An efficient method using ultrasound to accelerate aging in crabapple (Malus asiatica) vinegar produced from fresh fruit and its influencing mechanism investigation

Xinyu Zhai, Xu Wang, Xiaoyi Wang, Haoran Zhang, Yucheng Ji, Difeng Ren, Jun Lu

Abstract

In this study, a kind of crabapple vinegar was developed by the method of mixed bacteria fermentation. It showed that the total acids and total esters in the vinegar increased by 30.51% and 22.67%, respectively. Simultaneously, ultrasound was used to treat the vinegar to shorten the time of aging. In addition, the HS-SPME-GC-MS results showed that some volatile components had increased significantly, such as total esters, aldehydes and heterocycles. Combining OAV with radar chart of aroma active ingredients, the order of contribution to the characteristic aroma of crabapple vinegar was esters > alcohols > others > acids. Finally, ultrasonic cavitation and hydroxyl radicals were measured to further prove it could accelerate chemical reaction of crabapple vinegar. The results of FTIR showed that the hydrogen-bonded molecules had increased, while free molecules with irritating taste (such as ethanol and acetic acid) had decreased, which made the taste of crabapple vinegar softer. Results have showed that ultrasound is a promising technique for shortening aging time and it also provides the possibility to improve the taste of the vinegar.

1. Introduction

Malus asiatica, known as Chinese pear-leaved crabapple in China, is a popular fruit with various nutrients such as vitamins, organic acids, dietary fiber and minerals which are richest in selenium, zinc and iron and so on [1,2]. Some studies had reported that selenium is a strong antioxidant and it could protect cardiovascular and antitumor, preventing aging and tissue hardening caused by oxidation [3]. In addition, it also contains polysaccharides, polyphenols, flavonoids and other antioxidant factors, which provides therapeutic effect in treating high blood pressure and coronary heart disease. However, crabapple has a short shelf life so that it cannot be well utilized in food industry [4]. It is unpopular since it is rich in organic acids, amino acids, vitamins and certain aroma compounds, and also provides special positive health benefits [6,12]. Liquid state fermentation (LSF) is generally applied to prepare fruit vinegars. However, the content and activity of volatile compounds, organic acids, polyphenols and free amino acids in LSF vinegar was weak [13]. Thus, mixed bacteria fermentation technology in the alcohol fermentation process was used to enhance the flavor and quality of LSF fruit vinegar. It had found that furfural residues were simultaneously saccharified and fermented through mixed cultures of lactic acid bacteria and yeast, finally increasing the yield of lactic acid and ethyl lactate [14]. Besides, it had also reported that yeast co-cultured with lactic acid bacteria had a positive effect on milk fermentation [15]. Chen et al. [16], had reported that mixed cultures of Saccharomyces cerevisiae and Lactobacillus plantarum in alcoholic fermentation could effectively willing to explore new products of vinegar since its many beneficial effects on health like anticancer, antioxidation and antibacteria [7,8,9,10,11]. Recently, the demand for fruit vinegars has increased since it is rich in organic acids, amino acids, vitamins and certain aroma compounds, and also provides special positive health benefits [6,12].

Keywords:
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A B S T R A C T

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Liquid state fermentation (LSF) is generally applied to prepare fruit vinegars. However, the content and activity of volatile compounds, organic acids, polyphenols and free amino acids in LSF vinegar was weak [13]. Thus, mixed bacteria fermentation technology in the alcohol fermentation process was used to enhance the flavor and quality of LSF fruit vinegar. It had found that furfural residues were simultaneously saccharified and fermented through mixed cultures of lactic acid bacteria and yeast, finally increasing the yield of lactic acid and ethyl lactate [14]. Besides, it had also reported that yeast co-cultured with lactic acid bacteria had a positive effect on milk fermentation [15]. Chen et al. [16], had reported that mixed cultures of Saccharomyces cerevisiae and Lactobacillus plantarum in alcoholic fermentation could effectively
improve the flavor and quality of citrus vinegar.

 Newly brewed fruit vinegars usually need to be aged to promote fruit vinegar ripening after fermentation. However, there are some difficulties during natural aging such as long period and high cost [12]. Ultrasound, a technique that low frequency high energy power, often is used in various fields of food processing like extraction, sterilization, oxidation [17,18]. According to previous reports, acoustic cavitation of ultrasound is probably important during vinegar aging process [19,20]. The acoustic cavitation engendered by ultrasonic waves in liquids can cause some chemical reactions (oxidation, esterification, etc.) and improve reaction rates [21–23]. A high pressure and temperature environment is immediately provided by ultrasonic treatment, consequently accelerating the biochemical, physical and chemical reactions of vinegar, and then rapidly improves the sensory quality of vinegar [24]. It had been reported that the use of ultrasound in red wine aimed to accelerate aging of wine and increase the contents of anthocyanin and tannin [20],[25–27]. Besides, other alcoholic beverages also extensively apply ultrasound to accelerating the aging of beverages, shortening the aging stage from one year to a week or three days successfully [12,28].

The objective of this study is to investigate the effect of ultrasound on accelerating aging of crabapple vinegar and improving its quality. To begin with, fresh crabapple was used as raw material and a mixed-bacteria fermentation method (with Lactobacillus plantarum B7 added in the alcohol fermentation process) was used to prepare vinegar to enhance the flavor of the vinegar. Using HS-SPME-GC–MS technology combined with aroma activity value method and aroma active component radar chart to explore the effect of ultrasonic treatment on the physicochemical characteristics, volatile components and aroma active components of crabapple vinegar. Lastly, the iodine release method, methylene blue solution method and Fourier Infrared Transform
Spectroscopy were used to explore the mechanism of ultrasonic aging of crabapple vinegar. The results of this study not only make full use of crabapple resources, but also provide practical information for ultrasonic treatment to accelerate the aging of fruit vinegar.

2. Materials and methods

2.1. Materials

Fresh crabapples were produced from Keyousianqi, Inner Mongolia, China. The food grade of citric acid, ascorbic acid, glucose, sodium bicarbonate and chitosan were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). All chemical reagents (calcium carbonate, sodium hydroxide, concentrated sulfuric acid, sulfosalicylic acid, sodium acetate, methanol, formaldehyde, acetonitrile, ethanol, potassium iodide solution, carbon tetrachloride, sodium benzoate, methylene blue) were bought from Sigma-Aldrich Inc (St. Louis, MO, U.S.A.). Saccharomyces cerevisiae was supplied by Angel Yeast Co., Ltd (Beijing, China). Lactobacillus plantarum B7 and Acetobacter pasteurii CGMCC 1.41 were provided from China Microbial Culture Collection.
MRS medium, agar and yeast extract were purchased from Beijing.* E represents the essential amino acids and T represents the total amino acids.

### Table 1

| Index | FCV (mg/100 mL) | UFCV (mg/100 mL) | NFCV (mg/100 mL) | Taste |
|-------|-----------------|------------------|------------------|-------|
| Thr⁴  | 0.52            | 0.74             | 0.59             | Sweet |
| Ser⁴  | 0.33            | 0.48             | 0.41             | Sweet |
| Glu⁴  | 1.45            | 1.95             | 1.76             | Fresh |
| Gly⁴  | 0.46            | 0.52             | 0.46             | Strong |
| Val⁵  | 1.20            | 1.15             | 0.97             | Astringent |
| Ile⁵  | 0.70            | 0.71             | 0.68             | Bitter |
| Lys⁵  | 0.81            | 0.75             | 0.94             | Slight Sweet |
| Arg⁰  | 0.07            | 0.07             | 0.062           | Sour |
| Ala⁰  | 1.0             | 1.3              | 1.2              | Strong |
| Met⁰  | 0.18            | 0.23             | 0.23             | Sweet |
| Tyr⁰  | 0.23            | 0.24             | 0.22             | Slight bitter |
| Leu⁰  | 1.1             | 1.2              | 1.2              | Astringent |
| Phe⁰  | 1.0             | 1.1              | 1.0              | Sweet |
| His⁰  | –               | 0.36             | 0.19             | Slight bitter |
| Arg⁰  | 0.55            | 0.68             | 0.7              | Bitter |
| Pro⁰  | 0.99            | 0.94             | 0.82             | Sweet |
| Cys⁰  | 0.15            | 0.19             | 0.15             | |
| Total  | 10.74           | 12.612           | 11.582           | |
| E/T (%) | 51.30          | 49.48            | 50.08            | |

* E represents the essential amino acids and T represents the total amino acids.

### Table 2

**Effect of ultrasonic treatment on free amino acid content in crabapple vinegar.**

| Amino acid | FCV (mg/100 mL) | UFCV (mg/100 mL) | NFCV (mg/100 mL) | Taste |
|------------|-----------------|------------------|------------------|-------|

### 2.2. The properties of crabapple vinegar

#### 2.2.1. Crabapple juice and inoculum

Fresh ripe crabapple were washed with tap water and the cores were separated, putting them into a pulping machine (QMZ-PBJ-01, China). In this process, 0.4% citric acid and 0.4% ascorbic acid were added to inhibit enzymatic browning of crabapples. Additionally, 10% food-grade glucose and NaHCO₃ were added into crabapple juice and then pasteurization was used to sterilize at 85°C for 15 min. Finally, the juice was filtered through a multi-layer filter cloth. In order to prepare inoculums, Saccharomyces cerevisiae, Lactobacillus plantarum B7 and Acetobacter pasteurian CGMCC1.41 were cultured in corresponding activation mediums. The bacterial liquid obtained after two passages can be used as bacterial seed liquids, which would be inoculated into clarified crabapple juice.

#### 2.2.2. Production of crabapple vinegar

As shown in Fig. 1, the production process of crabapple vinegar includes two fermentation stages: (1) Alcohol fermentation: Saccharomyces cerevisiae and Lactobacillus plantarum B7 were added to crabapple juice for fermenting at 30°C, and the fermentation was terminated when ethanol content no longer increased. The obtained fermentation broth was clarified and centrifuged at 8000 r/min for 10 min, placing supernatant in a constant temperature water bath at 60°C for 30 min to obtain mixed bacteria fermented crabapple wine. (2) Acetic acid fermentation: Adding Acetobacter pasteurian seed liquid to the crabapple wine for fermenting at 30°C until the acetic acid content in the fermentation system no longer grew, which is regarded as the end of this fermentation. Subsequently, fermentation broth was centrifuged at 8000 r/min for 10 min and the supernatant obtained was crabapple vinegar. According to the above method, the wine and vinegar prepared by fermentation with only 0.2% Saccharomyces cerevisiae were used as control group.

#### 2.2.3. Soluble solid, pH, alcohol, lactic acid, total acid and total esters

In order to explore alcohol and acetic acid fermentation process, soluble solid content, pH, alcohol, lactic acid and total acid content were measured daily. The soluble solids were tested by means of an Abbe refractometer (Bel-lingham Stanley Limit 60/70 Refractometer, England). The pH values and ethanol contents in present research were measured with pH meter (Metter Toledo PHS-3C, USA) and alcohol meter (Dujardin-Salleron, France).

The concentration of lactic acid was passed HPLC (Shimadzu, Japan) using Agilent-ZORBXS8-C18 (4.6 mm × 250 mm, 5 μm), the detector temperature was 280°C and the detection wavelength was 210 nm. The mobile phase was methanol and 0.02 mol/L phosphoric acid water phase (15:85) with a flow rate of 0.8 mL/min, calibration with external standard.

A potentiometric titration method was used to measure total acids as an equivalent of acetic acid according to the Chinese national standard method GB/T12456-2008 (BCPCA, 2008). In brief, the vinegar was titrated with NaOH (0.05 mol/L) until the pH value reaches to 8.2. The volume of NaOH used for the sample and blank was recorded. The content of total acids was calculated by following equation:

\[
X = \frac{(V_1 - V_2) \times 0.05 \times 60}{10V}
\]

where, X represents the content of total acids, V₁ is the volume of NaOH solution used in the titration of sample, V₂ is the volume of NaOH solution used in the titration of control, V is the volume of the tested sample.

Furthermore, the above solution was placed in the dark for 24 h to completely saponify esters in the vinegar solution. Then H₂SO₄ (0.05 mol/L) was used until the pH value was 9.5, recording the consumed volume of H₂SO₄. Simultaneously, 40% ethanol solution was used as a blank control. The content of total acids was calculated by the following equation (calculated as an equivalent of ethyl acetate):

\[
Y = \frac{(V_1 - V_4) \times 0.05 \times 88}{V}
\]

where, Y signifies the content of total esters, V₃ is the volume of H₂SO₄ solution used in the titration of sample, V₄ is the volume of NaOH solution used in the titration of control, V is the volume of the tested sample.

#### 2.3. Ageing of vinegars

##### 2.3.1. Ultrasonic treatments

An ultrasonic cell disruptor (Biosafer3D, Saifei Technology Co., Ltd., China) was used to accelerate aging fresh crabapple vinegar (FCV). The ultrasonic power was 300 W, the amplitude was about 35 μm, the ultrasonic time was 45 min, and the amount of ethanol added was 0.6%. The probe is centered and immersed 2 cm in a 150 mL glass container containing 100 mL sample. During the long ultrasound process, the temperature was controlled by placing the sample in an ice bath and

\[
\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}
\]
measuring the temperature change with a thermometer. The ultrasonically processed fruit (UFVC) is naturally cooled and placed in a refrigerator at 4 °C to avoid possible changes in the case that analysis cannot be performed within a short period of time after sampling. Compared with its qualities, naturally aged for 30 days crabapple vinegar (NFCV) was also studied in present research.

2.3.2. Physicochemical properties of crabapple vinegars

Under ultrasonic treatment, use the 2.2.3 method to determine the pH, total acid and ester content of the sample. At the same time, use a viscometer and a colorimeter to measure the viscosity and color of the vinegar. The calculation formula of the color difference value is as follows:

![Fig. 3. Effect of ultrasound on the flavor quality of crabapple vinegar. Total ion flow diagram of volatile components of fresh vinegar (a), ultrasound treated vinegar (b) and natural aging vinegar (c). Comparison of the relative contents (d) and species (e) of various volatile substances in three kinds of vinegars. Radar chart of three active ingredients of crabapple vinegar (Complete aroma active ingredients (f); alcohol, acid, and other substances aroma active ingredients (g)). (FCV: Fresh crabapple vinegar; UFCV: Ultrasound treated fresh crabapple vinegar; NFCV: Natural aging fresh crabapple vinegar).]
\[ \Delta E = \sqrt{\left( L_n - L_0 \right)^2 + (a_n - a_0)^2 + (b_n - b_0)^2} \]

where, \( \Delta E \) represents color difference of crabapple vinegar sample, \( L_0 \), \( a_0 \), \( b_0 \) stand for the brightness, red-green value, yellow-blue value of fresh crabapple vinegar (FCV), respectively. \( L_n \), \( a_n \), \( b_n \) are the brightness, red-green value, yellow-blue value of vinegar after ultrasonic treatment (UFCV) or natural aging (NFCV), respectively.

Crabapple vinegar was mixed with 10% sulfosalicylic acid (1:1), placed at 4 \( ^\circ \)C for 24 h and centrifuged at 10,000 rpm for 15 min, and the supernatant was taken. After drying with nitrogen at 40 \( ^\circ \)C, add buffer solution (pH 2.2) to mix and filter for determination. A model L-8900 automatic amino acid analyzer (Hitachi, Tokyo, Japan) coupled with a Biochrom Li cation exchanger column(4.6 \( \times \) 20 mm), maintained 40 \( ^\circ \)C. The mobile phase is 20 mmol/L sodium acetate solution (Phase A) and mixture containing 20 mmol/L sodium acetate, methanol and acetonitrile (Phase B, 1:2:2) with a flow rate of 0.5 mL/min for 125 min. Chromatographic signals were determined at wavelengths of 440 nm and 570 nm. Identification and quantification of amino acids was performed based on standards. The results were expressed as mg/100 mL.

### 2.4. Volatile flavors of crabapple vinegars (FCV, UFCV and NFCV)

#### 2.4.1. Hs-SPME-GC-MS

The volatile compounds in three vinegars were detected using gas-chromatography-mass-spectrometry (GC/MS) coupled with the Head space solid-phase microextraction (HS-SPME) technique. A conditioned HS-SPME fiber coated with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS,50/30 \( \mu \)m) was maintained 2 cm above the liquid, and exposed to the headspace for 45 min. Separations were achieved by DB-WAX (60 m \( \times \) 0.25 mm \( \times \) 0.25 \( \mu \)m) capillary column. The program of column temperature was shown as follows: initial temperature 35 \( ^\circ \)C, maintaining for 5 min, followed by raises to 100 \( ^\circ \)C at a rate of 5 \( ^\circ \)/min, and increasing at 3 \( ^\circ \)/min to 200 \( ^\circ \)C, finally growing at a rate of 10 \( ^\circ \)/min to 220 \( ^\circ \)C and held constant for 15 min. The MS detector was operated in an EI mode (70 eV), with a mass scan range of 33–450 amu (m/z). The capillary direct interface temperature was 250 \( ^\circ \)C and MS source temperature was 230 \( ^\circ \)C. The detected volatile components in crabapple vinegars were compared with the NIST98 library to qualitatively analyse in combination with relevant literature. Each sample was analyzed in triplicate.

In this research, 2-octanol (CAS: 6169–06-8) was used as internal standard for quantification. Comparing the chromatographic peak area of volatile aroma substances with that of the internal standard substance, calculating the amount of each volatile fragrance compound relative to the internal standard substance, which is the relative content of the volatile components in the system (The substances with positive and negative matching degrees greater than 800 are reported). The calculation formula is as follows:

\[ C = \frac{A \times C_i}{A_i} \]

where, \( C \) is the concentration of volatile substance, \( A \) is the chromatographic peak area of volatile substance, \( C_i \) is the concentration of internal standard 2-octanol, \( A_i \) is the chromatographic peak area of internal standard 2-octanol.

#### 2.4.2. Odor activity values (OAVs)

The aroma threshold of aroma components in foods can evaluate the degree of contribution of the aroma components to the aroma quality of the food. Combining the aroma thresholds of the corresponding volatile
The calculation formula is as follows: 

\[ \text{OAV} = \frac{C_i}{O_T} \] 

OAV is the aroma activity value of substance i, and \( C_i \) is the mass concentration of substance i calculated according to the internal standard method, \( O_T \) is the aroma threshold value of substance i in water.

2.5. Preliminary study on the mechanism of ultrasonic accelerated aging of crabapple vinegar

The cavitation effect produced by ultrasound caused the vinegar to produce a large number of cavitation bubbles below ultrasound. The blasting of cavitation bubbles could generate instantaneous high temperature and high pressure, which led to the generation of a large number of free radicals to promote a series of physical and chemical reactions in the vinegar, thereby improving the quality of vinegar and shortening the aging time.

2.5.1. Ultrasonic cavitation yield

The iodine release method [29], was used to indirectly characterize the strength of ultrasonic cavitation effects. On the one hand, the prepared potassium iodide solution was sonicated for 15 min, 30 min, 45 min, 60 min, and 75 min under 300 W ultrasound power. On the other hand, the 0.2 mol/L potassium iodide solution added with carbon tetra-chloride was sonicated for 60 min at different ultrasonic powers (100 W, 200 W, 300 W, 400 W, 500 W). Using potassium iodide solution without ultrasound as a blank control, the absorbance of the system was measured at 354 nm immediately after the ultrasound was completed to study the effect of ultrasound power and ultrasound time on the yield of ultrasound cavitation.

2.5.2. Hydroxyl radical release during ultrasound

Studies have found that methylene blue can combine well with hydroxyl radicals into a stable and colorless product [30]. The methylene blue solution (15.00 µmol/L) was sonicated for 60 min under different ultrasonic powers (100 W, 200 W, 300 W, 400 W, 500 W). The maximum absorption wavelength of methylene blue solution was observed by UV-visible spectrophotometer scanning and the hydroxyl radical produced in the ultrasonic process was reflected by the drop of absorbance at the maximum absorption wavelength. Repeat the above operation to explore the amount of hydroxyl radicals generated by different ultrasound time (15 min, 30 min, 45 min, 60 min, 75 min) with 300 W.

In order to clarify that the hydroxyl radicals generated by ultrasound can promote the aging process of fruit vinegar, sodium benzoate (5 mmol/L) as a free radical scavenger was added to fresh crabapple vinegar and subjected to ultrasound treatment (300 W, 60 min). The content of total acid and total ester in crabapple vinegar was determined to verify the mechanism of hydroxyl free radicals.

2.6. Determination of the degree of hydrogen bond association

The degree of hydrogen bond association was measured on Nicolet iS5 FTIR spectrometer (Thermo Fisher, USA). The sampling station was outfitted with a multi-reflection attenuated total reflectance accessory (ATR, six bounces, Specac, Orpington, U.K.). Each spectrum was recorded in the range of 4000–400 cm\(^{-1}\) by an average of 32 scans at a resolution of 4 cm\(^{-1}\). A background spectrum was taken with an empty ATR crystal and recorded on the computer before scanning each sample. Each sample (FCV, UFCV and NFCV) was spread uniformly through the ATR crystal and analysed with OMNIC software. Each sample was analyzed in triplicate.

2.7. Statistical analysis

Samples were prepared in duplicate, and measurements were performed three times. Origin 9.0 software was used to process and analyze experimental data. Data were subjected to analysis of variance (ANOVA) using the software package SPSS 20.0 for Windows (SPSS-IBM Chicago, IL, USA). Differences were considered statistically significant when \( P < 0.05 \).
3. Results and discussion

3.1. Alcohol and acetic fermentation of crabapple vinegar

In this study, crabapple vinegar was produced by mixed bacteria fermentation method (adding Lactobacillus plantarum B7 in the alcohol fermentation process) to enhance the flavor of vinegar. Therefore, the alcohol fermentation and acetic acid fermentation process of crabapple vinegar were monitored to further explore the interaction between Saccharomyces cerevisiae and Lactobacillus plantarum B7. In the Fig. 2a, the soluble solid content of the two systems (single bacterium and mixed bacteria) decreased rapidly and the alcohol content increased on the first day of fermentation, indicating that strains began to adapt to the fermentation environment and produced a large amount of metabolites. However, in the middle of the fermentation, the consumption of soluble solids and the alcohol content in the mixed bacteria system slower than that of the single fermentation system. This might be because the two bacteria need to adapt to a more complex environment [16]. Furthermore, it is worth noting that the final ethanol yields in the two fermentation systems were almost not different (both reached 9.78 ± 0.21%). Therefore, the addition of Lactobacillus plantarum B7 affected the fermentation speed of Saccharomyces cerevisiae, but it had no effect on the final ethanol yield. This is consistent with the results by a previous report [31]. Moreover, the pH value of the mixed bacteria fermentation system had been lower than that of single fermentation system during the whole fermentation process, which was mainly due to the continuous accumulation of lactic acid in the mixed bacteria fermentation system. As shown in Fig. 2b, it was found that the alcohol content of two wines had the same decreasing trend and both dropped to greatly low levels. A small amount of lactic acid did not adversely affect the reproduction and metabolism of acetic acid bacteria. Vinegar fermented by mixed bacteria has higher acidity due to higher lactic acid content. Through mixed bacteria fermentation, the total acid content in crabapple vinegar increased by 30.51%, laying a foundation for enhancing the flavor of vinegar.

3.2. Physicochemical properties

The results on effect of ultrasonic treatments on pH, viscosity, total acidity, total ester and color in crabapple vinegar are shown in Table 1. The pH and viscosity of crabapple vinegar among three vinegars (FCV, UFCV and NFCV) had no obvious difference (p < 0.05). It could be related to the ultrasonic intensity and energy level applied to vinegars without changing the molecular structures of high molecular weight associated with these physicochemical properties [21]. The total acids content in both UFCV and NFCV was significantly reduced due to the fact that the acids in crabapple vinegar were further combined with alcohols to produce aromatic esters. The total ester content of UFCV (5.16 ± 0.21 g/L) was slightly higher than that of NFCV (4.82 ± 0.11 g/L), indicating that the ultrasonic treatment had a significant effect on accelerating aging of crabapple vinegar. However, no statistically significant change was detected (p < 0.05).

According to the classification of Cserhalmi et al. [32], FCV and NFCV showed obvious variation from FCV, showing the ‘slightly noticeable’ color change range (0.5 < δE < 1.5). This phenomenon might be due to the accumulation of unstable particles in vinegars that were partially precipitated [33,34]. In addition, the color of crabapple vinegars is mostly contributed by natural pigments, so the acceleration of isomerization caused by ultrasonic treatment could bring the loss of yellow color (decreased b value) [35]. Owing to the colored compounds formed were destroyed, the L value of UFCV and NFCV remarkably increased resulting in vinegars more transparent. The δE value of UFCV (1.13 ± 0.11) is slightly higher than that of NFCV (0.92 ± 0.24). The reason might be the substances in the vinegar underwent degradation, oxidation, Maillard reaction and other physicochemical changes during the ultrasonic process [36,37].

3.3. Free amino acids

Free amino acids are thought to be a significant contributor to the unique taste of vinegar [16]. The most abundant amino acid in crabapple vinegar was Glu, followed by astringent Val and slightly sweet Lys. It could be seen (Table 2) that the types of free amino acids in UFCV and NFCV had not changed but the total amount had increased. This might be due to the degradation of proteins by ultrasound, which led to an increase in some free amino acids. The Val decreased after two aging treatments, indicating that the astringent and acid taste of crabapple was weakened. In conclusion, ultrasonic treatment was beneficial to improve...
the taste of crabapple vinegar. It had reported that ultrasonic cavitation effect triggered extreme high temperature and pressure to produce a large number of hydroxyl radicals in the system, which could accelerate Maillard reaction in vinegar and affect the change of amino acid type and content [12].

3.4. Volatile aroma compounds in crabapple vinegars by flavor analysis

HS-SPME-GC–MS detected totally 55 kinds of volatile aroma compounds in crabapple vinegars, including acids, alcohols, esters, aldehydes, ketones, heterocycles, phenols and others (Fig. 3 and Table 3). In the Fig. 3b, the relative content of alcohols and acids in UFCV and NFCV significantly decreased, while the total esters increased to 41.38%, indicating that ultrasound can shorten the esterification reaction time and increase the esterification rate [38]. It reported that the addition of *L. plantarum* to the fermentation culture had improved the production of ester compounds [39]. The relatively high content of alcohols in crabapple vinegars were ethanol, isovaleryl alcohol, phenethyl alcohol, n-hexanol. Compared with FCV, the content of alcohols in NFCV and UFCV were reduced by 10.83% and 14.08% (Fig. 3), which might be
related to the esterification reaction, indicating that ultrasound could accelerate the esterification of alcohols and reduce the alcohols in vinegar [12,40]. As shown in Fig. 3d, the acidic compounds in FCV had the highest content (45.37%) of all volatile components, while the relative content of acids in NFCV and UFCV was reduced by 21.35% and 29.65%, respectively. It might be related to the oxidation-reduction between acid compounds and esters [5]. Besides, NFCV and UFCV had newly identified isobutyric acid, isovaleric acid, heptanoic acid, caprylic acid and other acidic compounds, which made the types of acids increase. To some extent, a large number of free radicals produced by the ultrasonic process could accelerate the reaction rate and promote the production of products [27].

As shown in Table 4, crabapple vinegar contained 22 kinds of volatile components that exceed its own aroma threshold, which meant they had obvious aroma activity. In FCV, the highest OAV is phenethyl butyrate (honey, 5038), followed by 9-Hexadecenoic acid ethyl ester (fruity, 272.668), ethyl caprate (fruity, 109.383). Moreover, phenethyl butyrate (honey) had the highest OAV (7210.8) in NFCV, followed by hexanoic acid (fruity, 2166.5), 9-Hexadecenoic acid ethyl ester (fruity, 452.925), ethyl caprate (fruity, 140.009). However, phenethyl butyrate was not detected in UFCV since the high temperature and pressure caused by ultrasound could promote Maillard reaction [41]. In UFCV, hexanoic acid (fruity, 3655.950) had the highest OAV, followed by 9-Hexadecenoic acid ethyl ester (fruity, 552.679), ethyl caprate (fruity, 137.176). Therefore, 9-Hexadecenoic acid ethyl ester and ethyl caprate were important aroma compounds in crabapple vinegars and hexanoic acid was essential aroma compound in aging crabapple vinegars. Above all, it could be known that fruity aromas were the major aroma quality of crabapple vinegars. Actually, the characteristic aroma in vinegar was created by the interaction among various volatile components, such as the redox, esterification, Maillard reaction and others [8,26,42]. The alcohols with higher OAV values in crabapple vinegar were 2-Ethyl-1-hexanol with citrus and rose fragrances, phenethyl alcohol with woody and gardenia fragrance, and Hexyl alcohol with pineapple sweet fragrance. Acids, generally had the flavor of cheese and fat, were the substances that played a negligible role in overall aroma of crabapple vinegar. Tetradecanoic acid was a new compound in UFCV. Additionally, other substances (like 2,4-di-tert-butyphenol and 3-hydroxy-2-butanone) had the smallest relative content but had strong aroma characteristics, which constituted the special flavor of crabapple vinegar.

A radar chart of aroma active components in crabapple vinegars was drawn (Fig. 3f & Fig. 3g) according to fragrance active ingredients with OAV ≥ 1. The cumulative aroma activity intensity of ester compounds was much higher than acids and alcohols, most likely due to the esterification reaction, indicating that ester compounds play an extremely important role in the characteristic aroma of crabapple vinegar. Moreover, the cumulative aroma activity intensity of alcohol and acid substances in UFCV and NFCV were considerably enhanced compared to FCV (Fig. 3g). In short, the contribution of aroma active ingredients to crabapple vinegar was in the order of esters > alcohols > others > acids. The overall aroma intensity of crabapple vinegar had been improved after aging, suggesting that vinegar needed to be aged for a period of time, which would reduce the irritation of vinegar and make aroma stronger. Simultaneously, it was found that the overall aroma intensity
and the aroma change trend of crabapple vinegar with ultrasonic treatment were basically consistent with natural aging, indicating that ultrasound had a positive effect on accelerating the aging process of crabapple vinegar.

3.5. The mechanism of ultrasound on accelerating aging

Since ultrasound has a cavitation effect, it has a wide range of applications in food industry. Some studies had reported that the mechanism of ultrasonic accelerating aging on vinegar and wine was mainly due to ultrasound cavitation, which could be divided into chemical and physical aspects [25,27,43]. On the one hand, ultrasound produces a large amount of hydroxyl radicals in the solution, which accelerates the oxidation, esterification, and Maillard reactions [20,26]. On the other hand, ultrasound changes the degree of association of hydrogen bonds (mostly hydrogen bonds of water molecules) in the solution molecules, thereby increasing the number of molecules involved in association and reducing the number of irritating free molecules, making the vinegar more soft and mellow. As shown in Fig. 4, the absorbance of the solution increased with increasing of ultrasonic time and ultrasonic power. It implied that the more iodine ions in the solution were oxidized into iodine to produce free radicals, which meant that the cavitation effect of ultrasound increased with the increase of ultrasonic time and ultrasound power. However, when the ultrasonic power was 300 W and the ultrasonic time was 60 min, the ultrasonic effect was most obvious because the total ester content was the largest at this time. In addition, it could be seen that the ultrasound cavitation firstly appeared increase and then slowly with the increase of ultrasonic power and ultrasonic time, while the total ester content decreased sharply after reaching the maximum. To some extent, ultrasonic treatment could accelerate the aging of crabapple vinegar. However, the excessive ultrasonic effect went against the accumulation of esters in the vinegar and made the quality of vinegar poor.

Ultrasound could produce cavitation bubbles in liquid system [17]. The large amount of energy released during the cracking of these small bubbles was able to change properties of substances in the system, such as the cracking of water molecules to generate free radicals [12,26,29,30]. It was shown that the spectrums of methylene blue solution under different ultrasonic power and ultrasonic time were exhibited in Fig. 5. It was found that the methylene blue solution had a maximum absorption value at 665 nm from Fig. 5. With the increase of ultrasonic power and the extension of ultrasonic time, the absorbance of methylene blue solution at 665 nm gradually decreased, indicating that hydroxyl radicals were produced. The solution created the most hydroxyl radicals when the ultrasonic power was 300 W. The increase in ultrasonic power caused an increase in sound amplitude, which promoted the enhancement of cavitation effect and ultimately led to an increase in the number of hydroxyl radicals [7]. Hydroxyl radicals played important roles in a series of oxidation reactions such as sugars, esters and amino acids, such as transferring electrons and depriving oxygen atoms [20,44].

As shown in Fig. 5, the total acid content of FCV without sodium benzoate (SB) was reduced by 17.64% after ultrasonic aging, and the total ester increased by 19.61%, indicating that the hydroxyl radicals generated by the ultrasonic process promoted the esterification reaction. Compared with FCV without SB, the content of total acid and total ester in UVCV with SB did not change significantly. It implied that the ultrasonic process generated free radicals and processes of some chemical reactions were relatively slow after the elimination of free radicals. It had reported that the mechanism of ultrasonic aging was that the cavitation effect produced by ultrasound promoted the formation, breaking and reorganization of molecular bonds of various substances in wine, accelerating various chemical reactions in wine to achieve the purpose of aging [20,26]. Above all, it was further shown that hydroxyl free radicals produced by ultrasound could positively promote chemical reactions in the aging process of crabapple vinegar.

3.6. Degree of hydrogen bond association

Most of flavoring substances in crabapple vinegar were polar molecules with hydroxyl groups, such as ethanol, acetic acid, ethyl acetate, etc., which easily formed unique hydrogen bonds with water. The vibrators spectra were analogue due to the hydrogen bond in these samples. It could be seen that three curves all have an obvious peak at the wavelength from 3000 cm$^{-1}$ to 4000 cm$^{-1}$ (Fig. 6), which was the absorption peak of hydroxyl stretching vibration. The water molecules in the solution easily associated with other polar molecules through hydrogen bonds to form molecular clusters. The larger the molecular cluster, the wider the peak width of hydroxyl in the spectrum. Conversely, when the associative balance in the solution was disrupted or there were more free molecules, the peak width of hydroxyl peak became smaller [49]. To some extent, it could suggest the strength of the hydrogen bond association in the solution. In Fig. 6, the hydroxyl peak of FCV was located at 3415.33 cm$^{-1}$, while that of UVCV and NFCV were at 3380.69 cm$^{-1}$ and 3373.94 cm$^{-1}$, respectively. It was found that hydroxyl peaks of crabapple vinegars with aging treatments (ultrasound and natural aging) all moved to low frequency region and the width of peaks increased slightly. Vinegar with ultrasound treatment could form an instantaneous high-temperature and high-pressure environment and then provide high energy for molecules, shorten the distance between molecules, improving the taste of vinegar. In other words, it was proved that ultrasound could accelerate the aging of crabapple vinegar and make its taste softer.

4. Conclusion

The mixed bacteria fermentation could increase the content of total acid and total ester in crabapple vinegar and make its flavor richer. Simultaneously, the ultrasonic treatment was confirmed to be a feasible method for the aging process of crabapple vinegar. It could shorten the time of aging and improve the quality of vinegar. A total of 22 aroma active ingredients that contributed to crabapple vinegar were identified by OAV value method and the order was esters > alcohols > others > acids. Moreover, the cavitation produced by ultrasound in the vinegar caused the system to produce a large amount of hydroxyl free radicals, which could induce certain chemical reactions and accelerate reaction rates (such as esterification, oxidation of alcohols or Maillard reactions). Therefore, it would be a helpful way to use ultrasonic aging technology as a hopeful processing for vinegar aging.

CRediT authorship contribution statement

Xinyu Zhai: Conceptualization, Investigation, Writing - original draft. Xu Wang: Conceptualization, Writing - original draft. Xiaoyi Wang: Resources, Validation, Formal analysis. Haoran Zhang: Resources, Validation, Formal analysis. Yucheng Ji: Resources, Formal analysis. Difeng Ren: Writing - review & editing. Jun Lu: Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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