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Extraction of active calcium from *Megalobrama Amblycephala* bone and optimization of process conditions

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Abstract

Megalobrama Amblycephala is an important fishery resource. The processing of *Megalobrama Amblycephala* often generates many underutilized leftovers including bone which contents abundant active calcium. This study investigated the optimization for extraction conditions of active calcium from *Megalobrama Amblycephala* bone. The active calcium was extracted from *Megalobrama Amblycephala* bone using citric acid and malic acid. The response surface methodology was applied to optimize the extraction technology parameters including extraction temperature, extraction time and ratio of citric acid and malic acid. Results showed that, the optimal conditions of extracting active calcium from *Megalobrama Amblycephala* bone were as follows: extraction temperature, 113 °C, extraction time, 0.8 h, ratio of citric acid and malic acid, 2: 1. Under these conditions, the yield of active calcium was 90.11%. This study has provided a reference for efficiently obtaining of active calcium and utilization of *Megalobrama Amblycephala* bone resource.

Keywords: *Megalobrama Amblycephala*; active calcium; extraction; response surface methodology.

Practical Application: This study has provided a reference for efficiently obtaining of active calcium and utilization of *Megalobrama Amblycephala* bone resource.

1 Introduction

Megalobrama Amblycephala, also known as Wuchang fish, is a freshwater cyprindae, which mainly inhabited in the Yangtze River, and the downstream of medium-sized lakes. *Megalobrama Amblycephala* is a hubei province specialty and one of the main aquatic farming species in China, with nearly 10,000 hectares of farm nationwide (Lu et al., 2014). As required, *Megalobrama Amblycephala*, before sales, is often processed into fish balls or fish meats, etc. The processing generated many underutilized fish leftovers. Not only its economic value underutilized, it also causes environmental pollution (Abbey et al., 2016). The main component of these leftovers is fishbone, which is a good source of calcium, with very high calcium content (Feng et al., 2015). Calcium, one of the most abundant mineral elements, is an essential nutrient for the human body. The insufficient food intake with calcium can cause the metabolic bone diseases, such as rickets in children and osteoporosis in older adults (Ji et al., 2022). Calcium deficiency is common among Chinese people, especially children and the elderly (Huang et al., 2018). People often need to take in active calcium for the prevention and treatment of disease caused by calcium deficiency (Veldurthy et al., 2016). As a result, the development of calcium has drawn the increased attention from society (Peterlik et al., 2009). Moreover, fishbone calcium mostly exists in the form of hydroxyapatite crystals, with very little dissolution amount during the extraction (Johnson et al., 2000).

The calcium from fishbone is often extracted using acid solvents. In comparison with extracting calcium using hydrochloric acid and lactic acid, the organic acids have advantages of high solubility, better taste, and no other side effects (Shiowatana et al., 2006). Meanwhile, the organic acids are highly absorbable calcium materials, as well as a good calcium nutrition fortifier, which have huge potential to tap. The response surface methodology adopts a multiple quadratic regression equation to fit the relation between multiple factors and the response value and seeks the best process parameters method through a regression equation (Zhu & Liu, 2013; He et al., 2014). In this study, the active calcium was extracted from *Megalobrama Amblycephala* bone using citric acid and malic acid. Based on single factor experiment, the response surface methodology was applied to optimize the extraction conditions.

2 Materials and methods

2.1 Pretreatment of Megalobrama Amblycephala *bone*

Megalobrama Amblycephala bone, which was collected from the local market of Tuanchengshan Street, Xialu district, Huangshi, Hubei province, was digested for 2 h by adding 50,000 U/g neutral protease (Hubei Yuanchengsaichuang Science and Technology Co., Ltd., Hubei, China) solution at 60 °C, and filtered and washed with hot water (60 °C). Then fishbone was

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soaked in 7% NaOH solution for 8 h to remove the salt soluble proteins. The solid portion was washed with diluted water 5-10 times to neutralize pH and then dried at 105 °C to a constant weight. Finally, the remaining solid was grinded into powder (80 meshes) by using universal mill.

2.2 Extraction of active calcium

Firstly, 50 mL mixture of citric acid and malic acid was added into 0.5 g of dried fish bone powder in a 100 mL beaker and then mixed using constant temperature heating magnetic stirrer DF-101S (Gongyi Yuhua Instrument Co., Ltd, Zhengzhou, China) at 60 °C (Pak et al., 1987; Wolters et al., 1993). After centrifuged at 3000 rpm for 20 min, the supernatant was collected, neutralized, and dried in the vacuum drying oven, and grinded it into powder, which is also known as the milk white active calcium powder. Finally, EDTA method was used to determine the calcium content. The formula was shown as follow: Calcium extraction rate (%) = (active calcium content/ total calcium content multiplying) * 100.

2.3 Single-factor experiment

As taking calcium extraction rate as the evaluation index, we tested the effect of extraction temperature, extraction time and ratio of citric acid and malic acid on yield of active calcium. Each factor was set with three levels: extraction temperature (100 °C, 110 °C, and 120 °C), extraction time (0.5 h, 1.5 h, 1 h), ratio of citric acid and malic acid (1: 1, 3: 2, 2: 1).

2.4 Response surface test

Based on the results of single factor experiment, we designed an experiment with three factors of three levels. Under the guidance of the response surface methodology included in the center of Box-Behnken Design (BBD) (To et al., 2022), three independent variables were represented by A, B and C, and expressed with the -l, 0, 1 to represent the low, medium and high levels of independent variables. The response surface design experimental factor level was shown in Table 1. The yield of active calcium Y was taken as the response value to determine the optimal conditions of extraction process of active calcium in *Megalobrama Amblycephala* bone.

2.5 Statistical analysis

All experiments were repeated at least three times. The data were presented as mean±SD. The response surface analysis was performed using Design Expert software 8.0 (Stat-Ease Inc., Minneapolis, USA). Statistical analysis was performed using the Student's t-test with SPSS software version 16.0 (SPSS Inc., Chicago, USA). A *P* value < 0.05 was considered to be statistically significant.

Table 1. Code and level of factors chosen for the trials.

Factor	Level		
	-1	0	1
Extraction temperature (°C)	100	110	120
Extraction time (h)	0.5	1	1.5
Ratio of citric acid and malic acid	1: 1	3: 2	2: 1

3 Results and discussion

3.1 Results of single-factor test

The single-factor test is often used to determine the main factors and the appropriate ranges for the BBD (Li et al., 2022). In this study, the extraction process was carried out at different temperatures of 90 °C, 100 °C, 110 °C, and 120 °C, while other parameters were extraction time of 1 h and ratio of citric acid and malic acid of 1: 1. The effect of extraction temperature on yield of active calcium was shown in Figure 1. It was shown that the yield of active calcium firstly increased and then decreased with the increase of extraction temperature. When the temperature was 110 °C, the yield of active calcium was the highest. Therefore, the best extraction temperature was 110 °C.

In order to observe the effect of extraction time on yield of active calcium, we set the citric acid to malic acid ratio at 1: 1, extraction temperature at 110 °C, and extraction time at 0.5 h, 1.5 h, 1 h, and 2 h, respectively. The results demonstrated that, the highest yield of active calcium was attained while the extraction time reached 1 h. When the extraction time exceeded 1 h, the yield decreased. Therefore, the best extraction time was 1 h (Figure 2).

To test and observe the effect of ratio of citric acid and malic acid on yield of active calcium, we set the ratio of citric acid and malic acid at 1: 2, 1: 1, 3: 2 and 2: 1, respectively, with extraction temperature of 110 °C and extraction time of 1 h. The results suggested that, the yield of active calcium firstly increased, and reached the highest value when the ratio of citric acid and malic

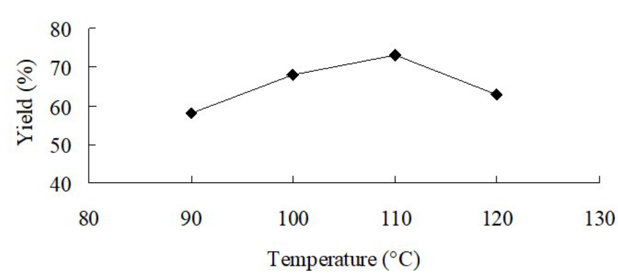

Figure 1. Effect of extraction temperature on yield of active calcium.

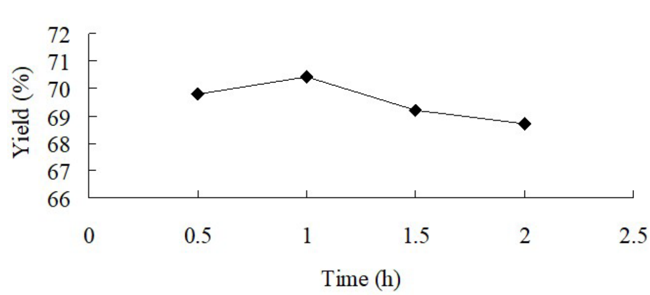

Figure 2. Effect of extraction time on yield of active calcium.

acid became 3: 2. Therefore, the best ratio of citric acid and malic acid was 3: 2 (Figure 3).

3.2 Results of response surface test

BBD is an important method to optimize the conditions (Yao et al., 2022). In this study, the results of active calcium extraction response surface test were shown in Table 2. By using the Design Expert software to undertake multiple regression and input the experimental data in the table, it was concluded that the quadratic multinomial regression model equation for the yield of active calcium, with the independent variables of extraction temperature (A), extraction time (B) and the ratio of citric acid and malic acid (C), is as follows: $Y=85.84+2.45A+3.95B+3.50C-5.62AB+2.15AC-7.90BC-13.25A^2-5.95B^2-0.42C^2$. The analysis of variance was adopted for this model, and the results were shown in Tables 2 and 3. It was shown that the total model corresponding to $P < 0.001$, which meant that the model reached the level of extreme significance, and that the established model and the experiment were significant. In variance analysis results, the P value of monomial item A, B, C, was less than 0.05, suggesting that three factors had a significant impact. In addition, the order of influence, from top to the bottom, was extraction time, ratio of citric acid and malic acid, and extraction temperature. On the other hand, the P value of interactive items AB (extraction temperature and extraction time), BC (extraction time and ratio

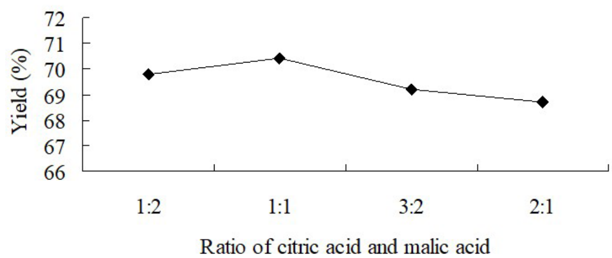

Figure 3. Effect of ratio of citric acid and malic acid on yield of active calcium.

Table 2. Response surface design and results.

No.	Extraction temperature (°C), A	Extraction time (h), B	Ratio of citric acid and malic acid, C	Yield of active calcium (%)
1	110	1	3: 2	85.82
2	120	1.5	3: 2	71.14
3	110	1.5	1: 1	85.98
4	120	1	2: 1	78.42
5	120	0.5	3: 2	70.78
6	110	1.5	2: 1	77.14
7	100	1.5	3: 2	73.74
8	100	0.5	3: 2	50.9
9	110	1	3: 2	85.86
10	110	1	3: 2	85.86
11	110	1	3: 2	85.8
12	120	1	1: 1	67.06
13	110	0.5	1: 1	65.98
14	100	1	1: 1	70.22
15	110	0.5	2: 1	88.74
16	110	1	3: 2	85.86
17	100	1	2: 1	72.98

Table 3. Results of the variance analysis of regression model for yield of active calcium.

Source	df	Sum of Squares	Mean Square	F Value	Prob > F
Model	9	1600.30	177.81	222.42	0.0002
A	1	47.82	47.82	6.43	0.0437
B	1	124.82	124.82	15.74	0.0054
C	1	98.24	98.28	12.39	0.0097
AB	1	126.34	126.34	15.91	0.0052
AC	1	18.49	18.49	2.33	0.1706
BC	1	245.86	245.86	31.48	0.0008
A^2	1	738.65	738.65	93.14	<0.0001
B^2	1	149.31	149.31	18.83	0.0034
C^2	1	0.76	0.76	0.096	0.7658
Residual	7	55.51	0.93		
Lack of Fit	3	55.51	18.50	23129.17	0.1620
Pure Error	4	3.200E-0.03	8.000E-0.04		
Cor Total	16	1655.82			

of citric acid and malic acid) was less than 0.01, representing that these factors have high impact. Quadratic term A^2 and B^2 also had a highly significant impact on response value, indicating that the proper fitting of the regression equation was also highly relevant. As representing a nonlinear relationship, the influence of the three factors on the active calcium extraction yield was difficult to explain with simple analysis method, while by applying the response surface method, we could better describe the relationship between them, and better optimize the extraction process.

Three-dimensional (3D) plots were highly recommended for the graphical interpretation of the interaction effect of independent variables on the response variables (Chua et al., 2009). The 3D figure and contour map of the response surface for all the interactive factors were shown in Figure 4. There was an extreme value within the selected scope, namely, the highest point in the response surface. Through comparing several figures, we could see that the effects of B (extraction time) and C (ratio of citric acid and malic acid) on yield of active calcium were significant, and the curves changes greatly. The following was A (extraction temperature). A flat curve change was presented, which corresponded to the regression analysis of the results.

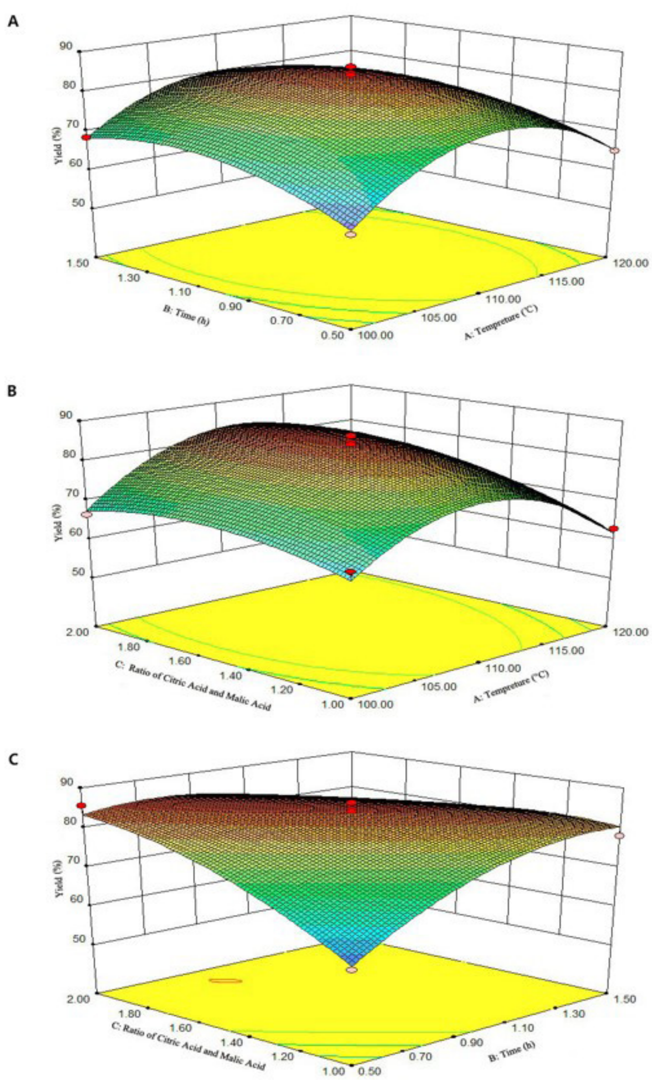

Figure 4. Response surface plot showing effects of extraction temperature (A), extraction time (B) and ratio of citric acid and malic acid (C).

3.3 Verification of model

According to the software analysis, the optimized extraction conditions were as follows: extraction temperature, 112.71 °C; extraction time, 0.77 h; ratio of citric acid and malic acid, 2: 1. In this case, the theoretical optimal extraction yield was 90.45%. In order to test the feasibility of the response surface method, the verification experiment was carried out using the best extraction conditions to extract active calcium. Meanwhile, for the convenience of practical operation and production, the optimized extraction condition was set at the extraction temperature of 113 °C, extraction time of 0.8 h, and ratio of citric acid and malic acid of 2: 1. The actual average extraction yield of three parallel experiments was 90.11%, with a variance of only 0.34% in comparison with the theoretical value. Therefore, optimizing the extraction technique with response surface method was feasible, and the specific extraction technique was practically applicable.

4 Conclusions

In conclusion, through the response surface methodology, the optimal conditions of extracting active calcium from the *Megalobrama Amblycephala* bone are as follows: extraction temperature, 113 °C, extraction time, 0.8 hs, ratio of citric acid and malic acid, 2: 1. Under these conditions, the yield of active calcium is 90.11%. This study has provided a reference for efficiently obtaining of active calcium and utilization of *Megalobrama Amblycephala* bone resource.

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