

# Different distillation stages Baijiu classification by temperature-programmed headspace-gas chromatography-ion mobility spectrometry and gas chromatography-olfactometry-mass spectrometry combined with chemometric strategies

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## ABSTRACT

Liquid-liquid microextraction (LLME) combined with gas chromatography-olfactometry-mass spectrometry (GC-O-MS) was used to detect the variations in volatile compounds during the distillation process (head, heart, and tail) of raw Baijiu produced by different layers of fermented grains; 47 aroma compounds were sniffed and identified. Moreover, temperature-programmed headspace gas chromatography-ion mobility spectrometry (TP-HS-GC-IMS) was applied to characterize the Baijiu distillation process for the first time. The 3D fingerprint spectrum clearly showed a variation in volatile compounds from different distillation stages, and most compounds showed a downward trend. In addition, multivariate statistical analysis, including principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), etc., confirmed ten aroma active markers related to classification, indicating that these markers had a great influence on the flavor of raw Baijiu.

## 1. Introduction

Baijiu is a traditional indigenous distilled spirit prepared from grain fermentation by a technique that has been used for over 2000 years. As the most popular alcoholic beverage in China, its annual output exceeded 4 million kiloliters (kL) in 2020 and has created a market profit of >700 billion RMB (data from the [China Alcoholic Drinks Association](#)). Due to the great diversity in the production environment, brewing materials, fermentation equipment and manufacturing processes, Baijiu is generally classified into 12 categories ([Liu & Sun, 2018](#)), among which the strong flavor type is popular with consumers due to its unique taste and aroma.

The flavor characteristics of strong flavor Baijiu depend on the fermentation technology and geographical environment or microenvironment of the fermentation pit. Additionally, the distillation technique is considered to be one of the most important factors in the production of Baijiu, which is closely correlated with quality and yield. The flavor

constituents of the matrix are transferred into raw Baijiu by vaporization, extraction and fractionation by distillation. The process is carried out in a batch pattern, and raw Baijiu is divided into several appropriate fractions to control quality by experienced workers ([Zheng, Liang, Wu, Zhou, & Liao, 2014](#)). Therefore, revealing the flavor features of raw Baijiu at different distillation stages may contribute to a better understanding of the effects of raw Baijiu on the quality of the final product and facilitate scientific classification.

Liquid-liquid microextraction (LLME), as a new type of pretreatment method, uses a small amount of liquid as an extractant to separate the target compounds, which overcomes the disadvantages of traditional methods. It is simple and fast to operate, consumes less organic solvent, has a low cost, and has a high enrichment factor ([Yamini, Rezazadeh, & Seidi, 2019](#)). It can be used in conjunction with chromatography to analyze target compounds quickly and easily ([Campillo, López-García, Hernández-Córdoba, & Viñas, 2018](#); [Zhou, Fan, & Xu, 2015](#)).

Meanwhile, In the last decade, some novel approaches have been

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developed for discriminating subtle differences in product aroma, such as gas chromatography-ion mobility spectrometry (GC-IMS). As an analytical instrument that combines the dual advantages of gas chromatography and migration chromatography, GC-IMS has the advantages of simple sample preparation, short incubation time, short analysis time and exceptional sensitivity (Charlotte, Rebecca, Esther, Kristina, & Albert, 2010; Hu et al., 2019; Zeng et al., 2019). However, GC-IMS is involved in less alcoholic beverage research due to the influence of ethanol, and most of these studies are limited to beverages with a low alcohol content, such as huangjiu (Huang et al., 2019; Qi et al., 2019), wines (Márquez-Sillero, Cárdenas, & Valcárcel, 2012). The earliest applications of GC-IMS for beverages with a high alcohol content such as Baijiu began in 2014. Peng et al. established a method for the rapid detection of trace phthalates in Baijiu using a homemade ion mobility spectrometer (Peng et al., 2014). In recent years, GC-IMS has been increasingly used for the detection of volatile compounds in alcohol. Zhu et al. used GC-IMS to establish a method for the analysis of volatile flavor compounds in Baijiu and constructed a flavor fingerprint of three different flavor types of Baijiu (Zhu, Cai, & Liu, 2020). Literature research has shown that the application of GC-IMS to Baijiu is in its preliminary stage, and there are almost no literature reports on the applications of temperature-programmed headspace gas chromatography-ion mobility spectrometry (TP-HS-GC-IMS) for Baijiu. Therefore, this article innovatively used TP-GC-IMS to analyze variations in the substances in raw Baijiu during the distillation process.

Due to the complexity and variability of Baijiu preparation, its classification represents an analytical challenge. With this aim, chemometric methods coupled with analytical techniques are increasingly being used for alcoholic beverage studies (Song et al., 2020; Dong et al., 2019). However, after literature searching, it was found that research on the distillation process of strong flavor Baijiu mainly focused on the change trend of some volatile substances (Ding, Wu, Huang, & Zhou, 2015; Lang, 2015; Xu et al., 2019), and no research has identified the key substances that cause the differences in raw Baijiu samples at different distillation stages from the perspective of aroma-active compounds. Therefore, the main objective of this study was to investigate the trend of aroma-active compounds and the differences between raw Baijiu samples and to further determine the significant compounds by LLME-GC-MS, TP-GC-IMS, and GC-O-MS coupled with multivariate analysis.

## 2. Materials and methods

### 2.1. Samples

Sampling was obtained from Bandaojing Baijiu Co., Ltd. (Shandong, China). A total of 12 fresh raw Baijius (4 heads, 4 hearts, and 4 tails), which were collected from three distillation stages of all layers of fermented grains from a fermentation cellar. Samples were sealed at 4 °C until analysis. The samples were detailed in Table 1.

**Table 1**  
Baijiu sample information.

Layer of fermented grains	No. <sup>a</sup>	Distillate position	Ethanol Content (V/V)
First floor (Top)	1-1	Head	74%
	1-2	Heart	66%
	1-3	Tail	38%
Second floor (Middle)	2-1	Head	76%
	2-2	Heart	64%
	2-3	Tail	50%
Third floor (Middle)	3-1	Head	80%
	3-2	Heart	56%
	3-3	Tail	31%
Fourth floor (Bottom)	4-1	Head	80%
	4-2	Heart	55%
	4-3	Tail	40%

<sup>a</sup> Sample number.

### 2.2. Chemicals

All standards were of GC quality with at least 95% purity. Compounds, including ethyl acetate, ethyl butanoate, butyl acetate, 2-methylpropanol, ethyl pentanoate, 1-butanol, ethyl 4-methylpentanoate, 3-methyl-1-butanol, ethyl hexanoate, furfuryl ethyl ether, ethyl lactate, 1-hexanol, hexyl butyrate, propyl lactate, ethyl octanoate, acetic acid, isopentyl hexanoate, furfural, ethyl nonanoate, butanoic acid, ethyl decanoate, phenylacetaldehyde, 2-furanmethanol, 3-methylbutanoic acid, diethyl butanedioate, 2-methoxyhexyl acetate, pentanoic acid, hexanoic anhydride, ethyl phenylacetate, 4-methylpentanoic acid, phenethyl acetate, hexanoic acid, ethyl 3-phenylpropanoate, 2-phenylethanol, heptanoic acid, creosol, phenol, 4-ethyl-2-methoxyphenol, *p*-cresol, 4-ethylphenol, isovaleraldehyde, ethyl isobutyrate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl heptanoate, and hexyl hexanoate were purchased from Sigma-Aldrich (Shanghai, China). Pentyl acetate, diethylacetic acid, and 4-octanol, from Sigma-Aldrich (Shanghai, China), were used as internal standards and labeled as IS1, IS2, and IS3, respectively. A C<sub>7</sub> – C<sub>40</sub> *n*-alkane mixture (Sigma-Aldrich, Shanghai, China) was employed for determining the linear retention indices (RIs). Absolute ethanol (CH<sub>3</sub>CH<sub>2</sub>OH), sodium chloride (NaCl) and dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>) were obtained from China National Pharmaceutical Group Corp. (Shanghai, China).

### 2.3. Aroma compound liquid–liquid microextraction (LLME) and fractionation

LLME was performed to extract aroma-active compounds from Baijiu. An appropriate amount of Baijiu sample was diluted to 15% in ethanol (*v/v*) with ultrapure water (Millipore, Bedford, MA), and NaCl was added to saturation. Internal standards were also added according to the proportion of 1 mL of original Baijiu with 20 ppm internal standards. Then, 5.0 mL of the above sample were transferred to a 10.0-mL centrifuge tube, and 1.0 mL of dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>, extraction solvent) was injected into the Baijiu solution. The mixture was shaken vigorously on an IKA VORTEX2 vortex agitator (Janke & Kunkel, Staufen, Germany) at 2800 rpm for 60 s. The emulsion was then divided into two phases by centrifugation at 8000 rpm for 5 min at room temperature, and the sample to be analyzed was the CH<sub>2</sub>Cl<sub>2</sub> phase, which was extracted for GC-MS and GC-O-MS analysis.

### 2.4. Identification by GC-MS and GC-O-MS

GC-MS analysis was performed on an Agilent 7890B gas chromatograph equipped with an Agilent 5977A mass selective detector (MSD) (Agilent Technologies, Santa Clara, CA). All samples were analyzed on a DB-WAX column (60 m × 0.25 mm i.d., 0.25 µm film thickness; J&W Scientific, USA). The flow of carrier gas (helium) was set to 1 mL/min, and the injection (1 µL) was performed in splitless mode at 250 °C. The oven temperature increased from 40 °C to 50 °C (held for 5 min) at a rate of 10 °C/min, then increased from 50 °C to 80 °C (held at 5 min) at a rate of 3 °C/min, and finally increased at a rate of 5 °C/min until it reached 230 °C (10 min).

GC-O-MS analysis was performed on an Agilent 7890B gas chromatograph equipped with an Agilent 5977A MSD and a sniff port (ODP3, Gerstel, Germany). All samples were analyzed with a DB-FFAP column (60 m × 0.25 mm i.d., 0.25 µm film thick; J&W Scientific, USA) and an HP-5MS column (60 m × 0.25 mm i.d., 0.25 µm film thick; J&W Scientific, USA). The temperature of the olfactory port was 250 °C, the full-scan range was *m/z* 35–400, the ion source temperature was 230 °C and the other GC conditions were the same as those for GC-MS. Three trained panelists from the Laboratory of Food, College of Applied Chemistry at Beijing Technology and Business University were chosen to perform the GC-O-MS analysis. During the GC operation, an evaluator put his/her nose close to the sniffing port and recorded the smell, intensity and retention time of the stimulus.

The aroma compounds were identified by comparing the mass spectrum, aroma and retention index (RI) with those of the pure standards. The RI was calculated by injecting the C<sub>7</sub>–C<sub>40</sub> *n*-alkane mixture under the same GC–MS conditions as those for Baijiu samples.

1-Hexanol, ethyl acetate, ethyl hexanoate, and ethyl lactate were quantified by gas chromatography with a flame ionization detector (GC–FID) because these compounds were present in high concentrations. Their separations were performed on a DB-FFAP column and the other GC conditions were the same as those for GC–MS: one microliter of Baijiu sample with 40 mg/L spiked pentyl acetate and 4-octanol was injected into the GC in splitless mode. Stock solutions were prepared by mixing the standard and diluting it with absolute ethanol, followed by 2-fold serial dilutions with 52% ethanol. Then, IS1 and IS2 were added to the standard solution, which was analyzed by GC–FID in the same way as the Baijiu sample. The standard curve was constructed by taking the ratio of the concentrations of the target compound to the internal standards as the abscissa and the ratio of the responses of the target compound to the internal standards as the ordinate. The concentrations of the target compounds in the Baijiu sample were calculated according to the calibration curves. A correction factor was estimated from the ratio of the response of the target compound to the internal standard compound at the same concentration. Other compounds were quantified by GC–MS using the internal standard method, and the analysis conditions were the same as those for the GC–MS analysis described above.

## 2.5. TP-HS–GC–IMS analysis

TP-HS–GC–IMS analysis was performed on an advanced IMS system manufactured by FlavourSpec® (G.A.S., Dortmund, Germany) coupled with an Agilent 7890B gas chromatograph. The system was equipped with an Acrichi Automatic Headspace Sampler (Beijing Juxinzhufeng Technology Co., Ltd., China) as the headspace sampling unit.

An appropriate amount of Baijiu sample was diluted 2.6-fold with ultrapure water. For volatile organic compound (VOC) analysis, 1 mL of diluted Baijiu sample was introduced into a 20-mL headspace vial and subsequently incubated at 60 °C for 10 min. The sampling time, injection time, pressure time and backflushing time were set to 20 s, 20 s, 30 s and 1 min, respectively, and the total analysis time was 50 min. A syringe temperature of 90 °C was used to avoid condensation effects. Nitrogen (99.99% purity) was used as the carrier gas. Then, the samples were driven into a DB-FFAP capillary column (30 m × 0.25 mm i.d.) by nitrogen at a constant flow rate of 1.5 mL/min. The GC oven temperature was programmed as follows: initial temperature 40 °C, hold for 5 min, temperature ramp up to 80 °C at 2 °C/min, hold for 3 min, then an increase to 230 °C at a rate of 10 °C/min.

The IMS drift time cell was mounted beside the GC. Following GC separation, the analytes were ionized in the IMS ionization chamber by a 3H ionization source (300 MBq activity). The drift tube length was 98 mm and was operated at a constant voltage of 5 kV and a temperature of 45 °C with a nitrogen flow rate of 150 mL/min. The IMS cell was operated in positive ion mode. Each spectrum represented the average of twelve scans using an injection pulse width of 150 μs, a repetition rate of 30 ms, and blocking and injection voltages of 110 mV and 2500 mV, respectively.

All analyses were performed in triplicate. Volatile compounds were identified by comparing the RI and drift time to standards in the GC–IMS library.

## 2.6. Calculation of the odor activity values (OAVs)

The OAVs of the aroma compounds were calculated from the ratio of the concentrations detected in the Baijiu samples compared to their threshold values (Grosch, 1994). The threshold values were obtained by searching relevant literature. (Fan, Fan & Xu, 2015; Gao, 2014; Zhao et al., 2018; Zheng et al., 2016; Sha, 2017; Gemert, 2003).

## 2.7. Statistical analysis

Statistical analysis was performed through one-way analysis of variance (ANOVA) to identify significant differences among raw Baijiu samples from different distillation stages using Origin version 9.0 software (OriginLab Co., Northampton, MA). The TP-HS–GC–IMS data were viewed and processed by LAV software (G.A.S., Dortmund, Germany). PCA and PLS-DA were carried out with SIMCA version 14.0 (Umetrics, Umeå, Sweden). Heatmap analysis and hierarchical cluster analysis (HCA) were conducted by Yihanbo Biological Information.

## 3. Results and discussion

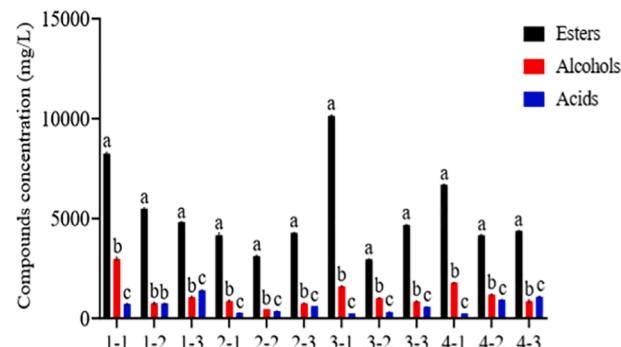
### 3.1. Variation in the ethanol content results and discussions

As shown in Fig. S1, the alcohol content showed a gradual decreasing trend with increasing distillation time, i.e., the head Baijiu samples had the highest alcohol content and the tail Baijiu samples had the lowest alcohol content. During distillation, the ethanol content of the Baijiu head was 74°–80°, the Baijiu heart was 55°–74°, and the Baijiu tail was 31°–50°.

### 3.2. Volatile compounds of raw Baijiu at different distillation stages

During Baijiu distillation, its temperature will continue to increase under the continuous heating of steam. Many trace flavor compounds in the fermented grains will also enter the raw Baijiu body with the steam during the distillation. Due to the differences in the numbers and contents of the flavor compounds, raw Baijiu had different flavor characteristics at different distilling stages. A total of 121 volatile flavor compounds were detected by GC–MS in 12 raw Baijiu samples during distillation, including 66 esters, 15 alcohols, 14 acids, 6 aldehydes, 3 ketones, 6 nitrogenous substances, 7 phenols and 4 other compounds. Among them, the esters, alcohols, and acids were the most diverse and highest-content volatile flavor substances detected. Therefore, this experiment mainly studied the changes in esters, alcohols (except ethanol), and acids that greatly contribute to the flavor of Baijiu.

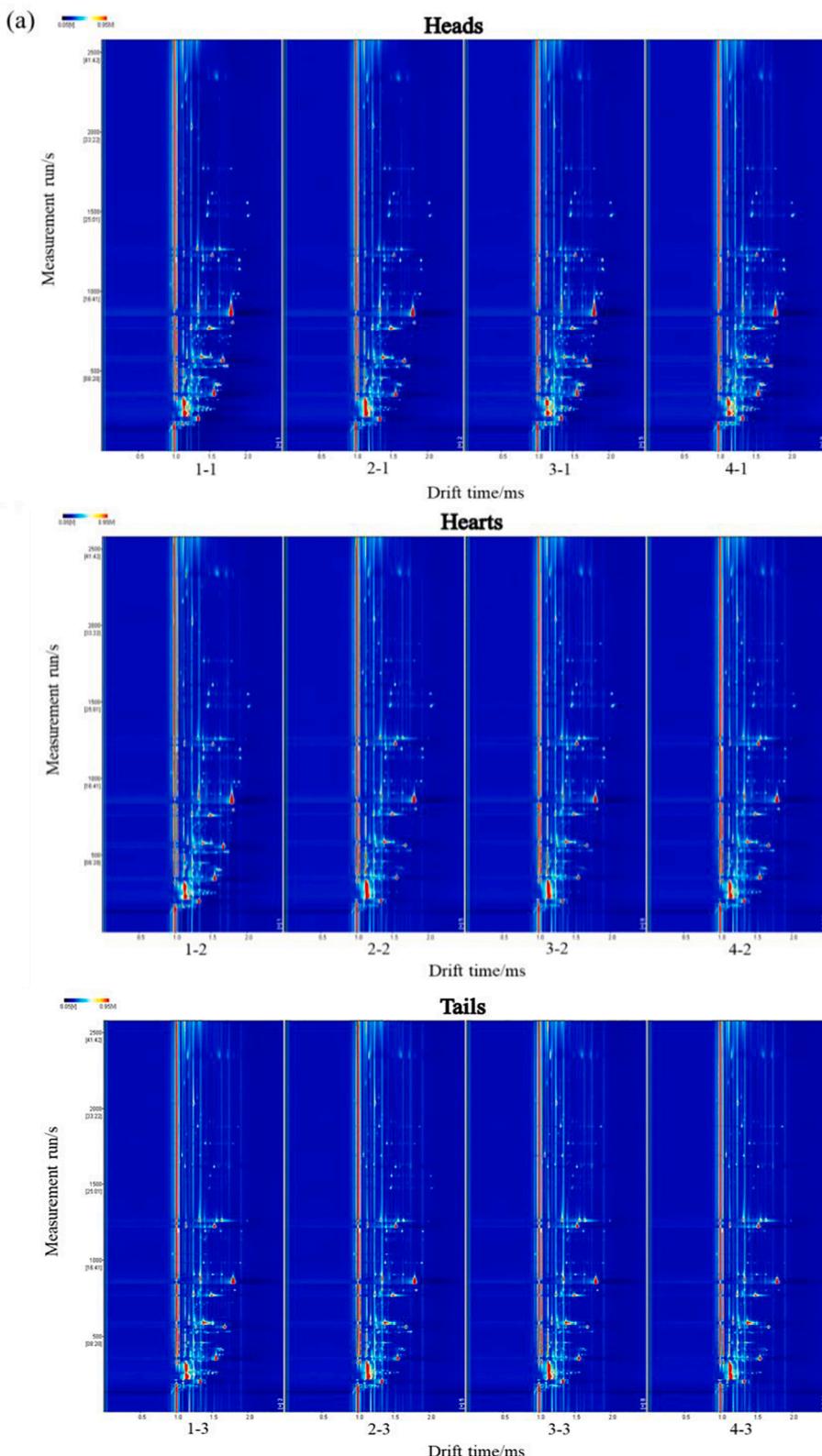
The total concentrations of esters, alcohols and acids from different distillation stages were quantified; the results are shown in Fig. 1. From Fig. 1, we can see that the contents of esters in each sample were significantly higher than those of the alcohols and acids ( $p < 0.05$ ). The contents of total esters gradually decreased from the head Baijiu to the tail Baijiu with the increase in the distillation time on the first layer of fermented grains, but there was a tendency to first decrease and then increase on the second, third and fourth layers of fermented grains. The head had the highest ester contents in each layer of fermented grains.



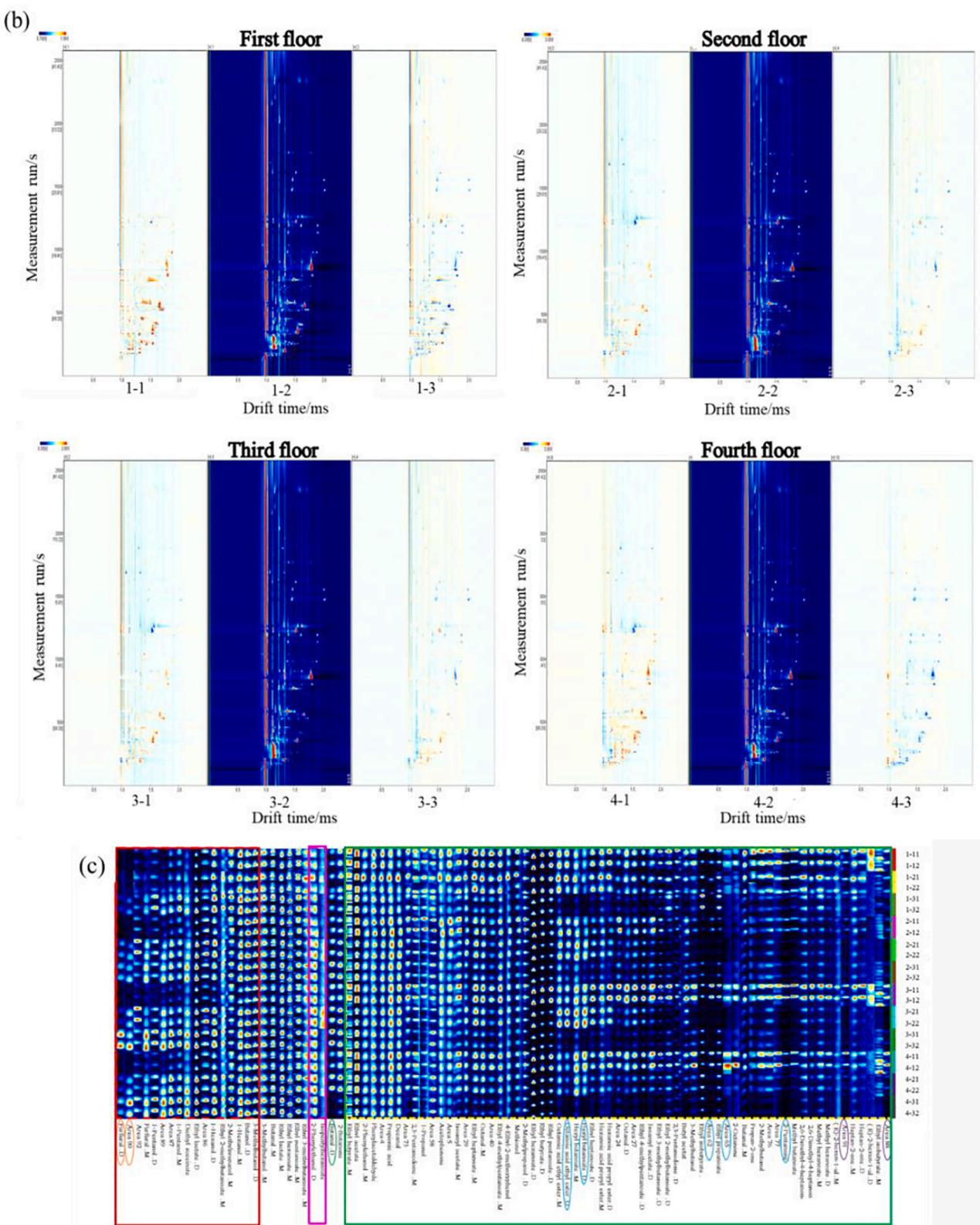
**Fig. 1.** Variation in total ester, alcohol and acid concentrations of raw Baijiu from different distillation processes. The concentrations of volatile compounds were represented as the mean and standard derivation (mean ± SD,  $n = 3$ ). Letters a, b and c represented the significant variation among total ester, alcohol and acid from raw Baijiu (one-way ANOVA with Bonferroni test,  $p < 0.05$ ).

This was because a large amount of ethanol in the fermented grains was steamed out at the beginning of the distillation, and the alcohol-soluble esters were steamed out together. Through research, it was also confirmed that there were a lot of alcohol-soluble and small molecular

weight esters in head Baijiu. Additionally, the first layer fermented grains produced the highest total ester content (18.6 g/L), followed by the third (17.8 g/L) and fourth layers (15.2 g/L), with the second having the lowest ester content (11.6 g/L).



**Fig. 2.** TP-HS-GC-IMS analysis based on raw Baijiu. (a) 2D topographic images of raw Baijiu samples in the three distillation periods; (b) 2D topographic images with differential comparison mode of raw Baijiu samples during different distillation stages produced by four layers of fermented grains; and (c) fingerprints of all samples generated using the Gallery Plot.



**Fig. 2.** (continued).

The changes in alcohol contents were similar to those of the esters. The total alcohol contents showed the trend of first decreasing and then increasing from the head Baijiu to the tail Baijiu of the first and second layer of fermented grains, but there was a decreasing tendency in the third and fourth layers of fermented grains with increasing distillation time. The head Baijiu samples had the highest alcohol contents of each

of the layers of fermented grains. Moreover, the raw Baijiu produced by the first layer of fermented grains had the highest total level of alcohols with a concentration of 4822 mg/L, which significantly decreased by 20.28% in the fourth layer, by 27.88% in the third layer, and by 56.50% in the second layer.

Total acids showed an upward trend overall with the extension of the

distillation. The acid contents were low in the early stage of distillation and reached their maximum in the tail Baijiu. Acid compounds were mostly concentrated in the late distillations because most organic acids were easily soluble in water but hardly soluble in ethanol, and their boiling points were high. In addition, the raw Baijiu produced by the first layer of fermented grains had the highest total acids with a concentration of 2.88 g/L, followed by the fourth layer with a concentration of 2.26 g/L, and the second and third layers had the lower concentrations of 1.26 g/L and 1.14 g/L, respectively.

### 3.3. TP-HS-GC-IMS analysis

The volatile components of different distilling stages with different layers of fermented grains were detected via TP-HS-GC-IMS analysis.

A FlavourSpec® instrument was used to generate data, as shown in Fig. 2 (a), (b) and (c). Fig. 2 (a) showed 2D topographic plots, which compares the compounds in the Baijiu samples of the same distillation stage of Baijiu produced from different layers of fermented grains. The background of all samples was blue, and the red vertical line at the horizontal coordinate of 1.0 was the reactive ion peak (RIP), which was normalized. Each point on the right side of the RIP symbolized a VOC. Then, to clearly compare the differences among samples from different distillations, differential comparisons mode could be adopted, as seen in Fig. 2 (b). That was, the spectral diagram of one sample (the heart Baijiu sample) was selected as the reference, while the spectral diagram of other samples was deducted from the reference. If two VOCs were identical, then the background after deduction was white. Red spots indicate that the concentration of the substance was higher than that of the reference, whereas blue spots indicate that the concentration of the substance was lower than that of the reference. In addition, a difference comparison of fingerprint maps that involved all plots and areas of the compounds at different distillation stages is shown in Fig. 2 (c), where each row represents a sample and each column represents a compound; each sample was analyzed twice in parallel. A total of 73 signal peaks were identified and marked with an existing name, as presented in Table S1.

As shown in Fig. 2 (a), the head group samples (1-1, 2-1, 3-1, 4-1) were similar in the peak number and area except for the 2-1 sample, which had a lower area than the other samples. The differences between the heart group samples (1-2, 2-2, 3-2, 4-2) and the tail group samples (1-3, 2-3, 3-3, 4-3) were small, indicating that the samples of the same distillation stage were similar in the number and contents of the compounds. Additionally, as shown in Fig. 2 (b), the head samples of the four groups showed large red spots and a small number of blue spots; that was, the contents of most compounds in the head samples were higher than those in the heart, and only a small number of compounds had lower contents. However, the tail samples showed a large number of blue spots and a small number of red spots; that was, the contents of most compounds in the tail samples were lower than those in the heart, and a small number of compounds had higher contents than those in the heart, indicating that the contents of most substances decreased with the distillation time extension, although a few substances increased. Combined with the fingerprint spectrum in Fig. 2 (c), we can conclude that: (1) the concentrations of ethyl acetate, butyl acetate, isoamyl acetate, ethyl propanoate, methyl butanoate, ethyl isobutyrate, ethyl butyrate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, hexyl butanoate, ethyl pentanoate, ethyl 4-methylpentanoate, methyl hexanoate, ethyl hexanoate, propyl hexanoate, ethyl heptanoate, ethyl octanoate, methionol, 1-propanol, 2-methyl-1-propanol, propionic acid, phenyl-acetaldehyde, 2-methylbutanal, 3-methylbutanal, (E)-2-hexenal, octanal, decanal, acetophenone, acetone, 2-pentanone, 2,3-pentanedione, 2-heptanone, 2,6-dimethyl-4-heptanone, 2-octanone, and 4-ethyl-2-methoxyphenol in the green frame gradually decreased with increasing distillation time; (2) the concentrations of 1-hexanol, ethyl lactate, 3-methyl-1-butanol, butanol, 1-pentanol, furfural and diethyl succinate in the red frames gradually increased with increasing

distillation time; and (3) isoamyl hexanoate and 2-phenylethanol in the pink frames showed a tendency to increase and then decrease with the extension of distillation time. In addition, 2-pentanone, area 65, area 62 and the dimers of compounds such as ethyl octanoate and hexyl butanoate in the blue circles existed in the head and heart samples but were hardly contained in the tail samples. The dimers of furfural and area 90 in the orange circles were hardly contained in the head samples. Areas 88 and 91 with purple circles were mostly present in the head samples and less prevalent in the heart and tail samples, and the dimers of butanal in the green circles were present in all Baijiu samples except 4-3.

### 3.4. Comparison of the results via TP-HS-GC-IMS and GC-MS

Table S1 indicates that in 12 raw Baijiu samples, 46 compounds were identified via TP-HS-GC-IMS, and 121 ones were identified via GC-MS. Also, 33 mutual compounds were identified via these two analysis methods as shown in Fig. S2.

Some compounds, including decanal, octanal, 2,6-dimethyl-4-heptanone, (E)-2-hexenal, 2,3-pentanedione, 2-butanone, butanal, acetone, methyl butanoate, 2-octanone, acetophenone, methionol and 2-methylbutanal could not be detected by GC-MS, but they were detected by TP-HS-GC-IMS. Due to the high sensitivity of GC-IMS, the compounds could be separated by GC column and chemical ions were characterized substances based on their differences in the rate of migration of the gas phase ions in an electric field (Shvartsburg, 2010). Although Fig. 2 showed the changing trends of compounds in different samples, some volatile compounds could not be confirmed due to the incomplete database. GC-MS identified more compounds than TP-HS-GC-IMS, but the sample must be pretreated and extracted via LLME for GC-MS analysis, otherwise the efficiency will be reduced. Hence, a combination of GC-IMS with GC-MS could more comprehensively identify and quantify the volatile compounds.

### 3.5. Qualitative analysis of aroma compounds via GC-O-MS

To identify compounds that contribute to the overall aroma profile, GC-O-MS was used to analyze the raw Baijiu samples. A total of 47 odorants (Table S2) were identified by mass spectrometry, aroma and RI, including 24 esters, 6 alcohols, 7 acids, 3 aldehydes, 5 aromatic compounds, 1 other compound and 1 unknown compound. Table S3 shows the concentrations of the compounds in each sample. As shown in Table S4, the OAVs of these 47 aroma compounds ranged from 0 to 46,521 according to GC-O-MS analysis. Based on Table S4, ethyl hexanoate contributed a strong fruity flavor to the aroma of Baijiu and had the highest OAVs (12984 to 46521) among the aroma-active compounds and reached its maximum in the head of the Baijiu produced by the third layer of fermented grains, which was consistent with previous research results. In addition, ethyl butanoate (247 to 11465; pineapple), ethyl pentanoate (439 to 7145; apple), ethyl octanoate (1093 to 7603; fruity), isovaleraldehyde (1021 to 6613; aldehydic chocolate), ethyl 4-methylpentanoate (110 to 1317; fruity), 4-ethyl-2-methoxyphenol (68 to 634; phenolic clove), butyl acetate (11 to 811; pear), butanoic acid (55 to 427; sweaty, rancid), ethyl 3-methylbutanoate (55 to 442; apple), ethyl isobutyrate (14 to 497; fruity alcoholic), and hexanoic acid (45 to 208; sweaty, rancid) had high OAVs. Most of the above substances reached their maxima in the head Baijiu produced by the third layer of fermented grains and the tail Baijiu produced by the first layer. 2-Phenylethanol (rosy, honey), ethyl nonanoate (wood), heptanoic acid (wood), furfural (wood), 3-methyl-1-butanol (fruity), hexyl butyrate (green waxy soapy), acetic acid (vinegar), ethyl heptanoate (apple), 4-ethylphenol (phenolic), 2-methyl-1-propanol (fruity), and phenethyl acetate (floral rose) had low OAVs, and the OAVs of most compounds were <1.

### 3.6. Statistical analysis

PCA was carried out based on the aroma compounds data to reveal the distribution of the samples, of which the PCA score and loading plots were shown in Fig. 3. A trend of intergroup separation was shown on the score plot of data obtained in both positive and negative modes. The principal components (PCs) PC1 and PC2 represented 45.4% and 21.7% of the total variance, respectively, with the cumulative contribution rate of the first two PCs accounting for 67.1%, which indicates that they were sufficient to explain the total variance in the dataset. The Baijiu samples clustered into three distinct groups in the dimensional graph based on the PC1 × PC2 score, as shown in Fig. 3 (a). PC1 was positively correlated with the head Baijiu samples but negatively correlated with the tail Baijiu samples. These results showed that there were significant differences between the Baijiu samples at different distillation stages.

Additional information can also be found from the loading plot in Fig. 3(b). As pictured, PC1 was strongly associated with the compounds in red circle, indicating higher concentrations in samples that showed positive scores (the head Baijiu). On the other hand, the compounds in blue circle were negative correlation with PC1, while the tail Baijiu samples with negative scores on PC1 corresponded to high concentrations of those compounds.

The results obtained above showed that the different distillations of baijiu caused significant differences with respect to the aroma compound profiles.

As a supervised multivariate statistical analysis, PLS-DA can be considered a good model for biological data and provide a powerful approach to discriminate samples with different characteristics. Thus, PLS-DA models were used in this study to identify specific marker compounds that led to the differences in the aromas of 12 Baijiu samples according to the data matrices of the detected aroma active compounds at these concentrations and OAVs. A variable importance in projection (VIP) value variable selection method was applied to identify the most statistically significant variables. First, logarithmic transformation was used for all the detected compounds, giving values in a smaller range without masking the effects of small values within the data. For concentration analysis, as seen in Fig. 4 (a), 65.6% of the total variance with  $R^2Y = 97.62\%$  and  $Q^2 = 92.65\%$  was explained, and three groups of samples were successfully differentiated in the quadrant diagram: (a) first and second quadrant sample dots for the heart samples except for sample 4-2; (b) third quadrant sample dots for the tail samples except for samples 2-3 and 3-3; and (c) fourth quadrant sample dots for the head samples except for sample 2-1. In addition, permutation tests ( $n = 200$ ) were performed to evaluate whether the discriminant models were overfitting the data. The permutation tests randomly rearranged the experiments by changing the sort order of the classification variables (Y)

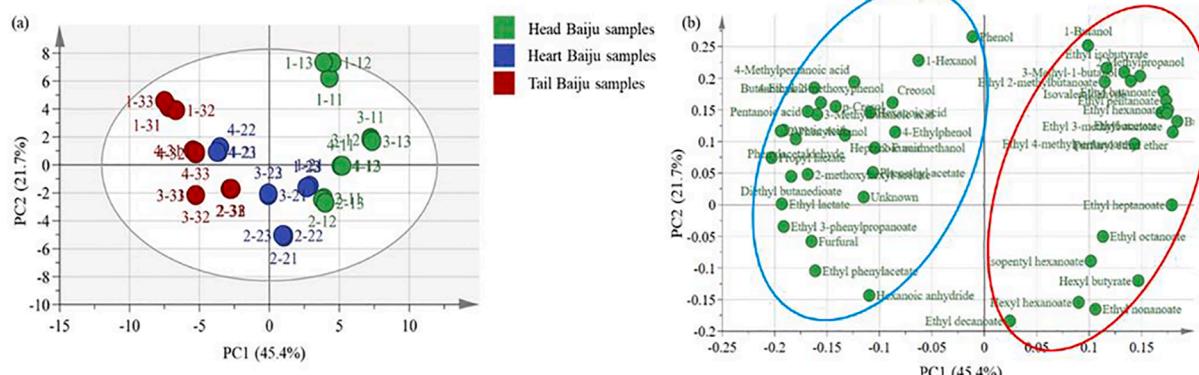
and randomly assigned Q2Y up to 200 times. The recommended values for good fitting of models have been described, as all blue Q2 values to the left are lower than the original points to the right or the blue regression line of the Q2 points intersects the vertical axis (on the left) at or below zero. Herein, the permutation tests results met the above conditions, which indicated that the models were not overfitting the data. So the initial model outperformed the randomly permuted models (Fig. 4b). Then 20 different compounds were selected from the initial 47 detected aroma active compounds that showed high VIP values ( $VIP > 1.0$ ) as seen in the red frame in Fig. 4 (c), namely, furfuryl ethyl ether, ethyl acetate, ethyl isobutyrate, 4-ethyl-2-methoxyphenol, ethyl 3-phenylpropanoate, ethyl lactate, ethyl heptanoate, isovaleraldehyde, hexanoic anhydride, phenol, 1-butanol, furfural, 2-methyl-1-propanol, 3-methyl-1-butanol, acetic acid, butyl acetate, propyl lactate, 4-ethylphenol, 2-methoxyhexyl acetate and ethyl butanoate.

By contrast, as seen in Fig. 4 (d), 55.6% of the total variance with  $R^2Y = 89.58\%$  and  $Q^2 = 76.73\%$  was explained by OAVs, and three groups of samples were successfully differentiated in the quadrant diagram similar to the results above. Also, the permutation tests results indicated that the models were not overfitting the data, and the initial model outperformed the randomly permuted models (Fig. 4 (e)). Moreover, 17 marker compounds, as seen in the red frame in Fig. 4 (f), namely, 4-ethyl-2-methoxyphenol, ethyl acetate, ethyl 3-phenylpropanoate, ethyl lactate, 3-methyl-1-butanol, acetic acid, ethyl butanoate, hexanoic acid, butyl acetate, furfural, isoamyl hexanoate, ethyl pentanoate, ethyl hexanoate, ethyl isobutyrate, ethyl 3-methylbutanoate, phenylacetaldehyde and 2-phenylethanol, were also identified with high VIP values ( $VIP > 1.0$ ).

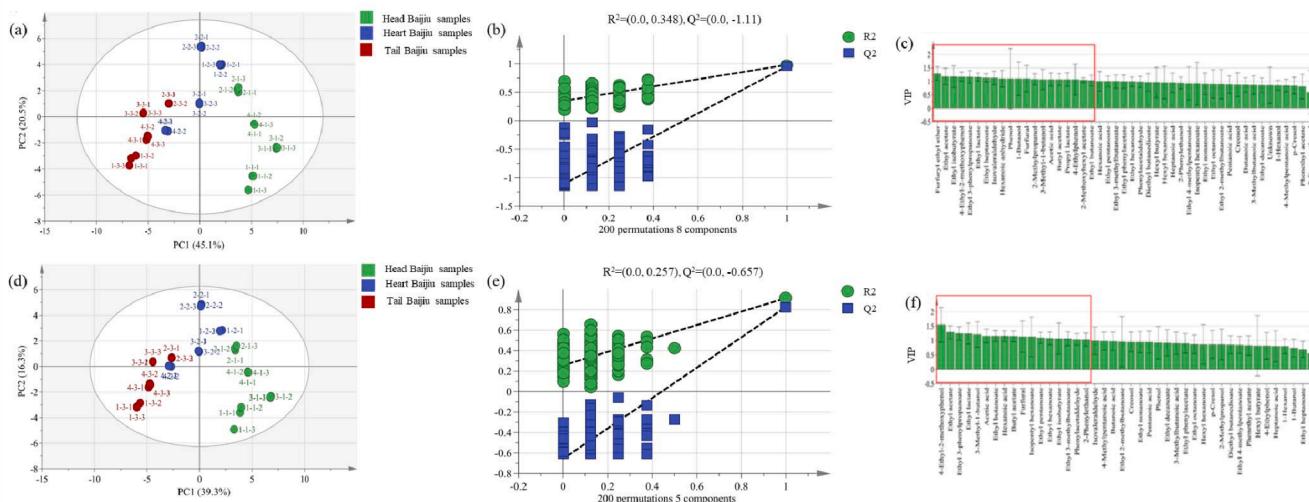
To make the results more convincing and the selected key substances more representative, the compounds common to the two VIP results were taken as the final key compounds. Overall, 10 discriminant compounds, namely, 4-ethyl-2-methoxyphenol (phenolic clove), ethyl acetate (pineapple), ethyl 3-phenylpropanoate (floral), 3-methyl-1-butanol (fusel oil), acetic acid (vinegar), ethyl butanoate (pineapple), butyl acetate (pear), ethyl lactate (fruity), furfural (sweet, almond) and ethyl isobutyrate (fruity alcoholic) were identified as the most potent volatiles contributing to discriminating the aroma perception of the raw Baijiu samples at different distillation stages.

### 3.7. Heatmap and HCA of marker compounds

To elucidate the associations among the quantitated marker compounds, the concentrations of the quantitated compounds in the 12 raw Baijiu samples were used to prepare a heatmap, and the color (from blue to red) indicated that the relative intensity changed from low to high. First, log 2 transformation was used for all marker compounds before



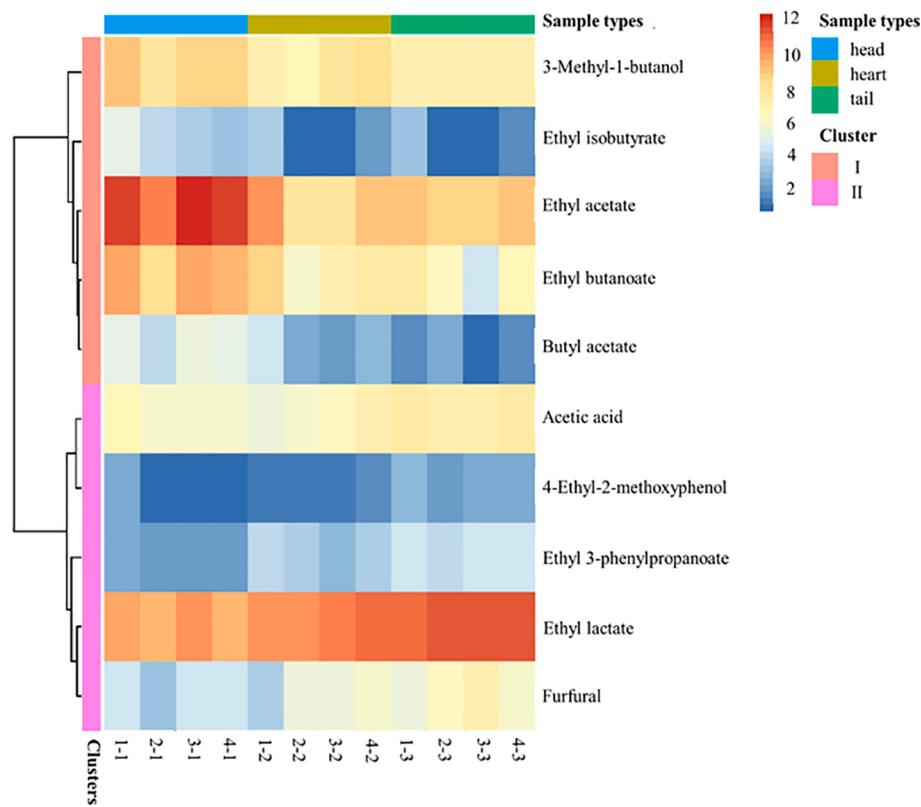
**Fig. 3.** PCA analysis based on aroma-active compounds. (a) scores and (b) loading plot of PCA for classification of samples from different distillations. Green, blue and red represent the head, heart and tail Baijiu samples, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Fig. 4.** PLS-DA analysis based on aroma active compounds. (a) scores, (b) permutation test and (c) VIP plot using the concentrations of aroma-active compounds, which summarized the importance of the variables to both explain the aroma-active compounds and correlate with Baijiu samples, sorted from high to low; (d) scores, (e) permutation test and (f) VIP plot using OAVs of aroma-active compounds. Green, blue and red represent the head, heart and tail Baijiu samples, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

construction of the heatmap. The differences between the marker compounds among the samples are shown in Fig. 5. The HCA clearly classified the quantitated markers into two clusters. Cluster I consisted of 3-methyl-1-butanol, ethyl isobutyrate, ethyl acetate, ethyl butanoate and butyl acetate, which had significantly higher mean concentrations in the 4 head Baijiu samples and showed a downward trend with the extension of distillation time. This was because these substances were easily soluble in ethanol, a large amount of ethanol was evaporated in the early stage of distillation and a large amount of alcohol-soluble

substances were steamed out together. With the extension of the distillation time, the concentration of ethanol decreased and the concentrations of alcohol-soluble compounds also gradually decreased. Cluster II consisted of 5 chemical markers, including acetic acid, 4-ethyl-2-methoxyphenol, ethyl 3-phenylpropanoate, ethyl lactate, and furfural, which had significantly higher mean concentrations in the 4 tail Baijiu samples and showed an increasing trend with the extension of distillation time. The reasons were that these compounds were more soluble in water and had a higher boiling point. A large amount of ethanol was



**Fig. 5.** Heatmap for the 10 quantitated marker compounds and HCA of the 12 raw Baijiu samples.

steamed out in the early stage of distillation, which was not conducive to the steaming of these water-soluble compounds. In the later stage, a large amount of water-soluble and high-boiling compounds gradually steamed out and accumulated in the tail with the ethanol concentration decreasing (Li et al., 2012).

Moreover, a heatmap and HCA were used to further analyze the changes in these 10 aroma active compounds from different distillations and their important contributions to the flavor of Baijiu. The result is helpful for the intelligent classification and to promote the control of the quality of strong flavor raw Baijiu.

#### 4. Conclusion

The changing trends in the flavor compounds in Baijiu from different distillations fermented from different layers were investigated by GC-MS and GC-IMS. The Baijiu samples from the same distillation had similar compound types and contents, but there were great differences between the different distillations of Baijiu samples. The flavor compounds were more abundant in the head Baijiu samples than in heart and tail samples. Most alcohol-soluble and low molecular weight compounds showed a downward trend with the extension of distillation time, but water-soluble, high molecular weight and high boiling compounds showed the opposite result. Moreover, the differences in compounds identification between the two detection methods were compared. Then, 47 compounds were sniffed by GC-O-MS, which were analyzed by PCA and PLS-DA to efficiently reveal three categories of raw Baijiu within different distillations, as well as their separation-related aroma active markers. They were 4-ethyl-2-methoxyphenol, ethyl acetate, ethyl 3-phenylpropanoate, 3-methyl-1-butanol, acetic acid, ethyl butanoate, butyl acetate, ethyl lactate, furfural and ethyl isobutyrate.

#### CRediT authorship contribution statement

**Fei He:** Investigation, Methodology, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Jiawen Duan:** Methodology, Formal analysis, Data curation. **Jiwén Zhao:** Conceptualization, Supervision, Resources. **Hehe Li:** Conceptualization, Writing - review & editing. **Jinyuan Sun:** Conceptualization, Writing - review & editing. **Mingquan Huang:** Conceptualization, Writing - review & editing. **Baoguo Sun:** Conceptualization, Writing - review & editing.

#### Declaration of Competing Interest

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

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2021.130430>.

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