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Quality Evaluation of *Fallopia multiflora* in Vietnam Based on HPLC-FLD and Chemometrics

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Abstract

In this study, a simple and sensitive HPLC method with fluorescence detector (FLD) was developed and validated for quantitation of three stilbenes (2,3,5,4'-tetrahydroxystilben-2-O-β-D-glucoside (1), resveratrol (2), piceid (3)) and two anthraquinones (emodin (4), physcion (5)) in *Radix fallopiae multiflorae*. The separation was carried out on column Ascentis C18 (250 mm × 4.6 mm; 5 µm). All calibration curves showed good linearity (R^2>0.999) within test ranges. The overall recovery was 95.0-106.0%. RSD for intra- and inter-day of 5 analytes were less than 4%. This proposed method has high sensitivity with the LODs for the determinations of 5 analytes (1-5) were 0.3 µg/mL, 0.4 µg/mL, 0.4 µg/mL, 4.9 µg/mL, 3.42 µg/mL, respectively. The validated method was successfully applied for quantification of 5 compounds in samples of *Radix fallopiae multiflorae* from different locations of Vietnam. Chemometrics such as principal component analysis (PCA) and clustering analysis (CA) were used to evaluate homogeneity of *Radix fallopiae multiflorae* in Vietnam, which suggested that their quality homogeneity was good.

Keywords: *Radix fallopiae multiflorae;* Stilbene; Anthraquinone; RP-HPLC-FLD; Chemometric

Introduction

Radix Fallopiae multiflorae (RFM) is a common medicinal plant in China (Figure 1). It has been also widely used as Vietnamese traditional medicine for treatment of depression, anemia, hair-loss and constipation. RFM contains anthraquinones (emodin, physcion, chrysophanol, citreorosein, chrysophanol-8-O-β-D-glucopyranoside, physcion-8-O-β-D-glucopyranoside, emodin-8-O-β-D-glucopyranoside, emodin-1,6-dimethylether, questin, questinol, 2-acetylemodin, 2-methoxy-6-acetyl-7-methyljuglone, emodin-8-O-(6'-O-malonyl)-glucoside) [1-3]; stilbene glucosides (2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucopyranoside, 2,3,5,4'-tetrahydroxystilbene-2,3-O-β-D-glucopyranoside [4]) and flavonoids (tricin [2], quercetin-3-O-galactoside, quercetin-3-O-arabinoside [5]), as well as gallic acid, catechin [6], torachrysone-8-O-β-D-glucopyranoside [4], N-transferuloyl tyramine, N-transferuloyl-3-methyldopamine [2] and 1,3-dihydroxy-6,7-dimethylxanthone-1-O-β-D-glucopyranoside [4]. Anthraquinones and stilbenes are considered to be the major constituents with pharmacological effects.

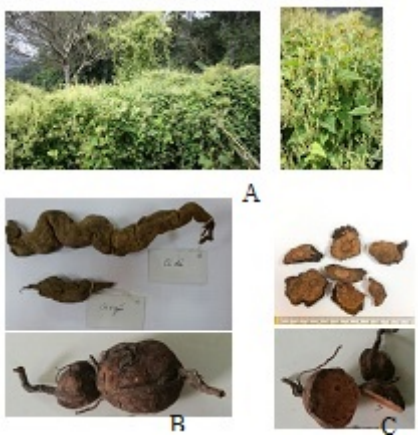

Figure 1: Image of *Fallopia multiflora* plant (A) and *Radix fallopiae multiflorae* (B,C).

In this study, a simple, rapid, accurate, specific, precise and high-sensitive RP-HPLC with fluorescence detection (RP-HPLC-FLD) method was developed and validated for simultaneous qualitative and quantitative analysis of 5 compounds (1-5) and combined anthraquinone (calculated as the total amount of emodin and physcion) in RFM sample. Based on chemical quantitative results in 48 RFM samples collected from different locations of Vietnam, multivariate analysis has been applied for classification of samples [5-7].

Experimental

Materials and chemicals

Forty-eight RFM samples (RFM 1-48) were collected from different parts of Vietnam and were authenticated by Department of Medicinal Material Resources, NIMM (Vietnam).

The chemical structures of analytes are shown in Figure 2. All primary standards were purchased from Sigma-Aldrich. All solvents for HPLC were purchased from Merck. Distilled water was produced by a Milli-Q purification system (Millipore, USA) [8,9].

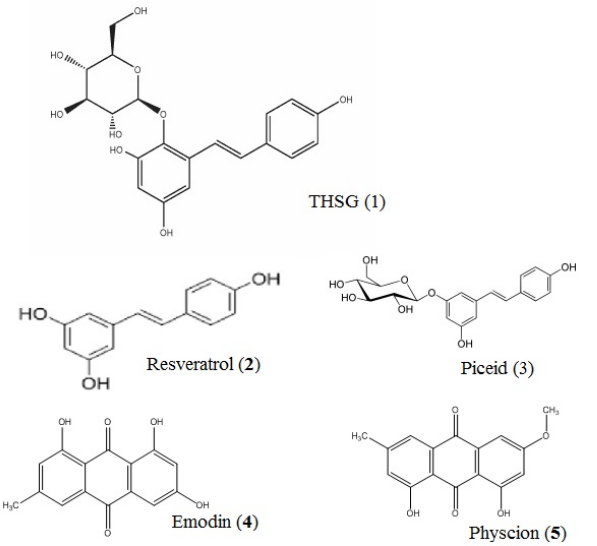

Figure 2: Chemical structure of 5 analytes (1-5).

Preparation of analytical samples

Sample solution A (for quantification of free anthraquinone): weigh accurately 1 g of the powder (through No.4 sieve) to a stoppered conical flask, accurately add 50 mL of methanol and weigh. Heat under reflux for 1 hour, cool and weigh again, replenish the loss of the solvent with methanol and mix well, filter, use the successive filtrate as the test solution A1 (for determination of free anthraquinones, calculated as the total amount of emodin and physcion) (FAQ) [10].

Measure accurately another 25 mL of the successive filtrate to a stoppered conical flask, evaporate to dryness on a water bath, add accurately 20 mL of 8% solution of hydrochloric acid, ultrasonicate (power, 100 W, frequency, 40 kHz) for 5 minutes, add 20 mL of chloroform, heat under reflux for 1 hour and cool immediately, then transfer to a separating funnel, wash the container with a small quantity of chloroform and combine the washings to the same separating funnel, separate the chloroform solution and extract the acidic solution again by shaking with three 15 mL quantities of chloroform, combine the chloroform solutions and evaporate to dryness. Dissolve the residue in methanol and transfer to a 10 mL volumetric flask, dilute with methanol to volume, mix well, filter and use the successive filtrate as the test solution A2 (for determination of total anthraquinones, calculated as the total amount of emodin and physcion) (TAQ) [10].

Content of total combined anthraquinones=content of TAQ-content of FAQ [10].

Sample solution B (for quantification of stilbene): Weigh accurately 0.5 g of the powder to a stoppered conical flask, accurately add 50 mL of ethanol 50% and weigh. Heat under reflux for 1 hour, cool and weigh again, replenish the loss of the solvent with ethanol 50% and mix well, filter, use the successive filtrate as the test solution B.

HPLC analysis

The method was performed on a Shimadzu (Kyoto, Japan) HPLC system equipped with a LC-20AD pump, DGU-20As degasser, SIL-20A HT autosampler, CTO-10AS VP column oven, and RF-20AXs fluorescence detector. Data acquisition and integration were performed using LC Solution software. Chromatographic separation was carried out on an Ascentis C18 column (250 mm × 4.6 mm; 5 µm).

Condition A (for quantification of anthraquinone): A reverse phase HPLC assay was carried out using an isocratic elution with a flow rate of 1 mL/min, a column temperature of 25°C, a mobile phase of acetonitrile and 0.5% (v/v) formic acid (pH=3) (70/30, v/v). The injection volume was 10 µl of each solution. The fluorescence detector was set at excitation wavelength of 435 nm and emission wavelength of 515 nm [7].

Condition B (for quantification of stilbene): A reverse phase HPLC assay was carried out using an isocratic elution with a flow rate of 0.5 mL/min, a column temperature of 25°C, a mobile phase of acetonitrile (A) and 0.5% (v/v) formic acid (pH=3) (B). The gradient was as follow: 0-22 min: 16% A; 22-45 min: 16-34% A; 45-50 min: 34-95% A. The injection volume was 10 µl of each solution. The fluorescence detector was set at excitation wavelength of 315 nm and emission wavelength of 395 nm [9].

Method validation

Calibration curves, LOD, LOQ: Stock solutions containing reference compounds were prepared and diluted to appropriate concentrations for the construction of calibration curves. At least 6 levels of concentrations of the solution were analyzed, and then the calibration curves were constructed by plotting the peak areas versus the concentrations of each analyte [11].

The method detection limit (LOD) and the method quantitation limit (LOQ) were estimated by diluting standard solutions until signal-to-noise ratios of 3 and 10, respectively.

Repeatability, accuracy and robustness: Intra- and inter-day variations were chosen to determine the precision of the developed method. For intra-day variability test, the samples were analyzed for six replicates within one day, while for inter-day variability test, the sample were examined in duplicates for consecutive two days. Variations were expressed by the relative standard deviations (RSD) for intra and inter-day.

The accuracy of the method was established by performing recovery experiment by using standard addition method at three different levels.

The robustness of the method is a measure of method's capacity to remain unaffected by small deliberate variation in method parameters during normal usage. The flow rate was set up at 1.1 mL/min (condition A) and 0.6 mL/min (condition B). From the observations, it was concluded that the method was robust as the deliberate changes made in the method did not affect the results.

Multivariate data analysis: Clustering analysis (CA) and principal component analysis (PCA) were performed by Minitab 14 for windows, which comprise a number of "procedures"-graphical, statistical, reporting, processing and tabulating procedures-that enable simple and rapid data evaluation. A method named as within groups linkage was applied, and Pearson correlation which is a pattern similarity measure, was selected as measurement for clustering analysis.

Results and Discussion

HPLC conditions

Different mobile phases were tested for separation of 5 analytes from other components of RFM.

To separate of emodin and physcion: a good separation was achieved by using acetonitrile and 0.5% (v/v) formic acid (pH=3) (70/30, v/v). The flow rate was 1 mL/min and the fluorescence detection had an excitation wavelength of 435 nm and emission wavelength of 515 nm (Figure 3).

To separate of THSG, resveratrol and piceid: a good separation was achieved by using acetonitrile and 0.5% (v/v) formic acid (pH=3). The gradient was as follow: 0-22 min: 16% A; 22-45 min: 16-34% A; 45-50 min: 34-95% A. The flow rate was 1 mL/min and the fluorescence detection had an excitation wavelength of 315 nm and emission wavelength of 395 nm (Figure 3).

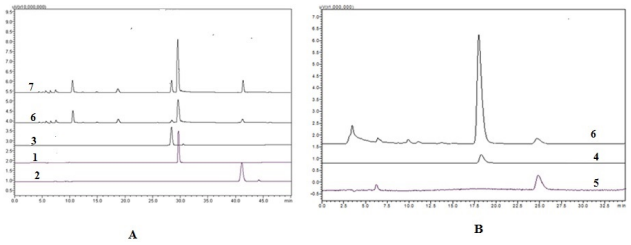

Figure 3: HPLC-FLD chromatograms analysis of three stilbenes (B) and two anthraquinones (A) in RFM sample (1-THSG; 2-resveratrol; 3-piceid; 4-emodin; 5-physcion; 6,7-RFM samples).

Method validation

The linearity, regression, and linear ranges of 5 analytes were determined using the developed HPLC-FLD method. The data indicated a good relationship between the investigated compounds concentrations and their peak areas within the concentration ranges of analytes (R^2>0.999) (Table 1).

The LODs of 5 analytes were 0.3, 0.4, 0.4, 4.88 and 3.42 μg/mL, respectively (Table 1).

The overall intra- and inter-day variations (RSD) of 5 analytes were less than 4.0%, respectively (Table 1).

The analytes recoveries were between 95.0% and 100.8% (Table 1).

Parameter	THSG (1)	Resveratrol (2)	Piceid (3)	Emodin (4)	Physcion (5)
Retention time, t_R	29.44	41.78	28.36	18.82	24.74
Resolution, R	2.27	1.67	2.57	3.52	3.17
Tailing factor, T_f	1.28	1.07	0.99	1.34	1.37
Theoretical plate, N	80794	53218	17872	6072	6503
Linear range (μg/mL)	1.5-60	1.0-40	1.0- 40	3.9-62.5	1.36-43.75
Correlation coefficient	0.9996	0.9995	0.9998	0.9995	0.9995
LOD	0.3 μg/g	0.4 μg/g	0.4 μg/g	4.88 μg/g	3.42 μg/g
LOQ	0.99 μg/g	1.32 μg/g	1.32 μg/g	16.1 μg/g	11.28 μg/g
Repeatability (%RSD)	0.62	1.68	2.43	0.77	2.75
Reproducibility Precision (%RSD_R)	0.89	2.12	2.98	1.63	3.00
Recovery (%)	96.67-101.67	95.00-108.33	95.45-106.36	98.64-102.27	97.73-104.09

Table 1: Method validation data for quantitation of 5 analytes (1-5).

As the flow rate changed, the resolutions, tailing factors and theoretical plates did not change much. The modifications did not affect the system suitability criteria. However, the slight variation in the retention time was observed, but did not affect the results.

The results showed that the developed HPLC method was sensitive, precise and accurate for quantitative determination of 5 investigated compounds in RFM sample.

Quantification of 5 compounds in RFM samples

The proposed method was applied to quantitatively analyze THSG, resveratrol, piceid, emodin, physcion and combined anthraquinone (calculated as the total amount of emodin and physcion) in RFM samples. The contents of 5 investigated compounds (1-5) and combined anthraquinone in 48 RFM samples were summarized in

Table 2. The results showed that their contents in the samples from different locations were greatly variable.

Sample code	Origins	Years old	THSG (g/100 g)	Resveratrol (mg/g)	Piceid (mg/g)	Emodin (mg/g)	Physcion (mg/g)	Combined anthraquinone (g/100 g)
RFM1	Van Giang, Hung Yen (12/2017)	2	2.88	-	0.011	0.50	0.16	0.12
RFM2	Van Giang, Hung Yen (12/2017)	2	2.71	-	0.015	0.42	0.12	0.10
RFM 3	Van Giang, Hung Yen (12/2017)	2	2.65	-	0.012	0.44	0.12	0.11
RFM 4	Khoai Chau, Hung Yen (11/2017)	2	2.43	-	0.009	0.37	0.11	0.13
RFM5	Khoai Chau, Hung Yen (11/2017)	2	2.68	-	0.014	0.41	0.12	0.14
RFM6	Khoai Chau, Hung Yen (11/2017)	2	2.37	-	0.01	0.35	0.13	0.12
RFM7	Thuong Tin, Hanoi (11/2017)	3	2.21	-	0.010	0.39	0.10	0.16
RFM8	Thuong Tin, Hanoi (11/2017)	3	2.18	-	0.009	0.30	0.11	0.13
RFM9	Thuong Tin, Hanoi (11/2016)	2	1.95	-	0.007	0.25	0.09	0.13
RFM10	Thuong Tin, Hanoi (11/2016)	2	2.04	-	0.009	0.26	0.07	0.12
RFM11	Thuong Tin, Hanoi (11/2015)	1	1.17	-	0.006	0.11	0.05	0.08
RFM12	Thuong Tin, Hanoi (11/2015)	1	1.25	-	0.006	0.08	0.05	0.07
RFM13	Dong Anh, Hanoi (12/2017)	3	2.67	-	0.014	0.40	0.13	0.24
RFM14	Dong Anh, Hanoi (12/2017)	3	2.52	-	0.017	0.33	0.15	0.20
RFM15	Dong Anh, Hanoi (11/2016)	2	2.17	-	0.014	0.31	0.11	0.13
RFM16	Dong Anh, Hanoi (11/2016)	2	2.02	-	0.011	0.27	0.09	0.13
RFM17	Dong Anh, Hanoi (11/2015)	1	1.31	-	0.006	0.20	0.07	0.07
RFM18	Dong Anh, Hanoi (11/2015)	1	1.16	-	0.004	0.16	0.07	0.08
RFM19	Quang Ninh (6/2018)	2	1.98	-	-	0.21	0.16	0.14
RFM20	Quang Ninh (6/2018)	2	1.59	-	-	0.18	0.11	0.11
RFM21	Quang Ninh (6/2018)	2	2.03	-	-	0.17	0.20	0.12

RFM22	Quang Ninh (6/2018)	2	1.75	-	-	0.19	0.18	0.11
RFM23	Quang Ninh (6/2018)	2	1.49	-	-	0.21	0.18	0.13
RFM24	Quang Ninh (6/2018)	2	2.00	-	-	0.25	0.15	0.14
RFM25	Ha Giang (5/2018)	-	4.10	-	0.016	0.51	0.21	0.10
RFM26	Ha Giang (5/2018)	-	4.42	-	0.021	0.29	0.20	0.08
RFM27	Ha Giang (5/2018)	-	4.29	-	0.025	0.63	0.33	0.13
RFM28	Ha Giang (12/2016)	-	4.05	-	0.020	0.53	0.32	0.11
RFM29	Ha Giang (12/2016)	-	3.99	-	0.019	0.50	0.25	0.11
RFM30	Ha Giang (12/2016)	-	4.14	-	0.024	0.44	0.34	0.09
RFM31	Thuan Chau, Son La (11/2017)	-	3.09	0.01	-	-	-	-
RFM32	Thuan Chau, Son La (11/2017)	-	3.73	0.02	-	-	-	-
RFM33	Thuan Chau, Son La (11/2017)	-	3.39	0.02	-	-	-	-
RFM34	Moc Chau, Son La (11/2016)	-	3.27	-	-	-	-	-
RFM35	Moc Chau, Son La (11/2016)	-	3.34	-	-	-	-	-
RFM36	Moc Chau, Son La (11/2016)	-	3.55	-	-	-	-	-
RFM37	Sin Ho, Lai Chau (8/2017)	-	1.92	0.035	0.068	-	-	-
RFM38	Sin Ho, Lai Chau (8/2017)	-	1.66	0.036	0.058	-	-	-
RFM39	Sin Ho, Lai Chau (8/2017)	-	2.19	0.026	0.061	-	-	-
RFM40	Sin Ho, Lai Chau (8/2017)	-	3.41	0.029	0.077	-	-	-
RFM41	Sin Ho, Lai Chau (8/2017)	-	3.18	0.031	0.065	-	-	-
RFM42	Sin Ho, Lai Chau (8/2017)	-	2.73	0.028	0.059	-	-	-
RFM43	Hải Dương (5/2018)	2	2.53	-	-	0.38	0.13	0.11
RFM44	Hải Dương (5/2018)	2	2.27	-	-	0.23	0.12	0.10
RFM45	Hải Dương (5/2018)	2	2.18	-	-	0.24	0.11	0.13

RFM46	Hải Dương (5/2018)	2	2.91	-	-	0.31	0.23	0.12
RFM47	Hải Dương (5/2018)	2	2.06	-	-	0.36	0.14	0.15
RFM48	Hải Dương (5/2018)	2	2.22	-	-	0.24	0.09	0.10

Table 2: The contents of 5 analytes (1-5) in RFM samples (n=3).

THSG presented in all tested samples with varying contents ranging from 1% to 4%. However, the contents of THSG in the samples from Ha Giang were above 3% level-these were much higher than other samples.

The contents of four components (2-5) and combined anthraquinone varies considerably in different samples. To determine if these components were specific to the small sample group in the large sample group, Principal components analysis (PCA) and Clustering analysis (CA) were applied to classify and analyze the major components for these samples.

Chemometric analysis

Principal component analysis (PCA): The analysis of chemical data by the technique of PCA permitted to group chemically the samples in four groups, in a way to express and demonstrate their similarities and differences. It was observed with the first main component (PC1), the second main one (PC2) and the third main one (PC3), it was possible to describe 93.3% of the data, being 56.3% of the total variance described by PC1 and 80.1% of the total variance describe by PC1 and PC2 (Figure 4) [8].

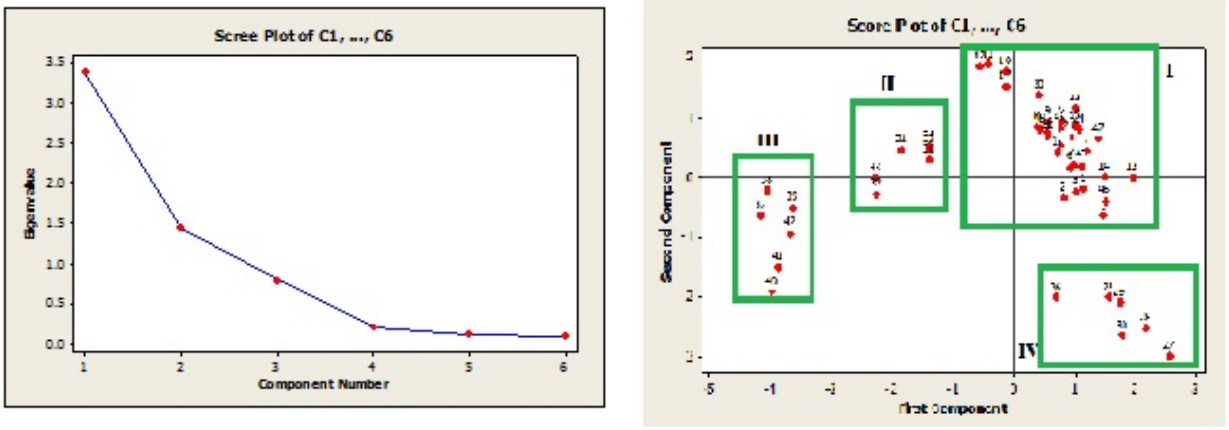

Figure 4: Scree plot (A) and Score plot (B) of PCA for 42 RFM samples. The sample codes were the same as in Table 2.

In the Figure 5, the biplot graphic PC1 × PC2 of the scores was presented, relating the data of chemical constituents of 48 samples of RFM, in order to correlate them, grouping them in 4 groups (I, II, III, IV).

All samples of group I (RFM1RFM24, RFM43→RFM48) were closer due to the similarities of the contents of the anthraquinone (including emodin, physcion and combined anthraquinone). These samples were in the Red River Delta. The contents of 5 analytes and combined anthraquinone in the two-years-old samples were significantly higher than these in the one-year-old samples, but these in the three-years-old samples were not significantly different from the two-years-old samples. Based on these results, it was recommended to use two-years-old samples of RFM.

The same way, the similarity between the RFM samples from Lai Chau and Son La can be observed (Groups II and III) due to their similarities in relation to the contents of two components resveratrol and piceid. Resveratrol appeared in the samples from Sin Ho (Lai Chau) and Thuan Chau (Son La) with the cultivation land's height of more than 1000 m, but did not appeared in the samples from Moc Chau (Son La) with the field's height of less than 1000 m.

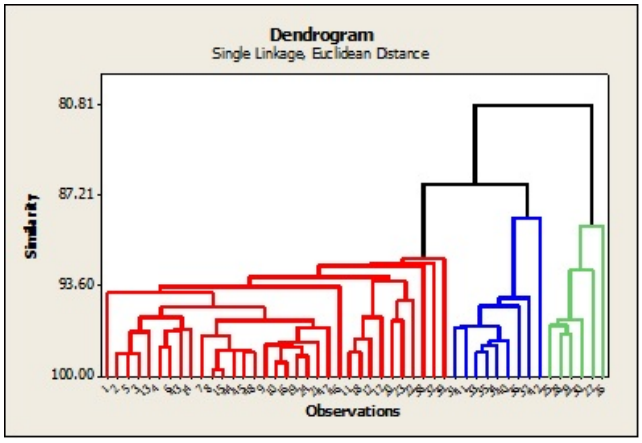

Figure 5: Dendrograms of cluster analysis for 48 tested samples of RFM based on 5 analytes (1-5). The sample codes were the same as in Table 2.

In relation to the components of the samples in Group IV, there was a similarity in relation to the content of THSG, piceid, emodin, physcion and combined anthraquinone, especially, the content of THSG above 3% level-these were much higher than other samples. This information contributed to the research on traceability of RFM from Son La, Ha Giang.

Clustering analysis (CA): Cluster analysis of 48 selected samples of RFM was performed using 5 analytes (1-5) as markers, respectively (Figure 4). Their results were very similar (Figure 3), which were also in accordance with that of PCA.

Conclusion

This was the first study of quantitation of 5 components (two anthraquinones and three stilbenes) and combined anthraquinone in *Radix Fallopiae multiflorae* using fluorescence detector in Vietnam. The methods were validated for precision, accuracy and robustness, linearity and sensitivity according to ICH guidelines. The data showed good sensitivity, a wide linear range, precision and accuracy. Application of the optimized conditions for analyzing 48 RFM samples in Vietnam was shown to be useful, and the method could be used for quality control of RFM.

Besides, their contents in samples from different locations of Vietnam were also compared based on chemometrics. Therefore, THSG should be used as marker for quality control of RFM samples in Vietnam and resveratrol could be used as a marker of RFM samples from Lai Chau and Son La with the land's height of more than 1000 m.

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