Journal Name: Journal of Food Engineering

Type of manuscript: Research Article

Influence of Micro Wet Milling Parameters on the Processing of Komatsuna (*Brassica rapa* var. *perviridis*) Juice with Rich Phosphatidic Acid

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Abbreviations Used

PLs, phospholipids; PC, phosphatidylcholine; PLD, phospholipase D; PA, phosphatidic acid; PLA_2, phospholipase A_2; LPA, lysophosphatidic acid; GI tract: gastrointestinal tract; MWM: micro wet milling.

ABSTRACT

The aim of this work was to study the effects of multiple micro wet milling (MWM) parameters on particle size reduction and phosphatidic acid (PA) content enrichment of Komatsuna juice. Through an investigation of MWM milling characteristics for Komatsuna juice with different milling conditions, the results showed that milling time, milling rotational speed, gap size, material feeding rate, and interactions among the milling parameters had different significant effects on reduction in particle size. Furthermore, reduction in particle size strongly promoted the increase of PA content in the milled Komatsuna juice, which could improve the bioaccessibility of PA that converts to lysophosphatidic acid in the GI tract for the restoration of GI disorders. The MWM system was able to produce Komatsuna juice with a smaller particle size (approximately 21 μm) and a higher PA content (approximately 70 μg/mL) in a continuous processing system compared to an ordinary grinding mixer.

Keywords: micro wet milling, particle size, phosphatidic acid, milling parameters, Komatsuna

1. Introduction

Japanese mustard spinach (*Brassica rapa* var. *perviridis*) is also known as Komatsuna in Japanese. This plant is a leaf vegetable that is traditional and popular in the Japanese diet, and it is normally eaten as a salad (boiled). Previous research were conducted mainly on the phospholipids of cabbage, egg, and soybean, whereas little research was performed on Komatsuna. The content of phosphatidic acid (PA) in fresh Komatsuna has been estimated to be approximately 43.6% of the total phospholipids, which is a high percentage amount the leafy vegetables belonging to the *Brassica* genus (Tanaka et al., 2012). Oral treatment with PA has showed potential for preventing gastric ulcers in mice due to the formation of lysophosphatidic acid (LPA) from PA in the gastrointestinal (GI) tract through the actions of gastric phospholipase A_2 (Tanaka et al., 2013). LPA plays an important role in the integrity of the GI tract epithelium (Tokumura, 2011). A previous study demonstrated that abundant PA was produced in masticated and milled foodstuff (Tanaka et al., 2012), and the PA content of raw cabbage increased after mastication compared to boiled cabbage (Tanaka et al., 2009). Li *et al.* (2017) also observed a similar phenomenon when PA in fresh Komatsuna rose sufficiently after effective milling, which occurred primarily due to the release of the native PA in Komatsuna from the biomembrane through particle splitting as well as through the release of endogenous phosphatidylcholine (PC) during the grinding process, which stimulated the hydrolysis of PC to PA through phospholipase D (PLD). Thus, there is a possibility to manufacture a healthy diet of PA-rich Komatsuna juice with milling processing for either preventive or therapeutic treatment for gastric ulcers.

There is wide utilization of wet milling in food processing and the pharmaceutical industry. Wet milling facilitates improvement of the physiochemical characteristics of materials, e.g., particle size, nutritional yield, water holding capacity, emulsion, foamability, and nutrition bioavailability (Aluko et al., 2009; Kethireddipalli et al., 2002; Maphosa and Jideani, 2016; Müller and Polke, 1999; Wall and Paulis, 1978). The most of the changes in properties arise from the reduction of particle size, which is a basic physical property of food products. Commercial yellow pea seed was processed to flour with a smaller particle size through wet milling, and the food emulsion subsequently showed high stability (Aluko et al., 2009). Grain was milled into smaller particles, which facilitated starch digestion (Al-Rabadi et al., 2009). Furthermore, the mean particle size was one of the dependent factors that influenced the bioavailability of β-carotene (Müller and Polke, 1999).

Although wet milling can achieve an efficient decrease in particle size (Kwade, 1999), some milling methods produce heat, which can have detrimental effects on the product. Vishwanathan *et al*. (2011) and Cerdeira *et al*. (2011) have reported on different wet milling methods including mixer grinder, stone grinder, and ball mill. While mixer grinding needed to be conducted intermittently to avoid much heat generation during grinding and ball milling lead to product contamination and heating, the temperature in the stone mill did not increase significantly during grinding and was maintained at 31 ^{o}C. The new wet milling technique used in this study is a modified electric stone mill system, named the Micro Wet Milling (MWM) system (Fig. 1). In the MWM system, the pump feeds materials into the stone mill with an adjustable feeding rate and the lower millstone with grooves can cleave materials to small particles efficiently at a certain milling rotation. Koyama and Kitamura (2014) applied MWM to develop the new rice slurry with fine rice particles smaller than 20 μm. A cheese-type food using rice milk milled by MWM system was developed (Nakamura et al., 2016). Likewise, concentrated orange juice with a smaller particle size and higher values of nutrition and antioxidant activity was also produced by using the MWM system (Islam et al., 2017). In this study, the fresh Komatsuna is processed into Komatsuna juice with smaller particles and higher PA content using the MWM system.

Reduction in particle size are influenced by milling parameters, such as the milling rotational speed, milling time, the volume fraction of the milling particles relative to the milling zone and flow rate during ball milling in a wall chamber (Cerdeira et al., 2011; Kwade, 1999). The MWM system is a continuous process that is similar to the colloid mill and the particle size also depends on the gap between the upper and lower millstones (Vishwanathan et al., 2011). The aim of this work was to study the influences of the milling parameters of MWM on particle size reduction and PA content enrichment in Komatsuna juice. Komatsuna juice with a smaller particle size and higher PA content was explored to be produced under the optimum MWM conditions and the resulting product could support a beneficial diet for the prevention or restoration of GI disorders. The obtained results for milling time, milling rotational speed, material feeding rate and gap size were expected to provide a reference for the MWM operation to produce different products with required properties.

2. Materials and Methods

2.1. Materials

Japanese mustard spinach (*B. rapa*; named Komatsuna in Japan) was purchased from a local market. Standard PA with a purity grade of 98% was purchased from Sigma-Aldrich (St. Louis, USA). Chemicals: chloroform, methanol, hydrochloric acid and ammonia solution (28%) were obtained from Wako (Osaka, Japan). TLC Silica gel 60 was purchased from Merck (Darmstadt, Germany).

2.2. Preparation of Komatsuna juice with MWM

To investigate the influences of MWM operational parameters on particle size and PA content of Komatsuna, a preparation was made from whole Komatsuna. After washing Komatsuna with tap water, it was mixed with water (4 ^{o}C) at a ratio of 1:1 (w/w) and blended by a mixer (SBC-1000J, Cuisinart, Japan) for 5 min at room temperature. The Komatsuna mixture obtained had a particle size (D50) of approximately 120 μm.

Komatsuna mixture was then milled by the MWM system (WM12A-R, Sincou Co., Ltd., Fig. 1). The mixture was kept in a tank and mixed continuously by a stirrer. The tubing pump (Fig.1. (3)) with an adjustable feeding rate successively fed the mixture into the stone mill of MWM system. The mill consists of two stones. Only the lower millstone (Fig.1. (5)) is rotated at a certain rotational speed by an electric motor (Fig.1. (7)). The distance between upper and lower millstones was maintained at 250 μm at the beginning of the operation (Islam et al., 2017) to protect the surface of millstones until the milling material filled the gaps between the millstones, and then reduced to 60 μm by a gap controller (Fig.1. (4)). The processed juice was collected simultaneously by a rubber spatula (Fig.1. (6)) into a receiver during the milling. Finally, the collected Komatsuna juice was blanched (approximately 100.4 °C) for 5 min to inhibit enzyme activity (Li et al., 2017) and then analyzed. The feed rate of the pump, milling rotation speed, gap size and milling time are discussed below.

2.3. Milling conditions for MWM

In this study, a milling stone with external contact surface area of 350 cm^2, a radius of 12 cm and a groove width of 4.5 mm was used, as shown in Fig. 2. The minimum inner distance between the upper and lower millstones is 60 μm (groove depth), and this distance was set as the initial gap size. The milling parameters that were investigated included: (i) milling time (0, 10, 20, 30, 40, 50 and 60 min); (ii) milling rotational speed (10, 20, 30, 40 and 50 rpm); (iii) material feeding rate (8, 10, 20, 30 and 40 mL/min); and (iv) gap size (60, 200, 300 and 400 μm). The details of experiment setup were summarized in a matrix, as shown in Table 1. Komatsuna juice was processed using the different milling parameters and the particle size, yield (production volume) as well as PA content of the Komatsuna juice samples were analyzed.

2.4. Measurement of particle size

The particle sizes were determined using a laser diffraction particle size analyzer (SALD-2200, Shimadzu Corporation, Japan) in wet measurement mode. A D50 value in micrometers, which was designated the median diameter, was determined for the particle size and was defined as the average particle size by mass. Three independent determinations were conducted and the results are presented as an average ± standard deviation.

2.5. Determination of Komatsuna juice yield

The yield in this study is the production volume (mL) of Komatsuna juice processed by MWM under different milling conditions. The Komatsuna juice was collected in a receiver with the scale. After finished the milling process, the volume of milled Komatsuna juice was recorded. The MWM operations of Komatsuna juice with the same parameters condition were repeated three times, accordingly, three independent determinations were recorded and the results were presented as an average ± standard deviation.

2.6. Measurement of phosphatidic acid

Lipids were extracted from the Komatsuna juice using the Bligh and Dyer method with minor modifications (Bligh and Dyer, 1959; Tanaka et al., 2009). Komatsuna juice (8 mL with approximately 97% moisture) was added to a mixture consisting of 10 mL chloroform, 20 mL methanol and 0.24 mL water to form a phase of chloroform/methanol/water (10:20:8, V/V/V). The mixture homogenate was vortexed (Vortex Mixer, VM-96B, Jeio Tech, Korea) for 20 s and centrifuged at 1300 × *g* for 10 min, and the supernatant portion was collected. The supernatant was diluted with 15.0

mL of chloroform and water (1:1, V/V) to produce a biphasic system for phase-separation. After adding 0.05 mL of 5N HCl for acidification, the mixed homogenate was vortexed for 20 s and centrifuged at 1300 × *g* for 10 min. The lipid extract was collected from the chloroform layer. PA was isolated from a lipid extract by TLC with a developing agent consisting of chloroform/methanol/28% ammonia (60:35:8, V/V/V). Then, a two-phase separation was conducted to extract PA from the scraped silica gel. The recovered PA was first dissolved in 5.7 mL of a mixed solvent consisting of chloroform/methanol/water (10:20:8, V/V/V), 3.0 mL of chloroform and water (1:1, V/V) were added for phase separation, and finally, 0.02 mL of 5N HCl was added. The mixture was vortexed and centrifuged, and then the purified PA was collected from the lower phase. The amount of PA was quantified by a colorimetrical method based on phosphomolybudenum-malachite green formation with a spectrophotometer of the wavelength 660 nm (Chalvardjian and Rudnicki, 1970). Three independent measurements were collected.

2.7. The optimization of MWM processing conditions for Komatsuna juice

According to the results of the investigation of milling parameters, the milling parameters show different impacts on reducing particle size and increasing PA content. Interactive effects of milling parameters on particle breakage and PA enrichment were also observed. Thus, the sequence of primary parameters and the interaction of milling parameters were investigated using an orthogonal array L_8 (2^7) to find the optimum MWM processing conditions for Komatsuna juice to obtain fine sensory acceptable particle size and high PA content. The three variables, including mill rotational speed, gap size, and feeding rate, are reported to have a substantial impact on particle size and PA content. The range and levels of the variables are listed in Table 2 and the design matrix for the 8 treatments of experiment is shown in Tables 3 and 5, followed by the results of response values (particle size and PA content) and the range analysis and ANOVA analysis. In addition, the range analysis was performed through the calculation of the values $\overline{K}$ and *R*. $\overline{K}$ is the average value of all the response value of one variable with one level, and *R* is an absolute value, which is the difference between $\overline{K_1}$ and $\overline{K_2}$. A high *R* value shows that the influence of the corresponding variable is more important. The primary and secondary importance parameters for reducing particle size and improving PA content were analyzed. ANOVA analysis shows the significance of variables on the response value.

2.8. Statistical analyses

A student's *t*-test was performed between the two groups for comparison. A one-way ANOVA was conducted using SPSS software (version 22.0; IBM, Armonk, NY, USA). A *p* value of < 0.05 was considered to be statistically significant.

3. Results and Discussion

3.1. Effects of milling time on the particle size and PA content of Komatsuna juice

To observe the influences of MWM adjustable parameters on the physiochemical properties of Komatsuna juice, a suitable milling time that assures stable MWM milling status needs to be found. According to Table 1, the particle size, yield and PA content of Komatsuna juice with different milling times were measured for the process conditions of milling rotation 40 rpm, a feeding rate of 10 mL/min and a gap size of 60 μm.

Figs. 3a and 3b show that the particle size (D50) clearly decreased in the first 10 min of milling time, then decreased slowly during a milling time of 10-50 min, but the particle size became constant after 50 min of milling time. The yield of Komatsuna juice tended to increase in a linear fashion with equal extension of milling time when the milling time exceeded 20 min. The PA content of Komatsuna juice increased with longer milling time, whereas the PA content were constant between milling times of 50 min and 60 min.

Compared to the properties of the initial material of Komatsuna juice ground by a mixer (0 min of MWM milling time), the properties of Komatsuna juice milled for 10 min were very different. The decrease in particle size was almost three-fold, and PA content increased by approximately two-fold. This outcome confirmed the effects of MWM on reducing particle size and improving PA content. The MWM system is a successive processing system, and the milled juice is collected simultaneously while the material is fed into the millstones. In the first 10 min of milling time, material was mainly filling into the grooves of the millstones and being milled at the same time. Therefore, the yield of milled juice was low, the particles of Komatsuna juice were not milled sufficiently, and the PA was not released and produced efficiently. The particle size and PA content of Komatsuna juice changed gradually with longer milling times, then no differences were observed between 50 min and 60 min of milling time. Similarly, the yield of processed Komatsuna juice was stable after 50 min, which meant the successive milling process was in a stable situation. Therefore, 50 min of milling time was adopted for the following experiments.

3.2. Effects of milling rotation on the particle size and PA content of Komatsuna juice

The particle size, yield and PA content of Komatsuna juice with different milling rotational speeds were measured under the process conditions of milling time at 50 min, a feeding rate of 10 mL/min and a gap size of 60 μm to investigate the effects of rotational speed on the properties of Komatsuna juice, as shown in Table 1.

As shown in Figs. 3c and 3d, the particle size (D50) of Komatsuna juice was reduced with higher milling rotational speeds, but increased when the rotational speed exceeded 40 rpm. The yield of Komatsuna juice was higher when the rotational speed was higher, but the yields did not increase linearly with the increase in rotational speed. The PA contents of Komatsuna juice with the milling rotation between 20 rpm and 40 rpm were higher compared to samples processed with milling rotational speeds of 10 rpm and 50 rpm.

The reduction of particle size and increase in yield to a certain extent with the higher milling rotational speed could be attributed to higher milling forces, which are predominantly compression and some shear force in stone mills (Sharma et al., 2008). However, when the milling rotation exceeded 40 rpm, the particle size was not reduced continuously, and the yield of juice increased, which shows that the materials were pushed out of the millstone due to the higher milling rotation, and the particles were not milled sufficiently before they were pushed out. This result could have occurred because the increasing rotational speed leads to an increase in centrifugal force and helix angle. This result was different compared to the results of Koyama *et al.* (2014), who found the particle size of rice decreased with a higher milling rotation due to strong shear stress. The particle size reduction also depends on the properties of the materials, and some excipients and stabilizers could improve the reduction of particle size by stabilizing the physical properties of the milling particles (Cerdeira et al., 2011). In addition, the results for PA content agreed with the phenomenon reported previously by Li *et al.* (2017) in which the particle size was smaller, and the PA content was higher. In this study, with the different milling rotational speeds, the PA contents changed within a limited range.

3.3. Effects of material feeding rate on the particle size and PA content of Komatsuna juice

Since the volume fraction of the milling particles relative to the milling zone influences the reduction in particle size, the material feeding rate, which is one of the parameters that affects the volume fraction, was investigated. Thus, the particle size, yield and PA content of Komatsuna juice with different feeding rates were detected under the process conditions of a milling time of 50 min, a milling rotation of 40 rpm and a gap size of 60 μm (Table 1).

Figs. 3e and 3f show that the particle sizes and yields of Komatsuna juice increased with a higher feeding rate, whereas the PA content decreased. The results of the feeding rate between 8 mL/min and 10 mL/min showed little difference compared to the results for higher feeding rates.

At the beginning of the MWM process, the milling zone was filled gradually by feeding the materials into the zone. Next, the volume fraction of milling particles in the milling zone increases, and the friction and collisions between the particles-millstone and particles-particles becomes stronger, which breaks up the particles. However, the higher feeding rate also influences the flow of Komatsuna juice in the milling system, which affects the effective exposure of particles to the milling zone (Sharma et al., 2008) and leads the particles to flow out before they are fully milled. MWM is a continuous milling system that is similar to a colloid mill (Vishwanathan et al., 2011), but is not similar to ball milling in a wall chamber (Cerdeira et al., 2011).

3.4. Effects of gap size on the particle size and PA content of Komatsuna juice

In this experiment, according to Table 1, the influences of gap size on the particle size, yield and PA content of Komatsuna were observed with process conditions of a milling time of 50 min, a material feeding rate of 10 mL/min and a milling rotation of 40 rpm.

Figs. 3g and 3h show that the particle size and yield of Komatsuna juice increased gradually and PA content decreased with the larger gap size. The gap size is also a parameter that influences the volume fraction of milling particles in the milling zone. If the gap size was larger and the volume fraction was lower, both the friction and collisions between milling particles were lower. The predominant milling force of stone grinders is compressive force (Vishwanathan et al., 2011), and when the volume fraction of particles in the milling zone was small, the effective milling force was low and the reduction in particle size was low. The particles could flow out of the milling zone through centrifugal force of rotation, compressive and flow forces, and the particles were not milled sufficiently. Vishwanathan *et al.* (2011) also suggested that the particle size of the ground material in a colloid mill depends on the gap between the grinding stones and the volume fraction of grinding particles in the grinding zone increased with the increased volumetric size of soaked beans. Therefore, an interaction of high feeding rate and small gap size was presumed to achieve a large reduction in particle size.

3.5. The optimization of MWM milling parameters for Komatsuna juice processing

Based on the above results, the particle sizes and PA contents of different milling rotational speeds changed in a limit range, whereas the particle sizes and PA contents of different feeding rates and gap sizes led to obvious changes. The milling parameters show different degrees of importance for reducing particle size and increasing PA content. Thus, the optimal sequence of primary parameters was investigated. In this study, interaction effects of milling parameters on particle breakage and PA enrichment were also observed. Islam *et al.* (2017) reported that a higher rotational speed and higher gap produced orange juice with larger particles, and Koyama (2014) also suggested that reducing the gap size and increasing the milling rotational speed was beneficial for the reduction of particle size. Therefore, the interaction effects of different milling parameters on particle size and the PA content of Komatsuna juice were investigated in this study, and the optimum process conditions were obtained.

The orthogonal test is a simple method that analyzes the influence of single variables and the interaction of variables on the response value through range analysis and ANOVA analysis, which determined the optimum combination of tested parameters among the different treatments. In this study, for optimizing the MWM processing conditions of Komatsuna juice, an orthogonal array L_8 (2^7) was designed, and 8 treatments were implemented with different combinations of three factors in two levels (Table 2). The results for the response values (particle size and PA content) and ANOVA analysis are shown in the Tables 3-6.

Table 3 shows the effects of milling rotational speed, gap size and material feeding rate on the particle size as well as the results of the range analysis expressed by calculating the values $\overline{K}$ and *R*. According to the explanation in Sect. 2.7, $\overline{K}$ is the average value of all the particle sizes of one variable with one level, and *R* is an absolute value, which is the difference between $\overline{K_1}$ and $\overline{K_2}$. Since a high *R*-value shows that the influence of the corresponding variable is more important. Therefore, the results of the range analysis shows the effects of primary and secondary sequences of different parameters on the reduction of particle size were in the order of X_2, X_2X_3, X_3, X_1, X_1X_3, X_1X_2 and $X_1X_2X_3$. The interaction between gap size and feeding rate had an importance effect on the reduction in particle size, which is similar to the results described in Sect. 3.3 and 3.4. By decreasing the gap size and increasing the material feed rate, the volume fraction of milling particles in the milling zone increases, and the friction, collisions and effective milling force of compression also increase while the particle size decreases. To reduce particle size effectively, the suitable variable combination in the tested range is $X_{1-1}X_{2-1}X_{3-1}$ or $X_{1-2}X_{2-1}X_{3-2}$.

To further investigate which factor significantly affects the reduction in particle size, ANOVA was conducted, and the results are shown in Table 4. Statistical analyses indicate that gap size, the interaction of gap size and feeding rate as well as feeding rate were the principal factors that have the most significant effects on the particle size ($p < 0.01$), whereas the milling rotational speed shows no significant effect on the particle size ($p > 0.05$). These results have good agreement with the range analysis. Based on above analysis, the parameters of gap size and feeding rate show an interactive effect on the particle size, and the optimal MWM processing conditions of Komatsuna juice with smaller particles can be set as a milling rotational speed of 20 rpm, a gap size of 60 μm and a feeding rate of 10 mL/min or a milling rotational speed of 40 rpm, a gap size of 60 μm and a feeding rate of 20 mL/min. The particle size was verified to 21.803 ± 0.2 μm and 22.084 ± 0.4 μm according to those conditions, respectively.

The orthogonal array L_8 (2^7) and ANOVA analysis were also conducted to investigate the effects of milling parameters on PA content. Similar to the above analysis, the results of the range analysis (Table 5) show that the importance of principal variables is in the order of X_2, X_1X_3, $X_1X_2X_3$, X_3, X_1, X_1X_2, X_2X_3, and the interactions between milling rotation, gap size and feeding rate have a large effect on the PA content. ANOVA analysis (Table 6) shows the primary parameter of gap size has a significant effect on the PA content ($p < 0.05$), whereas the interactions among the factors show no significant effect on the PA content ($p > 0.05$). The suitable combination of variables for high PA content is $X_{1-1}X_{2-1}X_{3-1}$ or $X_{1-2}X_{2-1}X_{3-2}$, which has good agreement with the analyzed results for particle size. These results verified the outcomes from a previous study reported by Li *et al.* (2017), which showed the increase of PA content depended on the reduction in particle size due to the high proportion of native Komatsuna PA released from the biomembrane through particle splitting as well as the release of endogenous PC during grinding that is converted to more PA by PLD. Moreover, the PA contents from the optimum conditions were verified to be 69.878 ± 2.6 μg/mL and 70.089 ± 3.4 μg/mL. Therefore, the MWM system is more effective for reducing the particle size and increasing the PA content of Komatsuna juice to approximately 21 μm and 70 μg/mL, which is a six-fold decrease in particle size and a two-fold increase in PA content compared to ordinary grinding with a mixer (Figs. 3a and 3b, 0 min of milling time). The smaller particle size and increased PA content are expected to improve the bioaccessibility of PA in the digestion system, which could facilitate PA hydrolysis to LPA (Al-Rabadi et al., 2009). These phenomena will be studied in future work using a simulated digestion system.

4. Conclusions

The influence of milling parameters for a new wet milling technique, known as the micro wet milling system, on particle size reduction and PA content enrichment of Komatsuna juice was studied. The evaluation of milling characteristics of Komatsuna juice with different MWM conditions revealed that the milling time, milling rotational speed, gap size, material feeding rate and interactions among the parameters had different significant effects on the reduction of particle size of Komatsuna juice, and the reduction of particle size had a profound benefit on the increase of PA content in the milled Komatsuna juice. This process will improve the bioaccessibility of PA that converts to LPA in the GI tract. Additionally, Komatsuna juice was processed with the obtained optimum MWM conditions, and the particle size of Komatsuna juice was approximately 21 µm, which is suitable for consumption (Inoue, 2011), and the PA content of Komatsuna juice was approximately 70 µg/mL, which is twice the amount found in Komatsuna juice ground with an ordinary mixer. Therefore, the MWM system is a successful processing method that can effectively produce Komatsuna juice with a smaller particle size and rich PA content. In future studies, the MWM system is expected to be a successive milling system of industrial scale to produce PA-rich foods from Komatsuna juice, which can be a beneficial diet for the restoration or prevention of certain kinds of GI disorders.

Acknowledgement

This study was supported by a scholarship to Xinyue Li from the China Scholarship Council.

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Table 1
The parameters and parameter levels of the MWM processing of Komatsuna juice

Parameters	Milling time [min]	Mill rotational speed [rpm]	Material feeding rate [mL/min]	Gap size [μm]
Variable: Milling time of MWM				
	0 10 20 30 40 50 60	40	10	60
Variable: Mill rotational speed				
	50	10 20 30 40 50	10	60
Variable: Material feeding rate				
	50	40	8 10 20 30 40	60
Variable: Gap size				
	50	40	10	60 200 300 400

Table 2

The Level and code of variables in the orthogonal array L_8 (2^7) for the MWM processing of Komatsuna juice

Codes levels	Variables		
	Milling rotational speed	Gap size	Material Feeding rate
	X_1 (rpm)	X_2 (μm)	X_3 (mL/min)
1	20	60	10
2	40	200	20

Table 3

The orthogonal array design and the analysis for the response value of particle size

Treatment	Independent variables							Respond value
	X_1	X_2	X_1X_2	X_3	X_1X_3	X_2X_3	$X_1X_2X_3$	Median diameter (μm)
1	1 (20)	1 (60)	1	1 (10)	1	1	1	21.271
2	1	1	1	2 (20)	2	2	2	22.997
3	1	2 (200)	2	1	1	2	2	37.704
4	1	2	2	2	2	1	1	90.641
5	2 (40)	1	2	1	2	1	2	24.865
6	2	1	2	2	1	2	1	22.614
7	2	2	1	1	2	2	1	42.369
8	2	2	1	2	1	1	2	93.444
$\overline{K_1}$	43.153	22.937	45.020	31.552	43.758	57.555	44.224	
$\overline{K_2}$	45.823	66.040	43.956	57.424	45.218	31.421	44.753	
R	2.670	43.103	1.064	25.872	1.460	26.134	0.529	

Table 4

Analysis of variance (ANOVA) showing the significance of independent variables on particle size

Source	SS	df	MS	F	Sig.
Corrected model	6434.642[a]	4	1608.661	681.044	0.000**
X_2	3715.694	1	3715.694	1573.080	0.000**
X_2X_3	1365.998	1	1365.998	578.310	0.000**
X_3	1338.695	1	1338.695	566.751	0.000**
X_1	14.255	1	14.255	6.035	0.091^{NS}
Error	7.086	3	2.362		

a. R^2=0.999

b. **: $p < 0.01$; *: $p < 0.05$; ^{NS}: non-significant.

Table 5
The orthogonal array design and the analysis for the response value of PA content

Treatment	Independent variables							Respond value
	X_1	X_2	X_1X_2	X_3	X_1X_3	X_2X_3	$X_1X_2X_3$	PA content (µg/mL)
1	1 (20)	1 (60)	1	1 (10)	1	1	1	66.403
2	1	1	1	2 (20)	2	2	2	61.282
3	1	2 (200)	2	1	1	2	2	47.197
4	1	2	2	2	2	1	1	50.859
5	2 (40)	1	2	1	2	1	2	55.449
6	2	1	2	2	1	2	1	65.194
7	2	2	1	1	2	2	1	46.550
8	2	2	1	2	1	1	2	50.701
$\overline{K_1}$	56.435	62.082	56.234	53.900	57.374	55.853	57.252	
$\overline{K_2}$	54.474	48.827	54.675	57.009	53.535	55.056	53.657	
R	1.962	13.255	1.559	3.109	3.839	0.797	3.594	

Table 6
Analysis of variance (ANOVA) showing the significance of independent variables on PA content

Source	SS	df	MS	F	Sig.
Corrected model	426.047[a]	4	106.512	23.103	0.014*
X_2	351.403	1	351.403	76.223	0.003**
X_1X_3	29.472	1	29.472	6.393	0.086^{NS}
$X_1X_2X_3$	25.837	1	25.837	5.604	0.099^{NS}
X_3	19.335	1	19.335	4.194	0.133^{NS}
Error	13.831	3	4.610		

a. R^2=0.969
b. **: $p < 0.01$; *: $p < 0.05$; ^{NS}: non-significant.

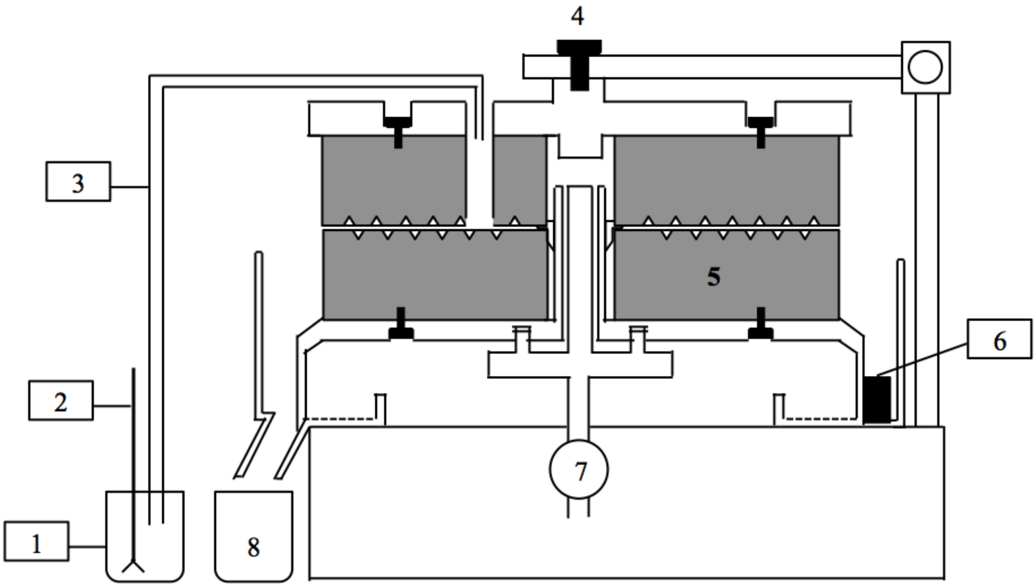


Fig. 1. Micro wet milling (MWM) system. (1) Raw material; (2) Stirrer; (3) Tubing pump; (4) Gap controller; (5) Lower millstone; (6) Rubber spatula; (7) Electric motor; (8) Sample receiver.

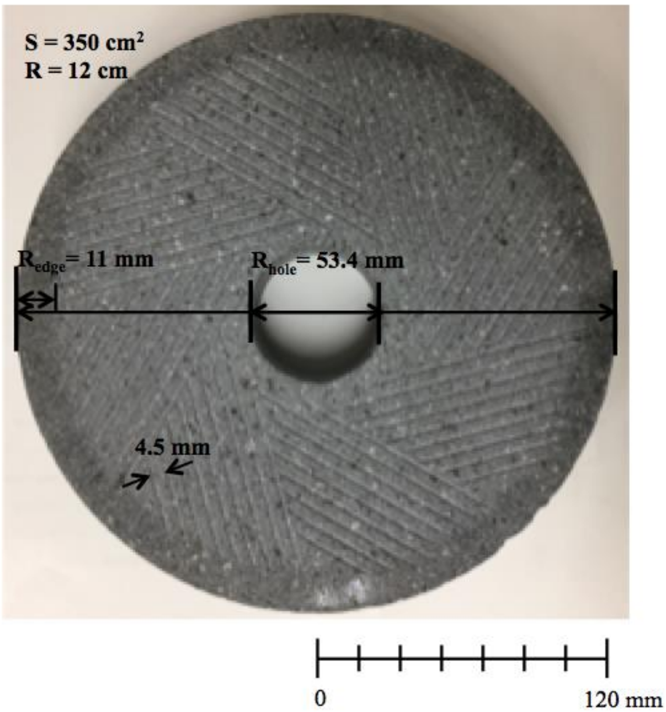
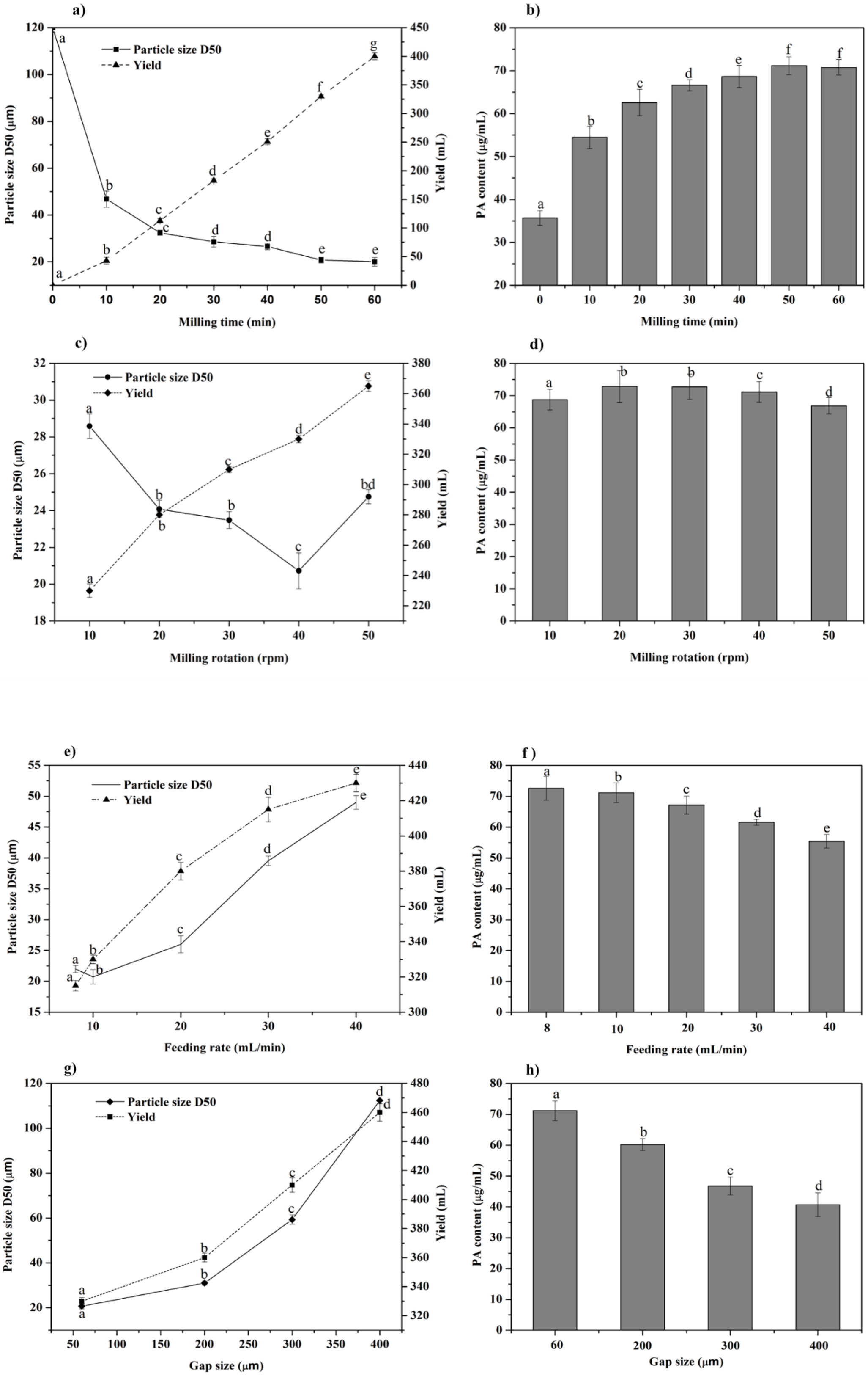


Fig. 2. The lower millstone of the Mill with contact surface area of 350 cm^2, millstone radius of 12 cm, edge distance of 11 mm, and groove width of 4.5 mm. The upper millstone is the same as the lower millstone.


a)
Particle size D50
Yield
Particle size D50 (μm)
Yield (mL)
Milling time (min)
b)
PA content (μg/mL)
Milling time (min)
c)
Particle size D50
Yield
Particle size D50 (μm)
Yield (mL)
Milling rotation (rpm)
d)
PA content (μg/mL)
Milling rotation (rpm)
e)
Particle size D50
Yield
Particle size D50 (μm)
Yield (mL)
Feeding rate (mL/min)
f)
PA content (μg/mL)
Feeding rate (mL/min)
g)
Particle size D50
Yield
Particle size D50 (μm)
Yield (mL)
Gap size (μm)
h)
PA content (μg/mL)
Gap size (μm)