Food Chemistry: X 23 (2024) 101716

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x

Unraveling the chemosensory characteristics on different types of spirits based on sensory contours and quantitative targeted flavoromics analysis

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ARTICLE INFO

Keywords:
Spirits
Sensory evaluation
Quantitative targeted flavoromics analysis
Multivariate statistical analysis
Differential markers
Chemical source

ABSTRACT

Due to differences in raw materials and production processes, different spirits exhibit various flavor even if undergo distillation operation. In this study, sensory analysis could clearly distinguish 5 types spirits, and had been validated through quantitative targeted flavoromics analysis. Consequently, 44 potential differential markers between 5 types spirits were screened. Among, 34 definite differential markers were further confirmed to be highly correlated with target sensory attributes and could effectively distinguish types of spirits. Ultimately, 14 key differential markers (including 2-methylbutane, linalool, acetaldehyde, D-limonene, β-myrcene, phenylethyl alcohol, phenethyl acetate, heptyl formate, ethyl octanoate, ethyl decanoate, ethyl pentanoate, ethyl hexanoate, hexanoic acid, and ethyl hexadecanoate) could reveal the chemical sources of spirit sensory and serve as targets for identifying different types of spirits. Overall, the results of flavoromic characterization of 5 types spirits provided a significant step forwards in understanding of differentiation of spirits by sensory coupled with quantitative, and statistical analysis

1. Introduction

Alcoholic beverages have been part of human civilization for thousands of years, and is deemed as an integral aspect of human culture. During the process of civilization development, various spirits are gradually emerged around the world, including whiskey, gin, rum, vodka and Baijiu(Zheng et al., 2017), which exhibit strong national and cultural characteristics and are loved by people all over the world. Today, spirits are high-value products, both commercially and culturally, and contribute significantly to total alcohol consumption.

Spirit is a beverage made of grain, fruit, and other raw materials through fermentation, distillation and blending. It has unique cultural connotation and drinking customs all over the world. Specifically (**Fig. S1**), gin developed in Northern Europe during the 17th century is mainly made by re-distilling a mixture of juniper seeds and other natural plant ingredients (such as cardamom seeds and lemon zest)(Vichi, Riu-Aumatell, Mora-Pons, Buxaderas, & López-Tamames, 2005). Rum is a rather tasteless and neutral spirit traditionally produced in Caribbean and Central American countries, where it is primarily derived from the fermentation and distillation of sugar cane juice before undergoing store in oak barrels(Pino, Tolle, Goek, & Winterhalter, 2012). For whiskey, malt or ground grains are mixed with water, then ferment with yeast, and the fermented wort is distilled to produce an alcoholic distillate, which is finally stored in barrels(Poisson & Schieberle, 2008). Vodka originated in Russia and Poland and is mainly fermented of grain with the process features being re-steaming and filtered(Lay-Keow, Hupe, Harnois, & Moccia, 1996). Baijiu is usually fermented from ground and cooked grains, fermented with qu (a saccharification and fermentation agents) for several months. Subsequently, the basic Baijiu is distilled with steam and then aged for several years to develop the flavor(Y. P. Zhao, Zheng, Song, Sun, & Tian, 2013). Compared with other spirits, Baijiu is the only spirit that uses qu (natural inoculation, multi-strain) as the saccharification and fermentation agents, and is also various in raw materials, including sorghum, corn, rice, wheat, and glutinous rice (Wang, Chen, Wu, & Zhao, 2022). Therefore, it is reasonable to infer that the composition and sensory quality on different types of spirits are

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https://doi.org/10.1016/j.fochx.2024.101716
Received 6 June 2024; Received in revised form 1 August 2024; Accepted 2 August 2024
Available online 4 August 2024

various due to differences in raw materials, production processes, etc.

According to research, the quality of spirits mainly depended on the distribution of trace components. The trace components currently founded were mainly divided into the following categories: alcohols, acids, esters, aldehydes, ketones, lactones, aromatics, terpenes, sulfur compounds, nitrogen compounds and furans, etc. These trace components not only expressed different flavor, but also interacted with others to produce new flavors. Studies have shown that the style characteristics and quality of different spirits depend on the type, concentration and quantity ratio of their trace components, which were closely related to brewing raw materials, fermentation technology, distillation and aging technology(Zheng et al., 2017). At present, some progress had been made in the exploration of trace components in spirits, but there was relatively little research on the differential markers between types spirits and their corresponding relationships with the characteristic quality of spirits. Based on, the identification of differential markers that differ between types spirits, especially the association between the components and sensory attributes, will help to fully understand the material basis that led to the diversity in the style of different spirits, and provide targeted markers for stable optimization of processes.

While The trace components with low mass concentrations in spirits exist in complex matrices, which poses a challenge to its identification. At present, various pre-treatment methods such as direct injection (DI) (Qin, He, Feng, Li, & Zhang, 2021; Song, He, Chen, & Yang, 2020), solvent-assisted flavor evaporation (SAFE)(Xie, Sun, Xiong, Li, & Yang, 2024), simultaneous distillation extraction (SDE)(He, Zhang, & Yu, 2013), and headspace solid phase microextraction (HS-SPME)(Li et al., 2022; H. Z. Wu et al., 2023) were widely used for the extraction of trace components. Although DI was simple to operate, it detected fewer components that was attributed to non-enrichment. SAFE required a large amount of sample, and some low boiling trace components would be lost during the concentration process. The heating temperature of SDE was relatively high, which could easily cause changes in the trace components. HS-SPME could avoid steps such as sample extraction and concentration, and could also avoid contamination of the chromatographic column by non-trace components in the sample. However, its enrichment rate was relatively low. In contrast, vacuum assisted adsorption extraction (VASE)(Hou et al., 2023) had the characteristics of large adsorption phase volume, high extraction efficiency, and high sensitivity, making it more suitable for the identification and analysis of trace components in complex matrices. It enhanced the static diffusion rate of components under vacuum and could collect much more trace components than under atmospheric pressure. After adsorption, it was directly analyzed at the gas chromatography injection port, eliminating the loss of secondary capture and sample transportation in traditional thermal analysis systems. A method of vacuum assisted adsorbent extraction (VASE) combined with gas chromatography–mass spectrometry (GC–MS) was developed to separate volatile phenolic components in beer(Jelen, Gaca, & Marcinkowska, 2018), confirming the great practicality of VASE in extracting components with stronger polarity and lower volatility. However, there were currently few reports on the application of VASE in the analysis of trace components in various spirits. Of note, not all trace components contributed directly to the sensory perception of spirits, thus, it was necessary to further screen and identify the large data sets by multivariate statistical analysis (principal component analysis (PCA)(Li et al., 2022), and partial least squares discriminant analysis (PLS-DA)(Huang et al., 2023)). In the previous research(Huang, Wu, et al., 2023), PLS-DA had been used to screen the key differential markers of sauce aroma style baijiu from 4 different regions, containing ethyl octanoate, ethyl 2-methylpropanoate, propyl acetate, ethyl heptanoate, 2-nonanone and butyl hexanoate. At the same time, PCA evaluated that the key difference markers had a strong ability to distinguish sauce aroma style baijiu from different regions. Hence, in this study, VASE was applied to extract volatile spectrum from spirit, quantitative targeted flavoromics analysis combined with multivariate statistical analysis (PLS-DA, PCA) was conducted to screen important trace components, and further to establish the correlation between trace components and sensory perception on different spirits, so as to reveal the chemical sources of sensory characteristics for the spirits.

The purpose of this study was to obtain extensive information on the chemical composition of the trace components of different spirits and to explore the relationship between trace components and sensory perception. To this end, a vacuum-assisted sorbent extraction (VASE) combined with gas chromatography–mass spectrometry (GC–MS) was developed and applied to characterize the distribution characteristics of volatile fractions in different spirits. Concretely: (1) to establish a volatility data set for spirits by combining VASE with GC–MS; (2) to compare the sensory and chemical characteristics of different spirits through sensory evaluation and quantitative targeted flavoromics analysis, and to identify potential differential markers through multivariate statistical analysis; (3) to verify the relationship between differential markers and sensory attributes to further understand the chemical sources of sensory attributes for spirits.

2. Material and methods

2.1. Sample collection

A total of 11 representative samples of different spirits were used in this study (**Table S1**). For baijiu, strong flavor Baijiu with the highest market share was selected, and due to the large regional differences within China, several regional representative Baijiu samples were used. These samples were purchased from the market and stored at 4 °C before further analysis.

2.2. Analytical reagents

Ethanol (chromatographically pure, 99.8%) was from Beijing InnoChem Science & Technology Co. Ltd. (Beijing, China). A C_3–C_{30} n-alkane mixture (Sigma–Aldrich, Shanghai, China) was used for determination of linear retention indices. Sodium chloride (analytically pure, 99.5%, NaCl) was from Chengdu Colon Chemical Co. Ltd. (Sichuan, China). The internal standard substances (2-ethylbutyric acid (IS1), 4-octanol (IS2), and n-amyl acetate (IS3)) were all purchased from J&K Scientific Company (Beijing, China). Water was purified using a Milli-Q purification system (Millipore, Bedford, MA).

2.3. Extraction of trace components by VASE

A diluted sample (2 mL), with a final ethanol concentration of 15% *v*/v was placed into a 20 mL headspace bottle, with a silicone rubber septum and saturated with sodium chloride (0.4 g). Added 40 μL mixed internal standards (including 2-ethylbutyric acid (IS1), 4-octanol (IS2), and n-amyl acetate (IS3)), all with a mass concentration of 20 mg/L. Inserted the sorbent pen into the headspace bottle and formed a seal with the bottle cap liner. Used a vacuum pump to vacuum the bottle to $<$0.01 atm through the sorbent pen. The headspace bottle was equilibrated in extraction system (5600 SPES; Entech Instrument, Simi Valley, CA) for 20 min at a temperature of 50 °C and a speed of 240 r/min. After extraction, placed the sorbent pen on a cold tray for 20 min of water management, and then placed it in an isolation sleeve for GC–MS analysis (Hou et al., 2023).

2.4. Analytical conditions for GC–MS

Gas chromatography-mass spectrometry (GC–MS) with a polar chromatographic column DB-FFAP(60 m × 0.25 mm × 0.25 μm; Agilent Technologies, Santa Clara, CA) was used to identify trace components. Each sample was analyzed in three replicates. Every sample (1 μL) was injected in a spitless mode and analyzed. Helium (99.999%) was used as a carrier gas at a constant flow rate of 1.0 mL/min, and the inlet temperature was set as 250 °C. Oven temperature was held at 40 °C at first, then raised to 50 °C at a rate of 10 °C/min and held for 10 min, then raised at 3 °C/min up to 80 °C and held for 10 min, finally raised at 5 °C/min up to 230 °C and held for 10 min. The total run time for each GC–MS analysis was about 73 min. The mass spectrometry (MS) was operated in an electron ionization (EI) mode at 70 eV. The temperature of the interface and the ion source were set at 150 and 230 °C, respectively. The identification of trace components was conducted in a full scan mode. The temperature of the transfer line was 245 °C. The mass range was set from 50 to 450 amu.

2.5. Identification and quantification of trace components

The similarity between the mass spectrometry information of each chromatographic peak and the mass spectrometry libraries of the National Institute of Standards and Technology (NIST) and Liquor (a team built mass spectrometry library) was at least 80%. Combined with the results of standard components comparison and retention index comparison, it was considered as the preliminary identification of this component.

The quantifying of target trace components was used internal standard relative peak area method (4-octanol was the internal standard for alcohol components, 2-ethylbutyric acid was the internal standard for acid components, and n-amyl acetate was the internal standard for esters and other components).

2.6. Sensory quantitative descriptive analysis (SQDA)

According to the methods reported in related studies(Huang, Wu, et al., 2023), the sensory evaluation team composed of 10 sensory evaluators (5 males and 5 females, aged from 21 to 28 years) with olfactory experience. The sensory quantitative descriptive analysis method was used to evaluate the sensory attributes of spirits samples. The descriptors with higher frequencies were screened out. All sensory evaluators were called together to discuss descriptors until an agreement was reached on sensory attributes. Sensory attributes were listed based on the description of aroma, taste and perception for spirits. Sensory attributes referred to “aroma intensity”, “sugarcane-aroma”, “fruity-aroma”, “oak-aroma”, “honey-aroma”, “juniper-aroma”, “alcohol-aroma”, “spice-aroma”, “ester-aroma”, “barley malt-aroma”, “grain-aroma”, “cellar-aroma”, “peat smoke-aroma”, “softness”, “sweet-taste”, “bitter-taste”, “sour-taste”, “juniper-taste”, “sugarcane-taste”, “oak-taste”, “essence-taste”, “barley malt-taste”. Finally, the evaluators were provided with spirits samples (10.0 mL) in glasses (25 mL) coded with 3-digit numbers and asked to score the sensory intensities using a 10-point scale (0 (none), 10 (very strong)). The sensory evaluation was performed in a sensory panel room at 25 ± 1 °C with the humidity of 35% - 50%, and each sample was evaluated in triplicates. The participants were informed and agreed to the above content. The experimental content obtained ethical permission from the Scientific Research Ethics Committee of Beijing Business and Technology University.

2.7. Flavor addition experiments

The addition experiment was conducted to verify the contribution of the differential markers screened to the sensory attributes of spirits. According to the actual concentration in sample tested, differential marker was added to the corresponding spirit. Then, as described in section 2.6, the sensory attributes of the samples were evaluated by the same evaluators.

2.8. Statistical methods

Statistical analysis was performed using SPSS 26.0 (Chicago, IL, USA). A one-way analysis of variance (ANOVA) was performed for the flavor profiling analysis. Principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), permutation tests, and variable importance in projection (VIP) were conducted using SIMCA 14.1 (Umetrics Inc., Sweden). The radar charts, heatmap, bar charts, violin charts, and venn charts were drawn by Origin 2021 (MicroCal Inc., MA, USA). The Pearson correlation coefficient was calculated, and the correlation network was visualized with Gephi (Version 0.9.2). The analysis of similarities (ANOSIM) was established through an online website (https://www.cloudtutu.com/#/index).

3. Results and discussion

3.1. Sensory evaluation

Selected 9 taste and perception attributes and 13 aroma attributes for sensory quantitative descriptive analysis (SQDA) of spirit samples, including “aroma intensity”, “sugarcane-aroma”, “fruity-aroma”, “oak-aroma”, “honey-aroma”, “juniper-aroma”, “alcohol-aroma”, “spice-aroma”, “ester-aroma”, “barley malt-aroma”, “grain-aroma”, “cellar-aroma”, “peat smoke-aroma”, “softness”, “sweet-taste”, “bitter-taste”, “sour-taste”, “juniper-taste”, “sugarcane-taste”, “oak-taste”, “essence-taste”, “barley malt-taste”. As shown in Fig. 1a, there were significant differences ($p < 0.05$) in the intensity of “sour-taste”, “juniper-taste”, “sugarcane-taste”, “oak-taste”, “barley malt-taste”, and “essence-taste” among different types of spirits, while the intensity of “sweet-taste” and “bitter-taste” were similar. The scores of all aroma attributes in these spirit samples showed significant differences ($p < 0.05$), but the difference of “aroma intensity” was not significant.

The flavor spectrum on different types of spirit samples had their distinctive characteristics, such as the unique “sugarcane-aroma” and “sugarcane-taste” in rum (L1), which may be related to its use of sugarcane as a raw material(F. H. Wu, Lin, & Chen, 2010). Gin (L2) had unique “juniper-taste” and “juniper-aroma” due to being produced by juniper berries (*Juniperus communis*) and other botanical ingredients such as coriander seeds, cardamom seeds, calamus root, angelica root (Vichi et al., 2005). All of these ingredients were rich in essential oils, which contributed to the aroma of most gins. For the vodka samples (L3-L4), unique “spice-aroma” and “essence-taste” were found, which may be related to the preparation and processing of the product before canning(Lay-Keow et al., 1996). In Scotland, whisky was mainly produced by barley sprouting(Y. Zhang et al., 2024). Therefore, in the whisky sample (L5), the unique “barley malt-aroma” and “barley malt-taste” were observed. The prominent feature of whisky, known as “peat smoke-aroma,” was unique among all spirits. “Grain-aroma” and “cellar-aroma” were the unique aroma in Baijiu (B1-B6), which originated from the brewing raw materials and the brewing environment(Du, Fan, & Xu, 2011). In terms of taste, obvious and harmonious “sweet-taste”, “bitter-taste”, and “sour- taste” was presented, which was consistent with the previous study(Huang et al., 2023) on sensory evaluation of Baijiu samples.

The PCA images of the sensory characteristics of these spirit samples were shown in Fig. 1b. The first principal component (PC1) accounted for 32.9% of the variance, and PC2 accounted for 28.0%. The total PCA explained 60.9% of the variance. All Baijiu samples (B1-B6) were distributed in the second quadrant, with obvious “grain-aroma”, “cellar-aroma”, and “sour-taste”. All vodkas (L3-L4) were distributed in the first quadrant, with “spice-aroma” and “essence-taste”. These conclusions were consistent with the sensory evaluation results.

Meanwhile, gin (L2) was clearly distinguished from other spirits, while rum (L1) and whisky (L5) cannot be independently distinguished due to their collective “oak aroma”, which was consistent with the sensory evaluation results in the literature(Y. P. Zhao et al., 2013). Roughly, based on SQDA, different types of spirits could only be roughly divided into four groups (i.e., S2, S3, S5 and others), and S1 and S4 samples cannot be clearly distinguished. In addition, sensory evaluation was subjective and greatly influenced by evaluators, so it was necessary to more scientifically distinguish different types of spirits.

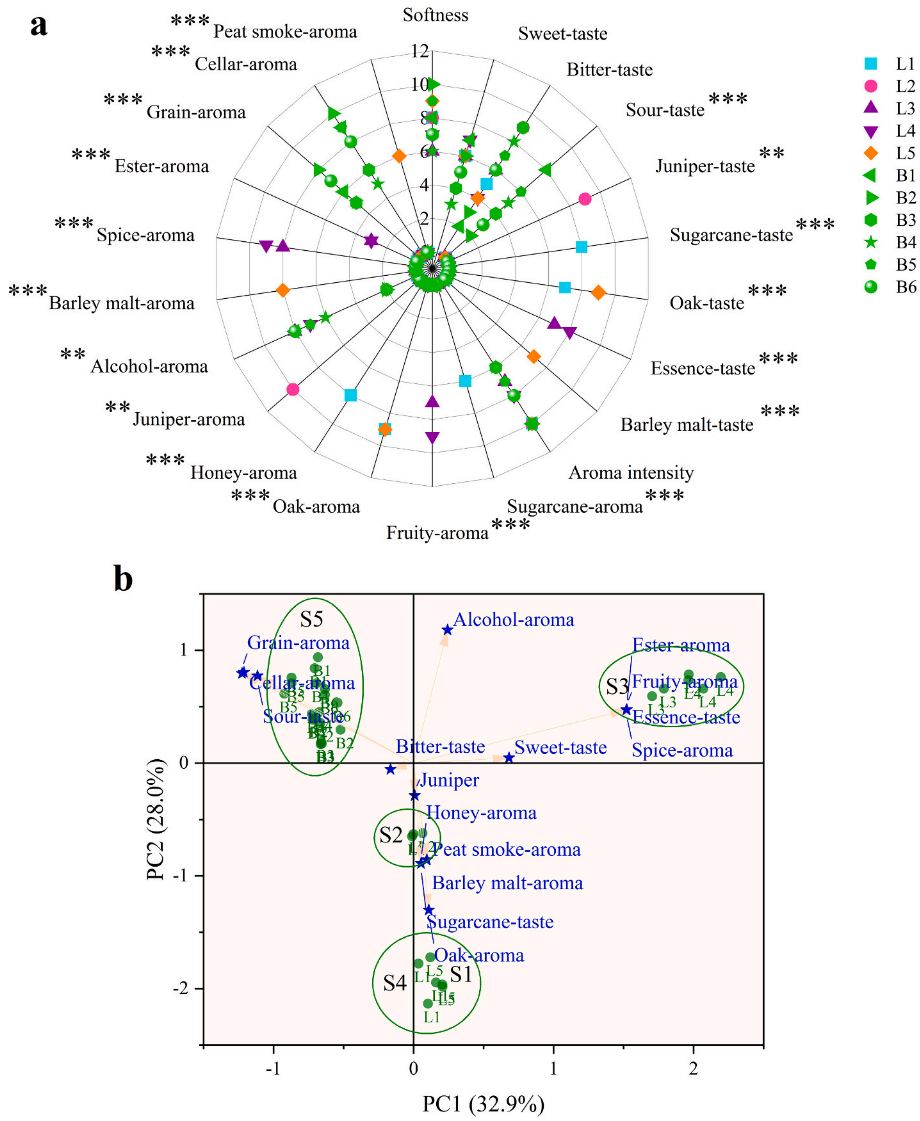

Fig. 1. The sensory evaluation results of spirits. **a**. The results of SQDA. The significant differences between groups were represented by * * * ($p < 0.001$), * * ($0.001 \leq p < 0.01$), and * ($0.01 \leq p < 0.05$); The color of the same type was consistent; **b**. PCA diagrams for sensory evaluation of spirits.

3.2. Quantitative targeted flavoromics analysis on volatile profiling of spirits

The sensory expression of flavor in spirits was closely related to the composition of trace components. These trace components had different flavor characteristics and influenced each other, together constituting the flavor diversity of spirits. In this study, 325 kinds of trace components found from 11 spirits and quantified by VASE combined with GC–MS (the total ion chromatograms were shown in Fig. S2), including 89 esters, 59 alcohols, 43 aromatics, 37 alkanes, 25 ketones, 18 furans, 17 aldehydes, 16 acids, 7 terpenes, 6 sulfur compounds, 5 lactones, and 3 nitrogen compounds. Fig. 2a showed that S1, S2, S3, S4, and S5 possessed 11 components in common, containing 4 esters, 4 aromatics, 1 acid, 1 alcohol and 1 ketone. The unique trace components in Baijiu samples are the most abundant both in individual samples or groups (S5). Multi strain fermentation and abundant raw materials provided the material basis for the rich volatile composition spectrum in Baijiu(Wang et al., 2022). Qualitatively speaking, only the generalized differences between spirits could be described, but further analysis was needed to scientifically reveal the underlying relationships.

PCA plots were generated based on the targeted trace components quantitative data, as shown in Fig. 2b. The PC1 accounted for 22.6% of the variance, PC2 accounted for 12.9%. The total PCA explained lower (35.5%) of the variance. The same types of spirits were closely grouped together, demonstrating significant differences between types of spirits. The results showed that the spirit samples could be well divided into 5 groups (S1, S2, S3, S4, and S5). It was worth noting that the samples in S5 were significantly different from those in the other groups, and the overall distribution was large, which may be due to the differences in brewing microorganisms and process control that leaded to the differences between Baijiu.

To reveal the material basis of differences in various spirits, the bar chart (Fig. 3a) and stacking diagram (Fig. 3b) were drawn based on the quantitative results of trace components in the spirits samples. The comparison shows that the total concentration of trace component varied among different types of spirits. The concentration of esters, lactones, acids, alcohols and alkanes in S5 was much higher than that in other groups. The rich material basis of Baijiu production was not only reflected in the quantity of trace components, but also in the concentration of trace components. Esters were one of the most important flavor components in spirits and were usually produced by esterification reactions between organic acids and alcohols during fermentation and aging(Xu, Yu, Ramaswamy, & Zhu, 2017). Except group S2, the total concentration of esters was the highest, which was consistent with the

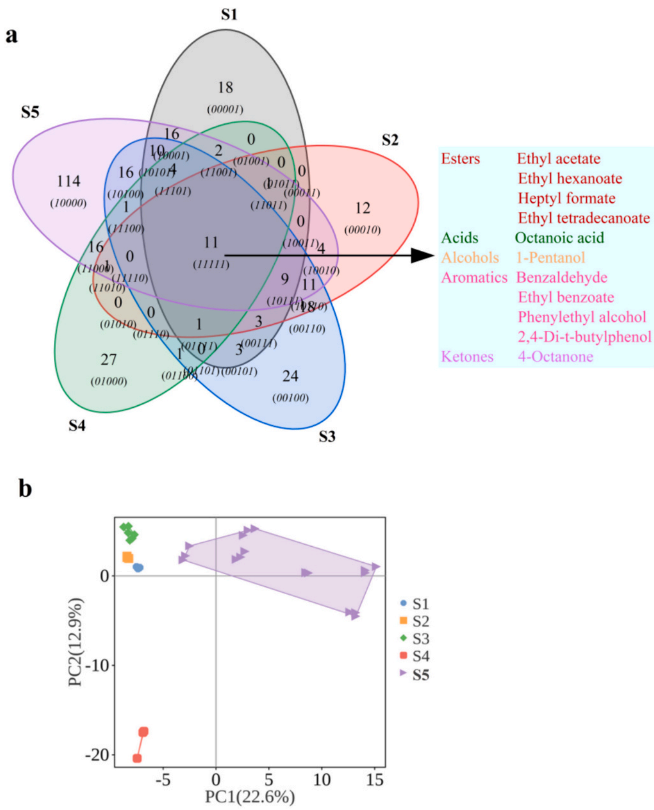

Fig. 2. Distribution of trace components in types of spirits. **a**. Venn diagram of trace components in types of spirits; **b**. PCA diagram of trace components.

literature(Fang, Du, Jia, & Xu, 2019). The concentration of esters mainly affected the intensity of fruity-aroma, and the total concentration of esters in groups S5 and S4 was relatively high, which was corresponding to the high score of "fruity-aroma" in sensory evaluation. Esters had been shown to have a significant correlation with yeast metabolism(Fan et al., 2019). Lactones were often used as edible essence to provide fruity-aroma for spirits, mainly fund in S5. Alcohols were precursors of esters, mainly generated through yeast fermentation metabolism, protein breakdown, etc., and exhibited floral fragrance(Poisson & Schieberle, 2008). Although the total concentration of alcohols in S2, S4, and S5 was higher than that in S1 and S3, as for higher alcohols, the concentration was higher in S2 and S3, such as spathulenol, α-terpineol, linalool, α-cadinol. Linalool showed a pure and sweet smell, which could endow spirit with flower and fruit flavors. It had been identified as the main contributor to the flavor of gin(Song et al., 2020). Acids were also precursors of esters and were one of the main contributors to the flavor of spirits. For example, 2-methylpropionic acid and butyric acid had pleasant fruit flavor, the generated methyl butyrate had apple flavor, ethyl butyrate and isopropyl butyrate expressed pineapple flavor, isoamyl butyrate showed snow pear flavor, isoamyl isobutyrate presented banana flavor, and octyl isobutyrate displayed grape flavor(Rollero et al., 2015). Alkanes had a spicy, green woody solvent odor and were widely present in spirits(Succoio et al., 2015). The total concentrations of acids and alkanes in S5, S1, and S3 were higher than those in S2 and S4. Aromatics were also the main trace components in spirits, mainly existed in S3, S4, and S5. Most aromatics had the fragrance of flowers and fruits, and were often used in the production of essence and spices (Malherbe, Tredoux, Nieuwoudt, & du Toit, 2012). For example, phenylethyl alcohol, with pleasant fruit aroma and sweet taste, was an important flavor component in Baijiu, whisky, vodka and rum(Song et al., 2020; Z. Zhao et al., 2021). Terpenes commonly found in S2, including β-pinene, D-limonene, β-phellandrene and γ-terpinene, which came from various materials soaked in gin during the brewing process. These components possessed a pleasant aroma and a very low olfactory thresholds, thus, they could be perceived even at low concentrations. The concentration of terpenes in S3 was second only to S2, with only one (longicycle) presented in S5, while no terpenes were found in the other groups. This pattern was consistent with literature research. Sulfur compounds with aromas similar to onions and rotten cabbage were mainly presented in S5, followed by S1. In spite of their low concentrations detected, they played an important role in the flavor perception of spirits for their relatively low sensory thresholds. Furans generated by the non-enzymatic browning reaction of sugars at high temperatures were important flavor components in spirits. The total concentration of furans in S5 was much higher than that in other groups. Among them, the concentration of 2-n-butyl furan and furfural was the highest, which had been identified in our previous studies(Huang, Wu, et al., 2023). Furfural presented sweet and almond-like aromas, with a higher concentration resulting in a more mellow taste(Zha, Yin, Sun, Wang, & Wang, 2017). In particular, the unique high-temperature production process of Daqu in Baijiu promoted the formation of furfural.

Concurrently, visual analysis was conducted on 11 trace components co-identified in different spirits. As shown in Fig. 3c, there were significant ($p < 0.05$) differences in the concentrations of ethyl hexanoate, heptyl formate, ethyl tetradecanoate, octanoic acid, 1-pentanol, benzaldehyde, ethyl benzoate, phenylethyl alcohol, and 2,4-di-t-butylphenol among different types of spirits. Most of them were highly expressed in S5, followed by S4. However, there was no significant difference in ethyl acetate, 4-octanone between different groups. Of note, 4 esters and 4 aromatics were co-identified, expressing floral, fruity and sweet flavors. In the previous sensory evaluation, it was found that the intensity of these sensory characteristics was not consistent among different spirits samples, that was, the sensory expression of the components may be varied in different substrates. This phenomenon may be due to the varying concentration levels of these components in different spirits.

In conclusion, the sensory expression of spirits was closely related to the distribution characteristics of trace components, and various trace components worked together to promote sensory expression of spirits. Overall, S5 had the most complex composition of trace components, supporting the rich flavor of Baijiu. The kind of trace components in group S4 was the second, followed by S1 and S2. The S3 group with the lowest total amount of trace components, showed pure and soft sensory characteristics as a whole, which was because of multiple distillations and the effective filtration during the production of vodka, resulting in a relatively pure vodka aroma. Obviously, groups S1, S2, S3, and S4 had their representative sensory attributes, but the correlation between sensory attributes and trace components was not clear. Multivariate analysis helped to resolve this problem and further distinguish the types of spirits.

3.3. Identification of potential differential markers between spirits

The method of identifying differences through all volatile data was less feasible in practice, and it was necessary to find the key that can truly define the differences of spirits quality expression through multivariate analysis. Then, supervised multivariate statistical analysis by PLS-DA was performed using the S1-S5 spirit samples volatility dataset to identify specific potential markers. As shown in Fig. 4a (R^2X [1] was 0.57 and R^2X [2] was 0.108), the explained variation (R^2Y) and the predictive capability (Q^2Y) of PLS-DA were 0.974 and 0.919, respectively. There were obvious differences between S4 and S5, and further analysis was made on S1, S2 and S3 with no obvious differences. As shown in Fig. 4b (R^2X [1] was 0.432 and R^2X [2] was 0.323), there were significant differences between groups S1, S2, and S3. The explained variation (R^2Y) and the predictive capability (Q^2Y) were 0.994 and 0.979, respectively. In addition, permutation tests ($n = 200$) were performed to evaluate whether the discriminant models were overfitting the data (Fig. 4c), and the plot strongly indicated that the original model was valid because all R^2 and Q^2-values to the left were lower than the

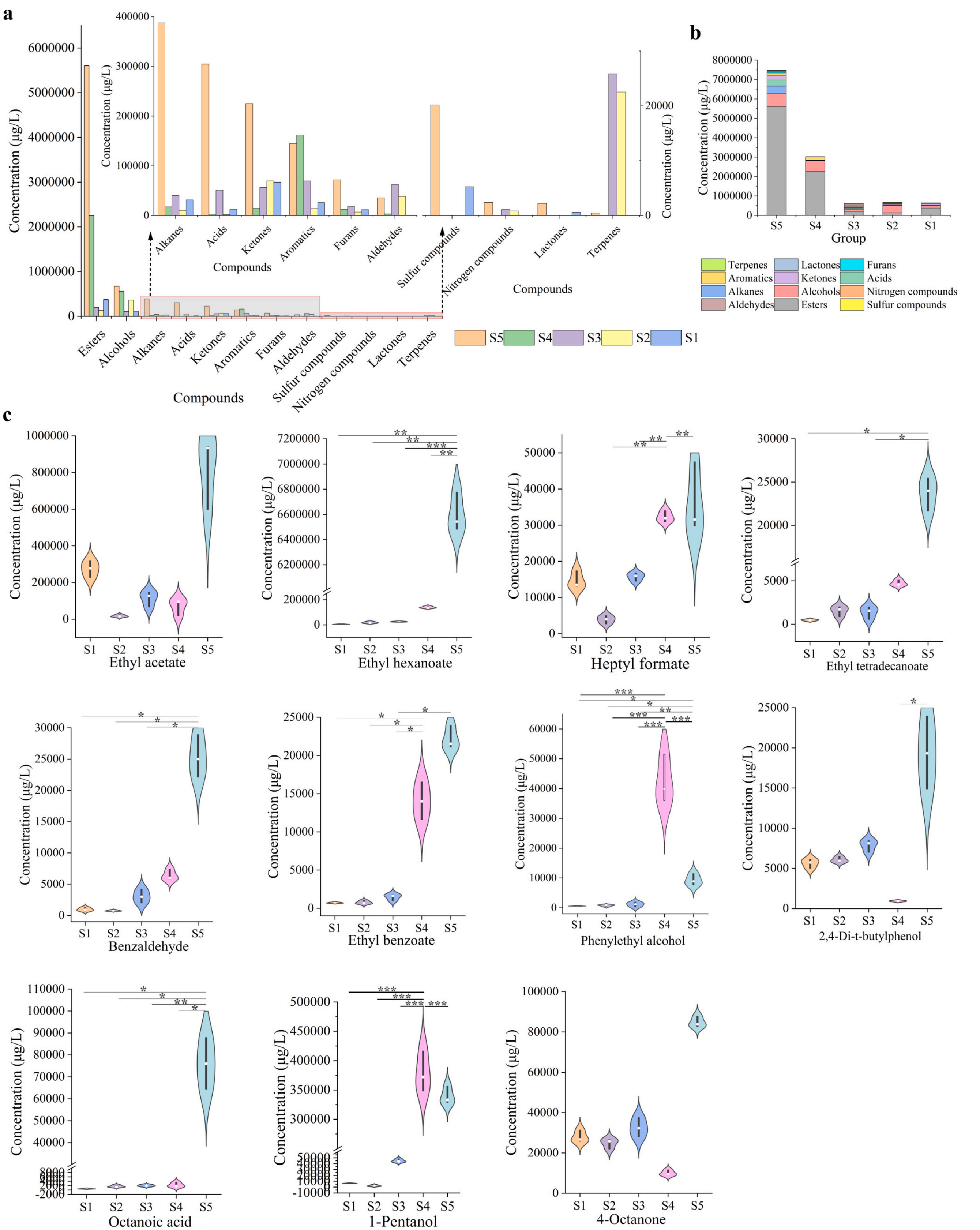

Fig. 3. Metabolic analysis of trace components for spirits. **a**. Bar chart on the total concentration of various trace components in different spirits; **b**. Stacking diagram on the total concentration of trace components in different spirits; **c**. The Violin plots described the concentrations and differences of 11 co-identified trace components in different spirits. The significant differences between groups were represented by * * * ($p < 0.001$), * * ($0.001 \le p < 0.01$), and * ($0.01 \le p < 0.05$).

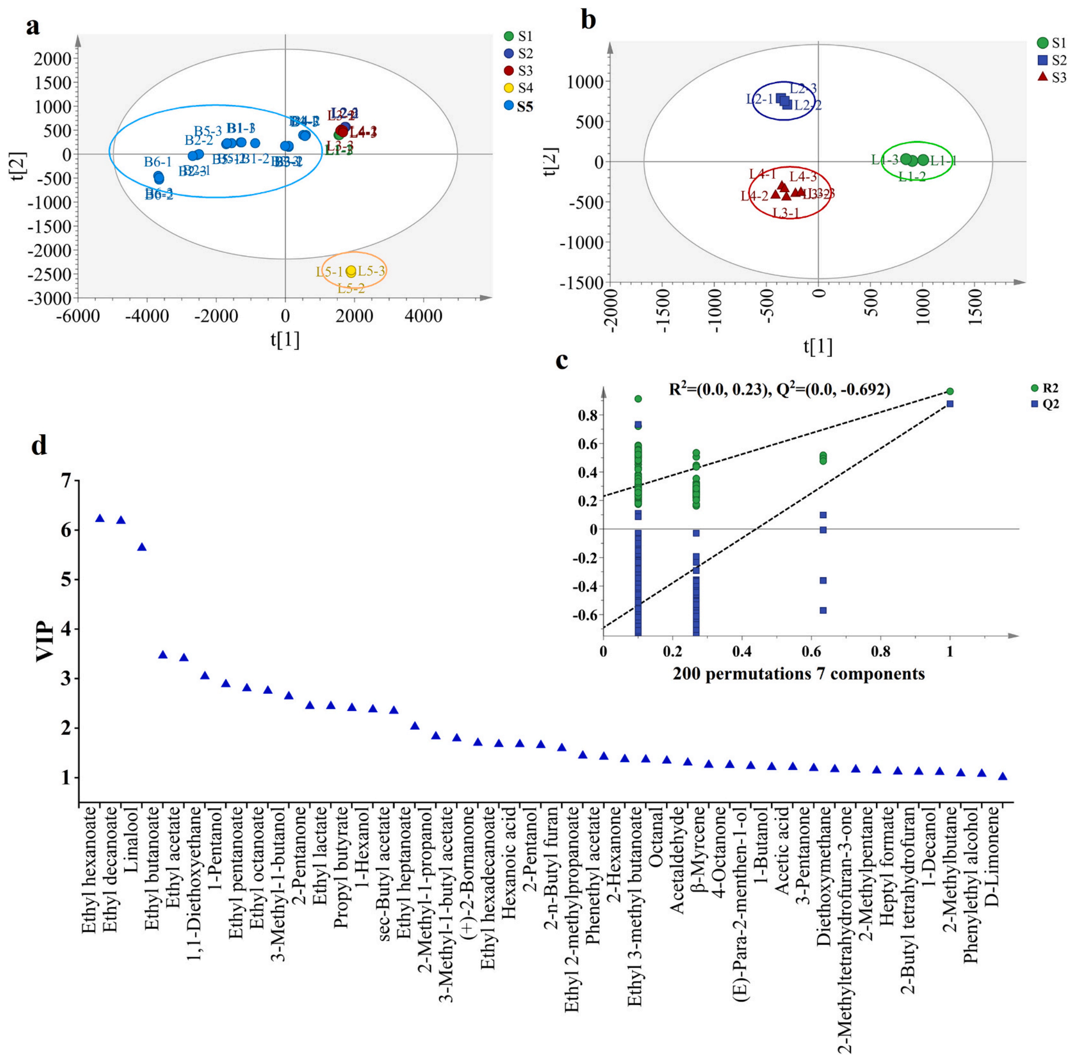

Fig. 4. Multivariate analysis of trace components in spirits. **a**-**b**. PLS-DA diagrams of different types spirits; **c**. Performance of the permutation test; **d**. Variable importance in projection values of 44 potential differential markers (VIP > 1).

original points to the right, and the blue regression line of the Q^2-points intersected the vertical axis below zero ($Q^2Y = -0.692$). The R^2 and Q^2 values of the permutation test revealed that the discriminant model did not overfit the data.

Further, variable importance in projection (VIP) values were calculated based on the PLS-DA model of different types of spirit samples. VIP analysis was commonly used to screen for potential differential markers, confirming that trace components with VIP > 1 have high identification potential. As shown in Fig. 4d, the VIP scores for the 44 trace components were >1, including 15 esters, 9 alcohols, 5 ketones, 4 alkanes, 3 furans, 2 acids, 2 aromatics, 2 aldehydes, and 2 terpenes. These trace components were thought to be potential markers for differences in different types of spirits. However, the correlation between potential differential markers and the sensory attributes of spirits was not clear. It was necessary to establish the corresponding relationship between potential differential markers and various sensory attributes of spirits, and further identify key differential markers of different spirits.

3.4. Relationships between the flavor perception and potential differential markers

In order to further elucidate the contribution of potential differential markers to the flavor perception of spirits, Pearson correlation analysis was used to analyze the relationship between various sensory attributes of spirits and 44 potential difference markers (VIP > 1). Consequently, 34 among them with coefficients (ρ) > 0.9 to at least one sensory attribute were considered as definite differential markers, including 14 esters, 7 alcohols, 3 alkanes, 2 aromatics, 2 ketones, 2 terpenes, 2 furans, 1 acid, and 1 aldehyde. There were 11 kinds of ethyl esters which were mainly formed by condensation of higher alcohols and acetyl-coA under the action of alcohol acetyltransferase(Plata, Mauricio, Millán, & Ortega, 2005), usually expressing a fresh fruit aroma.

As shown in Fig. 5a, the sensory attributes “grain-aroma”, “sweet-taste”, “cellar-aroma”, “sour-taste”, “bitter-taste”, and “alcohol-aroma” were significantly correlated with 19, 19, 17, 16, 15, and 11 kinds of differential markers, respectively. Particularly, hexanoic acid, phenethyl acetate, diethoxymethane were positively associated with the “grain-aroma” and “cellar-aroma”, together with 9 esters (ethyl pentanoate, ethyl heptanoate, ethyl octanoate, ethyl 2-methyl propanoate, ethyl butanoate, ethyl hexanoate, ethyl 3-methyl butanoate, 3-methyl-1-butyl acetate, ethyl hexadecanoate), 3 alcohols (2-methyl-1-propanol, 2-pentanol, 1-butanol). The results of sensory evaluation showed that “grain-aroma” and “cellar-aroma” were unique in Baijiu, so these differential markers may be important flavor components of Baijiu. Other sensory attributes were associated with only a few differential markers. Heptyl formate and ethyl octanoate jointly influenced the aroma and taste of barley malt, and ethyl octanoate was related to the “peat smoke-aroma”. Previous studies(Camara et al., 2007) have shown that ethyl

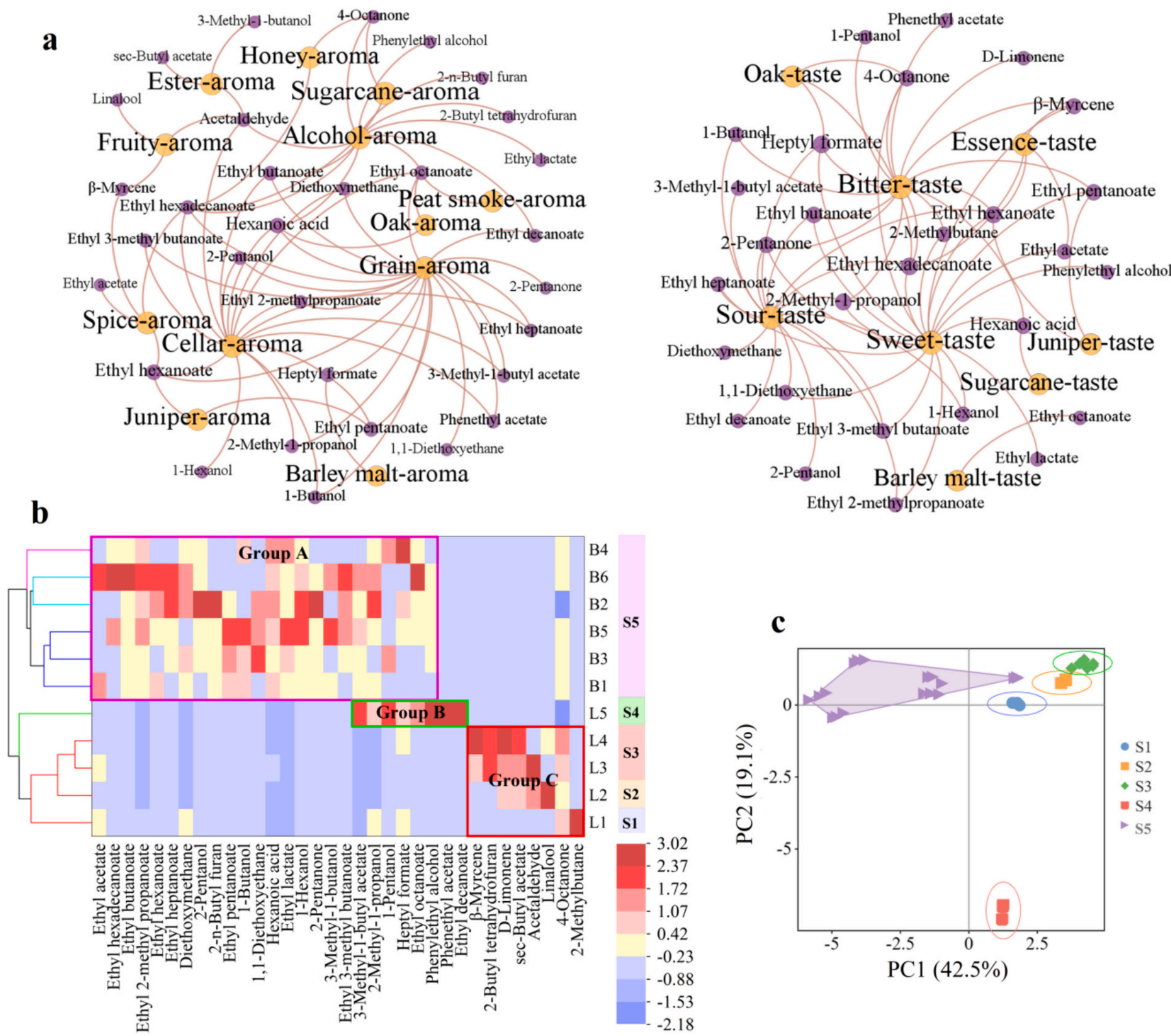

Fig. 5. Analysis of differential markers for different spirits. **a**. Networks of associations between sensory attributes and differential markers in spirits. Orange nodes represented sensory attributes, and purple nodes represented differential markers; **b**. Heat maps of 34 definite differential markers (VIP > 1 and (ρ) > 0.9) in different spirit samples; **c**. PCA analysis of spirits based on definite differential markers (VIP > 1 and (ρ) > 0.9). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

esters were an essential group of flavor components in whisky, to which they confer a pleasant aroma like "fruity". Ethyl pentanoate and ethyl hexanoate collectively influenced the expression of aroma and taste of juniper seeds. The "sugarcane" flavor was positively related to hexanoic acid, ethyl hexadecanoate, and 4-octanone. Hexadecanoic acid was the precursor of ethyl hexadecanoate, and it accounted for 4.038% and 4.972% respectively in unlenvened cane juice and rum(F. H. Wu et al., 2010). The "spice" flavor was positively related to β-myrcene, ethyl acetate, ethyl hexanoate, and ethyl hexadecanoate. β-Myrcene exhibited a light creamy flavor and was also related to "sweet taste", "bitter taste", and "fruity-aroma". Previous studies have shown that the main trace components in vodka were ethyl components, and different brands of vodka could be distinguished based on ethyl components and characteristic substances(Lay-Keow et al., 1996). Hexanoic acid, ethyl octanoate, ethyl hexadecanoate, 4-octanone and heptyl formate were related to "oak-taste", and further study was needed to determine whether these components each have an oak like taste. Current research showed that hexanoic acid was considered as flavor components in rum, and ethyl octanoate naturally existed in some fruits(Rollero et al., 2015). Besides, 9 esters (ethyl decanoate, ethyl 2-methylpropanoate, ethyl butanoate, ethyl hexanoate, ethyl heptanoate, heptyl formate, ethyl 3-methyl butanoate, ethyl hexadecanoate, 3-methyl-1-butyl acetate), 4 alcohols (2-pentanol, 1-butanol, 1-hexanol, 2-methyl-1-propanol), 2 alkanes (diethoxymethane, 1,1-diethoxyethane), and 2-pentanone were positively associated with the "sour-taste" scent. The combined of these components affected the "sour-taste", possibly due to the presence of complex perception interactions. Studies have shown that interactions between volatile, non-volatile, and substrate can all affect the volatility and release of odors, as well as the overall perceived intensity and quality of odors(Chen, Zhang, Quek, & Zhao, 2023). Linalool, with the aroma of citrus and floral(Y. P. Zhao et al., 2013), was closely related to the "fruity-aroma" scent, together with acetaldehyde which was often used to prepare essence such as citrus, apple and cream(del Barrio-Galan, Medel-Maraboli, & Pena-Neira, 2015). D-Limonene was positively associated with the "bitter-taste" scent, presenting a pleasant and fresh orange aroma(Niebler & Buettner, 2015). Furans (2-butyl tetrahydrofuran, 2-n-butyl furan) were strongly positively correlated with "alcohol-aroma". Phenylethyl alcohol, hexanoic acid, 4-octanone, and 3 esters (ethyl lactate, ethyl hexadecanoate, ethyl butanoate) were related to "alcohol-aroma" and "sweet-taste" at the same time. It could be seen that acids did not only exhibit acid-like senses, but were also related to sensory attributes such as sweetness. Significantly, 5 kinds of co-identified components in all types of spirits were screened as potential differential markers. It may be related to the fact that components may exhibit various flavor at different concentration levels or in different matrices(Niu, Li, & Xiao, 2021). The actual flavor contribution of these differential markers needed to be confirmed systematically through addition, omission and perceptual interaction test in further research.

3.5. Confirmation of validity

Studies had shown that not all components were involved in the formation of differences in spirit quality. In this study, interference and ineffective information were discarded through reasonable methods. Based on the potential differential markers with VIP > 1 in the spirit samples of different types, 34 of them were selected for (ρ) > 0.9, being acted as the definite differential markers of the flavor differences in spirit samples from different types. To further verify the contribution of definite differential markers to the effect of identification and sensory perception of various types of spirits, a heat map of these 34 definite differential markers was used to visualize the relationship between different types of spirits (Fig. 5b). In horizontal clustering, all the Baijiu samples (B1-B6) were grouped into one group, and the component group with higher concentration was defined as Group A. Components with higher concentrations were defined as Group B in whiskey (L5) which was separately divided. Similarly, rum (L1), gin (L2), and vodka (L3, L4) samples were grouped into their own categories, respectively. And the components with higher concentrations were defined as Group C due to the close proximity in horizontal clustering of L1-L4. Furthermore, the contribution of definite differential markers to sensory perception of spirits was verified by flavor addition experiments.

In the clustering results, the concentrations of definite differential markers in Group A were higher in S5, including hexanoic acid, diethoxymethane, ethyl pentanoate, ethyl heptanoate, ethyl octanoate, ethyl 2-methyl propanoate, ethyl butanoate, ethyl hexanoate, ethyl 3-methyl butanoate, 3-methyl-1-butyl acetate, ethyl hexadecanoate, 2-methyl-1-propanol, 2-pentanol, 1-butanol, which were positively related to "grain-aroma" and "cellar-aroma" scents, consistent with the results of sensory evaluation of S5, since Baijiu was fermented in mud cellars with a variety of grains as raw materials(Hu, Du, & Xu, 2015). Hexanoic acid was confirmed to be the definite odorants responsible for the mud-like and roasted odors(Dong et al., 2019). Hexanoic acid, ethyl pentanoate, ethyl butanoate, and ethyl hexanoate were confirmed as key flavor components in strong aroma type of Baijiu(Hong et al., 2023). Ethyl hexadecanoate, which was not detected in other spirits but had a high concentration in Baijiu, was considered to be one of the main reasons for the difference in flavor between Baijiu and other spirits. This phenomenon was consistent with previous research findings(Song et al., 2020). Further, the flavor addition experiments showed that ethyl pentanoate and ethyl hexanoate had strong positive correlation with sweet-taste" and "cellar-aroma", while hexanoic acid was positive correlation with "cellar-aroma", "sour-taste", and "bitter-taste". Thus, certain esters formed by fatty alcohol were potential indicators that may be used for differentiating Baijiu and other spirits(Fang et al., 2019).

Group B contained 8 definite differential markers with higher concentrations in S4, including 3-methyl-1-butyl acetate, 2-methyl-1-propanol, 1-pentanol, heptyl formate, ethyl octanoate, phenylethyl alcohol, phenethyl acetate, ethyl decanoate. Heptyl formate, ethyl octanoate were positively related to the "oak-taste" and the sensory of barley malt, consistent with the outstanding sensory aroma of S4. In previous study, phenethyl acetate, ethyl decanoate, and phenylethyl alcohol were identified as odor-active volatile constituents in Whisky (Poisson & Schieberle, 2008). Phenethyl acetate was trace components with peach aroma, giving whiskey a stronger rose and honey flavor. Ethyl octanoate could impart a fruity (banana, pear) flavor to whiskey, while ethyl decanoate could impart a sweet, fruity, and soapy flavor to whiskey. 3-Methyl-1-butyl acetate could give whisky nail polish and banana flavor (Y. Zhang et al., 2024). The synthesis of ester components was closely related to the unsaturated fatty acids in malt raw materials, saccharification process, and fermentation process. The low-temperature fermentation and the direct distillation process with fermentation broth helped with retaining more esters. 2-Methyl-1-propanol and 1-pentanol imparted a unique aroma to spirit, making it soft and rich (Huang, Huang, Wang, & Zhao, 2022). Phenylethyl alcohol, with a floral aroma could explain the differences in malt whisky among different brands, together with 3-methyl-1-butyl acetate, phenethyl acetate and ethyl octanoate(D. Q. Zhang et al., 2023). Further, the flavor addition experiments showed that phenylethyl alcohol and phenethyl acetate had strong positive correlation with "sweet-taste" and "fruity-aroma", while heptyl formate and ethyl octanoate was positive correlation with "fruity-aroma", and "oak-taste". These key differential markers could effectively reveal the chemical source of characteristic flavor for whiskey.

Definite differential markers in Group C had higher concentrations in S1-S3. In detail, the concentrations of 2-methylbutane and 4-octanone were higher in S1, while D-limonene, sec-butyl acetate, acetaldehyde and linalool were higher in S2. The concentrations of β-myrcene, 2-butyl tetrahydrofuran, D-limonene, sec-butyl acetate, acetaldehyde and 4-octanone were higher in S3, which existed some overlapping components with S2. 4-Octanone was related to the "oak-taste", "sugarcane-aroma", "honey-aroma" and "sweet-taste" scents, which was consistent with the results of sensory evaluation of S1. Ulteriorly, the flavor addition experiment showed that 2-methylbutane had strong positive correlation with "sweet-taste" and "honey-aroma". The higher quantities of monoterpenes and their oxygenated derivatives (D-limonene, linalool) may contribute significantly to the flavor of gin(Y. P. Zhao et al., 2013). Linalool has the highest concentration in S2 and has been identified as the main contributor to the flavor of gin in previous studies (Song et al., 2020). A similar assumption could be drawn for descriptors of floral (linalool, sec-butyl acetate) and citrus (acetaldehyde, D-limonene), which were also found typical for S2. Meanwhile, linalool was verified to be positive with "honey-aroma" and "sweet-taste" through flavor addition experiments, while acetaldehyde and D-limonene had strong positive correlation with "spice-aroma", "juniper-aroma" and "juniper-taste". The concentrations of D-limonene, sec-butyl acetate, acetaldehyde, and linalool were also high in S3, which may explain why the scent intensity of "essence-taste" and "spice-aroma" in S3 was prominent. β-Myrcene with a flavor of essence and spice also had higher concentration in S3, and been verified to had strong positive correlation with "spice-aroma", and "sweet-taste" through the flavor addition experiments. In addition, the concentrations of these definite differential markers which proved to be an important contributor to revealing the characteristic flavor of S1, S2, and S3 in Group C were higher than in S4 and in S5.

In order to verify the reliability of the selected definite differential markers, the quantitation data of these 34 definite differential markers (ρ > 0.9 and VIP > 1) was used for PCA (Fig. 5c). The PCA bi-plot explains 61.6% of the total variations in the first two dimensions, which was higher than that in Fig. 3a (35.5%). The spirits samples of different types were well separated in the PCA plots. Therefore, 34 definite differential markers could effectively identify different types of spirits. Comprehensive taking into account the VIP value, correlation coefficient, number of associations, uniqueness of association attributes and the results of sensory verification, 14 key differential markers (including 2-methylbutane, linalool, acetaldehyde, D-limonene, β-myrcene, phenylethyl alcohol, phenethyl acetate, heptyl formate, ethyl octanoate, ethyl decanoate, ethyl pentanoate, ethyl hexanoate, hexanoic acid, and ethyl hexadecanoate.) were ultimately confirmed to reveal the characteristic sensory of corresponding spirits (as shown in Fig. 6). These key differential markers could also serve as targets for identifying different types of spirits.

4. Conclusion

The volatile composition and sensory attributes of the five spirits

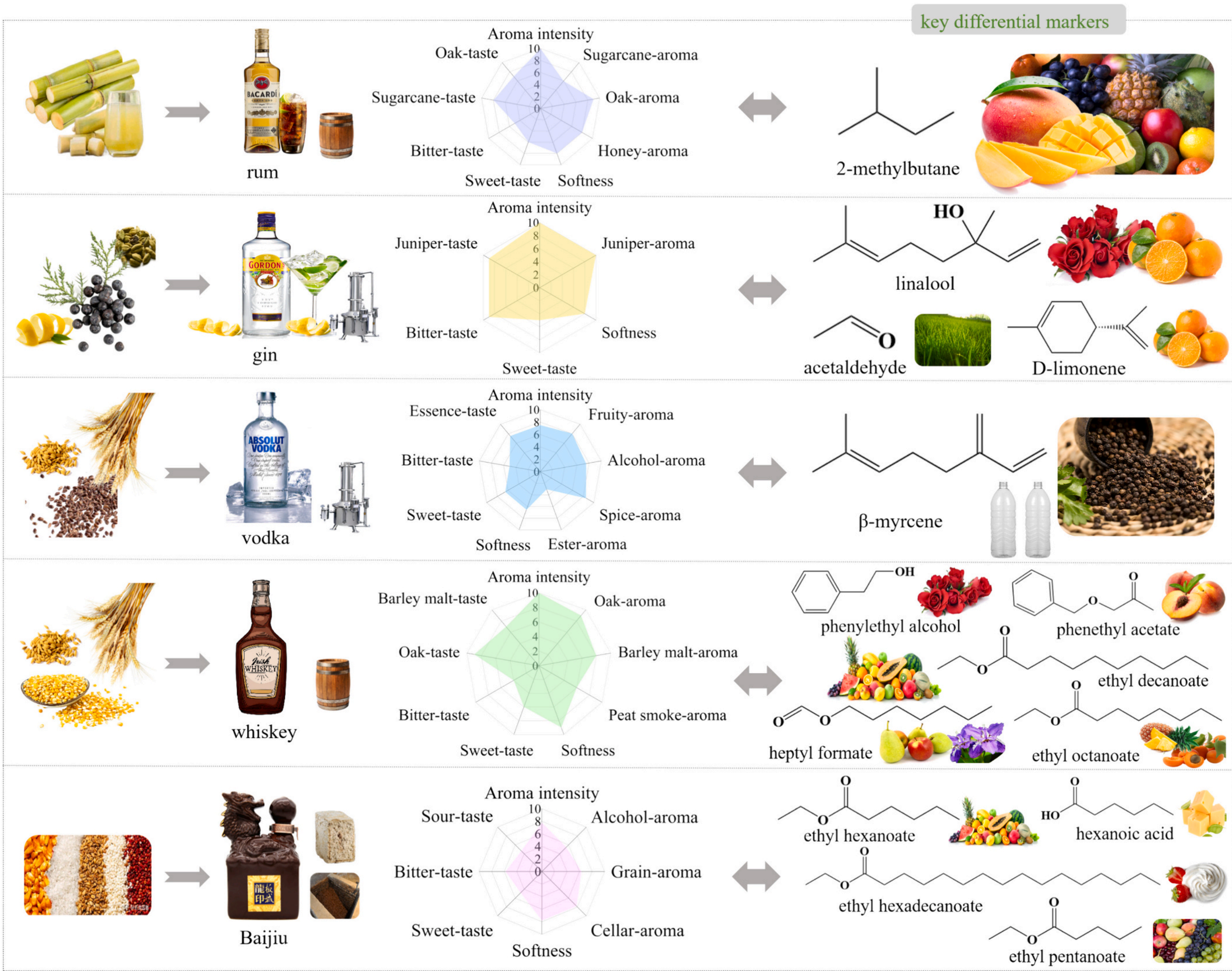

Fig. 6. The 14 key differential markers of spirits.

examined in this study were quite different. This was due to the significant differences in the variety and concentration of trace components related to different raw materials, fermentation conditions, distillation and aging processes. Specifically, chemical and sensory characteristics and their potential associations were evaluated. Samples of different types of spirits could be clearly classified by sensory characteristics. A total of 325 trace components were quantitatively analyzed by VASE combined with GC–MS, and the samples from different types of spirits were clearly divided into 5 groups according to quantitative targeted flavoromics analysis. In addition, 44 potential differential markers were obtained by PLS-DA and VIP. Correlation analysis further showed that 34 differential markers were highly correlated with sensory characteristics and could be used as definite differential markers to distinguish different types of spirit samples. Eventually, 14 key differential markers were ultimately confirmed to reveal the characteristic sensory of corresponding spirits, including 2-methylbutane, linalool, acetaldehyde, D-limonene, β-myrcene, phenylethyl alcohol, phenethyl acetate, heptyl formate, ethyl octanoate, ethyl decanoate, ethyl pentanoate, ethyl hexanoate, hexanoic acid, and ethyl hexadecanoate. These key differential markers could also serve as targets for identifying different types of spirits. This study evaluated the differences between mainstream spirits from multiple sensory dimensions, representing aspects such as craftsmanship, raw materials, and culture, which helped to fully understand the factors that lead to differences in the composition and style of different spirits. The research conclusion clarified the representative components of different types of spirit, providing an important lever for the differential expression and quality improvement of spirit in the future, and providing targets for stable optimization of processes.

CRediT authorship contribution statement

He Huang: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation. **Yiyuan Chen:** Project administration, Methodology, Investigation. **Jiaxin Hong:** Writing – review & editing, Methodology, Investigation. **Hao Chen:** Writing – review & editing, Investigation. **Dongrui Zhao:** Writing – review & editing, Resources, Methodology, Investigation. **Jihong Wu:** Resources. **Jinchen Li:** Writing – review & editing. **Jinyuan Sun:** Resources. **Xiaotao Sun:** Resources, Methodology. **Mingquan Huang:** Writing – review & editing, Investigation. **Baoguo Sun:** Resources.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgements

This research was funded by Beijing Elite Scientist Sponsorship Program by Bast (No. BYESS2023055), and the National Key Research & Development Program of China (grant no. 2022YFD2101205).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101716.

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