficiency for extraction purposes. Naringin and melitidine can be extracted using both 70% EtOH and MeOH; however, 70% EtOH was selected due to its safety and environmental advantages. Elevated temperatures produced increased peak areas, with 95°C identified as the optimal optimum. For extraction, 90 min reflux time resulted in the highest chemical concentration. Over 88% of the chemicals were removed in the first cycle, and up to 97% after the second cycle. From all the solvent quantities that were tested, the first cycle reacted with 35 mL and the second with 15 mL, giving the maximum yield for these two cycles.

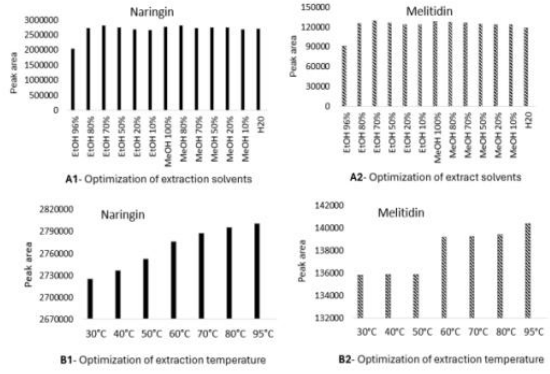


Fig. 5. The investigation results for sample preparation (solvents-A1, A2; temperature-B1, B2; and time C1, C2)

A1, A2 *(200.0 mg of sample, extracted at 80°C for 30 minutes with 50 mL of survey solvent);*
B1, B2 *(200.0 mg of sample, extracted at survey temperature for 30 minutes with 50 mL of 70% EtOH)*
C1, C2 *(200.0 mg of sample, extracted at 95°C for survey time with 50 mL of 70% EtOH).*

Table 1. Results of the investigation on the number of extractions and the extraction solvent ratio

Extraction	**Extraction volume** (Step 1, step 2, step 3) (20, 10, 10 mL)		**Extraction volume** (Step 1, step 2, step 3) (30, 5, 5 mL)		**Extraction volume** (Step 1, step 2, step 3) (35, 5, 5 mL)	
	Naringin	Metilidin	Naringin	Metilidin	Naringin	Metilidin
Step1 (S*)	15060316	818129	10668923	565104	8190783	434059
Step2 (S)	1519073	38946	657968	330651	433769	23759
Step3 (S)	423646	21613	264319	11161	172635	7707
Step1 (H%**)	88.57	93.11	92.04	92.74	93.11	93.24
Step2 (H%)	8.93	4.43	5.68	5.43	4.93	5.10
Step3 (H%)	2.49	2.46	2.28	1.83	1.96	1.66
Step1+Step2 (H%)	**97.51**	**97.54**	**97.72**	**98.17**	**98.04**	**98.34**

*Peak area (S); ** Extraction efficiency (H%).

3.3. Validation method

System suitability: Six consecutive injections of the mixture standard solution (naringin and melitidine in 70% EtOH) and the test sample solution (40 mg/mL in 70% EtOH) showed an RSD% below 2% for peak area and retention time. Other chromatographic parameters met analytical requirements according to ICH guidelines [9], confirming system suitability (**Table 2**).

Specificity: was confirmed by analysis of four samples. No target peaks were visible in the blank, while naringin (17.6 min) and melitidine (23.6 min) exhibited similar UV spectra and retention times in the test, standard, and spiked samples. Spiking increased peak height and area (**Fig. 6**).

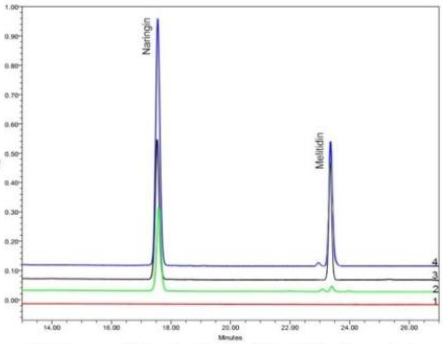
Linearity, LODs, and LOQs: The method displayed a wide linearity for naringin (50 - 1000 µg/mL, R^2 = 0.9999) and melitidine (25-500 µg/mL, R^2=0.9999). LODs and LOQs were evaluated on the reference solution (prepared in 70% EtOH) and sample solution (70% EtOH extract). The diluted test solutions were analyzed by using an HPLC method as described above. The LOD values were 0.03 µg/mL (naringin) and 0.05 µg/mL (melitidin) with an S/N ratio of 2-3. The LOQ values were calculated as LOQ = 3.3 × LOD [9], and the values were 0.1 µg/mL (for naringin) and 0.16 µg/mL (for melitidin) (**Table 3**).

Accuracy and Precision: For the precision study, intra-day RSD ranged from 0.44% to 1.68% for six samples processed on the same day, while inter-day RSD ranged from 0.61% to 1.92% for 12 samples processed on two days. The method showed accurate recovery rates of 102.03% to 103.97% for naringin (0.88% to 1.23%) and 95.08% to 102.94% for melitidine (0.55% to 1.82%). Naringin and melitidine levels in the test samples were determined according to a previously described analytical method. For the recovery study, standards were spiked into the matrix of herbs at three concentration levels (80%, 100%, and 120% of the quantification target level). Each spiking level was processed in triplicate. The spiked samples were subjected to extraction and subsequent analysis using the optimized chromatographic conditions. The recovery rate (Y) was then calculated using the following equation: Recovery (%) = 100 × (amount found - original amount)/amount spiked. As seen in **Tables 4** and **5**, the method used to measure naringin and melitidine in young pomelo is reliable and consistent.

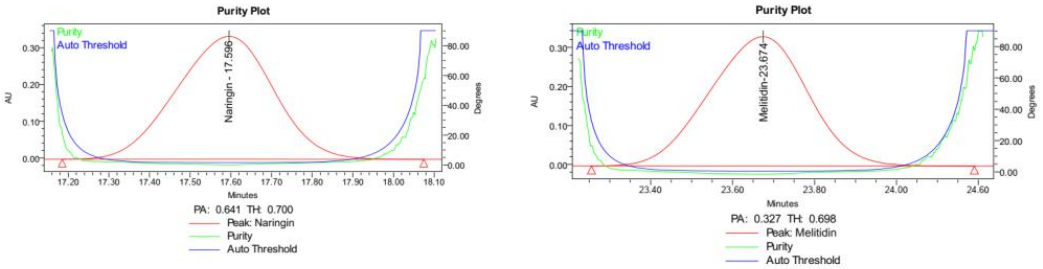
Table 2. System suitability of the naringin and melitidine quantification method according to ICH [9]

Compounds	Solution	t_R (min) (RSD %)	S (µV × s) (RSD %)	Rs	k'	T	N
Naringin	Sample	17.2 (0.67)	5582024 (0.10)	9.86	16.4	0.96	23504
Melitidin		23.0 (0.63)	238172 (1.84)	2.24	22.2	0.98	34577
Naringin	Standard	17.7 (0.37)	4284285 (0.19)	-	16.5	0.96	24716
Melitidin		27.7 (0.16)	3324345 (0.43)	2.36	22.6	0.97	38324
Requirement		RSD ≤ 2	RSD ≤ 2	Rs > 1.5	k' > 1.0	0.5 ≤ T ≤ 1.5	≥ 2000

Rs (resolution), t_R (retention time), S (peak area), k' (capacity factor), T (USP tailing), N (USP Plate count).



(A) - HPLC chromatogram at 283 nm of blank - 70% EtOH (1), the mixture of standard solution (3), young pomelo dry fruit extract (2), and standard-spiked sample (4)



(B)- Purity Plot of naringin and melitidin
PA (purity angle), PT (purity threshold). The purity angle (PA) is less than the purity threshold (PT) indicating that the peak is pure.

Fig. 6. The results of the specificity assessment

Table 3. Linearity, LODs, and LOQs of naringin and melitidin

Compounds	Regression equation[a]	R^2	Linear range (µg/mL)	Reference		Sample	
				LOD (µg/mL)	LOQ (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
Naringin	ŷ = 17030x + 33505	0.9999	50 - 1000	0.03	0.10	0.16	0.53
Melitidin	ŷ = 13211x - 1353.5	0.9999	25 - 500	0.05	0.16	0.17	0.56

[a]: The regression equation is compatible (F > F0.05), the coefficient *b* is not statistically significant, LOD (Limit of Detection), LOQ (Limit of Quantification).

Table 4. Results of the precision assessment of the HPLC method

Compounds	**Intra-day precision** (n=6)		**Inter-day precision** (n=12)	
	$\overline{X}$ ± SD	RSD (%)	$\overline{X}$ ± SD	RSD (%)
Naringin	8.99±0.04	0.44	8.97±0.06	0.61
Melitidin	0.47±0.01	1.68	0.47±0.01	1.92

Table 5. Results of the accuracy assessment of the HPLC method

Compounds	Spiking levels	Recovery (% mean)	RSD (%)
Naringin	80%	102.88	0.88
	100%	103.97	1.22
	120%	102.03	1.23
Melitidin	80%	95.08	1.82
	100%	98.28	0.55
	120%	102.94	0.63

3.3. Application of the quantification method

As seen in **Table 6**, the naringin content varied significantly among the young pomelo varieties. No melitidine signals were detected in Da Xanh (DX) and Oi (O), but Duong La Cam (DLC) had the greatest contents of naringin (9.0%) and melitidine (0.47%). Naringin content was the lowest in Oi pomelo (1.56%). The levels of flavanones were in the order of naringin (DLC > DDL > DX > O) and melitidine (DLC > DDL).

In terms of naringin and melitidine contents in young pomelo at different ages (1-, 2-, and 3-month-old), the results showed that the young pomelo of 1 month of age contained the highest levels (21.08% and 0.8%, respectively) (**Table 6** and **Fig. 7**). This suggests that the naringin and melitidine concentration is at peak levels at 1 month and slowly loses gradually during fruit maturation.

Table 6. The % yield (mean ± SD) of naringin and melitidine in young pomelo samples

Sample Name	Age (month)	Fruit Diameter (cm)	Harvested	Naringin (%)	Melitidine (%)
DLC	2	6-8	11/2022	**8.99 ± 0.04**	**0.47 ± 0.01**
DDL	2 months	6-8	11/2022	8.55 ± 0.011	0.32 ± 0.002
DX	2 months	6-8 cm	11/2022	5.99 ± 0.018	n.d
O	2 months	6-8 cm	11/2022	1.56 ± 0.013	n.d
DLC (1M0123)	1 month	2-4 cm	01/2023	21.19 ± 0.109	1.59 ± 0.009
DLC (2M0123)	2 months	6-8 cm	01/2023	10.34 ± 0.020	0.83 ± 0.002
DLC (3M0123)	3 months	10-12 cm	01/2023	4.89 ± 0.006	0.49 ± 0.003
DLC (1M0623)	1 month	2-4 cm	06/2023	21.08 ± 0.002	0.8 ± 0.004
DLC (2M0623)	2 months	6-8 cm	06/2023	9.33 ± 0.026	0.46 ± 0.005
DLC (3M0623)	3 months	10-12 cm	06/2023	5.16 ± 0.003	0.29 ± 0.003

DLC (Duong la cam pomelo); DDL (Duong da lang pomelo); DX (Da xanh pomelo); O (Oi pomelo).
n.d: not detected.

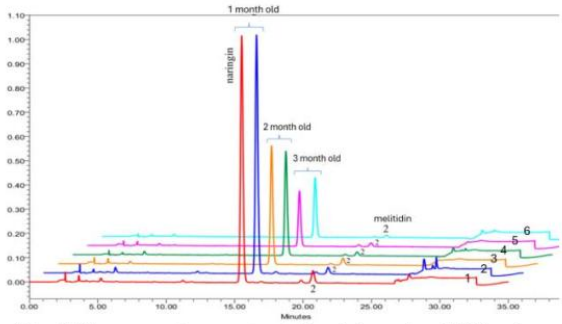


Fig. 7. The percentage content of narigin and melitidine in young pomelo varieties during the growth stages

1,2 - Young pomelo samples of the DLC cultivars, one month old, collected in January and June 2023. Sample codes: DLC (1M0123) and DLC (1M0623).

3,4 - Young pomelo samples of the DLC pomelo cultivars, two months old, collected in January and June 2023. Sample codes: DLC (2M0123) and DLC (2M0623).

5,6 - Young pomelo samples of the DLC pomelo cultivars, three months old, collected in January and June 2023. Sample codes: DLC (3M0123) and DLC (3M0623).

4. Discussion

Naringin, hesperidin, neohesperidin, and melitidine are flavonoids found in pomelo. Naringin is the major component, which represents 10.76% of Vietnamese young pomelo [10],[11]. In our study, the naringin concentration varied from 1.56% to 10.3% in four pomelo varieties, with a high level of 21.19% in 1-month-old young pomelo. The naringin content in young pomelo cultivated in Guangxi, China, was reported to be 26.5-28.3% in the pomelo peel [12]. The naringin levels in mature pomelo dramatically dropped, ranging from 0.2% to 0.9% in 35 varieties in China. Mature pomelo varieties in Thailand displayed high naringin levels, varying from 0.4% to 4.1% [7]. Most of the pomelo varieties from China and Thailand did not contain hesperidin and neohesperidin, which were only found in particular varieties [7]. The hesperidin content in Vietnamese young pomelo was very

low, at approximately 0.02% [10],[11]. The low concentration required the spiking of a reference standard into the sample for the simultaneous quantification hesperidin in immature pomelo by HPLC-DAD, ensuring precision and reproducibility [11]. Melitidine lacks documented data regarding its concentration in young pomelo; however, it has been found in mature pomelo from China at small concentrations, ranging from 0.005% to 0.06% [13]. In our investigation, melitidine content varied from 0.29% to 1.59% in the immature pomelo of DLC and DDL varieties, whereas it was undetected in pomelo of O variety and present only at trace levels in DX variety.

Numerous studies have shown that naringin possesses beneficial biological activities, including antioxidant, anti-inflammatory, and lipid-lowering effects. Conversely, hesperidin and neohesperidin are present at minimal levels in Vietnamese young pomelo, making their quantification challenging. Melitidin, although previously undocumented in immature pomelo, was clearly detected in the chromatograms of our investigation, with quantities ranging from 0.29% to 1.59%, especially in the DLC and DDL varieties. Moreover, melitidine with a structure similar to naringin and potential benefits in cholesterol reduction and cardiovascular protection, melitidine complements naringin in providing a comprehensive assessment of flavonoid quality in young pomelo. Therefore, simultaneous quantification of naringin and melitidine is essential for improving quality control standards in young pomelo.

5. Conclusions

This study successfully established a simultaneous determination of naringin and melitidine in the young pomelo fruit. The quantification assay was optimized for extraction and chromatographic conditions. The method fully met all the validation requirements for quantification procedures in accordance with ICH guidelines including chromatographic parameters, system suitability, specificity, linearity, intra-day precision, intermediate precision, and accuracy. Thus, this method can be used for the standardization of raw materials or young pomelo extracts. The method was then applied to analyze the contents of naringin and melitidine in young pomelo samples from Binh Duong, showing that their levels decrease as the fruit matures.

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