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Optimization of extraction process for ustiloxins A and B from rice false smut balls using an orthogonal array design

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Optimization of four factors, namely methanol concentration (*A*), extraction pH value (*B*), material-to-solvent ratio (*C*) and extraction times (*D*), for extraction of ustiloxins A and B from rice false smut balls was achieved by using an L_{18} orthogonal array design with three levels and four factors. The results show that the optimum conditions for ustiloxins A and B extraction should be $A_1B_2C_2D_3$ corresponding to methanol concentration at 10%, extraction pH value at 6, material-to-solvent ratio at 1:30 (g/mL) and extraction times as 3, respectively. Under the optimum extraction condition, the content of ustiloxins A and B in rice false smut balls was analyzed to be 0.80 and 0.57 mg/g, respectively, on a dry weight basis.

Key words: Rice false smut balls, *Villosiclava virens*, *Ustilaginoidea virens*, ustiloxins A and B, orthogonal array design, extraction optimization.

INTRODUCTION

The false smut of rice (*Oryza sativa* L.) is an emerging, increasingly significant and worldwide fungal disease (Brooks et al., 2009; Ashizawa et al., 2010; Ladhalakshmi et al., 2012; Tang et al., 2013). Its pathogen *Villosiclava virens* (Nakata) Tanaka and Tanaka (anamorph: *Ustilaginoidea virens* Takahashi) can produce ustiloxins which are cyclopeptide mycotoxins containing a 13-membered cyclic core structure (Koiso et al., 1994; Tanaka et al., 2008; Zhou et al., 2012). Ustiloxin A as well as the crude water extract of rice false smut balls were reported to cause liver and kidney damage in mice (Nakamura et al., 1994). This indicates that the false smut balls as well as false smut pathogen-infected rice food and forage create concerns for food and feed safety. Furthermore, ustiloxins are toxic to plants and animals especially with their antimitotic activity by inhibiting

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Table 1. Factors and levels of the orthogonal test.

Factor	Level 1	Level 2	Level 3
Methanol concentration (%, v/v)	10	30	70
Extraction pH value	2	6	10
Material-to-solvent ratio (g/mL)	1:20	1:30	1:40
Extraction times	1	2	3

microtubule assembly and cell skeleton formation (Luduena et al., 1994). As tubulin is an established chemotherapeutic target, mitotic arrest has been considered to be important in cancer chemotherapy. Therefore, ustiloxins provide an attractive entry to the study of antimitotic natural products and their biochemical effects at the molecular and cellular levels on the target. Ustiloxins have been regarded as a novel resource with their potential applications for medicinal and agrochemical purposes, acting as anticancer and anthelmintic agents (Li et al., 1995, 2006, 2008; Zhou et al., 2012).

Of five elucidated ustiloxins, both ustiloxins A and B are more structurally complicated and have been approved to be more effective to show cytotoxic activity than other ustiloxins (that is,, ustiloxins C, D and F) (Koiso et al., 1994). Both ustiloxins A and B were also the predominant toxin components in the rice false smut balls which were composed of the chlamydospores and mycelia of the pathogen (Shan et al., 2012). As ustiloxins A and B have not been successfully synthesized under *in vitro* till now (Zhou et al., 2012), rice false smut balls and cultured mycelia have been regarded as the only sources of ustiloxins A and B.

In order to speed up investigation and application of ustiloxins, one of the most important approaches is to efficiently obtain ustiloxins. To the best of our knowledge, it has not yet been reported in detail regarding the extraction process of ustiloxins A and B from rice false smut balls or cultured mycelia. The purpose of this investigation was to seek a practical method for ustiloxin extraction from the samples (that is false smut balls, cultured mycelia, grains, forage rice and their products). In this study, four factors, namely methanol concentration (that is, 10, 30 and 70%, v/v), extraction pH value (that is, 2, 6 and 10), material-to-solvent ratio (that is 1:20, 1:30 and 1:40, g/mL), and extraction times (that is, 1, 2 and 3) along with their three levels, for extraction of ustiloxins A and B from rice false smut balls were optimized by using the L_{18} orthogonal array design (OAD) based on the single-factor test. Verification experiment for the corresponding factors under the optimum condition was also carried out.

MATERIALS AND METHODS

Materials

The rice false smut balls, which were mainly composed of the chlamydospores and mycelia of the rice false smut pathogen (*Villosiclava virens*), were collected from the southwestern part of Shandong Province of China during cropping season. The materials were left to dry in shade at room temperature (23 ± 2°C) to a constant weight, and were then stored in the sterilized sealed plastic bags at -20°C until required.

Extraction procedure

The first step of the experimental design is to determine the important factors, whose variation has a critical effect on the extraction of ustiloxins A and B from rice false smut balls. 200 mg of rice false smut balls was powered by using pestle and motor, and was put into a 35-mL extraction tube under different extraction conditions. The basal extraction condition was at pH 6.0 and 60°C with extraction time as 30 min, material-to-solvent ratio at 1:30 (g/mL), and extraction times as 2. Methanol concentration (10-100%), extraction temperature (20-100°C), extraction pH value (2-10), extraction time (10-50 min), material-to-solvent ratio (1:10-1:50, g/mL), and extraction times (1-5) were evaluated for the extraction of ustiloxins A and B from rice false smut balls. The crude extract solution was filtered through Whatman no. 1 filter paper. The solvents were then removed by using a rotary evaporator at 50°C.

Orthogonal test

To further optimize the ustiloxin extraction from rice false smut balls, four parameters (methanol concentration, extraction pH value, material-to-solvent ratio, and extraction times) were used as the factors of the orthogonal experiment (Zhang et al., 2009; Guo et al., 2012; Khongsay et al., 2012). The selected factors and their levels in the experiment are shown in Table 1. The L_{18} (3^7) orthogonal design is shown in Table 2. The data were analyzed using the orthogonality experiment assistant software II V3.1 (Sharetop Software Studio, China).

HPLC analysis of ustiloxins A and B

Quantitative analysis of ustiloxins A and B by HPLC was carried out as previously described (Shan et al., 2012). The rice false smut balls sample was prepared with the optimum condition mentioned above. The concentrated extract was dissolved in 2 mL of methanol-water (15:85, v/v) and filtered through a filter (pore size, 0.22 μm) before analysis. Ustiloxin content was analyzed by a Prominence LC-20A high-performance liquid chromatography (HPLC) system (Shimadzu, Japan), which consisted of two LC-20AT solvent delivery units, an SIL-20A autosampler, an SPD-M20A photodiode array detector, and a CBM-20Alite system controller. Chromatographic separations were performed at 30°C using Synergi reversed-phase hydro-C_{18} column (250 mm x 4.6 mm, 5 μm, Phenomenex, Torrance, CA, USA). The mobile phase, composed of methanol with water containing 0.02% trifluoacetic acid (TFA) (15:85, v/v); was eluted at a flow rate of 1.0 mL/min, with

Table 2. The L_{18} orthogonal design.

Run	Methanol Concentration (%, *A*)	Extraction pH value (*B*)	Material-to-solvent ratio (g/mL, *C*)	Extraction times (*D*)	Blank 1 (*E*)	Blank 2 (*F*)	Blank 3 (*G*)
1	0	2	1:20	1	1	1	1
2	0	6	1:30	2	2	2	2
3	0	10	1:40	3	3	3	3
4	30	2	1:20	2	2	3	3
5	30	6	1:30	3	3	1	1
6	30	10	1:40	1	1	2	2
7	70	2	1:30	1	3	2	3
8	70	6	1:40	2	1	3	1
9	70	10	1:20	3	2	1	2
10	0	2	1:40	3	2	2	1
11	0	6	1:20	1	3	3	2
12	0	10	1:30	2	1	1	3
13	30	2	1:30	3	1	3	2
14	30	6	1:40	1	2	1	3
15	30	10	1:20	2	3	2	1
16	70	2	1:40	2	3	1	2
17	70	6	1:20	3	1	2	3
18	70	10	1:30	1	2	3	1

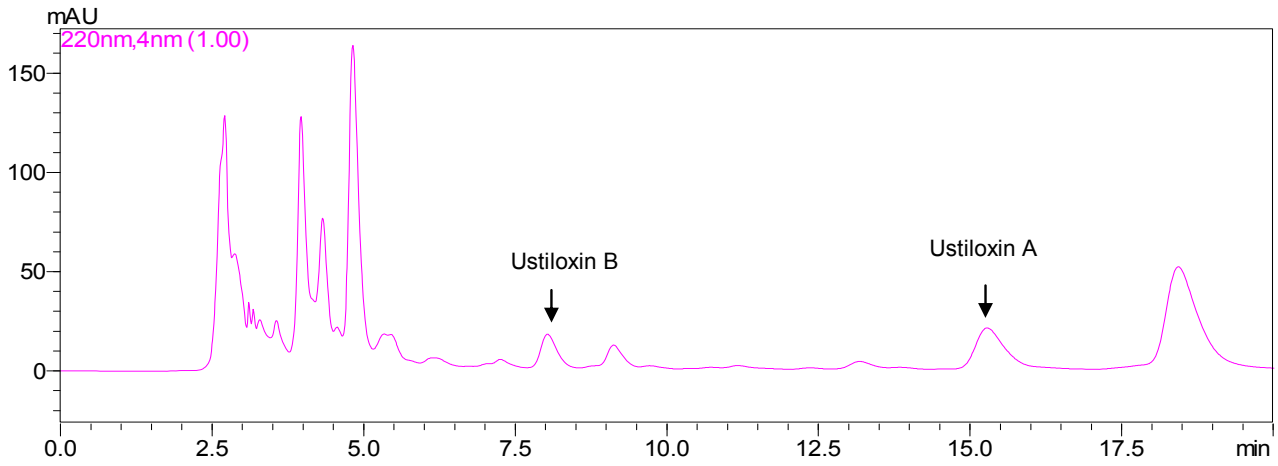

Figure 1. HPLC chromatogram of ustiloxins A and B in the extract of rice false smut balls.

UV detection at 220 nm and a total analysis time of 20 min. The LC solution multi-PDA workstation was employed to acquire and process chromatographic data. The HPLC chromatogram of ustiloxins A and B in the extract of rice false smut balls is shown in Figure 1.

Statistical analysis

All experiments were carried out in triplicate, and the results were represented by their mean values and the standard deviations (SD). Analysis of variance (ANOVA) was used in a completely random design. Duncan's multiple range test and Pearson's correlation coefficients were used to compare the data. The data were submitted to analysis of variance to detect significant differences by PROC ANOVA of SAS version 8.2.

RESULTS AND DISCUSSION

Single-factor test on extraction of ustiloxins A and B

Effects of each factor along with its five levels on

Table 3. Effects of different factors on extraction of ustiloxins A and B.

Factor and its levels		Content of ustiloxin A (mg/g)	Content of ustiloxin B (mg/g)
Methanol concentration (%, v/v)	10	0.71 ± 0.03[a]	0.61 ± 0.02[a]
	30	0.67 ± 0.01[b]	0.55 ± 0.01[b]
	50	0.58 ± 0.01[c]	0.46 ± 0.01[c]
	70	0.56 ± 0.00[c]	0.42 ± 0.01[d]
	100	0.40 ± 0.00[d]	0.30 ± 0.01[e]
Extraction temperature (°C)	20	0.50 ± 0.03[a]	0.41 ± 0.02[a]
	40	0.49 ± 0.01[a]	0.41 ± 0.01[a]
	60	0.49 ± 0.00[a]	0.41 ± 0.00[a]
	80	0.47 ± 0.01[a]	0.37 ± 0.02[b]
	100	0.19 ± 0.02[b]	0.16 ± 0.02[c]
Extraction pH value	2	0.49 ± 0.01[b]	0.36 ± 0.01[b]
	4	0.54 ± 0.02[ab]	0.42 ± 0.01[a]
	6	0.56 ± 0.01[a]	0.43 ± 0.01[a]
	8	0.55 ± 0.01[a]	0.43 ± 0.01[a]
	10	0.44 ± 0.03[c]	0.31 ± 0.01[c]
Extraction time (min)	10	0.52 ± 0.00[a]	0.39 ± 0.00[a]
	20	0.52 ± 0.01[a]	0.37 ± 0.03[a]
	30	0.52 ± 0.00[a]	0.39 ± 0.00[a]
	40	0.51 ± 0.04[a]	0.38 ± 0.01[a]
	50	0.50 ± 0.02[a]	0.36 ± 0.01[a]
Material-to-solvent ratio (g/mL)	1:10	0.15 ± 0.01[c]	0.12 ± 0.01[c]
	1:20	0.51 ± 0.02[b]	0.41 ± 0.01[b]
	1:30	0.58 ± 0.01[a]	0.46 ± 0.01[a]
	1:40	0.57 ± 0.01[a]	0.45 ± 0.00[a]
	1:50	0.59 ± 0.01[a]	0.47 ± 0.01[a]
Extraction times	1	0.58 ± 0.01[c]	0.46 ± 0.01[c]
	2	0.69 ± 0.07[b]	0.55 ± 0.06[b]
	3	0.76 ± 0.01[a]	0.61 ± 0.00[a]
	4	0.77 ± 0.04[a]	0.62 ± 0.04[a]
	5	0.77 ± 0.03[a]	0.63 ± 0.02[a]

The values are expressed as means ± standard deviations (n = 3). The values among the levels of each factor in the same column followed by different letters are significantly different at $p = 0.05$ level.

ustiloxins A and B extraction are presented in Table 3. Individually, both ustiloxins A and B content decreased with an increase of methanol concentration from 10 to 100% although, ustiloxins A and B content decreased observably when the temperature was at 100°C, while there were no significant differences in extraction of ustiloxins A and B content with an increase of temperature from 20 to 80°C and extraction time from 10 to 50 min at 60°C, respectively. So both temperature and time were considered to be non-significant factors for the extraction of ustiloxins A and B from rice false smut balls.

Ustiloxins A and B content increased with the increase of extraction pH value from 2 to 6, and achieved maximum at pH 6. Then the content was decreased as the pH value increased from 8 to 10. Both ustiloxins A and B content showed increasing tendency as the material-to-solvent ratio from 1:10 to 1:30 (g/mL), and nearly steady from 1:30 to 1:50 (g/mL). The extraction times as 3 achieved a maximum ustiloxins A and B content.

Among the six factors studied, extraction times and methanol concentration showed high impact on ustiloxin extraction followed by material-to-solvent ratio and extraction pH as compared to other factors (that is, extraction

Table 4. Results obtained under the experimental conditions using an L_{18} OAD.

Run	Content of ustiloxin A (mg/g)	Content of ustiloxin B (mg/g)
1	0.40 ± 0.02^{ef}	0.31 ± 0.00^{ef}
2	0.64 ± 0.01^{b}	0.44 ± 0.01^{b}
3	0.73 ± 0.01^{a}	0.47 ± 0.01^{ab}
4	0.49 ± 0.03^{d}	0.31 ± 0.04^{ef}
5	0.74 ± 0.01^{a}	0.49 ± 0.01^{a}
6	0.45 ± 0.05^{de}	0.35 ± 0.02^{d}
7	0.20 ± 0.02^{g}	0.12 ± 0.04^{j}
8	0.24 ± 0.02^{g}	0.24 ± 0.03^{hi}
9	0.46 ± 0.02^{de}	0.28 ± 0.01^{fg}
10	0.59 ± 0.02^{bc}	0.49 ± 0.04^{a}
11	0.48 ± 0.01^{d}	0.30 ± 0.01^{fg}
12	0.58 ± 0.03^{c}	0.49 ± 0.01^{a}
13	0.62 ± 0.02^{bc}	0.40 ± 0.01^{c}
14	0.57 ± 0.01^{c}	0.46 ± 0.00^{ab}
15	0.46 ± 0.04^{de}	0.34 ± 0.02^{de}
16	0.23 ± 0.04^{g}	0.20 ± 0.01^{i}
17	0.40 ± 0.04^{ef}	0.30 ± 0.02^{fg}
18	0.38 ± 0.01^{f}	0.27 ± 0.01^{gh}

The values are expressed as means ± standard deviations (n = 3). The values in the same column followed by different superscripted letters are significantly different at p = 0.05 level.

temperature and time) (Table 3). Therefore methanol concentration (10, 30 and 70%, v/v), extraction pH value (2, 6 and 10), material-to-solvent ratio (1:20, 1:30 and 1:40, g/mL) and extraction times (1, 2 and 3) were selected for further optimization of extraction process of ustiloxins A and B from rice false smut balls by using an orthogonal array design (OAD). The interaction effects of these factors were not found based on our previous central composite design (CCD) experiments (the data were not shown).

Optimization of ustiloxins A and B extraction

As various factors affected the extraction efficiency of ustiloxins A and B, optimization of the operating conditions most is crucial to obtain the higher yield of ustiloxins A and B from rice false smut balls. Based on the single-factor test, methanol concentration, extraction pH, material-to-solution ratio, and extraction times were selected for optimizing the ustiloxin extraction using an OAD of $L_{18}(3^7)$ (Zhang et al., 2009; Guo et al., 2012; Khongsay et al., 2012).

The obtained results (Table 4) show that the maximum content of ustiloxins A and B of the extracts was 0.74 and 0.49 mg/g, respectively. A further orthogonal analysis is given in Table 5. The influence of the extraction factors on ustiloxin A was in order of $A > D > B > C$. In other words, methanol concentration had the dominant effect on ustiloxin A, followed by extraction times, extraction pH and material-to-solvent ratio. The influence of the extraction factors on ustiloxin B was the same as on ustiloxin A (Table 5). ANOVA results show that methanol concentration and extraction times had a significant (p < 0.05) effect on the extraction of both ustiloxins A and B (Table 6). Extraction times were not a significant factor for ustiloxin B when the value of p was less than 0.01. The optimum condition obtained for extraction of ustiloxins A and B from rice false smut balls was $A_1B_2C_2D_3$ which means that optimum factors for obtaining the highest content of ustiloxins A and B from rice false smut balls were methanol concentration at 10%, extraction pH value at 6, material-to-solvent ratio at 1:30 (g/mL), and extraction times as 3.

Verification of experiments

According to the above analytical results, the optimum conditions for ustiloxin extraction from rice false smut balls were determined as $A_1B_2C_2D_3$, corresponding to methanol concentration at 10%; extraction pH value at 6, material-to-solvent ratio at 1:30 (g/mL), and extraction times as 3, respectively. Under the optimum extraction condition, the content of ustiloxins A and B in rice false smut balls was 0.80 ± 0.01 mg/g and 0.57 ± 0.01 mg/g (n = 3), respectively, on a dry weight basis.

In conclusion, this work firstly reported the optimized extraction of ustiloxins A and B from rice false smut balls by using L_{18} orthogonal array design which not only

Table 5. Range analysis of L_{18} orthogonal experiments for ustiloxin extraction.

Compound		Methanol concentration (*A*)	Extraction pH value (*B*)	Material-to-solvent ratio (*C*)	Extraction times (*D*)	Blank 1 (*E*)	Blank 2 (*F*)	Blank 3 (*G*)
Ustiloxin A	K_1	3.42	2.54	2.69	2.48	2.70	2.98	2.81
	K_2	3.32	3.06	3.15	2.63	3.12	2.74	2.87
	K_3	1.92	3.05	2.81	3.53	2.83	2.93	2.97
	k_1	0.57	0.42	0.45	0.41	0.45	0.50	0.47
	k_2	0.55	0.51	0.53	0.44	0.52	0.46	0.48
	k_3	0.32	0.51	0.47	0.59	0.47	0.49	0.50
	R	0.25	0.09	0.08	0.18	0.07	0.04	0.03
	Q	A_1	B_2	C_2	D_3			
Ustiloxin B	K_1	2.50	1.84	1.84	1.81	2.10	2.24	2.14
	K_2	2.37	2.22	2.21	2.03	2.25	2.04	1.97
	K_3	1.40	2.21	2.21	2.43	1.92	1.99	2.16
	k_1	0.42	0.31	0.31	0.30	0.35	0.37	0.36
	k_2	0.39	0.37	0.37	0.34	0.38	0.34	0.33
	k_3	0.23	0.37	0.37	0.41	0.32	0.33	0.36
	R	0.182	0.064	0.061	0.103	0.055	0.042	0.027
	Q	A_1	B_2	C_2	D_3			

K_i^A =Σthe amount of target compounds at A_i; $k_i^A = K_i^A/3$; R_i^A = max $\{k_i^A\}$ – min$\{k_i^A\}$.

Table 6. Analysis of variance (ANOVA) of the orthogonal experiments for ustiloxin extraction.

Compound	Source	Sum of squares	d.f.	Mean square	*F*-value	Fa	Significance
Ustiloxin A	*A*	0.235	2	0.117	30.337	$F_{0.05}(2,6)=5.140$	**
	B	0.030	2	0.015	3.907	$F_{0.01}(2,6)=10.900$	
	C	0.019	2	0.010	2.467		
	D	0.108	2	0.054	13.954		**
	Error	0.023	6	0.004			
	Total variation	0.415					
Ustiloxin B	*A*	0.119	2	0.060	19.244	$F_{0.05}(2,6)=5.140$	**
	B	0.016	2	0.008	2.580	$F_{0.01}(2,6)=10.900$	
	C	0.015	2	0.007	2.423		
	D	0.033	2	0.016	5.289		*
	Error	0.019	6	0.003			
	Total variation	0.201					

** Means significance at $p < 0.01$; * means significance at $p < 0.05$.

reduce time but also provide ideal ustiloxin extraction. From the analysis, the optimal design has been determined to be the combination of methanol concentration at 10%, extraction pH at 6, material-to-solvent ratio at 1:30 (g/mL), and extraction times as 3. It will provide the data supporting ustiloxins A and B preparation in large scale to meet their application in agriculture and medicine industry.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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