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Research Article

Characterization of Key Odorants in Soy Sauce and Their Concentration Changes during Storage

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To explore the key odorants and their concentration changes in high-salt liquid-state fermentation soy sauce (HLFSS) during storage, solvent extraction coupled with solvent-assisted flavor evaporation was applied to isolate the volatiles in HLFSS, and the volatiles were analyzed by gas chromatograph-mass spectrometry-olfactory and gas chromatograph-flame ionization detector-olfactometry combined with the aroma extract dilution assay. A total of 37 odor-active compounds with flavor dilution (FD) factors ranging from 1 to 1024 were identified, and 23 aroma components with odor activity values ≥1 were considered as key odorants. Methional, 3-methylbutanoic acid, phenethyl alcohol, sotolon, 4-hydroxy-2-methyl-5-ethyl-3(2*H*)-furanone (HEMF), and 4-hydroxy-2,5-dimethylfuran-3(2*H*)-one (HDMF) had the highest FD factors of 1024. The results of quantitative analysis through the internal standard curve showed that the concentrations of HEMF, HDMF, acetic acid, 1-hydroxy-2-butanone, acetol, and furfuryl alcohol varied greatly during storage, so these compounds might be used as the indicators to determine the shelf life of HLFSS. The recombination experiment also confirmed the important contribution of these compounds. This result will provide valuable information for understanding the flavor changes of HLFSS.

1. Introduction

Soy sauce (SS) originated from China with more than 3000 years of history, and its unique flavor was characterized by a potent umami, salty, and a characteristic aroma such as caramel-like and smoky-like [1], which could enhance the overall aroma of some kinds of dishes. SS is produced by microbial fermentation, enzymatic or nonenzymatic reactions using soybeans, wheat flour, salt, etc., as materials [2]. The production of SS in China has shown a gradual increase, with an annual output of 5.2 million tons, making the SS industry become one of the most prosperous industries in China [3]. According to the difference of the SS brewing process, it is divided into high-salt liquid-state fermentation SS (HLFSS) and low-salt solid-state fermentation SS (LSFSS) [4]. Because of HLFSS with some advantages, such as health protection functions, higher nutritional value, pleasant flavor, beautiful color, etc., the

fermentation process of HLFSS occupies a leading position in the SS industry in China at present [5, 6].

The aroma of SS is an indispensable indicator to determine its quality and consumer acceptance in the market [5]. SS contains various amino acids, sugars, and other substances, providing a good foundation for the occurrence of Maillard reactions [7]. During storage, some reactions occur in SS, such as Maillard reaction, oxidation reaction, esterification reaction, etc., resulting in the changes of concentration of odor constituents slowly in the process, which in turn affects the overall aroma of the SS [8]. However, there are very few research studies on the content changes of the aroma-active components in SS during storage.

At present, the reports on odor compound changes of SS focus on the changes under different heating conditions. Wang et al. [9] investigated the aroma changes of SS heated at higher temperature (220°C) and boiling (100°C) for a different

time, and the results suggested that the concentration changes of target aroma-active components led to vary caramel-like/ sweet, roasted/roasted nut-like, and spicy/burnt odors. Liang et al. [10] researched the changes on physicochemical properties, organic acids, and volatile components in SS heated at 125°C for 15 min, and the results showed that physicochemical properties and organic acids did not vary significantly, but the concentrations of volatiles rose more than 30%; the intensities of spicy, caramel-like, and fruity notes in heated SS were higher. Guo et al. [11] used liquid-liquid extraction combined with gas chromatograph-mass spectrometry-olfactory to analyze the volatile compounds in dark SS and explore the changes of the flavor characteristic from 0 days to 20 days, and they found that the aroma intensity scores of roasted and smoky notes increased. In addition, a metabolomic approach was used for investigating the odor changes of SS during storage, and the results obtained indicated the changes of concentrations of several key biomarkers resulted in the sensory decreases in fruity/grape and nutty/sesame notes and the increases in methional/potato note and astringent attributes [12].

Accelerated aging experiments are widely used to simulate the changes of food during long-term storage. Our previous study results showed that the overall flavor profile of SS kept at 37°C for one week was similar to that of SS stored at room temperature for three weeks [13]. In order to further explore the concentration changes of odor-active compounds in SS during a longer storage, accelerated aging experiments were also applied in the present study.

The purpose of this study is (i) to screen and identify the odor-active compounds in SS using gas chromatograph-flame ionization detector-olfactometry and gas chromatograph-mass spectrometry-olfactory combined with aroma extract dilution analysis; (ii) to quantitate the odor compounds identified by using the internal standard curve; (iii) to determine the key odorants by calculating odor activity values; (iv) to investigate the change law of aroma-active compounds in HLFSS during long-term storage.

2. Materials and Methods

2.1. HLFSS Samples.
The same batch of HLFSS samples was obtained from Jiajia Foods Co., Ltd. The raw materials for HLFSS samples were water, organic defatted soybeans, sugar, salt, wheat, yeast extract, sodium glutamate, disodium 5′-ribonucleotide, and sucralose. Samples 1–9 represent HLFSS samples stored at 37 ± 1°C for 1–9 weeks, respectively. All HLFSS samples that have reached storage time were kept at 4°C refrigerator until analysis.

2.2. Chemicals.
Furfuryl alcohol (98%), acetic acid (>99%), propionic acid (99%), isobutyric acid (99%), isobutanol (99%), 1-butanol (99.5%), maltol (99%), phenethyl alcohol (99%), guaiacol (99%), γ-butyrolactone (99%), ethyl vanillate (98%), methylpyrazine (98%), 3-methylbutanoic acid (99%), 3-methylvaleric acid (>98%), 4-methylpentanoic acid (99%), 2-acetylpyrrole (98%), methionol (98%), 2-acetylfuran (97%), 2,5-trimethylpyrazine (99%), methyl cyclopentenolone (99%),

4-ethyl-2-methoxyphenol (98%), 2,6-dimethylpyrazine (98%), and 2,3,5-trimethylpyrazine (98%) were bought from J&K Chemicals Ltd. (Beijing, China); 3-octanol (98%), benzoic acid (≥99%), phenylacetaldehyde (95%), 2 (5H)-furanone (98%), methional (98%), 1-hydroxy-2-butanone (98%) were obtained from Macklin Biochemical Co., Ltd. (Shanghai, China); acetol (>80%), and 4-ethylphenol (>97%) were purchased from TCI Chemical Ltd. (Shanghai, China); acetoin (97%) and 2-ethyl-6-methylpyrazine (>98%) were obtained from Adamas Reagents Co., Ltd. (Shanghai, China); HDMF (98%), HEMF (97%), phenylacetic acid (95%), ethyl lactate (99.8%), and sotolon (97%) were supplied by Aladdin Reagents Co., Ltd. (Shanghai, China), Ark Pharm Inc. (Chicago, IL, USA), Key Organics (Cornwall, England), Minda Technology Co., Ltd. (Beijing, China), respectively. C6~C30 n-alkanes were obtained from Aldrich Chemical Co., Ltd. (Shanghai, China); anhydrous sodium sulfate and dichloromethane were obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China).

[^{2}H$_3$]-Methionol and [^{2}H$_3$]-methional were prepared from [^{2}H$_3$]-methyl iodide reacted with ethyl 3-mercaptopropionate to give ethyl 3-[^{2}H$_3$]-methylthiopropionate, which was reduced by lithium aluminum hydride to obtain [^{2}H$_3$]-methionol; [^{2}H$_3$]-methional was formed by oxidizing [^{2}H$_3$]-methionol with pyridinium chlorochromate [14]. [^{2}H$_3$]-2,3,5-trimethylpyrazine was synthesized from [^{2}H$_3$]- iodomethane through the reaction of 3-chloro-2,5-dimethylpyrazine with the Grignard reagents, under the catalytic agent of [1,3-bis-(diphenylphosphino) propane] nickel (II) chloride according to the reference [15].

2.3. Extraction of Volatiles from HLFSS.
The volatiles were isolated based on the method used in our previous research, and some modifications were made [16]. HLFSS samples (100 mL) with dichloromethane (50 mL × 3) were extracted at room temperature by using an oscillator (ZWY-100H, Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd.) for 1 h, and then the extracts were centrifuged at 4°C, 8000 rmp by using a centrifugal (H1750R, Hunan Xiangyi Instrument Manufacturing Co., Ltd.); the organic phase was separated. Repeat the above operation 2 times. The resulting organic phase was distilled by solvent-assisted flavor evaporation (SAFE) (Edwards TIC Pumping Station from BOC Edwards, England) in a 40°C water bath, and the vacuum pressure was 5 w10^{-4} mbar. Anhydrous sodium sulfate (50 g) was added to the distillate obtained from SAFE to remove water, and then the distillate was placed in a −20°C refrigerator overnight.

Using Vigreux columns (50 cm × 1 cm; Beijing Jingxing Glassware Co., Ltd., Beijing, China) concentrated the distillate to about 3 mL at 50°C and then condensed to 1 mL under mild nitrogen flow. The obtained isolate was conducted to GC-MS-O and GC-FID-O analysis.

2.4. Gas Chromatograph-Mass Spectrometry-Olfactory (GC-MS-O) Analysis.
The Agilent model 7890B gas chromatography combined with the Agilent 5977A mass spectrometer detector (MSD) and an olfactory detector port

(OPD3, Gerstel, Germany) were used to analyze the volatiles in HLFSS. DB-WAX and HP-5 capillary columns (30 m × 0.25 mm, 0.25 μm film thickness) were utilized to analyze chromatographic separation of the volatiles in SS isolates. The oven temperature was held at 40°C for 2 min, then increased the temperature to 80°C at a rate of 8°C/min, then to 100°C at a rate of 4°C/min, and finally to 230°C at a rate of 6°C/min, and it was maintained at this temperature for 10 min. Helium was utilized as the carrier gas with a constant flow rate of 1.7 mL/min, the GC injector temperature was 250°C, and the injection volume was 1 μL (splitless mode), while the ion source temperature was 230°C. Mass spectra electronic impact (EI) ionization was 70 eV, with scanning range m/z 30–350. The temperature of quadrupole was set at 150°C. Nitrogen was used as a supplemental gas to ensure that the mass spectrometer and the olfactory detector could detect the signal simultaneously; the temperatures of transmission line and sniffing port were set at 250°C and 120°C, respectively, and moist air was introduced at the sniffing port to avoid dehydration of the nasal mucosa of the sniffers, which could affect the accuracy of the sniffing.

2.5. Gas Chromatograph-Flame Ionization Detector-Olfactometry (GC-FID-O) Analysis.

GC-FID-O analysis was conducted on a gas chromatography (Agilent model 7890B) coupled with an FID (Agilent Technologies, Inc.) and an olfactory detector port (OPD3, Gerstel, Germany). A polar capillary column DB-WAX (30 m × 0.25 mm, 0.25 μm film thickness) was utilized for chromatographic separation of volatile components. The temperature procedure was consistent with Section 2.4. The effluent from the GC was split 1 : 1 between the FID and sniffing port. Helium was utilized as carrier gas with a constant flow rate of 1 mL/min, and the temperature of the sniffing port was 120°C, and the capillary linked to the FID was maintained at 280°C. Three sensory experts were selected for GC-FID-O analysis.

2.6. Qualitative Analysis.

All of odor-active components in HLFSS were positively identified by comparing their mass spectra, the retention indices (RIs), and aroma characteristics with those of standard compounds. The RIs were calculated on the basis of the retention time of C_6–C_{30} hydrocarbon [17].

2.7. Quantitative Analysis

2.7.1. Internal Standard Curve Method.

After adding 3-octanol (300 μL, 10^{-4} g/mL) as an internal standard into 100 mL HLFSS samples, then the volatiles were extracted according to the method described previously. For the standard calibration curve, a series of mixed solution of internal standard and standard chemicals (five different concentrations) were analyzed by GC-MS-O in the selected ion monitoring (SIM) mode under the same conditions mentioned in Section 2.4. Each standard curve was obtained by plotting the ratios of the peak areas of the standard to the peak areas of the internal standard and its concentration ratio. The concentrations of the volatiles were calculated from the approximated curve using the linear least-squares method. The quantitative results were obtained by averaging data of the triplicate experiments.

2.7.2. Stable Isotope Dilution Assays (SIDA).

The separation of HLFSS volatiles was conducted as mentioned previously (Section 2.4) after the addition of the isotope-labeled standards. To calculate the response factors, the solutions containing the labeled and unlabeled standards with five different concentrations (1 : 5, 1 : 3, 1 : 1, 3 : 1, and 5 : 1) were analyzed by GC-MS-O under the same procedure as above; the SIM mode was used. The quantitative results were obtained by averaging the triplicate experiment data.

2.8. Aroma Extract Dilution Analysis (AEDA).

The concentrates of the HLFSS sample were twice diluted (1 : 2, 1 : 4, 1 : 8, 1 : 16…1 : 1024) with dichloromethane and then analyzed with GC-FID-O until no odorant was perceived by all panelists at the sniffing port. The maximum dilution of each odor-active compound that at least two team members could perceive was recorded as the flavor dilution (FD) factor. AEDA was performed twice at least for each HLFSS sample by all panelists.

2.9. The Calculation of Odor Activity Value (OAV).

The OAV of each odorant with aroma activity is calculated as a ratio of the component concentration in SS to its threshold in water [18–20]. The compound with the OAVs ≥ 1 manifests that it contributed significantly to the overall aroma.

2.10. Sensory Evaluation.

A total of 10 volunteers (6 women and 4 men, average age of 25) were recruited from the Beijing Key Laboratory Flavor Chemistry Beijing Technology and Business University. All volunteers were well in sensory evaluation of food and could correctly distinguish aromas of compounds. The six evaluation odor attributes were defined as follows: guaiacol for smoky aroma, methional for cooked-potato aroma, HEMF for caramel-like aroma, butanol for alcoholic aroma, 3-methyl-1-butanol for malty aroma, and 2-phenylethanol for floral aroma. Sensory experiments were conducted to evaluate the intensities of each aroma attribute of the HLFSS samples on a ten-point liner scale (0 is imperceivable, 10 is strongly perceivable). Sensory tests were carried out in a room temperature at 21°C, and each sample was placed in a 40 mL sealed glass vial, labelled with any three digits. The average score of all panelists was the score for each attribute.

2.11. Aroma Recombination Test.

The recombination experiments were conducted by merging 23 key odorants with OAVs ≥ 1 in the odorless matrix (including 8 g monosodium glutamate, 17 g table salt, 0.35 g $I + G$, 0.012 g sucralose, 0.12 g potassium sorbate, and 75 mL odorless water). The recombination sample (RS) and the original sample (OS)

were evaluated by the method mentioned previously (0 is not perceivable, 10 is strong perceivable). The average score of all panelists was the score for each attribute.

2.12. Statistical Analysis. All results were performed as the mean value ± standard deviation (SD) in triplicate. All obtained data were using Microsoft Office Excel 2021. Heatmaps were drawn by TB tool, and origin 2021b (Origin-Lab, Northampton, MA, USA) was used to gain the radar chart. One-way analysis of variance (ANOVA) and Duncan's multiple-range tests were conducted by using the statistical package SPSS software; the results were significant when $p < 0.05$.

3. Results and Discussion

HLFSS and its volatile isolate were evaluated to obtain an idea of the overall aroma of SS and check whether the extraction method was suitable. The results revealed that the isolate had almost the same odor characteristic with SS, which indicated that the odor compounds contributing to the overall odor profile had been separated successfully from SS. Both of them had smoky, cooked-potato, caramel, alcoholic, malty, and floral notes.

3.1. Identification of Odor-Active Compounds in HLFSS by Combining FD Factors. A total of 37 aroma-active compounds were identified and displayed in Table 1, including seven acids (**Nos.: 13, 16, 17, 21, 24, 25, 36**), six alcohols (**Nos.: 1, 2, 3, 6, 20, 28**), five furans (**Nos.: 15, 23, 32, 33, 35**), five pyrazines (**Nos.: 4, 7, 8, 11, 12**), four ketones (**Nos.: 5, 10, 26, 29**), three esters (**Nos.: 9, 19, 37**), three phenols (**Nos.: 27, 31, 34**), two sulfur-containing substances (**Nos.: 14, 22**), one aldehyde (**Nos.: 18),** and one heterocyclic compound (**Nos.: 30**), and most of the compounds were identified in the previous study [16, 21].

3.1.1. Acids. Seven acids were determined in those HLFSS samples, and these odorants were mostly described as unpleasant aroma during GC-O analyses. Acetic acid was a very essential volatile constituent with an FD factor of 64, which was mainly generated by the metabolism of by lactic acid bacteria [22]. In addition, other acids may be the result of degradation of amino acids or may be the products of oxidative degradation of fatty acids [16, 23]. 3-Methylbutanoic acid (FD value = 256) was usually described as an unpleasant, intolerable, or pungent smell, which contributed significantly to the overall flavor, and it was mainly produced by the oxidation reaction of amino acids and corresponding aldehyde intermediates [24].

3.1.2. Alcohols. Alcohols mainly exist in the early stage of fermentation in SS [25], which has an effect on the formation of the overall flavor of SS. The concentrations of 3-methylbutanol and phenylethyl alcohol were higher than those of the four alcohols identified. They were generated through the Ehrlich pathway, in which amino acids as

precursors conduct deamination, decarboxylation, and reduction to yield alcohols with one less carbon atom than the corresponding precursors, such as 3-methylbutanol from leucine and phenethyl alcohol from phenylalanine [26, 27]. Moreover, phenylethyl alcohol (floral note) with the FD factor of 256 was an important aroma-active compound, which had great contribution to SS aroma.

3.1.3. Furans. HEMF and HDMF, as representatives of furans with caramel-like odor, had been identified as crucial aroma-active compounds in SS in the previous study [20], which not only have high aroma intensity but also have the ability to improve aroma, enhance sweetness, and neutralize saltiness [28]. Moreover, the formation of HEMF was influenced by diverse factors, such as glucose concentration, yeast species, and fermentation temperature [21, 26]. The FD factors of HDMF, HEMF, and sotolon were all 1024, so these components were considered to be important aroma-active components in HLFSS and had a great impact on the unique flavor of HLFSS.

3.1.4. Pyrazines. A total of five pyrazines were detected in this study. 2,5-Dimethylpyrazine had a higher FD factor of 128 of them, followed by 2,6-dimethylpyrazine and 2,3,5-trimethylpyrazine with an FD factor of 16. Usually, pyrazines release an attractive nutty aroma, which is mainly produced during the fermentation process through Maillard reaction and heat sterilization [27].

3.1.5. Ketones. Four ketones including acetoin, 1-hydroxy-2-butanone, methylcyclopentenolone, and maltol were detected; 1-hydroxy-2-butanone had a higher concentration of 9148–18617 μg/L, followed by maltol with a concentration of 5449–5790 μg/L. Ketones mainly came from three sources, that is, raw materials, microbial metabolism, and Maillard reaction [16]; for example, methylcyclopentenolone and maltol could be formed from Maillard reaction; acetoin and 1-hydroxy-2-butanone were generated from microbial metabolism [29].

3.1.6. Esters. A total of three esters were identified, namely, ethyl lactate, γ-butyrolactone, and ethyl vanillate. Most of the esters exhibited a fruit-like aroma; they were generally formed by metabolism of yeast or by esterification reactions of organic acid with alcohols [30]. Among three esters, ethyl lactate had the higher FD value of 16 and it was widely found in all kinds of alcoholic drinks with a fruity odor [31]. Moreover, few studies have reported the presence of ethyl vanillate in SS, which have been reported to exist in wines [32]. Ethyl vanillate had a low aroma intensity and was not easily detected by olfaction.

3.1.7. Phenols. A total of three phenols were sniffed during GC-O analyses in this study, including 4-ethyl-2-methoxyphenol, guaiacol, and 4-ethylphenol, which exhibited strong smoky aromas. The FD factor of guaiacol and 4-

TABLE 1: Aroma-active compounds in HLFSS determined by using GC-MS-O and GC-FID-O combined with AEDA.

Nos.[a]	Compounds	Odor[b]	RI[c] DB-WAX	HP-5	CAS	FD[d]	Identification[e]
1	Isobutanol	Cheese	1090	-[f]	78-83-1	4	a, b, c, d
2	Butanol	Alcoholic	1137	—	71-36-3	<2	a, b, c, d
3	3-methyl-1-butanol	Malty	1201	732	123-51-3	4	a, b, c, d
4	Methylpyrazine	Roasted, nutty	1255	821	109-08-0	<2	a, b, c, d
5	Acetoin	Creamy	1272	—	513-86-0	4	a, b, c, d
6	Acetol	Sweet	1290	—	116-09-6	2	a, b, c, d
7	2,5-dimethylpyrazine	Roasted	1313	912	123-32-0	128	a, b, c, d
8	2,6-dimethylpyrazine	Roasted, nutty	1319	—	108-50-9	16	a, b, c, d
9	Ethyl lactate	Fruity, buttery	1330	—	97-64-3	16	a, b, c, d
10	1-hydroxy-2-butanone	Roasted, coffee	1359	—	5077-67-8	<2	a, b, c, d
11	2-ethyl-6-methylpyrazine	Roasted, coffee	1375	998	13925-03-6	<2	a, b, c, d
12	2,3,5-trimethylpyrazine	Nutty, peanut	1396	—	14667-55-1	16	a, b, c, d
13	Acetic acid	Sour	1409	—	64-19-7	64	a, b, c, d
14	Methional	Cooked potato-like	1435	907	3268-49-3	1024	a, b, c, d
15	2-acetylfuran	Sweet, coffee	1481	912	1192-62-7	64	a, b, c, d
16	Propionic acid	Cheesy	1510	—	79-09-4	<2	a, b, c, d
17	Isobutyric acid	Sour	1545	—	79-31-2	2	a, b, c, d
18	Phenylacetaldehyde	Floral, sweet	1581	1047	122-78-1	128	a, b, c, d
19	γ-butyrolactone	Creamy, caramel	1609	914	96-48-0	<2	a, b, c, d
20	Furfuryl alcohol	Sweet	1638	854	98-00-0	4	a, b, c, d
21	3-Methylbutanoic acid	Sour, cheese	1644	855	503-74-2	1024	a, b, c, d
22	Methionol	Cooked potato-like	1696	978	505-10-2	256	a, b, c, d
23	2(5H)-furanone	Buttery	1727	—	497-23-4	2	a, b, c, d
24	3-methylvaleric acid	Cheese	1780	—	105-43-1	16	a, b, c, d
25	4-methylpentanoic acid	Cheese	1784	—	646-07-1	16	a, b, c, d
26	Methyl cyclopentenolone	Caramel	1802	1028	80-71-7	<2	a, b, c, d
27	Guaiacol	Smoky	1830	1092	90-05-1	256	a, b, c, d
28	Phenethyl alcohol	Floral, rose	1885	1118	60-12-8	1024	a, b, c, d
29	Maltol	Sweet	1941	1117	118-71-8	4	a, b, c, d
30	2-acetylpyrrole	Nutty	1942	1068	1072-83-9	<2	a, b, c, d
31	4-ethyl-2-methoxyphenol	Smoky	1996	1284	2785-89-9	512	a, b, c, d
32	HDMF	Caramel	2005	—	3658-77-3	1024	a, b, c, d
33	HEMF	Caramel	2068	1142	27538-09-6	1024	a, b, c, d
34	4-ethylphenol	Smoky	2140	—	123-07-9	256	a, b, c, d
35	Sotolon	Caramel	2189	1105	28664-35-9	1024	a, b, c, d
36	Phenylacetic acid	Honey	2516	1263	103-82-2	<2	a, b, c, d
37	Ethyl vanillate	Burnt	2590	—	617-05-0	<2	a, b, c, d

[a]The volatile compounds were listed based on their elution orders on DB-wax capillary column. [b]Odor perception sensed by sniffing port. [c]Retention index; RI did not exceed ±10 of the standard databases. [d]Flavor dilution factor determined by AEDA. [e]a, identification by odor characteristic; b, identification by comparison mass spectrum with the NIST 14 database; c, identification by retention index; d, identification by authentic standards. [f]"-," indicates that the compound has not been identified on the HP-5MS column.

ethylphenol was 256, and that of 4-ethyl-2-methoxyphenol was 512, so these compounds were considered to be important aroma-active compounds and were crucial for the overall flavor enhancement of HLFSS [20, 33]. Research had shown that increasing the proportion of wheat flour in raw materials could raise the concentration of guaiacol in SS [34].

3.1.8. Sulfur-Containing Compounds. Sulfur-containing constituents were mainly derived from the decomposition of amino acids containing sulfur in raw materials [35]; although their peak areas were relatively small, their influence on the flavor of the SS should not be underestimated. These compounds often had an aroma similar to that of onions, potatoes, or garlic [36]. The FD factor of both methionol and

methional was 256 with a cooked potato-like, and they had been identified in fermented alcoholic beverages such as Qingke Jiu and wine [37, 38]. Normally, methionol and methional have significantly lower thresholds; therefore, they could be sniffed even at a low concentration.

3.1.9. Aldehydes. Only phenylacetaldehyde with a pleasant floral and honey aroma was identified as an odor-active compound in this study. In the AEDA experiment, it reached an FD factor of 128, which indicated it had a large contribution to the aroma of SS. Phenylacetaldehyde could be produced through amino acid degradation, so it could be speculated that the formation of this aldehyde might be related to the fermentation process and protein metabolism [21].

Table 2: Selected ions and response factors used in the quantitative analysis.

Nos.	Compounds	Ions (m/z)[a] Analyte	Standard curves	R^{2b}
1	Isobutanol	74	$y = 0.1575x + 0.0866$	0.9998
2	Butanol	56	$y = 0.1706x - 1.9943$	0.9971
3	3-methyl-1-butanol	70	$y = 0.9393x + 3.2194$	0.9983
4	Methylpyrazine	94	$y = 7.8231x - 10.281$	0.9900
5	Acetoin	88	$y = 0.5428x - 0.2305$	0.9967
6	Acetol	74	$y = 0.1618x + 0.9311$	0.9982
7	2,5-dimethylpyrazine	52	$y = 0.2669x - 0.0073$	0.9993
8	2,6-dimethylpyrazine	67	$y = 0.3173x - 0.0159$	0.9999
9	Ethyl lactate	75	$y = 0.488x - 0.1911$	0.9998
10	1-hydroxy-2-butanone	88	$y = 0.0514x - 0.0001$	0.9997
11	2-ethyl-6-methylpyrazine	122	$y = 1.5858x + 0.0364$	0.9998
12	2,3,5-trimethylpyrazine[c]	84	$y = 1.3559x + 11.665$	0.9999
13	Acetic acid	60	$y = 0.8918x - 1.337$	0.9930
14	Methional[d]	107	$y = 10.167x + 3.195$	0.9975
15	2-acetylfuran	110	$y = 3.8914x - 2.3708$	0.9941
16	Propionic acid	74	$y = 1.695x - 3.3806$	0.9930
17	Isobutyric acid	88	$y = 0.2993x - 0.0962$	0.9939
18	Phenylacetaldehyde	91	$y = 2.8919x + 1.2293$	0.9950
19	γ-butyrolactone	42	$y = 1.2185x - 2.0827$	0.9991
20	Furfuryl alcohol	98	$y = 0.3202x + 12.562$	0.9957
21	3-methylbutanoic acid	60	$y = 0.9937x + 9.0398$	0.9918
22	Methionol[e]	109	$y = 14.37x + 3.8688$	0.9919
23	2(5H)-furanone	84	$y = 2.0576x - 1.341$	0.9910
24	3-methylvaleric acid	87	$y = 0.4795x + 0.1068$	0.9973
25	4-methylpentanoic acid	39	$y = 0.6306x + 0.0381$	0.9916
26	Methyl cyclopentenolone	112	$y = 1.3368x + 0.7372$	0.9982
27	Guaiacol	124	$y = 3.4991x - 1.099$	0.9984
28	Phenethyl alcohol	122	$y = 1.1318x$	0.9925
29	Maltol	126	$y = 1.2064x + 30.159$	0.9954
30	2-acetylpyrrole	94	$y = 2.156x$	0.9907
31	4-ethyl-2-methoxyphenol	152	$y = 2.2447x + 0.1465$	0.9970
32	HDMF	128	$y = 0.1187x + 0.0159$	0.9987
33	HEMF	142	$y = 0.1525x - 0.3057$	0.9906
34	4-ethylphenol	77	$y = 1.1067x$	0.9990
35	Sotolon	128	$y = 0.3773x$	0.9995
36	Phenylacetic acid	91	$y = 3.1445x + 21.529$	0.9960
37	Ethyl vanillate	196	$y = 1.9382x - 0.1379$	0.9994

[a]Selected ions (m/z) used in quantitative analysis; [b]determination coefficient obtained through the linear least-squares method; [c,d,e]the selected internal standards were [^{2}H$_3$]-2,3,5-trimethylpyrazine, [^{2}H$_3$]-methional and [^{2}H$_3$]-methionol, respectively; and the internal standard of other compounds was 3-octanol. The selected ions of 3-octanol, [^{2}H$_3$]-2,3,5-trimethylpyrazine, [^{2}H$_3$]-methional, and [^{2}H$_3$]-methionol were 83, 81,104 and 106, respectively.

3.1.10. Others. Except for the odorants above, the remnant compound only included 2-acetylpyrrole. Usually, pyrroles have the higher thresholds, so they have the insignificant aroma characteristics at low concentrations and are not easily detected by GC-O analyses. The threshold of 2-acetylpyrrole in water can reach $58000\,\mu$g/L [18], so it not easily sniffed; in this study, the FD factor of 2-acetylpyrrole (nutty) was less than 2.

3.2. Quantitative Analysis of Odor-Active Compounds in HLFSS during Storage. To investigate the changes on concentrations of odor-active compounds during storage, the odorants in the samples with different storage time were isolated and quantitated and the related data and the results obtained are shown in Tables 2 and 3 and Figure 1. From the results, it could be seen that the relative contents of furans, acids, alcohols, ketones, and sulfur-containing substances were higher among the odor-active compounds of the brewing SS samples, accounting for about 44.40%, 35.19%, 13.54%, 3.9%, and 1.29%, respectively. The compound with the highest concentration was acetic acid, which could reach $238250-257825\,\mu$g/L, followed by HEMF ($207441 - 103\,763\,\mu$g/L), HDMF ($101325-157806\,\mu$g/L), acetol ($46319-54261\,\mu$g/L), butanol ($22204 - 19536\,\mu$g/L), and 1-hydroxy-2-butanone ($18617 - 9148\,\mu$g/L).

3.2.1. Aroma-Active Compounds with Increased Content during Storage in HLFSS. The concentration of acetic acid, propionic acid, isobutyric acid, 3-methylbutanoic acid, 3-methylvaleric acid, and 4-methylpentanoic acid rose during storage. This is because most of the microorganisms in SS have an active lipase system. Although the HLFSS is sterilized before leaving the factory, the enzymes in HLFSS are not completely inactivated. These enzymes can promote the degradation of amino acids and fat acid occurring in HLFSS

TABLE 3: The concentrations of aroma-active compounds in HLFSS during storage.

Nos	Compounds	Concentrations (μg/L)[a]								
		S1	S2	S3	S4	S5	S6	S7	S8	S9
1	Isobutanol	11930±2b	10963±182c	10683±220cd	10624±1cde	12385±15a	10143±26e	10343±33de	12431±55a	10378±40de
2	Butanol	22204±38d	22305±18d	22598±234c	21292±61e	24087±77a	23888±23a	20721±15f	23438±14b	19536±17g
3	3-methyl-1-butanol	4771±99f	5434±133d	4612±92g	4595±547g	5193±24e	5590±67b	5157±12e	5771±108a	5492±140c
4	Methylpyrazine	354±1ab	354±0.7ab	354±0.6ab	355±0.2ab	357±0.6ab	359±0.4ab	359±0.4b	360±0.5a	361±0.1a
5	Acetoin	2765±27b	2841±5a	2511±12e	2688±10c	2776±12b	2581±22d	2324±0.2g	2409±5f	2307±5g
6	Acetol	46319±218g	48312±2e	46786±431f	49757±65d	48690±283e	54467±58b	50813±418c	56263±333a	54261±178b
7	2,5-dimethylpyrazine	73.2±2c	73.7±0.2d	68.0±3g	69.0±2f	66.3±12i	77.1±2a	76.4±2b	67.5±0.1h	71.2±0.8e
8	2,6-dimethylpyrazine	131±5h	143±3f	136±2g	147±1e	137±2g	166±21b	153±12d	160±3c	182±3a
9	Ethyl lactate	3715±20d	3705±7f	3704±22f	3704±6g	3716±70d	3798±10a	3735±64b	3711±7e	3728±119c
10	1-hydroxy-2-butanone	18617±189a	9245±10d	8711±132i	9064±20g	9469±0.8b	9181±27e	8947±20h	9322±6c	9148±12f
11	2-ethyl-6-methylpyrazine	30.2±0.1g	32.4±0.6e	25.3±3h	40.2±0.4d	32.7±3f	47.7±0.8c	49.6±1b	52.5±3a	52.4±1a
12	2,3,5-trimethylpyrazine	8.53±0.1d	9.20±0.1c	9.35±0.1c	9.42±0.5c	8.36±0.4d	11.4±1a	10.2±1b	9.81±1c	8.04±1d
13	Acetic acid	238250±2138ab	227963±213ab	216250±4038ab	240375±438ab	261250±2363ab	254250±238b	255988±163ab	287925±1013a	257825±113ab
14	Methional	3139±89e	3163±48e	3113±15f	3549±148c	3876±13a	3575±46c	3490±26d	3673±92b	3569±2c
15	2-acetylfuran	356±0.1bc	350±0.5d	347±2f	347±1e	357±0.9ab	359±1a	354±0.2c	357±0.5ab	358±0.5ab
16	Propionic acid	2459±10e	2435±10f	2444±12ef	2553±5d	2689±3c	2677±22c	2729±2b	2842±5a	2839±7a
17	Isobutyric acid	946±10i	958±18h	964±6g	972±14f	1000±48e	1020±12d	1030±16c	1046±18b	1048±16a
18	Phenylacetaldehyde	69.2±0.1i	74.4±0.4h	77.2±0.9g	86.3±0.4f	99.0±0.3e	110±1d	112±1c	123±1b	132±0a
19	γ-butyrolactone	4356±19g	4336±83g	4365±51g	4378±6f	4400±7e	4644±24d	4808±5c	4875±17b	4940±28a
20	Furfuryl alcohol	1918±63i	2884±2g	1923±177h	2915±66f	4727±38e	6398±63c	6232±58d	6796±43b	6953±39a
21	3-methylbutanoic acid	227±3g	237±0.9f	211±21i	219±18h	395±24c	409±10b	382±4d	415±11a	367±10e
22	Methionol	5879±64a	5143±10i	5241±17f	5175±42h	5201±44g	5394±62d	5453±4c	5488±45b	5379±110e
23	2(5H)-furanone	245±5g	249±4f	280±4b	257±2d	285±14a	258±2d	252±2e	276±22c	257±4d
24	3-methylvaleric acid	106±2g	108±0.2f	89.2±0.4i	94.4±1h	115±0.2e	122±4d	122±2c	124±2b	129±1a
25	4-methylpentanoic acid	313±8g	306±12h	369±44f	377±8e	425±12c	409±36d	425±1c	460±52b	464±4a
26	Methyl cyclopentenolone	51.3±1b	49.7±0.1c	26.5±0.7e	46.6±0.2d	50.4±0.1bc	55.2±2a	49.5±0.9c	49.6±0.2c	47.4±0.2d
27	Guaiacol	109±0.2d	111±0.2cd	109±0.3d	112±0.2bcd	114±0.2abcd	115±0.7d	116±0.1abc	118±0.3a	117±0.3ab
28	Phenethyl alcohol	7338±46a	6897±3b	6276±34g	6005±41h	6551±27d	6849±47c	6419±17f	6466±14e	5890±14i
29	Maltol	5790±259c	5716±99d	5095±115f	4975±101g	6025±68b	6704±150a	6074±14b	5728±2d	5449±93e
30	2-acetylpyrrole	1521±8h	1967±13g	2093±31f	2640±17e	3215±27d	3965±21c	3981±39c	4255±4b	4519±0.3a
31	4-ethyl-2-methoxyphenol	889±10a	822±10d	825±5c	738±2h	805±14e	841±10b	796±2f	791±1g	726±10i
32	HDMF	101325±731b	118188±544ab	126525±388ab	142431±606ab	155000±631ab	170763±294a	167538±238ab	163856±5975a	157806.25±356ab
33	HEMF	207441±741	183404±985	165525±2762	151762±471	151842±549	149782±582	133002±610	115190±119	103763±330
34	4-ethylphenol	258±8b	233±5bc	233±2c	219±5cd	233±25cd	236±11cd	247±0.3e	230±8de	238±0.8e
35	Sotolon	547±3a	519±9d	525±10d	539±9f	508±3d	508±3c	494±2b	465±4e	448±5c
36	Phenylacetic acid	1799±29a	1611±12d	1452±18c	1513±31b	1598±17e	1743±5e	1503±10f	1467±9g	1268±1h
37	Ethyl vanillate	206±2b	183±2d	174±1g	168±1f	170±2e	180±2c	163±1f	161±1g	132±1h

[a]The average concentration of aroma-active components in HLFSS obtained from three parallel experiments' data. [b]a–i means different alphabets in the same row which indicate significant differences ($p < 0.05$).
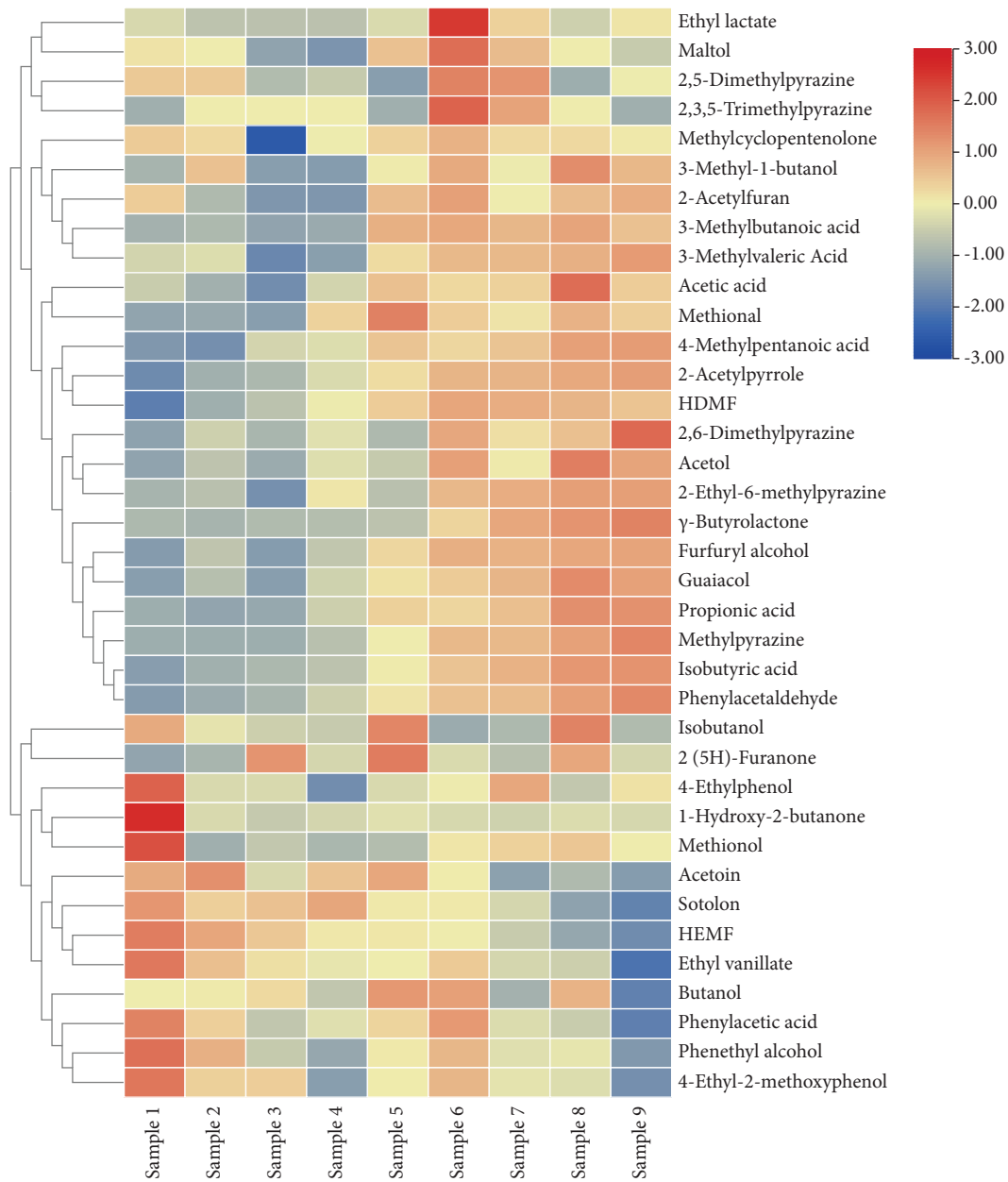
Figure 1: Heatmap of changes on aroma-active compound concentrations in HLFSS during storage.

[23], thereby increasing the concentration of some acids. Acetic acid (238250–257825 μg/L) continued to increase during storage in HLFSS, and it varied greatly throughout the storage process, so its content change could be used as an indicator to determine the storage time of HLFSS.

Pyrazines were mainly produced through Maillard reaction, and they were considered to be the main volatiles in acid-hydrolyzed SS formed after being heated [1]. The concentrations of 2-methylpyrazine, 2,6-dimethylpyrazine, and 2-ethyl-6-methylpyrazine also went up during storage, probably due to more precursors formed, which favored their formation.

Except that furfuryl alcohol was formed by microbial fermentation during the production [39], some reducing sugars could react with amino acids to generate furfural, which could be further converted into furfuryl alcohol,

leading to an increase in its concentration [11]. It should be noted that the concentration of furfuryl alcohol (1918–6953 μg/L) varied greatly throughout the storage process, and its content changes could be also used as an indicator to determine the storage period of HLFSS. In addition, the concentration of 3-methyl-1-butanol also rose; its formation was associated with the degradation of leucine [40], which could degrade during storage, resulting in increasing in its concentration.

The formations of furans were related to Maillard reaction to a certain extent [1]. Among five furans, the concentrations of 2-acetylfuran and HDMF increased during storage, especially that of HDMF exhibited significant changes (101325–157806 μg/L), so the content change of HDMF could be used as an indicator for determining the storage period of HLFSS.

TABLE 4: OAV of aroma-active compounds detected in HLFSS.

Nos.	Compounds	Odor threshold (μg/L)	OAV[a]								
			S1	S2	S3	S4	S5	S6	S7	S8	S9
33	HEMF	1.15	180383	159482	143935	131967	132037	130245	115654	100165	90229
14	Methional	0.45	6976	7029	6918	7887	8613	7944	7756	8162	7931
32	HDMF	22.3	4544	5300	5674	6387	6951	7658	7513	7348	7077
3	3-methyl-1-butanol	4	1193	1359	1153	1149	1298	1398	1289	1443	1373
35	Sotolon	1.7	322	305	309	317	299	299	291	274	264
5	Acetoin	14	198	203	179	192	198	184	166	172	165
20	Furfuryl alcohol	12.3	156	234	156	237	384	520	507	553	565
27	Guaiacol	0.84	130	132	130	133	136	137	138	140	139
2	Butanol	459.2	48	49	49	46	52	52	45	51	43
22	Methionol	123.23	48	42	43	42	42	44	44	45	44
29	Maltol	210	28	27	24	24	29	32	29	27	26
1	Isobutanol	550	22	20	19	19	23	18	19	23	19
34	4-ethylphenol	13	20	18	18	17	18	18	19	18	18
28	Phenethyl alcohol	564.23	13	12	11	11	12	12	11	11	10
31	4-ethyl-2-methoxyphenol	69	13	12	12	11	12	12	12	11	11
18	Phenylacetaldehyde	6.3	11	12	12	14	16	17	18	20	21
6	Acetol	10000	5	5	5	5	5	5	5	6	5
19	γ-butyrolactone	1000	4	4	4	4	4	5	5	5	5
13	Acetic acid	99000	2	2	2	2	3	3	3	3	3
11	2-ethyl-6-methylpyrazine	40	1	1	1	1	1	1	1	1	1
16	Propionic acid	2190	1	1	1	1	1	1	1	1	1
17	Isobutyric acid	1500	1	1	1	1	1	1	1	1	1
37	Ethyl vanillate	206	1	1	1	1	1	1	1	1	1
21	3-methylbutanoic acid	490	<1	<1	<1	<1	1	1	1	1	1
25	4-methylpentanoic acid	810	<1	<1	<1	<1	1	1	1	1	1
4	Methylpyrazine	30000	<1	<1	<1	<1	<1	<1	<1	<1	<1
7	2,5-dimethylpyrazine	1750	<1	<1	<1	<1	<1	<1	<1	<1	<1
8	2,6-dimethylpyrazine	718	<1	<1	<1	<1	<1	<1	<1	<1	<1
9	Ethyl lactate	50000	<1	<1	<1	<1	<1	<1	<1	<1	<1
12	2,3,5-trimethylpyrazine	350.12	<1	<1	<1	<1	<1	<1	<1	<1	<1
15	2-acetylfuran	15025.2	<1	<1	<1	<1	<1	<1	<1	<1	<1
23	2(5H)-furanone	714	<1	<1	<1	<1	<1	<1	<1	<1	<1
26	Methylcyclopentenolone	300	<1	<1	<1	<1	<1	<1	<1	<1	<1
30	2-acetylpyrrole	58585.25	<1	<1	<1	<1	<1	<1	<1	<1	<1
36	Phenylacetic acid	6100	<1	<1	<1	<1	<1	<1	<1	<1	<1
10	1-hydroxy-2-butanone	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
24	3-methylvaleric acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a]OAV: calculated by dividing the concentration by the corresponding odor threshold.

Phenylacetaldehyde was present at the lower level (69–132 μg/L) in HLFSS, and its concentration tended to increase during storage. It had been found in many fermented soy foods, and its concentration usually peaked during the later stages of SS fermentation. Long-term storage would lead to an increase in phenylacetaldehyde content, which was related to the degradation of phenylalanine.

Moreover, the concentrations of 2-acetylpyrrole, acetol, guaiacol, and γ-butyrolactone also increased during storage. Among them, acetol (46319–54261 μg/L) had a significant change in concentration throughout the storage process, which could be used as an indicator to determine the different storage periods of HLFSS.

3.2.2. Aroma-Active Compounds with Decreased Content during Storage in HLFSS. The contents of 4-ethyl-2-methoxyphenol and 4-ethylphenol decreased during

storage, and Wang et al. [9] also found that as heating time was prolonged, their concentrations in SS went down. Maybe they reacted with other compounds to form new compounds. Among all alcohols, the levels of 2-phenylethanol (from 7338 μg/L to 5890 μg/L) and butanol (from 22204 μg/L to 19536 μg/L) declined during storage. The reason was that both of them could react with organic acids to give ester compounds, and they could be oxidized into aldehyde and organic acid, which made their concentrations decease. From the results obtained previously, it could be seen that the content of phenylacetaldehyde increased indeed.

HEMF, with a strong caramel-like note, had the higher concentration (207441−103763 μg/L) among odor-active compounds identified. HEMF could be generated not only by the biosynthesis pathway of yeasts but also by Maillard reaction [28]. However, HEMF contained active groups in its structure, so it could take place degradation
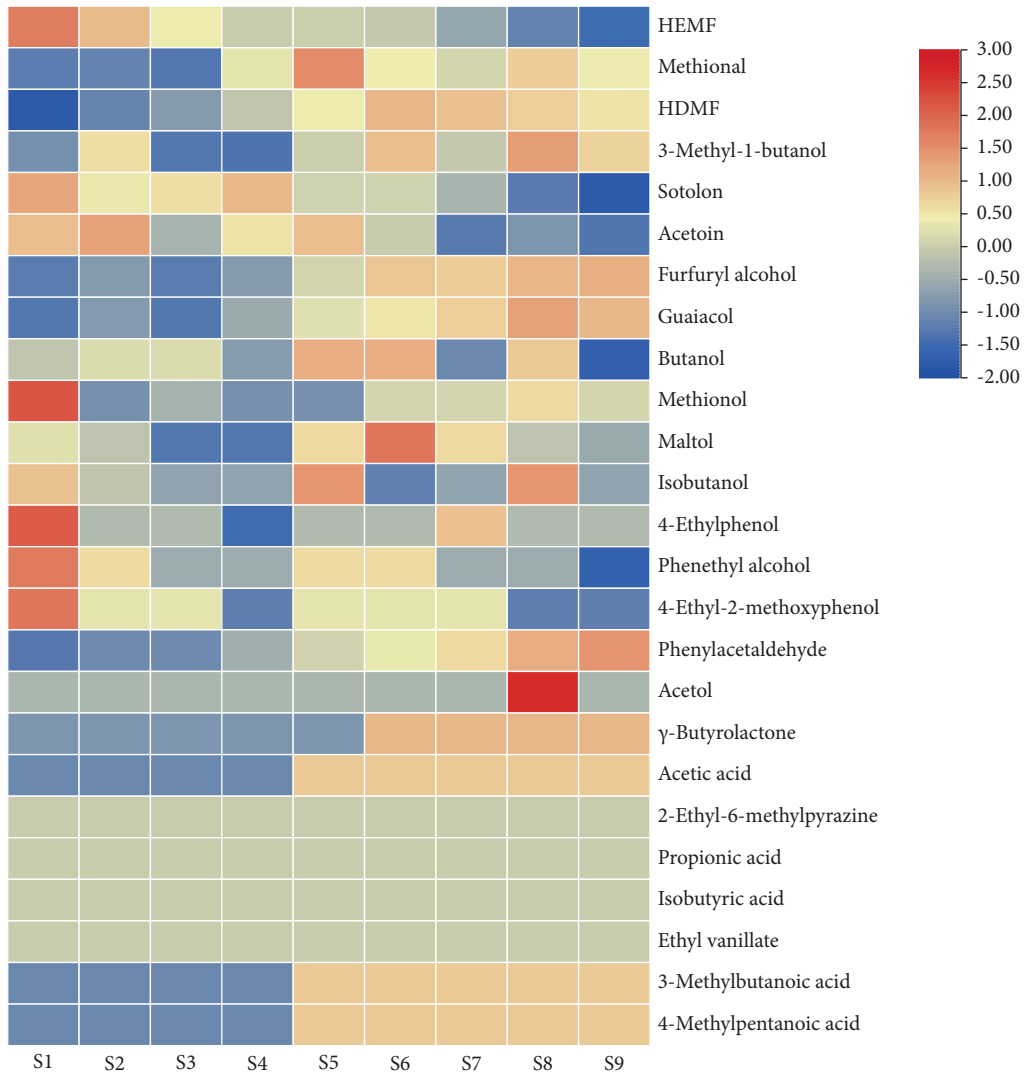
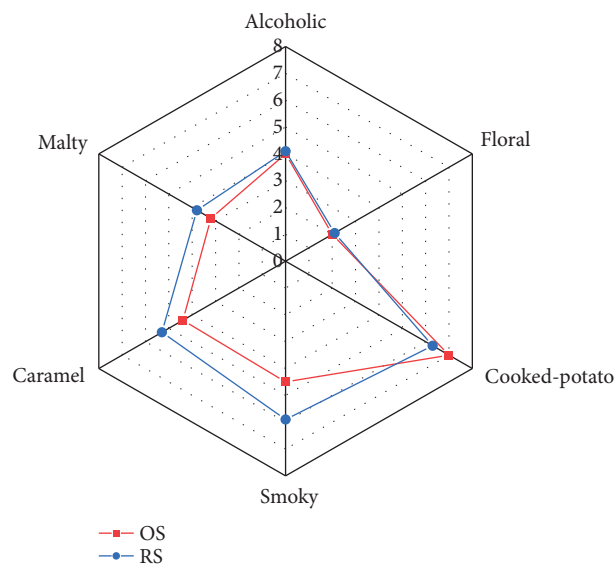Figure 2: Heatmap of OAVs of key odorants in HLFSS during storage.

Figure 3: Odor profile analyses. (a) Recombination sample (RS); (b) original sample (OS).

reaction during storage, which led to a decrease in its concentration. Changes in the concentrations of HEMF during storage could be used as an indicator to determine different storage periods for HLFSS. In addition, the contents of 1-hydroxy-2-butanone (from 18617 μg/L to 9148 μg/L) and acetoin (from 2765 μg/L to 2307 μg/L) dropped because they easily took place oxidation or polymerization reactions to form diketone or dimmer compounds. Because of the great change on the concentration of 1-hydroxy-2-butanone, these changes could be used as an indicator to determine the storage period of HLFSS.

Esters were widely present in fermented foods; their formations were closely related to the yeast metabolism [23]. In the production process of SS, yeast could degrade some precursors into alcohols and acids, which took place esterification reaction to give ester compounds [27]. Among the three esters detected, the concentration of ethyl vanillate (from 206 μg/L to 132 μg/L) in HLFSS decreased during storage, which might be due to the hydrolysis of ethyl vanillate. Furthermore, the contents of phenylacetic acid and sotolon also showed a gradual decrease during storage.

3.2.3. Aroma-Active Compounds with Fluctuating Content during Storage in HLFSS.

Sulfur-containing compounds had a low content in SS, but they had an important effect on the overall flavor. They were mainly produced by degradation of sulfur-containing amino acids or peptides in raw materials [3], and their threshold in water was very low [18], so these components were easily identified by GC-O analyses. The concentrations of methional and methionol fluctuated during storage; both of them could carry out interconversion under a certain condition, so it was speculated that there might be some dynamic equilibrium between them.

In addition, the concentrations of ethyl lactate, maltol, methylcyclopentenolone, 2(5H)-furanone 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, and isobutanol also showed a fluctuation trend during storage. The concentration of ethyl lactate (3715−3728 μg/L) was higher than other esters, which was consistent with previous studies [41]. Maltol was produced from Maillard reaction, and methylcyclopentenolone had been detected in the glucose-tyrosine model system and the glucose-histidine model system [42, 43]. It was presumed that these compounds might be in a state of transformation and formation during storage of HLFSS.

3.3. OAV.

Among the 37 aroma-active compounds detected, 23 odorants had OAV ≥ 1, so they were identified as the key odorants of HLFSS (showed in Table 4 and Figure 2). In addition, the OAVs of 3-methylbutanoic acid and 4-methylpentanoic acid ranged from less than 1 to 1 during storage. HEMF (OAV: 90229−180383), methional (OAV: 6976−7931), and HDMF (OAV: 4544−7077) had relatively higher OAVs, and the OAVs of HEMF were the highest among all aroma compounds, so it could be concluded that this component should contribute greatly to the unique flavor of SS. Both methional (6976−7931) and methionol (44−48) exhibited potent cooked potato-like notes. The smoky aroma was a typical characteristic aroma of phenols; three phenols identified in the present research had an OAV > 1, and guaiacol (130−139) got a higher OAV. At the same time, the FD factors of the two sulfur-containing compounds mentioned previously and phenolic compounds were ≥256, indicating that these compounds played an essential role in the formation of the overall aroma of SS. The OAVs of 2-phenylethanol and phenylethyl aldehyde ranged from 11 to 21, which imparted a floral note to SS. Among the components with OAVs ≥ 1, 3-methyl-1-butanol (1193−1373), butanol (48−43), and isobutanol (22 − 19) had alcoholic aroma, and they were the main contributors to the alcoholic aroma of HLFSS.

3.4. Aroma Recombination Experiment.

To investigate the contribution of aroma-active compounds with OAVs ≥ 1 to the overall aroma profile of SS, reconstitution experiments and sensory evaluation were conducted based on the detected key aroma components, and the results are shown in Figure 3. From Figure 3, it could be seen that the aroma profile of the recombinant sample (RS) was similar to that of the original sample (OS), and the scores for alcohol-like and floral attributes were closer. In addition, the RS had stronger intensities in malty, caramel-like, and smoky attributes compared to the OS; the reason might be that the matrix of HLFSS was more complex than that of the RS, resulting in the odorants in HLFSS that were not released. Therefore, it could be concluded that the RS better simulated the aroma profile of HLFSS.

4. Conclusion

GC-FID-O and GC-MS-O coupled with SE-SAFE were employed to investigate the flavor of HLFSS during storage. A total of 37 odor-active compounds were identified; their FD factors were measured through AEDA, and the FD factors of methional, 3-methylbutanoic acid, phenethyl alcohol, sotolon, HEMF, and HDMF were 1024. Twenty-three compounds with OAV ≥ 1 were considered as key odorants, and the recombination tests also confirmed the important contribution of these key odorants to HLFSS. The concentration of HEMF, HDMF, acetic acid, 1-hydroxy-2-butanone, acetol, and furfuryl alcohol vary greatly during storage, and the changes on content of these compounds could be used as the indicators to determine the storage time of HLFSS.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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