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Revealing the Changes in Compounds When Producing Strong-Flavor Daqu by Statistical and Instrumental Analysis

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Abstract: Daqu is not only a crucial starter in the production of baijiu, but it is also an important source of flavoring substances, so maintaining a stable quality is an important part of improving the quality of baijiu. Nonetheless, since the production of daqu is still a natural fermentation process, which is influenced by seasonal factors, the rapid testing of daqu quality is a problem that must be solved. In this study, headspace solid-phase microextraction technology (HS-SPME) was used to explore the volatile components in daqu, and a total of 115 volatile components were extracted. By constructing an untargeted statistical model, the variation in volatile compounds in dissimilar production processes of daqu was studied, and the differences between different maturation stages and the correlations between volatile compounds were analyzed. Subsequently, six compounds, including ethyl acetate, ethanol, phenylethanol, (R,R)-2,3-butanediol, ethyl caproate, and 2,3-butanediol, were further screened out by partial least squares discrimination analysis (PLS-DA), and the symbolic combination of daqu's maturity was speedily judged in accordance with the changes in marker compound concentrations to lay the foundation for the mechanization of baijiu production.

Keywords: baijiu; daqu; flavor; rapid judgment; marker compounds

1. Introduction

As a unique beverage in China with a long history of more than 2000 years, baijiu has always been deeply loved by people [1]. Baijiu can be divided into 12 flavor types in line with dissimilar production processes [2]. Traditionally, baijiu is made from grains as raw materials. After a series of complicated processes, such as cooking, adding qu, saccharification, fermentation, and distillation, a traditional Chinese brewed beverage with a complex aroma and rich flavor are ultimately obtained [3,4]. Baijiu uses a stater called "jiuqu" for saccharification and fermentation in the brewing process. In general, jiuqu can be categorized as daqu, xiaoqu, and fuqu [5]. One of the most commonly employed is daqu. In accordance with the culture temperature, daqu can be divided into three categories: low-temperature type, medium-temperature type, and high-temperature type [6], which are adopted to produce diverse flavor types of baijiu. Light-flavor baijiu uses low-temperature daqu with the highest product temperature of 40–50 °C. Strong-flavor baijiu generally uses medium-temperature daqu with the highest product temperature of 50–60 °C, and sauce-flavor baijiu generally uses high-temperature daqu with the highest product temperature greater than 60 °C. Daqu is made of grain and is shaped like rectangular bricks. After artificial caiqu, these bricks are placed in a daqu house. At this time, bacteria, mold, yeast, and other microorganisms began to work. [7]. Within one month, the anqu duration will normally vary in line with the climate and temperature. When daqu is in the anqu process,

rich flavors can be obtained through the fermentation and culture of microorganisms, making the flavor of baijiu diversified.

Apart from being employed as a fermentation starter during the production processes of baijiu, daqu can also be employed as a "flavoring agent" to directly or indirectly provide baijiu with various flavors [8]. Dissimilar fermentation environments will give rise to the diversification of microorganisms when producing daqu, which will affect the flavor and fermentation metabolism of daqu [9]. Strong-flavor baijiu is one of the important styles of baijiu, and some of its flavor components are provided by daqu. Discussing the changes in volatile components in the production process of daqu can provide ideas and references for the study of flavor substances in strong-flavor baijiu. Notwithstanding the fundamental fact that scholars have conducted a myriad of studies on the microbial community in daqu, primarily exploring the correlation between microbes and flavor components [10,11], but the variation in volatile compounds has been rarely discussed. In this study, the aim is to analyze the volatile compounds in the production process of daqu.

There are many methods currently applied to the study of volatile compounds in the field of baijiu. The direct injection method (DI) involves the direct injection of the sample into a gas chromatograph (GC) for separation and detection without or with simple treatment. Liquid–liquid extraction (LLE) is the extraction and concentration of trace components in baijiu using organic solvents to enrich volatile compounds. HS-SPME is a solvent-free extraction method for the extraction of volatile compounds from baijiu by coating adsorption. Solvent-assisted flavor evaporation (SAFE) is a combination of a distillation unit and a high vacuum pump to achieve the separation and analysis of flavor substances. In addition to the above methods, other methods for the extraction of volatile compounds include liquid–liquid microextraction (LLME), dynamic headspace adsorption (DHS), etc. Based on the characteristics of the various methods and the aim of this study, which is to achieve rapid detection of flavor and rapid determination of maturity level in the production process of daqu, HS-SPME, which is simple to operate and less expensive, was finally selected for this study.

As a consequence, this study is intended to analyze the types and contents of volatile components in daqu samples to reveal the change law of volatile compounds during the production process of strong-flavor daqu and to screen out the index compounds by various statistical methods so as to achieve a rapid determination of the maturity level of daqu, which in turn will lead to the improvement of the quality and stability of daqu and baijiu, and then to lay the foundation for the realization of the mechanized production of baijiu.

2. Materials and Methods

2.1. Sample Collection

Daqu samples were taken from a distillery fermentation workshop. Representative time points in the production process were selected, which were, respectively, 0, 4, 8, 12, 13, and 24 days, corresponding to six stages in the production process, which indicated that daqu was gradually matured. In addition, the samples at each stage were fermented independently in a qufang, and the temperature and humidity of the qufang were not fixed with the degree of fermentation but did not exceed 60 °C. In this study, several medium-temperature strong-flavor daqu samples were analyzed: A (production for 0 days), B (production for 4 days), C (production for 8 days), D (production for 12 days), E (production for 13 days), and F (production for 24 days, finished qu). Daqu samples were ground into powder and screened with a 40-mesh sieve, then were stored at −20 °C before the experiment. The RI were calculated on the basis of the modified Kovats method [12].

2.2. Chemicals

The following commercial standards (analytical reagent grade, ≥97% purity) were used: 4-octanol from Sigma-Aldrich (Shanghai, China) was employed as an internal standard. The ultra-pure water employed herein was obtained from a Milli-Q system. A

C8−C40 n-alkane mixture (Sigma-Aldrich, Shanghai, China) was employed for determining linear retention indices (RI). Absolute ethanol was from China National Pharmaceutical Group Corp (Shanghai, China).

2.3. Isolation of the Volatiles

A total of 1 g of ground daqu sample was taken; then, 5 μL of a 4-octanol solution was added in a 20 mL headspace bottle. It was covered, sealed, and equilibrated for 20 min at 40 °C. The fiber was 50/30 μm, DVB/CAR/PDMS. The fiber tip was placed above the sample and extracted for 40 min while stirring below 40 °C, and was then immediately analyzed for 5 min with a GC injector at 250 °C.

2.4. Gas Chromatography–Mass Spectrometry (GC-MS)

The volatile compounds were further evaluated by adopting GC-MS (7890B GC System, 5977A MSD). Each sample (1 μL each) was employed for analysis on a DB-FFAP capillary column (60 m × 250 μm × 0.25 μm, Agilent Technologies, Santa Clara, CA, USA). Helium (99.999%) was used as a carrier gas at a constant flow rate of 2.0 mL/min, and the inlet temperature was 250 °C. The oven temperature was maintained at 40 °C initially and was held for 3 min, ramped to 150 °C at a rate of 2 °C/min and was held for 2 min, and then finally was raised to 230 °C at a rate of 7 °C/min and held was for 8 min. The temperature of the transfer line was 250 °C, and that of the MS ion source was set at 230 °C. The ionization energy of the electron impact mass spectra was 70 eV, and the acquisitions were over an *m/z* scan range of 35–450 amu. The injection was performed in splitless mode.

Three parallel experiments were conducted for each sample, and the volatile compounds were identified by comparing their mass spectra in the NIST (2020) baijiu flavor compounds database developed by our group and their RI from the DB-FFAP column with those of the authentic standards. The retention indices of the compounds were calculated using alkanes (C8–C40). The n-alkanes of C8–C40 were configured in the n-hexane solution, which was selected to take into account the protection of the column. The solution concentration was 20 μg/L. The same heating procedure as the sample was selected. The injection mode was splitless, and the injection volume was 1 μL. After the RI were calculated according to the formula, they were compared with the values in the literature so as to realize the further characterization of the compounds.

2.5. Statistical Analysis

Statistical analysis and hierarchical cluster analysis (HCA) of volatile compounds in strong-flavor daqu were conducted by Origin version 9.0 software (Origin Lab Co., Northampton, MA, USA). SIMCA version 14.0 (Umetrics, Umea, Sweden) performed the principal components analysis (PCA) and PLS-DA.

3. Results and Discussion

3.1. Qualitative and Quantitative Analysis of Volatile Compounds

A total of 115 volatile compounds were detected in the strong-flavor daqu, including 31 esters, 13 alcohols, 7 acids, 13 aldehydes, 13 nitrogenous compounds, and so on. Ester compounds are mainly formed by microorganisms in daqu using alcohols and carboxylic acids. Almost all of the 31 ester compounds detected in this study were found in the flavor analysis of strong-flavor baijiu [13–15].

Figure 1 shows the variation in volatile compounds at dissimilar stages of the production process of strong-aroma daqu. As illustrated in Figure 1a, when elongating the production duration, the maximum change in volatile compound type was observed after 4 days of daqu production, which belongs to the material rapid growth stage. This is probably owing to the fact that multitudinous new substances are produced by microbial fermentation [16,17], such as through the Maillard reaction, and the reaction substrate is sufficient at this stage, thus achieving speedy growth. In the last four periods, the species of compounds changed were unremarkable, all within 10 species. At this time,

the variety of components was comparatively stable, but the concentrations of the compounds still changed. As depicted in Figure 1b, the species of acid compounds reached a maximum value in the first four periods after constant growth and then exhibited a decreasing trend before reaching relative stability. A similar trend existed for aldehydes, esters, and alcohols, which presented a comparatively balanced and harmonious level in the final period, suggesting that the daqu gradually matured. It is noteworthy that nitrogenous and sulphur-containing compounds are two extremely important categories in the study of baijiu flavor, with nitrogenous compounds usually showing a roasted aroma. Fan et al. [18] detected a variety of pyrazine compounds such as 2,6-dimethylpyrazine and 2-ethylpyrazine in strong-flavor baijiu such as Gujinggong and Jiannanchun. These pyrazines are often produced by the Maillard reaction. In addition to the above aroma, there also are certain health care effects [19]. Sulphur-containing compounds, on the other hand, have a considerably low olfactory threshold and usually have tremendously high odor activity values [20], thus making a considerably high contribution to the overall flavor development of baijiu. In this study, the nitrogenous compounds only appeared at stage C, and 13 species in total were detected. The sulfur compounds were only produced in the form of methyl mercaptan in the final stage, which indicates that in the latter stages, although the total types and concentrations of esters and alcohols changed very little, the overall flavor still changed significantly.
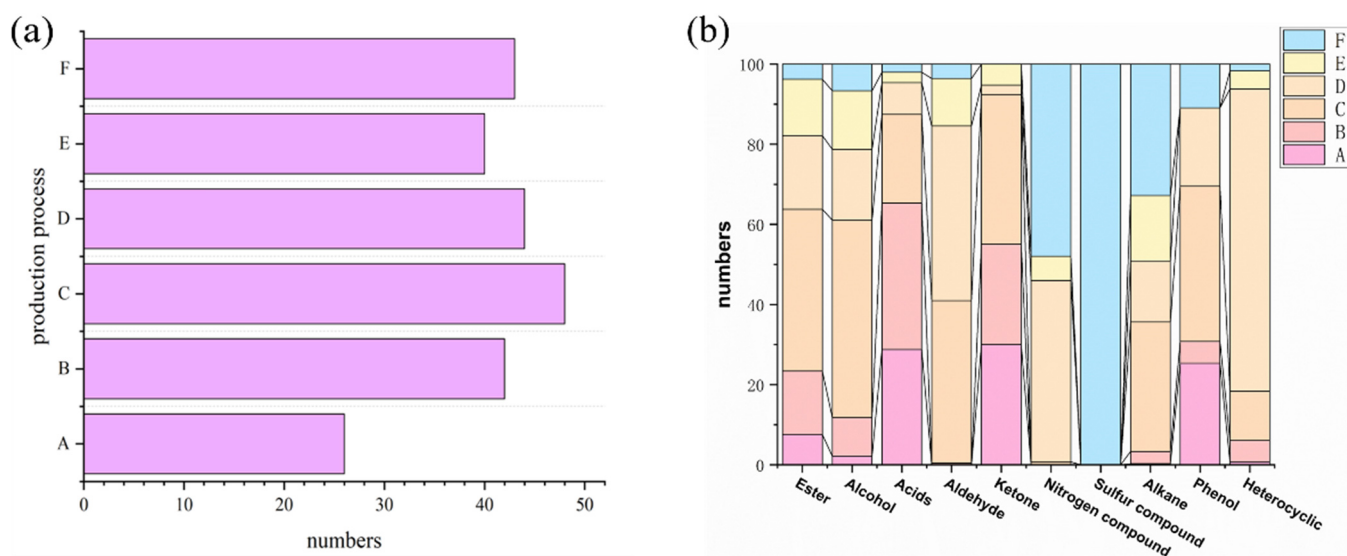

Figure 1. Variation in volatile compounds in dissimilar production process stages of strong-flavor daqu. (**a**) Changes in the total amount of volatile compounds when producing strong-flavor daqu. (**b**) Changes in the types and quantities of volatile compounds in dissimilar production processes of strong-flavor daqu. A, B, C, D, E, and F represent samples from different periods, respectively.

The concentration changes in volatile compounds in the different production processes of strong-flavor daqu are exhibited in Table 1. We found that some compounds could be detected in all the daqu production processes, such as ethyl acetate, ethyl caproate, ethyl pentadecanoate, ethyl octadecenoate, etc. Ester compounds give baijiu an important fruit aroma but also have certain health effects, such as ethyl acetate, which has a protective effect on acute liver injury. Similar to dibutyl phthalate, nonanoic acid, octanoic acid, 4-hydroxy-2-butanone, and 2,5-di-tert-butylphenol could only be detected in A; 12 species, such as ethyl valerate, hexyl formate, and ethyl heptanoate, were only present in stage B; 10 compounds, including isopentyl formate, 2,8-dimethylundecanoic acid, and methyl ester, were only detected in phase C. The mere compounds in D were methallyl alcohol, crotonyl alcohol, 2-methylhexadecanoic acid, 11,14-eicosadienoic acid, methacrolein, 2-vinyl-2-butenal, (E,E)-2,4-hexadienal, 2-Ethyl-5-methyl-pyridine, ethyl 5-amino-1,2,3-thiadiazole-4-carboxylate, 3,5-dimethylpyrazole, methyl anthranilate, 3,8-

dimethyl-undecane, and 2-ethyl-furan. The certain number of aldehydes in baijiu plays an indispensable role in regulating its aroma release [21], but excessive amounts will lead to a rough and unpleasant taste. Acid compounds not only directly affect the overall flavor of baijiu, for example, the existence of excessive lactic acid will cause the body taste to be sour and astringent, but also, acid compounds are precursors to the formation of ester compounds [22]. In the maturing process of daqu, acid compounds react with alcohol compounds to form ester compounds and further regulate the overall smell and taste of the baijiu body. They play an important role in the formation of daqu and baijiu flavor. 2-Butyloctanol, methyl 2-(propanoylamino)benzoate, 7,9-dimethyl-hexadecane, 2,6,10-trimethyl-dodecane, 1-methyl-2-(3-methylpentyl)-cyclopropane, 1,3,5,7-cyclooctatetraene, decene, and dihydroaplotaxene were merely found in E, while ethyl isobutyrate, ethyl isovalerate, diethyl phthalate, isobutyric acid, 2-pyrrolecarbaldehyde, 2-methyl-pyrazine, 4,6-dimethyl-pyrimidine, 2,3-dimethyl-pyrazine, trimethyl-pyrazine, 1,2-dimethyl-hydrazine, methanethiol, 2,2,4,6,6-pentamethyl-heptane, 2,2,11,11-tetramethyl-dodecane, pentadecane, 4,6-dimethyldodecane, 4-methyl-nonane, 2,3,3-trimethyl-pentane, 2,3,4-trimethyl-hexane, and (*E*)-2,3-epoxydecane are volatile compounds that could merely be detected in finished products. Through these compounds, we can also find that the volatile compounds in strong-flavor daqu have undergone tremendous changes with the prolonged production duration. The concentration of different volatile compounds also varied considerably at different stages of maturation. Ethyl acetate, ethyl butyrate, ethyl caproate, ethyl caproate, and 22 other compounds showed an increasing and then decreasing pattern of change in their concentration during the maturation of the daqu. Ethyl valerate isopentyl formate, hexyl formate, ethyl heptanoate, ethyl nonanoate, diethyl succinate, and 46 other compounds were only present in the middle stages, while ethyl isobutyrate, ethyl isovalerate, and methanethiol were only produced in the final stage. Based on the complex changes in the above compounds and their crucial flavor contributions to the existence of daqu and baijiu, it is necessary to deeply analyze the law between the changes in substances and the maturity level of daqu.

Table 1. Volatile compounds identified by HS-SPME-GC-MS in daqu.

No.	Compound	RI	CAS No	Molecule Formula	Odor Description	ID	Concentration (ng/g)					
							A	B	C	D	E	F
Esters												
1	ethyl acetate	877	141-78-6	$C_4H_8O_2$	fruity, sweet	MS/RI	1294.66 ± 70.65 [a]	1411.03 ± 61.80 [b]	2475.00 ± 254.71 [c]	845.03 ± 34.70 [d]	855.67 ± 59.28 [e]	589.01 ± 30.54 [f]
2	ethyl isobutyrate	936	97-62-1	$C_6H_{12}O_2$	fruity	MS/RI	-	-	-	-	-	31.49 ± 2.26 [a]
3	ethyl butyrate	1006	105-54-4	$C_6H_{12}O_2$	fruity, sweet	MS/RI	0.70 ± 0.09 [a]	-	36.62 ± 3.30 [b]	14.21 ± 1.46 [c]	-	-
4	ethyl isovalerate	1046	108-64-5	$C_7H_{14}O_2$	fruity, sweet	MS/RI	-	-	-	-	-	9.91 ± 1.95
5	isoamyl acetate	-	123-92-2	$C_7H_{14}O_2$	fruity, sweet	MS	28.01 ± 3.78 [a]	46.69 ± 1.32 [b]	114.17 ± 17.51 [c]	-	-	-
6	ethyl valerate	1108	539-82-2	$C_7H_{14}O_2$	fruity, sweet	MS/RI	-	3.66 ± 0.31	-	-	-	-
7	isopentyl formate	-	110-45-2	$C_6H_{12}O_2$	sharp, green	MS	-	-	670.93 ± 139.80	-	-	-
8	ethyl caproate	1259	123-66-0	$C_8H_{16}O_2$	fruity, sweet	MS/RI	189.22 ± 27.75 [a]	447.94 ± 22.87 [b]	1642.67 ± 50.04 [c]	509.17 ± 36.90 [d]	238.57 ± 5.47 [e]	135.83 ± 11.36 [f]
9	ethyl lactate	1323	97-64-3	$C_5H_{10}O_3$	fruity, sweet	MS/RI	91.13 ± 6.06 [a]	78.03 ± 6.03 [b]	63.40 ± 12.40 [c]	-	-	-
10	hexyl formate	1333	629-33-4	$C_7H_{14}O_2$	fruity, sweet	MS/RI	-	12.65 ± 1.58	-	-	-	-
11	ethyl heptanoate	1414	106-30-9	$C_9H_{18}O_2$	fruity	MS/RI	-	7.70 ± 1.28	-	-	-	-
12	ethyl caprylate	-	106-32-1	$C_{10}H_{20}O_2$	fruity	MS	29.73 ± 6.44 [a]	137.09 ± 4.21 [b]	126.28 ± 7.10 [c]	-	32.98 ± 20.53 [d]	-
13	ethyl nonanoate	-	123-29-5	$C_{11}H_{22}O_2$	fruity, rose	MS	-	14.31 ± 1.26	-	-	-	-
14	diethyl succinate	1702	123-25-1	$C_8H_{14}O_4$	fruity	MS/RI	-	27.39 ± 5.00	-	-	-	-
15	ethyl laurate	1901	106-33-2	$C_{14}H_{28}O_2$	floral, sweet	MS/RI	-	31.62 ± 12.42	-	-	13.65 ± 5.88	-
16	ethyl myristate	2016	124-06-1	$C_{16}H_{32}O_2$	sweet, waxy	MS/RI	-	132.55 ± 24.16 [a]	398.77 ± 36.97 [b]	266.77 ± 42.34 [c]	284.02 ± 73.29 [d]	78.02 ± 17.85 [e]
17	2,8-dimethylundecanoic acid methyl ester	-	55955-74-3	$C_{14}H_{28}O_2$	-	MS	-	-	13.56 ± 2.29	-	-	-
18	ethyl pentadecanoate	-	41110-00-5	$C_{17}H_{34}O_2$	honey, sweet	MS	9.22 ± 2.01 [a]	5.24 ± 0.26 [b]	107.33 ± 10.78 [c]	73.55 ± 11.33 [d]	60.67 ± 16.08 [e]	31.06 ± 7.46 [f]
19	ethyl pentadec-9-enoate	-	56219-09-1	$C_{17}H_{32}O_2$	-	MS	-	1.52 ± 0.94	-	-	-	7.45 ± 2.57
20	ethyl palmitate	2241	628-97-7	$C_{18}H_{36}O_2$	waxy, creamy	MS/RI	299.20 ± 57.75 [a]	2330.06 ± 512.63 [b]	4998.16 ± 455.92 [c]	3064.57 ± 461.17 [d]	2397.87 ± 402.49 [e]	-
21	methyl hexadecanoate	2280	112-39-0	$C_{17}H_{34}O_2$	waxy, fatty	MS/RI	534.02 ± 120.77	1.24 ± 0.81	-	-	-	-
22	diethyl phthalate	2409	84-66-2	$C_{12}H_{14}O_4$	-	MS/RI	-	-	-	-	-	8.89 ± 1.30
23	ethyl heptadecanoate	2351	14010-23-2	$C_{19}H_{38}O_2$	-	MS/RI	-	-	21.46 ± 4.91 [a]	15.48 ± 1.74 [b]	9.31 ± 1.21 [c]	-
24	ethyl octadecanoate	2482	111-61-5	$C_{20}H_{40}O_2$	mild, waxy	MS/RI	7.98 ± 0.56 [a]	41.39 ± 8.77 [b]	90.69 ± 11.36 [c]	64.75 ± 5.47 [d]	46.05 ± 3.85 [e]	13.94 ± 5.19 [f]
25	ethyl (9E)-9-octadecenoate	2575	6114-18-7	$C_{20}H_{38}O_2$	-	MS/RI	-	-	1481.96 ± 191.83	-	-	-
26	ethyl oleate	-	111-62-6	$C_{20}H_{38}O_2$	fatty, oily	MS	64.11 ± 17.13 [a]	452.81 ± 111.6 [b]	1563.12 ± 165.29 [c]	1052.85 ± 88.62 [d]	-	259.92 ± 96.10 [e]
27	9,12-octadecadienoic acid ethyl ester	2516	544-35-4	$C_{20}H_{36}O_2$	fatty, fruity	MS/RI	-	181.87 ± 66.89 [a]	46.61 ± 7.51 [b]	381.52 ± 138.72 [c]	858.89 ± 114.48 [d]	83.93 ± 30.55 [e]
28	ethyl linolenate	-	1191-41-9	$C_{20}H_{34}O_2$	rubber	MS	-	-	-	23.84 ± 5.49	17.92 ± 2.93	-
29	butyl isobutyl phthalate	-	17851-53-5	$C_{16}H_{22}O_4$	-	MS	-	13.89 ± 2.64	-	-	-	-
30	dibutyl terephthalate	-	1962-75-0	$C_{16}H_{22}O_4$	-	MS	-	46.92 ± 7.29	-	-	-	56.22 ± 21.07
31	dibutyl phthalate	2641	84-74-2	$C_{16}H_{22}O_4$	faint, odor	MS/RI	49.24 ± 17.06	-	-	-	-	-
Alcohols												
1	ethanol	909	64-17-5	C_2H_6O	medical, alcoholic	MS/RI	2176.89 ± 45.10 [a]	2207.31 ± 53.46 [b]	7162.04 ± 513.72 [c]	4502.20 ± 139.20 [d]	2989.47 ± 211.49 [e]	2727.54 ± 62.66 [f]
2	isobutyl alcohol	1144	78-83-1	$C_4H_{10}O$	fusel, whiskey	MS/RI	-	11.09 ± 0.57	41.51 ± 15.54	-	-	-
3	2-butyloctanol	1144	3913-02-8	$C_{12}H_{26}O$	-	MS/RI	-	-	-	-	41.45 ± 2.17	-
4	isopentyl alcohol	1264	123-51-3	$C_5H_{12}O$	fusel, fermented	MS/RI	-	-	160.85 ± 16.79	-	-	-
5	methallyl alcohol	-	513-42-8	C_4H_8O	-	MS	-	-	-	85.46 ± 11.57	-	-
6	crotonyl alcohol	-	6117-91-5	C_4H_8O	-	MS	-	-	-	63.24 ± 4.52	-	-
7	(R,R)-2,3-Butanediol	1502	24347-58-8	$C_4H_{10}O_2$	fruity, creamy	MS/RI	70.80 ± 4.89 [a]	542.10 ± 75.54 [b]	1928.62 ± 118.82 [c]	535.32 ± 10.39 [d]	464.15 ± 36.02 [e]	221.33 ± 14.38 [f]
8	2,3-butanediol	-	513-85-9	$C_4H_{10}O_2$	fruity, creamy	MS	187.71 ± 17.26 [a]	513.84 ± 42.72 [b]	923.56 ± 109.47 [c]	310.14 ± 22.14 [d]	425.51 ± 34.02 [e]	184.56 ± 18.51 [f]
9	3-furanmethanol	-	4412-91-3	$C_5H_6O_2$	-	MS	-	38.49 ± 4.53	-	-	3.75 ± 0.31	-
10	2-furanmethanol	1627	98-00-0	$C_8H_{10}O_3$	sweet, fruity	MS/RI	-	-	711.83 ± 66.18	16.75 ± 0.92	-	-
11	benzyl alcohol	1878	100-51-6	C_7H_8O	floral, balsamic	MS/RI	5.55 ± 1.64 [a]	8.94 ± 0.69 [b]	326.61 ± 27.24 [c]	75.89 ± 4.67 [d]	30.77 ± 2.08 [e]	17.32 ± 1.08 [f]
12	phenethyl alcohol	1934	60-12-8	$C_8H_{10}O$	floral, sweet	MS/RI	30.04 ± 1.52 [a]	193.85 ± 13.93 [b]	2620.92 ± 208.73 [c]	1322.73 ± 52.89 [d]	1025.98 ± 98.27 [e]	481.79 ± 18.46 [f]
13	linoleic alcohol	2520	506-43-4	$C_{18}H_{34}O$	Faint, fatty	MS/RI	-	4.67 ± 0.89	-	-	-	-
Acids												
1	acetic acid	1402	64-19-7	$C_2H_4O_2$	vinegar	MS/RI	1145.62 ± 61.79 [a]	1480.12 ± 99.13 [b]	860.98 ± 152.43 [c]	289.56 ± 44.01 [d]	106.02 ± 22.92 [e]	-
2	nonanoic acid	2164	112-05-0	$C_9H_{18}O_2$	sour, vinegar	MS/RI	13.97 ± 3.62	-	-	-	-	-
3	2-methylhexadecanoic acid	-	27147-71-3	$C_{17}H_{34}O_2$	fatty, waxy	MS	-	-	-	3.19 ± 1.11	-	-
4	isobutyric acid	1529	79-31-2	$C_4H_8O_2$	acidic, sour	MS/RI	-	-	-	-	-	82.02 ± 3.44
5	octanoic acid	2095	124-07-2	$C_8H_{16}O_2$	fatty, waxy rancid, oily	MS/RI	5.70 ± 2.12	-	-	-	-	-
6	11,14,17-eicosatrienoicacid	-	17046-59-2	$C_{20}H_{34}O_2$	-	MS	-	-	35.81 ± 2.50	-	-	-
7	11,14-eicosadienoicacid	2613	2091-39-6	$C_{20}H_{36}O_2$	-	MS/RI	-	-	-	28.27 ± 0.96	-	-

Table 1. *Cont.*

No.	Compound	RI	CAS No	Molecule Formula	Odor Description	ID	Concentration (ng/g)					
							A	B	C	D	E	F
Aldehydes												
1	acetaldehyde	-	75-07-0	C_2H_4O	pungent, fresh	MS	-	-	14.31 ± 3.03 [a]	1007.74 ± 14.97 [b]	447.04 ± 37.22 [c]	363.50 ± 32.27 [d]
2	isobutyraldehyde	-	78-84-2	C_4H_8O	aldehydic, herbal	MS	-	1.12 ± 0.14	17.40 ± 0.94	-	-	-
3	isovaleraldehyde	937	590-86-3	$C_5H_{10}O$	fruity, green	MS/RI	-	-	156.58 ± 9.04	-	20.01 ± 1.13	-
4	methacrolein	915	78-85-3	C_4H_6O	wild, foliage	MS/RI	-	-	-	4735.98 ± 66.05	-	-
5	2-butenal	1017	4170-30-3	C_4H_6O	floral	MS/RI	-	-	4884.79 ± 325.02 [a]	52.21 ± 5.31 [b]	1259.28 ± 86.49 [c]	-
6	crotonaldehyde	1095	123-73-9	C_4H_6O	-	MS/RI	-	-	18.47 ± 12.05 [a]	125.34 ± 32.10 [b]	10.93 ± 10.43 [c]	159.37 ± 49.03 [d]
7	2-vinyl-2-butenal	1348	20521-42-0	C_6H_8O	-	MS/RI	-	-	-	274.10 ± 16.60	-	-
8	(E,E)-2,4-hexadienal	1371	142-83-6	C_6H_8O	sweet, green	MS/RI	-	-	-	122.41 ± 59.23	-	-
9	furfural	1463	98-01-1	$C_5H_4O_2$	sweet, woody	MS/RI	-	-	-	512.70 ± 104.90	-	-
10	benzaldehyde	1511	100-52-7	C_7H_6O	sweet, fruity	MS/RI	-	35.37 ± 3.08 [a]	-	339.19 ± 8.26 [b]	55.09 ± 6.04 [c]	-
11	phenylacetaldehyde	1646	122-78-1	C_8H_8O	sweet, floral	MS/RI	-	29.59 ± 2.65	-	-	-	-
12	2-phenyl-2-butenal	1911	4411-89-6	$C_{10}H_{10}O$	green, vegetative	MS/RI	-	-	724.54 ± 37.73 [a]	144.81 ± 5.48 [b]	55.45 ± 3.84 [c]	18.98 ± 3.57 [d]
13	2-pyrrolecarbaldehyde	-	1003-29-8	C_5H_5NO	musty, beefy	MS	-	-	-	-	-	18.57 ± 2.38
Ketones												
1	4-hydroxy-2-butanone	-	590-90-9	$C_4H_8O_2$	-	MS	107.94 ± 9.01	-	-	-	-	-
2	acetoin	1299	513-86-0	$C_4H_8O_2$	sweet, buttery	MS/RI	-	39.98 ± 2.47 [a]	134.28 ± 22.66 [b]	-	15.27 ± 1.65 [c]	-
3	hexahydrofarnesylacetone	2156	502-69-2	$C_{18}H_{36}O$	oily, herbal	MS/RI	-	50.21 ± 8.08 [a]	-	8.79 ± 1.87 [b]	3.56 ± 1.06 [c]	-
Nitrogen compounds												
1	2-methyl-pyrazine	1392	109-08-0	C_5H_6N2	-	MS/RI	-	-	-	-	-	86.73 ± 12.09
2	2,6-dimethyl-pyrazine	1319	108-50-9	$C_6H_8N_2$	coffee, roasted	MS/RI	-	-	6.29 ± 0.55	-	-	-
3	4,6-dimethyl-pyrimidine	1320	1558-17-4	$C_6H_8N_2$	-	MS/RI	-	-	-	-	-	28.31 ± 0.81
4	2,5-dimethyl-pyrazine	1327	123-32-0	$C_6H_8N_2$	cocoa, roasted	MS/RI	-	-	-	-	25.14 ± 3.20	50.87 ± 2.37
5	2,3-dimethyl-pyrazine	1358	5910-89-4	$C_6H_8N_2$	coffee, roasted	MS/RI	-	-	-	-	-	41.23 ± 2.56
6	trimethyl-pyrazine	1435	14667-55-1	$C_7H_{10}N_2$	coffee, roasted	MS/RI	-	-	-	-	-	156.20 ± 8.36
7	1,2-dimethyl-hydrazine	-	540-73-8	$C_2H_8N_2$	-	MS	-	-	-	-	-	40.93 ± 3.00
8	2-ethyl-5-methyl-pyridine	1453	18113-81-0	$C_8H_{11}N$	-	MS/RI	-	-	-	162.87 ± 151.52	-	-
9	ethyl 5-amino-1,2,3-thiadiazole-4-carboxylate	-	6440-02-4	$C_{12}H_{11}N_3O_4S$	-	MS	-	-	-	7.97 ± 1.92	-	-
10	methyl 2-(propanoylamino)benzoate	-	25628-84-6	$C_{11}H_{13}NO_3$	-	MS	-	-	-	-	20.98 ± 3.34	-
11	3,5-dimethylpyrazole	-	67-51-6	$C_5H_8N_2$	-	MS	-	-	-	195.52 ± 26.05	-	-
12	methyl anthranilate	2219	134-20-3	$C_8H_9NO_2$	-	MS/RI	-	-	-	12.98 ± 1.99	-	-
13	2-acetyl pyrrole	1979	1072-83-9	C_6H_7NO	sweet, fruity musty	MS/RI	-	-	-	5.24 ± 0.52 [a]	5.50 ± 0.99 [b]	3.59 ± 0.48 [c]
Sulfur compounds												
1	methanethiol	-	74-93-1	CH_4S	sulfurous, alliaceous	MS	-	-	-	-	-	0.77 ± 0.06
Alkanes												
1	2,2,4,6,6-pentamethyl-heptane	953	13475-82-6	$C_{12}H_{26}$	-	MS/RI	-	-	-	-	-	84.90 ± 4.25
2	2,2,11,11-tetramethyl-dodecane	-	127204-12-0	$C_{16}H_{34}$	-	MS	-	-	-	-	-	3.89 ± 0.24
3	decane	-	124-18-5	$C_{10}H_{22}$	-	MS	1.85 ± 0.25 [a]	2.26 ± 0.21 [b]	28.77 ± 7.96 [c]	12.98 ± 1.78 [d]	.62 ± 0.39 [e]	11.72 ± 1.41 [f]
4	eicosane	-	112-95-8	$C_{20}H_{42}$	waxy	MS	-	-	4.72 ± 0.63	17.60 ± 5.53	-	-
5	pentadecane	-	629-62-9	$C_{15}H_{32}$	waxy	MS	-	-	-	-	-	40.25 ± 3.42
6	4,6-dimethyldodecane	-	61141-72-8	$C_{14}H_{30}$	waxy	MS	-	-	-	-	-	12.63 ± 1.92
7	7,9-dimethyl-hexadecane	-	21164-95-4	$C_{18}H_{38}$	-	MS	-	-	-	-	6.97 ± 0.28	-
8	4-methyl-nonane	1083	17301-94-9	$C_{10}H_{22}$	-	MS/RI	-	-	-	-	-	3.57 ± 0.72
9	2,3,3-trimethyl-pentane	1090	560-21-4	C_8H_{18}	-	MS/RI	-	-	-	-	-	6.18 ± 0.52
10	2,6,10-trimethyl-undecane	1133	3891-98-3	$C_{15}H_{32}$	-	MS/RI	-	-	-	-	4.44 ± 0.88	-
11	4,6-dimethyl-undecane	-	17312-82-2	$C_{13}H_{28}$	-	MS	-	-	-	19.08 ± 1.80	-	10.82 ± 0.55
12	dodecane	-	112-40-3	$C_{12}H_{26}$	alkane	MS	-	-	-	22.35 ± 7.59	-	-
13	3,8-dimethyl-undecane	-	17301-30-3	$C_{13}H_{28}$	-	MS	-	-	-	3.90 ± 1.36	-	-
14	2,3,4-trimethyl-hexane	-	921-47-1	C_9H_{20}	-	MS	-	-	-	-	-	2.62 ± 0.19
15	2,6-dimethyl-heptadecane	-	54105-67-8	$C_{19}H_{40}$	-	MS	-	-	9.59 ± 5.90	-	-	-

Table 1. *Cont.*

No.	Compound	RI	CAS No	Molecule Formula	Odor Description	ID	Concentration (ng/g)					
							A	B	C	D	E	F
16	dipentene	1204	138-86-3	$C_{10}H_{16}$	citrus, herbal	MS/RI	-	11.97 ± 1.68	-	-	-	-
17	styrene	1283	100-42-5	C8H8	floral, sweet	MS/RI	-	6.21 ± 1.40 [a]	66.83 ± 6.70 [b]	67.02 ± 2.71 [c]	-	41.98 ± 2.65 [d]
18	2-methyl-2,4-hexadiene	-	28823-41-8	C_7H_{12}	-	MS	-	-	19.33 ± 13.34	-	65.67 ± 36.09	-
19	caryophyllene	-	17627-40-6	$C_{15}H_{24}$	-	MS	-	-	44.17 ± 4.06	-	-	-
20	decene	-	872-05-9	$C_{10}H_{20}$	-	MS	-	-	-	-	3.66 ± 0.17	-
21	dihydroaplotaxene	-	56134-03-3	$C_{17}H_{30}$	-	MS	-	-	-	-	20.59 ± 3.04	-
Phenols												
1	mequinol	-	150-76-5	$C_7H_8O_2$	phenolic	MS	-	2.84 ± 0.44	-	-	-	-
2	phenol	2099	108-95-2	C_6H_6O	phenolic, plastic rubber	MS/RI	2.04 ± 0.28 [a]	-	20.25 ± 1.47 [b]	10.23 ± 1.71 [c]	-	5.71 ± 1.93 [d]
3	2,5-di-tert-butylphenol	2342	5875-45-6	$C_{14}H_{22}O$	-	MS/RI	11.21 ± 2.77	-	-	-	-	-
Heterocyclics												
1	(E)-2,3-epoxydecane	-	54125-39-2	$C_{10}H_{20}O$	-	MS	-	-	-	-	-	1.71 ± 0.15
2	2,5-dihydrofuran	-	1708-29-8	C_4H_6O	-	MS	-	-	11.87 ± 0.89	-	-	-
3	1-methyl-2-(3-methylpentyl)-cyclopropane	-	62238-07-7	$C_{10}H_{20}$	-	MS	-	-	-	-	3.94 ± 1.33	-
4	1,1-dimethylcyclopropane	-	1630-94-0	C_5H_{10}	-	MS	-	78.77 ± 3.79	-	-	-	-
5	1-methyl-5-(1-methylethenyl)-cyclohexene	1207	13898-73-2	$C_{10}H_{16}$	-	MS/RI	7.13 ± 2.10	-	-	-	-	-
6	2-pentylfuran	1216	3777-69-3	$C_9H_{14}O$	green, waxy	MS/RI	5.24 ± 2.62	-	95.72 ± 11.51	-	-	-
7	1,3,5,7-cyclooctatetraene	1289	629-20-9	C_8H_8	-	MS/RI	-	-	-	-	33.04 ± 1.90	-
8	2-ethyl-furan	-	3208-16-0	C_6H_8O	coffee, malty	MS	-	-	-	1160.40 ± 187.16	-	-
9	3-methyl-2-(2-methyl-2-butenyl)-furan	-	15186-51-3	$C_{10}H_{14}O$	caramel, minty	MS	-	7.49 ± 1.15	-	-	-	-
10	1,2-dimethoxybenzene	1705	91-16-7	$C_8H_{10}O_2$	-	MS/RI	-	-	90.23 ± 6.31 [a]	58.60 ± 2.78 [b]	35.88 ± 4.17 [c]	25.67 ± 1.37 [d]

Results are expressed as mean ± standard deviation; ID: the identification method was based on the mass spectra and retention index; RI: the retention index was compared with the literature value, and the source of the literature value is the NIST Chemistry Web Book; odor description: the source of odor description is the Good Scents Company Information System; [a], [b], [c], [d], [e], [f]: significant difference in substance content between samples at 5% level of significance. A, B, C, D, E, and F represent samples from different periods, respectively.

3.2. The Variation Rule of Volatile Compounds in Daqu Production

3.2.1. PCA Analysis

In order to identify the distribution correlation of the sample, principal component analysis was conducted in accordance with the data of volatile compounds in daqu detected by HS-SPME-GC-MS. The principal component analysis score and load diagram are depicted in Figure 2. The score graph of the data obtained in the positive–negative mode indicates the trend of separation between groups. PC1 and PC2 are in Figure 2a. PC1 accounted for 32.7% of the total variance, while PC2 accounted for 21.9% of the total variance. The cumulative contribution rate of the first two PCs accounted for 54.6%, which demonstrates that they are both sufficient to explain the total variance in the dataset. PC1 and PC2 are positively correlated with the C and D daqu samples and are negatively correlated with the A, B, E, and F daqu samples, as illustrated in Figure 2a. As demonstrated by the experimental results, with the prolongation of the production duration, the variability between samples heightens. In the middle stage of production (8–12 days), the distance between C and the other data points in the PAC diagram is the greatest, not only indicating that the content of volatile components in the strong-aroma daqu changed most noticeably, but also indicating that the substance concentration changed considerably during the maturation process, which is extremely important for the maturation of daqu. Combined with Figure 2b, it was further resolved that the special state of the C-stage daqu was correlated with dozens of volatile compounds, such as acetic acid, ethyl acetate, 2,3-butanediol, isoamyl acetate, ethyl palmitate, and isobutyl alcohol. In the early stages of maturation, i.e., the A and B periods, which are in the same quadrant of the PCA diagram, there is an overall consistency, but there are still some gaps between them because of the large variation in compound species at this time. In contrast, D and E are both on the axes, again with some similarity, while F is more independently positioned, indicating that matured macrographs are easily distinguished from those in process, which is related to substances such as acetaldehyde, crotonaldehyde, 2-acetyl pyrrole, and 2,5-dimethyl-pyrazine. PCA analysis further proved the evidence of compound types and content changes in daqu samples at each maturity stage and associated them with specific compounds.
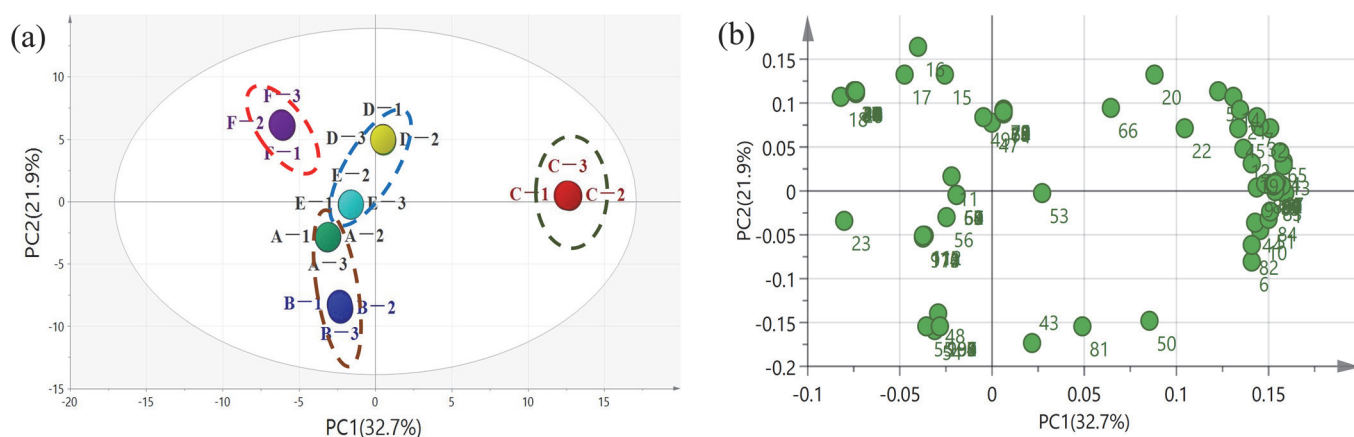

Figure 2. PCA analysis based on volatile compounds. (**a**) Scores and (**b**) loading plot of PCA for classification of samples of strong-flavor daqu with different production durations. The specific compounds represented by the numbers are shown in Table S1. A, B, C, D, E, and F represent samples from different periods, respectively.

This was mutually verified with the changes in the quantity and concentration of the above compounds. The above results show that the number of compounds in stage C was the largest, and the change was the largest compared with the adjacent stage. The number of substances in stages D and E is similar, while stage F is separated from stage D and E due to the appearance of some compounds, which indicates that the determination of the mature state of daqu is scientific.

3.2.2. HCA Analysis

HCA was adopted to quickly assess the correlations between dissimilar stages of daqu (Figure 3). In this study, the Pearson (*n*) correlation was applied to measure the correlation and classification in line with the association between groups. The position of the line on the scale indicates the distance between the clusters. HCA identified six kinds of strong-flavor daqu in dissimilar production processes. D is the daqu with the greatest difference from other samples, indicating that the daqu in the middle stage of production is the sample with the greatest difference, which is also consistent with the PCA results. However, this is merely a preliminary analysis of strong-flavor daqu through unsupervised statistical analysis methods. It needs further analysis.
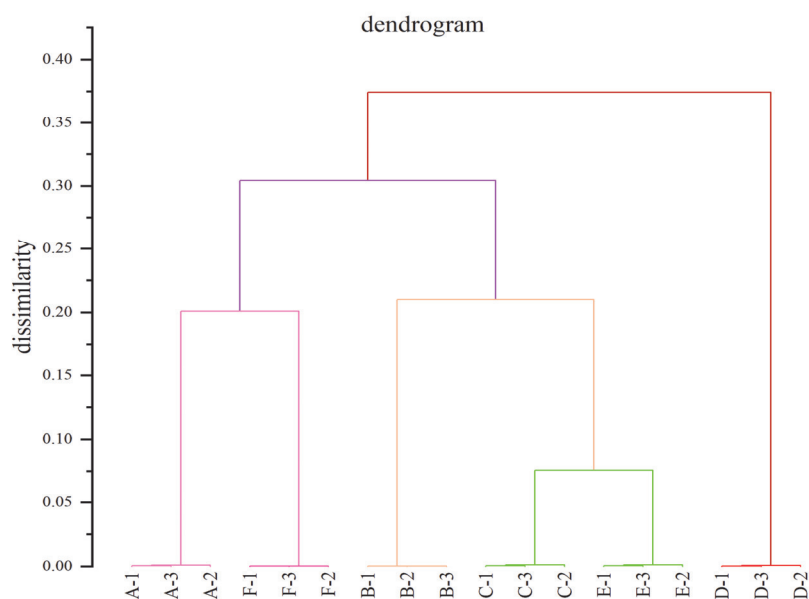
Figure 3. Dendrograms of strong-flavor daqu. A, B, C, D, E, and F represent samples from different periods, respectively.

3.2.3. PLS-DA

In order to probe deeper into the effects of volatile compounds in daqu, the supervised multivariate statistical method PLS-DA can establish a useful statistical model of the data, which can be adopted as an analysis method to distinguish the differences between samples. This experiment uses the PLS-DA model to screen out specific volatile compounds on the basis of the volatile compounds detected in strong-flavor daqu in dissimilar production processes.

The most representative variables were identified through variable importance for the projection (VIP) value. For strong-flavor daqu, as exhibited in Figure 4a, it explains 84.5% of the total variance, R^2 = 99.9% and Q^2 = 99.7%. Apart from D in the second quadrant and C in the third quadrant, the rest of the daqu samples are clustered in the fourth quadrant. This result is generally consistent with the PCA results. The samples in the mid-production period have the most noticeable difference compared to the samples in the early and late periods of production. Apart from that, as illustrated by the permutation plot results, the model does not overfit the data, the established PLS-DA model is valid, and the initial model is better than the predicted random arrangement model (Figure 4b). Aside from that, the permutation plot was applied to evaluate whether the PLS-DA model had a satisfactory predictive value. To put it in another way, it was used as the criteria for judging whether the model was successful. The permutation plot was compared on the basis of the fit of the data model for randomly sorted categorical variables (Y). This figure proves that the established PLS-DA model is valid, and there is no over-fitting of data because all the Q^2-value blue boxes on the left are lower than the original point (standard) on the right or the blue Q^2 point. The color regression line intersects the vertical axis (on

the left) at or below zero, as exhibited in Figure 4b. As a result, the performance of the initial model PLS-DA is significantly better than the predicted random array model. The volatile compounds with high VIP values (VIP > 1.0) were selected in strong-flavor daqu, as exhibited in the red box in Figure 4c, namely, methacrolein, ethyl palmitate, acetic acid, methyl hexadecanoate, 9,12-octadecadienoic acid ethyl ester, 2-butenal, ethanol, ethyl oleate, ethyl acetate, phenethyl alcohol, 2-ethyl-furan, acetaldehyde, trimethyl-pyrazine, ethyl (9E)-9-octadecenoate, (R,R)-2,3-butanediol, ethyl caproate, 4-hydroxy-2-butanone, 2,3-butanediol, crotonaldehyde, 2-methyl-pyrazine, 2-methyl-2,4-hexadiene, 2-furanmethanol, 2,2,4,6,6-pentamethyl-heptane, and isobutyric acid. In order to control the entire maturation process, the present study further analyzed the substances present in the six maturation stages and summarized their concentration changes. Six key compounds were selected as indicators of the production process, namely, ethyl acetate, ethanol, phenethyl alcohol, (R,R)-2,3-butanediol, ethyl caproate, and 2,3-butanediol.
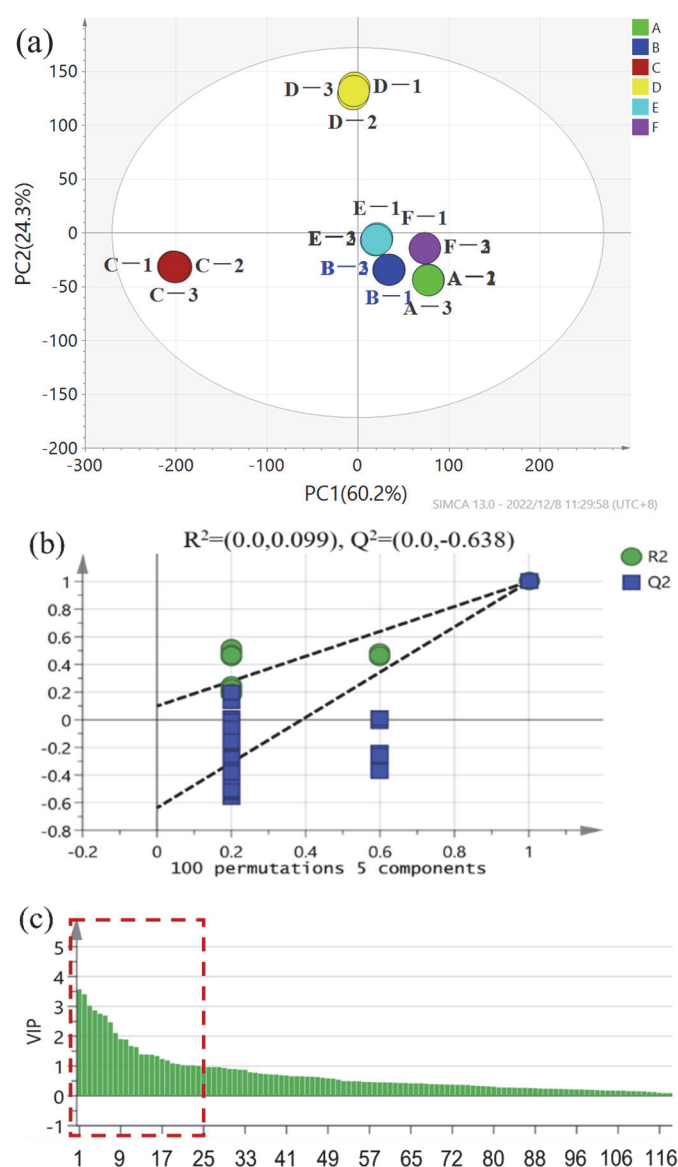
Figure 4. Schemes follow the same formatting. PLS-DA analysis based on volatile compounds. (**a**) Scores, (**b**) permutation test, and (**c**) VIP plot constructed by adopting the concentrations of strong-flavor daqu's volatile compounds. The consecutive numbers of the horizontal coordinates in (**c**) correspond to the different compounds, as shown in Table S2, the red boxed line indicates substances with VIP > 1. A, B, C, D, E, and F represent samples from different periods, respectively.

When the six indicator compounds are used to determine the different maturity stages of daqu and when ethyl acetate is detected in the range of 1000–1400 ng/g and ethyl caproate and 2,3-butanediol are both below 200 ng/g, the barley is still in the early stages of maturity and still needs a long time to be put into use. When the ethanol concentration is above 4000 ng/g and the concentrations of ethyl caproate as well as phenethyl alcohol are in the range of 500–2000 and 1200–2000 ng/g, respectively, it means that the daqu is in the middle stage of maturity, reaching the level of 8–12 days in this study. When the concentrations of ethyl acetate and ethyl caproate are below 600 and 160 ng/g, respectively, and the concentration of (R,R)-2,3-butanediol is around 200 ng/g, the sample is ready to produce baijiu.

4. Conclusions

In this study, a total of 115 volatile compounds were identified by HS-SPME-GC-MS in strong-flavor daqu. Subsequently, the types and concentrations of the compounds in different stages were analyzed. It was found that the volatile components change during the production processes. Aside from that, the acids and aldehydes showed a trend of increasing first and then decreasing. Moreover, all compounds changed very little in the last stage, reaching a balanced and stable system. It is worth noting that both nitrogenous and sulfur-containing compounds appeared in the later stages. Apart from that, PCA and PLS-DA analysis models were constructed to further explain the differences between daqu at dissimilar maturation stages and relate them to compounds. Finally, six indicator compounds, ethyl acetate, ethanol, phenethyl alcohol, (R,R)-2,3-butanediol, ethyl caproate, and 2,3-butanediol, were further screened by PLS-DA analysis combined with the concentration changes in compounds during the production processes, which were used for speedily determining the maturity level of the daqu. Through a systematic and in-depth analysis, this study provides a theoretical basis for the optimization of the production process and the speedy judgment of the maturity degree of strong-flavor daqu so as to improve the quality and stability of daqu and baijiu and further promote the mechanized production process of baijiu. Nevertheless, the level of aroma compounds has not been involved so far. On that account, the indicator compounds can be further verified and optimized in line with the aroma components.

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