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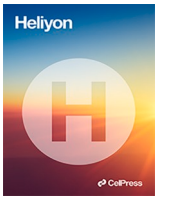

Research article

Optimization of hydrolysis conditions of alginate based on high performance liquid chromatography

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ABSTRACT

Alginate is the most abundant polysaccharide compound in brown algae, which is widely used in various fields. At present, the determination of the content of alginate is mostly carried out using sulfuric acid and trifluoroacetic acid hydrolysis followed by the determination of the content, but the results are not satisfactory, and there are problems such as low hydrolysis degree and low recovery rate. Therefore, in this study, based on the optimization of high performance liquid chromatographic conditions for pre-column derivatization of 1-phenyl-3-methyl-5-pyrazolone (PMP), the hydrolysis effects of sulfuric acid, trifluoroacetic acid (TFA), oxalic acid, and formic acid were compared and the hydrolysis conditions were optimized. The results showed that formic acid was the best hydrolyzing acid. The optimal hydrolysis conditions were 95 % formic acid at 110 °C for 10 h. The hydrolysis effect was stable, with high recovery and low destruction of monosaccharides, which made it possible to introduce formic acid into the subsequent polysaccharide hydrolysis. The pre-column derivatization high performance liquid chromatography method established in this study was accurate and reliable, and the hydrolysis acid with better effect was screened, which provided a theoretical basis for the subsequent determination of alginate content.

1. Introduction

Alginate is a kind of water-soluble acidic polysaccharide derived from the cell wall and intercellular matrix of brown algae, which is mainly extracted from kelp, Undaria pinnatifida and Sargassum. Alginate is a copolymer composed of α-L-guluronic acid (G) and β-D-mannuronic acid (M) [1–3], which has unique structure and biological activities [4,5], such as anti-tumor, immune enhancement, and plant growth promotion. Due to its unique structure and healthful effects [6], it has been widely used in pharmaceutical, food and textile applications [2,7,8]. The alginate content of various algae purchased on the market varies widely, so in the alginate industry, an accurate knowledge of the composition and structure of alginate is required [9], and purity is examined as an important quality indicator [10], and the analysis of the M and G content of alginate allows the examination of the quality of the product [11]. However, there are no uniform standards and specifications for its content analysis methods [12], and fewer reports on the optimization of alginate hydrolysis conditions and quality studies have been reported, which directly affects the assessment of its quality and limits its potential applications to a certain extent [13]. It is difficult to analyze the M and G content directly because alginic acid is a macromolecular compound with a disordered structure, and the M/G values of alginic acid from different seaweed sources vary,

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making it possible to have differences in rheological properties, which may affect the hydrolysis of alginate to a certain extent [14,15]. By degrading alginate to the monosaccharide forms of M and G, the content of the two uronic acid is measured to reflect the content of alginate [16], which in turn allows for the evaluation of product quality [17]. Therefore, the degradation of alginate becomes a key step in the determination of its content.

Currently, the degradation methods of alginate mainly include physical degradation, biodegradation, and chemical degradation [18]. The physical degradation method is mainly based on physical methods such as direct heating, radiation and ultrasonic treatment to degrade alginate [19,20]. This method is characterized by environmentally friendly and rapid and fast reaction, but the reaction of alginate degradation by physical method is still difficult to control, and the product is complex, costly, inefficient and not suitable for operation in the laboratory. Bioenzymatic digestion has the advantages of mild conditions and high specificity, but it has the problems of high enzyme cost and low enzyme catalytic activity [21]. Chemical degradation is a commonly used method to degrade alginate, mainly acid hydrolysis is the main, commonly used hydrolysis acids are TFA, sulfuric acid, etc. D. I. Sánchez-Machado et al. [22] used sulfuric acid to hydrolyze algal dietary fibers and established a high-performance liquid chromatography method for the determination of the M, G ratio. Although the hydrolysis effect of alginate varies under different acids [16], it is simple to operate, less costly and more stable in obtaining fragments with intrinsic structural features [23], so further studies on the hydrolysis efficiency of different acids and optimization of hydrolysis conditions are needed to improve the hydrolysis effect.

The main methods used to determine the uronic acid content of polysaccharides in current studies mainly include spectroscopy and chromatography [24]. Spectrometry is simple to operate, such as visible light spectrophotometry, but it is easily affected by other factors, such as water bath time and color development resting time, what's more, impurities in the reagents or the sample can bring bias to the experimental results [25]. Chromatography methods include gas chromatography and liquid chromatography. Gas chromatography has high accuracy in the determination of results, but it is very demanding for polysaccharide sample, which need to be maximally converted into substances with strong volatility [26]. Liquid chromatography is one of the main techniques for polysaccharide analysis, and in recent years, pre-column derivatization HPLC method with PMP has emerged [27–29], which can be further developed and utilized due to its good reproducibility and stability, high recoveries, and more accurate determination of content [30].

Studies have shown that alginate can produce good tolerance to inorganic acid hydrolysis, so it is difficult to break all the glycosidic bonds in alginate in acid hydrolysis reaction [16,31]. Therefore, it is necessary to further study the hydrolysis efficiency of different acids and optimize the hydrolysis conditions to improve the hydrolysis effect. In addition, it is important to compare the effects of both organic and inorganic acids of the hydrolysis of alginates as literature data is lacking. Therefore, in this study, formic acid, oxalic acid, sulfuric acid and TFA were used for the preliminary hydrolysis of alginate, and the contents of the two uronic acids in the hydrolysate were determined by HPLC to compare the hydrolysis effects. We first determined the appropriate acid concentration, hydrolysis time, and hydrolysis temperature range through single-factor experiments [32]. On this basis, orthogonal experiments [33] are carried out to judge the interaction between factors, so as to obtain the optimal hydrolysis conditions to improve the hydrolysis effect of alginate and provide technical support for the determination of alginate content.

2. Materials and methods

2.1. Materials

Alginate was purchased from Qingdao Hyzlin Biology Technology Co., Ltd. (Qingdao, China). β-D-mannuronic acid and α-L-guluronic acid standard (HPLC ≥98 %) were purchased from Qingdao BZ Oligo Biotechnology Co., Ltd. (Qingdao, China). 1-Phenyl-3methyl-5-pyrazolone (PMP ≥99 %), potassium dihydrogen phosphate (HPLC grade), acetonitrile (HPLC grade), methanol (HPLC grade) and trifluoroacetic acid were purchased from Shanghai Macklin Biochemical Co. Ltd. (Shanghai, China). Sulfuric acid, formic acid, oxalic acid and other reagents were analytically pure and were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Preparation of polysaccharide standard solution

We made appropriate modifications according to the research methods in the article of Chen et al. [34]. Preparation of M and G standard solutions: 0.5 mg samples of M and G standards were accurately weighed, and concentration of 1 mg/mL of uronic acid standard reserve solutions were prepared with ultrapure water.

Mixed standard solution: 1 mg/mL M standard solution and G standard solution were taken respectively to prepare a 0.5 mg/mL mixed standard solution.

2.3. Hydrolysis of alginate

As Lu et al. [16]described, we made minor modifications to the hydrolysis of alginate. Briefly, 0.1 g sample of alginate was accurately weighed and placed in a 25 mL hydrolysis tube, to which was added 10 mL of a 2 mol/L solution of trifluoroacetic acid. The tube was sealed and placed in an oven at 110 °C for 3 h. After the sample was hydrolyzed, the hydrolysate was removed and cooled to room temperature (25 °C), a volume of 2 mol/L sodium hydroxide solution was added, the pH was adjusted to neutral, and the sample was diluted to 25 mL with 0.1 mol/L phosphate buffer solution.

2.4. Establishment of high performance liquid chromatography (HPLC)

2.4.1. Optimization of derivatization conditions

In this study, four factors including reaction temperature, reaction time [35], sample/PMP molar ratio and sample/NaOH molar ratio were selected and L_9 (3^4) orthogonal tests were performed at three levels for each factor. The experimental scheme was presented in Table S1 of the supplementary material. The derivatization reaction steps were appropriately modified according to existing studies [36]. Briefly, 100 μL of standard solution was taken and 0.1 mol/L NaOH solution was added according to Table 1. After the solution was mixed well, different volumes of 0.25 mol/L PMP-methanol solution were added. The solutions were heated at various temperatures for various durations according to Table 1, cooled to room temperature and then neutralized with 0.1 mol/L HCl solution before adding 300 μL of chloroform. The solution was shaken well, centrifuged at 8000 r/min for 10 min, and the organic phase was discarded, and the extraction was repeated three times.

2.4.2. Single-factor optimization of chromatographic separation conditions

In this study, the separation conditions of M and G were further investigated and optimized by varying the pH of the phosphate buffer (5.5, 6.0, 6.5, 6.8 and 7.0), the percentage of acetonitrile (14 %, 16 %, 18 %, 20 %, and 22 %), the flow rates (0.5, 0.8, and 1 mL/min), and the column temperatures (20 °C, 25 °C, and 30 °C) [37,38].

2.4.3. Methodological examination

Plotting of standard curve: 0.5 mg samples of M and G standard substances were accurately weighed and prepared into uronic acid standard stock solutions with a concentration of 2.5 mg/mL with ultrapure water, followed by gradient dilution. After the sample was derivatized, the sample was injected for determination and the chromatogram was recorded. The standard curve was plotted with the concentration of the standard (X) as the horizontal coordinate and the peak area (Y) as the vertical coordinate, and the regression equation was derived. The limit of detection (LOD) was three times the signal-to-noise ratio, and the limit of quantification (LOQ) was ten times the signal-to-noise ratio [39].

Proprietary experiments: in this study, the effect of common Glucuronic Acid (GlcUA) on the determination of M and G was investigated. A certain volume of GlcUA standard solution was added to 1 mg/mL of mixed standard solution of M and G. Derivatization was carried out and 20 μL of samples were injected under the optimized conditions, and the effect of GlcUA on the determination of M and G was judged according to the time of peaks and the degree of separation.

Precision experiment: after 0.6 mg/mL standard monosaccharides mixed solution was derivatized, the sample was injected continuously for 6 shots, and the relative standard deviation RSD of each uronic acid peak area was calculated.

Stability experiment: 1 mg/mL of the standard monosaccharides mixture solution was derivatized and stored at 4 °C, and the sample was injected every 2 h for a total of 6 times. The relative standard deviation RSD of the peak area of each uronic acid was calculated.

Repeatability experiment: Six samples of the same batch of alginate were prepared in parallel, hydrolyzed and derivatized for determination, and each sample was injected three times consecutively, and the relative standard deviation RSD of the peak area of each uronic acid was calculated [40].

Recovery experiment: Monosaccharide standards were accurately weighed and prepared by dissolving in ultrapure water to obtain three mixed standard solutions at high (1 mg/mL), medium (0.5 mg/mL) and low (0.25 mg/mL) concentrations. 100 μL of sample solution was precisely aspirated in triplicate, and 100 μL of mixed standard solution at high, medium and low mass concentration were added respectively. After the samples were derivatized, three parallel assays were performed on the samples. The recoveries and relative standard deviations were calculated for each uronic acid.

2.5. Single-factor experiments of hydrolysis by different acids

25 mg of alginate was placed in an ampoule, 5 mL of different concentrations of H_2SO_4 (0.01, 0.05, 0.1, 0.2, 0.5 and 1 mol/L) was added and hydrolyzed for a certain period of time (2, 3, 4, 5 and 6 h) at different temperatures (90 °C, 100 °C, 110 °C, 120 °C and 130 °C). After the end of hydrolysis, the samples were removed and cooled to room temperature, vortexed and mixed, then 2 mL was aspirated in a centrifuge tube and 2 mL NaOH solution was added to neutralize the sample.

The single-factor experiments of TFA and oxalic acid were conducted separately according to the above methods. For TFA the effects of acid concentration (0.5, 1, 2, 3 and 4 mol/L), hydrolysis time (0.5, 1, 2, 3, 4, 5 and 6 h), and hydrolysis temperature (90 °C, 100 °C, 110 °C, 120 °C and 130 °C) on the alginate contents and recoveries were investigated. For oxalic acid the effects of acid concentration (0.5 %, 2 %, 6 %, 8 % and 12 %), hydrolysis time (2, 3, 4, 5, 6 and 7 h), and hydrolysis temperature (90 °C, 100 °C,

Table 1
The orthogonal experimental design of derivatization conditions.

Levels	Factors			
	A (Reaction temperature/°C)	B (Reaction time/min)	C (Molar ratio of sample to PMP)	D (Molar ratio of sample to NaOH)
1	50	30	1:9	1:2
2	70	60	1:12	1:4
3	90	90	1:15	1:6

110 °C, 120 °C and 130 °C) on alginate contents and recoveries were investigated.

For the single-factor experiment hydrolysis of formic acid, 25 mg of alginate was weighed and placed in a digestion tube. Then 5 mL of formic acid of different concentrations (50 %, 70 %, 80 %, 90 % and 95 %) was added and hydrolyzed for a certain period of time (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 h) at different temperatures (90 °C, 100 °C, 110 °C and 120 °C). After samples were hydrolyed, 25 mL of ultrapure water was added to dilute and hydrolyzed at 100 °C for 2 h. Then, the sample was cooled down to room temperature, vortexed and mixed, and 2 mL was aspirated into a centrifugal tube. 2 mL of NaOH solution was added to neutralize the hydrolysis solution, and then the contents of the alginate and the recoveries were determined.

2.6. Orthogonal experiments on the hydrolysis of different acids

Based on the results of the single-factor experiments, a three factors and three levels orthogonal experiment was design with hydrolysis time, acid concentration and hydrolysis temperature as the main influencing factors, and the recovery of alginate hydrolysis as the dependent variable, using the orthogonal table of L_9 (3^4). Two replications were set up at each place to determine the optimal hydrolysis conditions. The orthogonal experimental designs of the four acids were shown in Table 2, Table 3, Table 4 and Table 5. The recovery (%) of alginate was calculated according to the following equation.

$$R(\%) = \frac{(c_1 + c_2) \times V}{m} \times 100\% \quad (1)$$

where: R represents the recovery of alginate, %; c_1 represents the mass concentration of M in the hydrolysate, mg/mL; c_2 represents the mass concentration of G in the hydrolysate, mg/mL; V represents the total volume of the hydrolysate, mL; m represents the amount of alginate sampled, mg.

2.7. Validation of optimal hydrolysis conditions

The best hydrolysis conditions obtained by orthogonal experiments using four acids were optimized to hydrolyze the same kind of alginate, and the hydrolysis effects and recoveries of the four acids were compared.

2.8. Statistics

Unless otherwise specified, all experiments were carried out three times, and the data were expressed as average values ± standard error. All analyses were performed using Origin and GraphPad Prism 9. F-test was applied for comparisons between the experiment groups. $P < 0.05$ and $P < 0.01$ were considered to be significant differences (95 % confidence interval) and extremely significant differences (99 % confidence interval) between the data.

3. Results and discussion

3.1. Derivatization conditions

In this study, in order to explore the optimal derivatization conditions for M and G, the analysis of the results of orthogonal experiments using the peak area of G as a reference showed that the derivatization temperature and derivatization time were the main factors affecting the peak area of the monosaccharide derivatives ($R_A > R_B > R_C > R_D$), and the sample/NaOH molar ratio had the smallest effect among the four factors (Table S1). The optimal conditions for monosaccharide derivatization were 90 min of reaction at 70 °C, and the molar ratios of sample to PMP and NaOH were 1:12 and 1:4, respectively.

3.2. Chromatographic separation conditions

The experimental results showed that the pH of phosphate buffer had a significant effect on the retention time of M and G derivatives (Fig. 1A). When the percentage of acetonitrile was 18 %, with the increase of pH of phosphate buffer from 5.5 to 7.0, the retention time of M and G derivatives and separation were decreased. Jian Wu et al. [38] found that in the optimization of the chromatographic separation conditions of propylene glycol alginate sodium sulfate, the retention time of the two PMP derivatives was shortened with the increase of pH of phosphate buffer from 6.5 to 7.4, and the retention time of the two PMP derivatives was reduced

Table 2
The orthogonal experiment design of sulfuric acid hydrolysis condition.

Levels	Experimental factors		
	Hydrolysis time (h)	Acid concentration (mol/L)	Hydrolysis temperature (°C)
1	2	0.1	110
2	3	0.2	120
3	4	0.5	130

Table 3
The orthogonal experiment design of TFA hydrolysis condition.

Levels	Experimental factors		
	Hydrolysis time (h)	Acid concentration (mol/L)	Hydrolysis temperature (°C)
1	2	1	100
2	3	2	110
3	4	3	120

Table 4
The orthogonal experiment design of oxalic acid hydrolysis condition.

Levels	Experimental factors		
	Hydrolysis time (h)	Acid concentration (%)	Hydrolysis temperature (°C)
1	4	6	100
2	5	8	110
3	6	12	120

Table 5
The orthogonal experiment design of formic acid hydrolysis condition.

Levels	Experimental factors		
	Hydrolysis time (h)	Acid concentration (%)	Hydrolysis temperature (°C)
1	9	85	100
2	10	90	110
3	11	95	120

Notes: The hydrolysis of formic acid is divided into two steps: pre-hydrolysis and re-hydrolysis. The mentioned hydrolysis time is the pre-hydrolysis time, and the re-hydrolysis is fixed at 100 °C for 2 h.

and the column pressure is higher at higher pH. In summary, at pH 6.8, the M and G derivatives could be eluted in a shorter time with better separation and without higher pressure, so the phosphate buffer pH 6.8 was used as the experimental condition.

The effect of acetonitrile percentage on the of M and G derivatives was very significant (Fig. 1B), as the percentage of acetonitrile was increased from 14 % to 22 %, the retention time of M and G derivatives were advanced but the retention times were getting closer to each other and the separation was lower. This result is consistent with the effect of acetonitrile percentage on the retention behavior of the separation studied by Pu et al. [41]. Under the condition of low percentage of acetonitrile, the peaks of M and G derivatives were homogeneous, and the two monosaccharides could be better separated with 18 % (v/v) of acetonitrile in the mobile phase, and the peaks could be produced in a shorter time. In conclusion, the percentage of acetonitrile was selected as 18 %.

Under the mobile phase condition of phosphate buffer (pH 6.8) acetonitrile with the percentage of 82:18, the effect of flow rate on the retention time of M and G derivatives was more obvious (Fig. 1C), but the effect on the peak shape and the degree of separation was not obvious. When the flow rate was increased from 0.5 mL/min to 1 mL/min, the retention times of the two monosaccharide derivatives were advanced, but the separation was not much different. When the flow rate was high, the two monosaccharides could be separated well and completely separated from the impurity peak and the material peak of PMP, and the retention time was relatively short, and the flow rate of 1 mL/min was selected for comprehensive consideration.

Under the condition that the percentage of the mobile phase is phosphate buffer (pH 6.8)-acetonitrile is 82:18, the temperature has little effect on the retention time of the two monosaccharides (Fig. 1D), but the peaks of the two monosaccharides are relatively the highest and have a good peak shape at 30 °C. It may be that the higher temperature helps the buffer salts in the mobile phase to be solubilized, and the two mobile phases are mixed more homogeneously. It was also found [42] that increasing the column temperature can reduce the sample viscosity and thus the mass transfer resistance, but too high a temperature can damage the packing in the column. In summary, the column temperature was chosen to be 30 °C.

By comparing the peak plots before and after the optimization of the chromatographic conditions (Fig. 1E and F), it can be seen that there is a good separation of M, G and PMP after the optimization of the conditions, which proves that the optimization of the chromatographic conditions is necessary.

3.3. Methodological validation

3.3.1. Selectivity test

The calibration curve equations for M and G were $Y = 27433.82X+35.42$ ($R^2 = 0.9989$) and $Y = 31365X+661.63$ ($R^2 = 0.9995$), respectively. The limits of detection (LOD) of M and G measured by this method were 0.42 μg/mL and 0.35 μg/mL, respectively. The limits of quantification of M and G were 1.4 μg/mL and 1.2 μg/mL, respectively.

Algae contain a large number of polysaccharides, among which GlcUA is structurally similar to M and G. If the extraction is not

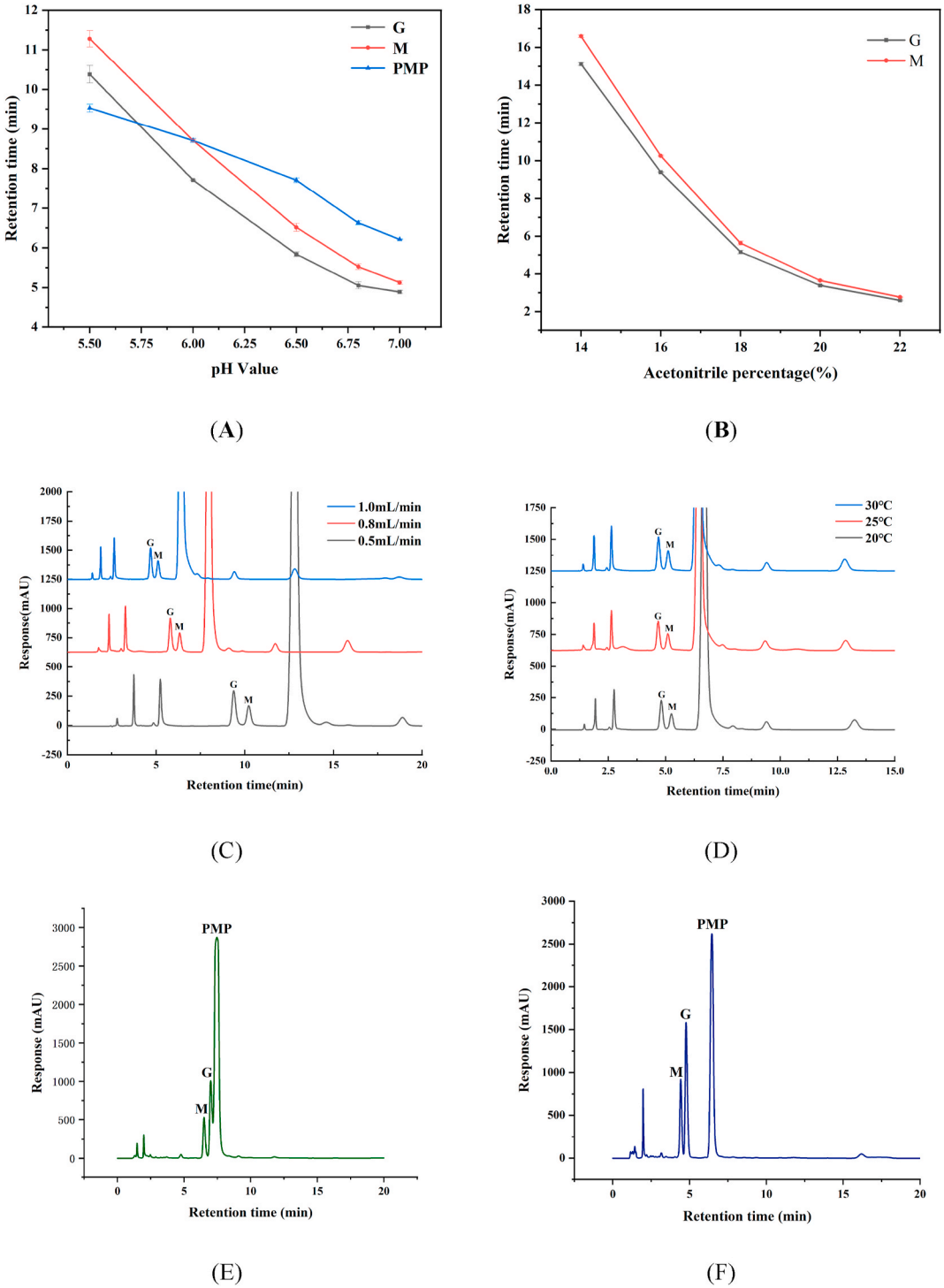


Fig. 1. Effects of chromatographic separation conditions on retention time and separation of PMP derivatives of M and G. (A) Effect of pH on phosphate buffer (acetonitrile percentage 18 %); (B) Effect of acetonitrile percentage (phosphate buffer pH = 6.8); (C) Effect of flow rate (phosphate buffer pH = 6.8, acetonitrile percentage 18 %); (D) Effect of column temperature (phosphate buffer pH = 6.8, acetonitrile percentage 18 %); (E) Separation of M and G from PMP before optimization of chromatographic conditions; (F) Separation of M and G from PMP after optimization of chromatographic conditions.

pure, it may have an effect on the determination of alginate content. Therefore, in this study, we mixed glucuronic acid with the standards of M and G to make a mixing standard, in order to observe the interference of glucuronic acid on the detection of M and G. From the overlapping peaks of the chromatograms with or without the addition of GlcUA (Fig. 2), peaks 1 and 2 were G and M, respectively, peak 3 was the derivatization reagent PMP, and peak 4 only appeared in the chromatograms with the addition of GlcUA, so it can be deduced that peak 4 was GlcUA. The area of peak 3 depends on the extent of PMP extraction during the extraction process, which is random in nature but does not affect the experimental results. The chromatograms showed that GlcUA was completely separated from M and G, and there was no interference with the determination of the two kinds of uronic acid, so the method is specific.

3.3.2. Precision, stability, repeatability results

As can be seen in Table 6, the RSDs of M and G peak areas after derivatization of the same standard monosaccharide mixture solution with six consecutive injections were 1.74 % and 1.98 %, respectively, which indicated that the precision of the instrument was good. The RSDs of M and G peak areas after injection of the same mixed solution at different times were 1.88 % and 1.80 %, respectively, indicating that the mixed solution had good stability within 12 h. The RSDs of M and G peak areas were 1.98 % and 1.96 %, respectively, which were less than 2 % after the injection of six parts of alginate hydrolyzed solution, indicating that the method has good reproducibility [43].

3.3.3. Recovery experiment results

The average spiked recoveries ranged from 99.97 % to 109.07 % for M and 99.49 %–109.80 % for G. The RSDs were less than 3 % (Table S2), indicating that the method has good recoveries.

3.4. Single factor analysis of acid hydrolysis of alginate

When the alginate was hydrolyzed with sulfuric acid and TFA for 3 h and 2 h, respectively, the hydrolysis of alginate was more complete, and the concentrations of M and G and the recovery of alginate reached the maximum value. However, with the increase of hydrolysis time, the recovery of alginate hydrolyzed in sulfuric acid and the concentrations of M and G tended to be stabilized, while a decreasing trend was observed in TFA (Fig. 3A(a) and 3B(a)). When investigating the effect of two acid concentrations on the hydrolytic capacity of alginate, it was found that the concentration of M and G increased and then decreased with the increase of the acid concentration, probably due to the fact that too high a concentration of sulfuric acid would carbonize the monosaccharides, which would lead to decrease in the concentration of the two monosaccharides. While TFA is a strong acid, increasing its concentration within a certain range can make the hydrolysis of the alginate more completely [44], but too high will destroy the structure of monosaccharides leading to a decrease in its concentration and recovery. Therefore, when the concentrations of sulfuric acid and TFA were 0.2 mol/L and 2 mol/L, respectively, the total mass concentrations and recovery reached the maximum (Fig. 3A(b) and 3B(b)).

With the increase of temperature, the hydrolysis ability of both sulfuric acid and TFA on alginate increased firstly and then decreased. When the hydrolysis temperature of sulfuric acid was 120 °C, the hydrolysis ability was obviously different from the other temperatures, and when the temperature continued to increase, the destruction rate of sulfuric acid on M and G was greater than the hydrolysis rate, resulting in a decrease in the total mass concentration (Fig. 3A(c)). The hydrolysis ability of TFA hydrolysis was comparable when the hydrolysis temperatures were at 110 °C and 120 °C, therefore, 110 °C with lower energy consumption was chosen (Fig. 3B(c)). In summary, the hydrolysis conditions of sulfuric acid and TFA were finally determined as follows: hydrolysis with 0.2 mol/L sulfuric acid for 3 h at 120 °C, and hydrolysis temperature of 120 °C, and hydrolysis with 2 mol/L TFA for 2 h at 110 °C.

Considering the low solubility of oxalic acid at room temperature, the highest concentration of oxalic acid applied in this study was 12 % in order to ensure better solubility of oxalic acid. The increase of both oxalic acid concentration and hydrolysis time increased the concentration of monosaccharides in the hydrolysate (Fig. 3C(a)(b)), and the better hydrolysis effect was achieved at the acid

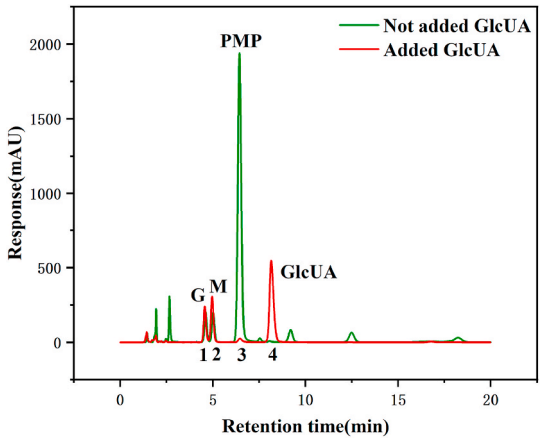


Fig. 2. Effects of glucuronic acid on M and G peaks. Notes: 1-G, 2-M, 3-PMP, 4-GlcUA.

Table 6
Precision, stability and repeatability for determination of M and G.

Component	Precision RSD/%	Stability RSD/%	Repeatability RSD/%
M	1.74 %	1.88 %	1.98 %
G	1.98 %	1.80 %	1.96 %

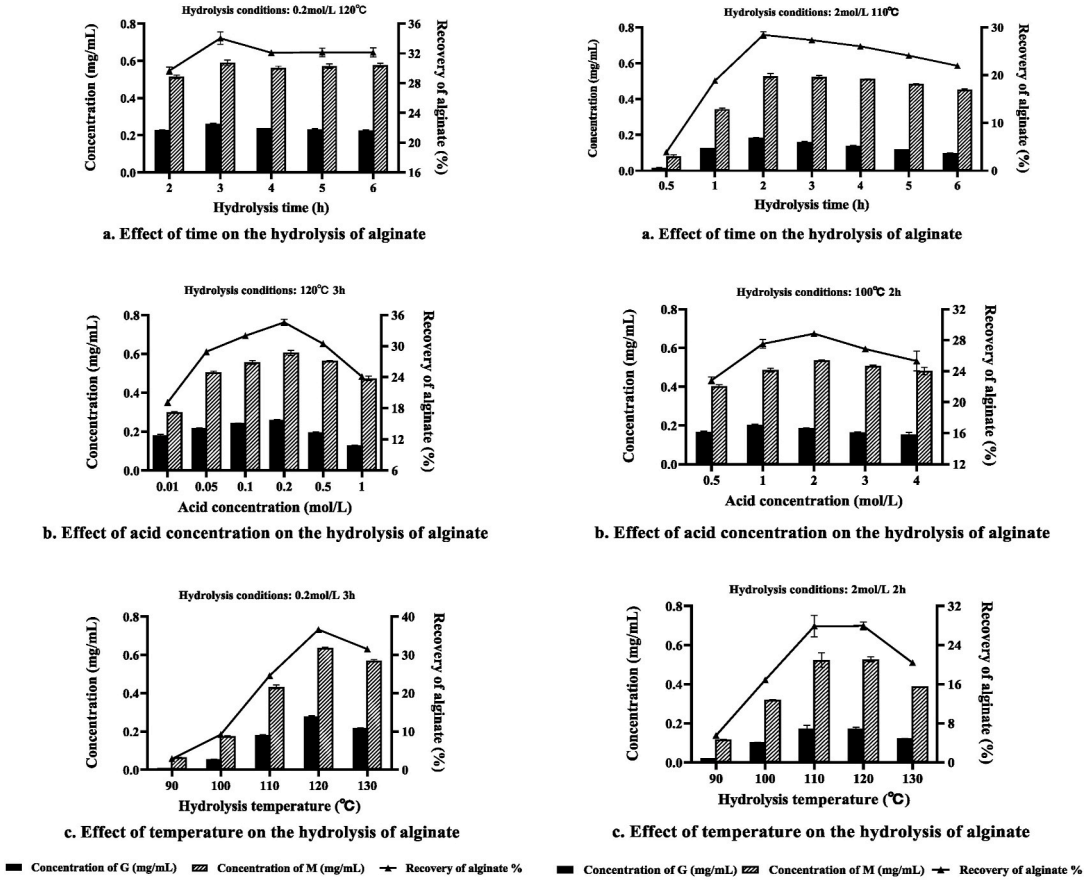
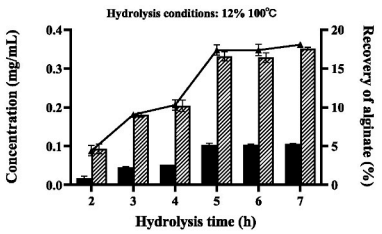


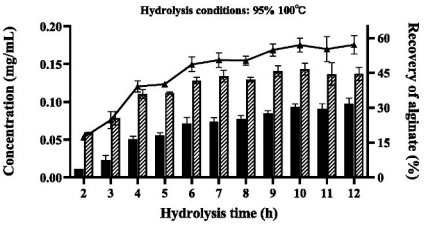
Fig. 3. Single factor analysis of sulfuric acid, TFA, formic acid and oxalic acid. (A) Sulfuric acid single-factor results; (B) TFA single-factor results; (C) Oxalic acid single-factor results; (D) Formic acid single-factor results.

concentration of 12 % and the hydrolysis time of 5 h. The hydrolysis temperature has a greater effect on the degree of hydrolysis, and mass concentration of M and G in the hydrolysate showed a trend of increasing and then decreasing with the increase of temperature, and the content of M and G reached the maximum value at 110 °C (Fig. 3C(c)). In summary, the hydrolysis conditions of oxalic acid were finally determined as follows: hydrolysis with 12 % oxalic acid for 5 h at 110 °C.

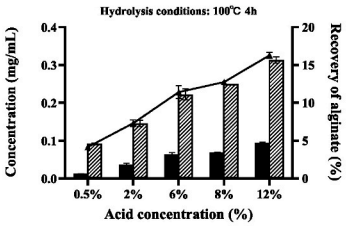
When Chandía et al. [39] studied the effect on hydrolysis of alginate by formic acid, 90 % formic acid was first used to pre-hydrolyze the alginate at 100 °C, and then 1.5 mol/L formic acid was used to rehydrolyze the alginate at 100 °C, which showed a better effect than that of sulfuric acid and hydrochloric acid. In this study, the pre-hydrolysis conditions were further optimized to obtain better hydrolysis effect using it as a reference. The prolongation of the hydrolysis time could increase the concentration of M and G in the hydrolysate, and the recovery of alginate showed a trend of slow growth after 6 h, and the recovery reached a kind of smooth fluctuation from 9 h to 12 h (Fig. 3D(a)). The highest monosaccharide concentration and recovery were achieved at 95 % formic acid concentration (Fig. 3D(b)). The hydrolysis effect could be enhanced to some extent when the hydrolysis temperature was increased, but the destructive power of acid on alginate was more obvious when it exceeded 110 °C, resulting in a significant decrease in the concentration of M and G (Fig. 3D(c)). In conclusion, the final hydrolysis conditions were 95 % formic acid hydrolysis at 110 °C for 10 h.



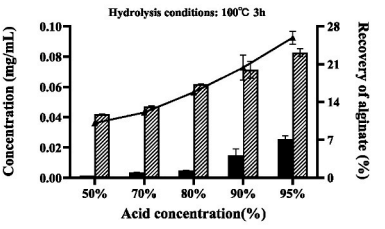
a. Effect of time on the hydrolysis of alginate



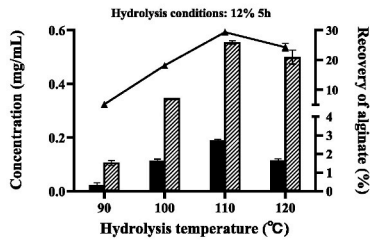
a. Effect of time on the hydrolysis of alginate



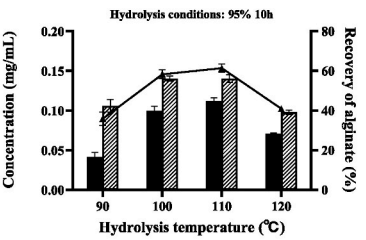
b. Effect of acid concentration on the hydrolysis of alginate



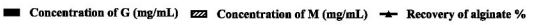
b. Effect of acid concentration on the hydrolysis of alginate



c. Effect of temperature on the hydrolysis of alginate



c. Effect of temperature on the hydrolysis of alginate



(C) (D)

Fig. 3. (*continued*).

3.5. Orthogonal analysis of the hydrolysis of four acids

By analyzing the range table of sulfuric acid orthogonal experiments, it can be seen that the order of influencing factors of sulfuric acid hydrolysis of alginate was as follows: hydrolysis temperature > acid concentration > hydrolysis time, and the optimal combination was $A_2B_2C_3$ (Table S3). The ANOVA results showed that the sulfuric acid concentration (B) and hydrolysis temperature (C) had a significant effect on the hydrolysis of alginate, and the difference was statistically significant ($P < 0.05$). The sum of squared deviations of the hydrolysis time (A) was smaller than the overall error e_1, which indicated that it had no significant effect on the hydrolysis efficiency and no further calculations were made (Table S4). In conclusion, the optimal hydrolysis conditions were 0.2 mol/L sulfuric acid hydrolysis at 130 °C for 3 h.

Range analysis of the orthogonal experiments of TFA showed that the factors affecting the hydrolysis of alginate by TFA were in the following order: hydrolysis temperature > hydrolysis time > acid concentration, and the best combination was $A_2B_2C_2$ (Table S5). The results of ANOVA showed that the effect of hydrolysis temperature (C) on the hydrolysis rate of alginate was significant, and the difference was statistically significant ($P < 0.05$). The sum of squared deviations of the acid concentration (B) and hydrolysis time (A) was less than the overall error e_1, so that the hydrolysis time (A) would have no significant effect on the efficiency of hydrolysis and no further calculations would be done (Table S6). In summary, the optimal hydrolysis conditions were 2 mol/L TFA hydrolysis at 110 °C for 3 h.

By comparing the magnitude of the polar deviation R in the range analysis of oxalic acid orthogonal experiments, it can be seen that the order of factors affecting the hydrolysis of alginate by oxalic acid was as follows: hydrolysis temperature > acid concentration > hydrolysis time, and the optimal combination was $A_3B_3C_3$ (Table S7). The results of ANOVA showed that the hydrolysis rate of alginate was highly significantly affected by hydrolysis time (A), acid concentration (B) and hydrolysis temperature (C), and the differences were statistically significant ($P < 0.01$) (Table S8). In conclusion, the optimal hydrolysis conditions were 12 % oxalic acid hydrolysis at 120 °C for 6 h.

The range analysis of orthogonal experiments with formic acid showed that the order of factors affecting the hydrolysis of alginate

by formic acid was: hydrolysis temperature > acid concentration > hydrolysis time, and the optimal combination was: $A_1B_3C_2$ (Table S9). The results of ANOVA showed that the hydrolysis rate of alginate was highly significantly affected by acid concentration (B) and hydrolysis temperature (C), and the differences were statistically significant ($P < 0.01$) (Table S10). The sum of squared deviations of the hydrolysis time (A) was smaller than the overall error e_1, so the hydrolysis time (A) had no significant effect on the hydrolysis efficiency and was no longer calculated. In summary, the optimal hydrolysis conditions were 95 % formic acid hydrolysis at 110 °C for 9 h.

3.6. Comparison of optimal hydrolysis conditions for four acids

The results of the optimal hydrolysis conditions validation experiments for four different acids were 61.38 %, 31.26 %, 33.34 % and 29.09 % in terms of recovery, respectively (Table 7). Among which the hydrolysis effect of formic acid was the best, and the hydrolysis recovery rate was nearly two times of the other acids, which probably because the acidity of formic acid was relatively weak and the destructive property of uronic acids were small, and the structure of the uronic acid could be better retained. The acidity of TFA was stronger, but the hydrolysis effect was the worst, and the hydrolysis effects of sulfuric acid and oxalic acid were similar. After comparison, the hydrolysis effects of the four acids were better than those in the orthogonal experiments, which were in line with the theoretical predictions, indicating that the hydrolysis conditions obtained in the orthogonal experiments were reliable.

The proportion of G content in the four hydrolysates was 43.92 %, 25.65 %, 28.02 %, and 24.16 %, respectively, and the proportion of G content in the formic acid hydrolysate was the highest relative to the other hydrolysates, again indicating that the hydrolysis process of formic acid proceeds more gently and destroys the monosaccharide G less. This is in agreement with Chandía et al. [39] who found lower M/G ratio of formic acid hydrolysis products in their experiments using sulfuric, formic and hydrochloric acids for the treatment of different tissues of brown algae.

By comparing the contents of M and G in each hydrolysate, it can be found that the contents of M were significantly higher than those of G, indicating that the hydrolysis effect of acid hydrolysis on the two kinds of uronic acid was different, which might be related to the structural properties of the two kinds of uronic acid. In conclusion, it can be seen that formic acid is more suitable for the hydrolysis of alginate than the other three acids, and the hydrolysis of alginate is more complete and less destructive.

3.7. Validation analysis of optimal acid hydrolysis

The recoveries after formic acid hydrolysis of six alginates with different ratios fluctuated around the theoretical predicted values (Table 8), which may be related to the proportion of the monosaccharide composition of different species of alginate and its structure [1], suggesting that the recoveries of different alginate hydrolyzed by formic acid are relatively stable. In conclusion, it can be seen that the hydrolysis of alginate with formic acid is more stable and can be used for the determination of M and G contents in different types of alginate.

4. Conclusion

In this study, the pre-column derivatization and chromatographic separation conditions of alginate were optimized. By optimizing the chromatographic conditions, experimental conditions were obtained that could lead to a good separation of M, G and PMP. On the basis of this study, the effects of hydrolysis time, acid concentration and hydrolysis temperature on the hydrolysis effect of alginate hydrolyzed by formic acid, oxalic acid, sulfuric acid and TFA were investigated and the hydrolysis conditions of different acids were optimized by orthogonal experiments. Comparison of the results showed that the hydrolysis effect of formic acid was better, which was able to hydrolyze alginate to a greater extent and preserves best the integrity of monosaccharides constitutive of alginate. The optimal hydrolysis conditions were 95 % formic acid at 110 °C for 10 h. Meanwhile, the recoveries of the samples after acid hydrolysis with formic acid were more stable, which can be used to deduced the alginate content of the samples by calculating the recoveries in practical applications, thus providing technical support for the determination of the alginate content.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Wenling Wang: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Yu Fu:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Jiachao Xu:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Xin Gao:** Writing – review & editing, Supervision, Resources. **Xiaoting Fu:** Writing – review & editing, Supervision, Resources. **Lei Wang:** Writing – review & editing, Supervision,

Table 7
The comparison of hydrolysis effect of four kinds of acids.

Types of acid	Hydrolysis condition	M Content (mg)	G Content (mg)	Recovery rate (%)	G content percentage (%)
Sulfuric acid	0.2 M, 130 °C, 3 h	0.24 ± 0.02	0.09 ± 0.02	33.34 ± 0.02	28.02
TFA	2 M, 110 °C, 3 h	0.22 ± 0.02	0.07 ± 0.02	29.09 ± 0.02	24.16
Formic acid	95 %, 110 °C, 9 h	0.34 ± 0.03	0.27 ± 0.03	61.38 ± 0.03	43.92
Oxalic acid	12 %, 120 °C, 6 h	0.24 ± 0.05	0.08 ± 0.04	31.26 ± 0.05	25.65

Note: M content and G content refer to the content of M and G in 1 mg of alginate hydrolysate (mg).

Table 8
Formic acid hydrolyzes different types of alginate.

Types of alginate	M Content (mg/mg)	G Content (mg/mg)	Recovery rate (%)
1	0.2849 ± 0.02	0.2808 ± 0.02	57.99 ± 0.02
2	0.3520 ± 0.03	0.2582 ± 0.02	62.54 ± 0.03
3	0.4311 ± 0.02	0.2038 ± 0.03	65.07 ± 0.03
4	0.4585 ± 0.02	0.1449 ± 0.02	61.85 ± 0.02
5	0.4311 ± 0.02	0.1115 ± 0.02	55.62 ± 0.02
6	0.4103 ± 0.02	0.1589 ± 0.02	58.34 ± 0.02

Notes: 1-M/G = 0.45, 2-M/G = 0.75, 3-M/G = 0.80, 4-M/G = 1.16, 5-M/G = 1.12, 6-M/G = 1.11.

Resources.

Declaration of competing interest

The authors declare that they have no known competing financial or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29738.

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