Application of Multivariate Data Analysis to the Determination of Multiclass Pesticide Residues in Fruits and Vegetables using Headspace Solid-Phase Microextraction Gas Chromatography-Mass Spectrometry

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Abstract

Design of experiment (DOE) was employed to develop a headspace solid phase microextraction gas chromatography-mass spectrometry (HS-SPME/GC-MS) method for pesticide residues analysis. The significance of SPME parameters was determined using Plackett-Burman (P-B) design. The main effect and the interaction effect of the significant factors were also determined followed by the optimization of the significant factors using central composite design (CCD). A Minitab® statistical software was used to generate both the 2^{7-4} Plackett-Burman and the central composite design matrix. The same statistical software was also employed in the determination of the optimum level of the significant parameters using surface response optimizer and desirability surface plot. The most significant factors are: extraction temperature (90%), extraction time (80%), the pH and stirring rate (50% and 60% respectively). The optimum parameters are: Temperature, 62 °C; time, 34 min; NaCl, 10%; stirring, 350 rpm; pH, 6; desorption time, 7 min; desorption temperature, 270 °C. The figures of merit of analytical methodologies were determined using an internal standard calibration method. The linearity of the developed method ranges from 1- 500 µg/kg with correlation coefficient (R^2) greater than 0.99. The average recovery was found to be between 74–115% and relative standard deviation ranges from 1.1–14%. The developed method was used to analyze 14 multiclass pesticide residues in two fruit (pear and grape) and two vegetable (lettuce and broccoli) samples, and the method was found to be satisfactory with LOD between 0.17–7.34 µg/kg and LOQ ranges from 0.55–24.50 µg/kg.

Keywords: *Design of experiment (DOE); Solid phase microextraction; Response surface optimizer; Pesticide residues; Central composite Design.*

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Introduction

Pesticides refer to all natural and synthetic chemicals that are used to prevent, destroy, repel or fight crop pest and vector of plant diseases.[1] They are mostly organic compounds with different functional groups, forming various types of isomeric compounds.[2] They differ in their substitution groups, degree of ionization, octanol/water coefficients, polarity, volatility and their solubility. The production and applications of pesticides in agriculture and non-agricultural purposes has in no doubt led to a steady increase in food production, high food quality and reduced incidence of illness due to insect-borne diseases. Pesticides are unavoidable inputs in agriculture and public health that are produced in large quantities since the end of World War II.[3,4] The benefits of using pesticides have been demonstrated through the increase in global agricultural production, eradication of insect borne and epidemic diseases as well as in conserving the ecosystem.[5] However, occupational and accidental exposure to pesticides has been observed to lead to a wide variety of chronic effects such as endocrine disorder, blood disorder and genetic change.

Solid phase microextraction is a solvent-free sample preparation method which combines sample preparation, isolation, concentration and enrichment into one step.[7] Pesticide residues analyses in fruit and vegetable samples have been investigated using different microextraction techniques and subsequent instrumental analysis using gas chromatography, liquid chromatography and capillary electrophoresis.[8,9] The Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method was used to analyze multiclass pesticide residues in fruits and vegetables produced in Shangai.[10] Two-phase hollow fiber solid phase microextraction was also used for the analysis of pyrethroid pesticides in fruits and vegetables.[11] The analysis of fungicides in water and fruit samples was also carried out using a combination of solvents extractants for dispersive liquid-liquid microextraction coupled to liquid chromatography with tandem mass spectrometry.[12] Other microextraction techniques do involve the use of microliter volumes of solvents, some of which are toxic.

Design of Experiment is a chemometric approach used to design experimental runs, identifies significant factors, and estimates the main and interaction effect of various factors under study.[13] It requires few experimental runs that are carried out in an orderly manner, saving thus analysis time and improving sample throughput.[14] The One Factor at a Time (OFAT) approach requires optimizing each factor at a time; the other factors are kept constant while varying the factor being optimized,[13] which has been shown to produce misleading results.[15] The use of chemometrics in the optimization of microextraction parameters for the analysis of pesticide residues in fruits and vegetables was also reviewed by our group.[16]

In our present study, the Plackett-Burman (P-B) Design is employed to determine the factors that significantly affect the efficient extraction of 14 multiclass pesticide residues in pear, grape, lettuce and broccoli, while central composite design (CCD) was used to determine the optimum values of the significant parameters.

Experimental

Reagents and Solutions

Pesticide standards (fenobucarb, ethoprop, diazinon, chlorothalonil, fenitrothion, methyl parathion, chlorpyrifos, thiobencarb, quinalphos, endosulfan I, endosulfan II, bifenthrin, fenpropathrin and permethrin) at 100 µg/mL and 1-chloro-3-nitrolbenzene (1000 µg/mL) used as internal standards were purchased in more than 95% purity from AccuStandard. A working standard solution containing the pesticides was prepared daily by diluting the stock solution in methanol to a concentration of 10 µg/mL; the solution was then kept at 4 ^{o}C until used. All solvents used (methanol, acetone and acetonitrile) were pesticide grade and were purchased from Fisher Scientific. Sodium chloride and ammonium chloride were purchased from Merck. The pH buffer solutions 4, 6, 8–10 and 5–7 were purchased from Fisher Scientific and Sigma-Aldrich, respectively. Millipore filtered (0.45 µm) deionized water was used for method development.

Sample Preparation

For the solid phase microextraction method development, 100 g of pesticide free fruits and vegetables obtained from Malaysian night and hypermarkets, were accurately weighed, finely chopped and homogenized in a blender. A known aliquot of the homogenized sample was then weighed into a separate 20 mL amber glass vial containing the internal standard and diluted accurately with Milli-Q filtered deionized water containing 10% of NaCl to make up a total mass of 5 g. The mixture was then spiked with a known amount of the working standard solution to prepare a concentration of 50 µg/kg used for validation studies. Fruit and vegetable samples used for method development, calibration and recovery studies were first analyzed to ensure the absence of the target pesticide residues.[17]

Headspace-Solid Phase Microextraction Procedure

The SPME fibers (100 µm PDMS), purchased from Supleco, were conditioned in the GC/MS injection at 250 ^{o}C for 30 min prior to their first use as recommended by the manufacturer. Optimization of parameters and analysis were performed in a 20 mL amber glass vial containing 5 mL of Milli-Q filtered deionized water containing 10% of NaCl and spiked with 50 µL of the working standard solution to give a concentration of 0.1 µg/mL. To exract the target pesticides, the vial containing the sample spiked with pesticide standard was shaken ultrasonically for 5 min, then agitated and incubated for 5 min at 60 ^{o}C in the autosampler agitator, followed by the exposure of the fiber to the

headspace of the sample in the vial sealed with PTFE/silicone septum. The analytes extracted onto the SPME coated fiber were desorbed into the injection port for GC-MS analysis

GC-MS Analysis

The extraction and analysis of pesticides was carried out with a CTC CombiPAL autosampler equipped with an agitator and needle heater (for fiber conditioning and inter-extraction clean up) coupled to a GC-MS (Shimadzu QP2010Series) and operated in the split/splitless mode at an injection temperature of 270 ^{o}C. The separation of target analytes was achieved on a DB-5MS fused capillary column containing 5% diphenyl and 95% dimethylpolysiloxane (30 m x 0.25 mm i.d. x 0.25 µm film thickness). The injection port of the GC was equipped with a high-pressure Merlin Microseal septumless injection kit and a silanized narrow bore liner (78.5 m x 6.5 mm o.d x 0.75 mm i.d). Helium (carrier gas) was set to a constant flow rate of 1.3 mL/min with linear velocity of 42 cm/sec. The GC column oven temperature program was set as follows: Initial temperature was set at 60 ^{o}C for 2 min, ramped at 30 ^{o}C/min to 180 ^{o}C, and then ramped to 270 ^{o}C at 5 ^{o}C/min where it was held constant for 5 min. The MS operation conditions include the transfer line at 300 ^{o}C, the ion source at 200 ^{o}C and electron ionization (EI) of 70 eV. A target ion (most abundance ion) and two other reference ions were monitored for the target analytes (Table 1). The investigated pesticides were identified by comparing the mass spectrum obtained for each analyte to that of the reference compound in GC-MS library using the US National Institute of Standard and Technology (NIST) and PESTANA libraries search. The Plackett-Burnan (P-B) and the central composite design matrices were performed and estimated with Minitab® statistical software package version 16 (Minitab Inc., State College, USA).

Results and Discussion

The data of the SPME analysis of pesticide residues in pear, grape, lettuce and broccoli are summarized in Tables 1-3.

Table 1: Chromatography data, Linearity range and R^2 of the developed HS-SPME/GC-MS method in pear, grape, lettuce and broccoli samples.

Analytes	Ret. Time (min)	Ion (m/z)	Linearity (µg/kg)	R^2			
				Pear	Grape	Lettuce	Broccoli
Fenobucarb	8.81	121, 91, 150	2.5 – 500	0.9918	0.9979	0.9955	0.9976
Ethoprophos	9.13	158, 97, 139	2.5 – 250	0.9979	0.9980	0.9961	0.9945
Diazinone	11.04	179, 137, 152	2.5 – 250	0.9980	0.9989	0.9978	0.9958
Chlorothalonil	11.31	266, 263, 268	10 – 500	0.9989	0.9964	0.9973	0.9978
Parathion-m	12.81	109, 79, 125	1 – 250	0.9964	0.9952	0.9963	0.9973
Fenitrothion	13.70	125, 79, 109	2.5 – 200	0.9952	0.9985	0.9983	0.9963
Chlorpyrifos	14.34	97, 125, 197	5 – 500	0.9985	0.9985	0.9983	0.9985
Thiobencarb	14.50	100, 125, 127	5 – 250	0.9977	0.9978	0.9981	0.9988
Quinalphos	16.37	146, 118, 156	2.5 – 125	0.9964	0.9968	0.9990	0.9964
Endosulfan I	17.26	195, 207, 241	5 – 250	0.9976	0.9976	0.9970	0.9976
Endosulfan II	18.61	195, 159, 207	10 – 250	0.9988	0.9987	0.9980	0.9988
Bifenthrin	20.14	181, 166,	1 – 500	0.9982	0.9983	0.9985	0.9982
Fenpropathrin	20.31	97, 125, 181	1 – 50	0.9972	0.9972	0.9986	0.9927
Permethrin	22.21	183, 91, 163	5 – 100	0.9976	0.9973	0.9990	0.9973

Table (2): LOD and LOQ (µg/kg) of the developed HS-SPME/GC-MS method in pear, grape, lettuce and broccoli samples.

Analytes	LOD (µg/kg)				LOQ (µg/kg)			
	Pear	Grape	Lettuce	Broccoli	Pear	Grape	Lettuce	Broccoli
Fenobucarb	2.19	2.17	2.47	2.44	7.31	7.22	8.22	8.13
Ethoprophos	2.51	1.20	0.34	0.21	8.36	4.00	1.14	0.70
Diazinone	0.51	1.05	0.23	0.21	1.84	3.50	0.77	0.68
Chlorothalonil	4.76	0.43	0.51	7.34	15.86	1.44	1.84	24.50
Parathion-m	0.27	0.22	0.59	0.55	0.89	0.72	0.59	0.55
Fenitrothion	0.23	0.20	0.20	0.27	0.88	0.66	0.67	2.24
Chlorpyrifos	3.17	2.79	3.52	3.32	10.58	9.29	11.75	11.08
Thiobencarb	3.42	3.19	3.19	3.67	11.40	10.62	10.62	12.23
Quinalphos	2.41	2.24	2.05	1.86	8.03	7.47	6.83	6.20
Endosulfan I	2.76	3.45	2.27	2.93	9.20	11.50	7.57	9.77
Endosulfan II	2.71	3.28	3.06	2.34	9.03	10.95	10.20	7.80
Bifenthrin	0.17	0.75	0.64	0.67	0.60	2.50	2.14	2.22
Fenpropathrin	0.22	0.55	0.34	0.49	0.74	1.83	1.13	1.65
Permethrin	2.03	1.94	1.65	1.95	6.78	6.44	5.50	6.50

Table (3): Accuracy (Relative recoveries) and precision of the pesticides in pear, grape, lettuce and broccoli samples.

Analytes	Spike (µg/kg)	Pear		Grape		Lettuce		Broccoli	
		Accuracy (%)	RSD[a] (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
Fenobucarb	50	99.1	8.9	90.4	4.9	85.5	12.5	79.9	7.2
	100	104.0	5.0	80.7	6.9	80.7	10.9	76.8	1.7
	150	103.5	5.8	86.8	5.0	94.8	4.2	83.4	1.8
Ethoprophos	20	106.7	7.1	76.0	9.2	105.9	8.0	75.8	4.9
	50	103.9	3.6	79.9	5.1	85.2	4.1	75.6	1.8
	100	106.4	4.7	81.9	3.1	93.1	3.7	93.9	2.3
Diazinone	50	97.8	7.7	86.7	9.0	92.5	5.0	88.0	5.9
	100	994	8.4	87.3	4.9	86.4	4.6	88.3	2.4
	150	104.2	5.8	96.7	2.7	88.3	4.3	94.0	1.9
Chlorothalonil	50	89.6	5.6	80.7	8.6	82.1	8.1	90.3	5.9
	100	82.0	10.6	78.9	5.4	88.1	4.4	86.4	2.7
	150	100.5	6.0	89.4	6.3	87.5	4.0	97.2	1.5
Parathion-m	50	76.6	7.1	83.2	5.5	79.3	5.6	78.4	6.1
	100	76.4	14.0	87.5	5.2	74.2	5.0	88.5	4.5
	150	88.6	4.1	87.3	2.2	80.1	4.2	86.0	2.6
Fenitrothion	5	109.1	5.4	95.8	6.0	75.7	8.1	76.4	2.8
	10	108.7	3.9	103.2	4.7	86.2	3.4	86.88	3.1
	20	107.5	4.1	103.5	3.7	89.5	3.1	93.4	2.5
Chlorpyrifos	20	99.8	7.9	100.7	7.6	74.0	6.7	85.0	2.9
	50	104.1	4.0	105.8	5.4	78.6	5.0	94.9	1.8
	100	102.2	3.6	111.0	2.5	86.6	3.7	98.9	1.2
Thiobencarb	50	88.0	9.7	94.8	6.2	77.7	9.3	94.7	3.1
	100	95.4	4.7	92.1	2.9	80.0	4.9	95.8	2.7
	150	95.0	5.4	98.2	3.2	85.3	2.2	97.4	1.7
Quinalphos	20	105.8	5.1	103.0	8.0	82.1	6.1	86.5	7.6
	50	105.9	2.6	108.5	1.9	82.7	5.2	94.7	4.5
	100	102.9	2.8	110.3	1.7	78.0	4.4	92.8	4.6
Endosulfan I	50	99.1	7.0	101.6	3.9	74.4	5.4	93.4	4.5
	100	102.4	5.6	102.4	2.2	76.4	2.6	95.6	2.0
	150	104.1	3.4	104.2	1.9	91.3	2.1	98.6	2.6
Endosulfan II	50	97.6	5.4	101.8	3.3	75.6	5.7	90.4	3.4
	100	103.1	4.4	105.6	2.7	85.1	3.1	95.3	3.1
	150	102.4	4.3	102.22	2.4	91.0	1.4	96.1	3.8
Bifenthrin	50	101.8	4.7	108.0	3.3	75.3	2.4	115.7	5.1
	100	104.6	3.0	107.5	3.1	85.1	2.9	101.3	5.4
	150	103.9	2.5	110.4	1.5	91.0	1.6	94.2	4.4
Fenpropathrin	5	79.9	3.4	93.3	2.9	113.6	4.0	109.8	9.4
	10	93.9	8.1	95.1	8.8	95.5	9.6	99.8	11.1
	20	96.2	9.8	98.5	7.9	83.9	7.3	95.8	2.4

Analytes	Spike (µg/kg)	Pear		Grape		Lettuce		Broccoli	
		Accuracy (%)	RSD[a] (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
Permethrin	20	95.7	11.2	102.4	8.8	80.6	8.0	95.4	8.0
	50	103.4	7.0	107.9	6.6	76.0	5.2	79.8	4.7
	100	104.6	3.3	109.9	3.1	83.5	5.8	99.7	4.1
Range	5-150	76.4-108.9	2.4-14	76-111	1.5-9	74-113.6	1.4-12.5	75.6-115.7	1.1-11.1

[a] RSD, Relative standard deviation.

The significant factors affecting the SPME analysis of pesticide residues in fruit and vegetables samples were developed in our previous study using the Plackett-Burman Design followed by optimization of the significant factors.[18] The ruggedness of the developed method was determined by using the developed method for the determination of 14 multiclass pesticide residues in pear, grape, lettuce and broccoli. The interaction effect of the significant factors was also analyzed.

Plackett-Burman (P-B) Design

The normal plot of standardized effect (Fig. 1a) shows that the extraction temperature has the most significant effect with about 90%, followed by the extraction time with 80%, the pH and stirring rate have an average effect (50% and 60%, respectively). The extraction temperature has been observed to have a dual effect on the extraction efficiency since it enhances the transport of analytes and, at the same time, causes the distribution coefficient to decrease,[19] the reason why it must be carefully optimized.

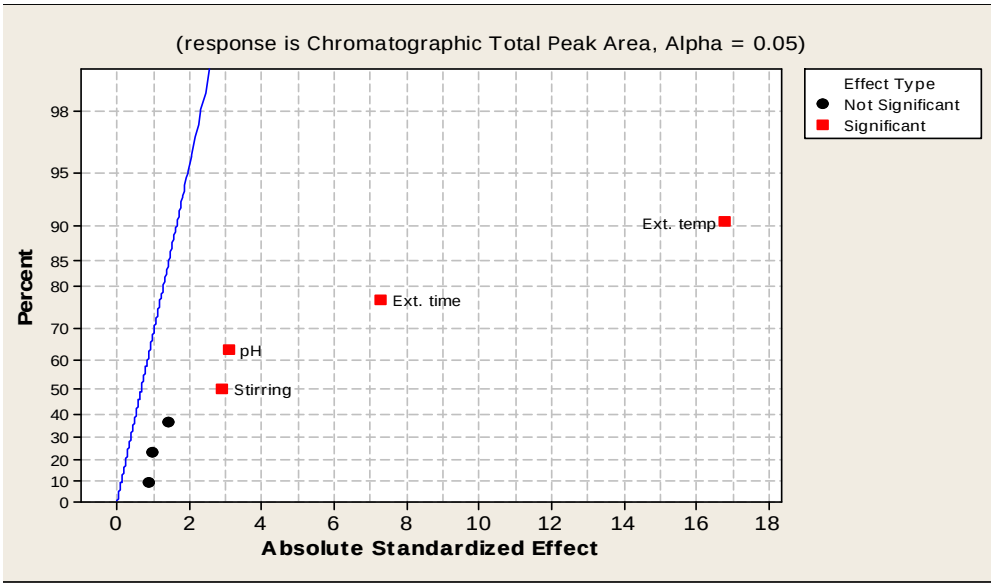


Fig 1 (a): Normal Plot of Standardized Effect of TCPA.

The interaction plot (Fig. 1b) of the significant factors shows that the total chromatographic peak area (TCPA) increases when the extraction time changes from low level to high level. This effect is however much larger at 60 ^{o}C than at 30 ^{o}C

reflecting the significance of temperature effect. The extraction temperature showed a negative effect on the stirring rate and pH at higher temperature (i.e. the total chromatographic peak area (TCPA) is reduced as the stirring rate and pH moved from low level to high level), while it showed a positive effect at lower temperature. The extraction time showed also a negative effect on the stirring rate and pH at high level, while it showed a positive effect at low level. The stirring rate showed a negative effect on the pH at high level, while the effect was negative at low level. It can be concluded that any interaction between the extraction parameters can either diminish or magnify the total chromatographic peak area (TCPA). Therefore, the significant factors must be carefully optimized.

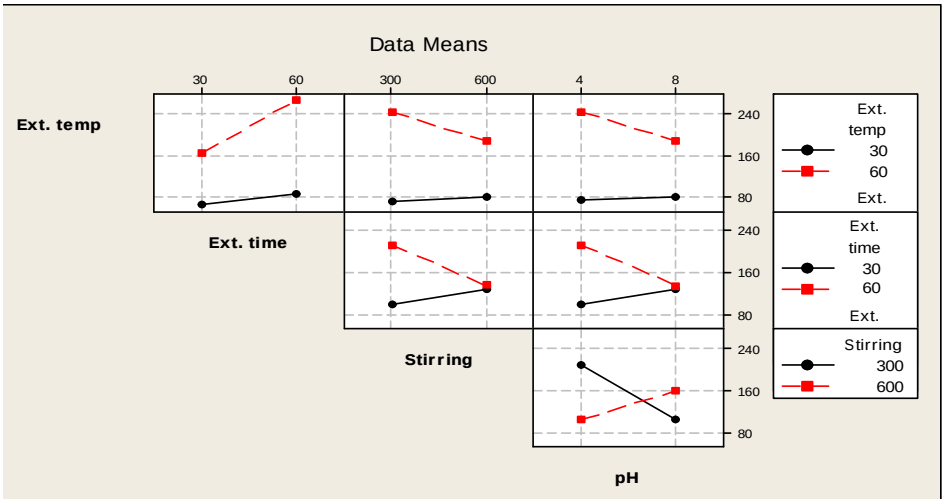


Fig 1 (b): Interaction Plot of TCPA.

Central Composite Design (CCD)

The significant factors were optimized using the CCD approach. The residual plot (Fig 1c) shows that the measurement deviation is randomly distributed around the mean; the global desirability surface response in 3D plot was obtained for the optimized parameters (Fig. 1d). In the contour plot of two-way interactions (Fig. 2), it is to notice that the factors that are not in the plot were held constant. The contour plot of pH and stirring rate showed that the highest response (TCPA) is obtained at pH 2-5 and a stirring rate greater than 700 rpm. The highest TCPA for the stirring rate versus extraction temperature lies at stirring rates between 200–600 rpm and an extraction temperature of approximately 70 ^{o}C. Optimal TCPA for pH versus extraction temperature is at temperatures greater than 70 ^{o}C and pH 2-10. The extraction time versus extraction temperature best TCPA is after 30–70 min of extraction at temperatures greater than 70 ^{o}C. The highest TCPA response for pH versus extraction time is in the pH range 2–10 at 50–60 ^{o}C, while the contour plot for the stirring rate versus extraction time shows the best TCPA at a stirring rate greater than 600 rpm and an extraction time greater than 60 min. The second order response is utilized because of its flexibility and ability to give an approximation of the true value; the parameters

can thus be easily estimated.[16] Taking into account the univariate and multivariate results, the optimal extraction conditions are: Temperature, 62 ^{o}C; time of extraction, 34 min; salt addition, 10%; stirring rate, 350 rpm; pH, 6; desorption time, 7 min; desorption temperature, 270 ^{o}C.

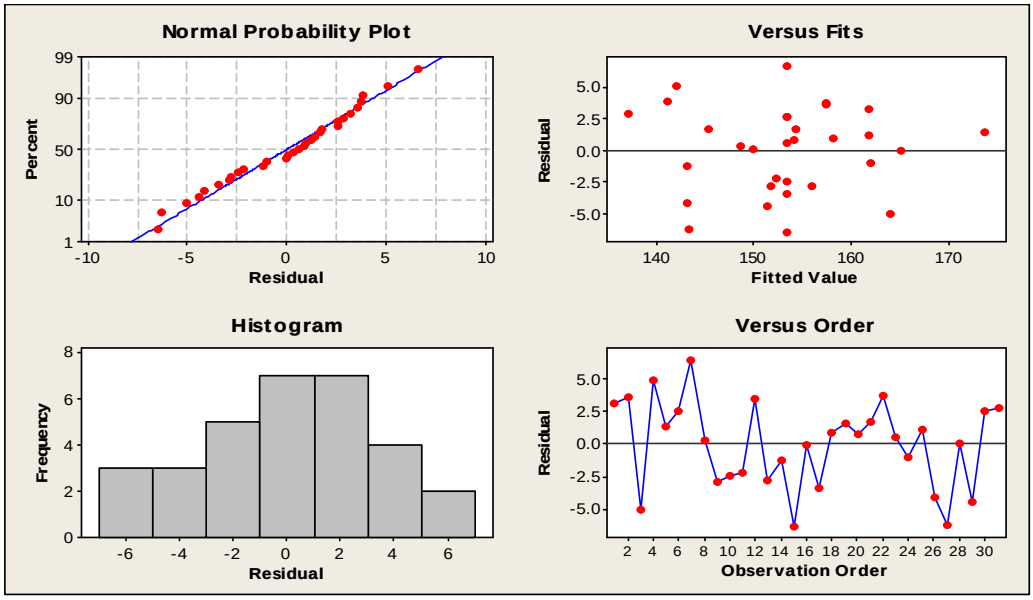
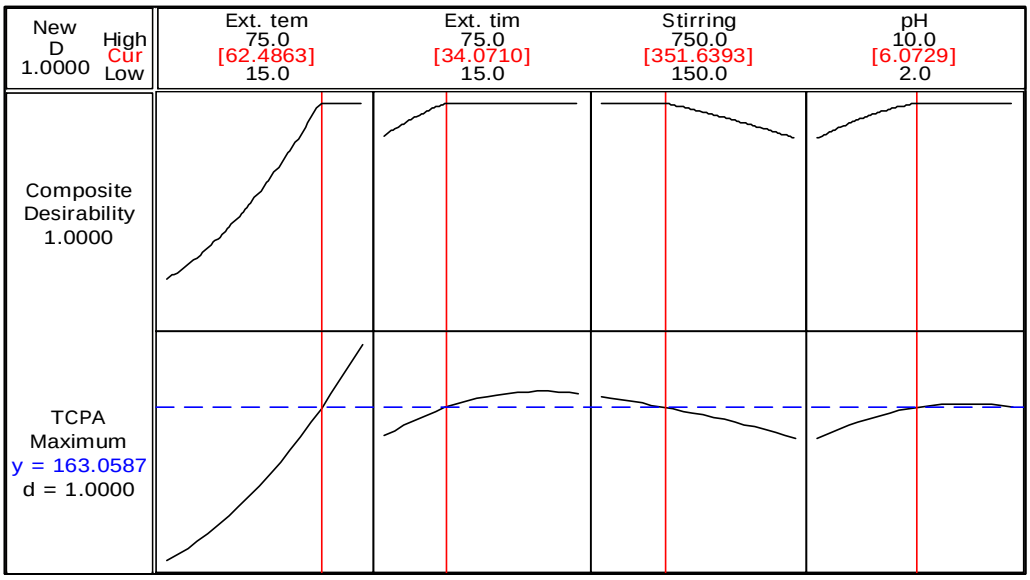


Fig 1 (c): Residual Plots for TCPA in Plackett-Burman Design.



N.B: Ext. time, Extraction time; Ext. temp, Extraction temperature; TCPA, Total chromatographic Peak Area

Fig 1 (d): Desirability Surface Plots of TCPA

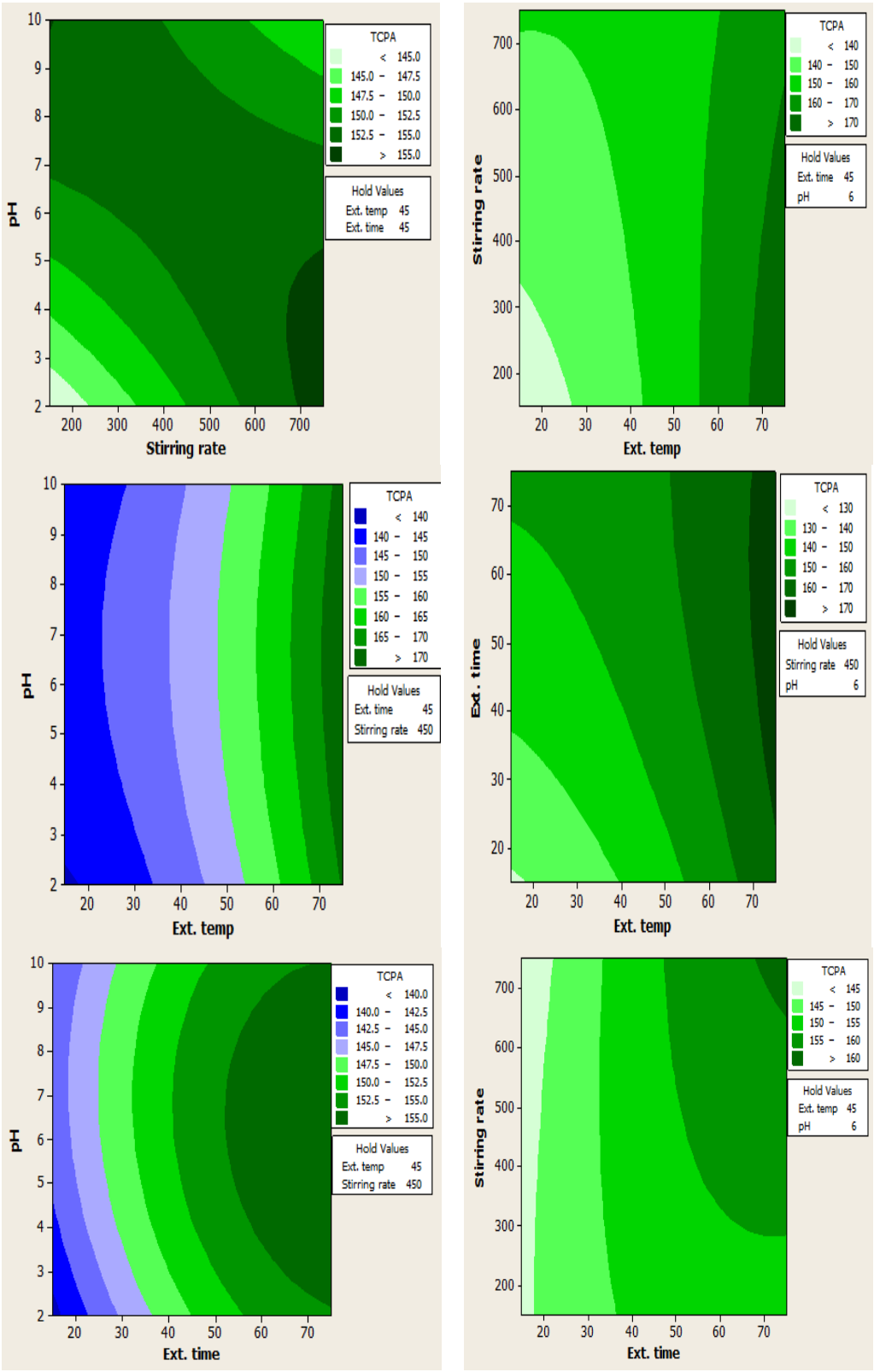


Fig. (2): Contour Plot of TCPA.

Validation of Analytical Parameters

The analytical data (Tables 1 – 3) of the optimized SPME method was validated for the determination of 14 target pesticides in pear, grape, lettuce and broccoli samples. The limit of quantitation (LOQ) was calculated experimentally from a signal-

to-noise ratio of 3, while the limit of detection (LOD) was calculated from a signal-to-noise ratio of 10 using the standard deviation of the y-intercept of the regression line of the calibration curve. The repeatability (n=9) was estimated by performing three extractions per day for three days. The calibration curve was obtained using the internal standard method. The ratio of chromatographic peak area of each target analyte to the chromatographic peak area of internal standard was plotted against the concentration of each analyte. The linearity ranged from 1- 500 µg/kg, the relative recoveries ranged from 74–115.7%, the LOD ranged from 0.17–7.34 µg/kg and the LOQ ranged from 0.55–24.50 µg/kg. Fig. 3 shows the chromatogram of pear sample spiked at 50 µg/kg with the pesticide standards, indicating no matrix effects as no interfering peaks around the peak of the target analytes can be recognized in the chromatogram.

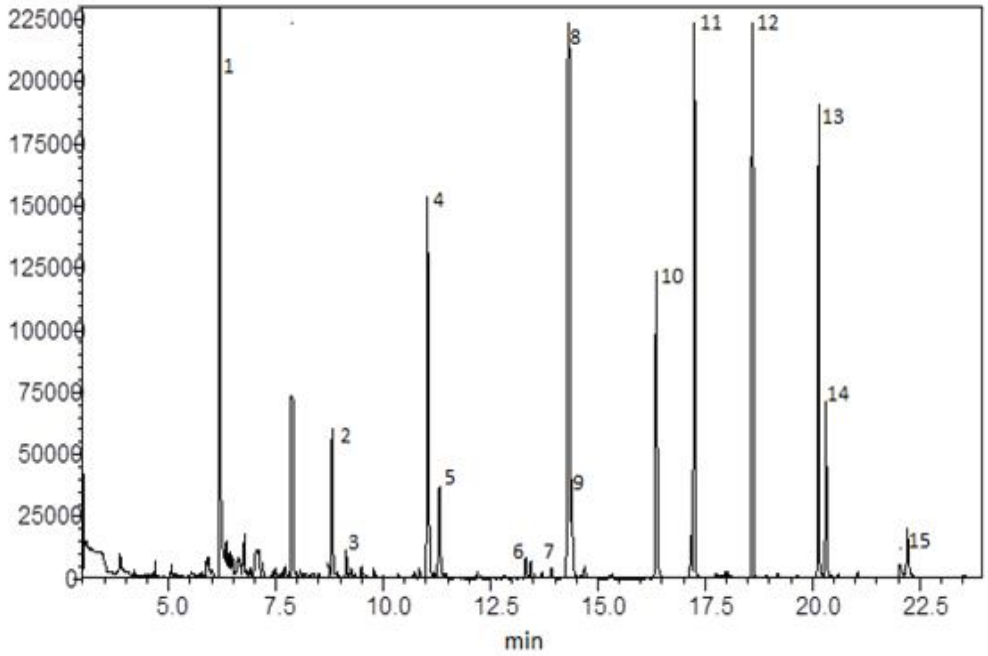


Fig. (3): GC-MS Chromatogram of Pear Sample spiked at 50 µg/kg of pesticide standard 1. I.S (Internal Standard); 2. Fenobucarb; 3. Ethoprophos; 4. Diaxinon; 5. Chlorothalonil; 6. Parathion Methyl; 7. Fenitrothion; 8. Chlropyrifos; 9. Thiobencarb; 10. Quinalphos; 11. Endosulfan I; 12. Endosulfan II; 13. Bifenthrin; 14. Fenpropathrin; 15. Permethrin.

Analysis of Real Samples

The developed method was applied in the analysis of 20 samples each of pear, grape, lettuce and broccoli purchased from Malaysian local and hypermarket markets (Table 4). This is done in order to verify further the reliability and robustness of the developed method. All samples were devoid of target analytes and were therefore not detected.

Table (S1): Analysis of Real Samples.

Pesticides	Pear (µg/kg)	Grape (µg/kg)	Broccoli (µg/kg)	Lettuce (µg/kg)
Fenobucarb	n.d	n.d	n.d	n.d
Ethoprophos	n.d	n.d	n.d	n.d
Diazinone	n.d	n.d	2.10 (±7.4)	n.d
Chlorothalonil	n.d	n.d	n.d	n.d
Parathion-methyl	n.d	n.d	n.d	n.d
Fenitrothion	n.d	n.d	n.d	n.d
Chlorpyrifos	n.d	n.d	n.d	n.d
Thiobencarb	n.d	n.d	n.d	n.d
Quinalphos	n.d	n.d	n.d	n.d
Endosulfan I	n.d	n.d	n.d	n.d
Endosulfan II	n.d	n.d	n.d	n.d
Bifenthrin	n.d	n.d	n.d	n.d
Fenpropathrin	n.d	n.d	n.d	n.d
Permethrin	n.d	n.d	n.d	n.d

N.B: n.d, not detected

Conclusions

In this study, a headspace solid phase microextraction method coupled online with gas chromatographic mass spectrometry detection was developed for simultaneous analysis of 14 pesticide residues in four fruit and vegetable samples. Design of Experiment was employed in the determination of significant factors and subsequent optimization of the factors for effective extraction of the target pesticide residues in the samples. The HS-SPME extraction has shown to be highly selective for the target analytes with little or no matrix interferences. It has been shown that the use of chemometrics combined with the SPME method is cheap, simple, robust and fast. It enhances recoveries and improves method validation. The figures of merit obtained were comparable or better than other methods of pesticide residues analysis as reported in our previous review.[19] The developed method could be used for routine analysis and monitoring of pesticide residues in other fruit and vegetable samples with complex matrices. Further studies should be focused on the use of other sorbents, such as sol–gel prepared sorbents, ionic liquids, nanomaterials, supramolecular molecules and molecularly imprinted polymer coatings as the extraction phase. This will increase the types of analytes, ranging from polar to non-polar, that can be extracted from a wide range of environmental samples.

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