Orthogonal experimental design optimizes the compound extraction process of sulforaphane from broccoli seeds

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Abstract

The broccoli seeds were used as experimental material, the enzymolysis condition of sulforaphane extraction from broccoli was optimized by orthogonal design method. According to orthogonal design experiment design principle and analysis of four factors and three levels, additives including ascorbic acid, temperature, time, pH. on the basis of the single factor to determine the impact of various factors on the extraction rate of sulforaphane. The optimum extraction conditions were as follows: Ethyl acetate was extracted, and the activity of mustard seed was 0.934 μmol/(min·g), ascorbic acid 0.02 mg/g, temperature 30°C, time 60 min, pH 4.The theoretical maximum extraction rate of sulforaphane was 1.56 mg/g, which indicated that the extraction process parameter above was feasible in sulforaphane extraction from broccoli seeds.

Keywords

Sulforaphane; Enzymolysis; Additive; Qrthogonal design.

1. Introduction

Sulforaphane can be obtained form cruciferous vegetables as one of the products of enzymatic or acid hydrolysis of glucoraphanin, which has been indentified the best anticancer activity. Molecular weight is 177.29, formula is $C_6H_{11}S_2NO$.

Are found in cruciferous plants together sulfur and its hydrolysis enzyme [1], but its distribution in different parts of the plant, the former is generally stored in the plant cell SAP [2], while the latter is located in a specific protein in the body[3], when the plant tissue damage caused by such as chewing, grinding, originally present in the protein body myrosinase has unleashed[4], glucosinolates indican, generating a precursor of the sulforaphane-isothiocyanate (Isothiocyanates). Huang Xiaoqin etc, Yuan Haina [5-6] and Wu Yuanfeng [7] in the extraction of sulforaphane, such as study, all is the use of cruciferous plant myrosinase itself hydrolysis of sulforaphane, but plant myrosinase content is low, cannot be fully released, digestion time, low efficiency, and the actual production operation, easy to microbial contamination. This experiment using broccoli seeds as raw material, by adding exogenous myrosinase, enzyme concentration, different additives, again with the aid of ultrasonic assisted organic extraction, enzymatic hydrolysis conditions were optimized by orthogonal design to establish effective method to extract sulforaphane, in order to of sulforaphane in broccoli seed extract provides technical reference and theoretical basis. This topic with broccoli seeds as raw material, studied the broccoli seeds in the enzymatic hydrolysis conditions and products of glucosinolates extraction, separation and purification technology of sulforaphane, and identification of its structure, laid the foundation for the development of sulforaphane related products.

2. Materials and methods

2.1 Materials and instruments

Broccoli seeds, purchased from hangzhou seed company, storage in cold storage; Sulforaphane standard products are purchased from the American Sigma company, with a purity of > 98%; 95% acetone (pure), ethyl acetate (pure), methanol (pure) were purchased from tianjin guangfu fine chemical industry. RE-52 rotary evaporator Shanghai arron biochemical instrument factory; DS-8510DTH ultrasonic wave oscillation instrument Shanghai shengli ultrasonic instrument co. LTD.

High performance liquid chromatograph system (equipped with variable wavelength ultraviolet detector and shimazu Lcsolution 15c chromatogram workstation) Japan shimon company.

2.2 HPLC analysis and determination method[8]

Chromatographic condition: Shimadzu RP-18Cs (250 x 4.6 un i.d.); Detection wavelength: 201 nm; Flow phase: methanol: water = 4:6(v/v); Flow rate: 1 mL /min; Column temperature 25 ℃; Sample size: 10 μL, analysis time: 20 min.

Standard curve drawing: take methanol 0.5 mL (chromatography pure) in the specifications for the 5 mg sulforaphane standard, the mixture concentration of 10 mg/mL sulforaphane standard solution, diluted mixture again, it was 0.5, 0.75, 1.0, l. 25, 1.5, 1.75, 2 mg/mL solution, HPLC detection for each concentration with sulforaphane concentration as the abscissa, The chromatogram is shown in figure 2. The corresponding peak area as the ordinate, make the standard curve (figure 1), y = 11750x + 620.73, R^2 = 0.997. Based on the prototype of the retention time qualitative and quantitative analysis and the standard curve.

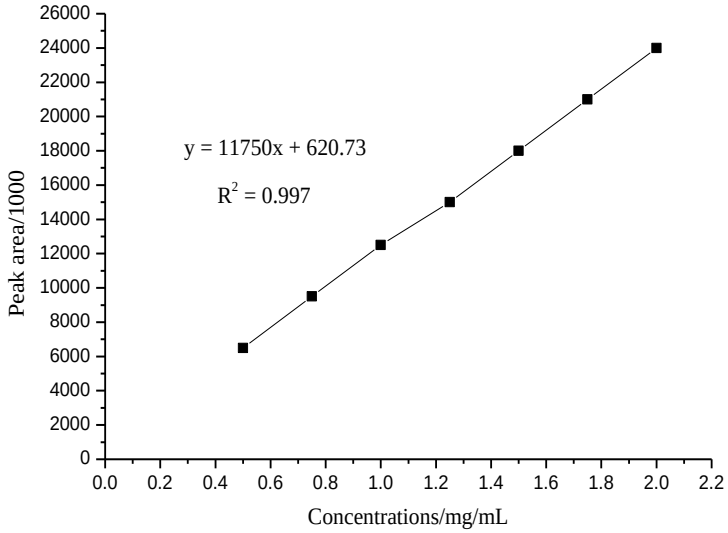

Fig.1. The linear relationship of sulforaphane standard solution was determined by H PLC.

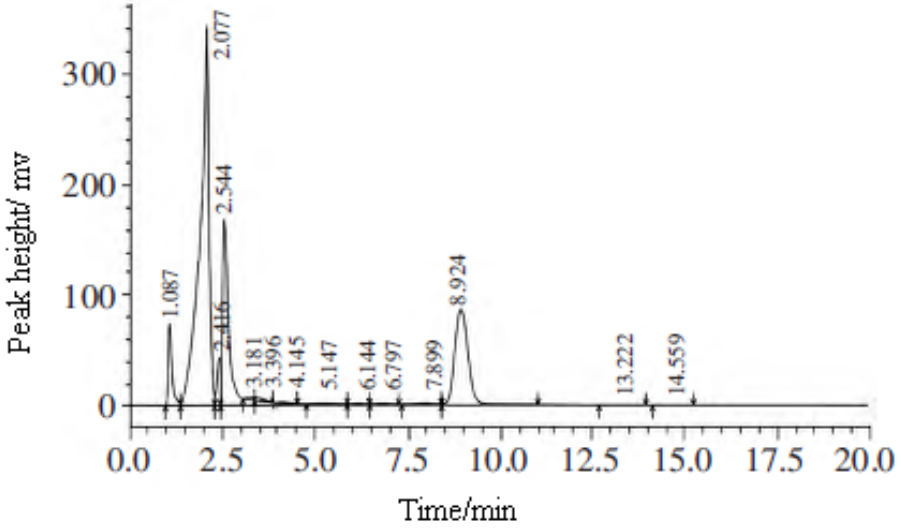

Fig.2 HPLC chromatogram of sample

2.3 Experimental method

2.3.1 Extraction process of sulforaphane

After the crushed broccoli seeds, 60 mesh screen, put the dry powder 5 g in the triangular bottle to be used. Another new harvest mustard seed, pulverized, over 60 mesh screen, take 0.5 g to the triangular bottle, add 25 mL distilled water, ultrasonic oscillation 20 min, filtered out of the crude enzyme solution. The activity of the measured crude enzyme was 0.934 mu/(min·g). 25 mL of crude enzyme solution was added to the dried broccoli powder, and the control group replaced the crude enzyme with distilled water and added different concentrations of additives (ascorbic acid) in the enzymatic hydrolysis system. Digestion after a certain amount of time under different temperature conditions, adding 95% ethyl acetate 100 mL 0.5 h ultrasonic assisted extraction, let stand for 0.5 h, over 40 mesh sieve to remove broccoli seeds, 65 ℃ steam to concentrate, sticky, according to the volume ratio 1:1 to join three ethyl acetate extraction, combined ethyl acetate extract, 60 ℃ steam to concentrate fully ethyl acetate, at last the capacity to 25 mL, the methanol under test.

2.3.2 Effects of different solvents on the extraction rate of sulforaphane

Take 10 g broccoli seed pieces, and add 30 mL of water in the table (room temperature) enzyme, freeze drying, , take 4 dry powder of 2 g respectively. then add 40 mL of acetone, ethyl acetate, ethanol, methylene chloride as extracting solvent, ultrasonic assisted extraction, gauze filter, concentrating filtrate. Samples with 2 mL water dissolve after 0.22μm drainage membrane, HPLC quantitative analysis, according to the standard curve and the sample of the concentration of sulforaphane, by comparing the size of the concentration of the extraction effects of different solvents and extraction solvent.

2.3.3 Effect of temperature on the extraction rate of sulforaphane

According to take 6 copies of the same amount of 2 g broccoli seed powder in small l0 mL beaker, add thick enzyme fluid and 6 mL distilled water, ascorbic acid and crude enzyme fluid mixing sealed with plastic wrap, respectively at 20, 30, 40, 50, 60 ℃ enzymatic hydrolysis for 60 min.

2.3.4 The effect of pH on the extraction rate of sulforaphane

According to take eight copies of the same amount of 2 g broccoli seed powder in l0 rnL small beaker, crude enzyme liquid and distilled water 6 mL, ascorbic acid, then use 1 moL/L HCL solution among them 7 sets of pH adjustment to 3.0, 4.0, 5.0, 6.0, 7.0, 8.0. After the adjustment, wrap it with plastic wrap and put it at room temperature for 60 min.

2.3.5 The effect of enzymatic hydrolysis time on the extraction rate of sulforaphane

According to take 5 copies of the same amount of 2 g broccoli seed powder in small l00 mL beaker, add thick enzyme fluid and 6 mL distilled water, ascorbic acid, mixing sealed with plastic wrap, respectively in the enzyme solution at room temperature 20, 40, 60, 80, 100 min.

2.3.6 Effect of ascorbic acid on the extraction rate of sulforaphane

Take 9 copies of the same amount of 2 g broccoli seed powder in small l00 mL beaker, add distilled water 6 rnL, then add 0, 0.04, 0.4, 4.0, 8.0, 12.0, 16.0, 20.0, 40.0 mg of ascorbic acid, sealed with plastic wrap, stir in the enzyme solution at room temperature for 60 min.

2.4 Orthogonal experimental design

Based on the results of single factor experiment, according to the principle of orthogonal experimental design, the selection of temperature, pH (B) (A) (C), time, concentration of ascorbic acid (D) the most significant factors as independent variables, the design of orthogonal experiment, the factor levels as shown in table 1.

Table 1. Factors and levels of orthogonal design test

Factors/level	A/°C	B/	C/min	D/mg/g
1	20	3	40	0.01
2	25	4	60	0.02
3	30	5	80	0.03

2.5 GC/MS analysis

Chromatographic column: HP-5 ms quartz capillary column (30 m x 0.25 mm x 0.25 μm); Carrier gas: N2, 1.0 mL/min; Injection port temperature: 250 ℃; Column temperature: temperature programmed: 50 ℃ for 2 min, and then to 10 ℃/min up to 250 ℃, keep 8 min; Temperament interface temperature: 250 ℃. EI ion source temperature: 200 ℃, EI energy: 70 eV, sweep frequency: 2 times/s, the scanning range: 35-500 amu.

3. Results and analysis

3.1 Effects of different solvents on the extraction rate of sulforaphane

The sulforaphane is less polar, the solvent is low toxicity, the common solvent is acetone and ethyl acetate and ethanol. The extraction effect is compared with dichloromethane as shown in the table below. The extraction effect of dichloromethane is the best, followed by ethyl acetate, but the extract has the highest purity, and the ethanol is the worst. Considering the toxicity of dichloromethane, ethyl acetate is preferred as solvent extraction solvent.

Table 2. Effects of different solvents on the extraction rate of sulforaphane

Extracting agent	Broccoli seed quality/g	Purity/%	Extraction yield/(mg/g)
Methylene chloride	2	88.5	1.2
Ethyl acetate	2	91.2	1.1
Acetone	2	85.8	0.96
Ethanol	2	82.7	0.88

3.2 Effect of different temperatures on sulforaphane extraction rate

The results of sulforaphane extraction at different temperatures are shown in figure 3.With temperature rise, sulforaphane extraction yield increased significantly, when the temperature 30 ℃ when the extraction yield reached the highest (the experimental group is up to 1.45 mg/g), and then with the increase of temperature, sulforaphane extraction yield declines. At the appropriate temperature, the activity of myrosinase was high, which was beneficial to hydrolysis of glucoside glucoside. When the temperature was too high, the activity of myrosinase decreased significantly. It may also be that the high temperature dissolves the sulforaphane part, leading to a decrease in the extraction rate.

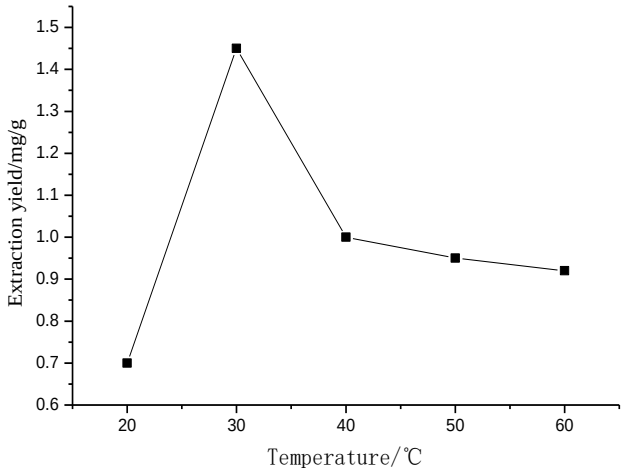

Fig 3. Effect of different temperatures on sulforaphane extraction rate

3.3 Effect of different pH on sulforaphane extraction rate

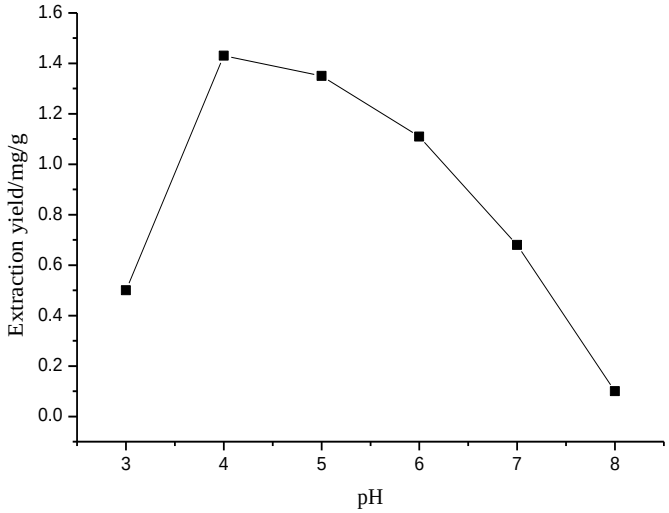

Fig 4. Effect of different pH on sulforaphane extraction rate

The effect of pH on the solution of thiosidase is shown in figure 4. When pH = 4, the solution is most complete. With the increase or decrease of pH, the enzymatic hydrolysis capacity of thioside is decreased. This is also consistent with the principle of producing isothiocyanate in the range of strong acids and weak bases.

3.4 Effect of different enzymatic hydrolysis time on sulforaphane extraction rate

The results of sulforaphane were extracted under different enzymatic hydrolysis, as shown in figure 5. When the enzymolysis time was less than 60 min, the extraction efficiency was significantly improved with the extension of the enzymatic hydrolysis time. The experimental group could reach 1.45mg /g, and then the extraction rate decreased with the extension of the enzymatic hydrolysis time. The time of enzymatic hydrolysis was too short, the sulforaphane was not sufficient, the extraction rate was low, and the sulforaphane was fully extracted and the extraction rate was high after the suitable enzyme solution time. The time of enzymatic hydrolysis is too long, and sulforaphane may start to degrade, resulting in a decrease in sulforaphane.

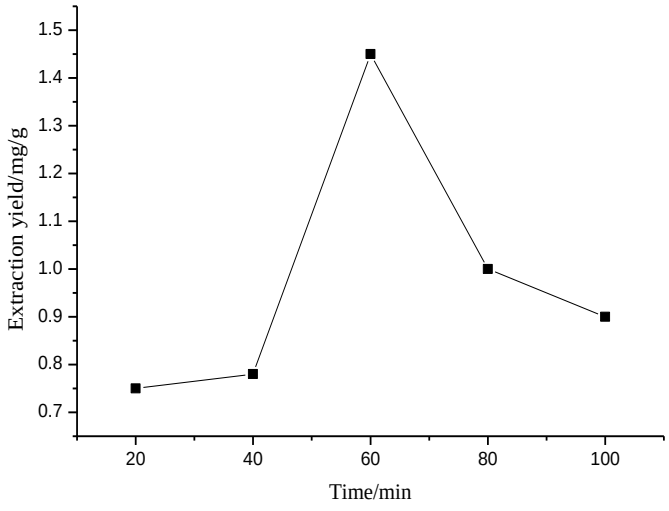

Fig. 5 Effect of different enzymatic hydrolysis time on sulforaphane extraction rate

3.5 Effect of different ascorbic acid concentrations on sulforaphane extraction rate

The effect of the addition of ascorbic acid on the solution of the glucosidase is shown in figure 6. It can be seen that adding a small amount of ascorbic acid can significantly improve the enzymatic hydrolysis of the glucoside. This is consistent with the literature report that ascorbic acid can increase the enzyme activity of the enzyme, and its activation mechanism is that ascorbic acid can cause slight changes in the conformation of the enzyme. The concentration of sulforaphane was highest at 0.02mg/g. However, with the increase of ascorbic acid, the concentration of sulforaphane decreased, and the reason was not clear.

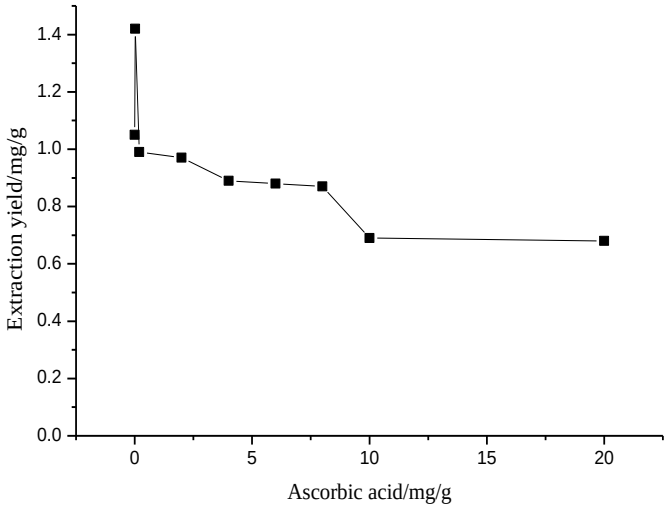

Fig 6. Effect of different ascorbic acid concentrations on sulforaphane extraction rate

3.6 The results of orthogonal experimental design

Table 3. Program and results of orthogonal experimental

Column	1	2	3	4	
Factors	A	B	C	D	Results/(mg/g)
Experiment 1	1	1	1	1	1.11
Experiment 2	1	2	2	2	1.45
Experiment 3	1	3	3	3	1.35
Experiment 4	2	1	2	3	1.25
Experiment 5	2	2	3	1	1.41
Experiment 6	2	3	1	2	1.31
Experiment 7	3	1	3	2	1.15
Experiment 8	3	2	1	3	1.21
Experiment 9	3	3	2	1	1.23
Average 1	1.303	1.170	1.210	1.250	
Average 2	1.323	1.357	1.310	1.303	
Average 3	1.197	1.297	1.303	1.270	
Poor	0.126	0.187	0.100	0.053	

From the orthogonal experimental results, it can be seen that the sequence of the factors affecting the extraction of sulforaphane is: B>A>C>D, which is the most influence of pH on the extraction rate, followed by the temperature and the amount of ascorbic acid.

4. Conclusion

In this study, HPLC was used for the analysis and detection of sulforaphane, and the results were accurate and reliable. By adding exogenous myrosinase, studied various factors influence on glucosinolates in broccoli seed enzyme solution, through the orthogonal experiment design, determined the optimum digestion conditions of glucosinolates are as follows: enzymolysis time 60 min,temperature 30 ℃, pH = 4, ascorbic acid content 0.02mg/g. And by using gas chromatography/mass spectrometry technology analyzes the sulfur yesterday enzymolysis products, identified the seven kinds of compounds, among them the content of sulforaphane, largest of glucosinolates enzymolysis products of volatile compounds in 42.23% of the total, the extraction and separation of sulforaphane and further research.

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