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QUALITY EVALUATION OF *PHELLODENDRON AMURENSE* COLLECTED IN VIETNAM USING HPLC-DAD AND QAMS METHODS

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Summary

Quality Evaluation of *Phellodendron amurense* Collected in Vietnam Using HPLC-DAD and QAMS Methods

A simple and sensitive high-performance liquid chromatography (HPLC-DAD) method has been developed to quantify phellodendrine, palmatine chloride, and berberine chloride in *Phellodendron amurense* collected in Vietnam. Based on this method, a quantitative analysis of multiple components with a single marker (QAMS) was carried out and the relative correction factors (RCFs) were calculated to determine the contents of phellodendrine and palmatine chloride according to berberine chloride (used as an internal standard). The RCF values of phellodendrine and palmatine chloride were 2.79 and 0.30, respectively, according to the internal standard. The accuracy of the QAMS method was verified by comparing it with the results of the external standard method (ESM), as well as the feasibility of the method applied to quality control of *Phellodendron amurense*. The %RSD of ***f*** values in different injection volumes were from 3.76% to 3.98%. The accuracies of QAMS were from 90% to 110% and no significant difference in results was found between the content of phellodendrine and palmatine chloride analyzed by QAMS and external standard method. The findings from QAMS indicated that it was a convenient and precise approach for identifying multiple components, particularly when certain genuine standard substances were inaccessible. It can potentially be utilized for controlling the quality of *Phellodendron amurense*.

Keywords: *Phellodendron amurense, Berberin, HPLC, QAMS.*

1. Introduction

The well-known traditional remedy *Phellodendri amurensis* cortex was developed from the dried bark of *Phellodendron amurense* Rupr. [1]. *Phellodendri amurensis* cortex has been shown through contemporary pharmacological studies to possess a range of biological activities, such as anti-inflammatory [3], anti-tumor [2], anti-microbial, anti-oxidant, and anti-herpes simplex virus [4], as well as hypoglycemic and neuroprotective properties [5]. Multiple constituents contribute to the effects of *Phellodendri amurensis* cortex; however, only palmatine chloride and berberine chloride were determined according to Chinese Pharmacopoeia 2015 [6], Hong Kong Chinese Materia Medica Standards [7], and Vietnamese Pharmacopoeia 2017 [1]. Despite being the active components of the cortex of *Phellodendri amurensis*, they are also present in certain other species. To guarantee the medication's effectiveness, it is crucial to set up comprehensive quality control procedures for *Phellodendri amurense*. The quantitative analysis of multiple components by single-marker (QAMS) method allows to determination of the concentrations of multiple components simultaneously and requires only a single reference standard. To some extent, this method could also significantly reduce the cost and time of the analysis process [8],[9],[10],[11],[12]. Thus, QAMS has been widely accepted and applied in the quality control of herbal medicine. This study selects three alkaloids, including phellodendrine, palmatine chloride, and berberine chloride, as markers for the simultaneous determination of *Phellodendri amurensis* cortex quality control in Vietnam using the HPLC-DAD method. The purpose of this study was to develop a QAMS method for simultaneously determining phellodendrine, palmatine chloride, and berberine chloride in *Phellodendri amurensis* cortex samples collected in Vietnam. Berberine chloride was used as an internal standard substance, and the RCF of other compounds was established. The developed method could simultaneously determine the contents of active components in 10 *Phellodendri amurensis* cortex samples collected in Vietnam, which provides a theoretical scientific basis for the comprehensive quality control and evaluation of *Phellodendri amurensis* cortex and its products.

2. Experimental

2.1. Materials

The *Phellodendri amurensis* cortex sample **(PAC-1)** was collected in 4/2023 in Sa Pa (Lao Cai province, Vietnam). The samples were identified by Prof. Dr. Pham Thanh Huyen (NIMM). These samples were cleaned, dried in the

oven at (60ºC) and ground into powder before using in this study. Some *Phellodendri amurensis* cortex samples **(PAC2-PAC10)** were purchased on the market.

2.2. Chemicals

Methanol and acetonitrile of HPLC grade were purchased from Merck (Germany). All other solvents were of analytical grade. Phellodendrine (CAS 6873-13-8; Lot: CFS202301, 99.5%); palmatine chloride (CAS: 10605-02-4, Lot: CFS202201, 98%) and berberine chloride (CAS: 633-65-8; Lot: CFS202202, 98%) were purchased from Chemfaces (China).

2.3. Preparation of standard solutions

Standard stock solutions of the three compounds (phellodendrine, palmatine chloride, and berberine chloride) were prepared by dissolving the primary standard in methanol. The stock solutions were then diluted to establish calibration curves in the ranges of 0.5 - 125 µg/mL for phellodendrine, 0.25 - 50 µg/mL for palmatine chloride, and 13 - 1300 µg/mL for berberine chloride. The stock and working solutions were stored at 4ºC.

Mixed standard solution: each reference stock solution was accurately, put into a 5 mL volumetric flask, and then methanol was added to the scale, and mixed thoroughly; the concentrations of phellodendrine, palmatine chloride, and berberine chloride were 25 µg/mL, 25 µg/mL, and 65 µg/mL, respectively. The solutions were stored at 4ºC in a refrigerator and filtered through a 0.45 µm membrane filter before injection.

2.4. Preparation of sample solution

Weigh accurately 0.2 g of the powdered sample and put it into a 50-mL centrifugal tube, then add accurately 10 mL of methanol and weigh. Sonicate the mixture for 30 min and weigh again. Add an appropriate amount of methanol to compensate for the weight loss. Mix and centrifuge at about 3000 x g for 5 min. Filter through a 0.45 µm filter [6],[7].

2.5. Instrument and Chromatographic Conditions

The HPLC system (Shimadzu, Japan) used for the analysis consisted of a binary pump LC-30AD, a SIL-30AC autosampler, a CTO-10AS column oven, and a diode-array detector (SPD-M20A). Quantitative estimation was performed with LabSolutions software programs. The separation of compounds was performed on an Agilent C_{18} column (250 x 4.6 mm, 5 µm). The mobile phase was a mixture of acetonitrile (ACN) and 0.1% trifluoroacetic acid. The elution program was optimized and conducted as follows: 0-20 min (10-90% ACN); 20-30 min (90% ACN); 30-35 min (90-10% ACN); 35-40 min (100% ACN). The solvent flow rate was kept at 0.8 mL/min. The injection volume was 10 µL. Detection was set at a wavelength of 270 nm [1],[14],[15].

2.6. External standard method (ESM)

The analytical method was developed and validated for system suitability, specificity, calibration curve, accuracy, precision, detection limit, and quantitation limit following the current ICH guidelines [13].

2.7. QAMS method

Methods for calculating the relative retention time (RRT) and the relative correction factor (RCF, ***f***) have been previously reported [8],[9],[10],[11],[12]. First, berberine chloride was selected as the internal standard, and a multipoint method was used to calculate ***f*** values for phellodendrine and palmatine chloride. The content of the multi-marker components measured by QAMS was compared with results from ESM, to validate the methods of QAMS.

The formula for calculating the RRT and RCF:

$$RRT_X = \frac{T_{Rx}}{T_{Rs}} \quad (1)$$

$$RCF = \frac{A_s \times C_x}{A_x \times C_s} \quad (2)$$

where

T_{Rs}, As, and Cs are the retention time, peak area, and concentration of the internal standard substance (berberine chloride);

T_{Rx}, A_x và C_x are the retention time, peak area, and concentration of the analyzed compound.

The RCF value was evaluated by analyzing the mixed standard solution under some different conditions in terms of sample injection volume (2-10 µL), types of HPLC C_{18} chromatography columns from 3 different brands (Agilent, Phenomenex, and Vertical) and analyzed on 2 different HPLC systems.

2.8. Calculation

The content of the measured component in *Phellodendri amurensis* cortex samples by the ESM method was calculated as follows:

$$X_{ESM}(\%) = \frac{C_t \times V \times P \times 100}{m \times 100 \times (100 - a)} \times 100 \quad (3)$$

where C_t: the concentration of analyzed compounds in the sample solution from the calibration curve equation (mg/mL);

V: volume of the sample solution (mL);

m: weight of sample taken to prepare the sample solution (mg);

a: the moisture of sample (%);

P: the purity of the standard compound (%).

The concentration of the measured component by QAMS was calculated as follows:

$$C_x = \frac{f_x \times A_x \times C_s}{A_s} \quad (4)$$

where A_x and C_x are the peak area and the concentration of the analyte in the test sample;

A_s và C_s are the peak area and the concentration of the internal standard substance;

f_x is the RCF value.

3. Results and Discussion

3.1. Validation HPLC-DAD method for quantification of phellodendrine, palmatine chloride, and berberine chloride in Phellodendri amurensis cortex

3.1.1. Specificity:

The experiment was performed with the blank, standard, and *Phellodendri amurensis* cortex sample solutions according to the analytical procedure. The retention times of phellodendrine, palmatine chloride, and berberine chloride were 12.6 min, 17.1 min, and 17.6 min, respectively. The peak of each compound was confirmed by comparing the retention time and UV spectrum of each marker constituent. The obtained results are shown in Fig. 1 and Table 1.

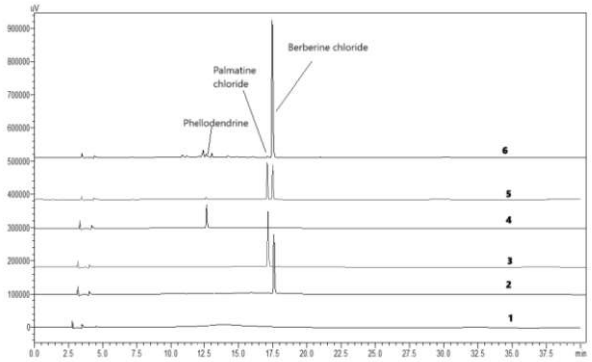

Fig. 1. HPLC-DAD chromatograms of the blank, standard, and *Phellodendri amurensis* cortex sample solutions
(1-blank sample; 2-berberine chloride; 3-palmatine chloride; 4-phellodendrine; 5- mixed standard solution sample; 6- Phellodendri amurensis cortex sample)

Table 1. Parameters of peaks of three components

	Phellodendrine	Palmatine chloride	Berberine chloride
Retention time (t_R, min)	12.6	17.1	17.6
Resolution (Rs)	2.11	1.98	2.89
Tailing factor (T_f)	1.23	1.18	1.25
Number of Theoretical Plates (N)	83709	97228	121096
Purity of peak	0.999	0.993	0.999
The similarity of the UV spectrum	0.997	0.998	0.999

3.1.2. Linearity, LOD, and LOQ:

The calibration curves of three components were established by using the peak area (y) as the vertical axis and the concentration of the analyte (x) as abscissa, respectively. All the correlation coefficients (R) were more than 0.999 for the concentration range, indicating acceptable linearity.

Table 2. Regression, equations, correlation coefficients, linearity ranges, limits of detection, and limits of quantification for three components

Compounds	Regression equation*	R^2	Linearity range (µg/mL)	LOD (µg/g)	LOQ (µg/g)
Phellodendrine	y = 5126.6x + 1968.2	0.9999	0.5 - 125	0.03	0.09
Palmatine chloride	y = 47279x + 10888	0.9999	0.25 - 50	0.01	0.04
Berberine chloride	y = 17188x - 191368	0.9992	13 - 1300	0.01	0.04

**In the regression equation y = ax + b, where y refers to the peak area and x refers to the concentration of the substance (µg/mL).*

Furthermore, the limit of detection (LOD) and the limit of quantification (LOQ) were calculated by diluting sample solutions until signal-to-noise ratios of 3 and 10, respectively. LOD and LOQ of three substances were also calculated as shown in Table 2.

3.1.3. System suitability:

It can be observed from Table 3 that the RSD% of retention time and peak area were all under 2%. These indicate that the developed method conforms to system suitability criteria.

Table 3. System suitability testing (n = 6)

Parameter	Phellodendrine 25 µg/mL		Palmatine chloride 25 µg/mL		Berberine chloride 65 µg/mL	
	Mean	RSD(%)	Mean	RSD(%)	Mean	RSD(%)
Retention time, t_R (min)	12.64	0.16	17.11	0.16	17.58	0.15
Peak area (mAu.s)	127659.8	1.36	1192262	0.26	1057784	0.34

3.1.4. Precision:

The intra- and inter-day precision was summarized in Table 4.

Table 4. The evaluated results of precision (intra-day and inter-day)

Time	Code	Weight (g)	Content (%)		
			Phellodendrine	Palmatine chloride	Berberine chloride
Day 1	M1	0.2011	0.60	0.15	7.45
	M2	0.2042	0.60	0.15	7.35
	M3	0.2006	0.61	0.14	7.41
	M4	0.2024	0.59	0.15	7.41
	M5	0.2014	0.60	0.15	7.42
	M6	0.1932	0.59	0.15	7.68
	Mean (n=6)		**0.60**	**0.15**	**7.45**
	RSD% (n=6)		**1.23**	**1.02**	**1.55**
Day 2	M7	0.2003	0.58	0.14	7.48
	M8	0.1908	0.55	0.15	7.86
	M9	0.2001	0.58	0.14	7.50
	M10	0.1957	0.59	0.15	7.66
	M11	0.1938	0.58	0.15	7.74
	M12	0.1922	0.57	0.15	7.80
	Mean (n=12)		**0.59**	**0.15**	**7.56**
	RSD% (n=12)		**2.88**	**1.41**	**2.32**

The evaluation was performed by calculating the relative standard deviation (RSD%). The criterion of acceptance was RSD% <3.7% for the mean contents of three compounds phellodendrine, palmatine chloride, and berberine chloride, respectively. The results showed that the method had good precision.

3.1.5. Accuracy:

The accuracy was evaluated by adding a known amount of three standards at four different levels (20%, 80%, 100%, and 120%) to the sample. Each experiment was repeated three times. Conduct HPLC-DAD analysis of spiked and unspiked samples, determine the amount of standard added based on the calibration curve equations, and calculate the recovery efficiency compared to the actual amount of standard added.

Table 5. The results of evaluating the accuracy (n=3)

Compound	Level	Recovery rate (%)
Phellodendrine	20% spiked	97.8 ± 0.6
	80% spiked	105.8 ± 1.2
	100% spiked	101.4 ± 1.7
	120% spiked	98.3 ± 0.9
Palmatine chloride	20% spiked	96.4 ± 0.5
	80% spiked	102.4 ± 3.4
	100% spiked	104.7 ± 2.5
	120% spiked	100.7 ± 4.4
Berberine chloride	20% spiked	99.9 ± 1.0
	80% spiked	96.2 ± 0.7
	100% spiked	96.2 ± 0.5
	120% spiked	97.3 ± 0.3

The results in Table 5 showed that the recovery rates of the three compounds were in the range of 96.2% -105.8%. The recovery values were 97.8-105.8% for phellodendrine, 96.4-104.7% for palmatine chloride, and 96.2-99.9% for berberine chloride. The results indicated that the accuracy of the developed method was acceptable.

3.2. QAMS method for quantification of phellodendrine, palmatine chloride, and berberine chloride in Phellodendri amurensis cortex

3.2.1. Determine RCF value according to different sample injection volumes:

Berberine chloride was selected as the internal standard, and the values of RCF (***f***) for the other two markers were computed in different concentrations according to Equation 2 mentioned in Section 2.7. The average RCF value of each compound is shown in Table 6.

Table 6. RCF values of two markers according to different sample injection volumes

Injection volumes	**Phellodendrine 25 µg/mL**			**Palmatine chloride 25 µg/mL**			**Berberine chloride 65 µg/mL**	
	Peak area	Concentration (µg/ml)	**RCF**	Peak area	Concentration (µg/ml)	**RCF**	Peak area	Concentration (µg/ml)
2	63278	11.96	**2.60**	477985	9.88	**0.28**	765675	55.68
3	78318	14.89	**2.67**	716639	14.93	**0.29**	859176	61.12
4	103531	19.81	**2.73**	955075	19.97	**0.30**	935482	65.56
5	127813	24.55	**2.77**	1194386	25.03	**0.30**	996999	69.14
6	164160	31.64	**2.82**	1438524	30.20	**0.31**	1085619	74.30
7	182894	35.29	**2.86**	1673909	35.17	**0.31**	1198897	80.89
8	209113	40.41	**2.87**	1917524	40.33	**0.31**	1215285	81.84
9	221606	42.84	**2.90**	2155463	45.36	**0.32**	1320972	87.99
10	247161	47.83	**2.93**	2387111	50.26	**0.32**	1426821	94.15
TB			**2.79**			**0.30**		
RSD%			**3.98**			**3.76**		

3.2.2. Determination of RRT and RCF values on different columns:

Table 7. RRT and RCF values of two markers according to different columns

Compounds		**Berberine chloride**	**Phellodendrine**			**Palmatine chloride**		
Instrument	Column	RT (min)	RT (min)	RRT	**RCF**	RT (min)	RRT	**RCF**
HPLC-DAD	A	17.50	12.63	0.72	**2.79**	17.09	0.98	**0.31**
		17.49	12.62	0.72	**2.80**	17.09	0.98	**0.31**
		17.49	12.62	0.72	**2.80**	17.09	0.98	**0.31**
	B	15.49	11.82	0.76	**2.80**	15.25	0.98	**0.30**
		15.49	11.88	0.77	**2.81**	15.25	0.98	**0.31**
		15.49	11.88	0.77	**2.77**	15.25	0.98	**0.30**
	C	19.03	14.12	0.74	**2.80**	18.64	0.98	**0.30**
		18.99	14.11	0.74	**2.79**	18.61	0.98	**0.30**
		18.97	14.10	0.74	**2.77**	18.58	0.98	**0.30**
Mean				**0.74**	**2.79**		**0.98**	**0.30**
RSD%				**2.55**	**0.47**		**0.34**	**0.49**

Column A (Eclipse Plus C_{18} Agilent, 4.6x250 mm, 5µm); Column B (Phenomenex: Luna® 5µm C_{18}, 4.6x250 mm, 5 µm); Column C (VertiSep TM UPS C_{18} HPLC Column, 4.6x250 mm, 5µm).

3.2.3. Determination of RRT and RCF values on different HPLC instruments:

Table 8. RRT and RCF values of two markers according to different HPLC instruments

Compounds	**Berberine chloride**	**Phellodendrine**			**Palmatine chloride**		
Instrument	RT (min)	RT (min)	RRT	**RCF**	RT (min)	RRT	**RCF**
HPLC-DAD 1	17.50	12.63	0.72	**2.79**	17.09	0.98	**0.31**
	17.49	12.62	0.72	**2.80**	17.09	0.98	**0.31**
	17.49	12.62	0.72	**2.80**	17.09	0.98	**0.31**
HPLC-DAD 2	12.18	9.10	0.75	**2.80**	0.97	0.97	**0.31**

Compounds	Berberine chloride	Phellodendrine			Palmatine chloride		
	12.19	9.01	0.74	**2.81**	0.97	0.97	**0.31**
	12.22	9.12	0.75	**2.81**	0.97	0.97	**0.31**
Mean			**0.73**	**2.80**		**0.98**	**0.31**
RSD%			**2.05**	**0.27**		**0.56**	**0.00**

The results of determining the RRT and RCF values of each substance phellodendrine (RRT = 0.74 and RCF = 2.79), palmatine chloride (RRT = 0.98; RCF = 0.30) compared to the berberine chloride standard showed good repeatability (RSD values < 3%) when using different stationary phases and different HPLC chromatography systems. This proves that the research method is stable and has good repeatability.

3.3. Analysis of samples and comparison of the results between QAMS and ESM methods

The contents of each component in 10 samples of *Phellodendri amurensis* cortex were calculated by QAMS and ESM, respectively. The results are shown in Table 9.

Table 9. Content of berberine chloride, phellodendrine, and palmatine chloride in 10 samples of *Phellodendri amurensis* cortex determined by QAMS and ESM (n=3)

Code	Method	Content (%)			P-value*
		Berberine chloride	Phellodendrine	Palmatine chloride	
PAC-1	ESM	7.92	0.71	0.11	0.295
	QAMS		0.68	0.10	
PAC-2	ESM	7.62	0.56	0.07	0.500
	QAMS		0.54	0.07	
PAC-3	ESM	5.76	0.84	0.17	0.374
	QAMS		0.79	0.16	
PAC-4	ESM	4.25	0.22	0.05	0.795
	QAMS		0.20	0.06	
PAC-5	ESM	6.16	0.36	0.02	0.545
	QAMS		0.35	0.01	
PAC-6	ESM	6.43	0.41	0.02	0.205
	QAMS		0.39	0.01	
PAC-7	ESM	5.21	0.28	0.03	0.500
	QAMS		0.27	0.03	
PAC-8	ESM	3.22	0.19	0.06	0.662
	QAMS		0.18	0.05	
PAC-9	ESM	5.32	0.55	0.23	0.206
	QAMS		0.54	0.21	
PAC-10	ESM	4.98	0.86	0.13	0.295
	QAMS		0.83	0.12	

3.5. Discussion

Vietnamese Pharmacopoeia V and Chinese Pharmacopoeia stipulated that the berberine chloride content in *Phellodendri amurensis* cortex must not be less than 0.33% [1],[6]. Hong Kong Chinese Materia Medica Standards Office stipulates that the berberine chloride content in *Phellodendri amurensis cortex* must not be less than 0.33% and the palmatine chloride content must not be less than 0.18% [7]. The results showed the mean contents of 3.22 - 7.92%, 0.18 - 0.86%, and 0.01 - 0.23%, for the berberine chloride, phellodendrine, and palmatine chloride, respectively. The results obtained in this study showed that all samples of *Phellodendri amurensis* cortex met the berberine chloride content criteria, however, only one sample (**PAC-9**) met the palmatine chloride content criteria. Sample **PAC-1** has the highest berberine chloride content (7.92%), higher than the *Phellodendri amurensis* cortex samples purchased on the market (**PAC-2** to **PAC-9**). The highest phellodendrine content is 0.86% in sample **PAC-10**, the lowest is 0.2% in sample **PAC-4**.

It was found that the percentage difference between these two methods was less than ±5.0%. Using Minitab 16 software, a paired sample *T-test* was performed on two contents of ten samples measured by QAMS and ESM. The results revealed that there were no significant variations between the results of two different determination methods (P-value > 0.05), indicating that the established approach was accurate and dependable. QAMS is a multi-component analysis method based on one standard substance, which has been applied in many studies on medicinal herbs, with advantages such as being able to analyze multiple substances simultaneously but only using a single standard substance, helping to reduce analysis time and costs [8],[9],[10],[11],[12]. In Vietnam, there are few studies on the simultaneous quantification of berberine chloride, palmatine chloride, and phellodendrine in herbs. In particular, there have been no studies applying the QAMS method to simultaneously quantify these substances according to a standard substance. The results obtained in this study contribute a new method in the field of standardizing the quality of *Phellodendri amurensis* cortex.

4. Conclusion

In this study, a method named QAMS was established to evaluate the quality of *Phellodendri amurensis* cortex using HPLC-DAD equipment. In this method, berberine chloride was used as the internal standard to determine the RCF between berberine chloride and other markers (phellodendrine, palmatine chloride). According to the obtained results, QAMS was accurate and feasible for quality evaluation, and there was no significant difference in the content results obtained by QAMS and ESM. Ten samples of *Phellodendri amurensis* cortex were determined, and the results showed that the mean contents of 3.22-7.92%, 0.18-0.86%, and 0.01-0.23%, for the berberine chloride, phellodendrine, and palmatine chloride, respectively. There were no significant variations between the results of two different determination methods QAMS and ESM (P-value >0.05). The obtained results indicated that the established QAMS method can be accurately, economically, simply, and quickly applied to the multiple components analysis of *Phellodendri amurensis* cortex.

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