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Development of Headspace Solid-Phase Microextraction Method for the Analysis of Pesticide Residues in Fruit and Vegetable Samples using OFAT Design

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ABSTRACT: A headspace solid-phase microextraction (HS-SPME) method was developed as a preliminary investigation using univariate approach for the analysis of 14 multiclass pesticide residues in fruits and vegetable samples. The gas chromatography mass spectrometry parameters (desorption temperature and time, column flow rate, interface temperature) and solid phase microextraction parameters (fiber coating type, extraction temperature and time, pH, salt addition, stirring rate, dilution factor, organic solvent type and amount) were all investigated and optimized. The optimum values for the optimized parameters are as follows: Injection Temperature, 270 °C; Interface temperature, 300 °C; Column flow rate, 1.3 mL/min; Fiber coating, PDMS/DVB; Extraction time, 30 mins; Extraction temperature, 60 °C; Stirring rate, 300 rpm; Salt addition, 10% (v/w) NaCl; pH, 7; Desorption time, 7 min; Desorption temperature, 270°C; Organic solvent 3 % (methanol/acetone, 21:79%). The optimization of the mixture of organic solvents was optimized using design of experiment (DOE) with simplex lattice, designed using Minitab Statistical Software®. The developed method was then applied to the analysis of samples of apple, tomato, broccoli, lettuce, grape, cucumber, cabbage and pear. The investigated pesticides were found to be below the maximum residue levels, while some were not found. This shows that the fruit and vegetable samples are safe for consumption and do not pose any health risk for consumers. © JASEM

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***Keywords*:** Univariate design, Simple lattice design, design of experiment, GC-MS, HS-SPME

Pesticides are chemical substances which are applied to crops at various stages of cultivation and are very important in pest control and management, and they play a vital role in agriculture to ensure sufficient production of food (Bakırcı, et al. 2014; Zheng, et al. 2013). Despite their benefit, their environmental persistence and their penetrating effect into tissue of food pose a potential health risk to animal and human (Abdulra'uf, et al. 2014; Araoud, et al. 2007). Since fruits and vegetables are sometimes consumed raw, and due to the health risk pose by the residue of pesticide on them, there is need to balance the expected benefits and possible health, there is a need to develop a method for the analysis of the pesticide residues which are present in trace levels.

Several methods have been developed for pesticide residue analysis in fruits and vegetables, but solid-phase microextraction (Arthur, et al. 1992), has been found to be environmental friendly due to the low volume of solvent used. In this study, a univariate method was developed for the analysis of 14 pesticide residues in 4 fruit and 4 vegetable samples, most of which are consumed raw without further processing. The optimized parameters was consequently used for the analysis real fruit and vegetables samples and was found to be suitable, effective and efficient for the extraction and analysis of target analytes.

MATERIALS AND METHOD

Standard solutions of each pesticide were prepared by diluting the stock standards (100 mg/L) in methanol to 10 µg/mL and stored at 4 0C. The working standard solution containing the 14 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 5 to 500 µg/kg, were prepared by spiking a calculated amount of the working standard directly in the sample matrix. A 100 g of chopped apple sample were weighted and homogenized in food processor

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and 5g aliquot was placed in sample vial, diluted with appropriate amount of water and was subjected to HS-SPME procedure.

The SPME fiber holder for autosampler and several replaceable fibers coated with polydimethylsiloxane (PDMS,100µm), olydimethylsiloxane/divinylbenzene (PDMS/DVB, 65µm) and Polyacrylate (PA, 85µm) purchased from Supelco (Bellefonte, PA, USA) were compared. The fibers were conditioned prior to their first use as recommended by the manufacturer. All analysis was performed in 20 mL amber glass vial with headspace volume of 10 mL. For the HS-SPME, 5 g of previously homogenized sample was weighed in 20 Ml amber glass vial, spiked with known amount of the standard mixture and allowed to rest for 2 hrs. Optimum dilution was made with 5 mL of distilled water containing 10 % NaCl and the mixture was shaken ultrasonically for 10 min. The analytes were then extracted with 100 µm PDMS in the headspace mode at 60 ^{0}C for 30 min. After the extraction, the fiber was placed in the GC injector for desorption at 270 ^{0}C for 7 min.

Extraction and analysis of pesticides were performed with CTC combiPAL autosampler, coupled to a GC-MS (Shimadzu QP2010 Series) and operated in the splitless mode at 270 ^{0}C. The capillary column was fused silica DB5-MS column (30 m x 0.25 mm x 0.25 µm i.d). The GC oven temperature program was as follows: 60 ^{0}C (2 min), ramped to 180 ^{0}C (0 min) at 30 ^{0}C/min, then to 210 ^{0}C (0 min) at 5 ^{0}C/min, and finally increased to 270 ^{0}C at 5 ^{0}C/min, where it was held for 5 min. The MS transfer line was 290 0C, ion source 200 ^{0}C and ionization model at 70 eV. The analyses were done in selected ion monitoring (SIM) mode.

RESULTS AND DISCUSSION

Optimization of GC-MS Parameters: The GC-MS parameters were first investigated in order to obtain the required sensitivity. Different parameters affecting the performance of the GC-MS system and require optimization in order to give a better chromatographic separation. The working standard solution containing the 14 pesticides were spiked into an aqueous solution at a concentration of 0.1 µg/mL and used to optimize the performance of the GC-MS system and were run in triplicate. The GC-MS was operated in the split/splitless mode.

Injection Temperature: The injection temperature of the GC injection port should be high enough to achieve column efficiency, consistent with the stability of the analyte to avoid thermal decomposition or chemical reaction. In the present study, the optimal injection temperature was determined by analyzing an aqueous solution spiked with the working standard solution at 0.1 µg/mL, containing the target analytes and desorbed at injection temperature between 240 and 280 ^{0}C, while keeping other conditions constant.

Fig. 1a, shows the plot of the total chromatographic peak area of all the investigated analytes at different injection temperatures. It shows that the maximum sensitivity, as measured by the total peak area of the GC-MS chromatogram obtained was achieved at a temperature of 270 ^{0}C. It implies that there was complete desorption of the analytes at this temperature and thus 270 ^{0} C was selected for further study in order to eliminate carry-over effect and minimize residence time of analytes in the injection liner. The results obtained are in agreement with results reported in other studies with different pesticide residues in fruits and vegetables, such as pyrethroid (Beltran, et al. 2003; Sanusi, et al. 2004), organochlorine and organophophorous (Cai, et al. 2006; Chai, et al. 2008; Yu, et al. 2004) pesticide residues.

Interface Temperature: The interface temperature is a critical parameter for a better system performance. The optimization of the condition is important in order to prevent the condensation of the analytes. The GC-MS interface temperature should be higher than the highest column temperature in the temperature programming. Thus, for this study, the interface temperature was investigated between 260 and 320 ^{0}C.

The plot of the total chromatographic peak area of the analytes and the GC-MS interface temperature (Fig. 1b), show the best interface temperature at 300 ^{0}C, and thus was selected for subsequent experiments. The ion source temperature was maintained at 200 ^{0}C. The analytes eluting from the GC column must pass through the ion source which must be maintained at a constant and reproducible temperature.

Column Flow Rate of Carrier Gas: The column flow rate was investigated between 0.8 to 1.8 mL/min. The linear gas velocity of the column which is a measure of the column efficiency is dependent on the flow rate. The optimization of the flow rate is essential because chromatographic analysis is based on the comparison of retention times and the flow rate determines the elution time of each analyte. The increase in flow rate decreases the analysis time, and thus the separation capacity of the column will be better at the optimized column flow rate. The column

flow rate was optimized in order to maximize the resolution of the chromatographic peaks.

The effect of column flow rate on the total chromatographic peak area is as shown in Fig 2a. It can be observed that the total peak area increases relatively with increase in the column flow. Although the retention time of each analyte varied slightly at the investigated flow rate, the optimal flow rate was found at 1.3 mL/min which gives the highest sensitivity in terms of chromatogram peak area and was selected for this study.

Optimization of Solid Phase Microextraction Parameters: The development of SPME method is described in this section. The selection of fiber coating was conducted as the preliminary optimization step. The SPME extraction conditions were optimized using one factor at a time (OFAT). The headspace extraction mode was adopted for this study due to the volatility of the target analytes and also to prolong the fiber lifetime.

Selection of Fiber Coating Type: The pesticides selected for this study are of different physico-chemical properties. Therefore, there is a need to investigate the extraction efficiency and performance characteristics of three commercial SPME fiber coatings. The result as indicated in Fig 2b, illustrated the extraction efficiency of the 3 investigated fibers. It showed that PDMS and PDMS/DVB were the most efficient fibers coating for the extraction of the multiclass pesticides under investigation, since they give the higher total chromatographic peak area compared to the PA. Further experiments were carried out to determine the best fiber coating for each pesticides and the results are as represented in Fig 3.

It can be seen that the PMDS fiber coating gave the best extraction efficiency for the target analytes. The PDMS/DVB showed relatively better extraction efficiency for pyrethroid pesticides, but since PDMS showed a better efficiency for all the investigated analytes, it was selected for further method optimization and was used for real sample analysis.

Optimization of Extraction Time: It has been shown that the SPME extraction is an equilibrium process which depends on the partitioning coefficient between the analytes and the fiber coatings. The extraction time was optimized by varying the time between 10 and 100 min, this range was selected because a longer extraction time favours pesticides of low diffusion coefficients.

The extraction time profile presented graphically in Fig. 4a, shows the extraction of the 14 pesticides residues in aqueous solution spiked with the standard mixture. It can be observed that an increase in extraction time increases the total peak area until 30 min, after which the peak area decreases with time, with no significant difference in the total peak area with increase in time, this may be due to unavailable adsorption space or displacement of the already extracted analytes due to competition for the available adsorption site. Since the extraction efficiency is a compromise between the sensitivity and extraction efficiency, 30 min was selected for subsequent analysis. The time was selected to reduce the total time of analysis, since efficient extraction can also be achieved prior to equilibrium provided all other factors are constant (Ai, 1997).

Optimization of Extraction Temperature: The diffusion coefficients of the analytes in the sample matrix onto the coated fiber and the distribution constant of analytes between the sample and fiber depend on the extraction temperature. Therefore an increase in extraction temperature, increases the diffusion coefficient and enhances the diffusion of analyte from the sample to the coated fiber and increase the extraction rate (Kataoka, et al. 2000). In order to maximize the amount extracted with respect to change in temperature, an optimal extraction temperature should be selected to achieve satisfactory sensitivity and faster extraction rate.

Fig. 4b, showed that the amount of pesticides extracted increases with increase in the extraction temperature, and an optimal temperature is reached at 60 ^{0}C. The optimized temperature is also favourable because higher extraction temperature may lead to the decomposition of some pesticides by hydrolysis and can also lead to the vaporization of the aqueous sample solution.

Optimization of Stirring Rate: The efficiency of SPME technique can also be improved by agitation, because stirring the sample matrix will reduce the diffusion layer and improves the mass transfer of analytes from the matrix to the headspace and then to the coated fiber. Increase in stirring rate increases extraction rate and decreases the equilibrium time. For this study, vial agitation was achieved using a CTC CombiPAL autosampler equipped with agitator and the agitation rate was varied between 250 and 750 rpm. The range was used as specified by the manufacturer.

As can be observed from Fig. 5, the amount extracted only increase between 250 rpm and 300 rpm after

which further stirring leads to the decrease in the amount of pesticide extracted. It showed that a higher stirring rate can lead to the vibration of the fiber which could lead to displacement of extracted analytes. Thus, an extraction rate of 300 rpm was selected for subsequent experiment.

Optimization of Salt Addition: The salting out effect can also be used to improve the extraction of pesticide residues from sample matrix, by saturating the sample matrix thereby increasing the analytes distribution constant. The addition of salt to sample matrix decreases the solubility of water-soluble pesticides, changes their ionic strength and also changes the physico-chemical properties of the pesticides. For this study, three salts (NaCl, $(NH_4)_2SO_4$ and Na_2SO_4) were tested for their effect on the extraction of the 14 investigated pesticides at 5 % (v/v) for each salt.

The results as shown in Fig. 6a indicates that NaCl enhances the extraction of the pesticides more than the other two salts, and was selected for further experiment. The amount of NaCl required to maximize the extraction of the pesticides was also investigated and the optimal amount was found at 10 % (v/v) as shown in Fig. 6b. Thus 10 % of NaCl was selected as the optimum concentration required for effective extraction of pesticides from the sample matrix.

Optimization of pH Value: The efficiency of SPME extraction is also improved by adjustment of sample pH, this is because, SPME involves the extraction of the dissociated and neutral species (Kudlejova, et al. 2012; Risticevic, et al. 2010). The adjustment of sample pH also helps to transform the analytes into their molecular state and significantly improves the extraction efficiency. In this study, the sample pH was varied between pH 4 and pH 10 and was adjusted by addition of known amounts of pH buffer solutions into the sample matrix to maintain the desired pH values. The result (Fig. 7a) shows the effect of adjusting the pH of sample matrix and the optimum pH value was found at pH 7, indicating that the extraction efficiency of the investigated pesticides is enhanced in neutral medium. Although it was observed that, there was only a slight difference in the extraction efficiency at pH 6 and 7, pH 7 was selected for subsequent experiments.

Optimization of Desorption Time: The time taken to completely desorb the analytes extracted on the coated fiber is also very essential and must be optimized. This will give the highest chromatographic sensitivity and eliminate the carry-over effect.

The, desorption time was varied between 2 and 10 min, while keeping all other chromatographic and SPME conditions constant. As shown in Fig. 7b, the optimal desorption time was found at 7 min, which implies that the SPME fiber should be left in the injection chamber of the GC for 7 min at 270 ^{0}C in order for the extracted pesticides to be completely desorbed into the injection chamber.

Optimization of Dilution Factor: It has been shown that dilution of samples enhances extraction efficiency of pesticides from the sample matrix (Lambropoulou and Albanis, 2003; Simplício and Vilas Boas, 1999). However, the dilution ratio should be limited in order not to reduce the concentration of the pesticides in an aqueous sample. The dilution will enhance the displacement of the pesticide bonded to the sample component and increases extraction efficiency. The optimum dilution ratio was investigated by adding different amounts of water to the sample, ranging from dilution factor of 1 to 5.

The addition of water to the sample matrix enhances the release of pesticide residues and reduces the effects of high molecular compounds present in the sample (e.g pectin and sugar), which can also adsorb the analytes leading to the formation of micelles and results in the reduction of pesticide extracted (Lambropoulou and Albanis, 2003; Simplício and Vilas Boas, 1999). As shown in Fig 8 (a-h), the optimum dilution factor is as follows: (ratio, sample/water(w:v): tomato, 1:2; grape, 1:3: Pear, 1:3; Cabbage, 1:4; Broccoli, 1:5, Apple, 1:3, Cucumber, 1:3 and Lettuce, 1:4).

Selection and Optimization of Organic Solvent: The addition of organic solvents increases the extraction efficiency by increasing the release of analytes from the sample matrix to the headspace. The addition of organic solvents also helps to reduce the adsorption of target analytes to the sample vial wall (Ochiai, et al. 2005).

Optimization of organic solvent was carried out using the design of experiment (DOE), by utilizing the simplex lattice design. The design was chosen because; it involves fewer experimental runs and spans the mixture space of solvents evenly (Brereton, 2003). It is assumed that the possible interactions of different mixture components can have both negative and positive effects on the extraction efficiency of the investigated pesticides. This was achieved by constructing a simplex lattice design matrix (Tab. 1),

consisting of three solvents using the Minitab® 16 Statistical Software package.

In this study three solvents (methanol, acetone and acetonitrile) were selected due to the difference in the polarity and solubility. The use of chlorinated solvents was not considered due to their health hazards, environmental pollution and cost of disposal. The simplex lattice design with 10 experimental points was performed in duplicate randomly at all points and the experimental data was fitted to a quadratic polynomial model. The simplex design plot in amounts of solvent was constructed as shown in Fig 9a. As shown in Fig. 9b, the optimum extraction as indicated by the higher chromatographic peak area (TCPA) was found between mixture of acetone and methanol, while the lowest TCPA was found between the mixture of methanol and acetonitrile. To determine the maximum desirability of the TCPA, the response optimizer was utilized, and it shows the main effect of each solvent on the TCPA. The mixture surface and contour plots for TCPA is as shown in Figs. 10(a and b).

As shown in the Fig. 11, the maximum desirability (0.99955) of component mixture, the optimal mixture consisting of approximately 21.32 % of methanol and 78.87 % of acetone, give the optimum extraction of the investigated pesticides. Therefore, further experiment was conducted using a mixture of methanol and acetone (21:79, v/v %).

The result obtained is in agreement with the recent study (Sang, et al. 2013), which showed that the use of binary solvents could accommodate a wide array of matrix characteristics. It has been shown that the presence of organic solvent can reduce the distribution constant of the analytes, therefore the addition of organic solvent should not exceed 5 % of the total sample weight or volume (Kudlejova, et al. 2012). The solvent percentage was varied between 1 – 5 % and the result (Fig. 12) shows that maximum chromatographic peak was observed at 3 % organic solvent and it was selected for further studies.

*Analysis of Real samples***:** The HS-SPME method developed in this study was subsequently applied to the analysis of 4 vegetable and 4 fruit samples obtained from Malaysian local wet market (Section 17 and Pantai) and hypermarket (Mid Valley Mall). Samples were analyzed in triplicate, in order to ascertain the applicability of the developed method and also to further verify the reliability and robustness of the developed method. All fruits and vegetables found to contain the target pesticides were far below the maximum residue levels allowed by the European Union and the Codex Alimentarius Commission (EU, 2005) and are thus safe for consumption.

Conclusion: The use of univariate optimization of parameters in microextraction techniques has found wide applicability in pesticide residue analysis. Its limitation lies in the large number of experiments and the fact that it does not consider the effect of interaction of the optimized parameters on the extraction efficiency. Further study should be carried out using design of experiment where the main effect of each factor and their interaction effect will be considered in order to give a more precise and accurate optimized parameters.

The use of univariate experiment design is commonly used in method development for pesticide residues analysis. It is recommended that multivariate experimental design should be introduced in order to determine the effect of interaction of various factors on extraction efficiency. It has been observed that no single parameter is independent in microextraction techniques, further studies involving development of microextraction methods should be based on multivariate design of experiment (Abdulra'uf and Tan, 2015; Leardi, 2009)

Acknowledgement: The University of Malaya Research Grant Management Center is gratefully acknowledged for the permission given to publish this research work and for the financial support through the IPPP Grant (PV09/2011A) and UMRG Grant (RG227/12AFR).

Table 1: Simplex Lattice Design Matrix [(a)]

RunOrder	PtType	Blocks	MeOH	AcO	ACN
1	2	1	50.00	50.00	0.00
2	1	1	0.00	100.00	0.00
3	1	1	100.00	0.00	0.00
4	1	1	0.00	0.00	100.00
5	2	1	50.00	0.00	50.00
6	-1	1	66.67	16.67	16.67
7	-1	1	66.67	16.67	16.67
8	-1	1	16.67	16.67	66.67
9	2	1	50.00	50.00	0.00
10	1	1	0.00	100.00	0.00
11	0	1	33.33	33.33	33.33
12	-1	1	16.67	16.67	66.67
13	1	1	100.00	0.00	0.00
14	-1	1	16.67	66.67	16.67
15	2	1	0.00	50.00	50.00
16	1	1	0.00	0.00	100.00
17	0	1	33.33	33.33	33.33
18	2	1	50.00	0.00	50.00
19	2	1	0.00	50.00	50.00
20	-1	1	16.67	66.67	16.67

[a] Generated using Minitab Statistical Software®

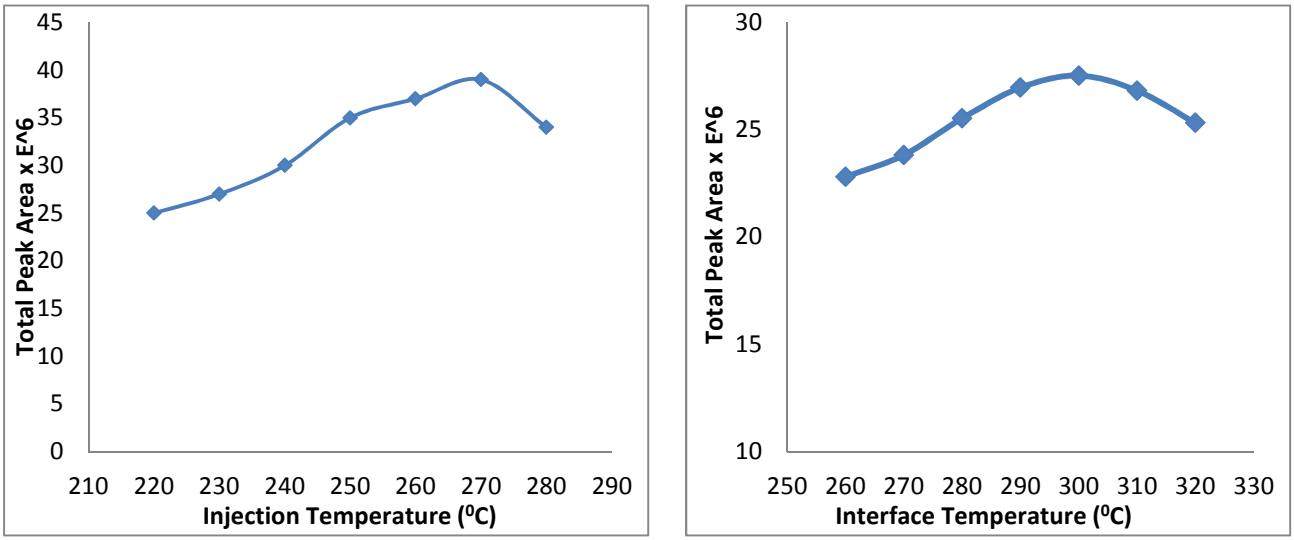


Fig. 1: (a) Optimization of Injection Temperature (b) Optimization of Interface Temperature

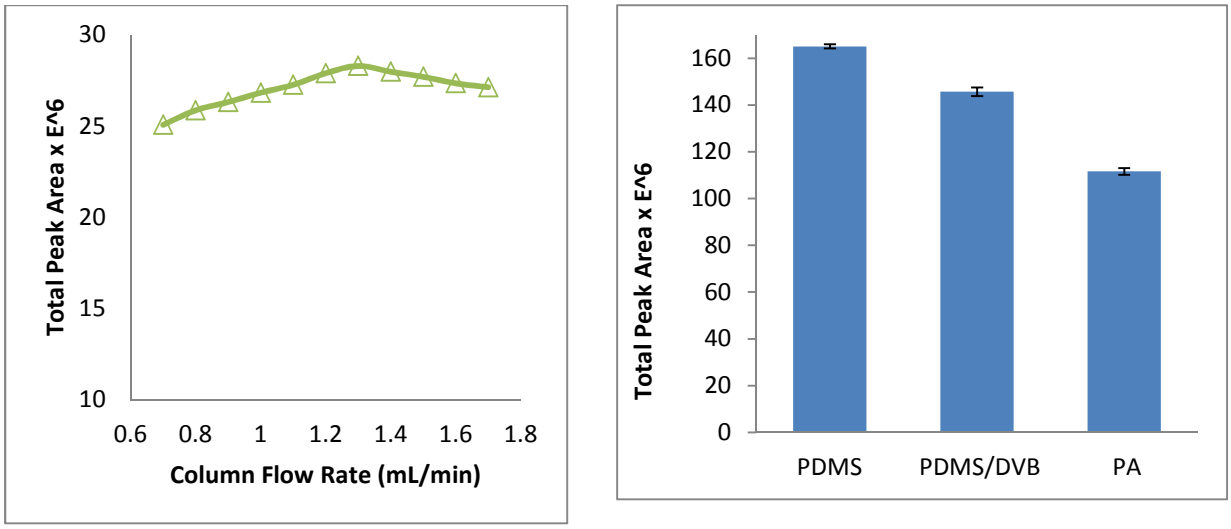


Fig. 2: **(a)** Optimization of the GC-MS Column Flow Rate **(b)** Optimization of Fiber Coating Type

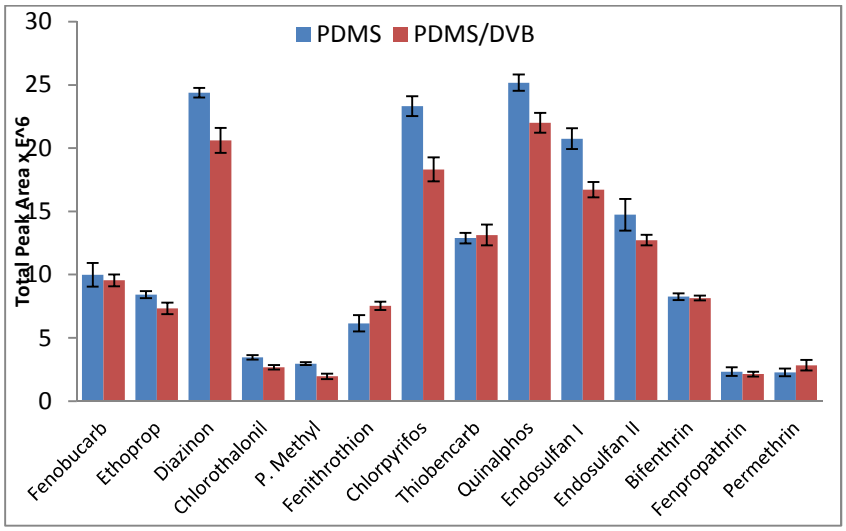


Fig. 3: Performance Characteristics of PDMS and PDMS/DVB Fibers

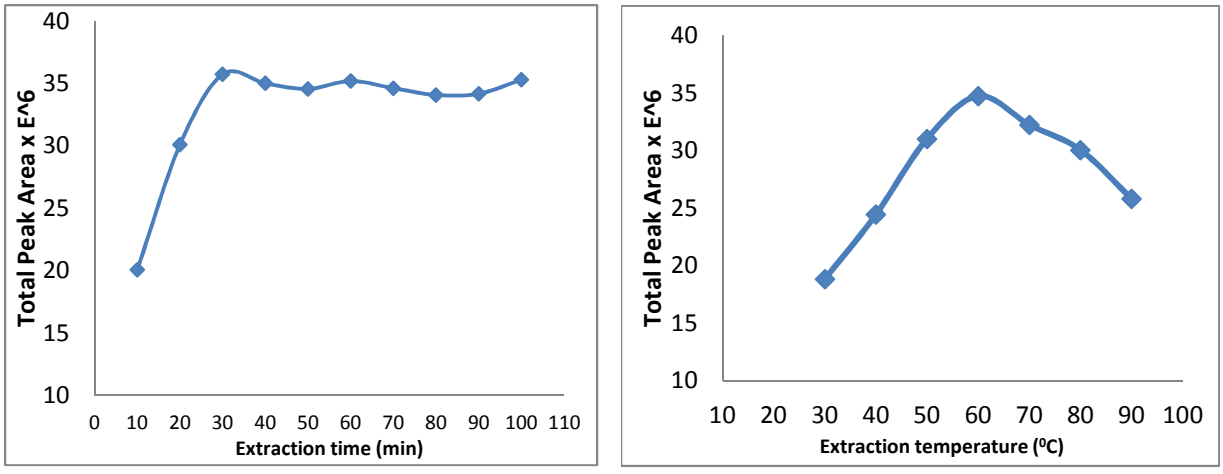


Fig. 4: (a) Optimization of Extraction Time **(b) Optimization of Extraction Temperature**

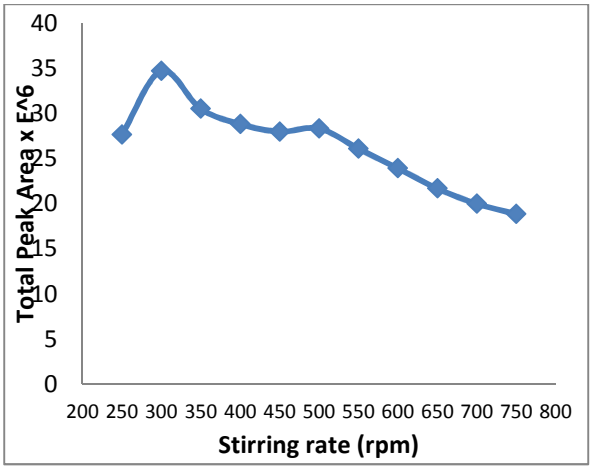


Fig. 5: Optimization of the Stirring Rate

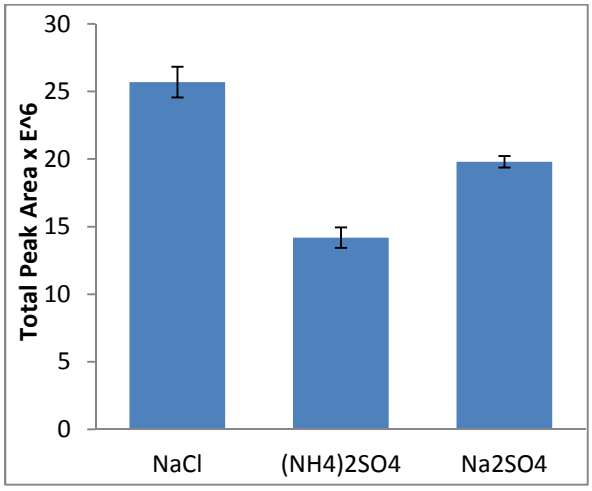
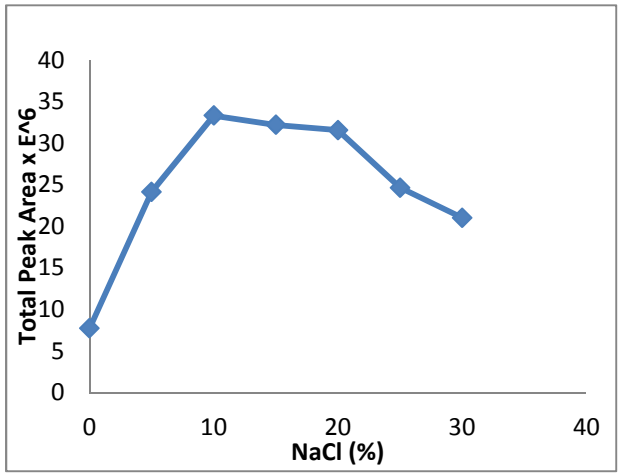


Fig. 6: (a) Effect of Types of Salt



(b) Optimization of Amount of NaCl (%)

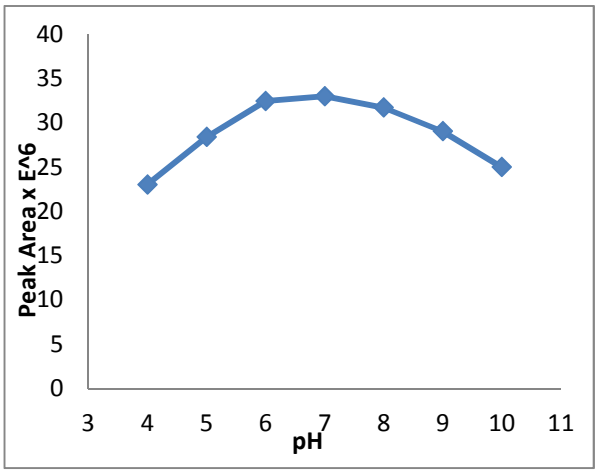
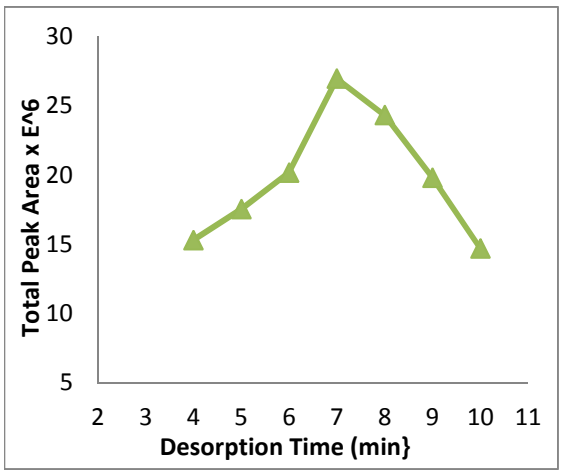
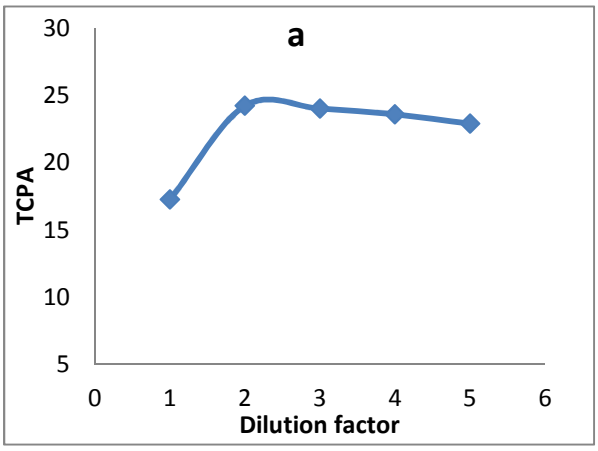
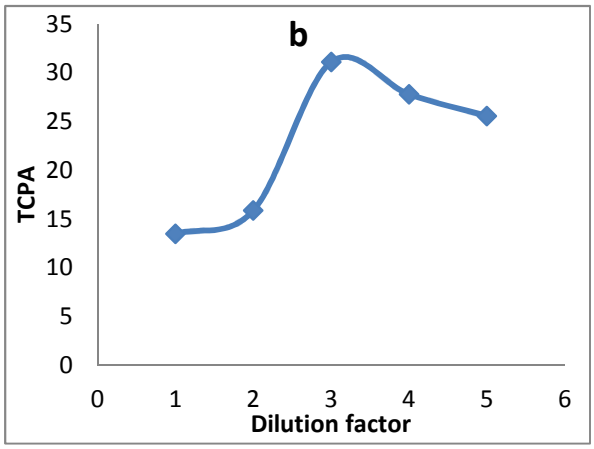


Fig. 7: (a) Optimization of pH Value



(b) Optimization of Desorption Time


a
TCPA
Dilution factor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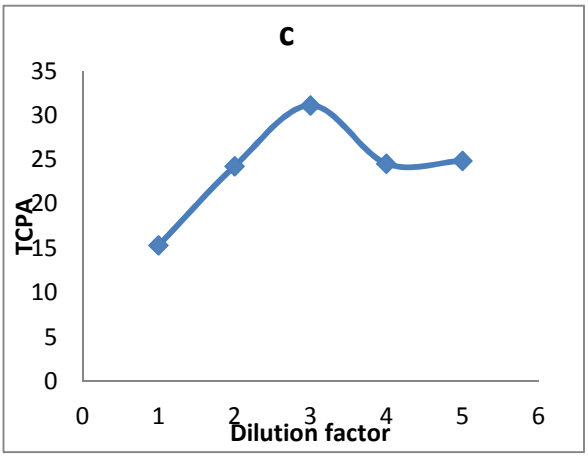
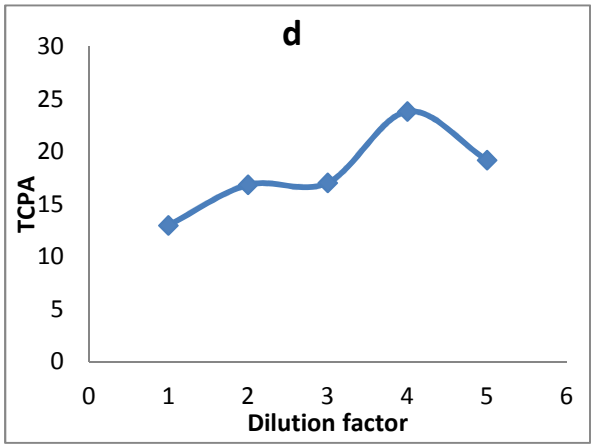
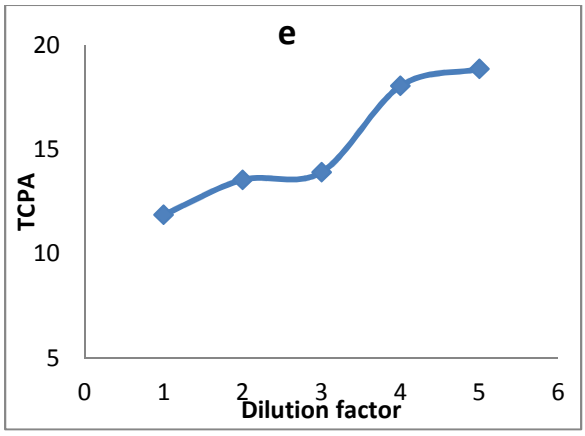
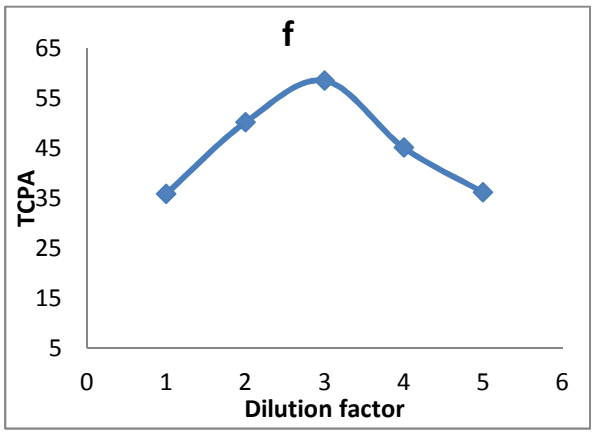
TCPA
Dilution factor


c
TCPA
Dilution factor


d
TCPA
Dilution factor


e
TCPA
Dilution factor


f
TCPA
Dilution factor

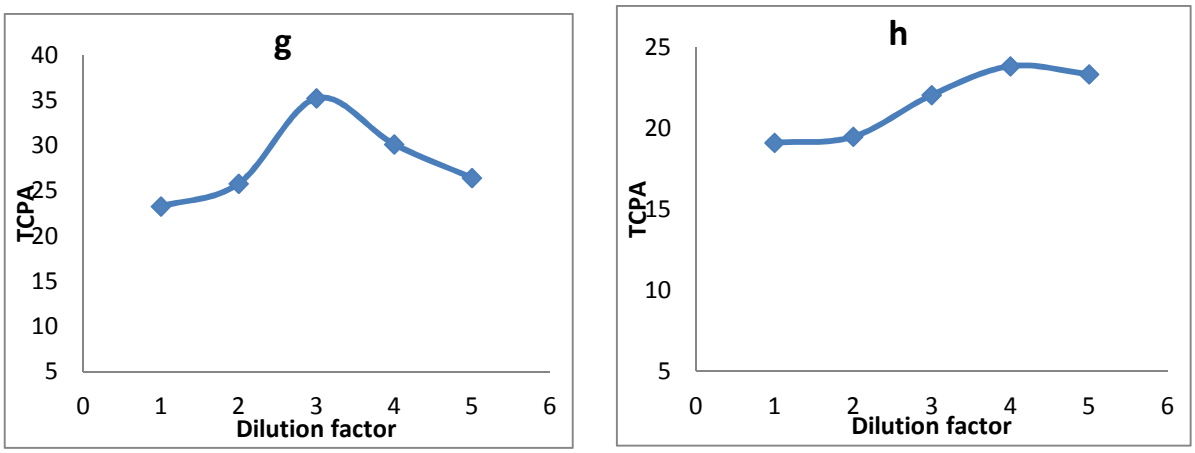


Fig. 8: Optimization of Dilution Factor (a) Tomato (b) Grape (c) Pear (d) Cabbage (e) Broccoli (f) Apple (g) Cucumber (h) Lettuce

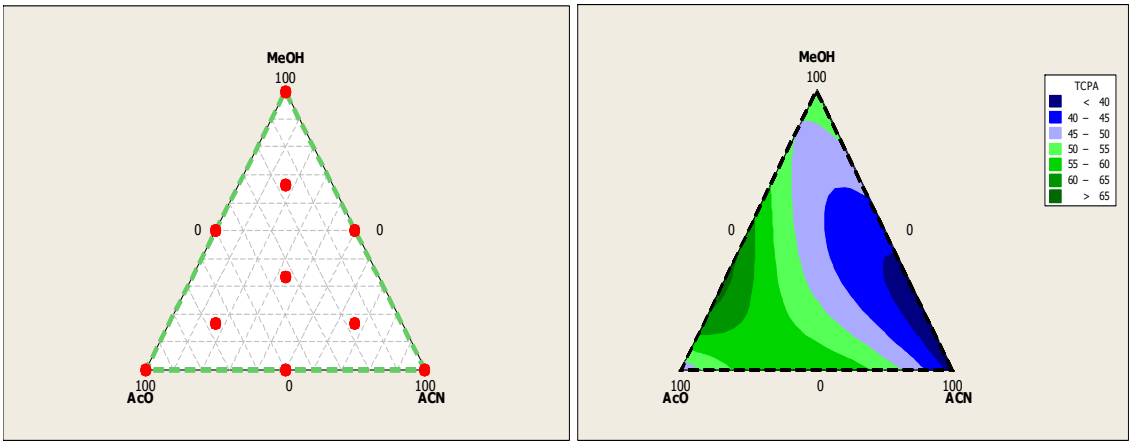


Fig. 9: (a) Simplex Design Plot in Amounts (b) Mixture Contour Plot for TCPA
N.B: MeOH, Methanol; AcO, Acetone; ACN, Acetonitrile

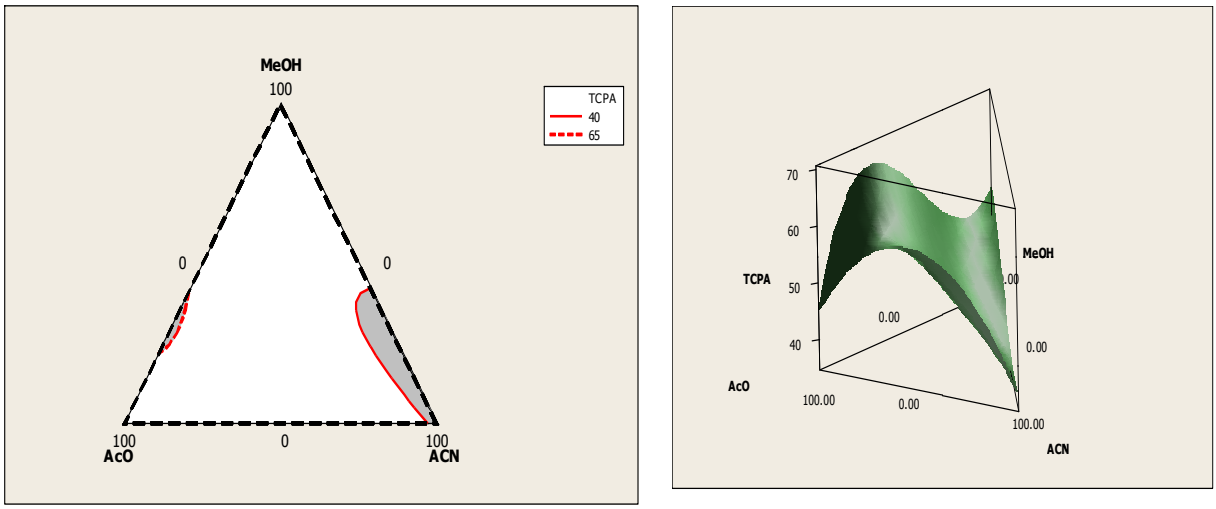


Fig. 10: (a) Mixture Surface Plot for TCPA (b) Contour Plot for TCPA

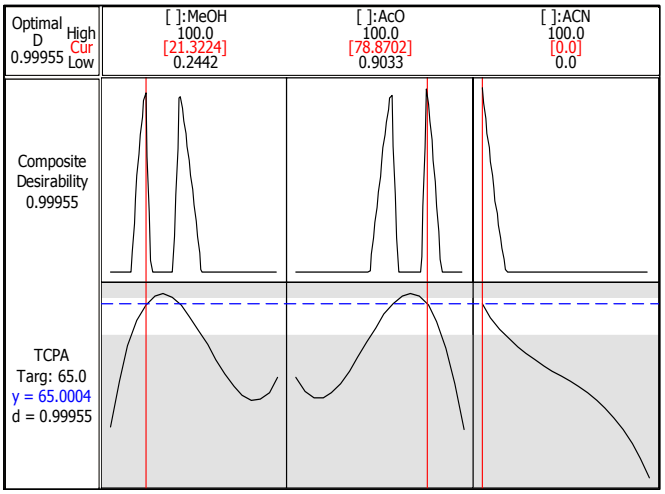


Fig 11: Response Optimizer for Maximum Component Desirability

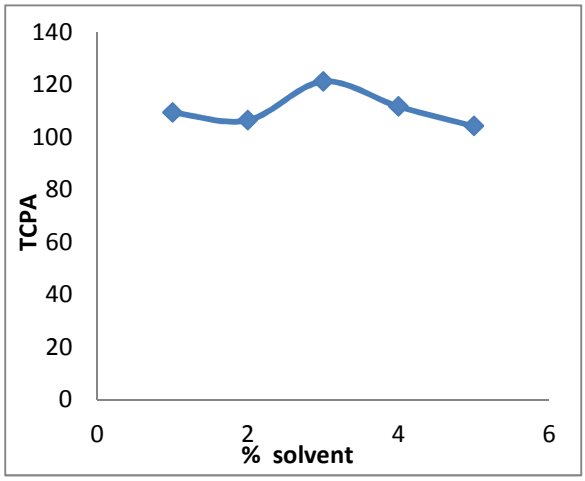


Fig 12: Optimization of the Percentage Solvent

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