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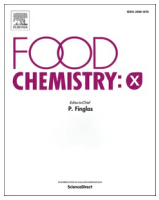

High-sensitivity profiling of dipeptides in sauce-flavor Baijiu Daqu by chemical derivatization and ultrahigh-performance liquid chromatography coupled with high-resolution mass spectrometry

Zixuan Wang [a,c,1], Liangqiang Chen [b,d,1], Fan Yang [b,d], Xiaolin Wang [a,c], Yang Hu [b,d], Ting Wang [a,c], Xin Lu [a,c], Jianjun Lu [b,d], Chunxiu Hu [a,c,*], Huabin Tu [b,d,*], Guowang Xu [a,c]

[a] CAS Key Laboratory of Separation Sciences for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China
[b] Kweichow Moutai Co., Ltd, Renhuai, Guizhou 564501, China
[c] Liaoning Province Key Laboratory of Metabolomics, Dalian 116023, China
[d] Kweichow Moutai Group, Renhuai, Guizhou 564507, China

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ABSTRACT

Dipeptides in sauce-flavor Baijiu Daqu are protein degradation products during the fermentation of Daqu, which are believed to play a crucial role in the flavor and quality of Baijiu. Herein, we integrated dansyl chloride derivatization with ultrahigh-performance liquid chromatography coupled with high-resolution mass spectrometry (UHPLC-HRMS) for comprehensively profiling dipeptides in Daqu. The derivatization efficiency was higher than 99.1 % for all 17 dipeptide standards under the optimized derivatization conditions. In total, 118 dipeptides were detected in Daqu. The method was validated and the analytical characteristics including the linearity (spanned across 2–4 orders of magnitude), precision (1.2–19.9 %), limit of detection (varied from 1.1 to 53.4 pmol/mL) and the stability (3.6–15.8 %) are satisfactory. The usefulness of the method was examined by studying the distribution characteristics of dipeptides in Daqu under different production conditions. The present method provides an effective and robust strategy for comprehensively analyzing dipeptide compounds in complex biological samples.

1. Introduction

Chinese Baijiu is a traditional alcoholic liquor with a rich history. Daqu is the brewing raw material made from wheat, sorghum, peas and other materials, and serves as the starter, saccharifying agent and fragrance agent in the brewing process (Zheng, Tabrizi, Nout, & Han, 2011). Attributed to the openness of Daqu-making process, there are complex synergies among microorganisms, enzyme systems and metabolic substances in the fermentation process of Daqu, which eventually produces a wide variety of special substances, and further impacts the quality and flavor of Baijiu (Gou, Wang, Yuan, Zhang, Tang, & Kida, 2015; Xiao et al., 2021). Protease is one of the most crucial enzymes in the fermentation process of Daqu (J. Liu, Chen, Fan, Huang, & Han, 2018). It is produced by bacillus and lactic acid bacteria, which dominate the early stages of Daqu production (Garcia-Cano, Rocha-Mendoza, Ortega-Anaya, Wang, Kosmerl, & Jimenez-Flores, 2019; Jin et al., 2019; Wei, Huang, Huang, Deng, Yang, & Qiao, 2014), causing active protein degradation and producing a variety of small peptides.

Dipeptides are small peptides containing two amino acids, which constitute important products of protein degradation during Daqu fermentation. It has been reported that dipeptides in fermented foods make a significant contribution to the flavor of food (Alejandro Heres, Yokoyama, Gallego, Toldrá, Arihara, & Mora, 2021; Kitagawa, Mukai, Furukawa, Adachi, Mizuno, & Iefuji, 2008; Takahashi, Tokuoka, Kohno, Sawamura, Myoken, & Mizuno, 2012). Dipeptides can produce tastes such as sweet, salty and umami (A. Heres, Gallego, Mora, & Toldra, 2022; Yamamoto et al., 2014), while their amino groups can react with the carbonyls of reducing carbohydrates to cause the Maillard reaction, further changing the color, aroma and nutritional value of the food (Van Lancker, Adams, & De Kimpe, 2010). Additionally, certain bioactive dipeptides may exhibit antagonistic effects on certain bacterial species, affecting the bacteria distribution in Daqu (R. Liu, Kim, Kwak, & Kang,

* Corresponding authors.
E-mail addresses: hucx@dicp.ac.cn (C. Hu), Tumoutai2023@163.com (H. Tu).
[1] Authors contributed equally to this work.

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2017). Therefore, a comprehensive characterization of dipeptides in Baijiu Daqu can provide valuable help for assessing Daqu quality and regulating the overall quality of Baijiu.

So far, there have been no reports on the establishment of a method for analyzing dipeptides in Baijiu Daqu. Currently, the analysis and determination of dipeptides in fermented foods mainly rely on liquid chromatography-tandem mass spectrometry (LC-MS/MS) technology. For example, Gallego et al. (Gallego, Toldra, & Mora, 2022) established a method using hydrophilic interaction LC combined to MS/MS (HILIC LC-MS/MS) for the quantification of 7 taste dipeptides in dry-cured ham. Zhao et al. (Zhao & Ganzle, 2016) reported a method for the identification of γ-Glutamyl dipeptides in sourdough by LC-MS/MS. However, only a limited number of dipeptide compounds in food were detected in these studies, which greatly restricted the observation of the overall composition and changes of dipeptides in sample matrix.

Dipeptides are polar small-molecule compounds that exhibit poor retention on reversed-phase liquid chromatographic columns and are susceptible to matrix interference, making it challenging to achieve highly sensitive MS detection. Dansyl chloride is a highly efficient and stable chemical derivatization reagent that can significantly improve the chromatographic separation and MS detection sensitivity of amino compounds (Goodwin et al., 2019; Jia, Kang, Park, Lee, & Kwon, 2012; Wu, Wang, Li, & Yu, 2019; Yu, Lewis, Gray, & Dalleska, 2020). Therefore, dansyl chloride may be an appropriate derivatization reagent for the analysis of dipeptides in Daqu. In a recent study, Cheng et al. (Cheng & Li, 2023) reported a method for analyzing approximately 400 dipeptides in rice wines and human serum utilizing dansyl chloride derivatization combined with LC coupled to high-resolution MS (LC-HRMS), proving the potential of dansyl chloride for derivatizing dipeptides in biological samples. However, many other amino compounds, such as amino acids present in Daqu, may compete with dipeptide derivatization reagents, resulting in incomplete reactions. Therefore, the traditional reaction system used in the dansyl chloride derivatization method for amino compounds in food is probably not fully applicable to dipeptides in Daqu.

In this study, we developed a highly sensitive method using dansyl chloride derivatization coupled with UHPLC-HRMS for the comprehensive analysis of dipeptides in Baijiu Daqu. First, the reaction conditions of dansyl chloride derivatization were optimized. Then, the dipeptides in Daqu were characterized comprehensively using a non-targeted metabolomics strategy based on UHPLC-HRMS. A total of 118 dipeptide compounds were detected in sauce-flavor Baijiu Daqu. The method was further applied to study the effects of different operation conditions during Daqu-making process on the metabolic characteristics of dipeptides in Daqu, which could serve as a valuable reference for controlling the operation conditions of Baijiu Daqu and evaluating its quality.

2. Materials and methods

2.1. Chemicals and reagents

Methanol and acetonitrile (HPLC-grade) were purchased from Merck (Darmstadt, Germany). Ultra-pure water was prepared by Milli-Q system (Millipore, Bedford, MA, USA). Formic acid (HPLC-grade) and dansyl chloride (>98 %) were obtained from J&K (Beijing, China). Chloroform (HPLC-grade) was purchased from Kermel (Tianjin, China). Sodium hydroxide (AR), sodium carbonate anhydrous (>99.8 %) and sodium bicarbonate (>99.5 %) were purchased from Meilun Biological Technology (Dalian, China). L-Serine-1-^{13}C (Ser-^{13}C) (98 %) and DL-Phenyl-D_5-Alanine (Phe-d_5) (98 %) were purchased from Sigma-Aldrich (St Louis, MO, USA). All 17 dipeptide standards including Gly-Gly, Ile-Ala, Leu-Ala, Ile-Ser, Ser-Ile, Val-Thr, Thr-Val, Thr-Thr, Leu-Pro, Ile-Val, Leu-Val, Asp-Val, Val-Asp, Thr-Leu, Leu-Thr, Thr-Asp and Asp-Asp were purchased from Nanjing Yuan-peptide Biotech Co. Ltd (Nanjing, China). The purity of all dipeptide standards is greater than 95 %.

2.2. Stock solution and calibrators

All dipeptide standard stock solutions were separately prepared in Milli-Q water at 2 mg/mL. Dipeptide mixture standard working solutions were obtained by mixing equal volume of the stock solutions and diluted stepwise to the concentrations of 10000, 5000, 2500, 1250, 500, 250, 100, 50, 25, 12.5, 5, 2.5, 1, 0.5, 0.25, 0.125, 0.05, 0.025, 0.01, 0.005, 0.0025, and 0.00125 ng/mL. Phe-d_5 and Ser-^{13}C were used as internal standards (IS) and separately prepared in Milli-Q water at 2 mg/mL.

2.3. Sampling and sample preparation

Daqu samples were obtained from Kweichow Moutai (Guizhou, China). All samples were ground using a grinder (Retsch MM440, Germany) at 25 Hz for 5 min and stored at −80 °C until sample preparation. QC samples were prepared by mixing equal weights of all ground Daqu samples. Detailed sample information is provided in Table S1 in Supporting Information.

Firstly, 5 mg of ground Daqu samples was weighed in a 2 mL Eppendorf tube. Then, 500 μL of methanol was added and the mixture was allowed to soak overnight at 4 °C. After that, 500 μL of chloroform and 200 μL of ultra-pure water were successively added to the tube. The obtained mixture was shaken for 30 min at 1000 rpm, 4 °C using a ThermoMixer C (Eppendorf, Hamburg, Germany). Subsequently, the mixture was subjected to an ultrasound bath in ice water using a 5510E-DTH ultrasonic cleaner (Branson, USA) for 10 min at a frequency of 40 kHz. After centrifugation at 14000*g*, 4 °C for 20 min, 300 μL of the upper aqueous layer was carefully transferred to a new 1.5 mL Eppendorf tube. Subsequently, 100 μL of IS solution was added and mixed well with the supernatant. The resulting mixture was then dried under a stream of nitrogen and the residue was stored at −80 °C prior to derivatization.

The chemical derivatization was performed according to a previously reported method with some modifications (Guo, 2009). First, 250 mg of dansyl chloride was suspended in 5 mL of acetonitrile to prepare the dansyl chloride suspension. Then, 50 μL of dansyl chloride-acetonitrile suspension, 50 μL of Na_2CO_3/$NaHCO_3$ buffer solution with a pH of 9 (0.5 M), and 2 μL of NaOH solution (2 M) were added to the dried sample residue. The mixture was vortexed for 1 min and then incubated at 4 °C for 75 min. After centrifugation at 14000*g*, 4 °C for 5 min, the supernatant was transferred for UHPLC-HRMS analysis.

2.4. UHPLC − HRMS analysis and dipeptide identification

The chromatographic separation was performed on a 1290 series UHPLC system (Agilent, Santa Clara, CA, USA) equipped with an ACQUITY BEH C8 (2.1 mm × 100 mm, 1.7 μm) column (Waters, Milford, MA, USA). The column temperature was 50 °C. The flow rate was 0.35 mL/min. Mobile phase A consisted of 0.1 % (v/v) formic acid in water while mobile phase B consisted of 0.1 % (v/v) formic acid in acetonitrile. The initial gradient was 5 % B and maintained for 1 min, then linearly increased to 100 % B in 23 min, held for 4 min. Finally, the gradient was reduced to 5 % B in 0.1 min and balanced for 1.9 min.

The untargeted MS data were acquired by a 6546 series quadrupole time-of-flight (Q-TOF) MS system (Agilent, Santa Clara, CA, USA). The spray voltage was 4.0 kV. The desolvent gas flow rate was 8 L/min and the temperature was 320 °C. The sheath gas flow rate was 11 L/min and the temperature was 350 °C. The untargeted data were acquired in positive ion mode. The full scan *m/z* range was from 100 to 1300 Da. The conditions for auto-MS/MS analyses were set as follows: the collision energies were 15, 30 and 45 eV; the max precursor per cycle was set as 5; the precursor threshold was set as 1000 counts; the number of injections for iteration was set as 20; and the *m/z* scan range for MS^2 was from 100 to 1000 Da.

Dipeptides in Daqu samples were identified by exact mass, retention time and peptide bond fragments based on the dipeptides/tripeptides

database built previously (Lu et al., 2023). The tolerance of exact mass and retention time were defined as 10 ppm and 0.46 min, respectively. Compounds that were not detected in more than 80 % of the samples, or those with a calibrated peak area relative standard deviation (RSD) greater than 30 % in QC samples, were excluded from the final quantitative dataset.

2.5. Data processing and statistical analysis

Peak detection was performed by Agilent MassHunter Qualitative Analysis 10.0 (Agilent, Santa Clara, CA, USA). Peak alignment and integration were performed by Agilent MassHunter Profinder 10.0. Principal component analysis (PCA) was performed by SIMCA-P 13 software (Umetrics, Umea, Sweden). Heatmap analysis and nonparametric test were performed by MultiExperiment Viewer (MeV.4.9.0) (Boston, MA, USA). Volcano plots were fulfilled by GraphPad Prism 5.0 software.

2.6. Method validation

A standard mixture containing 17 dipeptides was used for method validation. A series of dipeptide mixture standard working solutions (0.00125, 0.0025, 0.005, 0.01, 0.025, 0.05, 0.125, 0.25, 0.5, 1, 2.5, 5, 12.5, 25, 50, 100, 250, 500, 1250, 2500, 5000 and 10000 ng/mL) prepared in section 2.2 were used for linearity evaluation. Briefly, 100 μL of each dipeptide mixture standard working solution was mixed with 10 μL of IS solution (Phe-d_5 and Ser-^{13}C at 1 μg/mL). The resulting mixed solution was then dried under N_2 followed by derivatization with dansyl chloride. The linear range was evaluated by plotting the regression curve of IS-calibrated peak area of dipeptide derivates against their injection concentration. Given the wide range of concentrations, the weighted least squares (WLS) linear regression with a weighting factor of $1/x^2$ was adopted for linear fitting. Relative error (RE%) was calculated for evaluating the regression curve.

$$\%RE = |[(C_{meas}\text{-}C_{theo})/C_{theo}] \times 100|$$

where C_{meas} and C_{theo} are the measured and theoretical values of the substrate concentration, respectively.

The limit of detection (LOD) and the limit of quantification (LOQ) were determined as the minimum injection concentration detected at the signal-to-noise ratio was 3 and 10, respectively.

Regarding the inter-day and intra-day precisions, three different concentrations (i.e., low, medium, and high levels) of mixture standard solutions were respectively added into the QC samples. Intra-day precision was performed by analyzing samples prepared in six parallel in one day ($n = 6$) and inter-day precision was evaluated by analyzing samples prepared in triplicates in three continuous days ($n = 3 \times 3$). Six QC samples mixed with dipeptide standard solution (100 ng/mL) were processed in parallel for evaluating method repeatability ($n = 6 \times 3$).

The extraction recovery was evaluated by calculating the peak area ratio of the derivatives of dipeptide standards spiked in blank matrix solution (5 % methanol, v/v) before to after the extraction at low, medium, and high levels (25, 100, and 1250 ng/mL).

$$\text{Recovery } (\%) = (A_{pre}/A_{post}) \times 100$$

where A_{pre} and A_{post} represent the detected peak area of the derivatives of dipeptide standards which spiked before and after sample preparation, respectively.

The stability of the dipeptide derivatives of dansyl chloride in 48 h was also investigated. In brief, a Daqu QC sample mixed with dipeptide standard solution (100 ng/mL) was processed and placed at 4 °C after derivatization. After that, the peak areas of dipeptide derivates at 0 h, 8 h, 24 h, 32 h, and 48 h were determined, respectively. The RSDs of the IS-calibrated peak area of dipeptide derivates were calculated for investigating the stability of dansyl chloride derivates within 2 days.

3. Results and discussion

3.1. Selection of derivatization reagent

Benzoyl chloride and dansyl chloride have been well-recognized as derivatization reagents that can enhance the MS response of amino compounds. To determine the appropriate derivatization reagent for dipeptides, we compared these two acyl chloride reagents. Specifically, 100 μL of a mixture solution containing 17 dipeptide standards was dried under N_2 and then derivatized with benzoyl chloride and dansyl chloride, respectively. The derivatization of benzoyl chloride occurred under the following conditions: 50 μL of benzoyl chloride-acetonitrile solution (2 %, v/v) and 50 μL of ammonium bicarbonate buffer solution (150 mM, pH = 8.2) were added to the residue of dipeptide standard mixture. The reaction was carried out at room temperature for 30 min after a thorough vortex. As shown in Fig. S1, the MS response of the dansyl chloride derivatives was superior to that of the benzoyl chloride derivatives. This is probably attributed to the tertiary amine group present in dansyl chloride which is more easily ionized. Furthermore, it has been reported that at both 4 °C and room temperature, dansyl chloride derivatives display better stability compared to other acyl chloride reagents (S. J. Liu et al., 2018). Based on these findings, we selected dansyl chloride as the derivative reagent for dipeptides in Daqu.

3.2. Optimization of derivatization conditions

To obtain the best derivatization efficiency, we optimized the conditions of the derivatization reaction. Firstly, the pH of the reaction was optimized. Specifically, 50 μL of dansyl chloride-acetonitrile suspension (50 mg/mL) and an equal volume of $Na_2CO_3/NaHCO_3$ buffer solution were added to six dried Daqu QC samples. Then, 2 μL of NaOH solution at different concentrations ranging from 0.02 to 2 mM were spiked into the system. The mixture was then incubated at 60 °C for 60 min. To optimize the amount of derivatization reagent, varying volumes of dansyl chloride suspension were spiked into the system. The amount of dansyl chloride in the reaction system ranged from 0.09375 to 2.5 mg. An equal volume of buffer solution was added to the solution and then the mixture was incubated at 60 °C. The reaction time was investigated from 10 to 90 min. Finally, the reaction efficiencies were compared at both low (4 °C, 10 °C) and high temperatures (40 °C, 60 °C).

The chromatographic peak response of the derivatization products was used to evaluate the reaction efficiency. The results are shown in Fig. 1. For the pH, the largest peak response of the derivative products of dipeptide standards was obtained when 2 μL of NaOH solution at 2 mM was added to the system, resulting in a pH value of 8.0 (Fig. 1A). This could be attributed to the fact that the hydrochloric acid (HCl) produced during dansylation can be neutralized in an alkaline environment, thereby facilitating the reaction. As shown in Fig. 1B, the peak response of dipeptide derivates gradually increased with the increase of the amount of dansyl chloride, reaching a maximum response when the derivative reagent weight was 2.5 mg (50 μL of dansyl chloride suspension at 50 mg/mL). Regarding the reaction time, the peak response of the derived dipeptide standards increased slightly with an extension of the reaction time. Most of the dipeptides achieved optimal derivatization efficiency at 75 min (Fig. 1C). Furthermore, we compared the derivatization efficiency at both low and high temperatures. In contrast to the traditional reaction temperature for dansyl chloride derivatization at 60 °C, the lower temperature (4 °C, 10 °C) produced a higher reaction efficiency for dipeptides (Fig. 1D). This could be attributed to the instability of the dipeptides under higher temperatures. Moreover, the dipeptide derivatives with a reaction temperature of 10 °C exhibited a slightly higher peak response compared to those with a reaction temperature of 4 °C, however, the difference was not significant. Given that the refrigerators in most laboratories were set at 4 °C, we chose 4 °C as

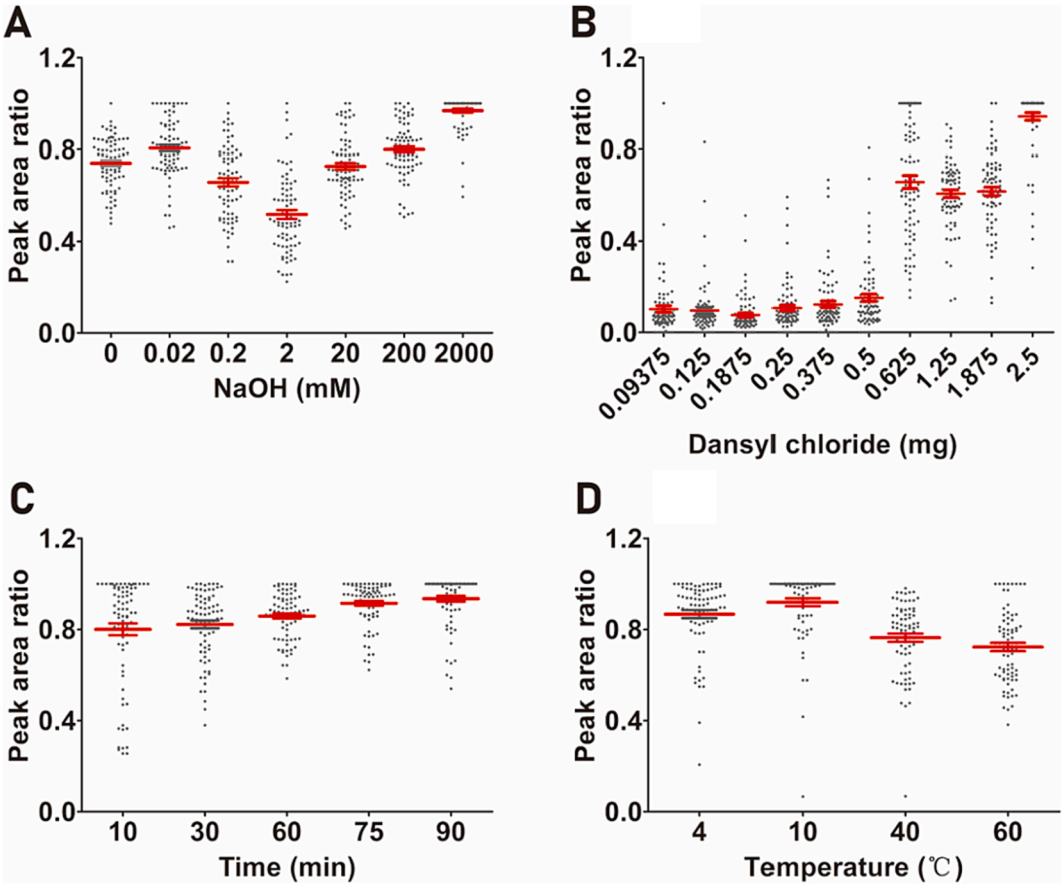

Fig. 1. Optimization of derivatization conditions. (A) Amount of NaOH, (B) Amount of dansyl chloride, (C) Reaction time and (D) Reaction temperature.

the temperature of the dansyl chloride derivatization for dipeptides. Typical extraction ion chromatogram of dipeptide dansyl chloride derivates at 4 °C and 60 °C is shown in Fig. 2.

Collectively, the optimal derivatization conditions of dansyl chloride derivatization for dipeptides were as follows: 2.5 mg of dansyl chloride (50 μL of dansyl chloride suspension at 50 mg/mL), 50 μL of $NaHCO_3$/Na_2CO_3 buffer (0.5 M), 2 μL of NaOH at 2 mM, reaction temperature at 4 °C and reaction time for 75 min.

Ultimately, the derivatization reaction efficiency of dansyl chloride was investigated under the optimized conditions. The reaction efficiency was evaluated by calculating the conversion rate of the dipeptide substrate.

$$\text{Conversion rate (\%)} = [(C_{pre}\text{-}C_{post})/C_{pre}] \times 100$$

where C_{pre} (5000 ng/mL) represents the concentration of dipeptide substrate in the reaction system before the derivatization, while C_{post} represents the concentration of the remaining dipeptide substrate after the reaction. C_{post} was calculated by standard regression curve of dipeptide substrate.

The results showed that the conversion rate for all dipeptides exceeded 99 % (Table 1). Meanwhile, approximately half of the residual substrates of the dipeptides were undetectable after derivatization, which indicated that a high reaction efficiency for dansyl chloride derivatization was achieved under the optimized conditions.

3.3. Profiling of dipeptides in sauce-flavor Baijiu Daqu

Both Full scan and auto-MS/MS data acquisitions for dipeptides in Daqu samples were performed using a UHPLC-HRMS-based non-targeted metabolomics strategy. The LC-MS conditions used for separating and detecting dipeptides in Daqu samples were the same as those described in our previous study. In that study, we developed a method for large-scale annotation of dipeptides/tripeptides in complex biological samples based on quantitative structure retention relationship (QSRR) (Lu et al., 2023) and established a dansyl chloride derivatized dipeptides/tripeptide library containing exact mass, retention time and peptide bond fragmentations. The dipeptides in Daqu samples were firstly matched with the exact mass and retention time in the library. Then the peptide bond fragmentations obtained by auto-MS/MS were used for further identification. In total, 118 dipeptides were detected in Daqu samples, most of them contained hydrophobic amino acids. This could be contributed to the large amount of hydrophobic amino acids that existed in wheat, the raw material of sauce-flavor Baijiu Daqu (Widyarani, Sari, Ratnaningsih, Sanders, & Bruins, 2016). The extracted ion chromatogram of the detected dipeptides is shown in Fig. 3. Detailed information on the 118 dipeptides is displayed in Table S2 in Supporting Information.

3.4. Method validation

To test the feasibility and effectiveness of the method for profiling dipeptides in complex biological sample matrix, 17 dipeptide standards were used to investigate the analytical characteristics in terms of linearity, LOD, LQD, intra-day and inter-day precisions, repeatability, recovery, and stability. Typically, ordinary least squares (OLS) regression is employed to construct linear regression models. However, the heteroscedasticity of instrument response could cause the predicted values of linear models to be unreliable in the low concentration range (Gu, Liu, Wang, Aubry, & Arnold, 2014). Weighted least squares (WLS) regression is one of the simplest and most effective methods, which could be a solution to this issue. Previous studies have compared OLS and WLS

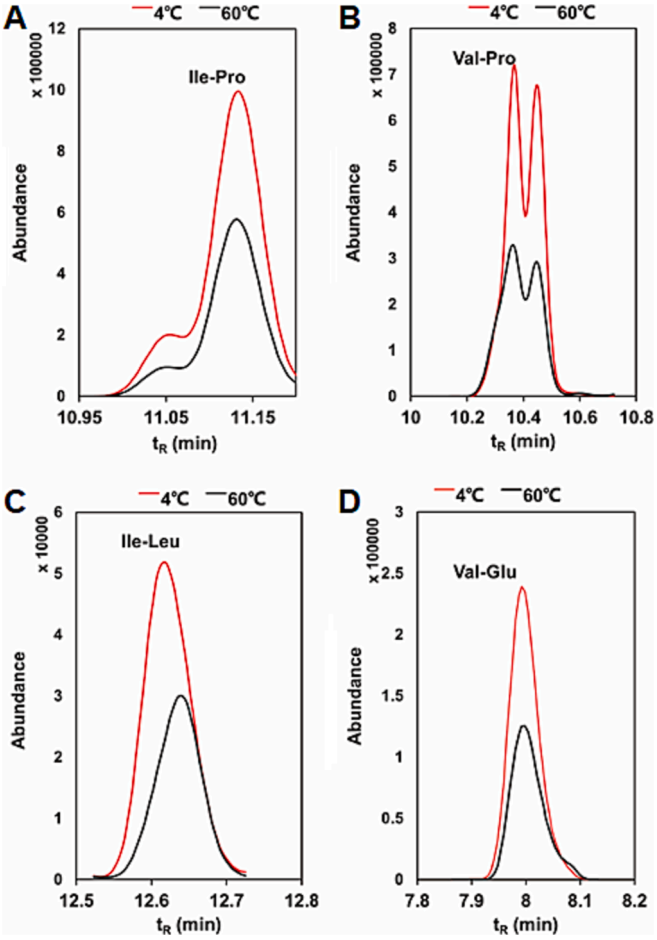

Fig. 2. The extracted ion chromatogram of four representative dipeptide dansyl chloride derivates including (A) Ile-Pro, (B) Val-Pro, (C) Ile-Leu and (D) Val-Glu at 4 °C and 60 °C.

Table 1
Conversion rate of dansyl chloride derivatization of 17 dipeptide standards.

Dipeptide standards	Standard curve of substrate			Conversion rate (%)
	Slope	Intercept	R^2	
Gly-Gly	727.5	9920.5	0.993	No residual substrate was detected
Ile-Ala	3987.5	36023.0	0.995	No residual substrate was detected
Leu-Ala	683.2	5331.4	0.997	No residual substrate was detected
Ile-Ser	7283.7	20696.0	0.996	99.5
Ser-Ile	223.1	3327.4	0.996	No residual substrate was detected
Val-Thr	14307.0	26734.0	0.998	99.8
Thr-Val	5482.4	26663.0	0.999	99.4
Thr-Thr	1605.5	6620.6	0.999	No residual substrate was detected
Leu-Pro	12814.0	43002.0	0.994	No residual substrate was detected
Ile-Val	13299.0	9093.0	0.990	No residual substrate was detected
Leu-Val	36537.0	43854.0	0.990	99.9
Asp-Val	1521.0	836.0	0.995	99.9
Val-Asp	1322.8	10327.0	0.990	99.1
Thr-Leu	8250.7	89268.0	0.996	99.8
Leu-Thr	2068.3	39858.0	0.999	No residual substrate was detected
Thr-Asp	258.4	5219.7	0.997	99.6
Asp-Asp	261.7	11051.0	0.995	No residual substrate was detected

regression and found that using $1/x^2$ as the weighting factor was optimal (Almeida, Castel-Branco, & Falcao, 2002; Gu et al., 2014; Sadray, Rezaee, & Rezakhah, 2003). In most studies, the determination coefficient (R^2) is the commonly used index to evaluate regression performance. However, R^2 can be misleading in assessing the adequacy of linear curves. A previous study summarized the limitations of using R^2 (Raposo, 2016). As an alternative metric, %RE represents the relative error between measured and theoretical values, which can better reflect the fitting quality of the linear curve at each concentration point. Therefore, %RE was proposed as a standard for evaluating linear curves (Almeida et al., 2002; Raposo, 2016). In the SW-846 manual published by the United States Environmental Protection Agency, %RE was suggested to be an alternative to R^2 for the evaluation of linear curves and was recommended to be less than 30 % (USA-EPA). In our study, we utilized WLS with a weight of $1/x^2$ to establish linear regression curves for the 17 dipeptide derivatives, and then calculated %RE for each concentration point within the linear range to evaluate the established regression curves.

As shown in Table S3, the linear range of the method spanned 2–4 orders of magnitude, with R^2 values ranging from 0.965 to 0.995. All % RE values for concentration points were less than 30 %, indicating that the linear curve was stable and reliable. The specific %RE for each concentration point is shown in Table S4 in Supporting Information. LOD of each dipeptide varied in the range of 0.25–12.5 ng/mL, while LOQ was in range of 1–50 ng/mL. The intra- and inter-day precision of all 17 dipeptides spiked at medium and high concentration levels was good with RSD lower than 15 %. At the low concentration level, the intra- and inter-day precision of 17 dipeptides were showing RSD lower than 20 % (Table S5). The outcome of intra- and inter-day precision indicated that the repeatability of the method was good.

In addition, the reproducibility outcome showed that the RSDs of all tested dipeptides were less than 20 % (8.9–19.9 %), indicating the method reproducibility was satisfactory (Table S5). Concerning the stability of the dipeptide derivatives, the peak area RSDs of all the dipeptide derivatives within 48 h were less than 20 % (3.6–15.8 %), indicating that the dipeptide derivatives remained stable after being placed at 4 °C for at least 2 days (Table S5).

The recovery of the dansyl chloride derivatization method for profiling dipeptides was also investigated. The results showed that the extraction recoveries of dipeptide were 68.9–112.0 %, 69.5–77.3 %, and 70.5–97.0 % at low (25 ng/mL), medium (100 ng/mL), and high (1250 ng/mL) concentration levels, and RSDs were all less than 15 % (Table S6).

Collectively, the established dansyl chloride derivatization method for profiling dipeptides in Daqu was stable and reliable.

3.5. Comparison of dipeptides in sauce-flavor Daqu under different production conditions

The process of Daqu-making starts with mixing the materials and pouring the materials into molds for shaping. Subsequently, either traditional manual stomping or mechanical compression is employed for preparing Daqu blocks. After a fermentation period of approximately one month, the Daqu blocks are transferred to the dry warehouse, stored for approximately six months, and finally ground for Baijiu brewing. Therefore, the variation in production conditions during Daqu-making could have a significant impact on microbial communities and enzymatic metabolism, consequently altering the composition of Daqu metabolites. It was reported that the machine-made Daqu showed a higher level of Bacillus than handmade Daqu at the whole fermentation period (Qiancheng Zuo, Huang, & MinGuo, 2020). In practice, however, handmade rather than machine-made high-temperature Daqu is the preferred starter of Maotai-flavor liquor. Till now, the effect of varied production conditions during Daqu-making on metabolic profiling of Daqu has remained unknown yet. To this end, we applied the developed dansyl chloride derivatization method to investigate the effects of two important production conditions (i.e., Daqu was produced by handmaking and machine-making, Daqu was produced in ventilated and unventilated warehouse) on the composition of dipeptides in Baijiu Daqu. Each subgroup contained 15 Daqu samples ($n = 4 \times 15$).

The dipeptide data from the four subgroups were first subjected to PCA analysis to obtain the overall differences in dipeptides between handmade and machine-made Daqu as well as warehouse ventilated and

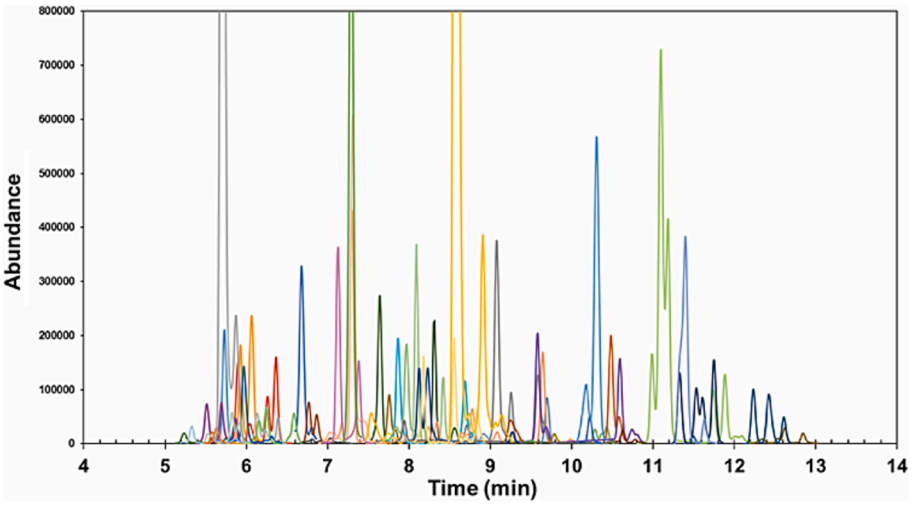

Fig. 3. The extracted ion chromatogram of the detected 118 dipeptides.

unventilated Daqu. As shown in Fig. 4, all groups were distinctly separated, indicating that both production conditions have significant effects on the metabolite composition of Daqu dipeptides. Nonparametric test was further performed to investigate the changes of individual dipeptides among the groups. Dipeptides with p-value < 0.05 were considered to have a significant alteration. The heatmaps of the significantly changed dipeptides between groups are shown in Fig. 5. Most of the differential dipeptides related to the production conditions included Val, Ala, Pro, Ile, Leu, Arg and Glu, with the majority being hydrophobic amino acids, indicating that the production conditions had a more significant impact on dipeptides containing hydrophobic amino acids. In mechanized Daqu, 36 dipeptides were found to exhibit significant differences, of which 9 dipeptides showed decreased contents and 27 dipeptides increased as compared to handmade Daqu.

In Daqu produced by dry warehouse-ventilated, 64 dipeptides were found to exhibit significant differences, in which the relative contents of 32 dipeptides decreased while 32 increased in contrast to unventilated Daqu. In detail, compared with hand-made Daqu, dipeptides with C-Arg exhibited reduced levels, while dipeptides with Val, Pro, or Ala showed elevated levels in Daqu produced through mechanical-making. Dipeptides containing Leu, Val, or Ala showed decreased levels, and dipeptides containing Pro showed increased levels in Daqu produced in ventilated warehouse.

Currently, the production of Daqu primarily relies on traditional manual molding, which is not only time-consuming and laborious but also may lead to the uneven quality of Daqu. With the advancement of technology, manual molding may be gradually replaced by mechanized Daqu production. Attributed to the differences in uniformity and exposure to air or moisture between Daqu produced by mechanical and hand-making, variations exist in microbial species, physicochemical indicators, and fermentation performance, which are further reflected in the metabolism of Daqu dipeptides (Tang et al., 2022). According to the results of non-targeted metabolomics and differential analysis, except for some dipeptides containing C-Arg, the majority of differential dipeptides exhibited higher levels in Daqu produced by mechanical-making than that produced by hand-making, particularly for those containing Pro, Val and Ala. This could be attributed to the higher abundance of Bacillus species in mechanically produced Daqu compared to manually molding Daqu (19.10 % in mechanical-making, 17.67 % in hand-making) (Q. Zuo, Huang, Guo, Hu, You, & Cheng, 2021). Bacillus is well known to be the dominant species in proteinase production. Similarly, it has been reported that mechanically produced Daqu exhibits a richer fungal species and microbial population compared to manually molding Daqu (Tang et al., 2022). These factors may potentially contribute to the higher dipeptide content in mechanically produced Daqu. As a matter of fact, it should be noted that mechanically produced Daqu contained a higher abundance of Pro-Gly, which could undergo the Maillard reaction with glucose to generate ample flavor compounds (Yu-Chiang, Thomas G, & Chi-Tang, 1992). These results could provide valuable insights for promoting Daqu production mechanization.

The oxygen content in the fermentation warehouse could be increased through ventilation. Thereby, the growth of certain aerobic bacteria could be altered and consequently influencing the composition of metabolites in Daqu. Our results indicated that dipeptides containing Lys (i.e., Lys-Pro, Gly-Lys, Ala-Lys) exhibited higher average abundance in ventilated Daqu. It has been reported that dipeptides containing Lys could react with carbonyl compounds to form pyrazine-like substances, which are typical flavor-contributing compounds in Baijiu (Van Lancker et al., 2010). Similarly, ventilated Daqu also contained a higher

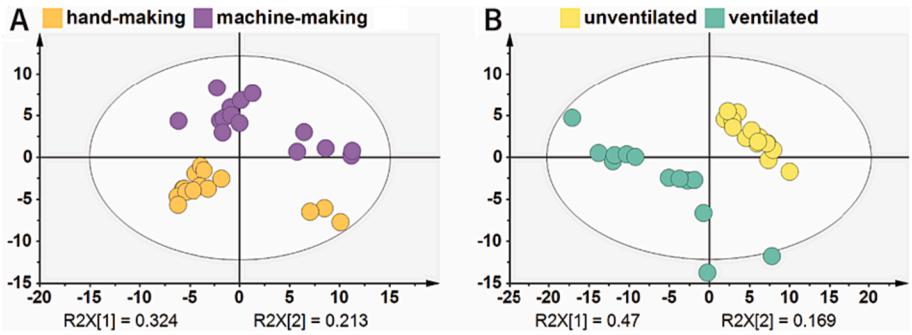

Fig. 4. PCA score plots of dipeptides in Daqu produced by (A) hand-making and machine-making and produced in (B) ventilated and unventilated warehouse.

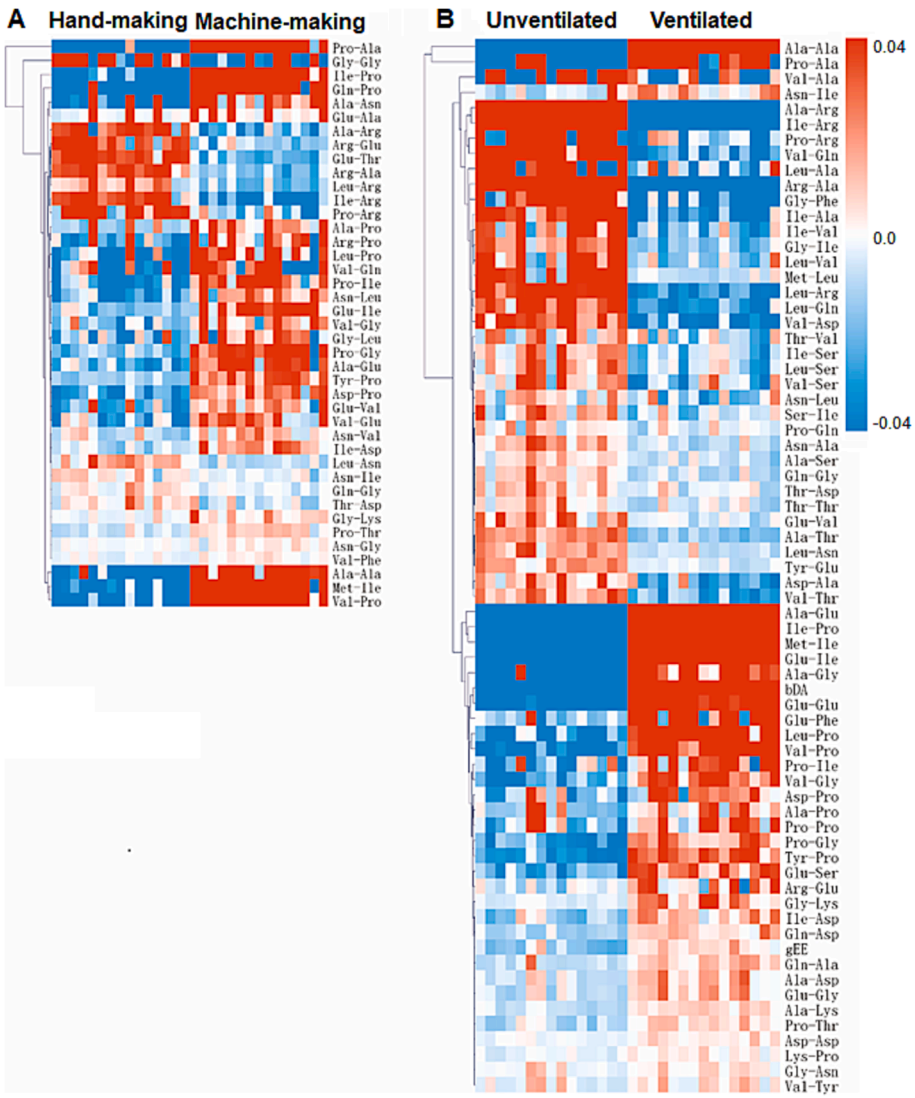

Fig. 5. Heatmaps of differential dipeptides in Daqu produced by (A) hand-making and machine-making and produced in (B) ventilated and unventilated warehouse.

abundance of two dipeptides such as Pro-Gly and γ-Glu-Glu. The former is an important participant in the Maillard reaction, while the latter contributes to a mellow flavor (Toelstede & Hofmann, 2009).

In short, variations in production conditions have different impacts on the composition of Daqu dipeptides. Daqu produced through mechanical-making and stored in a ventilated warehouse exhibited a higher abundance of flavor-contributing dipeptides. Therefore, regulating production conditions could alter the composition of dipeptide compounds in Daqu and ultimately impact the flavor of Baijiu. Our findings could offer valuable references to the selection and control of Daqu production conditions.

4. Conclusions

In this study, we developed a method utilizing UHPLC-HRMS in combination with dansyl chloride derivatization for the comprehensive profiling of dipeptides in Baijiu Daqu. The method exhibited high efficiency for dansylation at a low temperature and demonstrated good analytical characteristics in sensitivity, stability and reproducibility. A total of 118 dipeptides was detected in Baijiu Daqu, predominantly composed of hydrophobic amino acids. The applicability of the method was testified by studying the impact of various production conditions on the metabolic characteristics of dipeptides in Daqu. Our findings revealed that changes in production conditions exerted varying degrees of influence on the metabolism of Daqu dipeptides, which could provide valuable guidelines for selecting and controlling Daqu process conditions. To conclude, the developed method offered an effective strategy for the comprehensive analysis of dipeptides in complex biological samples.

CRediT authorship contribution statement

Zixuan Wang: Methodology, Software, Investigation, Writing – original draft. **Liangqiang Chen:** Writing – review & editing, Project administration. **Fan Yang:** Resources, Project administration. **Xiaolin Wang:** Software, Validation. **Yang Hu:** Formal analysis, Data curation. **Ting Wang:** Validation, Data curation. **Xin Lu:** Validation. **Jianjun Lu:** Resources. **Chunxiu Hu:** Writing – review & editing, Supervision. **Huabin Tu:** Conceptualization, Writing – review & editing, Project administration, Funding acquisition. **Guowang Xu:** Conceptualization, Writing – review & editing.

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

The authors do not have permission to share data.

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Appendix A. Supplementary data

The following supporting information is available online. Table S1: Information on real Daqu samples under different operation conditions; Table S2: The detailed information on the detected 118 dipeptides; Table S3: Linear range, LOD and LOQ of the 17 studied dipeptide standards; Table S4: Specific %RE for concentration points within the linear range; Table S5: Precision, repeatability and stability of 17 dipeptide standards; Table S6: Recovery of 17 dipeptide standards; Figure S1: Comparison of derivatization results between benzoyl chloride and dansyl chloride; Figure S2: Changes in dipeptide composition in Daqu produced under different production conditions. Figure S3. Volcano plots of dipeptides in Daqu produced by different production conditions. Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.101097.

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