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Investigation of the Distribution of 5-Hydroxymethylfurfural in Black Garlic from Different Regions and Its Correlation with Key Process-Related Biochemical Components

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Abstract

Black garlic is a thermally processed product derived from fresh garlic through controlled high-temperature and -humidity conditions. During this process, the formation of 5-hydroxymethylfurfural (5-HMF), a potentially harmful byproduct, is a major quality and safety concern in food processing. This study systematically investigated the distributions of 5-HMF and key process-related biochemical components in black garlic samples from three major production regions in China—Jiangsu, Yunnan, and Shandong. Additionally, correlations between 5-HMF and biochemical components—reducing sugars, amino acids, and organic acids—were analyzed to inform process optimization strategies. Results showed significant regional variation in 5-HMF content, with Jiangsu black garlic exhibiting the highest levels, followed by Yunnan and Shandong ($p < 0.05$). Partial least squares regression analysis (PLSR) indicated that the key biochemical factors regulating 5-HMF accumulation are primarily organic acids. Among them, citric acid was identified as the most important negative regulator (VIP = 3.11). Although acetic acid (VIP = 1.38) and malic acid (VIP = 1.03) showed positive correlations with 5-HMF, aspartic acid (VIP = 0.41) and fructose (VIP = 0.43) exhibited a weak positive correlation, and arginine (VIP = 0.89) showed weak negative correlations, their effects were far less significant than that of citric acid. Based on these findings, we propose a potential strategy for reducing 5-HMF content in black garlic—selecting raw material cultivars with higher endogenous citric acid levels or exploring the exogenous addition and regulation of citric acid during processing. This study provides a theoretical foundation for understanding the accumulation mechanism of 5-HMF in black garlic and suggests new potential regulatory directions for controlling its content.

Keywords: black garlic; 5-HMF; reducing sugar; organic acid; amino acid

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1. Introduction

Black garlic is a novel deep-processed food produced from fresh garlic (*Allium sativum* L.) under high-temperature (60–90 °C) and high-humidity (relative humidity 70–90%) conditions, through the interaction of various components [1]. Compared to fresh garlic, black garlic not only changes in appearance from white to black and develops a chewy, sweet, and sour taste but also becomes rich in a variety of bioactive compounds, such as

reducing sugars, amino acids, organic acids, and polyphenols. In recent years, black garlic has attracted widespread attention due to its unique flavor and health benefits [2–5].

5-Hydroxymethylfurfural (5-HMF) is an important non-enzymatic browning product formed in heat-processed foods. It not only imparts the characteristic color and flavor to black garlic but also is directly related to its nutritional activity and food safety [6–11]. However, toxicological studies have shown that 5-HMF can be metabolically converted to 5-sulfoxymethylfurfural (SMF), which induces the formation of DNA adducts and significantly increases apoptosis of renal proximal tubular epithelial cells, posing a potential carcinogenic risk [12,13]. Therefore, the precise detection and monitoring of 5-HMF levels in foods are of significant scientific importance for optimizing processing methods, reducing dietary exposure risk, and protecting consumer health.

There are two main pathways for the formation of 5-HMF (Figure 1), as follows: one is the dehydration of hexoses (pathway 1), and the other is the Maillard reaction (pathway 2) [14–16]. Its formation is regulated by various factors, including processing temperature and humidity, substrate content (such as reducing sugars and amino acids), pH, and the presence of metal ions [17–20]. Sasmaz et al. used response surface methodology to optimize the fermentation process of black garlic, effectively maintaining a low level of HMF in the final product [21]. Zhang et al. revealed that epigallocatechin gallate effectively reduces the production of 5-HMF during the heat treatment of black garlic [22]. Liu et al. developed a short-duration black garlic processing technology that can reduce the 5-HMF content to 5.6% of the original level [23]. It is evident that numerous studies have extensively investigated the effects of processing parameters and individual components on the formation of 5-HMF in black garlic. Literature reports indicate that soil composition, climatic conditions, and cultivation practices significantly influence the accumulation of carbohydrates, amino acids, and secondary metabolites in garlic [24–26]. Thus, geographical origin, as a critical factor determining variations in the initial composition of garlic (such as carbohydrates, amino acids, and organic acids), ultimately affects the 5-HMF content in black garlic from different regions. However, studies on 5-HMF in black garlic from different regions are still lacking, and further investigation is urgently needed.

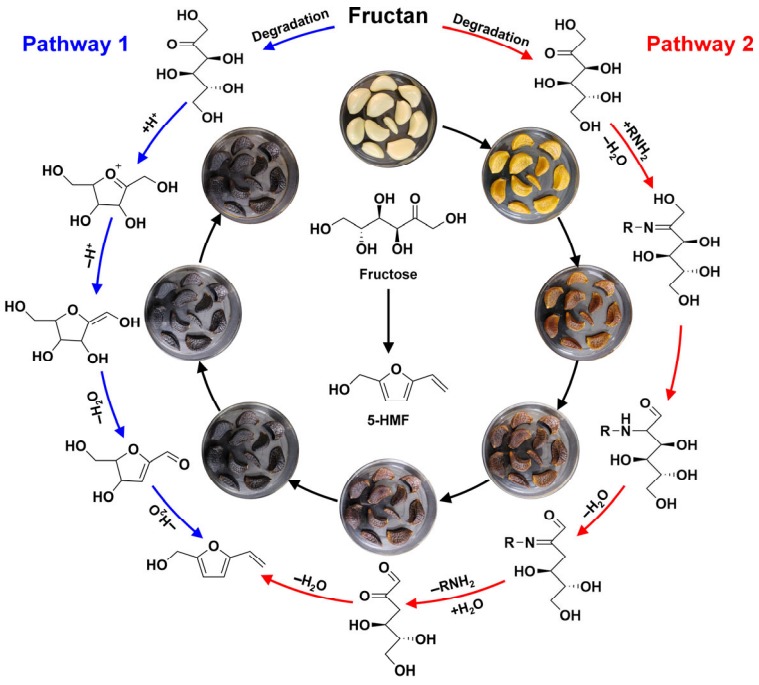


Figure 1. Possible mechanisms of 5-hydroxymethylfurfural (5-HMF) formation in black garlic.

China accounts for over 75% of the world's total garlic production, and its garlic cultivation exhibits significant regional concentration characteristics. The major production areas include the southern region, with Dali in Yunnan being a significant production area (renowned for single-clove garlic), and the northern regions of Jinxiang in Shandong and Pizhou in Jiangsu [27]. Notably, the garlic production in Shandong, Jiangsu, and Yunnan provinces accounts for approximately 80% of the total national output [28]. Furthermore, black garlic processing enterprises are highly concentrated in these regions—the number of enterprises in these three areas exceeds 65% of the national total (data source: Qichacha Technology Co., Ltd., Suzhou, China, http://www.qichacha.net/, accessed on 26 April 2025)—making their raw material varieties and processing techniques representative of the industry. More importantly, preliminary experiments revealed significant differences ($p < 0.05$) in the content of the key quality indicator, 5-HMF, in black garlic among these three major production areas. It is imperative to uncover the causes underlying this regional variation.

Therefore, this study analyzes black garlic samples from Shandong, Jiangsu, and Yunnan to systematically characterize the regional distribution of 5-HMF and key biochemical components and explores their correlations to elucidate the driving factors behind regional differences. This research will provide a theoretical basis for understanding the accumulation mechanism of 5-HMF in black garlic and offer new potential regulatory directions for controlling its content.

2. Materials and Methods

2.1. Chemical and Reagents

Standard substances, including fructose, 5-HMF, citric acid, malic acid, acetic acid, phosphoric acid, and mixed standard solution of 17 amino acids, were purchased from Anpel Scientific Instrument Co., Ltd. (Shanghai, China). Chromatographic-grade reagents, including methanol, acetonitrile, dipotassium phosphate, phosphoric acid, triethylamine, phenyl isothiocyanate, n-hexane, sodium acetate, gallic acid, were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Analytical-grade reagents, Folin-phenol reagent, sodium carbonate, potassium ferrocyanide ($K_4[Fe(CN)_6]$), zinc sulfate ($ZnSO_4$), and hydrochloric acid were purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). Ultrapure water was prepared using a Milli-Q system purchased from Millipore (Billerica, MA, USA).

2.2. Samples

Nine types of black garlic were sourced from Jiangsu, Shandong, and Yunnan province (Figure 2). For each type, three parallel samples were collected. Each parallel sample (1 kg) was first frozen at −80 °C for several hours and then subjected to freeze-drying (Scientz-18N, Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China) for 48 h. The freeze-dried black garlic samples were ground using a grinder (800-Y, Yongkang Bo'ou Hardware Products Co., Ltd., Yongkang, China) and stored at 4 °C for subsequent analysis (Figure 3).

All black garlic samples were produced using the solid-state fermentation method. The main processing steps are garlic selection, the removal of 1–2 outer layers of skin and roots, cleaning, high-temperature and high-humidity fermentation, and the packaging of finished products. The main differences in the processing procedures depend on the fermentation temperature, humidity, and duration. The specific process parameters for each type of black garlic are shown in Table 1.

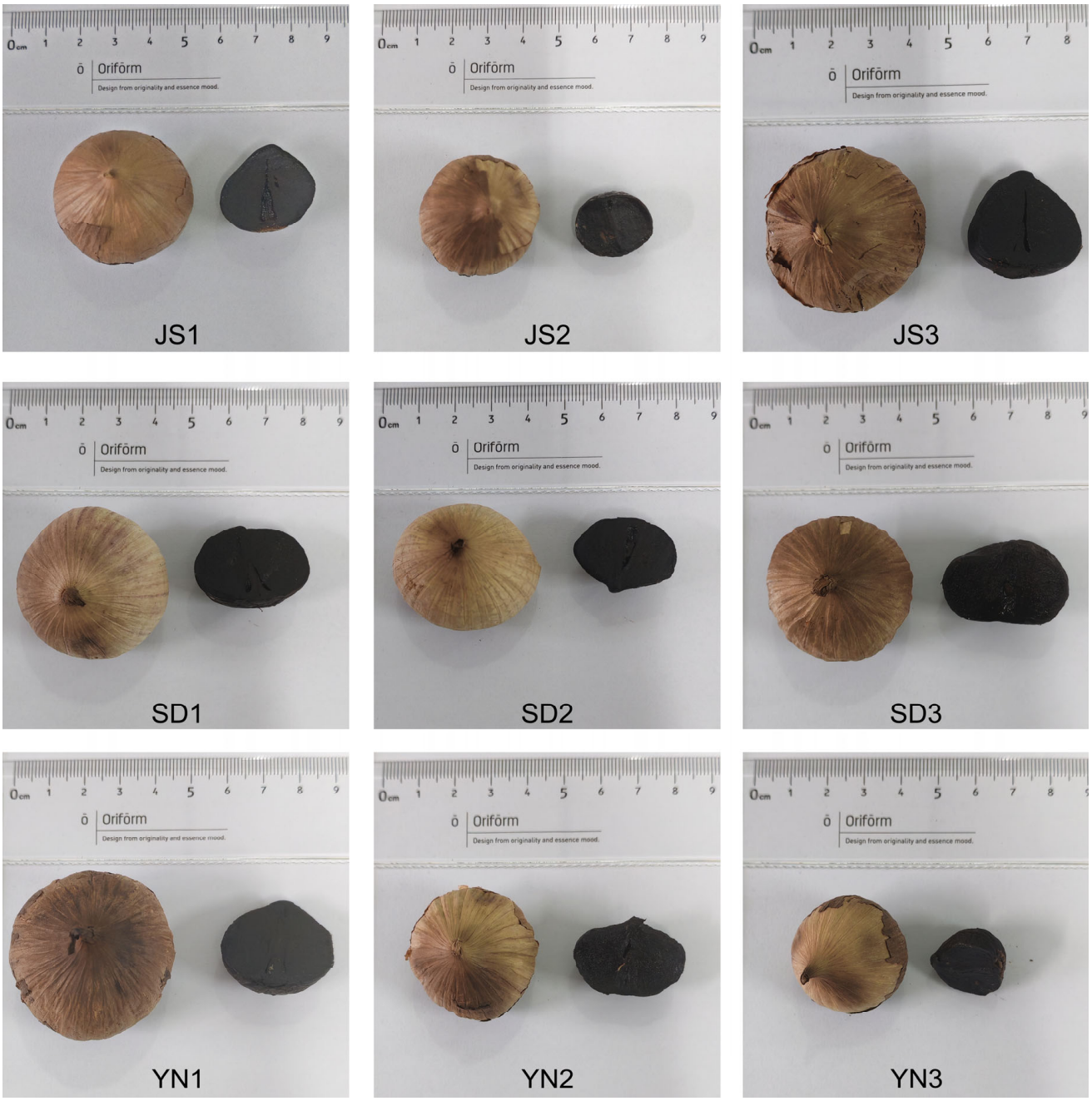


Figure 2. Photos of black garlic from different origins.

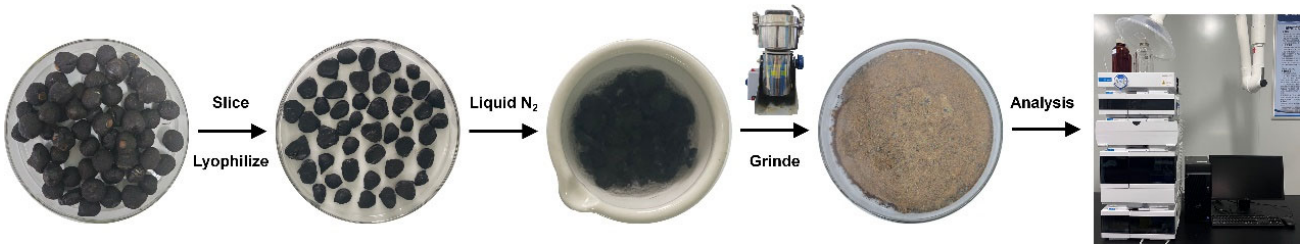


Figure 3. Sample preparation process.

Table 1. Samples from different places of origin.

No.	Code	Place of Origin	Moisture Content	Processing Parameters
1	JS1	Jiangsu Province	32%	75–80 °C, 25–30 d, 55%
2	JS2	Jiangsu Province	35%	70–75 °C, 20–25 d, 65%
3	JS3	Jiangsu Province	34%	70–80 °C, 20–25 d, 60%
4	SD1	Shandong Province	42%	60–65 °C, 15–25 d, 70%
5	SD2	Shandong Province	41%	65–70 °C, 15–20 d, 65–70%
6	SD3	Shandong Province	42%	60–65 °C, 15–20 d, 70–75%
7	YN1	Yunnan Province	37%	70–75 °C, 15–25 d, 65–70%
8	YN2	Yunnan Province	36%	70–80 °C, 15–25 d, 70%
9	YN3	Yunnan Province	37%	70–75 °C, 15–25 d, 65–70%

2.3. Determination of 5-HMF in Black Garlic

2.3.1. Pretreatment of the Samples

The 5-HMF content was measured based on the literature with some modifications [23,29,30]. A precisely measured 1.0000 g portion of powdered black garlic (FA2004, Sunny Hengping Scientific Instrument Co., Ltd., Shanghai, China) was vigorously agitated with 30 mL deionized water for 60 s. The resulting suspension was poured into a 50 mL conical centrifuge tube, then the blending vessel was rinsed with 10 mL deionized water, which was subsequently merged with the primary mixture. Sequential additions of 3 mL 10.6% (*m*/*v*) potassium ferrocyanide solution and 3 mL 21.9% (*m*/*v*) zinc sulfate solution were made to the tube contents with thorough mixing (VORTEX-5, Haimen Kylin-Bell Lab Instruments Co., Ltd., Haimen, China). The mixture underwent ultrasonic-assisted extraction (KH-800DE, Kunshan Hechuang Ultrasonic Instrument Co., Ltd., Kunshan, China) for 20 min before being subjected to centrifugal separation at 8000 rpm for an equivalent duration (H1580, Hunan Xiangyi Laboratory Instrument Development Co., Ltd., Changsha, China). The pelleted material underwent secondary extraction using 30 mL deionized water followed by repeat centrifugation. All clarified liquid fractions were pooled in a 100 mL volumetric flask and diluted to the calibration mark with additional deionized water.

2.3.2. Purification of the Samples and Chromatography Conditions

A 25 mL aliquot of the sample solution was introduced into an HLB Pro SPE cartridge (ANPEL Laboratory Technologies Inc., Shanghai, China). The column was first washed with deionized water to eliminate water-soluble contaminants, followed by elution with 6 mL diethyl ether. The obtained eluent underwent solvent evaporation at 55 °C using rotary vacuum evaporation (RE-5203, Shanghai Yarong Biochemical Instruments Co., Ltd., Shanghai, China). The dried residue was subsequently reconstituted with 25 mL methanol and then passed through a 0.22 μm syringe filters. This processed solution was then subjected to analytical procedures for 5-HMF quantification.

The 5-HMF contents in the samples were analyzed by an Agilent-1260 system equipped with diode array detector (DAD). A column (ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 μm, Agilent Technologies Inc., Santa Clara, CA, USA) was used, and the column temperature was set at 30 °C. The detection wavelength was 284 nm. The injection volume was set at 10 μL. The chromatographic eluent consisted of an unchanging ratio of 70% aqueous solution and 30% methanol (by volume), delivered at a constant flow rate of 1 mL/min. Analytical cycles were maintained for 10 min intervals, with subsequent data processing conducted through the OpenLab software (version 3.6).

2.4. Measurement of Reducing Sugar Contents in Black Garlic

The quantification of reducing sugars in black garlic followed established methodologies outlined in prior research [31]. The sample solution underwent filtration using a

0.22 μm syringe membrane filter. Analytical determination was performed on an Agilent-1260 HPLC system fitted with a refractive index detection system. Chromatographic separation was achieved using a TSKgel Amide-80 column (4.6 × 250 mm, 5 μm, Tosoh Bioscience, Tokyo, Japan) maintained at 35 °C. The analytical parameters included a 10 μL sample injection volume and a constant flow rate of 1 mL/min. The mobile phase consisted of an isocratically maintained solvent combination (30:70 *v/v* water–acetonitrile ratio) throughout the separation process.

2.5. Measurement of Amino Acids in Black Garlic

The amino acids contents were measured according to the literature with some modifications [32]. A 1.0000 g sample (±0.0001 g precision) of freeze-dried black garlic was precisely measured into a 50 mL centrifugation vessel. The material underwent extraction with 20 mL of 0.1 mol/L HCl solution through 30 min ultrasonic treatment. Following phase separation via centrifugation at 10,000 rpm for 20 min, the clarified supernatant was collected in a 50 mL volumetric flask and brought to volume using ultrapure water. For derivatization, 200 μL aliquots were combined with 100 μL each of 1 mol/L triethylamine in acetonitrile and 0.2 mol/L phenyl isothiocyanate in acetonitrile within 2 mL microcentrifuge tubes. After thorough vortex mixing, the reaction proceeded at ambient temperature (25 °C) for 60 min. Post-derivatization treatment involved vigorous shaking with 400 μL *n*-hexane for 10 s, followed by phase separation. The organic upper phase was removed, while 200 μL of the aqueous layer was diluted with 800 μL deionized water. The solutions were filtered through 0.22 μm syringe filters (Nylon, Tianjin Jinteng Laboratory Equipment Co., Ltd., Tianjin, China) before analysis.

An Agilent-1260 HPLC system equipped with a diode array detector (DAD) was employed to quantify amino acids in black garlic samples. Chromatographic separation was achieved using an Athena AAA C18 column (4.6 mm × 250 mm, 5 μm, ANPEL Laboratory Technologies Inc., Shanghai, China) maintained at 40 °C. Detection parameters included a fixed wavelength of 254 nm. Samples were introduced through a 10 μL injection volume. The mobile phase composition comprised two components; mobile phase A contained 50 mmol/L sodium acetate buffer (pH-adjusted to 6.5), and mobile phase B consisted of a ternary mixture of methanol, acetonitrile, and deionized water (20:60:20 $v/v/v$). A multi-step gradient elution program was executed as follows: linear reduction from 95% to 51% A over 0–39 min, rapid decrease to 0% A between 39–40 min, isocratic elution at 0% A for 10 min (40–50 min), immediate return to initial conditions from 0% to 95% A within one minute (50–51 min), followed by a 9 min equilibration period at 95% A. The mobile phase flow rate remained constant at 1.0 mL/min throughout the analysis. Chromatographic data acquisition and processing were performed using OpenLab 3.6 software.

2.6. Measurement of Organic Acids in Black Garlic

The organic acids' contents were measured according to the literature with some modifications [33]. Precisely 2.0000 g of black garlic powder was measured and combined with 20 mL of deionized water. The suspension underwent homogenization for two minutes using laboratory homogenization equipment. This mixture was then processed through centrifugation at 10,000 rpm for ten minutes. Following initial processing, the sediment was rehydrated with an additional 20 mL of purified water and the homogenization–centrifugation cycle was replicated. Collected supernatants from both extraction phases were transferred to a 50 mL volumetric flask, with deionized water added to achieve the calibration mark. The prepared solution underwent final filtration through a 0.22 μm aqueous membrane filter prior to analytical procedures.

The quantification of organic acids was performed using an Agilent-1260 HPLC system equipped with a diode array detector (DAD). Separation was achieved through a Venusil MP C18 column (4.6 × 250 mm, 5 μm, Agela Technologies Co., Ltd., Tianjin, China) maintained at 35 °C. Detection occurred at 210 nm wavelength with a 10 μL sample injection volume. The mobile phase consisted of 10 mM potassium phosphate dibasic solution (pH adjusted to 2.55 using phosphoric acid) delivered at 1.0 mL/min flow rate. The quantification of individual organic acids was achieved through calibration curves generated from certified reference standards. All chromatographic data were processed using Chemstation for LC system B.04.03 analytical software.

2.7. Measurement of Total Polyphenols in Black Garlic

The method for measuring total polyphenols in black garlic was previously described by Lee et al. [22] and Caper et al. [30] with some modifications. A precisely measured 1.0000 g portion of black garlic powder was homogenized with 50 mL of 40% (*v*/*v*) ethanol solution through 2 min vortex mixing. The resultant suspension was quantitatively transferred to a 100 mL volumetric flask and subjected to 30 min ultrasonic-assisted extraction. Following phase separation through centrifugation at 9500 rpm for 20 min, the clarified supernatant was carefully collected and diluted to the 100 mL mark with 40% (*v*/*v*) ethanol. For colorimetric analysis, 0.5 mL aliquots of the processed solution were combined with 0.5 mL deionized water in test tubes. The reaction sequence involved sequential addition of 5 mL Folin-phenol reagent followed by 4.0 mL of 7.5% sodium carbonate solution after a 5 min interval. After 60 min of ambient temperature incubation, absorbance measurements were conducted at 765 nm using a visible spectrophotometer (T6, Beijing Purkinje General Instrument Co., Ltd., Beijing, China) with 40% (*v*/*v*) ethanol as the reference solution. The results were expressed as milligram gallic acid equivalent (mg GAE/g).

2.8. Statistical Analysis

All measurements were repeated three times, and the mean values along with their corresponding standard errors were subsequently calculated. Statistical evaluations were performed through analysis of variance (ANOVA) methodology utilizing SPSS Statistics software (version 25.0, IBM Corp., Armonk, NY, USA). Comparative analyses between groups employed Duncan's multiple range test protocol, maintaining a 95% confidence threshold ($p < 0.05$) for determining statistical significance. Partial least squares regression (PLSR) modeling was executed through the SIMCA 14.1 program (Sartorius Stedim Data Analytics AB, Uppsala, Sweden).

3. Results and Discussion

3.1. Distribution of 5-HMF in Black Garlic from Different Regions

Given the prominent status of Shandong, Jiangsu, and Yunnan in China's garlic production, these regions not only provide geographical advantages for black garlic processing but also possess well-developed industrial chains for black garlic production. Based on this consideration, black garlic samples from these three regions were selected as research subjects to systematically analyze and compare their 5-HMF content differences through scientific methodologies.

The 5-HMF levels in nine black garlic samples are presented in Figure 4A, and the mean values of 5-HMF contents from different regions are summarized in Figure 4B. The 5-HMF content fluctuated between 202.13 μg/g and 550.46 μg/g, with a mean value of 347.88 μg/g. JS black garlic exhibited the highest 5-HMF levels (480.82 ± 35.59 μg/g), followed by YN samples (337.84 ± 13.54 μg/g), while SD samples showed the lowest concentrations (225.0 ± 20.90 μg/g). Statistical analysis revealed significantly higher 5-

HMF content in JS samples compared to both YN and SD samples ($p < 0.05$), with YN samples also demonstrating significantly elevated levels relative to SD samples ($p < 0.05$).

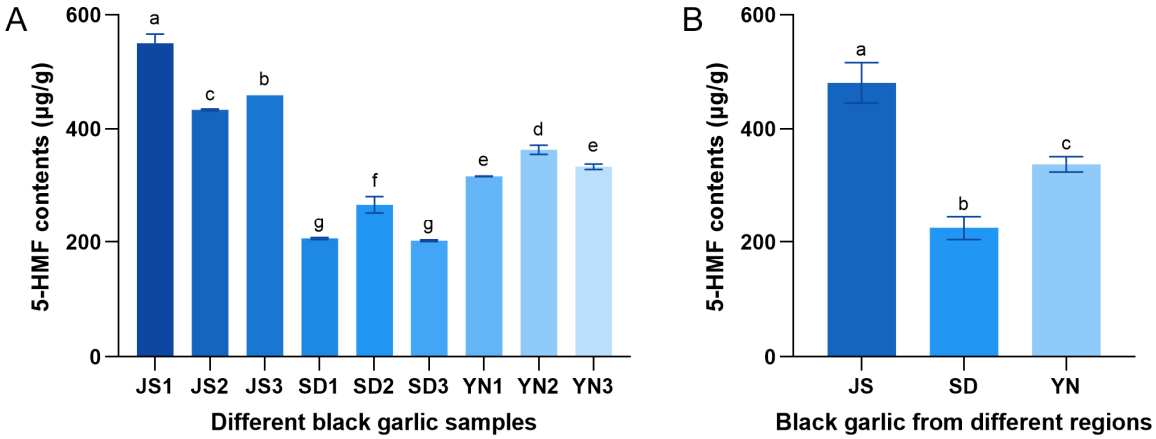


Figure 4. The contents of 5-hydroxymethylfurfural (5-HMF) in different black garlic samples (**A**), the mean values of 5-HMF in black garlic from different regions (**B**). Different lowercase letters indicate significant differences between groups ($p < 0.05$). Groups sharing the same letter are not significantly different ($p > 0.05$).

The main formation pathways of 5-HMF in black garlic are the intramolecular dehydration and cyclization of fructose, as well as the Maillard reaction [16,34]. Fructose is derived from the chain degradation of β-(2→1)-D-fructofuranosyl polymers during the thermal processing [35]. Garlic cultivars in JS production areas potentially contain higher fructan or amino acid content, as previous studies have demonstrated significant interspecific variations in carbohydrate composition among garlic varieties [24].

Furthermore, studies have shown that the formation of 5-HMF not only depends on the fructose concentration but may also be influenced by the synergistic effects of processing temperature, humidity, and time [12,19]. JS samples were processed at higher temperatures, a lower humidity, and longer times than SD and YN samples (Table 1). These conditions are more conducive to 5-HMF formation. This observation is consistent with previous research findings. Zhang et al. demonstrated accelerated 5-HMF formation rates at higher temperatures (60 °C samples contained 0.39–0.46 times 5-HMF compared to 70–90 °C processed samples) [14]. González-Ramírez et al. found that excessively high humidity reduces the 5-HMF content in black garlic (the 5-HMF content at 58% humidity is higher than at 71% and 96% humidity) [36]. In addition, shortening the processing time or presoaking garlic with tea polyphenols can effectively reduce the content of 5-HMF [21,22,29]. Based on the above reasons, we speculated that differences in processing parameters are one of the reasons for the significantly higher 5-HMF content in Jiangsu black garlic.

3.2. Changes in Reducing Sugar Content in Black Garlic from Different Regions

To investigate the regional differences in the content of reducing sugars in black garlic, fructose, glucose, maltose, and lactose were analyzed. Among them, fructose was found to have a relatively high content, while glucose, maltose, and lactose were not detected. The fructose content in black garlic samples from Jiangsu (JS1–JS3), Shandong (SD1–SD3), and Yunnan (YN1–YN3) was determined in this study (Figure 5A).

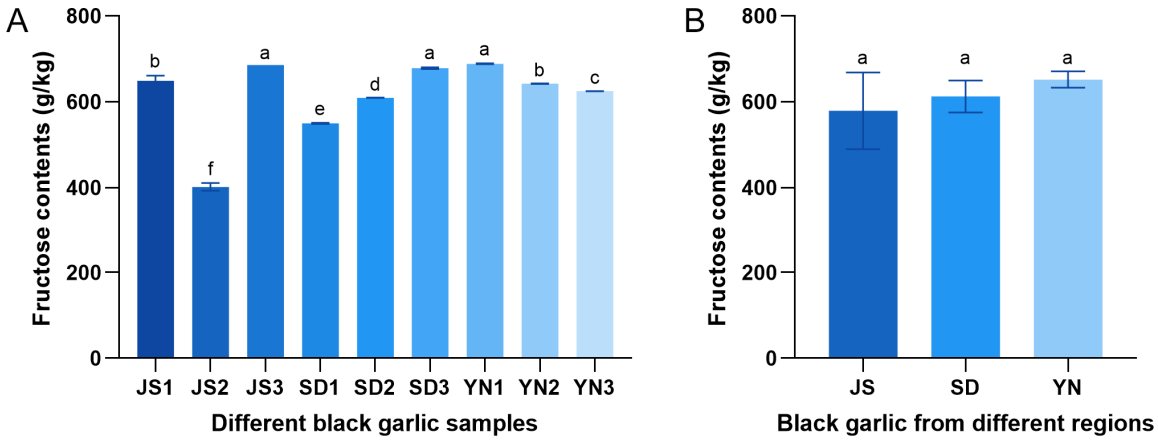


Figure 5. The contents of fructose in different black garlic samples (**A**), the mean values of fructose in black garlic from different regions (**B**). Different lowercase letters indicate significant differences between groups ($p < 0.05$). Groups sharing the same letter are not significantly different ($p > 0.05$).

Among the JS samples, JS1 and JS3 exhibited higher fructose contents, measured at 649.12 ± 11.91 mg/g and 685.80 ± 0.13 g/kg, respectively, while the fructose content in JS2 was significantly lower at 401.9 ± 8.88 g/kg. For the SD samples, SD3 had the highest fructose content (678.2 ± 1.84 g/kg), followed by SD1 and SD2, with values of 549.60 ± 1.31 g/kg and 609.30 ± 0.58 g/kg, respectively. In the YN samples, the fructose contents of YN1, YN2, and YN3 were similar, measured at 688.60 ± 0.93 g/kg, 642.30 ± 0.86 g/kg, and 624.9 ± 0.34 g/kg, respectively. As shown in Figure 5B, YN black garlic exhibited the highest fructose levels (651.93 ± 19.00 g/kg), followed by SD samples (612.37 ± 37.16 g/kg), while JS samples showed the lowest concentrations (578.90 ± 89.14 g/kg). Statistical analysis shows that there was no significant difference between the regions ($p > 0.05$).

The processing methods of black garlic have a significant impact on its fructose content [37]. In the early stages of processing, fructans are rapidly degraded into fructose, leading to a noticeable increase in fructose content in black garlic, especially under high-temperature conditions where this transformation is more pronounced [38,39]. However, as the processing continues, fructose is continuously consumed due to intramolecular dehydration and cyclization reactions, as well as Maillard reactions with amino compounds [40]. If the temperature is too high or the processing time is too long in the later stages, the consumption of fructose will further increase, resulting in a decrease in the fructose content of the final product [41]. It is worth noting that the fructose content in black garlic is affected not only by the processing methods but also by the initial fructan content in the raw garlic [24]. However, whether the processing method or the initial fructan content of the raw material has a more significant effect on the final fructose content in black garlic still requires further research.

3.3. Analysis of Amino Acids Contents in Black Garlic from Different Regions

According to the literature, the protein content of fresh garlic accounts for approximately 1.5–2.1% of its fresh weight [42], and it is rich in various free amino acids, whose specific content varies depending on the garlic variety and growth environment [25]. When fresh garlic is processed under high-temperature and high-humidity conditions for several days, the proteins in garlic are hydrolyzed due to the decrease in system pH, resulting in the formation of free amino acids. However, these free amino acids are partially consumed during the processing of black garlic through Maillard reactions with reducing

sugars [31,43]. Therefore, the amino acid content during black garlic processing exhibits a dynamic change.

In this study, the types and contents of amino acids in black garlic samples from different regions were analyzed. A total of ten amino acids were detected, namely proline, threonine, valine, alanine, serine, glycine, leucine, glutamic acid, arginine, and aspartic acid. The contents of these ten free amino acids and the total amino acids are shown in Figure 6. The results indicated that the total amino acids content was highest in SD samples (1259.383 ± 120.61 mg/kg), followed by YN (1008.62 ± 91.95 mg/kg), and lowest in JS (962.85 ± 53.69 mg/kg). Although differences in total amino acids content were observed among samples from different regions, statistical analysis showed that these differences were not significant ($p > 0.05$).

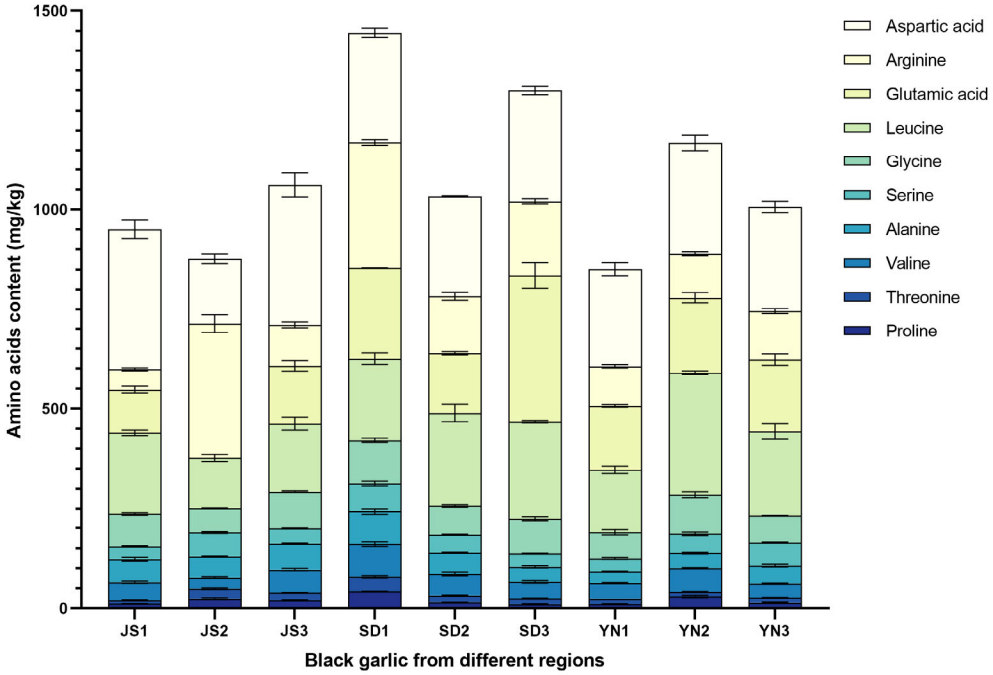


Figure 6. The contents of amino acids in black garlic from different regions.

As shown in Figure 6, the main amino acids in black garlic are aspartic acid, leucine, arginine, and glutamic acid. The higher contents of aspartic acid and glutamic acid may be attributed to their classification as acidic amino acids, which are less likely to undergo Maillard reactions with reducing sugars, thereby reducing their consumption. Additionally, glutamic acid is one of the most abundant free amino acids in fresh garlic [35]. Considering these factors, aspartic acid and glutamic acid are the most abundant amino acids in black garlic. The study by Choi et al. revealed that the leucine content in black garlic is higher than that in fresh garlic [44]. However, the strong bitterness of leucine may affect the flavor balance of black garlic [45]. Furthermore, it has been reported that the arginine content in fresh garlic decreases by approximately 90% after processing, which may be due to its involvement in Maillard reactions and subsequent consumption [25,38,44].

3.4. Distribution of Organic Acids in Black Garlic from Different Regions

Organic acids impart black garlic with a distinctive sourness and refreshing taste. They not only effectively neutralize its original pungency but also make the flavor of black garlic more mellow, complex, and appealing. The organic acid composition and total organic acid content of black garlic samples from Jiangsu (JS1–JS3), Shandong (SD1–SD3), and Yunnan (YN1–YN3) province were systematically analyzed in this study (Figures 7–10).

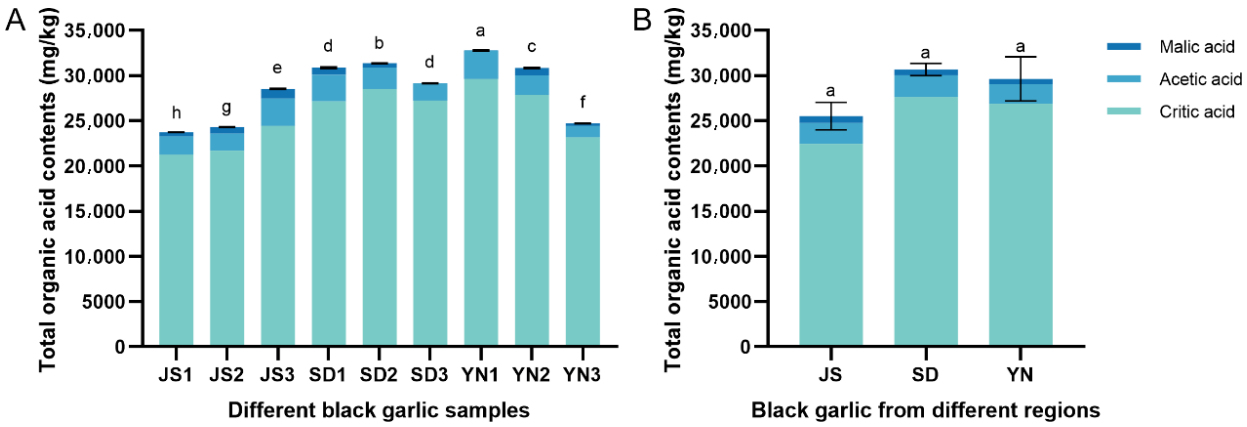


Figure 7. The contents of total organic acid in different black garlic samples (**A**), the mean values of total organic acid in black garlic from different regions (**B**). Different lowercase letters indicate significant differences between groups ($p < 0.05$). Groups sharing the same letter are not significantly different ($p > 0.05$).

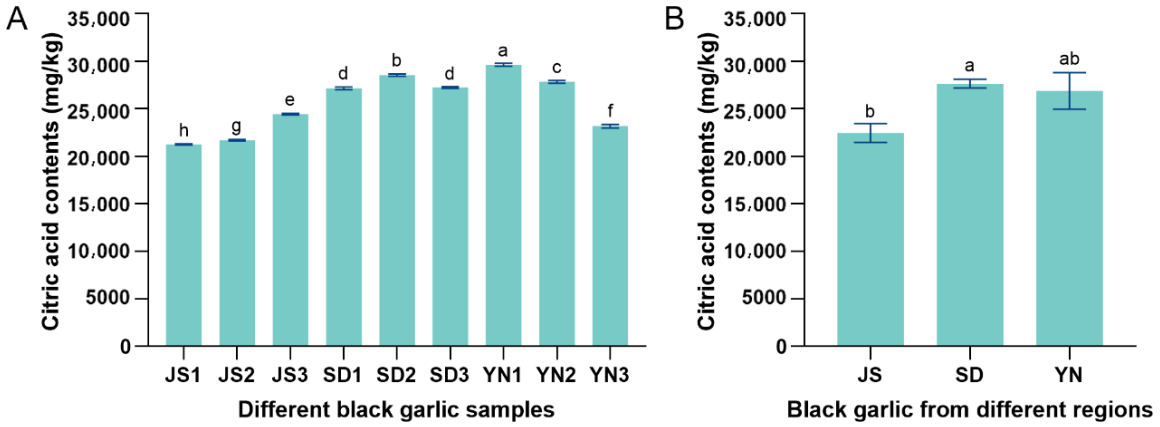


Figure 8. The contents of citric acid in different black garlic samples (**A**), the mean values of citric acid in black garlic from different regions (**B**). Different lowercase letters indicate significant differences between groups ($p < 0.05$). Groups sharing the same letter are not significantly different ($p > 0.05$).

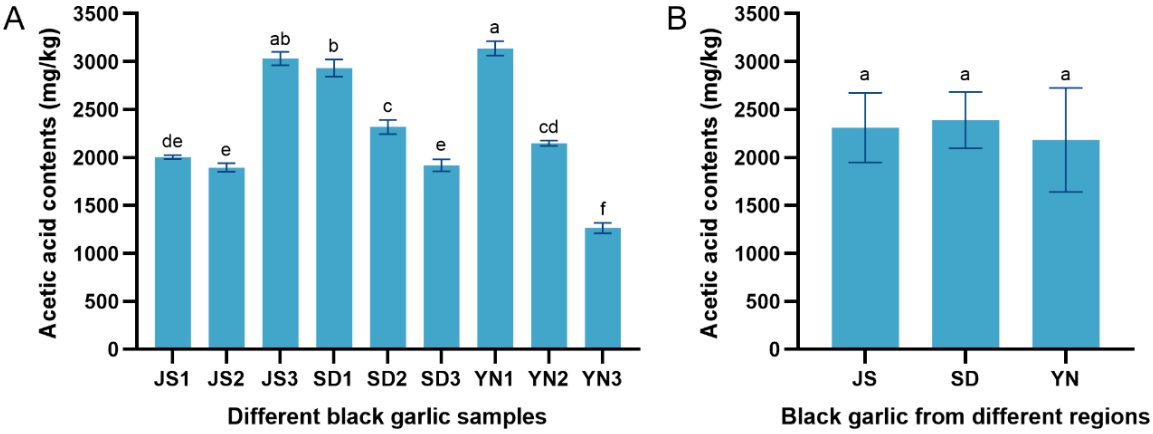


Figure 9. The contents of acetic acid in different black garlic samples (**A**), the mean values of acetic acid in black garlic from different regions (**B**). Different lowercase letters indicate significant differences between groups ($p < 0.05$). Groups sharing the same letter are not significantly different ($p > 0.05$).

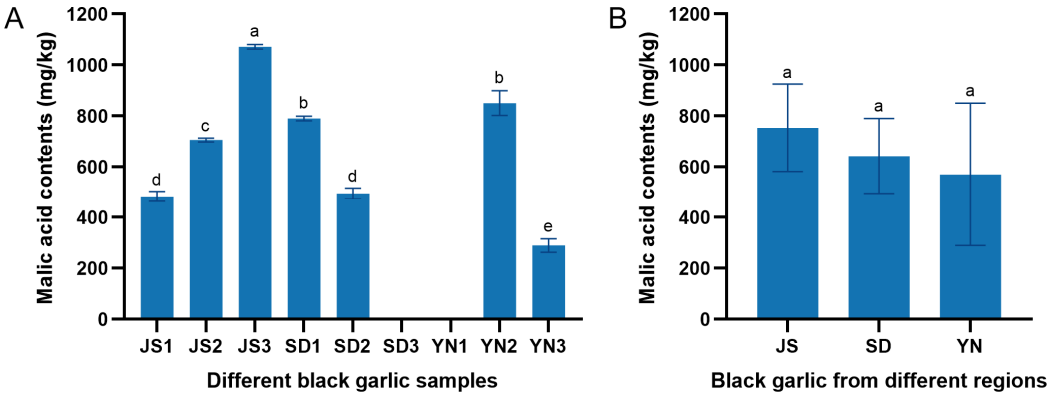


Figure 10. The contents of malic acid in different black garlic samples (**A**), the mean values of malic acid in black garlic from different regions (**B**). Different lowercase letters indicate significant differences between groups ($p < 0.05$). Groups sharing the same letter are not significantly different ($p > 0.05$).

As shown in Figure 7, significant variations ($p < 0.05$) were observed in malic acid, acetic acid, and citric acid contents. A stable concentration gradient was demonstrated, with citric acid constituting the highest proportion (78.3–85.6%), followed by acetic acid (8.1–12.7%), and malic acid showing the lowest levels (0–4.2%). Among the samples from the three regions, the total acid content was highest in SD samples, followed by YN, and lowest in JS. However, statistical analysis showed that the differences in total acid content among the regions were not statistically significant ($p > 0.05$).

Citric acid, as the main organic acid in black garlic, exhibits distinct geographical distribution characteristics. The results show significant differences between regions (Figure 8); Shandong has the highest citric acid content (27,635.67 ± 453.19 mg/kg), followed by Yunnan (26,870.03 ± 1929.25 mg/kg), and Jiangsu the lowest (22,450.53 ± 996.67 mg/kg). The difference between Shandong and Jiangsu is statistically significant ($p < 0.05$), while the differences between Yunnan and Jiangsu, Shandong and Yunnan are not significant ($p > 0.05$). The high citric acid content in SD samples may be attributed to the variety of raw materials and optimized control of fermentation temperature and humidity, while the lower content in JS could be related to insufficient precursor substances in the raw materials [46,47].

Figure 9 illustrates the acetic acid content in black garlic samples from three regions—Jiangsu (JS1–JS3), Shandong (SD1–SD3), and Yunnan (YN1–YN3). The results show that the average acetic acid content is highest in Shandong (2390.26 ± 294.86 mg/kg), followed by Jiangsu (2310.65 ± 361.84 mg/kg), and lowest in Yunnan (2183.32 ± 541.39 mg/kg). One-way ANOVA revealed no statistically significant differences between the regions ($p > 0.05$). Studies suggest that the formation of acetic acid may involve two potential mechanisms, as follows: (1) under alkaline conditions, the nucleophilic attack of 1-deoxy-2,4-hexodiulose induces a Claisen-like β-cleavage reaction [48,49]; (2) it is associated with sugar degradation and the Maillard reaction [14].

Malic acid distribution displayed anomalous characteristics (Figure 10), with JS samples showing the highest mean value (753.68 ± 171.43 mg/kg), while SD3 and YN1 samples showed no detectable levels. This dispersion was attributed to genetic variations in malate storage of raw garlic and microbial community-specific malic enzyme activities [50]. There are no statistically significant differences between the regions ($p > 0.05$).

3.5. Distribution of Polyphenols in Black Garlic from Different Regions

Polyphenols are recognized as essential bioactive components in black garlic, with their content being closely correlated to functional properties, including antioxidant and anti-inflammatory activities, and are established as a critical indicator for evaluating processing

effects on nutritional quality [51]. Therefore, polyphenol quantification is considered valuable for elucidating the accumulation mechanisms of functional constituents and optimizing production protocols.

The polyphenol contents in black garlic samples from different regions are shown in Figure 11. The highest content was detected in the SD2 sample (11.02 mg GAE/g), while the lowest value was recorded in the YN3 sample (4.40 mg GAE/g). Mean values for each region were determined as follows: Jiangsu 7.68 ± 1.94 mg GAE/g, Shandong 8.56 ± 1.28 mg GAE/g, and Yunnan 7.92 ± 1.83 mg GAE/g, with no significant differences ($p > 0.05$).

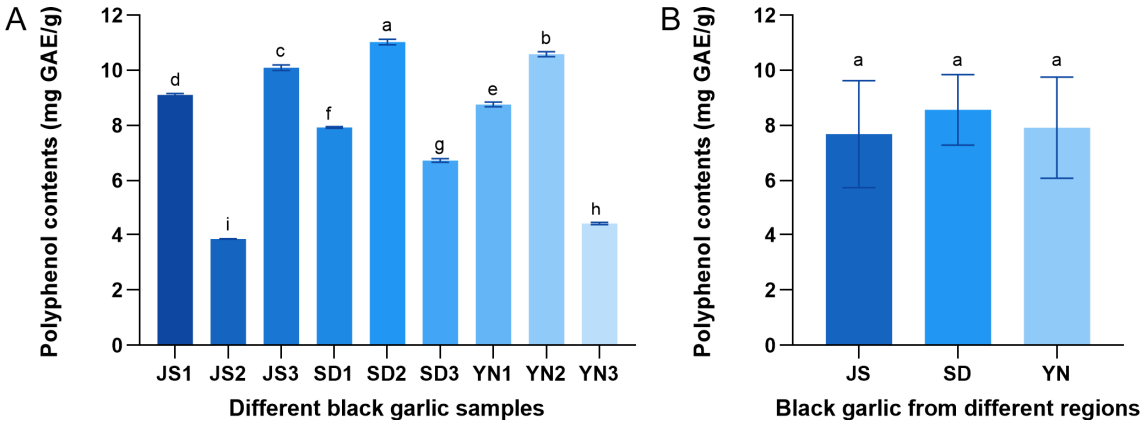


Figure 11. The contents of polyphenol in different black garlic samples (**A**), the mean values of polyphenol in black garlic from different regions (**B**). Different lowercase letters indicate significant differences between groups ($p < 0.05$). Groups sharing the same letter are not significantly different ($p > 0.05$).

Polyphenol accumulation mechanisms in black garlic have been documented to involve two pathways, as follows: (1) the release of bound phenolic compounds through plant cell wall disruption under high-temperature/humidity conditions (70–90 °C) [37]; and (2) the formation of phenolic substances via the Maillard reaction during processing [51,52]. For instance, Nam et al. reported peak total phenolic content (27.08 mg GAE/g) at 30 days of processing [53], while the SD2 sample (11.02 mg GAE/g) in this study, though lower than this value, still exceeded typical fresh garlic levels [54]. This result further confirms that the processing promotes polyphenol accumulation [35].

Substantial intra-regional variations were identified, exemplified by a range of 4.30 mg GAE/g (SD1–SD3) in Shandong and 6.25 mg GAE/g (JS1–JS3) in Jiangsu. These variations were speculated to originate from non-uniform processing conditions. For example, Maillard reaction progression has been shown to be highly sensitive to minor temperature fluctuations (± 2 °C) [52], while differential carbohydrate degradation rates may lead to uneven substrate availability for polyphenol synthesis [55]. Additionally, raw material variations (e.g., garlic maturity) could cascade into final product composition through altered initial sugar/phenol ratios [26].

3.6. PLSR-Based Quantification of Component Effects on 5-HMF Accumulation

Based on PLSR models, this study systematically elucidated the regulatory mechanisms of key components (fructose, organic acids, and amino acids) on 5-HMF content in black garlic. Significant influencing factors were screened through variable importance in projection (VIP > 1), with their interaction pathways clarified by loading plots (Figure 12). Model validation demonstrated cumulative explained variance of 90.2% (PLS1: 77.1%, PLS2: 13.1%) and reliable predictive capability ($Q^2 = 0.518$).

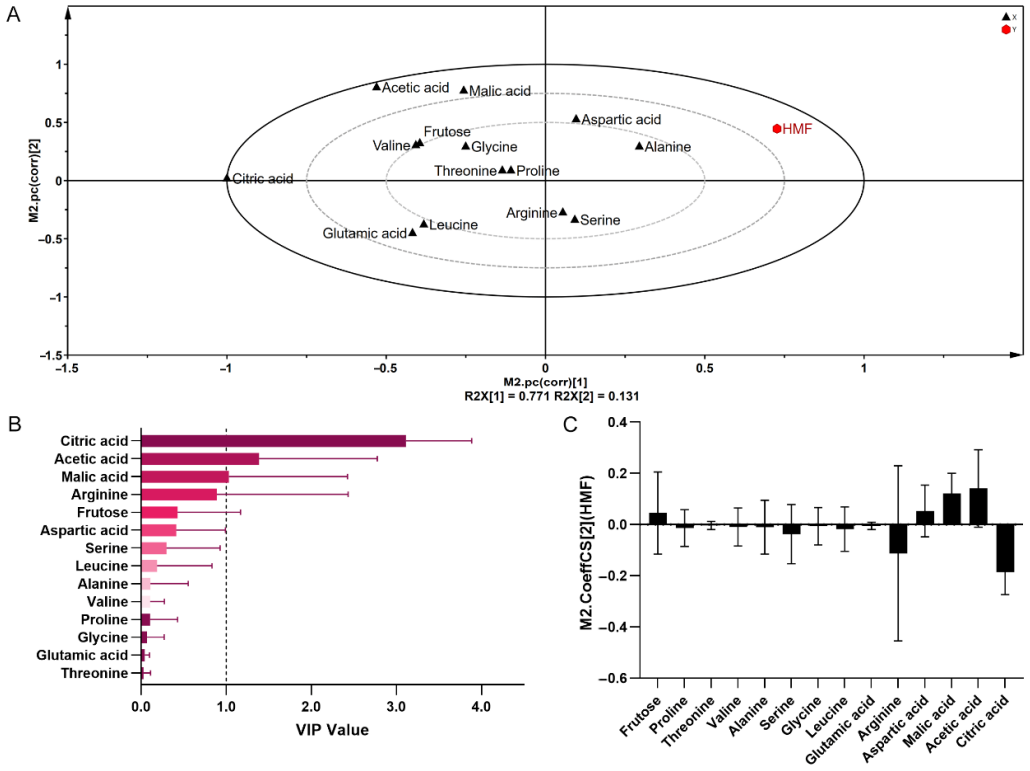


Figure 12. Partial least squares regression (PLSR) of the amino acids, sugars, organic acids, 5-HMF in black garlic ((**A**): loading plot, (**B**): VIP plot, (**C**): coefficient plot).

In the organic acid system, citric acid (VIP = 3.11, Figure 12B), acetic acid (VIP = 1.38, Figure 12B), and malic acid (VIP = 1.03, Figure 12B) exhibited significant regulatory effects on 5-HMF generation. Among them, acetic acid and malic acid show a significant positive correlation with 5-HMF (Figure 12C), which aligns with the classic mechanism that an acidic environment promotes the formation of 5-HMF; a decrease in pH not only accelerates the acid-catalyzed dehydration of fructose to produce 5-HMF but also enhances the yield of 5-HMF by facilitating the 1,2-enolization reaction of key intermediates, Amadori compounds, in the Maillard reaction [16,56,57].

Although citric acid has the highest VIP value, it shows a negative correlation with 5-HMF (Figure 12C). This negative correlation effect may be attributed to the metal-chelating properties of citric acid. It can bind transition metal ions, such as Fe^{2+} and Cu^{2+}, thereby partially inhibiting the formation of 5-HMF via the Maillard reaction pathway [16,34,58]. This finding is consistent with previous studies, as follows: Verma et al. found that soaking potatoes in 0.5–2% citric acid effectively suppressed 5-HMF formation in fries, with 2% concentration being the most effective [59]; Lim et al. also reported that in a soybean paste model system, 0.96% citric acid showed significantly better inhibition of 5-HMF compared to other anti-browning agents (such as sodium sulfite and sodium tripolyphosphate) [60], and similarly speculated that the mechanism was related to metal ion chelation. Notably, the effect of citric acid is concentration dependent. Some studies have shown that low concentrations of citric acid (0.1%) may promote 5-HMF formation [61]. In this study, the citric acid contents in black garlic ranged from 22,450.53 ± 996.67 to 27,635.67 ± 453.19 mg/kg (equivalent to 2.24% to 2.76%, *w*/*w*) (Figure 8), which is a relatively high concentration range. Combining the negative correlation results found in this study with the high-concentration inhibitory effects reported by Verma and Lim, the authors speculate that regulating endogenous citric acid levels or exploring the exogenous addition and regulation of citric acid during black garlic processing could be a potential approach to reduce 5-HMF content. However, the specific inhibitory mechanism

of citric acid in the complex black garlic system, the optimal concentration range, as well as the feasibility and impact of exogenous addition on product quality, still require systematic verification through dedicated experiments targeting the black garlic system.

Among amino acids, arginine (VIP = 0.89, Figure 12) shows a weak negative correlation with 5-HMF, while aspartic acid (VIP = 0.41, Figure 12) shows a weak positive correlation. This phenomenon may be related to protein hydrolysis during black garlic processing. Protein hydrolysis increases the initial levels of free amino acids, including aspartic acid and arginine. However, these two amino acids react differently in the Maillard reaction. Specifically, arginine, as a basic amino acid, has high reactivity [6]. During later reactions, arginine is consumed much faster than it is released by hydrolysis. This causes its net concentration to decrease. Meanwhile, 5-HMF is a product of the Maillard reaction, so its concentration rises. These reasons may account for the observed negative correlation between arginine and 5-HMF. In contrast, aspartic acid, an acidic amino acid, has low reactivity in the Maillard reaction [6,62]. Its consumption is negligible, and its increased concentration mainly reflects enhanced protein hydrolysis rather than directly causing 5-HMF production. Therefore, the positive correlation between aspartic acid and 5-HMF is mainly due to their common upstream factor, protein hydrolysis intensity, and not a direct causal relationship. Additionally, since the analysis involves different black garlic origins, varietal differences may affect protein hydrolysis and reaction progress. This could have some effect on the relationship between amino acids and 5-HMF. Further research is needed to clarify the role of varietal factors in these changes for a more comprehensive tunderstanding.

PLSR analysis showed a weak positive correlation between fructose and 5-HMF (VIP = 0.43, Figure 12), which is contrary to the strong correlation trend observed in traditional thermal processing systems. This may be due to the excessively high fructose content in black garlic (>60 g/100 g, Figure 5), which exceeds the optimal range for reaction kinetics, causing fructose to no longer be a key limiting factor for 5-HMF formation [26,43,63]. Meanwhile, other factors, such as organic acids, may have a more significant impact on the final accumulation of 5-HMF, as reflected by their VIP values.

In summary, the PLSR analysis revealed the key biochemical factors regulating 5-HMF accumulation in black garlic. Among them, citric acid was identified as the most important negative regulatory factor (with the highest VIP value), providing a novel and promising potential target for strategies aimed at reducing 5-HMF. Although acetic acid and malic acid showed positive correlations with 5-HMF, aspartic acid and fructose exhibited a weak positive correlation, and arginine showed weak negative correlations, their effects were all less significant than that of citric acid according to the VIP analysis. Overall, organic acids—especially citric acid—play a dominant role in regulating 5-HMF in black garlic. This provides a scientific basis and clear direction for the future optimization of black garlic processing by modulating citric acid levels to reduce 5-HMF formation and thereby enhance product safety.

4. Conclusions

This study systematically analyzed the distribution characteristics of 5-HMF and key biochemical components in black garlic from Jiangsu, Shandong, and Yunnan provinces and further explored the correlation between them, aiming to clarify the key driving factors leading to regional differences. The results showed that the 5-HMF content in black garlic from different production areas exhibited significant differences ($p < 0.05$). Jiangsu black garlic had the highest 5-HMF content (480.82 ± 35.59 μg/g), which was significantly higher than that of samples from Yunnan (337.84 ± 13.54 μg/g) and Shandong (225.0 ± 20.90 μg/g). Considering the typical processing parameters of each origin (Jiangsu samples generally

undergo higher temperature, lower humidity, and longer duration), it is speculated that differences in processing parameters are one of the reasons for the significantly higher 5-HMF content in Jiangsu black garlic. Through PLSR analysis, it was further found that key biochemical factors regulating 5-HMF accumulation were dominated by organic acids, among which citric acid was identified as the most important negative regulatory factor (VIP = 3.11). Its potential mechanism likely involves inhibition primarily through high concentrations of citric acid chelating metal ions (such as Fe^{2+}, Cu^{2+}). Although acetic acid (VIP = 1.38) and malic acid (VIP = 1.03) showed positive correlations with 5-HMF, aspartic acid and fructose (VIP = 0.43) showed a weak positive correlation (VIP = 0.41), and arginine (VIP = 0.89) showed weak negative correlations, their influence was significantly weaker than that of citric acid. This finding provides a new potential regulatory target for reducing 5-HMF content in black garlic—selecting raw material varieties with higher endogenous citric acid content or exploring the feasibility of exogenous addition and the regulation of citric acid levels during processing. Subsequent studies will focus on verifying the efficacy of citric acid regulation strategies, optimizing regulatory parameters, and further clarifying the relative contributions of processing parameters and citric acid strategies in reducing 5-HMF content in black garlic.

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Abbreviations

5-HMF	5-Hydroxymethylfurfural
PLSR	Partial least squares regression
SPE	Solid phase extraction
DAD	Diode array detector

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