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A Study on Improvement of the Analytical Method of Chlorantraniliprole Residue in Herbal Medicine (*Rehmannia glutinosa* Libosch) using HPLC-UVD

Jeong Yoon Choi[1], Yeong Jae Lee[1], Hun Ju Ham[2], Abd Elaziz Sulieman Ahmed Ishag[1,3], Jang Hyun Hur[1]*

[1]Department of Biological Environment, Kangwon National University, Chuncheon 24341, Korea
[2]Environment Friendly Agricultural Products Safety Center, Kangwon National University, Chuncheon 24341, Korea
[3]Department of Crop Protection, University of Khartoum, Khartoum North, Shambat 13314, Sudan

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Abstract This study was conducted to develop a possible method for chlorantraniliprole in *R. glutinosa* root using HPLC-UVD. Acetonitrile/*n*-hexane and dichloromethane/d-water partitioning methods were used to remove non-polar and polar interfering substances in *R. glutinosa* root extract, respectively. Unlike agricultural products that usually go through one-time refining process, *R. glutinosa* root had difficulty removing impurities and it could be analyzed only after two consecutive SPE refining processes. Thus double cleaning up using SPE-silica and SPE-NH_2 cartridges were continuously used. The respective *n*-hexane/ethyl acetate and *n*-hexane/acetone elution were used for SPE-silica and SPE-NH_2. Gradient conditions were used to establish the optimal HPLC-UVD-analysis conditions for quantitative-analysis. Wavelength of 230 nm was selected after scanning the absorbance of wavelengths for minimizing the effect of interfering substances. The MLOQ was 0.02 mg/kg, recovery rate ranged 87.9-105.2% after reviewed at 0.02 and 0.2 mg/kg and RSDs was 3.0%. Chlorantraniliprole was verified by LC-MS SIM mode and the recovery was 90.1-106.9%. Cross-laboratory validation of this method found that the average recovery and RSD were 88.4% and 0.3%, respectively. Chlorantraniliprole met the criteria of validation parameters (acceptable recovery 70-120% and RSD <10%). This study carried out two clean up processes methods and changing UV spectra, after two consecutive refining processes with two kinds of SPE, significant interference materials were eliminated, but found it was difficult to completely separate the chlorantraniliprole from *R. glutinosa* matrix. However, it is believed that this study could be used in some domestic and/or developing countries' laboratories using conventional instruments to research pesticide residues analysis in herbal medicines.

Key words Chlorantraniliprole, Clean-up, HPLC-UVD-analysis, *R. glutinosa*

Introduction

Consumers in modern society are increasingly interested in health and longevity as economic growth and income levels improve (Ahn et al., 2013; Hee et al., 2012). This social change has led to increased purchases of healthy functional foods and natural products, boosting the market for the distribution of various products using medicinal plants (Ahn et al., 2013). To meet the growing demand, naturally collected herbal medicines are rapidly being replaced by artificial cultivation methods in natural harvesting, and various kinds of pesticides are used to control insects, weeds, and other upsurgance pests during the growing season. It is also true that some people are concerned about the safety of pesticide residues in domestic as well as the imported medicinal herbs (Ahn et al., 2013; Kim et al., 2012; Hwang et al., 2011). According to the Ministry of Food and Drug Safety, MFDS (Notice No. 2018-68) currently, in Korea, there are 17 methods used for the analysis of 61 pesticides in herbal medicines (MFDS, 2018). Pesticide residues analytical methods in herbal materials are relatively insufficient compared to those in foods. The accredited analytical laboratories in Korea for analysis of pesticides in herbal materials followed the food methods for pesticide residues in herbal medicines. Therefore, it is essential to establish an optimized pesticide residue analytical method in herbal medicines (Kim

*Corresponding author
E-mail: jhhur@kangwon.ac.kr

et al., 1999; Cho et al., 2001). *R. glutinosa* L., selected in this study, is reported in previous work to have medicinal values (Lee et al., 2011; Oh et al., 2013). However, it's difficult to secure stable production due to Pythium root rots disease and insect pests attacks such as beet armyworms (*Spodoptera exigua*) and castor moths (*Agrotis segetum*) (Park et al., 2003; Lee et al., 2018; Kang et al., 2016). In particular, the larvae of the beet armyworm (*Spodoptera exigua*) appear before cultivation and throughout the growth period of *R. glutinosa* root, and the damage is more severe during the early stages of the growth. Therefore, chlorantraniliprole is registered and used as a specialized insecticide to control Lepidoptera, Coleoptera, Diptera, and Isoptera (Turner, 2015), but the Korean Pharmacopoeia does not list the residue analytical method for chlorantraniliprole in herbal medicines (An, 2012; KCPA, 2013). To analyze chlorantraniliprole in the *R. glutinosa* root, the method listed by the MFDS is also openly applied. However, this method resulted in fully saturated with interferences materials, because the method is specialized for pesticides analysis in agricultural products, and not for herbal medicines and therefore, not suitable for purified analyte samples in herbal medicine. In this case, due to the complex matrix of the *R. glutinosa* root, it is essential to study developing a new analytical method with a high efficiency using conventional analytical instruments. Thus, the current study aimed to establish an optimized analytical method for chlorantraniliprole residues in the *R. glutinosa* root and contribute to the safety of human health regarding pesticide residues in herbal medicines.

Materials and Methods

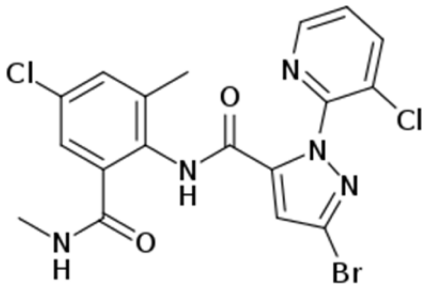
Reagents and instruments

The chlorantraniliprole standard (98.0% purity) used in this study was purchased from Sigma Aldrich (USA). Reagents used for analysis include acetonitrile, water (HPLC-grade), and other reagents used for residual analysis were purchased from Fisher Scientific (USA). SPE cartridges filled with silica and NH_2 were purchased from Phenomenex (USA). SPE Vacuum manifolds (visiprepTM) were purchased from Supelco (USA). A shaker machine for extracting samples was obtained from Lab Companion IS-97IR, JeioTech, Korea. The vacuum rotary evaporator (Eyella NE-1001) and centrifuge (Allegra X-1R, Beckman) used in this work were purchased from, Tokyo Rikakikai Co. Ltd., Japan, and Culter Life Sciences, USA, respectively. The chemical structure formula of chlorantraniliprole used is shown in Table 1 (Turner, 2015).

Herbal medicines

Dried *R. glutinosa* root was purchased from the herbal medicine bureau (Yaksan, North Jeolla Province, Korea) with distribution products that conform to the herbal medicine standard. The *R. glutinosa* root was used after the sensory evaluation done by herbal medicine expert according to the herbal medicines test criteria. In addition, to check the

Table 1. Physicochemical properties of chlorantraniliprole

	Chlorantraniliprole
Structure	
IUPAC name	3-Bromo-*N*-[4-chloro-2-methyl-6-(methylcarbamoyl)phenyl-1-(3-chloropyridin-2-yl)-1*H*-pyrazole-5-carboxamide
Mol. wt.	483.15
CAS No.	500008-45-7
V.p. (mPa)	$6.3\ 10^{-9}$ (20°C), $2.1\ 10^{-8}$ (25°C)
K_{ow}logP	2.76
Solubility in water	0.9~1.0 mg/L (20°C), pH 7

analysis of interference materials in *R. glutinosa*, dried herbal medicine from the distribution stores at Chuncheon and Seoul, Republic of Korea were purchased and used. *R. glutinosa* root uses its roots as a medicinal ingredient, and it is used as an analytical specimen after being prepared by grinding the dried roots with a pulverizer machine and sieved through a No. 2 mesh sieve to obtain a homogeneous sample.

Extraction and Clean up Procedure

The herbal medicine, *R. glutinosa* is traditionally used after preparing by poured boiling water on the roots; therefore, water and acetonitrile were used as extraction solvents. The extraction followed the method accredited by MFDS (2017); by adding 20 mL of distilled water to 5 g of *R. glutinosa* root and wetted for 1 hour in a polyethylene-centrifuged bottle 200 mL, and then 50 mL of acetonitrile was added and shaken for 30 minutes at 300 rpm. Afterward, 5 g of $MgSO_4$ and 7.5 g of NaCl were added to the bottle containing extract and subject to centrifugation for 10 minutes at 4°C and 400 rpm. The amount of 25 mL of centrifuged supernatant solution was transferred to a separatory funnel (capacity 500 mL) and cleaned with 100 mL of *n*-hexane. Then 100 mL of distilled water and 50 mL of saturated sodium chloride solution were added to a separatory funnel 500 mL, and the concentrated extract was distributed and extracted with 100 mL of dichloromethane. After dehydrating the lower layer of dichloromethane by passed through anhydrous sodium sulfate and further to fully utilized the remaining residues in the aqueous phase was re-extracted again with 100 mL of dichloromethane and passed through anhydrous sodium sulfate. The collected dichloro-methane layers were combined and fully concentrated in a vacuum rotary evaporator at 40°C and then re-constituted in a 5 mL mixture of *n*-hexane/ethyl acetate (90/10, v/v).

Clean-up by Silica SPE Cartridge

Clean-up was done by passing the extracts through the silica SPE cartridge (1 g). The SPE cartridge was activated by pre-washing with *n*-hexane 5 mL. The extracted sample was passed through the column and then the cartridge was washed with 15 mL *n*-hexane/ethyl acetate (80/20, v/v) and 15 mL *n*-hexane/ethyl acetate (70/30, v/v). Finally, the column was eluted with 10 mL *n*-hexane/ethyl acetate (50/50, v/v) mixture. The collected elution solvent was concentrated to dryness under a vacuum rotary evaporator at 40°C and re-dissolved in 5 mL of a mixed *n*-hexane/acetone (90/10, v/v) and used as a specimen for secondary clean-up.

Clean-up by NH_2 SPE Cartridge

The secondary clean-up was done using an NH_2 SPE cartridge. The NH_2 SPE cartridge (1 g) is pre-washed with 5 mL of *n*-hexane. The primary extract that is cleaned by Silica SPE passed through the NH_2 SPE cartridge column, then washed with 20 mL *n*-hexane/acetone (90/10, v/v) mixture, followed with 8 mL *n*-hexane/acetone (80/20, v/v), respectively which is discarded. The column was then eluted with 10 mL *n*-hexane/acetone (70/30, v/v) mixed solvent. The elution was concentrated to dryness under a vacuum

Table 2. The HPLC-UVD operating condition for analysis of chlorantraniliprole

HPLC	DIONEX Ultimate 3000 (Thermo Science, USA)		
Detector	Diode Array Detector (DAD)		
Column	Capcell pak-C_{18} (4.6 mm I.D. × 250 mm, 5.0 μm) Shiseido, Japan		
Oven temp.	40°C		
Mobile phase	A:B = water:acetonitrile = (v/v)		
	Min.	A (%)	B (%)
	0.0	60	40
	5.0	60	40
	10.0	30	70
	13.0	30	70
	15.0	60	40
	20.0	60	40
Flow rate	1.0 mL $min.^{-1}$		
Injection vol.	30.0 μL		
Wavelength	230 nm		

rotary evaporator at 40°C and re-dissolved in 10 mL of acetonitrile/water (50/50, v/v) mixture.

HPLC-UVD/MS Instrument Analysis

In this study, the analytical method of chlorantraniliprole was reviewed using HPLC-UVD, taking into consideration the physiochemical characteristics of the analyte (chlorantraniliprole), and the residue of chlorantraniliprole was reconfirmed by HPLC-MS. The HPLC DIONEX Ultimate 3000 (Thermo Science, USA) equipped with Shiseido Capcel Pak-C18 column (4.6 mm I.D. × 250 mm, 5.0 μm, Shiseido, Japan) was used to analyze chlorantraniliprole samples. The instrument condition was shown in Table 2. Moreover, the instruments HPLC DIONEX Ultimate 3000 (USA) and Mass Spectrometer TSQ Quantum Extra (USA) were used to verify the identity of chlorantraniliprole residue detected by HPLC DIONEX Ultimate 3000 (Thermo Science, USA). The Unison UK-C18 column (2.0 mm I.D. × 150 mm, 3.0 μm, Imtakt, USA) was used. The instrument condition was given in Table 3 and the selected reaction monitoring (SRM) condition for chlorantraniliprole is presented in Table 4.

Standard calibration curve

The stock solution of chlorantraniliprole standard (98.0% purity) was prepared by accurately taken 10.20 mg, placed in a 100 mL volumetric flask then topped up to the mark with acetonitrile. To improve the peak shape, the working solution was prepared with an acetonitrile/water (5/5, v/v) mixed

Table 3. The HPLC-MS operating condition for analysis of chlorantraniliprole

HPLC condition			
HPLC	DIONEX Ultimate 3000 (Thermo Science, USA)		
Column	Unison UK-C_{18} (2.0 mm i.d. × 150 mm, 3.0 μm), Imtakt, USA.		
Oven temp.	40°C		
Mobile phase	A:B = 0.1% formic acid in water:0.1% formic acid in acetonitrile = (v/v)		
	Min.	A (%)	B (%)
	1.0	80	20
	1.5	80	20
	3.0	5	95
	6.0	5	95
	6.1	80	20
	8.0	80	20
Flow rate	0.3 mL $min.^{-1}$		
Injection vol.	1.0 μL		
MS condition			
Detector	TSQ Quantum Ultra (Thermo science, USA)		
Ionization	Electrospray ionization (ESI), positive-ion mode		
Spray voltage	Positive polarity 3,500 V		
Capillary temp.	320°C		
Vaporizer temp.	300°C		
Ion sweep gas pressure	1.0 Arb		
Sheath gas pressure	45.0 units		
Aux gas pressure	5.0 units		
Mass range (m/z)	200~600		

Table 4. Selected reaction monitoring (SRM) condition for chlorantraniliprole

Compound	Molecular weight	Parent ion	Product ion	Collision Energy
Chlorantraniliprole	483.2	484.72	286.19[a)] 453.36[b)]	17 18

[a)] Qualitative ion
[b)] Quantitative ion

solution considering the 30 µL of the injection volume. Accordingly, the stock solution was serially diluted to concentrations of 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mg/L, and then 30 µL aliquot was injected into HPLC, and the response is used for the construction of the standard calibration curve.

Analytical limit of quantitation

After the establishment of the above analytical method, the limit of the quantitation of the analytical method was calculated by equation (1) taking into consideration the amount of the following; the minimum detection, sample (supernatant amount of extracted solvent), test solution, device injection, and dilution factor or enrichment drainage by analysis operation (Lee et al., 2017; Leem et al., 2019).

$$A(ng)\times\frac{B(mL)}{C(g)}\times\frac{D(mL)}{E(mL)}\times\frac{F}{G(\mu L)}=MLOQ(mg/kg) \quad (1)$$

A: Minimum amount of detection
B: Volume of extraction solvent
C: Sample amount
D: Final Volume
E: The supernatant amount taken from the extracted solvent
F: Dilution factor
G: Injection quantity

Recovery rate test

The recovery rate of the chlorantraniliprole was tested to verify the suitability and precision of the established analytical method. The recoveries were taken after triplicate times of chlorantraniliprole standard to the *R. glutinosa* root-free sample at 1 time MLOQ (0.02 mg/L) and 10 times MLOQ (0.2 mg/L) levels. The RSD was calculated after triplicate repetition.

Cross-laboratory validation

Cross-validation was carried out using the same standards and solvents that were used in the current study. The cross-laboratory followed all processes done in the tested sample under the study; Extraction and partitioning, double clean up with SPE cartridges (SPE-silica and SPE-NH_2), and instrumental analysis. The MLOQ, recovery rate and RSD were calculated.

Results and Discussion

HPLC-UVD Analysis Condition Setup

This study was conducted to develop a possible methods for the analysis of chlorantraniliprole in *R. glutinosa* root using HPLC-UVD analysis rather than GLC (MFDS, 2017; Tomlin, 2009). The reasons behind that the chlorantraniliprole are; a very low vapor pressure (6.3°C 10^{-9} mPa (20°C)), low volatility, and the amide groups in the molecule are vulnerable to heat in GLC analysis. For pesticides, residue analysis the MFDS recommended the analysis wavelength for HPLC at 254 nm; accordingly, the wavelength 254 nm was used in the analysis of chlorantraniliprole in this study (MFDS, 2017). The retention time of chlorantraniliprole was 14.07 minutes when analyzed with the single component analysis conditions of the MFDS method. However, this

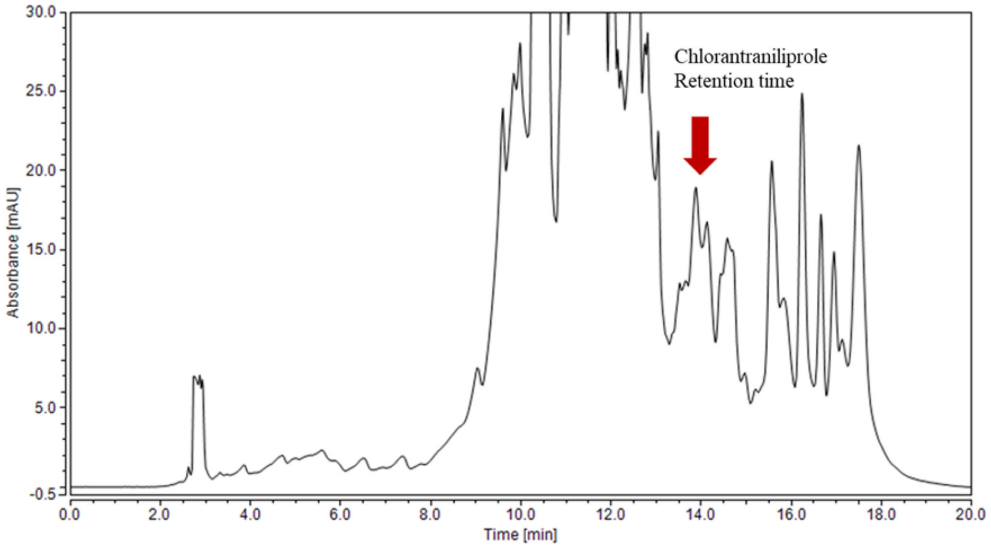


Fig. 1. HPLC-UV chromatogram of the *R. glutinosa* root sample analyzed by MFDS method.

method is specialized for agricultural products and not for herbal medicines so the clean up and analysis need optimization. For this, the results of the analysis conditions indicated that it is difficult to effectively separating the large number of interfering substances contained in the *R. glutinosa* root (Figure 1). Thus, the shape of the peak had to be improved. The improvement could be done by adjusting the ratio of movement; therefore, the chromatographic analysis was performed with a gradient condition in which the ratio of movement was held for five minutes with a mixture of acetonitrile/water (40/60 v/v), then held for eight minutes at the rate of acetonitrile/water (70/30, v/v), and finally held for seven minutes at acetonitrile/water (40/60, v/v). The applied analysis conditions resulted in a shape of the sharp peak and relatively improved the peak shape and resolution of interfering materials from that in the conventional analysis conditions. However, the retention time of the chlorantraniliprole analyzed under the optimized conditions was 12.3 min, but the complete separation of the targeted compound from the interference materials was not successfully achieved.

Extraction and Partitioning Process Setup

To select the optimal extraction solution of *R. glutinosa* root, among the various solvents (acetone, acetonitrile, and methanol) are commonly used in pesticide residues analysis, acetonitrile was selected (Jang et al., 2015; Lee et al., 2017). Acetonitrile is a universal solvent used to extract non-polar as well as intermediate-polar pesticides and has been recognized for it is efficiency, reproducibility in pesticide extraction and resulted in fewer interfering impurities (Choung, 2019; Tengfei et al., 2019). In addition to the target analyte, the liquid-liquid partitioning was applied during chlorantraniliprole extraction to remove interfering substances from the *R. glutinosa* root extract (Ahn 2015). The previous works revealed that *R. glutinosa* root contained a variety of ingredients, such as fatty acids, catalpol, and glucose, approximately 78% of which are carbohydrates and have slightly different general ingredients (Oh et al., 2012; Lee et al., 2004). To remove non-polar interfering substances from the *R. glutinosa* root, acetonitrile/*n*-hexane was used, while the polar substance was removed by using dichloromethane (Lee et al., 2009). Furthermore, the solubility of chlorantraniliprole is known as < 0.0001 g/L in *n*-hexane, 0.711 ± 0.072 g/L in acetonitrile, and 2.476 ± 0.058 g/L in dichloromethane (Turner, 2015; Tomlin, 2009). Thus, liquid-liquid partitioning was carried out using 100 mL of acetonitrile/*n*-hexane which confirmed that 101.8% of the acetonitrile layer was recovered. On the other hand, the dichloromethane/d-water partitioning achieved 111.9% recovery from the dichloromethane layer. Therefore, it was to prove that to perform a two-time partitioning process, primarily with acetonitrile/*n*-hexane and secondary with dichloromethane/d-water to remove various substances in *R. glutinosa* root.

Optimization of SPE Cartridge Clean-up Conditions

The extraction and partitioning process previously described by the MFDS was followed for chlorantraniliprole residue analysis in *R. glutinosa* root, but there were still many interfering substances, requiring additional clean-up processes. The method of cleaning up by adsorption chromatography generally used for pesticide residue analysis is a method in which adsorbent materials (such as silica gel, alumina, and

Table 5. Recovery rate by sequential elution of different elution mixtures

SPE cartridge (1 g)	Elution solvent mixture	Elution Solvent ratio (v/v)	Recovery rate (%)			
			1~5 mL	6~10 mL	11~15 mL	Total
Silica [a)]	*n*-hexane/ethyl acetate	80/20	<10	<10	<10	<10
		70/30	<10	<10	<10	<10
		50/50	91.2	7.9	<10	99.1
		Elution Solvent ratio (v/v)	Recovery rate (%)			
			1~10 mL	11~20 mL		Total
NH_2 [b)]	*n*-hexane/acetone	90/10	<10	<10		<10
		80/20	1~4 mL	5~8 mL		Total
			<10	<10		<10
		70/30	1~5 mL	6~10 mL		Total
			86.9	13.0		99.9

[a)] *n*-hexane 5 mL pre-washing, *n*-hexane/ethyl acetate (90/10) 5 mL (+ 5 mL) loading
[b)] *n*-hexane 5 mL pre-washing, *n*-hexane/acetone (90/10) 5 mL (+ 5 mL) loading

florisil) are filled in a glass tube, which takes a long time for pesticides to eluate. Hence, a solid-phase extraction (SPE) method was adopted to process a large number of samples quickly and effectively, and therefore, a vacuum manifold was used to maintain a constant discharge rate (1-2 mL/min) (Yim et al., 2013).

Silica SPE cartridge

In this study, the cleaning up conditions accredited by MFDS (2017) was applied using silica SPE cartridge for removing a large number of interfering substances contained in herbal medicines. For establishing optimal elution solvent conditions (MFDS, 2017) the elution solvent was performed

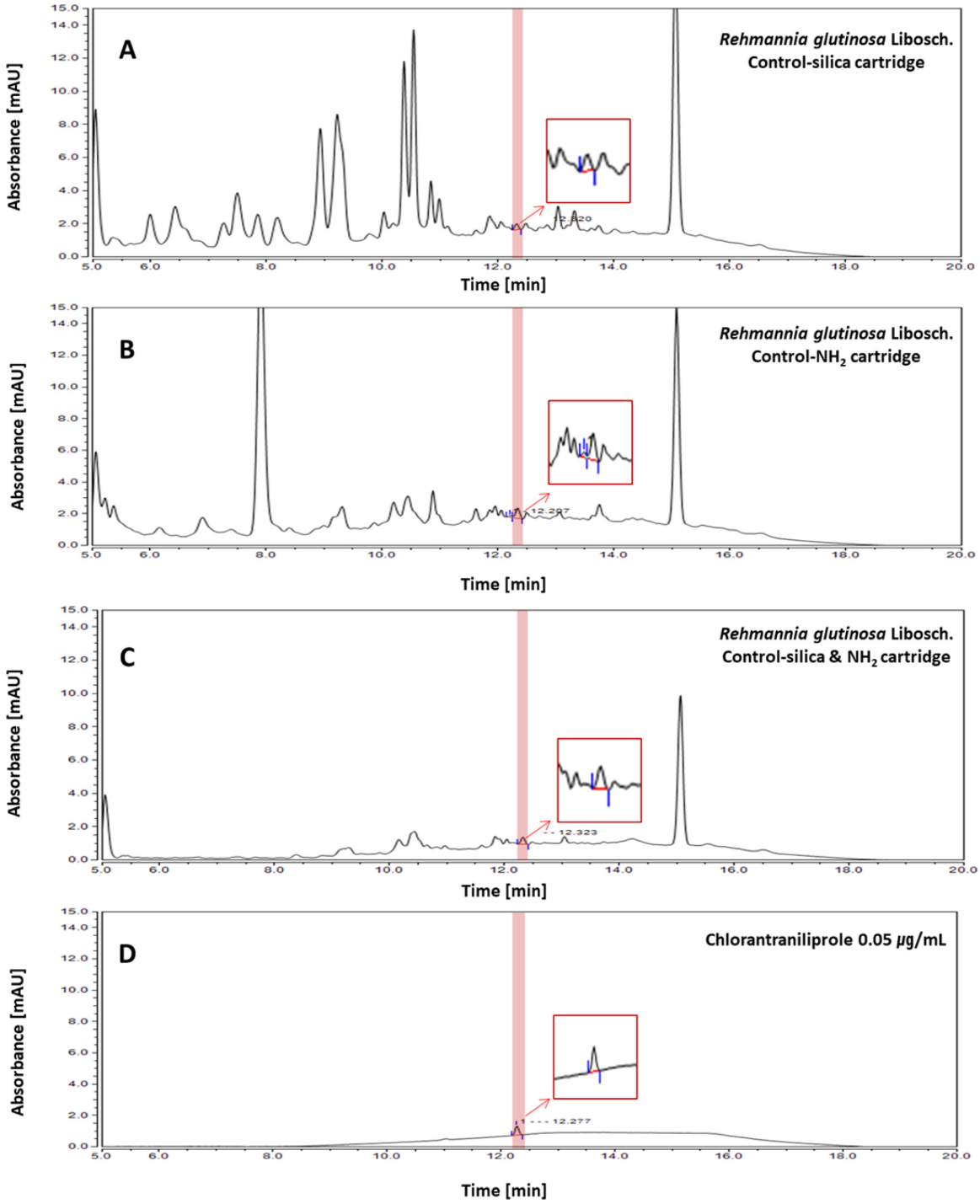


Fig. 2. HPLC chromatograms of using silica SPE cartridge, NH_2 SPE cartridge and Both SPE (silica & NH_2) cartridges at 254 nm [A: *R. glutinosa* root control after silica SPE cartridge cleanup, B: *R. glutinosa* root control after NH_2 SPE cartridge cleanup, C: *R. glutinosa* root control after silica SPE cartridge & NH_2 SPE cartridge cleanup, D: chlorantraniliprole 0.05 μg/mL].

by altering the *n*-hexane/ethyl acetate mixture, and no pesticides were detected when *n*-hexane/ethyl acetate = 80/20 and 70/30 (v/v) mixed solvents were passed, further when eluted with *n*-hexane/ethyl acetate = 50/50 (v/v) 99% or more were recovered (Table 5). However, many interfering substances were observed near the retention time (12.3 min.) of chlorantraniliprole and it was not possible to effectively exclude the interference when using a silica SPE cartridge alone (Figure 2 A). Therefore, it was essential to check the cleaning efficiency by using NH_2 SPE cartridge, which is a weak anion exchange cartridge, instead of silica SPE cartridge using adsorption power.

NH_2 SPE cartridge

NH_2 SPE cartridge is a method of utilizing a weak anion exchange cartridge in which ion or charged compounds binding with ion exchange resin by electrostatic forces to achieve equilibrium (Acikara, 2013). The weak ion exchange adsorbent aminopropyl was selected to remove a large number of interfering substances contained in *R. glutinosa* root. The elution solvent was performed by adjusting the elution strength of the *n*-hexane/acetone mixed solvent, and the analyte was not detected when the mixed solvent *n*-hexane/acetone = 90/10 and 80/20 (v/v) was used, and when eluted with *n*-hexane/acetone = 70/30 (v/v) satisfactory recovery rate was obtained (Table 5). However, it was indicated that it is not possible to effectively clean the interference substances even when the NH_2 SPE cartridge is used alone (Figure 2 B).

Silica SPE + NH_2 SPE cartridges

As a result, when silica SPE cartridge and NH_2 SPE cartridge were used alone, it was difficult to remove the interference substance from *R. glutinosa.* Therefore, a double clean-up method with consecutive use of SPE cartridges containing different absorbent materials was used. In other words, the primary clean-up was carried out with silica SPE cartridge, then NH_2 SPE cartridge was continuously applied to eliminate as much as possible complex matrix contained. The double clean-up conditions utilizing the two types of SPE cartridges considered in the above clean-up process are as follows; First, a 15 mL of the mixed *n*-hexane/ethyl acetate = 80/20 and 70/30 (v/v) passing each through silica SPE cartridge using adsorption strength, which is discarded, and then eluted with 10 mL of *n*-hexane/ethyl acetate = 50/50 (v/v) and the elution was concentrated. Afterward, NH_2 SPE cartridges, were continuously used to clean-up, and the mixture *n*-hexane/acetone = 90/10 and 80/20 passing through the column at 20 mL and 8 mL respectively, and discarded, and then eluted with *n*-hexane/acetone = 70/30 (v/v) 10 mL and the recovery rate was greater than 91%. However, even after the double clean-up process consecutively used, it was found that the degree of interference near the retention time of the chlorantraniliprole exceeded 30% of the minimum quantitation level (Figure 2 C). This result contradict with that of MFDS standard which stated, the analytical components should be within 30% of quantitative level during device analysis and therefore the current method is not met the criteria of residue analysis.

Vacuum manifold efficiency

A vacuum manifold is a device designed to provide increased efficiency and performance when used with many columns for purification. It is also capable of processing many samples simultaneously. In particular, in this analysis, vacuum manifold devices were essentially used to minimize the time required during the double clean-up process, and the elution rate was maintained at 1 mL/min. (or 68 to 70 drop/min). The elution time was approximately 1 hour, resulting in a reduction of clean-up time by approximately 3 times (20 minutes) compared to that simple used of gravity (Figure 3).

Effect of Change of Detection Wavelengths

According to the MFDS (2017), the detector wavelength for HPLC analysis was 254 nm when analyzing chlorantraniliprole in foods, but in the case of herbal medicines such as *R. glutinosa*, it was difficult to analyze due to interference that occurs during the same period of stay. In particular, the analytical method was insufficient to satisfy 30% of the minimum quantitation limit level due to interfering substances detected even after the cleaning up conditions were improved by the double clean-up method. To solve this problem, the absorbance of different wavelengths between 190 and 360 nm was scanned for optimal wavelengths that have low sensitivity of the interfering substances appears at the same retention time (12.3 min.) when chlorantraniliprole is detected and for improving the sensitivity of the analyte. As a result, the interfering substances showed a relatively low sensitivity compared to the recommended 254 nm in the wavelengths band around 230 nm and above 300 nm (Figure 4 A). It was also able to identify a tendency for the analysis analyte to show a higher absorption at 230 nm than 254 nm at the same retention time (12.3 min.) (Figure 4 B), and minimizing the

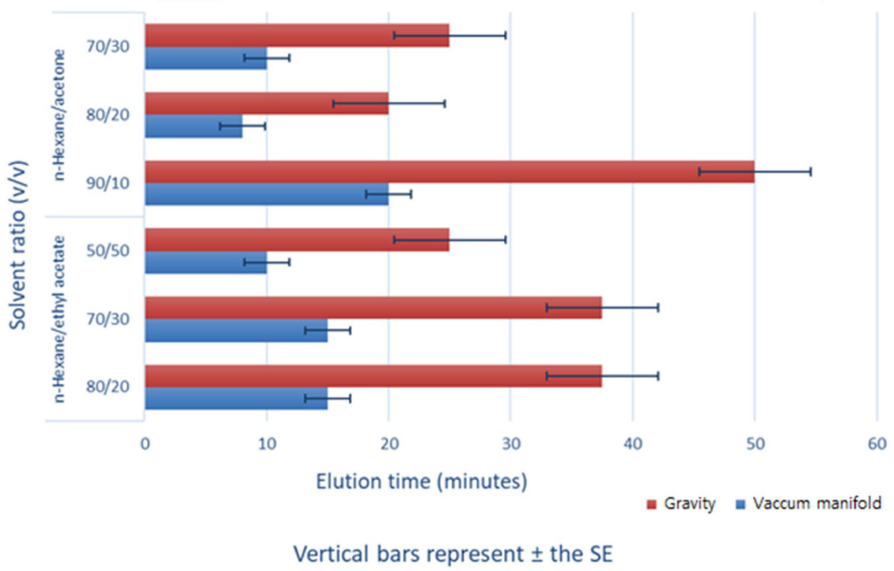


Fig. 3. Comparison of elution rates with manifold and gravity.

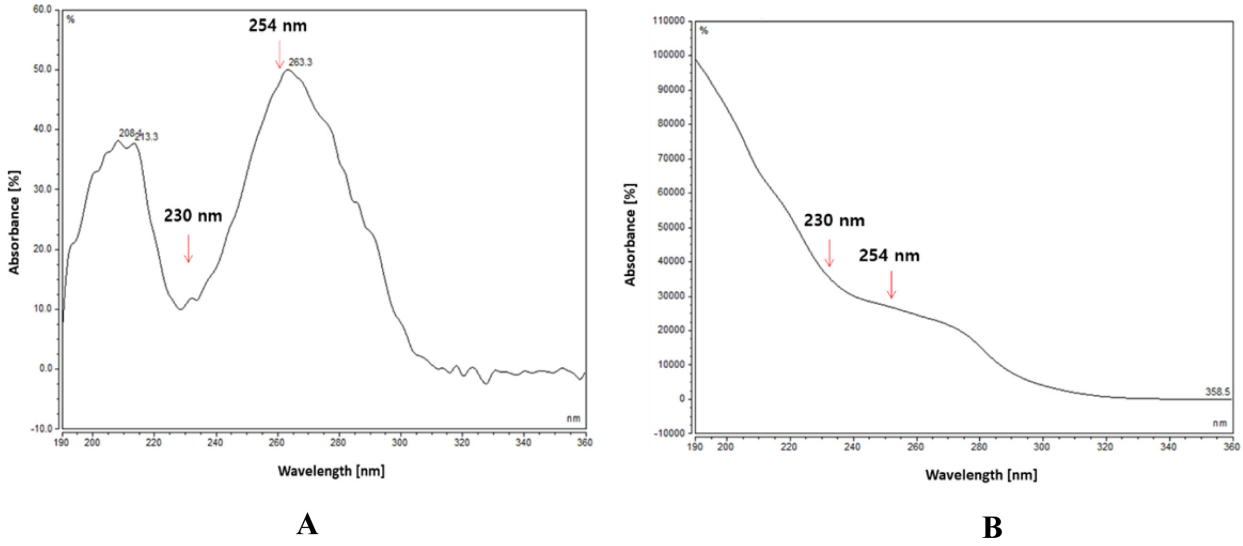


Fig. 4. A: Comparison of absorbance on interfering substance at wavelengths of 254 nm and 230 nm in HPLC, B: UV absorption spectrum of chlorantraniliprole.

effects of the detection interference. The application of the analysis conditions resulting in relatively reducing the sensitivity of the interfering substance and also the selectivity of the analyte was relatively improved approximately 1.5 times (Figure 5). Besides, the effects of changing wavelengths and the same interfering substances were confirmed in the other two types of dried *R. glutinosa* samples purchased from the market, confirming that the current method not suitable for chlorantraniliprole residue analysis in *R. glutinosa*. The study also found that the area value of the quantitatively limited level (0.05 ppm) of chlorantraniliprole was 0.0639, the same retention time of interference peak area value of untreated *R. glutinosa* was 0.0083, showing a difference of approximately 8 times, and finally 13.0% of the quantitatively limit was available for analysis. Furthermore, the number of the theoretical plate calculations when using UV absorption spectrum 254 nm the values were 95971.77, 47894.83, and 195663.37 for silica, NH_2 and double (silica+NH_2) clean up methods respectively (Figure 2). This indicates that the double clean up method is better than the single-use of SPE when using 254 nm, but the interfering peak is still in the same retention time of chlorantraniliprole. However, after

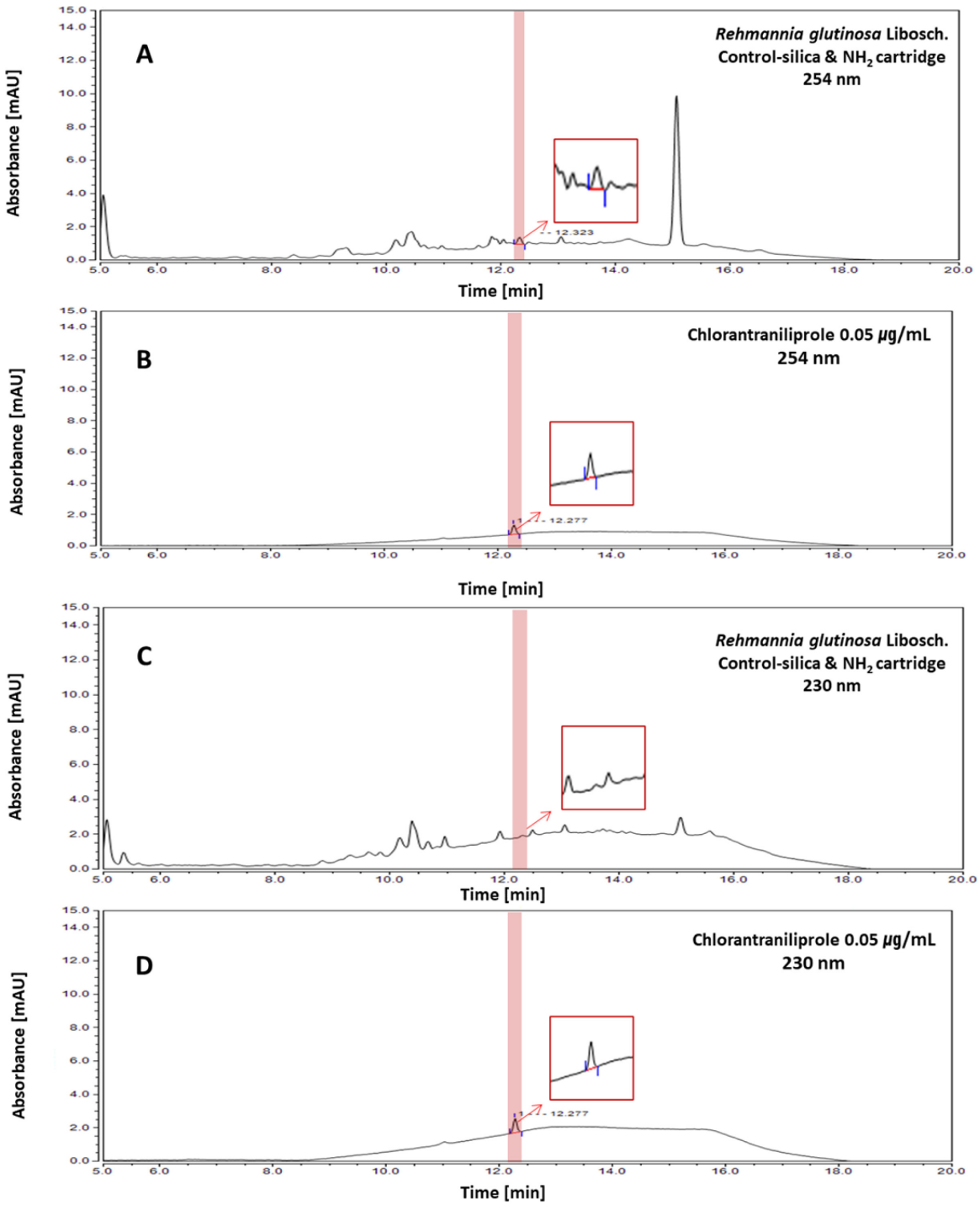


Fig. 5. HPLC chromatogram after cleanup using silica SPE cartridge & NH_2 SPE cartridge at 254 nm and 230 nm [A: *R. glutinosa* root. control after silica SPE cartridge & NH_2 SPE cartridge cleanup at 254 nm, B: chlorantraniliprole 0.05 μg/mL at 254 nm, C: *R. glutinosa* root control after silica SPE cartridge & NH_2 SPE cartridge cleanup at 230 nm, D: chlorantraniliprole 0.05 μg/mL at 230 nm].

improvement carried out by changing the UV absorption spectrum to 230 nm this interfering peak is significantly disappeared. The value of selectivity factor of the adjacent interfering substance was 1.0148, which indicates that the separation is considered relatively not good resolution, however the improvement of the method is not successfully achieved. In fact the analysis of *R. glutinosa* matrix need high level of refining method. Thus, it was difficult to completely separate chlorantraniliprole from the *R. glutinosa* matrix by using the current methods and therefore, further

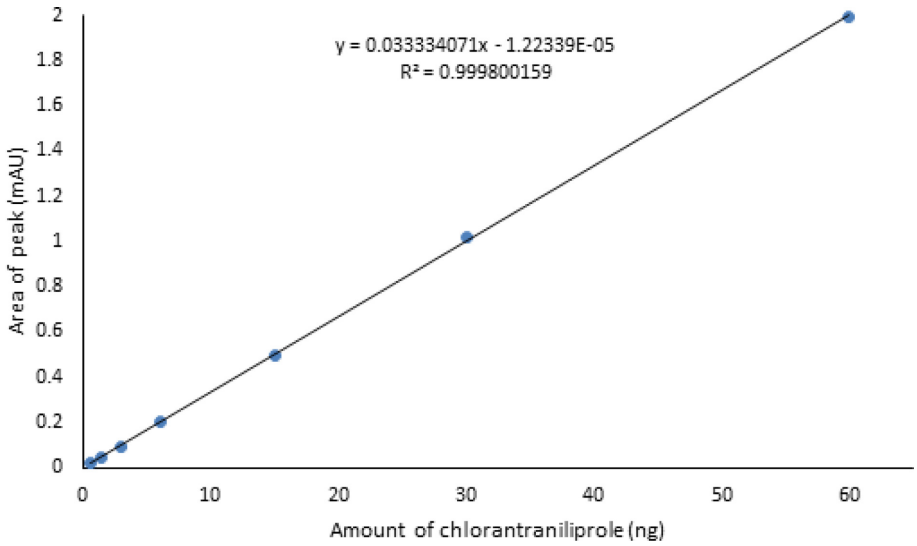


Fig. 6. Calibration curve of chlorantraniliprole standard solution using HPLC-UVD.

appropriate improvement measures of developing effective refining and separation method was suggested.

Standard Calibration Curve

The peak shape was improved by serial diluting the concentration of chlorantraniliprole standard solution to 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mg/L using acetonitrile/water (5/5, v/v). The injection volume was 30 μL. The regression equation of the calibration curve obtained under the conditions of the instrument analysis was $y = 0.033334071x - 1.22339E^{-05}$ ($R^2 = 0.9998$), showing excellent linearity and high correlation between the area of peak and the concentrations of the standard solution (Figure 6). The instrumental limit of detection (ILOD) and instrumental limit of quantification (ILOQ) are calculated by the minimum of the analyte providing an S/N ratio of > 3 and S/N ratio> 10, respectively. Thus, the ILOD of this analysis method was 0.02 ng (S/N >3), and ILOQ was 0.05 ng (S/N >10) and the minimum detection was 1.5 ng when the injection volume was 30 μL.

Analytical Limit of Quantitation

The method limit of quantitation (MLOQ) refers to the lowest concentration of the analyte that can be reliably quantified by considering the minimum quantitation limit, sample volume (supernatant amount), and dilution factor during analysis operation (Equation (2)). The manual of the pesticide residue analytical method in MFDS recommends that the detection should be less than 0.05 mg/kg of the standard for pesticide residue analysis, or less than a half of the MRLs (MFDS, 2017; Oh, 2012; Yoon et al., 2013). In this study, the analytical quantitation limit calculated by equation (1) was 0.02 mg/kg. In other studies, the LOQ values were found to be 0.10 mg/kg in vegetables (Singh et al., 2012) and 0.06 mg/kg in grapple (Malhat, 2012). In addition, the MLOQ of the current study is in agreement with that of the European Commission LOQ value (0.02 mg/kg) in herbal infusion (steaming from leaves and herbs) (ECMRL, 2021). This indicates that this method can be used in determination of chlorantraniliprole in other medicinal herbs and agricultural products (contain less active materials) that usually go through one-time refining process.

$$1.5(\mathrm{ng}) \times \frac{50(\mathrm{ml})}{5(\mathrm{g})} \times \frac{1(\mathrm{ml})}{25(\mathrm{ml})} \times \frac{1}{30(\mu\mathrm{l})} = 0.02(\mathrm{mg/kg}) \quad (2)$$

Recovery Rate Test

After spiking the chlorantraniliprole standard solution of 1 MLOQ (0.02 mg/kg) and 10 MLOQ (0.2 mg/kg) to the *R. glutinosa* root-free sample, the previously established method was triplicated and the analysis results were compared against throughput and evaluated for accuracy and reproducibility of the analytical method (MFDS, 2017). The chromatogram of *R. glutinosa* non-treated sample confirmed that there was no interfering substance found at the same retention time as that of chlorantraniliprole (Figure 7 C) compared to the MFDS methods (Figure 1). The recovery rate values of chlorantraniliprole were ranged from 102.4 to 105.2% at the 1MLOQ (0.02 mg/kg), and 87.9 to 93.1% at the 10 MLOQ (0.2 mg/kg) for HPLC-UVD, while it were 104.9 to 109.6% and 88.0

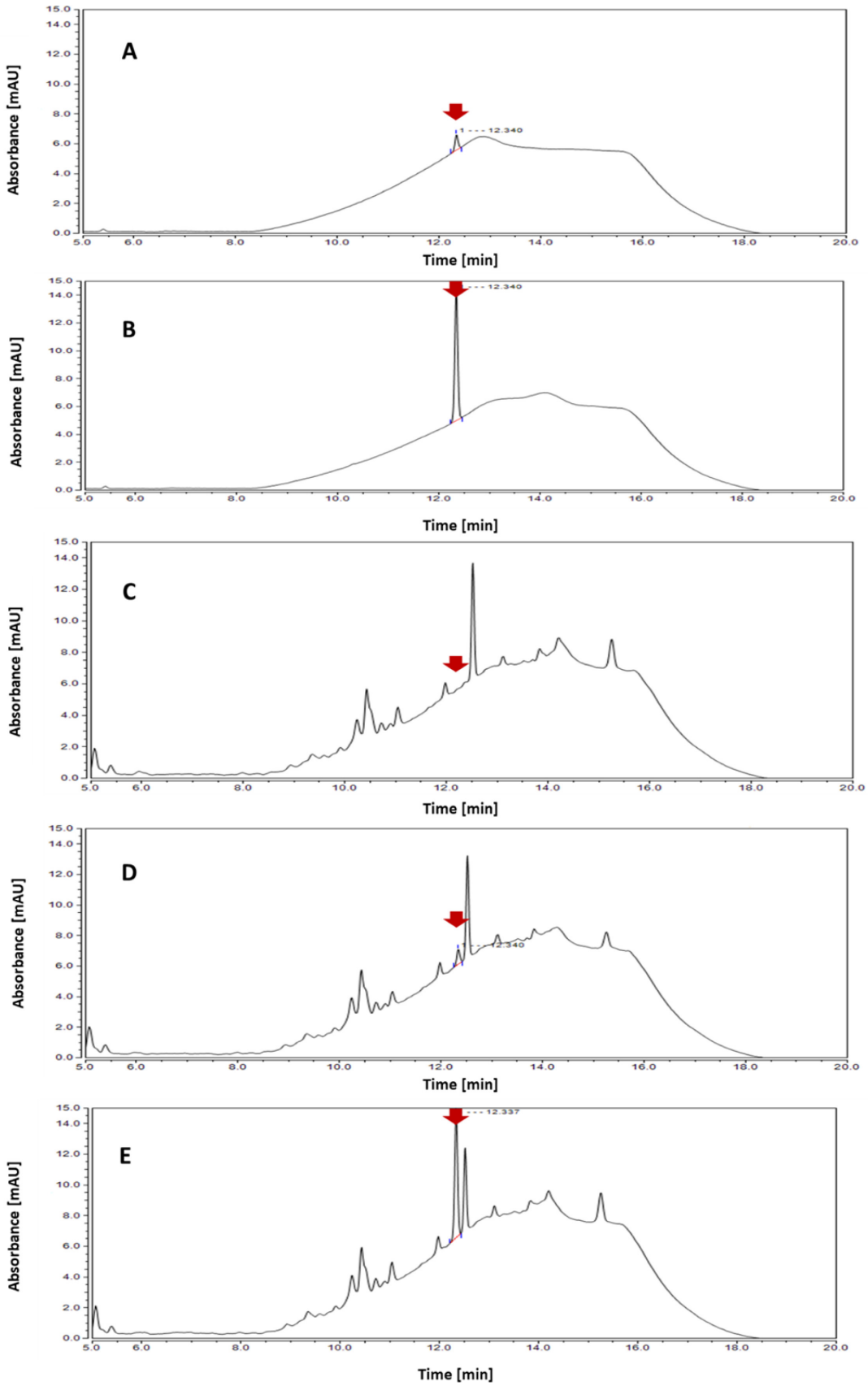


Fig. 7. HPLC chromatograms of residue analysis of chlorantraniliprole in *R. glutinosa* root. [A: chlorantraniliprole 0.05 μg/mL, B: chlorantraniliprole 0.5 μg/mL, C: control, D: 1 MLOQ (0.02 μg/mL), E: 10 MLOQ (0.2 μg/mL)].

Table 6. Recovery rate and MLOQ for chlorantraniliprole in *R. glutinosa* root

Fortification (mg/L)	Recovery (%)[a)]	RSD(%)[b)]	MLOQ (mg/kg)
0.02	104.3 ± 1.6	1.6	0.02
0.2	91.0 ± 2.7	3.0	

[a)] Mean values of triplicate samples with standard deviations
[b)] RSD (relative standard deviation, %) = standard deviation/average × 100

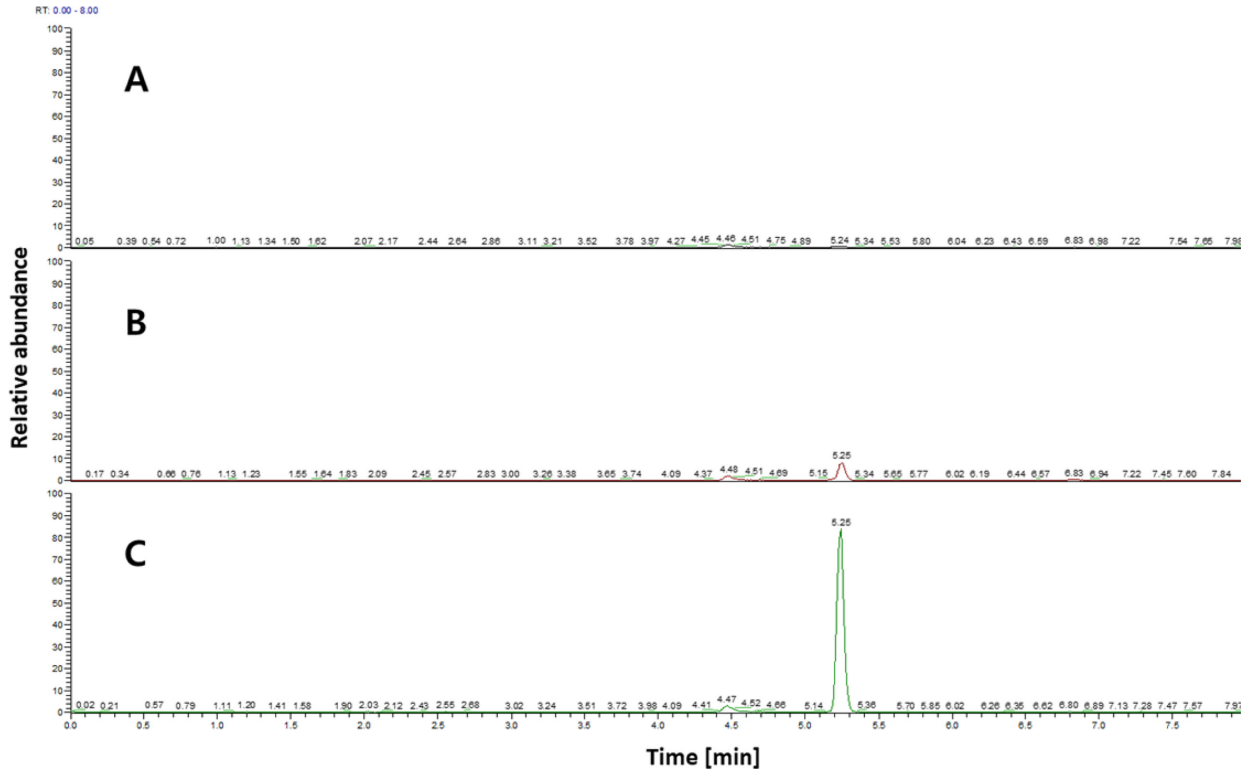


Fig. 8. SIM chromatograms of control and spiked samples of chlorantraniliprole in *R. glutinosa* root [A: control, B: 1 MLOQ (0.02 μg/mL), C: 10 MLOQ (0.2 μg/mL)].

to 92.8% for LC-MS. The RSD were 1.6-3.0% and 2.0% at a 0.02 and 0.2 μg/mL spiked concentrations for HPLC-UVD and LC-MS, respectively. This result is in line with other studies where the recovery rates were found to be 85.0-96.0% in vegetables (Singh et al., 2012), 95.2-102.0 % in grapple (Malhat, 2012), and 95.2-108.0% in fresh tea leaves (Tengfei, 2019) when they were using HPLC-PDA, HPLC-DAD, and GC-ECD, respectively. The results of the RSD satisfied the criteria for residual analysis with a recovery rate of 70-120% and an RSD of 10% or less within the repeated recovery rate values of the MFDS in the Republic of Korea (2017) (Table 6), but not successfully separate the interference peak from the chlorantraniliprole.

Confirmation Test

When analyzing substances whose type and composition of interference is unknown, such as in pesticide residue analysis, there is a possibility of qualitative trace even if a trace of interfering substances with similar characteristics are present, hence, a confirmation for qualitative reliability is essential and badly needed (Lee and Jang, 2010; Lee et al., 2011). To secure the qualitative reliability of chlorantraniliprole, molecular ions induced from the analytes were identified by LC-MS using the method established in the present study (Lee et al., 2011; Lee et al., 2019). A system operation conditions used for the HPLC-UVD analysis were also applied in the LC-MS analysis system, however, similar non-significant recovery values were obtained (104.9 to 109.6% at 0.02 mg/kg, and 88.0 to 92.8% at 0.2 mg/kg, respectively). The LC-MS operated in the positive ion mode using electron spray ionization (ESI) (Table 2). The positive ion has $[M+H]^+$ m/z = 484 ion, which has been reported to represent the

strongest ion (Singh et al., 2012). To improve the ionization under ESI positive ion conditions of chlorantraniliprole, 0.1% of formic acid was added to the mobile phase solvents (acetonitrile/water mixture) and protonated to form $[M+H]^+$ m/z = 484 ion (Lee et al. 2017; Lee et al. 2017). In the current study, the $[M+H]^+$ peak of chlorantraniliprole was used as the base peak, and the selected ion monitoring (SIM) mode m/z = 484 ion was used for sufficient qualitative verification. The results of the above analysis conditions showed no peak at the same retention time of chlorantraniliprole in the untreated *R. glutinosa* root, and only the residue of chlorantraniliprole at the same retention time was accurately identified in the spiked sample (Figure 8).

Cross-laboratory validation

Cross-laboratory cross-validation reconfirms the analysis criteria of the established methods. The results of the cross-validation analysis showed that the average recovery was 88.4% after triplicate repetition of 10 MLOQ and RSD was 0.3%. Accordingly, the cross-laboratory validation analysis reconfirms the results of the current method (recovery rate values ranged from 70-120% and the RSD was less than 10%).

Conclusions

The insecticide, chlorantraniliprole residue analytical method in *R. glutinosa* L. studied through this work by optimized to utilize HPLC instrument equipped with UVD detector at 230 nm wavelength. The double cleaning up method was adopted using SPE-silica and SPE-NH_2 cartridges, primary with silica-SPE. In this study, gradient conditions determined the ratio of water:acetonitrile (60:40, 60:40, 30:70, 30:70, 60:40, 60:40 v/v) with respective holding times intervals (0, 5, 10, 13, 15 and 20 min). The MLOQ was 0.02 mg/kg, recovery rate ranged from 87.9-105.2%, high correlation coefficient (R^2 = 0.9998) and low RSD value (3.0%). Although this work studied the purification process and the process of changing the UV spectrum twice where significant interference materials were removed, but small amount of interface peak at the same retention time of the targeted compound was detected, thus it was difficult to completely separate the *R. glutinosa* L. matrix and chlorantraniliprole when using conventional instruments, hence further appropriate improvement measures of developing effective refining and separation method was suggested. However, the use of LC/MS/MS following the pre-treatment and cleaning up methods presented in this study will be possible to perform a precise analysis more easily with high selectivity and resolution. It is expected that this analytical method may be listed in the Korean Pharmacopoeia, where the pesticide residue analytical method in herbal medicines is insufficient and thus may contribute to the safety management of the herbal medicines in some domestic and/or developing countries laboratories that still need to use conventional instruments.

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Authors Information and Contributions

Jeongyoon Choi, Department of Biological Environment, Kangwon National University, Ph.D. student, https://orcid.org/0000-0002-7931-6408

Yeongjae Lee, Department of Biological Environment, Kangwon National University, Master student, https://orcid.org/0000-0001-7611-8281

Hunju Ham, Environment Friendly Agricultural Products Safety Center, Doctor of Philosophy, https://orcid.org/0000-0001-9816-019X

Abd Elaziz Sulieman Ahmed Ishag, Department of Biological Environment, Kangwon National University & Department of Crop Protection, University of Khartoum, Khartoum North, Doctor of Philosophy, https://orcid.org/0000-0003-2605-7219

Janghyun Hur, Department of Biological Environment, Kangwon National University, Professor, https://orcid.org/0000-0003-2592-7147

Work and designed the experiment, Jang Hyun Hur

Experiments and analyzed the results, Jeong Yoon Choi, Yeong Jae Lee and Hun Ju Ham

Writing, Jang Hyun Hur, Jeong Yoon Choi and Abd Elaziz Sulieman Ahmed Ishag

Conflict of Interest

The authors declare that there is no conflict of interest.

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