

Changes in the physicochemical and volatile profiles during the winemaking of Marselan in the Eastern Foot of Helan Mountain, China

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Abstract

Marselan wine, one of the most important wines in the Ningxia Hui Autonomous Region of China, has attracted much attention due to its unique quality. This study focused on determining and analyzing the changes in volatile flavor compounds and antioxidant activity during different stages of Marselan winemaking. A total of 40 volatile aroma compounds were identified by headspace-gas chromatography-ion mobility spectrometry (HS-GC-IMS). Among these compounds, ethyl hexanoate, isoamyl acetate, ethyl formate, ethyl acetate, ethyl butanoate, ethyl octanoate, 3-methyl-1-butanol, ethanol, and 2-methyl-1-propanol showed significant increases after fermentation. Flavonoid and phenol contents in Marselan wine samples also significantly increased after fermentation, demonstrating high antioxidant capacity. Principal component analysis (PCA) successfully distinguished the fruit juice processing stage, alcohol fermentation stage, and malolactic fermentation stage, while the malolactic fermentation stage and wine stable stage could not be distinguished, This indicates that the formation of aroma profiles primarily occurs during the malolactic fermentation stage. The study successfully established flavor fingerprints of samples from different stages of Marselan wine production based on the detected volatile compounds.

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Introduction

Starting in the early 2000s, China has experienced rapid growth as an emerging wine market. It has now established itself as the world's second-largest grape-growing country in terms of vineyard surface area. Furthermore, China has also secured its position as the sixth-biggest wine producer globally and the fifth-most significant wine consumer in terms of volume^[1]. The Ningxia Hui autonomous region, known for its reputation as the highest quality wine-producing area in China, is considered one of the country's most promising wine regions. The region's arid or semiarid climate, combined with ample sunlight and warmth, thanks to the Yellow River, provides ideal conditions for grape cultivation. Wineries in the Ningxia Hui autonomous region are renowned as the foremost representatives of elite Chinese wineries. All wines produced in this region originate from grapes grown in their vineyards, adhering to strict quality requirements, and have gained a well-deserved international reputation for excellence. Notably, in 2011, Helan Mountain's East Foothill in the Ningxia Hui Autonomous Region received protected geographic indication status in China. Subsequently, in 2012, it became the first provincial wine region in China to be accepted as an official observer by the International Organisation of Vine and Wine (OIV)^[2]. The wine produced in the Helan Mountain East Region of Ningxia, China, is one of the first Agricultural and Food Geographical Indications. Starting in 2020, this wine will be protected in the European Union^[3].

Marselan, a hybrid variety of Cabernet Sauvignon and Grenache was introduced to China in 2001 by the French National Institute for Agricultural Research (INRA). Over the last 15 years, Marselan has spread widely across China, in contrast to its lesser cultivation in France. The wines produced from Marselan grapes possess a strong and elegant structure, making them highly suitable for the preferences of Chinese consumers. As a result, many wineries in the Ningxia Hui Autonomous Region have made Marselan wines their main product^[4]. Wine is a complex beverage that is influenced by various natural and anthropogenic factors throughout the wine-making process. These factors include soil, climate, agrochemicals, and human intervention. While there is an abundance of research available on wine production, limited research has been conducted specifically on local wines in the Eastern Foot of Helan Mountain. This research gap is of significant importance for the management and quality improvement of Chinese local wines.

Ion mobility spectrometry (IMS) is a rapid analytical technique used to detect trace gases and characterize chemical ionic substances. It achieves this through the gas-phase separation of ionized molecules under an electric field at ambient pressure. In recent years, IMS has gained increasing popularity in the field of food-omics due to its numerous advantages. These advantages include ultra-high analytical speed, simplicity, easy operation, time efficiency, relatively low cost, and the absence of sample preparation steps. As a result, IMS is now being applied more frequently in various areas of food analysis, such as food composition and nutrition, food authentication,

detection of food adulteration, food process control, and chemical food safety^[5,6]. The orthogonal hyphenation of gas chromatography (GC) and IMS has greatly improved the resolution of complex food matrices when using GC-IMS, particularly in the analysis of wines^[7].

The objective of this study was to investigate the changes in the physicochemical properties of Marselan wine during the winemaking process, with a focus on the total phenolic and flavonoids content, antioxidant activity, and volatile profile using the GC-IMS method. The findings of this research are anticipated to make a valuable contribution to the theoretical framework for evaluating the authenticity and characterizing Ningxia Marselan wine. Moreover, it is expected that these results will aid in the formulation of regulations and legislation pertaining to Ningxia Marselan wine in China.

Materials and methods

Plant materials

All the grapes used to produce Marselan wines, grow in the Xiban vineyard (106.31463° E and 38.509541° N) situated in Helan Mountain's East Foothill of Ningxia Hui Autonomous Region in China.

Chemicals and reagents

Folin-Ciocalteu reagent, (\pm)-6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,20-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ), anhydrous methanol, sodium nitrite, and sodium carbonate anhydrous were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Reference standards of (+)-catechin, gallic acid, and the internal standard (IS) 4-methyl-2-pentanol were supplied by Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). The purity of the above references was higher than 98%. Ultra-pure water (18.2 M Ω cm) was prepared by a Milli-Q system (Millipore, Bedford, MA, USA).

Preparation of Marselan juices and wines

Stage 1—Juice processing: Grapes at the fully mature stage are harvested and crushed, and potassium metabisulfite (5 mg/L of SO₂) was evenly spread during the crushing process. The obtained must is transferred into stainless steel tanks. Stage 2—Alcoholic fermentation: Propagated *Saccharomyces cerevisiae* ES488 (Enartis, Italy) are added to the fresh must, and alcoholic fermentation takes place, after the process is finished, it is kept in the tanks for 7 d for traditional maceration to improve color properties and phenolics content. Stage 3—Malolactic fermentation: When the pomace is fully concentrated at the bottom of the tanks, the wine is transferred to another tank for separation from these residues. *Oenococcus oeni* VP41 (Lallemand Inc., France) is inoculated and malic acid begins to convert into lactic acid. Stage 4—Wine stabilization: After malolactic fermentation, potassium metabisulfite is re-added (35 mg/L of SO₂), and then transferred to oak barrels for stabilization, this process usually takes 6-24 months. A total of four batches of samples during the production process of Marselan wine were collected in this study.

Total polyphenols and flavonoid content determination

Total polyphenols were determined on 0.5 mL diluted wine sample using the Folin-Ciocalteu method^[8], using gallic acid as

a reference compound, and expressed as milligrams of gallic acid equivalents per liter of wine. The total flavonoid content was measured on 0.05 mL of wine sample by a colorimetric method previously described^[9]. Results are calculated from the calibration curve obtained with catechin, as milligrams of catechin equivalents per liter of wine.

ABTS free-radical scavenging assay

The antioxidative activity was determined using the ABTS⁺ assay^[10]. Briefly, the ABTS⁺ radical was prepared from a mixture of 88 μ L of potassium persulfate (140 mmol/L) with 5 mL of the ABTS⁺ solution (7 mmol/L). The reaction was kept at room temperature under the absence of light for 16 h. Sixty μ L samples were mixed with 3 mL of ABTS⁺ solution with measured absorption of 0.700 ± 0.200 at 734 nm. After 6 min reaction, the absorbance of samples were measured with a spectrophotometer at 734 nm. Each sample was tested in triplicate. The data were expressed as mmol Trolox equivalent of antioxidative capacity per liter of the wine sample (mmol TE/L). Calibration curves, in the range 64.16–1,020.20 μ mol TE/L, showed good linearity ($R^2 \geq 0.99$).

FRAP free-radical scavenging assay

The FRAP assay was conducted according to a previous study^[11]. The FRAP reagent was freshly prepared and mixed with 10 mM/L TPTZ solution prepared in 20 mM/L FeCl₃·6H₂O solution, 40 mM/L HCl, and 300 mM/L acetate buffer (pH 3.6) (1:1:10; v:v:v). Ten ml of diluted sample was mixed with 1.8 ml of FRAP reagent and incubated at 37 °C for 30 min. The absorbance was determined at 593 nm and the results were reported as mM Fe (II) equivalent per liter of the wine sample. The samples were analyzed and calculated by a calibration curve of ferrous sulphate (0.15–2.00 mM/mL) for quantification.

HS-GC-IMS analysis

The volatile compounds were analyzed on a GC-IMS instrument (FlavourSpec, GAS, Dortmund, Germany) equipped with an autosampler (Hanon Auto SPE 100, Shandong, China) for headspace analysis. One mL of each wine was sampled in 20 mL headspace vials (CNW Technologies, Germany) with 20 μ L of 4-methyl-2-pentanol (20 mg/L) ppm as internal standard, incubated at 60 °C and continuously shaken at 500 rpm for 10 min. One hundred μ L of headspace sample was automatically loaded into the injector in splitless mode through a syringe heated to 65 °C. The analytes were separated on a MxtWAX capillary column (30 m \times 0.53 mm, 1.0 μ m) from Restek (Bellefonte, Pennsylvania, USA) at a constant temperature of 60 °C and then ionized in the IMS instrument (FlavourSpec®, Gesellschaft für Analytische Sensorsysteme mbH, Dortmund, Germany) at 45 °C. High purity nitrogen gas (99.999%) was used as the carrier gas at 150 mL/min, and drift gas at 2 mL/min for 0–2.0 min, then increased to 100 mL/min from 2.0 to 20 min, and kept at 100 mL/min for 10 min. Ketones C₄–C₉ (Sigma Aldrich, St. Louis, MO, USA) were used as an external standard to determine the retention index (RI) of volatile compounds. Analyte identification was performed using a Laboratory Analytical Viewer (LAV) 2.2.1 (GAS, Dortmund, Germany) by comparing RI and the drift time of the standard in the GC-IMS Library.

Statistical data analysis

All samples were prepared in duplicate and tested at least six times, and the results were expressed as mean \pm standard

error ($n = 4$) and the level of statistical significance ($p < 0.05$) was analyzed by using Tukey's range test using SPSS 18.0 software (SPSS Inc., IL, USA). The principal component analysis (PCA) was performed using the LAV software in-built 'Dynamic PCA' plug-in to model patterns of aroma volatiles. Orthogonal partial least-square discriminant analysis (OPLS-DA) in SIMCA-P 14.1 software (Umetrics, Umeå, Sweden) was used to analyze the different volatile organic compounds in the different fermentation stages.

Results

Changes in total polyphenol and flavonoid contents and antioxidant activity during fermentation

The results of the changes in the antioxidant activity of Marselan wines during the entire brewing process are listed in Table 1. It can be seen that the contents of flavonoids and polyphenols showed an increasing trend during the brewing process of Marselan wine, which range from 315.71–1,498 mg CE/L and 1,083.93–3,370.92 mg GAE/L, respectively. It was observed that the content increased rapidly in the alcoholic fermentation stage, but slowly in the subsequent fermentation stage. This indicated that the formation of flavonoid and phenolic substances in wine mainly concentrated in the alcoholic fermentation stage, which is consistent with previous reports. This is mainly because during the alcoholic fermentation of grapes, impregnation occurred to extract these compounds^[12]. The antioxidant activities of Marselan wine samples at different fermentation stages were detected by FRAP and ABTS methods^[11]. The results showed that the ferric reduction capacity and ABST⁺ free radical scavenging capacity of the fermented Marselan wines were 2.4 and 1.5 times higher than the sample from the juice processing stage, respectively, indicating that the fermented Marselan wine had higher antioxidant activity. A large number of previous studies have suggested that there is a close correlation between antioxidant activity and the content of polyphenols and flavonoids^[13–15]. Previous studies have reported that Marselan wine has the highest total phenol and anthocyanin content compared to the wine of Tannat, Cabernet Sauvignon, Merlot, Cabernet Franc, and Syrah^[13]. Polyphenols and flavonoids play an important role in improving human immunity. Therefore, Marselan wines are popular because of their high phenolic and flavonoid content and high antioxidant capacity.

Analysis of the change in volatile organic compounds (VOCs) during fermentation by GS-IMS

This study adopted the GC-IMS method to test the volatile organic compounds (VOCs) in the samples from the different fermentation stages of Marselan wine. Figure 1 shows the gas phase ion migration spectrum obtained, in which the ordinate represents the retention time of the gas chromatographic peaks and the abscissa represents the ion migration time (normalized)^[16]. The entire spectrum represents the aroma fingerprints of Marselan wine at different fermentation stages, with each signal point on the right of the relative reactant ion peak (RIP) representing a volatile organic compound detected from the sample^[17]. Here, the sample in stage 1 (juice processing) was used as a reference and the characteristic peaks in the spectrum of samples in other fermentation stages were

compared and analyzed after deducting the reference. The colors of the same component with the same concentration cancel each other to form a white background. In the topographic map of other fermentation stages, darker indicates higher concentration compared to the white background. In the 2D spectra of different fermentation stages, the position and number of peaks indicated that peak intensities are basically the same, and there is no obvious difference. However, it is known that fermentation is an extremely complex chemical process, and the content and types of volatile organic compounds change with the extension of fermentation time, so other detection and characterization methods are needed to make the distinction.

Fingerprint analysis of VOCs at different fermentation stages of Marselan wine

To visually display the dynamic changes of various substances in the fermentation process of Marselan wine, peaks with obvious differences were extracted to form the characteristic fingerprints for comparison (Fig. 2). Each row represents all signal peaks selected from samples at the same stage, and each column means the signal peaks of the same volatile compound in samples from different fermentation stages. Figure 2 shows the volatile organic compounds (VOCs) information for each sample and the differences between samples, where the numbers represent the undetermined substances in the migration spectrum library. The changes of volatile substances in the process of Marselan winemaking is observed by the fingerprint. As shown in Fig. 2 and Table 2, a total of 40 volatile chemical components were detected by qualitative analysis according to their retention time and ion migration time in the HS-GC-IMS spectrum, including 17 esters, eight alcohols, eight aldehydes, two ketones, one organic acid, and four unanalyzed flavor substances. The 12 volatile organic compounds presented dimer due to ionization of the protonated neutral components before entering the drift tube^[18]. As can be seen from Table 2, the VOCs in the winemaking process of Marselan wine are mainly composed of esters, alcohols, and aldehydes, which play an important role in the construction of aroma characteristics.

Esters are produced by the reaction of acids and alcohols in wine, mainly due to the activity of yeast during fermentation^[19], and are the main components of fruit juices and wines that produce fruit flavors^[20,21]. In this study, it was found that they were the largest detected volatile compound group in Marselan wine samples, which is consistent with previous reports^[22]. It can be observed from Table 2 that the contents of most esters increased gradually with the extension of fermentation time, and they mainly began to accumulate in large quantities during the stage of alcohol fermentation. The contents of ethyl hexanoate (fruity), isoamyl acetate (banana, pear), ethyl octanoate (fruity, pineapple, apple, brandy), ethyl acetate (fruity), ethyl formate (spicy, pineapple), and ethyl butanoate (sweet, pineapple, banana, apple) significantly increased at the stage of alcoholic fermentation and maintained a high level in the subsequent fermentation stage (accounting for 86% of the total detected esters). These esters can endow a typical fruity aroma of Marselan wine, and played a positive role in the aroma profiles of Marselan wine. Among them, the content of ethyl acetate is the highest, which is 5,153.79 $\mu\text{g/mL}$ in the final fermentation stage, accounting for

Table 1. GC-IMS integration parameters of volatile compounds in Marselan wine at different fermentation stages.

No.	Compounds	Formula	RI*	Rt [sec]**	Dt [RIPrel]***	Identification approach	Concentration (µg/mL) (n = 4)			
							Stage 1	Stage 2	Stage 3	Stage 4
Aldehydes										
5	Furfural	C ₅ H ₄ O ₂	1513.1	941.943	1.08702	RI, DT, IS	89.10 ± 4.05 ^c	69.98 ± 3.22 ^c	352.16 ± 39.06 ^b	706.30 ± 58.22 ^a
6	Furfural dimer	C ₅ H ₄ O ₂	1516.6	948.77	1.33299	RI, DT, IS	22.08 ± 0.69 ^b	18.68 ± 2.59 ^c	23.73 ± 2.69 ^b	53.39 ± 9.42 ^a
12	(E)-2-hexenal	C ₆ H ₁₀ O	1223.1	426.758	1.18076	RI, DT, IS	158.17 ± 7.26 ^a	47.57 ± 2.51 ^b	39.00 ± 2.06 ^c	43.52 ± 4.63 ^{bc}
17	(E)-2-pentenal	C ₅ H ₈ O	1129.2	333.392	1.1074	RI, DT, IS	23.00 ± 4.56 ^a	16.42 ± 1.69 ^c	18.82 ± 0.27 ^b	18.81 ± 0.55 ^b
19	Heptanal	C ₇ H ₁₄ O	1194.2	390.299	1.33002	RI, DT, IS	17.28 ± 2.25 ^a	10.22 ± 0.59 ^c	14.50 ± 8.84 ^b	9.11 ± 1.06 ^c
22	Hexanal	C ₆ H ₁₂ O	1094.6	304.324	1.25538	RI, DT, IS	803.11 ± 7.47 ^c	1631.34 ± 19.63 ^a	1511.11 ± 26.91 ^b	1526.53 ± 8.12 ^b
23	Hexanal dimer	C ₆ H ₁₂ O	1093.9	303.915	1.56442	RI, DT, IS	588.85 ± 7.96 ^a	93.75 ± 4.67 ^b	92.93 ± 3.13 ^b	95.49 ± 2.50 ^b
29	3-Methylbutanal	C ₅ H ₁₀ O	914.1	226.776	1.40351	RI, DT, IS	227.86 ± 6.39 ^a	33.32 ± 2.59 ^b	22.36 ± 1.18 ^c	21.94 ± 1.73 ^c
33	Dimethyl sulfide	C ₂ H ₆ S	797.1	193.431	0.95905	RI, DT, IS	120.07 ± 4.40 ^c	87.00 ± 3.82 ^d	246.81 ± 5.62 ^b	257.18 ± 3.04 ^a
49	2-Methylpropanal	C ₄ H ₈ O	828.3	202.324	1.28294	RI, DT, IS	150.49 ± 7.13 ^a	27.08 ± 1.48 ^b	19.36 ± 1.10 ^c	19.69 ± 0.92 ^c
Ketones										
45	3-Hydroxy-2-butanone	C ₄ H ₈ O ₂	1293.5	515.501	1.20934	RI, DT, IS	33.20 ± 3.83 ^c	97.93 ± 8.72 ^b	163.20 ± 21.62 ^a	143.51 ± 21.48 ^a
46	Acetone	C ₃ H ₆ O	836.4	204.638	1.11191	RI, DT, IS	185.75 ± 8.16 ^c	320.43 ± 12.32 ^b	430.74 ± 3.98 ^a	446.58 ± 10.41 ^a
Organic acid										
3	Acetic acid	C ₂ H ₄ O ₂	1527.2	969.252	1.05013	RI, DT, IS	674.66 ± 46.30 ^d	3602.39 ± 30.87 ^c	4536.02 ± 138.86 ^a	4092.30 ± 40.33 ^b
4	Acetic acid dimer	C ₂ H ₄ O ₂	1527.2	969.252	1.15554	RI, DT, IS	45.25 ± 3.89 ^c	312.16 ± 19.39 ^b	625.79 ± 78.12 ^a	538.35 ± 56.38 ^a
Alcohols										
8	1-Hexanol	C ₆ H ₁₄ O	1365.1	653.825	1.32772	RI, DT, IS	1647.65 ± 28.94 ^a	886.33 ± 32.96 ^b	740.73 ± 44.25 ^c	730.80 ± 21.58 ^c
9	1-Hexanol dimer	C ₆ H ₁₄ O	1365.8	655.191	1.64044	RI, DT, IS	378.42 ± 20.44 ^a	332.65 ± 25.76 ^a	215.78 ± 21.04 ^b	200.14 ± 28.34 ^b
13	3-Methyl-1-butanol	C ₅ H ₁₂ O	1213.3	414.364	1.24294	RI, DT, IS	691.86 ± 9.95 ^c	870.41 ± 22.63 ^b	912.80 ± 23.94 ^a	939.49 ± 12.44 ^a
14	3-Methyl-1-butanol dimer	C ₅ H ₁₂ O	1213.3	414.364	1.49166	RI, DT, IS	439.90 ± 29.40 ^c	8572.27 ± 60.56 ^b	9083.14 ± 193.19 ^a	9152.25 ± 137.80 ^a
15	1-Butanol	C ₄ H ₁₀ O	1147.2	348.949	1.18073	RI, DT, IS	157.33 ± 9.44 ^b	198.92 ± 3.92 ^a	152.78 ± 10.85 ^b	156.02 ± 9.80 ^b
16	1-Butanol dimer	C ₄ H ₁₀ O	1146.8	348.54	1.38109	RI, DT, IS	24.14 ± 2.15 ^c	274.75 ± 12.60 ^a	183.02 ± 17.72 ^b	176.80 ± 19.80 ^b
24	1-Propanol	C ₃ H ₈ O	1040.9	274.803	1.11042	RI, DT, IS	173.73 ± 4.75 ^a	55.84 ± 2.16 ^c	80.80 ± 4.99 ^b	83.57 ± 2.34 ^b
25	1-Propanol dimer	C ₃ H ₈ O	1040.4	274.554	1.24784	RI, DT, IS	58.20 ± 1.30 ^b	541.37 ± 11.94 ^a	541.33 ± 15.57 ^a	538.84 ± 9.74 ^a
28	Ethanol	C ₂ H ₆ O	930.6	231.504	1.11901	RI, DT, IS	5337.84 ± 84.16 ^c	11324.05 ± 66.18 ^a	9910.20 ± 100.76 ^b	9936.10 ± 101.24 ^b
34	Methanol	CH ₄ O	903.6	223.79	0.98374	RI, DT, IS	662.08 ± 13.87 ^a	76.94 ± 2.15 ^b	61.92 ± 1.96 ^c	62.89 ± 0.81 ^c
37	2-Methyl-1-propanol	C ₄ H ₁₀ O	1098.5	306.889	1.35839	RI, DT, IS	306.91 ± 4.09 ^c	3478.35 ± 25.95 ^a	3308.79 ± 61.75 ^b	3313.85 ± 60.88 ^b
48	1-Pentanol	C ₅ H ₁₂ O	1257.6	470.317	1.25222	RI, DT, IS	26.13 ± 2.52 ^c	116.50 ± 3.71 ^{ab}	112.37 ± 6.26 ^b	124.17 ± 7.04 ^a
Esters										
1	Methyl salicylate	C ₈ H ₈ O ₃	1859.6	1616.201	1.20489	RI, DT, IS	615.00 ± 66.68 ^a	485.08 ± 31.30 ^b	470.14 ± 23.02 ^b	429.12 ± 33.74 ^b
7	Butyl hexanoate	C ₁₀ H ₂₀ O ₂	1403.0	727.561	1.47354	RI, DT, IS	95.83 ± 17.04 ^a	62.87 ± 3.62 ^a	92.59 ± 11.88 ^b	82.13 ± 3.61 ^c
10	Hexyl acetate	C ₈ H ₁₆ O ₂	1298.6	524.366	1.40405	RI, DT, IS	44.72 ± 8.21 ^a	33.18 ± 2.17 ^d	41.50 ± 4.38 ^c	40.89 ± 4.33 ^b
11	Propyl hexanoate	C ₉ H ₁₈ O ₂	1280.9	499.577	1.39274	RI, DT, IS	34.65 ± 3.90 ^d	70.43 ± 5.95 ^a	43.97 ± 4.39 ^b	40.12 ± 4.05 ^c
18	Ethyl hexanoate	C ₈ H ₁₆ O ₂	1237.4	444.749	1.80014	RI, DT, IS	55.55 ± 5.62 ^c	1606.16 ± 25.63 ^a	787.24 ± 16.95 ^b	788.91 ± 28.50 ^b
20	Isoamyl acetate	C ₇ H ₁₄ O ₂	1127.8	332.164	1.30514	RI, DT, IS	164.22 ± 1.00 ^d	243.69 ± 8.37 ^c	343.51 ± 13.98 ^b	365.46 ± 1.60 ^a
21	Isoamyl acetate dimer	C ₇ H ₁₄ O ₂	1126.8	331.345	1.75038	RI, DT, IS	53.61 ± 4.79 ^d	4072.20 ± 11.94 ^a	2416.70 ± 49.84 ^b	2360.46 ± 43.29 ^c
26	Isobutyl acetate	C ₆ H ₁₂ O ₂	1020.5	263.605	1.23281	RI, DT, IS	101.65 ± 1.81 ^a	15.52 ± 0.67 ^c	44.87 ± 3.21 ^b	45.96 ± 1.41 ^b
27	Isobutyl acetate dimer	C ₆ H ₁₂ O ₂	1019.6	263.107	1.61607	RI, DT, IS	34.60 ± 1.05 ^d	540.84 ± 5.64 ^a	265.54 ± 8.31 ^c	287.06 ± 3.66 ^b
30	Ethyl acetate dimer	C ₄ H ₈ O ₂	885.2	218.564	1.33587	RI, DT, IS	1020.75 ± 6.86 ^d	5432.71 ± 6.55 ^a	5052.99 ± 9.65 ^b	5084.47 ± 7.30 ^c
31	Ethyl acetate	C ₄ H ₈ O ₂	878.3	216.574	1.09754	RI, DT, IS	215.65 ± 3.58 ^a	38.29 ± 2.37 ^c	71.59 ± 2.99 ^b	69.32 ± 2.85 ^b
32	Ethyl formate	C ₃ H ₆ O ₂	838.1	205.127	1.19738	RI, DT, IS	175.48 ± 3.79 ^d	1603.20 ± 13.72 ^a	1472.10 ± 5.95 ^c	1509.08 ± 13.26 ^b
35	Ethyl octanoate	C ₁₀ H ₂₀ O ₂	1467.0	852.127	1.47312	RI, DT, IS	198.86 ± 36.71 ^b	1853.06 ± 17.60 ^a	1555.51 ± 24.21 ^a	1478.05 ± 33.63 ^a
36	Ethyl octanoate dimer	C ₁₀ H ₂₀ O ₂	1467.0	852.127	2.03169	RI, DT, IS	135.50 ± 13.02 ^d	503.63 ± 15.86 ^a	342.89 ± 11.62 ^b	297.28 ± 14.40 ^c
38	Ethyl butanoate	C ₆ H ₁₂ O ₂	1042.1	275.479	1.5664	RI, DT, IS	21.29 ± 2.68 ^c	1384.67 ± 8.97 ^a	1236.52 ± 20.21 ^b	1228.09 ± 5.09 ^b
39	Ethyl 3-methylbutanoate	C ₇ H ₁₄ O ₂	1066.3	288.754	1.26081	RI, DT, IS	9.70 ± 1.85 ^d	200.29 ± 4.21 ^a	146.87 ± 8.70 ^b	127.13 ± 12.54 ^c
40	Propyl acetate	C ₅ H ₁₀ O ₂	984.7	246.908	1.48651	RI, DT, IS	4.57 ± 1.07 ^c	128.63 ± 4.28 ^a	87.75 ± 3.26 ^b	88.49 ± 1.99 ^b
41	Ethyl propanoate	C ₅ H ₁₀ O ₂	962.1	240.47	1.46051	RI, DT, IS	10.11 ± 0.34 ^d	107.08 ± 3.50 ^a	149.60 ± 5.39 ^c	167.15 ± 12.90 ^b
42	Ethyl isobutyrate	C ₆ H ₁₂ O ₂	971.7	243.229	1.56687	RI, DT, IS	18.29 ± 2.61 ^d	55.22 ± 1.07 ^c	98.81 ± 4.67 ^b	104.71 ± 4.73 ^a
43	Ethyl lactate	C ₅ H ₁₀ O ₃	1352.2	628.782	1.14736	RI, DT, IS	31.81 ± 2.91 ^c	158.03 ± 2.80 ^b	548.14 ± 74.21 ^a	527.01 ± 39.06 ^a
44	Ethyl lactate dimer	C ₅ H ₁₀ O ₃	1351.9	628.056	1.53618	RI, DT, IS	44.55 ± 2.03 ^c	47.56 ± 4.02 ^c	412.23 ± 50.96 ^a	185.87 ± 31.25 ^b
47	Ethyl heptanoate	C ₉ H ₁₈ O ₂	1339.7	604.482	1.40822	RI, DT, IS	39.55 ± 6.37 ^a	38.52 ± 2.47 ^a	28.44 ± 1.52 ^c	30.77 ± 2.79 ^b
Unknown										
1						RI, DT, IS	15.53 ± 0.18	35.69 ± 0.80	12.70 ± 0.80	10.57 ± 0.86
2						RI, DT, IS	36.71 ± 1.51	120.41 ± 3.44	198.12 ± 6.01	201.19 ± 3.70
3						RI, DT, IS	44.35 ± 0.88	514.12 ± 4.28	224.78 ± 6.56	228.32 ± 4.62
4						RI, DT, IS	857.64 ± 8.63	33.22 ± 1.99	35.05 ± 5.99	35.17 ± 3.97

* Represents the retention index calculated using n-kenones C4–C9 as external standard on MAX-WAX column. ** Represents the retention time in the capillary GC column. *** Represents the migration time in the drift tube.

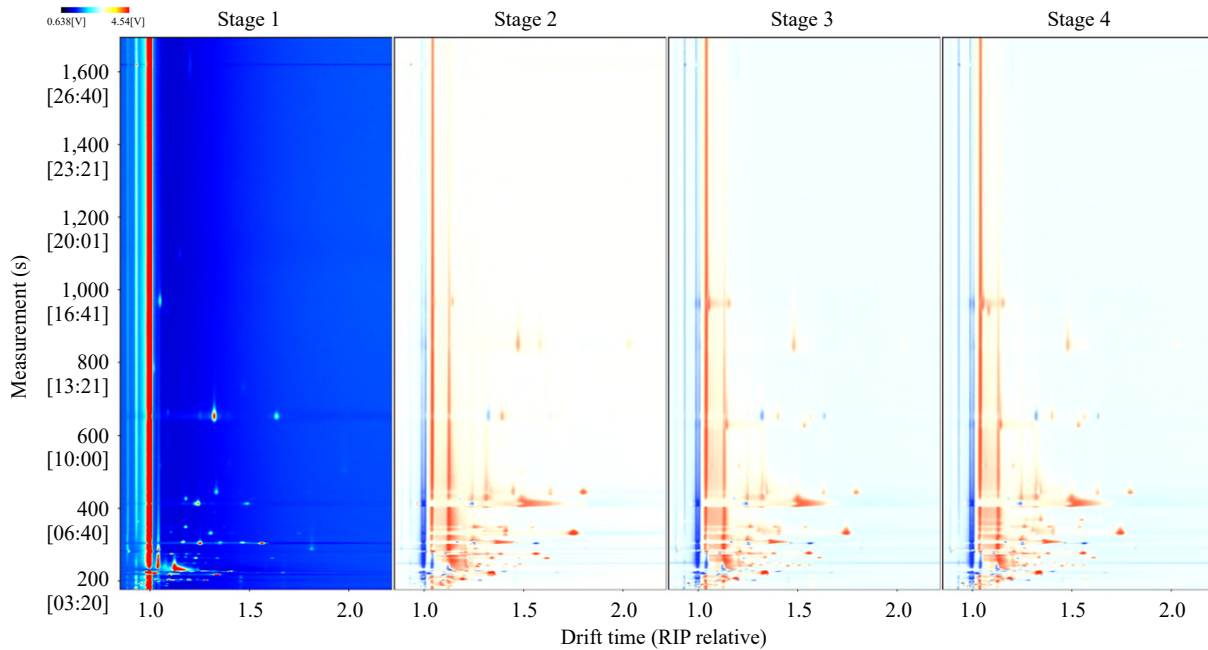


Fig. 1 2D-topographic plots of volatile organic compounds in Marselan wine at different fermentation stages.

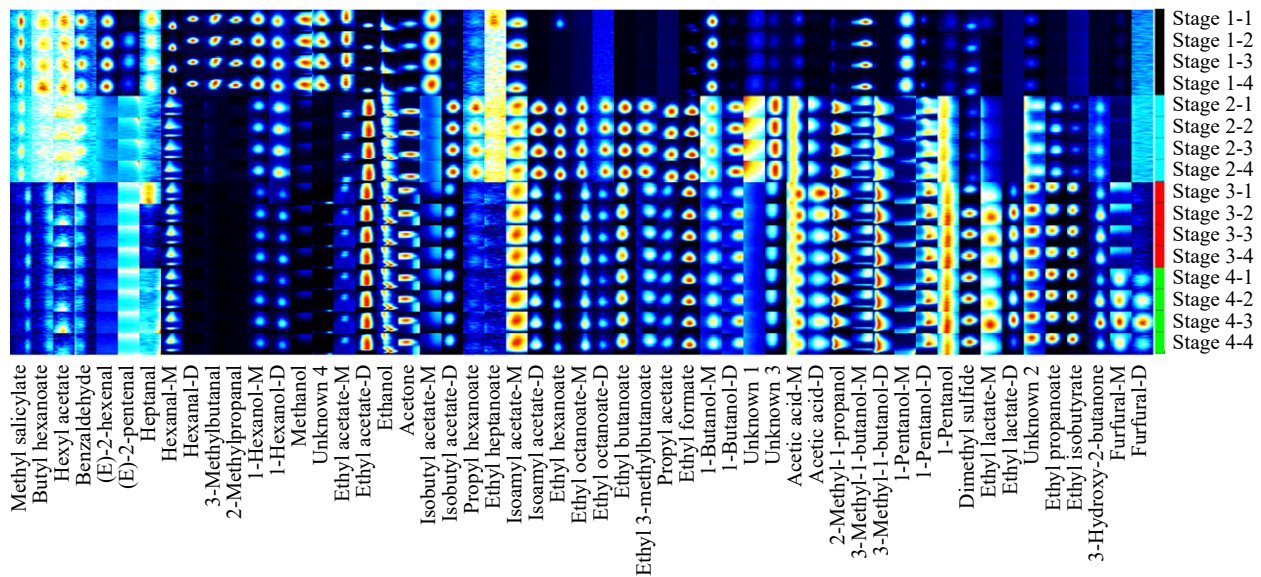


Fig. 2 Fingerprints of volatile organic compounds in Marselan wine at different fermentation stages.

Table 2. Antioxidant activity, total polyphenols, and flavonoids content of Marselan wine at different fermentation stages.

Winemaking stage	TFC (mg CE/L)	TPC (mg GAE/L)	FRAP (mM FeSO ₄ /mL)	ABTs (mM Trolox/L)
Stage 1	315.71 ± 0.00 ^d	1,083.93 ± 7.79 ^d	34.82 ^c	38.92 ± 2.12 ^c
Stage 2	1,490.00 ± 7.51 ^c	3,225.51 ± 53.27 ^c	77.32 ^b	52.17 ± 0.95 ^b
Stage 3	1,510.00 ± 8.88 ^a	3,307.143 ± 41.76 ^b	77.56 ^b	53.04 ± 0.76 ^b
Stage 4	1,498.57 ± 6.34 ^b	3,370.92 ± 38.29 ^a	85.07 ^a	57.46 ± 2.55 ^a

Means in the same column with different letters are significantly different ($p < 0.05$).

33.6% of the total ester. However, the content of ethyl acetate was relatively high before fermentation, which may be from the metabolic activity of autochthonous microorganisms present in the raw materials. Isobutyl acetate, ethyl 3-methyl butanoate,

propyl acetate, ethyl propanoate, ethyl isobutyrate, and ethyl lactate were identified and quantified in all fermentation samples. The total contents of these esters in stage 1 and 4 were 255.28 and 1,533.38 µg/mL, respectively, indicating that

they may also have a potential effect on the aroma quality of Marselan wine. The results indicate that esters are an important factor in the formation of flavor during the brewing process of Marselan wine.

Alcohols were the second important aromatic compound in Marselan wine, which were mainly synthesized by glucose and amino acid decomposition during alcoholic fermentation^[23,24]. According to Table 2, eight alcohols including methanol, ethanol, propanol, butanol, hexanol, amyl alcohol, 3-methyl-1-butanol, and 2-methyl-1-propanol were detected in the four brewing stages of Marselan wine. The contents of ethanol (slightly sweet), 3-methyl-1-butanol (apple, brandy, spicy), and 2-methyl-1-propanol (whiskey) increased gradually during the fermentation process. The sum of these alcohols account for 91%–92% of the total alcohol content, which is the highest content of three alcohols in Marselan wine, and may be contributing to the aromatic and clean-tasting wines. On the contrary, the contents of 1-hexanol and methanol decreased gradually in the process of fermentation. Notably, the content of these rapidly decreased at the stage of alcoholic fermentation, from 2,026.07 to 1,218.98 $\mu\text{g/mL}$ and 662.08 to 76.94 $\mu\text{g/mL}$, respectively, which may be ascribed to volatiles changed from alcohols to esters throughout fermentation. The reduction of the concentration of some alcohols also alleviates the strong odor during wine fermentation, which plays an important role in the improvement of aroma characteristics.

Acids are mainly produced by yeast and lactic acid bacteria metabolism at the fermentation stage and are considered to be an important part of the aroma of wine^[22]. Only one type of acid (acetic acid) was detected in this experiment, which was less than previously reported, which may be related to different brewing processes. Acetic acid content is an important factor in the balance of aroma and taste of wine. Low contents of volatile acids can provide a mild acidic smell in wine, which is widely considered to be ideal for producing high-quality wines. However, levels above 700 $\mu\text{g/mL}$ can produce a pungent odor and weaken the wine's distinctive flavor^[25]. The content of acetic acid increased first and then decreased during the whole fermentation process. The content of acetic acid increased rapidly in the second stage, from 719.91 to 3,914.55 $\mu\text{g/mL}$ reached a peak in the third stage (5,161.81 $\mu\text{g/mL}$), and decreased to 4,630.65 $\mu\text{g/mL}$ in the last stage of fermentation. Excessive acetic acid in Marselan wine may have a negative impact on its aroma quality.

It was also found that the composition and content of aldehydes produced mainly through the catabolism of amino acids or decarboxylation of ketoacid were constantly changing during the fermentation of Marselan wines. Eight aldehydes, including furfural, hexanal, heptanal, 2-methylpropanal, 3-methylbutanal, dimethyl sulfide, (E)-2-hexenal, and (E)-2-pentenal were identified in all stage samples. Among them, furfural (caramel bread flavor) and hexanal (grass flavor) are the main aldehydes in Marselan wine, and the content increases slightly with the winemaking process. While other aldehydes such as (E)-2-hexenal (green and fruity), 3-methylbutanal (fresh and malt), and 2-methylpropanal (fresh and malt) were decomposed during brewing, reducing the total content from 536.52 to 85.15 $\mu\text{g/mL}$, which might potently affect the final flavor of the wine. Only two ketones, acetone, and 3-hydroxy-2-butanone, were detected in the wine samples, and their contents had no significant difference in the fermentation process, which might not affect the flavor of the wine.

Multivariate statistical analysis

To more intuitively analyze the differences of volatile organic compounds in different brewing stages of Marselan wine samples, principal component analysis was performed^[26–28]. As presented in Fig. 3, the points corresponding to one sample group were clustered closely on the score plot, while samples at different fermentation stages were well separated in the plot. PC1 (79%) and PC2 (18%) together explain 97% of the total variance between Marselan wine samples, indicating significant changes in volatile compounds during the brewing process. As can be seen from the results in Fig. 3, samples of stages 1, 2, and 3 can be distinguished directly by PCA, suggesting that there are significant differences in aroma components in these three fermentation stages. Nevertheless, the separation of stage 3 and stage 4 samples is not very obvious and both presented in the same quadrant, which means that their volatile characteristics were highly similar, indicating that the volatile components of Marselan wine are formed in stage 3 during fermentation (Fig. S1). The above results prove that the unique aroma fingerprints of the samples from the distinct brewing stages of Marselan wine were successfully constructed using the HS-GC-IMS method.

Based on the results of the PCA, OPLS-DA was used to eliminate the influence of uncontrollable variables on the data through permutation test, and to quantify the differences between samples caused by characteristic flavors^[28]. Figure 4 revealed that the point of flavor substances were colored according to their density and the samples obtained at different fermentation stages of wine have obvious regional characteristics and good spatial distribution. In addition, the reliability of the OPLS-DA model was verified by the permutation method of "Y-scrambling" validation. In this method, the values of the Y variable were randomly arranged 200 times to re-establish and analyze the OPLS-DA model. In general, the values of R^2 (y) and Q^2 were analyzed to assess the predictability and applicability of the model. The results of the reconstructed model illustrate that the slopes of R^2 and Q^2 regression lines were both greater than 0, and the intercept of the Q^2 regression line was -0.535 which is less than 0 (Fig. 5). These results indicate that the OPLS-DA model is reliable and there is no fitting phenomenon, and this model can be used to distinguish the four brewing stages of Marselan wine.

VIP is the weight value of OPLS-DA model variables, which was used to measure the influence intensity and explanatory ability of accumulation difference of each component on classification and discrimination of each group of samples. In

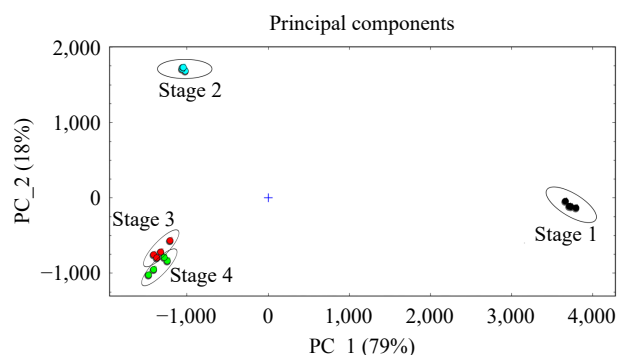


Fig. 3 PCA based on the signal intensity obtained with different fermentation stages of Marselan wine.

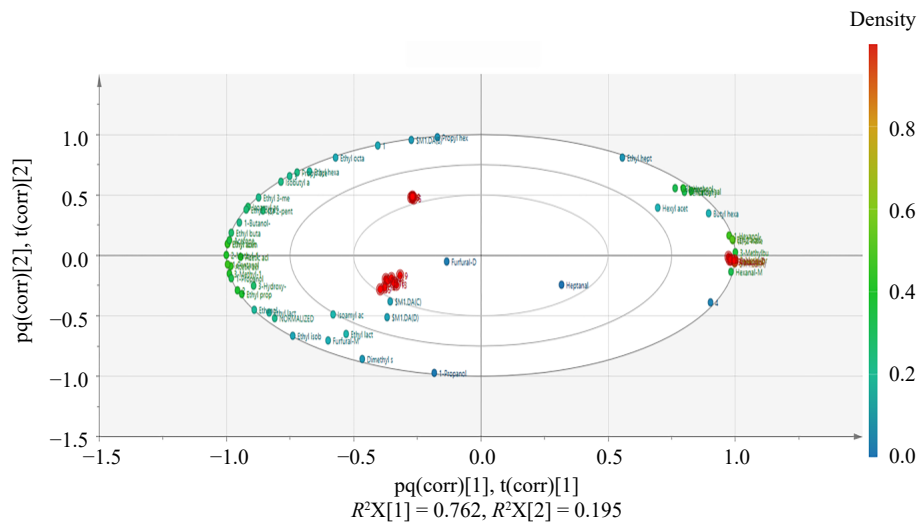


Fig. 4 Scores plot of OPLS-DA model of volatile components in Marselan wine at different fermentation stages.

previous studies, $VIP > 1$ is usually used as a screening criterion for differential volatile substances [28–30]. In this study, a total of 22 volatile substances had VIP values above 1, indicating that these volatiles could function as indicators of Marselan wine

maturity during fermentation (see Fig. 6). These volatile compounds included furfural, ethyl lactate, heptanal, dimethyl sulfide, 1-propanol, ethyl isobutyrate, propyl acetate, isobutyl acetate, ethanol, ethyl hexanoate, acetic acid, methanol, ethyl formate, ethyl 3-methylbutanoate, ethyl acetate, hexanal, isoamyl acetate, 2-methylpropanal, 2-methyl-1-propanol, and three unknown compounds.

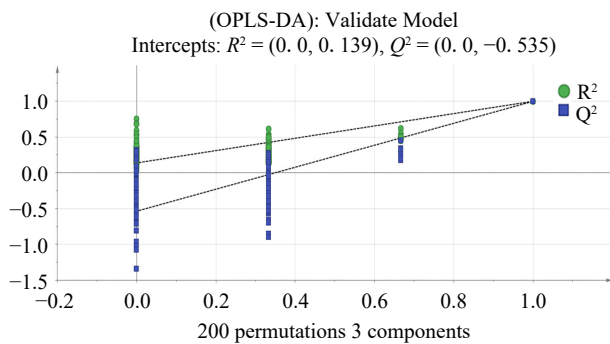


Fig. 5 Permutation test of OPLS-DA model of volatile components in Marselan wine at different fermentation stages ($n = 200$).

Conclusions

This study focuses on the change of volatile flavor compounds and antioxidant activity in Marselan wine during different brewing stages. A total of 40 volatile aroma compounds were identified and collected at different stages of Marselan winemaking. The contents of volatile aroma substances varied greatly at different stages, among which alcohols and esters were the main odors in the fermentation stage. The proportion of furfural was small, but it has a big influence on the wine flavor, which can be used as one of the standards to measure

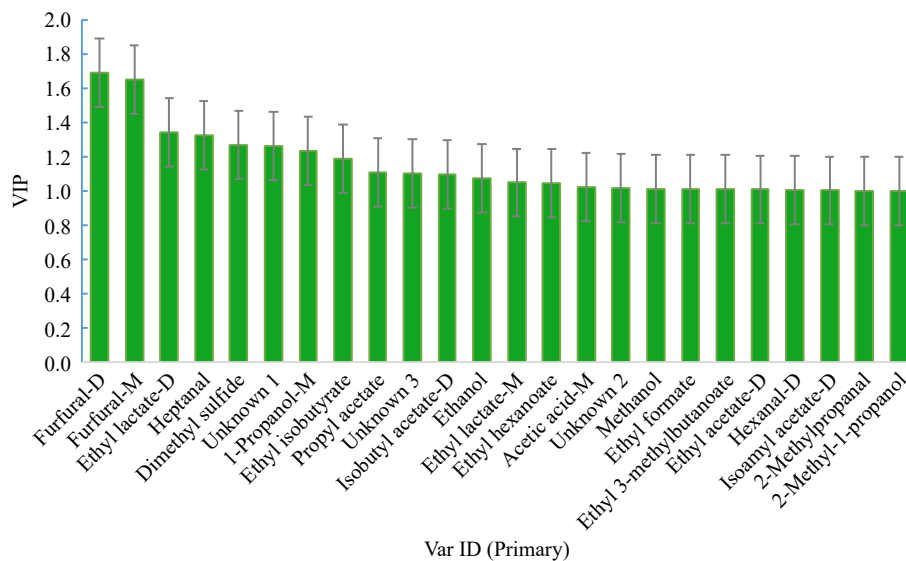


Fig. 6 VIP plot of OPLS-DA model of volatile components in Marselan wine at different fermentation stages.

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wine flavor. Flavonoids and phenols were not only factors of flavor formation, but also important factors to improve the antioxidant capacity of Marselan wine. In this study, the aroma of Marselan wines in different fermentation stages was analyzed, and its unique aroma fingerprint was established, which can provide accurate and scientific judgment for the control of the fermentation process endpoint, and has certain guiding significance for improving the quality of Marselan wines (Table S1). In addition, this work will provide a new approach for the production management of Ningxia's special wine as well as the development of the native Chinese wine industry.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Gong X, Fang L; data collection: Fang L, Li Y; analysis and interpretation of results: Qi N, Chen T; draft manuscript preparation: Fang L. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest.

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