Determination of biogenic amines in Shanlan liquor by HPLC

Ningli QI1, Chuyuan QIU2, Pei LI1, Jianzhi YE1, Wuhai CHEN1, Chunliang YANG1,*

1 Center for Food Quality Supervision & Testing of Ministry of Agriculture and Rural Affairs (Zhanjiang), Agricultural Products Processing Research Institute, Chinese Academy of Tropical Agricultural Sciences, Zhanjiang, 524001, China;
2 School of Chemistry and Chemical Engineering, Lingnan Normal University, Zhanjiang 524037, China.
*Corresponding author: zjyangcl@126.com (C. Yang)

Abstract: A survey of biogenic amine species and content of traditional Chinese Li Nationality Shanlan liquor was carried out. Eight major biogenic amines including histamine, cadaverine, tryptamine, putrescine, tyramine, phenylethylamine, spermidine and spermine were separated by reversed-phase HPLC-DAD on Inertsil ODS-SP column after pre-column derivatisation with dansyl chloride. The results showed the eight biogenic amines were not distributed in Shanlan liquor, and the content and type of biogenic amines in different Shanlan liquors were different. Overall, total content of biogenic amines ranging from 1.02 to 38.42 μg/g were within the safe level for human health.

1. Introduction
Shanlan liquor has very obvious geographical characteristics. It is known as “Shanlan Yuye” and Qiongjiang Yuye[1]. Shanlan liquor, also known as “biang” liquor, is a kind of fermented food produced in Hainan region with typical Li nationality characteristics. It uses the unique local dry rice (Shanlan rice) as raw material and is cooked. Saccharification, inoculation of koji with local special spices, made by traditional natural fermentation. The Shanlan liquor is also known as the “Moutai” of the Li people. Just like the champagne in foreign countries, it is usually taken out on a VIP or a major festival[2]. It is delicious, low in alcohol, sweet, and rich in a variety of glucose, amino acids, vitamins, and the right amount of organic acids, maltose and a variety of mineral elements, favored by consumers. The Shanlan liquor is mainly based on workshop-style processing. It belongs to open semi-solid fermentation. The process is simple. The artificial fermentation process is controlled by artificial experience. The quality of the koji used in brewing is also unstable[3]. As the aging time is extended, the liquor is red or even black, which is the top grade. The Shanlan liquor is deep and shallow, and it has a strong stamina. It is often used for longevity[4]. Some microorganisms in the Shanlan liquor produce harmful substances such as biogenic amines, which leads to uncontrollable brewing process and unstable quality, which poses potential harm to human health.

Biogenic amine (BA) is a general term for a class of bioactive, amino-containing low molecular weight organic compounds. According to the structure of biogenic amines (BAs), it can be divided into three parts: cadaverine, putrescine, spermine, spermine, and other aliphatic amines; phenylethyl, tyramine. An aromatic amine such as phenylethylamine; a heterocyclic amine such as histamine or tryptamine[5]. They are widely found in foods, such as frozen pork[6], and are precursors for the synthesis of proteins, hormones, nucleotides, alkaloids, and aromatic compounds. Appropriate intake
of BAs can promote growth, enhance metabolic activity, enhance immunity and scavenge free radicals, but excessive intake of BAs can cause adverse physiological reactions such as headache, abdominal cramps and vomiting. Its potential toxic effects and indication of food freshness have attracted the attention of a large number of researchers. Certain microorganisms with strong amino acid decarboxylase activity may produce large amounts of biogenic amines during their decomposition of amino acids [7]. Therefore, BAs are widely found in fermented foods such as yogurt [8], cheese [8], alcohol [9], soy sauce [10], stinky tofu [11], vinegar [12], and kimchi [13]. High concentrations of BAs are observed in foods with higher protein content, especially in fermented foods [14]. Biogenic amines have toxic effects. If the body ingests excessive amounts of foods containing BAs, it can cause poisoning and even endanger life [15].

At present, domestic and foreign scholars have done a lot of research on BA detection, in some fermented foods (such as soy products and alcohol) [16]. Studies have been conducted on the production of amines, such as Flasarová et al. [17], for the production of BAs from Lactococcus lactis subspecies. Some people have studied from the organism itself. For example, Zare et al. [18] evaluated the quality of fish meat by evaluating the quality of sardines based on the bio-amine-based fuzzy mathematical model. A fuzzy logic model based on BA content was proposed. Baciak et al. [19] studied the presence of BAs in the duckweed. The results showed that tetracycline had a certain toxic effect on the growth rate, yield and chlorophyll a and b contents of D. mongolica. Tetracycline had no significant effect on carotenoid content. The study found that tetracycline, as a water pollutant, is a stress factor that triggers an increase in amine synthesis. Baciak To date, there have been many studies on BAs in liquor. For example, Redruello et al. [20] used ultra-efficient convergent chromatography to analyze nine biotins in beer, which can be a valuable tool for monitoring beer safety and quality. Lirui Cao et al. [21] determined the determination of biogenic amines in rice liquor by high performance liquid chromatography. The results showed that 9 kinds of biogenic amines (pyridoxamine hydrochloride, tryptamine, putrescine, cadaverine, β-phenylethylamine, histamine, tyramine, Spermine and spermidine are well separated in 40 min. Some scholars have used fluorescent autochromatography to determine beer BAs and amino acids. This method has been validated and applied to liquor and beer, achieving a good recovery rate (72.3-138.4%). Conghan Chen et al. [25] used high performance liquid chromatography to determine the content of BAs in rice liquor. The results showed that β-phenylethylamine and putrescine were detected in the tested rice liquor. Spermidine and tyramine were only detected in some rice liquors. Both are below 0.5 mg/L, within the safety standards. Therefore, BA analysis has become an important and routine component of food contamination and quality analysis.

Shanlan liquor is an important traditional fermented liquor in Hainan, so its BA content should be paid enough attention. In this study, HPLC method was used to determine the BAs of Shanlan liquor, in order to provide basis for the BA control in Shanlan liquor.

2. Materials and methods

2.1. Chemicals and reagents

Ingredients: Different varieties of Shanlan liquor were taken from samples of 9 different varieties. Cadaverine (Cad), histamine (His), putrescine (Put), phenylethylamine (Phe), spermine (Spm), tyramine (Tyr), spermidine (Spd), tryptamine (Try), dansyl chloride (Dns-Cl) and 1,7-heptanediamine (internal standard, IS) were purchased from Fluka (Buchs, Switzerland). Ultrapure water (<18.2 MΩ cm⁻¹ resistivity) was prepared with a Millipore ultrapure system (Millipore, Bedford, MA, USA). Acetone and HPLC-grade solvents of acetonitrile were obtained from Fisher Scientific Co. (Pittsburgh, PA, USA).

2.2. Standard preparation and calibration

A standard stock solution was prepared by adding 50 mg of each standard to a 50 mL volumetric flask and diluting to a volume with 0.4 mol/L HClO₄. Calibration standards for each amine were constructed
using six concentrating stock solutions with appropriate HClO₄ dilutions.

2.3. Sample extraction and derivatisation

Different varieties of Shanlan liquor were extracted from the Li nationality in Hainan (In 2019). A representative sample was homogenized using a mixer (ZLD 500, Zonce, China), and then 5 g of the resulting slurry was weighed into a centrifuge tube and mixed with 10 mL of 0.4 mol/L HClO₄ containing a known amount of internal standard. The vortex was mixed for 5 minutes. After the resulting homogenate was centrifuged at 1800 g for 10 minutes (4 °C), the aqueous layer was collected as described above and the residue was re-extracted. The combined extracts were filtered and diluted to 25 mL with 0.4 mol/L HClO₄ and taken in volume flask. Derivatives of BAs were prepared according to the method of Ben-Giglrey et al.[26].

Derivatives of BAs were prepared according to the method of Ben Giglrey et al.. For the sake of simplicity, add 1 mL of extract or standard amine solution to 100 μL of internal standard (0.4 mg/mL) and in plastic test tube with 200 μL of 2.0 mol/L NaOH, 300 μL of saturated NaHCO₃ and 2.0 mL of Dns- Cl (10 mg/mL ) Mix in acetone. After complete mixing, the tubes were incubated at 40 °C for 45 minutes with continuous shaking in the dark. After that, 100 μL of 25% NH₄OH was added to each sample to remove residual Dns-Cl. After the resulting solution was allowed to stand at RT for 30 minutes, the mixture was made into a mixture having a final volume of 5.0 mL with acetonitrile. Prior to analysis, the supernatant was filtered through a 0.22 μm filter and maintained at -20 °C.

2.4. HPLC analysis

High performance liquid chromatography was carried out using LC-10A liquid chromatography (Shimadzu, Japan) and Inertsil ODS-SP column (150 mm × 4.6 mm, 5 μm; GL Scientific, Tokyo, Japan) and a PDA detector. A gradient elution system consisting of water (solvent A) and acetonitrile (solvent B) started at 65% B, increased to 80% B in 5 minutes by a linear gradient, and increased to 90% B in 10 minutes and remained for 5 minutes. Then return to the initial ingredients within 5 minutes. The total run time for the analysis was 23 minutes and the flow rate was 0.8 mL/ min. The column temperature was maintained at 30 °C. A volume of 10 microliters was injected into the HPLC system. The composition was initially determined by comparing the retention time with the retention time of the authentic standard under the same analytical conditions of 254 nm. The analytical method was validated and the following parameters were determined: linearity, LOD and LOQ, precision and accuracy. The linear range was obtained by injecting six concentrations of BAs standard solutions. The linearity of the calibration curve was evaluated by linear regression analysis, and the relationship between the peak area and the standard concentration was plotted. The correlation coefficient (R²) was calculated and used to evaluate linearity. LOD was considered to be the lowest analyte concentration and gived a 3:1 signal to noise ratio. LOQ was the lowest concentration with an S/N ratio of 9:1. The accuracy of this method was estimated by measuring the concentration of the simulated solution six times in succession. Accuracy was assessed by a recycling test.

2.5. Statistical analysis

Statistical analysis was performed with SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). All data were expressed as the average of at least three measurements per sample.

3. Results and analysis

3.1. Calibration curve, LOD, LOQ and recovery values
Table 1. Linearity, precision, LOD, LOQ and recovery values.

| BAs | Calibration curve | R²   | LOD  | LOQ  | Recovery (%) | RSD(%) |
|-----|-------------------|------|------|------|--------------|--------|
| Tyr | y=1074.9x-1033.9 | 0.995 | 0.20 | 0.61 | 89.9         | 2.59   |
| Phe | y=1331.9x-956.3   | 0.996 | 0.58 | 1.76 | 92.3         | 1.09   |
| Put | y=2699.9x-2678    | 0.995 | 0.10 | 0.31 | 92.6         | 1.47   |
| Cad | y=3069.2x-30482   | 0.997 | 0.09 | 0.29 | 93.5         | 1.22   |
| His | y=2401x-11799     | 0.998 | 0.15 | 0.46 | 95.2         | 1.53   |
| Try | y=1984.2x+7482.6  | 0.999 | 0.13 | 0.40 | 96.9         | 1.85   |
| Spd | y=2759.2x-2496.1  | 0.999 | 0.10 | 0.32 | 92.8         | 1.52   |
| Spm | y=3112.2x-24006   | 0.997 | 0.07 | 0.23 | 95.7         | 1.08   |

The correlation coefficient R² of the eight bioamine regression equations was greater than 0.999, indicating that the regression models of each biogenic amine standard were significant (Table 1). The recovery of BA in the mulberry sample was 89.9% (Tyr) to 96.9% (Try), and the corresponding RSD was 1.08-2.59% (Table 1). These advantages make the method of the present method meet the detection requirements of BA in conventional types of liquor.

3.2. Biogenic amines in Shanlan liquor

Table 2. Bioamine content in selected Shanlan liquor

| Variety | Tyr content mean±SD (μg/g) | Put content mean±SD (μg/g) | Cad content mean±SD (μg/g) | Spd content mean±SD (μg/g) | Try content mean±SD (μg/g) | Phe content mean±SD (μg/g) | Spm content mean±SD (μg/g) | His content mean±SD (μg/g) | Total content |
|---------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------|
| 1       | 0.89±0.18                 | 4.86±1.18                 |                          |                          |                           |                           |                           |                           | 5.78          |
| 2       | 1.12±0.23                 |                           |                          |                           |                           |                           |                           |                           | 1.32          |
| 3       | 0.86±0.19                 |                           |                          |                           |                           |                           |                           |                           | 1.07          |
| 4       | 0.73±0.10                 |                           |                          |                           |                           |                           |                           | 1.17±0.22      | 2.12          |
| 5       | 1.14±0.20                 |                           |                          |                           |                           |                           |                           |                           | 1.26          |
| 6       | 1.02±0.11                 |                           |                          |                           |                           |                           |                           |                           | 1.02          |
| 7       | 4.78±1.28                 | 7.50±1.46                 | 1.75±0.25                | 14.38±1.78                |                           |                           |                           |                           | 29.35         |
| 8       | 7.42±1.45                 | 7.45±1.45                 |                          |                          |                           |                           |                           |                           | 38.42         |
| 9       | 5.29±1.29                 | 4.95±1.20                 |                          |                          |                           |                           |                           |                           | 25.31         |

The eight biogenic amines studied in this experiment were not completely detected in the nine samples of Shanlan liquor, and one sample detected up to five biogenic amines. The results were summarised in Table 2. Among them, Put was present in nine kinds of ylang liquor samples, the content of which was 0.89-7.50 μg/g. It was known that Put was the main biogenic amine of mulberry. Try and Phe were not detected. His and Cad were only present in one sample, and their contents were 1.17 μg/g and 1.75 μg/g, respectively. The sixth sample only detected the presence of Put at a content of 1.02 μg/g. The Tyr content was 4.78-7.42 μg/g, the Spd content was 4.86-22.31 μg/g, and the Spm content was 0.03-1.24 μg/g. There were significant differences in biogenic amine content in different Shanlan liquor samples, and the total biogenic amine content was 1.02-38.42 μg/g. The content of biogenic amines in mulberry liquor may be related to the production conditions, storage time and storage conditions of Shanlan liquor, and further research is needed.

4. Conclusion

In conclusion, the proposed HPLC method after precolumn derivatisation with Dan-Cl for the analysis of BAs in Shanlan liquor showed high recoveries, good resolution and sensitivity, as well as no interferences in the samples. The content of BAs in the main traditional fermented foods produced in
Hainan Province was investigated. The results showed that there were different kinds of BAs in the mountain liquors, and the samples varied greatly.

Biogenic amines are toxic. Histamine has the greatest impact on human health, and tyramine is second. Excess histamine can cause food poisoning and even life-threatening. Therefore, it is very important to strictly control the production of mulberry liquor, control its raw materials and processing environment, and avoid microbial contamination. It is also possible to inhibit the growth of microorganisms or the activity of decarboxylase to control the content of biogenic amine by changing the production process and controlling factors such as pH and temperature.

Many countries have already made a limited range of histamine in wine [27] and biogenic amines in beer [28]. However, there is no uniform limit standard for biogenic amines in China. The average content of biogenic amines in Chinese traditional alcoholic rice liquor is as high as 115 mg/L [29], and the content of histamine in rice liquor is between 5.02 and 78.50 mg/L [30]. The total biogenic amine content of Shanlan liquor in this study was 38.42 μg/g, and the His content was 1.17 μg/g. Therefore, Shanlan liquor of this study was safe. In this study, to a certain extent, it can provide a certain reference value for the local production of Shanlan liquor in Hainan Li nationality, promote the large-scale production of mulberry liquor, and promote the economic benefits of Shanlan liquor.

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