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Characterization of flavor fingerprinting of red sufu during fermentation and the comparison of volatiles of typical products

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ABSTRACT

Traditional Chinese red sufu is a popular condiment, and typical flavor is an important indicator of sensory qualities in commercial products. In this study, the volatile aroma analysis of Wangzhihe red sufu (WRS) from different fermentation stages and four typical red sufu products from different locations was carried out by gas chromatography-mass spectrometry (GC–MS), gas chromatography-mass spectrometry/olfactory (GC–MS/O), electronic nose (E-nose) and sensory evaluation. Results showed that 106 volatile compounds were identified in Wangzhihe red sufu during fermentation process, in which phenolics and alcohols were dominant at molded and salted phetze stages, while esters and alcohols became predominate at post-fermentation stage. The volatile aroma substances varied at each fermentation stage. Furthermore, 86 volatiles, including 16 aroma-active compounds, were detected in four typical red sufu products. The multivariate analysis results showed the difference in samples from different fermentation stages and typical red sufu products according to GC–MS and E-nose analysis. Combined with flavor omics, discriminant model was established for effective discrimination of samples from different fermentation stages and locations, as well as references to sufu maturity extent. The study presented a new strategy for quality evaluation of red sufu, which could be the supplement to quality evaluation standards.

1. Introduction

As a Chinese traditional fermented soybean product, sufu has been consumed as an appetizer and condiment for centuries [1]. It is considered a nutritious and soft creamy product with a characteristic flavor profile and relatively high protein content [2]. Meanwhile, it has been authorized to be a health food on account of its beneficial bioactivities, such as antioxidative activity [3], antimutagenicity [4] and antihypertensive acitivity [5]. According to the different ingredients of dressing mixture, which affects its color and flavor, four types of sufu could be distinguished, including red sufu, white sufu, grey sufu and sauce sufu [6]. Red sufu is a large category of beancurd products, which is made from soybean, yellow rice wine, wheat flour, red koji, spices and other raw materials [7]. Varieties of red sufu products are available in China and the quality of each product depends on the fermentation conditions, microorganisms and ingredients used by the manufacture [8]. Among the famous red sufu, Wangzhihe red sufu stands out as a representative of the northern part of China.

Wangzhihe red sufu production (Fig. 1) starts with the grind of soybean, soybean curd preparation, molded phetze stage (pre-fermentation), salted phetze stage, and long-term post-fermentation [1]. The pehtze is prepared by cultivating *Actinomucor elegans* on the surface of tofu cubes, and then the pehtze is salted with sodium chloride and salt-saturated solution for about 5 days. Afterwards, the salted pehtze is bottled into wide-mouthed glass containers and ripened in the dressing mixture for approximately 3 months. The dressing mixture of red sufu is mainly composed of red kojic rice (cooked rice inoculated with *Monascus purpureus*), edible alcohol, sugar, chiang (flour paste fermented by *Aspergilus*

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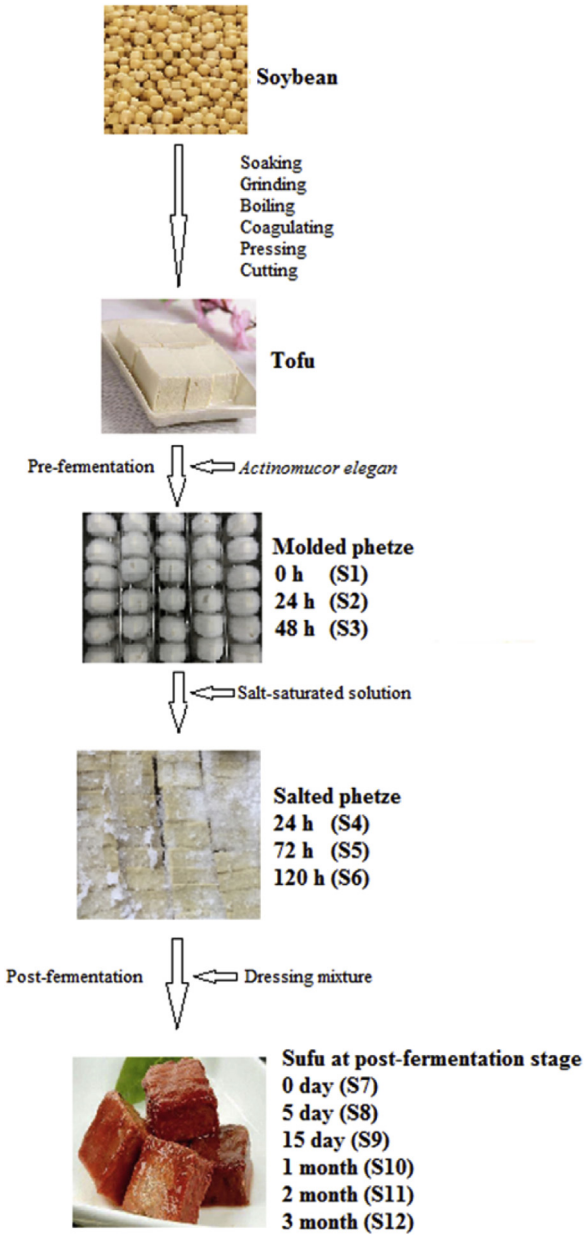

Fig. 1. The manufacture processing for red sufu fermentation and sample points collected for analyzation.

oryzae) and spices. Eventually, the fermentation proceeds into the maturation stage, at which the aroma of red sufu is reinforced. In fact, each single fermentation step is not completely independent and the final product is affected by the interaction together with above-mentioned fermentation process. In the whole process, the qualitative characteristics of red sufu vary in terms of the metabolites present, due to the physiochemical and enzymatic activities existed in each stage, which contribute to the taste and aroma.

The aroma of red sufu, an important indicator of the sensory qualities, often affects the acceptance and selection of consumers directly. In addition, the characterization of the aroma in sufu will contribute to explain the distinctions among various products from different locations of origin. It depends primarily on the availability of volatile flavor compounds, which are usually influenced by some factors, such as the starting materials, microorganisms and processing techniques [1]. Volatile compounds of red sufu have been investigated in previous studies. Huang et al. and Xie et al. [8,9] employed headspace solid phase micro-extraction (HS-SPME) combined with gas chromatography-mass spectrometry (GC–MS) to analyze red sufu products, 72 volatile flavors and 12 characteristic volatile components were identified respectively, prominently esters. 83 volatile compounds have been reported to be identified in Beijing red sufu by simultaneous distillation-extraction (SDE) coupled with GC–MS, including ethyl linoleate, ethyl hexadecanoate, benzeneacetaldehyde, 3-methylbutanal, 2,6-dimethylpyrazine and methylpyrazine mainly [10]. Home-made red sufu and two commercial red sufu were analyzed with the same method above, and 72 volatile compounds were detected totally, while long chain fatty acid (LCFA) esters accounted for 90% of volatiles content [11]. Chung [12] identified 89 volatile compounds (19 aroma-active compounds) in 3 different commercial red sufu products by SDE coupled with GC–MS/O. Ethyl 2-methylpropanoate, 2,3-butanedione, ethyl butanoate, ethyl 2-methylbutyrate, 3-(methylthio)-propanal, benzeneacetaldehyde, as well as ethyl 3-phenylpropionate were reported to be common aroma-active compounds contributing to the characteristic aroma of red sufu enormously. Volatile flavors, consisting of alcohols, esters, aldehydes and ketones, provided red sufu the specific flavor under synergistic effects [13]. However, up to now, most previous studies focused on volatile profiles of commercial sufu products and few works have been reported to investigate the dynamic change in flavor profiles of red sufu during the industrial manufacturing process. Moreover, the comparison of typical red sufu aromas and the corresponding respective volatile flavor compounds should be carried out. Furthermore, the current Chinese sufu industrial standard [14] takes physiochemical indices (amino acid nitrogen, water-soluble protein, total acid and salt content) as the basis of quality evaluation simply. Without the consideration of aroma evaluation, it will lead to the deficiency and irrationality of its quality evaluation system.

In this study, the dynamic changes of volatile profiles in WRS samples during fermentation were investigated, the characteristic and key flavor compounds of commercial red sufu were identified by the comparison of four typically commercial red sufu, and the characteristic volatile flavor fingerprint database was established. By HS-SPME-GC–MS, GC–MS/O, electronic nose (E-nose), and sensory evaluation, metabolites identified in samples were analyzed multidimensionally and multilevelly, and factors affecting the flavor of red sufu and the critical control points in WRS during fermentation were revealed with omics technologies. Based on statistical analysis, such as principal component analysis (PCA), linear discriminant analysis (LDA), partial least squares discrimination analysis (PLS-DA), hierarchical clustering analysis (HCA) and volcano plot, the discriminant model of red sufu flavor was established, which provide a new method for classification of red sufu flavor and discrimination of their quality characteristics according to volatile compounds.

2. Materials and methods

2.1. Samples

Molded phetze (MP) stage: 0 h (S1), 24 h (S2) and 48 h (S3); Salted phetze (SP) stage: 24 h (S4), 72 h (S5) and 120 h (S6); Post-fermentation (PF) stage: days 1 (S7), 5 (S8), 15 (S9), 30 (S10), 60 (S11) and 90 (S12). All samples collected from three batches were provided by Beijing Wangzhihe Foodstuff Group Co., Ltd. and stored at −70 °C until analysis. Four typically commercial red sufu, including R1 (Zhejiang, Xianheng), R2 (Guangdong, Guanghe), R3 (Taiwan, Jiangji) and R4 (Beijing, Wangzhihe), were purchased from a market in Beijing and each product were collected with three samples at the same product date. These red sufu are commonly consumed in China and they are the most famous red sufu brands in China.

The selection of different brands of red sufu was determined in terms of their popularity of preference among consumers. Zhejiang Xianheng red sufu, also known as "Gongfang", was one of the eight tributes for the imperial court of Ming and Qing Dynasties. Guangdong Guanghe red sufu, belonging to Cantonese cuisine, is a traditional and famous dish in Guangdong province. Taiwan Jiangji red sufu is sold well in Taiwan and exported overseas, including Australia, North America and Southeast Asia. Detailed information has been listed in Table S1.

2.2. Reagents and materials

3-Octanol (> 99%) and C8–C20 *n*-alkane analytical standard were purchased from Sigma-Aldrich Corporation (Shanghai, China). Sodium chloride and other reagents (> 99%) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Fused silica fibers of 65 μm DVB/PDMS were obtained from Supelco, Inc. (Bellefonte, PA, USA).

2.3. Sample extraction condition of flavor compounds

After optimization of all experimental conditions, the extraction method was established. Samples were drained at room temperature for 15 min and then homogenized. Exactly 4 g of sufu sample and 4 mL of water were transferred to a 20 mL vial, and the ionic strength of the solution was adjusted to 25% with NaCl. Prior to analysis, samples were mixed with 20 μL of 3-octanol solution as an internal standard to achieve a final concentration of 500 ng/g. Samples were vibrated for 2 min by a vortex mixer and then sonicated for 5 min. The PDMS/DVB fiber was selected to extract volatiles and conditioned prior to use according to the supplier's prescriptions among three fibers, including PDMS/DVB, DVB/CAR/PDMS and PA. The extraction temperature was set to 50 °C and the absorption time was 40 min.

2.4. GC–MS and GC–MS/O conditions

GC–MS analysis was conducted on an Agilent 5975C/7890A instrument (Agilent, USA) equipped with an Agilent GC 80 multimode sampler. The chromatographic column was Agilent J&W HP-5 ms (30 m ×0.25 mm ×0.25 μm, Agilent). The carrier gas was helium with the flow rate of 1.0 mL/min, and the fiber was injected in splitless injection mode at 250 °C for 3 min. The initial oven temperature was set to 40 °C for 3 min, increased from 40 °C to 100 °C at a rate of 5 °C/min, increased from 100 °C to 220 °C at a rate of 6 °C/min, and then held at 220 °C for 10 min. Electron impact mass spectra were scanned at 70 eV in the mass range of 35–400 amu, and temperatures of ion source and quadrupole were 230 °C and 150 °C, respectively.

GC–MS/O system consisted of a GC–MS system (Agilent 5975C/7890A) and a sniff port (Brechbühler Sniffer 9000, Switzerland). GC–MS conditions were identical to those described above. The effluent from the analytical column was split into two identical streams and the temperature of the transmission wire connected to the sniff port was 180 °C. Three panelists (18–30 years old) were selected and trained by standard compounds with different concentration. Retention time, odor intensity, and odor description were noted with rated odor intensity on a three-point scale (1: weak; 2: medium; 3: strong). The results were averaged for each sensory note.

2.5. Compound identification and semi-quantification

Identification was achieved by matching with the mass spectra of reference standards found in the National Institute of Standards and Technology mass spectrum library on the basis of their similarity (>800) and retention indices (RI). RI values were calculated using C8–C20 n-alkanes as standard references and processed under the same chromatographic conditions. The concentration of each compound was estimated as: peak area of analyte × (concentration of internal standard/peak area of internal standard).

2.6. E-nose measurement

PEN3 (Win Muster Airsense Analytics Inc., Schwerin, Germany) E-nose system consists of 10 metal oxide gas sensors: W1C (aromatic compounds), W5S (nitrogene oxides), W3C (aromatic compounds), W6S (hydrogen), W5C (alkenes and aromatic compounds), W1S (methane), W1W (sulfur-containing compounds), W2S (alcohols), W2W (aromatics compounds and sulfur organic compounds), and W3S (methane). Approximately 2 g of sufu sample, 2 mL of water, and 0.5 g of NaCl were added to a 10 mL airtight glass bottle, which was then sealed. The sample was agitated with a constant speed at 50 °C for a headspace generation time of 15 min. To obtain stable response signals, the measurement and flushing times were set to 90 s and 250 s, respectively. Each sample was determined for seven parallel trials and stable response signals obtained at 78 s were used for analysis.

2.7. Sensory evaluation

With reference to the sensory evaluation methods in the literatures [15–17], through several preliminary screening sessions, including personal information, matching and ranking test, training about sensory evaluation and scaling methods, definitions and references, as well as validation procedure, eight trained panelists (4 males and 4 females, 27 years old on average) were selected to evaluate sensory attributes of four typically commercial red sufu products by quantitative descriptive analysis (QDA). Twenty grams of homogeneous red sufu samples with three-digit random codes were prepared in a plastic cup to serve for sensory evaluation three sessions per day for two weeks and each session took at least 30 min. Sensory attributes were scored on the basis of 5-point linear scale from 0 (none) to 5 (strongest). The definitions and references of relevant odors listed in Table S2 were developed through group discussions and relevant literatures. Finally, the experimental results were collected and PCA was applied to confirm that data obtained from sensory evaluation was valid and reliable.

2.8. Statistics

All analyses were conducted in triplicate. Data normalization was carried out, and then PLS-DA was performed with SIMCA version 13 software (Umetrics, Umearing, Sweden) for reducing the dimensionality of the dataset to maximum the separation between classes. Between-group differences ($P < 0.05$) were performed by ANOVA and data were analyzed using SPSS version 21.0 software (SPSS, Chicago, IL, USA). The data collected from E-nose was analyzed by principal component analysis (PCA) and linear discriminant analysis (LDA) using Win Muster software. Volcano plot and heatmap of CA were performed with Metabo Analyst version 4.0 software (www.metaboanalyst.ca) for cluster and variance analysis of volatile flavors.

3. Results and discussion

Volatile compounds in red sufu samples were analyzed by HS-SPME-GC–MS and total ion chromatograms (TIC) of WRS samples at MP stage, SP stage, PF stage and four products are listed in Fig. 2A–D. The effect of fermentation time on the composition of volatile com-

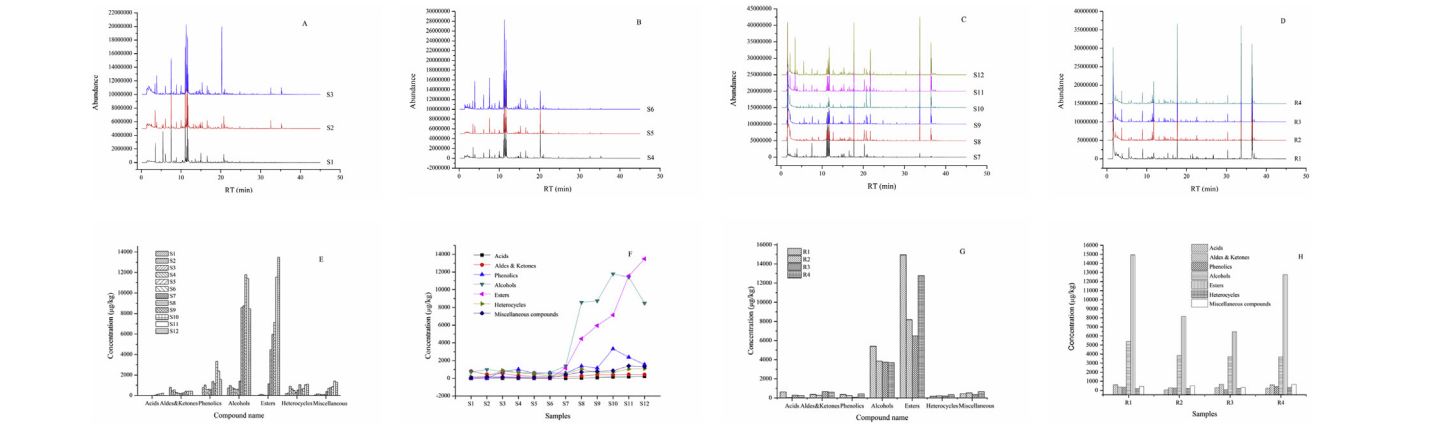

Fig. 2. Total ion chromatograms (TIC) of sufu samples at MP stage (A), SP stage (B), PF stage (C) and four sufu products (D), and changes in the contents of volatile compounds during fermentation process (E & F) and four commercial sufu products (G & H).

pounds are illuminated in Fig. 2E and F, while volatiles in four products are compared, as shown in Fig. 2G and H.

3.1. Analysis of volatile compounds identified over the course of fermentation in WRS

A total of 106 volatile compounds were identified in WRS samples over the course of fermentation, including esters (34), alcohols (17), aldehydes&ketones (25), acids (3), phenolics (4), heterocycles (5), and miscellaneous compounds (18), as listed in Supplementary Table S3.

3.1.1. Analysis of volatile compounds at MP and SP stages

According to Table S3, 59 compounds were detected at MP and SP stages, including aldehydes & ketones (23), alcohols (15), esters (7), heterocycles (4), acids (1), phenolics (1), and miscellaneous compounds (8).

Aldehydes & ketones are the largest group, which accounts for approximately 40% of total volatiles. 3-Methylbutanal, pentanal, 2,4-heptenal and benzeneacetaldehyde had significant increases in MP stage (P<0.05). On the other hand, there were significant decreases for hexanal, 2-hexenal, heptanal, nonanal, and 2,4-decadienal (P<0.05). These aldehydes have been reported to provide an undesirable beany flavor to soybean products [18].

Most alcohols detected at MP and SP stages were produced along with mold cultivation (S1-S3) [19], where seven more alcohols were identified, including 2-methylbutanol, 3-methylbutanol, 2,3-butanediol, 2-heptenol, benzyl alcohol, phenylethyl alcohol and 2,4-decadien-1-ol. Among alcohols, 1-octen-3-ol (343.582 μg/kg) and 1-hexanol (334.291 μg/kg), the primary volatile compounds at the earlier MP stage, had a green beany odor and decreased to 248.000 μg/kg and 154.750 μg/kg separately during mold cultivation [20].

Esters had lower amount and most esters identified were fatty acid methyl esters, except for ethyl hexadecanoate. All esters, except 3-methylphenylpropanoate, either generated or increased at MP stage, are likely synthesized by the action of fungal lipases in the soybean lipid [2,21].

Indole, the dominant heterocycle at MP and SP stages, is the important odor-contributing compound due to its unique unpleasant, fecal, and putrid aroma in grey sufu. It has been reported to be synthesized from tryptophan [22]. Only 1 acid and 1 phenolic compound existed at MP and SP stages. Phenol, which provides a phenolic, plastic, and woody odor, showed the largest content among volatile compounds. It is known to generate from the degradation of lignin glycoside and tyrosine during fermentation [22].

According to Fig. 2E and F, the total amount of volatile compounds at MP stage showed an increasing trend, while decreased at SP stage. Mold metabolism utilized certain volatile compounds with uncomfortable aroma and contributed special flavors to red sufu.

3.1.2. Analysis of volatile compounds at PF stage

There were 79 volatile compounds identified at PF stage, consisting of 29 esters, 13 aldehydes&ketones, 12 alcohols, 3 heterocycles, 3 acids, 4 phenolics and 15 miscellaneous compounds (Table S3).

Esters was the most important class of volatile compounds in WRS with 12-fold increases from S7 (1144.915 μg/kg) to S12 (13619.597 μg/kg) at PF stage (Fig. 2E and F). They are usually produced by esterification reaction and microbial metabolism [23]. Ethyl esters were the dominant esters in PF samples, in addition more short and medium-chain fatty acid (S&MCFA) ethyl esters (16) were identified than LCFA ethyl esters (13). Ethyl hexanoate, ethyl octanoate and ethyl acetate, as the predominant S&MCFA ethyl esters, exhibited fruity, brandy and pineapple flavor, respectively [24]. Compared with corresponding alcohols and acids, S&MCFA ethyl esters possess higher volatilities, lower perception thresholds and particular aroma, such as ethyl acetate (pineapple flavor), ethyl propionate (fruity), ethyl butanoate (fruity), ethyl pentanoate (fruity) and ethyl heptanoate (brandy) [24]. Ethyl hexadecanoate, ethyl linoleate and ethyl oleate, as the primary LCFA ethyl esters, afford fatty and oily aroma to red sufu. The presence of these esters is related to the metabolism of lipid by yeast, which are involved in the formation of free fatty acids to facilitate the synthesis of corresponding ethyl esters with alcohols [23]. Besides, ethyl propionate and ethyl 3-phenylpropionate increased 10 folds at the end of PF stage, presenting fruity and flowery notes to red sufu [24].

Among 12 alcohols identified, ethanol was the primary alcohol with largest amount among all volatiles, followed by phenylethyl alcohol, hexanol, 1-octen-3-ol, 2-methylbutanol and 3-methylbutanol. Ethanol plays an important role in aroma enhancement through synergistic effect with other flavor compounds, because of their good dissolubility in ethanol solution. Ethanol reached the peak at 1 month of PF stage and decreased along with fermentation, suggested that it was consumed largely to produce esters [2]. This result suggested that adequate addition of ethanol could strengthen aroma during fermentation period and control sufu flavoring substances with no adverse influence on sufu flavor. 2-Methylbutanol, 3-methylbutanol and phenylethyl alcohol, primary aroma-impact compounds in soy sauce to offer flowery and sweety notes [25,26], are the respective products of oxidative deamination of free amino acids precursors leucine, isoleucine,

and phenylalanine [19]. They had significant increases at 3 month of PF stage and these branched-chain alcohols have been proven to be produced from two different sources during yeast fermentation: carbohydrates via the EMP pathway and amino acids via the Ehrlich pathway [27,28]. Hence, increased demands for these alcohols could be promoted by the addition of corresponding amino acids. As the new alcohol identified in red sufu samples at PF stage, α-terpineol, which also exists in some herbs and black tea, exhibited an increasing tendency at PF stage and contributed the cloves flavor of sufu [28].

13 aldehydes and ketones were identified, and the amount showed certain increases, while the number decreased along with PF stage (Fig. 2E and F). Of these, benzeneacetaldehyde, α-ethylidene-benzeneacetaldehyde and benzaldehyde, generated by amino acid degradation, had higher contents and increased gradually as fermentation processed. Benzeneacetaldehyde and benzaldehyde, strecker aldehydes obtained from 2-phenylalanine and phenylalanine respectively [29,30], both of which are widely found in fermented soybean products, exhibit sweet and fruity notes [31]. α-Ethylidene-benzeneacetaldehyde, the only new aldehyde found at PF stage and identified in WRS for the first time, was discovered in cocoa as the key component of chocolate aroma and possesses a chocolatey, earthy, and floral aroma [32]. Most aldehydes related to the beany flavor disappeared gradually, such as 2,4-heptadienal, 2,4-nonadienal, 2,4-decadienal, 2-heptenal, 2-undecenal. Based on the dynamic change of alcohols, this observation may be explained by the reduction of unsaturated aldehydes into the corresponding unsaturated and saturated alcohols. Ketones could be generated by lipid auto-oxidation or amino acid degradation assisted by the Strecker reaction [19]. 2-Heptanone and 2-nonanone were dominates and exhibited remarkable changes at PF stage, imparting a fruity and floral flavor. They have been identified as key volatile compounds in red koji based on the metabolites of fungal [33].

Among four phenolic compounds identified, 2-methoxy-4-vinylphenol and 2-methoxy-4-(2-propenyl)-phenol had the relatively higher content. 2-Methoxy-4-vinylphenol, which contributes burnt and smoky notes to soy sauce [34], could be generated from the thermal degradation of lignin glycoside or associated ferulic acid. In addition, it has been reported to act as an anti-proliferative agent for cancer [35]. 2-Methoxy-4-(2-propenyl)-phenol and 2-methoxy-4-(1-propenyl)-phenol, also called eugenol and isoeugenol, respectively, are used as food preservative, food flavoring, and medical agents. They exist in spice plants and play an important role in the defense of herbivores, parasitic bacteria, and fungi [36].

3 Heterocycles, 3 acids and 15 miscellaneous compounds were identified at PF stage. 2-Pentylfuran was an abundant volatile compound, presenting a grassy or green bean-like odor. It showed an increasing trend and has been proved to be produced by the growth of *Staphylococcus saprophyticus* [20,37]. Acids could be synthesized due to the enzymatic decomposition of LCFAs or amino acids and microbial metabolism [21,38]. Acetic acid, as the metabolite of microorganisms, had relatively higher amount and showed increasing tendency at PF stage. Acids exhibited lower number and amount, but large numbers of esters were detected, indicating that most acids were esterified to corresponding esters during fermentation and conduced to esters formation importantly [23]. Among 15 miscellaneous compounds, dimethyl disulfide (stinky), methoxy-phenyl-oxime (fishy), naphthalene (mothball-like), eucalyptol (mint-like), caryophyllene (clove), and estragole (fennel), as volatile compounds with specific aromas, contributed to the flavor of red sufu [24,39], of which the latter three compounds were from spices in the dressing mixture.

According to The Flavor and Extract Manufacturers Association, 69 of the volatiles identified above have been declared for the use as food flavor additives. These compounds play an important role in the formation of flavor, and their synergistic effects endow red sufu with a unique flavor.

3.2. Analysis of volatile compounds and aroma-active compounds in four typically commercial red sufu products

3.2.1. Comparison of volatile compounds in four typically commercial red sufu products

For further identification, the aroma comparison was performed for typical regions' red sufu poducts (Zhejiang, Guangdong, Taiwan and Beijing). As is shown in Table S4, Fig. 2G and H, a total of 86 volatile compounds in four typical products from different locations were identified, including 38 esters, 11 alcohols, 12 aldehydes&ketones, 7 phenolics, 3 acids, 5 heterocycles and 10 miscellaneous compounds. Obviously, the fingerprint of volatiles had high similarity in R2, R3 and R4, whereas R1 had slight differences from others (Fig. 2D). 46 volatiles were identified as common compounds in four products, including 24 esters, 10 aldehydes&ketones, 6 alcohols, 2 heterocycles, 1 acid, 1 phenolic compound and 2 miscellaneous compounds. Ethanol was the predominant compound, followed by LCFA ethyl esters and S&MCFA ethyl esters, including ethyl hexadecanoate, ethyl lineate, ethyl oleate, ethyl octanoate, ethyl hexanoate, ethyl acetate and ethyl butanoate, which could afford special aroma to products. Among 10 aldehydes&ketones, benzeneacetaldehyde, α-ethylidene-benzeneacetaldehyde, benzaldehyde and 2-nonanone were the primary volatile compounds. Hexadecanoic acid, 2,4-bis(1,1-dimethylethyl)-phenol, 2-pentylfuran, dihydro-5-pentylfuranone, methoxy-phenyl-oxime and 2,4-dissocyanato-1-methylbenzene were the identical compounds in acids, heterocycles and phenolics classes. These compounds contributed to the common background aroma of red sufu. Compared with volatile flavors in white sufu [8,11,39], 2-octen-1-ol, 2-nonanone, α-ethylidene-benzeneacetaldehyde, α-(2-methylpropylidene)-benzeneacetaldehyde, 5-methyl-2-phenyl-2-hexenal, 2,4-bis(1,1-dimethylethyl)-phenol, ethyl nicotinate, ethyl-3-phenylpropionate and dihydro-5-pentylfuranone detected in four products as characteristic volatile compounds of red sufu, might have direct relationship with red kojic rice in red sufu.

According to Fig. 2G and H, contents of alcohols, acids and esters were higher than those in other products, while ethyl lineate, ethyl hexadecanoate, ethyl oleate, ethyl butanoate and ethyl octanoate were the dominating compounds in R1. As the representative volatile compounds, ethyl butanoate and phenylethyl alcohol had much higher contents than those in other products, which could provide intense fruity and rose aroma.

4-Ethylphenol, 4-ethyl-2-methoxy-phenol, methyl octanoate and ethyl nonadecanoate were its typical volatile compounds in R2. Ethyl octanoate, ethyl hexadecanoate, ethyl lineate and ethyl oleate were the main esters. In addition, as another representative compound, ethyl octanoate had much higher amount and presented more brandy flavor to R2. Meanwhile, acids had the least amount among four samples, and acetic acid was not identified in R2.

Benzyl alcohol, 3-phenylfuran, diallyl disulphide, 2-methyl-3-methoxy-4H-pyran-4-one and ethyl tiglate were particular volatile compounds in R3. As exhibited in Fig. 2G and H, R3 had the least amount of ester class, including ethyl hexadecanoate, ethyl lineate, ethyl benzoate, ethyl oleate and ethyl octanoate predominantly. As the microbial metabolite of *Actinomucor elegans* at MP stage, phenol was not detected in R3, which could be explained by molds applied in Taiwanese sufu. Volatile compounds with a benzene ring had higher amount, such as ethyl benzoate, ethyl phenylacetate, benzyl alcohol, α-ethylidene-benzeneacetaldehyde and α-(2-methyl propylidene)-benzeneacetaldehyde, supplying flowery note to R3.

Compared with others, there are special spices added into dressing mixture, according to the list of ingredients of R4. Ester was the principle group with the largest number of compounds among four products, consisting of ethyl hexadecanoate, ethyl lineate, ethyl oleate, ethyl octanoate and ethyl hexanoate mainly. Compared with others, LCFA ethyl esters had a large proportion, which accounted for 66% of total esters content and offer fatty and oily notes. Caryophyllene, eucalyptol, 2-methoxy-4-(2-propenyl)-phenol, 5-ethyldihydro-2(3 H)-furanone, ethyl 3-methyl butyrate, 2,3-butandienol and ethyl propionate were the characteristic volatile compounds in R4, of which first three compounds were closely correlated with special spices in dressing mixture, while others tends to be products of microbial metabolism during fermentation.

Obviously, although the production principle of various brands sufu is mostly the same, due to different climatic conditions, different microorganisms including monascus, yeast and mold strains, as well as various auxiliary materials such as wine and spices, affecting the production at post-fermentation stage, sufu flavor with different brands has its own characteristics.

3.2.2. *Analysis of aroma-active compounds among four typically commercial products*

16 aroma-active compounds were identified in four products by GC–MS/O with 13 in R3 and R4, 12 in R1 and 10 in R2 (Table S4). Ethyl butanoate (e4), 2-heptone (k1), ethyl pentanoate (e7), ethyl hexanoate (e9), ethyl heptanoate (e10), ethyl octanoate (e15), benzaldehyde (ad7) and phenylethyl alcohol (a13) were common aroma-active compounds contributing to the characteristic aroma of red sufu, which provide fruity, rum, almond and fruity notes. Esters exhibited high odor intensity, such as ethyl butanoate (apple), ethyl hexanoate (pineapple) and ethyl octanoate (brandy), contributing greatly to the aroma of red sufu. Furthermore, phenylethyl alcohol could provide rose flavor to red sufu with high odor intensity.

As listed at Table S4 [40,41], ethyl butanoate and ethyl hexanoate had relatively higher odor activity value (OAV), and they could have significant influence on the aroma [42–44]. OAVs of 2-heptanone, phenylethyl alcohol and benzaldehyde were less than 1. Nevertheless, they were identified as aroma-active compounds in four products with high odor intensity. The difference between GC-O results and OAV analysis could be explained that thresholds of compounds in water had deviations with those in sufu, or they were strengthened by synergistic effects of various aroma components [45].

3.3. *Multivariate statistical analysis*

PLS-DA were performed to distinguish WRS samples during fermentation (Fig. 3A & B) and four typically commercial red sufu products (Fig. 3C). Heatmap of CA (Fig. 3D & E) and volcano plots (Fig. 3F & H) were conducted simultaneously for cluster and variance analysis of volatile flavors, affected by fermenttion periods and places of origin.

PLS-DA is a multivariate statistical analysis method in metabolomics to reduce the complexity and enhance the explanatory ability of the model effectively without reducing its predictive ability. On the basis of PCA, it could improve a supervised grading intervention to amplify differences between groups. As Fig. 3A illustrated, samples were separated at two sides of the score plot with high dispersion degree. Besides, sample S10-S12 stayed far from zero and their dispersion degree got smaller, demonstrating that fermentation processes had significant influence on volatile flavors of red sufu and volatile flavors at latter PF stage become consistent gradually as fermentation processed. Except methyl hexanoate (e8), methyl octanoate (e11), methyl phenylacetate (e13), 3-methylphenylpropanoate (e18), methyl hexadecanoate (e26) and methyl linoleate (e30) lied on the right side, most esters located at the left side of score plot (Fig. 3B), which indicated that esters contributed to the flavor of red sufu greatly.

It can be seen in Fig. 3C, four typically commercial red sufu products differed significantly from each other, whereas dispersion degree was not quite large, indicating some differences existed in volatile compounds among 4 products. R2 and R3 clustered at the left side of score plot, while R1 and R4 reversed, demonstrating that R2 and R3 had similar volatile profiles, and R4 showed successively larger variation with R1, R2 and R3. Volatile compounds, such as 2,3-butanediol (a4), Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-(k6), 2-methoxy-4-(2-propenyl)-phenol (p3), ethyl propionate (e2) and ethyl 3-methyl butyrate (en1), exhibited higher concentration in R4 than others, and these compounds were nearly overlapped with R4, which indicated that they were strongly correlated with R4. Moreover, large numbers of esters clustered at the right area, elucidating that esters were important compounds in four red sufu products.

According to Fig. 3D, samples S1-S12 were separated into two groups based on different process stages. Samples at MP and SP stages (S1-S6) could be distinguished from those at PF stage (S7-S12) in the first cluster analysis. S1 and S2-S6, S7 and S8-S12 could be differed in the second cluster analysis. Moreover, S11 and S12 were classified as a group due to their little difference, which concurred with conclusion of PLS-DA (Fig. 3A). Volatile compounds, especially esters, were distinguished from others in the first cluster analysis. Obviously, they had significant increases along with PF stage and they were very important volatiles, contributing to the flavor of sufu. Fig. 3E presented R4 differed significantly from others in the first cluster analysis. In addition, in the second cluster analysis, R1 could be parted from R2 and R3. These were consistent with Fig. 3C. Furthermore, most esters and alcohols were located at the bottom of heatmap and they could be distinguished in cluster analysis. Besides ethanol (a1), ethyl hexadecanoate (e28), ethyl linoleate (e31), ethyl oleate (e32) and ethyl octadecanoate (e33), ethyl hexanoate (e9) and ethyl octanoate (e15) were importantly common compounds with relatively higher amount in four products.

In the statistical analysis of metabolomics, volcano plot is a sort of scatter plot with log2 (fold change) and -log10 (*P* value) of metabolites between groups as x and y axis. Based on their fold change and p value, volcano plot could detect variances and determine differential metabolites among a mass of dataset between groups quickly. According to Fig. 3F, there were 23 differentially expressed volatile flavors in R1 and R4, and five were located at the right upper area, including ethyl butanoate (e4), acetic acid (c1), phenylethyl alcohol (p1), phenylethyl alcohol (a13) and 2-ethyl-1-hexanol (an1), where e4 and c1 were volatile compounds with the most significant differences. Fig. 3G illustrated that 24 differentially expressed volatile flavors existed in R2 and R4, and 4 volatile compounds, which lied at the right upper area, had higher amount in R2, including benzyl nitrile (mn3), 4-ethyl-2-methoxy-phenol (pn3), 2-nonanone (k4), and 4-ethyl-phenol (pn2). Among 29 differentiate volatile flavors in R3 and R4 (Fig. 3H), 8 volatile compounds lied at the right upper area and ethyl benzoate (e12) was the most significant differential volatile compounds. Therefore, 2,3-butanediol (a4), 1-hexanol (a5), ethyl 4-octenoate (e14), ethyl nicotinate (e16), ethyl 3-methylbutyrate (en1), ethyl propionate (e2), dihydro-5-pentyl-2(3 H)-furanone (h5), 2,4-diisocyanato-1-methylbenzene (m13) and 2-methoxy-4-(2-propenyl)-phenol (p3) were differential volatile compounds among four products in common. Most volatile compounds had no significant differences and differential volatile compounds were key factors giving rise to the overall flavor differences in red sufu products.

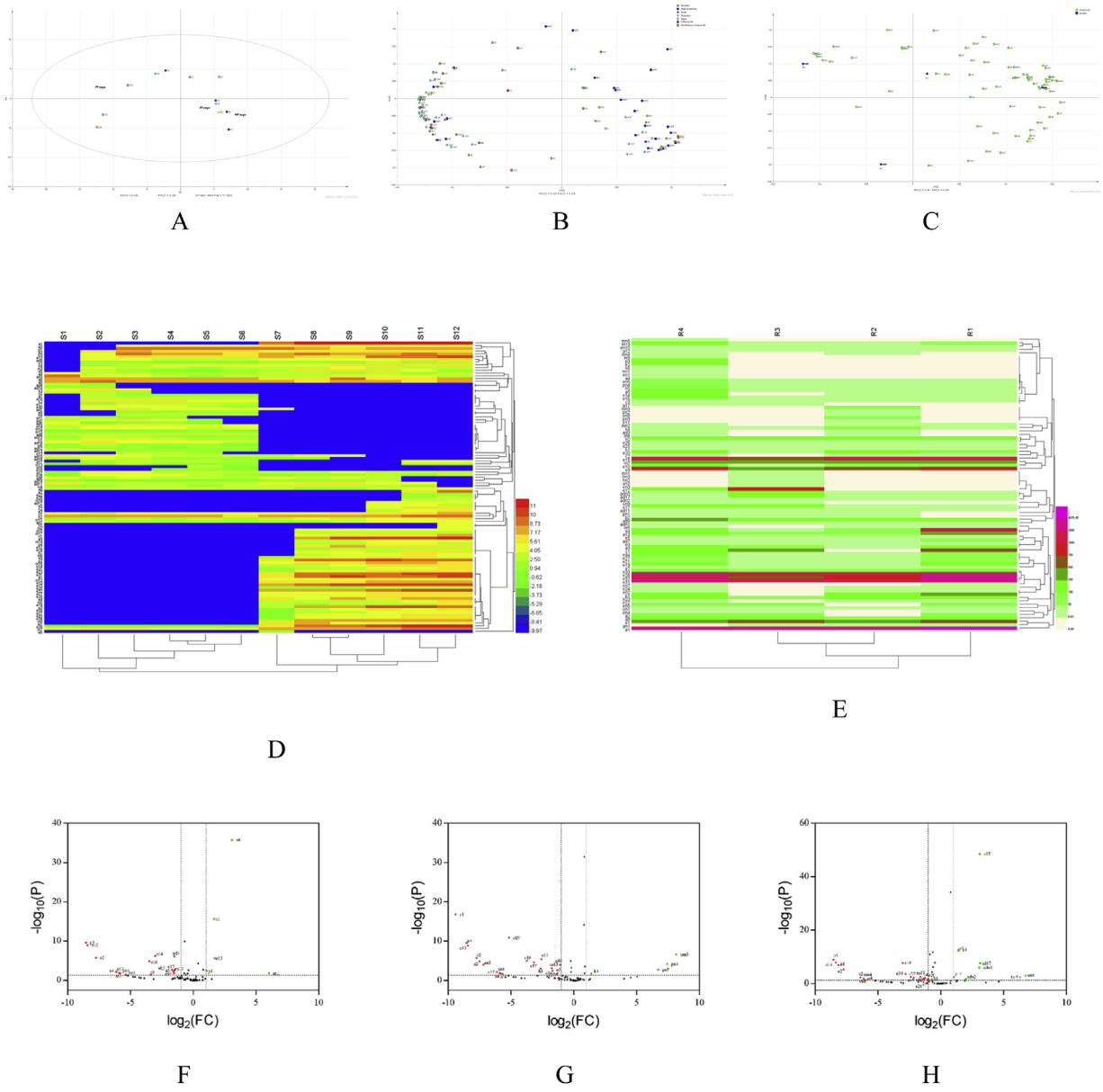

Fig. 3. PLS-DA score plots based on volatile compounds identified during WRS fermentation (A & B), four commercial products (C). Heatmap of CA was performed to analyze volatile compounds detected during WRS fermentation (D) and four commercial products (E). Volcano plot was performed for the identification of differentially expressed volatile compounds between R1 & R4 (F), R2 & R4 (G), R3 & R4 (H).

3.4. E-nose analysis

E-nose is another advanced technology to analyze aroma with chemical sensors, which detects families of chemicals, creates a pattern of sensor responses, and acquires overall volatile flavor profiles in samples without qualitative and quantitative analysis in minutes. Discrimination of WRS samples during fermentation (S1-S12) through E-nose was performed by PCA and LDA conducted on the E-nose dataset. According to Fig. 4A and B, volatile profiles of samples at MP stage, SP stage and PF stage could be distinguished from others, which concurred with GC–MS results (Fig. 3A). As seen in Fig. 4B, the cumulative variance contribution of LD1 and LD2 was 97.05%, indicating that E-nose could evaluate the volatile profiling of samples during fermentation to differentiate samples at PF stage. Obviously, a slight difference in the volatile profiles existed in samples S11 and S12. S12 could be distinguished from others (S7-S11), while overlapped with R4. Combined with GC–MS outcomes, it indicated that overall volatile profiles are gradually stable and 3 months is the maturation stage.

E-nose results of four typically commercial red sufu products (Fig. 4C and D) demonstrated that there were differences in the overall volatiles profiles among products. Hence, it could be applied to discriminate red sufu samples with different brands.

According to Fig. 5A, B, C and D, W5S, W1W and W2S had relatively higher response value among 10 metal oxide gas sensors, illustrating that alcohols, nitrogen oxides and sulfur-containing compounds were predominant volatile compounds in four commercial products and red sufu samples during fermentation.

3.5. Sensory evaluation

QDA was conducted to compare differences in sensory attributors among four products. As indicated in Fig. 6A and B, four products had their unique aroma, in terms of six sensory notes. Sauce flavor exhibited the highest score, followed by alcoholic,

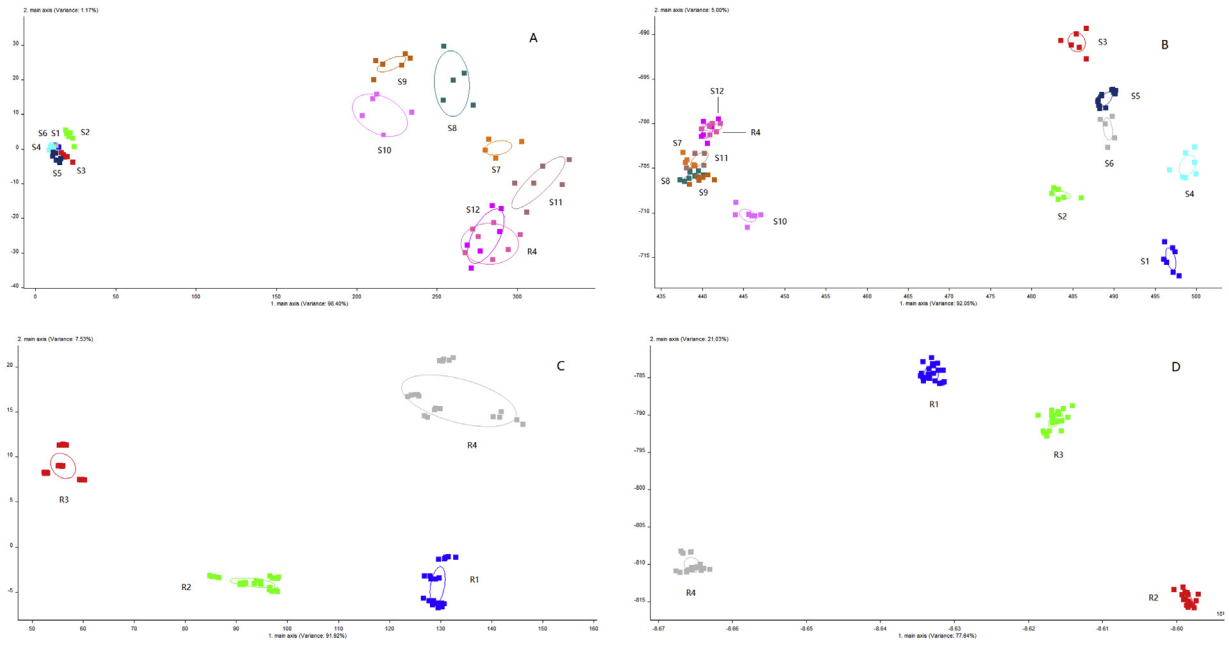

Fig. 4. PCA and LDA plots of E-nose analysis of sufu samples during fermentation process (A & B), and four sufu products (C & D).

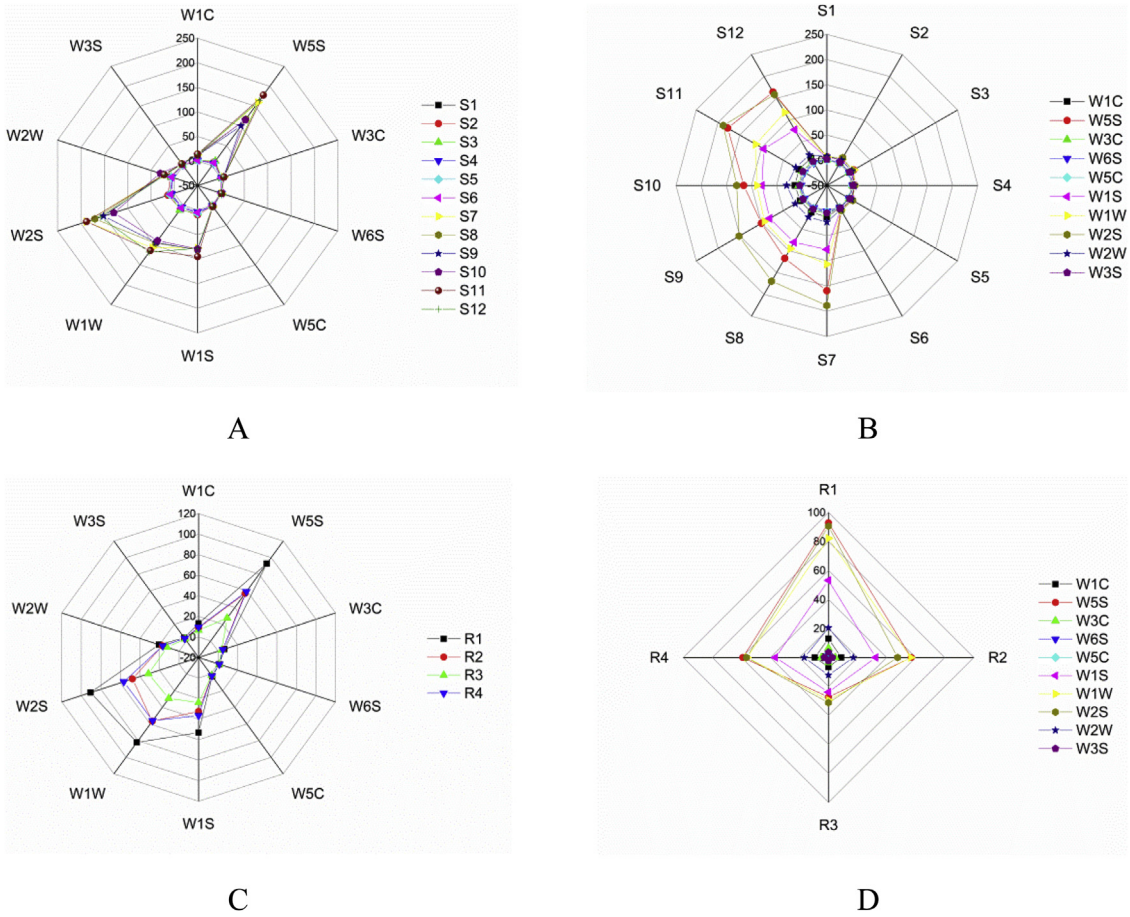

Fig. 5. Radar plot of E-nose analysis of samples during fermentation (A & B) and four sufu products (C & D).

ester, fruity, floral and sweety aroma. Sauce flavor showed the strongest intensity in R1, followed by alcoholic and floral flavor, whereas ester and sweety aroma had the lower intensities. It possessed relatively equilibrium flavors with fruity note, slightly larger than other attributors in R2. R3 also exhibited balanced attributors, except for sauce flavor, which was prominent. Ester and alcoholic aroma were the dominants in R4, followed by sauce and sweety flavor, and intensities of fruity and floral flavors were least

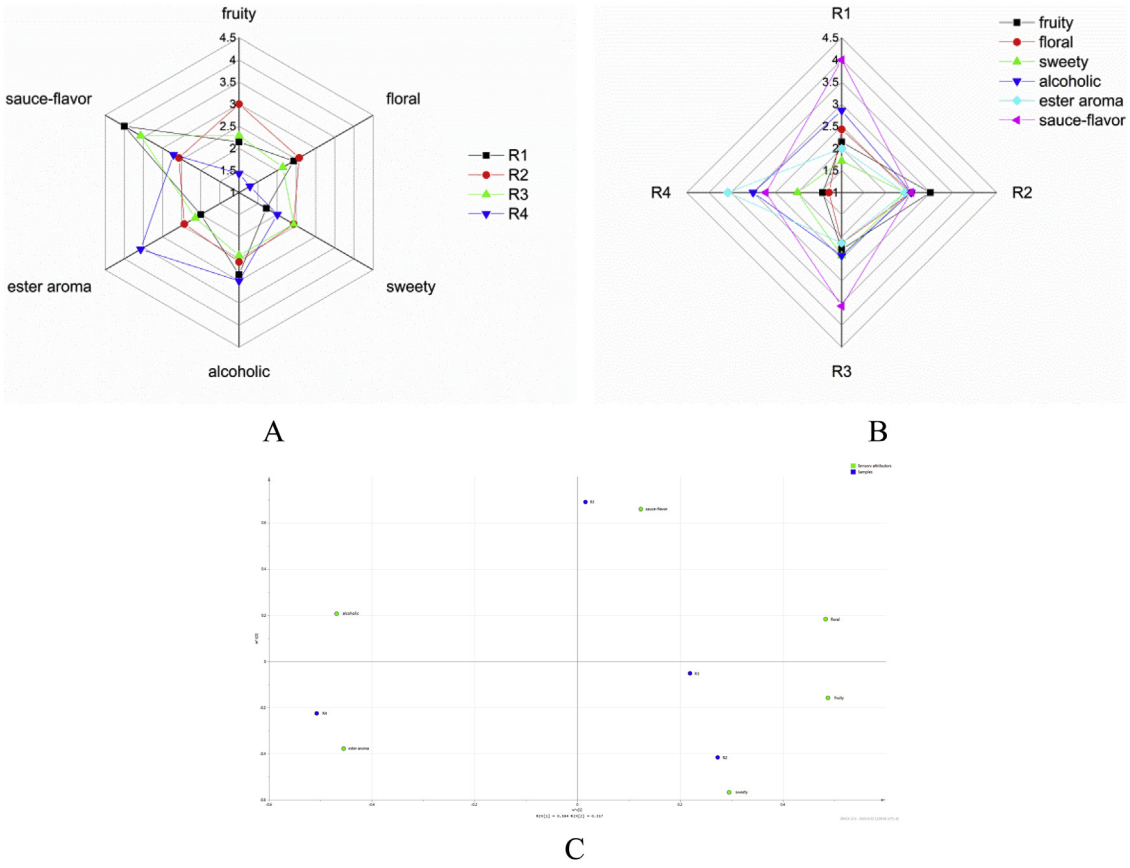

Fig. 6. Radar (A & B) and PLS-DA (C) plots based on sensory evaluation of four red sufu products.

among four products. PLS-DA was further performed to represent the separation and correlation between products and their sensory attributors, as shown in Fig. 6C. The result of PLS-DA illustrated that R1, R2 and R4 were strongly correlated with sauce, sweety and ester aroma respectively, which highly coincided with their sensory intensities. The result of variance analysis indicated that same sensory attributors in different products revealed significant difference ($P < 0.05$). Alcoholic and sweety aroma had largest significant difference, followed by sauce and floral notes, while ester and fruity flavor had the least difference. Thus, it demonstrated that six sensory attributors could explain aroma characteristics of different sufu better.

4. Conclusion

In this study, flavor omics were employed for the characterization and identification of volatile aromas of red sufu. Different fermentation stages of WRS were analyzed and evaluated. The correlation of the fermentation stages and the composition of the aroma compounds was investigated. Significant differences were identified between different fermentation stages, and primary aroma compounds were identified for each stage. The results indicated that phenolics and alcohols were dominant at molded and salted phetze stages, while esters and alcohols became predominate at post-fermentation stage.

In addition, four commercial typical products were compared to evaluate their discriminative characteristics. 16 aroma-active compounds were identified, which made the greatest contribution to the characteristic aroma profile of red sufu. Among them, 8 volatiles were identified as common substances, incuding ethyl butanoate, 2-heptone, ethyl pentanoate, ethyl hexanoate, ethyl heptanoate, ethyl octanoate, benzaldehyde and phenylethyl alcohol. By multivariate statistical analysis and flavor omics technologies, it was demonstrated that remarkable differences presented in volatile flavors among typical sufu products. 2,3-butanediol, 1-hexanol, ethyl 4-octenoate, ethyl nicotinate, ethyl 3-methylbutyrate, ethyl propionate, dihydro-5-pentyl-2(3 H)-furanone, 2,4-diisocyanato-1-methylbenzene as well as 2-methoxy-4-(2-propenyl)-phenol were identified as differential volatile compounds. The characteristic aroma compounds investigation would be potential guideline for typical the red sufu product identification, hence of practical significance for the red sufu quality improvement. Sensory evaluation also suggested products were strongly correlated with corresponding sensory attributes. The established discriminant model could not only apply in the differentiation of red sufu at the molecular level, but also determine its maturity in manufacturing process. The investigation could provide scientific strategy for the evaluation of red sufu quality, as well as perfecting the current quality evaluation system of sufu products.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.fshw.2019.11.004.

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