농학석사학위논문

강활과 백지 중 Hexythiazox 잔류분의 LC-MS/MS분석 및 HPLC에 의한 유효성분 분석

Analysis of hexythiazox residue by LC-MS/MS and active ingredient by HPLC in *Ostericum* root and *Angelica Dahurica* root

2021년 2월

서울대학교 대학원
농생명공학부 응용생명화학전공
주 보 은

A Dissertation for the Degree of Master of Science

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February 2021

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지도교수 김 정 한

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주 보 은

주보은의 석사학위논문을 인준함
2021년 1월

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부 위 원 장 _____ 김정한 _____ (인)

위 원 _____ 이상기 _____ (인)

Abstract

Hexythiazox is a carboxamide-based insectcide which has been used for controlling mites in melon, apple, strawberry and traditional herbal plants. Although the analytical method for hexythiazox residue in general food crops was well developed using HPLC or LC-MS/MS, there are no available analytical method for the traditional herbal medicines. The aim of this study was to develop an analytical method for hexythiazox residue in *Ostericum* root and *Angelica Dahurica* root which have traditionally been used in oriental medicine. The method limit of quantitation (MLOQ) was 0.02 mg/kg. A recovery test was performed to validate the established method at two fortification levels (MLOQ and 10 times of MLOQ). The recovery rate was in the range 70~105% with <10 % of the coefficient of variation. Therefore, the present method was proved to be reasonable for the quantitative determination of hexythoazox in the traditional herbal medicines *Ostericum* root and *Angelica Dahurica* root. Besides, an analytical method for the active ingredients in *Angelica Dahurica* root and *Ostericum* root was also established, based on the Korean Pharmacopoeia which is a statute for the analytical method of pharmaceuticals in Korea. A quantitative analytical method for the active ingredients in *Ostericum* root and *Angelica Dahurica* root was developed using HPLC-DAD. After optimizing several HPLC analytical parameters such as gradients program for the mobile phase and UV wavelength, four active

ingredients (oxypeucedanin, bisabolangelone, imperatorin, and isoimperatorin) for *Ostericum* root and five active ingredients (byakangelicol, oxypeucedanin, imperatorin, phellopterin, and isoimperatorin) for *Angelica Dahurica* root were successfully separated in the HPLC chromatograms. These active ingredients were identified by LC-MS/MS analysis considering their unique ionization properties.

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Key words: hexythiazox, herbal medicine, LC-MS/MS, active ingredient, *Ostericum* root, *Angelica Dahurica* root

***Student Number*: 2019-23419**

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List of Abbreviations

ACN	Acetonitrile
DL	Desolvation line
dSPE	Dispersive solid phase extraction
ESI	Electrospray ionization
GC	Gas-chromatography
GCB	Graphitizied carbon black
ILOD	Instrumental limit of detection
ILOQ	Instrumental limit of quantitation
LC	Liquid-chromatography
MLOQ	Method limit of quantitation
MRLs	Maximum residue limits
MRM	Multiple reaction monitoring
PSA	Primary secondary amine
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safety
RSD	Relative standard deviation
S/N	Signal to Noise
SPE	Solid phase extraction

Part 1

Analytical method for hexythiazox in *Ostericum* root and *Angelica Dahurica* root

Introduction

Characteristics of target pesticide ingredients and instrumental analysis plan

Traditional herbal medicines have been widely used to treat various diseases for thousands of years in Asia. Hexythiazox is an insecticide mainly used in traditional herbal medicines.

Hexythiazox is a carboxamide-based insecticide with low mammalian toxicity (LD50 for rats: >2000 mg/kg) and the main metabolite in urine is 5-(4-chlorophenyi)-N-(cis-4-hydroxycyclohexyl)-4-methyl-trans-2-oxothiazolidine -3-carboxamide in animal. It is degraded in soil [50% dissipation time (DT50) in clay loam at 15℃: 8 days], undergoes oxidation to the corresponding hydroxy and carbonyl compounds. Koc 8449. (The Pesticide Manual, 2015).

In Korea, the analytical method of hexythiazox residues in crops and food is listed in Food Code (Korea Food and Drug Administration, 2020) as an individual residue method using liquid chromatography-UV photometric detector (LC-UVD) or liquid chromatography-mass/mass (LC-MS/MS). However there is no analysis method for hexythiazox in herbal medicine, and conventional pesticide residue analysis methods have low recovery and high matrix, so quantification was not possible. The purpose of this study is the establishment of the standard analytical method for detection of hexythiazox residues in herbal medicine. Method validation was performed, and improvement for more efficient and simpler clean-up procedures than the other

existing methods was achieved.

Since there are no metabolites of toxicological importance in the herbal *Ostericum* root and *Angelica Dahurica* root, the target components for residual analysis were limited to the parent compound as an active ingredient. The chemical structure of hexythiazox is shown in Figure 1.

Table 1. shows the physicochemical properties of pesticide hexythiazox. It was expected that analysis using LC-UVD is easy due to the non-polar compound with a log Pow value of 2.75 and the absorption of π. However, it is required to remove large amounts of impurities, and it is impossible to separate from impurities through general distribution and purification, so high-sensitivity and high-resolution LC-MS/MS was used. Acetonitrile was extracted from *Ostericum* root and *Angelica Dahurica* root as an extraction solvent to minimize the extraction of impurities, and it was composed of a process of analyzing residuals by applying a purification method using SPE.

Figure. 1. Molecular structure of hexythiazox

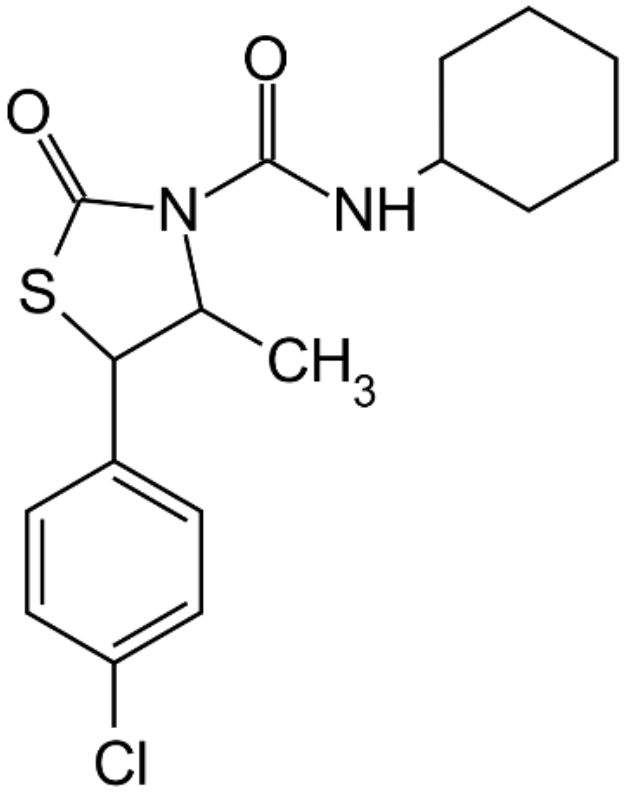

O
O
N
NH
S
CH3
Cl

Table 1. Physicochemical properties of hexythiazox

Property	Information
Common name	hexythiazox (BSI, E-ISO, (m) F-ISO)
IUPAC name	(4RS,5RS)-5-(4-chlorophenyi)-N-cyclohexyl-4-methyl-2-oxo-1 ,3-thiazolidine-3-carboxamide
CAS No.	78587-05-0
Molecular Formula	$C_{17}H_{21}ClN_2O_2S$
Molecular Weight	352.9 g/mol
Log Kow	2.75
Solubility in water (mg/kg)	0.41 (20-25 ℃)
Vapor Pressure	0.001333 (mPa) (20 ℃)
Organic solubility (g/l, 20-25℃)	Soluble in acetone (159), acetonitrile (34.5), chloroform (1379), n-hexane (4.64), methanol (17.6), xylene (230) Stability Stable to light, air and heat. Stable to 150 oc. Aqueous solution in sunlight,
Site of action	Mitochondrial complex Ill electron transport inhibitor (coupling site II). Inhibits cellular respiration.
Mammalian Toxicology	Acute oral (LD 50 , mg/kg) rats > 2000 Acute percutaneous (LD 50 , mg/kg) rats > 2000 Skin irritation Not an irritant (rabbits). Skin sensitisation Not a sensitiser (guinea pigs). Eye Not an irritant (rabbits). Inhalation (LC 50 , mg/1) rats >3.829 (4 h)

Environmental Fate	Animals The main metabolite in urine and faeces is 5-(4-chlorophenyi)-N-(cis-4-hydroxycyclohexyl)-4-methyl-trans-2-oxothiazolidine-3-carboxamide. Plants The major residue is unchanged hexythiazox, with formation of minor hydroxylated cyclohexyl metabolites.

*The Pesticide Manual Seventeenth Edition (J A Turner)

Material and Methods

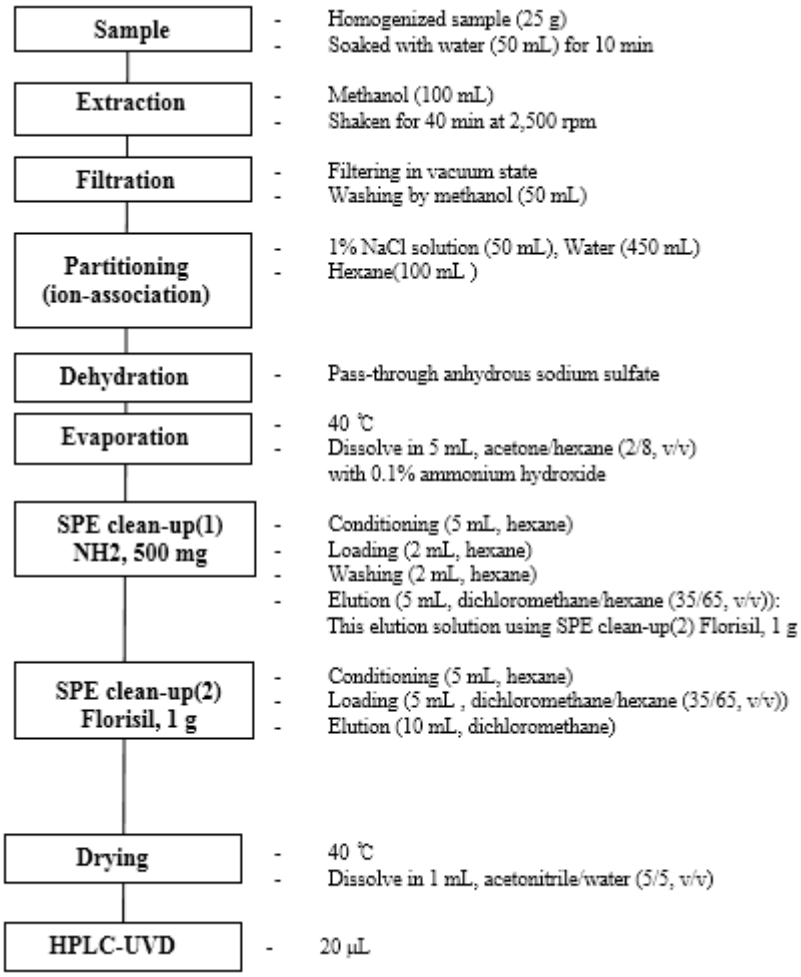
Standard material of hexythiazox (99%) was purchased from FUJIFILM Wako Pure Chemical Corporation. *Ostericum* root and *Angelica Dahurica* root were purchased from a pharmaceutical company, and it was used as a sample for this study only if it was a suitable product after it was verified by an expert recommended by the herbal medicine research department, which conforms to the sensory testing standards of herbal medicine. They were chopped, macerated,and kept in a freezer at a temperature below −20℃. Chemicals, reagents and standard solutions. Acetonitrile, acetone, n-hexane, dichloromethane and methanol were HPLC grade (Burdickand Jackson®, Korea). Sodium sulfate (GR grade) and sodium chloride (GR grade) were from Junsei Chemical Co. Ltd. (Japan). Florisil® (60-100 mesh), NH_2® (60-100 mesh), NH_2/GCB® (60-100 mesh) and Silica® (60-100 mesh) were purchased from FlukaTM. Formic acid (≥98%) was purchased from Tokyo Chemical Industry Ltd.

Filter paper (GF/A) were from Whatman International Ltd. (England). A stock solution of hexythiazox was prepared in acetonitrile of 1000 mg/L, and the working solutions were prepared by appropriate dilutions of the stock solutions with acetonitrile.

Korean Food Standards Codex Analysis Method by Series (7.1.3.14) applied

In this study, the pretreatment method of Korean Food Standards Codex series analysis method (7.1.3.14) using LC-UVD was reviewed, and the schematic diagram of pretreatment is shown in Figure 2. In particular, in the *Ostericum* root, a large amount of impurities was identified in the retention time of hexythiazox and it was confirmed that quantitative analysis was impossible. Therefore, additional purification methods were considered in addition to SPE using the existing NH_2/GCB cartridge.

Figure 2. Schematic diagram of Korean Food Standards Codex analysis method by series (7.1.3.14)


Sample
- Homogenized sample (25 g)
- Soaked with water (50 mL) for 10 min
Extraction
- Methanol (100 mL)
- Shaken for 40 min at 2,500 rpm
Filtration
- Filtering in vacuum state
- Washing by methanol (50 mL)
Partitioning (ion-association)
- 1% NaCl solution (50 mL), Water (450 mL)
- Hexane(100 mL)
Dehydration
- Pass-through anhydrous sodium sulfate
Evaporation
- 40 ℃
- Dissolve in 5 mL, acetone/hexane (2/8, v/v) with 0.1% ammonium hydroxide
SPE clean-up(1) NH2, 500 mg
- Conditioning (5 mL, hexane)
- Loading (2 mL, hexane)
- Washing (2 mL, hexane)
- Elution (5 mL, dichloromethane/hexane (35/65, v/v)): This elution solution using SPE clean-up(2) Florisil, 1 g
SPE clean-up(2) Florisil, 1 g
- Conditioning (5 mL, hexane)
- Loading (5 mL , dichloromethane/hexane (35/65, v/v))
- Elution (10 mL, dichloromethane)
Drying
- 40 ℃
- Dissolve in 1 mL, acetonitrile/water (5/5, v/v)
HPLC-UVD
- 20 μL

LC-UVD analysis

Instrument conditions using LC-UVD introduced in Korean Food Standards Codex Series Analysis Method 7.1.3.14 were confirmed as shown in Table 2. C18 column was used to separate the hexythiazox, water and acetonitrile were used, and the optimum wavelength was 228 nm. The peak of hexythiazox 1 μg/mL using LC-UVD.

Table 2. LC-UVD analysis conditions of hexythiazox

HPLC	Agilent 1100 series
Column	Kinetex C18 (150 × 3.9 mm, 4μm, phenomenex)
Mobile Phase	Water/Acetonitrile (30/70, v/v)
Column temperature	30 ℃
Detector	UVD
Wavelength	228 nm
Injection Volume	40 μL

Figure 3. Schematic diagram of the improved hexythiazox analysis method (7.1.3.14)

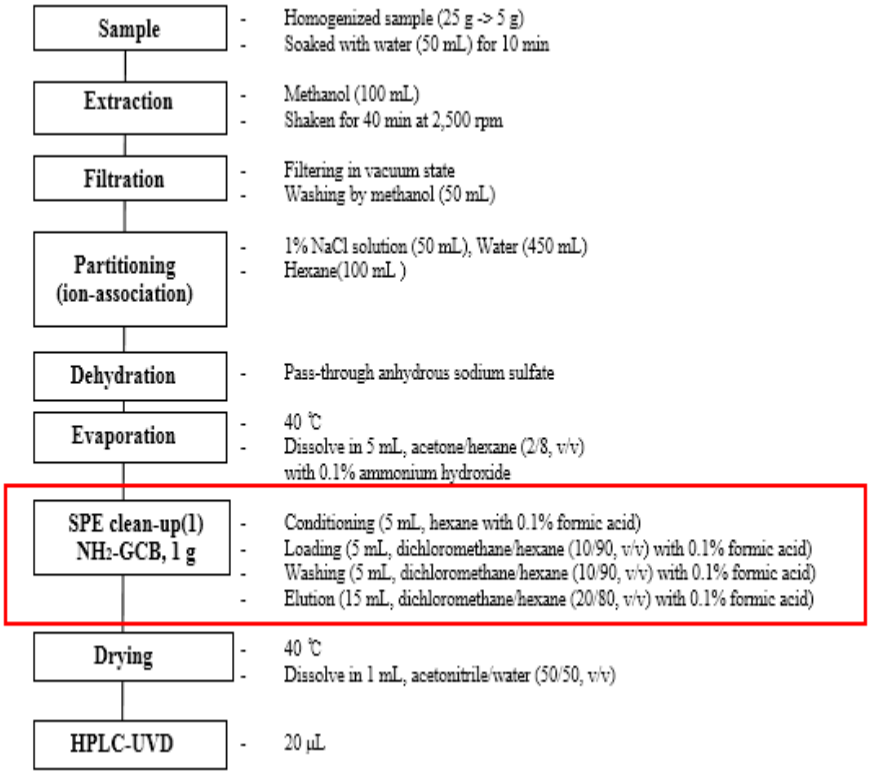
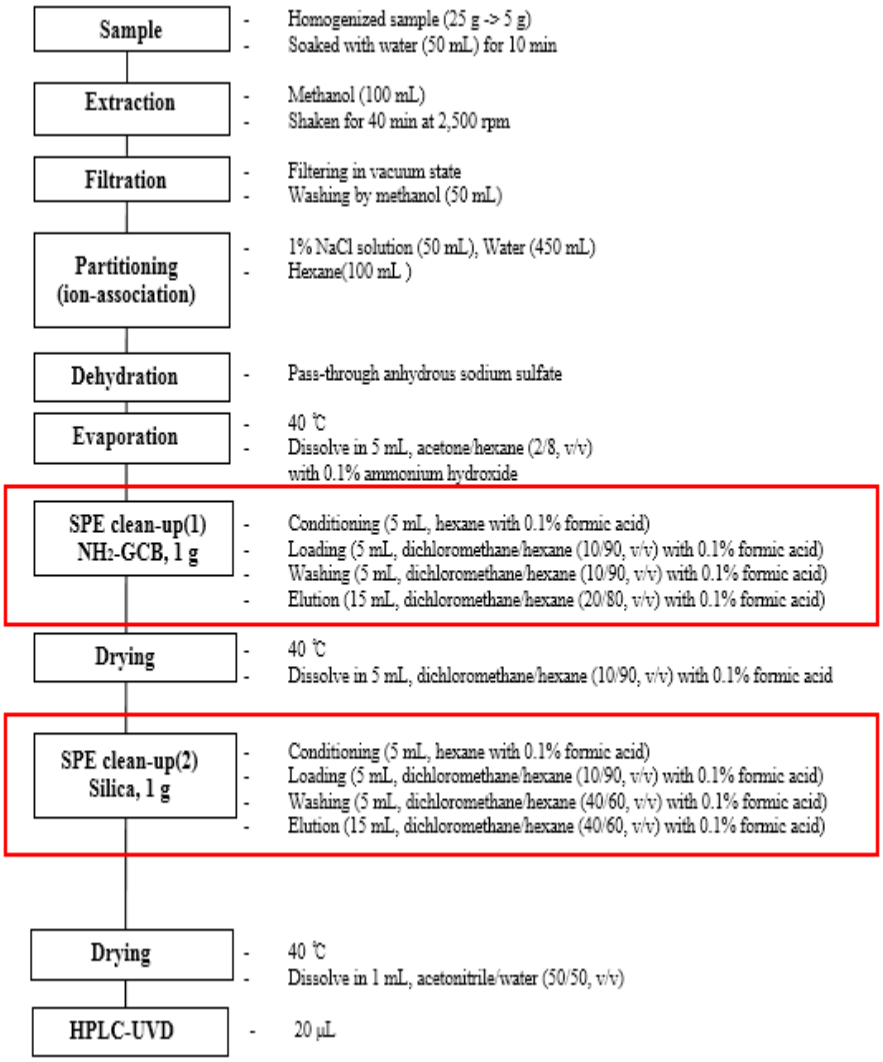

Sample
- Homogenized sample (25 g -> 5 g)
- Soaked with water (50 mL) for 10 min
Extraction
- Methanol (100 mL)
- Shaken for 40 min at 2,500 rpm
Filtration
- Filtering in vacuum state
- Washing by methanol (50 mL)
Partitioning (ion-association)
- 1% NaCl solution (50 mL), Water (450 mL)
- Hexane(100 mL)
Dehydration
- Pass-through anhydrous sodium sulfate
Evaporation
- 40 ℃
- Dissolve in 5 mL, acetone/hexane (2/8, v/v) with 0.1% ammonium hydroxide
SPE clean-up(1) NH2-GCB, 1 g
- Conditioning (5 mL, hexane with 0.1% formic acid)
- Loading (5 mL, dichloromethane/hexane (10/90, v/v) with 0.1% formic acid)
- Washing (5 mL, dichloromethane/hexane (10/90, v/v) with 0.1% formic acid)
- Elution (15 mL, dichloromethane/hexane (20/80, v/v) with 0.1% formic acid)
Drying
- 40 ℃
- Dissolve in 1 mL, acetonitrile/water (50/50, v/v)
HPLC-UVD
- 20 μL

Figure 4. Schematic diagram of the improved hexythiazox method


Sample
- Homogenized sample (25 g -> 5 g)
- Soaked with water (50 mL) for 10 min
Extraction
- Methanol (100 mL)
- Shaken for 40 min at 2,500 rpm
Filtration
- Filtering in vacuum state
- Washing by methanol (50 mL)
Partitioning (ion-association)
- 1% NaCl solution (50 mL), Water (450 mL)
- Hexane(100 mL)
Dehydration
- Pass-through anhydrous sodium sulfate
Evaporation
- 40 ℃
- Dissolve in 5 mL, acetone/hexane (2/8, v/v) with 0.1% ammonium hydroxide
SPE clean-up(1) NH2-GCB, 1 g
- Conditioning (5 mL, hexane with 0.1% formic acid)
- Loading (5 mL, dichloromethane/hexane (10/90, v/v) with 0.1% formic acid)
- Washing (5 mL, dichloromethane/hexane (10/90, v/v) with 0.1% formic acid)
- Elution (15 mL, dichloromethane/hexane (20/80, v/v) with 0.1% formic acid)
Drying
- 40 ℃
- Dissolve in 5 mL, dichloromethane/hexane (10/90, v/v) with 0.1% formic acid
SPE clean-up(2) Silica, 1 g
- Conditioning (5 mL, hexane with 0.1% formic acid)
- Loading (5 mL, dichloromethane/hexane (10/90, v/v) with 0.1% formic acid)
- Washing (5 mL, dichloromethane/hexane (40/60, v/v) with 0.1% formic acid)
- Elution (15 mL, dichloromethane/hexane (40/60, v/v) with 0.1% formic acid)
Drying
- 40 ℃
- Dissolve in 1 mL, acetonitrile/water (50/50, v/v)
HPLC-UVD
- 20 μL

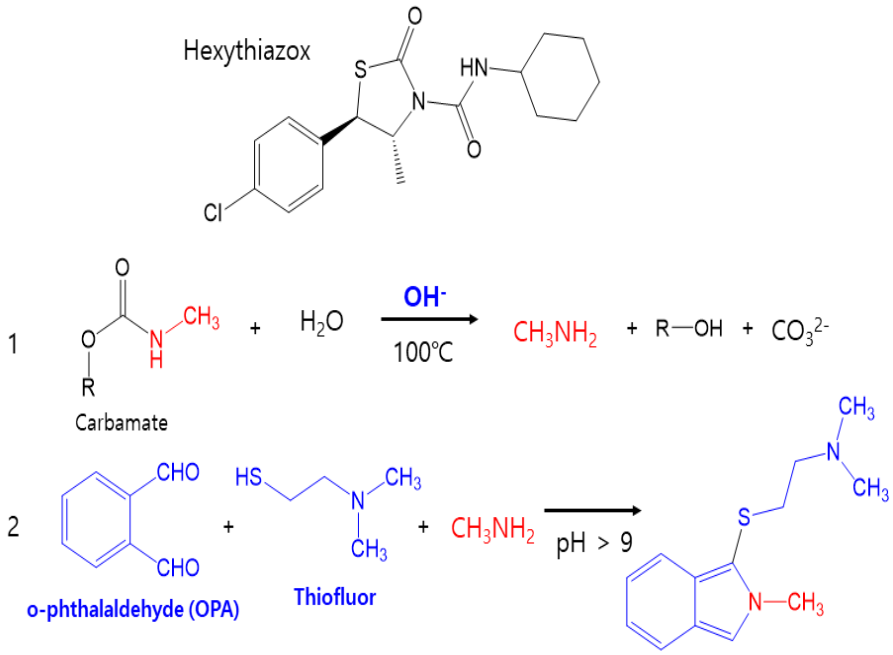
LC-FLD analysis

Hexythiazox could be analyzed using a fluorescence detector (FLD), which has relatively higher selectivity than a UV detector due to a large number of impurities. Because of the structure of the carbamate line, a more selective analysis method using a fluorescent derivative was applied to the carbamate with reference to the proposed EPA 531.2 and AOAC method's carbamate analysis method. The peak was separated from the C18 column by water and methanol mobile phase, and a fluorescent derivative reaction using post-column was applied. In the derivatization reaction, a fluorescent derivative reaction through o-phthaldialdehyde and thiofluor was performed under basic conditions using NaOH (1 M).

Table 3. LC-FLD analysis conditions of hexythiazox

HPLC		Agilent 1100 series
Column		Kinetex C18Kinetex C18 (250 × 4.6 mm, 5 μm, phenomenex)
Mobile Phase		Water/Methanol (3/97, v/v)
Column temperature		30 ℃
Post-column Derivatives reagent	**Reagent 1 (flow rate: 0.3 mL/min) (reaction temperature: 100 ℃)**	1 M NaOH solution
	Reagent 2 (flow rate: 0.3 mL/min) (reaction temperature: room temperature)	1% o-phthaldialdehyde in methanol (10 mL) + 0.2% thiofluor in OPA diluent (950 mL)
Detector		FLD
Wavelength		365 nm (Excitation), 470 nm (Emission)
Injection Volume		20 μL

Figure 5. Derivative principle using LC-FLD



Ref.: Pinnacle carbamet derivatization method

Figure 6. Schematic diagram of the hexythiazox derivative method

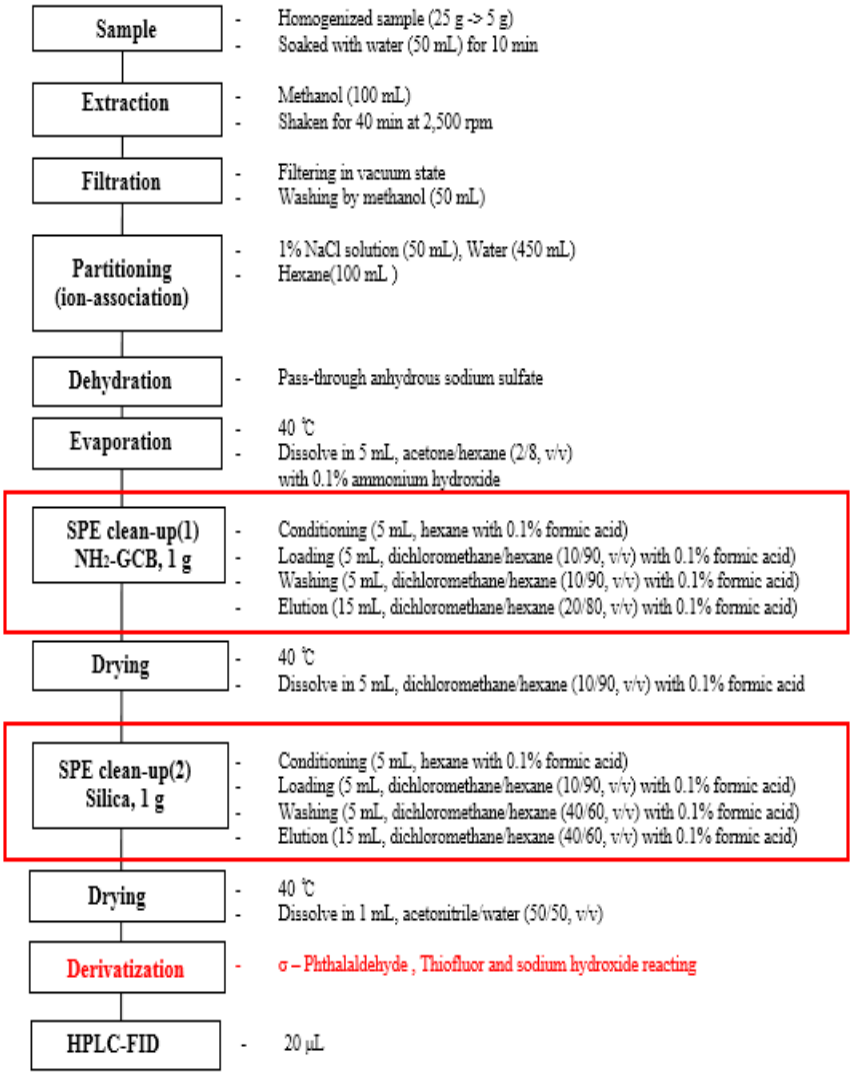

Sample
- Homogenized sample (25 g -> 5 g)
- Soaked with water (50 mL) for 10 min
Extraction
- Methanol (100 mL)
- Shaken for 40 min at 2,500 rpm
Filtration
- Filtering in vacuum state
- Washing by methanol (50 mL)
Partitioning (ion-association)
- 1% NaCl solution (50 mL), Water (450 mL)
- Hexane(100 mL)
Dehydration
- Pass-through anhydrous sodium sulfate
Evaporation
- 40 ℃
- Dissolve in 5 mL, acetone/hexane (2/8, v/v) with 0.1% ammonium hydroxide
SPE clean-up(1) NH2-GCB, 1 g
- Conditioning (5 mL, hexane with 0.1% formic acid)
- Loading (5 mL, dichloromethane/hexane (10/90, v/v) with 0.1% formic acid)
- Washing (5 mL, dichloromethane/hexane (10/90, v/v) with 0.1% formic acid)
- Elution (15 mL, dichloromethane/hexane (20/80, v/v) with 0.1% formic acid)
Drying
- 40 ℃
- Dissolve in 5 mL, dichloromethane/hexane (10/90, v/v) with 0.1% formic acid
SPE clean-up(2) Silica, 1 g
- Conditioning (5 mL, hexane with 0.1% formic acid)
- Loading (5 mL, dichloromethane/hexane (10/90, v/v) with 0.1% formic acid)
- Washing (5 mL, dichloromethane/hexane (40/60, v/v) with 0.1% formic acid)
- Elution (15 mL, dichloromethane/hexane (40/60, v/v) with 0.1% formic acid)
Drying
- 40 ℃
- Dissolve in 1 mL, acetonitrile/water (50/50, v/v)
Derivatization
- σ – Phthalaldehyde , Thiofluor and sodium hydroxide reacting
HPLC-FID
- 20 μL

Table 4. LC-MS/MS analysis conditions of hexythiazox

<table>
<tr><td rowspan="5">UHP LC</td><td>Instrument</td><td>Shimadzu LC-MS 8060
with UHPLC Nexera</td></tr>
<tr><td>Column (oven temp.)</td><td>KinetexC18 (2.1 x 100 mm, 2.6 um) (40℃)</td></tr>
<tr><td>Mobile phase</td><td>A: Water
(0.1% formic acid, 5 mM ammonium formate, 2% methanol)

B:Methanol
(0.1% Formic acid, 5 mM ammonium formate, 2% water)</td></tr>
<tr><td>Gradient</td><td><table><tr><th>Time (min.)</th><th>Mobile A (%, v)</th><th>Mobile B (%, v)</th></tr><tr><td>0.0</td><td>50</td><td>50</td></tr><tr><td>1.0</td><td>50</td><td>50</td></tr><tr><td>3.0</td><td>5</td><td>95</td></tr><tr><td>10.0</td><td>5</td><td>95</td></tr><tr><td>12.0</td><td>50</td><td>50</td></tr><tr><td>20</td><td>50</td><td>50</td></tr></table></td></tr>
<tr><td>Flow rate</td><td>0.2 mL/min
(20 min)</td></tr>
<tr><td rowspan="4">MS</td><td>ESI</td><td>Positive</td></tr>
<tr><td>DL temp.</td><td>250 ℃</td></tr>
<tr><td>Nebulizing gas</td><td>3 L/min</td></tr>
<tr><td>Heat block temp.</td><td>400 ℃</td></tr>
</table>

Drying gas	15 L/min	
Exact mass	352.101228 g/mol	
Ion mode	[M+H]+	
MRM (CE, eV)	353.00>228.00(-15)	353.00>168.05(-25)

Figure 7. Scan spectrum of hexythiazox

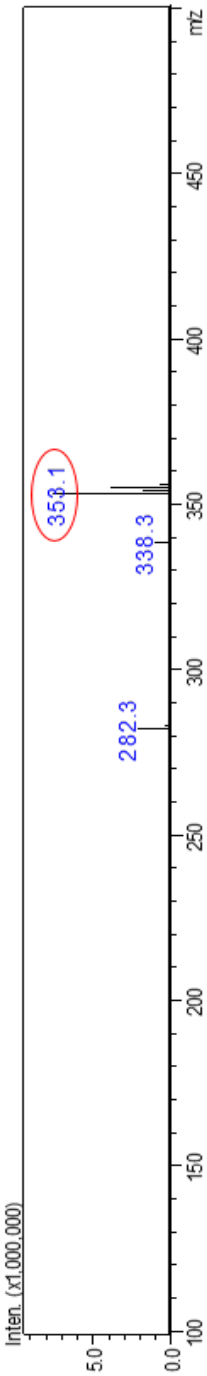
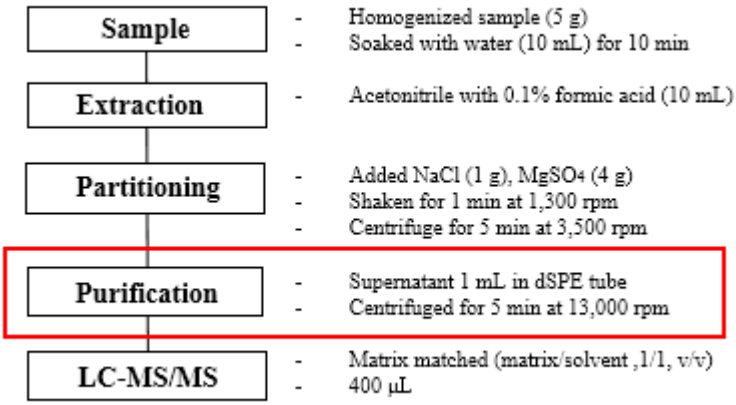

Inten. (x1,000,000)
5.0
0.0
100
150
200
250
300
350
400
450
m/z
282.3
338.3
358.1

Figure 8. Korean Food Standards Codex Multi-Pesticide Multi-Component Analysis Method Second Method (7.1.2.2) Reduction Improvement Schematic


Sample
- Homogenized sample (5 g)
- Soaked with water (10 mL) for 10 min
Extraction
- Acetonitrile with 0.1% formic acid (10 mL)
Partitioning
- Added NaCl (1 g), MgSO4 (4 g)
- Shaken for 1 min at 1,300 rpm
- Centrifuge for 5 min at 3,500 rpm
Purification
- Supernatant 1 mL in dSPE tube
- Centrifuged for 5 min at 13,000 rpm
LC-MS/MS
- Matrix matched (matrix/solvent ,1/1, v/v)
- 400 µL

Korean Food Standards Codex Multi-Pesticide Multi-Component Analysis Method (7.1.2.2) Established SPE conditions as a reduction and improvement method

Take 5 g of the sample into a 50 mL volume centrifuge tube, add 10 mL of water, wet for 10 minutes, add 10 mL of acetonitrile containing 0.1% formic acid, and shake vigorously at 1,300 rpm for 1 minute. After adding 4 g of anhydrous magnesium sulfate and 1 g of NaCl to the centrifuge tube, shake vigorously for 1 minute, and centrifuge at 3,500 rpm for 5 minutes. Take 5 mL of acetonitrile layer, blow off the solvent while passing nitrogen in a water bath below 40°C, and redissolve in 5 mL of hexane containing 0.1% formic acid and 10% dichloromethane.

Figure 9. Schematic of SPE-NH_2/GCB conditions for extraction + liquid partitioning using QuEChERS salt

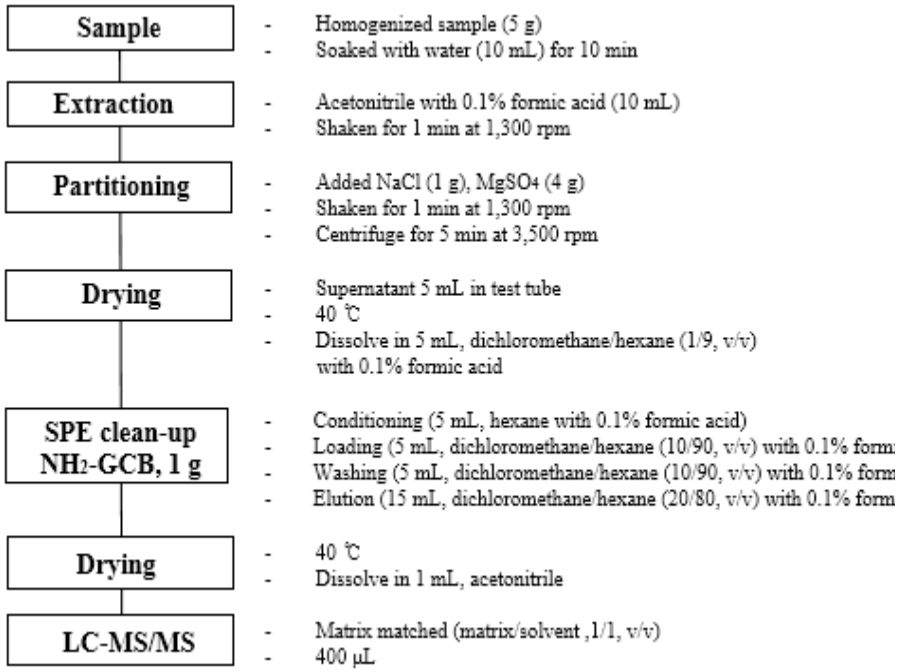
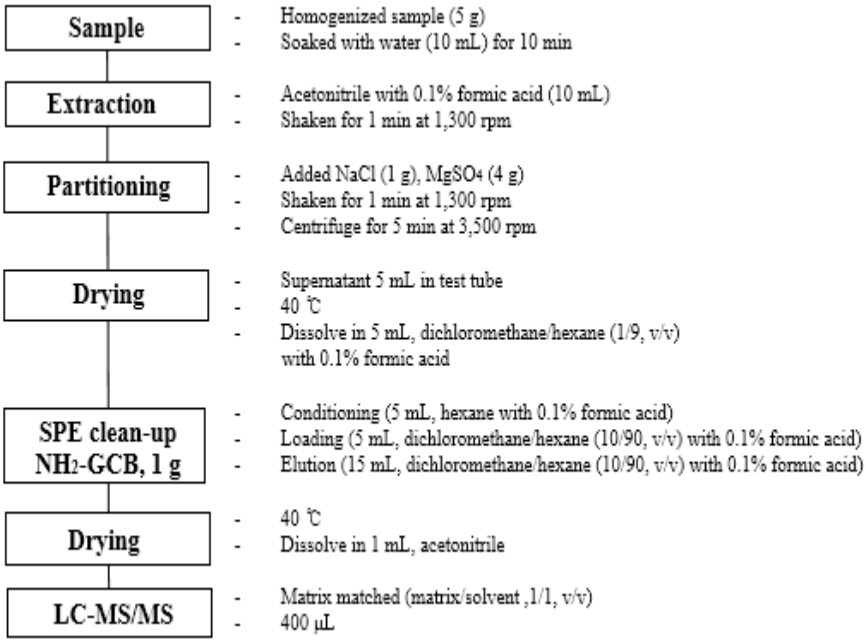

Sample
- Homogenized sample (5 g)
- Soaked with water (10 mL) for 10 min
Extraction
- Acetonitrile with 0.1% formic acid (10 mL)
- Shaken for 1 min at 1,300 rpm
Partitioning
- Added NaCl (1 g), MgSO4 (4 g)
- Shaken for 1 min at 1,300 rpm
- Centrifuge for 5 min at 3,500 rpm
Drying
- Supernatant 5 mL in test tube
- 40 ℃
- Dissolve in 5 mL, dichloromethane/hexane (1/9, v/v) with 0.1% formic acid
SPE clean-up NH2-GCB, 1 g
- Conditioning (5 mL, hexane with 0.1% formic acid)
- Loading (5 mL, dichloromethane/hexane (10/90, v/v) with 0.1% form
- Washing (5 mL, dichloromethane/hexane (10/90, v/v) with 0.1% form
- Elution (15 mL, dichloromethane/hexane (20/80, v/v) with 0.1% form
Drying
- 40 ℃
- Dissolve in 1 mL, acetonitrile
LC-MS/MS
- Matrix matched (matrix/solvent ,1/1, v/v)
- 400 μL

Figure 10. Schematic diagram of the improved hexythiazox method


Sample
- Homogenized sample (5 g)
- Soaked with water (10 mL) for 10 min
Extraction
- Acetonitrile with 0.1% formic acid (10 mL)
- Shaken for 1 min at 1,300 rpm
Partitioning
- Added NaCl (1 g), MgSO4 (4 g)
- Shaken for 1 min at 1,300 rpm
- Centrifuge for 5 min at 3,500 rpm
Drying
- Supernatant 5 mL in test tube
- 40 ℃
- Dissolve in 5 mL, dichloromethane/hexane (1/9, v/v) with 0.1% formic acid
SPE clean-up NH2-GCB, 1 g
- Conditioning (5 mL, hexane with 0.1% formic acid)
- Loading (5 mL, dichloromethane/hexane (10/90, v/v) with 0.1% formic acid)
- Elution (15 mL, dichloromethane/hexane (10/90, v/v) with 0.1% formic acid)
Drying
- 40 ℃
- Dissolve in 1 mL, acetonitrile
LC-MS/MS
- Matrix matched (matrix/solvent ,1/1, v/v)
- 400 μL

Results and Discussion

Sample preparation method for LC-UVD

The Korean Pharmacopoeia was applied to develop an analysis method for hexythiazox residues in the herbal medicine *Ostericum* root and *Angelica Dahurica* root. However, due to the large amount of impurities, quantitative and qualitative analysis was not possible with the method announced by the Korean Pharmacopoeia. Therefore, the purification process in the Korean Pharmacopoeia was modified. Since the Korean Pharmacopoeia used solid phase extraction (SPE) in the purification process, the conditions were set by changing the type of SPE in order to improve the purification process. The analysis method in the Korean Pharmacopoeia used the SPE-NH2 (500 mg) cartridge and SPE-florisil cartridge (1 g) under purification conditions, but the improved analysis method was the SPE-NH2/GCB (1 g) cartridge and the SPE-florisil cartridge (1 g) was used. However, even though the purification conditions were improved, it was confirmed that quantitative and qualitative analysis was still impossible in LC-UVD as impurities.

Figure 11. Hexythiazox 1 µg/mL standard chromatogram using LC-UVD

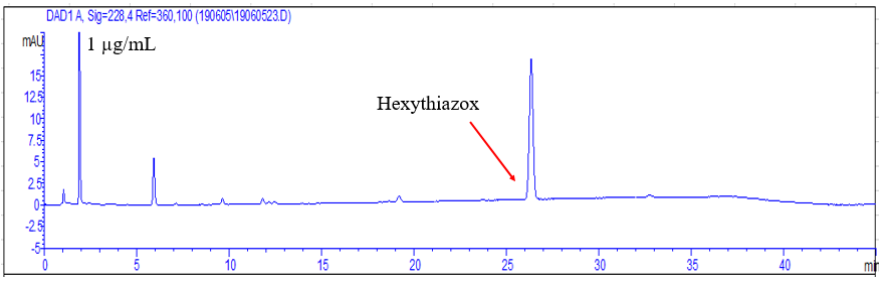
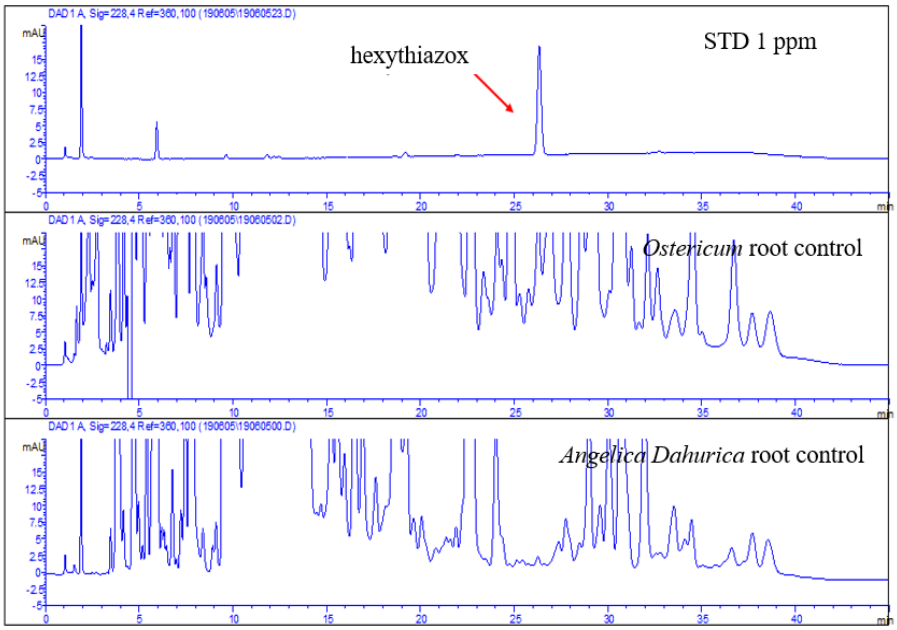

DAD1 A, Sig=228,4 Ref=360,100 (190605\19060523.D)
mAU
1 µg/mL
Hexythiazox
min

Figure 12. Chromatogram using pretreatment of Korean Food Standards method (7.1.3.14)


DAD1 A, Sig=228,4 Ref=360,100 (190605\19060523.D)
mAU
hexythiazox
STD 1 ppm
min
DAD1 A, Sig=228,4 Ref=360,100 (190605\19060502.D)
mAU
Ostericum root control
min
DAD1 A, Sig=228,4 Ref=360,100 (190605\19060500.D)
mAU
Angelica Dahurica root control
min

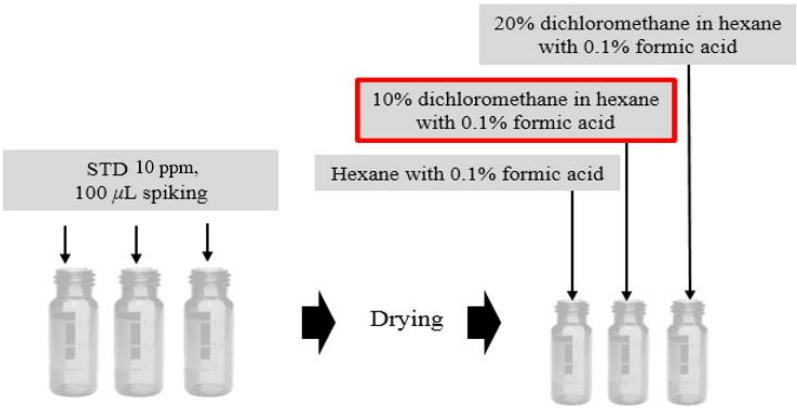
Optimization of clean-up method by SPE-NH_2/GCB

Considering the characteristics of herbal medicine samples containing a large amount of impurities. Besides to the florisil cartridge, analysis conditions were established in SPE-NH_2/GCB cartridge. In the case of adsorption chromatography, a method of normal phase, that is, a method of removing impurities while gradually increasing the polarity from non-polarity was used. Therefore, when selecting the initial solvent conditions, to select the minimum non-polar solvent capable of dissolving hexythiazox, treat 10 mg/mL, 100 μL of a standard solution. After drying it in a glass tube, dry it and dry it with hexane (0.1% formic acid), 10% dichloromethane in hexane (0.1% formic acid), 20% dichloromethane in hexane (0.1% formic acid) 1 mL was redissolved separately. To analyze each redissolved solution by LC-UVD, some solutions were transferred to another vial, dried again, and redissolved with acetonitrile to confirm solubility. Hexane (0.1% formic acid) was selected as the condition solvent for SPE, and 10% dichloromethane in hexane (0.1% formic acid) was selected as the loading solvent.

Table 5. Solubility results by solvent

	Selected dissolve solution		
	Hexane with 0.1% formic acid	**10% dichloromethane in hexane with 0.1% formic acid**	**20% dichloromethane in hexane with 0.1% formic acid**
Recovery (%)	99.6	106.8	109.4

Figure 13. SPE-NH_2/GCB redissolved solvent selection schematic


20% dichloromethane in hexane
with 0.1% formic acid
10% dichloromethane in hexane
with 0.1% formic acid
STD 10 ppm,
100 μL spiking
Hexane with 0.1% formic acid
Drying

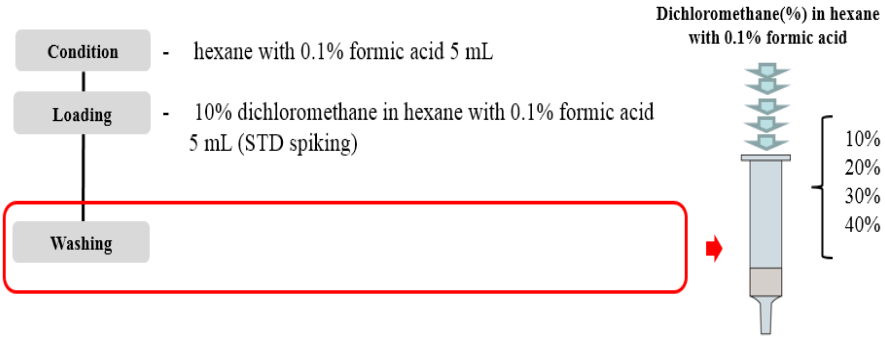
Optimization of additional purification SPE-NH_2/GCB conditions (selection of conditioning)

Conditioned in SPE-NH_2/GCB cartrdige with 5 mL of hexane (0.1% formic acid) to confirm the washing of impurities and the elution section of the component. Then, 10 μg/mL, 5 mL of a standard product dissolved in 10% dichloromethane in hexane (0.1% formic acid) was added. Subsequently, the ratio of dichloromethane was sequentially increased as shown in the table, and each fraction was separately received 10 mL to confirm the elution section. As a result, a recovery rate of 99.8% was confirmed in 10% acetone in hexane (0.1% formic acid) and 20% acetone in hexane (0.1% formic acid) section.

Table 6. Recovery results by washing fraction of dichloromethane (%) in hexane (0.1% formic acid) solvent combination (10 mL)

	dichloromethane (%) in hexane with 0.1% formic acid			
	10	**20**	**30**	**40**
Recovery (%)	52	43.8	_	_

Figure 14. SPE-NH_2/GCB washing + elution condition selection schematic


Condition
- hexane with 0.1% formic acid 5 mL
Loading
- 10% dichloromethane in hexane with 0.1% formic acid 5 mL (STD spiking)
Washing
Dichloromethane(%) in hexane with 0.1% formic acid
10%
20%
30%
40%

Optimization of additional purification SPE-NH_2/GCB conditions (selection of washing + elution conditions)

As 52% elution was performed in a 10 mL section of 10% dichloromethane in hexane (0.1% formic acid), a detailed 5 mL elution experiment was conducted, and it was confirmed that it did not elute in a 5 mL section. Therefore, 5 mL of 10% dichloromethane in hexane (0.1% formic acid) was determined as the washing solvent.

Table 7. Dissolution results by fraction of 10% dichloromethane in hexane (0.1% formic acid) solvent combination

Volume (mL)	Vol. of 10% dichloromethane in hexane & 0.1% formic acid					
	5	**10**	**15**	**20**	**25**	**30**
Recovery (%)	_	57	38	4.4	_	_

Table 8. Dissolution results by fraction of 20% dichloromethane in hexane (0.1% formic acid) solvent combination

Volume (mL)	Vol. of 20% dichloromethane in hexane & 0.1% formic acid					
	5	**10**	**15**	**20**	**25**	**30**
Component elution	O	O	O	_	_	_

Based on the above results, washing conditions were established with 5 mL of 10% dichloromethane in hexane (0.1% formic acid). In addition, the elution conditions were further established with 15 mL of 20% dichloromethane in hexane (0.1% formic acid).

Figure 15. SPE-NH_2/GCB washing volume selection schematic

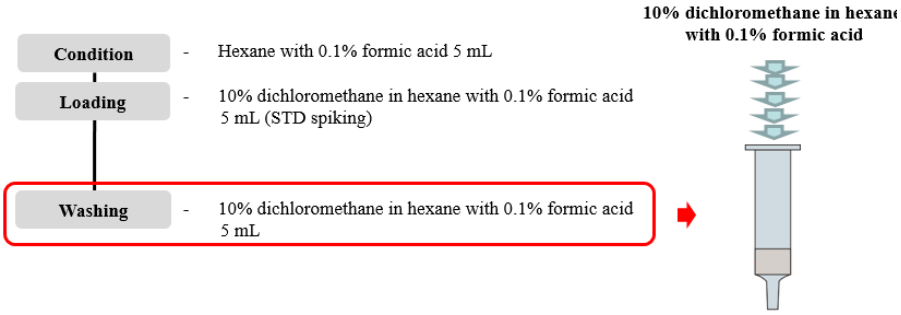

10% dichloromethane in hexane
with 0.1% formic acid
Condition
- Hexane with 0.1% formic acid 5 mL
Loading
- 10% dichloromethane in hexane with 0.1% formic acid
5 mL (STD spiking)
Washing
- 10% dichloromethane in hexane with 0.1% formic acid
5 mL

Figure 16. SPE-NH_2/GCB elution volume selection schematic

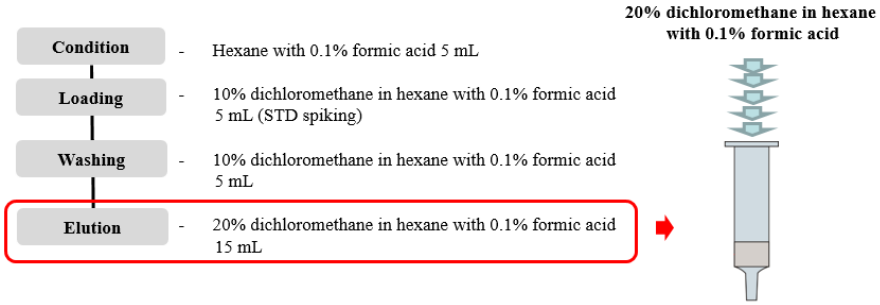
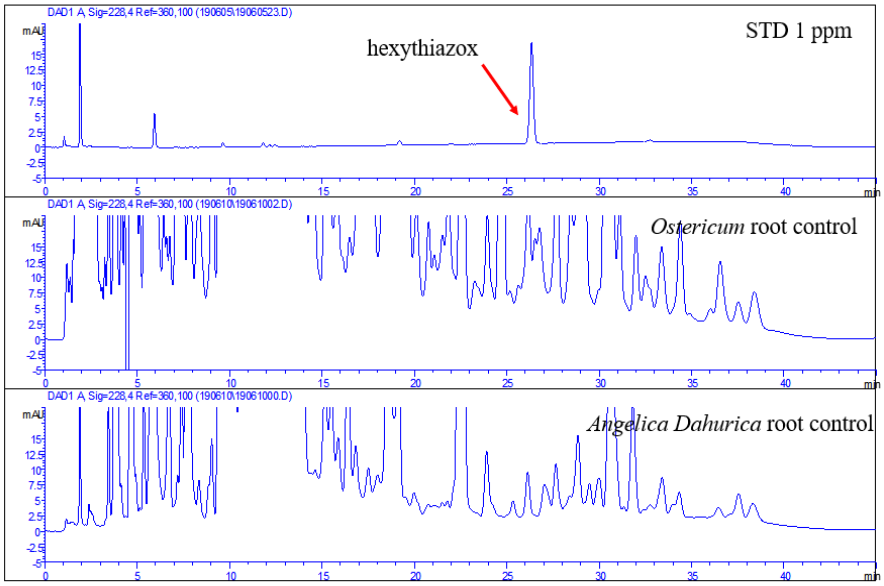

20% dichloromethane in hexane with 0.1% formic acid
Condition
- Hexane with 0.1% formic acid 5 mL
Loading
- 10% dichloromethane in hexane with 0.1% formic acid 5 mL (STD spiking)
Washing
- 10% dichloromethane in hexane with 0.1% formic acid 5 mL
Elution
- 20% dichloromethane in hexane with 0.1% formic acid 15 mL

Figure 17. Chromatogram of *Ostericum* root, *Angelica Dahurica* root samples after SPE-NH_2/GCB purification


DAD1 A, Sig=228,4 Ref=360,100 (190605\19060523.D)
STD 1 ppm
hexythiazox
DAD1 A, Sig=228,4 Ref=360,100 (190610\19061002.D)
Ostericum root control
DAD1 A, Sig=228,4 Ref=360,100 (190610\19061000.D)
Angelica Dahurica root control
mAU
min

Necessity of establishing double cartridge SPE conditions

Although the first SPE-NH_2 was confirmed by the Korean Food Standards Codex analysis method, it was confirmed that it was impossible to quantify due to the large amount of impurities in the hexythiazox retention time in the *Ostericum* root and *Angelica Dahurica* root samples. the second SPE-florisil method was applied according to the analysis method by the Korean Food Standards Codex analysis method but SPE-florisil had unstable results, therefore the SPE-silica cartridge was applied as the second purification process.

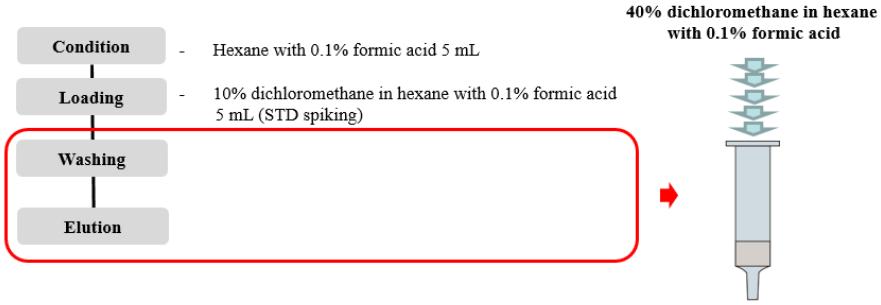
Optimization of additional purification SPE-silica conditions

5 mL of hexane (0.1% formic acid) was added to silica cartridge to confirm the elution section of the component with the conditioning solvent established before the first SPE -NH_2/GCB. Then, 10 μg/mL, 5 mL of a standard product dissolved in 10% dichloromethane in hexane (0.1% formic acid) was added to silica cartridge. Subsequently, the ratio of dichloromethane was sequentially increased and each fraction was separately received 10 mL to confirm the elution section. As a result, it was possible to confirm the recovery rate of 99.8% in the 40% acetone in hexane section.

Table 9. Recovery results by elution fraction of dichloromethane (%) in hexane (0.1% formic acid) solvent combination (10 mL)

	dichloromethane (%) in hexane with 0.1% formic acid			
	10	**20**	**30**	**40**
Recovery (%)	–	–	4.7	99.8

Figure 18. SPE-silica washing + elution condition selection schematic


40% dichloromethane in hexane
with 0.1% formic acid
Condition
- Hexane with 0.1% formic acid 5 mL
Loading
- 10% dichloromethane in hexane with 0.1% formic acid
5 mL (STD spiking)
Washing
Elution

Optimization of additional purification SPE-silica conditions (selection of washing + elution conditions)

Considering the elution section of 30-40% dichloromethane in hexane (0.1% formic acid), 40% dichloromethane in hexane (0.1% formic acid) was selected as the washing solvent and elution solvent, and the volume was monitored as shown in Table 10. and Table 11. Based on the results, established washing volume is 5 mL of 40% dichloromethane in hexane (0.1% formic acid) and elution volume is 15 mL of 40% dichloromethane in hexane (0.1% formic acid).

Table 10. Dissolution results by washing fraction of 40% dichloromethane in hexane (0.1% formic acid) solvent combination

Volume (mL)	Vol. of 40% dichloromethane in hexane & 0.1% formic acid					
	5	**10**	**15**	**20**	**25**	**30**
Recovery (%)	_	11.15	64.4	40.1		

Table 11. Dissolution results by elution fraction of 40% dichloromethane in hexane (0.1% formic acid) solvent combination

Volume (mL)	Vol. of 40% dichloromethane in hexane & 0.1% formic acid					
	5	**10**	**15**	**20**	**25**	**30**
Component elution	O	O	O	_	_	_

Figure 19. SPE-silica washing volume selection schematic

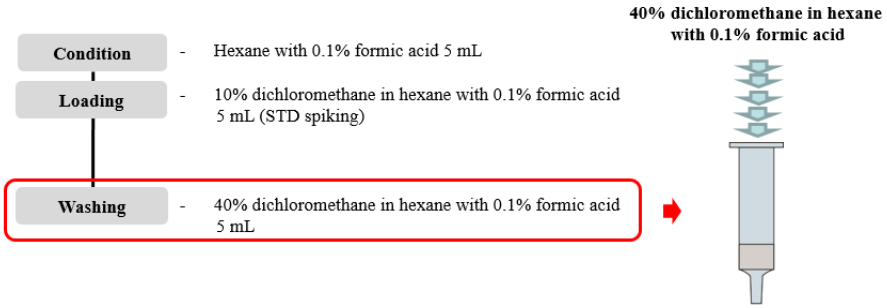

40% dichloromethane in hexane
with 0.1% formic acid
Condition
- Hexane with 0.1% formic acid 5 mL
Loading
- 10% dichloromethane in hexane with 0.1% formic acid
5 mL (STD spiking)
Washing
- 40% dichloromethane in hexane with 0.1% formic acid
5 mL

Figure 20. SPE-silica elution volume selection schematic

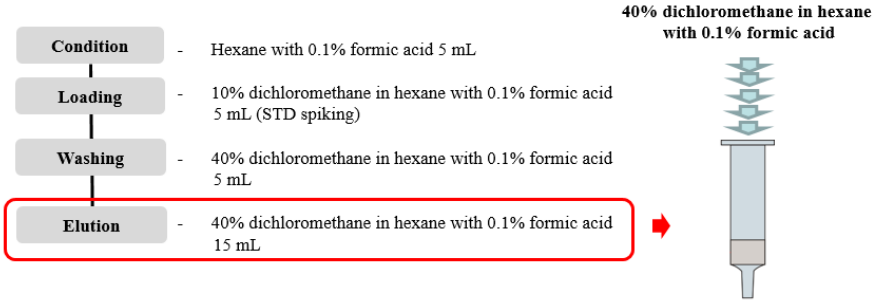
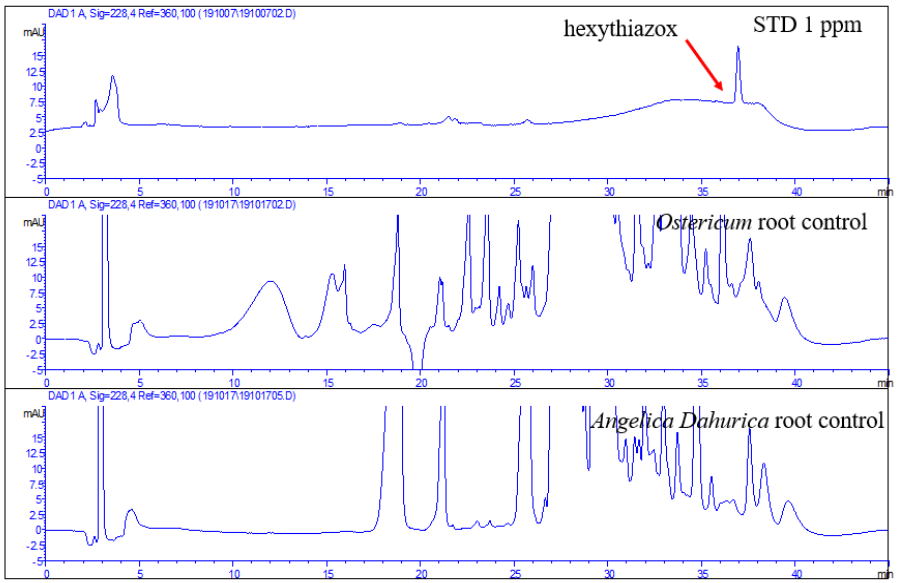

40% dichloromethane in hexane
with 0.1% formic acid
Condition
- Hexane with 0.1% formic acid 5 mL
Loading
- 10% dichloromethane in hexane with 0.1% formic acid
5 mL (STD spiking)
Washing
- 40% dichloromethane in hexane with 0.1% formic acid
5 mL
Elution
- 40% dichloromethane in hexane with 0.1% formic acid
15 mL

Figure 21. Chromatogram of *Ostericum* root and *Angelica Dahurica* root samples after primary SPE-NH_2/GCB and secondary SPE-silica purification


DAD1 A, Sig=228,4 Ref=360,100 (191007\19100702.D)
hexythiazox
STD 1 ppm
DAD1 A, Sig=228,4 Ref=360,100 (191017\19101702.D)
Ostericum root control
DAD1 A, Sig=228,4 Ref=360,100 (191017\19101705.D)
Angelica Dahurica root control
mAU
min

Applying derivatization method to develop analytical method using LC-FLD

Since quantitative and qualitative analysis could not be performed with LC-UVD, the analysis instrument was changed. As a result of pilot experiment using hexythiazox standard, it was confirmed that it was derivatized. Therefore, LC-FLD was used to use the analysis method modified by the Korean Pharmacopoeia. First, derivatization was attempted by reacting the standard with OPA (o-phthaldialdehyde) reagent, thiofluor and 1 M NaOH. After spiking the hexythiazox standard from the herbal medicine *Ostericum* root and *Angelica Dahurica* root, an improved analysis method was performed and detected with LC-FLD. However, although the purification effect was good, it was confirmed that the derivatization was not well done due to impurities, so the recovery rate was not good.

Table 12. Hexythiazox recovery using fluorescence derivatization

Sample	Spiking level (mg/kg)	Area	Recovery (%)
Astragalus root	STD 1 ppm	181.6	28.14
	Sample spiking	51.1	

Figure 22. Hexythiazox 1 µg/mL standard chromatogram using LC-FLD

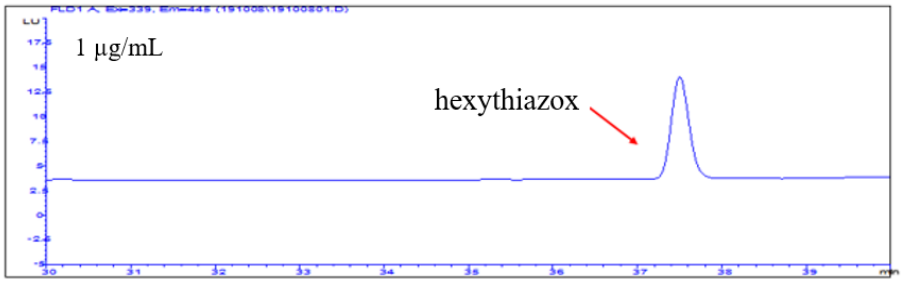
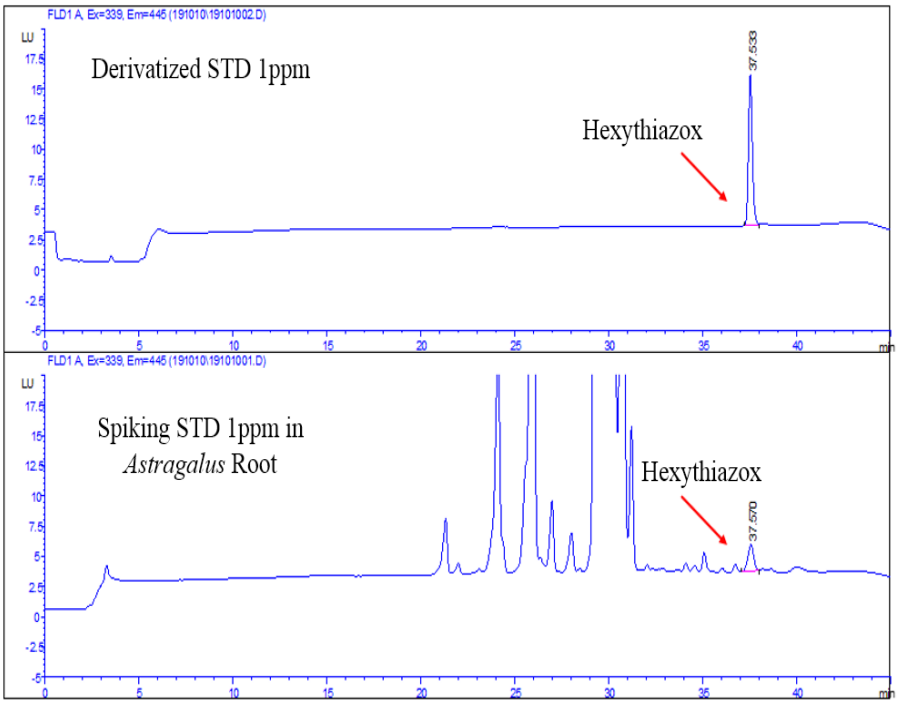

1 µg/mL
hexythiazox

Figure 23. Hexythiazox chromatogram using fluorescent derivatization


FLD1 A, Ex=339, Em=445 (191010\19101002.D)
Derivatized STD 1ppm
Hexythiazox
37.533
FLD1 A, Ex=339, Em=445 (191010\19101001.D)
Spiking STD 1ppm in
Astragalus Root
Hexythiazox
37.570

Korean Food Standards Codex Multi-Pesticide Multi-Component Analysis Method Second Method (7.1.2.2) Reduction

The residuals of hexythiazox in the sample were analyzed using the LC-UVD and LC-FLD identified above, but it was difficult to separate the peaks due to the large amount of impurities. Therefore, LC-MS/MS with high selectivity was used. To ensure the extraction efficiency of hexythiazox and to minimize the extraction of impurities, acetonitrile was selected as the extraction solvent, and the QuEChERS distribution salt, which is the minimum pretreatment method, was adopted in consideration of the high-resolution LC-MS/MS.

Multiple reactive monitoring (MRM) conditions selected ionized precursor ions of $[M+H]^+$ type and established product ions with optimal collision energy (CE). The instrumentation limit was confirmed to be 0.02 μg/mL, a concentration that satisfies the S/N ratio ≥ 10.

Table 13. Precursor ion and product ion conditions in LC-MS/MS

Precusor	Product	Dwell time	Q1	CE	Q3
353.1	228	100	-16	-15	-15
353.1	168.1	100	-16	-26	-17
353.1	116.1	100	-16	-49	-21

QuEChERS Extraction process

Ostericum root, *Angelica Dahurica* root sample was treated with 0.5 mg/kg of standard solution, and after the previous extraction and distribution process, the recovery rate was confirmed without dSPE, and the original salt (NaCl 1 g, $MgSO_4$ 4 g) was 93.23%, EN salt (NaCl 1 g, $MgSO_4$ 4 g, Trisodium citrate dehydrate 1 g, Disodium hydrogen citrate sesquihtdrate 0.5 g) was 88.87%, AOAC salt (NaOAc 1.5 g, $MgSO_4$ 6 g) was 87.37%, and the recovery from the original salt was the best.

Figure 24. Hexythiazox 0.5 µg/mL chromatogram using LC-MS/MS

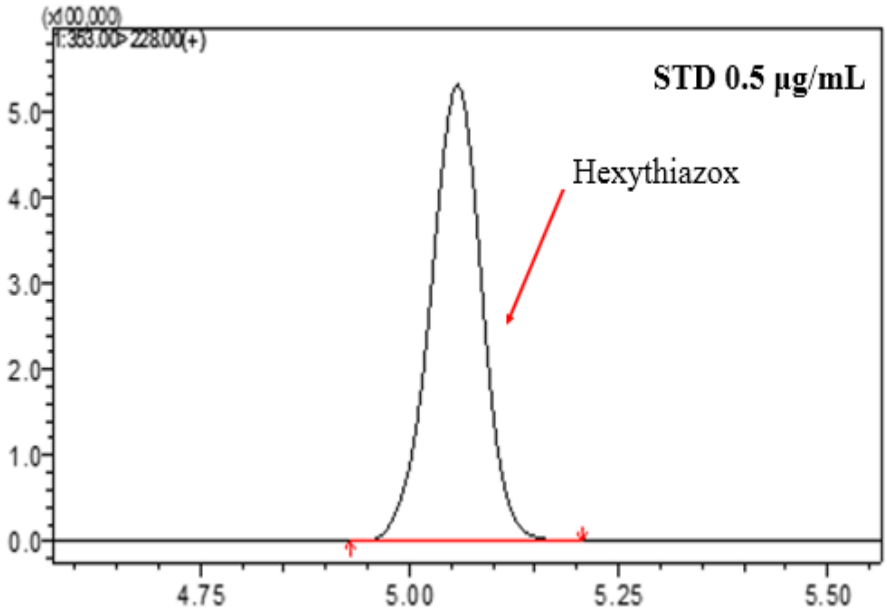
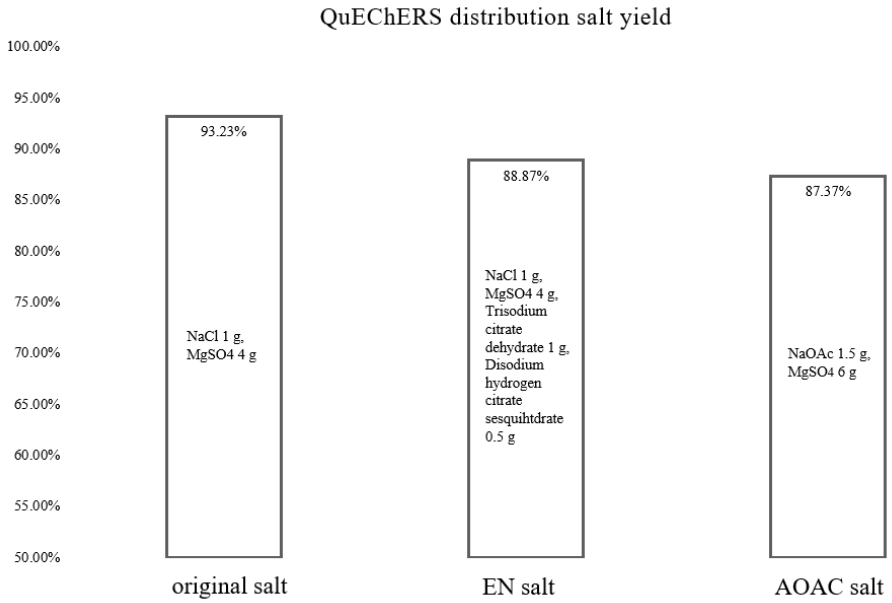

(x100,000)
T:353.00>228.00(+)
STD 0.5 µg/mL
Hexythiazox
5.0
4.0
3.0
2.0
1.0
0.0
4.75
5.00
5.25
5.50

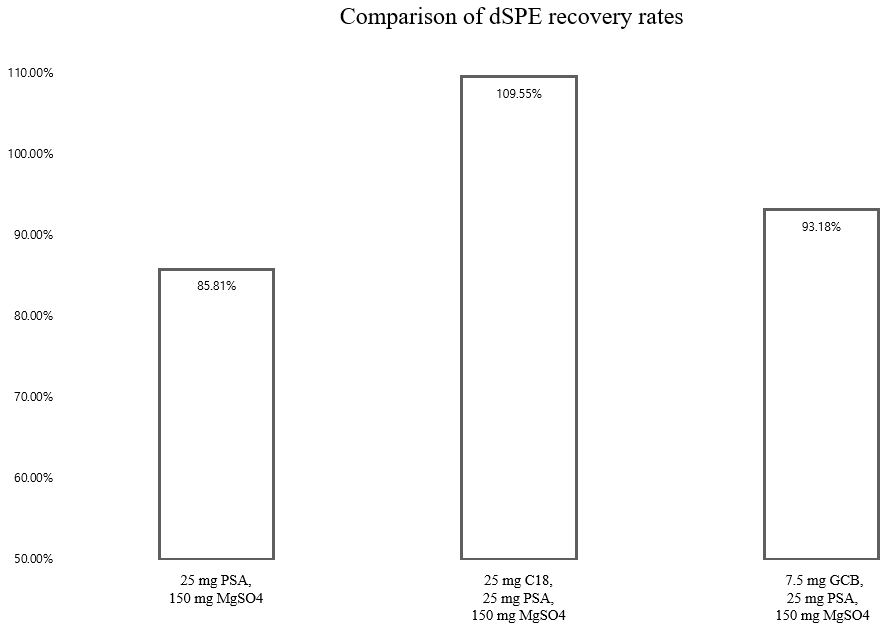
Figure 25. Comparison of QuEChERS distribution salt yield


QuEChERS distribution salt yield
100.00%
95.00%
90.00%
85.00%
80.00%
75.00%
70.00%
65.00%
60.00%
55.00%
50.00%
93.23%
NaCl 1 g, MgSO4 4 g
88.87%
NaCl 1 g, MgSO4 4 g, Trisodium citrate dehydrate 1 g, Disodium hydrogen citrate sesquihtdrate 0.5 g
87.37%
NaOAc 1.5 g, MgSO4 6 g
original salt
EN salt
AOAC salt

dSPE Purification process

Compared to GCB (graphite carbon black) sugar, which is excellent in removing pigments and structural planar impurities, and PSA (primary secondary amine), which is effective in purifying polar impurities, and C18, which is excellent in removing oily impurities, in order to at least remove a large amount of impurities in herbal samples. The purification efficiency was compared. Therefore, after extraction with original salt, dSPE with 7.5 mg GCB, 25 mg PSA, 150 mg $MgSO_4$, dSPE with 25 mg C18, 25 mg PSA, 150 mg $MgSO_4$, dSPE with 25 mg PSA, 150 mg $MgSO_4$ were compared respectively. As a result, it was confirmed that the dSPE containing 25 mg C18, 25 mg PSA, and 150 mg $MgSO_4$ was excellent at 109.55%. In dSPE, in order to confirm the recovery rate of the sample, the MLOQ was set to 0.05 mg/kg when performing the recovery rate test. However, it was confirmed that quantification was impossible due to the recovery rate and the peak of impurities at the quantitative limit level among the *Ostericum* root and *Angelica Dahurica* root samples. Therefore, purification was performed using SPE to maximize the purification effect.

Figure 26. Comparison of dSPE recovery rates


Comparison of dSPE recovery rates
110.00%
100.00%
90.00%
80.00%
70.00%
60.00%
50.00%
85.81%
109.55%
93.18%
25 mg PSA,
150 mg MgSO4
25 mg C18,
25 mg PSA,
150 mg MgSO4
7.5 mg GCB,
25 mg PSA,
150 mg MgSO4

Establishment of QuEChERS, dSPE and SPE conditions to develop methods using LC-MS/MS

In order to use LC-MS/MS, it was necessary to newly establish the conditions of QuEChERS and dSPE, which are easy to pretreatment. However, when dSPE was used, the sensitivity was lowered due to impurities and the recovery rate was also poor. Therefore, it was decided to establish the SPE condition instead of dSPE, and the previously established SPE-NH_2/GCB cartridge was used. However, it was confirmed that the previously established SPE-NH_2/GCB cartridge conditions can be applied when long time extraction and distribution conditions using separatory funnel are used.

In LC-MS/MS, the extraction and distribution conditions using QuEChERS were used, so when the previously established SPE-NH_2/GCB cartridge conditions were used, the recovery rates were also very low, 51% and 58%. Since QuEChERS was used, a new SPE-NH_2/GCB cartridge condition was established in consideration of changes in extraction and distribution conditions. As a result, the herbal medicine *Ostericum* root and *Angelica Dahurica* root were able to satisfy the recovery rate.

Figure 27. Recovery of dSPE from *Ostericum* root and *Angelica Dahurica* root

***Ostericum* Root** ***Angelica Dahurica* Root**

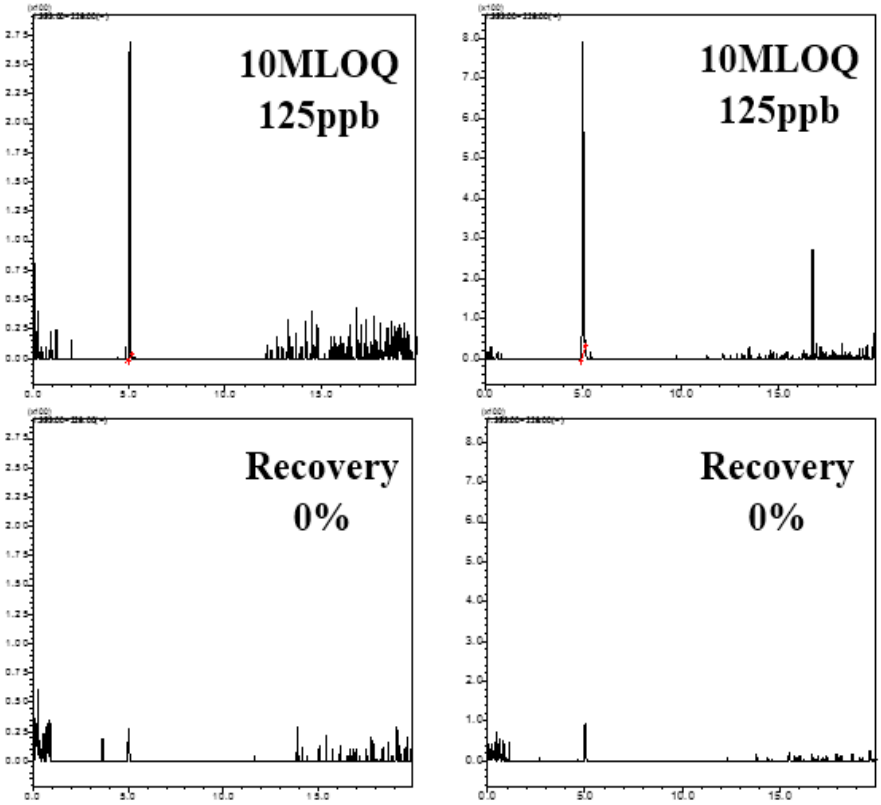
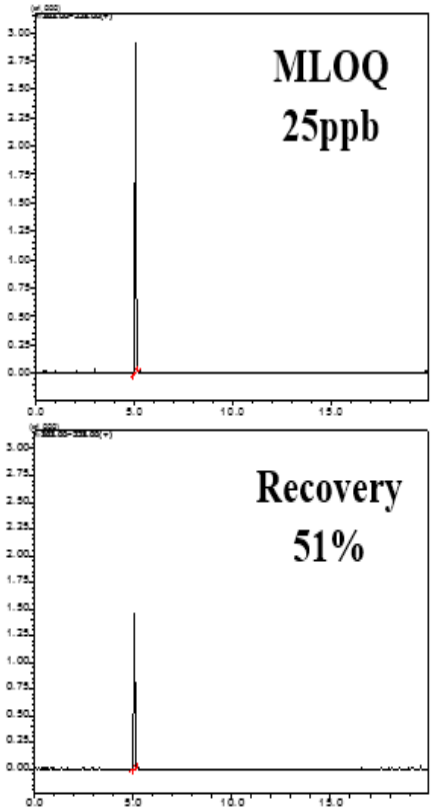
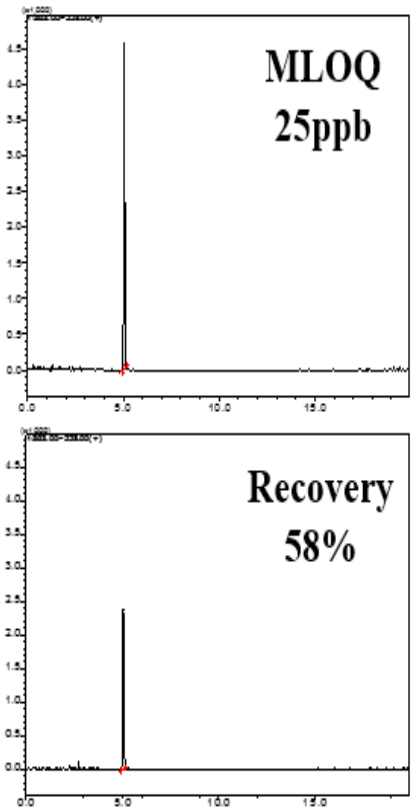


Figure 28. Recovery of SPE from *Ostericum* root and *Angelica Dahurica* root

Ostericum Root



Angelica Dahurica Root



Optimization of purification (SPE-NH_2/GCB) conditions (selection of washing + elution conditions)

Since the extraction and distribution methods are different from the improved series-specific test methods, the results of the purification conditions were also changed. Therefore, an extract with improved Korean Food Standards Codex Multi-Pesticide Multi-Component Analysis (7.1.2.2) was used. Subsequently, SPE-NH_2/GCB conditions were reestablished. Cartridge was conditioned with 5 mL of hexane (0.1% formic acid) to confirm the washing of impurities and the elution section of the component. Standard 10 μg/mL dissolved in 10% dichloromethane in hexane (0.1% formic acid), 5 mL was added. Thereafter, 5 mL of each 10% dichloromethane in hexane (0.1% formic acid) was separately received to confirm the elution section.

Table 14. Recovery result by fraction of 10% dichloromethane in hexane (0.1% formic acid) solvent combination

	10% dichloromethane in hexane & 0.1% formic acid			
	Loading, 5mL	**Elution-1, 5mL**	**Elution-2, 5mL**	**Elution-3, 5mL**
Recovery (%)	–	19.76	87.53	1.27

As a result of the experiment, the recovery rate of 108.6% was confirmed when 15 mL of the eluent was received without a washing section. Based on the above results, 15 mL of 10% dichloromethane in hexane (0.1% formic acid) was established as the elution section.

Figure 29. SPE-NH_2/GCB washing + elution condition selection schematic

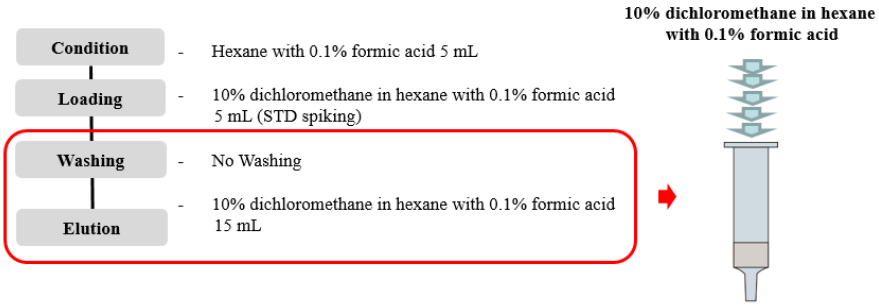
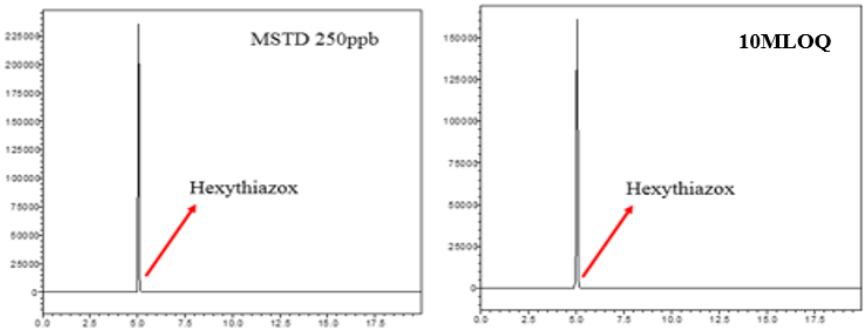
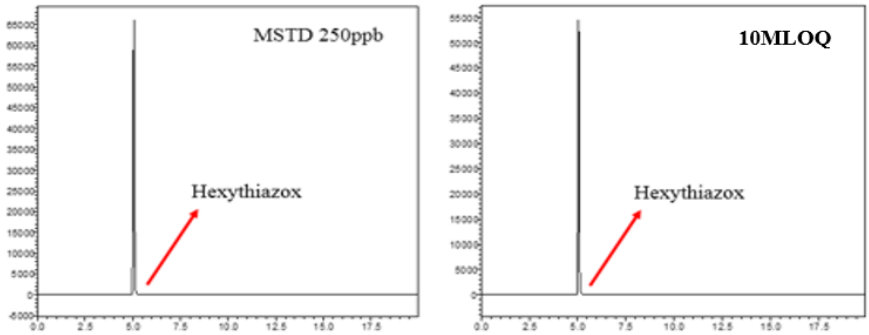

10% dichloromethane in hexane
with 0.1% formic acid
Condition
- Hexane with 0.1% formic acid 5 mL
Loading
- 10% dichloromethane in hexane with 0.1% formic acid
5 mL (STD spiking)
Washing
- No Washing
Elution
- 10% dichloromethane in hexane with 0.1% formic acid
15 mL

Figure 30. Recovery of SPE-NH_2/GCB from *Ostericum* root and *Angelica Dahurica* root

***Ostericum* Root**



***Angelica Dahurica* Root**



The final established analytical method

A small amount of water was added to the homogenized dry herbal sample (*Ostericum* root and *Angelica Dahurica* root) to moisten it, and then acetonitrile was added to shake extract to extract hexythiazox from the sample. A portion of the extracted supernatant was taken in SPE-NH_2/GCB cartridge and purified and analyzed by LC-MS/MS.

Quantitative limits and calibration curves for established analytical methods

In this study, a matrix matched calibration curve was prepared during LC-MS/MS analysis to quantify the sample extract. The instrument quantitative limit (S/N≥10) of hexythiazox was 0.02 μg/mL, and the coefficient of determination (r^2) of the standard calibration curve was 0.99 or higher for each medium, showing excellent linearity. In the case of hexythiazox analysis among the Ostericum root and Angelica Dahurica root established in this study, the Method Limit of Quantitation (MLOQ) was calculated as 0.02 mg/kg.

Table 15. Calibration curve and linearity

Matrix matched Standard solution	**MSTD 1 (0.02 µg/mL)**	**MSTD 2 (0.025 µg/mL)**	**MSTD 3 (0.05 µg/mL)**	**MSTD 4 (0.1 µg/mL)**	**MSTD 5 (0.25 µg/mL)**
Standard solution	0.04µg/mL 200 µL	0.05 µg/mL 200 µL	0.1 µg/mL 200 µL	0.2 µg/mL 200 µL	0.5 µg/mL 200 µL
Sample matrix	200 µL	200 µL	200 µL	200 µL	200 µL

Figure 31. Hexythiazox calibration curve in *Ostericum* root

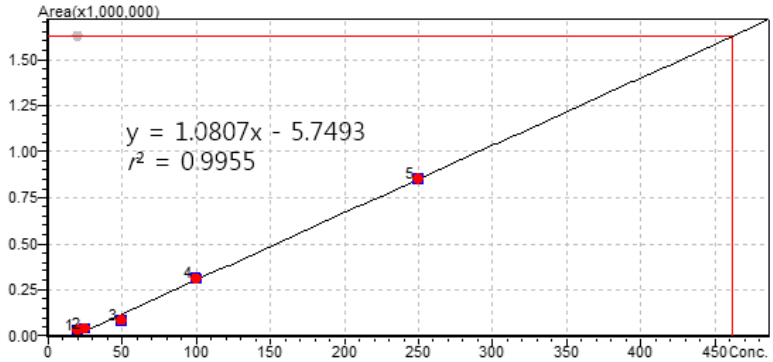
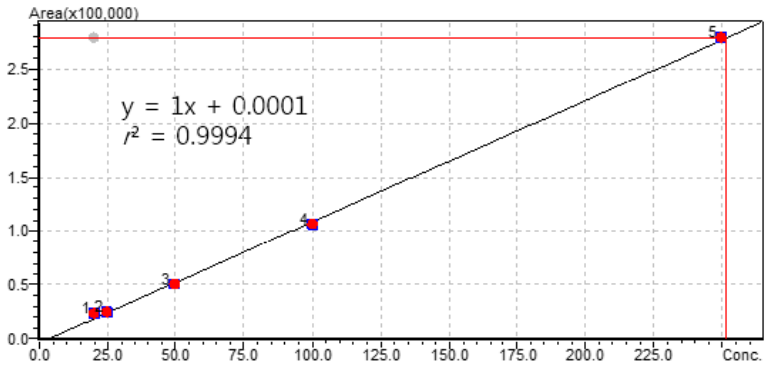

Area(x1,000,000)
y = 1.0807x - 5.7493
r² = 0.9955
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
50
100
150
200
250
300
350
400
450
Conc.
1
2
4
5

Figure 32. Hexythiazox calibration curve in *Angelica Dahurica* root


Area(x100,000)
y = 1x + 0.0001
r² = 0.9994
2.5
2.0
1.5
1.0
0.5
0.0
0.0
25.0
50.0
75.0
100.0
125.0
150.0
175.0
200.0
225.0
Conc.
1
2
3
4
5

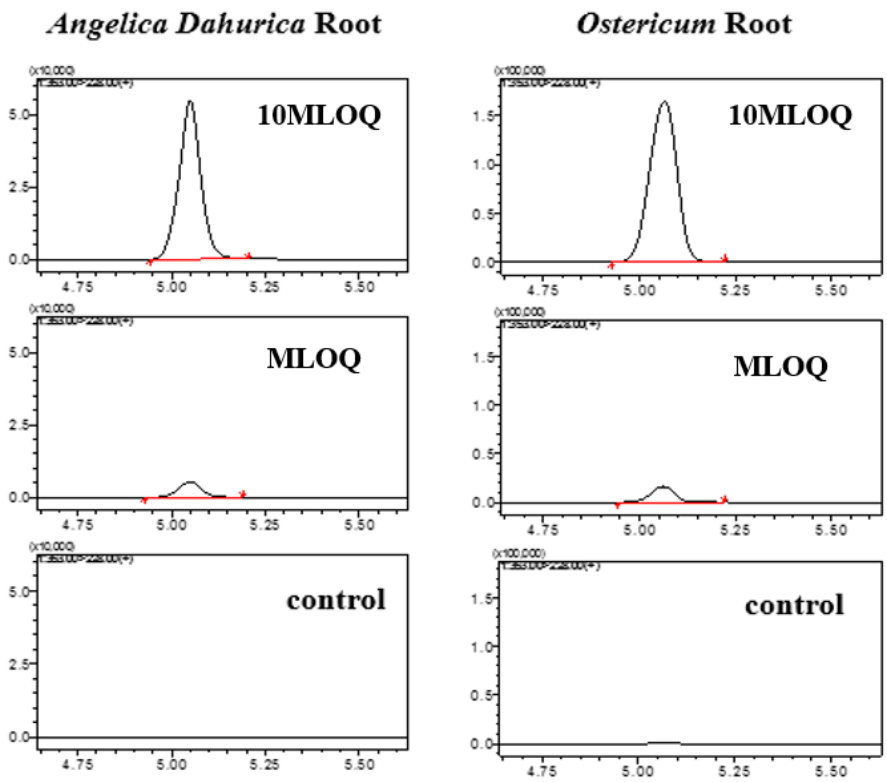
Verification of established analytical methods

The analysis of hexythiazox method established in this study. The accuracy and precision of the analysis were confirmed by confirming the recovery rate of hexythiazox in *Ostericum* root and *Angelica Dahurica* root according to the established method. The control samples were treated with MLOQ (0.02 mg/kg) and 10 MLOQ (0.2 mg/kg) for the standard treatment level, and then a recovery experiment was performed. Three repetition experiments were performed for each treatment level. The results of the recovery rate are shown in Table 16. and satisfy the conditions of the standard procedure guidelines prepared for the Codex Guideline (CAC/GL 40) test method.

Table 16. Recovery rate and quantitative limit of hexythiazox in *Ostericum* root and *Angelica Dahurica* root (n=3)

Sample	Fortified level (mg/kg)	Recovery (%)	RSD (%)	ILOQ (μg/mL)
Ostericum root	0.02	103.06	0.87	0.02
	0.2	88.17	2.18	
Angelica Dahurica root	0.02	93.37	8.78	
	0.2	77.79	3.71	

Figure 33. LC-MS/MS representative chromatogram of hexythiazox in *Ostericum* root and *Angelica Dahurica* root


Angelica Dahurica Root
Ostericum Root
10MLOQ
MLOQ
control
10MLOQ
MLOQ
control

Cross validation between laboratories at Seoul National University and Kangwon National University

In this study, cross validation of the analysis method for hexythiazox was conducted. The reliability of the analysis was verified by confirming the recovery rate of hexythiazox for *Ostericum* root and *Angelica Dahurica* root according to the established analysis method. According to the standard solution addition method to the untreated sample, a standard solution of hexythiazox was added so that the treatment level was 10 MLOQ (0.2 mg/kg) to prepare a recovery sample, and the experiment was repeated three times. As a result of cross validation between the laboratories (Seoul National University and Kangwon National University), the residual analysis criteria of 70-120% recovery rate and the coefficient of variation within 20% were satisfied, confirming the suitability of the process analysis method.

Table 17. Cross validation of hexythiazox analytical method between laboratories in *Ostericum* root and *Angelica Dahurica* root (n=3)

Sample	Fortified level (mg/kg)	Seoul National University Recovery (%)	Kangwon National University Recovery (%)	ILOQ (μg/mL)
Ostericum root	0.2	88.2	81.2	0.02
Angelica Dahurica root	0.2	77.8	93.8	

Conclusion

In this experiment, an analysis method was developed in the herbal medicine *Ostericum* root and *Angelica Dahurica* root, and this analysis method will be published in the Korean Pharmacopoeia issued by the Ministry of Food and Drug Safety. It is necessary to support the pesticide residue method in order to use the pesticide management method in policy. But the method notified in the existing Korean Pharmacopoeia is not suitable for applying pesticide analysis. In addition, the currently announced residue method has a very low analysis efficiency. Therefore, it was necessary to optimize and develop method for residual pesticides of herbal medicines containing a large amount of organic active substances. The newly developed analysis method is capable of quantitative and qualitative analysis in herbal medicines, and this study is believed to be able to utilize the pesticide management method in policy.

Part 2

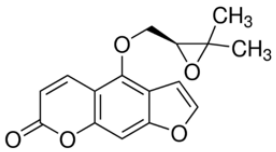
Analysis of active ingredient in *Ostericum* root and *Angelica Dahurica* root

Introduction

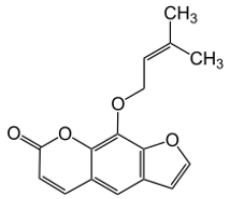
Active ingredient analysis plan

Korean Pharmacopoeia has a active ingredient method. *Angelica Dahurica* root, a herbal medicine, has active ingredient analysis method and active ingredient information, but *Ostericum* root does not have active ingredient analysis method and active ingredient information. Therefore, Korean Pharmacopoeia was used to confirm the active ingredient of *Angelica Dahurica* root, and the experiment was conducted in the same way as *Ostericum* root. As a result of using Korean Pharmacopoeia, there were many impurities and the peak was not separated. However, as a result of using Korean Pharmacopoeia, overlapping peaks were found in *Ostericum* root and *Angelica Dahurica* root. As a result of literature research, it was found that the active ingredient overlapped with the *Angelica Dahurica* root in the *Ostericum* root. In Korean Pharmacopoeia, oxypeucedanin, Imperatorin, and isoimperatorin were suggested as the active ingredient of *Angelica Dahurica* root. As a result of the analysis, it was confirmed that a peak recognizable as a active ingredient appeared. Likewise, three peaks of oxypeucedanin, Imperatorin, and isoimperatorin appeared in the *Ostericum* root, and it was confirmed that the sensitivity was excellent enough to be designated as a active ingredient.

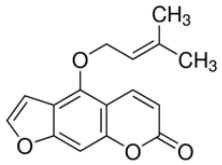
Figure 34. Molecular structure of oxypeucedanin, imperatorin, isoimperatorin



oxypecedanin



imperatorin



isoimperatorin

Material and Methods

Principle of analytical method

Weigh about 1 g of the sample (*Ostericum* root and *Angelica Dahurica* root) precisely, add 50 mL of methanol, ultrasonically extract for 1 hour, and filter. Add 50 mL of methanol to the residue and operate in the same way. Combine all and concentrate, then add 10 mL of methanol and analyzed by HPLC-UVD.

LC-UVD instrumental analysis condition

The instrument conditions using LC-UVD introduced in Korean Pharmacopoeia were confirmed as shown in Table 18. For the separation of oxypeucedanin, imperatorin, isoimperatorin, a C18 column was used, water and methanol were used as mobile phases, and an optimum wavelength of 254 nm was confirmed.

Table 18. oxypeucedanin, imperatorin, isoimperatorin LC-UVD analysis conditions

HP LC	**Instrument**	**Agilent 1100 series**
	Column (oven temp.)	Luna C18 (250 × 4.6 mm, 5 μm, phenomenex) (25℃)
	Mobile phase	A: Water, B:Methanol
	Isocratic	Water : Methanol (65:35)
	Wavelength	254 nm
	Detector	UVD
	Injection vol.	10 μL

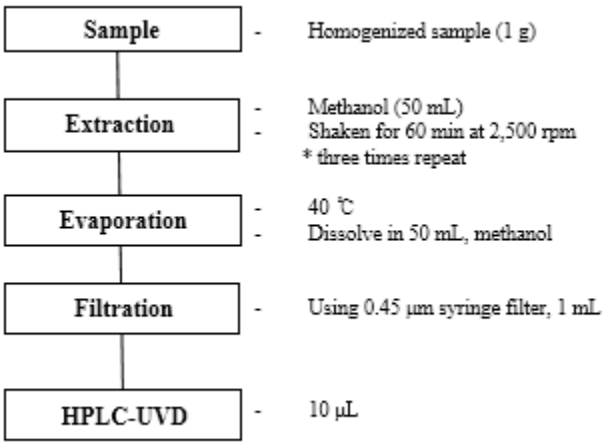
Changes in experimental procedures and instrument conditions for peak separation at *Angelica Dahurica* root

In Korean Pharmacopoeia, since peak separation of *Angelica Dahurica* root did not occur, quantitative analysis was not possible. Therefore, instead of Korean Pharmacopoeia, there was a need for a method to isolate the active ingredient of *Angelica Dahurica* root. As a result of referencing several documents, the extraction and purification process could be established, and the instrument condition was also changed from isocratic to gradient, allowing separation of two overlapping peaks at the *Angelica Dahurica* root.

Table 19. oxypeucedanin, imperatorin, isoimperatorin LC-UVD improved analysis conditions

<table>
<tr><td rowspan="6">HP LC</td><td>Instrument</td><td>Agilent 1100 series</td></tr>
<tr><td>Column (oven temp.)</td><td>XBridge C18 (250 × 4.6 mm, 5 μm, phenomenex) (30℃)</td></tr>
<tr><td>Mobile phase</td><td>A: Water, B:Methanol</td></tr>
<tr><td>Gradient</td><td><table>
<tr><td>Time (min.)</td><td>Mobile A (%, v)</td><td>Mobile B (%, v)</td></tr>
<tr><td>0</td><td>70</td><td>30</td></tr>
<tr><td>3</td><td>70</td><td>30</td></tr>
<tr><td>30</td><td>20</td><td>80</td></tr>
<tr><td>40</td><td>20</td><td>80</td></tr>
<tr><td>45</td><td>70</td><td>30</td></tr>
<tr><td>50</td><td>70</td><td>30</td></tr>
</table></td></tr>
<tr><td>Wavelength</td><td>254 nm</td></tr>
<tr><td>Detector</td><td>UVD</td></tr>
<tr><td>Injection vol.</td><td>10 μL</td></tr>
</table>

Figure 35. Schematic diagram of the analysis applied improved Korean Pharmacopoeia method


Sample
- Homogenized sample (1 g)
Extraction
- Methanol (50 mL)
- Shaken for 60 min at 2,500 rpm
* three times repeat
Evaporation
- 40 ℃
- Dissolve in 50 mL, methanol
Filtration
- Using 0.45 μm syringe filter, 1 mL
HPLC-UVD
- 10 μL

Results and Discussion

Qualitative analysis of active ingredients detected in herbal medicine *Ostericum* root and *Angelica Dahurica* root

In order to analyze the active ingredients of the herbal medicine *Ostericum* root and *Angelica Dahurica* root, the analysis method suggested by the Korean Pharmacopoeia was performed. However, it was confirmed that the peak of the active ingredient in the root of the herbal medicine *Angelica Dahurica* was not separated. In addition, since the sensitivity to active ingredients was low, the analysis method was decided to be improved. As a result of improving the method of the Korean Pharmacopoeia, the overlapping peaks at the root of the herbal medicine *Angelica Dahurica* were separated. In addition, it was confirmed that the sensitivity of the active ingredient in the herbal medicine *Ostericum* root and *Angelica Dahurica* root was improved. The active ingredients of the herbal medicine *Angelica Dahurica* root announced by the Korean Pharmacopoeia were oxypeucedanin, imperatorin and isoimperatorin. However, the peaks of oxypeucedanin, imperatorin and isoimperatorin were also confirmed in *Ostericum* root. After confirming three common peaks by LC-UVD, qualitative analysis was performed on the peaks at *Ostericum* root and *Angelica Dahurica* root using LC-MS/MS. The main four peaks in the *Ostericum* root were analyzed qualitatively, and the five main peaks in the *Angelica Dahurica* root were qualitatively analyzed.

Figure 36. Optimal wavelength of active ingredient using LC-UVD (254nm)

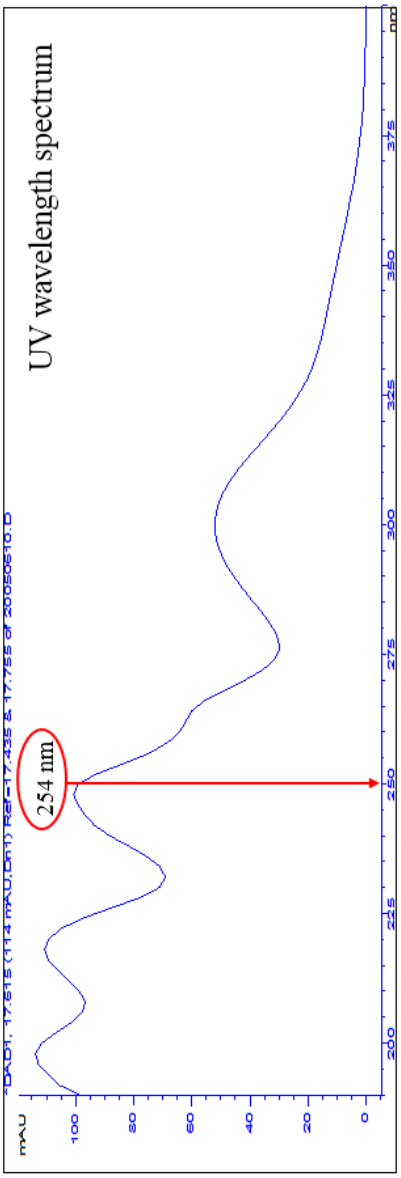

UV wavelength spectrum
254 nm
mAU
100
80
60
40
20
0
200
225
250
275
300
325
350
375
nm

Figure 37. Chromatogram of *Ostericum* root and *Angelica Dahurica* root active ingredient using Korean Pharmacopoeia

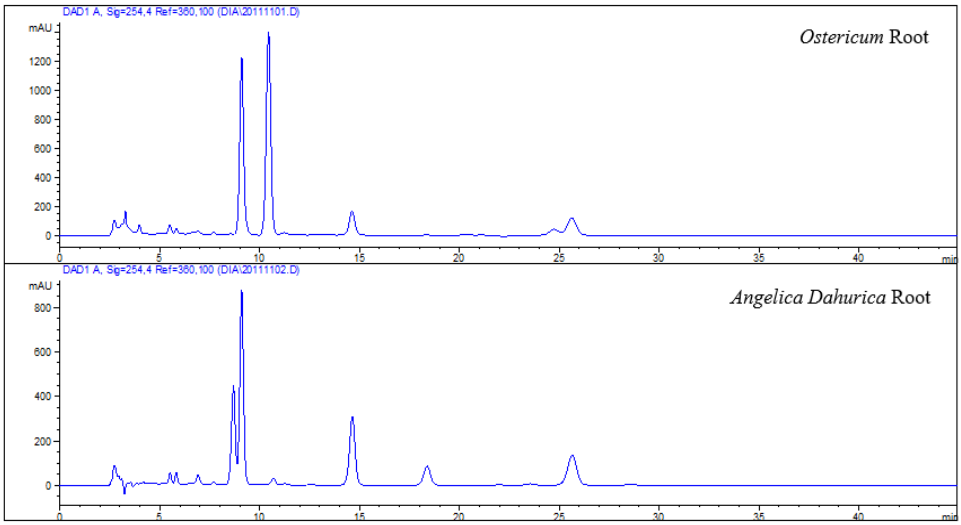

DAD1 A, Sig=254,4 Ref=360,100 (DIA\20111101.D)
mAU
Ostericum Root
DAD1 A, Sig=254,4 Ref=360,100 (DIA\20111102.D)
mAU
Angelica Dahurica Root

Figure 38. Chromatogram using the improved Korean Pharmacopoeia method

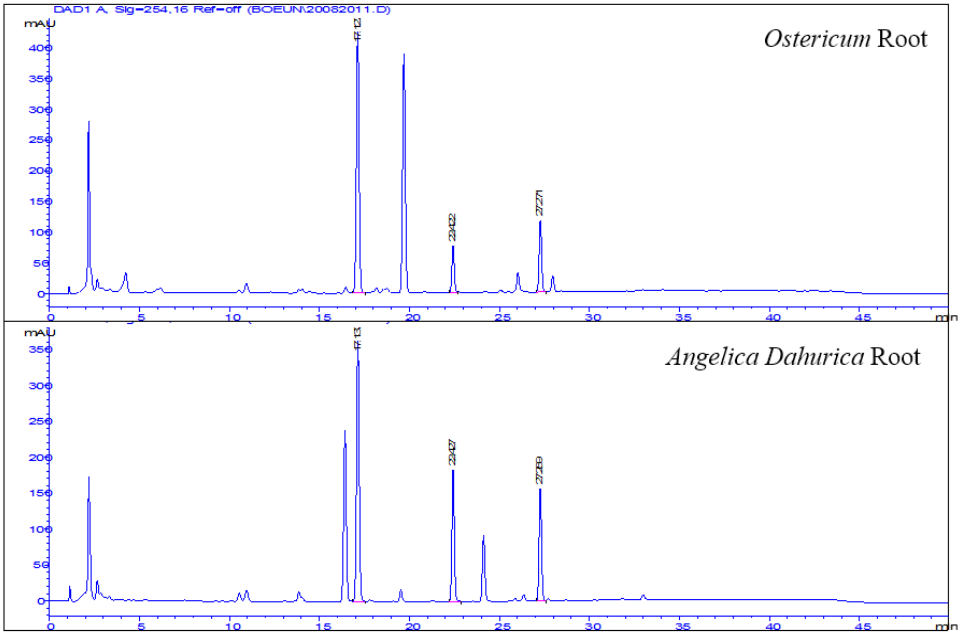
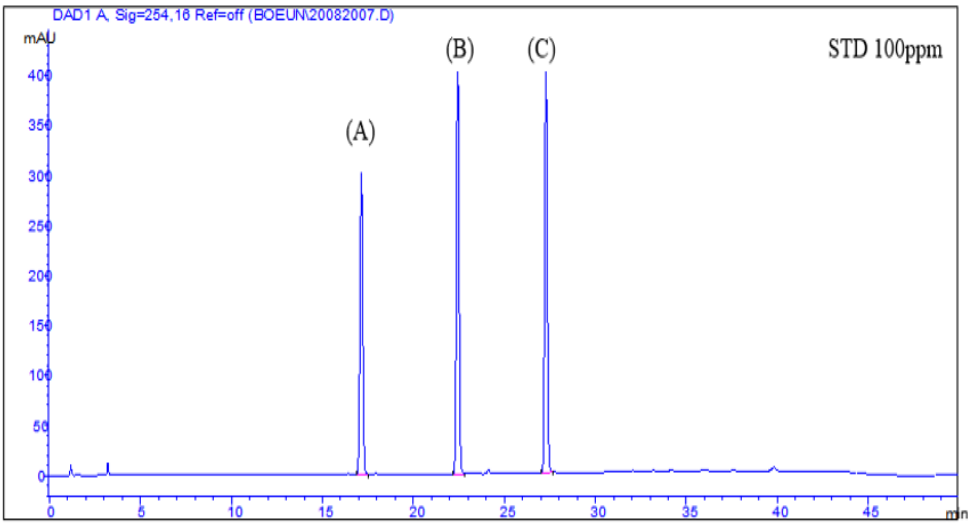

DAD1 A, Sig=254,16 Ref=off (BOEUN\20082011.D)
mAU
Ostericum Root
17.13
19.42
22.71
min
mAU
Angelica Dahurica Root
17.13
19.47
22.69
min

Figure 39. Chromatogram of 100ppm standard solution of oxypeucedanin, imperatorin, isoimperatorin

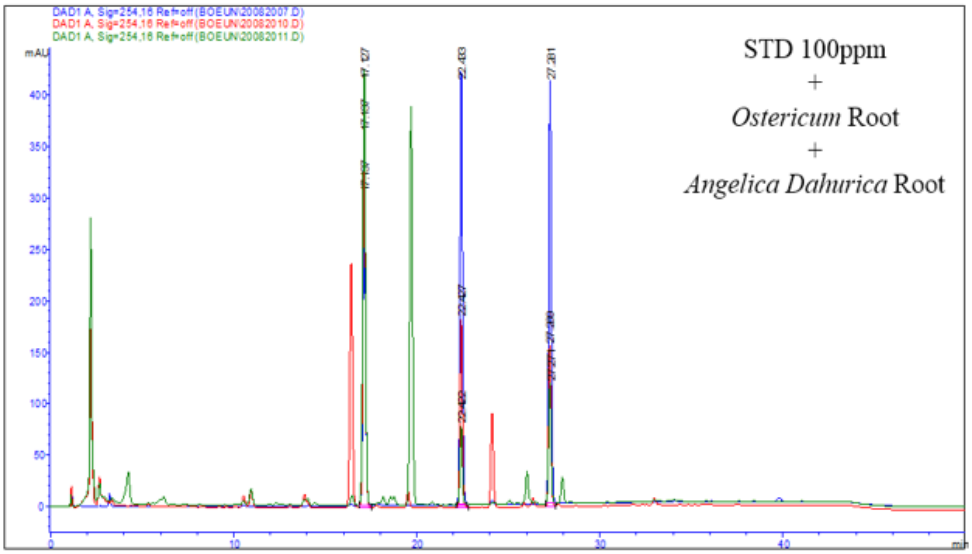


(A) oxypeucedanin

(B) imperatorin

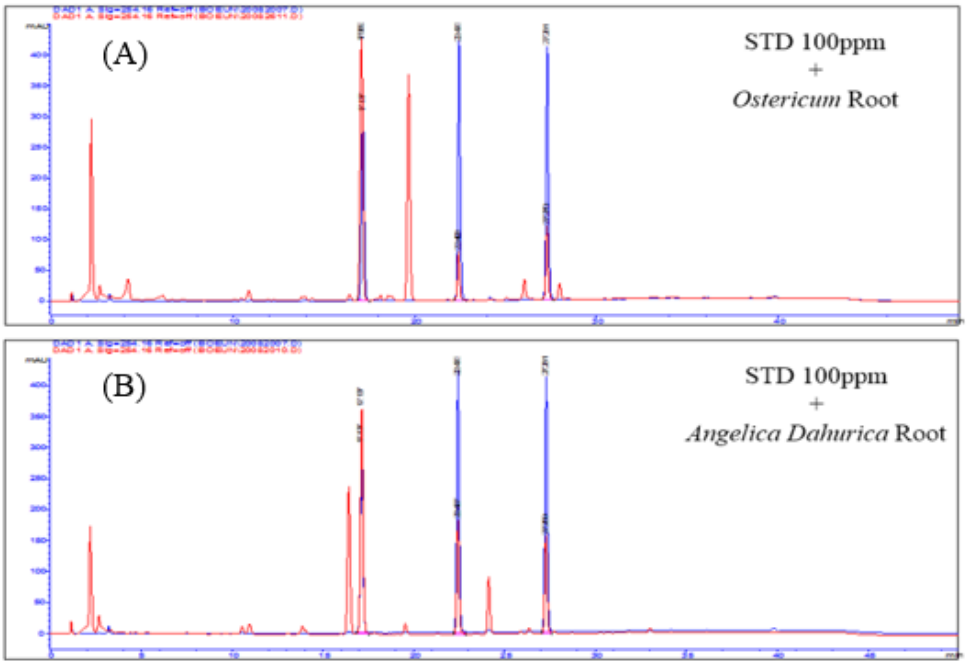
(C) isoimperatorin

Figure 40. Chromatogram of *Ostericum* root, *Angelica Dahurica* root, standard solution 100ppm



Blue: STD 100ppm, Green: *Ostericum* Root, Red: *Angelica Dahurica* Root

Figure 41. Chromatogram of *Ostericum* root + 100 ppm standard solution and *Angelica Dahurica* root + 100 ppm standard solution



(A) Blue: STD 100ppm, Red: *Ostericum* Root

(B) Blue: STD 100ppm, Red: *Angelica Dahurica* Root

Analysis of active ingredient results at *Ostericum* root

It was confirmed that 4 active ingredient peaks were found in the *Ostericum* root. As a result of scanning by LC-MS/MS, four active ingredients were found to be oxypeucedanin, bisabolangelone, imperatorin, and isoimperatorin. Among them, it was found that the proportions of oxypeucedanin, bisabolangelone, imperatorin, and isoimperatorin four substances in quality control were 43%, 39%, 7% and 11%, respectively.

Identification of active ingredient of *Ostericum* root using LC-MS/MS

In order to identify the components identified as the active ingredient of *Ostericum* root using HPLC, scan was performed using LC-MS/MS. These compounds were characterized by the presence of abundant [M+H]+ ions in positive ion mode ESI-MS analysis, due mainly to protonation at oxygen atom bonded furocoumarin ring.To elucidate the chemical structures of furocoumarins, LC-MS/MS scan technique was applied. The MS/MS spectra of [M+H]+ ions for imperatorin and isoimperatorin produced common product ion at m/z 203 ($C_{11}H_7O_4$) that is formed through the loss of side-chain from furocoumarins. This characteristic ion can be used as diagnostic ion for the presence of a furocoumarins ring moiety in complicate herbal extract. In the case of MS/MS spectrum for bisabolangelone, the characteristic ions were formed at the ions m/z 231 through dehydration from [M+H]+ ion.

Table 20. Percentage of active ingredient in *Ostericum* root

Retention Time (min)	Area	Area (%)
17.127 (oxypeucedanin)	4382.4	43
19.695 (bisabolangelone)	4060.8	39
22.422 (imperatorin)	736.7	7
27.271 (isoimperatorin)	1116	11

Figure 42. Scan spectrum of active ingredient of *Ostericum* root using LC-MS/MS

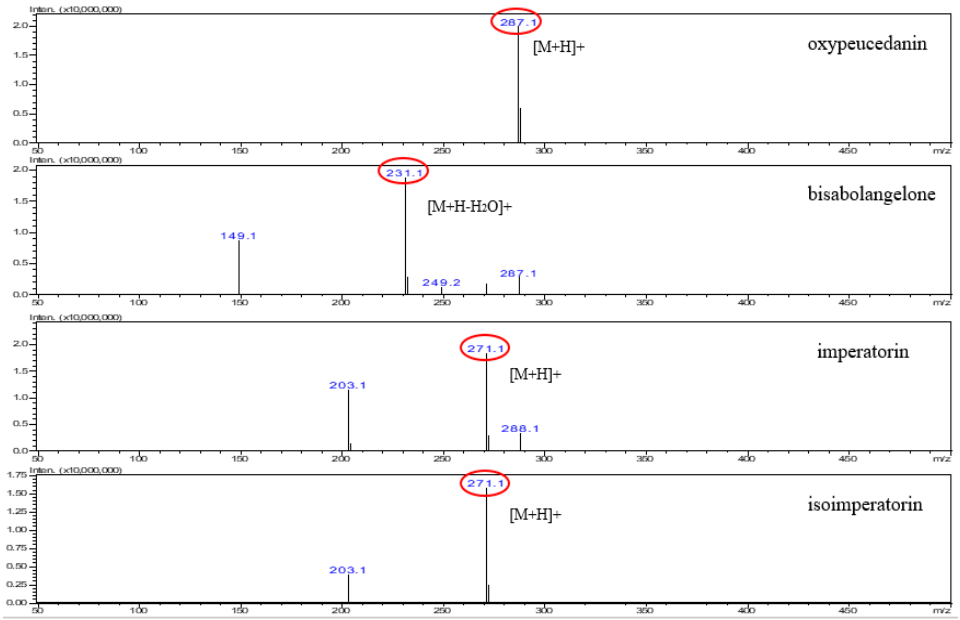
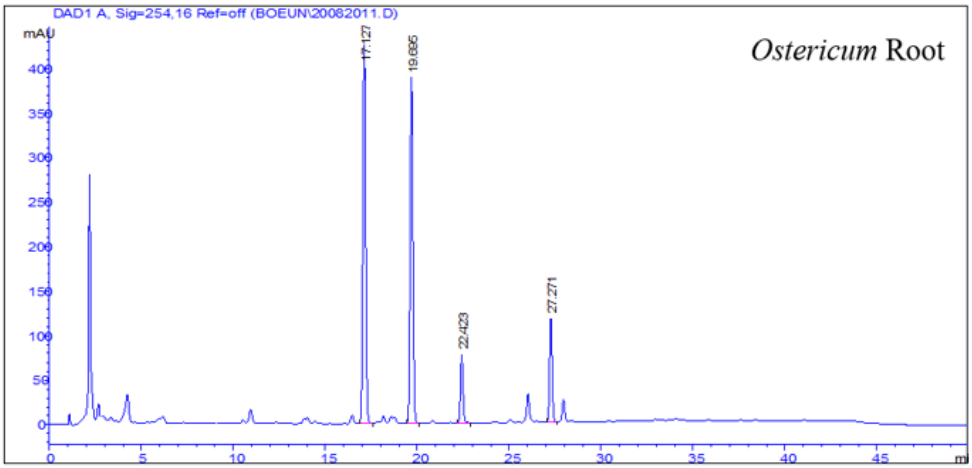

Inten. (x10,000,000)
287.1
[M+H]+
oxypeucedanin
231.1
[M+H-H2O]+
bisabolangelone
149.1
249.2
287.1
271.1
[M+H]+
imperatorin
203.1
288.1
271.1
[M+H]+
isoimperatorin
203.1
m/z

Figure 43. Chromatogram of all active ingredient integration at *Ostericum* root



oxypeucedanin	17.127 min
bisabolangelone	19.695 min
imperatorin	22.422 min
isoimperatorin	27. 271 min

Analysis of active ingredient results at *Angelica Dahurica* root

It was confirmed that 5 active ingredient peaks were found in *Angelica Dahurica* root. As a result of scanning by LC-MS/MS, five active ingredients were found to be byakangelicol, oxypeucedanin, imperatorin, phellopterin and isoimperatorin. Among them, it was found that the proportions of byakangelicol, oxypeucedanin, imperatorin, phellopterin and isoimperatorin five substances in qualiyt control were 24%, 36%, 17%, 9% and 14%, respectively.

Identification of active ingredient of *Angelica Dahurica* root using LC-MS/MS

In order to identify the components identified as the active ingredient of *Angelica Dahurica* root using HPLC, scan was performed using LC-MS/MS. These compounds were characterized by the presence of abundant [M+H]+ ions in positive ion mode ESI-MS analysis, due mainly to protonation at oxygen atom bonded furocoumarin ring. To elucidate the chemical structures of furocoumarins, LC-MS/MS scan technique was applied. Byakangelicol, oxypeucedanin, imperatorin, phellopterin and isoimperatorin were identified as [M+H]+ ions in MS/MS scan spectrum.

Pharmacological activity study on active ingredient of *Ostericum* root and *Angelica Dahurica* root

Isoimperatorin, imperatorin, byakangelicol, oxypeucedanin, and phellopterin were found to have anti-inflammatory effects. Oxypeucedanin showed the strongest anti-allergic effect in vitro. Imperatorin and phellopterin significantly inhibited mucin secretion in the state of secretion stimulation by ATP. It can be seen that imperatorin and phellopterin have respiratory disease inhibitory activity. Regarding the osteoporosis inhibitory effect, isoimperatorin showed a cell proliferative effect in MG-63 cells, an osteoblastic cell line involved in bone regeneration. It was confirmed that imperatorin and isoimperatorin inhibited hepatocyte death by TAA. These studies are thought to be related to the protective action of tacrine-induced liver damage in furocoumarins. The 70% ethanol extract of *Ostericum* root showed weak antioxidant activity. Byakangelicol showed antibacterial activity against H. pylori. Bisabolangelone shows the acaricidal effect of Dermatophagoides farinae and Dermatophagoides pteronyssinus against adult insects.

Table 21. Percentage of active ingredient in *Angelica Dahurica* root

Retention Time (min)	Area	Area (%)
16.420 (byakangelicol)	2461.7	24
17.137 (oxypeucedanin)	3714.5	36
22.427 (imperatorin)	1770.5	17
24.122 (phellopterin)	883.7	9
27.269 (isoimperatorin)	1493.3	14

Figure 44. Scan spectrum of active ingredient of *Angelica Dahurica* root using LC-MS/MS

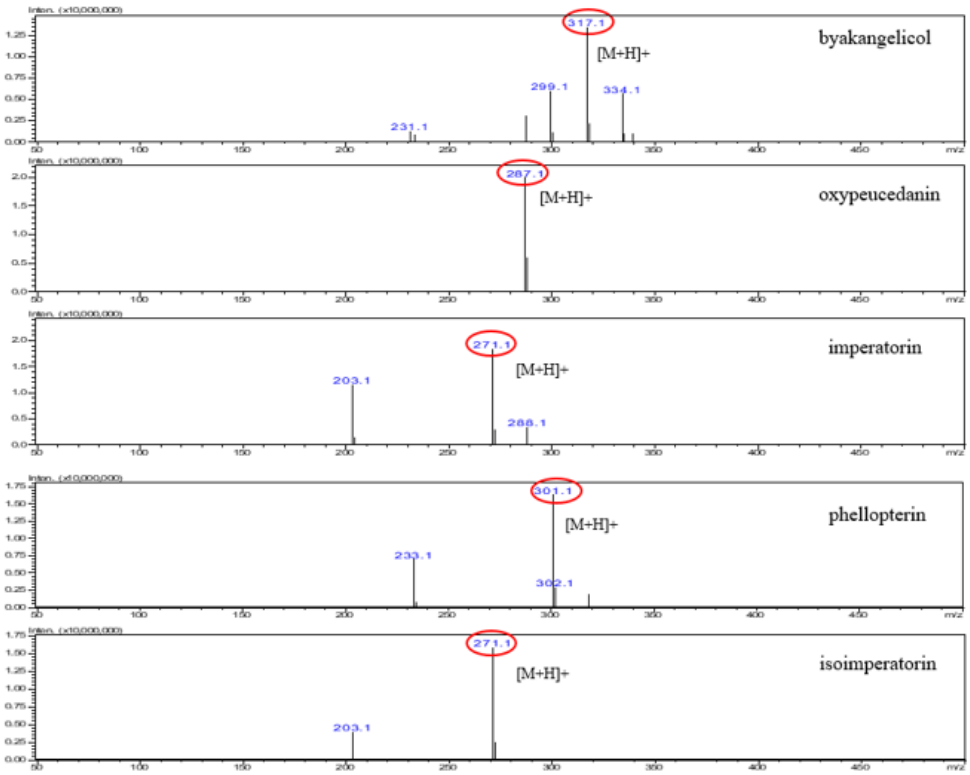
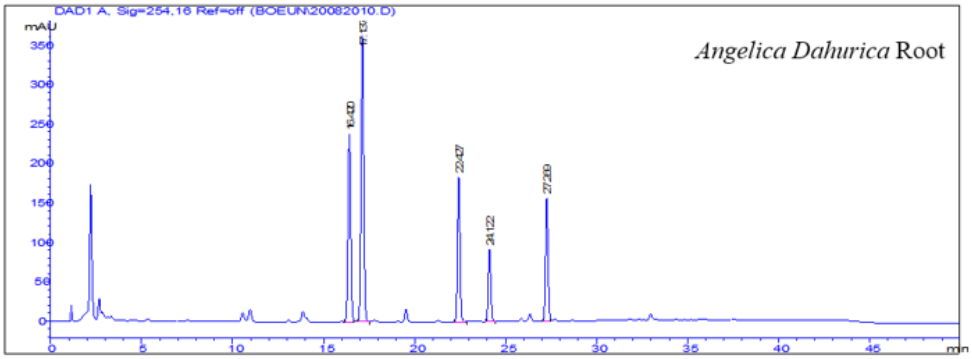
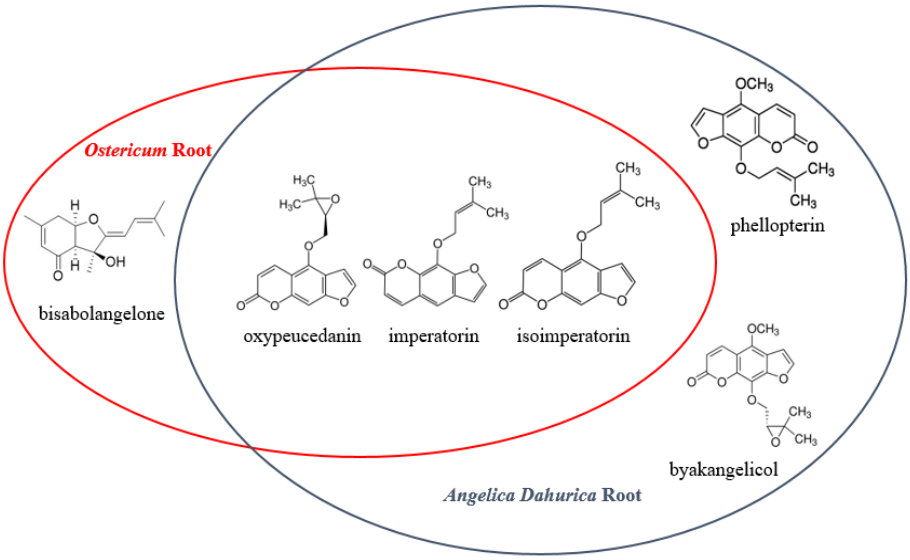

byakangelicol
317.1
[M+H]+
299.1
334.1
231.1
oxypeucedanin
287.1
[M+H]+
imperatorin
271.1
[M+H]+
203.1
288.1
phellopterin
301.1
[M+H]+
233.1
302.1
isoimperatorin
271.1
[M+H]+
203.1

Figure 45. Chromatogram of all active ingredient integration at *Angelica Dahurica* root



byakangelicol	16.420 min
oxypeucedanin	17.137 min
imperatorin	22.427 min
phellopterin	24.122 min
isoimperatorin	27.269 min

Figure 46. Active ingredient diagram in *Ostericum* root and *Angelica Dahurica* root


Ostericum Root
bisabolangelone
oxypeucedanin
imperatorin
isoimperatorin
phellopterin
byakangelicol
Angelica Dahurica Root

Conclusion

In this study, active ingredients were improved and developed in *Ostericum* root and *Angelica Dahurica* root. The content of active ingredients in herbal medicinal herbs is not constant depending on soil, climate, harvesting time and storage conditions. Therefore, the Korean Pharmacopoeia has notified the standard for herbal medicines and there are information on active ingredients and analysis methods for active ingredients. Therefore, I tried to analyze the active ingredients in the herbal medicine *Ostericum* root and *Angelica Dahurica* root. However, although the Korean Pharmacopoeia has information on active ingredients and analysis methods for active ingredients on *Angelica Dahurica* root, there is no information on *Ostericum* root. Therefore, the optimal analysis method was established by improving the method of analyzing active ingredients of *Angelica Dahurica* root in the Korean Pharmacopoeia.

In addition, the method for analyzing the active ingredient of *Ostericum* root did not exist in the Korean Pharmacopoeia, so it was newly developed by the HPLC-UVD method. In the process of improvement and development of the analysis method, three main ingredients were identified in addition to the active ingredient. I believe that this experiment can provide information on active ingredients to oriental medicine doctors and consumers who use herbal medicines.

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초 록

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주보은

Hexythiazox는 멜론, 사과, 딸기 및 생약(한약)에서 진드기를 제어하는 데 사용되는 살충제이다. Hexythiazox는 일반 식량작물에서 HPLC 또는 LC-MS / MS로 분석이 가능하며 분석법 또한 잘 확립되어 있으나 생약(한약)에서는 분석법 확립이 잘 되어 있지 않았다. 따라서 본 연구의 목적은 LC-MS / MS를 이용하여 전통 약재 인 강활(Ostericum) 및 백지(Angelica Dahurica)에 대한 분석 방법을 개발하는 것이다. 정량 분석 한계 (MLOQ)는 0.02 mg / kg 이며 두 개의 처리수준(MLOQ, 10 MLOQ)에서 확립 된 방법을 검증하기 위해 회수율 실험을 수행하였다. 회수율은 70 ~ 105 % 범위 였고 표준편차는 10 % 미만이었다. 따라서 본 방법이 생약(한약)인 강활 및 백지에서 hexythoazox의 정량분석에 유효함을 입증하였다.

대한민국 약전에는 유효성분 분석법이 있다. 한약재인 백지는 유효성분 분석법 및 유효성분에 대한 정보가 존재하지만 강활은 유효성분 분석법 및 유효성분의 정보가 존재하지 않았다. 따라서 백지의 유효성분을 확인하기 위해 대한민국 약전법을 이용하였으며, 강활 또한 백지와 같은 방법으로 실험하였다. 대한민국 약전법을 이용한 결과 백지에서 유효성분의 피크의 분리가 되지 않아 기기조건을 개선함과 동시에 강활과 백지에서 겹치는 피크를 관찰하였고 문헌조사를 한 결과 강활에서도 백지와 유효성분이 겹침을 확인할 수 있었다. 대한민국 약전에서는 백지의 유효성분으로 oxypeucedanin, imperatorin, isoimperatorin을 제시하였으며, 기기분석결과 유효성분으로 인지할 수 있는 피크가

나왔음을 확인하였다. 마찬가지로 강활에서도 oxypeucedanin, imperatorin, isoimperatorin의 3 개의 피크가 나왔으며 유효성분으로 지정되어도 무방할 만큼 감도가 뛰어남을 확인하였다. LC-MS/MS에서 Q3 scan 결과 강활에서는 oxypeucedanin, imperatorin, isoimperatorin, bisabolangelone을 유효성분으로 확인할 수 있었고, 백지에서는 oxypeucedanin, imperatorin, isoimperatorin, byakangelicol, phellopterin을 유효성분으로 확인할 수 있었다.

주요어 : 헥시티아족스, 한약, 생약, 액체크로마토그래피, LC-MS/MS, 유효성분, 강활, 백지

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