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ORIGINAL ARTICLE

Effect of adding ball-milled achenes to must on bioactive compounds and antioxidant activities in fruit wine

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Abstract This study reports the utilization of ball-milled achenes in fermentation to increase the levels of ellagic acid and total phenol content, as well as to enhance the antioxidant capacity of strawberry wine. Achenes were micronized using ball-milling process, and then added to strawberry must prior to fermentation. The effects of the addition of ball-milled achenes on the ellagic acid and total phenol content in strawberry wine were determined, and the free radical scavenging and iron chelation activities were also analyzed. Quality attributes and acceptance were studied in comparison with a leading commercial strawberry wine for market application. The particle sizes of achenes were reduced from 1.1 mm to 400 nm after 30 min of ball-milling, and this led to an increase in the amount of extracted ellagic acid from 550.72 to 915.24 μg/g. The addition of ball-milled achenes to must led to a 19.72 % and 52.37 % increase in ellagic acid and total phenol content in strawberry wine, respectively. The increase in bioactive compounds resulted in increases of 54.09 %, 51.49 % and 56.97 % in ABTS and DPPH radical scavenging, and ferrous ion chelating activities, respectively. Although the commercial strawberry wine showed greater aroma intensity, no significant differences in overall quality and acceptance among the conventional process, added ball-milled achenes and the leading commercial strawberry wines were found. This study demonstrates that supplementation of ball-milled achenes in fermentation can be beneficial in increasing the levels of bioactive compounds and antioxidative capacity, indicating a good market potential.

Keywords Ellagic acid · Achene · Ball milling · Antioxidant · Strawberry wine · Sensory evaluation

Highlights
- Ball-milled achenes was used in must during fermentation to increase the levels of phenolic compounds and bioactivities in strawberry wine.
- A 30-min ball milling of achenes effectively led to an increase in extracted ellagic acid and total phenolic compounds.
- Significant increase in antioxidant capacity in the strawberry wine was found in the presence of ball-milled achenes.
- The addition of ball-milled achenes in must did not cause quality loss in organoleptic properties of strawberry wine.

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Introduction

Epidemiological studies have demonstrated that phenolic bioactive compounds in fruits can prevent a number of chronic diseases (Hannum 2004; Landete 2011). Berries of the *Rosaceae* family (such as raspberry, cherry, strawberry etc.) contain relatively high levels of phenolic compounds, such as anthocyanins, flavonoids, ellagitannins, as well as ellagic acid and its methylated or glycosylated derivatives (Hakkinen et al. 2000; Aaby et al. 2005; Aaby et al. 2007a, b; da Silva Pinto et al. 2008; Landete 2011). Among those bioactive compounds, both ellagic acid and ellagitannins have been demonstrated to contribute to anticancer, antioxidant and anti-inflammatory activities (Hannum 2004; Landete 2011). Ellagic acid is a lactonic gallic acid dimer derivative of hexahydroxydiphenic acid, which is derived from

ellagitannins via hydrolysis (Sims and Bates 1994; Shi et al. 2005; Aguilera-Carbo et al. 2008; Landete 2011). However, owing to its low solubility in water, most of the ellagic acid is found in the sediment in the preparation of juice (Sims and Bates 1994).

Many berries are consumed fresh, however, large amount of those fruits are wasted during peak harvest period, due to high temperature and relative humidity, poor handling and inadequate storage, as well as microbial contamination. The use of ripe fruit for wine production has long been regarded as an attractive method to utilize surplus and over-ripened fruits (Noller and Wilson 2009). Research indicates that tropical fruit wine markets are emerging in a number of Asian countries, thus creating a potentially large market for such products (Noller and Wilson 2009). Many reports have suggested that the moderate consumption of fruit wine contributes to a reduced risk of cardiovascular disease and cancer (Heninonen et al. 1998; Cheel et al. 2007; Dey et al. 2009; Jeong et al. 2010; Gambacorta et al. 2011; Yoo et al. 2010; Johnson and de Mejia 2012; Negi et al. 2013; Kelebek and Selli 2014). Generally, berries have high flavonoid and phenolic contents, thus, wines based on berries would be expected to be beneficial in terms of increasing biofunctionalities, which creates a new dimension to the area of non-grape wines (Heninonen et al. 1998; Dey et al. 2009; Jeong et al. 2010; Yoo et al. 2010; Johnson and de Mejia 2012; Negi et al. 2013). Those facts have encouraged researchers to explore the potential use of berries, apart from grapes, for the production of fruit wines.

High contents of phenolic compounds and antioxidant activities have been reported in achenes (Ayala-Zavala et al. 2004; Hannum 2004; Aaby et al. 2005; Bakkalbasi et al. 2009; Ariza et al. 2010; Landete 2011), however, the relative thick pericarp limits applications of achenes in food products. Though strawberries contain 1 % achenes on a fresh weight basis, the achenes contribute more than 10 % of the total phenolic compounds and 14 % of the antioxidative activities in whole fruit, reflecting its significant biofunctional value (Aaby et al. 2005). Typically, after strawberry fruits are processed into juice and purees, a substantial amount of the remaining waste contains high levels of achenes. Such waste represents a potential source of bioactive ingredients, and could be processed for use in functional foods, instead of being utilized as animal feed or sent to a landfill (Aaby et al. 2005). To increase the value of achenes used in food products, micronization has attracted the attention of the food industry. Several micronization techniques, including ball milling, jet milling, and high-pressure treatments, are widely used (Chau et al. 2006; Wang et al. 2009; Buaban et al. 2010). Among those techniques, ball milling has been shown to effectively pulverize hard solid materials. Buaban et al. (2010) reported that ball milling for 2 h is sufficient to convert cellulose into an accessible form for enzymatic hydroplysis, resulting in high product yields during its subsequent fermentation.

As more attention is being paid to the health aspects of beverages nowadays, like wine, functional facts are needed in our knowledge base. It was hypothesized that the use of ball-milled achenes in must would likely increase the release of functional compounds, and, therefore, might lead to an increase in the levels of phenolic compounds and bioactive activities of the final wine. Food processing associated with ball milling produces finely ground, homogeneous particles that are more amenable to mixing and extraction, resulting wider applications. Strawberry wine is one of the most popular fruit wines in Taiwan as well as in many other Asian countries, and the addition of ball-milled achenes to strawberry must may increase the biofunctionality of this product. In this study, the effects of the addition of ball-milled achenes during fermentation on the levels of bioactive compounds and antioxidant activities in strawberry wine were investigated. Additionally, the sensory quality and acceptance of added ball-milled achenes to the product were also analyzed for market potential.

Materials and methods

Chemicals

Ellagic acid, formic acid, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and 2,2-diphenyl-1-picryhydrazyl (DPPH) were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Gallic acid, Folin, and Ciocalteu's phenol reagents were provided by Merck Chemicals (Darmstadt, Germany). Pectinase, used for degradation of pectin and clarification of strawberry must, was obtained from Trump Chemical Corporation (Taipei, Taiwan). Potassium metabisulfite ($K_2S_2O_5$), yeast (*Saccharomyces cerevisiae* var. *bayanus*) and nutrients (Fermaid K, Lallemand Australia Pty. Ltd., Australia) were supplied by Whole Earth Trading Company (Taipei, Taiwan).

Collection of achenes

Fresh strawberry fruits (*Fragaria* x *ananassa* Duch. cv. TY. No.1) were obtained through the courtesy of the Dahu Wineland Resort (Miaoli County, Taiwan). The berries were directly shipped from the farm after harvesting. Upon arrival, berries were washed using 120 ppm $K_2S_2O_5$ solution to remove microbial contamination on surface, rinsed with distilled water, and then stored in a –20 °C freezer prior to ball-milling. The composition of the fruit including moisture, protein, lipid and minerals were determined according to AOAC (2007). Soluble solids were measured using a refractometer, and titratable acids were also analyzed as g of citric acid/100 mL fruit juice. As we prepared for ball-milling, frozen berries were thawed in a 4 °C cooler for 48 h, and then

mixed with $K_2S_2O_5$ (150 mg/kg fruit). The resulting material was transferred to a Waring blender to be pureed. To preserve phenolic compounds in the achenes, freeze-drying was used in this study, as described in the literature (Aaby et al. 2005). The puree was freeze-dried, under a vacuum of 250–300 × 10^{-3} Torr, by a benchtop type freeze-dryer (FD24-3S-12P, Kingmech Co., New Taipei City, Taiwan) at -20 °C for 4 days. Separation of the dried substance and achenes were achieved through the use of a sieve (Tyler 20 mesh, 850 μm). The weights of the achenes were recorded and the materials then stored in a -20 °C freezer prior to ball-milling.

Ball-milling of achenes

In order to pulverize the hard pericarp and reduce particle size for further applications, the achenes were subjected to planetary ball milling (Retsch PM100, Haan, Germany). Achene samples and zircon (zirconium dioxide) balls (0.7 mm diameter) (a volume ratio of 1:1) were put into a 125 mL grinding vessel, and processed at 300 rpm for 0.5–3 h at 25 ± 2 °C (Wang et al. 2009). The ball-milled achenes were then collected, and the particle size was determined using a Dynamic Light Scattering Analyzer (Brookhaven 90 Plus, Brookhaven Instruments Corporation, New York, USA).

Preparation of strawberry wine

Thawed fruits were crushed using a juicer, and filtered with a cloth sieve (mesh 200). Strawberry must was prepared by adding pectinase up to a level of 60 ppm, and then sugar content and titratable acidity were then adjusted to 20°Brix and 0.6 % citric acid, respectively, followed by adding 400 ppm yeast nutrients and 1 % (*v*/*v*) a precultured (10^8 CFL/mL) yeast (*Saccharomyces cerevisiae* var. *bayanus*). Strawberry wine was prepared by transferring 800 mL of must to a 1.5 L fermentor, and underwent fermentation at 18 °C for 15 d. After fermentation, the liquid was placed in a 4 °C cooler and allowed to settle down solid and yeast culture for 3 d. The upper wine juice was transferred by siphoning to a clean glass container, and placed still for maturation at 4 °C for 3 d. The clear wine was siphoned to another clean glass container, and aged at 4 °C for 15 day. The achene supplemented strawberry wine was achieved by formulating with 1 % (*w*/*w*), according to the content in fresh fruit, ball-milled achenes in the must.

Extraction and purification of bioactive compounds

The ellagic acid and phenolic compounds in achenes were extracted following a previously described procedure (da Silva Pinto et al. 2008). The achene samples were mixed with 10 mL 70 % (*v*/*v*) aqueous acetone and sonicated for 10 min. After centrifugation, the supernatant was collected, and the insoluble material was re-extracted three times with 6 mL acetone. Then, the pooled extracts were mixed with a chloroform-water mixture (1:1, *v*/*v*), and aqueous and lipophilic phases were separated after centrifugation (1500 × *g* for 20 min) at 4 °C. Finally, the aqueous phase was collected, and residual acetone was removed using a nitrogen flush.

The removal of sugars and acids in the juice and/or wine was achieved using a solid-phase extraction (SPE) technique. C18 Sep.-Pak cartridges (Waters Associates Co., Taiwan) were first activated with 5 mL of acidified methanol (with 0.01 % HCl, *v*/*v*). Then, 1 mL sample was added to the cartridge, followed by 2 mL of 0.01 % HCl solution. Sugars, acids, and other water-soluble compounds were eluted with 2 mL of 0.01 % HCl, and the total polyphenols were recovered with 10 mL of acidified methanol. The methanolic extract was concentrated to 1 mL using a nitrogen flush, and stored in a glass vial at -20 °C prior to HPLC analysis.

Quantification of ellagic acid by HPLC

Contents of the ellagic acid in the achenes and strawberry wine were determined using a Waters 600 controller liquid chromatography, equipped with a Waters 486 Tunable Absorbance Detector (Waters Associates Co., Taiwan) (Klopotek et al. 2005; Aaby et al. 2007a; Garcia-Estevez et al. 2010). Chromatographic separation was performed using a C18 column (250 mm × 4.6 mm i.d., 5 μm particle size) (Phenomenex, USA) equipped with a guard column (4.0 mm × 4.6 mm i. d., 5 μm ODS coating) (Phenomenex, USA). The column temperature was held at 25 °C using a PID controller. The extract was filtered through a 0.45 μm filter (HP 13 mm, Advantec, Taiwan) prior to injection, and a 20 μL aliquot was used in each analysis. The mobile phase for the separation consisted of 2.5 % (*v*/*v*) formic acid in water (solution A) and 2.5 % formic acid in acetonitrile (solution B). A polarity gradient was programmed using 2 % (*v*/*v*) B for up to 5 min, and then from 5 to 38 % (*v*/*v*) in 59 min. Ellagic acid was identified based on its absorbance at 260 nm, and quantification was accomplished using calibration graphs prepared using standard ellagic acid diluted with methanol at 9 concentration levels (1–100 μg/mL) in triplicate.

Determination of total phenolic compounds

Total phenol content in the extract was determined according to the Folin-Ciocalteu procedure, as described by Aaby et al. (2005). An aliquot of 0.1 mL of a purified extract, or strawberry wine, was mixed with 0.6 mL of d-H_2O. Then 0.5 mL of Folin-Ciocalteu reagent and 1.5 mL 20 % sodium carbonate (*w*/*v*, in d-H_2O) were added to the mixture. Next, the mixture was diluted to 10 mL with d-H_2O, and incubated at 20 °C for

2 h. The amount of total phenolic compounds was determined from the absorbance at 765 nm (Du 640, UV-Vis spectrophotometer, Beckman Coulter, California, USA). All analyses were conducted in triplicate.

Analysis of antioxidant activity

Antioxidant activities of fortified strawberry wine were measured using ABTS cation radical ($ABTS^{\bullet+}$) and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) scavenging assays, as well as ferrous ion chelating capacity.

The $ABTS^{\bullet+}$ scavenging activity of the wine samples was measured as described previously (Sariburun et al. 2010; Jun et al. 2012). Preparation of $ABTS^{\bullet+}$ was completed by 5 mL of 7 mM aqueous ABTS solution mixing with 88 μL of 140 mM (2.45 mM final concentration) potassium persulphate, and the bluish-green reagent was kept in the dark over night at room temperature before assay. In the $ABTS^{\bullet+}$ assay, 2.25 ml of reagent was added with 0.25 ml of sample, and the absorbance at 734 nm was measured against deionized water used as blank. The $ABTS^{\bullet+}$ scavenging activity is given as:

$$ABTS^{\bullet+}\ \text{scavenging activity}(\%) = \left[1-\left(A_{734\ \text{nm, sample}}/A_{734\ \text{nm, control}}\right)\right] \times 100\%$$

The free radical scavenging ability of strawberry products was also determined with the DPPH reagent (Buaban et al. 2010; Jun et al. 2012). Aliquots of 0.1 mL of sample added with 5 ml of freshly prepared 0.1 mM DPPH methanolic solution were thoroughly mixed, and kept for 50 min in the dark. The absorbance of the reaction mixture at 515 nm was read with a spectrophotometer, where methanol was used as the blank. The percentage of DPPH free radical scavenging effect was calculated as follows:

$$\text{DPPH Scavenging ability}(\%) = \left[1-\left(A_{515\text{nm, sample}}/A_{515\text{nm, blank}}\right)\right] \times 100\%$$

Ferrous ion chelating capacity assay was accomplished according to previous reports (Decker and Welch 1990; Cheel et al. 2007; Jun et al. 2012). An aliquot of 1 mL sample was sequentially mixed with 3.7 mL of methanol, 0.1 mL of 2 mM $FeCl_2.4H_2O$, and 0.2 mL of 5 mM ferrozine (3-(2-pyridyl)-5,6-bis- (4-phenylsulfonic acid) - 1,2,4-triazine) in a test tube. The mixture was shaken vigorously and left to react for 10 min at ambient temperature. The absorbance at 562 nm was measured against methanol as blank, where a lower absorbance indicated a higher ferrous ion chelating capacity. The chelating ability was calculated as follows:

$$\text{Ferrous ion chelating capacity}(\%) = \left[1-\left(A_{562\ \text{nm, sample}}/A_{562\ \text{nm, blank}}\right)\right] \times 100\%$$

Sensory evaluation of strawberry wine

To analyze the impact of the addition of ball-milled achenes on the organoleptic properties of strawberry wine, sensory analyses were carried out to determine whether there were sensory and acceptance differences among the strawberry wines. Fifteen panelists (including eight females and seven males, between 22 and 32 years of age) from the Department of Food Science at Fu Jen Catholic University were recruited. The selection of panelists was based on previous experience in course work and participation in wine sensory projects. Those panelists were then trained on the color, aroma, taste and texture of wine for four sessions on colour, taste, aroma and overall flavour using standards. The quality of strawberry wine was analyzed based on a 20-point scale system developed by the American Wine Society (2014), and quality attributes including Appearance (graded 0–4 scores), Aroma (0–5), Taste (0–8), and Overall Impression (0–3) were analyzed and graded. The quality was based on the total score 18–20 as extraordinary, 15–17: excellent, 12–14: good, 9–11: commercially acceptable, and ≤8: deficient, poor or objectionable. Additionally, acceptance was determined on a 9-point scale, where 9: like extremely, 7: like moderately, 5: neither like nor dislike, 3: dislike moderately, 1: dislike extremely. Thirty mL of each wine sample was served in glass, and tested by panelists in separated booths in the Sensory Laboratory at Fu Jen Catholic University. Panelists were also provided with a cup of distilled H_2O and unflavoured steam bread to cleanse their palates between samples. To analyze the potential market acceptance, two commercial strawberry wines, including a leading product in Taiwan retail market, were also used in the sensory evaluation.

Statistical analysis

Variance analysis (ANOVA) was used to analyze the effect of ball-milling on extractable active compounds and antioxidative capacity, and $p < 0.05$ was considered to be a threshold for statistical significance. Duncan's multiple range test was used to determine whether the differences among treatments were significant.

Results and discussion

Characteristics of strawberry fruit and achenes

Strawberry is considered to be a health promoting fruit due to the content of its bioactive compounds. The carbohydrate, protein, fat, mineral, fibre content and titratable acidity were 7.60 ± 0.01 % (*w*/*w* total fruit), 0.83 ± 0.03 %, 0.14 ± 0.01 %, 0.53 ± 0.01 %, 2.20 ± 0.01 % and 0.86 ± 0.01 %, respectively (Table 1). Achenes were collected and weighed after freeze-drying, and the content was determined to be 1.16 ± 0.04 % (*w*/*w*) in whole fruit, which was one quarter of the dry matter (Table 1).

The strawberry achenes (Fig. 1a) were collected and determined approximately 1.1 mm in length using a calliper (Fig. 1b). The particle sizes of the pulverized achenes were less than 400 nm after 30 min of ball milling (Fig. 1c). However, increasing the milling duration for up to 3 h, failed to result in any further size reduction. The average particle size of achenes was 322 ± 184 nm after 3 h ball milling. The achene content determined in the present study was in agreement with the 1 % in ripe strawberries reported by Aaby et al. (2005). The strawberry achenes made up ca. 25 % of the dry weight of the fruit, and clearly contained a variety of functional ingredients. Since mature achenes were covered by a hard and relatively thick shell, a micronization process is needed to pulverize the seeds for further applications.

Table 1 Characteristics of the strawberry purée[a]

	% (*w*/*w* berries)	% (*w*/*w* dry weight basis)
Composition		
Moisture	88.99 ± 0.26	
Carbohydrate	7.60 ± 0.01	
Protein	0.83 ± 0.03	
Lipid	0.14 ± 0.01	
Minerals	0.53 ± 0.01	
Fibre	2.20 ± 0.01	
Titratable acids	0.86 ± 0.01	
pH	3.43 ± 0.02	
Part		
Flesh	3.85 ± 0.23	76.05 ± 4.35
Achenes	1.16 ± 0.04	23.95 ± 4.33

[a] Each value represents mean ± standard deviation ($n = 3$)

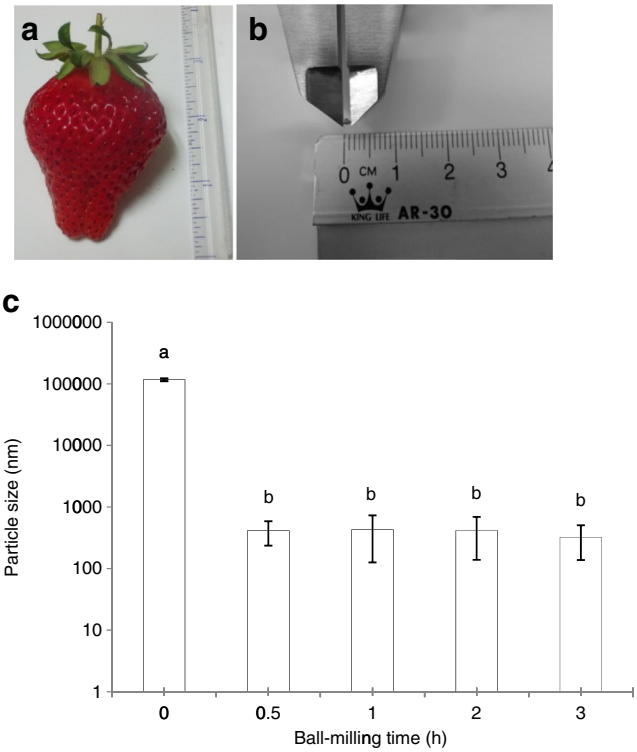

Fig. 1 Particle sizes of strawberry achenes (**a**) on fruit skin, (**b**) freshly collected, (**c**) after ball-milling (0 h means freshly collected). Each value represents mean ± standard deviation ($n = 3$), and different lower-case letters indicate a significant difference ($p < 0.05$) among milling durations

The ball-milling process increased the surface area of the fruit seed, which, in turn, facilitated solvent penetration, and enhanced the extraction of functional compounds. The milling rate in the present study was limited to 300 rpm, because higher milling rates caused the development of a brown colour and scorched appearance in pilot studies. The present study showed that a 30 min milling resulted in a significant ($p < 0.05$) size reduction in achene particles. However, no further reduction occurred when the duration was extended. Chau et al. (2006) reported that micronization led to improvements in the functionality of insoluble fibre and enhanced the applications. The significance of the ball-milling micronization on strawberry achenes in this study could be directly attributed to the pulverization of strawberry's hard and relatively thick pericarp, which was accomplished within a short period of time (30 min).

Ellagic acid and phenolic compounds in achene supplemented strawberry wine

Identification and quantification of ellagic acid was completed by HPLC after purification using SPE method, and the quantification of the total extracted ellagic acid was based on a concentration response curve as $Y = 3.2 \times 10^5$–7.8×10^5

(R^2 = 0.9996), where Y is the peak area and x is the ellagic acid content in 1–100 μg/mL methanol, and the limit of detection was determined to be 1 μg/mL. The extractable ellagic acid in raw strawberry achenes was determined to be 550.72 ± 15.82 μg/g. The level of extracted ellagic acid increased 66 % up to 915.24 ± 155.04 μg/g with ball-milling for 30 min, as compared to intact achenes (Fig. 2). However, no further difference (ranging 790.04 ± 57.59–874.65 ± 84.78 μg/g) was found for an extended duration of ball-milling for up to 3 h. The present results revealed that the extracted ellagic acid content is with respect to particle size, that a 30 min ball-milling process effectively increased the extraction of bioactive compound from achenes.

Increase of bioactive compounds and antioxidant capacity were determined to evaluate the benefits of ball-milled achenes in fermentation. The total phenolic compounds and ellagic acid content in strawberry must were 558.93 ± 10.29 μg GA/mL and 4.48 ± 0.19 μg/mL, respectively (Fig. 3). No significant differences in both functional compounds were observed between conventional strawberry must and those added with ball-milled achenes. Although micronization enhanced the leaching of ellagic acid, the addition of 1 % ball-milled achenes to the must did not significantly increase the ellagic acid content due to a dilution effect. After fermentation, the total phenol content decreased significantly ($p < 0.05$) to 253.84 ± 23.09 and 386.93 ± 12.28 μg GA/mL in traditional and ball-milled achene supplemented samples, respectively. Apparently, wine prepared with ball-milled achenes resulted in significantly ($p < 0.05$) less total phenol loss (35.40 %) than the traditional method (54.45 %) (Fig. 3a). In addition, a significant ($p < 0.05$) increase in ellagic acid was observed in the presence of ball-milled

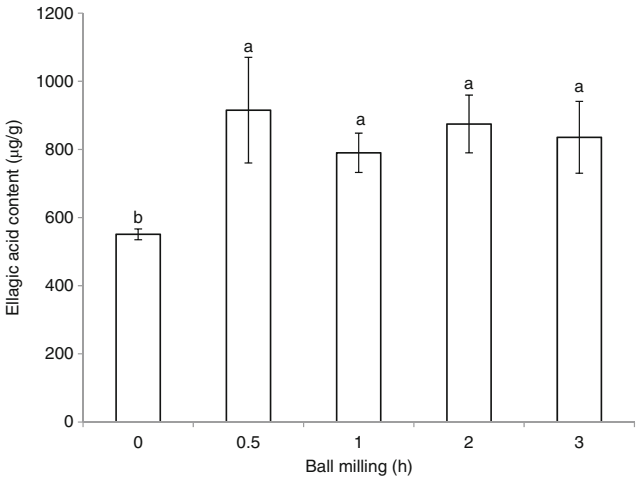

Fig. 2 Effect of ball-milling duration on the extraction of ellagic acid from strawberry achenes. Control (subjected to 0 h ball-milling) was accomplished by using methanol to extract intact achenes for 30 min. Each value represents mean ± standard deviation (n = 3), and different lowercase letters indicate a significant difference ($p < 0.05$) among ball-milling durations

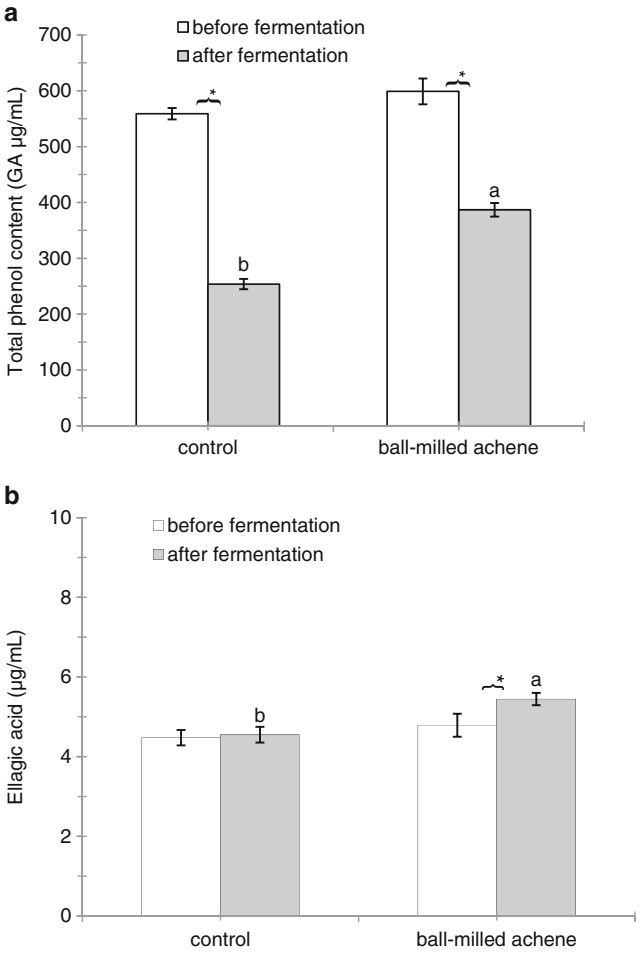

Fig. 3 Amounts of total phenols (**a**) and ellagic acid (**b**) in strawberry must (before fermentation) and wine (after fermentation). Each value represents mean ± standard deviation (n = 3). The asterisk (*) indicates a significant difference ($p < 0.05$) between before and after fermentation, and different lowercase letters indicate a significant difference ($p < 0.05$) between controls (conventional process) and ball-milled achene added samples

achenes. The ellagic acid levels in the ball-milled samples increased from 4.55 ± 1.97 to 5.46 ± 1.54 μg/mL (19.72 % increase) after fermentation, while no significant changes were found in the controls (Fig. 3b). With ball-milled achenes, the wine samples contained significantly higher levels of bioactive compounds than traditional products.

The ellagic acid content in strawberry achene was determined to be in the range from 500 to 800 μg/g, and this was in agreement with a previous study that reported 344 ± 37 to 873 ± 146 μg/g (Aaby et al. 2005). Although strawberry achenes have been shown to be a rich source of phenolic compounds, including ellagic acid, anthocyanins, and flavanols (Hakkinen et al. 2000; Aaby et al. 2005; Aaby et al. 2007b), their hard pericarp restricts the release of active compounds, and, as a result, also limits its potential applications in the food industry. Since ellagic acid in achenes is typically obtained by solvent extraction, pulverization would

lead to better solvent (e.g. ethanol) penetration and a greater yield. In the present study, the average particle size of the strawberry achenes was reduced from 1.1 mm to 400 nm, after a 30 min ball-milling, which resulted in an increase in ellagic acid content of the extract (Fig. 3).

Fermentation has been demonstrated to cause loss of total phenolic compounds, possibly due to the polymerization and condensation of monomeric phenol compounds (such as anthocyanins), and thus result in the loss of total phenols up to 69–79 % in wines (Parley et al. 2001; Klopotek et al. 2005; Aguilera-Carbo et al. 2009; Garcia-Estevez et al. 2010). Ball-milling was employed to enhance the penetration of solvent into achenes in this study, which resulted in a greater yield of active compounds after fermentation. Aaby et al. (2005) reported that ellagitannins and ellagic acid glycosides were precursors of ellagic acid in achenes, and a β-glucosidase was characterized as the key enzyme in this production (Sims and Bates 1994; Shi et al. 2005; Aguilera-Carbo et al. 2008; Aguilera-Carbo et al. 2009). The hydrolysis of ellagitannins produces hexahydroxydiphenic acid, which spontaneously rearranges into ellagic acid. Therefore, processes used to rupture the pericarp of achenes would favour hydrolysis and the production of greater ellagic acid contents in fermented products. In the present study, strawberry wine added with ball-milled achenes showed less total phenol compounds loss and greater ellagic acid contents. Active precursors (such as ellagitannins) released after ball-milling might be a contributing factor for such findings. Therefore, fermentation with ball-milled achenes led to a greater level of bioactive compounds, and might impart enhanced antioxidant, anti-inflammatory and other functionalities in fruit wine.

Antioxidant activity of ball-milled achene supplemented strawberry wine

Since antioxidant activities are highly related to phenolic compounds, increases in ellagic acid and other phenolic compounds due to the addition of ball-milled achenes is beneficial for antioxidant capacity. Effect of ball-milled achene supplementation on antioxidant activities was analyzed in this study, and significant increases ($p < 0.05$) in ABTS$^{\bullet+}$ and DPPH scavenging ability, and Fe^{+2} chelating capacity were found in the strawberry wine (Figs. 4, 5, and 6).

The ABTS$^{\bullet+}$ scavenging activities of control and ball-milled achene supplemented must were 99.31 ± 0.25 % and 96.67 ± 0.46 %, respectively. Ball-milled achene supplementation imparted ca. a 50 % increase of ABTS$^{\bullet+}$ scavenging capacity up to 148.12 ± 1.79 % in strawberry wine. However, no significant increase in radical scavenging activities was found in controls after fermentation (Fig. 4). Additionally, significantly greater DPPH scavenging activities (1.5 folds) in supplemented samples were found compared to control (190.03 ± 3.78 % vs. 125.43 ± 0.60 %), where no

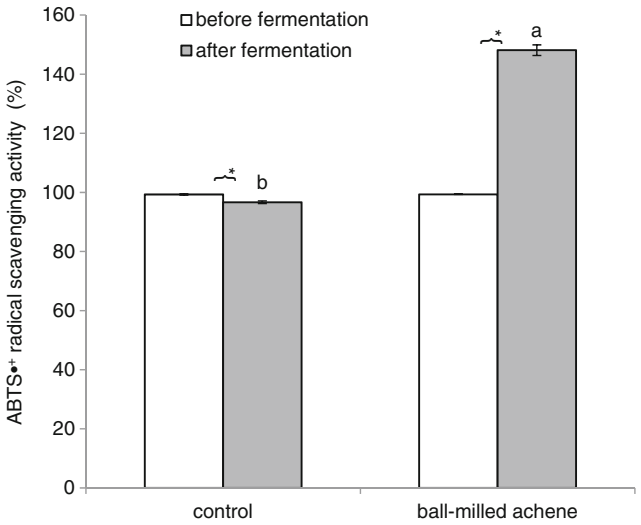

Fig. 4 ABTS$^{\bullet+}$ scavenging activity (%) of strawberry must (before fermentation) and wine (after fermentation). Each value represents mean ± standard deviation ($n = 3$). The asterisk (*) indicates a significant difference ($p < 0.05$) between before and after fermentation, and different lowercase letters indicate a significant difference ($p < 0.05$) between controls (conventional process) and ball-milled achene added samples

significant difference was found between those two samples prior to fermentation (Fig. 5). In DPPH assay, less radical scavenging activities were found in controls, this could be due to the loss of total phenolic compounds during fermentation (Parley et al. 2001; Aguilera-Carbo et al. 2009). A similar trend in ferrous ion chelating capacity between control and supplemented samples was also noticed. A range of 40.80 ± 0.03–44.95 ± 3.33 % for chelating capacity was determined in those samples before fermentation, and a

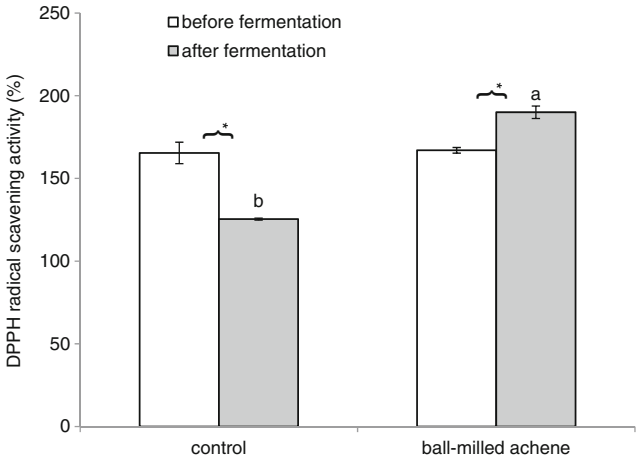

Fig. 5 DPPH radical scavenging activity (%) of strawberry must (before fermentation) and wine (after fermentation). Each value represents mean ± standard deviation ($n = 3$). The asterisk (*) indicates a significant difference ($p < 0.05$) between before and after fermentation, and different lowercase letters indicate a significant difference ($p < 0.05$) between controls (conventional process) and ball-milled achene added samples

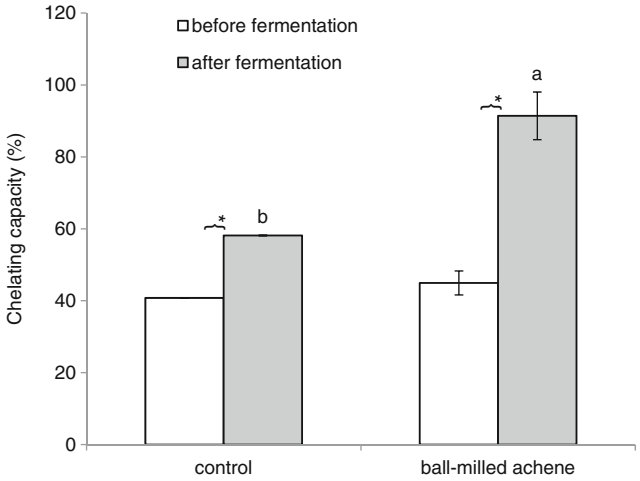

Fig. 6 Ferrous ion chelating activity (%) of strawberry must (before fermentation) and wine (after fermentation). Each value represents mean ± standard deviation (n = 3). The asterisk (*) indicates a significant difference ($p < 0.05$) between before and after fermentation, and different lowercase letters indicate a significant difference ($p < 0.05$) between controls (conventional process) and ball-milled achene added samples

significant difference ($p < 0.05$) of increasing chelation by 43.5 % and 125 %, up to 58.14 ± 0.14 % and 91.42 ± 6.63 % Fe^{+2} chelating capacities, were determined in control and supplemented wines, respectively (Fig. 6).

The ball-milled achene-supplemented strawberry wine showed higher radical scavenging and metal ion chelating capacities, and the fortification of antioxidant activity was due to the higher levels of ellagic acid and phenolic compounds (Fig. 3). Generally, high levels of total phenolic and ellagic acid contents cause an elevation in antioxidant capacity (Hakkinen et al. 2000; Ayala-Zavala et al. 2004; Aaby et al. 2005; Aaby et al. 2007a, b; da Silva Pinto et al. 2008; Ariza et al. 2010). Achenes make up only 1 % of whole strawberry fruit, but contribute to a great amount of antioxidant activities (Aaby et al. 2005). Cheel et al. (2007) also concluded strawberry achenes, on average, contributing to 50 % of total phenols, 43 % of total flavonoids, and 37 % of total anthocyanins content of the whole fruit. The ellagic acid and its derivatives, and various phenolic compounds in achenes are characterized as the main contributors to the antioxidant activities. Therefore, the supplementation of ball-milled achenes in must leads to greater bioactive compounds and higher antioxidant activities in strawberry wine.

Sensory evaluation of wine

Sensory evaluation is a key process in product development and consumer acceptance, therefore, the effect of the addition of ball-milled achenes in the fermentation process on quality attributes and acceptance was analyzed. In the sensory analysis, a leading commercial strawberry wine in Taiwan was also used and coded as commercial 1, in order to analyze the difference between it and the ball-milled added product. The quality scores of strawberry wines showed no significant difference ($p > 0.05$) among the control, ball-milled achenes added and commercial 1 samples, whereas those three wines were rated as good to excellent (Table 2). Among those 3

Table 2 Sensory evaluation of strawberry wines

	Sensory score			
	Control	Ball-milled	Commercial 1	Commercial 2
Attributes[1]				
Appearance	3.8 ± 0.4^{a}	3.3 ± 0.6^{a}	3.6 ± 0.9^{a}	3.4 ± 0.7^{a}
Aroma	3.0 ± 1.0^{b}	3.3 ± 1.0^{b}	4.3 ± 0.8^{a}	2.5 ± 1.9^{bc}
Taste	6.8 ± 1.1^{a}	6.0 ± 1.4^{ab}	6.0 ± 1.8^{ab}	5.1 ± 2.1^{b}
Overall impression	1.9 ± 0.7^{a}	1.7 ± 0.7^{a}	1.8 ± 0.9^{a}	1.3 ± 0.9^{a}
Total score	15.3 ± 2.6^{a}	14.3 ± 2.8^{ab}	15.9 ± 3.1^{a}	12.3 ± 4.6^{b}
Acceptance[2]				
Appearance	7.1 ± 0.9^{a}	6.2 ± 1.5^{ab}	7.3 ± 1.5^{a}	5.9 ± 1.7^{b}
Aroma	5.9 ± 1.5^{b}	5.8 ± 1.7^{b}	7.9 ± 1.5^{a}	4.7 ± 2.3^{b}
Taste	6.3 ± 1.2^{a}	5.7 ± 1.6^{a}	6.1 ± 1.8^{a}	5.7 ± 2.4^{a}
Overall	6.5 ± 1.2^{a}	5.9 ± 1.5^{ab}	6.1 ± 1.7^{ab}	5.1 ± 1.9^{b}

[1] Sensory attributes were analyzed using the evaluation chart developed by American Wine Society. Evaluation was based on a 20 points scale in 4 categories, including Appearance (0–4 points), Aroma (0–5), Taste (0–8), and Overall Impression (0–3). Where total score 18–20: extraordinary, 15–17: excellent, 12–14: good, 9–11: commercially acceptable, and ≤8: poor

[2] Acceptance was determined on a 9-point scale, where 9: like extremely, 7: like moderately, 5: neither like nor dislike, 3: dislike moderately, 1: dislike extremely

[a,b] Values with different lowercase letters are significantly different ($p < 0.05$) among samples within the same sensorial attribute or hedonic test (n = 15)

wines, no significant differences ($p > 0.05$) were observed in appearance and overall impression. Commercial 1 showed a higher aroma score than the control and ball-milled achenes added samples, and this sample was characterized as having an intense bouquet. Additionally, no significant difference ($p > 0.05$) in taste was determined between ball-milled and commercial 1 samples. The other commercial strawberry wine (commercial 2) was rated significantly lower total quality score among all samples, due to lower score in taste (Table 2). In the hedonic analysis, no significant difference ($p > 0.05$) in overall acceptance was found among control, ball-milled achene added and commercial 1 samples, though aroma of commercial 1 showed the greatest acceptance among those three wines. Additionally, the commercial 2 was found least acceptable ($p < 0.05$), what is similar to the previous quality analysis (Table 2). Commercial 2 sample was also least accepted in appearance and aroma, those could be due to lower quality scores.

Though, achene is characterized rich in bioactive compounds, no reports have demonstrated the application of this substance in food products. This study demonstrates potential of ball-milled achenes in fermentation. Ellagitannins are characterized to cause astringency and harshness in wine (Sims and Bates 1994), no off-taste was determined in ball-milled achene added strawberry wine. This could be due to hydrolysis of ellagitannins to ellagic acid, where greater ellagic acid content in wine was determined. The addition of ball-milled achenes in must did not cause quality loss in organoleptic properties of strawberry wine, as compared to a leading commercial product in Taiwan. The results demonstrated no sensorial attributes changed in the presence of ball-milled achenes, indicating the potential in food industry.

Conclusions

For a food product to be considered as a functional product, it should have high levels of nutritionally desirable compounds imparting physiological effects. The addition of ball-milled achenes to must significantly increases the levels of phenolic compounds and bioactivities in strawberry wine, therefore, leads to greater antioxidant activities. Additionally, no significant differences in overall flavour and acceptance in the presence of ball-milled achenes were determined. These findings suggest that ball-milling improves the extracted yield of active compounds in achenes, and supplementation of ball-milled achenes is beneficial to antioxidant content and activity in strawberry wine. In future studies, bioactive compounds derived from ball-milled achenes will be analyzed using HPLC-ESI-MS, and their biofunctions of anti-inflammatory and anti-proliferative will be also taken into account.

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