

Effects of acidified apple juice before fermentation on ethyl carbamate and volatile components of apple distillate

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Abstract

In order to eliminate ethyl carbamate (EC) content in apple distillate, Fuji apple juice was acidified to pH 3.0 by sulfuric acid (ST), malic acid (MT), lactic acid (LT), or citric acid (CT). The acidified juice was inoculated with yeast, fermented at room temperature, and distilled by double distillation. Acid treatment by ST (3.23 µg/L), MT (3.20 µg/L), LT (2.93 µg/L), and CT (3.57 µg/L) significantly eliminated EC from apple distillate. Combined with the EC content and sensory evaluation, it was suggested that the high-quality apple distillate could be obtained with lower EC if apple juice was treated with ST or MT before fermentation.

Keywords: apple distillate, ethyl carbamate, sulfuric acid, malic acid, lactic acid, citric acid

Introduction

Apple is one of the main fruits in China. Its annual output is on the top in the world. However, some low-quality apples are not able to meet the market demand of fresh sales, resulting in a serious waste of resources. Consequently, the preparation of apple distillate not only reduces apple waste but also increases new apple products and greatly improves the economic and social benefits. However, some harmful components, such as ethyl carbamate (EC) and cyanide, are formed during alcoholic fermentation and distillation. EC, a potentially genotoxic and carcinogenic substance (Tu *et al.*, 2018), has been recognized as a group 2A carcinogen by the World Health Organization's (WHO) International Agency for Research on Cancer. In France, the maximum allowable level of EC is set as 150 µg/L for distilled spirits. The upper limit for EC in Canada is 400 µg/L in fruit spirits, 30 µg/L in wines, and 150 µg/L in wine spirits, brandies, and whiskies (Jia *et al.*, 2022). Some studies have shown

high levels of EC in distillates, for example, plum brandy contains up to 4750 µg/L of EC in tail during distillation (Balcerek *et al.*, 2017), and the EC content in some sugarcane spirits is between 42 µg/L and 5589 µg/L (Alcarde *et al.*, 2011). It is essential to reduce the content of EC in apple distillate; however, no research has been conducted on the content of EC in apple distillate.

Ethyl carbamate is found in many fermented food products and alcoholic beverages, such as wine, sake (rice beer), whisky, brandy, etc. (European Food Safety Authority, 2007). EC has the effects of oral toxicity, immunosuppression, and heart rate inhibition (Jiao *et al.*, 2014). Thus, the presence of EC in apple distillate is objectionable, and the EC content is expected to be as low as possible.

In the process of making wine, five pathways of EC derived from urea, citrulline, cyanide, and 3a,6a-dimethylglycoluril react with ethanol (Wang *et al.*, 2014b;

Zimmerli and Schlatter, 1991). Concerning distillation, urea is decomposed at a high temperature to cyanic acid, which reacts with ethanol to form EC (Schaber *et al.*, 2004; Taki *et al.*, 1992). Many studies have shown that EC production can be affected by controlling pH in wine (Uthurry *et al.*, 2006). Lower pH affects citrulline metabolism (Arena and Nadra, 2005) and thus reduces EC production during fermentation. Meanwhile, amygdalin in apple is hydrolyzed to hydrocyanic acid by β -glucosidase, which is then oxidized to isocyanic acid, and isocyanic acid reacts with ethanol to form EC, and low pH inhibits β -glucosidase activity. As far as we know, no study has focused on reducing EC content in fruit distillates by adjusting juice's pH to 3.0 prior to fermentation.

Sulfuric acid (ST), an inorganic acid is widely used in the preparation of ethanol (Sun *et al.*, 2011). It is a listed food additive, as a flocculant agent, which can be used in fermentation processes in China (GB/T 2760) (National Health and Family Planning Committee of China, 2014). Malic acid (MT), lactic acid (LT), and citric acid (CT), as acidity regulators (GB/T 2760) (National Health and Family Planning Committee of China, 2014), are widely used in the food industry (Marques *et al.*, 2020; Won *et al.*, 2015). Hence, it is meaningful to apply ST, MT, LT, and CT to adjust the pH of apple juice before fermentation.

The objective of this study was to investigate the effect of ST, MT, LT, and CT on cyanide and EC in apple distillate. Apples were washed and juiced. Then ST, MT, LT, or CT was added to apple juice to adjust its pH to 3.0. The treated juice was fermented at room temperature to get fermented juice. The juice was distilled by a double-distillation method to obtain apple distillate. EC, cyanide, and volatile components present in the distillate were investigated.

Materials and methods

Materials

Fuji apple (*Malus domestica* Borkh. cv. "Red Fuji") fruits were purchased from a local fruit market (Tai'an, China). Commercial wine yeast Lalvin CY 3079 was purchased from Shanghai Jatou Industry and Commerce Co. Ltd. (Shanghai, China). ST was purchased from Laiyang Kant Chemical Co. Ltd. (Laiyang, China). DL-malic acid (Anhui Xuelang Biotechnology Co. Ltd, China) was ordered from a local food additives store. CT was purchased from the local food additive store, and LT was purchased from Henan Jindan Lactic Acid Technology Co. Ltd. (Henan China). Volatile standards (chromatographic grade) were obtained from China National Research Institute of Food and Fermentation Industries

(Beijing, China). All the reagents used were of analytical grade.

Apple distillate making

Apple juice preparation

Well-matured apple fruits were selected, washed, crushed, and squeezed to obtain apple juice. The resulting juice was collected, divided into five samples (control, ST, MT, LT, and CT). Each juice sample (35 L) was transferred into sterile fermenters (40 L) and treated as follows. Nothing was added to control. Other four juices were prepared by adding 10% ST, MT, LT, and CT to adjust their pH to 3.0. All the above-mentioned processes were carried out in triplicate (Figure 1).

Fermentation

Wine yeast CY 3079, 0.20 g/kg of juice, activated by apple juice was added into each of the above-prepared apple juices. Subsequently, the inoculated juice samples were fermented at room temperature. The fermented apple juice was obtained after the completion of fermentation, that is, the residual sugar in the juice did not decrease over 3 consecutive days. The fermented juice was sampled daily for further analysis.

Distillation

The fermented apple juice was distilled by a double-distillation method. The first distillation was carried out in a 35-L Dibosk distiller comprising a stainless steel pot and condensing unit. The pot was heated on an induction stove. The first distillate was obtained with 30% (v/v) alcohol.

The second distillation was carried out in a 5-L glass conical flask heated on an electric furnace (Satora and Tuszynski, 2010). During the second distillation, the

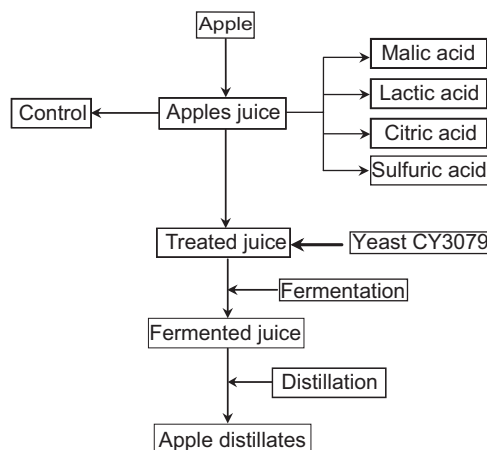


Figure 1. Process of making apple distillates.

following three fractions were collected: the head (1% ethanol), the heart (83% ethanol), and the tail (16% ethanol). A final alcoholic concentration of 68%–72% (v/v) was reached in the second distillate (SD). In order to avoid loss of volatiles, all samples were sealed and kept at 4°C until analysis. Before analysis, the heart was standardized and the alcohol was diluted to about 40% (v/v). All analyses were performed on 40% (v/v) samples.

Physicochemical analysis

Physicochemical analysis was carried out according to the National Standards of the People's Republic of China, GB/T 15038-2006. Alcohol content (% by volume) and dry extract (g/L) were measured based on the Pycnometer method. Titrable acidity (g/L, tartaric acid equivalent) was determined by potentiometric titration. pH values were directly measured using a laboratory recording pH meter (FE20, Mettler Toledo, Zurich, Switzerland). Sugar content was titrated with Fehling's reagent.

Determination of ethyl carbamate

Ethyl carbamate was analyzed by gas chromatography–mass spectrometry (GC-MS) method according to our previous study (Han *et al.*, 2021) with some modifications. Apple distillate samples, 2 mL, were mixed with 100 µL of EC-D5 (2 µg/mL in methanol solution), followed by addition of 0.30-g sodium chloride (NaCl). After ultrasonic dissolution for 10 min, the mixture was directly applied to solid phase extraction (SPE) cartridge (SBEQ-CA3999, CNW technology, Germany), and allowed to remain for 10 min for adequate absorption. The column was then washed with 10 mL n-hexane. Next, the analytes were extracted using 10-mL 5% ethyl acetate and diethyl ether solution. The eluate was mixed in a test tube and reduced to approximately 0.5 mL by a gentle stream of nitrogen. Subsequently, the residual eluents were adjusted to 1 mL with methanol and directly injected into a GC-MS system (GCMS-TQ8030, Shimadzu, Tokyo, Japan).

Substances were separated on a fused-silica capillary column (VF-WAS, 60 m × 0.25 mm × 0.5 µm; Agilent, USA). Helium was used as a carrier gas at a constant flow rate of 1 mL/min. The injector port was kept at 220°C in splitless mode. The starting temperature was held at 50°C for 1 min, then increased to 180°C at a rate of 8°C/min and held for 5 min. Finally, the temperature was increased to 240°C at a rate of 20°C/min and held for 5 min. The MS detector port and ion source temperature were set at 250°C and 230°C, respectively. GC-MS experiments were based on in-source collision-induced dissociation (CID)

occurring in the collision cell of triple quadrupoles, with an argon collision gas pressure of approximately 2.0 m Torr and an offset voltage of 20 eV. For quantitative analysis, the chosen fragments were monitored in multiple reaction monitoring (MRM) modes: 74, 44, 62, and 89 m/z for EC, and 64 and 76 m/z for EC-D5. Then, selective ion monitoring (SIM) of 62 m/z (EC) and 64 m/z (EC-D5) was used for the purpose of quantification. For quantification, peak area ratios of EC to EC-D5 were calculated as a function of the concentration of substances.

Determination of cyanide

The determination of cyanide is according to the Chinese national standard (National Health and Family Planning Committee of China, 2016; GB/T 5009.36). In a 50-mL beaker, 1 mL sample was taken; 5-mL of 2 g/L sodium hydroxide (NaOH) solution was added to the sample taken in beaker and allowed to remain for 10 min. Then the beaker was heated on electric heating plate at 120°C till the solution was reduced to about 1 mL. It was transferred into 10-mL stopper colorimetric tube and the volume was adjusted to 5 mL by adding 2 g/L NaOH solution.

Two drops of phenolphthalein indicator were added to the sample and the standard tube separately. Acetic acid was added to make the red color of the solution to fade; 2 g/L NaOH solution was used to adjust the color to near red. Phosphate buffer solution, 2 mL, and chloramine T solution, 0.2 mL, were added in turn with continuous shaking for 3 min. Then, 2 mL of isonicotinic-pyrazolone solution was added to the sample and diluted to 10 mL with water. Incubation was performed for 40 min in a water bath at 37°C constant temperature. Following incubation, the sample was taken out and the zero point was adjusted with a 1-cm colorimetric cup with a blank tube to measure absorbance at 638 nm. After the absorbance of 0-, 0.4-, 0.8-, 1.2-, 1.6-, and 2.0-mL cyanide standard intermediate solution into 10-mL stopper colorimetric tube, colorimetry was conducted according to sample determination to draw a standard curve. The cyanide content of the sample was measured by comparing to the standard curve of cyanide ion standard intermediate.

Determination of methanol

Methanol content was determined by gas chromatography with internal standard added according to the official reference method of Association of Official Analytical Chemists (AOAC, 1994; 940.06). The 100-mL sample was added to 50-mL deionized water and distilled to 100 mL. Internal standard (*tert*-amyl alcohol of 162 mg/L), 1 mL, was added to 10 mL of distillate; 1.0 µL of

sample was directly injected into gas chromatography system equipped with a capillary column PEG-20 M (30 m × 0.5 mm × 0.25 µm; Dalian Zhonghuida Scientific Instrument, China) and a flame ionization detector. The temperature of injector and detector was 220°C. The oven temperature procedure was as follows: the initial temperature was maintained at 40°C for 4 min; it was then increased to 200°C at a rate of 3.5°C/min and held at 200°C for 10 min. The carrier gas was nitrogen with a flow rate of 1.0 mL/min. The split ratio was 50:1. Methanol quantification was determined by external standard method, but internal standard method was also used to improve the accuracy of results.

Volatile compounds analysis

The volatile compounds were analyzed by gas chromatography (López-Vázquez *et al.*, 2010) with some modifications. Internal standard (10 µL), including tert-amyl alcohol and n-butyl acetate 162 mg/L, as well as 2-ethyl butyrate 186.6 mg/L, was added into 1 mL of sample. Then, 1 µL of sample was directly injected into a Shimadzu 2010 chromatograph system with flame ionization detector. A capillary column CP-WAX 57CB (50 m × 0.25 mm × 0.2 µm; Agilent, USA) was used for this analysis. The temperatures of detector and injector were 260°C and 240°C, respectively. The oven temperature program was maintained at 35°C for 4 min, increased to 60°C at a rate of 2°C/min, continued to rise to 130°C at a rate of 10°C/min, and finally increased to and maintained at 205°C at a rate of 15°C/min. The carrier gas was nitrogen with a flow rate of 1.35 mL/min, and the split ratio was 40:1. The qualitative analyses of volatile compounds were based on the comparison of retention time read from the chromatograms of both samples and standards. The quantitative analysis was performed according to the internal standard method. All detected carbonyl compounds, esters, higher alcohols, and acids were analyzed quantitatively.

Sensory analysis

The panel included three female and eight male analysts, with experience in the evaluation of fruit distillates and were trained to describe and recognize the evaluated odor qualities. The panelists were trained according to the ISO 8586 standard (International Organization for Standardization 2012). Prior to and during this study, monthly training was conducted to evaluate multiple flavor standards (acetoin, isoamyl acetate, ethyl acetate, ethyl lactate, acetaldehyde, 1-propanol, 1-hexanol, acetic acid, and head and tail distillation fractions) and different spirits distilled from cider, hawthorn wine, and persimmon wine. The sensory analysis of the samples

was performed according to the Chinese national standard (General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China. 2008; GB/T 11856) with some modifications. The evaluators were provided with 45-mL apple distillate in standard glass cups, coded with random numbers. Apple distillates were sniffed and tasted. Three aspects, including olfactory, gustatory, and typicality, were used to measure the quality of apple distillates. The descriptors of olfactory were fruity and vinous; for gustatory evaluations, considered descriptors were alcoholic and balance. During each session, expert judges first assessed the smell, and then they evaluated gustatory attributes after a short break. According to the characteristics of the samples, the experts scored each descriptor with the highest score of 20 points. After descriptive analysis, the panelists assessed all samples for typicality according to their olfactory and gustatory tests and rated them with a maximum score of 20 points. Finally, the experts wrote their comments on the samples after all the scores were completed. The total score of the samples were the sum of all the scores. All samples were diluted with distilled water to an alcohol concentration of 40% (v/v). Mouthwash was used by tasters between analyses of two samples.

Statistical analysis

All the data were processed using SPSS Statistics 22. All pictures were drawn using Origin 2022. In Figure 3, red indicates positive correlation and blue shows negative correlation; the deeper the color, the greater the absolute value of correlation and stronger the correlation between them. Mean differences at $p < 0.05$ were considered as significant using Tukey's test. All data were the average values of three replicates, analyzed in triplicate for each condition, and presented as mean values and standard deviations.

Results and discussion

Changes of titrable acidity and pH during fermentation

All apple juice samples were fermented for 7 days, and the total sugar content was reduced to less than 1.4 g/L. In order to explore the influence of different acid treatments on fermented apple juice, titrable acidity and pH were tracked during fermentation. The pH of control was increased to 4.25 on the second day of fermentation and then decreased to 4.06 (Figure 2A). On the contrary, the titrable acidity content decreased on the second day of fermentation and then increased (Figure 2B). However, the pH of apple juice samples treated with different organic acids increased, but the corresponding titrable acidity content decreased during fermentation, which

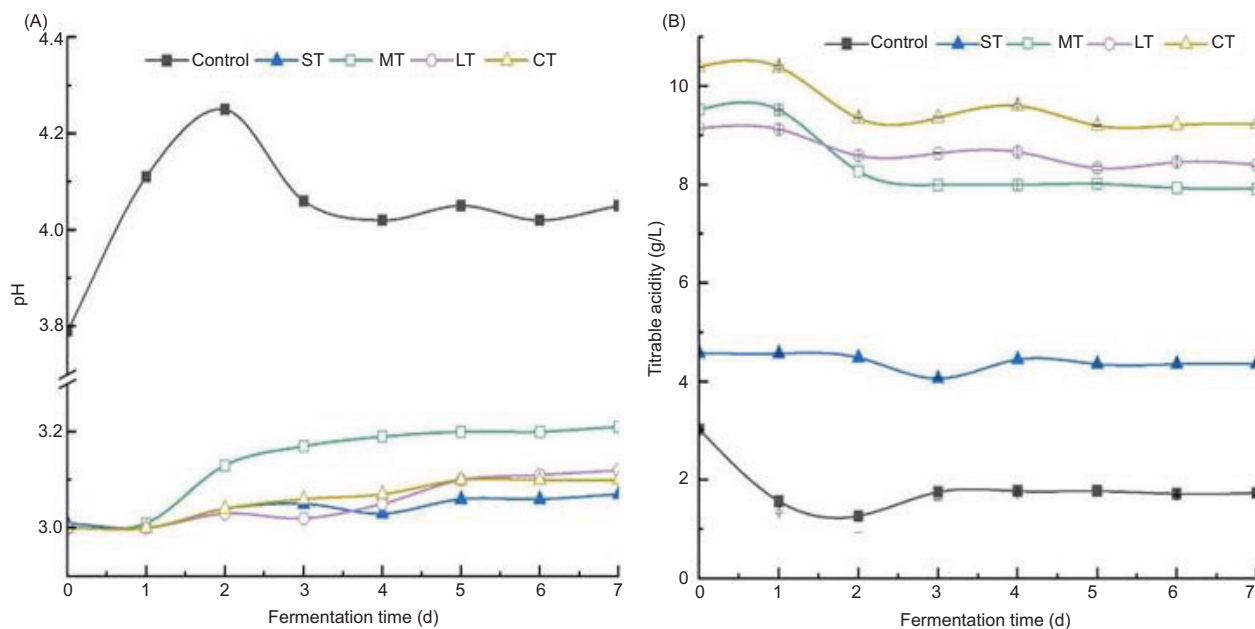


Figure 2. Changes in pH (A) and titrable acidity (B) during fermentation. Control: original apple juice; ST: sulfuric acid-treated juice; MT: malic acid-treated juice; LT: lactic acid-treated juice; CT: citric acid-treated juice.

might be caused by the degradation of organic acids by yeast and LT bacteria (Lerena *et al.*, 2016; Zhong *et al.*, 2020). In all organic acid-treated samples, maximum change in titrable acidity was observed in MT sample, which decreased from 9.53 g/L to 7.92 g/L during the fermentation. The titrable acidity of LT- and CT-treated samples was reduced by 0.74 g/L and 1.16 g/L, respectively. Conversely, the pH of MT increased from 3.00 to 3.21. In summary, the titrable acidity of MT-, LT-, and CT-treated samples showed an overall decreasing trend during the fermentation process, while the titrable acidity of the control and ST-treated sample showed an insignificant decreasing trend.

Physicochemical indices of fermented apple juices

The basic physical and chemical indexes of fermented apple juices are shown in Table 1. All the fermented apple juice samples achieved complete fermentation, with the total sugar content ranging from 0.9 to 1.3 g/L and the alcohol content from 7.66 to 7.73% (v/v). This proposed that different treatments of apple juice samples before fermentation did not affect the fermentation capacity of yeast. The methanol content of all fermented apple juice samples was between 5.28 mg/L and 6.42 mg/L. The methanol content of ST, MT, LT, and CT was significantly lower than the control ($p < 0.05$), indicating that the methanol content could be significantly reduced by different acid treatments. In addition, EC was not detected in all the fermented apple juices.

Volatile component of fermented apple juice samples

In the fermented apple juice samples, 20 volatile compounds, such as carbonyl compounds, esters, higher alcohols, and acids, were detected by gas chromatography (Table 2). Three carbonyl compounds were identified in all samples. These could be produced by oxidation of alcohols or decarboxylation of acids (Xiao *et al.*, 2015). Acetaldehyde contributed to the flavors of fermented apple juice samples with fruity, nut, and dried fruits aroma. However, acetaldehyde could impart pungent aroma if higher quantity is added. It was determined in all fermented apple juices; however, the maximum amount was determined in CT (123.26 mg/L). The lowest acetaldehyde content was observed in ST, which was 26.69 mg/L. Acetoin was the only ketone detected and it provided a buttery and cream aroma to the fermented apple juice samples (Welke *et al.*, 2014). In this study, the content of acetoin in ST, MT, LT, and CT were significantly reduced, compared to the control ($p < 0.05$). Acetoin reached the lowest level of 1.61 mg/L in ST. Although the amount of acetoin in fermented apple juices varies significantly, it is difficult for them to have a significant impact on the flavor of fermented apple juice samples because of its high sensory threshold.

Esters are one of the most important volatile constituents of fermented apple juices, and were formed by the reaction of alcohol and free organic acids during fermentation (Villière *et al.*, 2015). In this study, the contents of ethyl acetate and isoamyl acetate were above the threshold,

Table 1. Physicochemical indices of fermented apple juice samples.

	Control	ST	MT	LT	CT
Alcohol (% v/v)	7.71 ± 0.04 ^a	7.66 ± 0.06 ^a	7.70 ± 0.03 ^a	7.73 ± 0.04 ^a	7.66 ± 0.01 ^a
Total sugar (g/L)	1.3 ± 0.1 ^a	0.9 ± 0.0 ^d	0.9 ± 0.0 ^d	1.2 ± 0.0 ^b	1.1 ± 0.0 ^c
Titrate acidity (g/L)	2.2 ± 0.0 ^e	4.3 ± 0.0 ^d	7.9 ± 0.0 ^c	8.3 ± 0.0 ^b	9.5 ± 0.0 ^a
pH	4.03 ± 0.01 ^a	3.08 ± 0.00 ^e	3.24 ± 0.00 ^b	3.17 ± 0.00 ^c	3.15 ± 0.00 ^d
Dry extract (g/L)	16.7 ± 0.0 ^d	19.9 ± 0.2 ^c	25.4 ± 0.0 ^a	24.9 ± 0.0 ^b	24.7 ± 0.0 ^b
Methanol (mg/L)	6.42 ± 0.08 ^a	5.41 ± 0.00 ^b	5.28 ± 0.14 ^b	5.48 ± 0.69 ^b	5.37 ± 0.18 ^b
Ethyl carbamate (µg/L)	–	–	–	–	–

All values are expressed as mean values ± standard deviations (n = 3); different superscripted lowercase letters in the same row indicate significant difference (p < 0.05).

Control: fermented apple juice from original apple juice; ST: fermented apple juice from sulfuric acid-treated juice; MT: fermented apple juice from malic acid-treated juice; LT: fermented apple juice from lactic acid-treated juice; CT: fermented apple juice from citric acid-treated juice. – indicates not detected.

Table 2. Volatile component in fermented apple juice samples (mg/L).

Compound	Threshold	Control	ST	MT	LT	CT
Carbonyl compounds						
Acetaldehyde	0.5 ⁽¹⁾	48.76 ± 5.72 ^b	26.69 ± 0.25 ^c	46.48 ± 7.08 ^b	52.56 ± 4.87 ^b	123.26 ± 6.23 ^a
Acetal	0.05 ⁽¹⁾	ND	ND	0.85 ± 0.14 ^b	0.89 ± 0.09 ^b	1.49 ± 0.07 ^a
Acetoin	150 ⁽²⁾	31.85 ± 0.98 ^a	1.61 ± 0.11 ^c	12.72 ± 2.71 ^b	12.21 ± 0.22 ^b	14.08 ± 0.68 ^b
Esters						
Ethyl acetate	7.5 ⁽¹⁾	77.03 ± 4.08 ^a	22.41 ± 2.32 ^b	79.50 ± 3.49 ^a	76.95 ± 0.73 ^a	15.95 ± 0.05 ^c
Isoamyl acetate	0.03 ⁽³⁾	2.36 ± 0.17 ^a	1.04 ± 0.04 ^c	1.62 ± 0.07 ^b	1.43 ± 0.02 ^b	0.49 ± 0.13 ^d
Ethyl lactate	150 ⁽²⁾	7.18 ± 0.07 ^c	4.13 ± 0.51 ^c	31.77 ± 7.65 ^b	73.39 ± 2.42 ^a	13.19 ± 0.46 ^c
Ethyl decanoate	0.5 ⁽⁴⁾	0.07 ± 0.00 ^a	0.04 ± 0.01 ^b	0.07 ± 0.02 ^a	0.05 ± 0.00 ^{a,b}	0.04 ± 0.00 ^b
Higher alcohols						
1-Propanol	50 ⁽³⁾	62.78 ± 0.97 ^a	19.87 ± 0.16 ^e	39.75 ± 0.34 ^c	41.58 ± 0.52 ^b	38.16 ± 0.21 ^d
2-Methyl-1-propanol	40 ⁽¹⁾	16.5 ± 0.06 ^d	21.69 ± 0.20 ^b	18.43 ± 0.11 ^c	22.87 ± 0.09 ^a	18.41 ± 0.40 ^c
1-Butanol	150 ⁽³⁾	2.02 ± 0.04 ^a	1.96 ± 0.09 ^a	2.16 ± 0.17 ^a	1.99 ± 0.06 ^a	2.05 ± 0.02 ^a
2-Methyl-1-butanol	0.32 ⁽⁴⁾	14.85 ± 0.12 ^c	17.51 ± 0.19 ^a	13.39 ± 0.42 ^d	17.54 ± 0.02 ^a	16.24 ± 0.04 ^b
3-Methyl-1-butanol	30 ⁽⁴⁾	65.38 ± 0.39 ^e	78.98 ± 1.07 ^a	68.36 ± 0.88 ^d	75.33 ± 0.32 ^b	71.68 ± 0.79 ^c
1-Hexanol	8 ⁽¹⁾	2.39 ± 0.02 ^a	2.16 ± 0.02 ^a	2.1 ± 0.03 ^a	2.55 ± 0.53 ^a	1.95 ± 0.01 ^a
2,3-Butanediol	–	4.59 ± 0.00 ^c	4.18 ± 0.02 ^d	5.67 ± 0.08 ^b	6.30 ± 0.05 ^a	2.94 ± 0.12 ^e
2-Phenylethanol	10 ⁽¹⁾	0.53 ± 0.14 ^b	0.8 ± 0.07 ^b	0.82 ± 0.09 ^b	0.76 ± 0.20 ^b	1.99 ± 0.08 ^a
Acids						
Acetic acid	200 ⁽¹⁾	244.87 ± 4.79 ^a	108.3 ± 1.33 ^c	254.22 ± 7.12 ^a	230.22 ± 7.29 ^b	46.68 ± 0.44 ^d
Propanoic acid	8.1 ⁽³⁾	2.26 ± 0.30 ^a	1.33 ± 0.15 ^b	2.57 ± 0.35 ^a	2.81 ± 0.50 ^a	1.99 ± 0.53 ^{a,b}
Butyric acid	10 ⁽¹⁾	1.31 ± 0.23 ^b	1.97 ± 0.02 ^a	1.68 ± 0.06 ^a	1.92 ± 0.08 ^a	1.87 ± 0.11 ^a
Hexanoic acid	3 ⁽¹⁾	5.73 ± 0.4 ^a	4.47 ± 0.06 ^c	4.56 ± 0.12 ^c	4.82 ± 0.10 ^{b,c}	5.36 ± 0.45 ^{a,b}
Octanoic acid	0.2 ⁽³⁾	14.92 ± 2.08 ^{a,b}	10.89 ± 0.68 ^c	13.34 ± 0.18 ^{a,b,c}	12.06 ± 1.22 ^{b,c}	15.18 ± 0.60 ^a

All values are expressed as mean values ± standard deviations (n = 3); different superscripted lowercase letters in the same row indicate significant difference (p < 0.05).

Control: fermented apple juice from original apple juice; ST: fermented apple juice from sulfuric acid-treated juice; MT: fermented apple juice from malic acid-treated juice; LT: fermented apple juice from lactic acid-treated juice; CT: fermented apple juice from citric acid-treated juice; ND: not detected (Guth, 1997;⁽¹⁾ Peinado *et al.*, 2004;⁽²⁾ Wang *et al.*, 2017;⁽³⁾ Wei *et al.*, 2020⁽⁴⁾).

and had a great influence on the aroma of apple juices. However, compared to the control, the content of ethyl acetate in ST and CT was significantly reduced to 54.62 mg/L and 61.08 mg/L, respectively, whereas the content of isoamyl acetate in other treatment samples was significantly decreased ($p < 0.05$). Significantly, although the content of ethyl lactate did not exceed the threshold, the MT- and LT-treated apple juice before fermentation could obviously increase the content of ethyl lactate in fermented apple juice samples. This result might be caused by the addition of MT and LT before fermentation, which increased LT content during fermentation, thus increasing the content of ethyl lactate.

Higher contents of alcohols in cider was synthesized by yeast through the glucose synthesis pathway or the corresponding amino acid catabolism pathway, which was characterized by strong and pungent smell and had an important role in the aroma of fermented apple juices (Qin *et al.*, 2018). Eight higher alcohols were detected in all treatment samples, of which 3-methyl-1-butanol was the most predominant alcohol in all fermented apple juices and its concentration was in the range of 65.38–78.98 mg/L. Furthermore, both 3-methyl-1-butanol and 2-methyl-1-butanol were above the threshold in all fermented apple juices and provided the smell of alcohol, nail polish, and whiskey to fermented samples (Arcari *et al.*, 2017). Compared to the control, the contents of both 3-methyl-1-butanol and 2-methyl-1-butanol in ST, LT, and CT were significantly increased ($p < 0.05$). As for 1-propanol, only the control exceeded the threshold of 1-propanol content, which was 62.78 mg/L, indicating that different acid treatments could significantly affect the production of 1-propanol.

Five different volatile fatty acids were identified across fermented apple juices. Among these, acetic acid was the predominant volatile acid in all fermented apple juices. However, the content of acetic acid in ST and CT was significantly lower than that in the control ($p < 0.05$) and did not exceed the threshold, especially its content was only 46.68 mg/L in CT. Furthermore, the contents of hexanoic acid and octanoic acid exceeded the threshold in all

fermented apple juices. They were described with cheese, rancid, and fatty notes, and were important for the balance of complexity and fruity aromas of fermented apple juices (Sun *et al.*, 2013).

Physicochemical indices of apple distillates

Cyanide and EC were not detected in the fermented apple juice or first distillates. As shown in Table 3, the highest concentration of cyanide was detected in the control (0.035 mg/L). The cyanide content of other treatment samples was significantly lower than that of the control. In this study, the content of EC in different treatments was in the range of 9.12–2.93 $\mu\text{g/L}$. Compared to the control, the content of EC in ST, MT, LT, and CT was significantly reduced ($p < 0.05$). The EC content of the control was 9.12 $\mu\text{g/L}$, which was 2.12–3.11 times that of other treatment samples. LT had the best effect on reducing EC by 67.87% compared to the control. The EC reduction effects of ST, MT, and CT were 64.58%, 64.91%, and 60.86%, respectively. Generally, the concentration of cyanide and EC in apple distillates could be effectively reduced by treating apple juice with different acids before fermentation.

Volatile components of apple distillates

As shown in Table 4, 24 volatile compounds were detected in the apple distillates by gas chromatography, including carbonyl compounds, esters, alcohols, and acids. Among carbonyl compounds, a total of four aldehydes and one ketone were detected. Only the contents of acetaldehyde and acetal exceeded the threshold in all apple distillates. Acetaldehyde was the most important aldehyde; its concentration in different apple distillates ranged from 47.64 to 468.48 mg/L, and its content was significantly different ($p < 0.05$) in all distillates. The highest acetaldehyde content was detected in CT, and the lowest was observed in ST. In the control, the contents of acetaldehyde and acetal were 126.60 mg/L and 74.60 mg/L, respectively. It is worth mentioning that except for ST, the contents of

Table 3. Physicochemical indices of apple distillates.

	Control	ST	MT	LT	CT
Alcohol (% v/v)	40.2 \pm 0.0 ^a	40.2 \pm 0.0 ^a	40.2 \pm 0.0 ^a	40.1 \pm 0.1 ^a	40.2 \pm 0.0 ^a
Cyanide (mg/L anhydrous ethanol)	0.035 \pm 0.004 ^a	ND	0.015 \pm 0.000 ^c	0.010 \pm 0.000 ^c	0.028 \pm 0.004 ^b
EC ($\mu\text{g/L}$)	9.12 \pm 0.40 ^a	3.23 \pm 0.16 ^b	3.20 \pm 0.27 ^b	2.93 \pm 0.09 ^b	3.57 \pm 0.22 ^b

All values are expressed as mean values \pm standard deviations ($n = 3$); different superscripted lowercase letters in the same row indicate significant difference ($p < 0.05$).

Control: apple distillate from original apple juice; ST: apple distillate from sulfuric acid-treated juice; MT: apple distillate from malic acid-treated juice; LT: apple distillate from lactic acid-treated juice; CT: apple distillate from citric acid-treated juice; ND: not detected.

The concentrations of cyanide and EC were recalculated on the basis of 100% (v/v) ethanol.

Table 4. Volatile components of apple distillates (mg/L).

	Threshold	Control	ST	MT	LT	CT
Carbonyl compounds						
Acetaldehyde	19.2 ⁽¹⁾	126.60 ± 0.64 ^d	47.64 ± 0.18 ^e	191.39 ± 1.20 ^c	207.92 ± 2.42 ^b	468.48 ± 8.21 ^a
2-Methylpropanal	1.3 ⁽⁴⁾	0.52 ± 0.03 ^e	1.97 ± 0.04 ^b	1.82 ± 0.03 ^c	1.84 ± 0.03 ^c	2.07 ± 0.05 ^a
Acetal	0.719 ⁽¹⁾	74.60 ± 0.45 ^d	59.54 ± 0.46 ^e	92.55 ± 0.03 ^c	111.95 ± 0.60 ^b	148.33 ± 0.92 ^a
Acetoin	–	6.70 ± 0.21 ^b	0.45 ± 0.04 ^e	4.98 ± 0.31 ^c	16.03 ± 0.19 ^a	3.77 ± 0.13 ^d
Furfural	44 ⁽⁴⁾	1.77 ± 0.12 ^{b,c}	1.59 ± 0.06 ^c	2.21 ± 0.02 ^b	3.33 ± 0.39 ^a	1.91 ± 0.04 ^{b,c}
Esters						
Ethyl formate	–	1.46 ± 0.10 ^d	3.01 ± 0.00 ^c	3.78 ± 0.37 ^b	2.84 ± 0.12 ^c	5.31 ± 0.09 ^a
Ethyl acetate	32.6 ⁽³⁾	274.22 ± 3.08 ^a	101.63 ± 0.64 ^d	205.82 ± 1.17 ^c	219.37 ± 0.60 ^b	79.55 ± 1.07 ^e
Isoamyl acetate	0.245 ⁽³⁾	19.95 ± 2.29 ^b	17.59 ± 0.74 ^{b,c}	14.73 ± 0.39 ^{c,d}	13.09 ± 0.10 ^d	26.08 ± 1.56 ^a
Ethyl lactate	128 ⁽²⁾	8.21 ± 0.17 ^d	5.59 ± 0.16 ^e	21.65 ± 0.04 ^b	74.74 ± 0.30 ^a	18.76 ± 0.20 ^c
Ethyl octanoate	0.147 ⁽³⁾	1.45 ± 0.02 ^a	0.91 ± 0.03 ^c	0.80 ± 0.05 ^d	1.00 ± 0.01 ^b	0.88 ± 0.01 ^c
Ethyl decanoate	1.120 ⁽²⁾	0.02 ± 0.00 ^b	0.02 ± 0.00 ^a	0.01 ± 0.00 ^c	0.02 ± 0.00 ^b	0.01 ± 0.00 ^c
Alcohols						
1-Propanol	54 ⁽³⁾	394.49 ± 3.29 ^a	124.89 ± 0.22 ^e	252.3 ± 0.95 ^b	237.39 ± 0.76 ^c	228.41 ± 0.41 ^d
2-Methyl-1-propanol	28.3 ⁽³⁾	107.88 ± 0.76 ^d	152.56 ± 2.72 ^a	125.52 ± 0.07 ^c	144.24 ± 0.40 ^b	122.75 ± 0.32 ^c
1-Butanol	2.73 ⁽²⁾	12.60 ± 0.46 ^a	12.87 ± 0.29 ^a	12.94 ± 0.04 ^a	12.74 ± 0.08 ^a	13.18 ± 0.17 ^a
2-Methyl-1-butanol	45 ⁽¹⁾	126.30 ± 4.58 ^{a,b}	139.34 ± 1.78 ^a	115.09 ± 4.00 ^b	132.14 ± 7.11 ^a	123.45 ± 10.48 ^{a,b}
3-Methyl-1-butanol	179 ⁽³⁾	470.29 ± 1.84 ^c	614.23 ± 8.07 ^a	513.65 ± 4.54 ^b	524.47 ± 4.88 ^b	515.86 ± 0.81 ^b
1-Hexanol	8 ⁽³⁾	21.04 ± 0.56 ^a	20.77 ± 0.01 ^a	19.43 ± 0.13 ^b	20.73 ± 0.28 ^a	19.37 ± 0.17 ^b
2,3-Butanediol	–	1.47 ± 0.03 ^b	0.95 ± 0.18 ^c	1.35 ± 0.09 ^b	2.87 ± 0.00 ^a	0.24 ± 0.02 ^d
2-Phenylethanol	2.6 ⁽³⁾	1.74 ± 0.07 ^b	1.76 ± 0.02 ^b	1.45 ± 0.09 ^c	1.95 ± 0.04 ^a	1.14 ± 0.02 ^d
Methanol	–	25.54 ± 0.14 ^a	22.58 ± 0.52 ^c	23.86 ± 0.44 ^b	23.47 ± 0.28 ^{b,c}	22.45 ± 0.56 ^c
Acids						
Acetic acid	75.521 ⁽³⁾	4.60 ± 0.07 ^a	2.27 ± 0.60 ^c	1.11 ± 0.04 ^d	3.66 ± 0.40 ^b	ND
Butanoic acid	1.2 ⁽³⁾	1.85 ± 0.24 ^a	0.92 ± 0.14 ^{b,c}	1.21 ± 0.15 ^b	0.37 ± 0.03 ^d	0.68 ± 0.18 ^{c,d}
Hexanoic acid	2.52 ⁽³⁾	8.47 ± 0.35 ^a	5.80 ± 0.31 ^b	5.42 ± 0.25 ^b	5.66 ± 0.29 ^b	4.46 ± 0.33 ^c
Octanoic acid	2.7 ⁽³⁾	46.87 ± 0.77 ^a	17.96 ± 3.48 ^{c,d}	24.96 ± 0.28 ^b	21.1 ± 0.30 ^{b,c}	13.83 ± 2.53 ^d

All values are expressed as mean values ± standard deviations (n = 3); different superscripted lowercase letters in the same row indicate significant difference ($p < 0.05$).

Control: apple distillate from original apple juice; ST: apple distillate from sulfuric acid-treated juice; MT: apple distillate from malic acid-treated juice; LT: apple distillate from lactic acid-treated juice; CT: apple distillate from citric acid-treated juice; ND: not detected.

– indicates not found.

The concentrations of volatile compounds were recalculated on the basis of ethanol 40% (v/v) (Gao *et al.*, 2014;⁽²⁾ Wang *et al.*, 2014a;⁽⁴⁾ Willner *et al.*, 2013;⁽¹⁾ Xiang *et al.*, 2020⁽³⁾).

acetaldehyde and acetal were significantly increased in other samples ($p < 0.05$). Compared to fermented juices, 2-methylpropanal and furfural were newly observed natural products observed in apple distillates; these were formed due to the chemical reactions caused by high temperature during distillation (Awad *et al.*, 2017).

Six esters were also discovered in apple distillates (Table 4), among which the contents of ethyl acetate, isoamyl acetate, and ethyl octanoate were above the threshold in all samples. Ethyl acetate was the most important ester, and its presence was consistent with the results in other apple distillates (Ledauphin *et al.*, 2010; Versini

et al., 2009). The lowest content of 79.55 mg/L was discovered in CT. The content of ethyl acetate in ST, MT, and LT was 101.63, 205.82, and 219.37 mg/L, respectively. Its concentration in apple distillates with different treatments was significantly different ($p < 0.05$). The content of ethyl acetate in apple distillates was significantly reduced by acid treatments.

Alcohols were the most important volatiles observed in distillates, and nine compounds were discovered in this study. The contents of 1-propanol, 2-methyl-1-propanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 1-hexanol in all samples exceeded the threshold,

but 3-methyl-1-butanol had the highest concentration as observed previously for apple distillates (Ledauphin *et al.*, 2003). Compared to the control, content of 1-propanol in the acid-treated sample was decreased significantly ($p < 0.05$), and the lowest content found in ST was only 124.89 mg/L. However, the concentrations of 2-methyl-1-propanol and 3-methyl-1-butanol in different acid-treated samples were higher than that in the control ($p < 0.05$), and the highest contents of these two substances in ST-treated sample were 152.56 mg/L and 614.23 mg/L, respectively. However, the content of methanol in different treatment samples was decreased significantly ($p < 0.05$).

Four different volatile acids were identified in all samples. The content of acetic acid in apple distillates was significantly reduced compared to that in fermented apple juice; this could be due to the removal of tail during distillation. The contents of hexanoic acid and octanoic acid were above the threshold in all samples. In addition, the highest contents of hexanoic acid and octanoic acid in the control were 8.47 mg/L and 46.87 mg/L, respectively. Furthermore, the concentrations of these substances in other treatment samples were significantly decreased, and the lowest concentrations of hexanoic acid and octanoic acid were detected in CT sample, which were 4.46 mg/L and 13.83 mg/L, respectively.

Sensory evaluation of apple distillates

According to the sensory descriptors mentioned in Table 5, we observed that MT had the highest fruity score, which was also consistent with the expert's description of MT. ST had the lowest scores for fruity and typicality, but both vinous and alcoholic had high scores. The results indicated that although ST could affect the typicality of apple distillate, but ST would not have a seriously adverse effect on the quality of distillate. Concerning LT and CT, most of their scores of sensory descriptors were significantly lower than the control hence, LT and CT could have bad effects on the quality of distillate. In sensory evaluation, the control had the highest score of 92.27, but the CT score was lowest (85.73). The sensory evaluation scores from low to high were: LT (86.64), ST (88.64), and MT (91.09). The scores of control and MT were close to each other, which could be due to the close content values of esters and alcohols. ST had the highest 3-methyl-1-butanol content of 614.23 mg/L (Table 4), which could be responsible for its strong alcoholic taste. Although CT had the highest acetaldehyde content (468.48 mg/L), it had the lowest content of ethyl acetate (79.55 mg/L), which could be the reason for its flavor defect and bitter taste.

In summary, although the treatment of ST, MT, LT, and CT on apple juice before fermentation could reduce the EC

Table 5. Effects of different treatments on sensory scores of apple distillates.

Sample	Sensory descriptor				
	Olfactory		Gustatory		Typicality
	Fruity	Vinous	Alcoholic	Balance	
Control	17.91 ± 0.54 ^b	17.55 ± 0.52 ^a	18.09 ± 0.54 ^b	19.27 ± 0.47 ^a	19.45 ± 0.52 ^a
ST	17.09 ± 0.83 ^c	17.55 ± 0.69 ^a	19.18 ± 0.40 ^a	18.27 ± 0.47 ^b	16.55 ± 0.52 ^c
MT	18.82 ± 0.40 ^a	17.82 ± 0.60 ^a	16.91 ± 0.54 ^c	18.27 ± 0.47 ^b	19.27 ± 0.47 ^a
LT	17.73 ± 0.47 ^b	16.64 ± 0.50 ^b	16.27 ± 0.47 ^d	17.82 ± 0.60 ^c	18.18 ± 0.40 ^b
CT	16.91 ± 0.54 ^c	17.45 ± 0.52 ^a	16.73 ± 0.47 ^c	16.82 ± 0.40 ^d	17.82 ± 0.40 ^b
Sensory evaluation					Score
Control	The distillate had a pleasant aroma, a full-bodied palate, and no miscellaneous flavors, and had the typical characteristics of apple distillate.				92.27 ± 0.90 ^a
ST	The aroma of apple was weaker than that of the control and had a plastic odor. Strong alcoholic taste in the mouth. The overall coordination was good.				88.64 ± 1.12 ^c
MT	It had the best apple aroma, no odor, the mouth was clean and refreshing, but the overall coordination was lower than the control. It also had the typical characteristics of apple distillates.				91.09 ± 0.94 ^b
LT	It had a strong aroma of apple, but a weaker vinous. It also had a faint alcoholic taste in the mouth, which was unpleasant.				86.64 ± 1.21 ^d
CT	The aroma of the wine was flawed compared to that of the control, with a lighter apple aroma, a bitter taste, and the heaviest wine. The overall coordination was not good.				85.73 ± 0.65 ^e

All values are expressed as mean values ± standard deviations ($n = 11$); different superscripted lowercase letters in the same column indicate significant difference ($p < 0.05$).

Control: apple distillate from original apple juice; ST: apple distillate from sulfuric acid-treated juice; MT: apple distillate from malic acid-treated juice; LT: apple distillate from lactic acid-treated juice; CT: apple distillate from citric acid-treated juice.

content in the final distillates, it also impacted the aroma, flavor, and taste. Therefore, the stored fermented apple juice would be treated with the acids to find their influence on EC and volatiles on the final distillate in of the following research.

Correlation analysis of volatile constituents in apple distillates and basic physicochemical indexes of fermented apple juice

In order to further analyze the effects of different treatments on cyanide, EC, and volatiles found in apple distillates, the correlation analysis chart was used to analyze the relationship of total sugar, titrable acidity, pH, dry extract, and alcohol in fermented apple juice, and that of cyanide, EC and volatiles in distillates. As shown in Figure 3, a significantly positive correlation is observed between the EC content in apple distillates and the pH of fermented apple juice (0.98***). It indicates that lowering the pH of fermented apple juice significantly reduces the EC content of apple distillates. In addition, the EC and cyanide contents

in distillates had a significantly positive correlation (0.72). It has been reported that as a precursor substance of EC, cyanide content is significantly correlated with EC content (Wang *et al.*, 2021). Therefore, the EC content of apple distillate can be decreased by reducing the cyanide content. Furthermore, pH affected the production of ethyl acetate (0.73*), ethyl octanoate (0.93***), 1-propanol (0.91***), acetic acid (0.65*), butanoic acid (0.83**), hexanoic acid (0.91***), and octanoic acid (0.96***). In the meantime, pH was negatively correlated with 2-methylpropanal (-0.99***), ethyl formate (-0.69*), 2-methylpropanol (-0.77**), and 3-methyl-1-butanol (-0.70*). Overall, the pH of fermented apple juice plays an important role in the formation of volatiles in apple distillates.

Conclusion

Acid treatment by ST, MT, LT, and CT significantly removed EC from apple distillates produced by Fuji acidified apple juice. The lower the pH of fermented apple

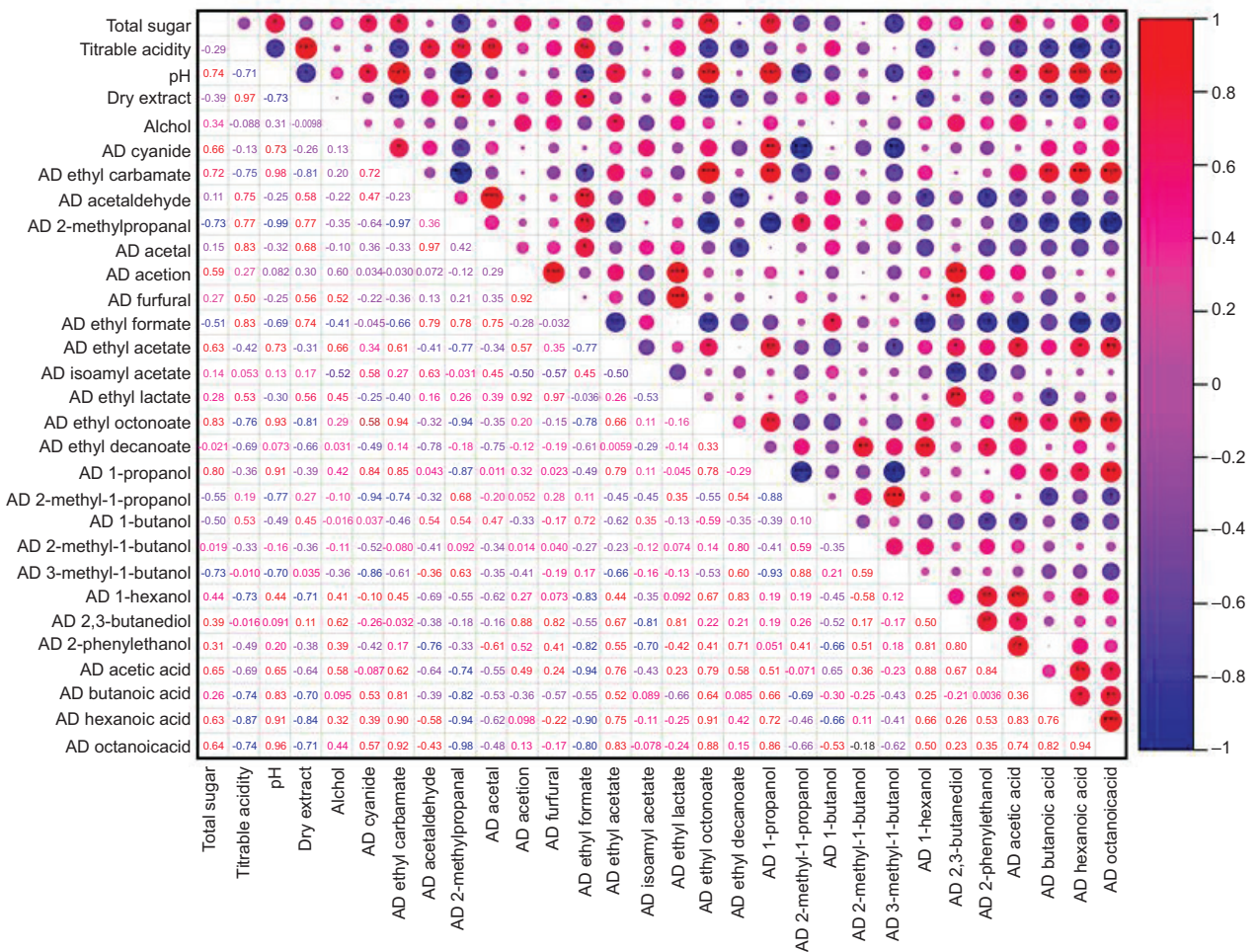


Figure 3. Correlation analysis of volatile constituents in apple distillates and basic physicochemical indexes of fermented apple juice. Red indicates positive correlation and blue shows negative correlation. The deeper the color, the greater the absolute value of correlation and the stronger the correlation between them. AD: apple distillates. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.0001.

juice, the lower the EC residual in distillate (0.98^{***}). However, the acid treatment of apple juice affected the volatile profiles of fermented apple juice and the profiles in apple distillate, which affected the distillate's flavor and taste. Different acid treatments resulted in different pH values of fermented apple juices. The pH of fermented apple juice affected volatile contents in distillate. Through sensory evaluation, distillate MT obtained a higher score, followed by ST. Summarily, it is an excellent option to adjust the pH of Fuji apple juice to 3.0 by both ST and MT to obtain apple distillate with lower EC.

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Author Contributions

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