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Study for Residue Analysis of Pinoxaden in Agricultural Commodities

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Abstract

BACKGROUND: Pinoxaden is the phenylpyrazoline herbicide developed by Syngenta Crop Protection, Inc. and marketed on 2006. The maximum residue levels for wheat and barley were set by import tolerance. Thus, Ministry of Food and Drug Safety (MFDS) official analytical method determining Pinoxaden residue was necessary in various food matrixes. Satisfaction of international guideline of CODEX (Codex Alimentarius Commission CAC/GL 40) and National Institute of Food and Drug Safety Evaluation-MFDS (2017) are additional pre-requirements for analytical method. In this study, liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was investigated to analyze residue of Pinoxaden (M4), which is defined as pesticide residue in Korea, in foods.

METHODS AND RESULTS: Pinoxaden (M4) was extracted followed by acid digestion (2hr reflux with 1N HCl) and pH adjusting (pH 4-5 with 3% ammonium solution). To remove oil, additional clean-up step with hexane saturated with acetonitrile was required to high oil contained sample before purification. HLB cartridge and nylon syringe filter were used for purification. Then, samples were analyzed by LC-MS/MS using reserve phase column C_{18}. Five agricultural group representative commodities (mandarin, potato, soybean, hulled rice, and red pepper) were used to verify the method in this study. The liner matrix-matched calibration curves were confirmed with coefficient of determination (r^2) > 0.99 at calibration range 0.002-0.2 mg/kg. The limits of detection and quantitation were 0.004 and 0.01 mg/kg, respectively, which were suitable to apply Positive List System (PLS). Mean average accuracies of pinoxaden (M4) were shown to be 74.0-105.7%. The precision of pinoxaden and its metabolites were also shown less than 14.5% for all five samples.

CONCLUSION: The method investigated in this study was suitable to CODEX (CAC/GL 40) and National Institute of Food and Drug Safety Evaluation-MFDS (2017) guideline for residue analysis. Thus, this method can be useful for determining the residue in various food matrixes in routine analysis.

Key words: Analytical method, *Pinoxaden*, M4

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Introduction

Pinoxaden (IUPAC name: 8-[5-(2,6-diethyl-p-tolyl) -1,2,4,5-tetrahyrodro-7-oxo-7H-pyrazolo[1,2,-d][1,4,5]ox adiazepin-9-yl 2,2-dimethylpropanoate) is phenylpyrazoline herbicide developed by Syngenta Crop (Hofer et al., 2006, Muehlebach et al., 2011). Pinoxaden is systemic grass herbicide applied post-emergence control with broad spectrum grass weed (*Alopecurus, Apera, Avena, Lolium, Phalaris* and *Setaris* spp.). This chemical acts as inhibitor fatty acid synthesis resulting in necrosis. Pinoxaden inhibits the Acetly-CoA-Carboxylase (ACCase), interrupts lipid metabolism and consequently deforms membrane lipid and cuticle waxes (Hofer et al., 2006, Yu et al., 2010, Muehlebach et al., 2011).

The Pinoxaden metabolism results in various metabolites depending on post-treatment days and chemical reactions (Muehlebach et al., 2011). Briefly, mother molecule Pinoxaden is turned into metabolite M2 on the applied day. Then, M2 is hydrolyzed into M4 up to 21 days. Depending on several chemical reactions such as glucose conjugation, oxidation, and hydroxylation, M4 can be turn to various metabolites. Due to several metabolites' existence, definition of residue is different among the countries. It is Pinoxaden and its metabolites M2 and M4 in USA (Pesticide fact sheet, EPA, 2005) and Australia (Evaluation report, APVMA, 2006), while Pinoxaden only in EC (EFSA, 2013) and Japan. CODEX designates it as sum of free and conjugated M4 (IUPAC name: 8- (2, 6-Diethyl-4-hydroxy methyl-phenyl)-9-hydroxy-1, 2, 4, 5-tetrahydro-pyrazole [1, 2-d] [1, 4, 5] oxadiasepin-7-om). However, MFDS decided same residue definition as CODEX, sum of free and conjugated M4. Harmonization of MRL with Codex will facilitate food trade internationally. Additionally, parent molecule Pinoxaden is rapidly metabolized and metabolites M2 and M6 are not detected at 21 days post-application.

The application of its maximum residue level setting for only two crops, barley and wheat was to Ministry of Food and Drug Safety (MFDS) on 2017 as import tolerance. However, MFDS has been established simultaneous analytical method for multi-residue using simple sample preparation and detection processing. Therefore, analytical method for Pinoxaden in various food matrixes was needed. The purpose of this study is to develop a MFDS food code method for Pinoxaden. Five representative produces (mandarin, potato, red pepper, soybean and hulled rice) were
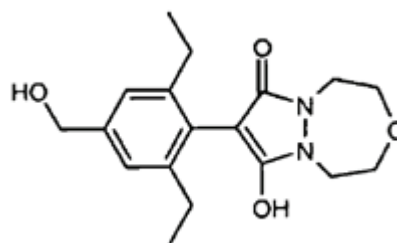

Fig. 1. molecular structure of pinoxaden (M4)

used for matrix-matched calibration. The accuracy and specification of analytical method were followed by guidelines of CODEX (CAC/GL 40) and National Institute of Food and Drug Safety Evaluation-MFDS (NIFDSEMFDS, 2017).

Materials and Methods

Reagents and materials

Pinoxaden (M4, Fig. 1.), purity 84 %, was supplied by Syngenta Korea (Seoul, Korea) and its physicochemical characteristic summarized in Table 1. Hydrophilic-lipophilic balance (HLB) cartridge was bought from Waters (Leinster, Ireland). HPLC grade of acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Other reagents used in this study were purchased from Wako Pure chemical industries (Osaka, Japan), Junsei (Tokyo, Japan) or Dae Jung (Kyungki-do, Korea). All reagents and organic solvents were of analytical grade.

Sample preparation

In this study, five representative agricultural commodities were chosen based on NIFDSEMFDS (2017), and domestic food consumption (Lee et al., 2010): mandarin orange for citrus group, potato for root vegetables group, red pepper for vegetable group, hulled rice for cereal group, and soybean for legumes. Non-pesticide treated representative agricultural commodities from local market (Seoul, Korea) were purchased. Sample preparation was followed by MFDS-Food Code method (MFDS, 2017). Briefly, samples (at least 1 kg per sample) were chopped, homogenized and kept at -50°C until further analysis. For hulled rice and soybean, samples were crushed enough to passing through standard sieve 420 μm.

Table 1. Physicochemical characteristics of mother molecule Pinoxaden and M4

Property	Pinoxaden	M4
CAS no.	243973-20-8	-
IUPAC name	8-(2,6-diethyl-4-methylphenyl)-7-oxo-1,2,4,5-tetrahydropyrazolo[1,2-d][1,4,5]oxadiazepin-9-yl] 2,2-dimethylpropanoate	8-(2,6-diethyl-4-hydroxymethyl-phenyl)-tetrahydropyrazolo[1,2,-d] [1,4,5] oxadiaepine-7,9-dione
Molecular formula	$C_{23}H_{32}N_2O_4$	$C_{18}H_{24}N_2O_4$
Molecular weight	400.519 g/mol	332.4 g/mol
Exact mass	400.236 g/mol	332.174 g/mol
Log Pow	Log P_{ow}3.2	
pKa	-	
Vapor pressure	4.6×10^{-7}Pa	
Solubility (in water)	200 mg/L	
Solubility (in organic solvent)	Acetone 250 g/L Dichloromethanee>500 g/L Ethyl acetate 130 g/L Hexane 1.0 g/L Methanol 260 g/L Octanol 140 g/L Toluene 130 g/L	
Hydrolysis	25°C pH 4 $t1/2$=18.1 days 25°C pH 5 $t1/2$=17.5 days 25°C pH 7 $t1/2$=9.9 days 25°C pH 9 $t1/2$=0.2 days 15°C pH 7 $t1/2$=23.7 days 15°C pH 9 $t1/2$=0.7 days	
Photolysis	30-50°N $t1/2$=14 days 60°N $t1/2$=20.6 days	

Standard solution

Stock standard solution for Pinoxaden (M4, 10.4 mg) was prepared in 10 mL acetonitrile as final concentration 1,000 mg/L. After preparing of stock working solution (1.0 μg/mL) by adding 100 μL at 900 μL of control sample extraction, series of solutions (the concentration levels 0.002, 0.01, 0.05, 0.1 and 0.2 μg/mL containing sample matrix background (> 90% v/v), while soybean case 0.005, 0.01, 0.05, 0.1, and 0.25 μg/mL) were made. All solutions were stored in amber vials at 4°C until further analysis.

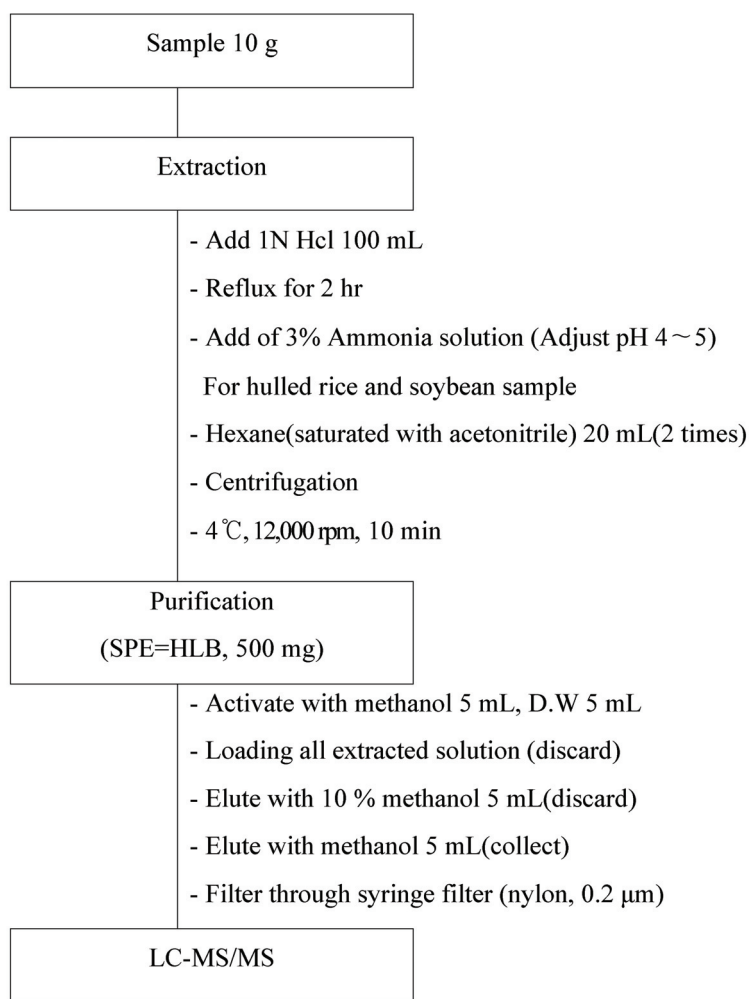## Optimal extraction and purification condition

Sample extraction and purification were slightly modified QuEChERS method (Akiyama et al., 2009, Muhammad et al., 2017). Reflux, pH, and fat removal conditions were investigated. First, three different reflux conditions by using hydrogen chloride (HCl) and acetonitrile (ACN) were tested: 1N HCl, 1 N HCl: ACN (9:1), and 1 N HCl: ACN (8:2). Also, adjusted pH conditions were compared. Next, fat removal

condition was investigated for high-oil samples such as soybean and hulled rice. For increasing the recovery, comparison of two cartridges (C_{18} and HLB) was investigated along with several solvents.

Extraction

Ten gram of homogenized sample, expect soybean (5 g), was placed 200 mL round flask. For high oil contained samples such as soybean and hulled rice samples, 10 mL of distilled water was added and waited for 30 min for moisture control. After adding 100 ml of 1N HCl, samples were connected to reflux cooler and heated for 2 hr. Then, sample was cooled to room temperature. After filtration by using Buhner funnel, 10 mL of filtrated samples were transferred to falcon tube. pH of samples were adjusted to pH 4-5 by using 3% ammonium solution.

For hulled rice and soybean samples, addition step is necessary for eliminating oil. 20 mL of acetonitrile saturated n-hexane was added and mixed vigorously for 20 min. After centrifugation (12,000 rpm, 10 min),

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┌─────────────────────────────┐
│        Sample 10 g          │
└─────────────────────────────┘
              │
┌─────────────────────────────┐
│        Extraction           │
└─────────────────────────────┘
              │  - Add 1N Hcl 100 mL
              │  - Reflux for 2 hr
              │  - Add of 3% Ammonia solution (Adjust pH 4∼5)
              │    For hulled rice and soybean sample
              │  - Hexane(saturated with acetonitrile) 20 mL(2 times)
              │  - Centrifugation
              │  - 4℃, 12,000 rpm, 10 min
┌─────────────────────────────┐
│        Purification         │
│     (SPE=HLB, 500 mg)       │
└─────────────────────────────┘
              │  - Activate with methanol 5 mL, D.W 5 mL
              │  - Loading all extracted solution (discard)
              │  - Elute with 10 % methanol 5 mL(discard)
              │  - Elute with methanol 5 mL(collect)
              │  - Filter through syringe filter (nylon, 0.2 μm)
┌─────────────────────────────┐
│         LC-MS/MS            │
└─────────────────────────────┘
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Fig. 2. Flow chart for Pinoxaden (M4) extraction and purification

aqueous layer were collected. This step was repeated one more time.

Purification

HLB cartridges were activated with 5 mL of methanol following by 5 mL of water at rate of 2-3 drops/ sec. All extracted solution was applied total rate of 1-2 drops/sec. After washing with 5 mL of 10 % methanol, the elution was collected by adding additional 5 mL of methanol. Then, the elution was filtrated by using membrane flier (Nylon, 0.2 μm). Brief flow chart for sample preparation was shown at Fig. 2.

LC–MS/MS

LC-MS/MS system consisted of Acuity UPLC (Waters, Millford, MA) and Xevo TQ-S tandem quad ruple mass spectrometer (Waters, Milford, MA) were used in this study. LC separation was carried out in

Unison UK-C_{18} column (100 x 2.1 mm, 3 μm, Imtakt, USA). The injection volume was 2 μL, and the analyte was eluted in 0.1% formic acid-acetonitrile at the flow rate of 0.3 mL/min.

Limits of quantitation

After confirming no interference from non-pesticide treated samples, limits of quantitation (LOQ) was calculated based on limits of detection (LOD) and concentration ratio (Ahn et al., 2014). To validate accuracy and precision, recovery test was investigated.

Results and Discussion

In this study, M4, residue definition of Pinoxaden in Korea, was analyzed using LC-MS/MS. Although different analysis method such as GC (Shah et al., 2010), HPLC (Lin et al., 2007), and LC-MS (Diez et al., 2008), LC-MS/MS method has not reported yet. Many

Table 2. Comparison of recovery of Pinoxaden (M4) depending on reflux conditions

Compound	Recovery ± RSD[a] (%)		
	1N HCl	1N HCl : ACN (9:1)	1N HCl : ACN (8:2)
M4	95.02 ± 3.61	85.37 ± 0.70	117.83 ± 4.43

[a]Mean values of 3 times repetitions with relative standard deviation.
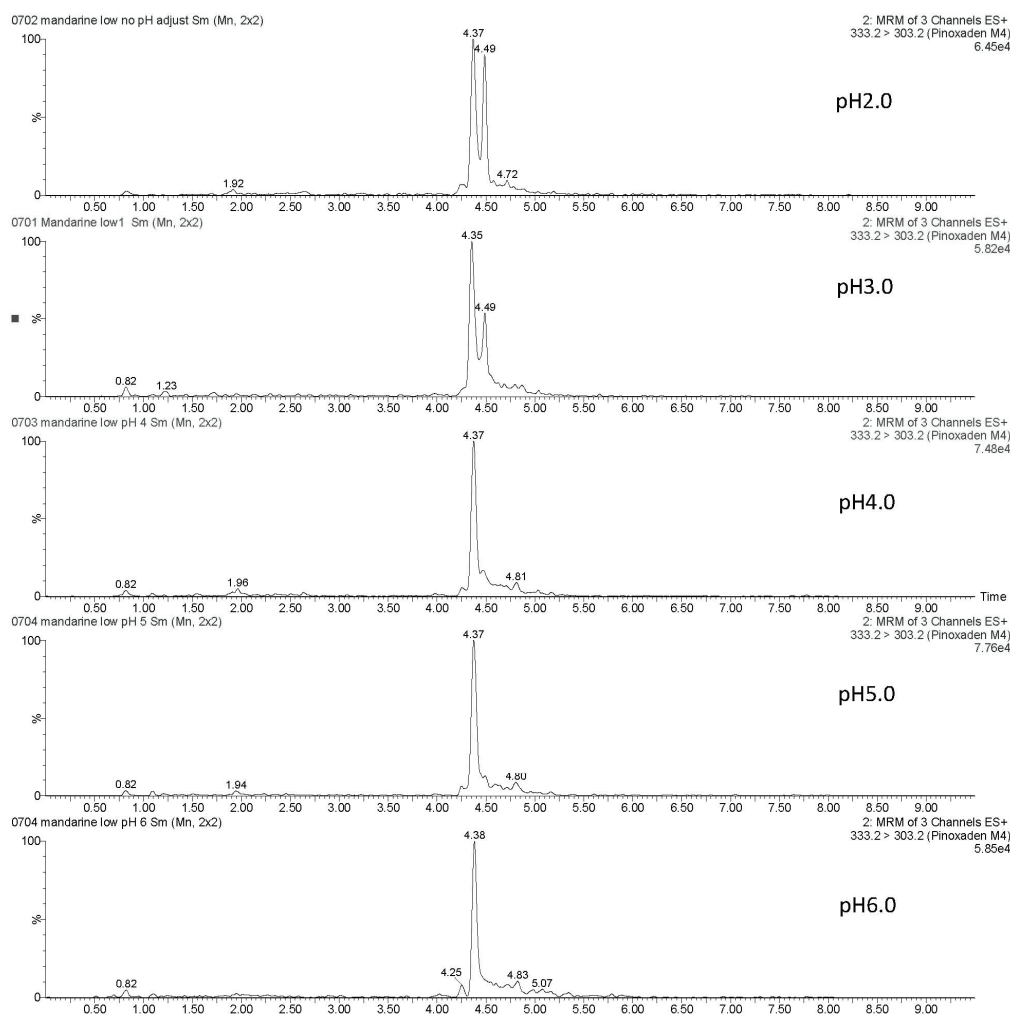
Fig. 3. Chromatogram of Pinoxaden (M4) spiked at mandarin depending on various pH

pesticide analysis methods in Food code are based on LC-MS/MS. Additionally, LC-MS/MS provides high sensitivity in the multi-residue screening (Akiyama et al., 2009). Thus, LC-MS/MS setting for Pinoxaden (M4) will be helpful for daily routine analysis for users.

Sample preparation

Extraction Free M4 molecule is transformed as conjugated one by using 1 N HCl reflux. This method is commonly used including CODEX (2005) and Australia

(2006). In this study, 3 different reflux conditions (1N HCl, 1N HCl: ACN (9:1), 1N HCl: ACN (8:2)) were investigated (Table 2). Although 1 N HCl: ACN (8:2) showed highest recovery, 1N HCl were chosen because ACN is evaporated during heating step.

For mandarin, split of chromatogram peak was observed at low pH (Fig. 3.). Thus, various pHs were investigated to find the optimal condition for mandarin orange. As the result (Fig. 3.), pH 4-5 showed better recovering without split of peak in chromatogram.

Table 3. Recovery comparison of different concentration of Pinoxaden (M4) depending on liquid-liquid separation by using Dichloromethane

Compound	Recovery ± RSD[a] (%)		
	Low	Mid	High
M4	72.1 ± 19.7	46.5 ± 21.8	44.5 ± 19.2

[a]Mean values of 3 times repetitions with relative standard deviation.

Table 4. Comparison of recovery of Pinoxaden (M4) at various elution solvent, its ratio and cartridges

Solvent	Solvent ratio (v/v)	Recovery (%)	
		HLB	C_{18}
Acetonitrile/Water/Formic acid	0/100/0.5 (5 ml)	0.0	55.2
	10/90/0.5 (10 ml)	45.4	52.0
	20/80/0.5 (10 ml)	50.8	49.8
Acetone/Water/Formic acid	0/100/0.5 (5 ml)	0.0	36.2
	10/90/0.5 (10 ml)	75.8	31.9
	20/80/0.5 (10 ml)	82.4	37.4
Hexane/Acetone/Formic acid	0/100/0.5 (5 ml)	65.6	66.6
	10/90/0.5 (10 ml)	67.5	57.7
	20/80/0.5 (10 ml)	49.8	54.2
Dichloromethane/Acetone/Formic acid	0/100/0.5 (5 ml)	67.2	74.5
	10/90/0.5 (10 ml)	49.6	49.8
	20/80/0.5 (10 ml)	52.3	59.2
Dichloromethane/Ethyl acetate/Formic acid	0/100/0.5 (5 ml)	86.0	54.5
	10/90/0.5 (10 ml)	59.0	51.0
	20/80/0.5 (10 ml)	62.8	54.6

Thus, optimal pH of extraction is pH 4-5. Previous studies also reported that lower pH condition is suitable for Pinoxaden extraction due it is base-sensitive pesticide (Muhammad et al., 2017, Lehotay et al., 2010). Additional fat removal step was required for soybean sample which contain high fat. First, liquid-liquid separation by using dichloromethane was used. However, this method showed low recovery rate (Table 3). Furthermore, it increased viscosity of sample which was not suitable for further analysis (data not shown). Thus, clean-up by using cartridge was studied.

Clean-up To investigating the proper solvent for simultaneous multiple residue detection, 5 different solvents (Acetonitrile/Water/Formic acid, Acetone/Water/Formic acid, Hexane/Acetone/Formic acid, Dichloromethane/acetone/Formic acid, and Dichloromethane/Ethyl acetate/Formic acid) were tested in this study (Table 4) summarized recoveries depending on solvents and their ratio. In addition, reflux step allows water in samples. Thus, we thought that it might be more efficient to use reversed phage cartridges rather than normal one. Two different reversed phage cartridges C_{18} and HLB were investigated. Recoveries between two cartridges were similar when using Acetonitrile/Water/Formic acid, Hexane/Acetone/Formic acid, and Dichloromethane/Acetone/Formic acid. However, recovery from HLB was higher than ones from C_{18} by using Acetone/Water/Formic acid (expect 0/100/0.5 trails) and Dichloromethane/Ethyl acetate/Formic acid (Table 4). As the result, HLB cartridge was chosen for further analysis.

Although Dichloromethane/Ethyl acetate/Formic acid with 0/100/0.5 (5 mL) showed the highest recovery rate (86.0 %), Methanol, which showed similar recovery rate (Table 5), was chosen as elution solvent for this study because of the inconvenience to apply pressure during Dichloromethane/Ethyl acetate/Formic acid elution.

Next, activation solution (Methanol : Water) ratio were tested for increasing purification. Only 50/50 Methanol : Water showed result (Table 6). Also, we

Table 5. Comparison of recovery of Pinoxaden (M4) at various Methanol elution condition

Solvent		Recovery ± RSD[a] (%)
		HLB
Methanol	load 10 ml	0.0
	fraction 1 (5 ml)	80.2 ± 3.3
	2	0.99 ± 7.8
	3	0.0
Total	-	81.6 ± 2.7

[a]Mean values of 3 times repetitions with relative standard deviation.

Table 6. Comparison of recovery of Pinoxaden (M4) at various cartridge activation conditions

Solvent	Solvent ratio(v/v)	Recovery± RSD[a] (%)
		HLB
Methanol/Water	load 10 ml	0.0
	10/90	0.0
	30/70	0.0
	50/50	81.2 ± 9.2
	70/30	0.0
	90/10	0.0
	100/0	0.0
Total		81.2 ± 9.2

[a]Mean values of 3 times repetitions with relative standard deviation.

found out the first there was no elution of M4 with the first 10% Methanol. Finalized extraction and purification were shown at Fig. 2.

Optimization of Instrument Condition

Due to low vapor pressure (4.6 x 10^{-7} mmHg at 25°C) and high boiling point, Pinoxaden is not suitable for Gas Chromatography (GC). Although the analysis of HPLC-UVD system is possible due to benzene and ketone group of M4, LC-MS/MS system was chosen because of PLS adaptation which has low LOD as 0.01 mg/kg. The separation was conducted with reverse-phase column UK-C_{18} column (100 x 2.1 mm, 3 μm) with gradient of mobile phase. Formic acid in a moving phase acted as a protonization enhancer and helped ion response in $[M+H]^+$ of Pinoxaden. To ionic each molecule, the positive-ion mode of electro-spray ionization (ESI) were used. The optimal specific ion was chosen by analyzing selected-ion monitoring with total ion chromatogram and mass spectrum. As the result of mass analysis by using Pinoxaden standard solution (Mass 332.74, concentration 0.1 μL/mL), value

of $[M+H]^+$ was confirmed as 333.2 mass. MS/MS analysis with MRM (multiple reaction monitoring) mode was used for investigating analysis selectivity and sensitivity at the optimized cone voltage. The best precursor/production ion pair was chosen by controlling collision energy at collision cell (Murray et al., 2013). The product ion shown the best selectivity was used for quantification ion, while the production ion shown the best sensitivity for qualification ion.

Optimal analyzing condition and selected-ion of LC-MS/MS for Pinoxaden (M4) summarized in Table 7 and Table 8. Matrix-matched calibrations of commodities were investigated for method validation.

Method validation

Limit of detection and quantitation The detected level of Pinoxaden (M4) were quantified as followed. First, residue levels in sample were determined by substituting at standard calibration curve and considering conversion factor 1.2 (molecular weight ratio between mother molecule (400.5) and M4 (332.4)). The limit of detection (LOD) and quantitation (LOQ) were deter-

Table 7. Analytical conditions for the determination of Pinoxaden (M4)

Condition	Content
Instrument	LC: AcquityUPLC(Waters, Milford, MA, USA) MS/MS: Xevo TQ-S(Waters, Milford, MA, USA)
Chromatographic separation	
Column	UK-C_{18} column (100 x 2.1 mm, 3 μm)
Flow rate	0.3 mL/min
Injection volume	2 μL
Oven temp.	40 ℃
Mobile phase	A: 5 mM Ammonium formate, 0.1% formic acid in water B: 0.1% formic acid in acetonitrile
Gradient	

Time(min)	A(%)	B(%)
0.0	90	10
3.0	90	10
5.0	60	40
5.1	10	90
7.0	10	90
7.1	90	10
10.0	90	10

Condition	Content
MS/MS condition	
Interface temp.	150 ℃
Desolvation temp.	400 ℃
Heating gas flow	10.0 L/min
Nebulizer gas flow	7.0 L/min
Cone voltage	30 V

Table 8. Selected-ion of LC-MS/MS for Pinoxaden (M4)

Compound	Retention time (min)	Molecular weight	Exact Mass	Precursor ion (m/z)	Production (m/z)	Collision energy (eV)
Pinoxaden (M4)	4.38	332.40	332.17	333.2	101.1	26
					303.2[b]	13
					315.2	12

[b]Quantification ion

mined by the ratio of signal-to-noise (S/N, Shrivastava & Gupta, 2011). The values of LOD were 0.001 μg/mL (S/N>3) as minimum instrumental detection con centration and 0.004 mg/kg. The values of LOQ were 0.005 μg/mL (S/N>10) as the lowest detection concentration and 0.01 mg/kg when calculated below.

Linearity Matrix-matched calibration curves were constructed by plotting the peak area vs. the six concentrations (0.002-0.2 mg/kg range) of the analyte. Good linearity for Pinoxaden ($r^2 > 0.99$) was observed for all five food matrices

Selectivity The selectivity was confirmed by comparing representative chromatogram of standard working solution, blank, and fortified sample extracts. No endogenous components were observed at the retention time and *m/z* of the analyte. As the result,
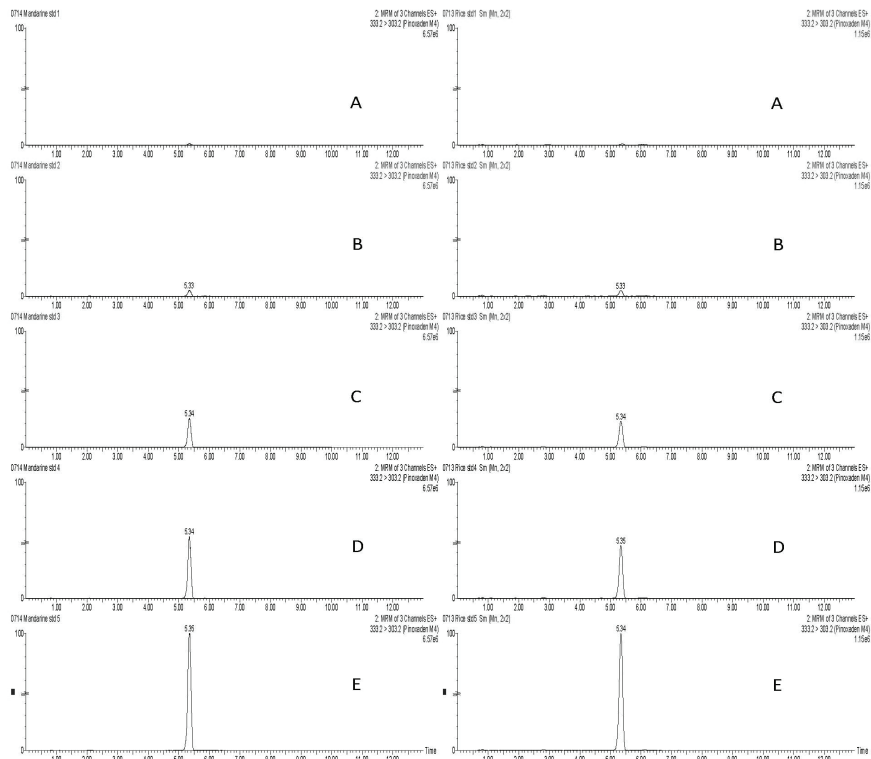
Fig. 4. LC-MS/MS Standard chromatograms of Pinoxaden (M4) in mandarin (left), hulled rice (right) at (A) 0.002 mg/kg, (B) 0.01 mg/kg, (C) 0.05 mg/kg, (D) 0.1 mg/kg, and (E) 0.2 mg/kg
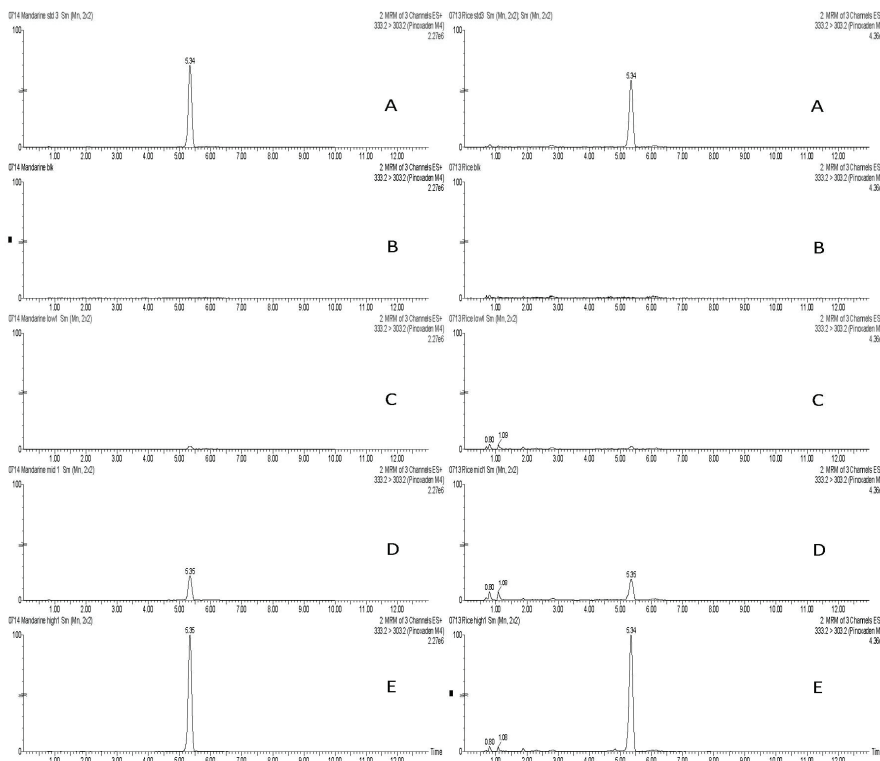

Fig. 5. Representative MRM (quantification ion chromatograms of Pinoxaden (M4)) in mandarin (left), hulled rice (right) corresponding to: (A) standard solutionat 0.05 mg/kg, (B) control, (C) spiked at 0.01 mg/kg, (D) spiked at 0.1 mg/kg and (E) spiked at 0.5 mg/kg.

Table 9. Validation results of analytical method for the determination of Pinoxaden (M4) in samples

Sample	Fortification (mg/kg)	Ave. ± RSD[a] (%) Pinoxaden (M4)	LOQ (mg/kg)
Mandarin	0.01	88.0 ± 12.3	
	0.1	90.5 ± 10.4	
	0.5	80.8 ± 7.3	
Potato	0.01	105.1 ± 9.0	
	0.1	86.9 ± 8.2	
	0.5	84.4 ± 5.9	
			0.01
Red pepper	0.01	85.7 ± 6.1	
	0.1	75.0 ± 4.8	
	0.5	74.0 ± 4.6	
Hulled rice	0.01	91.2 ± 13.7	
	0.1	87.8 ± 7.3	
	0.5	86.1 ± 3.9	
Soybean	0.05	105.7 ± 14.5	
	0.5	77.9 ± 6.4	0.05
	2.5	100.0 ± 13.2	

[a]Mean values of 5 times repetitions with relative standard deviation.

this method is confirmed to have a good separation and selectivity (Fig. 4).

Accuracy and precision Recovery test was performed with three different levels (1, 10, and 50-fold of LOQ) 0.01, 0.1 and 0.5 mg/kg, for testing accuracy and precision (Fig. 5, Table 9). It was done with five replicates. Mean average accuracies were shown 74.0-105.7 %. The precision (shown as relative standard deviation) were less than 14.5 % for all five agricultural commodities. Thus, this method is acceptable to guideline for CODEX (CAC/ GL 40) and NIFDSEMFDS (2017).

Conclusions

The analytical method for Pinoxaden (M4) developed from this study to be possible for applying for all food matrixes. Additionally, this analytical method analysis is suitable for routine analysis with reliable selectivity, sensitivity and accuracy.

Note

The authors declare no conflict of interest.

Acknowledgement

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