


Cite this: *RSC Adv.*, 2021, 11, 4547

# Ultrasound-assisted enzymatic extraction of hydroxy-sanshool compounds from the hydrodistillation residue of two Sichuan peppers: optimization, quantification and pungent taste contribution evaluation

Yuanlong Chi,<sup>id</sup> Yanglong Deng, Shenghui Pu, Yao Ren, Zhifeng Zhao<sup>id\*</sup> and Qiang He<sup>id</sup>

Hydroxy-sanshool compounds were extracted from the hydrodistillation residue of *Z. bungeanum* Maxim. pericarp (ZBM) and *Z. armatum* DC. pericarp (ZADC) by using ethanol–water extraction and extractions assisted with (i) enzymes, (ii) ultrasound, and (iii) enzymes and ultrasound. The yields of the hydroxy-sanshool extracts under these four extraction conditions were calculated and compared. The ultrasound-assisted enzymatic extraction was then optimized based on a Box–Behnken design. Furthermore, five hydroxy-sanshool compounds in the extracts were quantified and their pungent taste contribution value (PCV) was calculated. Oral sensory evaluation was finally performed. Compared with that of the hydroxy-sanshool extracted with ethanol–water, the yields of the samples from the extraction assisted with enzymes, ultrasound, and enzymes and ultrasound increased by 4.2–7.6%, 9.3–9.8%, and 21.5–26.2%, respectively. A maximum yield of 7.87% (w/w) was achieved by using ultrasound-assisted enzymatic extraction under the optimal conditions: 3.1% (w/w) amount of enzyme, an incubation temperature of 50.3 °C, and an ultrasound irradiation power of 207 W. The PCV of hydroxy- $\alpha$ -sanshool, hydroxy- $\beta$ -sanshool, hydroxy- $\gamma$ -sanshool, hydroxy- $\varepsilon$ -sanshool, and hydroxy- $\gamma$ -isosanshool was 1153, 447, 461, 139, and 51 ml g<sup>-1</sup> for the ZBM extract, respectively, while the PCV of these five hydroxyl-sanshools was 659 ml g<sup>-1</sup>, 575 ml g<sup>-1</sup>, not detected, 119 ml g<sup>-1</sup>, and not detected for the ZADC extract, respectively. Tingling and salivation were the main taste sensations of ZBM, whereas numbing and tingling sensations were dominant in ZADC.

Received 29th October 2020  
Accepted 11th January 2021

DOI: 10.1039/d0ra09234g

rsc.li/rsc-advances

## 1. Introduction


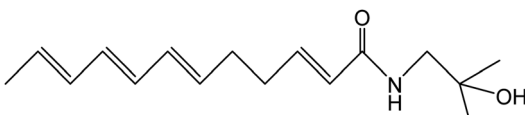

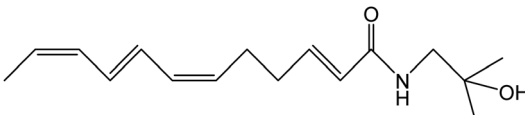

*Z. bungeanum* Maxim. and *Z. armatum* DC., two traditional cash crops belonging to the *Zanthoxylum* genus of the family Rutaceae, are widely cultivated in Asian countries including China, Japan, India, Korea, Nepal, etc. They have attracted increasing attention in recent decades because of their pharmaceutical properties, typical pungency and tingling sensation.<sup>1,2</sup> The pericarps of *Z. bungeanum* Maxim. and *Z. armatum* DC., ZBM and ZADC for short, are traditionally used in herbal medicine and stir-fried or processed with vinegar and salt-water. More than 140 chemical compounds have been isolated and identified from ZBM, including alkaloids, terpenoids, flavonoids, and free fatty acids. These bioactive constituents are responsible for the high medicinal value of the analgesic,<sup>2</sup> anti-inflammatory,<sup>3,4</sup> antibacterial,<sup>5</sup> antioxidant,<sup>6</sup> and anti-tumor activities.<sup>7</sup>

ZBM and ZADC are also known as Sichuan peppers or *Huajiao* and are used as condiments in cooking. Notably, the primary pungent and tingling substances in *Zanthoxylum* fruits have been identified as hydroxy-sanshool compounds such as hydroxy- $\alpha$ -sanshool, hydroxy- $\beta$ -sanshool, hydroxy- $\gamma$ -sanshool, hydroxy- $\varepsilon$ -sanshool and hydroxy- $\gamma$ -isosanshool.<sup>8,9</sup> Their chemical structure and molecular formula are shown in Table 1. The pungent nature of the hydroxy-sanshool compounds could be mediated *via* interactions with the transient receptor potential ankyrin 1 (TRPA1) and vanilloid 1, two chemosensory ion channels.<sup>10</sup> The *cis*-configured double bonds in the hydroxy-sanshools were reported to be essential for the activation of TRPA1.<sup>11</sup> A structure–pungency activity relationship study revealed that a minimal structure including an *N*-(2-methyl-2-hydroxypropyl) amide and a (CH=Z=CH–CH<sub>2</sub>–CH<sub>2</sub>–CH=E=CH) sequence motif plays an important role in the pungent and tingling sensations.<sup>12</sup> Moreover, hydroxy-sanshool and its isomers could show different pungent sensations and intensity. For example, hydroxy-sanshools ( $\alpha$ ,  $\gamma$ , and  $\varepsilon$ ) caused the oral sensations of tingling and salivation, while hydroxy- $\beta$ -sanshool and hydroxy- $\gamma$ -isosanshool produced a numbing sensation.<sup>8</sup> To

College of Biomass Science and Engineering, Healthy Food Evaluation Research Center, Sichuan University, Chengdu 610065, P. R. China. E-mail: 57109137@qq.com; Fax: +86 28 85404298; Tel: +86 28 85404298



Table 1 Chemical structure and molecular formula of five kinds of hydroxy-sanshool compounds

Compounds	Chemical structure	Molecular formula
Hydroxy- $\alpha$ -sanshool		$C_{16}H_{25}NO_2$
Hydroxy- $\beta$ -sanshool		$C_{16}H_{25}NO_2$
Hydroxy- $\gamma$ -sanshool		$C_{18}H_{27}NO_2$
Hydroxy- $\epsilon$ -sanshool		$C_{16}H_{25}NO_2$
Hydroxy- $\gamma$ -isosanshool		$C_{18}H_{27}NO_2$

the best of our knowledge, the composition of hydroxy-sanshools and the pungent taste contribution of each hydroxy-sanshool in the *Zanthoxylum* species have rarely been reported. So, extraction and quantification of these five hydroxy-sanshools and the evaluation of their pungent taste contributions would help to recognize the pungent and tingling characteristics of ZBM and ZADC at the molecular level and to deeply understand the structure–activity relationship between hydroxy-sanshool and pungent taste sensations.

In our previous work, the aromatic essential oil of ZBM and ZADC was obtained by using the hydrodistillation method and a residue was produced.<sup>13</sup> If this residue was discarded, it would be a waste of crop resources owing to the fact that the residue is rich in some valuable non-volatile alkylamides such as hydroxy-sanshool compounds.<sup>14</sup> So, the hydrodistillation residue of ZBM and ZADC was used as the raw material in this study, and extraction of the hydroxy-sanshool compounds was firstly performed with ethanol–water and then assisted with (i) enzymes, (ii) ultrasound, and (iii) enzymes and ultrasound. The cell destruction of ZBM after these four extractions were observed with scanning electron microscopy. Furthermore, ultrasound-assisted enzymatic extraction of the hydroxy-sanshool compounds was optimized using the Box–Behnken design of response surface methodology. The interactions between three extraction variables (enzyme amount, incubation temperature and ultrasound irradiation power) on the yield were discussed. In addition, five hydroxy-sanshool compounds in the extracts were quantitatively determined and their pungent taste contribution evaluated and the primary hydroxy-sanshool and the typical pungent sensation of ZBM and ZADC were identified. These results were finally verified by oral sensory evaluation.

## 2. Materials and methods

### 2.1. Materials and chemicals

ZBM and ZADC were picked in September 2017 from Ya'an, Sichuan Province, China. They were air dried at room temperature, cleaned of impurities and sealed in an aluminum foil bag and stored at  $-18\text{ }^{\circ}\text{C}$  until use. Their contents ( $\text{mg g}^{-1}$ ) of crude protein, crude fat, crude fiber, total phenols, total flavonoid and moisture were  $111.4 \pm 10.8$  and  $117.7 \pm 5.3$ ,  $38.1 \pm 1.6$  and  $48.7 \pm 8.0$ ,  $312.8 \pm 8.8$  and  $366.6 \pm 10.8$ ,  $11.6 \pm 0.6$  and  $6.5 \pm 0.1$  gallic acid equivalent,  $2.9 \pm 0.1$  and  $2.3 \pm 0.0$  rutin equivalent, and  $115.9 \pm 1.3$  and  $135.6 \pm 0.6$ , respectively.

Hydroxy- $\alpha$ -sanshool, hydroxy- $\beta$ -sanshool, hydroxy- $\gamma$ -sanshool, hydroxy- $\epsilon$ -sanshool, and hydroxy- $\gamma$ -isosanshool standards were purchased from Chengdu Mai Desheng Technology Co., Ltd. (Chengdu, China). Gallic acid and rutin standards were purchased from Beijing Suolaibao Biotechnology Co., Ltd. (Beijing, China). Commercial cellulase (EC 3.2.1.4,  $5000\text{ U g}^{-1}$ ), hemicellulase (EC 3.2.1.78,  $5000\text{ U g}^{-1}$ ) and pectinase (EC 3.2.1.15,  $5000\text{ U g}^{-1}$ ) were purchased from Nanning Pangbo Biological Co., Ltd. (Nanning, China). Food grade alcohol was procured from Chron Chemical Reagent Co., Ltd. (Chengdu, China). Other chemical reagents were of analytical grade.

### 2.2. Preparation of the hydrodistillation residue of ZBM and ZADC

The hydrodistillation residue of ZBM and ZADC was prepared according to the method described in our previous study with minor modifications.<sup>13</sup> 50 g of dry ZBM (or ZADC) was crushed and sieved through a mesh size 40 and then dispersed into 500 ml



water and heated to the boil. After distillation for 1.5 h, the vapor was collected as an aromatic essential oil. The residue was collected and weighed to about 100 g after vacuum filtration. Extraction and quantification of the hydroxy-sanshool compounds in these two residues were performed in the following study, and their pungent taste contributions were evaluated.

### 2.3. Extraction of hydroxy-sanshool compounds with four different conditions

The hydrodistillation residue of 20 g of ZBM (or ZADC) was soaked in 180 ml water for 3 h under the conditions of 50 °C and pH 4.8. The pH level was adjusted using 0.05 M citric acid. Food grade alcohol (200 ml) was then added and extraction was performed at 50 °C for 3 h. The hydroxy-sanshool crude extract was obtained after filtration and vacuum distillation. After addition of 100 ml food grade alcohol, the mixture was centrifuged at 4000 rpm for 15 min, and the supernatant was collected. Finally, the hydroxy-sanshool extract of ZBM, identified as HSE-ZBM, was obtained after vacuum distillation and weighed as  $M_1$ . In addition, the ethanol–water extractions assisted with (i) enzymes, (ii) ultrasound, and (iii) enzymes and ultrasound were investigated. For the enzyme-assisted ethanol–water extraction, 2.5% (w/w) mixed enzymes (the dosage ratio of cellulose : hemicellulose : pectinase was 1 : 1 : 1) was added during soaking for the hydroxy-sanshool extraction, and the other steps were the same as mentioned above. For the ultrasound-assisted ethanol–water extraction, ultrasonic irradiation at 150 W was carried out for 30 min and the other steps were the same as the above. For the ethanol–water extraction assisted with enzymes and ultrasound, 2.5% (w/w) mixed enzymes were added during soaking, and the extraction was assisted with ultrasound at 150 W for 30 min. The other steps were the same as above. The yield (% w/w) of HSE-ZBM (or HSE-ZADC) was calculated as  $(M_1/20) \times 100$ .

### 2.4. Optimization of the ultrasound-assisted enzymatic extraction by the Box–Behnken design

Based on the single extraction factor results in our preliminary experiments (data not shown), three factors, *i.e.*, enzyme amount ( $X_1$ ), incubation temperature ( $X_2$ ) and ultrasound irradiation power ( $X_3$ ), had a stronger influence on the yields of hydroxy-sanshool extract and were chosen as the independent variables. The yield of hydroxy-sanshool extract was the

response value. Optimization of the ultrasound-assisted enzymatic extraction from the ZBM residue was performed using a Box–Behnken design. The analysis of experimental data was performed using analysis of variance (ANOVA). Design-Expert software was employed for experiment design, regression and graphical analysis of the results obtained.

### 2.5. Scanning electron microscopy (SEM)

Cell destruction of ZBM after the four extraction conditions mentioned in Section 2.3 was observed using scanning electron microscopy (JSM-7500F, JEOL, Japan). The ZBM residue was fixed with glutaraldehyde solution, dehydrated with ethanol solutions, dried by a critical point drying process, fixed on adhesive tape and then sputtered with gold using a sputter coater (JFC-1600, JEOL, Japan) prior to observation.

### 2.6. Quantification and pungent taste contribution evaluation

**2.6.1. High performance liquid chromatography (HPLC).** The contents of hydroxy- $\alpha$ -sanshool, hydroxy- $\beta$ -sanshool, hydroxy- $\gamma$ -sanshool, hydroxy- $\epsilon$ -sanshool, and hydroxy- $\gamma$ -iso-sanshool in HSE-ZBM (or HSE-ZADC) were determined using an HPLC system (HPLC-6AD, Shimadzu, Japan) equipped with a 5  $\mu$ m (250  $\times$  4.6 mm i.d.) ZORBAX Eclipse Plus C18 column (Agilent, United States). For the sample preparation, 1 g HSE-ZBM (or HSE-ZADC) was dissolved in methanol and filtrated through an alkaline alumina column. After vacuum distillation, the remaining substance was collected and re-dissolved in methanol to 10 ml. For the sample determination, the injection volume was 20  $\mu$ l and the detection wavelength was 270 nm. Ultrapure water was used as solvent A and acetonitrile was solvent B. The solvents were delivered at a total flow rate of 0.5 ml min<sup>-1</sup>. The solvent gradient was from 45% B to 50% B linearly in 10 min, a linear increase to 65% B in 20 min then to 100% B in 1 min, followed by 9 min isocratic elution, and then a return to 45% B in 5 min. The five hydroxy-sanshools in the samples were identified by comparing their retention time with that of the reference standards and quantified with an external standard method.

**2.6.2. Determination of the pungent taste contribution value (PCV).** To recognize the pungent taste contribution and characteristic sensation of each hydroxy-sanshool in ZBM and ZADC, the PCV (ml g<sup>-1</sup>) was introduced and calculated as  $C \times$

Table 2 The yields of HSE-ZBM and HSE-ZADC by using four different extraction conditions

Methods	Yields (% w/w)	
	HSE-ZBM	HSE-ZADC
Ethanol–water extraction	5.12 $\pm$ 0.19 <sup>Aa</sup>	3.35 $\pm$ 0.04 <sup>a</sup>
Enzyme-assisted ethanol–water extraction	5.51 $\pm$ 0.28 <sup>AB</sup>	3.49 $\pm$ 0.05 <sup>a</sup>
Ultrasound-assisted ethanol–water extraction	5.62 $\pm$ 0.05 <sup>BC</sup>	3.66 $\pm$ 0.07 <sup>b</sup>
Ultrasound-assisted enzymatic ethanol–water extraction	6.46 $\pm$ 0.21 <sup>C</sup>	4.07 $\pm$ 0.06 <sup>c</sup>

<sup>a</sup> A, B, and C means that different letters within a column were significantly different ( $P < 0.05$ ) in HSE-ZBM; a, b, and c means that different letters within a column were significantly different ( $P < 0.05$ ) in HSE-ZADC.



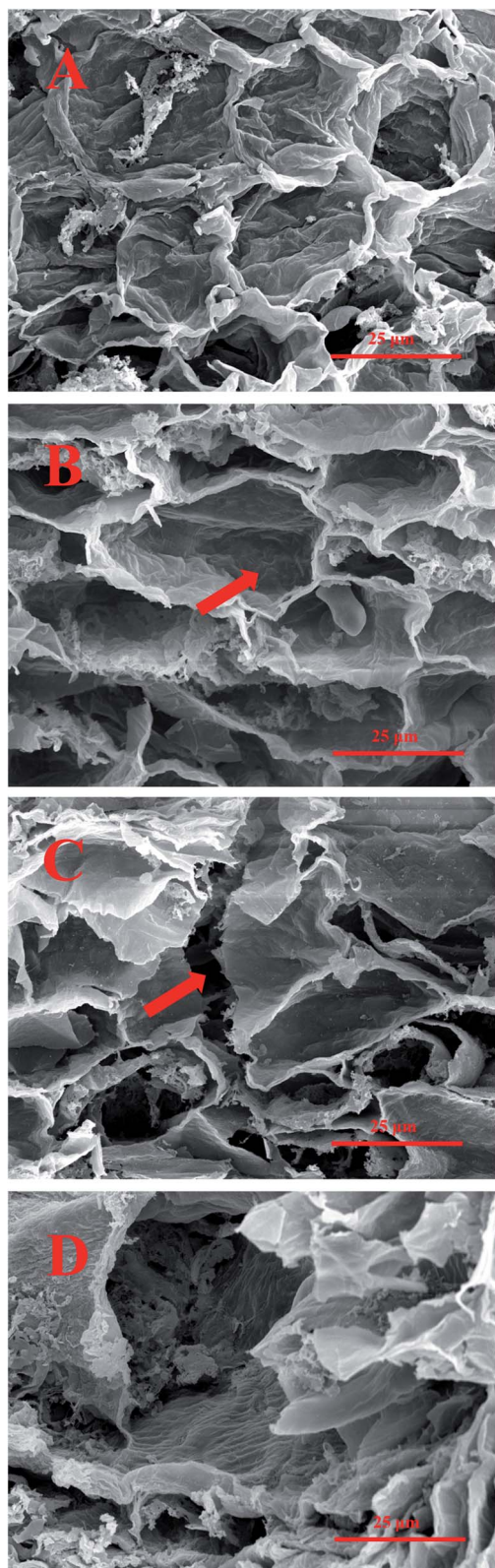


Fig. 1 Electron microscopy of ZBM cells with four extraction conditions. Ethanol–water extraction (A) and extraction assisted with enzymes (B), ultrasound (C), and enzymes and ultrasound (D).

$\text{SPU} \times 10^{-3}$ , where  $C$  is the content of each hydroxy-sanshool or its isomer in HSE-ZBM or HSE-ZADC. Scoville Pungency Unit (SPU,  $\text{ml g}^{-1}$ ) was the dilution factor per gram of substance, in which its pungent taste could be simply perceived. The SPUs of hydroxy- $\alpha$ -sanshool, hydroxy- $\beta$ -sanshool, hydroxy- $\gamma$ -sanshool, hydroxy- $\epsilon$ -sanshool, and hydroxy- $\gamma$ -isosanshool were  $2.31 \times 10^4 \text{ ml g}^{-1}$ ,  $4.86 \times 10^4 \text{ ml g}^{-1}$ ,  $2.78 \times 10^4 \text{ ml g}^{-1}$ ,  $4.63 \times 10^4 \text{ ml g}^{-1}$ , and  $2.78 \times 10^4 \text{ ml g}^{-1}$ , respectively.<sup>15</sup>

### 2.6.3. Oral sensory evaluation of ZBM and ZADC powder.

Sensation testing was performed according to the literature protocol.<sup>16</sup> Quantitative descriptive analysis was used to evaluate the performance of the sensory pungency characteristics of the ZBM and ZADC samples. Ten assessors (aged 23–27 years old) were invited and trained. The attributes and intensities of three pungency sensations, *i.e.*, numbing, tingling and salivation, were perceived quantitatively by using a standard solution containing hydroxy- $\alpha$ -sanshool and hydroxy- $\beta$ -sanshool with a ratio of 1 : 1. Six pungency intensities of the solution including hydroxy-sanshool contents ( $\text{mg ml}^{-1}$ ) of 0, 0.02, 0.08, 0.14, 0.2, 0.4, were scored at 0 (no sensation), 1 (very weak), 2 (weak), 3 (moderate), 4 (strong) and 5 (very strong), respectively. Assessors were requested not to eat and drink for at least 1 h before the test. Firstly, they were instructed to rinse their oral cavity with 5 ml of water and 7.25 mg ZBM or ZADC powder was taken inside the mouth for 10 s. Secondly, the assessors were asked to spit and to wait for 2 min before gargling with 5 ml of water and scoring the sensation intensity. The assessors repeated the steps described above and the test interval between each sample was 5 min. The tests were conducted in triplicate and the sensory score was expressed as an average value.

### 2.7. Statistical analysis

Design-Expert software version 8.0 (Stat-Ease company, USA) for the Box–Behnken design was used to determine the optimum extracting process. ANOVA and Duncan's multiple range tests were used to determine the significant differences ( $p < 0.05$ ) between the means. All the experimental results were expressed as mean  $\pm$  standard deviation.

## 3. Results and discussion

### 3.1. Effects of the extraction conditions on the yield of hydroxy-sanshool extract

ZBM and ZADC, generally known as “*honghuajiao*” and “*qinghuajiao*”, are popular spices used worldwide, especially in Asia. They possess different aroma and pungent characteristics, which could be correlated with the composition and content of the volatile and non-volatile flavor compounds. In our previous work, the essential oil of volatile aroma components of ZBM and ZADC was extracted with hydrodistillation, and the potent odorants and typical aroma were identified.<sup>13</sup> Meanwhile, the hydrodistillation residue of ZBM and ZADC was produced, and some valuable active compounds, such as hydroxy-sanshools, were retained. So, the hydroxy-sanshool compounds were extracted from the residue by using four conditions, and their extraction yields are shown in Table 2. The content of hydroxy-



**Table 3** Experimental design matrix to screen for the variable that determines the hydroxy-sanshool extract yield from the ZBM and ANOVA results

No.	Box-Behnken design				ANOVA						
	$X_1$	$X_2$	$X_3$	Y (%)							
				Actual	Predicted	Source	Sum of squares	Degree of freedom	Mean square	F-Value	p-Value
1	3.0	45	180	7.08	7.07	Model <sup>a</sup>	3.22	9	0.36	98.5	<0.01 <sup>b</sup>
2	3.0	45	240	6.74	6.79	$X_1$	0.08	1	0.08	20.82	<0.01
3	3.0	50	210	7.80	7.82	$X_2$	0.07	1	0.07	18.34	<0.01
4	2.5	50	240	7.05	7.00	$X_3$	0.03	1	0.03	6.97	0.03
5	3.0	50	210	7.76	7.82	$X_1X_2$	0.08	1	0.08	22.36	<0.01
6	3.0	50	210	7.89	7.82	$X_2X_3$	0.03	1	0.03	7.96	0.03
7	2.5	50	180	7.06	7.11	$X_1^2$	0.61	1	0.61	168.24	<0.01
8	3.0	50	210	7.79	7.82	$X_2^2$	1.27	1	1.27	348.69	<0.01
9	3.5	55	210	7.20	7.22	$X_3^2$	0.26	1	0.26	71.57	<0.01
10	2.5	45	210	6.30	6.28	$X_1X_2^2$	0.12	1	0.12	33.05	<0.01
11	3.0	50	210	7.87	7.82	Residual	0.03	7	0.01		
12	3.5	50	180	7.38	7.39	Lack of fit	0.02	3	0.01	1.43	0.36
13	3.5	45	210	7.35	7.33	Pure error	0.01	4	0.01		
14	3.0	55	180	7.14	7.09	Corrected total	3.25	16			
15	3.5	50	240	7.28	7.27	Credibility analysis of the regression equations					
16	2.5	55	210	6.72	6.74	Standard deviation	Mean	Coefficient of variation	$R^2$	Adequacy precision	
17	3.0	55	240	7.14	7.15	0.06	7.27	0.83%	0.9922	33.44	

<sup>a</sup>  $X_1$  is the amount of enzyme (%),  $X_2$  is the incubation temperature (°C),  $X_3$  is the ultrasound irradiation power (W), and  $Y$  is the total yield.

<sup>b</sup> Significant at  $p < 0.05$ .

sanshool compounds in ZBM was much higher than that in ZADC, suggesting a stronger pungent intensity of ZBM. In addition, compared with that of the hydroxy-sanshool extracted with ethanol–water ( $5.12 \pm 0.19\%$  for HSE-ZBM and  $3.35 \pm 0.04\%$  for HSE-ZADC), the yields of the samples for which the extraction was assisted with enzymes, ultrasound, and enzymes and ultrasound increased by 4.2–7.6%, 9.3–9.8%, and 21.5–26.2%, respectively. Ultrasound-assisted enzymatic extraction achieved a higher yield than the other three extraction conditions ( $p < 0.05$ ). This result could be attributed to the synergistic effects between mixed enzymes and ultrasound. The mixture of cellulase, hemicellulase and pectinase could disrupt and degrade the cell wall and release the intracellular constituents.<sup>17</sup> Sonication not only improved the affinity between the enzyme and substrate, but also enhanced the conversion of the enzyme/substrate complex into the product.<sup>18</sup>

Several literatures reported the extraction of alkylamides from *Zanthoxylum*. Yang and co-workers reported 6% (w/w) yield of the alkylamide extract with hexane/ethyl acetate from the dried pericarps of *Z. bungeanum* and *Z. schinifolium*.<sup>19</sup> Bhatt *et al.* obtained 15.1% (w/w) methanol extract of flavonoids, lignans, coumarin and amides from the dried leaves of *Z. armatum*.<sup>20</sup> Nevertheless, the yields of alkylamides or other active compounds depend not only on the extraction methods, but also on the *Zanthoxylum* growing area, variety, climate, cultivation conditions and sample preparation processes.<sup>21,22</sup> Some solvents, including methanol, ethanol, chloroform, petroleum ether, diethyl ether, vegetable oil and water, have been applied for the extraction of pungent and tingling

compounds from *Zanthoxylum*.<sup>23,24</sup> Ethanol and water are generally considered as safe and eco-friendly options, whereas organic solvent is not used at an industrial scale due to the toxicity associated with the solvent residue.

### 3.2. SEM analysis

To investigate the influence of extraction conditions on the matrix, SEM was employed to observe the microscopic structure changes of the ZBM tissues. The cell wall breakage of the sample treated with four extraction conditions is shown in Fig. 1. After ethanol–water extraction (Fig. 1A), the cell shape of the sample remained unchanged and intact, although the cell wall surface was rough and wrinkled and some cell fragments were observed. In the case of the enzyme-assisted ethanol–water extraction (Fig. 1B), huge perforations in the internal surface of the sample cell were observed, suggesting the disruption of the cell wall structure under the combined effects of cellulase, hemicellulase and pectinase. The shape of the sample cells became irregular. Notably, after ultrasound-assisted ethanol–water extraction (Fig. 1C), a jagged edge appeared on the cell wall and the cell structure was partly damaged, which indicated that the cell walls were attacked by the cavitation effects of the ultrasound. As for the extraction assisted with enzymes and ultrasound (Fig. 1D), compared with the other three treatments, the cell walls incurred more serious damage. The cell structure almost disappeared and a mass of cellular fragments was produced. It revealed that the combined effects of enzymes and ultrasound could greatly enhance the disruption of the ZBM cell structure, which facilitated the release of hydroxy-sanshools.



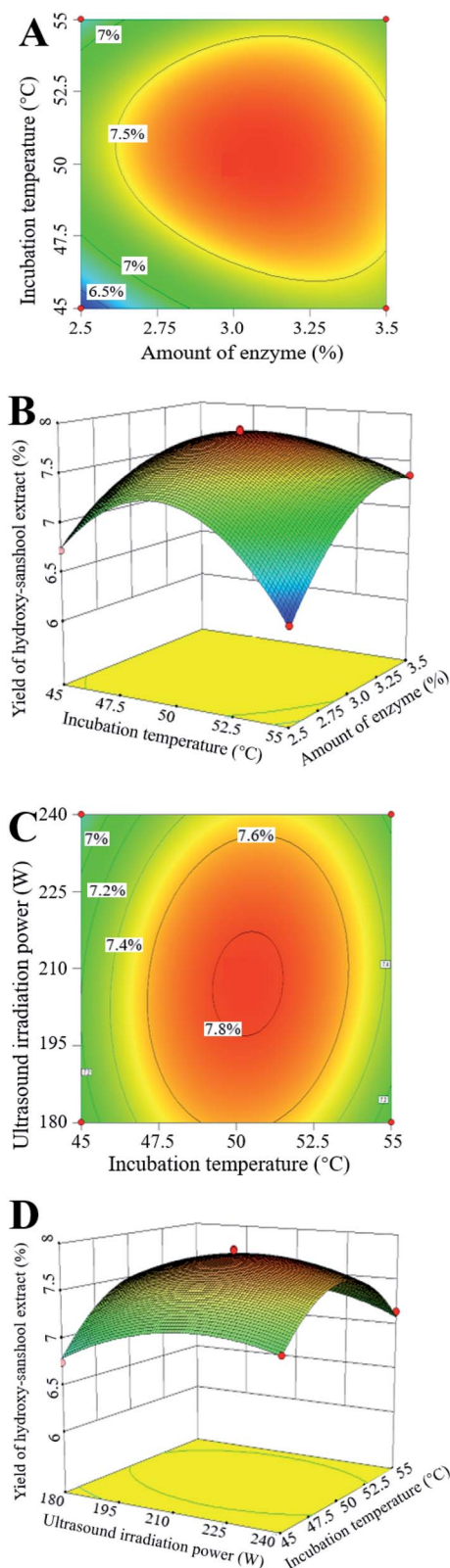


Fig. 2 Contour and response surface plots for the hydroxy-sanshool extraction yield from ZBM showing variable interactions. Contour plot between enzyme amount and incubation temperature (A), response surface plot between enzyme amount and incubation temperature (B), contour plot between incubation temperature and ultrasound irradiation power (C), and response surface plot between incubation temperature and ultrasound irradiation power (D).

This finding was also in good agreement with the high yield of HSE-ZBM extracted with enzyme and ultrasound mentioned above.

### 3.3. Optimization of the ultrasound-assisted enzymatic extraction of hydroxy-sanshool compounds

In our preliminary experiments, significant variables in the hydroxy-sanshool extraction from the ZBM residue were firstly selected from seven variables, including enzyme amount, incubation pH, incubation temperature, incubation time, ultrasound irradiation power, liquid–solid ratio and ultrasound irradiation time, based on a Plackett–Burman design (data not shown). Three variables, *i.e.*, enzyme amount ( $X_1$ ), incubation temperature ( $X_2$ ) and ultrasound irradiation power ( $X_3$ ), played important roles in the hydroxy-sanshool extraction and were selected for further optimization of the extraction process. A total of 17 experimental conditions were trialed and the results were applied to determine the influence of the variables, as shown in Table 3. The model developed had a  $p$ -value of <0.01 and a non-significant lack of fit value of 0.36. The regression equation from the ANOVA had a determination coefficient  $R^2$  of 0.9922, which is an estimate of the data variation of the model. “Adequacy precision” measures the signal to noise ratio. A ratio greater than 4 is desirable, and the ratio of 33.44 shows an adequate signal. These model characteristics indicated that the model was significant and could be used to efficiently determine the optimal conditions. Sufficient input levels were used to fit a third-order polynomial equation. The hydroxy-sanshool extract yield ( $Y$ ) was calculated as follows:

$$Y = 7.82 + 0.14X_1 + 0.09X_2 - 0.06X_3 - 0.14X_1X_2 + 0.09X_2X_3 - 0.38X_1^2 - 0.55X_2^2 - 0.25X_3^2 + 0.25X_1X_2^2$$

The ANOVA indicated that the model items  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_1X_2$ ,  $X_2X_3$ ,  $X_1^2$ ,  $X_2^2$ ,  $X_3^2$ , and  $X_1X_2^2$  are statistically significant ( $p < 0.05$ ). The interactions between variables (enzyme amount *vs.* incubation temperature and incubation temperature *vs.* ultrasound irradiation power) had a significant influence on the extraction yield, and their contour and response surface plots are shown in Fig. 2. The optimal conditions to maximize hydroxy-sanshool yields could be found from these interactions. The optimum extraction conditions according to the response surface model are: 3.1% (w/w) amount of enzyme, an incubation temperature of 50.3 °C, and an ultrasound irradiation power of 207 W. The predicted highest yield under this condition is 7.84%. In order to verify the reliability of the theoretical model, an experiment was performed using the optimal conditions. The obtained yield of  $7.87 \pm 0.32$  (% w/w) is almost consistent with the value predicted by the regression model. So, the results predicted by the model were reliable and practical.

### 3.4. Quantification and pungent taste contribution evaluation

Pungency and tingling are the characteristic taste sensations of Sichuan pepper as a spice. Hydroxy-sanshools were considered as the primary pungent active compounds and possessed





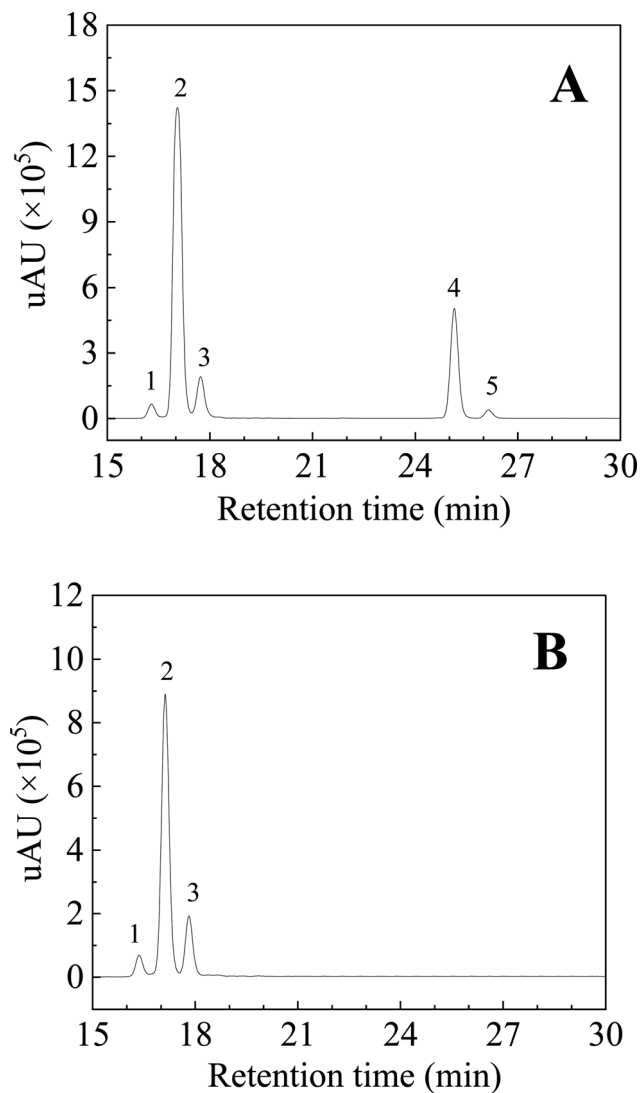


Fig. 3 HPLC chromatograms of HSE-ZBM solution (A) and HSE-ZADC solution (B) at 270 nm. The peaks of 1, 2, 3, 4 and 5 were identified as hydroxy- $\epsilon$ -sanshool, hydroxy- $\alpha$ -sanshool, hydroxy- $\beta$ -sanshool, hydroxy- $\gamma$ -sanshool, and hydroxy- $\gamma$ -isosanshool.

a potent influence on the pungent taste of ZBM and ZADC.<sup>9,15</sup> Five hydroxy-sanshool compounds in both HSE-ZBM and HSE-ZADC were quantitatively measured and their pungent taste

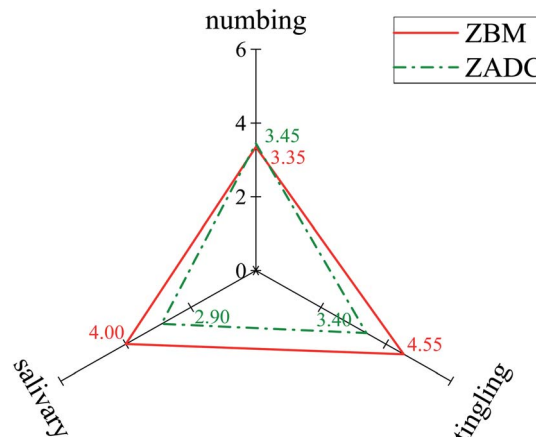


Fig. 4 Oral sensory evaluations of ZBM and ZADC.

contribution value was calculated according to the method described in Section 2.6.2. As shown in Fig. 3 and Table 4, hydroxy- $\alpha$ -sanshool was determined as the main pungent taste component in ZBM with the highest content of  $49.92 \pm 0.10 \text{ mg g}^{-1}$  and a PCV of  $1153 \text{ ml g}^{-1}$ , followed by hydroxy- $\gamma$ -sanshool ( $16.57 \pm 0.58 \text{ mg g}^{-1}$ ,  $461 \text{ ml g}^{-1}$ ) and hydroxy- $\beta$ -sanshool ( $9.19 \pm 0.43 \text{ mg g}^{-1}$ ,  $447 \text{ ml g}^{-1}$ ). With regard to ZADC, hydroxy- $\alpha$ -sanshool ( $28.53 \pm 0.46 \text{ mg g}^{-1}$ ,  $659 \text{ ml g}^{-1}$ ) and hydroxy- $\beta$ -sanshool ( $11.84 \pm 0.44 \text{ mg g}^{-1}$ ,  $575 \text{ ml g}^{-1}$ ) were the main pungent taste components. Hydroxy- $\epsilon$ -sanshool and hydroxy- $\gamma$ -isosanshool seemed to play a less important role and had a much lower content and PCV in both ZBM and ZADC. According to the oral sensations of five hydroxy-sanshools reported in the literature,<sup>8,25</sup> the characteristic pungent sensations of ZBM and ZADC were predicted. ZBM had the major sensations of tingling and salivation, for which the total PCV ( $1753 \text{ ml g}^{-1}$ ) was more than thrice that of the numbing sensation ( $498 \text{ ml g}^{-1}$ ). In comparison, ZADC shared similar PCVs for the tingling, salivation ( $778 \text{ ml g}^{-1}$ ), and numbing ( $575 \text{ ml g}^{-1}$ ) sensation.

The characteristic taste sensations were verified using an oral sensory evaluation for ZBM and ZADC powder, and the results are summarized in Fig. 4. The numbing score of ZBM (3.35) is nearly equal to that of ZADC (3.45) with no significant difference ( $p > 0.05$ ). The salivation and tingling scores of ZBM were 4.00 and 4.55, respectively, which were significantly higher

Table 4 Quantification and pungent taste contribution value (PCV) evaluation of five hydroxy-sanshool compounds in HSE-ZBM and HSE-ZADC

Compounds	Content ( $\text{mg g}^{-1}$ )		PCV ( $\text{ml g}^{-1}$ )		Oral sensation <sup>a</sup>
	HSE-ZBM	HSE-ZADC	HSE-ZBM	HSE-ZADC	
Hydroxy- $\alpha$ -sanshool	$49.92 \pm 0.10$	$28.53 \pm 0.46$	1153	659	Tingling, salivation
Hydroxy- $\beta$ -sanshool	$9.19 \pm 0.43$	$11.84 \pm 0.44$	447	575	Numbing
Hydroxy- $\gamma$ -sanshool	$16.57 \pm 0.58$	ND <sup>b</sup>	461	ND	Tingling, salivation
Hydroxy- $\epsilon$ -sanshool	$2.30 \pm 0.02$	$2.56 \pm 0.04$	139	119	Tingling, salivation
Hydroxy- $\gamma$ -isosanshool	$1.85 \pm 0.06$	ND	51	ND	Numbing

<sup>a</sup> The data of oral sensation is from the reference by Bader *et al.* (2014). <sup>b</sup> ND represents that the level is lower than the limit of detection.



than those of ZADC ( $p < 0.05$ ). This result implied that ZBM was inclined to the sensations of tingling and salivation and ZADC had nearly equal shares in tingling and numbing sensations. This finding was in accordance with the PCV analysis mentioned above.

## 4. Conclusions

An efficient approach for the extraction of hydroxy-sanshool compounds from the hydrodistillation residue of ZBM and ZADC was developed and optimized by Box-Behnken design. The optimal ultrasound-assisted enzymatic extraction conditions were as follows: 3.1% (w/w) amount of enzyme, an incubation temperature of 50.3 °C, and an ultrasound irradiation power of 207 W. The yield of HSE-ZBM was  $7.87 \pm 0.32\%$  (w/w) under the optimal conditions. Furthermore, five hydroxy-sanshools in HSE-ZBM and HSE-ZADC were quantified and their pungent taste contribution was recognized. ZBM had the primary pungent taste components of hydroxy- $\alpha$ -sanshool and hydroxy- $\gamma$ -sanshool, giving the main oral sensations of tingling and salivation. On the other hand, ZADC possessed the dominant pungent compounds of hydroxy- $\alpha$ -sanshool and hydroxy- $\beta$ -sanshool, exhibiting nearly equal shares in tingling and numbing sensations.

## Conflicts of interest

The authors state no conflict of interest.

## Acknowledgements

This work was supported by the Key Research and Development Projects of Sichuan Province (no. 2020YFN0151 and no. 2019YFS0063). We would like to acknowledge the volunteers for actively participating in the sensory tests and Dr Qixian Zhang for the helpful discussion.

## References

- 1 L. Ranawat, J. Bhatt and J. Patel, *J. Ethnopharmacol.*, 2010, **127**, 770–780.
- 2 M. M. Zhang, J. L. Wang, L. Zhu, T. Li, W. D. Jiang, J. Zhou, W. Peng and C. J. Wu, *Int. J. Mol. Sci.*, 2017, **18**, 2172.
- 3 Y. Lan, Q. Wu, Y. Q. Mao, Q. Wang, J. An, Y. Y. Chen, W. P. Wang, B. C. Zhao, N. Liu and Y. W. Zhang, *J. Zhejiang Univ., Sci., B*, 2014, **15**, 153–164.
- 4 X. L. Lei, S. R. Cheng, H. L. Peng, Q. He, H. Y. Zhu, M. Q. Xu, Q. Q. Wang, L. Liu, C. S. Zhang, Q. Z. Zhou, F. Zeng, J. Ying, H. Y. Yin and S. G. Yu, *Afr. J. Tradit., Complementary Altern. Med.*, 2016, **13**, 45–52.
- 5 H. Lei, J. Wu, Q. Wang, A. X. Su, M. Xue, Q. Liu and Q. H. Hu, *Food Funct.*, 2017, **8**, 1569–1576.
- 6 L. C. Yang, R. Li, J. Tan and Z. T. Jiang, *J. Agric. Food Chem.*, 2013, **61**, 1772–1778.
- 7 K. Y. Li, R. Zhou, W. W. Jia, Z. Li, J. Z. Li, P. F. Zhang and T. C. Xiao, *J. Ethnopharmacol.*, 2016, **186**, 351–361.
- 8 M. Bader, T. D. Stark, C. Dawid, S. Losch and T. Hofmann, *J. Agric. Food Chem.*, 2014, **62**, 2479–2488.
- 9 Z. F. Zhao, R. X. Zhu, K. Zhong, Q. He, A. M. Luo and H. Gao, *J. Food Sci.*, 2013, **78**, C1516–C1522.
- 10 S. Murakami, Y. Sudo, K. Miyano, H. Nishimura, M. Matoba, S. Shiraishi, H. Konno and Y. Uezono, *J. Pharmacol. Sci.*, 2016, **130**, 72–77.
- 11 C. E. Riera, C. Menozzi-Smarrito, M. Affolter, S. Michlig, C. Munari, F. Robert, H. Vogel, S. A. Simon and J. le Coutre, *Br. J. Pharmacol.*, 2009, **157**, 1398–1409.
- 12 C. C. Galopin, S. M. Furrer and A. Goeke, *Challenges in taste chemistry and biology*, 2004, 867, pp. 139–152.
- 13 R. X. Zhu, K. Zhong, W. C. Zeng, X. Y. He, X. Q. Gu, Z. F. Zhao and H. Gao, *Afr. J. Microbiol. Res.*, 2011, **5**, 4631–4637.
- 14 S. Wang, J. C. Xie, W. Yang and B. G. Sun, *J. Liq. Chromatogr. Relat. Technol.*, 2011, **34**, 2640–2652.
- 15 L. L. Zhang, L. Zhao, H. Y. Wang, B. L. Shi, L. Y. Liu and Z. X. Chen, *J. Sci. Food Agric.*, 2019, **99**, 1475–1483.
- 16 L. L. Zhang, S. S. Xu, B. L. Shi, H. Y. Wang, L. Y. Liu, K. Zhong, L. Zhao and Z. X. Chen, *J. Sens. Stud.*, 2018, **33**, e12465.
- 17 K. X. Hou, X. B. Yang, M. L. Bao, F. L. Chen, H. Tian and L. Yang, *Ind. Crops Prod.*, 2018, **117**, 205–215.
- 18 S. Singh, M. Agarwal, A. Bhatt, A. Goyal and V. S. Moholkar, *Bioresour. Technol.*, 2015, **192**, 636–645.
- 19 X. G. Yang, *J. Agric. Food Chem.*, 2008, **56**, 1689–1696.
- 20 V. Bhatt, S. Sharma, N. Kumar, U. Sharma and B. Singh, *J. Pharm. Biomed. Anal.*, 2017, **132**, 46–55.
- 21 X. H. Wang, S. C. Liang, D. M. Ma, C. Xu, H. H. Liu, Z. Z. Han, W. F. Wei and Q. S. Guo, *Ind. Crops Prod.*, 2020, **150**, 112–345.
- 22 Z. C. Wu, W. Wang, L. W. Sun, A. Z. Wei and D. M. Wang, *Ind. Crops Prod.*, 2020, **145**, 111998.
- 23 Y. Ma, X. Li, L. X. Hou and A. Z. Wei, *Ind. Crops Prod.*, 2019, **142**, 111872.
- 24 C. H. Wijaya, F. I. Napitupulu, V. Karnady and S. Indarini, *Food Rev. Int.*, 2019, **35**, 1–19.
- 25 E. Sugai, Y. Morimitsu, Y. Iwasaki, A. Morita, T. Watanabe and K. Kubota, *Biosci., Biotechnol., Biochem.*, 2005, **69**, 1951–1957.

