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Study on the relationship between flavor components and quality of ice wine during freezing and brewing of 'beibinghong' grapes

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ABSTRACT

Ice wine has prominent fruity sweetness and unique, rich aroma compared to wine. The sweetness was accumulating, the acidity and astringency tended to soften of grape berry during the freezing period. The process gave the ice wine balanced taste, with prominent honey sweetness, accompanied by refreshing alcoholic taste, soft acidity and astringency. Eleven key aroma compounds were identified in ice wine through GC–MS and ROAV values. The key aroma compounds were analyzed with Pearson correlation coefficient and fragrance mechanism were speculated. Ethyl acetate and 1-octen-3-ol derived from the aroma of grape, are produced by anaerobic metabolism and lipoxygenase pathways of pyruvate and linoleic acid, respectively. Ester aromas, 2-phenylethanol and 2-methylbutanal were derived from the brewing process, were produced by octanoic acid, caproic acid, phenylalanine and isoleucine through lipid metabolism, Ehrlich pathway and Strecker pathway, respectively. Proposed corresponding control methods based on factors that affect the formation of ice wine aromas.

Introduction

Ice wine is a sweet wine which is made from the fermentation of naturally frozen grapes juice left on the vine when the temperature falls below − 7 °C to − 8 °C (OIV, 2014). At present, Canada and Germany are the main producers of ice wine, while China, Austria and the United States are also producing ice wine in large quantities. In recent years, The Chinese ice wine industry has developed rapidly, with an annual output of 30,000 million L, especially in Huanren County of Liaoning Province and the Yalu River Basin of Jilin Province (Lan et al., 2016). 'Beibinghong' grape is the first wild grape variety (*Vitis amurensis Rupr*) in the world to brew ice wine and is very popular in Northeast China (Wang et al., 2018). It has the advantages of strong cold resistance and stable yield. Compared to unfrozen grapes, frozen grapes contain high concentrations of sugars, aroma and flavor compounds, which endow ice wine with a rich and fruity aroma. After alcohol fermentation, ice wine still contains a rich concentration of residual sugar, which gives ice wine a strong sweetness. 'Beibinghong' ice wine has a round taste and mellow aroma than 'Beibinghong' wine. Because of its characteristic flavor, ice wine has gained widespread attention.

The main flavor substances of wine include sugar, sweet amino acids, alcohol, organic acids, tannins and other substances, which make the wine taste sweetness, alcoholic taste, acidity and astringency. The sweetness of wine comes from glucose and fructose in grape berry (Shehadeh et al., 2020). Another part of sweetness comes from sweet amino acids, which come from methionine, alanine and serine in grape berry. In the fermentation process of wine, proline, glycine and lysine are produced by yeast fermentation metabolism, the side chain group of these amino acids is less hydrophobic, mainly sweet, and the autolysis of yeast cells after fermentation, so that the wine not only retains the original amino acids, but also generates new amino acids (Khalafyan et al., 2023). The taste of alcohol comes from ethanol, the main metabolite produced during fermentation. Ethanol has a certain effect on the convergence of red wine. Ethanol can interfere with the hydrophobic interaction between the protein and the tannin, resulting in reduced tannins precipitation and reduced astringent. Ethanol has a sweetness in an aqueous solution containing a low content of ethanol (0 ~ 4 %). However, it has a bitter taste in high content ethanol (10 ~ 22 %) (Cretin et al., 2018). The acidity comes from organic acid. There is a concentration-effect relationship between tartaric acid and the astringency of wine. When the concentration of tartaric acid is higher than 4.0 g/L, the high acidity will mask the expression of astringency (Zhao et al., 2023). The sharp malic acid is converted by MLF to a softer lactic acid and carbon dioxide, making the wine taste soft. Proper acidity can bring

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a refreshing taste to the wine. The astringency comes from tannins. Tannins enter the wine body through the impregnation of dregs, which increases the astringency of the wine (Pittari et al., 2022). Above most of the research on taste was based on standard wine. The difference between ice wine and standard wine was which grapes used for making ice wine underwent a series of oxidation, biosynthesis, or degradation metabolism during long-term maturation and freezing. The changes in the composition of grape berries themselves will in turn significantly affect the physical and chemical composition of ice wine, making it unique, mellow, and rich in taste.

Aroma is also one of the important sensory qualities of wine, which determines the style and typicality of the product. The aroma sources of wine mainly include variety aroma, fermentation aroma and aging aroma (He et al., 2022). These aromas are derived from grape berry and metabolic pathways of amino acids, fatty acids and carotenoids. Variety aroma comes from grape berry, and floral, fruit and herbaceous aroma all fall into this category. Fermented aroma substances are produced in the stages of alcohol fermentation and malolactic fermentation in the process of wine making, which represents mellow aroma, citrus, whiskey and so on. In the process of wine fermentation, amino acids can be converted into α-ketoacid through the Ehrlich pathway, which is further metabolized to produce higher alcohols such as isobutanol and isoamyl alcohol (Hong et al., 2021). Phenylethanol with the aroma of honey and flowers is a typical representative of the degradation and metabolism of phenylalanine and tyrosine through the Ehrlich pathway (Lukić et al., 2016). In the process of alcohol fermentation, volatile fatty acids such as acetic acid, which provide sour flavor, is mainly decarboxylated by pyruvate to acetaldehyde through glycolysis, and part of acetaldehyde is reduced to ethanol and partly oxidized to acetic acid (Lu, Cheng, Lan, Duan, & He, 2024). In the wine fermentation and aging process, esters will be formed, so that the wine has wet leaf aromas, almonds, oak aromas and other unique aging aromas (He et al., 2022). In contrast to standard wines, the aroma of ice wine was studied much less. Lan et al. (2019) studied the impact of the freezing period of the 'Beibinghong' grape in different years on the key aroma compounds of ice wine, and discovered that ice wine that has undergone freezing has a richer aroma. But they did not study the compositional changes and aroma precursor substances of grape berries during the freezing process, and the mechanism of aroma formation. This study will comprehensively discuss the impact of freezing process on the taste and aroma formation of ice wine, and explore its aroma formation mechanism. The key aroma compounds can be affected by grape breeding, planting environment and brewing technology, so as to obtain ice wine with target aroma characteristics.

Materials and methods

Reagents and chemicals

Analytical grade solvents include sodium chloride (AR), potassium hydroxide (AR) and formic acid (AR), purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Chromatographic grade solvents include methanol (GR), acetonitrile (GR), 2-octanol (GR), *n*-hexane (GR), 2-di-*tert*-butyl p-cresol (GR) and *n*-hexane (GR), purchased from Merck (Merck Sharp, USA). Standard products were purchased from Yuanye Biotechnology Co., Ltd (Shanghai, China).

Sample preparation

The grapes were taken from the grape planting base in Xiatao Village, Qingshi Town, Ji'an City, Northeast China (125°E, 41°N). The periphery 5 rows and the row end 7 m were not taken, the grape berries were randomly cut. The dehydration-freezing time was from the theoretical maturity period (September 10) to the last grape berry harvest (October 29), The time of Frosts Descent in 2022 was October 23, the ambient temperature was about −10 °C. Picking time: Starting from the theoretical maturity period, it was collected every seven days, a total of seven times. In the first and seventh sampling, we harvested 100 kg of grapes to produce standard wine and ice wine, respectively. During other harvesting stages, approximately five kilograms were taken each time. The selected grape berries had the same size and color. The 'Beibinghong' grape were divided into 7 sampling stages (TM + 0, TM + 7, TM + 14, TM + 21, TM + 28, TM + 35, TM + 42) (Table S1). The grape berries were transported to the laboratory for further analysis.

After the last sampling, the 'Beibinghong' grapes were fermented into ice wine. According to the previous experimental research of the research group (Fig. S1, Table S2 and S3), the optimized brewing technology of 'Beibinghong' ice wine was the inoculation amount of yeast was 0.25 g/L, initial pH 3.2 and fermentation temperature 13 °C, we obtained initial fermented ice wine (X1). The fermented ice wine was aged (X1), ice wine aged for six months (X2) and ice wine aged for one year (X3) were selected for further analysis.

Grape juice and wine samples were filtered through 0.45 μm cellulose acetate syringe filters (Jinlong, China).

Basic compositional analysis

Alcohol content, total sugar, total acidity and tannins were determined following the OIV standard (OIV, 2014).

Determination of monosaccharides by HPLC

Determination by high performance liquid chromatography (Agilent, Technologies, USA) (Yeganeh-Zare, Farhadi, & Amiri, 2022). The mobile phase was a mixture of DI water: acetonitrile (10:90 v/v). The flow rate was kept at 1 mL/min. The regression equation and correlation coefficient were obtained: $y_{Glucose} = 1{,}697{,}598.59\ x - 31{,}302.93$, $R^2 = 0.9998$; $y_{Fructose} = 1{,}269{,}140.34\ x + 14{,}259.34$, $R^2 = 0.9999$. Linear range: 0.1 mg/mL–2 mg/mL.

Determination of amino acid by HPLC

The content of amino acids was determined by high performance liquid chromatography (Agilent, Technologies, USA) (Liyanaarachchi et al., 2018). The supernatant of the sample was centrifuged at 4 °C and 12000 rpm. By using a ZORBAX Eclipse XDB-C_{18} column (4.6 mm × 250 mm,Agilent Technologies, USA). The injection volume was 5 μL.The column temperature was 40 °C. The mobile phase was 10 % methanol water and 50 % methanol water.

Determination of organic acid by HPLC

The contents of tartaric acid, malic acid and citric acid were determined by high performance liquid chromatography (Agilent, Technologies, USA) (Dong et al., 2023). Column Agilent ZORBAX Extend-C_{18} (250 mm × 4.6 mm, Agilent Technologies, USA). Using binary solvent gradient, including 0.1 % (v/w) phosphoric acid solution A. Mobile phase B containing 99.9 % (v/v) methanol, injection volume 20 μL, column temperature 40 °C and detection wavelength 210 nm. The regression equation and correlation coefficient were obtained: $y_{Tartaric\ acid} = 1228.2x + 100.35$, $R^2 = 0.9997$; $y_{Malic\ acid} = 683.35x + 8.4887$, $R^2 = 0.9997$; $y_{Citric\ acid} = 910.18x - 36.743$, $R^2 = 0.9989$. Linear range: 0.05 mg/mL – 0.08 mg/mL.

Determination of of volatile aroma compounds by GC–MS

The relative content of volatile aroma compounds was determined by headspace solid phase microextraction gas chromatography-mass spectrometer (Agilent, Technologies, USA) (Zhang et al., 2023). 5 mL samples were taken in a 20 mL headspace flask and shaken at 50 °C for 15 min at a shaking speed of 250 rpm. Inserted the extraction needle into the headspace bottle to keep it at a distance of 1 cm from the liquor surface and extracted for 30 min until the distribution was balanced. After desorbing in GC injector at 220 °C for 5 min, aromas were separated on a DB-5 GC column (50 m × 0.25 mm × 0.25 μm, Agilent, USA) with helium carrier gas at a flow rate of 1.5 mL/min. An original temperature of 40 °C was held for 3 min, then risen to 240 °C at the rate of 3 °C /min and held for 10 min. And ion source temperature was 230 °C. Analyses were conducted with GC–MS QP2020 (Shimadzu, Kyoto, Japan), scanned in a mass acquisition range of 35 ~ 750 *m/z* for quantitative analysis of detections.

Determination of fatty acids by GC–MS

Fatty acids were analyzed by gas chromatography-mass spectrometry (Agilent, Technologies, USA) (Hoving et al., 2018). The injection volume was 1 μL and the split ratio was 8:1 on a Thermo TG-FAME capillary column (50 m × 0.25 mm ID × 0.20 μm, Thermo Fisher Scientific, USA). The 15 mL sample was mixed with 2 mL 1 % methanol sulfate solution, then esterified in a water bath at 80 °C for 30 min, cooled, extracted with 1 mL *n*-hexane, shaken for 30 s, placed for 5 min, washed and centrifuged at 12000 rpm 4 °C for 10 min. The temperature of injection port was 250 °C, the temperature of ion source was 300 °C and the temperature of transmission line was 280 °C. The carrier gas was helium with a flow rate of 0.63 mL/min. Used Thermo ISQ 7000 mass spectrometer, electron bombardment ionization (EI) source, SIM scanning mode, electron energy 70 eV.

Determination of carotenoids by LC-MS

Liquid chromatography tandem mass spectrometry (Agilent, Technologies, USA) was used to analyze carotenoids. The sample pretreatment method was the same as the determination method of amino acid content (Geyer et al., 2004). The YMC C_{30} column was used (3 μm, 100 mm × 2.0 mm, Green Baicao Technology Co., Ltd., Beijing, China). The supernatant of the sample was centrifuged at 4 °C and 12000 rpm. The mobile phase was phase A, methanol acetonitrile was added with 0.01 % BHT and 0.1 % formic acid, phase B, methyl *tert*-butyl ether was added with 0.01 % BHT, the flow rate was 0.8 mL/min, the column temperature was 28 °C, the injection volume was 2 μL, the mass spectrometry condition was atmospheric pressure chemical ion source temperature 350 °C, air curtain gas 5 psi.

Sensory evaluation of taste

The sensory assessment team consists of 20 experienced and professionally trained members (10 males and 10 females, aged 23 ~ 60). They are faculty or students majoring in food science and engineering of Jilin Agricultural University. All participants were informed that they were completely voluntary and free to do these subjects, and formal consent was obtained from them in full accordance with the 1975 Declaration of Helsinki. According to the literature reports and the opinions of the participants, the description attributes were divided into five groups: honey sweet, fruit sweet, alcohol taste, sour taste and astringent taste. The team members evaluated the taste properties of frozen 'Beibinghong' grapes and ice wine during the brewing process and scored their strength (Lan et al., 2019). Assessed by a 5-point scale, 0 indicates no taste intensity, 3 indicates moderate taste intensity, and 5 indicates strong taste intensity (0 = 'absence', 3 = 'very low', and 5 = 'very high'). Each sample was evaluated for 3 times, and the average value was the taste strength value.

Evaluation of key aroma compounds

Relative odor activity value (ROAV) was calculated by the formula ROAV = 100 × C_A/C_{Tan} × T_{stan} /T_A, where C_A is the compound content in the ice wine sample and C_{Tan} is odor threshold. T_{stan} is the relative content of volatile flavor compounds that contribute most to the overall flavor. T_A is the threshold of volatile flavor compounds that contribute most to the overall flavor (Zhang et al., 2021).

Data analysis

All data results were expressed in the form of mean ± standard deviation (x ± SD) of three measurements. The data of physical and chemical indexes of flavor substances were analyzed by SPSS 27 software (SPSS, Chicago, IL, USA). The aroma were identified based on the NIST library. Radar map and cluster heat map of taste sensory evaluation, and the correlation between aroma precursors and key aroma were analyzed by Origin 2022 software (Microcal, Northampton, MA, USA).

Results and discussion

Analysis of taste

We mainly analyzed the flavor substances such as glucose, fructose, sweet amino acid, ethanol, tartaric acid, malic acid and tannins (Table 1), which were the important reasons for the sweetness, alcoholic taste, acidity and astringency of ice wine.

Glucose, fructose and 7 kinds of sweet amino acids were detected, which constitute the main source of sweetness of ice wine. Sugar content and sweet amino acids were the main substances that constituted the sensory characteristics such as softness and roundness of the wine. The sugar content of grape berry was concentrated during the freezing period, and the total sugar content reached the maximum at the last sampling during the freezing period. During the fermentation of ice wine, glucose and fructose were shared by yeast to produce compounds such as carbon dioxide, ethanol. Because the affinity of transporters to glucose was greater than that to fructose, yeast gave priority to the use of glucose catabolic metabolites. The results showed that the ratio of fructose to glucose changed from 1:1 to 1.8:1. Fructose was about twice as sweet as glucose, and high fructose content made ice wine taste sweet and had obvious honey sweetness (Reis et al., 2018). Amino acids also played an important role in the flavor formation of ice wine, participating in the formation of fruit flavor substances, thus making the fruit show a unique flavor. The contents of proline, serine, glycine and lysine accumulated continuously with the prolongation of the freezing period. The content of proline was the highest before fermentation, which provided honey sweetness for grape berry. This may be due to the amino acid metabolism in the fruit, which produced esters, alcohols and other substances through the ammonia transfer reaction, which in turn affected the taste of the grapes, and the honey sweetness continued to strengthen. In the process of ice wine brewing, the content of amino acids decreased significantly, because amino acids, as nitrogen sources for microbial growth, were consumed to produce other new substances in the fermentation process. With the extension of aging time, the contents of seven sweet amino acids increased gradually, including glycine, alanine, serine, proline, threonine, lysine and methionine (Fig. 3A), which were due to the biological reaction between various metabolites and the autolysis of yeast cells after fermentation. Proline and alanine were the most abundant, it can be inferred that proline and alanine were the main flavor amino acids of ice wine, which made ice wine sweet and mellow (Tian et al., 2021). The changes in sugar content and sweet amino acids were closely related to the sweetness, alcohol content and acidity of ice wine, which affected the final taste of ice wine.

The taste of alcohol in ice wine came from ethanol. In the process of ice wine brewing, the sugar of grape berry converted into alcohol by yeast (Reis et al., 2018). When the alcohol content of the wine is less than 14 %, it can enhance the expression of the sweetness of the wine, which can not only supplement the taste of the wine itself, but also integrate with its characteristics and enhance the fullness of the wine. When the alcohol content is more than 14 %, the wine will obviously show a stimulating alcohol taste, that is, the bad taste and bitterness of ethanol (Cretin, et al., 2018). The alcohol content of ice wine produced

Table 1
Changes of physical and chemical indexes during freezing period and brewing process of 'Beibinghong' grapes.

Name	Freezing period							Brewing process		
	TM + 0	TM + 7	TM + 14	TM + 21	TM + 28	TM + 35	TM + 42	X1	X2	X3
Total sugar (mg/mL)	184.63± 7.8^{h}	195.78± 13.3^{gh}	233.60± 13.6^{e}	270.55± 11.3^{d}	320.48± 13.2^{c}	353.10± 10.4^{b}	379.87± 8.2^{a}	212.39± 10.16^{f}	210.53± 10.47^{g}	218.8± 12.3^{f}
Fructose (mg/mL)	81 ± 5.5^{f}	82.2 ± 6.7^{f}	106.3 ± 5.3^{e}	121.9 ± 6.2^{d}	122.2 ± 5.6^{d}	173.1 ± 4.2^{b}	188.3 ± 4.8^{a}	135.41 ± 3.5^{cd}	134.42 ± 2.8^{d}	138.50 ± 3.2^{c}
Glucose $C_6H_{12}O_6$ (mg/mL)	71.2 ± 5.5^{e}	72.8 ± 6.3^{e}	103.2 ± 4.6^{d}	110.5 ± 5.6^{c}	111.2 ± 6.2^{c}	152.1 ± 5.8^{b}	175.18 ± 6.4^{a}	73.69 ± 2.9^{e}	74.38 ± 4.1^{e}	74.55 ± 3.5^{e}
Total acid (mg/mL)	1.79 ± 0.04^{a}	1.68 ± 0.04^{b}	1.59 ± 0.04^{c}	1.3 ± 0.03^{d}	1.01 ± 0.04^{g}	1.23 ± 0.03^{e}	1.1 ± 0.03^{f}	1.31 ± 0.03^{d}	1.27 ± 0.03^{d}	1.23 ± 0.03^{e}
Malic acid (mg/mL)	0.60 ± 0.03^{a}	0.57 ± 0.02^{b}	0.54 ± 0.03^{c}	0.49 ± 0.02^{d}	0.41 ± 0.03^{g}	0.48 ± 0.03^{d}	0.43 ± 0.04^{f}	0.45 ± 0.02^{e}	0.41 ± 0.02^{ef}	0.37 ± 0.01^{g}
Tartaric acid (mg/mL)	1.06 ± 0.03^{a}	0.96 ± 0.03^{b}	0.85 ± 0.03^{c}	0.70 ± 0.02^{d}	0.50 ± 0.04^{g}	0.65 ± 0.03^{d}	0.56 ± 0.03^{f}	0.62 ± 0.03^{e}	0.56 ± 0.01^{ef}	0.51 ± 0.02^{fg}
Citrate (mg/mL)	0.08 ± 0.02^{b}	0.06 ± 0.02^{b}	0.07 ± 0.025^{b}	0.08 ± 0.02^{b}	0.07 ± 0.017^{b}	0.08 ± 0.018^{b}	0.083 ± 0.02^{b}	0.14 ± 0.01^{a}	0.14 ± 0.02^{a}	0.14 ± 0.02^{a}
Tannins (mg/L)	243.75± 20.14^{i}	350.24± 26.45^{h}	564.43± 25.17^{g}	623.76± 26.05^{f}	681.47± 26.41^{e}	711.19± 17.32^{e}	733.26± 15.27^{d}	1016.46± 21.36^{c}	991.71± 18.44^{b}	845.24± 16.43^{a}
Flavor-presenting amino acids (μg/mL)	772.98± 0.06^{e}	536.87± 0.06^{g}	412.90± 0.08^{h}	802.04± 0.1^{c}	1420.69± 0.13^{a}	411.41± 0.12^{i}	638.88± 0.05^{f}	651.88± 0.08^{f}	774.98± 0.01^{d}	892.80± 0.01^{b}
Alcoholic strength (vol%)	nd	nd	nd	nd	nd	nd	nd	12.07 ± 0.02^{a}	12.1 ± 0.02^{b}	12.26 ± 0.02^{c}

Note: nd stands for not detected. Lowercase letters following the results represent significant differences under peer ($p < 0.5$) .

was 12 % in this study (Table 1). The moderate alcohol content did not increase the bitterness of the wine, nor concealed the expression of other flavors, and formed the balance of the wine with sweetness, acidity and tannins. Ice wine had high sweetness, which to a certain extent reduced the irritation of alcohol and made ice wine taste refreshing.

The acidity of ice wine came from tartaric acid, malic acid and citric acid. Their contents showed a downward trend. Tartaric acid had a sharp sour taste. Some tartaric acid precipitated as salt substances, which was relatively stable and weakened the sour taste of ice wine. When the concentration of tartaric acid in wine is higher than 4.0 g/L, high acidity will reduce the astringency of tannins and mask the expression of astringency of tannins to some extent (Zhao et al., 2023). In this study, the content of tartaric acid was always higher than 4.0 g/L (Table 1), tartaric acid was not only the key substance for the acidity of ice wine, but also contributed to covering up the astringency. Malic acid was decomposed into softer lactic acid in the process of alcohol fermentation, which reduced the acidity of the wine. The taste of ice wine gradually changed from rough to soft (Vion et al., 2023). Citric acid came from the grape berry. The content of citric acid detected in grape freezing and ice wine was lower (Lima et al., 2022). The changes in acid content made the taste of ice wine softer and more delicate, at the same time, it adjusted the balance of sweet and sour taste and retained the aroma of wine.

The astringency of ice wine came from tannins. The content of tannins increased gradually in the process of freezing. In ice wine, ethanol destroyed the outer fat layer of grape seeds and fused more tannins into the wine, so the fermented new wine had the highest tannins content. In the process of aging, the evaluation value of astringency decreased gradually (Fig. 1). According to previous studies, we speculated that tannins and anthocyanin condensed to form a stable tannin-anthocyanin complex, which could reduce the astringency and bitterness of wine and change the taste of ice wine from astringency to softness (Ju et al., 2021). The aging time was negatively correlated with the concentration of condensed tannins. With the extension of aging time, the condensed tannins would be hydrolyzed, and the astringency weakened (Lei et al., 2023).

Sensory analysis of the taste

The taste and sensory analysis of grape frozen period and ice wine were carried out. Taste properties were described as honey sweetness, fruit sweetness, alcohol taste, acidity and astringency. The effect of the interaction of flavor substances on sensory changes was verified (Fig. 1).

During the freezing period, the evaluation values of honey sweetness and fruit sweetness showed an upward trend. This was because in the process of grape freezing, glucose, fructose and sweet amino acids gradually accumulated, which made the grapes more sweet, rich and fruity. The evaluation value of sour taste decreased gradually (Fig. 1). We speculate that tartaric acid formed a more stable tartrate as the grape berries mature, malic acid was continuously consumed, and the content

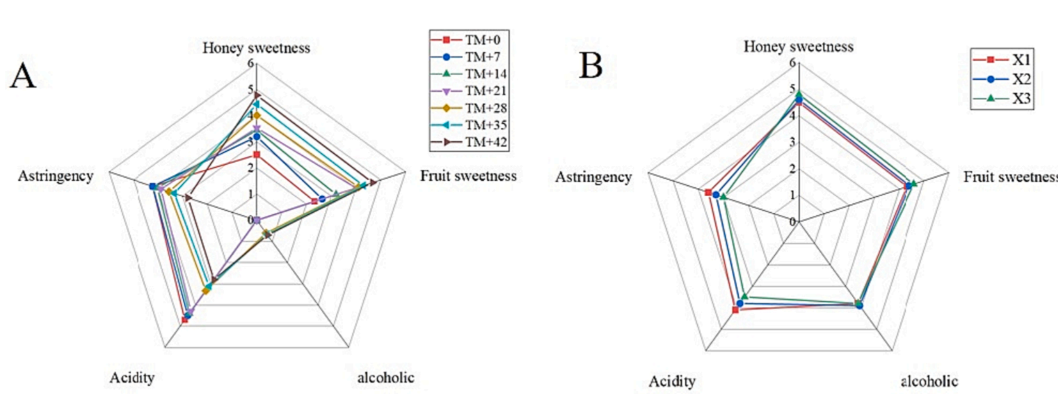


Fig. 1. Sensory evaluation electronic radar map. A: Rader chart of sensory evaluation of 'Beibinghong' grapes during freezing period during freezing and brewing of 'Beibinghong' grapes; B: Rader chart of sensory evaluation of ice wines during freezing and brewing of 'Beibinghong' grapes.

of both decreased. The evaluation value of astringency decreased gradually because the free tannins were polymerized and precipitated with the change in fruit maturity, which weakened the convergence. While the acidity and astringency continued to weaken, it was also partially masked by the prominent sweet taste and sweet taste of the grape berry, making the overall taste of the grape berry gradually soft and harmonious. The results of sensory evaluation of taste were consistent with the changes in the content of taste substances during the freezing period.

In the brewing process of ice wine, the evaluation values of honey sweetness and fruit sweetness were stable. Although part of the sugar is consumed by yeast to produce ethanol, the residual high concentration of fructose still makes the ice wine taste sweet. Internal redox reactions continued to occur due to the accumulation of matrix effect during the aging process of wine, and the changes of various substances made the taste of ice wine gradually balanced and mellow. The evaluation value of alcohol taste had no significant change, because the fermentation of yeast stopped. The evaluation value of sour taste decreased gradually. This was because the tartrate in the wine was relatively stable in the brewing process. The sharp malic acid was transformed into softer lactic acid by MLF fermentation, which gradually weakened the acidity of the wine. The evaluation value of astringency decreased gradually. The strong astringency of the newly fermented wine was due to the high concentration of condensed tannins. With the extension of aging time, the condensed tannins was hydrolyzed and the astringency was gradually weakened (Wang et al., 2022). In the process of ice wine brewing, the taste of honey and fruit was strong, there was a certain alcohololic taste and balance. The acidity and astringency tended to soften gradually under the cover of sweetness. Under the joint action of various substances, the taste of ice wine was gradually balanced, delicate and mellow (Fig. 1).

Analysis of volatile aroma compounds

The volatile aroma compounds produced during the freezing period and brewing process of grapes play an important role in evaluating the aroma of grape wine. In this study, 100 volatile compounds were identified during the freezing period of 'Beibinghong' grapes, and 56 volatile compounds were identified during ice wine making, including esters, volatile fatty acids and alcohols (Fig. 2).

Seven identical esters such as ethyl acetate, ethyl pyruvate, ethyl octanoate, ethyl propionate, ethyl caproate, ethyl dodecane and ethyl decanoate were detected during freezing and brewing. During the freezing process, the contents of ethyl acetate and ethyl pyruvate decreased, which may be due to hydrolysis (Table S4). In the process of ice wine brewing, ethyl acetate accumulated further due to esterification reaction. The relative content of ethyl pyruvate decreased because its synthetic precursor pyruvate participated in glucose metabolism and could not be esterified to accumulate ethyl pyruvate. The relative content of the other five esters increased gradually. This was because yeast produces a large amount of alcohol in the process of fermentation, which promoted the formation of esterification. Ethyl isobutyrate, isoamyl acetate, isoamyl acetate and *n*-propyl acetate were detected only during aging. This may be because the biological metabolism of carbon and nitrogen or the biosynthesis of fatty acids took some time to produce new esters, providing rich aromas for grapes (Liszkowska and Berlowska, 2021).

Acid substances increased at first and then decreased in the process of grape freezing (Table 1). This was because the grape berry was in a state of low water activity and low oxygen, and the activity of lipoxygenase decreased, which inhibited the production of acids. In the brewing process, the relative content of acids decreased, but the relative content of acetic acid increased significantly. This was because acetic acid was the main volatile acid produced during alcohol fermentation. The content of other acids decreased slowly, which was due to the

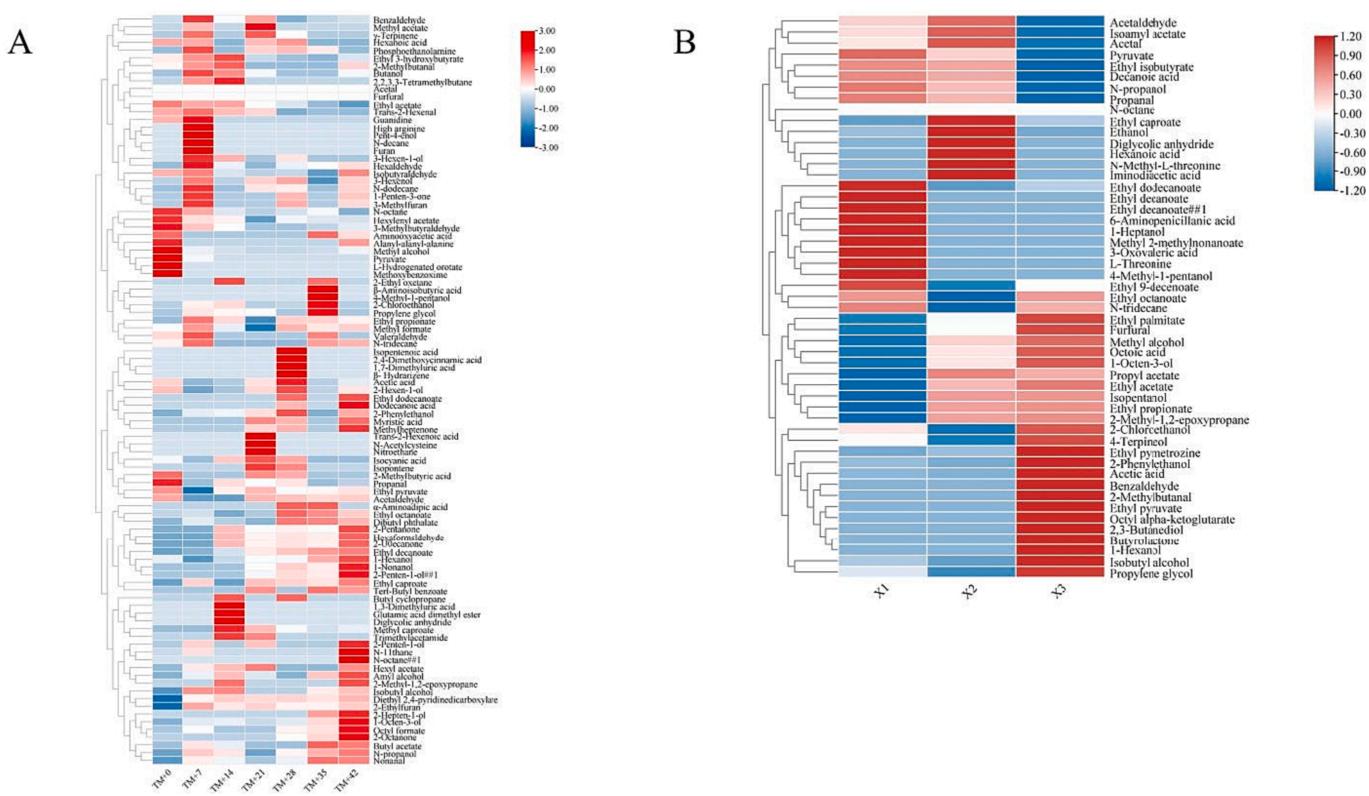


Fig. 2. Cluster heat map of volatile aroma compounds. A: Cluster heat map of volatile aroma compounds during freezing period of 'Beibinghong' grapes; B:Cluster heat map of volatile aroma compounds during brewing process of 'Beibinghong' grapes.

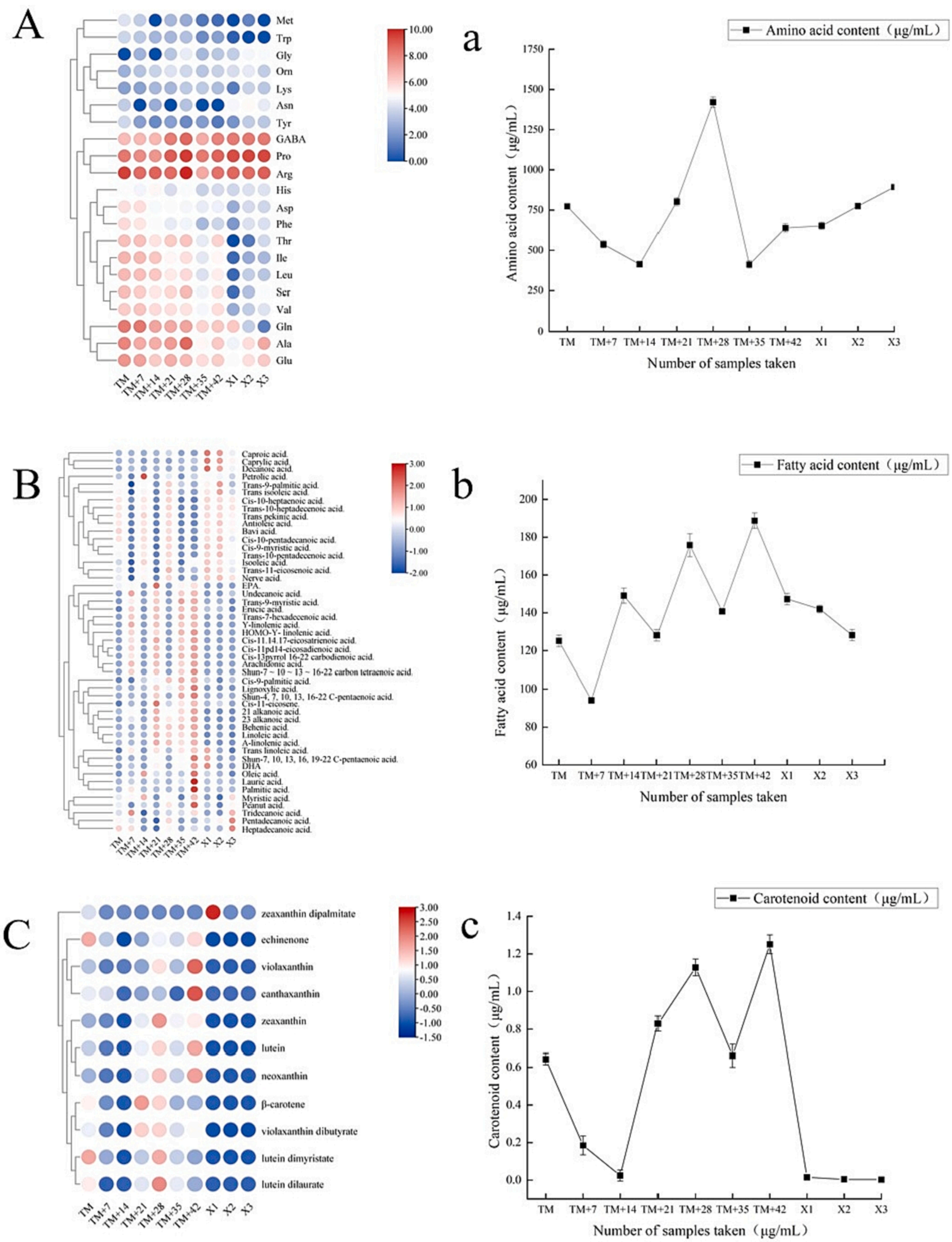


Fig. 3. Cluster thermogram and content changes of aroma precursors. A: Cluster heat map analysis of amino acids contents; a: Changes of the contents of total amino acids; B: Cluster heat map analysis of fatty acid contents; b: Changes of the contents of total fatty acid contents; C: Cluster heat map analysis of carotenoid contents; c: Changes of the contents of total carotenoid contents.

esterification reaction in the wine. During the aging period, seven kinds of new acids were produced, such as quinic acid and caprylic acid, indicating that not only new esters were produced during aging, but also accompanied by hydrolysis, which increased the content of acids, which was beneficial to maintain the basic structure of wine and made the wine flavor soft and harmonious.

The relative total content of alcohol in grape berries was relatively low. In the brewing process, the relative contents of ethanol, fatty alcohol and aromatic alcohol increased. The increase in the relative content of ethanol was due to the fact that glucose assists diffusion into yeast cells, converted to pyruvate through glycolysis, and produced acetaldehyde and carbon dioxide under the action of pyruvate decarboxylase. Acetaldehyde produced ethanol under the action of ethanol dehydrogenase. Fatty alcohols, aromatic alcohols and other higher alcohols were fermented alcohols as by-products in the process of synthesizing amino acids from branched amino acids through the Erlich pathway, which made isobutanol, isoamyl alcohol and 2-phenylethanol produced a large number of aromatic alcohols, which were important characteristic aroma of wine.

The relative total contents of aldehydes and ketones in grape berries decreased gradually. This was because acetaldehyde was oxidized to acetic acid under osmotic stress with high glucose concentration. The relative total content of aldehydes in ice wine was low (Table S4). Acetaldehyde, 2-butyraldehyde and furfural were detected in the aging process of ice wine, and their relative contents increased gradually, accumulating the aroma of ice wine. Acetals were formed by the biochemical conversion of branched chain amino acids with free aromatic substances under the action of microorganisms. The sources of furfural included the degradation of sugar and the Aldol condensation of C_3. Only a small part of ketones was detected in grape berries, which did not detected in the brewing process. The relative total content of alkanes was lower in grapes and ice wine. Because alkanes have a low aroma threshold and make little contribution to the flavor of ice wine, they were not discussed too much in our study.

Analysis of key aroma compounds in ice wine

The intensity of aroma is usually expressed in terms of the minimum concentration of aroma at the time of odor production (called threshold) (Sun et al., 2021). The odor activity value (ROAV) of aroma compounds in ice wine aged for one year was calculated to evaluate the effect. Through the calculation of relative odor activity, it was found that these 11 volatile aroma compounds made the greatest contribution to the sporadic flavor of ice wine (Table 2). The ROAV ≥ 1 of ethyl caprylic acid, isoamyl acetate, ethyl caproate, 2-phenylethanol and ethyl acetate were the key aroma compounds of ice wine. The other six substances ($0.1 \leq$ ROAV < 1) played an important role in modifying the aroma (Fig. 3).

These five substances provided the main properties of the sensory characteristics of wine aroma. The interaction of esters affected the aroma complexity of wine samples in varying degrees, and ice wine showed fruit aromas such as pineapple, apple, banana and so on. Compared with the corresponding base wine, the ester content of ice wine was higher. Ethyl acetate, isoamyl acetate, ethyl caproate and ethyl caprylic acid were the reasons for the aroma of fresh fruit in ice wine (Gallo et al., 2023). 2-phenylethanol was also an important source of the characteristic aroma of ice wine. 2-phenylethanol made ice wine showed a pleasant honey and flower aroma.

Table 2
ROAV of key aroma compounds in ice wine.

NO.	Flavor matter	Odor Description	Thresholds (μg/kg)	ROAV X3
1	Ethyl acetate	Pineapple	12	2.5773
2	Isoamyl acetate	Banana	30	9.6
3	Ethyl caproate	Apple	2	3.7765
4	Ethyl octanoate	Fruit	0.24	100
5	Ethyl isobutyrate	Pineapple	0.15	0.6848
6	Ethyl propionate	Fruit	165	0.4142
7	Octoic acid	Cream	10	0.286
8	1-Octen-3-ol	Mushroom	1	0.8045
9	2-Phenylethanol	Honey, floral	45	2.8782
10	2-Methylbutanal	Cocoa,almonds	4.4	0.6196
11	Furfural	Bread,almonds	9.562	0.1636

These six substances played an important role in modifying ice wine. They enhanced some existing aromas by synergism with other compounds. Ethyl isobutyrate and ethyl propionate had strong aromas of apples. Bitterness had a creamy aroma and enhanced the aroma of fruit. 1-octene-3-ol had aromas of mushroom and hay. 2-Methylbutyraldehyde showed the aroma of cocoa and coffee, which acted in synergy with other aromas, and showed slightly fruity and nutty aromas. Furfural had malt flavor, fermentation flavor and almond flavor (Zhang et al., 2023). The aroma of ice wine was attributed to the interaction of the above key aroma compounds, which showed pleasant aromas such as fresh fruit, flowers and cream. The aroma formation mechanism of ice wine needs to be further studied.

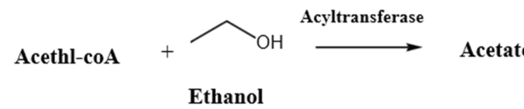
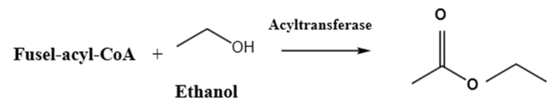
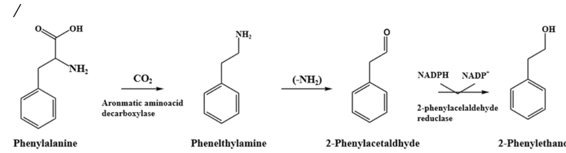
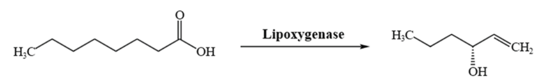
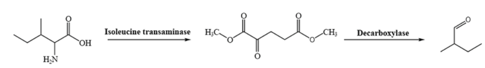
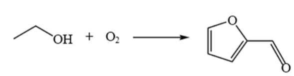
Speculation on the formation mechanism of key aroma compounds

We analyzed the relationship between amino acids and key aroma of ice wine, as well as the relationship between fatty acids and key aroma compounds of ice wine using Pearson correlation coefficient (Table S5 and S6), and the formation mechanism of key aroma compounds was explored (Table 3).

Among the eleven key aroma compounds, the contents of ethyl acetate and 1-octene-3-ol in the freezing period were higher than those in the brewing process (Table S1). These two aroma compounds were derived from grape berry. The aroma precursor of ethyl acetate was acetic acid. Acetic acid was not only produced by photosynthesis, but also from the decomposition of glucose by microorganisms, and pyruvate formed acetyl-CoA under the action of lyase. Acetyl coenzyme A and ethanol produced ethyl acetate under the action of acetyltransferase (Wang et al., 2023). There was a significant correlation between 1-octene-3 alcohol and linoleic acid. It was inferred that linoleic acid is the aroma precursor of 1-octene-3-ol. 1-octene-3-ol was formed by the formation of hydrogen peroxide derivatives of linoleic acid under the action of lipoxygenase. Hydrogen peroxide derivatives were formed by the action of hydrogen peroxide lyase to form a variety of aldehydes, which were further decomposed by the lipoxygenase pathway (Delgado et al., 2022).

The contents of ethyl caprylic acid, ethyl caproate, isoamyl acetate, ethyl isobutyrate, caprylic acid and 2-phenylethanol increased rapidly after the last grape juice was fermented into ice wine. These six key aroma compounds came from the fermentation process. Among them, the four ester aroma compounds were derived from their aroma precursors, such as caprylic acid, caproic acid and other fatty acids. Fatty acids such as caprylic acid and caproic acid undergo lipid metabolism with ethanol or isoamyl alcohol under the action of acyl-coenzyme A to form ester aroma compounds. Caprylic acid may also be formed by the oxidation of fatty acids. There was a significant correlation between 2-phenylethanol and phenylalanine. It was speculated that phenylalanine is the aroma precursor of 2-phenylethanol. 2-phenylethanol was formed by phenylalanine forming phenylethylamine under the action of aromatic amino acid decarboxylase and further dehydrogenation of phenylacetaldehyde under the action of monoamine oxidase. It was the Erlich degradation pathway of amino acids (Lu, Cheng, Lan, Duan, & He, 2024). The contents of caprylic acid, 1-octene-3-ol and 2-phenylethanol increased slightly in the aging stage, indicating that the above reactions were still taking place. Ethyl propionate, 2-methylbutyraldehyde and furfural only appeared in the aging stage, and their contents increased gradually. In the aging stage, a large amount of ethyl acetate may be hydrolyzed and fatty acids were oxidized to form propionic acid, which promoted the synthesis of ethyl propionate. There was a significant

Table 3
Speculation on the formation pathway of key aroma compounds.

Aroma source	Aroma category	Key aroma compounds	Aroma precursor	Synthesis pathway
Grape berry	Esters	Ethyl acetate Ethyl caproate	Isoleucine Caproic acid	
Fermentation process	Esters	Isoamyl acetate Ethyl isobutyrate Ethyl propionate Ethyl octanoate	Acetic acid Butyric acid Propionic acid Caprylic acid	
	Volatile fatty acid	Caprylic acid	Caprylic acid	/
	Alcohols	2-phenylethanol	Phenylalanine	
		1 octene 3 alcohol	Caprylic acid	
	Aldehydes	2-methyl butyraldehyde	Isoleucine	
		Furfural	/	

correlation between 2-methylbutyraldehyde and isoleucine (Table S5). Isoleucine reduced a carbon atom to aldehyde in the presence of amino acid decarboxylase. It was speculated that furfural was formed by the oxidation of alcohol. Aldehydes could not be stably present in wine. Under the action of reductase and yeast dehydrogenase, aldehydes can be easily reduced to corresponding alcohols. Phenolic substances were not detected in ice wine aged for one year. In the future, we will continue to study the changes of phenols and carotenoids components with the extension of aging time.

We compared the volatile aroma compounds of ice wine aged for one year (X3) with the volatile aroma compounds of standard wine fermented from the first sampled grape (TM + 0) (Tables S1 and S7). The key aroma compounds such as ethyl caproate, ethyl propionate, 1-octen-3-ol, and 2-methylbutyraldehyde in ice wine were not found in standard wine. The relative content of ethyl acetate, octanoic acid, and furfural in ice wine was higher than that in standard wine. This may be because the grapes are dehydrated and concentrated during freezing and the accumulation of caproic acid, propionic acid, linoleic acid, acetic acid, caprylic acid and isoleucine, resulting in an increase in the synthesis rate of these esters and alcohols (Noguerol et al.,2013). Maillard reaction can occur in high glucose environment and promote the formation of aldehydes. Ice wine has more aromas of apple, cream and cocoa than standard wines. The content of ethyl isobutyrate, isoamyl acetate, ethyl octanoate, and 2-phenylethanol in ice wine was lower than that of standard wine. This may be due to the exposure of yeast to high glucose permeation, which decreased the activity of lipoxygenase during the metabolism of butyric acid, acetic acid, caprylic acid and ethanol, isoethanol, isoamyl alcohol, as well as the activity of phenylalanine decarboxylase in the Ehrlich pathway, thus affecting the production of secondary metabolites such as esters and alcohols (Franco et al., 2004). The threshold of these three esters and 2-phenylethanol was large, and the aromas of fruit, honey and flowers were still prominent, and showed the unique aroma of ice wine under the coordination of other aromas.

The main factors affecting the aroma formation of ice wine varieties included variety selection and planting environment (climate, light, soil). Grapes making ice wine need to select aroma varieties with strong cold resistance and disease resistance. North American riparian grapes (*V. riparia*) hybrided with *V. labrusca* and *V. vinifera* by Dami et al. (2016) has a strong resistance to cold. Moreover, the brewed ice wine has a rich aroma. This is an excellent variety for making ice wine. When planting grapes, methods such as leaf picking, film covering, and adding external light sources can be used to increase the content of combined aroma components in grape fruits by increasing light intensity. The treatment of picking leaves should be carried out between the flowering stage and the color conversion period. This mainly affects the accumulation of glucose and the activity of enzymes in plants. Sunlight exposure to fruits is beneficial for the accumulation of norisoprene and monoterpenes (Skinkis et al., 2010). Sasaki et al. (2016) used plastic film covering during grape growth. The results showed that film mulching can increase the content of linalool in grapes, as direct scattered light from the sun can accelerate the activity of enzymes related to sucrose metabolism in the fruit, which helps to improve the aroma quality of wine. The blue wavelength ranges from 400 to 520 nm, which can significantly increase anthocyanins, aroma (alcohols and phenols), and soluble sugars (glucose and fructose). The red wavelength is 610–720 nm, which can enhance organic acids (citric acid and malic acid). Hui et al. (2021) found that foliar spraying of 0.26 g/m^2 ammonium sulfate and 0.12 g/m^2 urea can significantly increases the concentration of total anthocyanins, total flavonols in wine grape skins, significantly affect the aroma and sensory properties of grapes and wine.

In the brewing process, improved yeast varieties and optimized brewing technology were used to control the formation of aroma compounds. Mutation breeding, protoplast fusion breeding and genetic engineering breeding can improve the yeast strain and significantly enhance the fermentation aroma of ice wine. Ji et al. (2021) showed that hybridization of *V. vinifera* and *V. labrusca* produced *Kyoho* grape with a strawberry smell, and mutation breeding of *V. vinifera* produced 87–1 grape variety with a rose smell. The flash evaporation process is to use instantaneous high temperature to increase the temperatures of the grape to 70 ~ 90 °C, so that the grape skin can be quickly broken. Then instantly reduced to a suitable fermentation temperature under low pressure, so that the aroma, color and phenols can be fully leached

(Sebastian and Nadeau, 2002). The use of micro-oxygen, microwave and ultra-high pressure techniques in the aging process can improve the rough and sour body of the original wine and make it rich in aroma. Sánchez-Córdoba et al. (2021) found that after microwave treatment of Tempranillo wine (microwave conditions: Mars 6 machine; from 25 °C to 60 °C in 4 min, power 350 W, total time 10 min). The contents of isoamyl alcohol, furfural and valeric acid increased significantly. In the process of wine making, adding an appropriate concentration of sensory flavor protectant, such as glutathione, can effectively increase the content of alcohols and organic acids to increase the aroma of ice wine.

Conclusion

We found that grapes accumulated fructose, proline and alanine during the freezing period. The content of organic acids decreased and the content of tannins increased. In the brewing process, the content of fructose was still high. Part of the sugar was converted into alcohol. Tartaric acid precipitation reduced the acidity. Malic acid was converted into lactic acid. The tannins were hydrolyzed. The ice wine was harmonious and soft after the taste evaluation. The volatile aroma compounds of ice wine were analyzed and eleven key aroma compounds were identified, including esters, alcohols, acids and aldehydes. Five main aroma compounds formation pathways: Ethyl acetate and ethyl caproate came from the secondary metabolic pathway and caprylic acid came from the anaerobic metabolic pathway of grape. In the brewing process, Ethyl octanoate was derived from the lipid metabolic pathway of fatty acids such as caprylic acid and acyl-coenzyme A. 2-phenylethanol was derived from the Erlich degradation pathway of phenylalanine. 2-methylbutyraldehyde came from the Strecker degradation pathway of isoleucine. This gave the ice wine fresh floral, fruity and honey aromas. The other six aromas came from the Strecker degradation pathway or the lipid metabolism pathway or the biooxidation reaction. They enhanced some of the existing aromas while providing aromas of cocoa and almonds in synergy with other compounds.

CRediT authorship contribution statement

Penghui Li: . **Yuanlong Jia:** Formal analysis, Writing – review & editing. **Donglin Cai:** Formal analysis, Writing – review & editing. **Xinyuan Wang:** Investigation, Writing – review & editing. **Jiahua Liu:** Investigation, Writing – review & editing. **Rongchen Zhu:** Investigation, Writing – review & editing. **Zhitong Wang:** Conceptualization, Formal analysis, Supervision, Writing – review & editing. **Yang He:** Conceptualization, Formal analysis, Writing – review & editing. **Liankui Wen:** Conceptualization, Project administration, 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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.101016.

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