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Research Article

Polygonatum sibiricum Red. With Steaming Yellow Rice Wine: Processing Technology, the Content of 5-Hydroxymethylfurfura and Safety Assessment

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Abstract: To investigate the content and safety evaluation of 5-Hydroxymethylfurfural (5-HMF) in the processing of *Polygonatum sibiricum* Red, the steaming technology was optimized by response surface method combined with analytic hierarchy process of multiple indexes. The content of 5-HMF was quantitatively determined by HPLC. Safety assessment was conducted using a zebrafish model. The optimized conditions were a steaming time of 6.3 hours, a drying temperature of 74°C, a drying time of 13 hours and two cycles of processing. The contents of 5-HMF in one processing cycle (P_1) and two processing cycles (P_2) were 0.07±0.04 and 2.07±0.01 mg/g, respectively. As the number of steaming increased, the numbness of the tongue disappeared, the taste and antioxidant activity improved, and the 5-HMF content also increased. However, the 5-HMF content of P_2 exceeded the EU Food Safety Standard. The safety assessment using zebrafish revealed that the processed products exhibited some acute toxicity. 5-HMF, as a harmful component, may act alone or synergistically to affect zebrafish embryo survival, hatchability and teratogenicity. 5-HMF may pose a potential health risk to humans. Therefore, the content of 5-HMF should be carefully considered during the processing food and medicinal plants to ensure food safety and minimize health risks.

Keywords: *Polygonatum sibiricum* Red., Processing, 5-Hydroxymethylfurfural

Introduction

Polygonatum sibiricum Red. (PSR) is a plant of the genus Polygonatum in the Liliaceae family (Li *et al.*, 2023), with a long history as a food and as a traditional medicinal and edible plant (Zhang *et al.*, 2021). It is mainly distributed in Siberia, North Korea and China, with a large planting area and high output. Its rhizome has the functions of tonifying qi, tonifying spleen, moistening lung and tonifying kidney (Luan *et al.*, 2023). Modern pharmacological studies have shown that PSR has many beneficial effects such as lowering blood sugar, reducing blood lipids, delaying aging, improving memory, enhancing human immunity, and exhibiting anti-inflammatory, anti-aging, antiviral and anti-tumor activities (Liu *et al.*, 2024). Therefore, the rhizome is widely used in food and medicine. PSR is rich in polysaccharides, saponins, phytosterols and amino acids (Fan *et al.*, 2020). Because the raw product causes a numbing sensation feeling on the tongue, it shall be processed before eating. Since past dynasties, the processing methods have included the nine-steaming and nine-exposure process (about 80%) with water or wine, wine boiling, and black bean boiling. Wine-processed Polygonatum pieces (WIPP) are the main processed products of raw PSR Pieces (P_0) currently (Jin *et al.*, 2018). After 4 hours of steaming, 10 hours of braising, and drying at 80 °C , WIPP can reduce a numbing sensation feeling on the tongue, improve its taste, and enhancing its medicinal effects (Bi *et al.*, 2024). The steaming time from 0 to 24 hours, with samples collected every 4 hours and dried at 65 °C, the color started as bright yellow and became darker with increasing treatment time (Deng *et al.*, 2024). After two cycles (P_2), the colour of WIPP changed from light yellow to dark brown, and the bitterness and sweetness decreased (Wang *et al.*, 2022). Thermal processing stages promote protein denaturation, caramelization, and the Maillard reaction as a result of heating sugar-rich foods. Both Maillard reaction and caramelisation release toxic compound, 5-Hydroxymethylfurfural (5-HMF), which pose potential health risks to humans (Bachir *et al.*, 2023; Martins *et al.*, 2022).

5-HMF is a heterocyclic organic compound that has been known to exist in high-temperature processing for many years (Nguyen *et al.*, 2016). 5-HMF irritates to mucous membranes, eyes, and skin (Zappala *et al.*, 2005). It can bind to human proteins to cause toxicity, resulting in transverse muscle paralysis and visceral damage, and is neurotoxic, with potential genetic and reproductive toxicity (Alpözen & Üren, 2013). The National Honey Standard (GHT18796-2012) in China stipulates that the 5-HMF content cannot exceed 40 mg/kg. The European Commission on Food Safety has established that the upper limit of 5-HMF intake per person per day is 1.6 mg (Li *et al.*, 2019). In recent years, several studies have focused on 5-HMF safety in bakery or fried products. For example, date fruit fillings in baked goods contained 623.4 mg/kg of 5-HMF after storage (Karadeniz *et al.*, 2024), and coffee beans deeply roasted at 190 °C contained 544.44 mg/kg of 5-HMF (Zhu *et al.*, 2022). Therefore, excessive amounts of 5-HMF may pose a potential health risk to humans, making food safety a significant concern.

PSR is relatively high in sugar and easily produces 5-HMF after prolonged heating and drying. The current studies mainly focus on the processing technology, including changes in flavor, steaming times and chemical composition. To date, there are no recommendations on upper limits for HMF in WIPP, and there are no reports on the exposure and risk assessment of 5-HMF from WIPP in the literature. Therefore, the processing is optimized to mitigate the content of 5-HMF in order to reduce the risk of exposure.

In this study, to control the content of 5-HMF, the processing technology of WIPP was optimized. The contents of total sugar, saponin, and 5-HMF, as well as the comprehensive score of color, quality, and taste, were assessed by using multiple indexes, including steaming time, temperature, cycles, drying time, drying temperature and drying cycles. Under optimal conditions, the effectiveness of the process was verified by antioxidant experiments. The harmful ingredient of 5-HMF was quantitatively determined by HPLC. Safety assessment was conducted by zebrafish experiment. These studies provide valuable insights into the rational control of 5-HMF in the subsequent processing to ensure food safety and minimize potential health risks.

Materials and Methods

Materials and Reagents

Fresh PSR purchased from Liuhe County, Tonghua City, Jilin Province (125°73′ E, 41°28′ N) was identified as *Polygonatum sibiricum* Red. by Associate Professor Xiudong Yang (Jilin Institute of Chemical Technology, China). Fresh PSR was steamed for 40 min, cut 0.4-0.6 cm thick slices, and dried at 60°C for 12 hours to obtain a raw product. The raw product was marked as P_0 and the processed polygonatum made with yellow rice wine after one, two cycles were marked as P_1, P_2, respectively, up to nine cycles and beyond. 5-HMF (> 99%) and other standard substances were purchased from Chengdu Push Bio-Technology Co., Ltd. (Sichuan, China). Zebrafish were purchased from AB wild-type zebrafish, courtesy of Shandong YiXiYue Biotechnology Co. Other reagents used were of analytical grade.

The Contents of Total Sugar, Saponins, 5-HMF Polysaccharide and Saponins Contents

The total sugar content was determined by the phenol-sulfuric acid method using glucose as the standard (y = 36.4635x - 0.0107, R^2 = 0.9992, linearity range: 2.5-20.0 μg/mL) (Yue *et al.*, 2022).

The saponin contents were determined by the vanillin sulfuric acid method using diosgenin as the standard (y = 9.9137x + 0.0187, R^2 = 0.9995, linearity range: 0.005-0.04 μg/mL) (V. Le *et al.*, 2018).

5-HMF Contents

The 5-HMF content was determined by the HPLC method (Gülcan *et al.*, 2020). The conditions were as follows: Phenomenex Luna C_{18} (250×4.6 mm, 5μm) column, the mobile phase 0.1% formic acid-methanol (80: 20), detection wavelength 284 nm, the flow rate of 1.0 mL/min, column temperature at 30 °C , and the injection volume of 20 μL. The standard curve was: y=2112.8x+0.1962; R^2=0.9999, linearity range: 7.6-121.9 μg/mL, LOD=7.13×10^{-5} μg/L.

0.1 g of WIPP was mixed with 25 mL of methanol, and ultrasonic extraction was carried out at 60°C for 30 min. Then, 1 mL of the supernatant was accurately fixed to a 5 mL volumetric flask with 4 mL of 80% methanol. The diluted sample solution was filtered through a 0.45 μm microporous filtration membrane to obtain the 5-HMF test solution (Sun *et al.*, 2020).

Optimization of Processing Technology for WIPP

The water-rich rice wine is composed of a ratio of 1:1.875 rice wine to water (g/g). The ratio of PSR and water-rich rice wine is 5:1, with 10 g of rice wine added for every 50 g of PSR. The water-rich rice wine was fully stirred and absorbed for 80 min. It was then steamed with isolated boiling water under atmospheric pressure and dried. This process, referred to as P_1, was repeated nine times to produce P_1 through P_9.. The total amount of the water-rich rice wine was evenly divided according to the number of steaming times. On the basis of a single factor, the Response Surface Method (RSM) was optimized to combine with the Analytic Hierarchy Process (AHP).

Single-Factor Experimental Design

The effects of steaming time, drying temperature, drying time, and steaming times on the contents of total sugar, saponin, and 5-HMF were studied. One of the factors was changed while the other three factors were fixed (steaming time for 8 hours, drying temperature at 60ºC, drying time for 8 hours, and steaming times once). The steaming time was set at 4, 6, 8, 10 and 12 hours. The drying temperature was set at 50, 60, 70, 80 and 90 °C. The drying time was 10, 11, 12, 13 and 14 hours (Yao *et al.* 2022). The steaming times were 1 to 9 times (Sun *et al.*, 2020).

Analytic Hierarchy Process (AHP) of Calculated Weights

The priority weights of total sugar content, saponin content, and 5-HMF content as well as the comprehensive score of color-quality-taste were calculated by an exponential scaling method.

First, a standard judgment matrix was established. The ratios of the four evaluation indexes of total sugar, saponins, 5-HMF content, and color-quality-taste were 1, 5, 3, and 1, respectively.

Second, the consistency was checked. The consistency index CR was calculated as follows:

$$CI = (\lambda - 1)/(n - 1) \tag{1}$$

CI = 0, and CI close to 0 suggests complete and satisfactory consistency respectively. The larger CI indicates a higher inconsistency (Cheng *et al.*, 2020).

The random consistency index RI is introduced to measure the size of CI:

$$RI = (CI1 + CI2 + \ldots + CI_n)/n \tag{2}$$

According to the formula, RI is related to the order of the judgment matrix. The higher order of the matrix means the greater possibility of consistency random deviation. The corresponding RI of the relationships were 0, 0, 0.58, 0.90, 1.12, 1.24, 1.32, 1.41, 1.45, 1.49.

Since random deviation also leads to consistency deviation, it is necessary to compare CI with RI to verify whether the consistency of the judgment matrix is satisfactory. The formula is as follows:

$$CR = \frac{CI}{RI} \tag{3}$$

At CR < 0.1, in the case of comparison matrix A, the decision pairs have satisfactory consistency, or their degree of inconsistency is acceptable.

The initial weight coefficient is:

$$w_1^{'} = \sqrt{a_{i1}a_{i2}a_{i3}\cdots a_{in}} \tag{4}$$

A: the value in the matrix; m: the number of targets

The normalized weight coefficient is:

$$W_i = W_i^{'} / \sum_{i=1}^{n} w_i^{'} \tag{5}$$

Optimized Design by AHP Combined With Response Surface Method (RSM)

According to the design principle of Box-Behnken central composite test and the results of single-factor test, the steaming time (A), drying temperature (B), drying time (C) and steaming times (D) were taken as independent variables, and the comprehensive score of total sugar, saponin, 5-HMF and color-quality-taste was taken as response value. Table 1 shows the scoring criteria. The response surface analysis of 29 points with four factors and three levels was established by using Design Expert 8.0.6.1, as shown in Table 2. Quadratic multinomial regression equation fitting and optimization analysis were carried out (Cai *et al.*, 2018).

Table 1: Scoring criteria of the color-quality-taste

Classify	Marking scheme	Score
Colour	Black	19-30
	Tan	7-18
	Brown	1-6
Texture	Sweet taste, sticky, no tongue irritation	30-40
	Slightly sweet, slightly sticky, slightly numbing tongue irritation	9-29
	Basically no sweetness, no stickiness, numbness and irritation of the tongue	1-8
Scent	Wine aroma and sweetness	19-30
	Slightly wine aroma and sweet	7-18
	Basically, there is no wine aroma or sweetness	1-6

Table 2: Factors and levels for the Box–Behnken experimental design

Level	Independent variable			
	(A) Steaming time (h)	(B) Drying temperature (°C)	(C) Drying time (h)	(D) Steaming times (Times)
1	4	60	12	1
0	6	70	13	2
-1	8	80	14	3

Validation Experiments

Based on the optimal processing conditions above, three consecutive experiments were conducted and compared with the theoretical calculations.

In Vitro Antioxidant Activity Analysis

The free radical scavenging rate of ABTS was determined using the method by Wang *et al.* (2022). The concentration gradients were 0.4-2.0 mg/mL for P_0, 0.1-0.8 mg/mL for P_1, 0.03-0.13 mg/mL for P_2 and 0.01-0.09 mg/mL for VC.

The free radical scavenging rate of DPPH was based on a modified method by Wei *et al.* (2019). The concentration gradients were 0.2-1.0 mg/mL for P_0, 0.00625-1.0 mg/mL for the P_1, 0.05-0.25 mg/mL for the P_2, and 0.0005-0.004 mg/mL for VC.

The total reducing power assay was determined according to the method by Yang *et al.* (2022). The concentration gradients of 2-10 mg/mL for P_0, 0.2-1.0 mg/mL for P_1 and P_2, respectively and 0.009-0.016 mg/mL for VC.

Safety Assessment

Zebrafish Embryo Collection

Zebrafish have a high degree of physiological homology with humans and rodents, and therefore it may be used to evaluate the potential health risk of 5-HMF to humans (Zabegalov *et al.*, 2019). Zebrafish were reared under 28 °C, pH 7.8-8.0, with a 14/10 hours light/dark cycle. The zebrafish were fed twice a day. The night before the experiment, males and females in a 1:2 ratio were placed in a mating tank with a sieve at the bottom in an incubator separated by a transparent partition, and the embryos were obtained from natural spawning induced by switching on the light in the morning for 24 hours and were collected within 30 min, which were then cleaned and sterilized with 0.01% (v/v) methylene blue solution. Fertilized and developed embryos were selected for cardiac developmental toxicity experiments under a somatic vision microscope (Zhong *et al.*, 2022).

The 5-HMF extract powder (section 2.3.3) was dissolved in DMSO and then diluted with zebrafish embryo dilution medium to the concentration gradients of 20, 40, 80, 100, and 200 mg/mL.

Assessment of Zebrafish Embryo Developmental Toxicity

Embryos were selected under a microscope at 4 hpf and randomly placed in sterile 12-well plates at a density of 15 embryos per well. Solvent control (0.005% DMSO) and sample solutions for each group were added to each well in a final volume of 2 mL. The exposure solutions were replaced after 24 hours. The mortality rate in each well plate was recorded, including the number of embryos surviving at different time points after exposure at 24, 48, 72 and 96 hpf (Draut *et al.*, 2017), as well as the hatching rate at 48 hpf and 72 hpf (repeated experiments, n = 3).

Statistical Analysis

Experimental data were statistically tested by IBM SPSS Statistics (ANOVA). Results were expressed as mean ± standard error. $p < 0.05$ indicated significant effects (n = 3).

Results and Discussion

Processing Technology Optimal Results

Single-Factor Experiment

Results in Fig. 1 (A) showed that the total sugar content gradually decreased, and the 5-HMF content nearly increased twice after 8 hours. To control 5-HMF content, 4, 6 and 8 hours were selected as the steaming time parameters for the RSM analysis.

As shown in Figure 1B, when the drying temperature reached 90 °C , WIPP turned red-brown with a large number of burnt spots formed on the surface. As the temperature rose, 5-HMF accumulated and participated in the condensation reaction of Maillard reaction to form melanin. 5-HMF is not only a potential condition for pigment deposition, but also an important indicator of Maillard reaction and nonenzymatic browning (Chen *et al.*, 2019). The total sugar content of WIPP was the highest at 70 °C when the 5-HMF content was zero, so the drying temperature was set at 70°C.

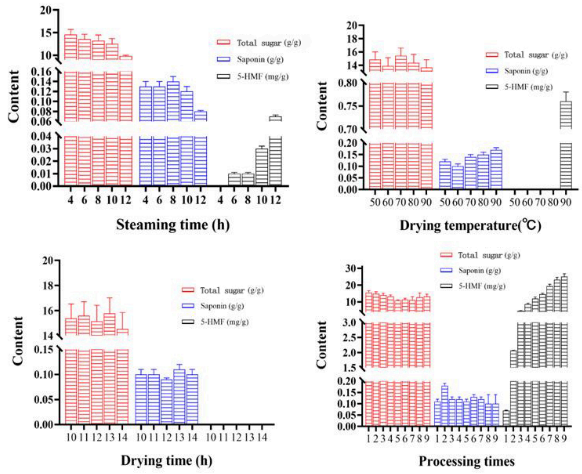

Fig. 1: Results of single factor experiment

As shown in Figure 1C, with the increase in drying time, there was almost no effect on 5-HMF or any significant change in polysaccharide content, but the moisture of the slices dried for 10, 11 and 12 hours was higher than 15%, which was not in accordance with the Pharmacopoeia of the People's Republic of China I (ChP 2020 I). The moisture of the slices was lower than 15% at 13 hours of drying, so 12, 13 and 14 h were chosen as the level parameters of the RSM analysis.

The total sugar content gradually decreased with the increase of processing times (Fig. 1D), while the 5-HMF content rose slowly (Fig. 2). The 5-HMF limit is 40 mg/kg according to the National Honey Standard (GHT18796-2012), and the 5-HMF content of P_1 and P_2 were 0.07 mg/g (70 mg/kg) and 2.07 mg/g (2.07 g/kg) respectively, so both exceeded the limit. The EU Food Safety Commission stated that the upper limit of 5-HMF per person per day was 1.6 mg, and the daily dose of WIPP in ChP 2020 I ranges from 9-15 g. The levels of 5-HMF in P_1 and P_2 were 0.63-0.98 mg and 18.63-28.98 mg, respectively, and the level of 5-HMF in P_2 exceeded the EU safety limit. As the color-quality-taste of P_1 did not comply with the standards of the ChP 2020 I, in which the surface is brown to black, glossy, with a brown to light brown center and visible veinlets, the quality is

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soft. sweet taste, and slightly wine aroma (Chinese Pharmacopoeia Commission, 2020), it also does not meet marking criteria Table 1, therefore, the processing times were determined to be twice.

Collectively, the abundance of sugars in PSR was reduced because of the long steaming time and high temperature, thereby leading to an increase in 5-HMF content through the Maillard reaction (Chen *et al.*, 2019).

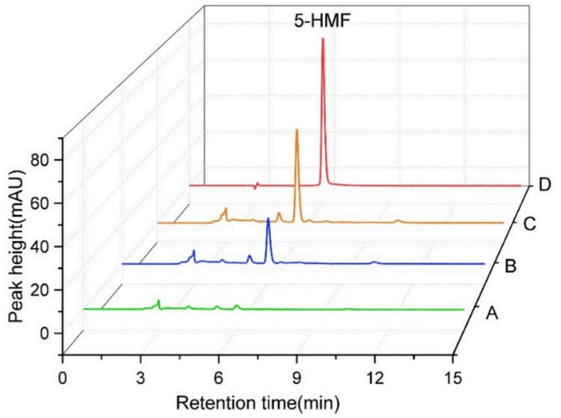

Fig. 2: 5-HMF content in WIPP with different steaming times (A) P_0; *(B)* P_1; *(C)* P_2; *(D) 5-HMF standard*

Weight Calculation by AHP

The maximum eigenvalue obtained from the standard matrix (λmax) is 4.0340. Consistency is compared as follows: at RI = 0.90, Cr = 0.0126 < 0.1, the normalized weight coefficient is calculated:

$$Comprehensive\ score = 0.3829 \times total\ sugar\ content + 0.0920 \times saponin\ content + 0.0945 \times 5 - HMF\ content + 0.4306 \times color\ quality\ taste\ score \quad (6)$$

Obviously, CI<0.1 means the comparisonmatrix is acceptable. The model also has good performance in weight fitting (b = 0.00001).

Optimal Processing by Response Surface Design

Model Fitting and Statistical Analysis

The four factors of steaming time (A), drying temperature (B), drying time (C) and processing times (D) were determined. The designed experimental conditions and the comprehensive scores of WIPP were shown in Table 3. Through multiple regression analysis of 29 experimental data, the reaction variables (comprehensive score of processing technology, Y) and test variables were related in the following second-order polynomial equation:

$$Y = 81.80 - 0.027A + 3.17B - 1.49C - 2.73D - 1.89AB + 0.75AC - 1.83AD - 0.35BC - 0.29BD - 2.08CD - 8.53A^2 - 5.70B^2 - 4.65C^2 - 10.86D^2 \quad (7)$$

Table 3: Response surface design and experimental data

No.	Steaming time	Drying temperature	Drying time	Processing times	Comprehensive score
1	4	60	13	2	64.52
2	8	60	13	2	67.33
3	4	80	13	2	66.21
4	8	80	13	2	61.44
5	6	70	12	1	68.37
6	6	70	14	1	67.81
7	6	70	12	3	63.54
8	6	70	14	3	54.67
9	4	70	13	1	68.28
10	8	70	13	1	68.75
11	4	70	13	3	66.77
12	8	70	13	3	59.93
13	6	60	12	2	71.67
14	6	80	12	2	80.80
15	6	60	14	2	69.84
16	6	80	14	2	77.58
17	4	70	12	2	67.40
18	8	70	12	2	69.90
19	4	70	14	2	64.19
20	8	70	14	2	69.70
21	6	60	13	1	58.90
22	6	80	13	1	72.19
23	6	60	13	3	57.24
24	6	80	13	3	69.37
25	6	70	13	2	80.03
26	6	70	13	2	82.27
27	6	70	13	2	83.00
28	6	70	13	2	81.30
29	6	70	13	2	82.43

A complex interactive relationship was found between various factors and the response target value. Through the comparison of *p*-value, the influence of single factors on the model ranks as steaming time > drying time > processing times > drying temperature. According to the coefficient of determination R^2=0.9038, the model has high reliability. The mismatch term *p* = 0.0654 > 0.05 revealed that the mismatch test of the model was not significant, indicating a strong correlation between the experimental results and the theoretical values inferred by fitting the corresponding polynomial s (Wang *et al.*, 2021). The experimental data suggested the model was suitable for analyzing and predicting the processing technology of WIPP.

Analysis of Response Surface Plots and Contour Plots

When the steaming time was 4-6 hours, the response surface seemingly had an obvious slope and turned flat at its highest point, suggesting that the steaming time greatly impacted the processing technology and was significantly different (Figure 3a). When the steaming time was 6 hours and the drying time was 13 hours, the response surface was close to the extreme value, and the surface diagram had an obvious slope (Figure 3b), indicating that the interaction between steaming time and drying temperature significantly impacted the processing results. When the drying time was 13 hours, the drying temperature had a significant impact before 70°C, and the slope decreased significantly after 70°C (Figure 3c), indicating that the impact was weakened after the drying temperature reached the extreme value. When the number of steaming times was 2-3, the surface had an obvious slope (Figure 3d), indicating that the processing times greatly impacted the processing technology.

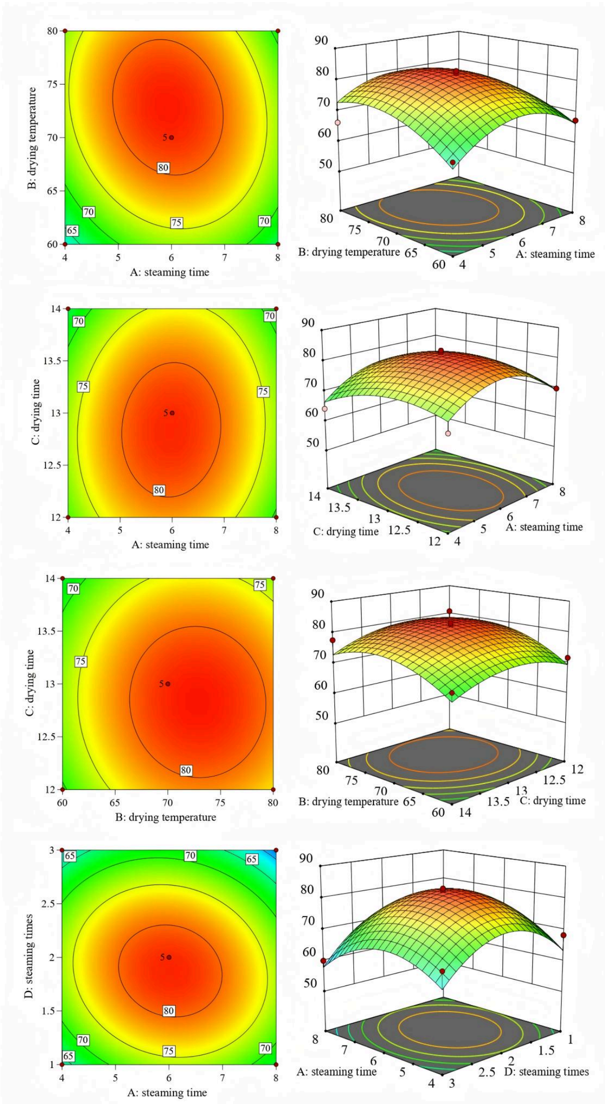

Fig. 3: Response surface diagrams about the effects of steaming time (A), drying temperature (B) drying time (C) and steaming times (D) on WIPP

Based on the above results, the key factors of the processing technology of WIPP were steaming time and processing times. Hot-processed foods often undergo a series of nonenzymatic browning reactions, such as caramelization reaction and Maillard reaction, forming abundant 5-HMF (Zhao *et al.*, 2020). Therefore, the steaming time and processing times have a great influence. Due to the abundance of carbohydrate compounds in P_0, when heated at high temperatures, it is easier to hydrolyze the glycoside bond hydrolysis and sugar dehydration reaction to produce 5-HMF (Zhao *et al.*, 2019).

Verification of Prediction Model

The best-predicted conditions and the actual conditions after correction were as follows: steaming time of 6.31 and 6.3 hours, drying temperature at 73.79°C and 74°C, drying time of 12.96 h and 13 h, both processing times of 2 times, and the comprehensive score was 82.40. The average total sugar, saponin and 5-HMF contents were 17.82±1.79%, 0.18±0.05%, and 0.14±0.01 mg/g, respectively, and the WIPP was black, of high quality, and had a fragrant taste. According to the P_0 daily dose of 9-15 g in ChP, 5-HMF is 1.26-2.10 mg. Therefore, according to the 1.6 mg limit of 5-HMF from the EU Food Safety Commission , the part of P_2 processed in this study was out of range.

The content of 5-HMF in P_2 was 0.14±0.01 mg/g in the response surface process, and 18.63-28.98 mg in the one-factor test, indicating that this optimisation process can effectively reduce the production of toxic ingredients. Combined with ChP, it is suggested that the daily dosage of WIPP shall be less than 11.4 g. Therefore, the processing parameters are feasible and have practically applicable. However, we have not conducted comprehensive testing and analyses of other potentially harmful substances. In the future, we can expand the scope for hazardous substances to comprehensively assess the safety of products and further improve the practical application value.

Antioxidant Activity Results

ABTS Radical Scavenging Assay Results

The ABTS assay is a common test to evaluate antioxidant activity that has been widely applied in plant extracts or food products (Pérez-Jiménez *et al.*, 2008). In the tested concentration range, the scavenging capacities of the P_0, P_1, P_2 and VC were increased with increasing concentrations (Fig. 4a), with IC_{50} values of 4.77, 0.215, 0.047, 0.0026 mg/mL, respectively, indicating that the scavenging capacities of the processing product for ABTS were higher than those of the P_0 ($p<0.05$) and lower than that of VC .

DPPH Radical Scavenging Assay Results

The scavenging of DPPH radicals by the P_0, P_1, P_2, and VC gradually increased with an increasing mass concentration in the tested concentration range (Fig. 4b) and the IC_{50} values of the four were 9.46, 0.144, 0.089, and 0.0047 mg/mL, respectively. Although the P_0, P_1 and P_2 had a certain capacity to scavenge DPPH radicals, and the scavenging effects of P_1 and P_2 were better than that of the P_0 ($p < 0.05$), the scavenging effect was lower than that of VC solution. This indicates that the processing improved the activity against DPPH radicals (Zhong *et al.*, 2013).

Total Reducing Power Assay Results

The total reducing power group determined the absorbance of the P_0, P_1, and P_2 at a concentration of 1 mg/mL as 0.0414, 0.2324, and 0.2713, and the absorbance of VC was 0.23 at a concentration of 0.016 mg/mL (Fig. 4c). The result demonstrated that P_1 and P_2 enhanced the antioxidant activity.

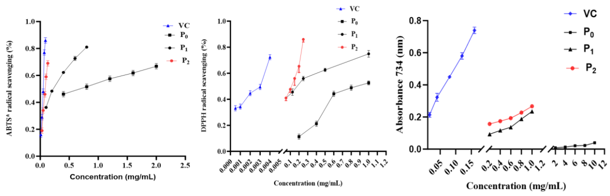

Fig. 4: The antioxidant activities of P 0 and P 2 using VC as positive control in vitro. (A) ABTS radical-scavenging activity; (B) DPPH radical-scavenging; (C) hydroxyl radical-scavenging activity; Data was expressed as the means ± SD (n = 3)

Among the three antioxidant assays, P_2 showed relatively high antioxidant activity. Morphological results showed that the surface became much tighter during the steaming process, which enhanced the antioxidant activity of PSR (Bian *et al.*, 2022). The 5-HMF content of P_2 was higher than that of P_0 and P_1. 5-HMF has antioxidant activity and may act synergistically with polysaccharides to enhance the antioxidant activity of P_2 (Boulebd *et al.*, 2020). Therefore, the processing technology decreased the sensation of a numb tongue and increased the antioxidant activity. It is necessary to carry out the study of structure-activity relationships in the future and cannot ignore the study of toxicity.

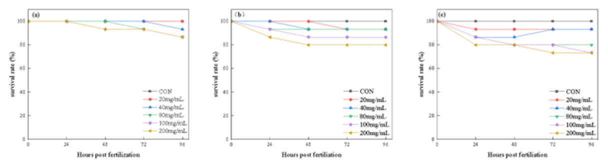

Fig. 5: The effect of different concentrations of drugs on the survival rate of zebrafish embryos (A) P_0; *(B) P_1; (C) P_2*

Safety Assessment

Zebrafish Toxicity

The survival rates of zebrafish embryos (15 embryos per condition, three independent experiments) at different concentrations at 0-96 hpf are shown in Figure 5(a-c). The mortality of zebrafish embryos increased in a time-dependent and dose-dependent manner, with the survival rate of P_2 being significantly lower than those of all other groups. There was no significant decrease in survival at lower concentrations of 20 and 40 mg/mL. Survival of P_1 group significantly decreased to 73% (p<0.01) at 48, 72 and 96 hpf in 100 and 200 mg/mL, respectively. At 96 hpf, the survival rate for all groups administered at a concentration of 200 mg/mL was as follows: control > P_0 > P_1 > P_2 group ($p < 0.05$).

At 48 hpf, the hatching rate of zebrafish embryos in different groups was significantly lower than that of the control group. At 72 hpf, the hatching rate of zebrafish embryos with P_0 in 200 mg/mL was significantly lower than that of the control group (Fig. 6a), but there was no significant difference. The hatching rates of zebrafish embryos at 200 mg/mL of P_1, and P_2 were 66.67 ± 2.80% (p<0.01) (Fig. 6b). and 60.0 ± 6.0% (p<0.01) (Fig. 6c), respectively.

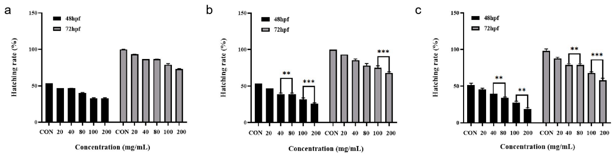

Fig. 6: The effect of different concentrations of drugs on the hatching rate of zebrafish embryos. hpf: 48 h and 72 h (A) P_0; *(B) P_1; (C) P_2*

The results showed that high concentrations of 5-HMF (200 mg/mL) affected the hatchability of zebrafish, while low concentrations of 5-HMF (20 mg/mL) had no effect on the survival of embryos. However, low concentrations of 5-HMF (25 µg/mL) may result in abnormal cardiovascular development in zebrafish larvae (Jiang *et al.*, 2022a). Therefore, 5-HMF may pose a potential risk to human health. Building on the success of the preliminary screening of zebrafish experiments, the mechanism of 5-HMF from PSR on safety in the rodent experiments will be carried out in the future.

Developmental Toxicity

Zebrafish embryos in all sample groups showed no obvious malformations at 24 hpf. At 48 hpf, P_1 and P_2 in the 200 mg/mL administration group showed pericardial oedema and obvious malformations. At 72 hpf, the P_2 group at 80, 100, and 200 mg/mL exhibited varying degrees of pericardial edema. At 96 hpf, pericardial oedema was more severe at 40, 80, 100, and 200 mg/mL in P_1 and P_2 (Fig. 7 a-c). Control groups showed no obvious malformations at 96 hpf.

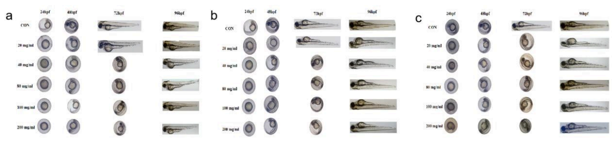

Fig. 7: The effect of the tested samples at different sample on the morphological changes of zebrafish embryos. (A) P_0; *(B) P_1; (C) P_2*

Experimental studies have shown that the toxicity is more pronounced as the processing cycle increases. P_1 and P_2 exhibited acute toxicity and teratogenic to the embryonic development of zebrafish, which may be due to changes in toxicity resulting from alone and the double interaction of 5-HMF and/or other analogues. High concentrations of 5-HMF (100 µg/mL) can disrupt zebrafish behaviour, inhibit cartilage development and reduce bone mineralisation, leading to increased mortality and malformations in zebrafish larvae. (Jiang *et al.*, 2022b). Therefore, it is necessary to find an optimal process with good activity and low toxicity through processing technology in the future.

Conclusion

The process was optimized using RSM combined with the analytic hierarchy process of multiple indices. The taste and antioxidant activities of P_1 and P_2 were significantly better than those of P_0, but the 5-HMF contents of P_1 and P_2 were also increased and exceeds European standards safety limits. There were pericardial oedema and apparent malformations in some zebrafish embryos. It is necessary that the content of 5-HMF is considered as an index component for the processing technology of WIPP, so as to provide a basis for the quality of food and also help to establish a more scientific food quality evaluation system. Therefore, in order to better explore the changes of sugar and the dose-effect relationship between 5-HMF and toxicity, it is necessary to study the production of 5-HMF under different processing conditions and carry out cell and animal experiments.

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Author's Contributions

Shiyu Ji and Jiale Shan: Responsible for experimental data analysis, chart processing and manuscript writing.

Shuo Li: The subsequent revision of the manuscript and replied to the comments.

Zhenlei Wang: Responsible for experimental operation and manuscript format adjustment.

Hongli Zhou: Investigation.

Hepeng Zhao: Responsible for experimental design and manuscript modification.

Ke Sun: Sctivity and safety evaluation operations.

Jingli Zhao: The theoretical derivation of the paper and provided valuable assistance in the "writing-reviewing-editing-funding" process.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved. The authors declare no conflict of interest.

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