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DEVELOPMENT OF A SCALABLE EXTRACTION PROTOCOL FOR RECOVERY OF TETRODOTOXIN FROM PUFFERFISH VISCERA

PHÁT TRIỂN QUI TRÌNH CHIẾT XUẤT NÂNG CẤP ĐỂ THU HỒI TETRODOTOXIN TỪ NỘI TẠNG CÁ NÓC

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ABSTRACT

Tetrodotoxin analogues (TTXs) have been broadly applied in many fields. Viscera of pufferfish from Viet Nam was reported to contain a high level of TTXs, indicating a promising source for isolation of TTXs. In this work, critical factors affecting TTX recovery yield from pufferfish viscera collected from fishermen in the Can Gio coast of Viet Nam were optimized using response surface methodology (RSM). Data revealed that the RSM model displayed a highly precise prediction of TTX recovery yield with R^2 of 99.01% and the maximal TTX recovery yield predicted of 99.0% at solvent/solid ratio, extraction temperature and extraction time of 6.8mL/g, 100°C and 41 min, respectively. Validation the reliability of the regression model was implemented on a scalable protocol for extraction and recovery of TTX from 10 kg pufferfish viscera. Data revealed that the first extraction achieved a TTX recovery yield of 95.5%, which was lower than predicted level of 3.5%. However, the second extraction further improved TTX recovery yield to 98.8% very closing to the predicted response. Therefore, repeating extraction twice was recommended to include in the scalable protocol for maximal recovery of TTX from pufferfish viscera for large scale extraction.

Keywords: *Pufferfish viscera, tetrodotoxin, liquid chromatography, response surface methodology, optimization.*

TÓM TẮT

Các dẫn xuất của tetrodotoxin (TTXs) đã được ứng dụng rộng rãi trong nhiều lĩnh vực. Nội tạng của cá nóc của Việt Nam đã được báo cáo là có chứa hàm lượng TTXs cao, cho thấy một nguồn đầy hứa hẹn để phân lập TTXs. Trong nghiên cứu này, các yếu tố quan trọng ảnh hưởng đến hiệu suất chiết và thu hồi TTXs từ nội tạng cá nóc thu thập từ ngư dân ở bờ biển Cần Giờ của Việt Nam đã được tối ưu hóa bằng phương pháp đáp ứng bề mặt (RSM). Số liệu cho thấy mô hình RSM thể hiện khả năng dự đoán có độ chính xác cao hiệu suất thu hồi TTX với hệ số R^2 là 99,01% và hiệu suất thu hồi TTX cực đại được dự đoán là 99,0% ở tỷ lệ dung môi/pha rắn, nhiệt độ chiết và thời gian chiết xuất lần lượt là 6,8mL/g, 100°C và 41 phút. Độ tin cậy của mô hình hồi quy đã được kiểm chứng trong một thí nghiệm nâng cấp chiết TTXs từ 10 kg nội tạng cá nóc. Dữ liệu cho thấy lần chiết đầu tiên đạt được hiệu suất thu hồi TTXs là 95,5%, thấp hơn mức dự đoán là 3,5%. Tuy nhiên, lần chiết thứ hai tiếp tục cải thiện năng suất phục hồi TTXs lên 98,8% rất gần với giá trị dự đoán. Do đó, việc chiết xuất lặp lại hai lần được khuyến nghị đưa vào qui trình chiết nâng cấp để thu hồi tối đa lượng TTXs từ nội tạng trên quy mô lớn.

Từ khoá: *Nội tạng cá nóc, tetrodotoxin, sắc ký lỏng, phương pháp đáp ứng bề mặt, tối ưu hoá.*

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1. INTRODUCTION

Tetrodotoxin analogues (TTXs) have been documented to variously distribute in aquatic animals (e.g., puffer fish, bacteria, xanthid crab, mangrove horseshoe crab, snail, etc.) and terrestrial animals (e.g., frogs, newt, etc.) [1, 2]. One of the most recognizable species containing TTX toxin and its analogs is pufferfish (e.g., *Takifugu niphoble*, *Takifugu pseudommus*) [3]. Recently, Le Ho Khanh Hy et al. reported that ovaries of pufferfish *Lagocephalus inermis* contained 7.59µg TTXs/g fresh weight [2]. This indicates that Vietnamese pufferfish is a promising sources for TTX purification [4, 5].

Factors such as temperature, solvent/solid ratio and extraction time are considered as critical factors influencing TTX extraction efficiency [6, 7]. Single-factor investigation was commonly applied for obtaining optimal condition of every variable. However, this traditional approach is scanned one factor but fixed other factors, leading to the interaction effect of different factors on response is not fully understood [7]. To overcome this disadvantage, statistical design solely relied on response surface methodology (RSM) is usually adopted to find out the optimal conditions of different factors for and generate regression model for precise prediction of response from random experiments [3]. To date,

there is no study reporting optimization of TTX extracting conditions for 1 - 10kg pufferfish's soft tissue scale using response surface methodology.

Therefore, the objectives of this study are to (i) optimize the conditions of the main factors (solvent/solid ratio, extraction temperature, and extraction time) for maximal recovery of TTX from pufferfish viscera using response surface methodology and generate a regression model, (ii) to validate reliability of the RSM model using extraction experiments at pufferfish viscera scale of 10kg/batch and (iii) to develop a scalable protocol for extraction and recovery of TTX for scales of larger 10kg pufferfish viscera.

2. MATERIALS AND METHODS

2.1. Materials

Trichloroacetic acid (TCA) of analytical grade was obtained from Sigma-Aldrich (Munich, Germany). Acetic acid (99.8%), hexane (99.5%), diethyl ether (99.5%) and ethanol (99.9%) were obtained from Samchun Chemical Co., Ltd. (Seoul, Republic of Korea). Tetrodotoxin citrate (1 mg) was purchased from Tocris Bioscience (Bristol, UK); 4epi-TTX and Anh-TTX were granted by Prof. Shigeru Sato, Kitasato University, Japan.

2.2. Pufferfish viscera sources

Pufferfish viscera were collected from fishermen in the Can Gio coast, Ho Chi Minh City, Viet Nam in May 2022. The viscera samples were immediately refrigerated and transferred to the Laboratory of Technology of Bioactive Compounds, Institute of Chemistry, Vietnam Academy of Science and Technology. Then, the viscera soft tissues were frozen and stored below -20°C until use.

2.3. High shear blending-assisted extraction (HSBAE) method

For toxin extraction, pufferfish viscera soft tissues were thawed at room temperature, followed by grinding with extracting solvent using a laboratory blender (LBC15, Thomas Scientific, LLC, New Jersey, USA) at a speed of 15,000rpm to homogenize soft tissues, particularly eggs. The homogenized mixture was stirred by a magnetic stirrer at 150rpm and heated in a heating-controlled water bath for toxin extraction. The aqueous phase was separated from the mixture by centrifugation at 9000×g under ambient temperature for 10 min with a centrifuge (Z36HK, HERMLE Labortechnik GmbH, Wehingen, Germany). Proteins and lipids in the resulted supernatant were sequentially removed by 30% trichloroacetic acid and hexane/diethyl ether (2/1, v/v), respectively. The final extract was preserved under 2 - 4°C in a refrigerator for further quantitative determination of toxin.

2.4. Optimization of TTX recovery yield from pufferfish viscera using response surface methodology

Response surface methodology was used for optimizing the effects of solvent/solid ratio, extraction temperature and extraction time on TTX recovery yield from pufferfish viscera. Solvent/solid ratio, extraction temperature and extraction time were denoted as A, B and C, respectively, with their levels are listed in Table 1. Box-Behnken design was adopted for three factors-three levels design, which contains totally 15 runs (e.g., 12 axial and 3 central point runs). Response was recovery yield of TTX (Y, %) which was determined from each experiment and listed in Table 2. The TTX recovery yield response was determined using a following second-order polynomial equation [8].

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum \sum \beta_{ij} X_i X_j + \varepsilon \quad (1)$$

Where Y is the predicted response; β are the coefficients of the equation, and X_i and X_j are the uncoded level of parameters i and j, respectively; k is the number of studied factors; and ε is random experimental error. The RSM analysis was implemented using Minitab 18 (Minitab, LLC, Pennsylvania, USA) for determining correlation through non-linear regression. The significance of each variable was determined using Student's t-test.

Table 1. Coded and uncoded levels of solvent/solid ratio, extraction temperature and acetic acid concentration in Box-Behnken design

Factors	Factor level		
	-1	0	1
Solvent/solid ratio (A, mL/g)	3	6	9
Extraction temperature (B, °C)	60	80	100
Extraction time (C, min)	20	40	60

Table 2. Box-Behnken design for optimization the effects of solvent/solid ratio, extraction temperature and extraction time on TTX recovery yield (Y)

No.	Uncoded levels (Coded levels)			Experimental response (Y, %)	Predicted response (Y, %)
	A	B	C		
1	3 (-1)	60 (-1)	40 (0)	84.9	85.6
2	6 (0)	100 (1)	60 (1)	96.8	96.7
3	9 (1)	60 (-1)	40 (0)	84.0	83.1
4	6 (0)	60 (-1)	20 (-1)	91.1	91.2
5	9 (1)	100 (1)	40 (0)	94.6	93.9
6	6 (0)	60 (-1)	60 (1)	91.8	91.9
7	6 (0)	80 (0)	40 (0)	95.3	95.1
8	3 (-1)	100 (1)	40 (0)	82.9	83.8
9	6 (0)	80 (0)	40 (0)	95.0	95.1
10	9 (1)	80 (0)	60 (1)	83.2	84.0
11	3 (-1)	80 (0)	60 (1)	84.3	83.6
12	6 (0)	100 (1)	20 (-1)	95.5	95.4
13	9 (1)	80 (0)	20 (-1)	85.8	86.5
14	6 (0)	80 (0)	40 (0)	95.2	95.1
15	3 (-1)	80 (0)	20 (-1)	80.0	79.2

2.5. Scalable extraction of TTX

In this part of the study, a scale-up extraction was implemented with 10kg pufferfish viscera employing the optimal conditions which were obtained from previous Sections. TTX recovery yield was measured for three extraction times. Moreover, the data for TTX recovery yield was used to certify reliability of the regression model. Finally, a scalable extraction protocol for recovery of TTX from pufferfish viscera was proposed.

2.6. Analysis

TTXs were extracted according to the modified procedure that described in Brillantes et al. [9]. Briefly, 2 g of mixed soft tissues (muscle, viscera and eggs) was mixed with 8 mL of 1% acetic acid and homogenized for 20 min, followed by heating for 30 min in a boiling water bath to precipitate protein. The mixture was cooled down to room temperature and centrifuged at 11000×g for 10 min. The resulted extracts was treated using an ENVI-carb SPE cartridge 250 mg (Sigma Aldrich Japan, Tokyo, Japan), diluted by four-fold diluted acetonitrile [10]. Then, TTX was quantified by using a protocol and hydrophilic interaction liquid Chromatography-Mass spectrometer (HILIC/MS-MS) coupled with Shimadzu system triple-quadrupole mass spectrometer (LCMS-8040; Shimadzu Corporation, Kyoto, Japan) that were described by Dao et al. [11].

2.7. Determination of TTX recovery yield

TTX recovery yield (Y) was determined as proportion of amount of all detected TTX derivatives (mg) measured from each treatment method compared to the amount measured by the analytical procedure (mg).

2.8. Statistical analysis

Experiments were conducted in triplicate and data was reported as mean ± standard deviation (SD). Experimental run for the central composite design (Table 2) was carried out in one replicate. Statistical analysis was done using one-way ANOVA followed by post hoc Tukey's test (Graph pad V7) and a p-value of < 0.05 was declared as significant. The statistical analysis was conducted using software package MiniTab18 (Minitab Pty Ltd., Sydney, Australia).

3. RESULT AND DISCUSSION

3.1. Identification and quantitative determination of TTX

By comparing HPLC chromatograms of TTX standards and the pufferfish viscera extract, it was determined that pufferfish viscera extract contained three derivatives of TTX e.g., TTX, 4epi-TTX and Anh-TTX (Figure 1). TTX derivatives content of pufferfish viscera extract is shown in Table 3.

Table 3. Level of TTX content in pufferfish viscera extract sample collected from the Can Gio coast, Ho Chi Minh City, Viet Nam in May 2022 (n = 3)

Index	4epi-TTX	TTX	Anh-TTX
Concentration (μM)	1.15 ± 0.05	7.75 ± 0.08	1.89 ± 0.11

Contents of 4epi-TTX, TTX and Anh-TTX in the pufferfish viscera extract were determined as 0.15, 7.75 and 1.89μM, respectively. This data is agreement with TTX contents determined in the pufferfish extract conducted by Le Ho Khanh Ly et al. [2]. This is a promising source for isolation and purification of TTX. As the pufferfish viscera contained high level of TTX, they were used for further experiments in optimization of TTX recovery yield and development of a scalable extraction protocol for recovery of TTX from larger 10kg scale.

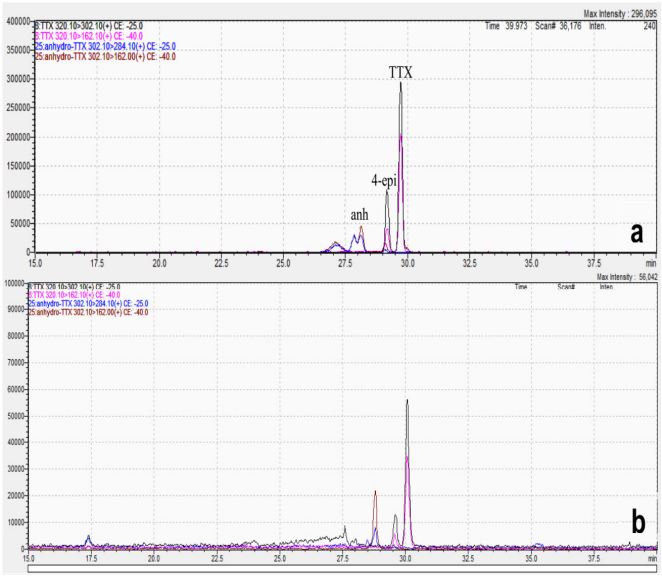


Figure 1. HPLC chromatograms of TTX standards (a) and pufferfish viscera's extract (b)

3.2. Response surface model fitting and surface contour plot analysis

0.5% acetic acid was used as solvent for all experiments. Total fifteen experimental runs at different combinations of solvent/solid ratio, extraction temperature, and extraction time resulted in TTX recovery yields as listed in Table 2. The outcomes from fitting the quadratic equation with experimental data yielded a regression model (2) and predicted responses were also listed in Table 2.

$$Y = 69.9 + 10.22\ A - 0.619\ B + 0.620\ C - 1.0523\ A\times A + 0.00251\ B\times B - 0.00568\ C\times C + 0.05250\ A\times B - 0.02875\ A\times C + 0.00037\ B\times C \quad (2)$$

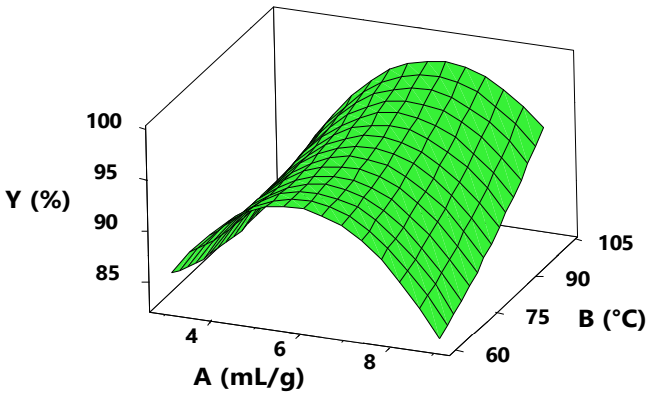
The significance of single factors and their synergistic interactions on TTX recovery yield is shown in Table 3. Data reveals that solvent/solid ratio and extraction temperature are all significant to TTX recovery yield because of p-values < 0.05. Nevertheless, extraction time was identified as insignificant to TTX recovery yield in the studied range (20 - 60 min, $p = 0.238 > 0.05$). Reparably, F-values calculated from model for solvent/solid ratio, extraction temperature, and extraction time were 31.48, 42.46, and 1.79, respectively, implying that extraction temperature is the most significant among three factors for TTX recovery yield, followed by the solvent/solid ratio and extraction time. Square terms of solvent/solid ratio ($A\times A$, $p = 0.000 < 0.05$) and extraction time ($C\times C$, $p = 0.007 < 0.05$) were significant, but square of extraction temperature

(C×C) is insignificant to TTX recovery yield ($p = 0.105 > 0.05$). Similarity, two-way interaction terms of solvent/solid ratio and extraction temperature (A×B, $p = 0.001 < 0.05$) and solvent/solid ratio and extraction time (A×C, $p = 0.017 < 0.05$) are significant, whereas interaction of extraction temperature and extraction time (B×C) is in significant to TTX recovery yield ($p = 0.771 > 0.05$). The obtained data demonstrated that the relationship between each of these variables and the response is not a simple linear relationship. Moreover, according to analysis of variance, the F-value and p-value of the regression model are 55.52 and 0.000, respectively, demonstrating that the model is accurate and reliable. This is further confirmed by correlation coefficient of R^2 at values of 99.01 (Table 3). The F-value and p-value of lack-of-fit index determined as 1.56 and 0.081 (> 0.05), respectively, indicating that unknown factors have less influence on the experiment. This data also confirmed that the lack-of-fit item was not significant to the pure error and the error of the experiment was small. The analyses recommend that the model fitted well with experimental data, and it thus can be applied for precise prediction of experimental results. The maximal TTX recovery yield predicted from RSM model was 99.0% which was obtained under solvent/solid ratio of 6.8mL/g, extraction temperature of 100°C and extraction time of 40 min.

Table 3. Analysis of variance for Box-Behnken design-based experiments

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	476.627	52.959	55.52	0.000
Linear	3	72.243	24.081	25.25	0.002
A	1	30.031	30.031	31.48	0.002
B	1	40.500	40.500	42.46	0.001
C	1	1.711	1.711	1.79	0.238
Square	3	352.702	117.567	123.26	0.000
A×A	1	331.188	331.188	347.22	0.000
B×B	1	3.723	3.723	3.90	0.105
C×C	1	19.040	19.040	19.96	0.007
2-Way Interaction	3	51.682	17.227	18.06	0.004
A×B	1	39.690	39.690	41.61	0.001
A×C	1	11.902	11.902	12.48	0.017
B×C	1	0.090	0.090	0.09	0.771
Error	5	4.769	0.954		
Lack-of-Fit	3	4.742	1.581	1.56	0.081
Pure Error	2	0.027	0.013		
Total	14	481.396			
R^2	99.01%				

Surface and contour plots displayed in Figures 2 - 4 are 2-D graphs describing relationship between TTX recovery yield and solvent/solid ratio, extraction temperature and extraction time. The surface and contour patterns show in Figure 2 reveals that the condition at points in the regions of solvent/solid ratio of 6 - 8mL/g and extraction temperature of 95 - 100°C will yield TTX recovery yield of over 95% when extraction time is fixed at 40 min. Moreover, steepness analysis for the contour graph of Figures 2 - 4 display that extraction temperature and solvent/solid ratio are significant factors for TTX recovery yield, among them extraction temperature is the most influential one, followed by solvent/solid ratio, while extraction time is insignificant (Figures 3, 4). As illustrated in Figure 3, when extraction temperature is fixed at 80°C, TTX recovery yield can reach over 92% when solvent/solid ratio and extraction time varied between 5.5 and 7.5mL/g and 20 and 60 min, respectively.



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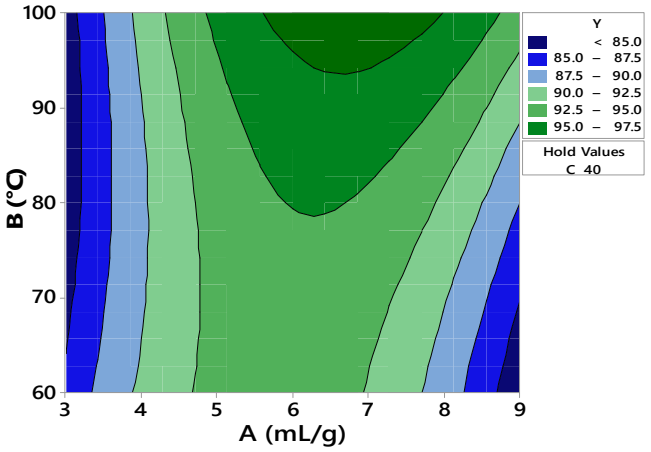
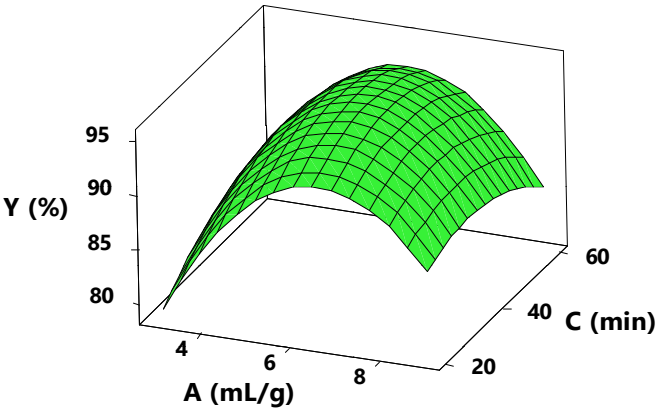


b)

Figure 2. Surface (a) and contour plots (b) of TTX recovery yield versus solvent/solid ratio and extraction temperature at extraction time of 40 min



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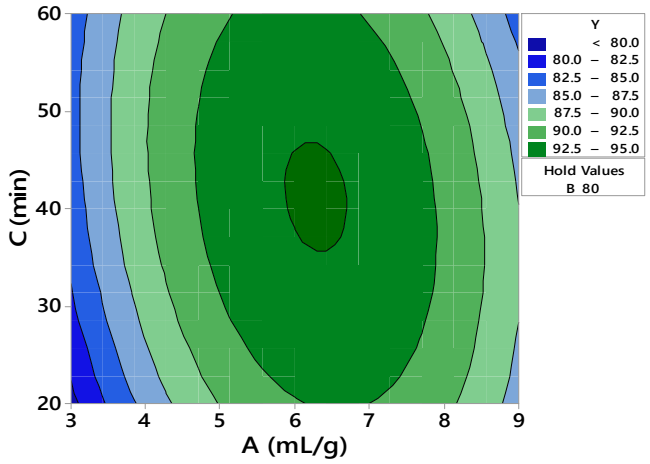
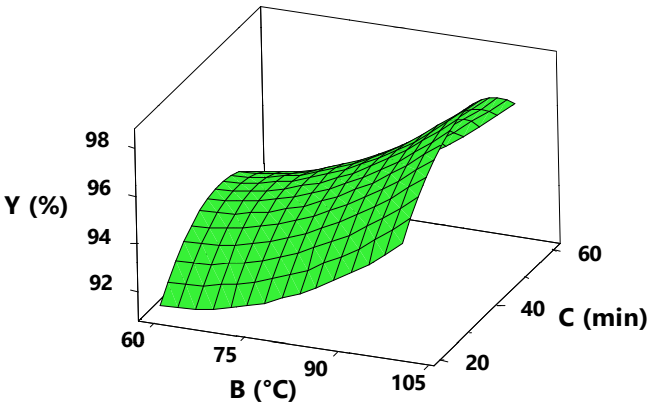


b)

Figure 3. Surface (a) and contour plots (b) of TTX recovery yield vursus solvent/solid ratio and extraction time at extraction temperature of 80°C



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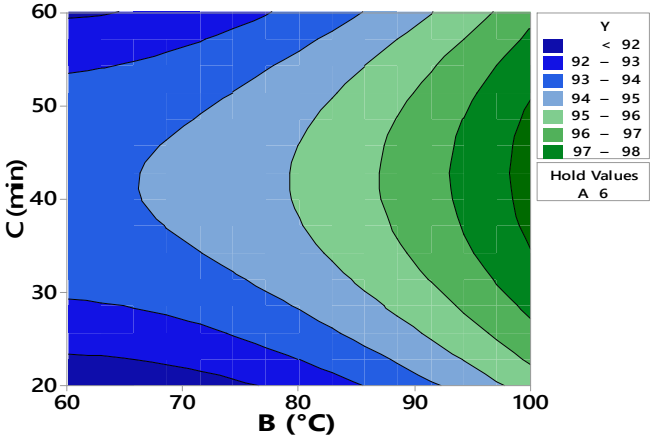


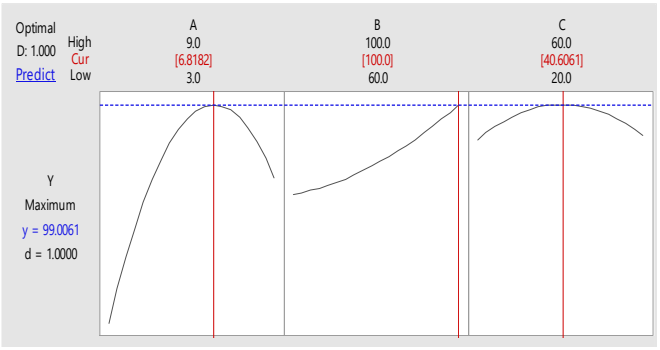
b)

Figure 4. Surface (a) and contour plots (b) of TTX recovery yield versus extraction temperature and extraction time at solvent/solid ratio of 6mL/g

Similarity, one solvent/solid ratio is fixed at 6 mL/g, over 96% TTX recovery yield can be achieved when extraction temperature and extraction time varied in the range of 95 - 100°C and 30 - 50 min (Figure 4), respectively. In order to further enhance TTX recovery yield to over 97%, extraction temperature and extraction time varied in the range of 97 - 100°C and 35 - 50 min (Figure 4), respectively. Compared with single-factor investigation and orthogonal experimental design as implemented in the literature [12-14], the response surface methodology adopted in this study is more intuitively and precisely described the synergistic effect between factors to the response [3, 15].

3.3. Validation of RSM model and scalable extraction of TTX

The optimal conditions for extraction of TTX from pufferfish viscera were solvent/solid ratio of 6.8mL/g, extraction temperature of 100°C and extraction time of 40.6 min (Figure 5a). Under the optimal conditions, the TTX recovery yield from 10kg scale extraction was 95.5%, which was lower than the predicted values of 99% by 3.5% (Figure 5b). This is reasonable because the predicted level of TTX recovery yield was resulted from RSM model which was constructed from experiments with 10g solid scale, at which mass and heat transfer is negligible. However, increasing weight of solid to 10kg, the resistance of mass and heat transfer between extracting solvent and heat to the solid matrix is improved, resulting in reduction of TTX recovery yield.



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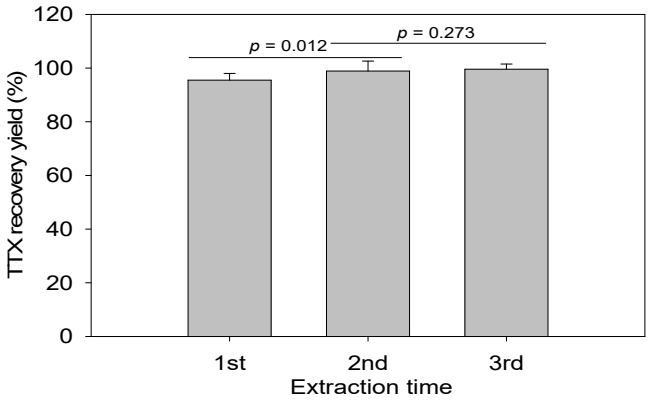

b)

Figure 5. TTX recovery yields predicted from RSM regression model (a) and measured from 10kg pufferfish viscera scale (b)

Therefore, increase number extraction is necessary to maximally recover TTX from the matrix. As data shown in Figure 5b, the TTX recovery yield from 10kg scale extraction is increased from 95.5% (for the 1st extraction) to 98.8% and

99.6% when number of extraction increased to 2 and 3 times, respectively. Among these three times of extraction, TTX recovery yields measured for 1st and 2nd extractions were statistically different ($p = 0.012 < 0.05$), while those levels were not statistical difference for 2nd and 3rd extractions ($p = 0.273 > 0.05$). These results suggested that number extraction should be increased to two times to maximally recovery of TTX from pufferfish viscera.

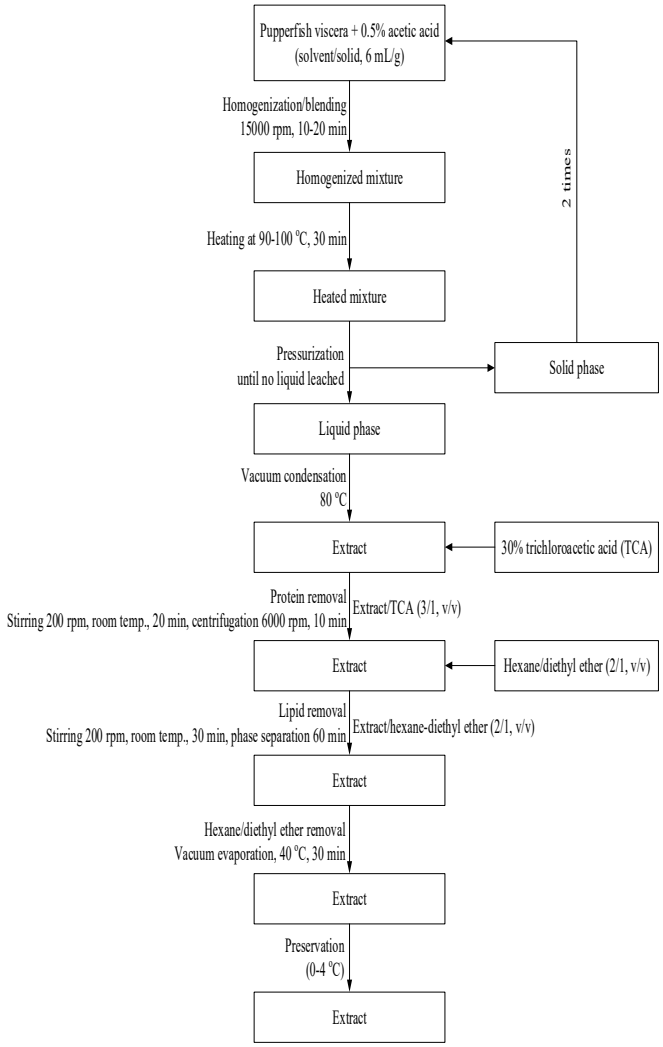

Figure 6. Scalable protocol for extraction and recovery of TTX from pufferfish viscera

The scalable protocol for extraction and recovery of TTX from scales of larger 10kg pupperfish viscera is proposed as in Figure 6. Firstly, grinding/homogenization of pupperfish viscera with 0.5% acetic acid at solvent/solid ratio of 6mL/g is performed by a high speed blender/homogenizer at 15000rpm for 10 - 20 min. Secondly, the homogenized mixture is stirred at 90 - 100°C for 30 min for TTX extraction. Thirdly, the heated mixture is pressurized with a filter bag (PE, pore size of 0.5µm) to separate aqueous and solid phases. The solid portion is re-extracted with the same proportion of 0.5% acetic acid (6mL/g) under the same temperature and extraction time conditions. Fourthly, the liquid portions of two extractions are summed and condensed to the volume equal to the acetic acid volume used of each extraction under 80°C using a vacuum rotary evaporator. Fifthly, the resulted extract is treated with 30% trichloroacetic acid at volumetric ratio of 3 volume of the extract and 1 volume of 30% trichloroacetic acid under stirring of 200rpm for 20 min at room temperature. The treated mixture is centrifuged at 6000rpm under room temperature for 10 min with a centrifuge to separate TTX-rich phase and protein-rich phase. The supernatant containing TTX is obtained while the bottom phase containing protein is discarded. Sixthly, the supernatant is further treated with hexane/diethyl ether (2/1, v/v) at volume ratio of 2 volume of the extract and 1 volume of hexane/diethyl ether under stirring rate of 200rpm for 30 min at room temperature. The mixed solution is then loaded into a funnel for phase separation at room temperature for 60 min. The bottom phase containing TTX and residual hexane/diethyl ether is obtained. The residual hexane/diethyl ether in the extract is removed by the vacuum rotary evaporator at 40°C for 30 min. Seventhly, the final extract is preserved under 0 - 4°C until use for TTX purification.

4. CONCLUSION

This work has completed optimization of TTX recovery from pufferfish viscera collected from fishermen in the Can Gio coast of Viet Nam. The RSM model built with the most significant factors e.g., solvent/solid ratio, extraction temperature and extraction time showed highly precise prediction of TTX recovery yield with correlation coefficient of 0.9901 with maximal TTX recovery yield reached 99.01% at solvent/solid ratio, extraction temperature and extraction time of 6.8mL/g, 100°C and 41 min, respectively. A scalable protocol for extraction and recovery of TTX from 10 kg pufferfish viscera was developed to verify the reliability of the regression model. Data revealed that the first extraction achieved a TTX recovery yield of 95.5%, which was lower than predicted level of 3.5%. However, the second extraction further improved TTX recovery yield to 98.8% closing to the predicted response. Therefore, repeating extraction twice was recommended to include in the scalable extraction protocol for maximal recovery of TTX from pufferfish viscera for large scale.

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