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DEVELOPMENT OF ANALYTICAL METHOD FOR DETERMINATION OF TRIAZOLE AND PYRETHROID PESTICIDES IN MUSTARD GREENS (*Brassica juncea*)

Nguyen Dang Giang Chau[1]*, Nguyen Thi Minh Hieu[2]

[1] HU – University of Sciences, 77 Nguyen Hue St., Hue, Vietnam

[2] Pham Van Dong high school, Phong Hoa, Tay Hoa, Phu Yen Province, Vietnam

Abstract. Triazole and pyrethroid are two of the most commonly used pesticides in vegetable cultivation nowadays. In this study, a multi-residue analytical method employing gas chromatography-mass spectrometry (GC–MS) technique was successfully developed for the quantification of two triazole fungicides, namely propiconazole and difenoconazole, and pyrethroid insecticide cypermethrin (including 4 isomers) in mustard greens. The GC–MS running program performed good separation with a low detection limit and short analysis time (24 minutes). Target compounds in mustard green samples were ultrasonically extracted in 15 minutes with 60 mL acetone, followed by clean-up steps using solid phase extraction in a packed activated carbon column with 40 mL acetone:toluene (v:v, 1:1) for elutions, and florisil cartridge with an elution mixture of 15 mL acetone:n-hexane (v:v, 1:5). The method quality control was conducted before applying for a preliminary screening of triazole and pyrethroid residue levels in mustard greens collected in Phu Yen province, Vietnam.

Keywords: triazole, pyrethroid, residues levels, GC–MS

1 Introduction

Mustard greens have been considered as one of the most common vegetables in Vietnam. During their growth, the vegetables are damaged by many insects such as *Delia brassicae, Phyllotreta spp., Ceutorhynchus spp., Thrips tabaci, Brevicoryne brassicae, Contarinia nasturrii, Agrotinae* [1]. Accordingly, different pesticides (insecticides, fungicides, and herbicides) are applied to control insects and diseases of the mustard greens. Since organochlorine pesticide use is banned, new generations of pesticides have been synthesized and introduced to the market, in which triazole and pyrethroid pesticides are the most extensively used. Therefore, health risk assessment on pesticide exposure from vegetable consumption is of concern worldwide [2–5]

Triazole compounds containing one or more 1,2,4-triazole rings have been shown to have some of the most potent antifungal properties [6]. Synthetic pyrethroids are pesticides derived from naturally-occurring pyrethrins, taken from pyrethrum of dried *Chrysanthemum* flowers [7]. This new generation of pesticides is chemically designed to be more toxic with a faster break down time.

* *Corresponding: titan.envi@gmail.com*

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In Vietnam, the number of studies on either multi-residue method development or risk assessment of pesticides in vegetables is very limited. Hoai et al. [8] conducted a study concerning some organochloride pesticides (OCPs), trichlorfon, fenobucarb, cyfluthrin, and cypermethrin detection in vegetables and tea. In this study, the authors employed ethylacetate as an elution solvent for the ultrasonic extraction step, activated carbon packed column (ACPC) and florisil cartridge for the cleanup step, and finally gas chromatography for qualitative and the quantitative analysis. Recently, Chau et al. [9] published an analytical method to quantify different pesticide classes, namely fenobucarb, fipronil, quinalphos, isoprothiolane and cypermethrins in onion leaves, in which acetone was used for extraction, ACPC and florisil cartridge were used to clean up the matrix. An ACPC was also employed in combination with aluminium oxide in the study of Akoto et al. [10] to extract 37 pesticides from maize and cowpea, while florisil cartridge was successfully used with 5 mL acetone:hexane (2:8, v:v) as an elution solvent to analyze 253 pesticides in 13 different vegetables in Korea [11]. However, in most of the cases, triazole has not been considered.

This study targeted to develop a method to quantify the fungicide triazole, viz. propiconazole and difenoconazole and the insecticide cypermethrins (4 isomers) in mustard greens.

2 Materials and methods

2.1 Target pesticides

The selection of target pesticides was based on the following criteria: (1) the new-generation pesticides in the groups of triazole and pyrethroid (old-generation and prohibited pesticides were not the focus of this study); (2) measurable by GC–MS instrument; (3) potential to cause health risk to human health (based on the toxicity and maximum residue level (MRL) of individual pesticide). General properties and toxicology of the selected pesticides are shown in Table 1.

Table 1. General physical-chemical properties [12], toxicities [13], acceptable daily intake (ADI) [14] and MRLs of the target pesticides

	Propiconazole	**Difenoconazole**	**Cypermethrins**[b]
Chemical class	Triazole	Triazole	Pyrethroid
Melting point (°C)	–23	82–83	81–84
Boiling point (°C)	180	101	200
Vapour pressure (mmHg, 20 °C)	1.3×10^{-6}	3.3×10^{-8} (25 °C)	3.1×10^{-9} (25 °C)
Solubility in water (mg/L, 20 °C)	1.3×10^{-6}	3.32×10^{-8} (25°C)	0.009
Octanol-water partition coefficient	3.72	4.4	5.3
Toxicity [(a)]	II	II	II
Half-life in soil (days)	96–229	15	60
MRL (mg/kg)	1.5 (rice grain) [15]	2 (brassica) [16]	0.7 (leafy vegetables) [16]

	Propiconazole	Difenoconazole	Cypermethrins[b]
Chemical class	Triazole	Triazole	Pyrethroid
ADI (μg/kg bw)	70	10	20

(a) WHO toxicity classes: class II: moderately hazardous, class III: slightly hazardous, U: unlikely to present an accute hazard; (b) cypermethrins include 4 isomeres α-cypermethrin, β-cypermethrin, γ-cypermethrin, and θ-cypermethrin

Additionally, p,p′-DDT and fluorene-D10 were used as a surrogate and internal standard, respectively.

2.2 Chemicals and reagents

Pesticide standards, the surrogate standard (p,p′-DDT) and the internal standard (fluorene-D10) with purity higher than 97 % were obtained from Sigma Aldrich (USA). Stock solutions (1000 μg/mL) of pesticides and surrogate were prepared in acetone and stored at −20 °C. Working solutions were prepared in toluene. Fluorene–D10 (stock solution of 1000 μg/mL) was kept in toluene. All employed solvents were HPLC grade, including n-hexane, acetone, acetonitrile, ethylacetate, toluene, dichloromethane, methanol and water (J. T. Baker, Deventer, The Netherlands). Sodium chloride and sodium sulphate were obtained from Sigma Aldrich (USA). Glass fiber filters (Whatman, 47 mm, pore size 1.6 μm) and florisil (500 mg/6 mL) silica-based reversed phase cartridges for solid phase extraction from Sigma Aldrich (USA), and activated carbon from Merck (Darmstadt, Germany) were used.

2.3 Method development

The analytical method was developed based on the reported results [8, 9], in which a typical analytical process includes the following steps: 1) solvent extraction to separate the target pesticides from vegetable samples; 2) activated carbon packed column extraction to remove green color of chlorophyll; 3) florisil solid phase extraction (SPE) to remove polar compounds; and 4) chromatographic analysis. Based on Chau et al. [9], this study applied without modification of the extraction solvent of 60 mL acetone, 15 min ultrasonic extraction for step 1, and the elution solvent for florisil SPE of 15 mL acetone:n-hexane (v:v, 1:5) (step 3).

To identify the retention time and target ions of the individual pesticides, the NIST–05 database (NIST/EPA/NIH Mass Spectral Database) was used in combination with single standard injection. The GC–MS (7890A–5975C, Agilent, USA) conditions were: helium as carrying gas with a constant flow mode at the rate of 0.8 mL/min, DB–5 fused silica capillary column (30 m × 0.25 mm; film thickness 0.25 μm), splitless injection mode, injection volume 3 μL, inlet temperature 270 ^{o}C, interface temperature 150 ^{o}C, ion source temperature 230 ^{o}C with an EI (electron ionization) mode. The oven temperature was as followed: 85 ^{o}C – initial temperature kept for 2.5 min, increased to 220 ^{o}C at a rate of 20 ^{o}C/min, then increased to 228 ^{o}C at a rate of 3 ^{o}C/min, continued increasing to 232 ^{o}C at a rate of 2 ^{o}C/min, then to 260 ^{o}C at a rate of 10 ^{o}C/min, to 279 ^{o}C at a rate of 2 ^{o}C/min, and finally increased to 280 ^{o}C at a rate of 0.2 ^{o}C/min, held for 3 min. The total running program time was 24.4 minutes.

To define the eluting conditions for ACPC extraction, 5 solvents were investigated (40 mL for each solvent), namely: 1) ethanol, pH 3–4; 2) dichloromethane:toluene (v:v, 8:1); 3) acetonitrile, 4) ethylacetate:acetone (v:v, 1:1); and 5) acetone:toluene (v:v, 1:1). The applied elution rate was 1 mL/min. The initial concentration of target pesticides was 500 ppb. The extract was then concentrated with N_2 gas and filled up to approximately 1 mL with toluene before being injected into the GC–MS system.

The recovery performance of the ACPC extraction-and-florisil SPE combination applying the selected solvents was then tested.

Surrogate p,p′-DDT, 500 ng, was added from the very beginning of each experiment while the internal standard fluorene–D10, 100 ng, was spiked into the final extract before being injected into the GC–MS. The calibration curve method was used for quantification.

2.4 Method of quality control

The instrument limit of detection (LOD) for each target compound was calculated using eight replicated injections of the standard solutions at the lowest concentrations in the calibration curve, which was delivered by multiplying the *t*-distribution by the determined standard deviation (SD), which is: LOD = 3.14 × SD (3.14 is the *t* value for a 99 % confidence interval, with six degrees of freedom) [17]. Accordingly, the respective limit of quantification (LOQ) of the method for an individual compound is LOQ = 10 × SD (Table 3). Only detected concentrations higher than the specific LOQ values were used for further assessment. The calibration curves for the studied compounds were developed from seven concentrations (10, 50, 100, 200, 500, 1000, 5000 ppb) based on the commonly detected concentrations of pesticides in vegetable samples in the literature.

The trueness of the analytical method was achieved by calculating the recovery for the three-replicate analysis of a practical mustard green sample spiked with 500 ppb of the studied pesticides. The repeatability of the method was checked via relative standard deviation (RSD): if RSD was lower than $\frac{1}{2}$ $RSD_{Horwitz}$, in which the $RSD_{Horwitz}$ was calculated from the Horwitz equation [18]: $RSD_{Horwitz} = 2^{(1 - 0.5 \cdot \log C)}$. In addition, this study accepted a recovery of the surrogate p,p′-DDT from 70 % to 120 %.

2.5 Sampling

The developed method was then applied to quantify triazole and pyrethroid compounds in the mustard green samples collected from four different mustard green cultivation areas in Phu Yen province. 0.5 kg of each sample was taken, wrapped in aluminium foil and transported within 24 hours under cold condition using an ice box to the Laboratory of POPs analysis at the Department of Chemistry, HU – University of Sciences, Hue, Vietnam. The samples were then frozen at –40 °C before analysis. To keep the natural condition of the mustard green samples, only the roots were removed while water was not used to flux the samples.

2.6 Statistical method

Chromatographic peak integration and statistic analysis were achieved using the Agilent G1701EA (GC–MSD ChemStation) software. Microsof–Excel 2010 was also used to perform the statistical analysis.

3 Results and discussion

3.1 Chromatographic identification and quantification conditions

Retention times, target ions

The specific retention time and target ions of the individual pesticide were shown in Table 2. Additionally, Fig. 1 demonstrates a chromatogram of a mixture of studied pesticides each of which has a concentration of 500 ppb.

Table 2. Retention times and target ions of propiconazole, difenoconazole, and cypermethrins

No	Compound	Retention time (min)	Target ion (m/z)	Reference ion (m/z)
1	Fluorene–d10 (*)	6.850	176	174; 146
2	p,p′-DDT (**)	12.480	235	237; 165
3	α-Propiconazole	13.031	69	173; 259
4	β-Propiconazole	13.142	69	173; 259
5	α-Cypermethrin	17.348	183	181; 165
6	β-Cypermethrin	17.527	183	181; 165
7	γ-Cypermethrin	17.629	183	181; 165
8	δ-Cypermethrin	17.705	183	181; 165
9	α-Difenoconazole	20.193	265	267; 323
10	β-Difenoconazole	20.354	265	267; 323

(*): internal standard; (**): surrogate

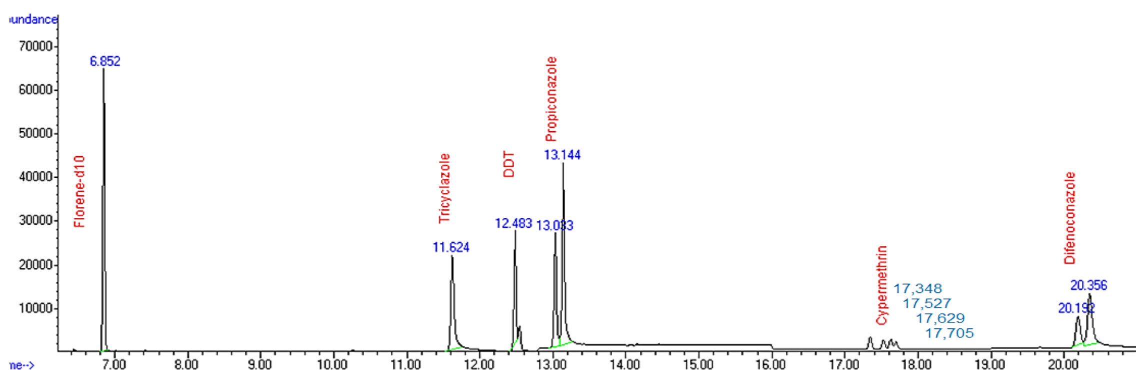

Fig. 1. Chromatogram of a mixture of the pesticides each of which has a concentration of 500 ppb

Detection limits and calibration curves

The limit of determination of the studied pesticides varied from 0.7 (for p,p′-DDT) to 5.9 ppb (for β-difenoconazole). Accordingly, LOQ values fluctuated from 2.3 to 19.6 ppb (Table 3).

Table 3. LODs, LOQs of the analyzed compounds

No	Compound	Injection conc. (ppb)	Detected conc. of 8 replicates (ppb)								LOD (ppb)	LOQ (ppb)
1	p,p′- DDT (*)	5	4.7	4.9	5.2	5.4	5.1	4.9	4.9		0.7	2.5
2	α-Propiconazole	10	10.2	9.9	10.6	10.7	9.6	10.0	8.9	9.9	1.7	5.7
3	β-Propiconazole	10	10.1	9.9	9.7	9.9	9.7	9.8	10.4	9.9	0.7	2.3
4	α-Difenoconazole	20	23.3	18.4	22.1	18.1	20.0	19.8	19.9	18.3	5.6	18.7
5	β-Difenoconazole	20	22.8	22.6	20.9	19.4	19.7	18.9	18.2	17.2	5.9	19.6
6	α-Cypermethrin	20	17.8	20.3	23.1	18.7	21.7	20.6	19.1	18.6	5.4	17.0
7	β-Cypermethrin	20	18.2	19.6	19.6	18.1	20.4	21.9	20.9	22.1	4.2	14.1
8	γ-Cypermethrin	20	19.5	21.1	22.4	19.2	21.8	20.1	18.1	17.6	5.1	17.1
9	δ-Cypermethrin	20	20.7	19.6	23.3	20.0	20.6	19.8	17.6	18.5	5.1	17.1

(*): surrogate, $n = 7$

Table 4 demonstrates the calibration curves for an individual pesticide. The x variable demonstrates the concentration while the y variable is the ratio between the chromatographic peak area of the target pesticide and the peak area of the internal standard. Since the two propiconazole isomers and the two difenoconazole isomers have usually existed together in every commercial product, therefore their calibration curves were derived from the total of the two isomers. The high R^2 values were also obtained.

Table 4. Calibration curves for the studied compounds

No	Compound	Calibration curve	Correlation coefficient (R^2)
1	Propiconazoles	$y = 0.0024 \cdot x - 0.0967$	0.9999
2	Difenoconazoles	$y = 0.0011 \cdot x - 0.1694$	0.9964
3	α-Cypermethrin	$y = 6.10^{-5} \cdot x - 0.0066$	0.9984
4	β-Cypermethrin	$y = 5.10^{-5} \cdot x - 0.0057$	0.9979
5	γ-Cypermethrin	$y = 4.10^{-5} \cdot x - 0.0055$	0.9976
6	δ-Cypermethrin	$y = 3.10^{-5} \cdot x - 0.0036$	0.9976

3.2 Cleanup conditions

Cleanup condition for ACPC extraction

Activated carbon is usually used as a stationary phase in low-pressure chromatographic separation because of its large surface area, or in other words, the high degree of microporosity to retain high molecular weight substances. Green extracts from vegetable samples usually contain a large amount of chlorophyll, therefore, it would be eliminated when passing through the activated carbon packed column [8, 10, 19].

Among the 5 investigated solvents (see 2.3), the mixture of acetone:toluene (v:v, 1:1) has got the highest recovery for the studied compounds between 86 % and 101 % (Table 5). Therefore, this mixture was chosen for further experiments.

Table 5. Recovery performance of ACPC

	Recovery (%)			
Elution solvent	**Propiconazoles**	**Difenoconazoles**	**Cypermethrins**	**p, p-DDT(*)**
Ethanol pH 3–4	–	–	–	–
DCM:toluene (8:1)	–	–	76	–
Acetonitril	–	–	–	–
EA:acetone (1:1)	94	97	66	90
Acetone:toluene (1:1)	101	99	86	9

Spiked concentration: 500 ppb; DCM: dichloromethane; EA: ethylacetate; – : lower than LOQ; (*): surrogate

Recovery performance of ACPC-and-florisil cartridge combination

1 mL of the target pesticides at 500 ppb each and the surrogate were introduced directly into the ACPC and eluted with 40 mL acetone:toluene (v:v, 1:1) at the rate 1 mL/min. The extract was concentrated in vacuum to 1 mL and put into the florisil cartridge, which was then eluted with 15 mL acetone:n-hexane (v:v, 1:5), and the eluate was concentrated to 1 mL by the same way. 100 ng of the internal standard was added before injecting the extract to the GC-MS system. The studied pesticides were rather well recovered (from 91 % to 103 %) after eluting through the ACPC followed by florisil cartridge (Table 6). This suggests the suitability of the cleanup step for the analytical method.

Table 6. Recovery for the studied pesticides using ACPC-and-florisil cartridge combination

No	**Compound**	**Initial-conc.** (ppb)	**Ave. detected conc.** (ppb) (n = 3)	**Ave. recovery (%)** (n = 3)
1	Propiconazoles	500	476	95
2	Difenoconazoles	500	517	91
3	Cypermethrins	500	457	103
4	p,p′-DDT (*)	500	499	100

(*) Surrogate

3.3 Analytical procedure

Based on the obtained results, a comprehensive analytical method to quantify triazole and pyrethroid pesticides in mustard greens was suggested as shown in Fig. 2.

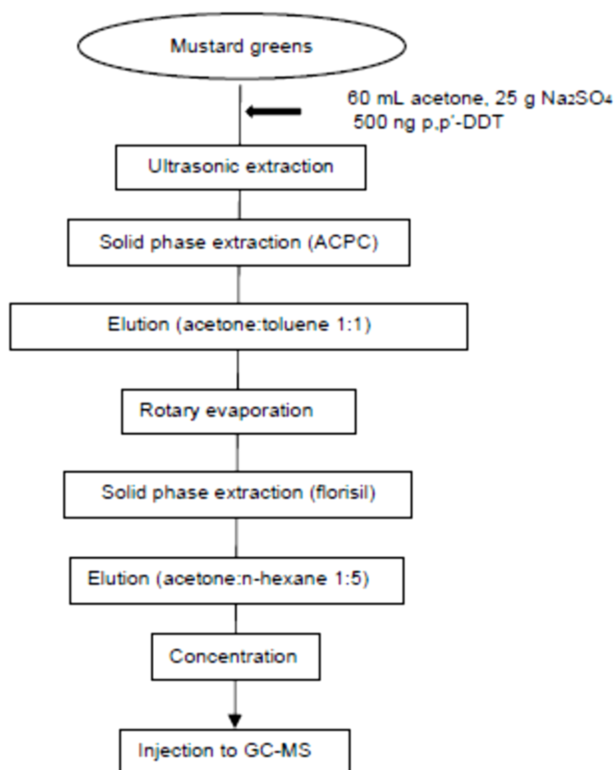

Fig. 2. Analytical scheme to quantify pesticide residues in mustard greens

To evaluate the trueness and repeatability of the developed method, 500 g of a mustard green sample was collected in a cultivation area in Tuy Hoa City – Phu Yen province and was named as R1. A three-replicate analysis was conducted for the raw sample (with a surrogate application and without pesticide spiked to see the practical concentration of the studied pesticides, named as R_1blank) and other three-replicate measurements of the sample spiked with 500 ppb pesticide (named as R_1S1, R_1S2, R_1S3). The developed method performed good trueness (n = 3) for the target pesticides (Table 7), with the recovery varied from 87 % (for difenoconazoles) to 101 % (for propiconazoles), while p,p'-DDT recovery was 82 %. In the meantime, the method showed a good repeatability with RSD ranging from 3.3 % (for p,p'-DDT) to 7.9 % (difenoconazoles), lower than the ½ $RSD_{Horwitz}$ (8.9 %). These results indicate that the analytical method could be applied to quantify the residues of propiconazoles, difenoconazoles and cypermethrins in mustard greens.

Table 7. Trueness and repeatability of the developed method

		Detected conc. (ppb wet weight)			
		Propiconazoles	**Cypermethrins**	**Difenoconazoles**	**p,p′- DDT (*)**
Sample	**R_1blank**	–	–	–	410
	R_1S1	531	457	470	395
	R_1S2	464	497	401	430
	R_1S3	520	524	437	395
Average (ppb) (n = 3)		505	493	436	408
Recovery (%) (n = 3)		101	99	87	82
RSD (%) (n = 3)		7.1	6.8	7.9	3.3

(*): surrogate; – : lower than LOQ; Spiked concentration: 500 ppb

3.4 Practical application for detection of pesticides in mustard green samples

The developed method was applied to analyze several mustard green samples (0.5 kg for each sample) collected from four mustard green cultivation areas in Tuy Hoa City – Phu Yen province in July 2016, named as R_1, R_2, R_3 and R_4. The pesticide residue levels were shown in Table 8.

Table 8. Pesticide residues (ppb wet weight) in mustard greens collected in Phu Yen province

No	Sample	**Detected conc.** (ppb wet weight)				**p,p′-DDT recovery, (%)**
		Propiconaozles	Difenoconazoles	α-cypermethrin	β-cypermethrin	
1	R_1	–	–	–	–	84
2	R_2	–	–	–	105	97
3	R_3	–	–	–	–	82
3	R_4	–	–	–	112	80

– : lower than LOQ

The target pesticides were not detected in samples R1 and R3. Meanwhile, β-cypermethrin was found in both R2 and R4 mustard green samples at the residue levels of 105 ppb and 112 ppb wet weight, respectively. However, in comparison with the MRL for leafy vegetables (700 ppb wet weight, Table 1), these concentrations were acceptable. In addition, compared with the ADI value for cypermethrin (20 μg/kg body weight, Table 1, corresponding to 1.2 mg per day for a person of 60 kg), the detected residues were not likely to cause a health risk.

4 Conclusions

The developed analytical method helps to simultaneously quantify the currently used triazole fungicides, namely α-propiconazole, β-propiconazole and α-difenoconazole, β-difenoconazole,

and cypermethrin (including 4 isomers) – one commonly used pyrethroid insecticide – in mustard greens. This method exhibited low detection limits and high accuracy, suggesting its applicability in any analytical laboratory equipped with a GC–MS system.

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