ORIGINAL ARTICLE

Optimization of High Antioxidant Smoothie from A Mixture of Milk, Fruits and Vegetables by Response Surface Methodology (RSM)

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ABSTRACT

Introduction: Smoothie which is mainely prepared from fruits and vegetables is a good source of health-promoting bioactive compounds, primarily antioxidants, which actively modulate disease development by inhibiting ROS-mediated reactions in the body. Smoothies represent an excellent and convenient alternative to promote the daily consumption of fruits and vegetables. **Methods:** The optimum combination of the five factors (carrot, beet, lettuce, pineapple, and banana) used to obtain the highest yield of total phenolic content (TPC), DPPH, and FRAP was analyzed using the central composite design by response surface methodology. These frutirs and vegetables used due to their availability and well-known health benefits. The effects of carrot (X1: 25-60g), beet (X2: 25-60g), lettuce (X3: 25-60g), pineapple (X4: 30-70g), and banana (X5: 25-60g), on the three variables (Y1, Y2, and Y3) were tested. **Results:** RSM generated 50 formulations**.** The experimental outcomes were adequately fitted into a second-order polynomial model regarding TPC (R^2 = 0.9436, *p* = 0.0001), DPPH (R^2 = 0.9292, *p* = 0.001), and FRAP (R^2 = 0.0.9176, *p* = 0.001). The optimum combination was 25 g of carrot,25 g of beet, 25.55 g of lettuce, 70 g of pineapple, and 30.05 g of banana. The predicted results for TPC, DPPH, and FRAP were 21.87 mg GAE/100 g, 37.17 mmol TE /100g, and 54.12 mmol TE /100g, respectively. The experimental outcomes were close to the predicted results: 21.97±0.99 mg GAE/100 g, 36.86±0.76 mmol TE /100g, and 52.26±1.52 mmol TE /100g, respectively. **Conclusion:** As a result, RSM successfully optimized the range of variables. Consequently, the optimal combination of fruits and vegetables provided the highest antioxidant content and activities, which can be used as a functional smoothie.

Keywords:
Smoothie; RSM; antioxidant; phenolic content

INTRODUCTION

Due to the increased prevalence of lifestyle diseases and awareness of the significance of a healthy lifestyle by the public, the market for functional foods and beverages has been growing and developing very quickly (Gayathry & John, 2021).

Milk contains several essential nutrients and is applied in beverage preparation to optimize nutritional content, texture, and overall consumer acceptability (Panda et al., 2023). Bananas, pineapple, carrot, beet, and lettuce are known to have several health benefits due to their bioactice compounds (Abd Halim et al., 2023; Netshiheni et al., 2019).

Despite the well-known health benefits of consuming fruits and vegetables, Malaysians are not consuming enough (Rodríguez-Verástegui et al., 2016). Thus, consuming fruits and vegetables should be promoted

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through the development of ready-to-eat with minimal and nonaggressive treatments. Accordingly, smoothies represent an excellent and convenient alternative to promote the daily consumption of fruits and vegetables. Therefore, high-antioxidant smoothies could be supplementary products for managing and preventing diseases and an alternative natural product of artificial fake functional food in the market (Tkacz et al., 2021).

Response Surface Methodology (RSM) is a combination of statistical and mathematical methodologies to improve processes, design, and formulate a product (Pinheiro et al., 2020). As such, this research aimed to develop and formulate a high-antioxidant smoothie from a mixture of milk, carrot, beet, lettuce, pineapple, and banana using response surface methodology.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals were from analytical grades obtained from Sigma, Merck, and Fisher Scientific.

Smoothie Preparation and Antioxidant Extraction

Figure 1 shows the ingredients used in smoothie preparation. The smoothie mixture consisted of two parts. The first is milk, which represents 35% of the whole mixture. The second part of the mixture contains a combination of fruits and vegetables, making up 65% of the whole mixture. Smoothie preparation was conducted at the Food Analysis Laboratory in Kulliyyah of Allied Health Sciences, International Islamic University Malaysia. The mixture was blended until the mixture was homogeneous. After that, the smoothie mixture was kept in the freezer for further analysis. However, antioxidant extraction was according to the method described by Rodríguez-Verástegui et al. (2016).

Figure 1: The ingredients used in smoothie preparation

Experimental Design

Response surface methodology was used to determine the optimum levels of carrot, beet, lettuce, pineapple, and banana for maximizing the antioxidant content and activities of the smoothie mixture on three dependent variables (responses), namely, total phenolic content (TPC), DPPH, and FRAP. The relationship between the process variables and the optimized formulation of the smoothie, in terms of its TPC, DPPH, and FRAP, was identified by adopting two factors inscribed central composite design (CCD). The independent variables investigated were carrot (X_1:25-60 g), beet (X_2: 25-60 g), lettuce (X_3: 25-60 g), pineapple (X_4:30-70 g), and banana (X_5: 30-70 g). The optimized independent variables were coded at 3 levels -1, 0, +1 (Table 1). Fifty randomized experiments were constructed.

Total Phenolic Content and antioxidant activities

The TPC was determined based on the method described by Rodríguez-Verástegui et al. (2016). DPPH and FRAP assays was conducted according to Abdullah et al. (2021).

Table 1: Coded and actual value levels of independent variables used for the optimization of high antioxidant smoothie by RSM

Independent variables	Unit	Factor	Coded level				
			-1	0	1	Axial (-α)	Axial (+α)
Carrot (X_1)	Gram	X1	25	42.5	60	0.88	84.12
Beet (X_2)	Gram	X2	25	42.5	60	0.88	84.12
Lettuce (X_3)	Gram	X3	25	42.5	60	0.88	84.12
Pineapple (X_4)	Gram	X4	30	50	70	2.43	97.57
Banana (X_5)	Gram	X5	30	50	70	2.43	97.57

Statistical Analysis

The statistical analysis used the Design-Expert Version 6.0.10 (Minneapolis, MN) software. The results were expressed as mean values. The response surface analysis was utilized to verify the regression coefficient and statistical significance of the experimental data models intended to optimize the response variables. The adequacy of the model was predicted through the regression analysis (r^2) and the ANOVA analysis (*$p<0.05$).* The desired aim was set in numerical optimization to generate the optimal conditions and point prediction outcomes of the model.

Model verification

The experimental data for TPC, DPPH, and FRAP were calculated based on the optimum conditions suggested by RSM software. The response surface model was verified by comparing the independent factors' experimental value with the optimized model's predicted value.

RESULTS AND DISCUSSION

Fitting the Model

The experimental values of TPC (Y1), DPPH (Y2), and FRAP (Y_3) were employed in multiple linear regression analysis performed using response surface analysis to fit the polynomial equation. The minute difference between the experimentally obtained response values and the predicted values indicates that an adequate model was obtained. The coefficient of the determination (R^2), adjusted (R^2), predicted (R^2), probability values (p), coefficient of variation (CV), and lack-of-fit values for response variables are tabulated in Table 2**.** The coefficients of determination (R^2) obtained were 0.94, 0.93, and 0.92 for TPC, DPPH, and FRAP, respectively, therefore indicating that approximately (91-94%) of the variations described by the model (Fan et al., 2008). In this study, the probability (p values) were less than < 0.01 for all of the response models suggesting that the models for the responses are statistically significant. None of the models displayed a significant lack of fit, suggesting that all the second-order polynomial models correlated well with the obtained results . The coefficient of variation (CV) is a measure of deviation from the mean values, which shows the reliability of the experiment. In general, CV<10% indicates better reliability. From the present findings, the TPC, DPPH, and FRAP showed low CV values (<5). Moreover, it is desirable to have sufficient precision (signal-to-noise ratio) greater than 4 (Nissar et al., 2017). In the current study, all parameters displayed a high degree of adequate precision.

Table 2 : Statistical parameters obtained after the implementation of a two-factor central composite experimental design

Coefficient	TPC	DPPH	FRAP
R^2	0.94	0.93	0.92
Adj R^2	0.90	0.88	0.86
Pred R^2	0.81	0.75	0.75
(p value)	< 0.01	< 0.01	< 0.01
Lack of fit	0.13	0.56	0.63
C.V	4.82	4.53	3.77
Adequate precision	19.64	16.15	18

Effect of The Independent Variables on TPC, DPPH, and FRAP

The second-order polynomial regression equation explained the effect of five independent variables on TPC, DPPH, and FRAP through the significant (p<0.05) coefficient. For TPC (Y1), the combination of fruits and vegetables showed a significant (p<0.05) effect regarding the first-order linear effect (X_1, X_2, X_3, X_4, and X_5), second-order quadratic effect (p<0.05) (X_1^2, X_2^2, X_4^2 *and* X_5^2), and interaction effect (p<0.05) ($X_1.X_4$, $X_2.X_4$, $X_2.X_5$, $X_3.X_5$ and $X_4.X_5$).

The predicted model observed for TPC (Y_1) Eq. (2) was:

$$TPC = +13.56 - 0.49 (X_1) -0.72 (X_2) - 0.55(X_3) + 1.30 (X_4) - 1.21(X_5) + 0.39 (X^2{}_1) + 0.61(X^2{}_2) + 0.65(X^2{}_4) + 0.55 (X^2{}_5) - 0.35 (X_1.X_4) + 0.39(X_2.X_4) + 0.54 (X_2.X_5) + 0.38(X_3.X_5) - 0.68(X_4.X) \quad (2)$$

Based on Eq. (2), carrot, beet, lettuce, and banana had shown a negative effect on total phenolic content. Meanwhile, pineapple exhibited a positive effect on TPC. The total phenolic content of the formulations decreases as the proportions of carrot, lettuce, beet, and banana increase. On the other hand, the total phenolic content (TPC) increases as the proportion of pineapple increases, which causes the most significant rise in TPC. Eq. (2) showed that TPC positively related to the quadratic effect of independent variables (carrot, lettuce, banana, and pineapple). In terms of interactions between factors, X_4 (pineapple) exhibited a significant negative effect with X_1(carrot) and X_5 (banana), while the interaction effect with X_3 (lettuce) was significantly positive ($p<0.05$). Subsequently, the individual quantity of each component used in smoothie production significantly affected the total phenolic content.

The total phenolic content for the 50 formulations varied from 12.481 mg/100g – 22.065 mg/100g gallic acid. The lowest concentration of TPC was measured when the formulation was set at (X_1= +1, X_2= +1, X_3= +1, X_4= -1 and X_5 = -1). Meanwhile, the highest concentration was measured when the formulation was at (X_1= -1, X_2 = -1, X_3= -1, X_4= +1 and X_5= -1). This indicated that the presence of pineapple in the mixture had a more significant impact than other variables on the increase in the phenolic content of the samples.

3D response surface plots were built to interpret the interactive effects of independent variables based on multiple linear regression equations, which can further assist in process optimization, help decide the optimal process conditions, and explain the cumulative effect of input variables on response values (Yang et al., 2019). Figure 2 reveals a linear and quadratic effects of variables in total phenolic content.

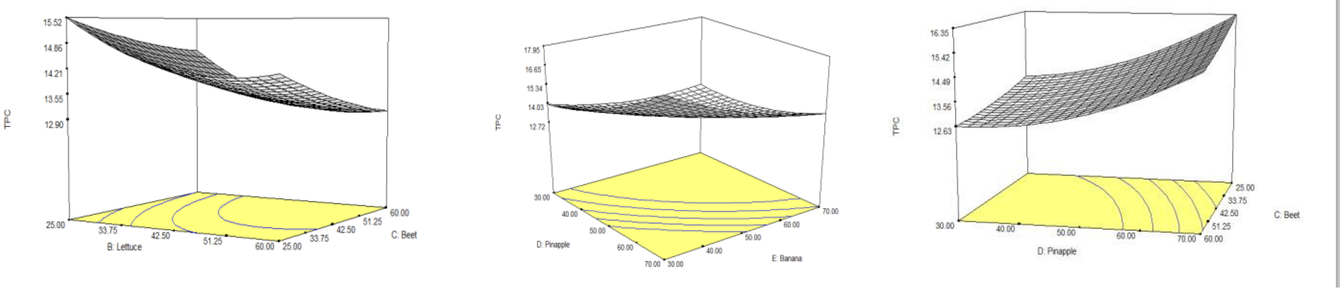

Figure 2 : Three-dimensional effect of variables on TPC

For DPPH, the combination of fruits and vegetables showed significant ($p<0.001$) effect regarding first-order linear effect (X_1, X_2, X_3, X_4 and X_5), second-order quadratic effect ($p<0.05$) (X_1^2, X_2^2,X_3^2 X_4^2, *and* X_5^2) and interaction effect ($p<0.05$) ($X_1.X_4$, $X_2.X_4$ and X_4X_5) towards DPPH (Y_2). The predicted model observed for DPPH (Y_2) was calculated according to Eq. (4)

DPPH = $+25.59 - 0.87X_1 - 1.75X_2 - 0.94X_3 + 2.04X_4 - 1.33X_5 + 0.80 (X^2{}_1) + 0.37 (X^2{}_2) + 1.23 (X^2{}_3) + 0.78 (X^2{}_4) + 0.98 (X^2 5) - 0.64X_1X_4 + 0.85X_2X_4 - 0.69X_4X_5$ (4)

Except for pineapple, which had a ($p<0.05$) significant positive correlation with DPPH, other variables had a significant ($p<0.05$) negative correlation with DPPH.
The negative correlation of the four variables (carrot, beet, lettuce, and banana) with DPPH indicates that as the concentration of these variables increases, DPPH decreases. In contrast, pineapple showed a positive correlation with DPPH. Any increase in pineapple causes a rise in DPPH level. It can be seen from Eq. (4) that DPPH is positively related to the quadratic effect of the five independent variables. Interaction terms between factors showed that X4 (pineapple) had a significant ($p<0.05$) negative effect with X1(carrot) and a significant positive effect with X_2 (beet). Subsequently, the individual quantity of each component used in smoothie production had a significant effect on the DPPH.
The DPPH values for the 50 formulations varied from 22.38-37.86 mmol/100g Trolox. The results showed that DPPH exhibited the lowest value (22.38 mmol/100g Trolox) when the formulation was set at (X_1= 0, X_2= 2.378, X_3=0, X_4=0 and X_5 = 0). Meanwhile, the highest level of DPPH value measured when the formulation was at (X_1= -1, X_2 = -1, X_3= -1, X_4= +1 and X_5= -1).
Figure 3 shows a linear and quadratic effects of variables on DPPH values. This might be due to the interactions between phytochemical compounds due to various factors (Educational & Panchor, 2020).

For FRAP, the combination of fruits and vegetables showed significant ($p<0.001$) effect regarding first-order linear effect (X_1, X_2, X_3, X_4, and X_5), second-order quadratic effect ($p<0.05$) (X_1^2, X_2^2, X_4^2 *and* X_5^2) and interaction effect (p<0.05) ($X_1.X_4$, $X_1.X_5$, X_3X_4, X_3X_5 and X_4X_5). The predicted model observed for FRAP (Y_3) was calculated based on Eq.(5)

$$\textbf{FRAP} = +45.81 -1.23 (X_1) -2.36 (X_2) +1.02 (X_3) + 2.20 (X_4) - 1.15 (X_5) + 0.77 (X^2_1) - 0.58 (X^2_2) + 1.05(X^2_4) + 0.99 (X^2_5) - 1.11 (X_1X_4) + 1.01 (X_1X_5) - 0.91 (X_3X_4) -1.60 (X_3X_5) +1.21 (X_4X_5) \quad (5)$$

The significant quadratic showed that three independent variables (carrot, beet, and banana) showed a significant (p<0.05) negative effect on FRAP. On the other hand, lettuce and pineapple showed a significant (p<0.05) positive impact on FRAP. FRAP values decrease with an increase in carrots, beet, and bananas. Meanwhile, FRAP values increase with an increase in pineapple and lettuce. Considering the quadratic effects of variables, Equation 5 showed that carrot, pineapple, and banana showed a significant (p<0.05) positive correlation with FRAP, while lettuce showed a negative quadratic effect (p <0.05). The interaction terms between ($X_1.X_4$), (X3.X4), and (X3.X5) showed a negative significant (p<0.05) effect on FRAP value, while (X1.X5) and (X4.X5) showed a positive significant effect on FRAP (p<0.05).

The FRAP value for the 50 formulations varied from 36.48 – 60.85 mmol/100g Trolox. The lowest level of FRAP value (36.48 mmol/100g Trolox) was measured when the formulation number was (X 1= 0, X2 = 2.37, X3= 0, X4= 0, and X_5= 0). Meanwhile, the highest value of FRAP was measured when the formulation was (X_1= -1, X_2 = -1, X_3= 1, X_4= 1 and X_5= -1). Figure 4 reveals a linear and quadratic effects of variables on FRAP values

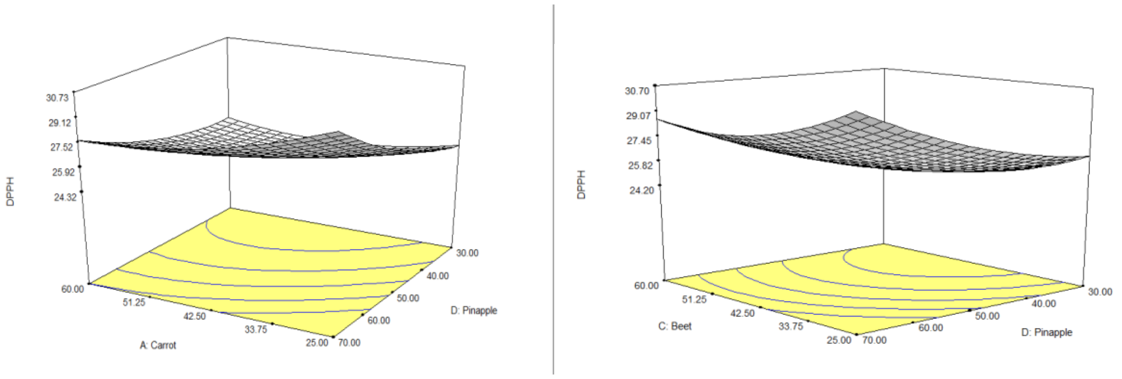

Figure 3 : Three-dimensional effect of variables on DPPH

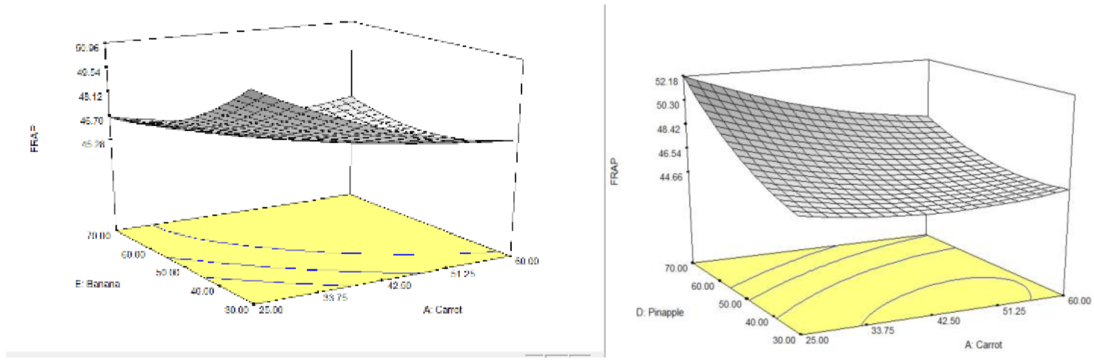

Figure 4 : The three-dimensional effect of variables on FRAP

The present global trend toward a healthy lifestyle has increased demand for convenient fresh meals that are rich in nutritional content. Thus, preparing a mixture of fruit and vegetables rich in antioxidants and having a good taste is the goal of this research. To date, fruits and vegetables such as , carrot, lettuce, beet, pineapple, and banana have been reported to contain a significant amount of phytochemical compounds that can prevent several diseases (Castillejo et al., 2016).

The phenolic content in the abovementioned fruits and vegetables is effective in absorbing and neutralizing free radicals. In this study, the observed positive and negative effects of variables and their interactions on total phenolic content might be due to the interactions between phytochemicals compounds in each factor (Stig et al., 2009). Furthermore, one study conducted by Ibrahim et al. (2022) revealed that a smoothie with a higher ratio of pineapple exhibited a higher value of total phenolic content. Also, the increase in TPC with the reduction of carrot, beet, lettuce, and pineapple might be due to the pro-oxidant activity, which may occur on factors (Michaëlsson et al., 2018). As far as reviews are concerned, pineapple is reported to be a novel antioxidant fruit that is rich in phenolic and flavonoid content. It also shows a strong antioxidant activity. Thus, higher phenolic content could be attributed to the inherent antioxidant properties of pineapple itself. Also, a consideration of the synergistic effects of different factors in the smoothie mixture and how all factors together contribute to the phenolic content and antioxidant capacity of the smoothie should be taken (Uduwana et al., 2023).

Several methods are used to measure antioxidant activity, with the DPPH assay being the most common. Another assay is FRAP (ferric-reducing/antioxidant power), which measures the conversion of a Fe_{3+}/ferricyanide complex to the ferrous form (Zou et al., 2015). The presence of pineapple in the mixture had a greater impact than other variables on the increase in the antioxidant activities measured by DPPH and FRAP. The antioxidant activity of the 50 formulations increases with total polyphenol contents. Also, geographical and climate conditions may affect the concentration of antioxidants in each factor. Additionally, the synergistic effects of each factor in the smoothie mixture contribute to the antioxidant activities (Uduwana et al., 2023).

Optimization of Responses and Verification of Model

Each industrial process requires optimization as it has a direct influence on product quality and process efficiency. Each of the investigated responses might be optimized independently on a target value; however, each has its optimum parameters, but not all of them are in great correlation, indicating that improving one response could have the opposite effect on another. However, by using RSM approach and desirability function (D), several responses can be optimized simultaneously (Saikia et al., 2020).

In this study, the independent variables were studied in range; meanwhile, the responses were maximized to obtain a mixture with high TPC, DPPH, and FRAP values. Therefore, the TPC, DPPH, and FRAP values of the mixture were simultaneously optimized according to the target presented in Table 3. Numerical optimization has been used to determine the best condition for independent variables from various solutions generated. Considering the degree of desirability (D) (0.905), the optimum combination was determined to be 25 g of carrot,25 g of lettuce, 25.55 g of beet, 70 g of pineapple and 30.05 g of banana with the predicted response values for TPC, DPPH and FRAP 21.87 mg/100g gallic acid, 37.17 mmol/100g Trolox, and 54.12 mmol/100g Trolox, respectively.
The combination that yielded the optimum condition was repeated to test the response surface models' ability to predict the optimal response values. The observed values of the total phenolic content, DPPH, and FRAP were 21.97±0.99 mg/100g gallic acid, 36.86±0.76 mmol/100g Trolox, and 52.26±1.52 mmol/100g Trolox, respectively. The experimental and predicted values were compared to verify the response surface model. The predicted and the experimental values were compared by the degree of difference. The differences for TPC, DPPH, and FRAP were 0.46%, 0.8%, and 3.43%, respectively. Therefore, the experimental values were close to the predicted values, and the model was verified (Table 3).

Table 3 : Simultaneously optimized conditions with target and predicted values of responses.

Response	Target	Predicted value	Experimental value	% Difference
TPC mg GAE/100g	Maximized	21.87	21.97±0.99	0.46%,
DPPH mmol TE /100g	Maximized	37.17	36.86±0.76	0.8%
FRAP mmol TE /100g	Maximized	54.12	52.26±1.52	3.43%

Experimental results were expressed as mean ±standard deviation (n=3)

CONCLUSION

The optimum combination that produced the highest TPC, DPPH, and FRAP from a mixture of fruits and vegetables was determined using a central composite design by response surface methodology. An adequate model equation was generated to predict the influences of the independent variables and their optimum level in the combination. TPC, DPPH, and FRAP successfully verified the high antioxidant combination. The final combination to prepare 100 grams of this smoothie is as follows: Milk: 35 grams, carrot: 9.25-gram, beet: 9.25-gram, lettuce: 9.5-gram, pineapple: 25.9 gram and banana: 11.1 gram. Thus, this combination can be considered optimal for the research's desired objective, which is to obtain a combination with high antioxidant content and activities.

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