농학석사학위논문

백출과 지황 중 Fosthiazate 및 기장 중 Tricyclazole 과 Tebuconazole 의 LC-MS/MS 와 GC-MS/MS 분석법 개발

Development of LC-MS/MS and GC-MS/MS Analytical Method for Fosthiazate in *Atractylodes* rhizome white and *Rehmannia* root and, Tricyclazole and Tebuconazole in *Panicum miliaceum*

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서울대학교 대학원

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고 락 도

A Dissertation for the Degree of Master of Science

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February 2020

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지도교수 김 정 한

이 논문을 농학석사학위논문으로 제출함

2019년 12월

서울대학교 대학원

농생명공학부 응용생명화학전공

고 락 도

고락도 석사학위논문을 인준함

2020년 1월

위 원 장 오 기 봉 (인)

부 위 원 장 김 정 한 (인)

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Abstract

Fosthiazate is an organophosphorus nematicide and insecticide. *Atractylodes* rhizome white and *Rehmannia* root is herbal medicine listed in the Korean Pharmacopeia (KP). This study develops a method for analyzing fosthiazate using GC-NPD. Cleanup using an SPE Cartridge was performed twice. This method using GC-NPD was complex to extract, partition and cleanup. After that, the method was developed by QuEChERS method using LC-MS/MS to improve unnecessary preparation procedures. LC-MS/MS on electrospray mode was used for the analysis of fosthiazate. In multiple reaction monitoring, m/z=228 and m/z=104.15 were engaged as the quantification ion and qualification ion, respectively. Method limit of quantitation (MLOQ) was 0.01 mg/kg. The linearity of matrix-matched calibration curve (r^2) was ≥0.99 at the calibration range of 0.001-0.05 mg/kg. For the recovery test, 5 g of macerated *Atractylodes* rhizome white or *Rehmannia* root was treated with standard solutions at MLOQ and 10MLOQ levels. Recovery rates were in the range of 70-120% (RSD≤20%) at two spiked levels (MLOQ and 10MLOQ) for *Atractylodes* rhizome white or *Rehmannia* root.

Proso millet (*Panicum miliaceum* L.) is the longest used summer cereal in human in addition to wheat and barley. Tricyclazole and tebuconazole is a fungicide which has been used for various crops including proso millet. LC-MS/MS on electrospray mode was used for the analysis of tricyclazole. In multiple reaction monitoring, m/z=136 and m/z=163.15 were engaged as the quantification ion and qualification ion, respectively. GC-MS/MS on electron ionization mode was used for the analysis of tebuconazole. In multiple reaction monitoring, m/z=125 and m/z=99.1 were engaged as the quantification ion and qualification ion, respectively. Method limit of quantitation (MLOQ) was 0.01 mg/kg. The linearity of matrix-matched calibration curve (r^2) was ≥0.99 at the calibration range of 0.0025-0.25 mg/kg. For the recovery test, 10 g of macerated grain or straw of *proso millet* was

treated with standard solutions at 10MLOQ and 50MLOQ levels. Recovery rates were in the range of 70-120% (RSD≤20%) at two spiked levels (10MLOQ and 50MLOQ) for grain and straw. In field study, tricyclazole 20 % suspension concentrate formulation was treated on each 4 plots (50/40/30 days, 40/30/21 days, 30/21/14 days and 21/14/7 days before harvest) with three replicates. After harvest, grain and straw were analyzed to show that the residue levels of trycyclazole decreased according to treatment time. Maximum levels of residue were 5.68 mg/kg and 1.13 mg/kg for grain and straw in treatment plot of 21/14/7 days before harvest. In field study of tebuconazole, tebuconazole 20 % suspension concentrate formulation was treated on each 4 plots (50/40/30 days, 40/30/21 days, 30/21/14 days and 21/14/7 days before harvest) with three replicates. After harvest, grain and straw were analyzed to show that the residue levels of tebuconazole decreased according to treatment time. Maximum levels of residue were 1.66 mg/kg and 1.20 mg/kg for grain and straw in treatment plot of 21/14/7 days before harvest.

Key words: Herbal medicine, GC-NPD, SPE, QuEChERS, Fosthiazate, LC-MS/MS, GC-MS/MS, Minor crop, Proso millet, Residue

***Student number*: 2018-24170**

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List of Abbreviations

ACE	Acetone
ACN	Acetonitrile
CAS	Chemical abstract service
CID	Collision induced dissociation
DL	Desolvation line
dSPE	Dispersive solid phase extraction
EC	Emulsifiable concentrate
ESI	Electrospray ionization
GC	Gas-chromatography
GCB	Graphitized carbon black
GR	Granules
HX	Hexane
ILOD	Instrumental limit of detection
ILOQ	Instrumental limit of quantitation
IUPAC	International union of pure and applied chemistry
LC	Liquid-chromatography
LC50	Lethal concentration 50

LD50	Lethal dose 50
ME	Matrix effect
$MgSO_4$	Magnesium sulfate
MLOQ	Method limit of quantitation
MOA	Mode of action
MRLs	Maximum residue limits
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MSTD	Matrix-matched standard
Na_2SO_4	Sodium sulfate
NaCl	Sodium chloride
NPD	Nitrogen phosphorus detector
PHI	Pre-harvest interval
PSA	Primary secondary amine
QuEChERS	Quick, Easy, cheap, effective, rugged and safety
RSD	Relative standard deviation
S/N	Signal to Noise
SC	Suspension concentrate

SPE	Solid phase extraction
SRM	Selected reaction monitoring
SSTD	Solvent standard
WG	Dispersible granule
WP	Wettable powder

Part 1

Development of GC-NPD and LC-MS/MS Analytical Method for Fosthiazate in *Atractylodes* rhizome white and *Rehmannia* root

Introduction

Herbal medicine

Herbal medicines have been used for thousands of years around the world. The history of herbal medicine has been proven to be effective over many years and has been used traditionally for many years.

In korea, herbs have been recognized for health care and disease treatment. Thesedays, they are the most widely manufactured and marketed in the pharmaceutical market for disease treatment and public health promotion in Korea. Korean traditional medicine is based on the philosophy of ancient medical science, and began with 11 oriental books that documented more about ancient remedies. Among these books, DongEu-Bo-Gam (by Hur Joon, AD1613) is the greatest masterpiece. This book is considered as a bible of Korean traditional medicine up to the present in Korea (Choi et al. 2002).

Among 520 herbal drug materials (HDMs) registered in Korea Pharmacopeia (KP) and Korean Herbal Pharmacopeia (KHP), more than 418 HDMs are mainly derived from plants that are mostly under cultivation like agricultural crops. Consumers could be exposed to residual pesticides through food and HDMs at the same time(Oh 2009).

Atractylodes rhizome white

Atractylodes rhizome white is a compositae herbal medicine listed in the Korean Pharmacopeia (KP). The origin of *Atractylodes* rhizome white is defined as the root stem of *Atractylodes japonica* or *Atractylodes macrocephala.*

Atractylodes rhizome white uses its roots as herbal medicine. Young stems and leaves have a unique aroma and bitter taste and have been used for a long time in korea. Especially for herbal medicine, it is a very important medicine used in prescription drugs to promote digestion and gastrointestinal protection (Bang et al. 2004). For preventing pest of *Atractylodes* rhizome white, some pesticides allowed to use (table 1).

Table 1. Allowed pesticides of *Atractylodes* rhizome white

Pesticide	Usage	MRL (mg/kg)
Aldrin	**Insecticide**	**0.01**
BHC	**Insecticide**	**0.2**
Captan	**Fungicide**	**2**
DDT	**Insecticide**	**0.1**
Dieldrin	**Insecticide**	**0.01**
Endrin	**Insecticide, Rodenticide**	**0.01**
Procymidone	**Fungicide**	**0.1**

*Korean pharmacopoeia (Ministry of food and drugs safety)

(*Article 2 Medicine*, 2019)

***Rehmannia* root**

Rehmannia root is a herbal medicine of scrophulariaceae and is listed in the Korean Pharmacopeia (KP). The origin of *Rehmannia glutinosa* is defined as *Rehmannia glutinosa* ex steudel.

Rehmannia glutinosa has a variety of pharmacological actions and chemical compositions. In recent decades, there have been many reports of the pharmacological functions and activities of *Rehmannia glutinosa* and active principles of blood system, immune system, endocrine system, cardiovascular system and nervous system and of anti-tumor, anti-senescence, etc (Zhang, Li, and Jia 2008). For preventing pest of *Atractylodes* rhizome white, some pesticides allowed to use (table 2)

Table 2. Allowed pesticides of *Rehmannia* root

Pesticide	Usage	MRL (mg/kg)
Aldrin	**Insecticide**	**0.01**
BHC	**Insecticide**	**0.2**
DDT	**Insecticide**	**0.1**
Dieldrin	**Insecticide**	**0.01**
Endrin	**Insecticide, Rodenticide**	**0.01**
Iminoctadine tris (albesilate)	**Fungicide**	**0.1**
Kresoxim-methyl	**Fungicide**	**0.1**
Pyrimethanil	**Fungicide**	**0.2**
Thiram	**Fungicide**	**0.5**

*Korean pharmacopoeia (Ministry of food and drugs safety)

(*Medicine; second part; Article 2,* 2019)

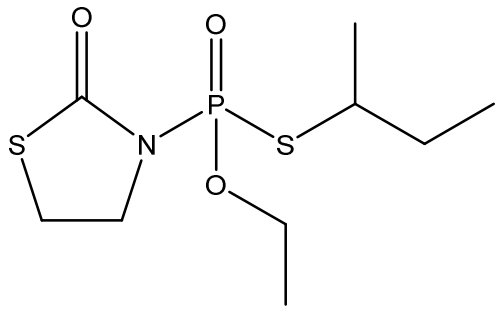
Fosthiazate

Fosthiazate is an organophosphorous insecticide and nematicide (Fig 1.). Oragnophosphorus pesticides are organic compounds containing phosphorus. Phosphorus can adopt a variety of oxidation states. IUPAC name is 3-[butan-2-ylsulfanyl(ethoxy) phosphoryl]-1,3-thiazolidin-2-one. It also has been called "Nemathorin". The chemical formula of fosthiazate is $C_9H_{18}NO_3PS_2$. The physical form is a slightly yellow and clear liquid.

Mode of action (MOA) of fosthiazate is known to inhibition of acetylcholine esterase in nematodes and other insects. Its excellent systemic actions also provide high performance against nematodes and foliar insect pests. It acts on the motility and larval stages of nematodes in the soil and prevents invasion to roots of crops (ISK, 1992).

Fosthiazate is applied on crops such as potato, tomato and banana. Its MRL is set at 0.05-0.5 mg/kg for crops including chili pepper and garlic. In Korea, fosthiazate was first registered in the form of emulsion in 1995 (MRLs for Pesticides in Foods, May 31, 2016).

Figure 1. Structure of fosthiazate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P
S
N
S
O

Table 3. Physicochemical properties of fosthiazate

Property	Information
Common name	Fosthiazate (BSI, E-ISO, (m) F-ISO)
IUPAC name	(RS)-[S-(RS)-sec-butyl O-ethyl 2-oxo-1,3-thiazolidin-3-ylphosphonothioate]
CAS No.	98886-44-3
Molecular formula	$C_9H_{18}NO_3PS_2$
Molecular weight	283.3
Boiling point	198 ℃/0.5 mmHg
Vapor pressure	0.53 mpa (25 ℃)
K_{ow}	Log P = 1.68
Solubility in solvent	In water 9850.0 mg/L (20-25 ℃). Soluble in hexane (15.14 g/L, 20-25 ℃), isopropanol, NMP, xylene
Mode of action	Cholinestrease inhibitor
Toxicology	**Acute oral (LD_{50}, mg/kg)** male rats 73, female rats 57 **Acute percutaneous (LD_{50}, mg/kg)** male rats 2396, female rats 861 **Skin irritation** Not an irritant (rabbits). **Eye** Not an irritant (rabbits). **Inhalation** (LC_{50}, mg/1) male rats 0.832, female rats 0.558 (4 h)
Environmental fate	**Animals** Rapidly and extensively absorbed, with >90% excretion, mainly via urine and air, within 48 h. **Soil/Environment** Terrestrial field dissipation $_{DT50}$ 10-17 d. DT_{50} in aerobic soil 45 d, in anaerobic water sediment 37 d. Mean K_{roc} 59

*The Pesticide Manual Seventeenth Edition (J A Turner)

Cleanup using solid phase extraction

As an alternative medicine, the use of herbal products is becoming increasingly popular. However, the analysis of active compound present in herbal products faces great difficulties because of the complexity of trace amounts of pharmacologically active compounds and matrices. In addition, as the demand for solving quality-related problems in herbal products, the development of simple and reliable methods for the purification and, sensitive and selective determination of active compounds of herbal products is essential (Sun et al. 2002)

To cope with this problem, plant extracts must go through several cleanup steps, using unrelated separation mechanisms to increase orthogonality and purification efficiency. Common cleanup procedures such as column chromatography, solid phase extraction(SPE), liquid-liquid extraction, etc. are used (Dobrev et al. 2005).

The choice of method for a particular problem should be chosen based on the experience and skills available, the analyte and the matrix characteristics. Nevertheless, the popularity of SPE has increased in recent years due to its ease of automation and the availability of various stages. It is also considered eco-friendly as large volumes of solvents are not used, as in liquid–liquid extraction (Stevenson 1999).

QuEChERS methodology

Anastassiades, Lehotay, Stajnbaher, and Schenck (2003) developed an original analytical method that combines the extraction/isolation of pesticides from the food matrix with extract cleanup. They coined the acronym QuEChERS for it, i.e. Quick, Easy, Cheap, Effective, Rugged and Safe. This technique is the process of purifying extracts using fine scale extraction with ACN (acetonitrile) and dispersive solid-phase extraction (d-SPE). Since the development and publication of this method, QuEChERS has been gaining considerable popularity. It is the method of choice for food analysis, as it combines several steps and extends the range of recovered pesticides with older and more boring extraction techniques. This method has undergone various modifications and improvements over the years since its first introduction. These have been designed to improve recovery for certain types of foodstuff or types of pesticides (Wilkowska and Biziuk 2011).

The purpose of studies

This study is to develop the residual test method for fosthiazate in herbal medicine to establish the maximum residue limits (MRLs). The samples used in this study are *Atractylodes* rhizome white and *Rehmannia* root. The herbal medicine samples are to selectively analyze fosthiazate by GC-NPD through solid phase extraction and by LC-MS/MS through dispersive solid phase extraction purification in various matrices such as secondary metabolites and active compounds.

Materials and Methods

Analytical standard

Standard material of fosthiazate (Purity: 99.1 %) was purchased from Wako (Osaka, Japan).

Standard solutions

Standard stock solution of fosthiazate to be used in method using GC-NPD was prepared at the concentration of 1,000 mg/L with ACE (acetone). The working solutions were prepared by serial dilution of stock solution with acetone.

Standard stock solution of fosthiazate to be used in method using LC-MS/MS was prepared at the concentration of 1,000 mg/L with acetonitrile. The working solutions were prepared by serial dilution of stock solution with acetonitrile.

Subject herbal medicine

Atractylodes rhizome white (*Atractylodes japonica*) and *Rehmannia* Root (*Rehmannia glutinosa*) which are the species in South Korea was used for experiments

Sampling

Acquisition of the herbal medicine sample was performed through a series of procedures. First, the sample is provided by a pharmaceutical company that sells herb sample. Next, the received sample is inspected by the herbal medicine pharmacist and the product is purchased. The herbal medicine sample purchased is subjected to sensory evaluation and used as a sample. After evaluation, the obtained sample was homogenized by food processor. Every sample was stored at -20 ℃ in polyethylene bags.

Analytical instruments and conditions

1) GC-NPD condition

GC-NPD analysis for fosthiazate was carried out on an Agilent 7890B equipped with Agilent 7693 auto sampler (Santa Clara, USA). A capillary column was DB-5MS Ultra Inlet (30m x 0.25mm, 0.25μm). The heater of Inlet was 260 ℃. Injection mode was pulsed-splitless mode (20 psi, until 0.75 min). The oven temperature program was initialized at 80 ℃ (held for 2min), increased to 300 ℃ at 10 ℃/min (held for 10). Total run time was 34 min. Detector was NPD (Nitrogen Phosphorus Detector) using blos bead (Agilent, USA). Nitrogen (≥ 99.999%) was used as carrier gas at a makeup gas flow (10 mL/min) and hydrogen and air (≥ 99.999%) was used as fuel gas to make flame. Hydrogen and air flow was 3 mL/min and 60 mL/min, respectively.

2) LC-MS/MS condition

LC-MS/MS analysis for fosthiazate was carried out on a LC-MS 8040 with UHPLC-Nexera (ESI positive mode). The oven temperature was 40 ℃ and the column was Raptor^{TM} ARC-18 (100mm x 2.1 mm, 2.7 μm). Mobile phases were 0.1% formic acid & 5mM ammonium formate in water/methanol (98/2, v/v) (A) and methanol/water (98/2, v/v) (B). The mobile phase (B) was initially hold at 5% for 1 min. Then (B) increased 5% to 95% for 8.5 min and hold 95% for 0.5 min. Finally decreased it to 5% for 0.5 min and hold 5% for 5 min. The flow rate was set at 0.2 mL/min and the injection volume was 5 μL. Desolvation line (DL) temperature was 250 ℃ and heat block temperature was 400 ℃. The nebulizing gas, drying gas was nitrogen and collision energy was used argon gas. Nebulizing gas flow was 3 L/min drying gas flow was 15 L/min. For MS/MS analysis, two transitions (quantifier and qualifier) were chosen for scheduled MRM mode after an automatic optimization procedure.

Method Validation

1) ILOQ (Instrumental Limit of Quantitation)

Standard solution (0.05 ~ 2 μg/mL) were analyzed by GC-NPD.

After matrix matched standard solution (0.001 ~ 0.05 μg/mL) were analyzed by LC-MS/MS. The ILOQ was settled as the concentration where the S/N (Signal-to-Noise) ratio was higher than 10.

2) MLOQ (Method Limit of Quantitation)

MLOQ was calculated by equation below

$$\text{MLOQ (mg/L)} = \frac{\text{LOQ (ng)} \times \text{Final volume (mL)} \times \text{Dilution factor}}{\text{Injection volume (μL)} \times \text{Initial sample weight (g)}}$$

3) Calibration curve and linearity

Solvent standard solution	SSTD 1 (0.05 μg/mL)	SSTD 2 (0.1 μg/mL)	SSTD 3 (0.2 μg/mL)	SSTD 4 (0.5 μg/mL)	SSTD 5 (1 μg/mL)	SSTD 6 (2 μg/mL)

A series of fosthiazate solvent standard solution with concentration of 0.05, 0.1, 0.2, 0.5, 1, 2 μg/mL were prepared with serial dilution in acetone for GC-NPD analysis. The relative standard deviation (RSD) was calculated at the calibration curve.

Matrix matched standard solution	MSTD 1 (0.001 μg/mL)	MSTD 2 (0.0025 μg/mL)	MSTD 3 (0.005 μg/mL)	MSTD 4 (0.01 μg/mL)	MSTD 5 (0.025 μg/mL)	MSTD 6 (0.05 μg/mL)
Standard solution	0.002 μg/mL 200 μL	0.005 μg/mL 200 μL	0.001 μg/mL 200 μL	0.02 μg/mL 200 μL	0.05 μg/mL 200 μL	0.1 μg/mL 200 μL
Sample matrix	200 μL	200 μL	200 μL	200 μL	200 μL	200 μL

A series of matrix-matched fosthiazate solvent standard solution with concentration of 0.001, 0.0025, 0.005, 0.01, 0.025, 0.05 μg/mL were prepared with serial dilution in acetonitrile for LC-MS/MS analysis. The relative standard deviation (RSD) was calculated at the calibration curve.

4) Recovery test of fosthiazate analytical method by GC-NPD

The homogenized free pesticide *Atractylodes* rhizome white and *Rehmannia* root samples (5 g) in 250 mL centrifuge bottle were treated with the standard solution of fosthiazate at spiking level following: For *Atractylodes* rhizome white and *Rehmannia* Root, 0.04 mg/kg and 0.4 mg/kg (MLOQ, 10 MLOQ). The samples of *Atractylodes* rhizome white and *Rehmannia* Root were moistened with 20 mL of deionized water for 10 minutes. 50 mL of acetonitrile was added to each bottle, and the bottles were shaken vigorously (MMV-1000W, EYELA, Tokyo, Japan). After that, 7.5 g of sodium chloride (NaCl) were added to the bottles. The bottles were vigorously shaken for 5 min and centrifuged at 3,500 rpm for 5 minutes (Combi 408, Hanil Science industrial, Korea). Twenty-five milliliter of the supernatant was transferred into round flasks and evaporated (Water bath B-480, BUCHI, Oldham UK / Rotavapor R-114, BUCHI, Oldham, UK / Laborota 4000 efficient, Heidolph, Schwabach, Germany and Minichiller, Huber, Germany). The residues were dissolved in 50 mL deionized water for partition. Fifty milliliter of deionized water with residues was transferred into separate funnels. Next, the residues in round flask were dissolved in 50 mL hexane. Fifty milliliter of HX with residues was transferred into separate funnels. After that, the residues in round flask were dissolved in 50 mL saturated sodium chloride solution. Fifty milliliter of saturated sodium chloride solution with residues was transferred into separate funnels. After shaking separate funnels for 1 minute, the 50 mL hexane was filtered using sodium sulfate (Na_2SO_4), transferred to round flasks and evaporated. The residues were dissolved in 4 mL of acetone/hexane (10/90, v/v) for clean-up. A silica solid phase extraction (SPE) cartridge (1,000 mg, 6 mL) was preconditioned with 5 mL of acetone/hexane (10/90,

v/v). After precondition, 4 mL of the extract was loaded on the cartridge (The sample was collected in loading step). The cartridge was washed with 20 mL of acetone/hexane (10/90, v/v). The cartridge was eluted with 10 mL of acetone/hexane (20/80, v/v) in test tubes. The eluates in test tubes were evaporated under nitrogen stream and re-dissolved with 4 mL of 0.1% formic acid in Ethyl acetate/hexane (10/90, v/v). An amino-propyl (NH_2) solid phase extraction (SPE) cartridge (1,000 mg, 6 mL) was preconditioned with 5 mL of 0.1% formic acid in hexane. After precondition, 4 mL of the extract was loaded on the cartridge (The sample was collected in loading step). The cartridge was washed with 10 mL of 0.1% formic acid in Ethyl acetate/hexane (20/80, v/v). The cartridge was eluted with 10 mL of 0.1% formic acid in Ethyl acetate/hexane (30/70, v/v) in test tubes. The eluates in test tubes were evaporated under nitrogen stream and re-dissolved with 1 mL acetone. 2 μL of final sample was injected into GC-NPD.

5) Recovery test of fosthiazate analytical method by LC-MS/MS

Samples were extracted using the modified QuEChERS method. The homogenized free pesticide *Atractylodes* rhizome white and *Rehmannia* root samples (5 g) in 50 mL centrifuge tube were treated with the standard solution of fosthiazate at spiking level following: For *Atractylodes* rhizome white and *Rehmannia* root, 0.01 mg/kg and 0.1 mg/kg (MLOQ, 10 MLOQ). The samples of *Atractylodes* rhizome white and *Rehmannia* root were moistened with 10 mL of deionized water for 10 minutes. 10 mL of acetonitrile was added to each tube, and the tubes were shaken vigorously (1600 MiniG™, SPEX® SamplePrep, New Jersey, USA). After that, 4 g of magnesium sulfate ($MgSO_4$) and 1 g of sodium chloride (NaCl) were added to the tubes. The tubes were vigorously shaken for 1 min and centrifuged at 3,500 rpm for 5 minutes (Combi 408, Hanil Science industrial, Korea). One milliliter of the supernatant was transferred into dSPE (Dispersive Solid Phase Extraction) tube containing 25 mg of PSA, 7.5 mg of GCB and 150 mg $MgSO_4$ (RESTEK, Germany). After voltexing for 1 minute, the d-SPE tubes were centrifuged at 13,000 rpm for 4 minutes. For matrix matching, 0.2 mL of supernatant was mixed with 0.2 mL of acetonitrile followed by analyzed using LC-MS/MS.

Matrix effect

Matrix effects (ME, %) was calculated by comparing the slope of calibration curve between solvent standard and matrix matched calibration curve using the following equation:

$$\text{ME, \%} = \left(\frac{\text{slope of matrix matched calibration curve}}{\text{slope of solvent standard calibration curve}} - 1\right) \times 100$$

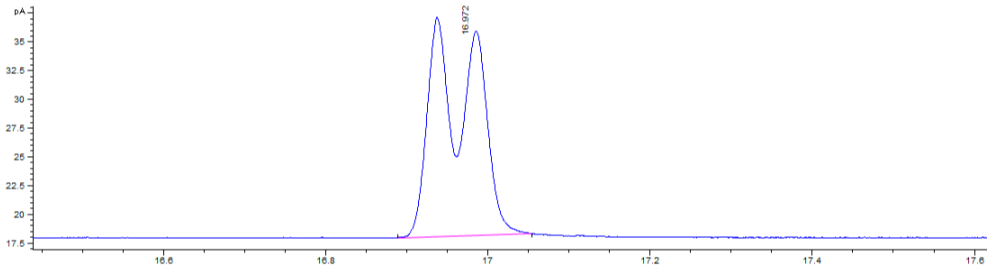
A negative value of matrix effect indicates signal suppression, a positive value indicates signal enhancement in matrix contained environment (Caban et al. 2012)

Results and Discussion

GC-NPD condition

GC-NPD is an acronym for Gas Chromatography-Nitrogen Phosphorus Detector. The fosthiazate contains nitrogen and phosphorus, so NPD was selected. Injector temperature and injection mode were set to analyze the fosthiazate. The proper output was set by flowing air and hydrogen and increasing the voltage of NPD. After changing the temperature of the oven, fosthiazate was separated from the inside of the column and then chromatogram was confirmed through NPD. Comparing the acetone with the fosthiazate standard solution and confirming the retention time of the fosthiazate, the compound was quantified. Area values of fosthiazate peaks were calculated and quantified. The chromatogram of fosthiazate was found to be followed by two peaks, and integration was performed using the area sum function (Fig. 2.).

Figure 2. Chromatography of fosthiazate analyzed by GC-NPD


pA
35
32.5
30
27.5
25
22.5
20
17.5
16.972
16.6
16.8
17
17.2
17.4
17.6

ILOD, ILOQ, MLOQ and calibration curve of fosthiazate analytical method by GC-NPD

Instrumental limit of detection (ILOD) and Instrumental limit of quantitation (ILOQ) are values that represent the sensitivity of analytical instrument. ILOD is determined by the minimum concentration of analyte providing S/N ratio of > 3 and ILOQ is determined by S/N ratio of > 10 (Fig. 3.) (Fong et al. 1999). In this experiment, ILOQ was checked from the results of analysis of several concentration standard solutions, 0.1 mg/L was observed as practicable ILOQ in GC-NPD.

Based on MLOQ calculating equation, MLOQ of fosthiazate in *Atractylodes* rhizome white and *Rehmannia* root is 0.04 mg/L.

Standard curves of fothiazate has a good linearity in samples of *Atractylodes* rhizome white and *Rehmannia* root. The range was between 0.05 to 2 mg/L of fosthiazate standard solution (Fig. 4.).

The regression equations were y = 1688.7x – 10.846. Coefficients of determination (r^2) were over 0.999 in each samples.

Figure 3. Concepts of ILOD and ILOQ

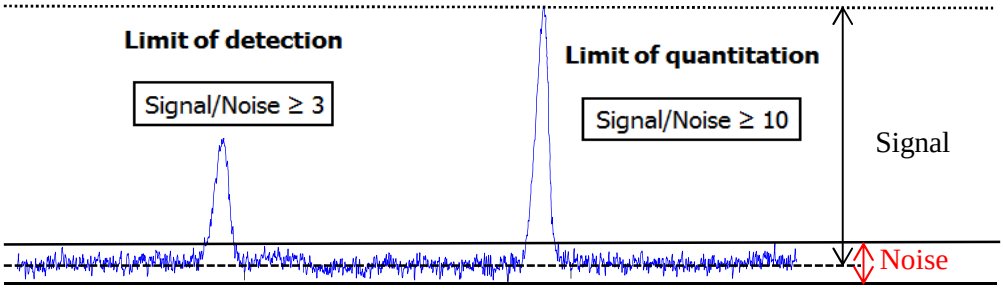
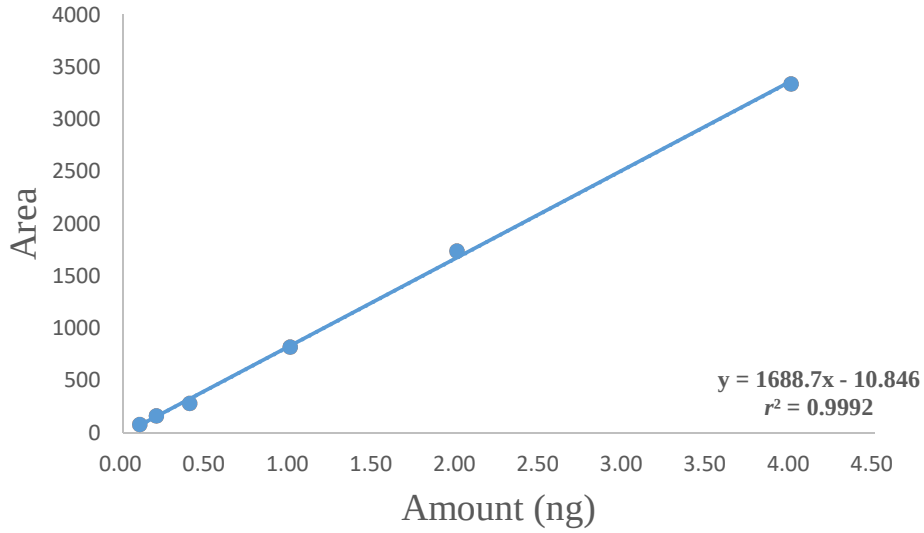

Limit of detection
Signal/Noise ≥ 3
Limit of quantitation
Signal/Noise ≥ 10
Signal
Noise

Figure 4. Calibration curve of fosthiazate


4000
3500
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1000
500
0
Area
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
Amount (ng)
y = 1688.7x - 10.846
r² = 0.9992

Optimization of cleanup using SPE cartridge

In order to effectively remove various matrix of analytical disturbances in herbal samples, various SPE cartridge cleanup processes were reviewed in SPE. Cleanup processes was added to washing section of the SPE cartridge and examined for the washing volume and elution section. The cleanup method was first established as fosthiazate standard solution. Then, the treated samples of the *Atractylodes* rhizome white and *Rehmannia* root were confirmed.

1) Florisil SPE cartridge (1 g, 6 cc)

Fosthiazate solution 1 mL of 1 mg/L was added to a round bottom flask, concentrated and dissolved in acetone/*n*-hexane (10/90, v/v). The elution of fosthiazate was investigated using acetone/*n*-hexane mixture as the eluent. As a result, 5 mL of *n*-hexane and 5 mL of acetone/*n*-hexane (10/90, v/v) were allowed to flow for conditioning SPE cartridge. When loading the solution 4 mL of fosthiazate dissolved in acetone/*n*-hexane (10/90, v/v) and eluting into 10 mL of acetone/*n*-hexane (10/90, v/v), the fosthiazate was not eluted at all. When 10 mL of acetone/*n*-hexane (20/80, v/v) was poured out, 101% was eluted. In order to select the washing volume in the washing section, acetone/*n*-hexane (10/90, v/v) was fractionated by 5 mL. In the third fraction, 27.2% was eluted (Table 4). Thus, the florisil SPE cartridge was then conditioned by flowing 5 mL of *n*-hexane and 5 mL of acetone/*n*-hexane (10/90, v/v). After loading fosthiazate solution 0.25 mg/L, 4 mL of acetone/*n*-hexane (10/90, v/v), SPE cartridge was washed with 5 mL of acetone/*n*-hexane (10/90, v/v) and eluted with 10 mL of acetone/*n*-hexane (20/80, v/v). The eluate was concentrated using a nitrogen evaporator and dissolved in 1 mL of acetone to obtain the final solution.

Table 4. Elution characteristics of fosthiazate using SPE-florisil cartridge

Solvent	Recovery (%)					
	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Total
acetone/*n*-hexane (10/90, v/v)	-	-	**27.2**	**26**	**4**	**57.2**

2) Silica SPE cartridge (1 g, 6 cc)

Fosthiazate solution 1 mL of 1 mg/L was added to a round bottom flask, concentrated and dissolved in acetone/*n*-hexane (10/90, v/v). The elution of fosthiazate was investigated using acetone/*n*-hexane mixture as the eluent. As a result, 5 mL of acetone/*n*-hexane (10/90, v/v) were allowed to flow for conditioning SPE cartridge. When loading the solution 4 mL of fosthiazate dissolved in acetone/*n*-hexane (10/90, v/v) and eluting into 10 mL of acetone/*n*-hexane (10/90, v/v), the fosthiazate was not eluted at all. When 10 mL of acetone/*n*-hexane (20/80, v/v) was poured out, 87% was eluted. In order to select the washing volume in the washing section, acetone/*n*-hexane (10/90, v/v) was fractionated by 5 mL. In the all fractions, the fosthiazate was not eluted at all (Table 5). Thus, the silica SPE cartridge was then conditioned by flowing 5 mL of acetone/*n*-hexane (10/90, v/v). After loading fosthiazate solution 0.25 mg/L, 4 mL of acetone/*n*-hexane (10/90, v/v), SPE cartridge was washed with 20 mL of acetone/*n*-hexane (10/90, v/v) and eluted with 10 mL of acetone/*n*-hexane (20/80, v/v). The eluate was concentrated using a nitrogen evaporator and dissolved in 1 mL of acetone to obtain the final solution.

Table 5. Elution characteristics of fosthiazate using SPE-Silica cartridge

Solvent	Recovery (%)					
	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Total
acetone/*n*-hexane (10/90, v/v)	-	-	-	-	-	-

3) Alumina SPE cartridge (1 g, 3 cc)

Fosthiazate solution 1 mL of 1 mg/L was added to a round bottom flask, concentrated and dissolved in acetone/*n*-hexane (10/90, v/v). The elution of fosthiazate was investigated using acetone/*n*-hexane mixture as the eluent. As a result, 3 mL of *n*-hexane and 3 mL of acetone/*n*-hexane (10/90, v/v) were allowed to flow for conditioning SPE cartridge. When loading the solution 3 mL of fosthiazate dissolved in acetone/*n*-hexane (10/90, v/v) and eluting into 6 mL of *n*-hexane, 3% was eluted. Serially, the same cartridge was eluted into 6 mL of acetone/*n*-hexane (10/90, v/v) and acetone/*n*-hexane (20/80, v/v), 27% and 30% was eluted, respectively (Table 6). Because a total of 60% were eluted, formic acid was used to increase the elution output.

Fosthiazate solution 1 mL of 1 mg/L was added to a round bottom flask, concentrated and dissolved in acetone/*n*-hexane (10/90, v/v) with 0.1 % formic acid. As a result, 3 mL of *n*-hexane with 0.1 % formic acid and 3 mL of acetone/*n*-hexane (10/90, v/v) with 0.1 % formic acid were allowed to flow for conditioning SPE cartridge. When loading the solution 3 mL of fosthiazate dissolved in acetone/*n*-hexane (10/90, v/v) with 0.1 % formic acid and eluting into 6 mL of *n*-hexane with 0.1 % formic acid, 5% was eluted. Serially, the same cartridge was elutied into 6 mL of acetone/*n*-hexane (10/90, v/v) with 0.1 % formic acid and acetone/*n*-hexane (20/80, v/v) with 0.1 % formic acid, 45% and 50% was eluted, respectively (Table 6). Washing volume cannot be selected in the washing section. Thus, the alumina SPE cartridge was then conditioned by flowing 3 mL of *n*-hexane with 0.1 % formic acid and 3 mL of acetone/*n*-hexane (10/90, v/v) with 0.1 % formic acid. After loading fosthiazate solution 0.25 mg/L, 3 mL of acetone/*n*-hexane (10/90, v/v), SPE cartridge was eluted with 6 mL of acetone/*n*-hexane (20/80, v/v). The eluate was concentrated using a nitrogen evaporator and dissolved in 1 mL of acetone to obtain the final solution.

Table 6. Elution characteristics of fosthiazate using SPE-Alumina cartridge

Solvent	Recovery (%)			
	(0/100, v/v)	(10/90, v/v)	(20/80, v/v)	Total
acetone/*n*-hexane	3	27	30	60
acetone/*n*-hexane with 0.1 % formic acid	5	45	50	100

4) NH_2 SPE cartridge (1 g, 6 cc)

Fosthiazate solution 1 mL of 1 mg/L was added to a round bottom flask, concentrated and dissolved in ethyl acetate/*n*-hexane (10/90, v/v). The elution of fosthiazate was investigated using ethyl acetate/*n*-hexane mixture as the eluent. As a result, 5 mL of *n*-hexane and 5 mL of ethyl acetate/*n*-hexane (10/90, v/v) were allowed to flow for conditioning SPE cartridge. When loading the solution 4 mL of fosthiazate dissolved in ethyl acetate/*n*-hexane (10/90, v/v) and eluting into 10 mL of ethyl acetate/*n*-hexane (10/90, v/v) and 10 mL of ethyl acetate/*n*-hexane (20/80, v/v) serially, the fosthiazate was not eluted at all. When 10 mL of acetone/*n*-hexane (30/70, v/v) was poured out, 30% was eluted (Table 7). Because a total of 30% were eluted, formic acid was used to increase the elution output.

Fosthiazate solution 1 mL of 1 mg/L was added to a round bottom flask, concentrated and dissolved in ethyl acetate/*n*-hexane (10/90, v/v) with 0.1 % formic acid. As a result, 5 mL of *n*-hexane with 0.1 % formic acid and 5 mL of ethyl acetate/*n*-hexane (10/90, v/v) with 0.1 % formic acid were allowed to flow for conditioning SPE cartridge. When loading the solution 4 mL of fosthiazate dissolved in ethyl acetate/*n*-hexane (10/90, v/v) with 0.1 % formic acid and eluting into 10 mL of ethyl acetate/*n*-hexane (10/90, v/v) with 0.1 % formic acid and 10 mL of ethyl acetate/*n*-hexane (20/80, v/v) with 0.1 % formic acid serially, the fosthiazate was not eluted at all. When 10 mL of ethyl acetate /*n*-hexane (30/70, v/v) with 0.1 % formic acid was poured out, 108% was eluted. In order to select the washing volume in the washing section, ethyl acetate/*n*-hexane (20/80, v/v) with 0.1 % formic acid was fractionated by 5 mL. In the fourth fraction, 62% was eluted (Table 7). Thus, the NH_2 SPE cartridge was then conditioned by flowing 5 mL of *n*-hexane (10/90, v/v)

with 0.1 % formic acid and 5 mL of ethyl acetate/*n*-hexane (10/90, v/v) with 0.1 % formic acid. After loading fosthiazate solution 0.25 mg/L, 4 mL of ethyl acetate/*n*-hexane (10/90, v/v) with 0.1 % formic acid, SPE cartridge was washed with 10 mL of ethyl acetate/*n*-hexane (20/80, v/v) with 0.1 % formic acid and eluted with 10 mL of ethyl acetate/*n*-hexane (30/70, v/v) with 0.1 % formic acid. The eluate was concentrated using a nitrogen evaporator and dissolved in 1 mL of acetone to obtain the final solution.

Table 7. Elution characteristics of fosthiazate using SPE-NH_2 cartridge

Solvent	Recovery (%)					
	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Total
acetone/*n*-hexane (20/80, v/v) with 0.1% formic acid	-	-	-	62	46	108

5) Serial cleanup in *Atractylodes* rhizome white and *Rehmannia* root

In case of the *Atractylodes* rhizome white samples, one-time cleanup did not completely remove the interferences, and thus quantitative analysis could not be performed. An analysis methods for various herbal medicine including *Atractylodes* rhizome white and Rehmannia root samples are needed. It was confirmed that Atractylodes rhizome *white* contains more interferences in GC-NPD than in *Rehmannia* root (Fig. 5). So, *Atractylodes* rhizome white samples was used to establish the cleanup method.

Cleanup conditions of SPE florisil – NH_2 cartridge and SPE alumina – NH_2 cartridge were applied to *Atractylodes* rhizome white. When analyzed under the GC-NPD condition, the cleanup efficiency was higher than that of the above mentioned cleanup conditions. Thus, the cleanup of SPE florisil – NH_2 cartridge and SPE alumina – NH_2 cartridge was not possible to quantify fosthiazate (Fig. 6).

Cleanup conditions of SPE silica – NH_2 cartridge cartridge were applied to *Atractylodes* rhizome white. When analyzed under the GC-NPD condition, purification efficiency was higher than that of the above mentioned cleanup conditions. It was consistent with the retention time of fosthiazate in standard solution and samples. Thus, the purification of SPE silica – NH_2 cartridge cartridge was effective as a cleanup capable of quantitative analysis of fosthiazate in *Atractylodes* rhizome white (Fig. 7).

Figure 5. Chromatogram of interferences of fosthiazate in *Atractylodes* rhizome white and *Rehmannia* root by GC-NPD

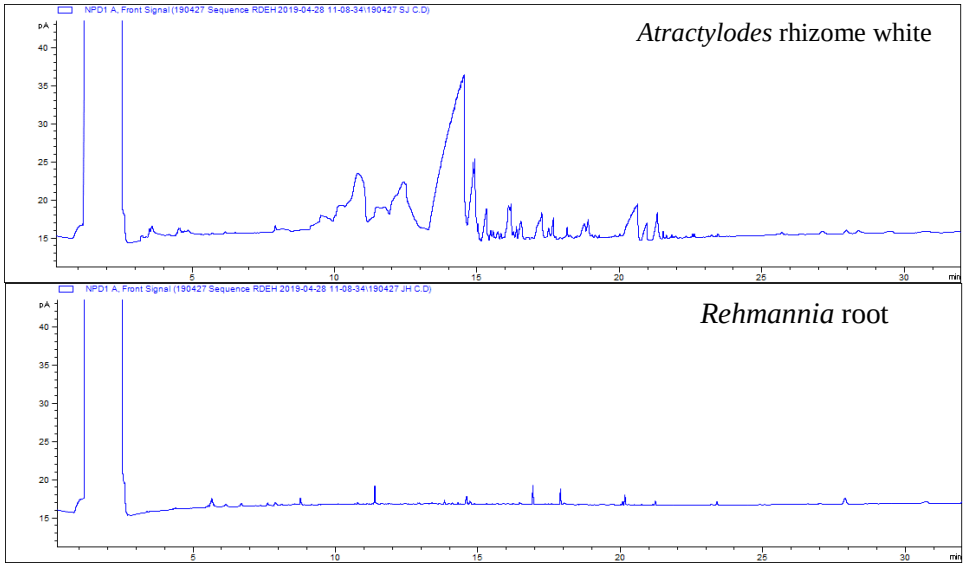

NPD1 A, Front Signal (190427 Sequence RDEH 2019-04-28 11-08-34\190427 SJ C.D)
pA
40
35
30
25
20
15
5
10
15
20
25
30
min
Atractylodes rhizome white
NPD1 A, Front Signal (190427 Sequence RDEH 2019-04-28 11-08-34\190427 JH C.D)
pA
40
35
30
25
20
15
5
10
15
20
25
30
min
Rehmannia root

Figure 6. Chromatogram of SPE florisil – NH_2 cartridge and SPE alumina – NH_2 cartridge test of fosthiazate in *Atractylodes* rhizome white by GC-NPD

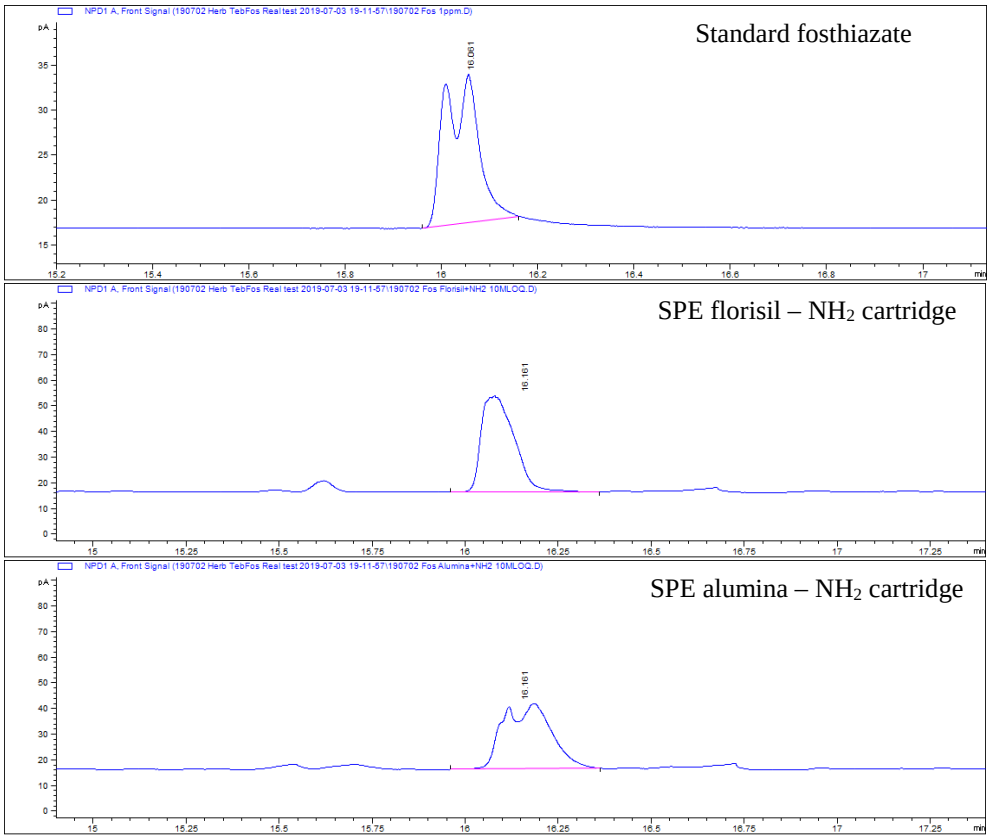
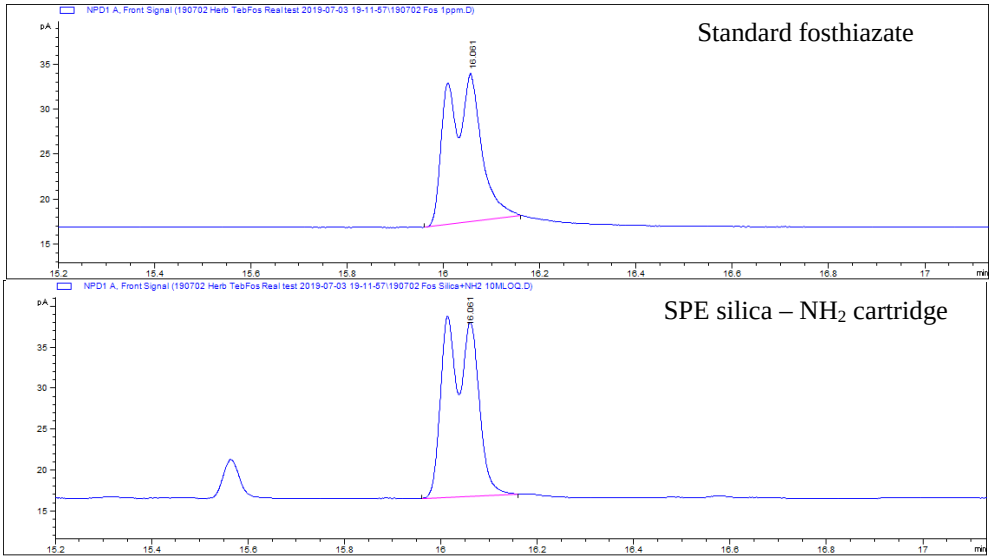

Standard fosthiazate
SPE florisil – NH2 cartridge
SPE alumina – NH2 cartridge

Figure 7. Chromatogram of SPE silica – NH_2 cartridge test of fosthiazate in *Atractylodes* rhizome white by GC-NPD


NPD1 A, Front Signal (190702 Herb TebFos Real test 2019-07-03 19-11-57\190702 Fos 1ppm.D)
Standard fosthiazate
16.061
pA
35
30
25
20
15
15.2
15.4
15.6
15.8
16
16.2
16.4
16.6
16.8
17
min
NPD1 A, Front Signal (190702 Herb TebFos Real test 2019-07-03 19-11-57\190702 Fos Silica+NH2 10MLOQ.D)
SPE silica – NH2 cartridge
16.061
pA
35
30
25
20
15
15.2
15.4
15.6
15.8
16
16.2
16.4
16.6
16.8
17
min

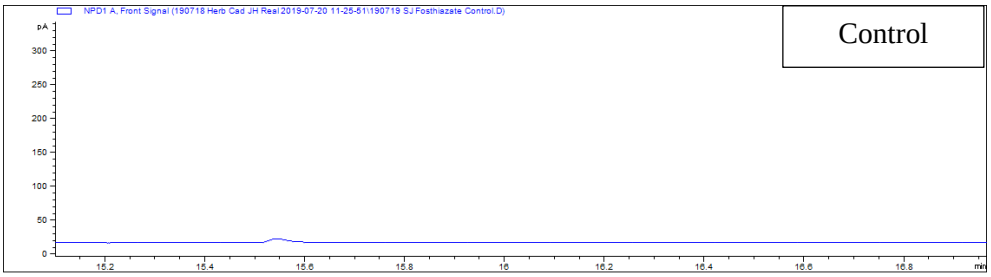
Recoveries of fosthiazate in *Atractylodes* rhizome white and *Rehmannia* root by GC-NPD

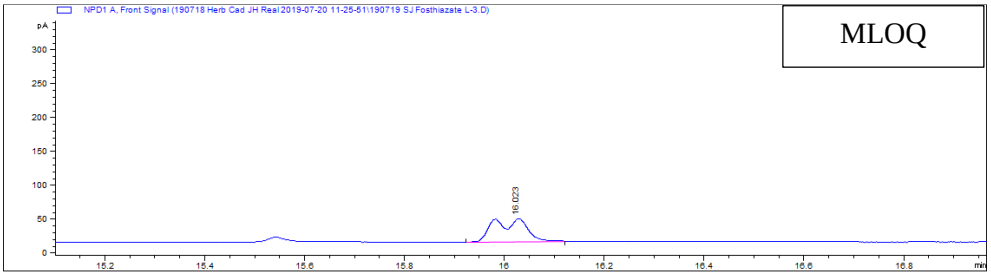
Recovery test providing accuracy and precision of sample preparation method with recovered rate (accuracy, %) and RSD (precision, %)(Fong et al. 1999). Untreated samples were spiked with MLOQ and 10 MLOQ levels of fosthiazate (*Atractylodes* rhizome white and *Rehmannia* root). And the analysis was peformed using the established method. Table 8 shows results of recovery test in *Atractylodes* rhizome white and *Rehmannia* root. In case of *Atractylodes* rhizome white, the range of recoveries were 95.3 % ~ 103.6 % at MLOQ level and 100.6 % ~ 102.5 % at 10 MLOQ level, and RSD was 4.2 and 0.9, respectively (Fig. 8). In case of *Rehmannia* root, the range of recoveries were 99.6 % ~ 110.9 % at MLOQ level and 100.3 % ~ 108.4 % at 10 MLOQ level, and RSD was 4.1 and 5.5, respectively (Fig. 9.)

Table 8. Recoveries test of fosthiazate in *Atractylodes* rhizome white and *Rehmannia* root by GC-NPD

Sample	Fortified level (mg/kg)	Recovery (%)	RSD (%)
***Atractylodes* rhizome white**	0.04	99.3	4.2
	0.4	101.4	0.9
***Rehmannia* root**	0.04	105.2	4.1
	0.4	106.1	5.5

Figure 8. Chromatogram of recovery test of fosthiazate in *Atractylodes* rhizome white by GC-NPD


NPD1 A, Front Signal (190718 Herb Cad JH Real 2019-07-20 11-25-51\190719 SJ Fosthiazate Control.D)
pA
300
250
200
150
100
50
0
15.2
15.4
15.6
15.8
16
16.2
16.4
16.6
16.8
min
Control


NPD1 A, Front Signal (190718 Herb Cad JH Real 2019-07-20 11-25-51\190719 SJ Fosthiazate L-3.D)
pA
300
250
200
150
100
50
0
16.023
15.2
15.4
15.6
15.8
16
16.2
16.4
16.6
16.8
min
MLOQ

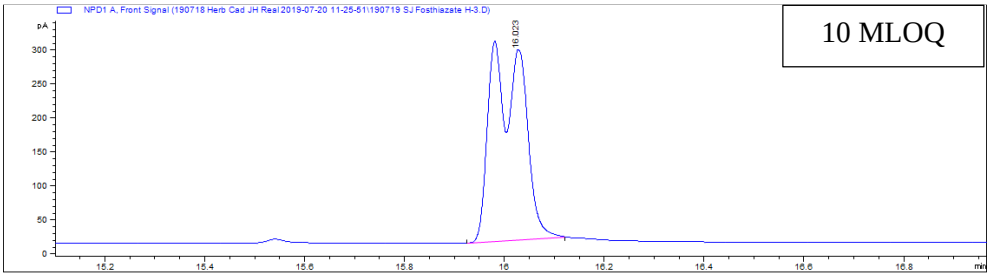
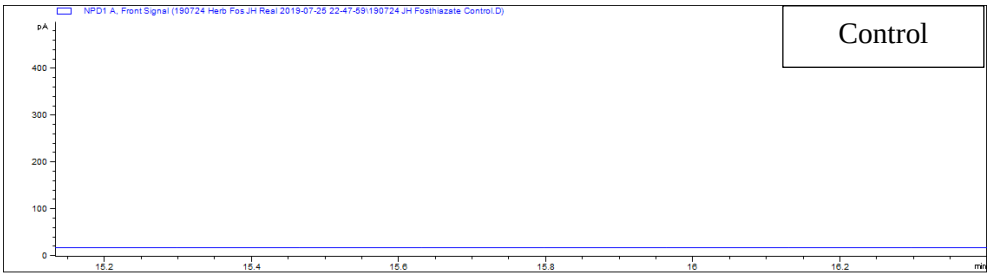
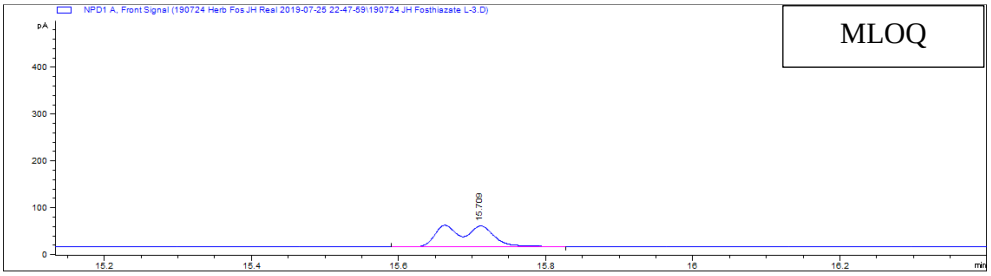
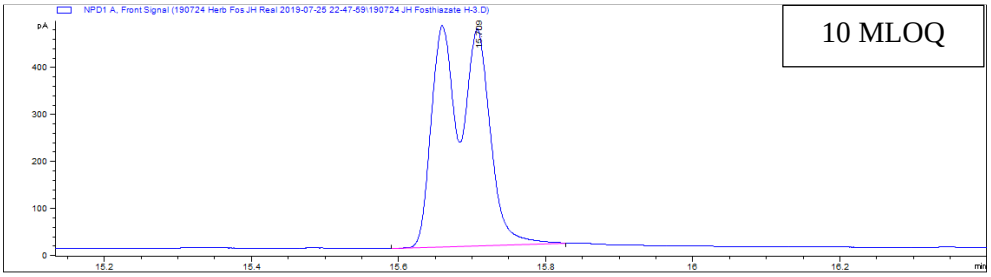

NPD1 A, Front Signal (190718 Herb Cad JH Real 2019-07-20 11-25-51\190719 SJ Fosthiazate H-3.D)
pA
300
250
200
150
100
50
0
16.023
15.2
15.4
15.6
15.8
16
16.2
16.4
16.6
16.8
min
10 MLOQ

Figure 9. Chromatogram of recovery test of fosthiazate in *Rehmannia* root by GC-NPD


NPD1 A, Front Signal (190724 Herb Fos JH Real 2019-07-25 22-47-59\190724 JH Fosthiazate Control.D)
pA
400
300
200
100
0
15.2
15.4
15.6
15.8
16
16.2
min
Control


NPD1 A, Front Signal (190724 Herb Fos JH Real 2019-07-25 22-47-59\190724 JH Fosthiazate L-3.D)
pA
400
300
200
100
0
15.709
15.2
15.4
15.6
15.8
16
16.2
min
MLOQ


NPD1 A, Front Signal (190724 Herb Fos JH Real 2019-07-25 22-47-59\190724 JH Fosthiazate H-3.D)
pA
400
300
200
100
0
15.709
15.2
15.4
15.6
15.8
16
16.2
min
10 MLOQ

LC-MS/MS condition and multiple reaction monitoring optimization

GC-NPD detects nitrogen and phosphorus selectivity, but its sensitivity is low when the compound to be analyzed is fosthiazate. The fosthiazate analysis method using GC-NPD showed an ILOQ of 0.01 mg/kg. LC and MS (Mass Spectrometry) were used to improve the complex preparation to remove interferences from various matrices and to obtain improved methods by setting lower LOQ and reducing instrumental run time through high sensitivity.

Applying tandem MS with selected reaction monitoring (SRM) can significantly reduce the chemical background. Even if the co-extracted matrix compounds have the molecular mass of a pesticide, in general, the two isobaric ions can be separated in SRM experiments, since their fragmentation in the collision cell often results in different product ions. As a result, tandem mass spectrometers offer excellent sensitivity and selectivity (Picó, Blasco, and Font 2004).

LC-MS/MS provides more sensitive, selective and rapid analysis than conventional LC. This analysis used multiple reaction monitoring (MRM) mode. MRM is a tandem mass spectrometric technique that allow the monitoring of specific collision induced dissociation (CID) reactions. As a result, MRM mode significantly improves analysis selectivity. In this mode, the ions migrate to Q1 in an ion source that acts as a filter depending on their mass-to-charge (*m/z*) ratio, where the precursor ion is selected. And then, a specific product ion is selected and monitored in Q3 (Despina Tsipi, 2015). Full scan spectrum of foshitazate was obtained in the mass range of 50-500 *m/z*. Figure 10 shows a spectrum of fosthiazate and 3 ion mass representing fosthiazate that is *m/z* 117.1, 228.0 and 283.9.

Precursor ion of fosthiazate was set to 283 *m/z*. Through optimization of precursor ions, quantifier ion and qualifier ion of fosthiazate was set to 228 *m/z* (-9 eV) and 104.15 (-18 eV) respectively. Table 9 shows MRM transition parameters of fosthiazate in LC-MS/MS.

Figure 10. Scan spectrum of fosthiazate

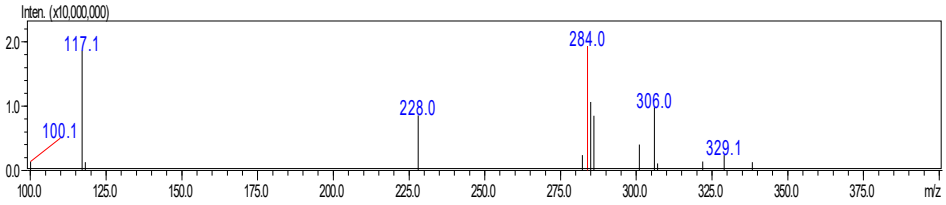

Inten. (x10,000,000)
2.0
1.0
0.0
117.1
284.0
228.0
306.0
100.1
329.1
100.0
125.0
150.0
175.0
200.0
225.0
250.0
275.0
300.0
325.0
350.0
375.0
m/z

Table 9. The MRM transition parameter

	Instrument	Monoisotopic Mass	Ionization	Precursor ion>Product ion (m/z)		Collision energy (eV)		Retention time (min)
				Quantitation	Qualification			
Fosthiazate	LC-MS/MS	283.046573	$[M+H]^+$	283.90>228.00	283.90>104.15	-9	-21	7.79

ILOD, ILOQ, MLOQ and calibration curve of fosthiazate analytical method by LC-MS/MS

Instrumental limit of detection (ILOD) and Instrumental limit of quantitation (ILOQ) are values that represent the sensitivity of analytical instrument. ILOD is determined by the minimum concentration of analyte providing S/N ratio of > 3 and ILOQ is determined by S/N ratio of > 10(Fong et al. 1999). In this experiment, ILOQ was checked from the results of analysis of several concentration standard solutions, 0.0025 mg/L was observed as practicable ILOQ in LC-MS/MS.

Based on MLOQ calculating equation, MLOQ of fosthiazate in *Atractylodes* rhizome white and *Rehmannia* root was 0.01 mg/L.

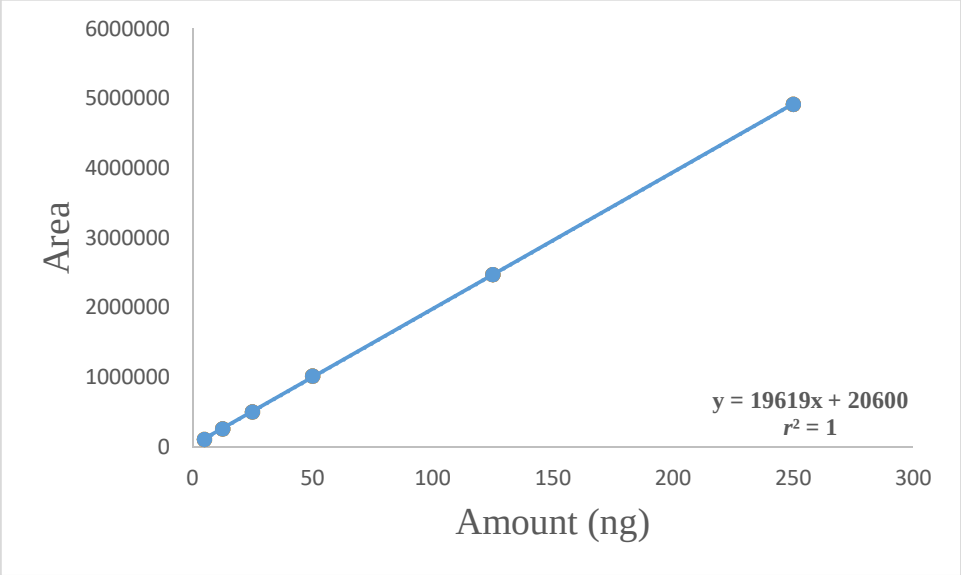
Matrix matched standard curves of fosthiazate has a good linearity in samples of *Atractylodes* rhizome white and *Rehmannia* root. The range was between 0.001 to 0.05 mg/kg of fosthiazate standard solution (Fig. 11).

The regression equations were y = 19619x + 20600 (*Atractylodes* rhizome white) and y = 19659x + 14199 (*Rehmannia* root) respectively. Coefficient of determination (r^2) were over 0.999 in each samples.

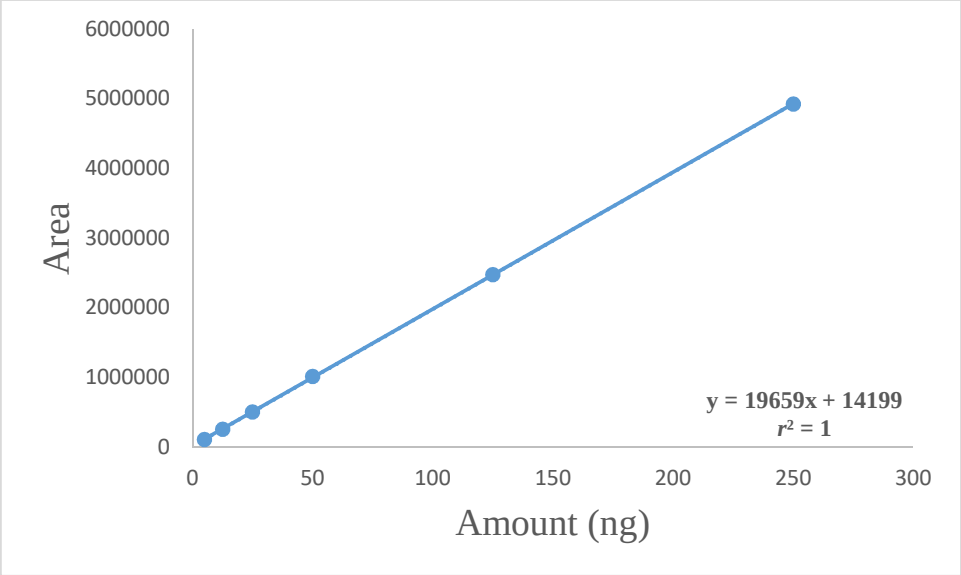
Figure 11. Matrix matched calibration curves of fosthiazate

(A) Calibration curve - *Atractylodes* rhizome white (Range: 0.001 – 0.05 mg/L), (B) Calibration curve - *Rehmannia* root (Range: 0.001 – 0.05 mg/L)

(A)


6000000
5000000
4000000
3000000
2000000
1000000
0
Area
0
50
100
150
200
250
300
Amount (ng)
y = 19619x + 20600
r² = 1

(B)


6000000
5000000
4000000
3000000
2000000
1000000
0
Area
0
50
100
150
200
250
300
Amount (ng)
y = 19659x + 14199
r² = 1

Optimization of sample preparation

To optimize a preparation method, a test was performed with *Atractylodes* rhizome white and *Rehmannia* root. The homogenized sample (5.0 ± 0.1 g) fortified at 0.01 mg/kg (n=3) was extracted with different combinations of extraction solvents, salts, and buffers based on the QuEChERS method as follows: (A) Original method (ACN), (B) AOAC method (Lehotay, 2007), (C) EN 15662 method (EN 15662, 2008). For optimization of the cleanup procedure, dSPE was used to three different dSPE sorbents as follows: (a) 150 mg $MgSO_4$, 25 mg PSA, (b) 150 mg $MgSO_4$, 50 mg C18, (c) 150 mg $MgSO_4$, 25 mg PSA, 7.5 mg GCB.

First, Samples were extracted by the methods of (A), (B) and (C). Then, after partitioning, 1 mL of the supernatant was transferred to (a), (b) and (c) dSPE, respectively. Extraction and cleanup efficiency of total nine methods were calculated on the basis of the matrix-matched standards employing single-point calibration (0.005 mg/kg).

Table 10 and Table 11 show results of extraction using QuEChERS method & dSPE cleanup recovery test in *Atractylodes* rhizome white and *Rehmannia* root. As the result of analysis *Atractylodes* rhizome white, the range of recoveries were 95.1 ~ 106.4 %. As the result of analysis *Rehmannia* root, the range of recoveries were 103.0 ~ 111.6 %. Recoveries of total nine methods in *Atractylodes* rhizome white and *Rehmannia* root was satisfied. All methods were valid. For the better purification, dSPE of 150 mg $MgSO_4$, 25 mg PSA, 7.5 mg GCB with comparatively less pigment (Fig.12) was chosen and the original QuEChERS method with relatively less salt was determined.

Table 10. Extraction using QuEChERS method & dSPE cleanup recovery test of fosthiazate in *Atractylodes* rhizome white by LC-MS/MS

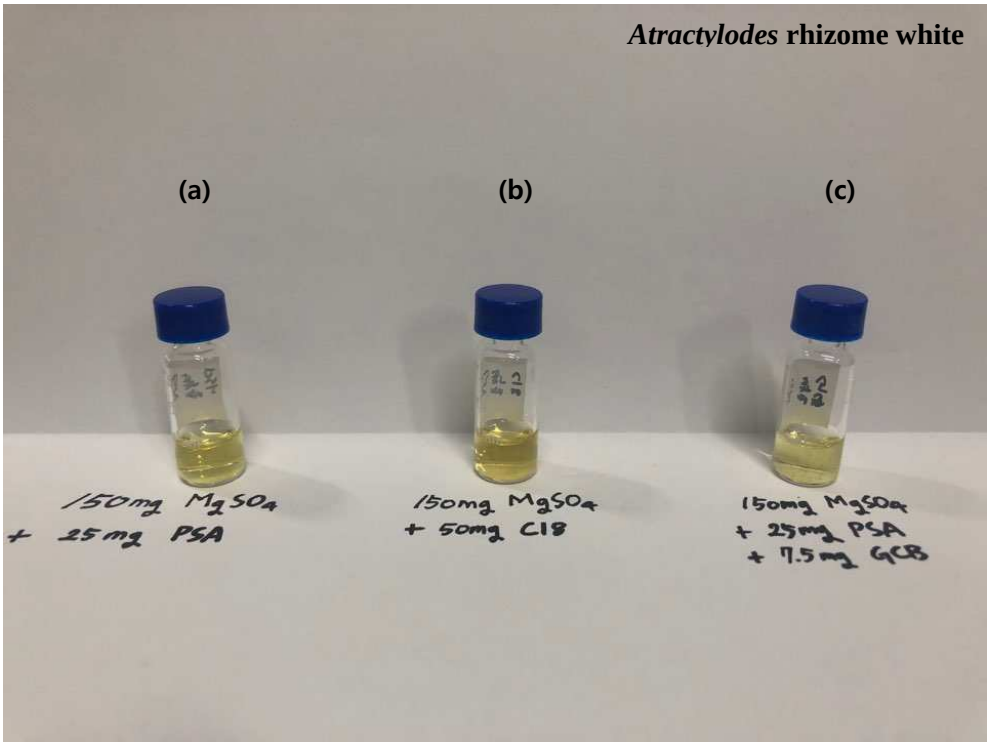
Extraction method	dSPE	Recovery (%)
Original method	150 mg $MgSO_4$, 25 mg PSA	100.3
	150 mg $MgSO_4$, 50 mg C18	95.1
	150 mg $MgSO_4$, 25 mg PSA, 7.5 mg GCB	98.3
AOAC method	150 mg $MgSO_4$, 25 mg PSA	101.2
	150 mg $MgSO_4$, 50 mg C18	100.4
	150 mg $MgSO_4$, 25 mg PSA, 7.5 mg GCB	97.2
EN 15662 method	150 mg $MgSO_4$, 25 mg PSA	104.4
	150 mg $MgSO_4$, 50 mg C18	103.4
	150 mg $MgSO_4$, 25 mg PSA, 7.5 mg GCB	106.4

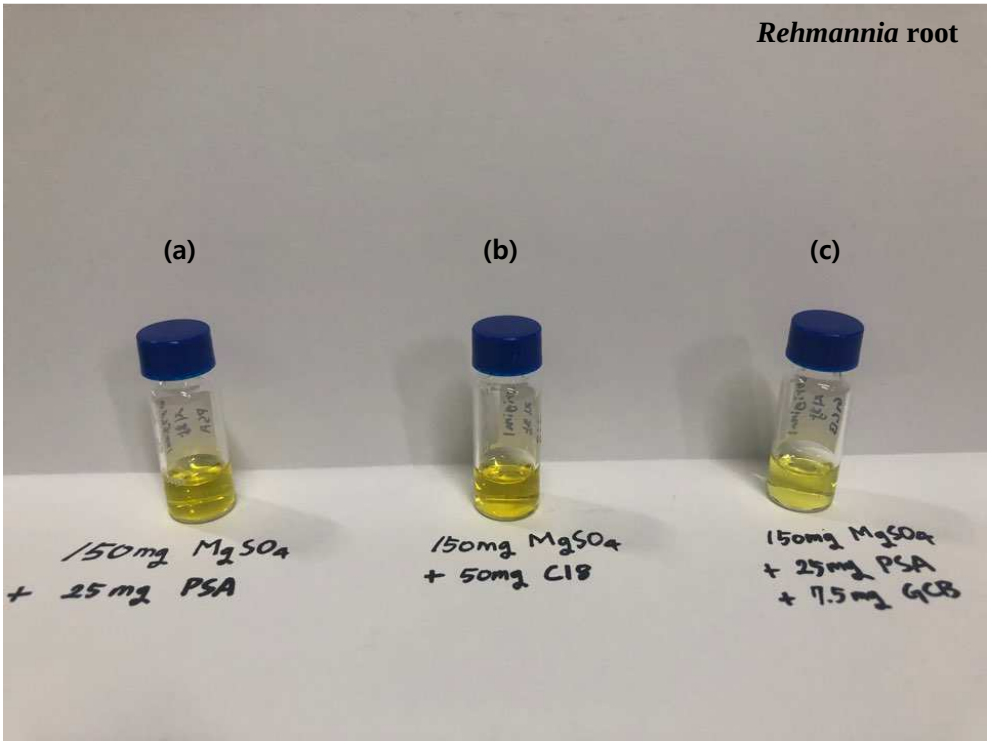
Table 11. Extraction using QuEChERS method & dSPE cleanup recovery test of fosthiazate in *Rehmannia* root by LC-MS/MS

Extraction method	dSPE	Recovery (%)
Original method	150 mg $MgSO_4$, 25 mg PSA	105.0
	150 mg $MgSO_4$, 50 mg C18	106.4
	150 mg $MgSO_4$, 25 mg PSA, 7.5 mg GCB	107.5
AOAC method	150 mg $MgSO_4$, 25 mg PSA	103.0
	150 mg $MgSO_4$, 50 mg C18	111.6
	150 mg $MgSO_4$, 25 mg PSA, 7.5 mg GCB	107.8
EN 15662 method	150 mg $MgSO_4$, 25 mg PSA	105.2
	150 mg $MgSO_4$, 50 mg C18	111.5
	150 mg $MgSO_4$, 25 mg PSA, 7.5 mg GCB	109.1

Figure 12. dSPE cleanup test in *Atractylodes* rhizome white and *Rehmannia* root

(a) 150 mg $MgSO_4$, 25 mg PSA, (b) 150 mg $MgSO_4$, 50 mg C18, (c) 150 mg $MgSO_4$, 25 mg PSA, 7.5 mg GCB


Atractylodes rhizome white
(a)
(b)
(c)
150mg MgSO4
+ 25mg PSA
150mg MgSO4
+ 50mg C18
150mg MgSO4
+ 25mg PSA
+ 7.5mg GCB


Rehmannia root
(a)
(b)
(c)
150mg MgSO4
+ 25mg PSA
150mg MgSO4
+ 50mg C18
150mg MgSO4
+ 25mg PSA
+ 7.5mg GCB

Matrix effect

In the electrospray ionization process in LC-MS/MS, simultaneous elution interference interacting with the target compound leads to a matrix effect resulting in signal suppression compared to the signal of the analyte injected only in the solvent (Lozano et al. 2016). Enhancement or suppression of analyte responses is accompanied by a reduced accuracy and precision of subsequent measurements. So, the matrix effect limits the utility of LC-MS/MS (Dams et al. 2003).

Some interference compounds were removed during the extraction. However, complex matrices can cause interference, which can lead to analytical errors, resulting in inaccurate results (Rubert, Soler, and Mañes 2011).

The matrix effects were calculated by comparing the slope of calibration curve between the solvent standard and the matrix matched standard. According to the equation mentioned in the method section, a positive value indicates signal enhancement, whereas a negative value indicates signal suppression.

In case of *Atractylodes* rhizome white, matrix effect was -35.6 % and *Rehmannia* root; -35.4 %. According to SANTE guideline's acceptability criteria (20%), matrix matched is necessarily needed in analysis of fosthiazate. All matrix induced suppression effect in *Atractylodes* rhizome white and *Rehmannia* root.

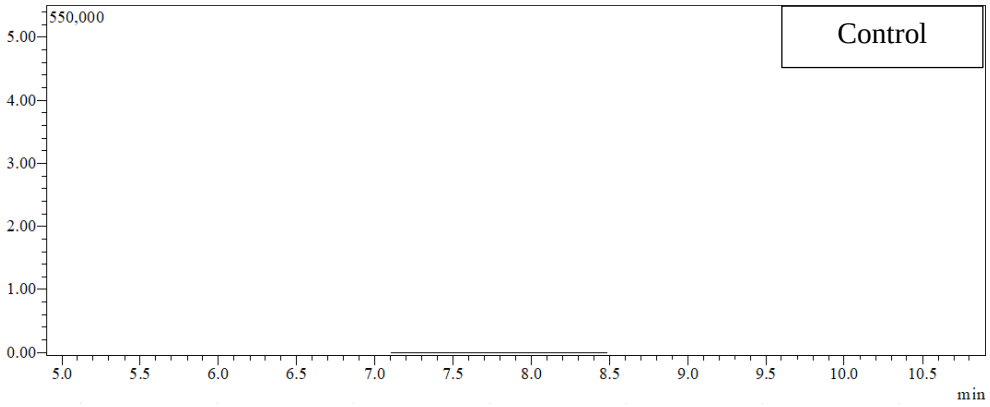
Recoveries of fosthiazate in *Atractylodes* rhizome white and *Rehmannia* root by LC-MS/MS

Recovery test provides accuracy and precision of sample preparation method by recovered rate (accuracy, %) and RSD (precision, %) (Fong et al. 1999). Untreated samples were spiked with MLOQ and 10 MLOQ levels of fosthiazate in *Atractylodes* rhizome white and *Rehmannia* root. And the analysis was performed using the established method. Table 12 shows results of recovery test in *Atractylodes* rhizome white and *Rehmannia* root. As the result of analysis *Atractylodes* rhizome white, the range of recoveries were 93.8 ~ 99.3 % at MLOQ and 96.0 ~ 99.6 % at 10 MLOQ level, and RSD was 2.9 and 2.1, respectively (Fig. 13). As the result of analysis *Rehmannia* root, the range of recoveries were 106.3 ~ 110.7 % at MLOQ and 103.8 ~ 107.2 % at 10 MLOQ level, and RSD was 2.1 and 1.7, respectively (Fig. 14).

Table 12. Recoveries test of fosthiazate in *Atractylodes* rhizome white and *Rehmannia* root by LC-MS/MS

Sample	Fortified level (mg/kg)	Recovery (%)	RSD (%)
***Atractylodes* rhizome white**	0.01	96.27	2.9
	0.1	97.30	2.1
***Rehmannia* root**	0.01	108.19	2.1
	0.1	106.1	1.7

Figure 13. Chromatogram of recovery test of fosthiazate in *Atractylodes* rhizome white by LC-MS/MS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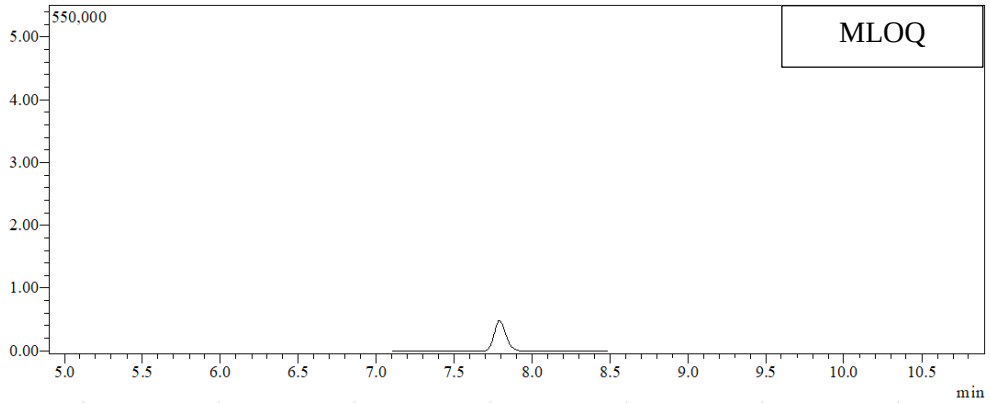
5.00
4.00
3.00
2.00
1.00
0.00
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
min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MLOQ
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4.00
3.00
2.00
1.00
0.00
5.0
5.5
6.0
6.5
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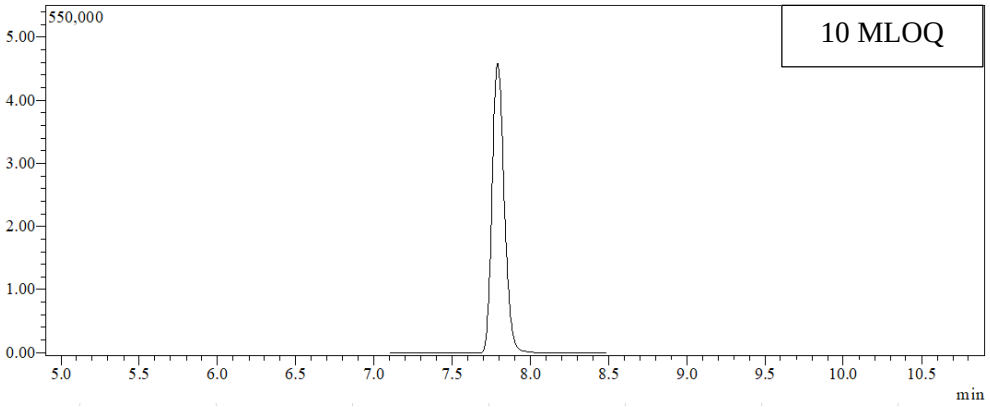
7.5
8.0
8.5
9.0
9.5
10.0
10.5
min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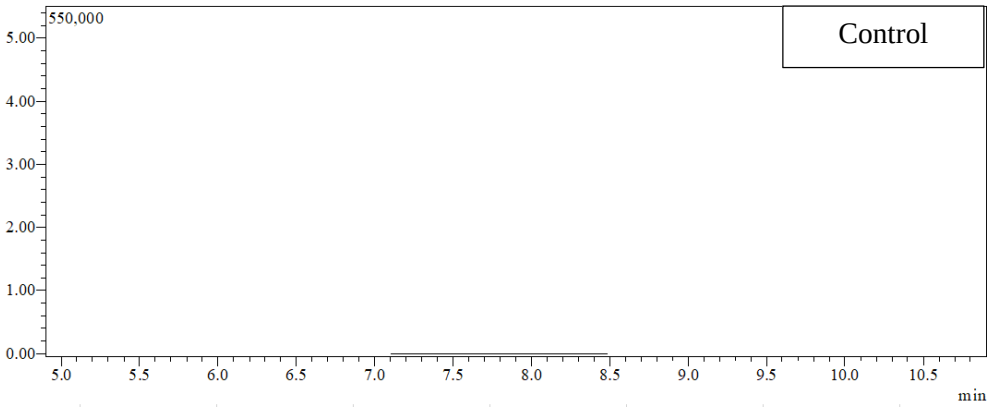
10 MLOQ
5.00
4.00
3.00
2.00
1.00
0.00
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
min

Figure 14. Chromatogram of recovery test of fosthiazate in *Rehmannia* root by LC-MS/MS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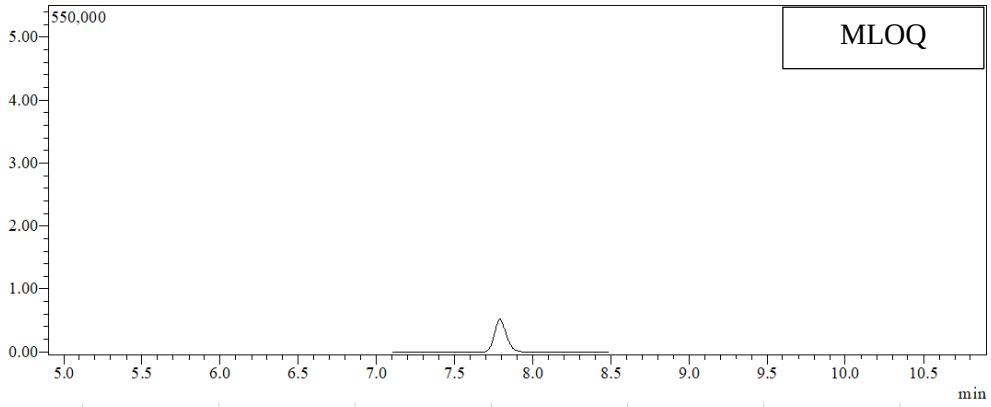
Control
5.00
4.00
3.00
2.00
1.00
0.00
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
min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MLOQ
5.00
4.00
3.00
2.00
1.00
0.00
5.0
5.5
6.0
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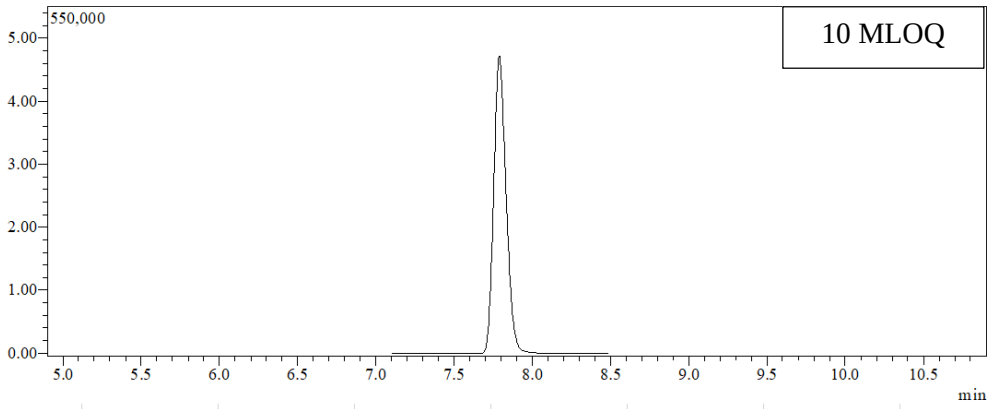
7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
min


550,000
10 MLOQ
5.00
4.00
3.00
2.00
1.00
0.00
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
min

Part 2

Dissipation of Tricyclazole and Tebuconazole Residues in Proso Millet using LC-MS/MS and GC-MS/MS during cultivation

Introduction

Minor crops

Due to the negligible cultivation area and limited markets of minor crops, plant breeding efforts have been directed to major crops, while discarding minor crops. Focusing on a small number of economically important primary crops, this process was further facilitated for commercial reasons. This may have resulted in bottlenecks where current low growth areas hinder investment in minor crop breeding programs, and the lack of competitive varieties prevents farmers from diversifying their crop systems and expanding the growth of minor crops (Peltonen-Sainio, Jauhiainen, and Lehtonen 2016). In korea, “Minor crop” is defined as a widely cultivated crops that cultivation area is less than 1,000 ha, based on Agricultural and Forestry Statistical Yearbook. Major crops include barley, groundnuts or peanuts, potatoes, rice, maize, millet, oil palm fruit, rye, sorghum, cassava, cotton, soybeans, sugar cane and wheat(Leff, Ramankutty, and Foley 2004).

Minor crops are grown in relatively small amounts compared with major crops. Therefore minor crops are considered of low economic importance. Pesticide manufacturers have been unwilling to do expensive research and development work required to register pesticides for use on the crop (Walorczyk, Drożdżyński, and Kierzek 2015). Because of the limited options for legal pesticide, farmers use un-registered pesticides to protect their crops from destroying by insects or pathogens. So, it is necessary to register pesticides that are not registered in the minor crop and to use pesticides legally and safely(de Oliveira, Pacheco, and Scherer 2016).

Proso millet is also one of the minor crops and has been in the same situation. To use pesticide safely in crop, PHI (Pre-harvest interval) and MRL (Maximum residue limit) must be established.

Proso millet

Proso millet (*Panicum miliaceum* L.) is the longest used summer cereal in human in addition to wheat and barley. It has very short growing season about 10–11 weeks. Thus, crops can grow at a wide range of altitude (Kalinova and Moudry 2006). Proso millet (*Panicum miliaceum* L.) is an important cereal and a valuable component of the human diet, especially in developing countries. Crops is salt-, alkali-, cold-, and drought-tolerant and can be grown in various types of soil and in poor growing conditions (Zhang, Liu, and Niu 2014).

The height of the proso millet is in the range from 0.5 to 1.2 m, depending on the growing conditions, and the stem is upright or the base is laid at an angle. Depending on the variety and cultivation environment of the millet, roughly 500 to 3,000 seeds per ear. 1,000 grains of millet seeds weigh 4-5 g and 1 L seeds weigh 500-530 g. For preventing pest of proso millet, some pesticides allowed to use (Table 13).

Table 13. Allowed pesticides of proso millet

Pesticide	Usage	MRL (mg/kg)
Glufosinate(ammonium)	Herbicide	0.05
Glyphosate	Herbicide	0.05
Deltamethrin	Insecticide	0.1
Dinotefuran	Insecticide	1.0
Difenoconazole	Fungicide	0.05
Bentazone	Herbicide	0.1
Bifenthrin	Insecticide	0.05
Cyantraniliprole	Insecticide	1.0
Acetamiprid	Insecticide	0.3
Emamectin benzoate	Insecticide	0.05
Etofenprox	Insecticide	0.05
MCPA	Herbicide	0.05
Oxolinic acid	Fungicide	0.05
Imidacloprid	Insecticide	0.05
Indoxacarb	Insecticide	2.0
Chlorantraniliprole	Insecticide	0.3
Tebufenozide	Insecticide	0.3
Tralomethrin	Insecticide	0.1
Fenitrothion	Insecticide	0.3
Phenthoate	Insecticide	2.0
Flufenoxuron	Insecticide	0.05
Pyraclostrobin	Fungicide	0.05

Tricyclazole

Tricyclazole is a triazolobenzothiazole fungicide (Fig 15.) Tricyclazole is a triazolobenzothiazole that is [1,2,4]triazolo[3,4-b][1,3]benzothiazole which is substituted at position 5 by a methyl group. IUPAC name is 5-methyl-1,2,4-triazolo[3,4-b][1,3]benzothiazole. The chemical formula of tricyclazole is $C_9H_7N_3S$. The physical form is a peach coloured granular solid. There are many kinds of formulation for tricyclazole products including wettable powder (WP), Suspension concentrate (SC) and Granules (GR).

Mode of action (MOA) of tricyclazole is known to inhibit melanin biosynthesis. Tricyclazole prevents the penetration of the epidermis by the fungus, protecting the plant from infection. The compound acts by inhibiting melanization within the appressorium, thus causing a lack of rigidity in the appressorial wall (Peterson 1990).

Figure 15. Structure of tricyclazole

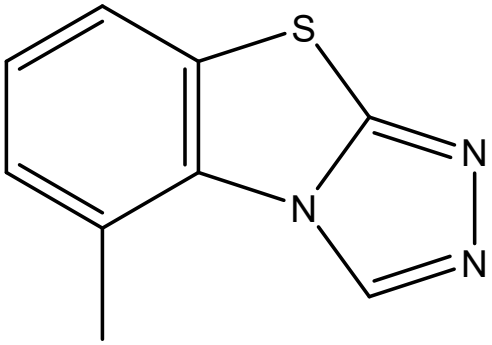

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Table 14. Physicochemical properties of tricyclazole

Property	Information
Common name	tricyclazole (BSI, E-ISO, (m) F-ISO, ANSI)
IUPAC name	5-methyl-1 ,2,4-triazolo[3,4-b][1 ,3]benzothiazole
CAS No.	41814-78-2
Molecular formula	$C_9H_7N_3S$
Molecular weight	189.2
Boiling point	184.6-187.2 ℃
Vapor pressure	0.027 mPa (20 ℃)
K_{ow}	Log P = 1.4
Solubility in solvent	In water 596 mg/L (20-25 ℃). Soluble in acetone (13.8), methanol (26.5), xylene (4.9)(g/L, 20-25 ℃)
Mode of action	Melanin biosynthesis inhibitor
Toxicology	**Acute oral (LD50, mg/kg)** rats 314, dogs >50, mice 245 **Acute percutaneous (LD50, mg/kg)** rabbits > 2000 **Skin irritation** Not an irritant (rabbits). **Eye** Slight irritant (rabbits). **Inhalation (LC50, mg/L)** rats 0.146 (1 h)
Environmental fate	**Animals** Rapid and extensive metabolism. **Plants** The principal metabolite in plants is the hydroxymethyl analogue. **Soil/Environment** K_d 4 (loamy sand, pH 6.5, 1.5% o.m.), 45 (loam, pH 5.7, 3.1% o.m.), 21 (clay loam, pH 7.4, 1.9% o.m.), 22 (silty clay loam, pH 5.7, 4.1% o.m.).

*The Pesticide Manual Seventeenth Edition (J A Turner)

Table 15. MRLs of tricyclazole in various agricultural products

Crop	MRL (mg/kg)	Registered date
Gondre	**0.2**	**2018-10-12**
Pepper	**3.0**	**2018-01-01**
Root and tuber vegetables	**0.05**	**2018-10-12**
Mung bean	**0.2**	**2018-10-12**
Perilla leaves	**0.2**	**2018-10-12**
Radish (roots)	**0.2**	**2018-10-12**
Radish (leaves)	**0.2**	**2018-10-12**
Water dropwort	**0.2**	**2018-10-12**
Ginger	**0.2**	**2018-10-12**
Watermelon	**0.2**	**2018-01-01**
Rice	**0.7**	**2001-04-01**
Crown daisy	**0.2**	**2018-10-12**
Aronia	**0.2**	**2018-10-12**
Cabbage	**0.2**	**2018-10-12**
Stalk and stem vegetable	**0.05**	**2018-10-12**
Leafy vegetable	**0.05**	**2018-10-12**
Millet	**0.7**	**2018-10-12**
Chamnamul	**0.2**	**2018-10-12**
Kale	**0.2**	**2018-10-12**
Seed spices	**0.2**	**2018-10-12**

*Pesticides and Veterinary Drugs Information (Ministry of food and drugs safety) (*Korean pesticides MRLs in Food*; 2019;, 2019) (Safety, 2019)

Tebuconazole

Tebuconazole is a triazole fungicide and plant growth regulator (Fig 16.). The compound is highly effective in controlling soil-borne and foliar fungal pathogens (Strickland, Potter, and Joo 2004). IUPAC name is (RS)-1-p-chlorophenyl-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol. The chemical formula of tebuconazole is $C_{16}H_{22}ClN_3O$. The physical form is a Colourless crystals. There are many kinds of formulation for tebuconazole products including wettable powder (WP), water dispersible granule (WG), emulsifiable concentrate (EC), suspension concentrate (SC) and granules (GR).

Mode of action (MOA) of tebuconazole is known to inhibit sterol demethylation. The main target site is cytochrome P450 sterol 14α-demethylase (CYP51). Tebuconazole can also inhibit other types of cytochrome P450 monooxygenases. Reduced sensitivity and resistance to DMIs (sterol 14α-demethylase inhibitors) in the field isolate of various plant pathogens have been reported (Leroux et al. 2000).

Figure 16. Structure of tebuconazole

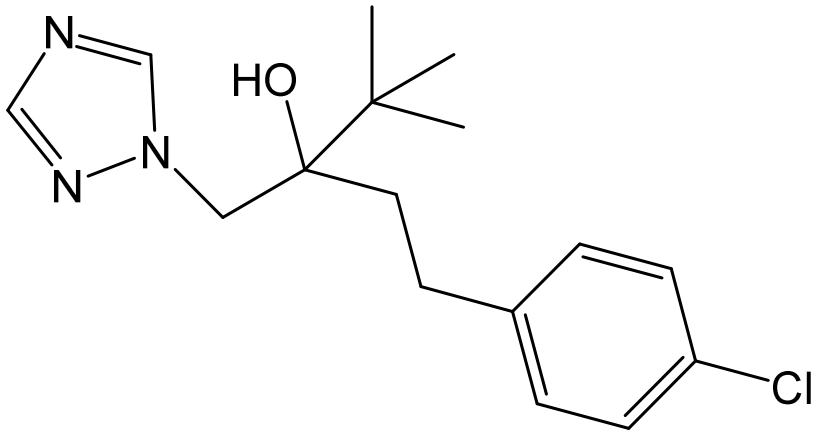

N
HO
N
N
Cl

Table 16. Physicochemical properties of tebuconazole

Property	Information
Common name	tébuconazole ((m) F-ISO); tebuco□a□ole (BSI, E-ISO)
IUPAC name	(RS)-1-p-chlorophenyl-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol
CAS No.	107534-96-3
Molecular formula	$C_{16}H_{22}ClN_3O$
Molecular weight	307.8
Boiling point	105 ℃
Vapor pressure	0.0017 mPa (20 ℃)
K_{ow}	Log P = 3.7
Solubility in solvent	In water 36.0 mg/L (20-25 ℃, pH 5-9). Soluble in dichloromethane (> 200), hexane (<0.1), isopropanol (50-1 00), toluene (50-1 00) (g/L, 20-25 ℃)
Mode of action	sterol demethylation inhibitor
Toxicology	**Acute oral (LD50, mg/kg)** male rats 4000, female rats 1700, mice c. 3000 **Acute percutaneous** (LD50, mg/kg) rats > 5000 **Skin irritation** Not an irritant(rabbits). **Eye** Mild irritant (rabbits). **Inhalation** (LC50, mg/1) rats 0.37 (aerosol), >5.1 (dust) (4 h)
Environmental fate	**Animals** In rats, after three days, elimination was almost complete (>99% of the recovered dose). Tebuconazole was excreted with the urine and the faeces. **Plants** Metabolism studies in representative crops show that tebuconazole is the major terminal residue in grapes and peanut and cereal straw. **Soil/Environment** The degradation of tebuconazole in soil was slow in laboratory studies. Under field conditions, the compound degraded much more rapidly, and did not accumulate in long-term studies (3-5 y).

*The Pesticide Manual Seventeenth Edition (J A Turner)

Table 17. MRLs of tebuconazole in various agricultural products

Crop	MRL (mg/kg)	Registered date
Persimmon	2	2012-12-27
Mandarin	2	2012-02-01
Kidney bean	0.1	2018-11-14
Ginseng(Dried)	1	2016
Raisin	6.0	2017-09-01
Nuts	0.05	2016-12-31
Cassia seed	0.05	2018-03-01
Sweet potato	0.05	2018-10-12
Sweet potato stalk	0.05	2018-10-12
Green & red pepper(Fresh)	3	2001-04-01
Green & red pepper(Dried)	5	2008-12-23
Fresh pepper leaves	5	2001-04-01
Chinese matrimony vine(Dried)	10	2010-10-29
Chard	15	2013-11-12
Mung bean	0.05	2018-11-14
Green Tea Extract	10	2007-09-06
Arguta kiwifruit	0.5	2018-11-14
Wild Garlic	2	2007-09-06
Carrot	0.4	2018-11-14
Soy bean	0.1	2018-10-12
Jujube	5	2018-10-12

Jujube(Dried)	5	2018-10-12
Bonnet bellflower	0.05	2003-04-01
Balloon flower	0.05	2018-11-14
Korean solomon's seal(Roots)	0.05	2018-11-14
Perilla leaves	15	2015
Strawberry	0.5	2010-07-23
Peanut	0.05	2017-09-01
Lemon	2.0	2018-11-14
Yams	0.1	2016-12-31
Yam(dried)	0.1	2018-10-12
Garlic	0.1	2001-04-01
Mango	0.05	2018-11-14
Korean Plum	1	2016
Melon	0.2	2018-03-01
Radish(Roots)	0.2	2018-11-14
Radish(Leaves)	5	2018-06-27
Fig	0.5	2018-11-14
Wheat	0.05	2016-12-31
(Banana	0.05	2016-12-31
Korean mint	0.3	2018-11-29
Korean cabbage	2	2018-08-16
Raspberry	0.5	2018-11-14
Peach	1.0	2017-09-01

Blueberry	0.5	2018-11-14
Beet root	0.09	2019-10-14
Apple	1	2016-12-31
Sugar beet	0.05	2018-11-14
Wild garlic leaf	0.1	2016-12-31
Apricot	2.0	2018-11-14
Lettuce, Leaf	0.05	2015-10-29
Sanghwang mushroom	0.05	2018-11-14
Ginger	0.05	2018-11-14
Celeriac	0.05	2018-11-14
Watermelon	1	2001-04-01
Ginseng(Fresh)	0.5	2016
Sorghum	0.05	2018-11-14
Turnip	0.05	2018-11-14
Spinach	3	2018-11-14
Rice	0.05	2004-04-01
Aronia	0.5	2018-11-14
Cabbage	5	2018-11-14
Lettuce, Head	0.05	2015-10-29
Onion	0.05	2009-05-07
Ssam cabbage	5	2018-08-16
Balsam pear	1	2018-06-27
Stalk and stem vegetable	5	2018-11-14

Leafy vegatable	3	2018-11-14
Mulberry	10	2018-03-01
Chinese magnolia vine	0.9	2018-11-14
Cucumber	0.2	2006-12-01
Corn	0.5	2018-03-01
Olive	0.05	2016-12-31
Burdock	0.05	2018-11-14
Korean Lemon : Citrus junos	2.0	2018-11-14
Rape seed	0.1	2018-06-27
Pome fruits	0.5	2012-02-01
Plum	0.9	2018-03-01
Horse bean	0.1	2018-11-14
Tea	5	2001-04-01
Korean melon	0.1	2018-03-01
Cherry	4.0	2017-09-01
Chwinamul	0.05	2018-03-01
Coffee bean	0.1	2016-12-31
Kiwifruit	2	2016-12-31
Tomato	1	2016-12-31
(Welsh Onion	3	2007-09-06
Parsnip	0.05	2018-11-14
Papaya	2.0	2018-11-14
Grapes	5.0	2017-09-01

Green garlic	2	2010-12-01
Unripe bean	0.5	2018-08-16
Sweet pepper	3	2004-04-01
Seed spices	0.05	2018-11-14
Herbs(fresh)	0.05	2018-11-14
Hop	40	2018-11-14
False saffron(Seeds)	0.05	2018-11-14

*Pesticides and Veterinary Drugs Information (Ministry of food and drugs safety) (*Korean pesticides MRLs in Food*; 2019; 2019) (Safety, 2019)

PHI (Pre-Harvest Interval)

Pesticides are substances that are useful for preventing pests. But they are potentially harmful to human health. Therefore, the use of pesticides requires strict rules. The pre-harvest interval (PHI) provide a reasonable pesticide that ensure acceptable pesticide residues on crops at harvest. It helps to produce safe agricultural products based on the limit number of spraying pesticide and final spraying period before harvest (Agency, 2007). It is a guideline for harvesting safe agricultural products and easy understanding to a farmer for use of pesticides. If the agricultural products and easy understanding to a farmer for use of pesticides. If a farmer uses pesticides according to this guideline, the residual amount of the pesticide in crops would be less than MRL. The PHI is registered in the pesticide spraying guidelines from Korea Crop Protection Association (Agency, 2007).

The purpose of studies

This studies were conducted to investigate the residual characteristics of tricyclazole and tebuconazole in proso millet to establish PHI. Tricyclazole 20 % suspension concentrate (SC) and tebuconazole 20 % SC were applied to proso millet according to the scheduled time (50/40/30 days, 40/30/21 days, 30/21/14 days and 21/14/7 days before harvest), respectively and the residues in grain and straw of proso millet were analyzed to find out the maximum residue level.

Materials and methods

Analytical standard and pesticide for spraying

Standard materials of tricyclazole (Purity: 99.49 %) and tebuconazole (Purity: 99.4 %) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and Sigma-aldrich (St. Louis, MO, USA), respectively. Tricyclazole 20+5 % suspension concentrate (SC) from Nonghyup chemical and tebuconazole 20% SC from Bayer Crop Science were purchased at pesticide market (Seoul, Korea).

Standard solutions

Standard stock solutions of tricyclazole and tebuconazole were prepared at the concentration of 1,000 mg/L with acetonitrile, respectively. The working solutions were prepared by serial dilution of stock solution with acetonitrile.

Subject crops

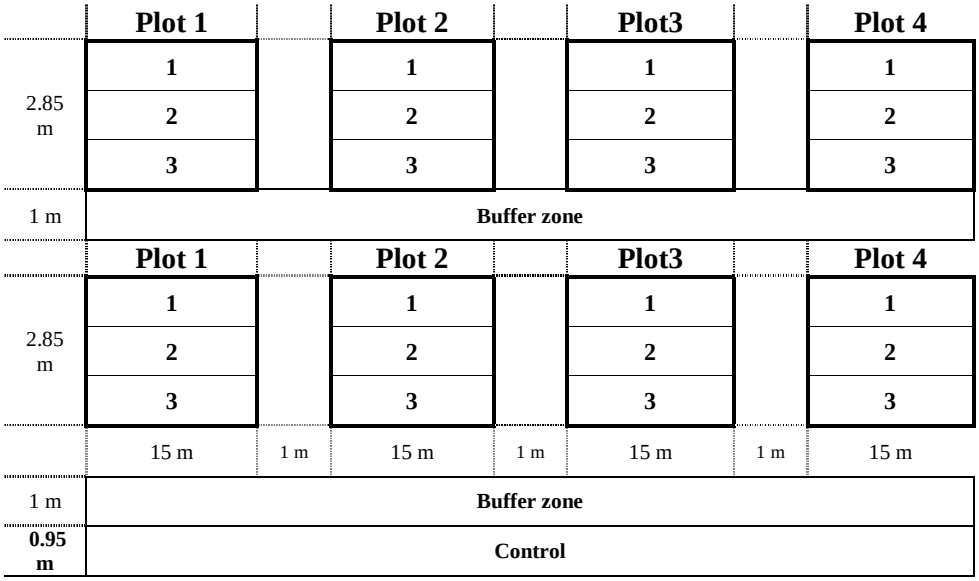
In several varieties of proso millet (*Panicum miliaceum* L.), 'Ibaekchal' which is the popular species in South Korea was used for field experiments.

Field trials

Test field was located in Hwaseong-si (Kyeonggi-do, Korea) and the field size was 63 m (length) x 3.8 m (width) (Fig. 17). Two treatment groups were divided in the field to test tricyclazole and tebuconazole. The field trial was divided into four plots depending on the date of pesticide treatment. The size of each plot was 42.75 m^2 containing 3 replicates of 14.25 m^2. Each plot was treated with the pesticide by 3 times as follows: Plot 1 was treated at 50/40/30 days before harvest, plot 2 was 40/30/21 days before harvest, plot 3 was 30/21/14 days before harvest and plot 4 was 21/14/7 days before harvest. To prevent cross contamination during spraying tricyclazole and tebuconazole, respectively, the buffer zones were installed between control and treated plots. The arrangement of field trial is illustrated in Figure 17.

Tricyclazole 20 % SC was diluted 1,000 times with water. In case of tebuconazole 20% SC, it was diluted 2,000 times with water. Pesticides using handgun sprayer reproducibility test for spraying was carried to check steady spraying capacity and speed. Diluted pesticide solution was sprayed onto crop until foliar were wetted sufficiently (Fig. 18).

Figure 17. Experimental plots in field



Control : Free pesticide; No treated
Plot 1 : Treated third time at 50/40/30 days before harvest
Plot 2 : Treated third time at 40/30/21 days before harvest
Plot 3 : Treated third time at 30/21/14 days before harvest
Plot 4 : Treated third time at 21/14/7 days before harvest

Sampling

The harvest of grain and straw of proso millet was conducted on September 27, 2018. Control plot (Free pesticide plot) was first harvested to prevent contamination. Other samples in Plot 1, 2, 3 and 4 were collected over 1.0 kg randomly. After harvest, the samples were immediately moved to laboratory. Grain and straw of proso millet was homogenized by food processor. Every sample was stored at -20 ℃ in polyethylene bags.

Figure 18. Preparation of pesticide solution, spraying in field and preparation sample

(A) Measure of tricyclazole product, (B) Measure of tebuconazole product, (C) Application of the pesticide solution on the sample (proso millet), (D) Grain collection, (E) Homogenization of grain

(A)

(B)

(C)

(D)

(E)

Analytical instruments and conditions

1) LC-MS/MS

LC-MS/MS analysis for tricyclazole was carried out on a LC-MS 8040 with UHPLC-Nexera (ESI positive mode). The column oven temperature was 40 ℃ and the column was Raptor^{TM} ARC-18 (100mm x 2.1 mm, 2.7 µm). Mobile phases were 0.1% formic acid & 5mM ammonium formate in water (A) and 0.1% formic acid & 5mM ammonium formate in methanol (B). The mobile phase (B) was initially hold at 5% for 1 min. Then (B) increased 5% to 70% for 2 min and hold 70% for 3 min. After that, (B) increased 70% to 98% for 1 min and hold 98% for 2.5 min. Finally, decreased it to 5% for 0.5 min and hold 5% for 5 min. The flow rate was set at 0.2 mL/min and the injection volume was 5 µL. DL temperature was 250 ℃ and heat block temperature was 400 ℃. The nebulizing gas, drying gas was nitrogen and collision energy was used argon gas.

For MS/MS analysis, two transitions (quantifier and qualifier) were chosen for scheduled MRM mode after an automatic optimization procedure.

2) GC-MS/MS

GC-MS/MS analysis for tebuconazole was carried out on a Shimadzu GCMS-TQ 8040 mass spectrometer coupled to a GC-2010 plus equipped with an AOD-20i auto sampler (Kyoto, Japan). A capillary column was BPX5 (0.25mm x 30 m, 0.25 µm). The oven temperature program was initialized at 80 ℃ (held for 2 min), increased to 300 ℃ at 22 ℃/min (held for 3 min). Total run time was 25.0 min. Helium (≥ 99.999%) was used as carrier gas at a constant flow (1.5 mL/min), and argon was used as collision gas. The interface temperature was 280 ℃. In case of ion source, temperature was 230 ℃. The electron ionization energy was -70 eV and the detector voltage was set at 1.4 kV. The Injection volume was 2 µL.

For MS/MS analysis, two transitions (quantifier and qualifier) were chosen for scheduled MRM mode after an automatic optimization procedure.

Method validation

1) ILOQ (Instrumental Limit of Quantitation)

After matrix matched standard solutions (0.0025 and 0.25 μg/mL) were analyzed by LC-MS/MS and GC-MS/MS. The ILOQ was settled as the concentration where the signal-to-noise ratio was higher than 10.

2) MLOQ (Method Limit of Quantitation)

MLOQ was calculated by equation below.

$$\text{MLOQ (mg/L)} = \frac{\text{LOQ (ng)} \times \text{Final volume (mL)} \times \text{Dilution factor}}{\text{Injection volume (µL)} \times \text{Initial sample weight (g)}}$$

3) Calibration curve and linearity

Matrix matched standard solution	**MSTD 1 (0.0025 μg/mL)**	**MSTD 2 (0.005 μg/mL)**	**MSTD 3 (0.01 μg/mL)**	**MSTD 4 (0.025 μg/mL)**	**MSTD 5 (0.05 μg/mL)**	**MSTD 6 (0.1 μg/mL)**	**MSTD 7 (0.25 μg/mL)**
Standard solution	0.005 μg/mL 200 µL	0.01 μg/mL 200 µL	0.02 μg/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	200 μL	200 μL

A series of matrix-matched standard solutions with concentration of 0.005, 0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 μg/mL were prepared with a blank extract. The relative standard deviation (RSD) was calculated at the calibration curve.

4) Recovery test of tricyclazole analytical method

Samples were extracted using the modified QuEChERS method. The homogenized free pesticide grain and straw of proso millet samples (10.0 ± 0.1 g) in 50 mL centrifuge tube were treated with the standard solution of tircyclazole at spiking level following: For grain and straw of proso millet, 0.1 mg/kg and 0.5 mg/kg (10 MLOQ, 50 MLOQ). The samples of grain and straw were moistened with 10 mL of deionized water for 10 minutes. 10 mL of acetonitrile with 0.1 % formic acid was added to each tube, and the tubes were shaken vigorously (1600 MiniG™, SPEX® SamplePrep, New Jersey, USA). After that, 4 g of magnesium sulfate ($MgSO_4$) and 1 g of sodium chloride (NaCl) were added to the tubes. The tubes were vigorously shaken for 1 min and centrifuged at 3,500 rpm for 5 minutes (Combi 408, Hanil Science industrial, Korea). One milliliter of the supernatant was transferred into dSPE (Dispersive Solid Phase Extraction) tube containing 25 mg of PSA, 7.5 mg of GCB and 150 mg $MgSO_4$ (RESTEK, Germany). After voltexing for 1 minute, the d-SPE tubes were centrifuged at 13,000 rpm for 4 minutes. For matrix matching, 0.2 mL of supernatant was mixed with 0.2 mL of acetonitrile followed by analyzed using LC-MS/MS.

5) Recovery test of tebuconazole analytical method

Samples were extracted using the modified QuEChERS method. The homogenized free pesticide grain and straw of proso millet samples (10.0 ± 0.1 g) in 50 mL centrifuge tube were treated with the standard solution of tebuconazole at spiking level following: For grain and straw of proso millet, 0.1 mg/kg and 0.5 mg/kg (10 MLOQ, 50 MLOQ). The samples of grain and straw were moistened with 10 mL of deionized water for 10 minutes. 10 mL of acetonitrile with 0.1 % formic acid was added to each tube, and the tubes were shaken vigorously (1600 MiniG™, SPEX® SamplePrep, New Jersey, USA). After that, 4 g of magnesium sulfate ($MgSO_4$) and 1 g of sodium chloride (NaCl) were added to the tubes. The tubes were vigorously shaken for 1 min and centrifuged at 3,500 rpm for 5 minutes (Combi 408, Hanil Science industrial, Korea). One milliliter of the supernatant was transferred into dSPE (Dispersive Solid Phase Extraction) tube containing 25 mg of PSA, 7.5 mg of GCB and 150 mg $MgSO_4$ (RESTEK, Germany). After voltexing for 1 minute, the d-SPE tubes were centrifuged at 13,000 rpm for 4 minutes. For matrix matching, 0.2 mL of supernatant was mixed with 0.2 mL of acetonitrile followed by analyzed using GC-MS/MS.

6) Storage stability test

The homogenized free pesticide samples were fortified with pesticide standard solution at spiking level following: For grain and straw of proso millet, 0.1 mg/kg (10MLOQ). This samples were stored in a freezer (-20 ℃) until analysis.

Grain samples of tricyclazole were stored for 28 days (Oct 2 ~ Oct 30, 2018) and straw samples of tricyclazole were stored for 29 days (Oct 2 ~ Oct 31, 2018).

Grain samples of tebuconazole were stored for 28 days (Oct 2 ~ Nov 06, 2018) and straw samples of tebuconazole were stored for 29 days (Oct 2 ~ Nov 07, 2018).

Matrix effects

Matrix effects (ME, %) was calculated by comparing the slope of calibration curve between solvent standard and matrix matched calibration curve using the following equation:

$$\text{ME}, \% = \left(\frac{\text{slope of matrix matched calibration curve}}{\text{slope of solvent standard calibration curve}} - 1\right) \times 100$$

A positive value refers signal enhancement and a negative value of matrix effect point to signal suppression in matrix contained environment (Caban et al. 2012)

Residual analysis of proso millet sample

The samples were prepared by established method through the recovery test and analyzed using established LC-MS/MS and GC-MS/MS conditions.

Results and discussion

The meteorological data at field

During cultivation of proso millet, temperature range of field was 9.2–39.2 ℃ (Table 18).

Table 18. The meteorological data

	Low temperature (℃)	Highest temperature (℃)	Average temperature (℃)	The amount of precipitation (mm)
5 / 28	12.7	29.4	21	
5 / 29	17.5	26.8	20.8	10.9
5 / 30	14.4	25.3	18.8	3.7
5 / 31	13.4	26.2	19.2	
6 / 1	14.9	29.9	22.1	
6 / 2	14.6	29.5	21.6	
6 / 3	14	30.9	22.4	
6 / 4	16.4	27.3	21.7	
6 / 5	18.6	30.1	23.3	
6 / 6	16.6	27.9	21.7	
6 / 7	16.5	29	20.7	
6 / 8	17.6	26.9	21.1	
6 / 9	17.1	29.5	23.5	0.1
6 / 10	17.2	25.8	21.7	1.4
6 / 11	18.2	23.8	20.8	
6 / 12	17.5	27.8	21	0.3
6 / 13	16.9	27.3	21.7	
6 / 14	18.5	25.9	22.4	3.4
6 / 15	17.7	26.3	22	0.3
6 / 16	16.3	29.1	21.8	
6 / 17	16.4	28.6	21.5	
6 / 18	18.7	30.6	23.8	
6 / 19	17.5	25	21.8	
6 / 20	18.1	28.9	22.3	
6 / 21	18.8	28.8	23	
6 / 22	16.9	32.6	24	
6 / 23	20.1	30.8	23.9	
6 / 24	19.9	33.3	24.8	
6 / 25	20	33.1	26.5	
6 / 26	19.6	26	22.2	95.5
6 / 27	18.8	28	22	0.2
6 / 28	20.7	26.3	23.4	3.1
6 / 29	21.5	29.9	24.5	1
6 / 30	20.7	29.7	24.9	1.7

7 / 1	20.8	23.9	21.9	105.7
7 / 2	20.4	28	23	76.3
7 / 3	22.6	33.5	26.5	17.7
7 / 4	22.6	32.3	26.4	
7 / 5	22.4	30.8	25.7	0.3
7 / 6	22.2	27.5	24.3	
7 / 7	20.4	29.6	24.3	
7 / 8	18.5	28.2	23.3	
7 / 9	20.3	23.6	21.1	11.5
7 / 10	20.5	27.9	24.7	2.9
7 / 11	24.2	30.9	27.2	4.2
7 / 12	25	31.1	27.4	
7 / 13	24	32.4	27.3	0.1
7 / 14	23.9	32.8	27.9	
7 / 15	23.4	34	27.9	
7 / 16	22.8	33.7	27.8	
7 / 17	22.3	32.4	27.2	
7 / 18	22.6	33.4	27.6	
7 / 19	22.1	33.4	27.6	
7 / 20	22.8	34	28.5	
7 / 21	23.1	36.7	29.3	
7 / 22	23.9	37.5	31.1	
7 / 23	28.2	36	31.2	
7 / 24	25.6	36.5	30.6	
7 / 25	27.3	33.9	29.6	
7 / 26	25.9	34.2	29.4	
7 / 27	26.4	35.7	29.8	
7 / 28	25.9	34.9	28.9	4
7 / 29	25.3	35.9	30.9	
7 / 30	26.6	36.3	31.6	
7 / 31	27.8	37.5	31.9	
8 / 1	26.6	39.3	32	
8 / 2	27.8	38.1	32	
8 / 3	28	37.6	32	
8 / 4	27.2	35	30.2	
8 / 5	26.2	34.3	29.7	
8 / 6	27.2	34.8	29.8	1
8 / 7	27.4	36.4	30.5	
8 / 8	26.1	36	30.2	

8 / 9	26.2	34.1	29.4	
8 / 10	26.7	37	31.2	
8 / 11	26.8	35.6	30.6	
8 / 12	25.8	35.6	30.3	2.8
8 / 13	26.2	36.7	31	
8 / 14	27.8	37.1	31.8	
8 / 15	28.6	39.2	32.4	
8 / 16	24.7	35.9	30	
8 / 17	22.3	32.5	26.9	
8 / 18	21.5	32.1	25.8	
8 / 19	22	33.8	27.1	
8 / 20	24.3	32.8	28.3	
8 / 21	23.2	30.1	25.6	5.7
8 / 22	23.1	37.2	30.6	
8 / 23	24.9	33.2	29.5	3.7
8 / 24	22.9	28	24.7	12.2
8 / 25	21	29.6	24.7	
8 / 26	19	25.9	21.7	4
8 / 27	19.3	24.6	22.4	53.1
8 / 28	22.3	25.4	23.5	124.8
8 / 29	22.8	29.6	26.1	8.5
8 / 30	24.4	31.4	26.7	1.9
8 / 31	21.3	30	24.7	0.9
9 / 1	19.9	30.7	25.3	
9 / 2	20.2	29.9	25.1	
9 / 3	22.5	28.5	24.3	17.7
9 / 4	20	29.3	23.8	
9 / 5	18.1	29	23.6	
9 / 6	19.7	28.8	23.1	6.7
9 / 7	17.9	25.4	21.4	1
9 / 8	14	28.2	21.3	
9 / 9	14.2	28.3	21.2	
9 / 10	18.2	28	23	
9 / 11	16.2	25.7	21.4	
9 / 12	16.1	27.6	22.5	
9 / 13	20.4	28	23.5	
9 / 14	20.4	25.1	23.1	0.2
9 / 15	20.1	23.6	21.7	6.9
9 / 16	20.6	25.4	21.9	1.6

9 / 17	17.9	27.8	21.8	
9 / 18	16.4	26.9	21.3	
9 / 19	18.1	26.9	21.4	0.1
9 / 20	17.6	20.3	19	5.7
9 / 21	17.6	22.1	19.2	20
9 / 22	14.7	26.5	20.2	0.5
9 / 23	14.6	25.7	19	1.3
9 / 24	11.9	23.3	17.1	
9 / 25	9.2	24.1	17.3	
9 / 26	16	24.8	18.9	
9 / 27	11.6	24.8	18.5	

LC-MS/MS condition and MRM (Multiple Reaction Monitoring) optimization

Applying tandem MS with selected reaction monitoring (SRM) can significantly reduce the chemical background. Even if the co-extracted matrix compounds have the molecular mass of a pesticide, in general, the two isobaric ions can be separated in SRM experiments, since their fragmentation in the collision cell often results in different product ions. As a result, tandem mass spectrometers offer excellent sensitivity and selectivity (Picó et al. 2004).

LC-MS/MS provides more sensitive, selective and rapid analysis than conventional LC. This analysis used multiple reaction monitoring (MRM) mode. MRM is a tandem mass spectrometric technique that allow the monitoring of specific collision induced dissociation (CID) reactions. As a result, MRM mode significantly improves analysis selectivity. In this mode, the ions migrate to Q1 in an ion source that acts as a filter depending on their mass-to-charge (*m/z*) ratio, where the precursor ion is selected. And then, a specific product ion is selected and monitored in Q3 (Despina Tsipi. 2015). Full scan spectrum of tricyclazole was obtained in the mass range of 50-500 *m/z*. Figure 19 shows a spectrum of tricyclazole and 2 ion mass representing tricyclazole that is *m/z* 190.0 and 192.0.

Precursor ion of tricyclazole was set to 190 *m/z*. Through optimization of precursor ions, quantifier ion and qualifier ion of tricyclazole was set to 136 *m/z* (-28 eV) and 163.15 (-20 eV) respectively. Table 19 shows MRM transition parameters of tricyclazole in LC-MS/MS.

Figure 19. Scan spectrum of tricyclazole

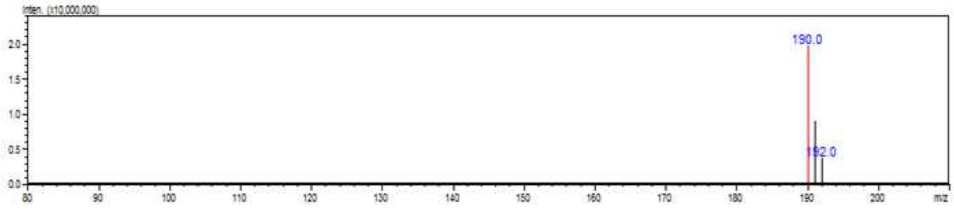

Inten. (x10,000,000)
2.0
1.5
1.0
0.5
0.0
190.0
192.0
80
90
100
110
120
130
140
150
160
170
180
190
200
m/z

Table 19. The MRM transition parameters of tricyclazole

	Instrument	Monoisotopic Mass	Ionization	Precursor ion>Product ion (m/z)		Collision energy (eV)		Retention time (min)
				Quantitation	Qualification			
Tricyclazole	LC-MS/MS	189.0361	$[M+H]^+$	190.00>136.00	190.00>163.15	-28	-20	5.00

ILOD, ILOQ, MLOQ and calibration curve of tricyclazole

Instrumental limit of detection (ILOD) and Instrumental limit of quantitation (ILOQ) are values that represent the sensitivity of analytical instrument. ILOD is determined by the minimum concentration of analyte providing S/N ratio of > 3 and ILOQ is determined by S/N ratio of > 10 (Fig. 20) (Fong et al. 1999). In this experiment, ILOQ was checked from the results of analysis of several concentration standard solutions, 0.0025 mg/L was observed as practicable ILOQ in LC-MS/MS.

Based on MLOQ calculating equation, MLOQ of tricyclazole in proso millet (grain and straw) was 0.01 mg/L.

Matrix matched standard curves of tricyclazole has a good linearity in samples of proso millet. The range was between 0.0025 to 0.25 mg/kg of tricyclazole standard solution (Fig. 21).

The regression equations were y = 1634.8x + 27018 (grain) and y = 2011.6x + 16559 (straw) respectively. Coefficient of determination (r^2) were over 0.999 in each samples.

Figure 20. Concepts of ILOD&ILOQ

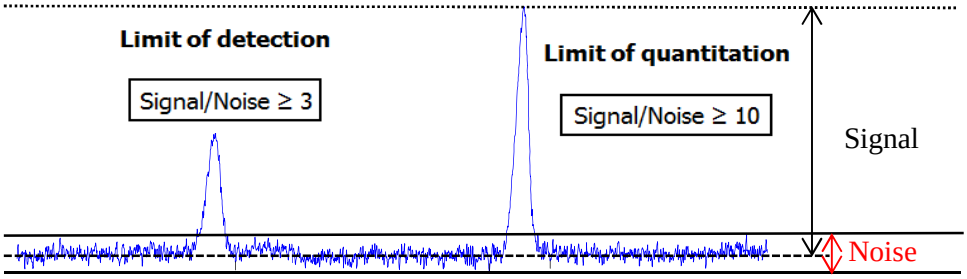
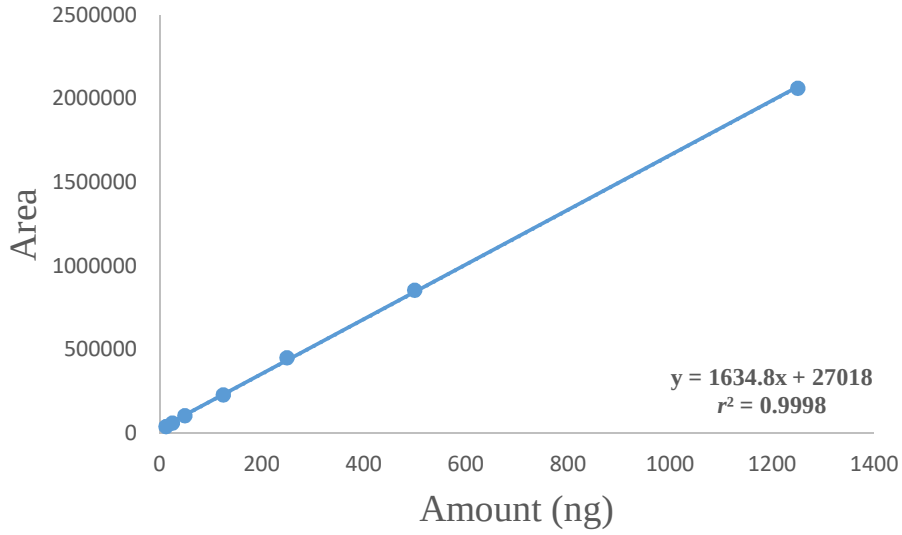

Limit of detection
Signal/Noise ≥ 3
Limit of quantitation
Signal/Noise ≥ 10
Signal
Noise

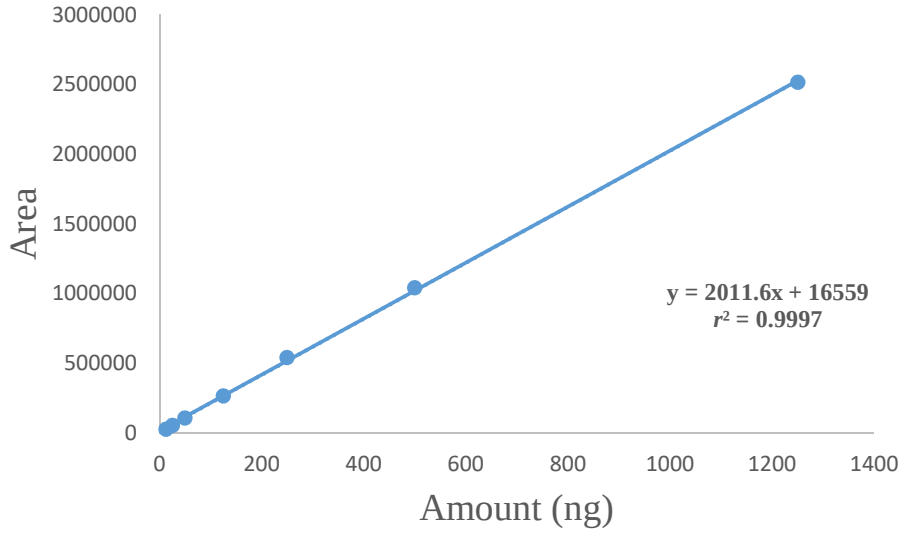
Figure 21. Matrix matched calibration curves of tricyclazole

(A) Calibration curve – grain (Range: 0.0025 – 0.25 mg/kg), (B) Calibration curve – straw (Range: 0.0025 – 0.25 mg/kg)

(A)


2500000
2000000
1500000
1000000
500000
0
Area
0
200
400
600
800
1000
1200
1400
Amount (ng)
y = 1634.8x + 27018
r² = 0.9998

(B)


3000000
2500000
2000000
1500000
1000000
500000
0
Area
0
200
400
600
800
1000
1200
1400
Amount (ng)
y = 2011.6x + 16559
r² = 0.9997

Matrix effects

In the electrospray ionization process in LC-MS/MS, simultaneous elution interference interacting with the target compound leads to a matrix effect resulting in signal suppression compared to the signal of the analyte injected only in the solvent (Lozano et al. 2016). Enhancement or suppression of analyte responses is accompanied by a reduced accuracy and precision of subsequent measurements. So, the matrix effect limits the utility of LC-MS/MS (Dams et al. 2003).

Some interference compounds were removed during the extraction. However, complex matrices can cause interference, which can lead to analytical errors, resulting in inaccurate results (Rubert et al. 2011).

The matrix effects were calculated by comparing the slope of calibration curve between the solvent standard and the matrix matched standard. According to the equation mentioned in the method section, a positive value indicates signal enhancement, whereas a negative value indicates signal suppression.

In case of grain, matrix effect was -61.6 % and straw; -52.7 %. According to SANTE guideline's acceptability criteria (20%), matrix matched is necessarily needed in analysis of tricyclazole. All matrix induced suppression effect.

Recoveries of tricyclazole in grain and straw of proso millet

Recovery test provides accuracy and precision of sample preparation method by recovered rate (accuracy, %) and RSD (precision, %) (Fong et al. 1999). Untreated samples were spiked with 10 MLOQ and 50 MLOQ levels of tricyclazole in proso millet (grain and straw). And the analysis was performed using the established method. Table 20 shows results of recovery test in grain and straw. In case of grain, the range of recoveries were 79.5 ~ 84.2 % at 10 MLOQ and 91.5 ~ 93.8 % at 50 MLOQ level, and RSD was 3.1 and 1.3, respectively. In case of straw, the range of recoveries were 86.1 ~ 88.5 % at 10 MLOQ and 80.4 ~ 82.5 % at 50 MLOQ level, and RSD was 1.5 and 1.4, respectively (Fig. 14).

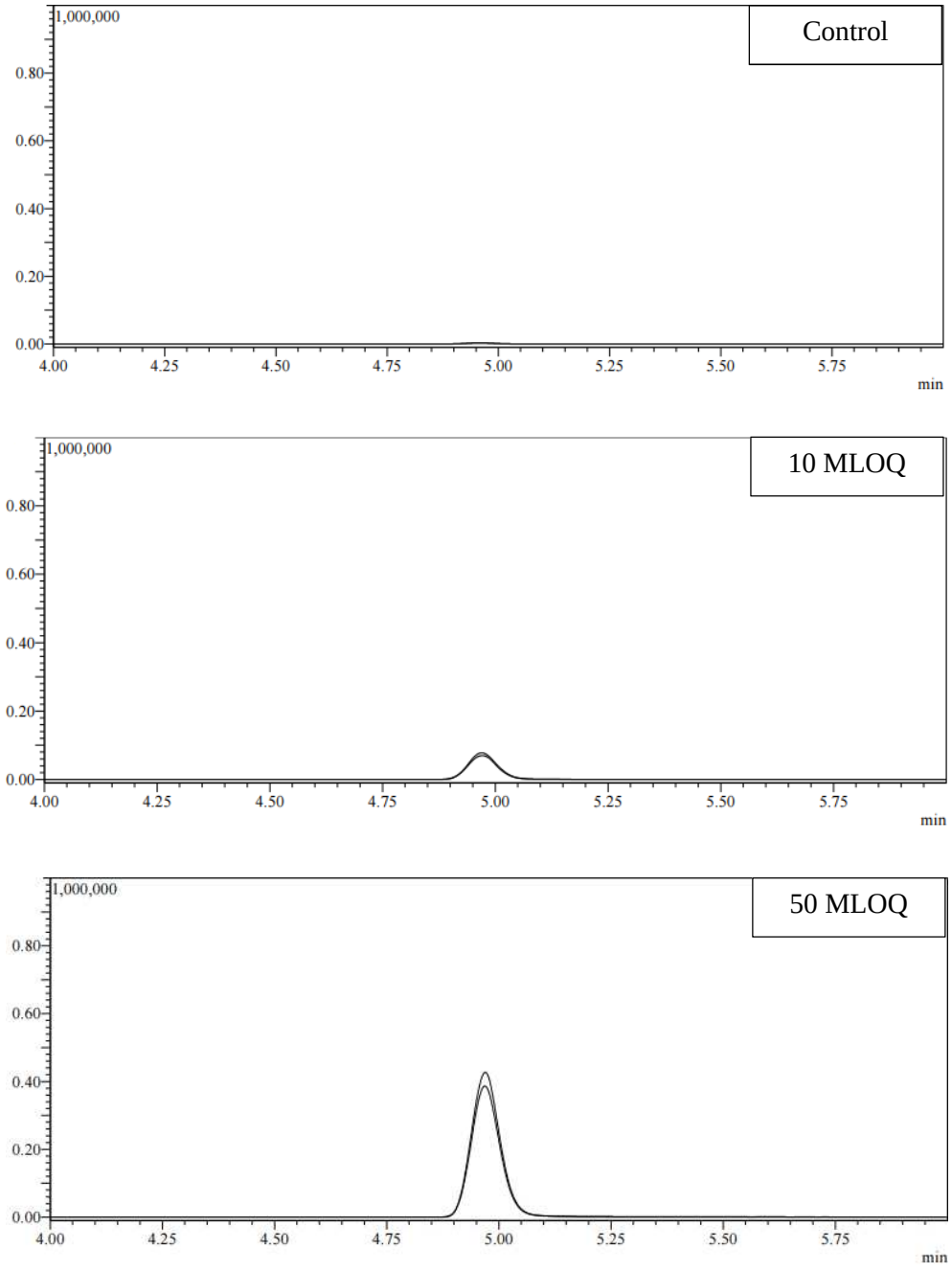
Table 20. Recoveries test of tricyclazole in grain and straw of proso millet

	Sample	Fortified level (mg/kg)	Recovery (%)	RSD (%)
Proso millet	Grain	0.1	82.5	3.1
		0.5	92.5	1.3
	Straw	0.1	87.7	1.5
		0.5	81.6	1.4

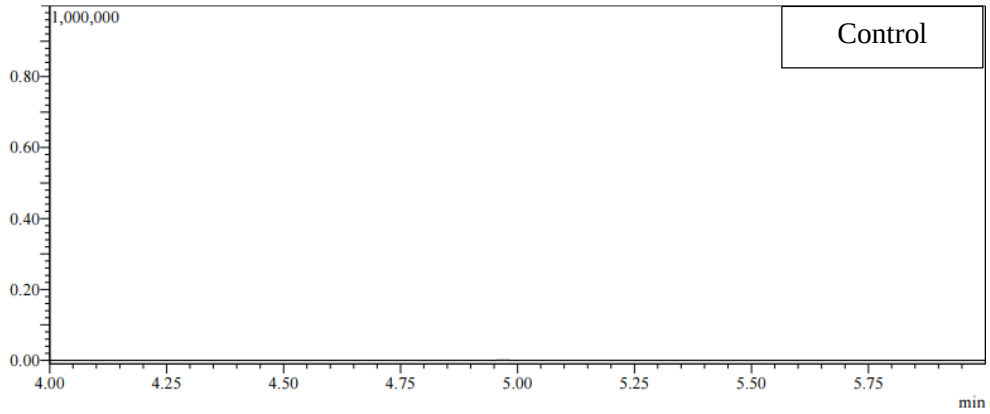
Figure 22. Chromatogram of recovery test of tricyclazole in grain and straw of proso millet

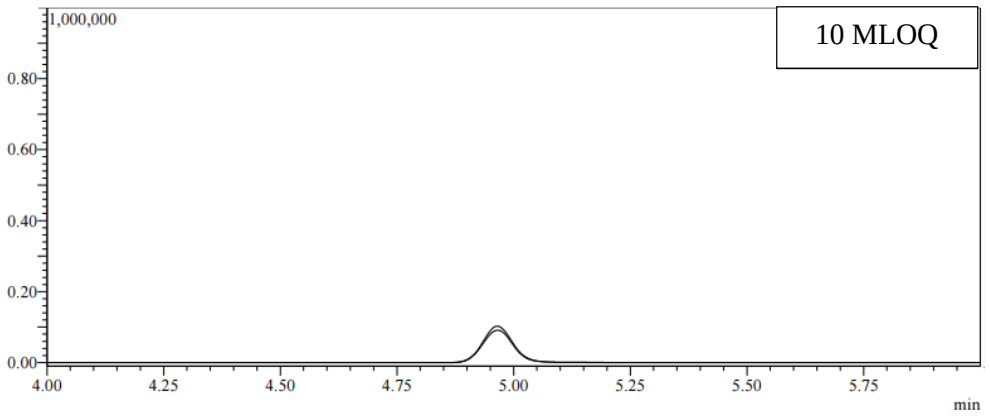
(A) Grain of proso millet (B) Straw of proso millet

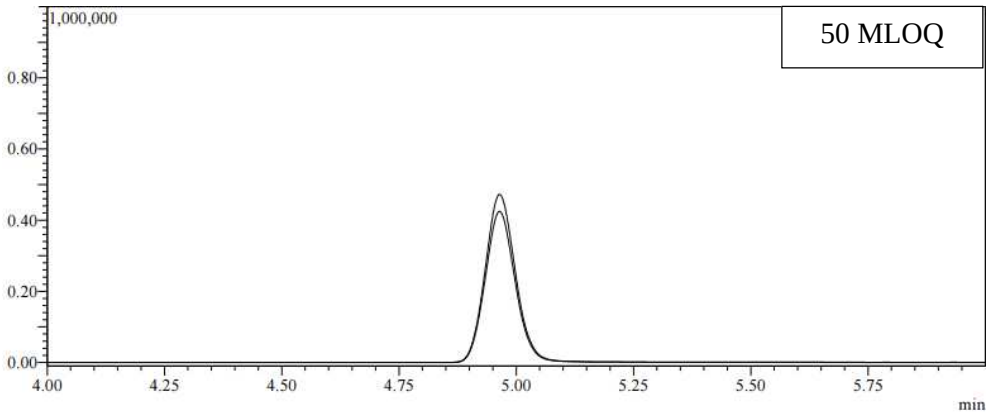
(A) Grain


Control
1,000,000
0.80
0.60
0.40
0.20
0.00
4.00
4.25
4.50
4.75
5.00
5.25
5.50
5.75
min
10 MLOQ
1,000,000
0.80
0.60
0.40
0.20
0.00
4.00
4.25
4.50
4.75
5.00
5.25
5.50
5.75
min
50 MLOQ
1,000,000
0.80
0.60
0.40
0.20
0.00
4.00
4.25
4.50
4.75
5.00
5.25
5.50
5.75
min

(B) Straw


1,000,000
0.80
0.60
0.40
0.20
0.00
4.00
4.25
4.50
4.75
5.00
5.25
5.50
5.75
min
Control


1,000,000
0.80
0.60
0.40
0.20
0.00
4.00
4.25
4.50
4.75
5.00
5.25
5.50
5.75
min
10 MLOQ


1,000,000
0.80
0.60
0.40
0.20
0.00
4.00
4.25
4.50
4.75
5.00
5.25
5.50
5.75
min
50 MLOQ

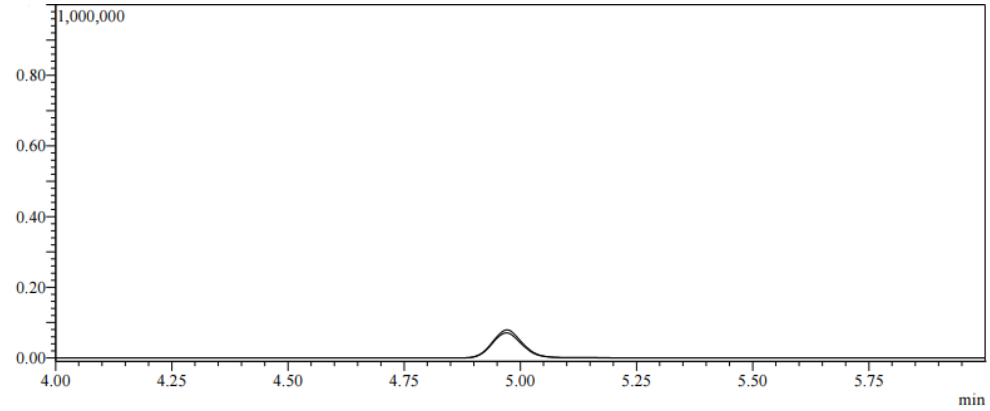
Storage stability test of tricyclazole

Storage stability tests demonstrate that the target compound is not degraded while the sample is stored. In pesticide residual analysis, it is generally difficult to perform sample preparation immediately after sampling (Fu et al. 2016). Therefore, the sample should be stored in the laboratory freezer. Samples usually are deep frozen, but the question arises whether residues are degraded during storage. In this experiment, the fortified samples of grain and straw of proso millet were analyzed using the optimized method. The results showed that recovery of grain samples ranged from 77.0 to 79.5 % and RSD was 1.6 %. In case of straw samples, recovery results ranged from 81.9 to 84.4 % and RSD was 1.7 % (Fig. 23). These accuracy and precision tests indicated that the target compounds were not degraded during the storage period (Table 21).

Figure 23. Representative chromatograms of storage stability test tricyclazole

(A) Grain of proso millet (B) Straw of proso millet

(A)


1,000,000
0.80
0.60
0.40
0.20
0.00
4.00
4.25
4.50
4.75
5.00
5.25
5.50
5.75
min

(B)

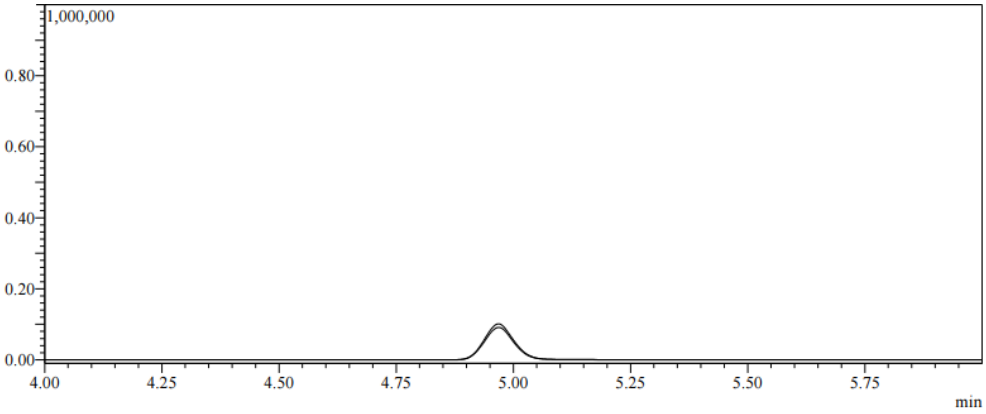

1,000,000
0.80
0.60
0.40
0.20
0.00
4.00
4.25
4.50
4.75
5.00
5.25
5.50
5.75
min

Table 21. Storage stability of tricyclazole in grain and straw of proso millet

	Sample	Fortified level (mg/kg)	Recovery (%)	RSD (%)
Proso millet	Grain	0.1	78.3	1.6
	Straw	0.1	83.5	1.7

Residual characteristics of tricyclazole in grain and straw of proso millet

No residue was detected in control sample and the results of tricyclazole residue in field trials were presented in Table 22. The residue in plot 1 (50/40/30 days before harvest) was 1.69 and 0.62 mg/kg in grain and straw, respectively. In plot 2 (40/30/21 days before harvest) was 3.08 and 0.68 mg/kg in grain and straw, respectively. In plot 3 (30/21/14 days before harvest) was 4.08 and 0.87 mg/kg in grain and straw, respectively. In plot 4 (21/14/7 days before harvest) was 5.68 and 1.13 mg/kg in grain and straw, respectively.

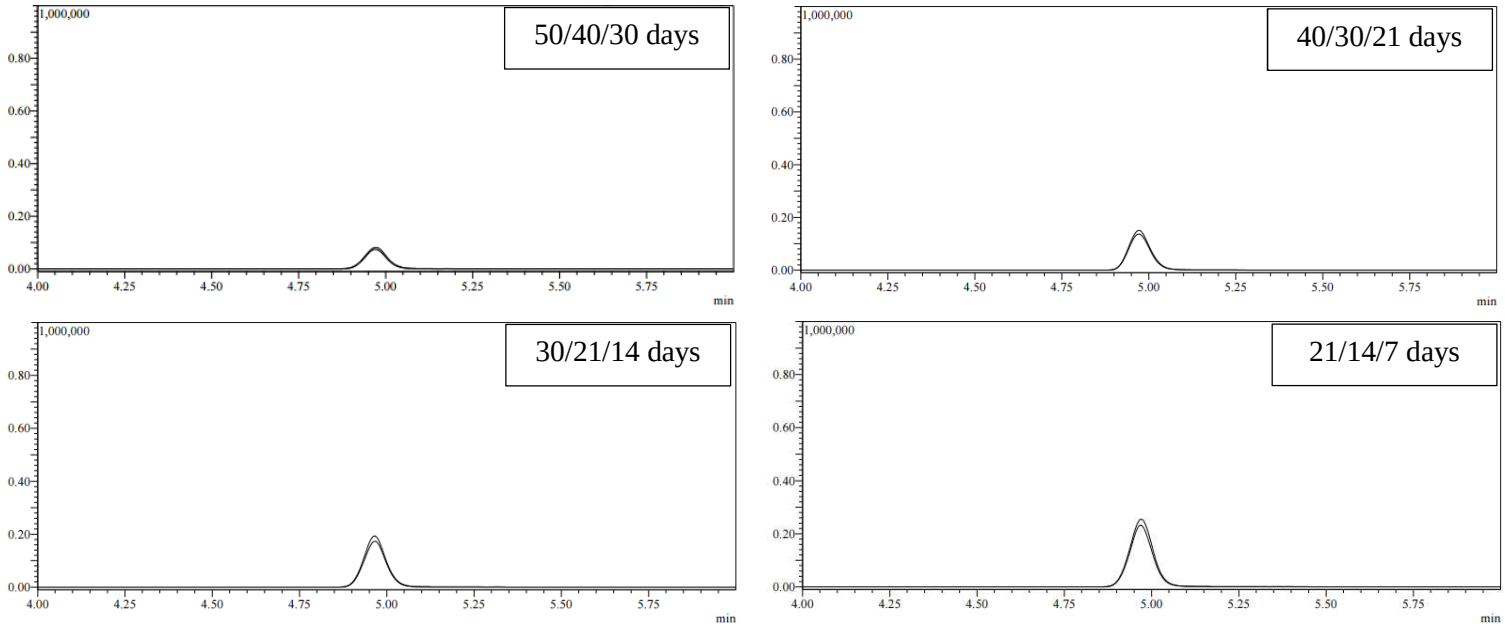
Table 22. Maximum residue of tricyclazole in grain and straw of proso millet

	Sample	Plot (before harvest)	Residual maximum amount of tricyclazole (mg/kg)
Proso millet	Grain	1 (50/40/30)	1.69
		2 (40/30/21)	3.08
		3 (30/21/14)	4.08
		4 (21/14/7)	5.68
	Straw	1 (50/40/30)	0.62
		2 (40/30/21)	0.68
		3 (30/21/14)	0.87
		4 (21/14/7)	1.13

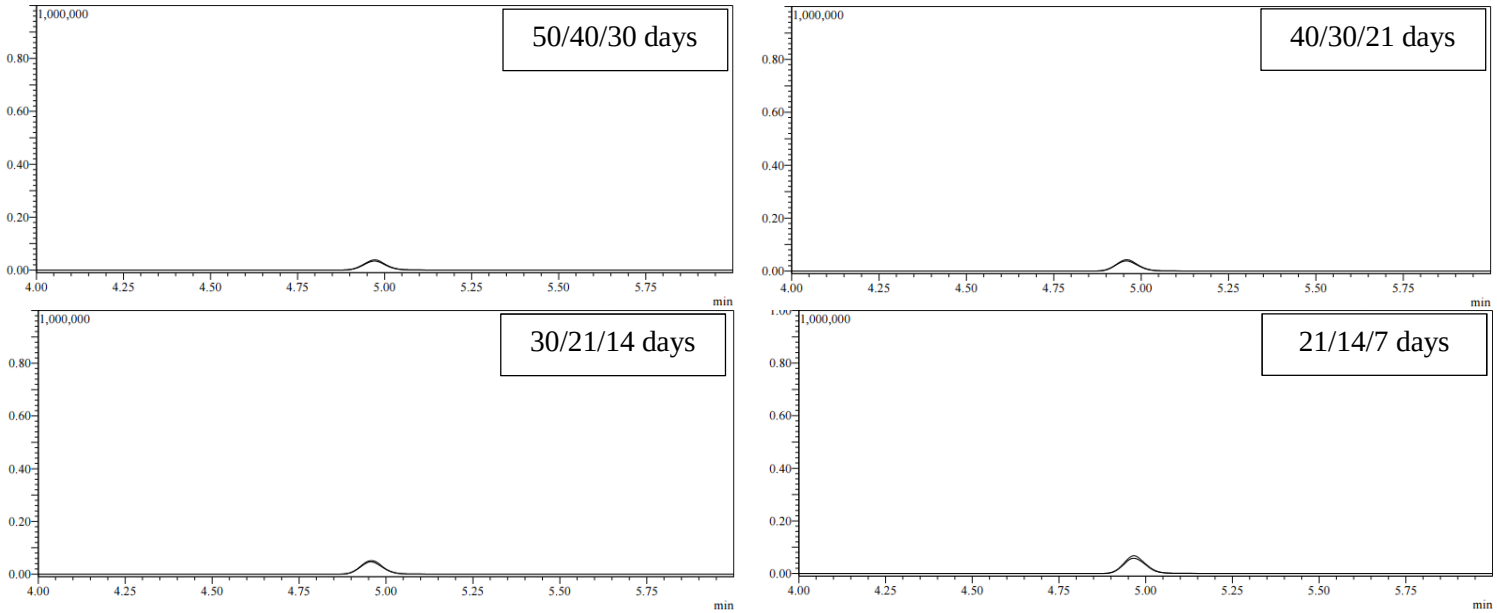
Figure 24. Chromatogram of residue analysis tricyclazole in grain and straw of proso millet

(A) Grain of proso millet, (B) straw of proso millet

(A) Representative chromatogram of samples (Grain)



(B) Representative chromatogram of samples (Straw)



GC-MS/MS condition and MRM (Multiple Reaction Monitoring) optimization

GC-MS/MS provides more sensitive, selective and faster analysis compared with the conventional GC. This analysis used multiple reaction monitoring (MRM) mode. MRM is a tandem mass spectrometric technique that allows the monitoring of specific collision induced dissociation (CID) reactions. As a result, MRM mode significantly improves in analytical selectivity. In this mode, the ions go from the ion source to Q1 operated as filter according to their mass-to-charge (*m/z*) ratio, where the precursor ion is selected. And then, a specific product ion is selected and monitored in Q3 (Despina Tsipi). Full scan spectrum of tebuconazole were obtained in the mass range of 50-500 *m/z*. Figure 25 shows a spectrum of tebuconazole and 3 ion mass representing tebuconazole that is *m/z* 83,125 and 250.

Quantifier ion and qualifier ion of tebuconazole was 125.0 m/z (-24 eV) and 99.1 (-18 eV) respectively. Table 23 shows MRM transition parameters of tebuconazole in GC-MS/MS.

Figure 25. Scan spectrum of tebuconazole

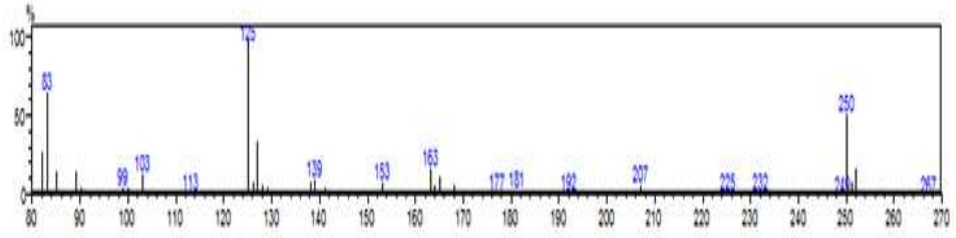

%
100
50
0
83
125
250
99
103
113
139
153
163
177
181
192
207
225
232
240
267
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230
240
250
260
270

Table 23. The MRM transition parameters of tebuconazole

	Instrument	Monoisotopic Mass	Ionization	Precursor ion>Product ion (m/z)		Collision energy (eV)		Retention time (min)
				Quantitation	Qualification			
Tebuconazole	GC-MS/MS	307.1451	$[M+H]^+$	250.0>125.00	125.00>99.1	-24	-18	11.8

ILOD, ILOQ, MLOQ and calibration curve of tebuconazole

In this experiment, ILOQ was checked from the results of analysis of several concentration standard solutions, 0.0025 mg/L was observed as practicable ILOQ in GC-MS/MS.

Based on MLOQ calculating equation, MLOQ of tebuconazole in proso millet (grain and straw) was 0.01 mg/L.

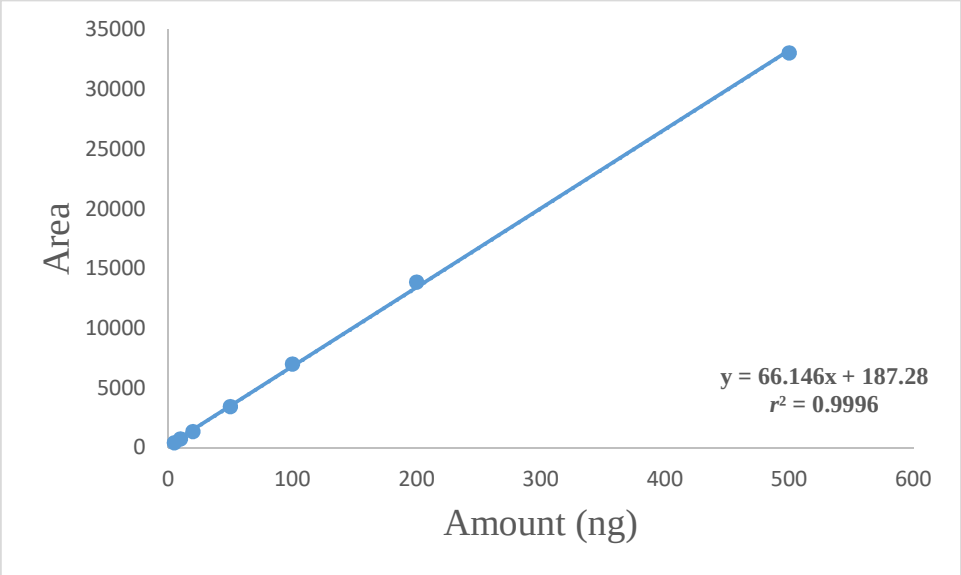
Matrix matched standard curves of tricyclazole has a good linearity in samples of proso millet. The range was between 0.0025 to 0.25 mg/kg of tebuconazole standard solution (Fig. 26).

The regression equations were y = 66.146x + 187.28 (grain) and y = 65.098x – 124.11 (straw) respectively. Coefficient of determination (r^2) were over 0.999 in each samples.

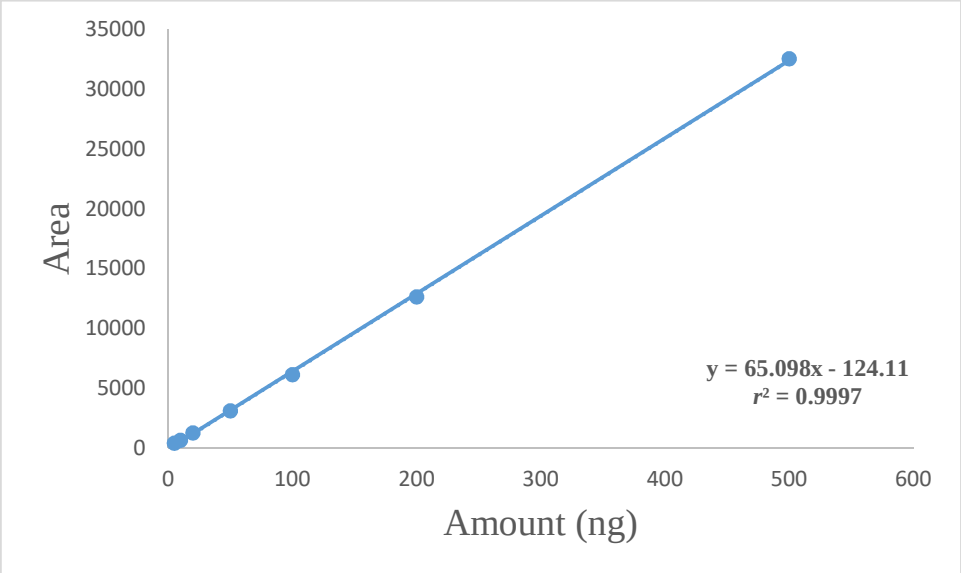
Figure 26. Matrix matched calibration curves of tebuconazole

(A) Calibration curve – grain (Range: 0.0025 – 0.25 mg/kg), (B) Calibration curve – straw (Range: 0.0025 – 0.25 mg/kg)

(A)


35000
30000
25000
20000
15000
10000
5000
0
Area
0
100
200
300
400
500
600
Amount (ng)
y = 66.146x + 187.28
r² = 0.9996

(B)


35000
30000
25000
20000
15000
10000
5000
0
Area
0
100
200
300
400
500
600
Amount (ng)
y = 65.098x - 124.11
r² = 0.9997

Matrix effects

To ensure the accuracy and reliability of experimental results, it is essential to fully extract, efficiently eliminate and compensate for matrix interferences. Matrix effect (ME) can come from the sample matrix, sample preparation procedure, chromatographic separation quality, and ionization type. All impurities cannot be completely removed, matrix effects still exist and become one of the main factors affecting accurate quantification and repeatability in GC–MS/MS detection (Dong et al. 2014).

In case of grain, matrix effect was 30.9 % and straw; 28.9 %. According to SANTE guideline's acceptability criteria (20%), matrix matched is necessarily needed in analysis of tebuconazole. All matrix induced enhancement effect.

Recoveries of tebuconazole in grain and straw of proso millet

Untreated samples were spiked with 10 MLOQ and 50 MLOQ levels of tebuconazole in proso millet (grain and straw). And the analysis was performed using the established method. Table 24 shows results of recovery test in grain and straw. In case of grain, the range of recoveries were 95.6 ~ 98.6 % at 10 MLOQ and 92.6 ~ 97.6 % at 50 MLOQ level, and RSD was 1.6 % and 2.7 %, respectively. In case of straw, the range of recoveries were 104.8 ~ 107.6 % at 10 MLOQ and 96.7 ~ 103.6 % at 50 MLOQ level, and RSD was 1.3 % and 3.4 %, respectively (Fig. 27).

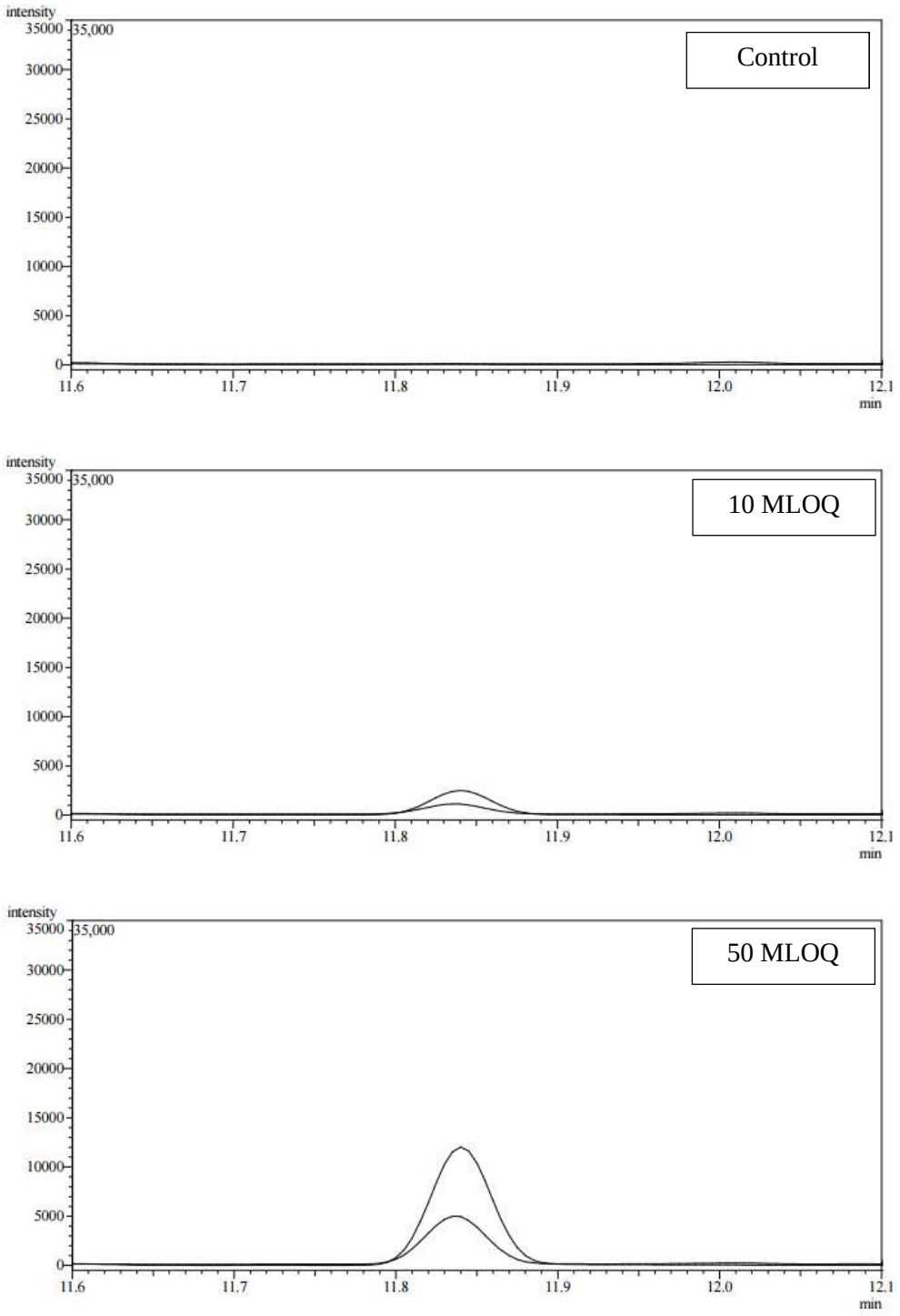
Table 24. Recoveries test of tebuconazole in grain and straw of proso millet

	Sample	Fortified level (mg/kg)	Recovery (%)	RSD (%)
Proso millet	Grain	0.1	96.9	1.6
		0.5	95.4	2.7
	Straw	0.1	106.1	1.3
		0.5	100.2	3.4

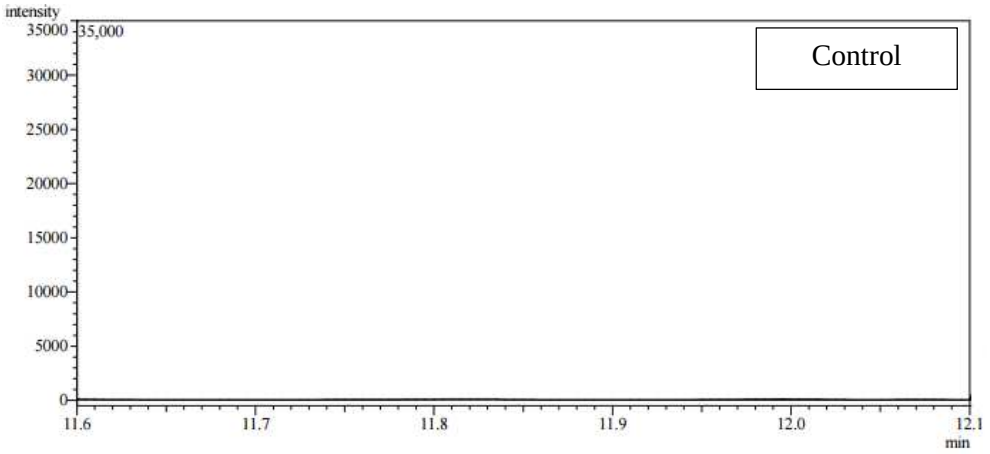
Figure 27. Chromatogram of recovery test of tebuconazole in grain and straw of proso millet

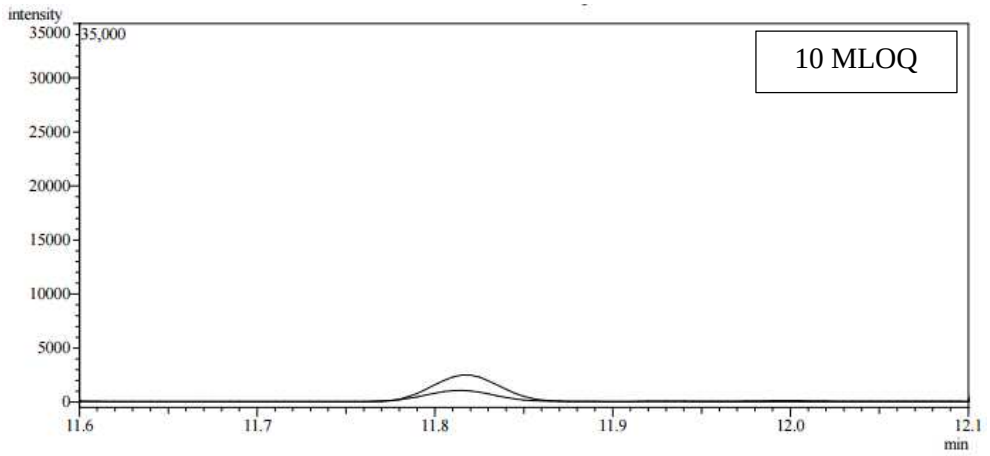
(A) Grain of proso millet (B) Straw of proso millet

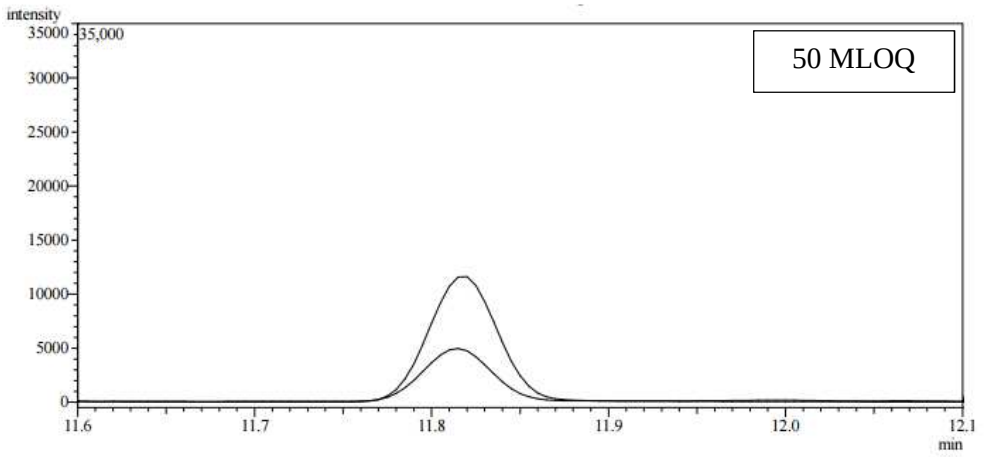
(A) Grain


intensity
35,000
Control
10 MLOQ
50 MLOQ
35000
30000
25000
20000
15000
10000
5000
0
11.6
11.7
11.8
11.9
12.0
12.1
min

(B) Straw


intensity
35000
35,000
30000
25000
20000
15000
10000
5000
0
11.6
11.7
11.8
11.9
12.0
12.1
min
Control


intensity
35000
35,000
30000
25000
20000
15000
10000
5000
0
11.6
11.7
11.8
11.9
12.0
12.1
min
10 MLOQ


intensity
35000
35,000
30000
25000
20000
15000
10000
5000
0
11.6
11.7
11.8
11.9
12.0
12.1
min
50 MLOQ

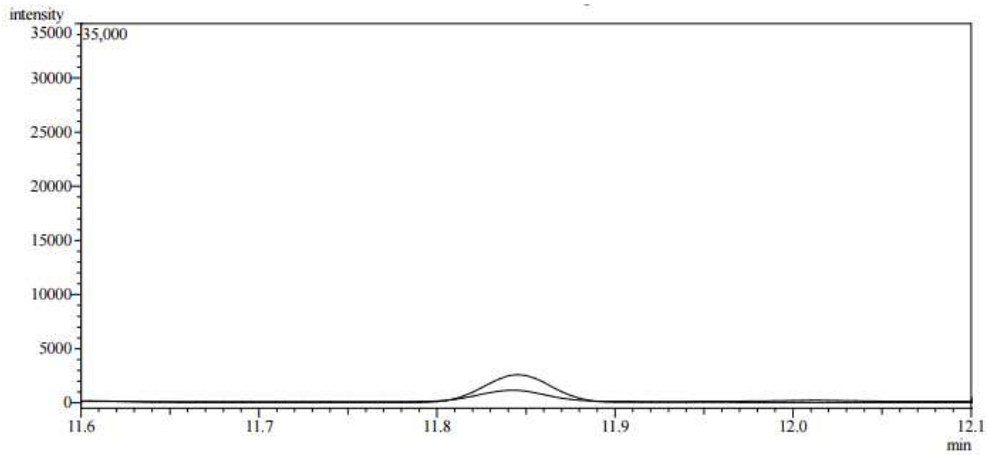
Storage stability test of tebuconazole

Samples of grain and straw should be stored in the laboratory freezer. Samples are usually deep frozen, but the question is whether the residues are degraded during storage. In this experiment, the fortified samples of grain and straw of proso millet were analyzed using the optimized method. The results showed that recovery of grain samples ranged from 95.0 to 100.0 % and RSD was 2.6 %. In case of straw samples, recovery results ranged from 106.1 to 107.8 % and RSD was 0.8 % (Fig. 28). These accuracy and precision tests indicated that the target compounds were not degraded during the storage period (Table 25).

Figure 28. Representative chromatograms of storage stability test tebuconazole

(A) Grain of proso millet (B) Straw of proso millet

(A)


intensity
35000
35,000
30000
25000
20000
15000
10000
5000
0
11.6
11.7
11.8
11.9
12.0
12.1
min

(B)

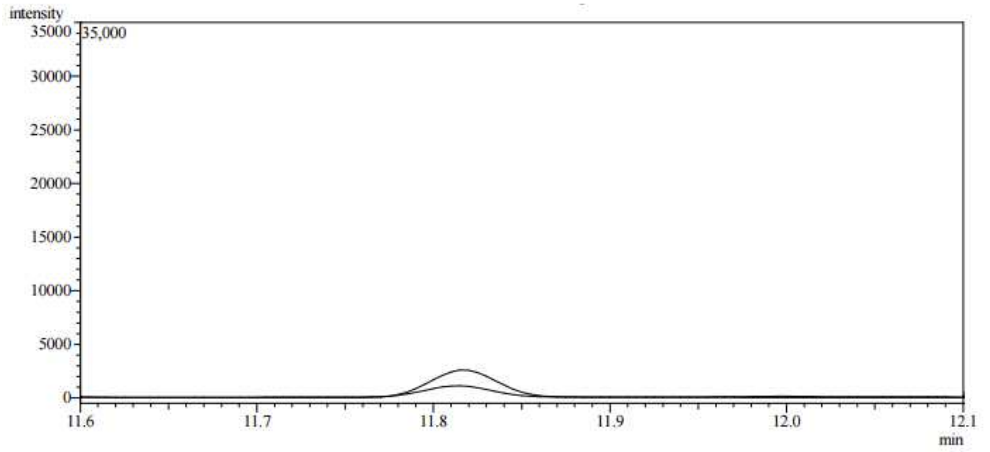

intensity
35000
35,000
30000
25000
20000
15000
10000
5000
0
11.6
11.7
11.8
11.9
12.0
12.1
min

Table 25. Storage stability of tebuconazole in grain and straw of proso millet

	Sample	Fortified level (mg/kg)	Recovery (%)	RSD (%)
Proso millet	**Grain**	0.1	97.3	2.6
	Straw	0.1	106.8	0.8

Residual characteristics of tebuconazole in grain and straw of proso millet

No residue was detected in control sample and the results of tebuconazole residue in field trials were presented in Table 26. The residue in plot 1 (50/40/30 days before harvest) was 0.39 and 0.53 mg/kg in grain and straw, respectively. In plot 2 (40/30/21 days before harvest) was 0.75 and 0.76 mg/kg in grain and straw, respectively. In plot 3 (30/21/14 days before harvest) was 1.19 and 0.89 mg/kg in grain and straw, respectively. In plot 4 (21/14/7 days before harvest) was 1.66 and 1.20 mg/kg in grain and straw, respectively.

This indicates that due to the protection role of proso millet of grain and straw against pesticide, contamination of residues does not likely to occur in proso millet

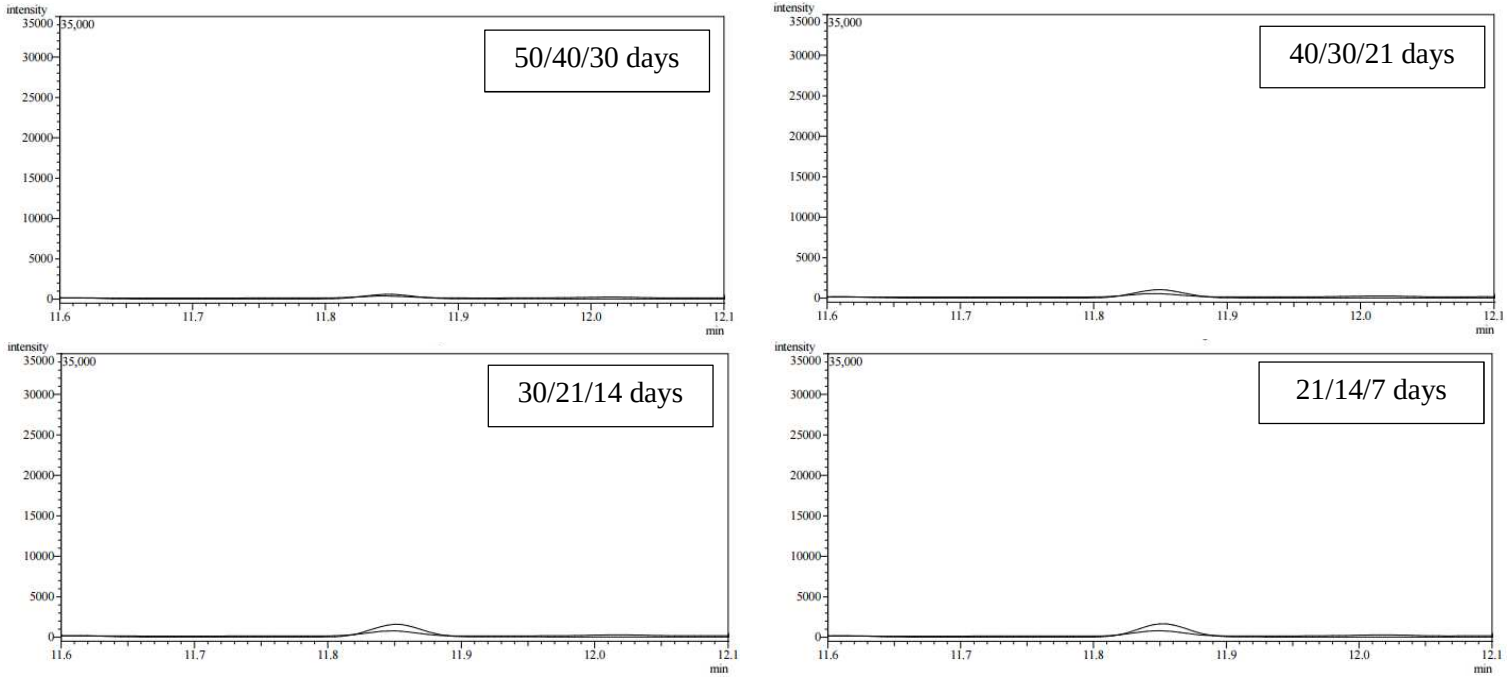
Table 26. Maximum residue of tebuconazole in grain and straw of proso millet

	Sample	Plot (before harvest)	Residual maximum amount of tebuconazole (mg/kg)
Proso millet	**Grain**	1 (50/40/30)	0.39
		2 (40/30/21)	0.75
		3 (30/21/14)	1.19
		4 (21/14/7)	1.66
	Straw	1 (50/40/30)	0.53
		2 (40/30/21)	0.76
		3 (30/21/14)	0.89
		4 (21/14/7)	1.20

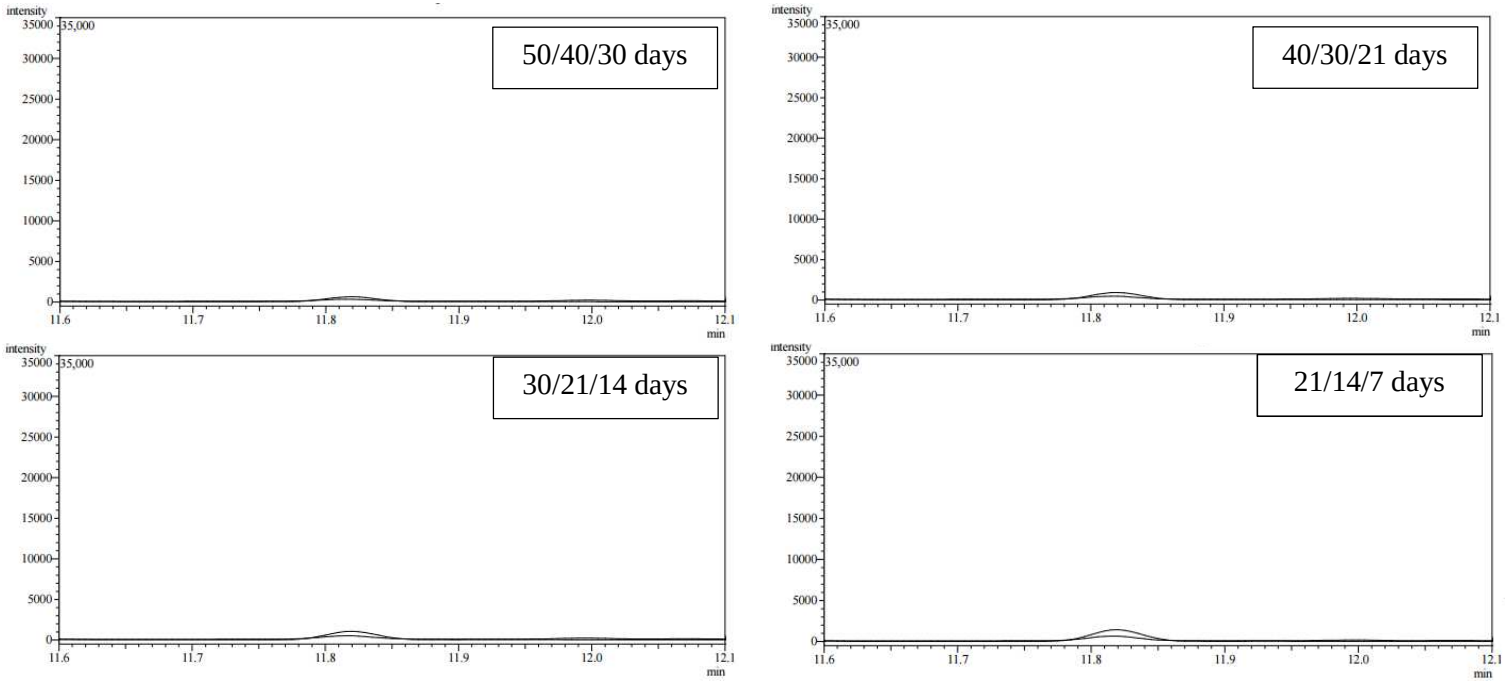
Figure 29. Chromatogram of residue analysis tebuconazole in grain and straw of proso millet

(A) Grain of proso millet, (B) straw of proso millet

(A) Representative chromatogram of samples (Grain)



(B) Representative chromatogram of samples (Straw)



Conclusion

In conclusion of Part 1, this study established a method that utilizes GC-NPD and purifies twice using an SPE cartridge in herbal medicines. The recoveries of *Atractylodes* rhizome white and *Rehmannia* root were satisfied with 70 ~ 120%. In case of RSD, 20 or less were satisfied. Coefficients of determination (r^2) were over 0.999 in each samples. So, this method can actually be used. In order to improve the method using GC-NPD, the method using LC-MS/MS was established by introducing QuEChERS method. The recoveries of *Atractylodes* rhizome white and *Rehmannia* root were satisfied with 70 ~ 120%. In case of RSD, 20 or less were satisfied. Coefficients of determination (r^2) were over 0.999 in each samples. Thus, this method can be used in practice. This method applied more easily and simply with time savings than those of GC-NPD.

As a conclusion of Part 2, it could be applied as a useful data for establishing PHIs of tricyclazole and tebuconazole during cultivation of proso millet. The results of analysis residual amount of tricyclazole and tebuconazole, all grains and straws were above the MLOQ level of 0.01 mg/kg. First of all, in the case of tricyclazole, the residue in plot 1 (50/40/30 days before harvest) was 1.69 and 0.62 mg/kg in grain and straw, respectively. In plot 2 (40/30/21 days before harvest) was 3.08 and 0.68 mg/kg in grain and straw, respectively. In plot 3 (30/21/14 days before harvest) was 4.08 and 0.87 mg/kg in grain and straw, respectively. In plot 4 (21/14/7 days before harvest) was 5.68 and 1.13 mg/kg in grain and straw, respectively. In case of tebuconazole, the residue in plot 1 (50/40/30 days before harvest) was 0.39 and 0.53 mg/kg in grain and straw, respectively. In plot 2 (40/30/21 days before harvest) was 0.75 and 0.76 mg/kg in grain and straw, respectively. In plot 3 (30/21/14 days before harvest) was 1.19 and 0.89 mg/kg in grain and straw, respectively. In plot 4 (21/14/7 days before harvest) was 1.66 and 1.20 mg/kg in grain and straw, respectively.

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Abstract in Korean

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고 락 도

Fosthiazate 는 유기인계 살충제이고 *Atractylodes* rhizome white 및 *Rehmannia* root 는 대한민국약전에 등재 된 약초이다. 이 연구는 GC-NPD 를 사용하여 해당 생약에서 fosthiazate 분석법을 개발하였다. SPE 카트리지를 사용한 정제가 두 번 수행되었으며 GC-NPD 를 사용하는 이 분석법은 추출, 분배, 정제 모두 복잡했다. 이 분석법을 개선하기 위해 LC-MS/MS 를 사용하는 QuEChERS 법으로 분석법을 확립하여 불필요한 준비 절차를 개선하였다. 회수율 시험을 위해, 5 g 의 *Atractylodes* rhizome white 또는 *Rehmannia* root 는 MLOQ 및 10MLOQ 수준에서 표준 용액으로 처리하였고. 2 개의 처리수준 (MLOQ 및 10MLOQ)에서 회수율은 70-120 % (RSD≤20 %)였다.

기장은 밀과 보리 외에 인간에서 가장 오래 사용되는 여름 곡물이다. Tricyclazole 과 tebuconazole 은 기장을 포함한 다양한 작물에 사용되는 살균제이다. 이 연구에서 정량한계(MLOQ)는 0.01 mg/kg 이었다. 회수율 시험을 위해, 10g 의 알곡 또는 짚의 기장을 10MLOQ 및 50MLOQ 수준의 표준 용액으로 처리 하였다. 알곡 및 짚에 대한 2 개의 처리수준 (10MLOQ 및 50MLOQ)에서 회수율은 70-120 % (RSD≤20 %) 범위였다. 살균제 tricyclazole 20 % 액상수화제를 3 회 반복하여 각각의 4 개의 구역 (50/40/30 일, 40/30/21 일, 30/21/14 일 및 21/14/7 일)에서 3 회 반복 처리 하였다. 수확 후, 알곡 및 짚을 분석하여 tricyclazole 의 잔류 수준이 처리 시간에 따라 감소 함을 보여 주었다. 수확 전

21/14/7 일의 처리구역에서 알곡 및 짚에 대한 최대 잔류 물 수준은 5.68 mg/kg 및 1.13 mg/kg 이었다. 살균제 tebuconazole 20 % 액상수화제를 각각의 4 개의 구역 (수거 전 50/40/30 일, 40/30/21 일, 30/21/14 일 및 21/14/7 일)으로 처리하였다. 수확 후, 알곡 및 짚을 분석하여 처리 시간에 따라 tebuconazole 의 잔류 수준이 감소 함을 보여 주었다. 수확 전 21/14/7 일의 처리구역에서 알곡 및 짚에 대한 최대 잔류 물 수준은 1.66 mg/kg 및 1.20 mg/kg 이었다.

주요어: Herbal medicine, GC-NPD, SPE, QuEChERS, Fosthiazate, LC-MS/MS, GC-MS/MS, Minor crop, Proso millet, Residue

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