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Effects of ultrasound frequency and process variables of modified ultrasound-assisted extraction on the extraction of anthocyanin from strawberry fruit

Jianqing LIAO[1*], Hongkun XUE[2], Junling LI[3], Ling PENG[3]

Abstract

The aim of this work was to evaluate the effects of ultrasound frequencies from 18 to 90 kHz on the extraction of anthocyanin from strawberry fruit by using an approach of modified ultrasound-assisted extraction (MUAE) that was based on a frequency search device with six extraction tanks controlled by a computer. According to the obtained optimal frequency of 62-64 kHz, the effects of other different process variables, such as treatment time, temperature, ultrasound power and solvent to solid ratio, on the extraction of anthocyanin were also studied in comparison with heat assisted extraction (HAE) and ultrasound-assisted extraction (UAE). The obtained results showed that the maximum yield of 796.9 μg/g of anthocyanin was obtained at the optimized conditions of ultrasound frequency of 62-64 kHz, solvent to solid ratio of 6 mL/g, ultrasound power of 120 W, temperature of 45°C and treatment time of 12 min with 55% ethanol as the solvent. The application of MUAE showed that anthocyanin yield increased by about 42% and 22% higher than that of HAE and UAE, respectively. These findings demonstrated MUAE is not only an effective and feasible method for extracting anthocyanin from strawberry fruit, but also promising for extracting active components from natural materials in industry.

Keywords: anthocyanin; strawberry fruit; ultrasound-assisted extraction; process variables.

Practical Application: The modified ultrasound-assisted extraction by seeking the optimal frequency provided an efficient approach to improve the extraction yield of anthocyanin from strawberry fruit.

1 Introduction

Strawberry fruit (*Fragaria × ananassa Duch.*) has been shown to possess high content of anthocyanin that is a type of polyphenols quantitatively most important in strawberry (Giampieri et al., 2014). Anthocyanin is known as flower pigments and water soluble natural pigments responsible for the blue, purple and red colors of vegetables, fruits and flowers (Einbond et al., 2004; Petroni& Tonelli, 2011). Recently, anthocyanin studies have received growing interests due to their health benefits against neurodegenerative diseases, cardiovascular diseases and cancer (Wrolstad, 2004). Moreover, anthocyanin is also found to be remarkable bioactive effects such as anti-inflammatory, anticancer, anti-bacteria, antioxidant, antidiabetic, hepatoprotective activities and memory enhancing (Wrolstad et al., 2005; Welch et al., 2008). In view of this, it would be interesting to use strawberry fruit to obtain high-added-value anthocyanin by extraction approach.

Conventional extraction technologies, such as maceration, solvent extraction, heat assisted extraction, Soxhlet extraction (Monrad et al., 2010; Ćujić et al., 2016; Leichtweis et al., 2019; Ochoa et al., 2020) etc., have been traditionally used for extracting anthocyanin from various plant materials. Nonetheless, the main drawbacks of those extraction processes are of large amount of solvents, longer time of treatment, higher consumption of energy, and lower extraction efficiency (Castañeda-Ovando et al., 2009; Silva et al., 2017; Scudino et al., 2020; Oliveira et al., 2022). Consequently, in order to overcome these shortcomings associated with conventional extraction methods, various new techniques, such as ultrasound-assisted extraction (Fernandez-Barbero et al., 2019; Xue et al., 2021b; Nyuydze & Martínez-Monteagudo, 2021; Scudino et al., 2022), microwave-assisted extraction (Duan et al., 2015; Xue et al., 2021a), supercritical carbon dioxide extraction (Tyśkiewicz et al., 2018), have been developed for the extraction of anthocyanins from various plant materials.

Among these extraction techniques, ultrasound-assisted extraction (UAE) has been proven to be a very effective and promising extraction technology due to improving the mass transfer, liquid turbulence and circulation currents produced by cavitation, which make UAE technology to benefit from many advantages including lower temperature, shorter processing time, smaller solvent consumption and higher extraction rate (Jiao & Kermanshahi pour, 2018). During ultrasonic irradiation, process variables are able to deeply affect the extraction process. Among them, ultrasound frequency is a key variable for the extraction of bioactive compounds from plant materials (Liao et al., 2015; Chemat et al., 2017; Hu et al., 2021). Generally, the extraction rate of bioactive compounds might reach a peak value at the most appropriate frequency. It is beneficial to promote cavitation bubbles into resonance, and to collapse violently in liquid medium, so that more extraction yields are

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[1] *College of Physical Science and Engineering, Yichun University, Yichun, Jiangxi, China*
[2] *College of Traditional Chinese medicine, Hebei University, Lianchi District, Baoding, China*
[3] *College of Chemistry and Bioengineering, Yichun University,Yichun, Jiangxi, China*
*Corresponding author: jndxljqbs@126.com

achieved (Lian et al., 2021). With that in mind, evaluating the role of ultrasound frequency in influencing the extraction of anthocyanin from plant materials is of important significance. Previous researches generally focused on the optimization or impact of other different process variables, whereas studying the effect of ultrasound frequency with wide range on the extraction of anthocyanin from plant materials (especially from strawberry fruit) is scarce.

For this purpose, the aim of this work was to research whether extraction efficiency of anthocyanin from strawberry fruit is deeply affected by process variable in terms of ultrasound frequency and to asses other different process variables to achieve the highest yield of anthocyanin from strawberry fruit.

2 Materials and methods

2.1 Plant material and reagents

Fresh strawberry fruit samples were collected from a farm product market in Yuanzhou (Yichun City, Jiangxi Province, China). The fruit samples were washed with water and cut into slices with about 0.5 cm granule in diameter. The obtained samples were carefully mixed to ensure sample equality and store in a black container to protect against light at 6°C until analysis.

Folin-Ciocalteu (FC) reagent was purchased fromShanghai Qiangshun Chemical Co. Ltd. (Shanghai, China). Sodium acetate, hydrochloric acid,potassium chloride,citric acid, ethanol (reagents of analytical grade) were obtained from Tianjin Chemical Factory (Tianjin, China). Acetonitrile and methanol (reagents of HPLC grade) were obtained by mail order from sinopharm chemical Reagent Co., Ltd. (Shanghai, China).All other chemicals and solvents were kindly provided from Aladdin Industrial Co., Ltd. (Shanghai, China).

2.2 The apparatus for MUAE

Schematic diagram of the apparatus for MUAE was illustrated in Figure 1. This apparatus was composed of a computer and six identical cylindrical extraction tanks where six continuous frequency bands were correspondingly configured to six extraction tanks from #1 to #6. Each extraction tank was constituted by a circular stainless steel bath with the diameter of 250 mm. The desired temperature was controlled by re-circulating the constant temperature water through a coil. A generator and an ultrasound power supply were applied to generate the desired power intensity to drive nine transducers (>0.1 W/mm^2) that were mounted under a circular aluminum vibrating plate with the diameter of 220 mm. To ensure high accuracy of ultrasound frequency for each vibrating plate, the difference of natural frequency of each transducer was kept within 1 kHz. Thus, even if there was frequency drift, ensure that at least one transducer can work well.

2.3 Extraction methods

Modified ultrasound-assisted extraction (MUAE) procedures

Extraction experiments were performed in each extraction tank at different frequency bands. The extraction procedures were divided into two steps.

Step 1: Six strawberry fruit samples with the same weight (3.0 g) and the predetermined volume of extracting solvent were respectively added into six conical flasks, and then six mixed solutions were respectively immersed into the six extraction tanks for ultrasound irradiation under different extraction conditions including ultrasound power (50, 80, 120, 150, 200 and 300 W), solvent to solid ratio (1, 3, 6, 9, 12 and 15 mL/g), extraction temperature (25, 35, 45, 55, 65 and 75°C) and treatment time (3, 6, 9, 12, 15, 18, 21, 24 and 27 min). The mixtures were irradiated by ultrasound for different times at the required solvent to solid ratio, ultrasound power levels and temperature with ethanol of 55% as the solvent. The desired ultrasound power can be regulated by varying input AC voltage through an autotransformer. Also, the desired temperature was controlled by re-circulating the constant temperature water through a coil. The extracts were passed through a C-18 DSC-18Lt 60 mL, 10 g cartridge (Supelco, Bellefonte, PA) activated with acidified ethanol followed by 1% HCl (v/v) in deionized water for all extraction tanks. Anthocyanins were adsorbed onto the column while sugars, acids, and other water-soluble compounds were removed by flushing with 1% HCl. Anthocyanins were recovered with ethanol containing 1% HCl (v/v). After extraction experiments finished, the acidified ethanol fractions were evaporated using a rotary evaporator (RE-300A, Shanghai Yitian Scientific Instrument Co., Ltd, China) at 40 °C and the solids were stored at 4 °C until analysis. All experiments were conducted and all samples analyzed in triplicates.

Step 2: Extraction experiments were carried out by using the optimum frequency band (e.g., 54-66 kHz) obtained from the results of the above step. Other process variables were fixed at the optimized conditions. In this step, the optimum frequency band was further divided into six narrow bands. The next experimental procedures were the same as mentioned in Step 1.

Heat assisted extraction (HAE)

For HAE experiments, 3.0 g sample of strawberry fruit and 60 mL of ethanol solution (55% v/v) were mixed evenly and placed into a reactor for extracting. A thermostatic water-bath with continuous electro-magnetic stirring agitation was used to control the extraction temperature. The controlled variables and tested ranges were: temperature (25 to 75°C), solvent to solid ratio (1 to 15 mL/g), and time (0 to 27 min). After extraction experiment finished, the supernatants was collected and all solutions were evaporated. The extracts were stored at 6 °C in the dark for further analysis. All experiments were conducted and all sample analyzed in triplicates.

Table 1. The configuration of ultrasound frequency band of 18-90 kHz.

Tanks	#1	#2	#3	#4	#5	#6
Ultrasound frequency band (kHz)	18~30	30~42	42~54	54~66	66~78	78~90

Ultrasound-assisted extraction (UAE)

To further evaluate the effectiveness of MUAE, the conventional UAE method was applied for the comparative experiment. For UAE, a rectangular ultrasonic bath (300×240×150 mm) was purchased from Kunshan Ultrasonic Instrument Co. Ltd. (Jiangsu, China). 3.0 g sample of strawberry fruit was mixed with 60 mL of ethanol solution (55% v/v). The mixed solution were put into a conical flask and immersed into the ultrasonic bath for ultrasound irradiation with 250 W at 40 kHz for 18 min. The temperature was maintained at 50 °C in a constant temperature tank. Then, the mixed solutions were further processed according to the description in section 2.3.2.

2.4 Determination of yield of anthocyanin

The yield of anthocyanin was determined according to a pH differential method. For each sample, it was mixed with potassium chloride buffer (pH = 1.0) and sodium acetate buffer (pH = 4.5). An UV-Vis spectrophotometer (751-GW, Shanghai Analytical Instrument Overall Factory, China) was used to measure the absorbance at 510 and 700 nm. To obtain the yield of anthocyanin, Equation 1 was used:

$$\text{Anthocyanin yield} = \{[(A_{510} - A_{700})pH_{1.0} - (A_{510} - A_{700})pH_{4.5}] \cdot M \cdot DF \cdot V\} / \varepsilon \cdot L \cdot m_f \quad (1)$$

where yield of anthocyanin is represented as cyanidin-3-glucoside equivalent (CGE) (mg CGE/g); A_{510} and A_{700} are the absorbancies at 510 nm and 700 nm, respectively; *M*, *DF* and *V* represent the molecular weight of cyanidin-3-glucoside (449.4 g/mol), the dilution factor and the volume of extract (mL), respectively. ε is the extinction coefficient of cyanidin-3-glucoside (26,900 L/mol·cm^{-1}); *L* and m_f represent the width of the cuvette (1 cm) and the sample's weight (g), respectively.

2.5 Statistical analysis

Three trails were performed for all experiments. All data were expressed as means ± SD (standard deviation). Duncan's multiple range test was applied for determining the significant differences among treatments at p-values<0.05.

3 Results and discussion

3.1 Optimization of ultrasound frequency and effect on anthocyanin extraction

In order to obtain the optimal ultrasound frequency and investigate its influence on the extraction of anthocyanin, extraction experiments were carried out by MUAE method under extraction conditions including ethanol of 55%, power of 120 W, extraction temperature of 45°C and solvent to solid ratio of 6 mL/g in 12 min. The configuration of ultrasound frequency band for each extraction tank was shown in Table 1. The result was obtained and represented in Figure 2A.

It was observed that when ultrasound frequency increased from 18 to 54 kHz, the extraction yield of anthocyanin significantly increased. The maximum of anthocyanin yield occurred at 54-66 kHz (Namely in tank #4). Subsequently, anthocyanin yield slowly descended as the ultrasound frequency increased. The possible reason was that when frequency was less than 36 kHz, the cavitation intensity in liquid medium would become stronger along with the increase of frequency, and therefore cavitation time lasted longer and target ingredients generated easily. However, when ultrasound frequency was higher than 44 kHz, the cavitation intensity would be weaker owing to the decrease in the size of cavitation bubble, therefore the yield of anthocyanin was lower. Based on the above results, 54-66 kHz was considered to be the best ultrasound frequency band.

In order to further accurately determine the optimal frequency for the extraction process of anthocyanin from strawberry fruit, in the following experiments, the optimal ultrasound frequency band of 54-66 kHz was further divided into six narrow frequencies and showed in Table 2. The effect of ultrasound frequency on the extraction of anthocyanin from strawberry fruitwas performed under the same conditions as

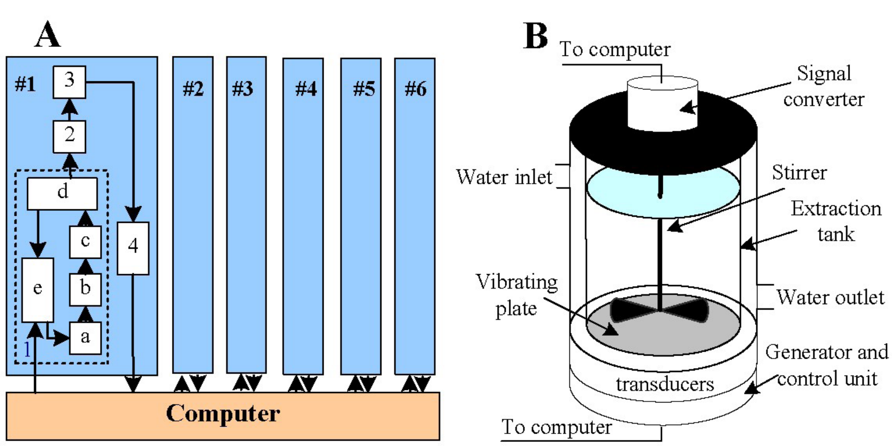

Figure 1. Schematic diagram of the apparatus for MUAE: (A)1. ultrasound power generation unit (a. ultrasonic signal generator b. Isolated driver c. power amplifier d. matcher of power and frequency e. controller of power and frequency);2. transducer work unit; 3. detection unit; 4. signal conversion unit; (B) single extraction tank.

Table 2. The configuration of ultrasound frequency band of 54-66 kHz.

Tanks	#1	#2	#3	#4	#5	#6
Ultrasound frequency (kHz)	54~56	56~58	58~60	60~62	62~64	64~66

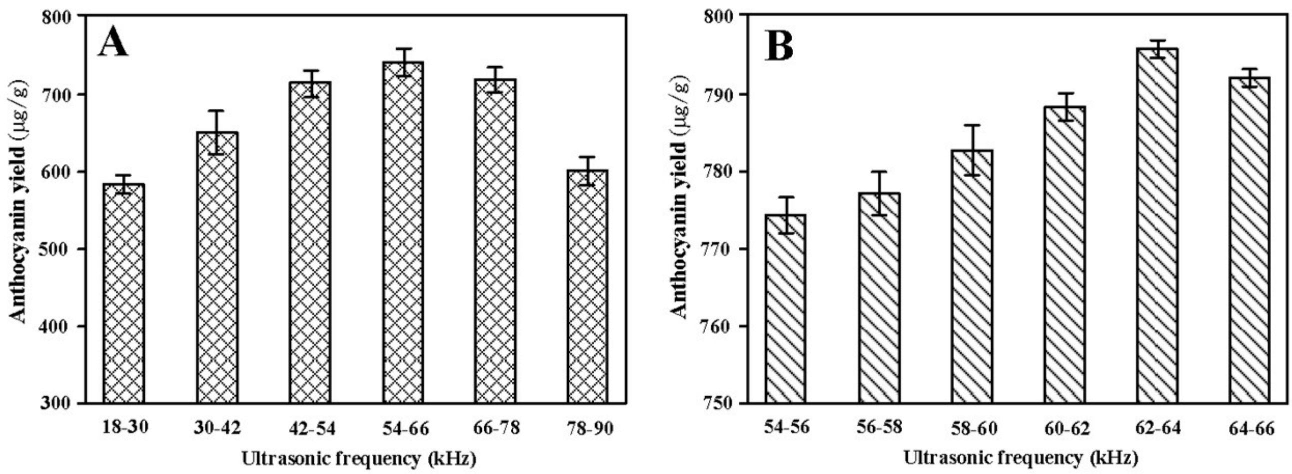

Figure2. Effect of ultrasound frequency: (A) 18-90 kHz, and (B) 54-66 kHz, on the extraction of anthocyanin from strawberry fruit at power of 120 W, temperature of 45°C, solvent to solid ratio of 6 mL/g and treatment time of 12 min with ethanol of 55% as the solvent.

the above experiment, and the effect of ultrasound frequency of 54-66 kHz on the extraction of anthocyanin was obtained and represented in Figure 2B.

It can be seen that the extraction of anthocyanin from strawberry fruit gradually increased with the increase in ultrasound frequency from 54 to 62 kHz, and anthocyanin yield reached the highest value of 796.9 μg/g at 62-64 kHz (Namely in tank #5). Subsequently, the extraction yield of anthocyanin decreased as ultrasound frequency further increased. It was also noteworthy that the yield of anthocyanin obtained at 54-66 kHz in this experiment exhibited higher values as compared to 18-90 kHz in the above experiment. Moreover, the maximum of anthocyanin yield obtained at 62-64 kHz was obviously higher than that of other frequencies in this experiment. These results indicated that the optimal ultrasound frequency could result in a longer swelling time of cavitation bubbles, so that there was more time to collapse fully, which would lead to an increase in cumulative effects of cavitation under the same irradiation intensity. In addition, more shear forces that could break more cell walls would be occurred at an optimum frequency, which would facilitate more active ingredients of anthocyanins into the ethanol solvent without chemical degradation. Similar results to that were observed by our research group on the effect of ultrasound frequency on the extraction of lycopene from tomatoes (Liao et al., 2016). Therefore, ultrasound frequency of 62-64 kHz with a positive effect on extraction of anthocyanin from strawberry fruit could give more extraction efficiency. Consequently, it suggested that 62-64 kHz was the optimum ultrasound frequency for the extraction of anthocyanin from strawberry fruit.

3.2 Effects of other process variables on the extraction of anthocyanin

Effect of treatment time

The strawberry fruit sample was extracted with 55% ethanol as the solvent by keeping other constant conditions as power of 120 W, temperature of 45°C, solvent to solid ratio of 6 mL/g and ultrasound frequency of 62-64 kHz. The obtained results from MUAE and HAE method for the variation in the amount of anthocyanin extracted per g of strawberry fruit with time have been shown in Figure 3A.

For MUAE, it was clearly observed that a rapid increase in anthocyanin yield was exhibiting at the beginning of extraction time, and the maximum yield of 796.9 μg/g of anthocyanin was found at 12 min. After that, the extraction process was slow along with a slight reduction in anthocyanin yield. For HAE, the yield of anthocyanin gradually increased as the treatment time from 3 to 21 min. When anthocyanin yield reached the maximum (462.6 μg/g) at 21 min, however, the rate of increase declined, and little increase between 21 and 27 min was observed. These results suggested that HAE method is more time-consuming than MUAE method, which proved MUAE could efficiently decrease the treatment time. This was attributed to the fact that the extraction process of solid-liquid in MUAE is faster than that of HAE. In fact, there was no significant change in the yield of anthocyanin for HAE after the diffusing of anthocyanin extracts arrived at equilibrium at 21 min. For this phenomenon, the possible reason is that the extraction process mainly came from interior parts to

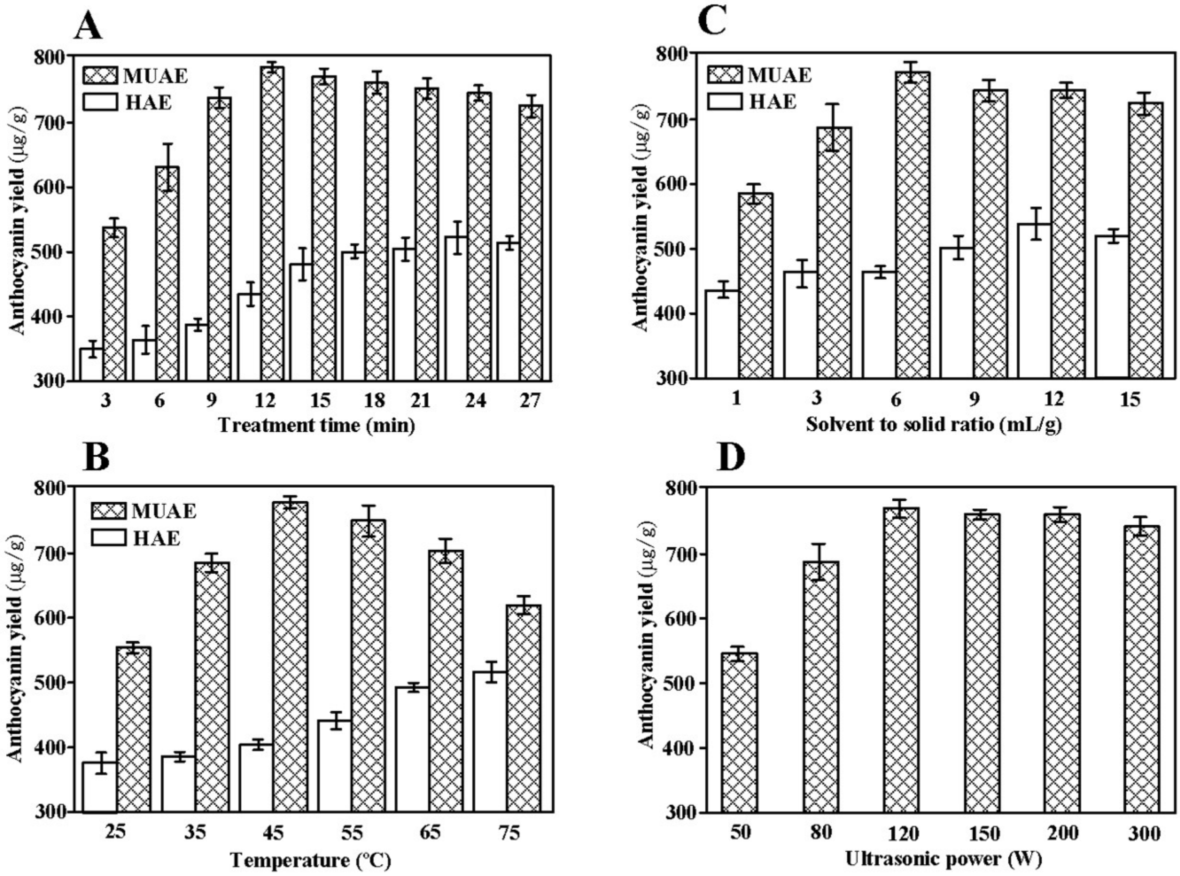

Figure 3. Effects of process variables: (A) treatment time, (B) extraction temperature, (C) solvent to solid ratio, and (D) ultrasound power, on the extraction of anthocyanin from strawberry fruit under HAE and MUAE at 54-66 kHz with ethanol of 55% as the solvent.

phloem region and nearby pith. It is well known that usage of longer time resulted in higher extraction yield, whereas the extension of time may cause a downward trend in yield owing to the degradation of anthocyanin.

Compared with the HAE method, the MUAE has exhibited higher extraction yield that was obtained approximately 1.72 times at 12 min than HAE. As already discussed, treatment time has an important impact on the extraction process of anthocyanin from strawberry fruit. Although Increasing appropriately the treatment time may help to improve the extraction process, too long time is not conducive to the yield of anthocyanin due to the occurrence of degradation and isomerization. Therefore, 12 min was selected as the most suitable treatment time for the extraction of MUAE in the following studies.

Effect of extraction temperature

Extraction experiments were performed at different temperatures ranging from 25 to 75 °C. Other constant conditions used in this work were 55% ethanol as the solvent, solvent to solid ratio of 6 mL/g, ultrasound power of 120 W, ultrasound frequency of 62-64 kHz and extraction time of 12 min. The obtained results have been shown in Figure 3B.

It was found that anthocyanin yield for HAE was not significant increase when the temperature was below 65°C, but anthocyanin yield increased obviously with the increase of temperature from 65 to 75 °C. Comparison with HAE, anthocyanin yield for MUAE has already reached the maximum value of 796.9 μg/g at 40 °C. Moreover, in the whole temperature range, the anthocyanin yield for MUAE was much higher than that of HAE. For HAE, the change of anthocyanin yield at extraction temperature may depend on the increased solubility and diffusivity of anthocyanin with the increase of temperature as well as the acceleration of molecular thermal motion. The influence of temperature on the extraction process under MUAE may be explained from the following two aspects. On the one hand, the increase in solubility and solute diffusivity would give an improved extraction as the extraction temperature increased. Moreover, the increase in extraction temperature might also promote the cell matrix of natural substance easier to open, and as a result, the extraction efficiency of anthocyanin increased. On the other hand, higher temperature has a negative effect on extraction of anthocyanin because of the evaporation of ethanol solvent and degradation of anthocyanin. Based on the above results and considering energy consumption, 45°C was taken as the optimal temperature and all experiments were performed at this temperature.

Effect of solvent to solid ratio

The effect of solvent to solid ratio from 1 to 15 mL/g on extraction of anthocyanin from strawberry fruit was studied under extraction conditions including 55% ethanol, power of 120 W, temperature of 45 °C and ultrasound frequency of 62-64 kHz for 12 min. The obtained results were given in Figure 3C,

For HAE, the increment of anthocyanin extraction yield was very small within 9 mL/g of solvent to solid ratio, and then the extraction yield increased significantly. When the solvent to solid ratio reached 6 mL/g, the anthocyanin extraction yield reached maximum, subsequently it began to decline slightly. For MUAE, as the solvent to solid ratio increased from 1 to 6 mL/g, the extraction yield of anthocyanin significantly increased. After that, the anthocyanin yield significantly decreased along with the further increase in solvent to solid ratio. It may be ascribed to the fact that the larger volume of ethanol and the increased surface area for solute-solvent contact could effectively promote swelling of the sample by water. Moreover, ultrasound wave could cause the establishment of equilibrium for dissolution of total anthocyanin between the extraction solvent and plant cell wall. Similar result using the ratio of solvent to solid was previously reported (Zhang et al., 2021). Thereby the solvent to solid ratios of 6 mL/g was chosen for the extraction of anthocyanin for MUAE.

Effect of ultrasound power

Extraction experiments were carried out at different ultrasound powers over the range of 50 to 300 W. Other constant conditions were fixed as solvent to solid ratio of 6 mL/g, temperature of 45 °C, ultrasound frequency of 62-64 kHz and treatment time of 40 min with 55% ethanol as the solvent. As illustrated in Figure 3D, the yield of anthocyanin increased as the ultrasound power increased from 50 to 120 W and reached the peak at 120 W. It was most likely that the increase of ultrasonic amplitude travelling through the solvent was beneficial to collapse the cavities seriously, so that to enhance physical effects including increasing solute diffusion, interfacial turbulence and cracked cell wall. All these physical effects were responsible for the increased yield of anthocyanin at increasing ultrasonic power. However, it was seen that subsequent increase caused a slight reduction in the yield of anthocyanin. A possible reason was that higher ultrasound power may result in the degradation of some anthocyanins, so the extraction yield of anthocyanin exhibited a slight decrease. The similar effect of ultrasound power on the extraction of flavonoids from peanut shells was previously reported (Liao et al., 2021). Hence, 120 W was selected as the optimal value for the extraction of anthocyanin from strawberry fruit.

3.3 Comparison of MUAE with UAE

To further understanding the advantage of MUAE, the comparison study on anthocyanin extraction from strawberry fruit by using MUAE and conventional ultrasound-assisted extraction (UAE) were carried out under the optimal constant conditions. The comparison results of anthocyanin yield obtained with different extraction time were shown in Figure 4.

It was observed that the maximum extraction yield of 796.9 μg/g for MUAE at 62-64 kHz was significantly higher than 621.7μg/g for UAE at 50 kHz. Namely, compared with UAE method, the extraction yield caused by MUAE was increased by about 22%. Moreover, the treatment time required by MUAE was only 12 min as compared to 18 min by UAE. Additionally, for all cases, ultrasound power of 200 W used by UAE was higher than 120 W used by MUAE method, but anthocyanin yield extracted by UAE was lower as compared to MUAE. Those results indicated that the degree of cavitation activity may determine the extraction efficiency. Generally, the optimum frequency was capable of causing more mass transfer and more disintegration of cell, which lead to the increase of extraction rate. Moreover, bubbles would violently collapse because the optimum frequency was closer to the resonance frequency of bubble, which was also beneficial to improve extraction efficiency. Another possible reason was that all transducers in MUAE were able to run at a resonant state that also can be helpful to generate higher output efficiency. So, the high extraction efficiency, shorter treatment time and lower ultrasound power were the major advantage offered by MUAE as against the UAE method.

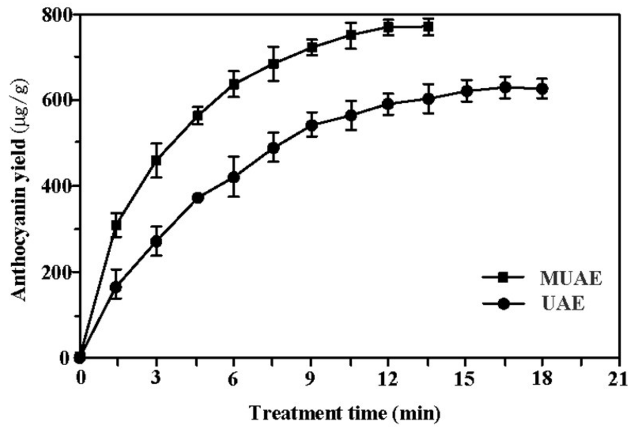

Figure 4. Comparison of the results between UAE and MUAE at different treatment time with ethanol of 55% as the solvent. Other conditions of UAE were solvent to solid ratio of 6 mL/g, temperature of 50°C, ultrasound power of 250 W and ultrasound frequency of 40 kHz; other conditions of MUAE were: solvent to solid ratio of 6 mL/g, temperature of 45°C, ultrasound power of 120 Wand ultrasound frequency of 62-64 kHz.

4 Conclusions

The MUAE has been applied successfully for the extraction of anthocyanin from strawberry fruit in this work. The optimal ultrasound frequency of 62-64 kHz was obtained by a two-step procedure. The effects of ultrasound frequencies of 18-90 kHz and other independent process variables on the extraction of anthocyanin were evaluated. The results showed that the optimized frequency of 62-64 kHz has exhibited a most positive impact and higher extraction rate, and the maximum yield of anthocyanin as 796.9 μg/g was achieved at the optimized variables of solvent to solid ratio of 6 mL/g, ultrasound power of 120 W, extraction temperature of 45°C and treatment time of 12 min with 55% ethanol as the solvent. Moreover, HAE and UAE were more time-consuming than MUAE. For all cases, ultrasound power used by UAE (200 W) was higher than that used by MUAE (120 W), but anthocyanin yield extracted by UAE was lower as compared to MUAE, which indicated MUAE is more energy efficient than UAE.

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