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SIMULTANEOUS DETERMINATION OF CHLOROGENIC ACID AND ISOCHLOROGENIC ACID A IN *PHARBITIDIS SEMEN* BY HPLC METHOD

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Summary

High-performance liquid chromatography (HPLC) was employed to quantify chlorogenic acid (CGA) and isochlorogenic acid A (ICGA) in *Pharbitidis Semen,* owing to its high sensitivity and suitability for quantitative analysis. An Agilent Eclipse Plus C_{18} column (250 × 4.6 mm, 5 µm) and a DAD detector at 327 nm were employed for separation and detection. The mobile phase consisted of 0.1% formic acid in water and acetonitrile, with a flow rate of 1.0 mL/min and a 5 µL injection volume. The method demonstrated linearity over the ranges of 4.6 – 557.62 µg/mL for CGA and 4.3 – 557.62 µg/mL for ICGA. Validation parameters, including accuracy, system suitability, linearity, repeatability, precision, limit of detection (LOD), and limit of quantification (LOQ), met the AOAC requirements according to ICH guidelines. The findings are useful for quality control of *Pharbitidis Semen* in Vietnam.

Keywords: *Pharbitidis Semen*; *HPLC-DAD; Acid chlorogenic; Isochlorogenic acid A.*

1. Introduction

Pharbitis nil (L.) Choisy, originating in South America, is now globally distributed, occurring in China, Australia, and several Southeast Asian countries. In Vietnam, it is found from the northern provinces of Lang Son and Hoa Binh to the southern province of Ba Ria-Vung Tau [1]. Studies have demonstrated that *Pharbitidis Semen* possesses a range of biological activities, including antifungal, antibacterial, anti-inflammatory, antioxidant, antitumor, and memory enhancement [2],[3],[4],[5]. The chemical composition of *Pharbitidis Semen* has been extensively studied, revealing the presence of triterpenoid saponins, flavonoid glycosides, phenolic compounds, lignans, amino acids, and glycosides [2],[3],[4],[5],[6]. The significant phenolic acid content in *Pharbitidis Semen* is of particular interest, owing to the broad spectrum of biological, pharmacological, and practical effects associated with these compounds. Chlorogenic acid (CGA) and its isomers (isochlorogenic acid A (ICGA), isochlorogenic acid B, isochlorogenic acid C, cryptochlorogenic acid) are key dietary polyphenols with demonstrated biological activities such as antioxidant, antibacterial, hepatoprotective, cardioprotective, anti-inflammatory, antipyretic, neuroprotective, anti-obesity, antiviral, antihypertensive, free radical scavenging, and central nervous system stimulation [7],[8].

The Vietnamese Pharmacopoeia V (2017) addresses quality control of *Pharbitidis Semen* and its preparations by providing specifications for morphology, microscopic characteristics, resin identification, moisture (≤ 12.0%), total ash (≤ 6.0%), foreign matter (≤ 1%), and resin content (≥ 1.2%) [9]. Relevant quality standards are also available in the Chinese Pharmacopoeia (CP 2020) [10]. Nevertheless, global research concerning comprehensive *Pharbitidis Semen* standardization remains limited, particularly regarding quantitative analysis of characteristic compounds using modern chromatographic techniques such as HPLC–DAD or LC-MS. Furthermore, in the chemical composition study, CGA and ICGA were identified as the major compounds in the HPLC chemical fingerprint of *Pharbitidis Semen*. This study aims to address this gap by developing an HPLC-DAD method for quantifying CGA and ICGA in *Pharbitidis Semen* samples collected in Vietnam.

2. Materials and Methods

2.1. Materials

Pharbitidis Semen was harvested in June 2023

from a GACP-WHO-certified cultivation site in Thach Dong commune, Thanh Thuy district, Phu Tho province, Vietnam. The material was supplied by Traphaco Joint Stock Company and taxonomically identified by the Center of Medicinal Material Resources, National Institute of Medicinal Materials (NIMM).

2.2. Chemicals

CGA (Lot No: CFN99116, 98.0% purity) and ICGA (Lot No: CFS202401, 98.6% purity) reference standards, purchased from ChemFaces (China), were used in this study. Solvents and chemicals used for HPLC analysis (acetonitrile and methanol) were purchased from Merck. All reagents used in sample extraction and preparation were of analytical grade (P.A.).

Preparation of CGA stock solution 1 mg/mL: Accurately weigh about 5 mg of CGA standard into a 5 mL volumetric flask. Add approximately 3 mL of methanol (60%), shake until completely dissolved, and bring to volume with the solvent mixture to obtain a CGA stock solution with a concentration of approximately 1115.24 µg/mL (corrected for standard purity).

Preparation of the ICGA stock solution was similar to the CGA stock solution, resulting in a solution with a concentration of approximately 1115.24 µg/mL (corrected for standard purity).

Calibration standards: The stock solution was diluted with methanol (60%) to obtain a series of calibration standards with CGA and ICGA concentrations ranging from 4.36 to 557.62 µg/mL.

2.3. Instruments

The main equipment employed in this research was a Shimadzu HPLC system (Japan) consisting of an LC-20AD pump, a SIL-20AHT autosampler, and a DAD detector. Data acquisition and processing were performed using LabSolutions software. An Agilent Eclipse Plus C_{18} column (250 × 4.6 mm, 5 µm) was used for chromatographic separation.

2.4. Methods

2.4.1. Optimization of chromatographic conditions:

Selection of the detection wavelength (λ_{max}): determined by examining the UV-Vis spectra to identify the maximum absorbance of CGA and ICGA, in conjunction with literature references.

Mobile phase optimization: The elution program was investigated using a mobile phase consisting of acetonitrile and 0.1% formic acid in water, or acetonitrile and water, at varying ratios and flow rates.

For the mobile phase investigation, the test sample solution was prepared according to the following reference procedure: Weigh accurately 1.0 g of powdered *Pharbitidis Semen* and place it in a 50 mL centrifuge tube, then add 25 mL of methanol. Sonicate (500 W, 60 kHz) the mixture for 30 min, then let stand for 10 minutes. Filter and transfer the filtrate to the 25 mL volumetric flask and make up to the mark with methanol. Filter through a 0.45 µm PTFE filter.

The chromatographic conditions were held constant as follows: Stationary phase, Agilent C_{18} column (250 × 4.6 mm, 5 µm); Flow rate of 1 mL/ min. The injection volume and mobile phase (see Optimization of chromatographic conditions).

The results obtained from each investigation were evaluated and compared based on retention time (t_R) (not excessively long, but with adequate separation), resolution (Rs ≥ 1.5), and tailing factor (T_f) within the range of 0.8 – 1.5 (optimally approaching 1).

2.4.2. Optimization of the sample extraction procedure:

Optimization of sample preparation conditions involved investigating the following parameters: extraction solvent (water-ethanol and water-methanol mixtures at varying ratios), extraction method (ultrasonic and reflux), extraction time (ranging from 15 to 120 minutes), and number of extractions (1 to 3). A univariate approach, varying one parameter at a time while holding others constant, was employed.

2.4.3. Method validation:

After optimizing the sample preparation and chromatographic conditions, the method was validated according to ICH guidelines and AOAC recommendations [11],[12]. Validation parameters included specificity, system suitability, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, and repeatability.

2.5. Calculation equation

The content (%) of CGA (or ICGA) in the test sample was calculated using the following formula:

$$X(\%) = \frac{C \times V \times 100}{m \times 10^6 \times (100 - B)} \times 100\%$$

Where:

X: The content (%) of CGA (or ICGA) (%)

C: Concentration of CGA (or ICGA) in the test solution, as determined from the calibration curve (µg/mL)

m: Mass of the test material (g)

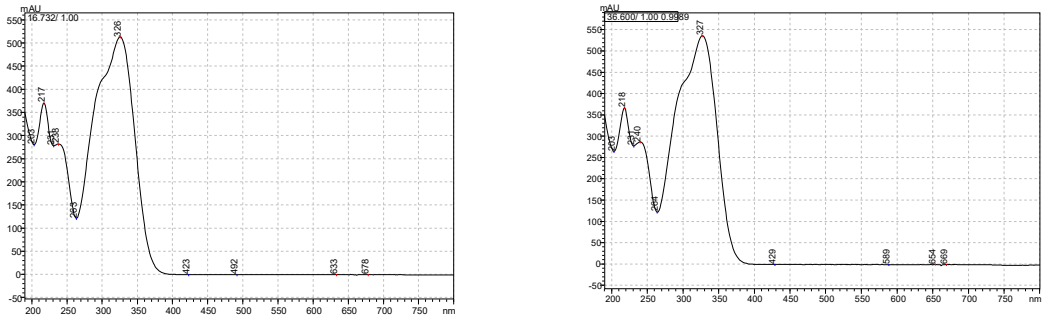
B: Moisture content of the test material (%)

V: Rated volume (mL)

3. Results and Discussion

3.1 Optimization of HPLC method conditions

3.1.1. Detection wavelength:



CGA ICGA

Fig. 1. UV-Vis spectrum of CGA and ICGA

To determine the maximum absorbance wavelength for each compound, UV-Vis spectra of individual standard solutions were obtained. The results, shown in **Fig. 1**, indicated a maximum absorption wavelength (λ_{max}) of 327 nm, which was selected for the quantification of CGA and ICGA.

3.1.2. Chromatographic conditions:

The chromatographic program optimization results are shown in **Table 1**.

Table 1. Chromatographic method development

Condition 1 (Acetonitrile - water)		**Condition 2 (Acetonitrile - 0.1% formic acid)**		**Condition 3 (Acetonitrile - 0.1% formic acid)**	
Time (min)	% ACN	Time (min)	%ACN	Time (min)	%ACN
0-30	10-50	0-30	5-30	0-30	5-20
30-50	50-90	30-40	30-95	30-45	20-30
50-55	90-100	40-45	95	45-50	30-95
55-60	100-10	45-47	95-5	50-55	95
70	stop	57	stop	55-57	95-5
				67	stop

Table 2. Chromatographic parameters for the analytes in the chromatographic program study

Conditions	**Retention time (min)**		**Resolution (Rs)**		**Theoretical plates**		**Tailing factor (T_f)**	
	CGA	**ICGA**	**CGA**	**ICGA**	**CGA**	**ICGA**	**CGA**	**ICGA**
1	7.754	15.232	0.537	3.101	17416	53940	-	1.300
2	13.689	25.823	0.848	0.661	40506	129005	1.432	1.168
3	**16.712**	**36.580**	**3.329**	**2.081**	**34245**	**157604**	**1.258**	**1.264**

The results from Tables 1 and 2 demonstrated that Condition 3 exhibited improved separation performance compared to the other two conditions tested. Although it exhibited a longer retention time, the resolution and peak shape were superior, and separation from the sample was enhanced. Therefore, Condition 3 was advanced as the mobile phase program for subsequent optimization.

Injection volume optimization: The influence of different injection volumes (5 µL, 10 µL, 15 µL, and 20 µL) on the analytical conditions was investigated. The results of this investigation are presented in **Table 3**.

The results indicated that the injection volume had a significant influence on peak parameters. Specifically, increasing the injection volume from 5 to 20 µL resulted in a decrease in resolution and theoretical plate number. Based on these findings, a 5 µL injection volume was chosen for subsequent experiments.

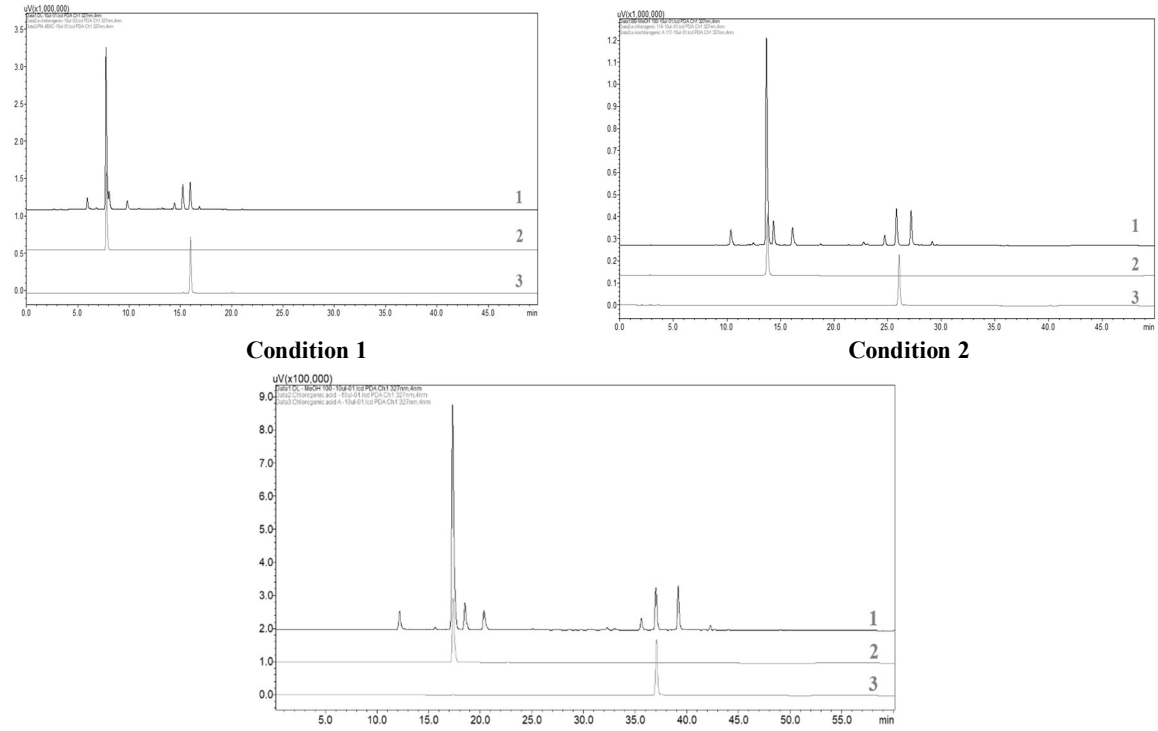


Fig. 2. Chromatograms of the chromatographic program survey
(1 - Test sample; 2 - CGA; 3 - ICGA, with an injection volume of 10 µL)

Table 3. Chromatographic parameters used to determine the sample injection volume for the analytes

Injection volume (µL)	Retention time (min)		Resolution (Rs)		Theoretical plates		Tailing factor (T_f)	
	CGA	ICGA	CGA	ICGA	CGA	ICGA	CGA	ICGA
5	**16.712**	**36.580**	**3.329**	**3.336**	**34245**	**103483**	**1.258**	**1.235**
10	16.193	36.053	2.929	2.781	29303	134206	1.258	1.264
15	15.950	35.746	1.938	2.618	22017	96457	1.458	1.202
20	16.219	36.101	0.930	2.500	21784	89076	-	1.092

3.2. Optimization of extraction conditions

Optimization of the extraction process involved the evaluation and comparison of the following key factors: extraction solvent, extraction method, extraction time, and the ratio of the sample mass to the solvent volume.

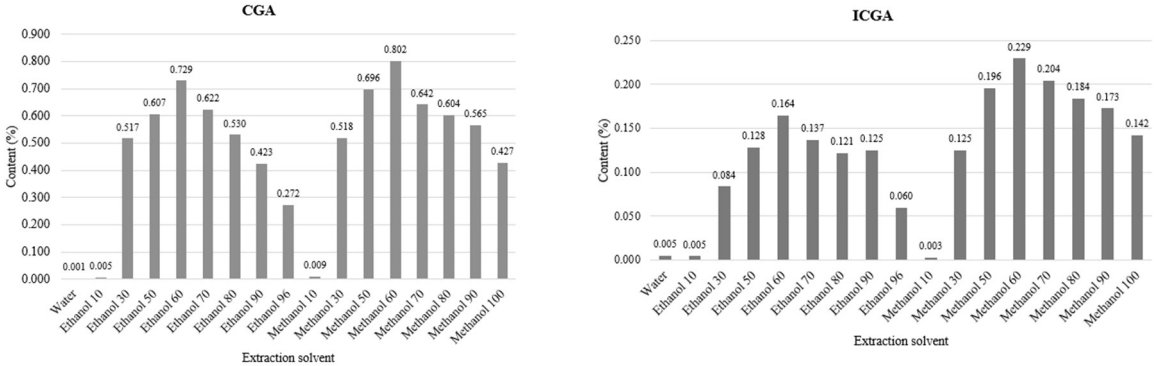


Fig. 3. Charts showing the investigation of CGA and ICGA using different solvents

The results showed that the highest CGA and ICGA contents were 0.802% and 0.229%, respectively, when methanol (60%) was used as the extraction solvent. Based on these results, we selected methanol (60%) as the extraction solvent for subsequent investigations.

Optimization of extraction method and time: To determine the optimal extraction method and duration, both ultrasonic and reflux extraction techniques were investigated. These solutions were analyzed by HPLC-DAD under previously optimized conditions, with the results presented in **Fig. 4**.

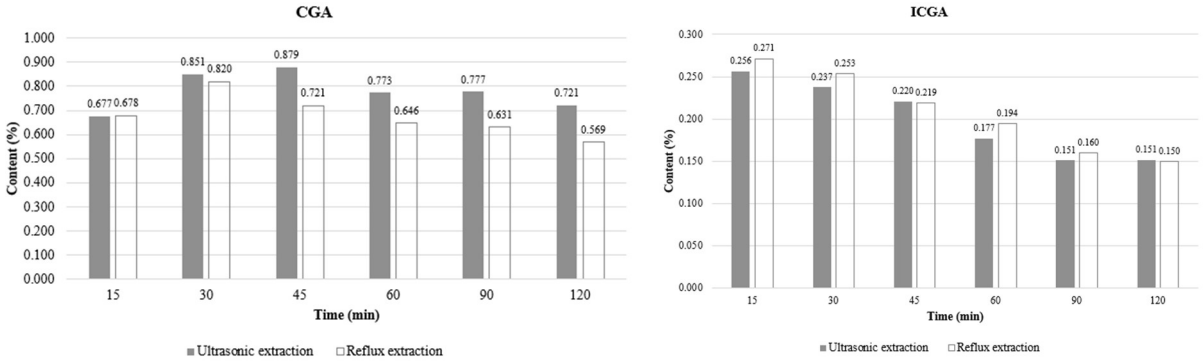


Fig. 4. Charts of CGA and ICGA content versus extraction method and extraction time

Optimization of extraction time and method for CGA revealed that increasing the ultrasonic extraction time from 15 to 45 minutes increased the measured CGA content, reaching a maximum at 45 minutes. Conversely, for ICGA, increasing the extraction time from 15 to 120 minutes resulted in a gradual decrease in the measured ICGA content. Because this method aims to simultaneously quantify both CGA and ICGA, the analytical and extraction conditions must be optimized concerning both analytes. Selecting a 15-minute ultrasonic extraction would result in a significantly lower CGA content (approximately 23% below its maximum). Furthermore, the difference in ICGA extracted by ultrasonic and reflux extraction methods was not significant (less than 10%), considering the combined extraction efficiency for both CGA and ICGA, a 30-minute ultrasonic extraction time was ultimately chosen for quantifying CGA and ICGA in *Pharbitidis Semen*.

Investigation of the solvent-solids ratio revealed that higher ratios resulted in greater extraction efficiency. However, increasing the solvent volume from 50 to 100 mL provided only a marginal increase in analyte content. Consequently, a single extraction using 50 mL of methanol (60%) was chosen as the optimal condition.

Table 4. The content of the extracted compounds concerning the solvent-solvent ratio

Ratio (g/mL)	**Content (%, Mean ± SD, n = 3)**	
	CGA	**ICGA**
1:10	0.613 ± 0.003	0.166 ± 0.000
1:25	0.763 ± 0.017	0.210 ± 0.004
1:50	**0.869 ± 0.009**	**0.229 ± 0.003**
1:100	0.871 ± 0.010	0.248 ± 0.006

Based on the results, the optimized sample preparation procedure is as follows: Weigh accurately 1.0 g of powdered *Pharbitidis Semen* and place it in a 100 mL round-bottomed flask, then add 50 mL of methanol (60%). Sonicate (500 W, 60 kHz) the mixture for 30 min, then let stand for 10 minutes. Filter and transfer the filtrate to the 50 mL volumetric flask and make up to the mark with methanol (60%). Filter through a 0.45 µm PTFE filter.

3.3. Method validation

3.3.1. Specificity:

The specificity evaluation results are presented in **Figures 5** and **6**.

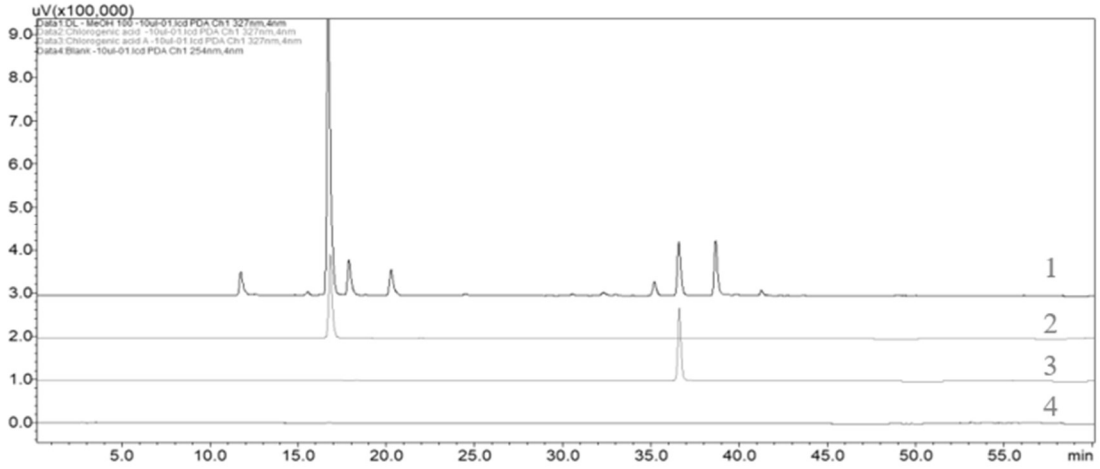


Fig. 5. Chromatograms of the method selectivity
(1- Test sample; 2- CGA; 3- ICGA; 4- Blank sample)

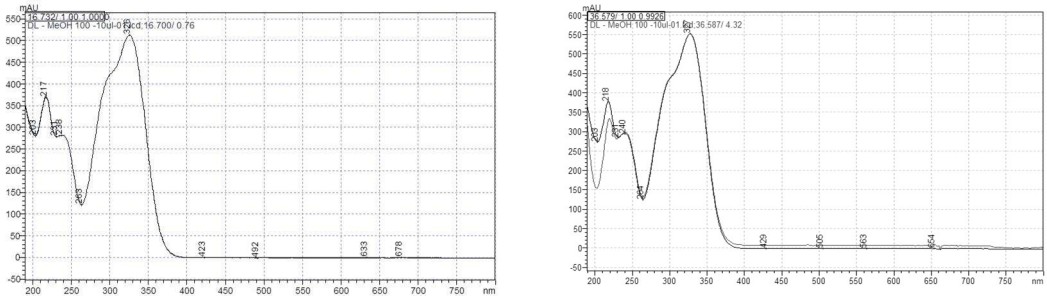


Fig. 6. UV spectra of the standard solution and test solution

The chromatograms of the mixed standard solution and the *Pharbitidis Semen* extract (**Fig. 5**) showed complete separation of the CGA and ICGA peaks. Furthermore, the match ratios of CGA and ICGA in the sample solution compared to the standard solutions were both greater than 0.99 (**Fig. 6**). These findings demonstrate that the analytical method met the requirements for specificity and is therefore suitable for the simultaneous quantification of CGA and ICGA in *Pharbitidis Semen*.

3.3.2. System suitability:

System suitability was assessed by injecting a mixed standard solution of CGA and ICGA (69.71 µg/mL and 139.42 µg/mL, respectively) 6 times under the optimized chromatographic conditions. The retention times and peak areas were recorded. The data presented in **Table 5**, which showed that the RSDs of retention times and peak areas for both compounds were below 2%, indicated that the method and HPLC system met system suitability criteria. This guaranteed the stability of the analysis.

Table 5. System suitability study of the proposed method

	CGA		ICGA	
	Retention time (min)	**Peak area (mAU.s)**	**Retention time (min)**	**Peak area (mAU.s)**
Mean (n=6)	16.762	545335	36.551	577165
RSD (%)	0.071	0.346	0.058	0.327

3.3.3. Calibration curve:

Calibration curves were constructed by plotting peak area against the concentration of the analytes, as shown in **Fig. 7**. The results demonstrated linearity within the concentration range of 4.6 – 557.62 µg/mL for CGA and 4.36 – 557.62 µg/mL for ICGA.

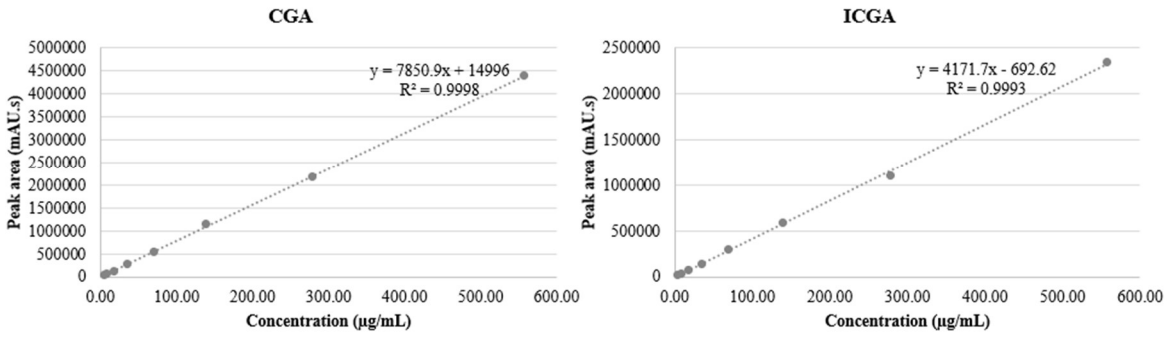


Fig.7. Calibration curves for CGA and ICGA

Table 6. Linearity study data

CGA			ICGA		
Concentration (µg/mL)	**Peak area (mAU.s)**	**Deviation (%)**	**Concentration (µg/mL)**	**Peak area (mAU.s)**	**Deviation (%)**
557.62	4395501	0.06	557.62	2346983	0.86
278.81	2179208	-1.13	278.81	1112389	-4.42
139.41	1155633	4.22	139.41	589454	1.24
69.70	547269	-2.73	69.70	299421	2.73
34.85	294837	2.28	34.85	145804	-0.19
17.43	141559	-7.49	17.43	75391	2.76
8.71	82301	-1.61	8.71	39254	6.09
4.36	45145	-11.85	4.36	20041	6.46

The results of the calibration curve revealed a strong linear correlation between the concentrations of both analytes and their peak areas ($r > 0.99$ and bias < 15%), and the linearity range was sufficiently wide, making it suitable for the analysis of CGA and ICGA in the *Pharbitidis Semen* samples.

3.3.4. Limit of detection (LOD) and limit of quantification (LOQ):

The quantified test solution was serially diluted to the lowest detectable concentration by chromatography to determine the S/N ratio. LOD was defined as the concentration with an S/N ratio of 3 (signal 2 – 3 times background noise). LOQ was accepted with an S/N ratio of 10 (signal 9-10 times background noise). These values are presented in **Table 7**.

Table 7. LOD and LOQ of the method

		CGA	ICGA
LOD	Concentration (µg/mL)	0.35	0.19
	Content (%)	0.002	0.001
LOQ	Concentration (µg/mL)	1.17	0.62
	Content (%)	0.006	0.003

3.3.5. Precision:

The results of the intra-day and inter-day repeatability assessment are presented in **Table 8**. These results demonstrate that the established method exhibits good repeatability (as per AOAC guidelines, with an active ingredient content > 0.1%, RSD% < 3.7%).

Table 8. Precision study of the proposed method

No.	CGA content (%)		ICGA content (%)	
	Day 1	**Day 2**	**Day 1**	**Day 2**
1	0.875	0.840	0.231	0.238
2	0.834	0.828	0.236	0.244
3	0.820	0.896	0.229	0.248

No.	CGA content (%)		ICGA content (%)	
	Day 1	Day 2	Day 1	Day 2
4	0.831	0.865	0.235	0.249
5	0.812	0.835	0.232	0.248
6	0.841	0.850	0.246	0.234
Mean (n=6)	0.835	0.852	0.235	0.244
RSDr (%)	2.215	2.674	2.192	2.343
Mean (n=12)	0.844		0.239	
RSD_R (%)	3.623		3.689	

3.3.6. Accuracy:

The accuracy of the method was assessed using recovery experiments involving the standard addition technique at several spike levels, specifically 10%, 30%, and 60% for CGA and 50%, 100%, and 150% for ICGA. Both spiked and unspiked samples were then quantified using HPLC, with each spike level analyzed in triplicate. The obtained results (**Table 9**) revealed recovery values within the range of 96.59% to 103.97%, indicating that the developed method possesses high accuracy and is appropriate for the quantitative determination of CGA and ICGA.

Table 9. Accuracy study of the proposed method

No.	CGA			ICGA		
	Amount spiked (mg)	Amount found (mg)	Recovery (%)	Amount spiked (mg)	Amount found (mg)	Recovery (%)
1	0.88	0.85	96.59	1.16	1.14	98.13
2	0.88	0.89	101.59	1.16	1.15	99.70
3	0.88	0.88	100.14	1.16	1.18	102.29
4	2.63	2.71	102.84	2.31	2.41	103.97
5	2.63	2.55	96.96	2.31	2.35	101.68
6	2.63	2.58	98.02	2.31	2.26	97.53
7	5.26	5.36	101.8	3.47	3.36	96.82
8	5.26	5.19	98.69	3.47	3.45	99.27
9	5.26	5.12	97.29	3.47	3.47	100
Mean			**99.32**			**99.93**
RSD (%)			**2.22**			**2.22**

3.4. Sample analysis

The developed method was applied to simultaneously quantify CGA and ICGA in the collected samples. The results are shown in **Table 10.**

Table 10. The CGA and ICGA content in the *Pharbitidis Semen* samples (n=3)

No.	Samples	CGA content (%)	ICGA content (%)
1	M1	1.027 ± 0.031	0.317 ± 0.009
2	M2	0.610 ± 0.027	0.272 ± 0.011
3	M3	0.852 ± 0.023	0.244 ± 0.006

The analysis revealed that the CGA content varied significantly, ranging from 0.610% to 1.027%, while the ICGA content showed less variation, ranging from 0.244% to 0.314%. The CGA content was consistently higher than that of ICGA in the *Pharbitidis Semen* samples. Based on these findings, the *Pharbitidis Semen* samples collected in Vietnam primarily contain CGA and ICGA.

3.5. Discussion

Pharbitidis Semen is a widely used medicinal material found in numerous commercially

available products that support the treatment of liver and kidney-related conditions, such as Liverbil, Altamin, Bibiso, BAR, Artisonic, Boliveric, and Kahagan; particularly in the Boganic product from Traphaco. However, the *Pharbitidis Semen* monograph in the Vietnamese Pharmacopoeia V provides only rudimentary quality standards. Therefore, research and development of quantitative methods for active compounds in *Pharbitidis Semen* is necessary and of significant importance, contributing to the improvement of quality standards for this medicinal material.

CGA and ICGA have been confirmed to be present in *Pharbitidis Semen* and possess characteristic biological activities, including: enhancing liver function, protecting the liver from damage, antioxidant, antiviral, and antibacterial properties. Currently, there are no studies in Vietnam on the quantification of these two compounds in *Pharbitidis Semen*. Therefore, the development of an HPLC-DAD method for the simultaneous quantification of CGA and ICGA in *Pharbitidis Semen* ensures novelty and is of significant importance for the quality standardization of *Pharbitidis Semen* in Vietnam. The developed method utilizes a simple extraction technique (ultrasonic), a common extraction solvent (methanol 60%), and conventional HPLC analytical conditions (mobile phase: acetonitrile and 0.1% formic acid in water, C_{18} column), ensuring feasibility and widespread applicability across various laboratories.

4. Conclusion

This study successfully developed an HPLC-DAD method for the simultaneous quantification of CGA and ICGA in *Pharbitidis Semen*. The analytical method was validated according to ICH guidelines, demonstrating: system suitability (RSD < 2%); repeatability (RSD < 4.0%); recovery (95 – 105%); and calibration curves ($R^2 > 0.99$, $\Delta \leq 15\%$). The LOD for CGA and ICGA were 0.002% and 0.001%, respectively, and the LOQ were 0.006% and 0.003%, respectively. Application of the developed method to *Pharbitidis Semen* samples yielded CGA and ICGA contents of 0.610 – 1.027% and 0.244 – 0.314%, respectively. These results indicate that CGA and ICGA are biomarker compounds in *Pharbitidis Semen* and contribute significantly to its quality standardization.

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