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Using GC–O–MS, GC–IMS, and chemometrics to investigate flavor component succession regularity in the Niulanshan Erguotou Baijiu brewing process

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ABSTRACT

The volatile compounds in Dacha liquor (DL) and Ercha liquor (EL) from Niulanshan Erguotou Baijiu (NEB) were analyzed. The results demonstrated that a total of 34 odorants were identified. For the first time, the products of different brewing stages were analyzed using temperature-programmed headspace gas chromatography-ion mobility spectrometry (TP-HS-GC–IMS). The 3D fingerprint obtained revealed that the compounds exhibited different change patterns during the brewing process. Furthermore, the results of principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) revealed that hexanal, 3-hydroxy-2-butanone, trans-2-pentenal, and ethyl hexanoate could be used to distinguish different types of fermented grains; and hexanal, 1-pentanol, methyl isovalerate, isoamyl acetate, 3-hydroxy-2-butanone, ethyl hexanoate, ethyl acetate, ethyl 2-methylbutanoate, and ethyl pentanoate could be used to distinguish different types of distilled spirits. This study serves as a useful reference for enhancing quality control measures in the production of NEB.

1. Introduction

Baijiu, China's national liquor, is one of the world's six major distilled spirits, along with rum, gin, vodka, whiskey, and brandy (Liu & Sun, 2018). Light flavor Baijiu (LFB) belongs to 1 out of the 12 major flavor liquors in China. LFB has a rich history and remarkable floral and fruity flavor, making it widely popular among consumers (Wang, Ye, et al., 2022). Representative products of LFB include Niulanshan Erguotou, Hongxing Erguotou, Fenjiu, Baofeng Jiu, and Qingke Jiu. Niulanshan Erguotou Baijiu (NEB), with its clear flavor and refreshing taste, is highly favored by people in northern China (Wang, Ye, et al., 2022). The production process of NEB originates from the traditional Erguotou process (removing the head and tail, leaving the middle part of the liquor), with grains (e.g., sorghum) as raw materials, Daqu as the saccharification starter, Digang as the fermentation vessel, and *Zeng* as the distillation device.

The fermentation cycle of NEB generally lasts 28–30 d. After fermentation, the fermented grains are removed and placed on a *Zeng* (Fig. S1) for distillation. During this process, most of the aroma compounds and alcohol in Jiupei (fermented grains) are distilled to form the liquor. In recent years, research has expanded beyond the analysis of flavor components in Baijiu to an increased focus on flavor compounds in Jiupei, with numerous studies investigating this aspect. Sun et al. (2016) used solvent-assisted flavor evaporation combined with a gas chromatography–mass spectrometer (GC–MS) to identify 148 volatile aroma compounds in Gujinggong Jiupei. Cao et al. (2010) used GC–MS to analyze the volatile aroma compounds in Fenjiu Jiupei. The authors discovered that the content of most aroma components in Jiupei tends to increase as fermentation progresses. Hu et al. (2014) identified a total of 82 substances in the Jiupei of strong flavored Baijiu, with esters and acids being the most abundant. Finally, Shang et al. (2016) investigated the volatile aroma compounds in Jiupei sauce flavor Baijiu using headspace-solid phase microextraction (HS-SPME) combined with GC–MS, and 32 aroma compounds were identified. Currently, there are

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few studies on the changes in volatile compounds during the Jiupei fermentation of NEB.

Ion mobility spectroscopy (IMS) is a novel analytical technique developed in recent years. IMS characterizes various chemical substances by measuring the mobility of gaseous ions to detect them. Unlike other techniques, the ionization of sample molecules under an electric field does not require a vacuum environment. This technique has high sensitivity and a rapid response time, which makes it a powerful tool for detecting changes in food under different conditions. He et al. (2021) used gas chromatography-ion mobility spectrometry (GC–IMS) to clearly identify the changes in volatile compounds at different stages of distillation. Their findings demonstrated that the majority of the compounds exhibited a downward trend throughout the distillation process. Zhou et al. (2021) used headspace and gas chromatography–ion mobility spectrometry (HS-GC–IMS) to establish the characteristic flavor profiles of five of Baiyunbian-aged liquors. The results indicated that the characteristic peaks of the flavor compounds in the GC–IMS 3D spectra could effectively reveal the differences in flavor compounds among five Baiyunbian-aged liquors. Zhang et al. (2023) used GC–IMS and SPME-GC–MS to analyze the volatile components of 6 different flavors of Baijiu, and a total of 56 and 77 compounds were detected by GC–MS and GC–IMS, respectively. In summary, GC–IMS has been proven to be a potent method for monitoring changes in the volatile compound due to its intuitive nature. Up to now, there has been no relevant research using GC–IMS to comprehensively characterize the changes in volatile compounds during Jiupei fermentation.

Therefore, this study used temperature-programmed headspace gas chromatography–ion mobility spectrometry (TP-HS-GC–IMS) to analyze the volatile flavor components in the Jiupei of NEB at different brewing stages, and to elucidate the variations of these flavor components during the brewing process. Moreover, this study used liquid–liquid extraction and gas chromatography–olfactory mass spectrometry (GC–O–MS) to investigate the aroma active compounds in NEB distillate at different distillation stages. In addition, a combination of chemometric and analytical technologies was used to identify the aroma compounds that significantly contribute to the differences among different brewing stages. The study provided valuable insights into the brewing process of NEB and provided a reference for product quality control and process improvement in NEB.

2. Materials and methods

2.1. Samples

All samples were obtained from Niulanshan Distillery, Beijing Shunxin Co., Ltd. (Beijing, China). There were seven samples, including cooked grains (CG), Jiupei before the first fermentation (JBFF), Jiupei after the first fermentation (JAFF), Dacha liquor (DL, 64% ethanol by volume), Jiupei before the second fermentation (JBSF), Jiupei after the second fermentation (JASF), and Ercha liquor (EL, 60% ethanol by volume). The relationships among the seven samples are shown in Fig. 1. Jiupei (CG, JBFF, JAFF, JBSF, and JASF) were stored at −40 °C until analysis. Liquors (DL, EL) were stored at 4 °C before analysis.

2.2. Chemicals

Every chemical used in the analysis was of GC grade, with a purity of no less than 97%. Detailed specifications can be found in Table S1. Dichloromethane [high-performance liquid chromatography (HPLC) grade], ethanol (HPLC grade), sodium carbonate (AR), anhydrous sodium sulfate (AR), sodium chloride (AR), and hydrochloric acid (AR) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China).

2.3. Isolation of the volatiles

This methodology was adapted and refined from He et al. (2021). The DL and EL samples (25.0 mL) were each diluted with Milli-Q water (Millipore) to an ethanol volume fraction of 10% and thoroughly shaken. Subsequently, these diluted mixtures underwent 3 rounds of extraction using dichloromethane (50 mL for each extraction). The resultant organic layers, labeled O_1 and totaling approximately 150 mL, were then divided into neutral/basic fractions (NBFs) and acidic fractions (AFs). This separation involved initially washing the O_1 layers thrice with a sodium carbonate solution (pH 10.0, 50 mL/time), yielding organic layers O_2. The pooled aqueous solutions were acidified to pH 2.0 using a 2.0 mol/L hydrochloric acid solution and saturated with sodium chloride. Afterward, 3 further extractions were conducted using dichloromethane (70 mL/time), leading to the formation of organic layers O_3. After adding a sufficient amount of anhydrous sodium sulfate to the O_2 and O_3 organic phases and allowing them to stand for 3–5 h, the samples were stored overnight at −20 °C for 12 h to remove residual moisture. After filtration, the filtrates were first reduced to approximately 1.0 mL via a rotary evaporator and then further to 500 μL under a nitrogen stream (99.999%, 10 mL/min), resulting in concentrated NBF and AF samples. These concentrated samples were then stored at −20 °C until required for GC-O-MS analysis.

2.4. Identification of odorants by GC–O–MS

The aroma-active compounds were analyzed using GC–MS (Agilent 7890B GC/ 5977 A MSD, Agilent, USA) with an olfactory detection port

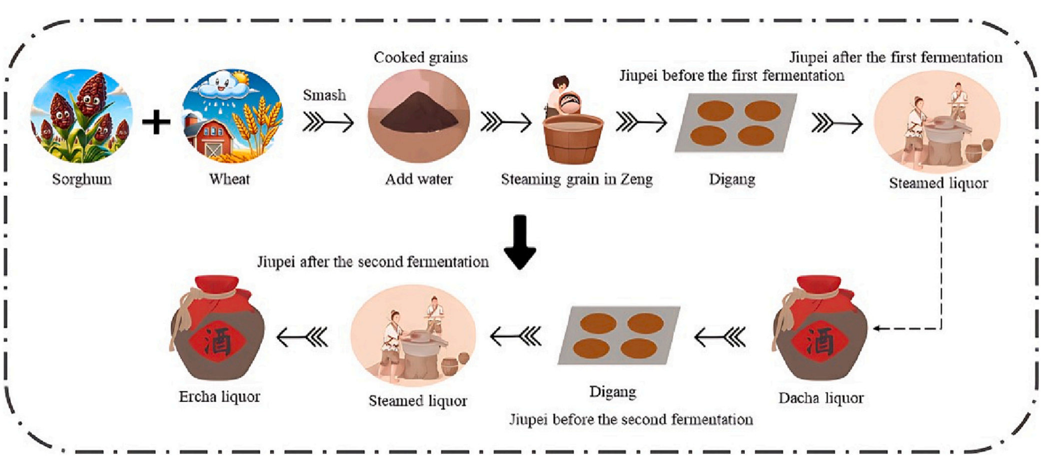

Fig. 1. Relationships among cooked grains (CG), Jiupei before the first fermentation (JBFF), Jiupei after the first fermentation (JAFF), Dacha liquor (DL, 64% ethanol by volume), Jiupei before the second fermentation (JBSF), Jiupei after the second fermentation (JASF), and Ercha liquor (EL, 60% ethanol by volume).

(ODP3, GERSTEL GmbH & Co.KG, Germany), coupled with a DB-WAX (60 m × 250 μm i.d., 0.25 μm film thickness, Agilent Technologies, Santa Clara, CA, USA) or HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies). Helium gas (purity 99.999%) was used as the carrier, maintaining a constant flow rate of 1.5 mL/min. The inlet temperature was set at 250 °C. The temperature program for the columns was adapted from Wang, Wang, et al. (2022). The temperature program started at 40 °C, increased to 50 °C at a rate of 10 °C/min and held for 5 min, then to 80 °C at 3 °C/min for another 5 min, and finally to 230 °C at 5 °C/min, where it was held for 15 min. The effluent from the capillary column was split evenly between the mass spectrometer and the ODP3. Mass spectra were generated in electron ionization mode at 70 eV and a scanning range of *m/z* 30–450. The ion source and transmission line temperatures were maintained at 230 °C and 250 °C, respectively. A solvent delay of 8–10 min was set. Qualitative analysis of the compounds was conducted using various resources: the NIST library 2020 for mass spectrometry data, retention index (RI), standards, the self-built database (SBD) of Baijiu flavor compounds from Beijing Technology and Business University, and aroma characteristics assessment.

A panel of 6 individuals (3 males and 3 females, aged 21–25) with experience in olfactory experiments was selected from the Key Laboratory of Brewing Molecular Engineering of the China Light Industry. The individuals underwent a training period of 2 weeks, 40 min/day. Afterward, three students with the most acute sense of smell were selected to evaluate the aroma of compounds via GC–O–MS. The intensity of each compound's aroma was assessed by these three evaluators and quantified using the Osme value, a scale that ranges from 0 (no aroma) to 1 (weak), 2–3 (moderate), and 4–5 (strong). This value represents the average aroma intensity as perceived by the evaluators.

2.5. TP-HS-GC–IMS analysis

The TP-HS-GC–IMS analysis of Baijiu samples was conducted using a state-of-the-art setup. The instrumentation comprised a FlavorSpec® IMS system (G.A.S., Dortmund, Germany), an Agilent 7890B gas chromatograph, and an Acrichi Automatic Headspace Sampler (Beijing Juxinzhuifeng Technology Co., Ltd., China).

Diluted the appropriate amount of Baijiu samples (DL and EL) with ultrapure water to create a solution with an ethanol volume fraction of 10%. Afterward, 1.0 mL of these diluted solutions was placed in 20 mL headspace vials, with 0.3 g of sodium chloride added. Similarly, 1.00 g of each of the other samples (CG, JBFF, JAFF, JBSF, and JASF) was placed into separate 20 mL vials. All samples were then incubated at 50 °C for 30 min. The analysis parameters included a sampling time of 20 s, an injection time of 20 s, a pressure time of 30 s, and a rinsing time of 60 s, totaling a 56-min analysis period. Nitrogen gas (99.999% purity) was used as the carrier, flowing through an HP-5 capillary column (15 m × 0.250 mm × 0.25 μm i.d.) at a constant rate of 1.5 mL/min. The temperature program started at an initial temperature of 35 °C, held for 2 min, increased to 50 °C at 0.5 °C/min and held for another 2 min, then raised to 70 °C at 6 °C/min for 1 min, and finally ramped up to 220 °C at 18 °C/min.

After GC separation, the compounds were ionized in the IMS ionization chamber using a 3H ionization source (300 MBq activity). The drift tube, measuring 98 mm, was operated at a constant 5 kV and 45 °C, with a nitrogen flow rate of 150 mL/min. The IMS cell functioned in positive ion mode. The spectrometric analysis involved averaging 12 scans per sample with an injection pulse width of 150 μs, a repetition rate of 30 ms, and blocking and injection voltages set at 110 and 2500 mV, respectively. All analyses were replicated three times for consistency. The identification of volatile compounds was achieved by comparing their RIs and drift times against standards in the GC–IMS library.

2.6. Statistical analysis

The TP-HS-GC–IMS data were viewed and processed using LAV software (G.A.S., Dortmund, Germany). Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were conducted using SIMCA version 13.0 (Umetrics, Umeå, Sweden).

3. Results and discussion

3.1. Results of GC–O–MS odor identification

A total of 34 aromatic active compounds and 1 unknown compound were detected in NEB using GC–O–MS. Thirty-four odorants were identified using MS, SBD, RI, standards, and aroma characteristics, including 16 esters, 6 alcohols, 3 acids, 6 aldehydes, 1 ketone, 1 phenol, and 1 other compound. In previous studies on NEB (Wang, Wang, et al., 2022), a total of 59 aroma active compounds were identified in the Baijiu with longer storage time. This suggests that the number of aroma-active compounds in NEB may increase with longer storage time. The specific changes that occur during the aging process of NEB warrant further investigation. In addition, in studies of other aroma types of Baijiu, He et al. (2021) used GC–O–MS to analyze the aroma active components in the distillates of the strong flavor "Bandaojing" during their brewing process. Integrating mass spectrometry, aroma assessment, and RI, they identified a total of 47 aroma active compounds, including 24 esters, 6 alcohols, 7 acids, and 3 aldehydes, with the quantity of esters and acids surpassing that in the light-flavor NEB. On the other hand, Zheng et al. (2023) conducted an analysis of Jian flavor "Lidu" of different years and quality levels, identifying 26 aroma-active compounds through GC–O–MS. The esters were the most abundant, with 18 identified types, along with 2 alcohols and 4 aldehydes. These results indicate that esters are the dominant and relevant flavor components in various types of distilled spirits, and play a critical role in flavor formation during the brewing process. The Osme values for each odorant are listed in Table 1. There were 22 esters and alcohols, which implied that they were crucial to the flavor of DL and EL. In previous studies on NEB, esters and alcohols accounted for a high proportion of volatile aroma compounds (Wang, Wang, et al., 2022). These showed that esters and alcohols were the main volatile aroma compounds in NEB. A total of 34 and 30 aroma compounds were detected in DL and EL, respectively. Among the aroma compounds, 3-ethoxy-1-propanol, undecan-4-olide, 2-methyl butyric acid, and acetic acid were only smelled in DL.

The Osme values of ethyl acetate (4/4, fruity), isovaleraldehyde (4.5/3, aldehydic), 3-methyl-1-butanol (4/3, fusel), and phenylacetaldehyde (4, floral) were higher in DL. Conversely, the Osme values of ethyl acetate (4/4, fruity), (*E, E*)-2,4-nonadienal (4, fatty, cucumber), and geosmin (4, soil) were higher in EL. This indicated that these compounds could have a significant contribution to the original liquor of NEB. Many studies have proven that ethyl acetate is the key odorant in LFB, which has been found in a large number of LFB (Wang, Ye, et al., 2022), such as Fenjiu, Baofeng Jiu, Qingke Jiu, and Caoyuanwang (Gao et al., 2014). In our previous studies, isovaleraldehyde and geosmin were identified as the major aroma components in NEB, and 3-methyl-1-butanol also had a considerably high Osme (4.60) in NEB stored for many years (Wang, Wang, et al., 2022). These showed that 3-methyl-1-butanol was the relevant odorant for NEB, whether it was a new base or aged liquor. Phenylacetaldehyde is a substantially relevant floral fragrance substance in LFB, which is found in almost all light-flavored Baijiu (Wang, Ye, et al., 2022).

3.2. Analysis of GC–IMS results for volatile components from different brewing stages

3.2.1. Differential spectrum analysis of volatile components from different brewing stages

The GC–IMS difference spectra of Jiupei and distilled spirits at

Table 1
Aroma active compounds identified by GC-O-MS in DL and EL [a].

No.	Aroma compounds	CAS	Odor descriptor	DL				EL				RI [c]		Identification [d]
				Fraction	Osme Values [b]	DB-WAX	HP-5MS	Fraction	Osme Values [b]	DB-WAX	HP-5MS	DB-WAX	HP-5MS	
1	Ethyl acetate	141–78-6	fruity	NBF, AF	4/4	√	√	NBF, AF	4/4	√	√	898	602	MS, Aroma, RI, SBD, S
2	Isovaleraldehyde	590–86-3	aldehydic	NBF, AF	4.5/3	√	√	NBF, AF	3/3	√	√	920	642	MS, Aroma, RI, SBD, S
3	Propyl acetate	109–60-4	pungency	NBF	2.5	√	–	NBF	3	√	–	980	–	MS, Aroma, RI
4	1-Hexanol	111–27-3	fusel oil	NBF	2	–	√	NBF	2	–	√	–	870	MS, Aroma, RI, SBD, S
5	Ethyl isovalerate	108–64-5	fruity	NBF	2	√	–	NBF	2	√	–	1060	–	MS, Aroma, RI, SBD, S
6	Ethyl acrylate	140–88-5	plastic	NBF	3	√	–	NBF	3	√	–	1000	–	MS, Aroma, RI, SBD, S
7	Ethyl butyrate	105–54-4	fruity	NBF	2	√	√	NBF	3	√	√	1010	800	MS, Aroma, RI, SBD, S
8	Ethyl 2-methylbutyrate	7452-79-1	fruity	NBF	2	√	√	NBF	2.5	√	√	1050	856	MS, Aroma, RI, SBD, S
9	2-Methyl-1-propanol	78–83-1	fusel	NBF, AF	2/2	√	–	NBF, AF	3.5/2	√	–	1091	–	MS, Aroma, RI, SBD, S
10	Ethyl valerate	539–82-2	sweet	NBF	1	√	√	NBF	2	√	√	1514	1073	MS, Aroma, RI, SBD, S
11	Unknown	–	green grass	NBF	3	√	–	NBF	2	√	–	–	–	–
12	3-Methyl-1-butanol	123–51-3	fusel	NBF, AF	4/3	√	√	NBF, AF	3.5/2	√	√	1201	730	MS, Aroma, RI, SBD, S
13	Ethyl hexanoate	123–66-0	fruity	NBF	3.5	√	√	NBF	3	√	√	1240	990	MS, Aroma, RI, SBD, S
14	3-Ethoxy-1-propanol	111–35-3	unpleasant	NBF	1	√	–	–	–	–	–	–	–	MS, Aroma
15	1-Nonanal	124–19-6	waxy	NBF	2	√	–	NBF	2	√	–	1394	–	MS, Aroma, RI
16	2-Hydroxy-3-methylbutanoic acid ethyl ester	2441-06-7	pineapple	NBF	1.5	√	√	NBF	2	√	√	–	–	MS, Aroma, SBD, S
17	Furfural	98–01-1	almond	NBF	3	√	–	NBF	3	√	–	1471	–	MS, Aroma, RI, SBD, S
18	Ethyl 2-hydroxy-4-methylvalerate	10,348–47-7	fruity	NBF	1	√	–	NBF	3	√	–	1512	–	MS, Aroma, RI, SBD, S
19	Trans, trans-2,4-nonadienal	5910-87-2	fatty, cucumber	NBF	3	√	–	NBF	4	√	–	1688	–	MS, Aroma, RI, SBD, S
20	Diethyl succinate	123–25-1	fruity	NBF	2.5	√	–	NBF	3	√	–	1674	–	MS, Aroma, RI, SBD, S
21	Phenethyl acetate	103–45-7	floral	NBF	3	√	–	NBF	2.5	√	–	1811	–	MS, Aroma, RI, SBD, S
22	*β*-Damascenone	23,696–85-7	sweet	NBF	2	√	–	NBF	2	√	–	1820	–	MS, Aroma, RI, SBD, S
23	Geosmin	16,423–19-1	soil	NBF	3	√	–	NBF	4	√	–	1805	–	MS, Aroma, RI, SBD, S
24	Ethyl 3-phenylpropionate	2021-28-5	rum	NBF	2	√	–	NBF	3	√	–	1903	–	MS, Aroma, RI
25	Phenylacetaldehyde	122–78-1	floral	NBF	4	√	√	NBF	3	√	√	1660	1040	MS, Aroma, RI, SBD, S
26	Phenethyl alcohol	60–12-8	floral	NBF	2.5	√	–	NBF	3	√	–	1934	–	MS, Aroma, RI, SBD, S
27	4-Ethyl-2-methoxyphenol	2785-89-9	bacon	NBF	2.5	√	–	NBF	3	√	–	2041	–	MS, Aroma, RI, SBD
28	*γ*-Nonanolactone	104–61-0	coconut	NBF	2	√	–	NBF	3	√	–	2028	–	MS, Aroma, RI, SBD, S
29	Ethyl myristate	124–06-1	sweet	NBF	2	√	–	NBF	2	√	–	2052	–	MS, Aroma, RI, SBD, S
30	Methyl palmitate	112–39-0	waxy	NBF	3	√	–	NBF	3	√	–	–	–	MS, Aroma
31	Undecan-4-olide	104–67-6	fatty	NBF	2	–	√	–	–	–	–	–	–	MS, Aroma
32	Isovaleric acid	503–74-2	sour stinky	AF	3	√	–	AF	3	√	–	1689	–	MS, Aroma, RI, SBD, S
33	2-Methyl-1-butanol	137–32-6	fusel alcoholic	AF	2	–	√	AF	2	–	√	–	750	MS, Aroma, RI, SBD, S
34	2-Methyl butyric acid	116–53-0	pungent acid	AF	3	–	√	–	–	–	–	–	840	MS, Aroma, RI
35	Acetic acid	64–19-7	sour	AF	2	√	–	–	–	–	–	1415	–	MS, Aroma, RI, SBD, S

[a] The selected aroma-active regions were detectable by all three sensory assessors.
[b] Osme is the mean value of the aroma perceived by the sensory assessors and the value is the olfactory result on a DB-WAX column. Indicates the strength value on HP-5MS if only HP-5MS columns are available; "-" no aroma detected.
[c] RI, linear retention indices.
[d] MS, aroma compounds were identified by MS; SBD, the self-built database of Baijiu flavor compounds from Beijing Technology and Business University; S, compounds were identified by pure standards.

different brewing stages of NEB are shown in Fig. 2. As shown in Fig. 2(a), the compounds in Jiupei and liquor samples at different brewing stages are significantly different. Fig. 2(b) shows a difference spectrogram with CG as the reference. When each comparison sample shows more red dots, it represents a higher content of compounds. The volatile compounds detected in CG are of fewer types and lower content. This was because the processing of CG is relatively simple; it is only steamed and not yet fermented. Fig. 2(c) shows a difference spectrum with JAFF as the reference. As shown in Fig. 2(c), it shows that adding Daqu and cooling can slightly increase the content of some compounds in CG, and the change is not significant, either. This is because these processes mainly involve physical changes, with fewer chemical changes, and are not enough to cause significant changes in the type or amounts of compounds. However, after fermentation for 28–30 d, the type and content of compounds in JAFF increase significantly, as shown in Fig. 2(d). This is because many complex chemical changes have occurred in the sugars, starch, and other substances in Jiupei, mainly under the action of microorganisms during the fermentation in Digang. For example, the dominant microorganisms in LFB, such as *Pichia*, *Hanseniaspora*, and *Lactobacillus*, can produce a variety of ester compounds by esterification (Gao et al., 2014; Liu & Sun, 2018). *Bacillus* can produce aldehydes and ketones by fermentation, so the type and content of compounds in Jiupei after microbial fermentation are often higher than those in Jiupei before fermentation (Yang et al., 2012). The type and

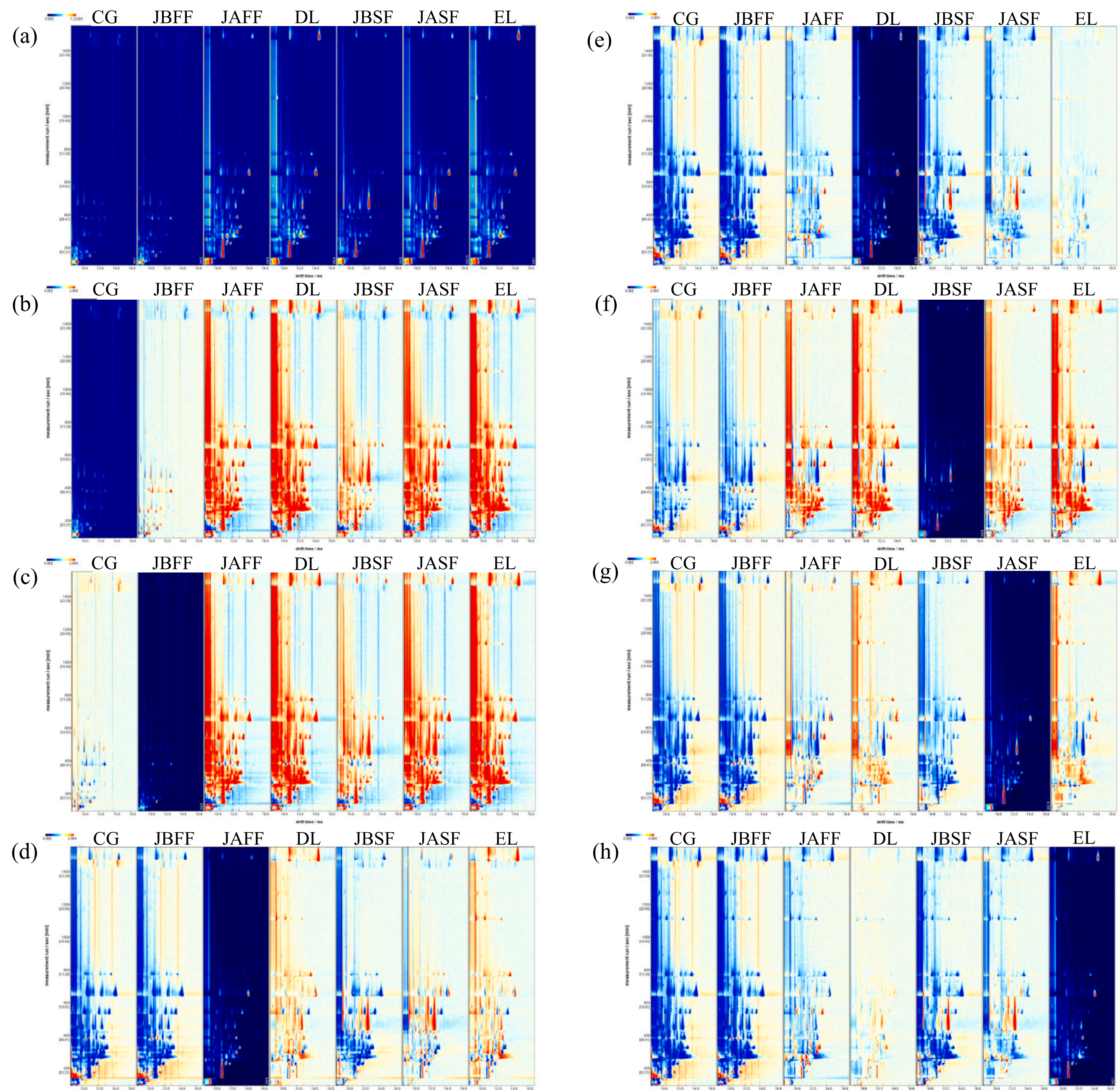

Fig. 2. The difference spectra of fermented grains at different brewing stages in Niulanshan Erguotou Baijiu (NEB). (a) 2D images of different brewing stages in NEB; (b) The difference spectrum, with CG as the comparison object; (c) The difference spectrum, with JBFF as the comparison object; (d) The difference spectrum, with JAFF as the comparison object; (e) The difference spectrum, with DL as the comparison object; (f) The difference spectrum, with JBSF as the comparison object; (g) The difference spectrum, with JASF as the comparison object; (h) The difference spectrum, with EL as the comparison object. The compound contents in the red parts are higher than those in the blue parts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

content of compounds in DL are extremely high (Fig. 2(e)), because new substances are generated during the distillation process, and distillation is also a concentration process, which leads to an increase in compound content (He et al., 2021). In addition, JBSF in Fig. 2(f) is similar to JBFF, whereas JASF in Fig. 2(g) is similar to JAFF. Finally, EL (Fig. 2(h)) was obtained after a second distillation. Similar to DL, the content of compounds in EL remains at a high-level. Thus, it was clear that the differential spectrum analysis could show the effect of each step in the brewing process on volatile compounds, making the brewing process more visible.

To further reflect the differences in aroma compounds in samples at different brewing stages, several important compounds in Baijiu were selected for specific analysis according to previous studies (Gao et al., 2014; Wang, Wang, et al., 2022; Wang, Wei, et al., 2022). The compounds were isoamyl acetate, ethyl lactate, hexanal, butyl acetate, ethyl 2-methylpropanoate, 1-pentanol, ethyl propanoate, and nonanal, respectively (Figs. 3, 1–8). These substances have been widely reported in LFB because of their rich content and great contribution to Baijiu flavor. For example, hexanal is a common volatile aldehyde in Baijiu with a fruity and green grass aroma (Wang, Wei, et al., 2022). Ethyl isobutyrate has a high content in Baijiu, presenting sweet and fruity aroma (Gao et al., 2014). 1-Pentanol with a fruity aroma contributes significantly to the overall aroma of Baijiu (Wang, Wei, et al., 2022). Ethyl propanoate and butyl acetate also have a certain impact on the overall aroma characteristics of Baijiu, with banana and pear aromas, respectively (Fan & Qian, 2006; He et al., 2021). Ethyl lactate and isoamyl acetate are the main aroma substances in LFB (Du et al., 2021; Tian et al., 2021).

As shown in Fig. 3, the content of compounds **1** (isoamyl acetate), **3** (hexanal), **4** (butyl acetate), **6** (1-pentanol), and **8** (nonanal) increases significantly after 2 fermentations. A similar phenomenon was found in the Fenjiu by Cao et al. (2010). The authors found that the content of alcohol in the Fenjiu increased with fermentation. This was due to the relatively strong metabolic activity of *Bacillus* sp., which has a population advantage during fermentation (Yang et al., 2012). *Bacillus vulgus* produces more alcoholic compounds, whereas *Bacillus subtilis* and *Bacillus cereus* mainly produce esters, aldehydes and ketones (Yang et al., 2012). In addition, the content changes of compounds **5** (ethyl 2-methylpropanoate) and **7** (ethyl propanoate) showed the same trend after 2 rounds of fermentation in Digang, but the content changes were not apparent in the distillation process, because the environment during the distillation process significantly inhibits the metabolic activity of yeasts (e.g., *Pichia anomala, Candida, and Hansenula polymorpha*) (Park et al., 2009; Xu et al., 2021). On the other hand, the content of compound **2** (ethyl lactate) in JBSF and JASF was found to be significantly higher than that in JBFF and JAFF. In the fermentation process of Fenjiu and the strong flavor Baijiu, the content of ethyl lactate is also highest in the later stage of fermentation (Cao et al., 2010; Gao et al., 2019). This could be attributed to the high alcohol content during the first fermentation period and the limited solubility of ethyl lactate in alcohol, resulting in a lower detected content. During the second fermentation stage, a substantial amount of alcohol was produced as a result of the initial distillation, leading to a lower alcohol content and a relatively higher water content. However, ethyl lactate exhibits strong water solubility, which consequently results in a significant increase in its detected content (Wang & Li, 2022). As above, the further analysis results of these typical aroma compounds in Baijiu were consistent with those of differential spectrum analysis.

3.2.2. *Fingerprint analysis of volatile components from different brewing stages*

A total of 36 peaks (including monomers and dimers of the same compound) were identified using GC–IMS, and there were a total of 26 compounds, as shown in Table S2, including 12 esters, 2 alcohols, 7 aldehydes, 3 ketones, 1 nitrogen-containing compound, and 1 oxy derivative of alkene.

The ester compounds in Jiupei were the most abundant, which was consistent with LFB (Wang, Ye, et al., 2022). They were mainly from the

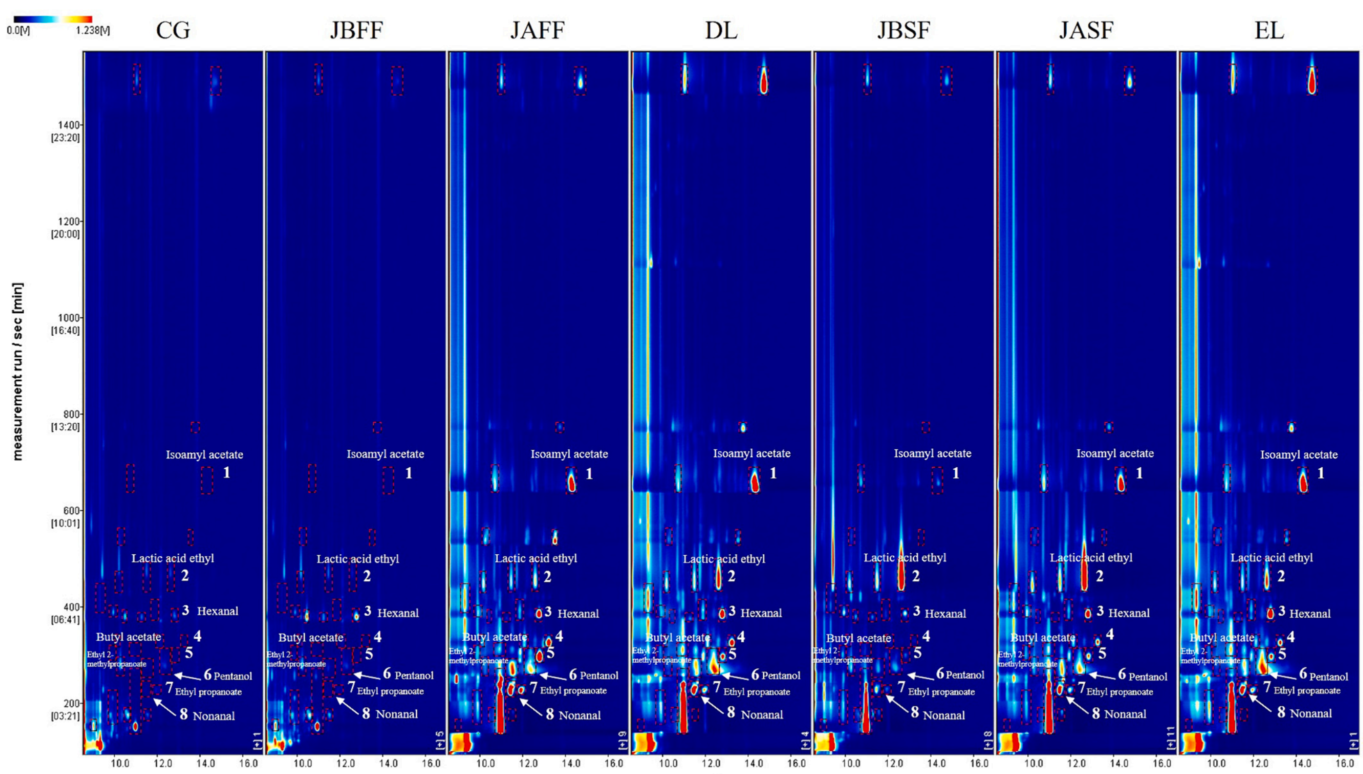

Fig. 3. Differential spectrograms of samples from different brewing stages of Niulanshan Erguotou Baijiu (NEB) (including marker compounds).

yeasts in Digang (Wang et al., 2012). The yeasts include *Candida* and *H. polymorpha*, which can convert sugars, acids, aldehydes, and other compounds into ester compounds (Zhu et al., 2016). Wang (Wang, 2019) found that the yeasts, *Pichia kudriavzevii* and *Wickerhamomyces anomalus*, had a strong ability to produce ethyl acetate in the fermented grains of NEB. Esters are the main flavor substances that can not only produce aroma and make the liquor more harmonious, but also have beneficial effects on the human body. For example, ethyl butyrate can calm the nerves after being absorbed by the body (Aoshima et al., 2006; Guo et al., 2020).

A total of 10 aldehyde and ketone compounds were identified. Aldehydes and ketones can add aroma to the liquor, but if the levels are too high, they can make the liquor taste bitter (Lei et al., 2020). The metabolic activity of microorganisms is a relevant source of aldehydes (Guo et al., 2020). Ketone compounds can inhibit the expression of Hp-related urease genes, thereby suppressing the activity of Hp urease. This action can reduce the adhesion and colonization ability of Hp bacteria to cells, ultimately leading to the inhibition of Hp-induced cellular damage (Luo et al., 2021). The identified alcohol compounds are pentanol and 3-methyl-1-butanol, also called higher alcohols. Studies have shown that most higher alcohols are produced by *Saccharomyces cerevisiae* (Eden et al., 2001). Guo et al. (2020) summarized the metabolism of alcohol compounds involved by *S. cerevisiae* in the process of Baijiu brewing. It is mainly produced through two pathways: one is the *Ehrlich* pathway, and the other is the anabolic pathway (Avalos et al., 2013; Hazelwood et al., 2008). In addition to giving Baijiu a certain flavor, the higher alcohols can also directly affect people's condition after drinking. The high alcohol content may lead to an increased risk of intoxication (Gou et al., 2016).

By drawing the fingerprint, the content of each compound in each sample and its change trend throughout the Baijiu brewing process were intuitively compared. The results are shown in Fig. 4. Different types of compounds show different content trends during the brewing process. Hexanal. M (purple box in Fig. 4) shows a higher content in CG and JBFF, but their content decreased significantly after fermentation in Digang. Aldehydes are indispensable aroma components in Baijiu, but if the content is too high, a strong, irritating taste will appear, which is harmful to the human body and causes headaches (Wang, Wei, et al., 2022). The raw materials used in brewing, such as rice husks and bran, are important sources of aldehyde compounds in liquor (Guo et al., 2020). Therefore, they may lead to a high content of aldehyde compounds before fermentation. After the initial fermentation, their content significantly decreases, possibly due to decomposition by certain microorganisms. The specific reasons warrant further investigation. On the contrary, the content of ethyl 2-methylbutanoate. M (red box in Fig. 4) significantly increased after fermentation in Digang, and maintained a high relative content in subsequent stages. This is mainly because *S. cerevisiae* is a dominant strain that can produce esters and other aromatic compounds through typical biochemical pathways under the catalysis of specific enzymes (Avalos et al., 2013). For example, yeast can synthesize ethyl acetate from alcohols and aldehydes through the oxidation of hemiacetals under tangible conditions, catalyzed by ethanol dehydrogenase (Park et al., 2009). The compounds ethyl lactate. M, nonanal. M, and ethyl butyrate (green box in Fig. 4) also increase in content after two fermentation cycles, and have considerably higher contents in DL and EL. The generation and content changes of these compounds are related to the metabolism of microorganisms in the brewing environment. The formation of ethyl lactate. M is related to *Candida* and may be a by-product of ethanol metabolism by yeast (Wei et al., 2021).

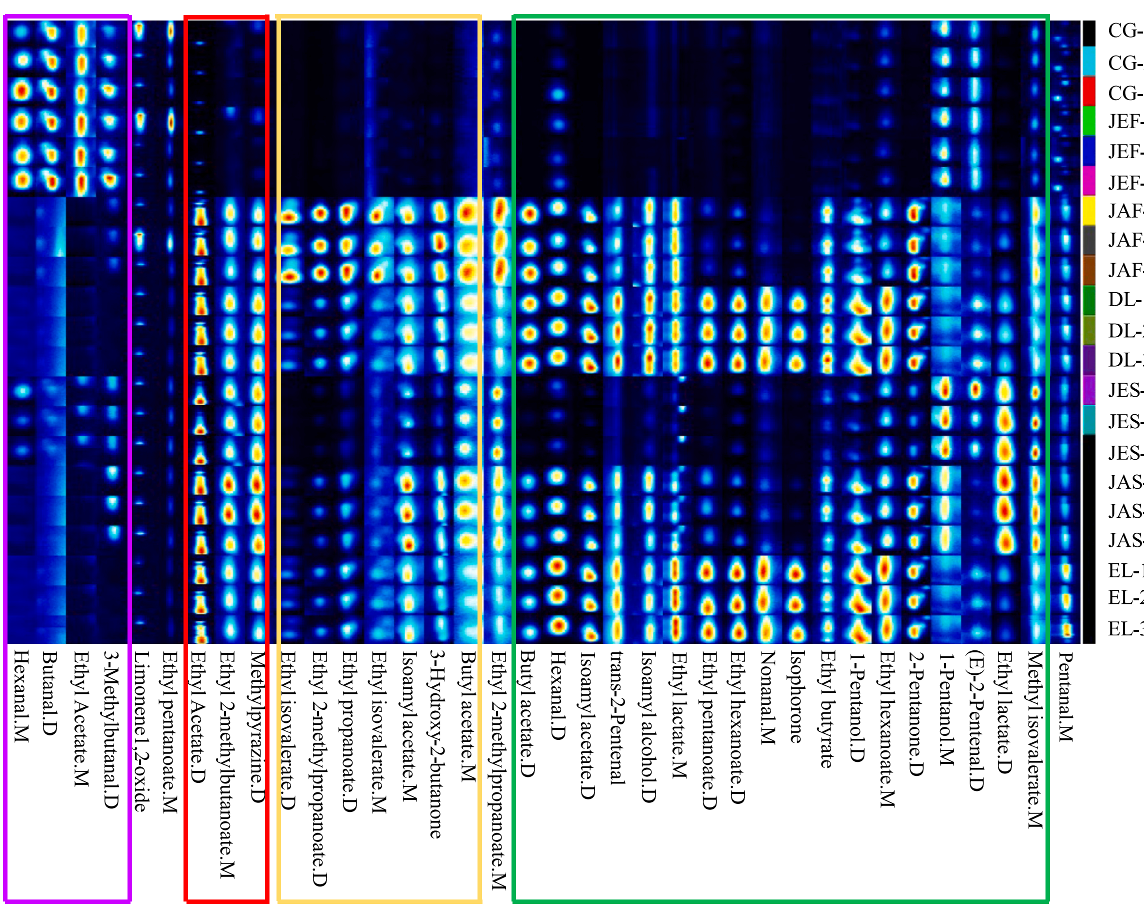

Fig. 4. Fingerprint of volatile compounds in different brewing stages of Niulanshan Erguotou Baijiu (NEB).

3.3. Statistical analysis

The multivariate statistical analysis method can provide an effective means to distinguish samples in different brewing stages, and provide effective theoretical support for analysis and research. He et al. (2021) used multivariate statistical analysis, including PCA and PLS-DA, to analyze Baijiu at different distillation stages, and confirmed that 10 aroma-active compounds were related to the classification, indicating that these markers have a great influence on the flavor of raw Baijiu. In addition, the production of Baijiu is different from that of other distilled liquors (Liu & Sun, 2018). Its brewing combines two different processes of fermentation and distillation, so it is particularly necessary to classify and discuss these two processes.

3.3.1. PCA based on volatile compounds

According to the four groups of experimental data (JBFF, JAFF, JBSF, and JASF), the influence of the fermentation process on the change of aroma compounds was analyzed. Seventeen relevant odorants frequently analyzed in Baijiu were screened according to the results of GC–IMS analysis, and the internal relationship among these samples in different brewing stages was revealed through PCA analysis. The scoring chart is shown in Fig. 5(a). The first 2 principal components, PC1 and PC2, account for 26.8% and 19.9% of the total variance, respectively, which can explain nearly half of the total variance. According to the PC1 × PC2 score chart, PC1 is positively correlated with JASF samples and negatively correlated with JBFF samples. JBFF and JASF samples can be clearly distinguished.

DL and EL were the products obtained after the distillation of fermented grains. The compounds in the two distilled liquors are significantly different from those in Jiupei. Therefore, PCA analysis was conducted on the distilled liquor and Jiupei separately. Therefore, a separate PCA analysis was performed for DL and EL to effectively analyze the effect of Baijiu distillation. The score chart is shown in Fig. 5 (b). The principal components PC1 and PC2 account for 31.5% and 27.5% of the total variance, respectively, and the cumulative contribution rate of the first 2 principal components is 59%, which can effectively explain the content information of most of the aroma compounds in the DL and EL groups. As shown in Fig. 5(b), there is a certain degree of difference in the distribution of compounds between the DL and EL, reflecting the low similarity of aroma compounds in the liquor samples obtained by two distillation processes. This may be the result of the combined effects of fermentation and distillation processes.

3.3.2. PLS-DA based on volatile compounds

As a supervised multivariate statistical analysis, PLS-DA can be used to establish good data models and provide a powerful way for distinguishing samples with different characteristics. Therefore, by constructing a PLS-DA model, the variable importance in projection (VIP) is used to identify compounds that can significantly contribute to discriminating samples from different brewing stages.

First, the volatile compounds in four groups of samples (JBFF, JAFF, JBSF, and JASF) before and after the two fermentation processes were analyzed, and the results are shown in Fig. 6(a). PC_1 and PC_2 explain 45.2% of the total variance, with $R^2Y = 97.67\%$ and $Q^2 = 76.21\%$, reflecting a good fit of the model. JBFF samples are distributed in the fourth quadrant; JAFF samples are primarily distributed in the first quadrant; JBSF samples are primarily distributed in the second quadrant; JASF samples are all distributed in the third quadrant, and the differentiation degree of each group of samples is relatively apparent. In addition, the rationality of PLS-DA model construction relies on the permutation test, which can be used to verify whether the model is tangible or not. The test randomly rearranges the experiment by changing the sorting order of the classification variable (Y) and randomly assigning Q^2Y up to 200 times ($n = 200$). The results of the test are shown in Fig. 6(b). All the blue points (Q^2) on the left are lower than the original points on the right, and the regression line composed of all the blue points (Q^2) intersects the Y axis on the negative half axis, suggesting that the model is valid and there is no overfitting. Finally, using the VIP plot obtained (Fig. 6(c)), the compounds with a VIP greater than 1.0 were screened to determine those compounds that have important contributions to the differentiation of liquor samples at different stages of fermentation. A total of four volatile compounds were identified: hexanal. M (VIP 1.3760), 3-hydroxy-2-butanone (VIP: 1.3335), trans-2-pentanone (VIP 1.0784), and ethyl hexanoate. M (VIP: 1.0397) (Fig. 6(c), in the purple box).

Second, the response values of aroma active compounds in the liquor samples after two distillation treatments were analyzed, and the results are shown in Fig. 6(d). PC1 and PC2 explain 54% of the total variance together, with $R^2Y = 99.86\%$ and $Q^2 = 85.30\%$, which proves that the model fits well. The two groups of samples, DL and EL, are separated from each other, with a good degree of differentiation (Fig. 6(d)), which is similar to the previously reported results. In addition, the results of the permutation test (n = 200) performed on the model are shown in Fig. 6 (e), suggesting that there is no overfitting of the model. Finally, using the VIP plot (Fig. 6(f)), 9 volatile compounds with a VIP greater than 1.0 were screened, namely hexanal. M (VIP: 1.6905), 1-pentanol. M (VIP: 1.3581), methyl isovalerate. M (VIP: 1.3311), isoamyl acetate. M (VIP: 1.3146), 3-hydroxy-2-butanone (VIP: 1.3132), ethyl hexanoate. M (VIP: 1.2797), ethyl acetate. M (VIP: 1.1810), ethyl 2-methylbutynoate. M (VIP: 1.1771), and ethyl pentanoate. M (VIP: 1.0494) (Fig. 6(f), in the purple box).

4. Conclusion

In the present study, GC–O–MS was used to investigate the aroma-active compounds in NEB distilled liquor at different distillation stages, identifying a total of 34 aroma-active compounds. Specifically, 34 compounds were identified in DL, whereas 30 compounds were found in EL. Additionally, the study further employed TP-HS-GC–IMS to analyze the products obtained at different brewing stages of NEB. The results indicated that the number of volatile compounds was highest in the distilled liquor, with the quantity in Chuchi Jiupei being higher than

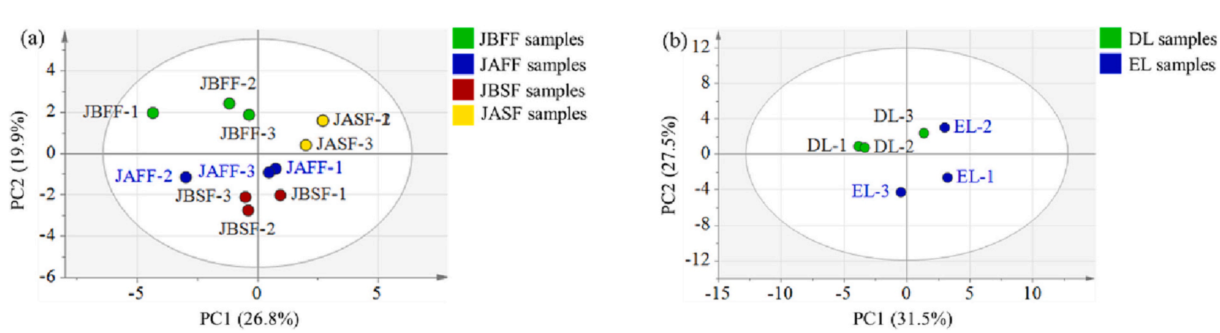

Fig. 5. PCA analysis based on volatile compounds. (a) analysis of volatile compounds in Jiupei during Baijiu brewing; (b) derived from the analysis of volatile compounds in distilled liquor during the distillation process.

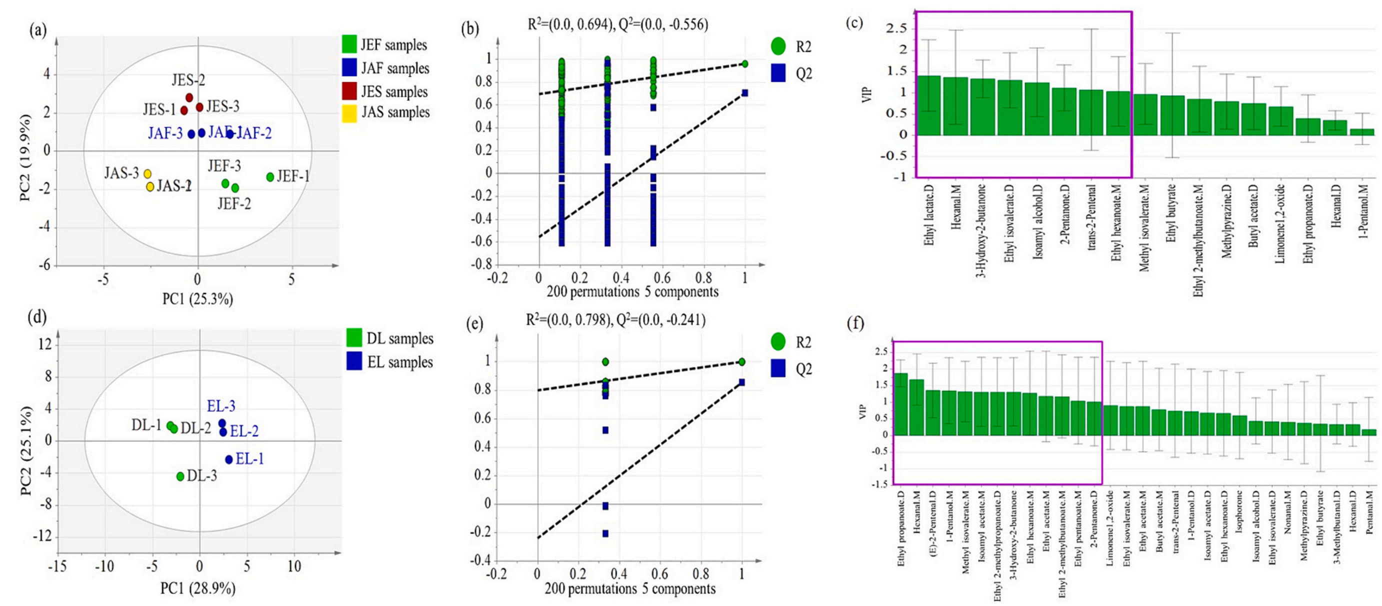

Fig. 6. PLS-DA analysis based on volatile compounds. (a), (d) is a score chart; (b), (e) is a permutation test chart; (c) VIP chart is obtained using the response value of GC-IMS to the determination of volatile compounds in Jiupei; (f) VIP chart is obtained using the response value of GC-IMS to the determination of volatile compounds in liquor.

that in Ruchi Jiupei. PCA effectively differentiated the volatile flavor components at different brewing stages, and PLS-DA effectively revealed the differences in compounds at these stages, along with their related aroma activity markers. Among them, the compounds that had important contributions to the differentiation of different fermentation stages in Jiupei were hexanal. M, 3-hydroxy-2-butanone, trans-2-pentenal, ethyl hexanoate. M; The compounds that had made important contributions to the differentiation of two spirits (DL and EL) were hexanal. M, 1-Pentanol. M, methyl isovalerate. M, isoamyl acetate. M, 3-hydroxy-2-butanone, ethyl hexanoate. M, ethyl acetate. M, ethyl 2-methylbutanoate. M, and ethyl pentanoate. M. This study aids in the in-depth understanding of aroma compounds present throughout the brewing stages, providing a reference for further exploration and optimization of the production process to enhance the aroma of NEB.

Ethical statement

All our work is carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). Finally, ethical approval for the involvement of human subjects in this study was granted by Beijing Technology and Business University Research Ethics Committee, Reference number 05, 02/20/2024. We will take all necessary measures to ensure that this study complies with ethical and legal standards, and protect the rights and privacy of participants.

CRediT authorship contribution statement

Zhen Wang: Writing – review & editing, Writing – original draft, Software, Investigation, Formal analysis. **Wenjun Hao:** Investigation. **Jinghao Wang:** Software. **Ying Wang:** Supervision. **Xinan Zeng:** Supervision. **Mingquan Huang:** Supervision. **Jihong Wu:** Resources. **Baoguo Sun:** Resources.

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

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