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Analytical Methods

Multivariate study of parameters in the determination of pesticide residues in apple by headspace solid phase microextraction coupled to gas chromatography–mass spectrometry using experimental factorial design

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ABSTRACT

Solid-phase microextraction (SPME) is a solvent-less sample preparation method which combines sample preparation, isolation, concentration and enrichment into one step. In this study, multivariate strategy was used to determine the significance of the factors affecting the solid phase microextraction of pesticide residues (fenobucarb, diazinon, chlorothalonil and chlorpyrifos) using a randomised factorial design. The interactions and effects of temperature, time and salt addition on the efficiency of the extraction of the pesticide residues were evaluated using 2^3 factorial designs. The analytes were extracted with 100 μm PDMS fibres according to the factorial design matrix and desorbed into a gas chromatography–mass spectrometry detector. The developed method was applied for the analysis of apple samples and the limits of detection were between 0.01 and 0.2 μg kg^{-1}, which were lower than the MRLs for apples. The relative standard deviations (RSD) were between 0.1% and 13.37% with average recovery of 80–105%. The linearity ranges from 0.5–50 μg kg^{-1} with correlation coefficient greater than 0.99.

1. Introduction

Fruits and vegetables provide the body with micronutrients (minerals and vitamins) that are very vital to the body but are required in small quantities (Lewis & Ruud, 2004). This has led to their production in large quantities to meet the ever growing demand. To achieve this, pesticides were introduced to protect fruits and vegetables on the farm and during storage. Pesticides, including organophosphorous pesticides (OPP), organochlorine pesticides (OCP), carbamate pesticides (CP), phenyl urea pesticides (PUP), pyrethroid pesticides (PP) and carbamate pesticides (CP) have been used effectively in controlling pests, fungi and weeds, thereby beneficial to the steady increase in agricultural production (Chai & Tan, 2010; Vazquez, Mughari, & Galera, 2008). However, they persist in the food chain, due to their penetrating effect into the tissues of fruits and vegetables and therefore there is a need to analyse the level of pesticide residues in food.

Sample preparation is the most crucial and critical steps in the analysis of pesticide residues from complex fruit and vegetable matrices (Menezes Filho, dos Santos, & de Paula Pereira, 2010). The introduction of solid phase microextraction (SPME) technique in 1990 (Arthur & Pawliszyn, 1990), has helped to overcome the problems inherent in the solvent-based sample preparation techniques. Solid phase microextraction is a solvent free sample preparation and extraction technique developed by Pawliszyn and his co-workers (Arthur & Pawliszyn, 1990). It is an efficient, simple, versatile and effective adsorption/absorption and desorption technique with minimum matrix interference. It eliminates the use of toxic solvents and combines sampling, isolation, concentration and enrichment in one step (Ouyang & Pawliszyn, 2008; Pawliszyn, 1997). It was developed to overcome the problems associated with solvent-based, time consuming techniques, such as, liquid–liquid extraction (LLE), solid phase extraction (SPE), supercritical fluid extraction (SFE), and accelerated solvent extraction (ASE) (De Koning, Janssen, & Th. Brinkman, 2009). The traditional techniques also required a large volume of sample and solvents which impose environmental pollution and health hazards (Kataoka, Lord, & Pawliszyn, 2000). The SPME technique is based on the use of a fused silica or metal alloy that is coated on the outside with an appropriate polymerized stationary phase, attached to a stainless steel, mounted on a fibre holder housed in a modified syringe (Arthur, Killam, Buchholz, Pawliszyn, & Berg, 1992; Beltran, Peruga, Pitarch, & Lopez, 2003). The SPME process involves two basic steps which

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are the partitioning of analytes between the coating and the sample matrix, and desorption of the concentrated extracts into analytical instruments (Pawliszyn, 1997). There is no need for any clean-up step (Lord & Pawliszyn, 2000), thus preventing loss and contamination of target analytes.

Previous studies in the development of SPME methods for the analysis of pesticide residues in fruit and vegetable samples have used the univariate approach (Cortes-Aguado, Sanchez-Morito, Grenido Frenich, Vidal, & Arrebola, 2007; Cortés-Aguado, Sánchez-Morito, Arrebola, Grenido Frenich, & Vidal, 2008; del Castillo, Rodriguez-Valenciano, de la Peña Moreno, & Blanch, 2012; Zambonin, Cilenti, & Palmisano, 2002; Zambonin, Quinto, Vietro, & Palmisano, 2004), in which each factor is optimised once at a time and involve many experiments (Carrillo, Bravo, & Zufall, 2011). The univariate approach is time consuming and does not allow the determination of interactions between the extraction conditions. Multivariate approach requires few experimental runs and can be used for quantitative and qualitative modeling of mathematical relationships between factors and response (Brereton, 2007). The objective of the present research is to study the significance of the factors that affect the extraction of 4 pesticides from apples, their main and interaction effects on the extraction efficiency.

2. Materials and methods

2.1. Standard reagents and solutions

Pesticide standards (fenobucarb, diazinon, chlorothalonil and chlorpyrifos) were obtained from Accustandard Inc., New Haven CT, USA with percentage purity greater than 97%. All the solvents used for this study were pesticide grade and were purchased from Fisher Scientific, Loughborough, UK. Stock standard solutions of 100 μg mL^{-1} of each compound was prepared in pesticide grade methanol and stored at −18 °C. Working standard solutions containing the pesticides were prepared daily by diluting the stock solution in methanol to concentration of 10 μg mL^{-1}.

For this experiment, extraction temperature, time and salt addition were varied at two levels selected for the factorial design: 30 and 60 °C, 30 and 60 min, and 5 and 10% of NaCl, respectively (Table 1). To study the effect of these factors and their possible interactions, a 2^3 randomised-block experimental design was conducted.

The figure of merit of the analytical methodology was estimated by external standardization. Minitab statistical package software version 15 (Minitab Inc., State College, USA) was used for the design of experiment, analysis and data processing.

2.2. Sample preparation

Standard solutions of each pesticide were prepared daily by diluting the stock standards (100 μg mL^{-1}) in methanol to 10 μg mL^{-1} and stored at 4 °C. The working standard solution containing the 4 pesticides was prepared daily in methanol and the working standard was used to spike the matrix to a required concentration for the optimization of extraction parameters. Calibration standards with concentrations of 0.5 to 50 μg kg^{-1} were prepared by spiking a calculated amount of the working standard solution directly in the sample matrix. A 100 g of chopped apple sample was weighed and homogenised in a food processor and 5 g aliquot was placed in a 20 mL amber glass vial containing 5 mL of deionized water, spiked with an appropriate amount of standard and was subjected to the following HS-SPME procedure.

Table 1
Factors and levels of the variables.

Variables	Low	High
(A) Extraction temperature (°C)	30	60
(B) Extraction time (min)	30	60
(C) Salt concentration (%, w/v)	5	10

2.3. Headspace – solid phase microextraction procedure

The SPME fibre holder for autosampler and replaceable fibres coated with polydimethylsiloxane (PDMS, 100 μm), purchased from Supelco (Bellefonte, PA, USA) was employed for the extraction of target analytes. This fibre coating was selected based on prior studies (Chai & Tan, 2009). The fibres were conditioned in the GC/MS injector at 250 °C for 30 min, prior to their first use as recommended by the manufacturer. All analyses were performed in a 20 mL amber glass vial with the headspace volume of 10 mL. For the HS-SPME, 5 g of previously homogenised sample was weighed in a 20 mL amber glass vial, spiked with a known amount of the standard mixture and allowed to rest for 2 h. Optimum dilution was made with 5 mL of deionized water containing 10% NaCl, and the mixture was shaken ultrasonically for 10 min. The analytes were then extracted with 100 μm PDMS, by exposing the fibre coating to the sample headspace, according to the experimental factorial design matrix (Table 2). After the extraction, the fibre was placed in the GC injector for desorption at 270 °C for 5 min.

2.4. GC–MS Analysis

The extraction and analysis of target analytes were performed with CTC combiPAL autosampler, coupled to a GC–MS (Shimadzu QP2010 Series) and operated in the splitless mode at 270 °C. The capillary column used was DM-5MS of 30 m × 0.25 mm × 0.25 μm i.d. The GC oven temperature program was as follows: initial oven temperature 60 °C, held for 2 min then ramped to 180 °C at 30 °C min^{-1} and finally increased to 270 °C (10 min) at 5° C min^{-1}.

Table 2
Factorial design matrix.[a]

Run order	Std order	Block	Time (min)	Temp. (°C)	Salt (%)
1	3	1	30	60	5
2	2	1	60	30	5
3	1	1	30	30	5
4	8	1	60	60	10
5	7	1	30	60	10
6	5	1	30	30	10
7	4	1	60	60	5
8	6	1	60	30	10
9	10	2	60	30	5
10	12	2	60	60	5
11	14	2	60	30	10
12	13	2	30	30	10
13	16	2	60	60	10
14	15	2	30	60	10
15	9	2	30	30	5
16	11	2	30	60	5

[a] Generated using Minitab statistical software.

Table 3
Fragment ions of pesticides used for quantification.

Pesticides	Mol. Wt.	Ret. time. (min)	Ion (*m*/*z*)
(1) Fenobucarb	207.3	24.25	121, 91, 150
(2) Diazinon	304.35	28.40	179,137,152
(3) Cholorothalonil	265.91	29.64	266, 263, 268
(4) Chlorpyrifos	213.66	32.37	97, 144, 197

Table 4
Main effect, interactions between factors for pesticide residues.[a]

Pesticides	T	t	S	T, t	T, S	t, S	T,t,S
Fenobucarb	+	+	+	+	+	–	–
Diazinon	+	+	+	+	+	+	+
Cholorothalonil	+	+	+	+	+	+	+
Chlorpyrifos	+	+	+	+	+	+	–

[a] Factors: T, temperature; t, time; S, salt addition (NaCl); –, negative effect; +, positive effect.

The carrier gas was helium (99.999%), at a constant flow rate of 1.3 ml min^{-1}. The MS transfer line temperature was 290 °C, ion source 220 °C and ionisation model 70 eV. The analysis of target analytes was carried out in the selected ion monitoring (SIM) mode. Pesticide characteristic mass fragments used for the determination and the relative retention time used for each determination of pesticide are shown in Table 3. One target and two qualifier ions were monitored, the first ion is the target and other two are reference ions.

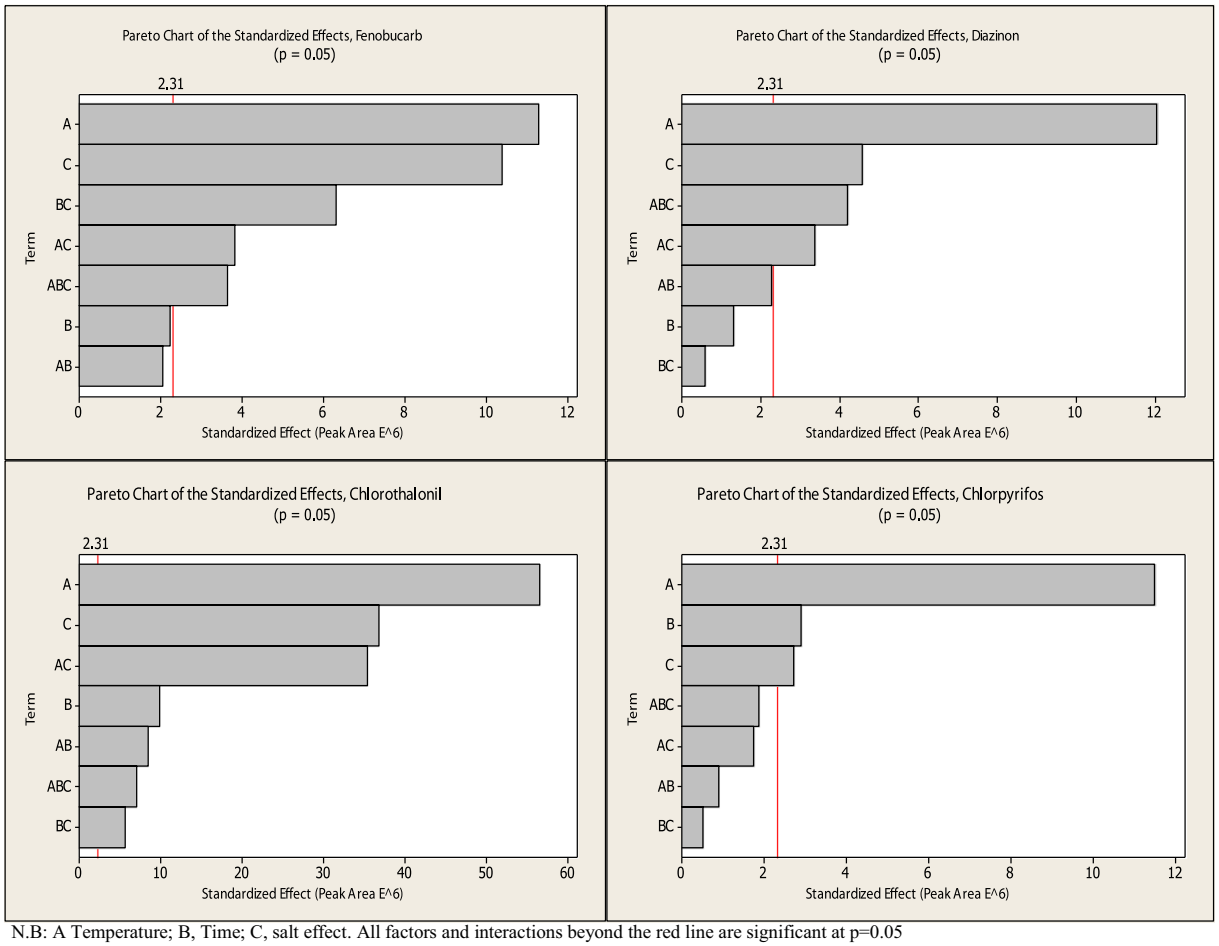
3. Results and discussion

3.1. Optimization of parameters

The effectiveness and efficiency of extraction of pesticides by SPME technique depend on the type, thickness and the coating volume of the fibres (Lord & Pawliszyn, 2000; Wardencki, Michulec, & Curyło, 2004), while the sensitivity is dependent on the distribution constant between the stationary phase and the sample matrix (Abdulra'uf, Hammed, & Tan, 2012). The matrix related condition is varied by salt addition, extraction temperature and time. The three factors were chosen based on the thermodynamic and kinetic theories of SPME. The thermodynamic theory is used to evaluate the amount of analyte extracted, while the kinetic theory estimates the rate of extraction. The extraction is a dynamic partitioning process between the target analytes and the fibre, and there is a maximum sensitivity at equilibrium (Ai, 1997). Kinetically, increase in temperature increases the extraction efficiency, by increasing the diffusion coefficient of analytes which enhances mass transfer of

Table 5
Method Validation.

Pesticides	Linearity (μg kg^{-1})	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	R^2	Recovery (%)	RSD (%)
(1) Fernobucarb	1–50	0.2	1.0	0.998	105.5	13.7
(2) Diazinon	0.5–50	0.01	0.05	0.999	89.48	1.7
(3) Cholorothalonil	0.5–50	0.01	0.05	0.999	83.78	0.1
(4) Chlorpyrifos	0.5–50	0.01	0.05	0.999	80.20	3.33



N.B: A Temperature; B, Time; C, salt effect. All factors and interactions beyond the red line are significant at p=0.05

Fig. 1. Pareto Charts of standardized effect for the investigated pesticides.

the analytes into the fibre (Kudlejova, Risticevic, & Vuckovic, 2012; Risticevic, Lord, Górecki, Arthur, & Pawliszyn, 2010).

To study the effect of extraction temperature, time and salt addition (NaCl) on the extraction of pesticide residues, a factorial 2^3 randomised-block experimental design was applied. The response variables were peak areas of the selected pesticides. The design was executed in two blocks, each daily. This 2-block design allowed the elimination of sources of daily variability. The design matrix is shown in Table 2. The design allows the assessment of the main effect, block effect and interactions between the selected conditions (Table 4).

The experimental design model was confirmed using ANOVA assumptions for the response variables of each pesticide. The significance of the studied variables in the experimental design is shown in Fig. 1 in the form of a Pareto chart. The chart illustrates the influence each variable (main effect) has on the response of the studied pesticides. This corresponds to the length of the bar, i.e. the length of the bar is proportional to the significance of the variables. The chart also shows the effect of the second- and third-order interactions among the variables. The results showed that temperature, time and salt addition were significant for chlorothalonil and chlorpyrifos, while only temperature and salt addition were significant for fenobucarb and diazinon (Fig 1). This is due to their low polarity and high affinity to the PDMS fibre. The interactions of most of the factors were also significant except in chlorpyrifos, estimated from the significance value ($p = 0.05$), of the interactions of various factors. The main effects and interactions of all the factors are significant for chlorothalonil, this is attributed to it low solubility in water.

As can be observed from Fig 1; temperature showed the strongest positive effect for all the investigated pesticides and that increase in sampling time causes a significant increase in peak response at higher temperature. The addition of salt was also found to have positive effect, while extraction time showed a positive effect on all the pesticides investigated, but the effect was not significant for diazinon and fenobucarb. All investigated factors are significant for chlorothalonil (Fig 1). All the second-order interactions are significant on the response of chlrothalonil (all are significant), fenobucarb (temperature/time, not significant) and diazinon (time/salt addition, not significant), except in chlorpyrifos where none of the second order interaction are significant, but they all show positive effect on chromatographic response of all investigated pesticides. Third-order interaction are significant for all pesticides except chlropyrifos, but also showed a positive effect. As shown in Table 4, a positive effect implies that the factors enhance extraction efficiency and gave better peak areas, while a negative effect showed that the interaction reduced extraction efficiency.

The plots showed that more analytes were extracted at higher extraction temperature and in a shorter time and with a higher percentage of salt addition. The overall conditions found based on the peak area responses of individual analyte was observed to be similar in the sample matrix. The factors considered and their interactions at different levels was used to construct a calibration curve, which was used for the determination of the limits of detection and quantification (Miller & Miller, 2010), and were found below the MRL values for the sample analysed. Consequently, a single factor was used for all the pesticides at 60 °C for 30 min in the presence of 10% NaCl for the extraction of target pesticides in apple sample.

3.2. Validation of analytical figure of merit

A developed method needs to be validated to confirm its performance characteristics and its suitability for the intended purpose. The analytical figures of merit of the developed method (Table 5), was validated under the best sampling conditions established above (30 min, 60 °C, 10% NaCl) by determining the repeatability, recoveries and linearity at 10 μg kg^{-1}. The external standard calibration curve was constructed by a-five point concentration level prepared in the sample matrix, each analysed in triplicate, using the same sampling procedure and chromatographic condition as used for the sample matrix. External standard calibration was employed due to lack of matrix effect on the extraction efficiency (Ouyang, 2012; Ouyang & Pawliszyn, 2008), this was achieved by carefully optimised dilution factor. The limits of quantification and detection values were estimated experimentally using a signal-to-noise ratio of 3 and 10, respectively. The precision expressed as the repeatability (% RSD) was estimated by three consecutive extraction of the selected pesticides from spiked apple sample. The method linearity ranged from 0.5 to 50 μg kg^{-1}, with correlation coefficient greater than 0.99. The limit of detection ranged from 0.01 to 0.2 μg kg^{-1} and limit of quantification were between 0.05 and 1 μg kg^{-1}. The accuracy of the method was determined in terms of recovery experiments by extracting the selected pesticides in apple sample at two concentration levels. The relative recovery calculated by comparing the peak areas of spiked sample with that of standard solution at the same concentration and extraction conditions ranged from 80% to 105% with an RSD less than 15% for all pesticides investigated.

3.3. Analysis of commercial apple samples

The HS-SPME procedure was routinely applied to apple samples purchased in the local Malaysian markets. In order to ascertain the applicability of the method, analyses were made in triplicate. A fibre blank was also carried out in order to check the carry-over effect, while calibration curves are prepared daily to ensure linearity in the working concentration range in order to avoid errors in quantitation caused by possible instrumental fluntuation, which was found to be stable. The pesticide chlorpyrifos was detected

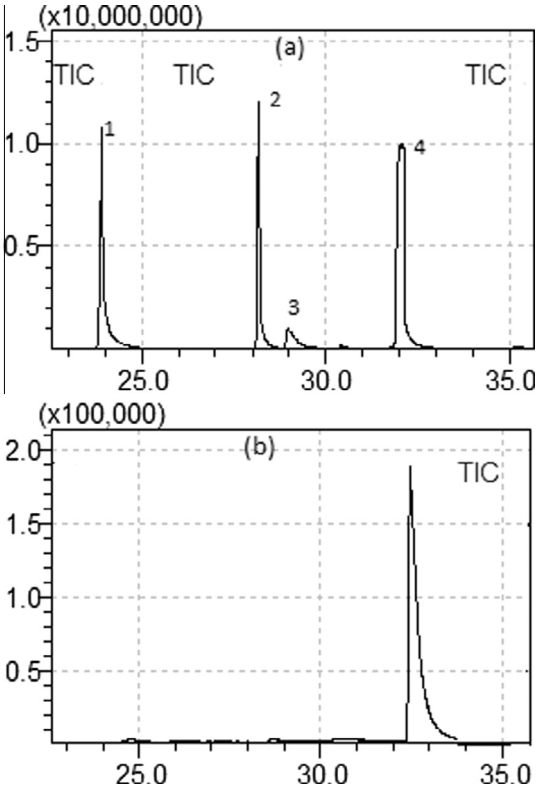


Fig. 2. GC–MS Chromatograms (a) spiked apple sample, (b) unspiked sample.

(Fig 2) at concentration of 0.2 μg kg^{-1}, which is 2-fold less than the MRL (0.5 mg kg^{-1}) for apples (European Union (EU), 2011).

4. Conlusions

The experimental factorial design was successfully used for the extraction of fenobucarb, diazinon, chlorothalonil and chlorpyrifos in apple samples obtained in the Malaysian market. As observed, the optimum conditions are dependent on temperature, time, and salt addition after carefully optimised dilution factor. The recovery and relative standard deviation obtained were comparable or better when compared with other method for pesticide residue analysis such as LPME and other SPME techniques, reviewed in our previous papers (Abdulra'uf, Chai & Tan, 2012; Abdulra'uf, Sirhan & Tan 2012).

The developed factorial design method has shown that it can be applied for the study of conditions affecting the extraction of pesticide residues from fruits and vegetables. It has been demonstrated in this study that the application of factorial design to HS-SPME technique, allows a simple, cheap and fast method for the simultaneous determination of factors affecting extraction conditions and their interactions. The sample preparation time is drastically reduced compared to the univariate method which involves many experiments and optimization of each factor at a time (Kudlejova et al., 2012) and also does not allow the determination of interaction of various factors.

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