A VALIDATED METHOD FOR INVESTIGATION OF THE EFFECT OF TIME AND TREATING CONCENTRATION ON THE PESTICIDE RESIDUES IN MUSTARD BASED ON THE MULTILEVEL FACTORIAL MODEL AND GC-MS/MS

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ABSTRACT

This work assesses the validated analytical method for investigation of the effect of time and treating concentration of some pesticides on their residue level in vegetables for further prediction of the harvesting time of safety products. The analytical method for the determination of pesticides in the vegetable was validated based on GC-MS/MS. The obtained merits of the method showed high accuracy in the simultaneous determination of chlorpyrifos and carbosulfan in mustard. A multilevel factorial model was designed with two factors, time and treatment concentration. The using concentration was from 32.62 mg/kg to 90.52 mg/kg for chlorpyrifos and was from 13.11 mg/kg to 43.66 mg/kg for carbosulfan. The experimental model resulted in the P-values for time and concentration that were considerably lower than 0.05, confirming that these two factors were closely associated with the pesticide residue in the vegetables at the 95.0% confidence level. Additionally, the correlation coefficients (R^2) were above 95.4%, demonstrating that the selected experimental model is a good fit. Based on the maximum residue levels acceptable for each pesticide, the vegetables can be harvested only 14 days after applying the pesticides containing chlorpyrifos and carbosulfan. Changing target pesticides or agricultural products will permit the use of this experimental model in investigations of other pesticides or other types of crops. The results provide an effective and accurate experimental model that can be used in relevant studies.

Keywords: pesticide residues, carbosulfan, chlorpyrifos, experimental design, GC-MS/MS.

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INTRODUCTION

Pesticides are widely used to control weeds and insect infestation in agricultural fields. Pesticides are an integral part of intensive agriculture due to the need to increase food supply.¹ Improper use of pesticides in cultivation is the leading cause of the pesticide levels in the products exceeding the maximum residue limits. Although the use of the majority of pesticides is regulated, some uncontrolled cultivation conditions may influence the pesticide residues of the products. Consequently, the dissipation of the pesticide residues and their residue levels in vegetables need to be investigated.

Recently, numerous studies have relied on the assessment of the levels of pesticides in vegetables and many types of crops.²⁻⁶ These studies described the statistics of the pesticide residues on each specific agricultural product, and pesticide contamination is specific for each study. Furthermore, the dissipation of the pesticide residue in vegetables is a complex process that depends on various crops and various field conditions, including concentration and application time.⁷⁻¹⁰ However, the effects of time and concentration on pesticide residue dissipation in vegetables have not been investigated by a multilevel factorial analysis to predict harvesting time for a safe product. The present study is based on an experimental design using GC-MS/MS determination of pesticides in vegetables.^{11,12} A multilevel factorial model is selected to assess the effects of time and treatment concentration on pesticide residue. The preharvest time for vegetables can be

calculated from the correlation equations based on the maximum acceptable residue limits and initial using dose of pesticides, which will be helpful to control the harmful effects of pesticide residues in vegetables.

The experimental design used chlorpyrifos, a non-systemic organophosphorus insecticide, as a conventional pesticide.¹³ This pesticide is highly toxic to fish and aquatic invertebrates, and the hydrolysis rate of this pesticide increases with temperature and alkalinity.^{14,15} Another pesticide used in the experimental model was carbosulfan. It is a benzofuranyl methylcarbamate pesticide used to control insects, mites, and nematodes.^{16,17} Although carbosulfan is banned in Europe, it is still widely used in other countries, such as Mexico, Brazil, Thailand, and Vietnam.

The multilevel factorial model was designed for chlorpyrifos and carbosulfan in mustard under real weather conditions of cultivation. Simultaneous changes in time and treatment concentration permit a small number of experiments that can save considerable time. The waiting period for harvesting mustard at a specific treatment concentration can be easily calculated using the highly accurate correlation equation. Additionally, this experimental model can be easily extended by changing target pesticides or other toxicological parameters to investigate the residue of pesticides or accumulation of toxicants in the target agricultural products.

EXPERIMENTAL

Materials and Reagents

All reagents used in this work were of analytical grade. Chlorpyrifos ($\geq 99.3\%$) and carbosulfan ($\geq 99.4\%$) were purchased from Sigma Aldrich (Singapore). Trifluraline-D14 ($\geq 98.6\%$) was supplied by Dr Ehrenstorfer™ (Germany). Ethylacetate ($\geq 99\%$) and acetonitrile (ACN) ($\geq 99\%$) were purchased from Merck (Merck, Darmstadt, Germany). An Agilent QuEChERS extraction kit (4 g MgSO₄, 1 g NaCl) was purchased from Agilent Technologies (USA). Dispersive SPE 2 ml clean-up tubes (150 mg MgSO₄, 50 mg PSA, 50 mg GCB, and 50 mg C18) and part number SBEQ-CA8642 were purchased from CNW Technologies (China). BOP 600 EC commercial pesticide used in this work contained 400 g/L chlorpyrifos and 200 g/L carbosulfan and was supplied by ADC Ltd. (Vietnam).

GC-MS/MS Analysis

Vegetables were sampled according to the guidelines of the FAO and United States Department of Agriculture.^{18,19} Five single mustard samples were collected according to the zigzag rule (average ~250 g per single mustard). These single samples were chopped and mixed into a mixture sample. The mixture was ground and divided into two PE bags. One bag was used for sample preparation and analysis within 24 h, and another bag was stored at -18°C.

Residues of pesticides in vegetables were extracted and analyzed according to the methods of AOAC 2007.01 and 5991-6054EN.^{20,21} 10mL of ACN was added to a 50 ml centrifuge tube containing 5 g of ground vegetable sample and 100 μ l of 1 μ g/L trifluraline-D14 (internal standard), and then the mixture was vortexed for 1 min by a mixer (Talboys, USA). The mixture was shaken again for 1 min after adding 10 ml ACN to confirm that the pesticides in the vegetable samples are diluted into ACN. The ACN phase was separated from the water phase by vortexing with 6.5 g of Agilent QuEChERS for 1 min and centrifuging at 5,000 rpm for 5 min. One millilitre of the ACN extract was cleaned by an SPE column (p/n: SBEQ-CA8642-H). The obtained solution was centrifuged at 5,000 rpm for 5 min and then filtered through a PTFE membrane with a pore diameter of 0.45 μ m (p/n: SBEQ-CF3101). The final solution was analyzed by GC/MS/MS under the operating conditions shown in Table-1.

Table-1: GC-MS/MS Operating Conditions for the Determination of Pesticides in Vegetable

Gas Chromatography	Trace 1300 Thermo Scientific
Autosampler	TriPlus RSH Thermo Scientific
Column	Agilent J&W DB -5MS UI, 30 m $\times$ 0.32 mm, 0.5 μ m (p/n 123-5536UI)
Inlet mode	Splitless
Inlet pressure	165.47 KPa
Inlet temperature	250 °C
Purge flow	5 mL/min
Gas saver flow	20 mL/min

Carrier gas	Helium, 2.5 mL/min
Oven temperature	75 °C (0 min); 25 °C/min to 150 °C (0 min); 10 °C/min to 280 °C (4 mins)
Injection volume	2 µL
Mass spectrometer	TSQ 8000 Thermo Scientific
Mode	Electron Impact (EI)
Transfer line temperature	290 °C
Ion source temperature	250 °C
Collision gas	Argon

Method Validation

The merits of the method were consecutively evaluated according to the guidelines of ISO 17025:2017 and AOAC 2016.^{22,23} The specificity of the method was evaluated by analyzing blank and standard-spiked samples. The selected reaction monitoring (SRM) chromatograms were used to confirm that precursor or product fragment signals did not interfere with pesticide detection. The blank matrix was used to conduct calibration that minimized the matrix effect on the analytical signal. Various concentrations of chlorpyrifos and carbosulfan and 100 µl of 1 µg/L trifluraline-D14 were simultaneously spiked to the blank matrix before extraction and analysis under optimal conditions (Table-1). MDL was calculated as three times the standard deviation observed from eleven replicate experiments at three spiked levels of pesticides and corrected by the relative ratio of the quantification ion response signal to the confirmation ion signal. The trueness was determined from the recovery assay results of samples spiked with pesticides at three levels, including 0.01, 0.05, and 0.10 mg/kg (n = 6) for chlorpyrifos and 0.05, 0.10, and 0.15 mg/kg for carbosulfan, on three different days by two analysts. Recoveries were calculated by comparing the concentrations of the extracted pesticides with those of the calibration curves. These data were also used to determine the repeatability and intermediate precision of the method and to quantify the measurement uncertainty (MU).

Experimental Model Design

The multilevel factorial model was designed with two factors: concentration and treatment time of mustard with pesticides. Treatment time was designed with six levels, including 0, 7, 10, 12, 14, and 16 days after pesticide treatment. Mustard was treated with pesticides on day 30 after seed drilling. The pesticide concentration was designed with three initial levels of 32.62, 62.82, and 91.52 mg/kg for chlorpyrifos and 13.11, 24.23, and 43.66 mg/kg for carbosulfan. These concentrations were residues of pesticides in mustard immediately after application (day 0). The application dosages were prepared by diluting 20, 40, and 60 ml of commercial pesticide (BOP 600 EC, ADC Ltd, Vietnam) in 10 l of water. The first level corresponded to the application dosage described in the instructions for the use of this commercial pesticide. Mustard was cultivated under actual weather conditions from October to December 2019 in Binh Duong Province (southern Vietnam). This period corresponds to the dry season with the average temperature ranging from 26.1 °C to 29.0 °C and humidity from 75% to 79%.²⁴

RESULTS AND DISCUSSION

Analytical Method Validation

The method specificity was demonstrated by recoding chromatography spectra in the full scan mode with a mass scan range of 50-560 and the selected reaction monitoring (SRM) mode for pesticides. Each analyte was defined by a search of the database of NIST MS version 2.2. The retention time, specific fragments, precursor fragments, and product fragments are presented in Table-2.

Table-2: Characterization of Analytes by GC-MS/MS

Analyte	Retention Time (min)	Full Scan Fragment	Quantification Transition (CE ^a , V)	Confirmation Transition (CE ^a , V)
Trifluraline-D14	8.99	163, 267, 315	315 > 267 (10)	315 > 163 (25)
Chlorpyrifos	12.40	97, 107, 169, 197, 258, 314	197 > 169 (14)	197 > 107 (40)
Carbosulfan	16.61	76, 107, 118, 135, 163, 164	164 > 149 (10)	164 > 103 (25)

^aCollision energy

SRM chromatograms showed that there was no signal of the quantification fragment (197 > 169) at retention time from 11.5 min to 13.5 min for the blank sample. In contrast, spiking 0.003 mg/kg chlorpyrifos into the blank sample resulted in the appearance of a single peak at 12.42 min (Fig.-1a). This result confirmed that there was no interference signal of the blank matrix. A similar result was obtained for carbosulfan, the chromatogram of the qualification fragment (164 > 149) was recorded, and there was no peak at retention time from 15.7 min to 17.8 min. A single peak was observed at 16.64 min during the analysis after spiking the sample with 0.010 mg/kg carbosulfan (Fig.-1b). To further verify the method specificity, chromatograms of the confirmation fragments were also recorded (197 > 107 for chlorpyrifos and 164 > 103 for carbosulfan). There were no signals in the blank sample, and the signals appeared in the spiked sample (Fig.-2). These results confirm the high specificity of the method of detection of chlorpyrifos and carbosulfan in the mustard samples.

Fig.-1: Representative GC-MS/MS chromatograms for specificity verification: (a) Chromatogram monitoring of the quantification fragment 169 (197>169) of the blank sample and blank sample spiked with 0.003 mg/kg chlorpyrifos. (b) Chromatogram monitoring of the quantification fragment 149 (164>149) of the blank sample and blank sample spiked with 0.010 mg/kg carbosulfan

The calibration curves, method quantitation limit (MQL), recovery, and uncertainty measurement were estimated in Table-3. The regression coefficient of the calibration curves for both pesticides was 0.999, and the values were stable on different days or different analysts performed the assay. MDL was 0.003 mg/kg for chlorpyrifos and 0.013 mg/kg for carbosulfan. These values of MDL were below the MRL of each pesticide. The quantification ion fragment signal ratio relative to the confirmation ion fragment signal at the MDL level was 0.63 for chlorpyrifos and 0.81 for carbosulfan, which were united with the quantitation concentration range. The trueness was validated by intra-laboratory reproducibility (% recovery). The recovery of two pesticides ranged from 90 to 98%, and the repeatability within-day (RSD%) was from

1.74% to 4.60%. The inter-day repeatability was 3.72 - 5.16%. The uncertainty measurement was calculated based on the "top-down" approach.^{25,26} The main sources of uncertainty for the method were precision, bias, and calibration curve. The uncertainty of the measurements was 12.9% and 14.5% for chlorpyrifos and carbosulfan, respectively. All of the merits show the high accuracy analytical method for determining chlorpyrifos and carbosulfan in mustard.

Fig.- 2: Representative GC-MS/MS Chromatograms for the Specificity Verification: (a) Chromatogram monitoring of the quantification fragment 107 (197 > 107) of the blank sample and blank sample spiked with 0.003 mg/kg chlorpyrifos. (b) Chromatogram monitoring of the quantification fragment 103 (164 > 103) of the blank sample and blank sample spiked with 0.010 mg/kg carbosulfan

Table-3: Multilevel Factorial Experimental Design for the Residue of Pesticides in Mustard

Parameters	Chlorpyrifos	Carbosulfan
Calibration range (mg/L)	0.01 – 0.2	0.05 – 0.3
Correlation Coefficient, R^2	0.999	0.999
Method detection limit, MDL (mg/kg)	0.003	0.013
Intra-laboratory reproducibility, recovery (%)	91 – 97	90 – 98
Repeatability within-day, RSD (%)	1.74 – 3.49	2.83 – 4.60
Repeatability inter-day, RSD (%)	3.49 – 4.88	3.72 – 5.16
Uncertainty measurement (%), $k=2$	12.9	14.5

Multilevel Factorial Experimental Design and Analysis

The experimental matrix was designed based on the multilevel factorial model. For each pesticide, eighteen experiments were carried out at three concentrations and six levels of application time. The experimental data are presented in Table-4.

Table-4: Multilevel Factorial Experimental Design for the Residue of Pesticides in Mustard

Experiment	Time (Days)	Residues (mg/kg)	
		Chlorpyrifos	Carbosulfan
1	0	32.622	13.111
2	0	62.816	24.229
3	0	91.523	43.659
4	7	3.545	1.701
5	7	7.458	3.013
6	7	12.353	4.625
7	10	0.302	0.145
8	10	0.631	0.256
9	10	1.268	0.474
10	12	0.161	0.085
11	12	0.414	0.189
12	12	0.761	0.268
13	14	0.015	0.043
14	14	0.087	0.089
15	14	0.139	0.112
16	16	0.013	0.027
17	16	0.062	0.032
18	16	0.087	0.040

The data were analyzed by Statgraphics-18@ software to calculate the regression equations for the residue of pesticides as a function of the concentration and time of application. These equations were derived based on the least-squares method and are shown as Eqn.-1 and Eqn.-2.

$$\text{Residue}_{\text{Chlo.}} = 11.010 - 6.27385 \cdot \text{Time} + 0.84265 \cdot \text{Conc} + 0.39854 \cdot \text{Time}^2 - 0.061921 \cdot \text{Time} \cdot \text{Conc} - 0.000299 \cdot \text{Conc}^2 \quad (1)$$

$$\text{Residue}_{\text{Carbo.}} = 5.309 - 2.75603 \cdot \text{Time} + 0.80022 \cdot \text{Conc} + 0.17502 \cdot \text{Time}^2 - 0.061003 \cdot \text{Time} \cdot \text{Conc} - 0.00026 \cdot \text{Conc}^2 \quad (2)$$

The quadratic equations show the correlation between the residues with time and concentration for chlorpyrifos and carbosulfan. The multiplier of the concentration is 0.84265 for chlorpyrifos and 0.80022 for carbosulfan. This coefficient indicates that dosage increases the residue of pesticides. Time coefficients of -6.27385 and -2.75603 correspond to a rapid reduction in the residue levels of pesticides. The quadratic factors (time squared and concentration squared) included only the effect of time squared on the residue with the multipliers of 0.39854 and 0.17502. This result confirms the time and application concentrations that can be used to control the residues of pesticides in vegetable products. The estimated response surface for residues is illustrated in Fig.-3.

Fig.-3: Estimated Response Surface of the Regression Equation. The residues of Chlorpyrifos (a) and Carbosulfan (b) depend on Time and Treatment Concentration

Table-5: ANOVA of Residue Levels of Chlorpyrifos and Carbosulfan

Source	Sum of squares	Df	Mean square	F-Ratio	P-Value
Chlorpyrifos					
A: Time	3438.5	1	3438.5	121.34	0.0000
B: Conc	622.708	1	622.708	21.98	0.0005
AA	1925.24	1	1925.24	67.94	0.0000
AB	1096.98	1	1096.98	38.71	0.0000
BB	0.268688	1	0.268688	0.01	0.9240
Total error	340.042	12	28.3368		
Total (corr.)	11059.9	17			
Carbosulfan					
A: Time	719.299	1	719.299	83.47	0.0000
B: Conc	153.182	1	153.182	17.78	0.0012
AA	371.272	1	371.272	43.09	0.0000
AB	294.397	1	294.397	34.16	0.0001
BB	0.012436	1	0.012436	0.00	0.9703
Total error	103.405	12	8.61708		
Total (corr.)	2227.64	17			

The correlation coefficients (R^2) of Eqns.-1 and 2 were 96.93% and 95.36%. This indicates a strong correlation between the residues of pesticides and time and concentration and confirms that the experimental model design is a very good fit. ANOVA estimates a statistical significance of each effect by comparison of the mean square to an estimate of the experimental error (Table-5). In this case, four effects of time, conc, time.conc, and time squared in the regression equation for both pesticides have P-values less than 0.05 (variable significant if p-value < 0.05). This result demonstrates that the effects are significantly different from zero at the 95.0% confidence level (highlighted in red). The concentration squared effect has a P-value higher than 0.924, indicating that it does not influence the residue of pesticides. The effects of

these factors are visualized in Fig.-4, and there are four columns over the red line at the confidence level of 95%. Overall, the results demonstrate a good fit of the experimental model for investigating the effect of time and concentration of the treatment on pesticide residue in mustard.

Fig.-4: Standardized Pareto Chart illustrating the effect of time and concentration on the residues of chlorpyrifos and carbosulfan. The factors associated with a decrease in the residues of pesticides are blue and the factors associated with an increase are grey.

Prediction of the Safety Waiting Period for Harvesting Mustard

The obtained regression equations were used to predict the waiting period of harvesting mustard after pesticide application to apply the experimental model. The preharvest interval can be calculated with known pesticide residue (MRL) and the initial dosage (according to the instructions for commercial pesticides). Based on the maximum level residue acceptable for chlorpyrifos of 0.01 mg/kg,²⁷ the application dosage of chlorpyrifos was estimated to be 32.62 mg/kg, and the preharvest time was calculated to be 13.94 days. The waiting period for carbosulfan was 13.82 days if the applied concentration was 13.111 mg/kg, and the residue was 0.05 mg/kg.²⁸ The calculated time indicates that vegetables should be harvested only after 14 days after the application of a commercial pesticide containing chlorpyrifos and carbosulfan to ensure the safety of the products. The observed preharvest time for mustard is shorter than for rice and tomato fruits by the difference in cultivation conditions.^{29,30}

CONCLUSION

A multilevel factorial experiment model has been designed for investigation of the effects of concentration and treatment time on the pesticide residues based on GC-MS/MS. The obtained correlation equations with highly significant correlation coefficients show that the experimental design is a good fit. From analyzing the model, the safety preharvest time for vegetables after treatment with pesticides can be easily calculated based on known application dosage and maximum level residue acceptable of the pesticides. The model can save considerable time for observation and can be used to accurately detect the effects of simultaneous changes in the treatment time and application concentration. Changing target pesticides or other toxins

enables the use of this experimental model for the investigation of the residues or accumulation of toxicants in agricultural products.

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