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# Effect of Ultrasonication Process on Microbial Content and Antioxidant Activities of Black Mulberry-Pineapple-Mango Functional Drink

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Fruit juice may function as functional drink to improve human immune system. Fresh fruit-based functional drink contains high number of bioactive compounds, but it also may contain harmful microorganisms. The first objective of this work was to study the effect of ultrasonic amplitude and processing time on the microbial content and antioxidant activities of novel black mulberry-pineapple-mango functional drink. The second objective was to optimise the ultrasonic processing condition of the juice. Ultrasonic method was chosen in this study because it is a non-thermal processing technique which requires less energy consumption with shorter processing time. The study was conducted using Response Surface Methodology with ultrasonic amplitude (50 to 100 %) and processing time (4 to 12 min) as the investigated factors. The results show that ultrasonic treatment was able to reduce the microbial content yet maintained the antioxidant properties of the juice. The microbial content was significantly ($p < 0.05$) decreased, total phenolic content (TPC) and antioxidant activities (DPPH scavenging and FRAP) were significantly increased ($p < 0.05$) when high amplitude was applied. Increasing the processing time beyond 8.00 min was shown to cause negative impact towards total phenolic content and antioxidant activities of the drink. From optimisation, the best sonication condition for obtaining black mulberry-pineapple-mango functional drink with the lowest microbial content of 0.002 CFU/ml and highest TPC of 632.54 mg GAE/100 g, DPPH scavenging activity of 92.81 % and FRAP of 0.5127 mmol $Fe^{2+}$/100 g is at amplitude of 100 % and processing time of 8.00 min. From the study, it can be concluded that ultrasonic processing is a potential alternative for fruit-based functional drink pasteurization.

## 1. Introduction

Functional drinks are defined as drinks which have advantageous physiological effects beyond their basic nutritional levels which provides essential nutrients for humans. Functional drinks are manufactured and processed to enrich nutrients such as vitamins, minerals and other natural antioxidants (Wang et al., 2016). Nowadays, there is an increasing demand for healthy drinks. This trend has led to the growth of functional drink industry (Szakaly et al., 2012). According to Technavio.com (2020), the market of functional drink is expected to increase as much as 179.28 B USD over 4 y period from 2020 – 2024.

Among all types of functional drinks, fruit-based functional drink is high in demand. Recently, this drink has become a trend in the market due to different type of nutrients and beneficial compounds available in it which serves different functions in the body. Based on this trend, a novel fruit-based functional drink from black mulberry-pineapple-mango was formulated. Black mulberry (*Morus nigra*) is a fruit which contains high health beneficial compounds such as anthocyanins and other biologically active substances with antioxidant, antimutagenic and anticancer properties (Atmakuri et al., 2009). Pineapple (*Ananas comosus L.*) has been found to be effective as anticoagulant, analgesic agent and tumor suppressor (Wali, 2019). Pineapples contain vitamin C, β-carotene, phenolic compounds, enzymes, minerals and fibers. Mango (*Mangifera indica L.*) is a

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good source of vitamin B3, B5, B6 and C (Braga et al., 2019), and it also contains phenolic compounds such as gallic acid and quercetin (Robles-Sanchez et al., 2009).
The problem with fruit-based functional drink is, it has a short shelf-life because it is susceptible to microbial and enzymatic attack. A proper treatment is required to extend the shelf-life of fruit-based functional drink. Conventional treatment methods available which are based on high temperature and long processing duration are not suitable for fruit-based functional beverages because these processes can degrade the quality of the juices. Fresh juice contains various heat sensitive phytochemicals which can easily be destroyed due to long exposure to high heat. Ultrasonic process, which is a non-thermal treatment, has been showed having no significant detrimental effect on bioactive compounds in kiwi juice (Wang et al., 2019). Ultrasonic process was selected in this study to extend the shelf-life and retain the phenolic content and antioxidant properties in this novel black mulberry-pineapple-mango mixed fruit juice. The objectives of this study were to evaluate effect of ultrasonic processing factors (amplitude and time) on the mulberry-pineapple-mango mixed juice and subsequently optimize this process. The findings from this study may be useful for small and medium beverage producers who are seeking for affordable alternative pasteurization process for their products.

## 2. Materials and methods

### 2.1 Experimental design and statistical analysis

This experiment was designed using Face Centered Central Composite Design (CCD) under Response Surface Methodology (RSM) concept. Two factors studied were ultrasonic amplitude and processing time, and these factors were selected based on literature review. Table 1 shows the factors and their levels. The experimental design and statistical analysis via Analysis of Variance (ANOVA) on the data obtained were conducted using the trial version of Design Expert software (version 7.1.5, Stat-Ease, USA).

*Table 1: Factors studied in ultrasonic processing of black mulberry-pineapple-mango mixed juice*

| Factor name | Factor | Factor level | | |
|---|---|---|---|---|
| | | -1 | 0 | 1 |
| Ultrasonic Amplitude (%) | A | 50 | 75 | 100 |
| Processing Time (min) | B | 4 | 7 | 12 |

The optimization was performed using data obtained in the experimental design shown in Table 1. The optimization study was conducted to obtain an optimum ultrasonic processing condition which can produce black mulberry-pineapple-mango functional drink with low microbial content and high TPC and antioxidant activities.

### 2.2 Preparation of black mulberry-pineapple-mango juice

Fresh black mulberries were purchased from Zenxin Organic Park (Kluang, Johor, Malaysia). Fresh pineapples and mangoes were procured from Taman Universiti wet market in Skudai, Johor. The fresh mulberries were washed under tap water and then they were strained using a plastic strainer to remove excessive water. To obtain the juice of black mulberries, the fruits were ground by using a domestic blender (Khind, BL 1515, Malaysia). For the extraction of pineapple juice, the fruits were cleaned with tap water. Then, the crown, skin and core of the pineapple fruits were removed by using a clean stainless-steel knife. The flesh was sliced into small pieces and ground using the same blender used for black mulberries. For the extraction of mango juice, the fruits were cleaned with tap water. Then, the skin and the seed the mango fruit was removed by using a clean stainless-steel knife. The flesh was sliced into small pieces and ground using the same blender mentioned previously. Each of the extracted juice was filtered twice using muslin cloth and stored in separate glass bottles in a refrigerator at 4°C for further experiments. The new formulated black mulberry-pineapple-mango functional drink was prepared by mixing 70 % black mulberry juice with 20 % pineapple juice and 10 % mango juice. This ratio was the best formulation obtained from the work of Tan (2020).

### 2.3 Ultrasonic processing of juice

An ultrasonic processor equipped with 13 mm diameter probe was used to sonicate the juice (Sonics, Fisher Scientific, USA). The equipment has the frequency of 20 kHz and power of 750 W. The samples were processed at ultrasonic amplitude between 50 to 100 % and processing time between 4 to 12 min, following the experimental design mentioned in Section 2.1. 100 mL of this functional drink was used for each experimental treatment. The probe was submerged 25 mm in the sample. During the experimental treatment,

the sample was put in ice bath to avoid the temperature of the sample from exceeding 50 ºC, which may cause degradation of compounds in the samples (Oliveira et al., 2018).

### 2.4 Determination of total phenolic content (TPC)

Folin–Ciocalteu reagent and gallic acid calibration curve were used to determine the concentration of total phenols in the samples. 0.2 mL of sample and 0.2 mL of Folin–Ciocalteu reagent was blended and mixed thoroughly. After 4 min, 1 mL of 15 % sodium carbonate ($Na_2CO_3$) was added, and then the mixture was kept for 2 h at room temperature. The absorbance was measured at 760 nm using a spectrophotometer (Jenway Genova, United Kingdom). The concentration of the total phenolics were determined as mg of gallic acid equivalent by using an equation obtained from gallic acid calibration curve (Hossain and Rahman, 2011).

### 2.5 Determination of antioxidant activity via DPPH assay

The method used for determining this antioxidant activity was according to method described in Ahmad et al. (2020). Reaction solution for each formulation was prepared by mixing 4.5mL of diluted fruit juice sample with 4.5 mL of 0.1 mM ethanolic DPPH. The solution was kept in dark at room temperature for 30 min. Then, the absorbance ($A_{juice}$) was measured at 517 nm using UV-vis spectrophotometer (Jenway Genova, United Kingdom). At the same time, a DPPH blank solution (4.5 mL of 0.1 mM ethanolic DPPH, 1.5 mL of water) was prepared. Absorbance ($A_{DPPH}$) was measured at 517nm using the same spectrophotometer. Percent inhibition of DPPH radical was calculated for each sample according to the Eq(1) below:

$$\% \text{ inhibition} = \frac{A_{DPPH} - A_{juice}}{A_{DPPH}} \times 100 \quad (1)$$

### 2.6 Determination of antioxidant activity via FRAP assay

The samples were added to 3.0 mL of freshly prepared FRAP reagent and 300 µL of ultrapure water. The reaction mixture was incubated at 37 °C for 10 min. Then, the absorbance the mixture was determined at 593 nm using a spectrophotometer (Jenway Genova, United Kingdom). In FRAP assay, the antioxidant potential of sample was determined via a standard curve plotted using the $FeSO_4 \cdot 7H_2O$ linear regression equation to calculate the FRAP values of the sample. BHT was used as the positive control (Zhu et al., 2019).

### 2.7 Determination of microbial content

The microbial content in the functional drink was determined via spread plate method using nutrient agar. For this test, a 0.1 mL of sample from each serial dilution (10-1 to 10-5) was spread onto the solidified agar. The nutrient agar plates were incubated inverted for 3 d at 37 °C. The colonies were counted using a colony counter (Chin, 2018).

## 3. Results and Discussion

### 3.1 Effect of ultrasonic amplitude and processing time on microbial content, total phenolic content (TPC), antioxidant activities (DPPH and FRAP) of mixed juice

ANOVA results for microbial content and TPC are tabulated in Table 2. From ANOVA, amplitude and time have significant effect ($p<0.05$) on the microbial content in the functional drink. The interaction effect between amplitude and time on this drink was also significant.

*Table 2: ANOVA for microbial growth and total phenolic content (TPC) models*

| Source | | Microbial Growth | | | | TPC | | | |
|---|---|---|---|---|---|---|---|---|---|
| $R^2$ Values | | $R^2$ = 0.9559; Adj $R^2$ = 0.9118 | | | | $R^2$ =0.9179; Adj $R^2$ =0.8359 | | | |
| Factors | DF | SS | MS | F-value | P-value | SS | MS | F-value | P-value |
| Model | 5 | 0.9500 | 0.1900 | 21.68 | 0.0021 | 12980.0 | 2596.16 | 11.19 | 0.0096 |
| A - amplitude | 1 | 0.4100 | 0.4100 | 46.94 | 0.0010 | 11786.10 | 11786.10 | 50.78 | 0.0008 |
| B - time | 1 | 0.2700 | 0.2700 | 30.72 | 0.0026 | 146.32 | 146.32 | 0.63 | 0.4632 |
| $A^2$ | 1 | 0.0036 | 0.0036 | 0.41 | 0.5502 | 364.09 | 364.09 | 1.57 | 0.2658 |
| $B^2$ | 1 | 0.0001 | 0.0001 | 0.0016 | 0.9694 | 821.87 | 821.87 | 3.54 | 0.1186 |
| AB | 1 | 0.2700 | 0.2700 | 30.31 | 0.0027 | 85.73 | 85.73 | 0.37 | 0.5699 |
| Lack of fit | 3 | 0.0420 | 0.0140 | 22.36 | 0.0500 | 853.06 | 284.35 | 1.85 | 0.3697 |
| Pure error | 2 | 0.0013 | 0.0006 | | - | 307.36 | 153.68 | | - |

*SS: sum of squares; DF: degree of freedom; MS: mean square

The effect of amplitude and processing time on the microbial growth can be observed in 3D graph (Figure 1a). Amplitude has linear negative impact on microbial content while processing time has positive linear impact. Gao et al. (2016) stated that more bacteria will be inactivated at high ultrasonic amplitude because more energy is released at this condition to cause bacterial destruction. Higher reduction of microbial content due to high amplitude was also reported in Cruz-Cansino et al. (2016). Ultrasonication is an innovative method which could be utilized as an alternative food processing technique to enhance the stability and microbiological safety of food (Oliveira, et al., 2018).

From ANOVA (Table 2), the total phenolic content (TPC) was significantly affected by ultrasonic amplitude ($p<0.05$). Processing time has no significant effect on TPC. The effect of amplitude and time on TPC can be seen in Figure 1b. The total phenolic content of formulated juice mixture was high when the process was conducted at high amplitude. This situation happened plausibly because of the extraction of bound phenolics from suspended particles in the mixed juice due to ultrasonic treatment (Mohideen et al., 2015). Processing time has quadratic effect on TPC. TPC became higher as time 4 min increases to 8 min. Exposing the juice beyond 8 min caused gradual reduction in TPC. Murcia et al. (2009) stated that the reduction of phenolic compound might be caused by the heat generated by the sonication during long processing time. The ultrasonic treatment time should be kept short at a suitable range to avoid potential TPC degradation.

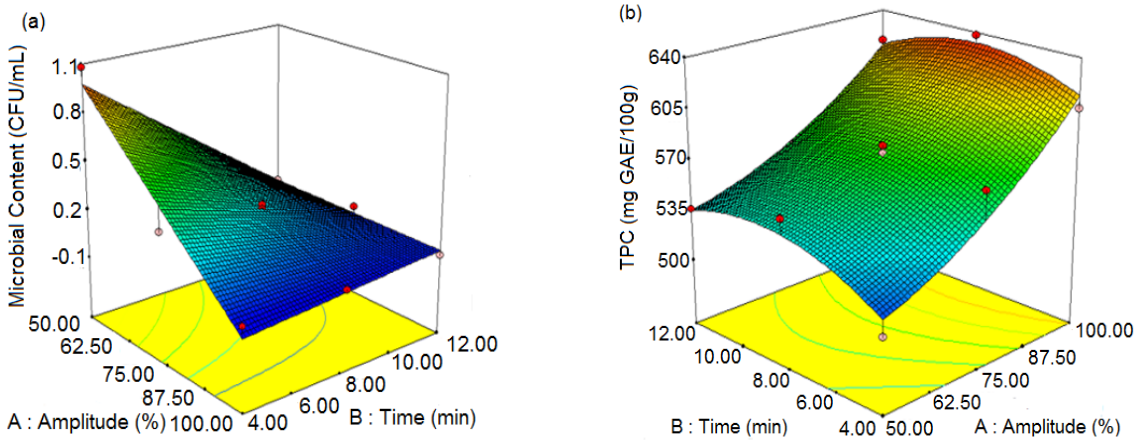


*Figure 1: Effect of ultrasonic amplitude and processing time on (a) microbial content and (b) TPC. The microbial content and TPC of untreated (control) sample were 1.0 CFU/mL and 595.93 mg GAE/100 g.*

Besides analysing impact of factors, ANOVA also generates models (in coded form) which relates microbial content and TPC to amplitude (A) and processing time (B) as shown in Eq(2) and Eq(3) :

$$Microbial\ Content = 0.23 - 0.26A - 0.21B + 0.26AB + 0.038A^2 - 0.002B^2 \quad (2)$$

$$Total\ Phenolic\ Content = 576.32 + 44.32A + 4{,}94B - 4.63AB + 11.99A^2 - 18.01B^2 \quad (3)$$

*Table 3: ANOVA for antioxidant (DPPH and FRAP) activities models*

| Source | | DPPH | | | | FRAP | | | |
|---|---|---|---|---|---|---|---|---|---|
| $R^2$ Values | | $R^2$ = 0.8913; Adj $R^2$ = 0.7825 | | | | $R^2$ =0.8401; Adj $R^2$ =0.6801 | | | |
| Factors | DF | SS | MS | F-value | P-value | SS | MS | F-value | P-value |
| Model | 5 | 155.30 | 31.06 | 8.20 | 0.0188 | 0.020 | 0.0040 | 5.25 | 0.0463 |
| A - amplitude | 1 | 107.08 | 107.08 | 28.26 | 0.0031 | 0.013 | 0.013 | 17.36 | 0.0088 |
| B - time | 1 | 1.66 | 1.66 | 0.44 | 0.5373 | 0.0001 | 0.0001 | 0.19 | 0.6786 |
| $A^2$ | 1 | 3.50 | 3.50 | 0.92 | 0.3807 | 0.0026 | 0.0026 | 3.35 | 0.1267 |
| $B^2$ | 1 | 31.33 | 31.33 | 8.27 | 0.0348 | 0.0018 | 0.0018 | 2.36 | 0.1848 |
| AB | 1 | 15.07 | 15.07 | 3.98 | 0.1027 | 0.0032 | 0.0032 | 4.17 | 0.0966 |
| Lack of fit | 3 | 10.59 | 0.0140 | 0.85 | 0.5820 | 0.0028 | 0.0009 | 1.83 | 0.3721 |
| Pure error | 2 | 8.35 | 0.0006 | | - | 0.0010 | | | - |

*SS: sum of squares; DF: degree of freedom; MS: mean square

From ANOVA (Table 3), the antioxidant activities of the juice were mainly affected by ultrasonic amplitude ($p<0.05$). The effect of time was not significant on antioxidant activities. From Figure 2a and Figure 2b, the DPPH and FRAP values were the highest when the process was conducted at the highest amplitude. The antioxidant activities portray similar trend as TPC. According to Nguyen and Nguyen (2018), TPC was probably the main antioxidant in mulberry juice since a strong positive correlation between TPC and antioxidant activity was observed in their study.The antioxidant activity via DPPH shows an increasing trend when the process was conducted from 4 min to 8 min. Exposing the juice to the ultrasonic process from 8 min to 12 min caused gradual reduction in DPPH values. As for FRAP values, in terms of processing time, the effect was quadratic, however the values start to decrease gradually when juice was exposed to the ultrasonic process beyond 10 min. Murcia et al. (2009) reported that the phenolic compounds and other phytochemicals in fruits and vegetables contribute to antioxidant activities in the fruits and vegetables. As explained previously, heat generated during the process may have degraded the phenolic compounds. It is plausible that the antioxidant activities in this formulated functional drink decreases due to the degradation of the phenolic compounds and other heat sensitive phytochemicals availabe in the drink. The ultrasonic process should be conducted at high amplitude for short processing time in order to retain the health benefits of this fruit-based functional drink.

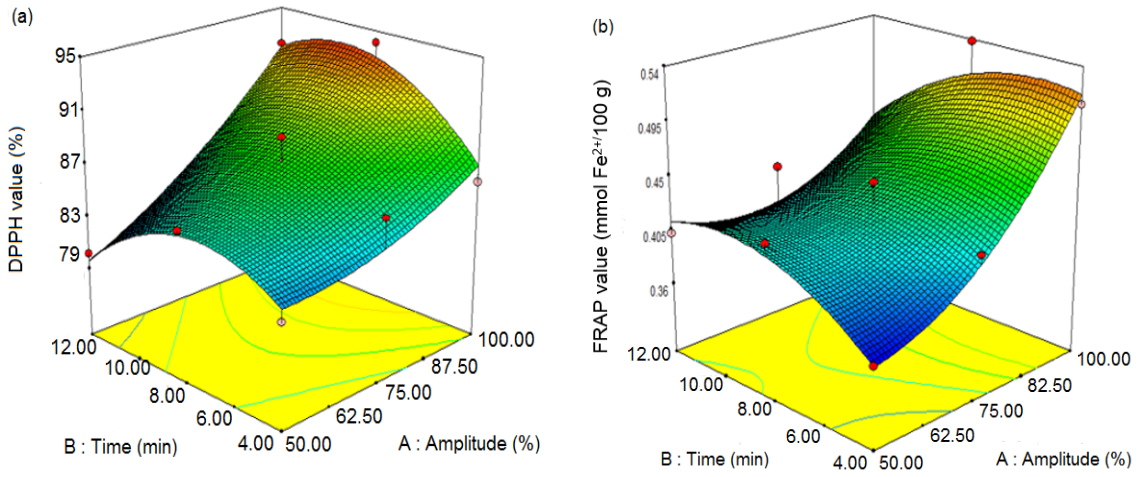


*Figure 2: Effect of ultrasonic amplitude and processing time on (a) DPPH and (b) FRAP activities. The DPPH and FRAP activities for untreated (control) sample were 88.10 % and 50.03 mmol $Fe^{2+}$/100 g.*

In addition to factors effect analysis, ANOVA also generates models (in coded form) for predicting DPPH and FRAP values in this drink as shown in Eq(4) and Eq(5) :

$$DPPH = 87.57 + 4{,}22A + 0.53B + 1.94AB + 1.18A^2 - 3.52B^2 \quad (4)$$

$$FRAP = 0.43 + 0{,}047A - 0.005B - 0.028AB + 0.032A^2 - 0.027B^2 \quad (5)$$

### 3.2 Optimization

From the desirability function in the software, the optimum ultrasonic processing condition with the highest desirability value of 92.8 % for this formulated functional drink is at amplitude of 100 % and processing time of 8.00 min. This optimum condition may produce functional drink with microbial content of 0.002 CFU/ml, TPC value of 632.54 mg GAE/100 g, DPPH value of 92.81 % and FRAP value of 0.5127 mmol $Fe^{2+}$/100 g.

## 4. Conclusions

From this experiment, it can be observed that ultrasonic processing can reduce microbial content without significant negative impact on TPC and antioxidant activities of this formulated mixed fruit juice. The effect of amplitude was significant on microbial growth, TPC, DPPH and FRAP, and most effective when the highest amplitude was utilized. The time factor has no significant impact on all responses, except for microbial content. Formulated black mulberry-pineapple-mango functional drink with low microbial content of 0.002 CFU/ml and high TPC, DPPH and FRAP value of 632.54 mg GAE/100 g, 92.81 % and 0.5127 mmol $Fe^{2+}$/100

g, can be obtained at the optimum ultrasonic processing condition of 100 % amplitude and 8.00 min operating time. The effect of this optimum processing condition on the shelf-life of this fruit-based functional drink during storage will be evaluated in the future work.

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