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Optimization of Headspace Solid Phase Microextraction (HS-SPME) for the Extraction of Volatile Organic Compounds (VOCs) in MD2 Pineapple

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ABSTRACT

Headspace solid phase microextraction (HS-SPME) was employed for the extraction of volatile organic compounds (VOCs) in MD2 pineapple (*Ananas comosus* L. var. comosus cv. MD2). Optimisation of HS-SPME operating parameters was conducted using three-factor, three-level Box–Behnken response surface experimental design to evaluate the interactive effects of temperature (30 – 50 ºC), extraction time (10 – 30 min) and salting effect (1 – 3 g of salt addition) on the amount of selected VOCs. Determination of VOCs was done using gas chromatography with spectrometry detector (GC-MSD). Extraction temperature was found to be significant ($p < 0.05$) in increasing the amount of selected VOCs (ethyl acetate, methyl isobutyrate and butanoic acid methyl ester). Based on the maximum amount of these VOCs, the optimum operating extraction conditions for HS-SPME were set up at temperature of 30 °C, time of 29 min and salt addition of 1 g. The optimized HS-SPME conditions were employed for the extraction of VOCs from pineapple of different varieties.

Keywords: *Headspace solid phase microextraction; Volatile organic compounds; Response surface experimental design; Pineapple*

INTRODUCTION

Pineapple (*Ananas comosus* L.) contributes to over 20% of the tropical fruit produced in the world and ranked third as the most important tropical fruit in the world after banana and citrus [1]. This fruit is categorized as one of the most popular subtropical fruits cultivated and consumed worldwide. The fruits are consumed fresh and largely used in food industry for the production of canned fruit, jam and concentrated juice [2]. Volatile organic compounds (VOCs) are important as they contribute to the quality of the fruits. About more than 280 volatile compounds have been reported in generating the pineapple flavor [3]. Generally, fruit aroma is one of the most significant attributes that affects the tropical and subtropical fruit consumption. Pleasant aroma of pineapple fruit is very much appreciated by consumers and it is strongly affected by fruit maturity [4]. Beside varietal differences [5] and ripening stage [6] the extrinsic factors such as the weathers [7], and soil acidity may contribute to variance of VOCs in pineapple.

Aroma in fruit comprises a wide range of VOCs with different volatilities, polarities and concentrations. Hence, the sample preparation is a crucial step in volatile compounds analysis for complex matrix which requires efficient sample extraction technique. Many extraction techniques have been applied to isolate these compounds from the sample matrix prior to their identification by instrumental analysis. The conventional methods for extracting VOCs involved such as distillation and solvent extraction that are time consuming, used large amount of solvent, and it suffers analyte loss due to the heating process [2]. Solid-Phase microextraction (SPME) has been used extensively to extract VOCs from the fruit samples [8]. The application of SPME to extract aroma constituents from various food and beverages offers significant advantages as it can isolate, concentrate and clean of target analytes in a single and uninterrupted sampling phase.

Ouyang & Pawliszyn [9] reported many factors such as extraction time, extraction temperature, stirring effect, desorption temperature and time, sample amount, and salt addition that may affect the efficiency of SPME. Optimizing of these factors is crucial in order to obtain maximum amount of VOCs from samples. Traditionally, method optimization can be achieved by monitoring one separate variable at a time while others are kept at a constant level. However, this approach has several drawbacks such as it provides less information about the influence of factors on the response and it does not represent the complete interactive effects among the variables studied [10]. Multivariate design is able to overcome this shortcoming by using experimental software to identify the significant factors of the SPME parameters that maximize the response obtained. One of the widely used multivariate designs to optimize extraction of VOCs in food is the response surface methodology (RSM) [11].

In this study, three SPME conditions (extraction temperature, extraction time and salt addition) were evaluated. It is important to investigate how these factors can affect the extraction efficiency in order to achieve the optimum extraction conditions of VOCs in pineapple. Design of experiment (DoE) approach involved statistical tools and method to study various factors in a single experiment and the level of factors changed at a time. With the fewest number of experiment runs, it helps conduct and plan experiments in order to provide more information in which all relevant factors are varied systematically. The aim of this study is to obtain the optimum SPME operating parameters (temperature, extraction time and salting effect) in extracting volatile organic compounds in pineapples.

EXPERIMENTAL

Sample preparation

Fresh MD2 pineapple was purchased from local orchards in Selangor, Malaysia. The fruit was sliced and homogenized using a wet blender.

Reagent and materials

Sodium chloride was purchased from Merck (Darmstadt, Germany). Headspace solid-phase microextraction (HS-SPME) was conducted using manual solid-phase microextraction holder. SPME fiber assembly was equipped with crosslinked phase, 65 µm polydimethylsiloxane–divinylbenzene (DVB/PDMS) supplied by (Supelco, Bellefonte, USA). The fiber was thermally conditioned as recommended by the manufacturer. Extraction vials (15 mL) with silicon septa were purchased from Supelco (Bellefonte, USA).

Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS conditions applied in this study were adopted from the study conducted by Zakaria et al. [12] on optimization of headspace solid phase microextraction (HS-SPME) for the extraction of volatile organic Compounds (VOCs) in mangoes (Harumanis cv.). The SPME fiber containing the volatile compounds was thermally desorbed for 5 minutes at 280 ºC in split mode (1:10). The composition of VOCs from the pineapple pulp were analyzed on Agilent 6890 gas chromatograph coupled to an Agilent 5975N mass spectrometer (Agilent, Santa Clara, CA, USA). Carrier gas, helium was applied at a constant flow of 1.2 mL/min and the separation of volatile compounds was performed on HP-5MS (Agilent) capillary column (30 m x 0.25 mm I.D x 0.25 µm film thickness).

GC-MS detection was accomplished with 70 eV ionization energy in the 50– 550 a.m.u. mass range. Column temperature was increased from 40 °C to 250 °C with temperature program: starting at 40 °C withhold time 2 minutes, subsequently was increased to 150 °C (5 °C min^{-1}) with hold time of 1 minute. Finally, the temperature was increased to 250 °C (10 °C min^{-1}) with a hold time of 5 min and total run time of 40 min. The ion source temperature was 230 °C and the interface temperature was 280 °C. The volatile compounds were identified by comparing their mass spectra to those of commercial spectra databases available in NIST14 Mass Spectral Library, version 2.0; National Institute of Standards and Technology, Gaithersburg, MA.

Headspace Solid Phase Microextraction (HS-SPME) procedure

In this study, response surface methodology (RSM) using Box Behnken design was utilized to investigate the HS-SPME operating parameters in obtaining the ideal operating parameters on the control variables which result into immediate optimization of the predicted values of the response within a region of interest. The effects of three independent variables namely temperature (30 – 50 ºC), extraction time (10 – 30 min) and salting effect (1 – 3 g of salt addition) on the amount of selected VOCs were evaluated. Vial (15 mL) containing 5 g of the homogenized pineapple pulp (with salt addition) was sealed with a silicon screw cap incubated for 5 min with agitation of 400 rpm on a heating unit to allow equilibrium partition of analytes between the sample matric and the headspace. SPME fiber (65 µm PDMS/DVB) was inserted into the vial containing sample for the extraction of the volatile organic compounds. In order to diminish any carryover between each sample, SPME fiber was pre-conditioned at 250 °C for 7 min prior to next analysis as suggested by Chmiel et al. [13].

Experimental Design Approach

Response surface methodology (RSM) is an empirical optimization technique to assess the interaction of variables and experimental responses that usually used in combination with factorial design methods such as Box–Behnken designs and central-composite designs [14]. This approach was implemented to identify the optimum conditions of three HS-SPME operating parameters having a significant effect on the amount of selected VOCs which coded as extraction temperature (X_1), extraction time (X_2) and salting effect (X_3). A second-order polynomial was applied to define responses as a function of the independent variables using an equation as presented in the following equation (1).

$$Y = \beta_0 + \sum_{i=1}^{3} B_i X_i + \sum_{i=1}^{3} B_{ii} X_i^2 + \sum_{i=1}^{3} B_{ij} X_i X_j \qquad (1)$$

Where Y is measured response (peak area of selected VOCs); X_1, X_2, X_3 are the independent variables; β_0, B_i, B_{ii}, B_{ij} are the regression coefficients of variables for intercept, linear, quadratic and interaction terms, respectively.

The experimental design and data analysis for HS-SPME operating parameters were generated using the Design-Expert 12.0.3 software (State-Ease Inc., Minneapolis, MN, USA). Box-Behnken design (BBD) suggested 17 experimental runs as tabulated in Table 1 with five replicates at the center point in order to indicate the pure errors. Box-Behnken was employed due to minimum experimental runs suggested without lowering the accuracy of the models.

Table 1: The three variables BBD matrix and experimental data of selected VOCs

Experiment number	**Extraction temperature**	**Extraction time**	**Addition of salt**	**Response (Peak area of selected VOCs) mAU**		
				Ethyl acetate	**Methyl isobutyrate**	**Butanoic acid, methyl ester**
1	30	20	3	45337597	6723870	47850796
2	30	20	1	66157309	8311991	65784187
3	40	10	3	71808198	20569709	30630772
4	50	30	2	10769937	2494032	23468982
5	30	10	2	85206074	10117205	29197093
6	40	20	2	12710276	3737203	21796407
7	50	20	3	44831713	4995100	24278261
8	50	20	1	13961287	2673922	32358413
9	40	20	2	28546340	5502358	26469827

10	40	20	2	38642310	3877889	15824174
11	40	10	1	21540383	3422319	16396423
12	30	30	2	98782681	16886425	35086634
13	40	20	2	18405668	2736798	14854979
14	40	30	3	14012132	1877232	9794896
15	40	30	1	87585652	16683196	29135427
16	40	20	2	10948217	3291910	18645537
17	50	10	2	89302076	6750035	28553740

RESULTS AND DISCUSSION

Fruit aroma is the essential marker that indicates the fruit quality where it is made up of a complex mixture of various esters, alcohols, aldehydes, terpenoids compounds, etc [15]. In this study, a total of 27 volatile organic compounds were identified in MD2 pineapple, which consist of 14 esters, 12 terpenes and one organosilicon compound. Esters are the most abundant aroma-active compounds found, followed by terpenes. Analyses using GC-MSD identified various ester presented in the fresh MD2 pineapple namely, methyl ethanoate, ethyl ethanoate, methyl propanoate, methyl 2-methylpropanoate, ethyl propanoate, methyl butanoate, ethyl 2-methylpropanoate, ethyl butanoate, methyl pentanoate, ethyl 2-methylbutanoate, methyl hexanoate, ethyl hexanoate, methyl octanoate, ethyl octanoate.

Zhang et al. [16] reported that ethyl ethanoate, ethyl octanoate, ethyl butanoate, and ethyl hexanote were found in fresh-cut pineapple which was in agreement with the present study. The aroma volatile compounds represented significant characteristics since it contributed to the quality of fresh and processed fruit where it has been reported that ethyl hexanoate and methyl hexanoate have the highest contribution to the pineapple aroma [8]. They also reported that major aroma volatile compounds in pineapple are made of esters.

Table 2: Odor characteristic of the selected VOCs

Compound (IUPAC NAME)	Common Name	Odor Characteristic
Ethyl ethanoate	Ethyl acetate	Ethereal, sweet, fruity, weedy [17]
Methyl 2-methylpropanoate	Methyl isobutyrate	Fruity, apple-like odor and an ethereal type flavor [18]
Methyl butanoate	Butanoic acid, methyl ester	Ether, fruity, sweet odor like that reminisce apples and pineapples [19].

In this study, headspace solid phase microextraction (HS-SPME) mode was implemented in extracting selected VOCs from MD2 pineapple sample in order to avoid interferences of nonvolatile compounds in sample matrix. The amount of selected VOCs (ethyl acetate, methyl isobutyrate and butanoic acid methyl ester) were used as responses to optimize the HS-SPME conditions. These VOCs (Table 2) were selected due to they are consistently found in pineapple due to their well-known importance to the aroma of pineapple varieties.

In obtaining optimum HS-SPME conditions, the variables (extraction temperature, extraction time and the amount of salt added) were set in range, while the goals were set at maximum for the responses (VOCs). The analysis of variance (ANOVA) was implemented to evaluate the validity of the model suggested by the software, which illustrates the data was significant ($p<0.05$) accompanied by a non-significant ($p>0.05$) lack of fit, summarizes in model equation as shown in Table 3. The coefficient of determination, R^2 obtained for selected VOCs; ethyl acetate, methyl isobutyrate and butanoic acid methyl ester are 0.91, 0.96 and 0.88, respectively. The R^2 above 0.8 proved that the model is adequate to be used to navigate the design space and quadratic model was chosen to fit the data.

Table 3: Model equation or selected VOCs based on Box–Behnken Design

Compounds	**Transform**	**Model**	**Lack of fit**	**R^2**	**Equation**
Ethyl acetate	None	Quadratic	Not significant	0.91	Ethyl acetate = 2.19E+07 - 1.71E+07A - 7.09E+06B - 1.66E+06C - 2.30E+07AB + 1.29E+07AC - 3.10E+07BC + 2.15E+07A^2 + 2.77E+07B^2 - 7.79E+05C^2
Methyl isobutyrate	Square root	Quadratic	Not significant	0.96	Sqrt (Methyl isobutyrate) = 1943.36 - 589.83A - 127.62B + 35.1C - 486.85AB + 222.44AC - 1349.95BC + 150.03A^2 + 773.45B^2 + 243.17C^2
Butanoic acid, methyl ester	Square root	Quadratic	Not significant	0.88	Sqrt (Butanoic acid, methyl ester) = 4392.75 - 693.9A - 129.43B - 342.16C - 254.76AB + 108.04AC - 938.33BC + 1434.58A^2 - 448.61B^2 + 583.65C^2

The experimental data for extraction of selected VOCs were statistically analyzed by analysis of variance (ANOVA) and the results are presented in Table 4. In order to determine significant factors, the F-values, sum of the squares and p-values are used. The lack of fit with value of 0.2198, 0.262 and 0.2577 for ethyl acetate, methyl isobutyrate and butanoic acid, methyl ester respectively, indicate that the value for lack of fit were insignificant relative to pure error. From the ANOVA results, all responses had a non-significant lack of fit F-value which were desirable.

Table 4: Analysis of variance (ANOVA), regression coefficient estimate and test of significant for selected VOCs

Compounds (Responses)	**Source**	**Sum of square**	**df**	**Mean square**	***F*-value**	***p*-value**
Ethyl acetate	Model	1.48E+16	9	1.65E+15	7.84	0.0064
	X_1	2.33E+15	1	2.33E+15	11.1	0.0126
	X_2	4.02E+14	1	4.02E+14	1.91	0.2093
	X_3	2.20E+13	1	2.20E+13	0.1045	0.756
	Residual	1.47E+15	7	2.10E+14		
	Lack of fit	9.31E+14	3	3.10E+14	2.29	0.2198
	Pure error	5.41E+14	4	1.35E+14		
	Corrected Total	1.63E+16	16			
Methyl isobutyrate	Model	1.44E+07	9	1.60E+06	17.25	0.0006
	X_1	2.78E+06	1	2.78E+06	30.01	0.0009
	X_2	1.30E+05	1	1.30E+05	1.4	0.2746
	X_3	9855.83	1	9855.83	0.1063	0.754
	Residual	6.49E+05	7	92754.78		
	Lack of fit	3.86E+05	3	1.29E+05	1.96	0.262
	Pure error	2.63E+05	4	65709.89		
	Corrected total	1.51E+07	16			
Butanoic acid, methyl ester	Model	1.97E+07	9	2.19E+06	5.55	0.0171
	X_1	3.85E+06	1	3.85E+06	9.75	0.0168
	X_2	1.34E+05	1	1.34E+05	0.3392	0.5785
	X_3	9.37E+05	1	9.37E+05	2.37	0.1675
	Residual	2.77E+06	7	3.95E+05		
	Lack of fit	1.66E+06	3	5.52E+05	1.99	0.2577
	Pure error	1.11E+06	4	2.77E+05		
	Corrected total	2.25E+07	16			

Note: X_1 – extraction temperature (°C); X_2 – extraction time (min); and X_3 – addition of salt (g)

The efficiency of SPME often relies on several factors such as extraction temperature and time, fiber coating, pH, volume of sample, salting effect, agitation effect, desorption temperature and time [10]. Generally, the significant coefficient term can be determined by referring to highest F-values and smaller p-values. The coefficients for the quadratic terms for the extraction temperature are shown to be very significant in all responses. Extraction temperature had the most significant impact ($p < 0.05$) on SPME performance since the partition coefficients are temperature-dependent [13] followed by extraction time. Increases in temperature did not result in any further increase in the amount VOCs extracted. The results concurred with the study done by Sores et al. [20]. Extraction time also contributes to extraction efficiency of SPME as it influences the equilibrium of the analyte between the headspace and the fiber coating [5].

Zakaria et al. [12] found that the addition of salt in sample matrix showed no significant effect in increasing the amount of volatile extracted from harumanis sample. However, Pino and Queris [2] indicated that the addition of salt (salting effect) improved extraction efficiency by decreasing the analyte solubility for pineapple wine samples, thus allowing it to be absorbed by the fiber. Previous study conducted by Wei et al. [21] stated sodium chloride (NaCl) as the most common salt used. Therefore, in this study, NaCl was added during preparation and it was found that extraction efficiency was significantly improved with the increased of VOCs extracted from pineapple matrices. However, the amount of salt need to be optimized due to further salt addition will no longer improve the extraction.

In this study, 1 g of salt addition in sample was the optimum amount in providing the salting effect to reduce the matrix-analyte interaction by increasing the sample viscosity which contributes to decrease in the mass transfer from the pineapple sample into the headspace. The interactive effect between independent variables (extraction conditions) and dependent ones (peak are of selected VOCs) was presented by response surface plot illustrated on the three dimensional space (Figure 1) by varying extraction temperature and time by holding the third constant at zero level (center point). In addition, 3D plot generated by quadratic model can be used to determine the point of optimum condition to extract selected VOCs as well as to study the effect of each parameter. Extraction temperature was found to be significant ($p< 0.05$) in increasing the amount of VOCs (ethyl acetate, methyl isobutyrate and butanoic acid methyl ester) followed by extraction time.

Similar finding was observed in the study conducted by Chmiel et al. [13] where the extraction temperature had the most significant effect on SPME performance since it is responsible for headspace capacity and analyte diffusion coefficients. It can be observed from the three dimensional (3D) plots (Figure 1) the amount of selected VOCs decreased with increasing of extraction temperature. Increasing the extraction temperature beyond 30 ºC did not result in any further increase in the amount of selected VOCs. In fact, it caused a significant decrease of volatile compounds extracted [20]. Study by Chmiel et al. [13] indicated that extraction temperature less than 50 ºC was favorable to determine the aroma constituents such as terpenes in food matrices to avoid thermal degradation. Apart from extraction temperature, the extraction time might affect the HS-SPME efficiency as it influences the equilibrium of the analyte between the headspace and the fiber coating [5]. The surface plots showed that the amount of selected VOCs in this study increased significantly with increase of extraction time and the area of VOCs reached at maximum value at 29 min.

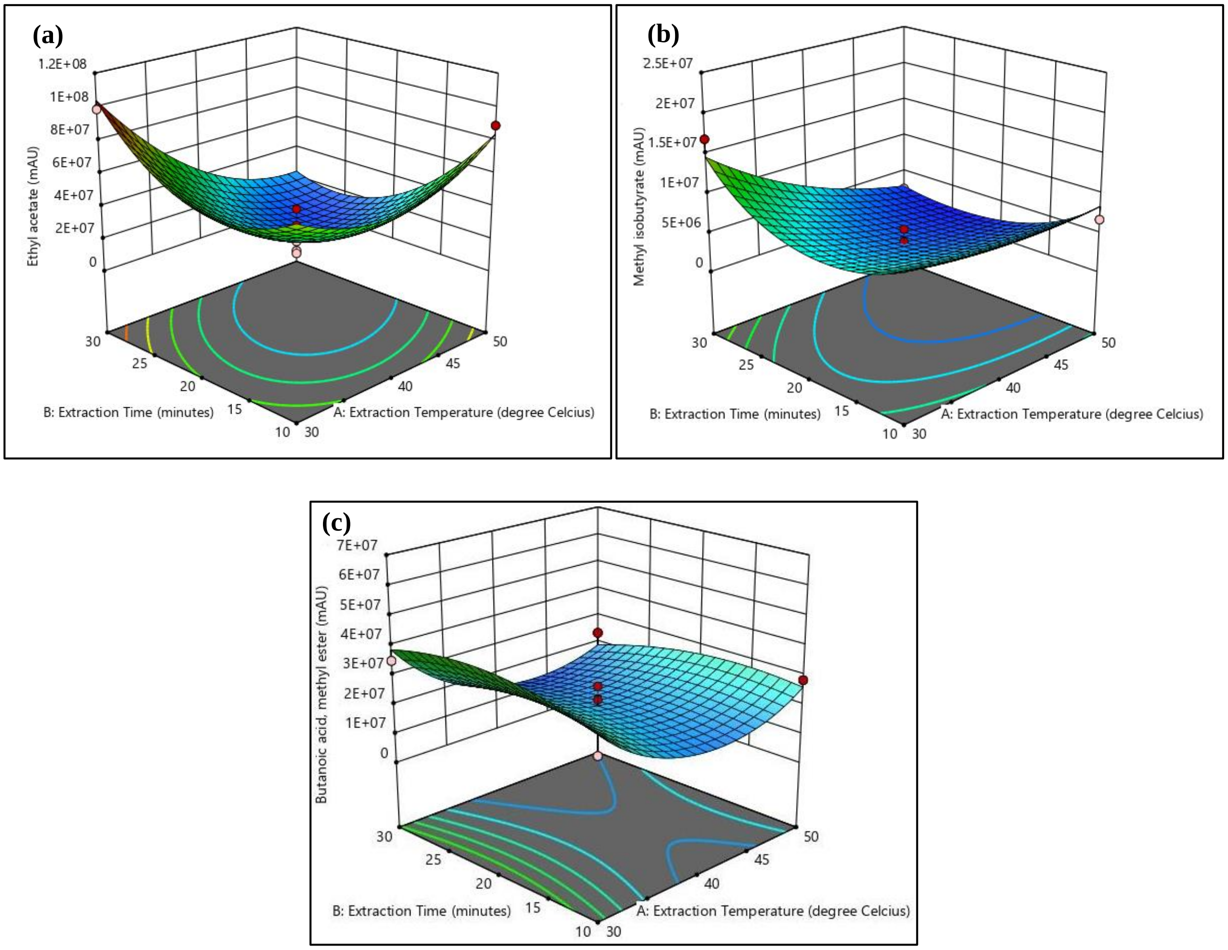

Figure 1: 3D surface plot of peak area for selected VOCs: (a) ethyl acetate, (b) methyl isobutyrate and (c) butanoic acid, methyl ester

For the generation of an adequate models, an accuracy check can be determined by comparing the value of predicted and actual peak are for selected VOCs as shown in Figure 2. The graphical visualization indicated a linear relationship between predicted and actual values as it can be seen the values are close to each other. Furthermore, a normal plot of residuals between the normal probability (%) and the internally studentized residuals was generated in order to determine whether the model sufficient to fit the assumptions of ANOVA. The normal probability plot of residuals for the model (Figure 3) was obtained, where each point located apparently closed to the straight line that indicates the validation of model.

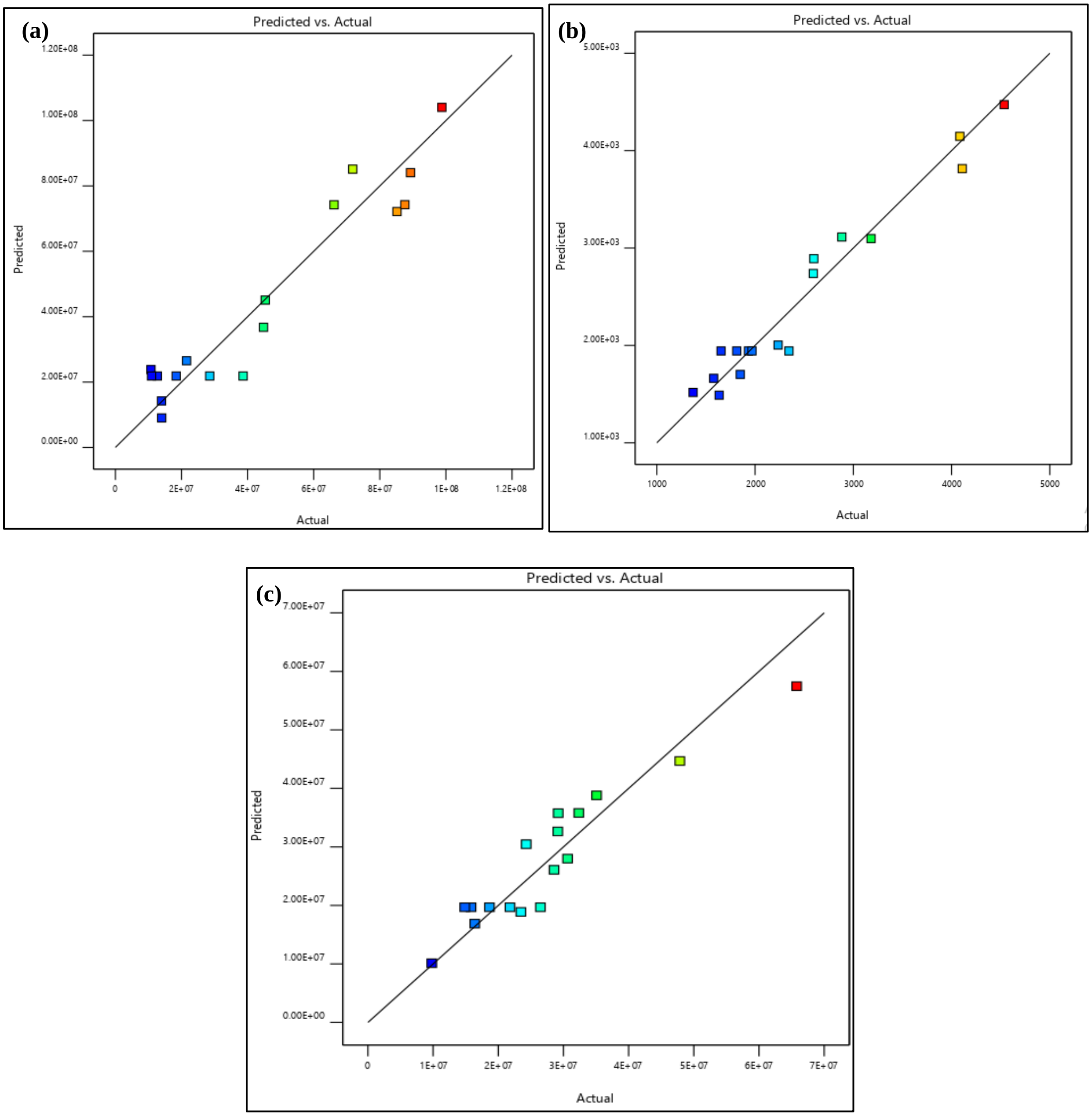

Figure 2: Plot of the relationship between predicted and actual of peak area for selected VOCs: (a) Ethyl acetate, (b) methyl isobutyrate and (c) butanoic acid, methyl ester

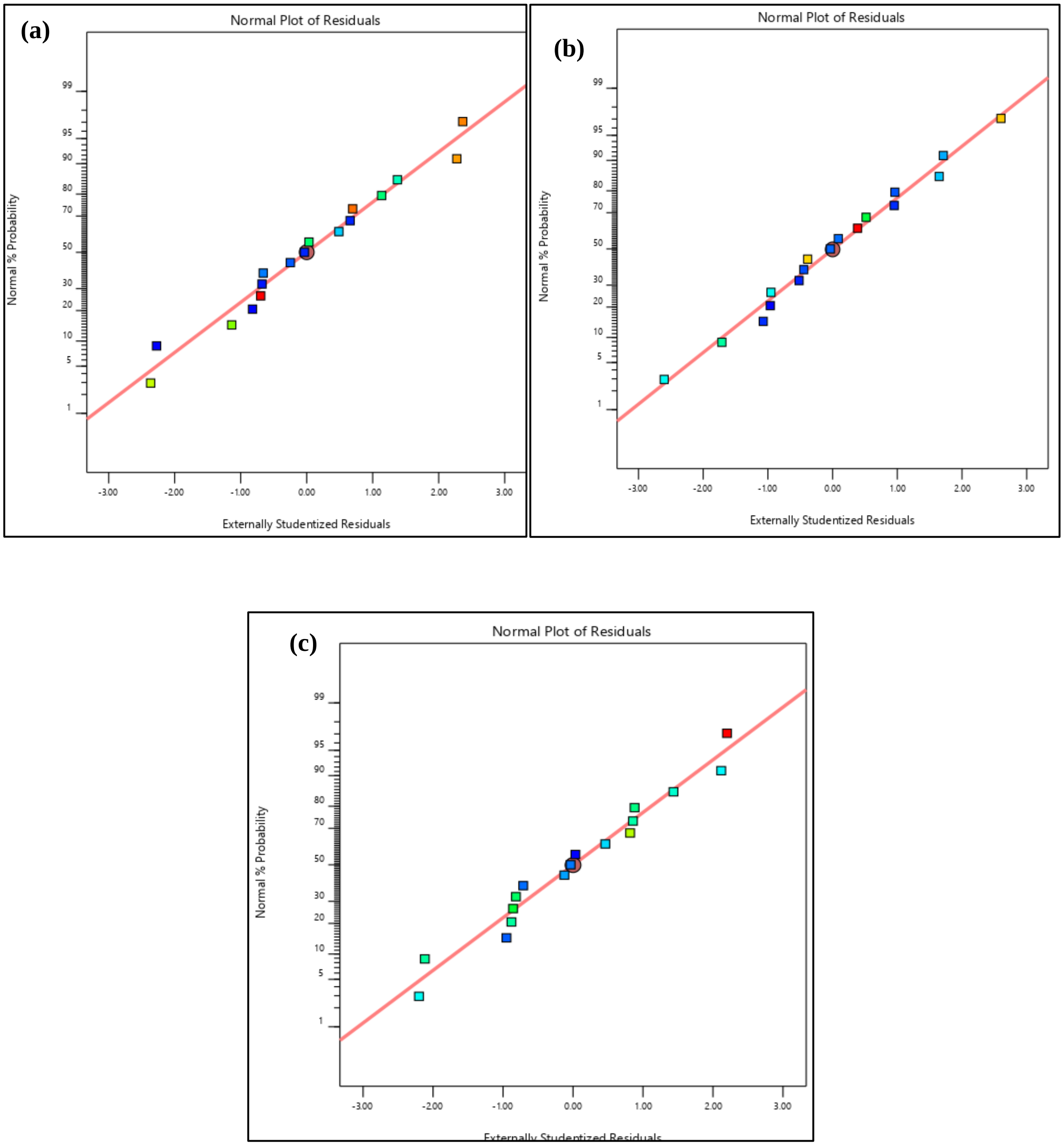

Figure 3: Normal plot of residuals for peak area of selected VOCs: (a) Ethyl acetate, (b) methyl isobutyrate and (c) butanoic acid, methyl ester

The desirability function is very much important in computing optimization of desired goal for each factor and responses. Figure 4 shows the desirability function obtained in this study. The results indicated that the optimum HS-SPME conditions for the extraction of selected VOCs in MD2 pineapples were at temperature of 30 °C, time of 29 minutes and salt addition of 1 g) with a desirability value of 1.00.

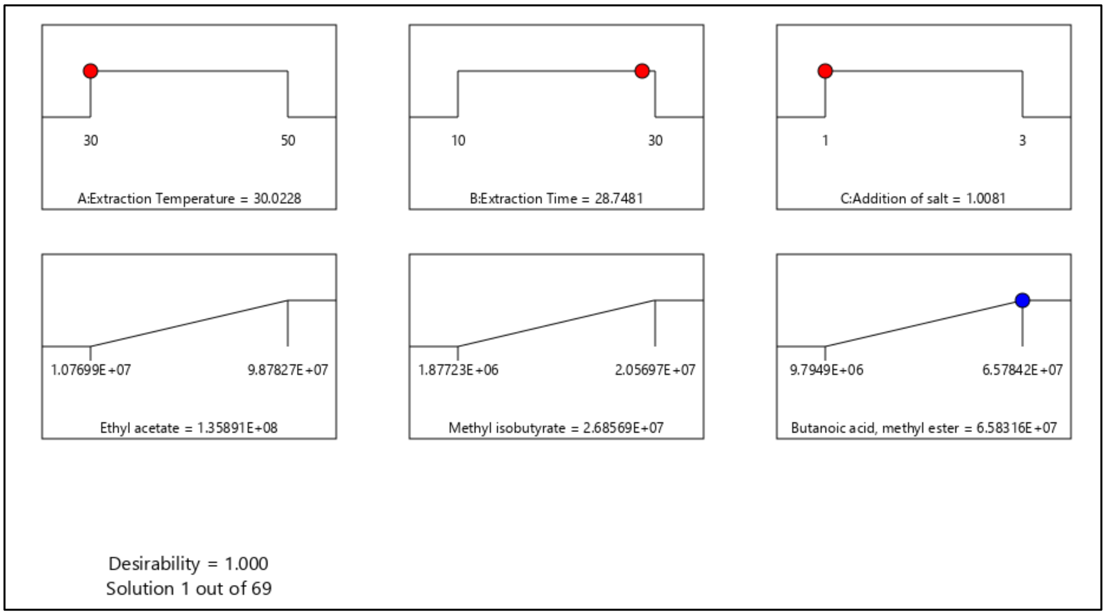

Figure 4: The ramps from Box-Behnken experimental design

CONCLUSIONS

Volatile organic compounds in pineapple are valuable parameters for the assessment of fruit quality. In this study, analytical procedure for extraction of selected VOCs in MD2 pineapple using HS-SPME was developed. The HS-SPME conditions (extraction temperature, extraction time and salt addition) were optimized using response surface method (RSM) conditions based on maximum amount of VOCs obtained. Extraction temperature of 30 ºC, extraction time of 29 min and 1 g amount of salt (NaCl) gave maximum amount of VOCs selected in this study. Therefore, these optimum conditions were utilised for the extraction of VOCs in pineapple of different varieties.

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