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Quality evaluation of *Sojae Semen Praeparatum* by HPLC combined with HS-GC-MS

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ABSTRACT

Sojae Semen Praeparatum is a popular fermented legume product in China, with a delicious flavour and health benefits. However, the quality control methods for *Sojae Semen Praeparatum* are now incomplete, and there are no standards for defining its degree of fermentation. In this study, we introduced colour, acid value, ethanol-soluble extractives and six flavonoid components' content to evaluate the quality of *Sojae Semen Praeparatum* comprehensively. Multiple linear regression was used to streamline the 11 evaluation indicators to 4 and confirm the evaluating feasibility of the four indicators. The degree of fermentation and odour of *Sojae Semen Praeparatum* were analyzed on headspace-gas chromatography-mass, and two types of odours, 'pungent' and 'unpleasant', could distinguish over-fermented *Sojae Semen Praeparatum*. Our research developed fermentation specifications and quality standards for *Sojae Semen Praeparatum*.

1. Introduction

The popularity of fermented foods has surged in recent years due to their health benefits. Postulated mechanisms for the health effects of fermented foods include the potential probiotic effects of microorganisms, the conversion of bioactive peptides, biogenic amines, and phenolic compounds into bioactive compounds from fermentation, and the reduction of anti-nutrients. Fermented legume products are widely consumed globally [1]. Through fermentation, the taste, appearance, nutrient digestibility, nutritional value, texture and shelf life of beans are improved [2], while protease inhibitors, lectins, oligosaccharides and phytates (non-nutritional compounds) present in bean seeds are reduced [3]. In addition, the fermentation of legumes leads to an increase in phenolic compounds in legume seeds [4]. A study has shown that fermented legumes exhibit anti-diabetic and anti-cancer properties by acting as antioxidants and modulating some enzymes such as acetylcholinesterase, glucosidase and amylase [5–7]. Fermented legume products first originated in China, and to this day, Chinese people still intake many fermented legume products in their diets, such as pickled tofu, soybean paste and *Sojae Semen Praeparatum* [8,9]. As a fermented soy product, it can be used as a seasoning or cooked as a dish.

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Sojae Semen Praeparatum is a fermented processed product of the mature seeds of the *Glycine* max (L.) Merr., with a strong fresh flavour [10]. *Sojae Semen Praeparatum* can be further processed into flavored Douchi, which has gained popularity all over the world.

Sojae Semen Praeparatum needs to be fully fermented to fulfil its health functions. It is considered both a food and a medicine by the Chinese Traditional Medicine Administration [11], with a high concentration of polyphenols, mainly isoflavones and isoflavone glycosides, which show hepatoprotective and antioxidant properties [12]. Many studies have shown that *Sojae Semen Praeparatum* improves depression-like behaviour in chronically stressed rats [13,14]. However, its quality varies greatly due to the quality of raw materials and the different fermentation conditions. The existing quantitative means of evaluating the quality indicator of *Sojae Semen Praeparatum* is mostly based on the whole content of two flavonoid compounds, daidzein and genistin [15,16]. However, it was found that most of the fully fermented, semi-fermented and over-fermented *Sojae Semen Praeparatum* contained flavonoid glycosides that could meet the requirements. Its fragrance, which may be due to pyrazine compounds [17] is another traditional quality indicator. Studies on the odour-presenting compounds of *Sojae Semen Praeparatum* are scarce, especially the flavour constituents of *Sojae Semen Praeparatum* with bad odour. *Sojae Semen Praeparatum* odour is related to the degree of fermentation the odour of *Sojae Semen Praeparatum* becomes more intense along with the fermentation, and excessive fermentation can produce a bad odour. But there is no volatile compounds data for explaining complete or excessive fermentation.

Laboratory studies have found that during controlled fermentation with the same batch of raw material, the flavonoid content of *Sojae Semen Praeparatum*, acid value, ethanol-soluble extractives content and redness increases, and flavonoid glycoside content and brightness decreases. The odour changes gradually during fermentation. It is therefore hypothesized that the above indicators may show their significance in evaluating the quality of *Sojae Semen Praeparatum* purchased from the market. In this study, the above characterization data of 36 batches of commercial and laboratory prepared *Sojae Semen Praeparatum* were analyzed. The indicators that were suitable for the quality assessment of *Sojae Semen Praeparatum* were selected by multiple linear regression. The flavour components related to the degree of fermentation were detected by headspace-gas chromatography-mass (HS-GC-MS).

2. Materials and methods

2.1. Source of Sojae Semen Praeparatum samples

Glycine max (L.) Merr. (purchased from Anguo Herbal Market); *Artemisia annua* L. (lot no. C427210202), *Morus alba* L. (lot no. C449210402) were purchased from Anguo Shenhao Pharmaceutical Co., Ltd. and identified by Yanlin Chen, a researcher from China Traditional Chinese Medicine Co. Information on 33 batches of *Sojae Semen Praeparatum* samples from markets and companies is given in Table S1. All *Sojae Semen Praeparatum* samples were stored under seal until analysis.

2.2. Sample preparation and characterization methods

2.2.1. Colour determination

The colour was determined according to methods published previously [18]. Briefly, *Sojae Semen Praeparatum* powder was passed through a 60-mesh sieve to determine brightness (L), redness (a) and yellowness (b).

2.2.2. Acid value

The acid value was performed referring to the previously published method [19]. Briefly, 1.5 g of *Sojae Semen Praeparatum* (sieved through 60 mesh), was placed in a 250 mL conical flask, added 50 mL of ethanol-petroleum ether (1:1) mixture, left for 30 min, filtered, washed the filtrate twice with 20 mL of ethanol-petroleum ether (1:1) mixture, combined the filter, added 5 drops of phenolphthalein indicator and titrated with potassium hydroxide solution until the pink colour persisted for 30 s without fading.

2.2.3. Ethanol-soluble extractives

Sojae Semen Praeparatum was crushed (sieved through 60 mesh) and 2 g was placed in a conical flask, added 100 mL of 70% ethanol, was sealed and weighed the flask. Allow to stand for 1 h, then refluxed for 1 h. Cool, and make up the weight with 70% ethanol. Take 25 mL of the filter, placed it in an evaporating dish, evaporated it in a water bath and dry it at 105 °C for 3 h. Cool in a desiccator for 30 min and weighed precisely. As a dried product, calculate the content of ethanol-soluble extractives (%).

2.2.4. Content of six flavonoid components

The method used was referred to in the literature [20]. Briefly, the chromatographic conditions were as follows: Agilent Zobax SB-C18 (5 μm 4.6 × 250 mm) column, mobile phase: methanol (A) - 0.5% acetic acid solution (B); gradient elution: 0–25 min, 30%–45% A, 25–50 min, 45%–60% A, flow rate 1.0 mL/min, column temperature 30 °C, detection wavelength 260 nm. The sample was extracted by soaking 1 g of powder (60 mesh sieve) in 5 mL of petroleum ether (60–90 °C) for 3 h and filtered. The filter residue was added to 25 mL of 80% methanol and ultrasound (500 W, 50 Hz) for 30 min. After cooling, the supernatant was filtered through a 0.45 μm microporous membrane, and the filter solution was injected into HPLC for analysis The linear ranges were 1.44–72.00, 0.4104–20.52, 2.88–144.00, 1.272–63.60, 0.2066–10.33, 1.314–65.70 μg/mL for Daidzin, Glycitin, Genistin, Daidzein, Glycitin and Genistein, respectively; with the recoveries of 107.59%, 101.17%, 99.70%, 101.60%, 96.08% and 101.99%, respectively.

2.2.5. Volatile components analysis

4g of *Sojae Semen Praeparatum* was placed in a 20 mL headspace vial, and the mass spectra of the peaks were searched in the NIST14

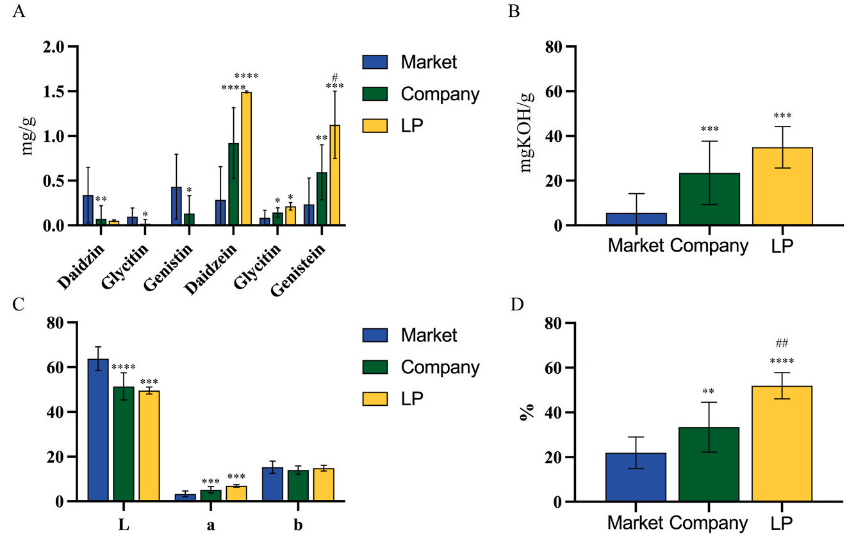

Fig. 1. The quantitative determination of quality control components of *Sojae Semen Praeparatum*. (A) Three flavonoid glycosides and three flavonoids contents; for all groups one-way ANOVA, F (2, 33) = 5.850, $p = 0.0067$; F (2, 33) = 5.658, $p = 0.0077$; F (2, 33) = 6.003, $p = 0.0060$; F (2, 33) = 19.68, $p < 0.0001$; F (2, 33) = 5.901, $p = 0.0064$; F (2, 33) = 12.94, $p < 0.0001$; (B) Acid value; for all groups one-way ANOVA, F (2, 33) = 13.76, $p < 0.0001$; (C) Colour; for all groups one-way ANOVA, F (2, 33) = 23.33, $p < 0.0001$; F (2, 33) = 13.60, $p < 0.0001$; F (2, 33) = 1.312, $p = 0.28$ (D) ethanol-soluble extractives; for all groups one-way ANOVA, F (2, 33) = 15.74, $p < 0.0001$. The significant difference is determined by $p < 0.05$ (*), 0.01 (**), 0.005 (***), 0.001 (****), respectively, compared with Market; $p < 0.05$ (#), 0.01 (##) compared with Company. Data are represented as means ± SD. Market: Sample from Market, n = 16; Company: Sample from Company, n = 17; LP: Laboratory preparation, n = 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

library based on the GC-MS total ion flow diagram. A match score ≥80 and reverse match index ≥700 were considered as the identified volatile component, moreover, the retention index (RI) was used for characterization. The detailed HS sampling, GC separation, and temperature ramp can be found in the supplementary materials.

2.3. Multivariate statistical analysis

The data were statistically analyzed and plotted using GraphPad Prism 9 and the R language. Data processing was carried out on RStudio. Principal component analysis (PCA) and stacked plots were plotted using the ggplot2 package. Correlation analysis was plotted using the corrplot package. Multiple linear regression was diagnosed using the gvlma package and plotted using ggpubr and ggplot2.

3. Results

3.1. Content of 6 flavonoids of Sojae Semen Praeparatum from different sources

As quality control components, Daidzin, Glycitin, Genistin, Daidzein, Glycitin and Genistein were detected. The most significant changes in *Sojae Semen Praeparatum* during fermentation were a gradual decrease of flavonoid glycosides whereas a gradual increase of flavonoids. This is the hydrolysis of flavonoid glycosides by microorganisms and suggested that fully fermented *Sojae Semen Praeparatum* should contain fewer flavonoid glycosides and more flavonoids. In the *Chinese Pharmacopoeia* (2020 edition), it is stipulated that the total amount of daidzein and genistein in *Sojae Semen Praeparatum* should not be less than 0.4 mg/g. Here, we have referred to this standard and added three flavonoid glycosides and one flavonoid component as quality control components (Table S2. Fig. 1 A). Ten *Sojae Semen Praeparatum* samples from the market (out of a total of 16 samples) had less than 0.4 mg/g of total daidzein and genistein and were considered inferior. All batches of *Sojae Semen Praeparatum* from the company and laboratory met the standard. The flavonoid glycoside content of *Sojae Semen Praeparatum* from the market was significantly higher ($p < 0.05$) and the glycoside content was lower ($p < 0.001$) than the samples from the company and laboratory, indicating that most samples from the market were not fully fermented. Some *Sojae Semen Praeparatum* had a significant amount of glycosides that had not been decomposed and had not reached full fermentation state, even though the glycoside content met pharmacopeial standards.

3.2. Acid value, colour, and ethanol-soluble extractives of Sojae Semen Praeparatum from different sources

The increase in ethanol-soluble extractives and acid values is due to the breakdown of macromolecules such as protein, cellulose and fat during the fermentation of *Sojae Semen Praeparatum* [21,22]. Along with the accumulation of enzymatic products [23], *Sojae*

Table 1
Eigenvalue and variance contribution ratio results of 11 indicators.

PC	Initial Eigenvalue		
	Eigenvalue	variance contribution ratio (%)	Cumulative variance contribution (%)
1	6.0810078	55.28189	55.28189
2	1.77728132	16.15710	71.43899
3	1.14996521	10.45423	81.89322
4	0.92537687	8.41252	90.30574
5	0.39303385	3.57304	93.87877
6	0.33386234	3.03511	96.91389
7	0.14514646	1.31951	98.23340
8	0.09064852	0.82408	99.05748
9	0.05573411	0.50667	99.56415
10	0.02680579	0.24369	99.80784
11	0.02113773	0.19216	1.00000

Table 2
Factor loading matrix of 11 indicators.

	PC1	PC2	PC3
Daidzin	−0.3088	−0.44814	0.110424
Glycitin	−0.29874	−0.47213	0.127573
Genistin	−0.30892	−0.46089	0.024852
Daidzein	0.365649	−0.19102	−0.0863
Glycitin	0.258075	−0.36526	−0.09228
Genistein	0.340962	−0.22404	−0.07904
Acid Value	0.315895	−0.1995	−0.05515
L	−0.3255	0.03072	−0.31694
a	0.309088	−0.01944	0.479807
b	−0.08131	0.06777	0.78378
Ethanol-soluble Extractives	0.309062	−0.3131	−0.02625

Semen Praeparatum slowly deepens in colour until the section is brownish-black. The acid value, redness (*a*) and ethanol-soluble extractives content of *Sojae Semen Praeparatum* from the market were significantly lower ($p < 0.001$, $p < 0.001$, $p < 0.001$) and the *L* was the opposite ($p < 0.001$) than the samples from company and laboratory, and the yellowness (*b*) varied between each sample did not differ significantly ($p > 0.05$) (Table S2, Fig. 1B–D). The incompletely fermented samples of *Sojae Semen Praeparatum* from the market, although qualified in terms of glycosides, had significantly lower acid value, ethanol-soluble extractives and redness than the samples from the company. Indicating that the above indicators can be a good characterisation of the degree of fermentation of the *Sojae Semen Praeparatum*.

3.3. Identification of volatile components from different sources of Sojae Semen Praeparatum

Using Headspace-GC-MS, 36 compounds were identified (Table S5). These included 4 nitrogen-containing organic compounds, 3 esters, 1 ether, 6 acids and anhydrides, 7 alcohols, 5 ketones, 6 aldehydes, 1 oxetanes and 3 sulphur-containing organic compounds. Nitrogenous organic compounds include amines (3), and nitrogenous heterocyclic compounds (1). Sulphur-containing organic compounds include thioethers (2), and sulphones (1). The relatively low levels of nitrogen and sulphur-containing compounds and the high levels of acids, alcohols, esters and aldehydes give *Sojae Semen Praeparatum* its characteristic aroma. A total of 22 compounds with a common number greater than 5 were found in 33 batches of *Sojae Semen Praeparatum*. (Table S6).

3.4. Identification of Sojae Semen Praeparatum quality based on flavonoids content, acid value, colour and ethanol-soluble extractives

To better evaluate the quality of *Sojae Semen Praeparatum*, principal component analysis (PCA) was performed on the above 11 indicators. The data were scaled before analysis. The top three principal components with eigenvalues greater than 1, cumulatively explained 81.89% of the total variation. PC1 component explained 55.28% of the variation and was significantly negatively correlated with flavonoid content and *L*; and significantly positively correlated with glycosides content, acid value, *a*, and ethanol-soluble extractives, which can be considered as indicators of fermentation degree. PC2 explained 16.16% of the variation and was positively correlated with flavonoid content, acid value, and ethanol-soluble extractives, which can be considered as an indicator of raw material quality. PC3 explains 10.45% of the variation and is mainly positively correlated with *b*, which can be considered as an indicator of *b* (Tables 1 and 2). Using the PCA with the above three principal components, the five highest-scoring batches of *Sojae Semen Praeparatum* were LP_1, C_14, LP_3, LP_2 and C_2, indicating that the laboratory-prepared *Sojae Semen Praeparatum* was more fully fermented and of better quality in the multi-indicator evaluation. The conversion rate of different components in the fermentation process is different, for example, the acid value of *Sojae Semen Praeparatum* will still increase and the colour will gradually deepen after the

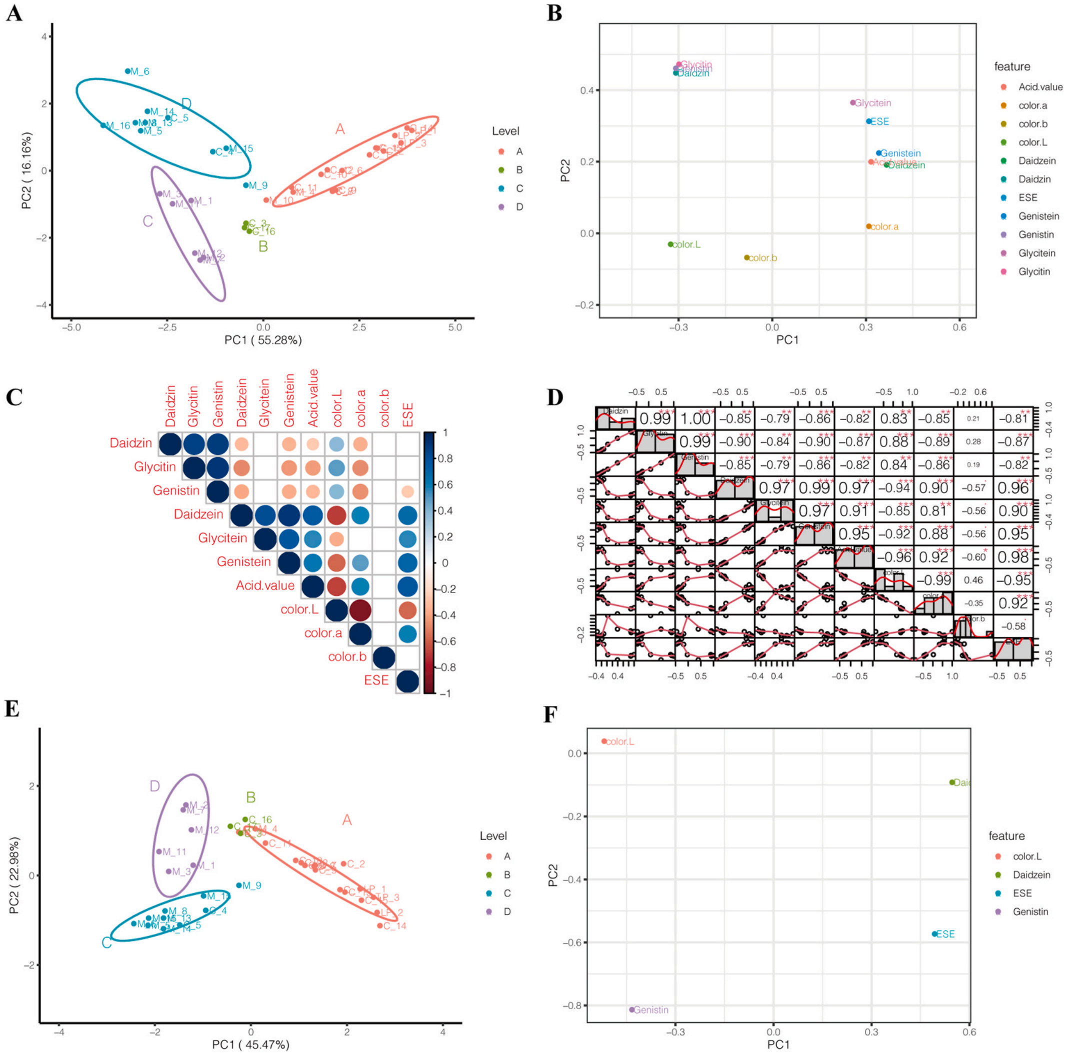

Fig. 2. Identification of *Sojae Semen Praeparatum* quality based on quality control components, acid value, colour and ethanol-soluble extractives. *Sojae Semen Praeparatum* quality PCA plots (A) and factor contribution plots (B) using 11 indicators; correlation coefficients and significant differences (C–D) for each indicator of *Sojae Semen Praeparatum*; Simplified indicators are plotted on the *Sojae Semen Praeparatum* quality PCA graph (E) and factor contribution graph (F). The significant difference is determined by $p < 0.05$ (*), 0.01 (**), 0.005 (***), 0.001 (****), respectively. ESE: ethanol-soluble extractives; Market: Sample from Market; Company: Sample from Company; LP: Laboratory preparation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

flavonoid glycosides were completely converted into flavonoids. Therefore, three flavonoids, three flavonoid glucosides, colour, acid value and ethanol-soluble extractives content were selected to evaluate the quality of *Sojae Semen Praeparatum* in a comprehensive manner.

Sojae Semen Praeparatum can be classified into 4 classes (Fig. 2A and B). The flavonoid glycosides in class A are completely converted into flavonoids for complete fermentation, with higher acid value, ethanol-soluble extractives content and redness, and lower brightness. The flavonoid glycosides in class B are also completely converted, but the acid value and ethanol-soluble extractives content are lower, which may be related to the fermentation conditions and quality of raw materials. High flavonoid glycoside content, light colour, low acid value and ethanol-soluble extractives content in class C indicate incomplete fermentation. Low content of flavonoid and their glycoside, acid value and ethanol-soluble extractives in class D showed the poor quality of raw material and incomplete fermentation. Most of the *Sojae Semen Praeparatum* from the same company are distributed in the same group. Most products from the company are in classes A and B.

There was a significant negative correlation within the content of the three glycosides and aglycone components; glycoside content was negatively correlated with acid value and *a*, and positively correlated with *L*; aglycone content was positively correlated with acid value, *a* and ethanol-soluble extractives, and negatively correlated with *L*, and *b* has no correlated with other indicators (Fig. 2C and D). To reduce the cost of evaluation, a multiple linear regression model was developed using the above 11 indicators as independent

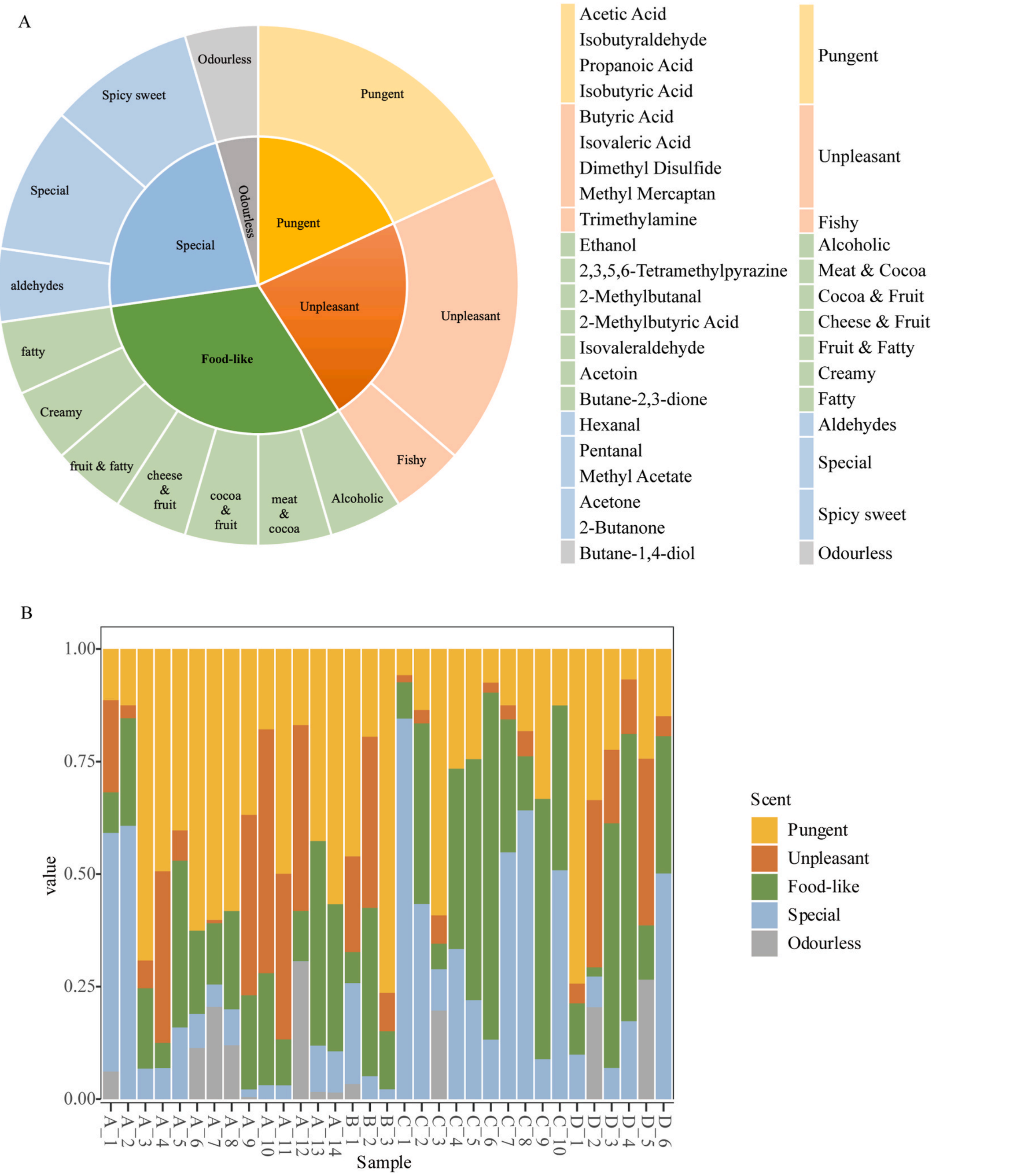

Fig. 3. Classification of *Sojae Semen Praeparatum* volatile organic compounds odours (A) and abundance of different classes of *Sojae Semen Praeparatum* odours (B).

variables and grades as dependent variables (class A, B, C and D denoted by 1, 2, 3 and 4 respectively) (Fig. S1 A). With this model, daidzein, genistin, *L* and ethanol-soluble extractives (ESE) were found to be used as simplified indicators to evaluate the quality of *Sojae Semen Praeparatum* with an accuracy of 77.82% (Table S3). Daidzein and ethanol-soluble extractives were negative indicators and were negatively correlated with *Sojae Semen Praeparatum* quality. Genistin and L were positively correlated with quality (Fig. S2). It was determined that genistin represented the glycoside component, daidzein represented the glycoside component, *L* represented the colour, and ethanol-soluble extractives represented the acid value and ethanol-soluble extractives. The simplification of the indicators still allowed *Sojae Semen Praeparatum* to be classified into 4 classes and the data distribution was more compact (Fig. 2E and F), indicating that the 4 simplified indicators were a good representation of the 11 indicators for quality evaluation of *Sojae Semen Praeparatum* (Table S4).

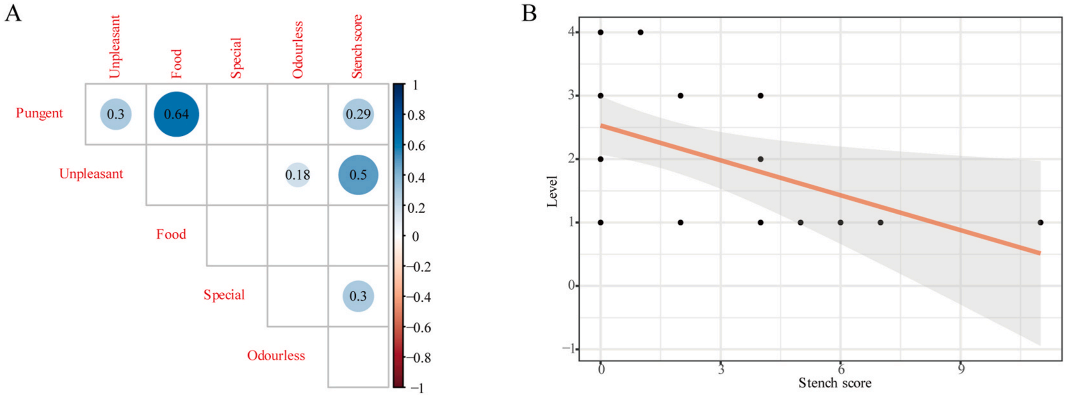

Fig. 4. Correlation of *Sojae Semen Praeparatum* stench scores with odour (A) and level (B). (B) $p < 0.05$, R=R = 0.382.

3.5. Correlation between volatile components and the degree of fermentation of Sojae Semen Praeparatum

We consider a good quality *Sojae Semen Praeparatum* to be fully fermented rather than over-fermented. However, the previous results do not allow a full determination of the degree of fermentation. Volatile components (VOCs) are produced during fermentation, some samples in class A showed undesirable odours through sensory evaluation. Thus, HS-GC-MS were used to further detection of VOCs. The 22 VOCs can be classified as pungent, unpleasant, food-like, special, and odourless (Fig. 3 A). The volatile content of *Sojae Semen Praeparatum* varied considerably between companies and batches, with the overall VOCs of *Sojae Semen Praeparatum* samples from the company being higher than that from the market (Table S7), suggesting that adequate fermentation may increase the odour of *Sojae Semen Praeparatum*. We considered samples in class A as a fully fermented product. However, there are more samples in class A containing a greater abundance of VOCs with pungent, unpleasant odours (Fig. 3 B). This suggests that although samples in class A has shown a good degree of fermentation, some of the samples were over-fermented and produced undesirable odours. This is also consistent with the sensory evaluation results, where the samples with the highest stench scores were all from class A (Table S7). Those from Classes C and D showed a lighter odour, which may be because these *Sojae Semen Praeparatum* had been stored for a long time before being purchased, causing their odour to dissipate. Overall, we found that the better quality of *Sojae Semen Praeparatum* generally had a stronger odour, and the lower grades had a lighter odour, which may be related to the degree of fermentation ($p < 0.05$, $R = 0.382$) (Fig. 4).

Volatile fatty acids have a strong odour. In an anaerobic, low-acid environment, the anaerobic bacterium Clostridium butyricum decomposes carbohydrates into short-chain volatile fatty acids, such as butanol, acetic acid and butyric acid. Butyric acid has a pungent and unpleasant smell, and a very dilute solution has a sweaty smell, which is one of the sources of odour in *Sojae Semen Praeparatum*. 2-methylbutyric acid and isovaleric acid have an irritating smell in high concentrations and can be used in edible flavours at low concentrations. They are also a source of odour in *Sojae Semen Praeparatum*, probably because they have a similar metabolic pathway to n-butyric acid, or because the superposition of different flavours can amplify the odour. Dimethyl disulphide and trimethylamine are both products of microbial fermentation of proteins and have a strong odour. Post-fermentation is the key stage for the production of flavour substances in *Sojae Semen Praeparatum*, and probes can be added to detect levels of pungent and unpleasant odour compounds such as n-butyric acid, dimethyl disulphide, and trimethylamine to better control fermentation.

4. Conclusion

The quality of fermented foods is influenced by raw materials and process conditions. This study provides an effective attempt to evaluate the quality of *Sojae Semen Praeparatum*. 11 landmark indicators were chosen to identify the grade of *Sojae Semen Praeparatum*. Using multivariate statistics, four representative indicators were selected, which could divide *Sojae Semen Praeparatum* into 4 quality categories with a correct rate of over 75%. For the over-fermentation of *Sojae Semen Praeparatum*, we used HS-GC-MS to identify the VOCs of *Sojae Semen Praeparatum*. Of the four categories of odours, we found two categories, 'pungent' and 'unpleasant', that could be associated with over-fermentation. Our study provides a reference for the development of more detailed fermentation specifications and quality standards for *Sojae Semen Praeparatum*.

Author contribution statement

Jiaqi Xie: Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Yibo Wang: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data. Rongrong Zhong; Zhenshuang Yuan: Performed the experiments; Analyzed and interpreted the data. Jie Du; Jianmei Huang: Conceived and designed the experiments.

Data availability statement

Data will be made available on request.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18767.

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