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Design of experiment approach for the process optimization of ultrasonic-assisted extraction of polysaccharide from mulberry leaves by response surface methodology

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Mulberry is considered as food-medicine herb, with specific nutritional and medicinal values. In this study, response surface methodology (RSM) was employed to optimize the ultrasonic-assisted extraction of total polysaccharide from mulberry using Box-Behnken design (BBD). Based on single factor experiments, a three level, three variable central composite designs were carried out to establish a quadratic regression model for the extraction efficiency of total polysaccharides as a function of extraction time, extraction temperature and material-water ratio. The optimum extraction conditions were obtained as follows: extraction temperature of 70°C, material-water ratio of 1:30 (g/ml), and extraction time of 40 min. Under these conditions, the predicted total polysaccharides extraction efficiency was 3.6%, while the experimental value was 3.56%. Analysis of variance (ANOVA) was used to examine the statistical significance of the developed model. The result indicates that the established model well predicted the extraction efficiency of total polysaccharides from mulberry leaves.

Key words: Mulberry leaves, total polysaccharides, extraction conditions, design of experiment.

INTRODUCTION

Natural products play a significant role in diet based therapies to cure various maladies (Butt et al., 2009). Genus *Morus* (mulberry) is one of such examples that consists of over 150 species, among these *Morus Alba* L. is the dominant one (Srivastavas et al., 2006). Mulberry leaves are composed of mineral, vitamins, food fibers, amino acid, plant sterols, flavones, alkaloids, polysaccharides, and so on. It has demonstrated significant effects as anti-oxidation (Doi et al., 2000; Doi et al., 2001; Butt and Suh, 2007) and anti-tumor (Kim et al., 2000; Masuda et al., 2002); other role of this product includes the capability to reduce low-density lipoprotein

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(LDL) and blood sugar level (Andallu et al., 2001;Andallu et al., 2002; Andallu et al., 2003; Jeszka-Skowron et al., 2014); and the cosmetic application to delaying ageing and beautifying skin (Halliwell, 1992; Tomoyuki et al., 2006; Wang et al., 2011). Numerous studies show that many polysaccharides works as anti-bacteria and anti-viruses, such as HIV, herpes simplex virus, influenza virus, and vesicular viral gastritis (Jia et al., 2012). Mulberry polysaccharides as well as flavonoids, alkaloids and other comonents in the hypoglycemic activity have a certain synergy (Yang et al., 2007). Polysaccharide also has some anti-radiation effect; the mechanism is generally considered for polysaccharide and activation of the hematopoietic system by strengthening the role of phagocytic cells to enhance the body's tolerance to radiation (Zhang et al., 2003).

In order to speed up investigations of polysaccharides and subsequent application, it is necessary to optimize the polysaccharides extraction process. Microwave-assisted extraction of polysaccharides has been used widely (Soria et al., 2014). To the best of our knowledge, the optimization of the polysaccharide extraction from mulberry leaves have only a few been reported (Ying et al., 2011; Liu et al., 2015; Thirugnanasambandham et al., 2015). In this study, Box-Behnken design (BBD) was selected to optimize the extraction parameters by response surface methodology (RSM) (Bezerra et al., 2008). Firstly, single-factor experimental designs (that is, extraction time, extraction temperature, ratio of water volume to raw material weight) were carried out before BBD experiments. Secondly, the more significant factors (that is, extraction time, extraction temperature, and ratio of water volume to raw material weight), with three levels, were chosen for further extraction optimization of polysaccharides by BBD experiment and RSM analysis. This method has been extensively used to optimize chemical and biochemical processes (Ge et al., 2014; Wang et al., 2014). This paper defined three factors namely extraction time, extraction temperature and material mass concentration by response surface methodology (RSM) to gain the optimum extraction condition.

MATERIALS AND METHODS

Pretreatment of mulberry leaves

Fresh mulberry variety Yu711 (*Morus multicaulis*) leave was harvested by hand from the National Mulberry Gene Bank of the Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, Jiangsu Province, China, and then were wind-dried. Dried mulberry leaves were crushed and passed through a 2.36 mm sieve, and used as the experimental sample.

Extract liquid preparation and glucose determination

The 3.0 g mulberry leaves powder was added to 120 ml 80% ethanol, and extracted for 40 min at 50°C by ultrasonic extraction method. After extraction, the residue was dried in an oven at 50°C, and each pretreated sample (1 g) was submitted to polysaccharide extraction according to the design of the experiment taking in consideration extraction times, temperatures and the ratio of water to raw material. After each reaction, samples were centrifuged for 5 min at 8000 rpm at room temperature. An amount of 1 ml of each supernatant was mixed with 5 ml 10% sulfuric acid anthrone solution. The mixture was strongly vortexed, and was heated until water boiled, held for 10 min, then water-cooled and stabilized 10 min at room temperature. The contents of D-(+)-Glucose were determined by sulfuric acid anthrone colorimetric method. D-(+)-Glucose was used as a standard for measurement of the total sugar content (Dubois et al., 1956). The absorbance of samples was measured by 722 UV-VIS spectrophotometry at 625 nm. The polysaccharide yield (%) was calculated using the following equation:

$$\text{Polysaccharides yield (\%)} = \frac{C \times N \times V}{W \times 1000} \times 100\%$$

Where, C is the concentration of polysaccharide calculated from the calibrated regression equation (mg/mL); N is the dilution factor; V is the total volume of extraction solution (mL); and W is the mass of raw material (g).

Single factor experiments

Effect of the ratio of water to raw material on polysaccharides

Adjustment of extraction time to 40 min, extraction temperature to 50°C and extract polysaccharides from mulberry leaves with different raw material to water ratio of 1:5, 1:15, 1:25, 1:35 and 1:45 g/ml, respectively were done. The extraction rate was determined with the above method described.

Effect of extraction time on polysaccharides

Fixed extraction temperature of 50°C, water-material ratio of 1:15 g/ml and different extract time of 10, 20, 30, 40 and 50 min, respectively were used. The extraction rate was determined with the above method described.

Effect of extraction temperature on polysaccharides

Fixed water-material ratio of 1:15, extraction time 20 min and extract polysaccharides from mulberry leaves with different temperatures were 40, 50, 60, 70 and 80°C，respectively were used. The extraction rate was determined with the above method described.

Experimental Box-Behnken design

After determining the preliminary range of the parameters by a single-factor experiment for the polysaccharides production, the extraction parameters were optimized using RSM. Based on the results of preliminary experiments, two levels, three variables Box-Behnken central composite design was carried out to establish a quadratic regression model for the extraction efficiency (y) of total polysaccharides as a function of extraction temperature (x_1), extraction time (x_2) and material-water ratio (x_3) as presented in Table 1. Based on the results of Box-Behnken design, optimization Box-Behnken design plan of extraction technology or predicted

Table 1. The factors and levels of Box-Behnken central composite design of extraction technology of polysaccharides from mulberry leaves by utrasonic-assisted

Level	Factor		
	X_1 temperature (°C)	X_2 time (min)	X_3 Material-water ratio (g /ml)
-1	60	10	1：15
0	70	20	1：25
+1	80	30	1：35

Table 2. Comparison between predicted and actual value on the yield of polysaccharides from mulberry leaves by Box-Behnken design.

Order	(X_1)	(X_2)	(X_3)	Actual value (y,%)	Predicted value (y,%)
1	-1	-1	0	1.94	1.92
2	0	0	0	3.52	3.35
3	-1	1	0	2.17	1.95
4	1	0	-1	0.95	0.84
5	0	1	1	1.99	2.11
6	0	0	0	3.53	3.35
7	0	0	0	3.43	3.35
8	0	-1	1	3.02	2.94
9	-1	0	-1	1.79	1.93
10	0	0	0	3.16	3.35
11	1	0	1	3.11	2.97
12	0	0	-1	1.33	1.41
13	1	-1	0	1.42	1.63
14	0	0	0	3.11	3.35
15	1	1	0	1.41	1.44
16	0	-1	-1	0.85	0.74
17	-1	0	1	2.58	2.69

value and actual value on the yield of polysaccharides from mulberry leaves was analyzed (Table 2).

Data analysis

Polysaccharide concentration was determined according to the colorimetric method describe aboved and the data were evaluated by using the Expert Design 8.0.6 for Windows software (SPSS Inc., USA). The values were calculated as the mean of individual experiments in triplicate. The statistical significance was analyzed by Student's t test and regression analysis and the mathematical model was performed and expressed as response of surface.

RESULTS

Effect of extraction temperature on polysaccharides

Extraction temperature range was 40 to 80°C, higher temperature leads to low extraction rate. Based on the extraction condition of polysaccharides from mulberry leaves, material-water ratio of 1:15, and time 20 min, temperature was set as 40, 50, 60, 70 and 80°C, respectively. Figure 1A shows that the extraction rate of polysaccharides from mulberry leaves reached the highest when temperature was set as 70°C.

Effect of water-material ratio on polysaccharides

As shown in Figure 1B, material-water ratio was selected as 1:5, 1:15, 1:25, 1:35, and 1:45 while the other factors were set as temperature of 50°C, and time of 20 min. The optimal material-water ratio for extraction rate of polysaccharides was 1:25.

Effect of extraction time on polysaccharides

With respect to time, the other factors were set as material-water ratio of1:15, and temperature 50°C and time was selected as 10, 20, 30, 40 and 50 min .The result in Figure 1C shows that the extraction rate increased rapidly from 10 to 20 min and the remaining little changed at later time. Therefore, the appropriate time was selected at 20 min.

Improvement of the extraction conditions

The response selection is a critical stage in optimization. The responses were directly related to the parameters that define the extraction yield of polysaccharides. Based

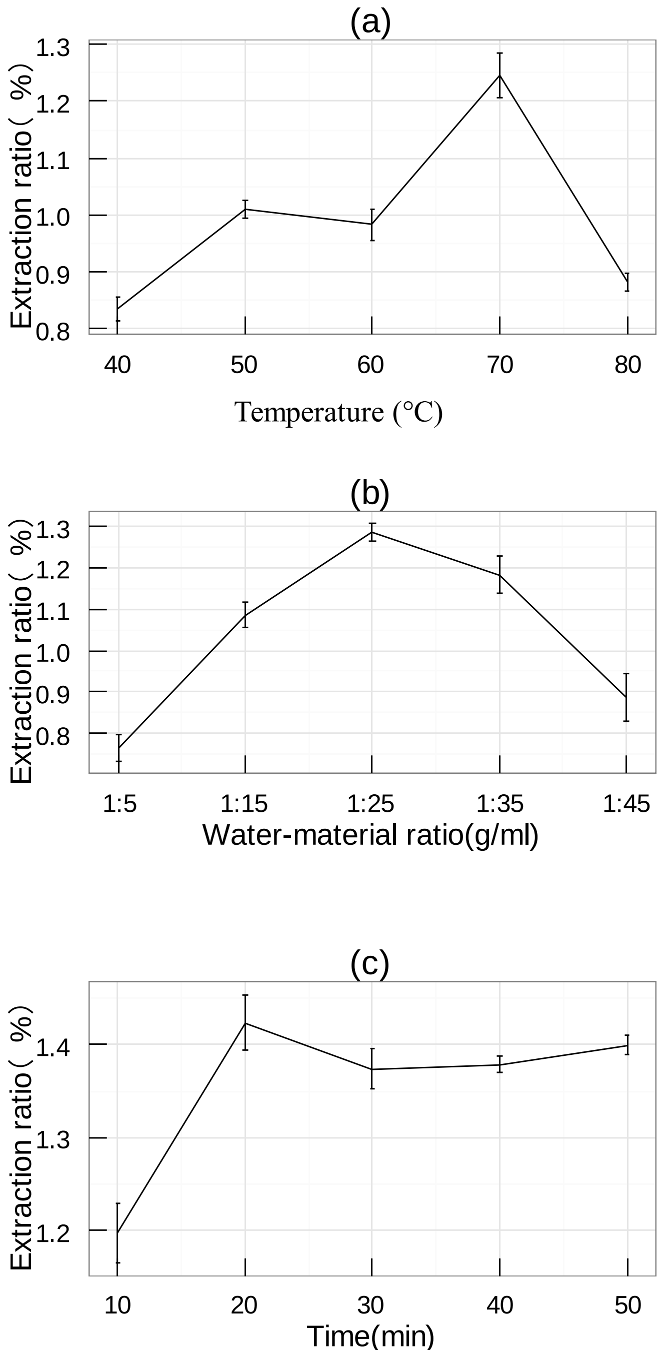

Figure 1. Effect of temperature, water-material ratio and extraction time on the yield of polysaccharides from mulberry leaves respectively. **(A)** extraction temperature. **(B)** Extraction water-material ratio. **(C)** Extraction time.

on the single factor experiments and the principle of Box-Beahnken design, temperature (x_1), time (x_2), and material-water ratio (x_3) were chosen to be as independent variables. Meanwhile, polysaccharide yield was regarded as response value. The response surface analysis was adapted to improve the process condition of extraction polysaccharides from mulberry leaves (Figure 2). According to statistical analysis, the result of regression analysis was shown in Table 3. By applying multiple analyses, the results were fitted to a second-order polynomial equation and the model was obtained as follows:

$$Y=3.35-0.20X_1-0.040X_2+0.72X_3-0.058X_1X_2+0.34X_1X_3-0.38X_2X_3-0.65X_1^2-0.96X_2^2-0.59X_3^2$$

Table 3 shows the significance of the linear relationship between dependent variable and independent one. The model could account for 97.46% of the response value, showing a good fit of the model. The non-significance of lack of fit showed that the quadratic regression equation could predict the response value. The significant terms of x_1, x_3, x_1x_3, x_2x_3, x_1^2, x_2^2,and x_3^2 shows that they could have a considerable influence on the response value. The actual response value was close to the predicted one, suggesting that the regression model for the design was available. The model “Prob > F” value was less than 0.001, showing that the model test is remarkable. Lack of fit was not significant. Model calibration coefficient *Adj* R^2 (0.9419) means that the model can explain the change in 94.19% response value, and only about 5.81% of the total variance was not explain with this model. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 15.174 indicates an adequate signal (Li et al., 2012). This model can be used to navigate the design space. x_1^2, x_2^2 and x_3^2 are significant model terms in Table 3.The results suggest the range of extraction time (x_2), extraction temperature (x_1), and material-water ratio (x_3).

In order to estimate the polysaccharides extraction factors, plotted graphs were made among the influencing parameters. The response surface graph depicted by the two out of three factors formed a series of approximate circles with one center regardless of diverse gradients; simultaneously vaults hanging down took shape at the three-dimensional chart and reached the maximum. The response values from exertion would be calculated by first order local deviation with equivalence to zero, when x^1, x^2 and x^3 were set to 70°C, 20 min and 1:30 (g/ml), respectively.

DISCUSSION

Morus is a genus of deciduous trees native to warm, temperate, and subtropical regions of Asia, Africa, North America, and Southern Europe. Mulberry leaves have historically been used for its foliage, to feed silkworm (*Bombyx mori* L.). In traditional Chinese medicine, Mulberry leaves has been used as a tonic and sedative, as dried mulberry fruits and root bark of the mulberry. Polysaccharides existed in mulberry leaves and exhibit anti-tumor, inhibition of enzymatic activity, anti-virus and

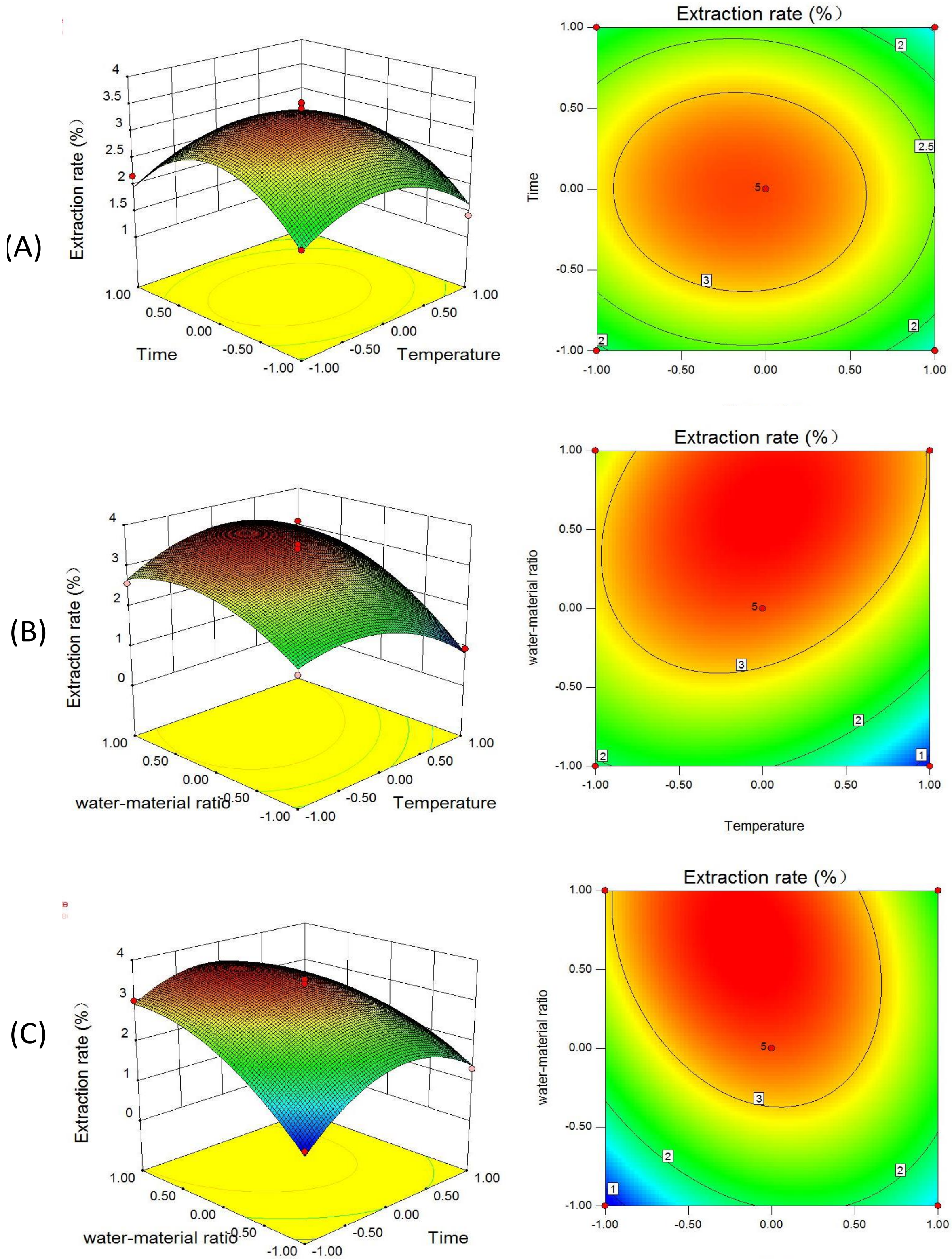

Figure 2. Response surface and contour plot showing the interactive extraction effects. **(A)** Temperature and time. **(B)** Water-material ratio and temperature. **(C)** Water-material ratio and time.

other biological activities, suggesting that the mulberry leaves have compounds with physiological functions that

Table 3. ANOVA for polysaccharide yield

Source	Sum of squares	df	Mean square	F value	*p*-value Prob > F	Significant
Model	13.48	9	1.50	29.83	< 0.0001	**
X_1	0.32	1	0.32	6.41	0.0391	*
X_2	0.013	1	0.013	0.26	0.6278	
X_3	4.18	1	4.18	83.15	< 0.0001	**
X_1x_2	0.013	1	0.013	0.27	0.6215	
X_1x_3	0.47	1	0.47	9.35	0.0184	*
X_2x_3	0.56	1	0.56	11.23	0.0122	*
X_1^2	1.79	1	1.79	35.61	0.0006	**
X_2^2	3.89	1	3.89	77.44	< 0.0001	**
X_3^2	1.46	1	1.46	29.10	0.0010	**
Residual	0.35	7	0.050		0.3208	Not significant
Lack of fit	0.19	3	0.064	1.61		
Pure error	0.16	4	0.040			
Cor total	13.83	16				
R-squared	0.9746					
Adj-squared	0.9419					
Adeq precision	15.174					

**Extremely significant difference at $p<0.01$, *significant difference at $p<0.05$.

offer protection to humans (Jeszka-Skowron et al., 2014).

There are a few reports about mulberry polysaccharide extraction. A water-soluble polysaccharide was extracted from *Morus abla* L. leaves by method of boiling water and ethanol deposition by Liu (2005). *M. abla* L. leaves polysaccharides were shown to be a homogenous component by the column chromatography and electrophoresis (Liu, 2005). Experiments showed that mulberry leaves polysaccharides are composed of a single polysaccharide，but the extraction temperature and extraction time was too long. Yin et al. (2011) studied the ultrasound-assisted extraction of polysaccharides from mulberry leaves and the results showed that optimum conditions were extraction temperature of 60°C, extraction time of 20 min and ratio of water to raw material of 15:1 (ml/g). Thirugnanasambandham et al. (2015) investigated the effects of three microwave-assisted extraction factors on the yield of polysaccharides using RSM, showing that optimum extraction conditions were determined as follows: weight of the sample of 20 g, microwave power of 170 W and extraction time of 10 min. In this paper, the optimum extraction conditions were obtained as follows: extraction temperature of 70°C, material-water ratio of 1:30 (g/mL), and extraction time of 40 min. Under these conditions, polysaccharide yield was found to be 3.56%; no significant difference was observed between the predicted yield and experimental one when the Student *t*-test was conducted, indicating that the model was satisfactory and adequate for reflecting the expected optimization.

In conclusion, based on single factor experiments, a 2 level, three variable central composite designs was carried out to establish a quadratic regression model for the extraction efficiency of total polysaccharides as a function of extraction time, extraction temperature and water-material ratio. The optimum extraction conditions were obtained as follows: extraction temperature of 70°C, material-water ratio of 1:30 g/mL and extraction time of 40 min. Under these conditions, the predicted total polysaccharides extraction efficiency was 3.6% and the experimental value was 3.56%. The result indicates that established model predicted the extraction efficiency of total polysaccharide from mulberry leaves and the optimized extraction conditions are a good reference for practice.

Conflict of interests

The authors have not declared any conflict of interests.

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