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The separation of microscale HA in aqueous solution by foam separation technique

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Hyaluronic acid (HA) has important applications in fields of health care products, cosmetics and clinical medical. However, the unique physiological properties of HA make cost of its traditional separation and extraction process relatively high. Foam separation technique has simple, gentle and efficient advantages on the separation of substances with surface activity by using bubbles as the separation medium. In this paper, natural surfactant CocamideBetaine (CAPB) was used as a foaming agent to explore the technology of microscale HA in aqueous solution by foam separation. The optimum process conditions were determined based on the recovery rate and enrichment ratio of HA by single factor and orthogonal experiment: at room temperature, pH = 7, separating air velocity (v) = 350 mL/min, HA concentration (C_{HA}) = 50 mg/L, adding liquid volume (V) = 200 mL, collecting time (t_{col}) = 10 min, CAPB concentration (C_{CAPB}) = 0.035 g/L. Under these conditions, HA enrichment ratio (E) equals 6.821 and HA recovery rate (R) equals 66.425%.

Keywords: Foam Separation; Hyaluronic Acid; Enrichment Ratio; Recovery Rate.

INTRODUCTION

Hyaluronic acid (HA) is an advanced acid straight-linked mucopolysaccharide composed of d-glucuronic acid and n-acetylglucosamine, which has important applications in health care products, cosmetics and clinical medicine[1–3]. At present, the main production mode of HA is microbial fermentation, and the separation and purification of hyaluronic acid from fermentation liquor mainly includes quaternary ammonium salt method, chloroform method, ion exchange chromatography, ethanol precipitation method, and mass dilution membrane filtration method, etc[4–6]. However, HA's unique physiological properties make its traditional separation and extraction process relatively expensive[7–9]. Therefore, it is of great significance to find an extraction method that is suitable for low concentration HA solution, with complete HA harvesting in the fermentation liquor, simple process equipment, low input and low material consumption[10–12]. As a new environmentally friendly extraction process, foam separation has the advantages of low solution concentration, high treatment capacity, high recovery rate, low consumption, low cost input, simple equipment, and the ability to separate the target products and additives, and has broad development prospects[13–15]. In this paper, we used CocamideBetaine (CAPB) as both a foaming agent and a gathering agent, discussing the effect of different factors in foam separation. On this basis we optimized the conditions of recycling HA with CAPB, which provided experiment gist for its effective separation of the microscale HA in aqueous solution.

MATERIALS AND METHODS

Main reagents and instruments

Hyaluronic acid was purchased from Beijing Solarbio Technology Co., Ltd. CAPB was purchased from Guangzhou Import and Export Trading Co.,Ltd. Other reagents were produced in Sinopharm Chemical Reagent Co., Ltd. The electronic scales were purchased from Sartorius Germany Co., Ltd. MODEL868 accurate acidity meter was purchased from Orion USA Co., Ltd. UV spectrophotometer was purchased from Shimadzu Japan

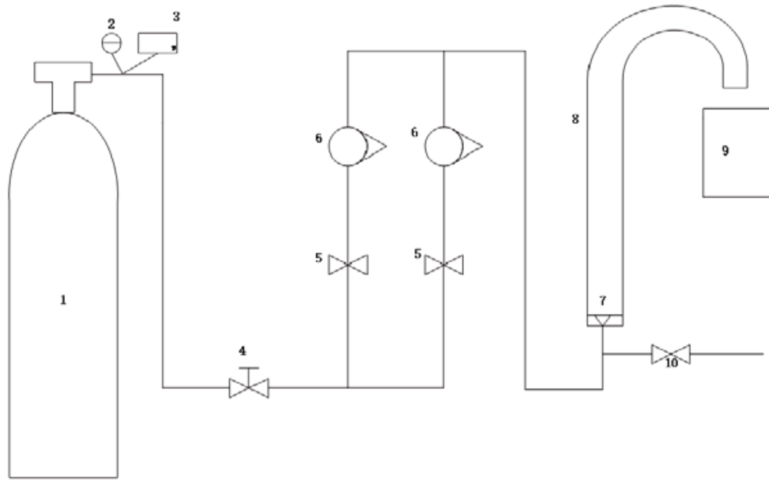

Figure 1. The schematic diagram of the device of foam separation. 1 – Air compressor, 2 – Reducing valve, 3 – Pressure gage 4 – Regulating valve, 5 – Stop valve, 6 – Rotor flow meter, 7 – Air distributor, 8 – Foam producing tube, 9 – Receiving flask, 10 – Discharging valve

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Co., Ltd. The device of foam separation was assembled in laboratory. The schematic diagram of the device of foam separation are showed in Fig.1.

Experimental methods

The evaluation indexes of the separating effect of HA solution

The separating effect of HA solution was measured by evaluation indexes of recovery rate (R) and enrichment ratio (E), which are defined as follows:

Recovery rate: $R(\%) = c_f v_f / c_0 v_0 \times 100\%$ (1)

Enrichment ratio: $E = c_f / c_0$ (2)

where, c_0 and c_f are the concentration of hyaluronic acid in the raw material liquid and the extracted foam liquid, v_f and v_0 and are respectively the volume of the raw material liquid and the extracted foam liquid (after foam fracture).

Steps of single-factor experiment

We designed single-factor experiment to explore the relationships of recovery rate (R) and enrichment ratio (E) versus pH, CAPB concentration (C_{CAPB}), separating air velocity (v), HA concentration (C_{HA}), adding liquid volume (V) and collecting time (t_{col}).

(1) Checking device: Ensure there was no liquid or air leaking.

(2) Blending HA solutions for separation: Blend HA solutions in different concentrations, add surface active agent, adjust pH of the solution by 6 mol/L NaOH, 1 mol/L NaOH, 6 mol/L HCl, 1 mol/L HCl.

(3) Add certain volume of HA solution into the foam producing tube in Fig. 1, open the air valve, adjust the stress meter and flow meter. Foam goes out from the top and be collected in the flask. Residual solution is discharged into the collecting bottle.

(4) The sample finishing collecting should be covered by preservative film and be stayed still to reduce the foam. Measure the volume of foam extracting solution and the concentration of HA in residual solution.

(5) Calculate recovery rate(R) and enrichment ratio(E) then graph them.

(6) Clean the separating column. Wash thoroughly by tap water then clean several times by distilled water. Dry after cleaning.

Orthogonal experiment designing

Based on the single-factor experiment, we designed an orthogonal experiment to analyze the results intuitively and signally, ensured the effect degree of different factors to HA recycling and then ensured the best technological condition. By doing the confirmatory experiment, we ensured the best recovery rate and concentration ratio.

ANALYSIS OF EXPERIMENTAL RESULTS

Analysis of single-factor experimental results

Experimental levels of investigated process parameters are showed in Table 1.

Effect of primary solution pH

The experimental results (Figure 2) showed that the pH value of the solution increased from 3 to 7, and the recovery and concentration ratio increased. When the pH value of solution increased from 7 to 11, the enrichment ratio and recovery rate decreased. When the pH value of solution is 1 and 13, the enrichment ratio increases greatly. When the pH value of the solution is between 3 and 7, the increase of recovery rate and enrichment ratio may be due to the increase of foam stability, surface adsorption and foam carrying HA during this period. When the pH value of the solution increased from 7 to 11, the bubbles formed during aeration were smaller, more dense, more stable and larger, and the water volume of HA and carried decreased. Under the condition of weak acidity and alkalinity, the equilibrium between hydrophilic and hydrophobic groups was destroyed, and the recovery rate of HA was reduced. As the stability of HA is significantly affected by the pH value of the

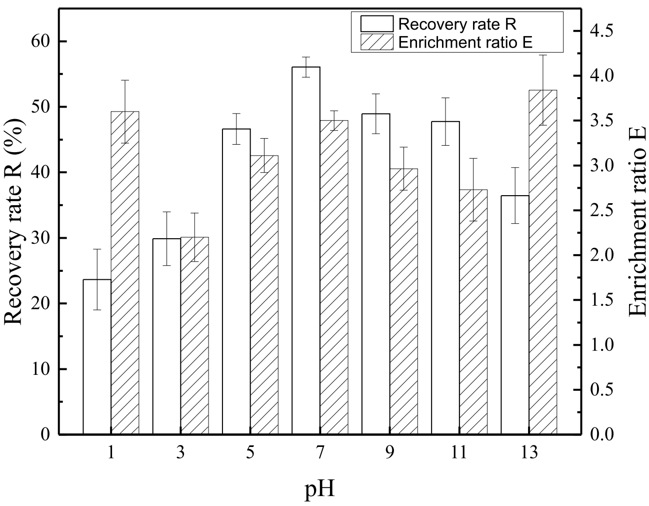

Figure 2. Relationship of recovery rate (R) and enrichment ratio (E) versus pH

Table 1. Experimental levels of investigated process parameters

Corresponding experiment	C_{HA}, mg/L	C_{CAPB}, g/L	V, ml	pH, -	v_{air}, mL/min	t_{col}, min
2.1.1	100	0.035	200	1, 3, 5, 7, 9, 11, 13	200	8
2.1.2	100	0.020, 0.030, 0.035, 0.040, 0.045, 0.050, 0.070	200	7	100	5
2.1.3	100	0.035	200	7	100, 200, 300, 400, 500, 600, 700	8
2.1.4	5, 15, 25, 50, 100, 200, 500, 800	0.035	200	7	400	8
2.1.5	50	0.035	50, 100, 150, 200, 250, 300	7	200	5
2.1.6	50	0.035	200	7	400	3, 5, 8, 10, 14, 18

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solution, the degradation of HA will be accelerated if the pH value is too high or too low, which has a great influence on the determination of HA concentration, and the enrichment ratio will suddenly increase. Secondly, when the solution is too low in acidity, the foam formed will become unstable and the recovery rate of HA will decrease. Moreover, the surface of HA shows electronegativity in the solution. When the pH value of the solution is alkaline, it will have no surface activity due to the formation of complex, which will affect the final extraction effect. Taken together, pH value should be around 7. At this point, R = 56.067% and E = 3.5.

Effect of CAPB's concentration in original solution (C_{CAPB})

As analyzed from Figures in Fig. 3, when concentration of CAPB added increased, HA recovery rate and HA enrichment ratio showed a tendency to increase first then decrease, enrichment ratio especially so. It is possible that when the concentration of CAPB is low, the gas is pumped, the foam stability is low and the quantity is small, which is difficult to collect. With the increase of CAPB dosage, the separation effect is gradually improved, and the recovery rate and enrichment ratio are gradually increased. When CAPB exceeds a certain limit, bubbles form into large bubbles, and the surface tension decreases, and HA adsorption volume decreases. However, the increase of water entrainment in the foam leads to a decrease in the enrichment ratio, resulting in a decrease in separation effect. After comprehensively analyzing rate of recovery and collection ratio, the best mass concentration of CAPB is 0.035 g/L. In this concentration, R = 51.50%, E = 4.68.

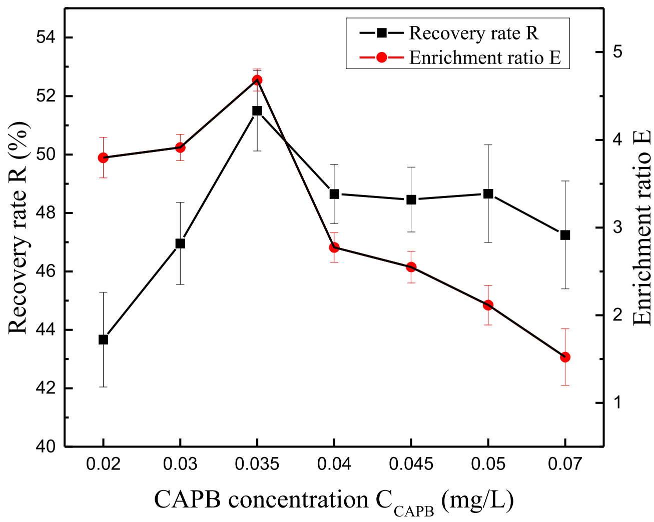

Figure 3. Relationship of recovery rate (R) and enrichment ratio (E) versus CAPB concentration (C_{CAPB})

Effect of separating air velocity (v_{air})

As analyzed from the Figure 4, with air speed's increasing, enrichment ratio of HA had an obvious decreasing tendency. A possible reason was that when the speed increased, bubbles would be produced faster, which meant shorter staying time in the bubble producing column. Meanwhile, with the level of backflow decreased, liquid discharged more slowly, leading to more water volume in foam phase and a volume growth of collecting liquid. As a result, enrichment ratio gradually decreased. However,

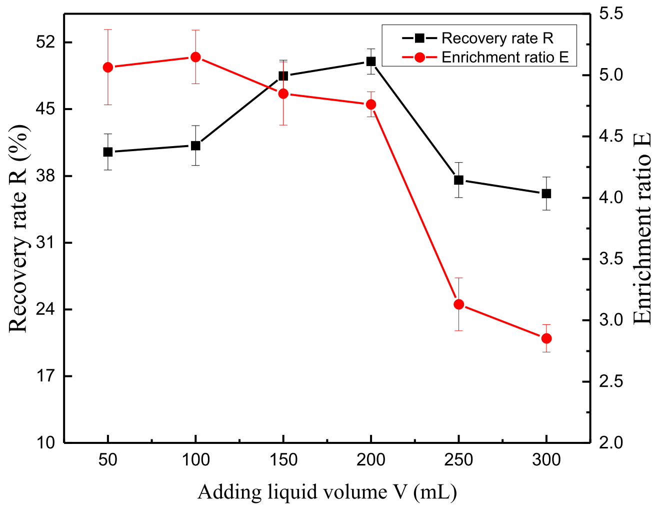

Figure 4. Relationship of recovery rate (R) and enrichment ratio (E) versus separating air velocity (v)

the recovery rate of HA first increases and then slowly decreases, mainly because of the increase of airflow velocity, the production of foam also increases, and the adsorption of HA also increases, and the recovery rate is improved. When the gas velocity reaches a certain value, the bubble size produced by CAPB is stable and the foam layer is tight, which can effectively take out the solute. However, if the gas velocity continues to increase, the bubble will deform and break. The residence time in the solution phase is too short, which is not conducive to the adsorption of surfactant and HA, resulting in the inability to be taken out in time, and the collection rate is relatively reduced. After comprehensive analyzing, the best separating air speed is 400mL/min. Meanwhile, R = 62.52%, E = 3.05.

Effect of HA concentration in original solution (C_{HA})

The obtained results are shown in Fig. 5. As the concentration of HA in the original solution increased from 5 to 50 mg/L, the recovery and enrichment ratio increased. As the concentration of HA in the original solution increased from 50 to 800 mg/L, the concentration ratio of HA decreased significantly, while the recovery rate decreased less. And the high concentration ratio has a higher collecting rate and a lower concentration ratio. A possible reason was that when the content of HA is low in the solution, the volume of individual

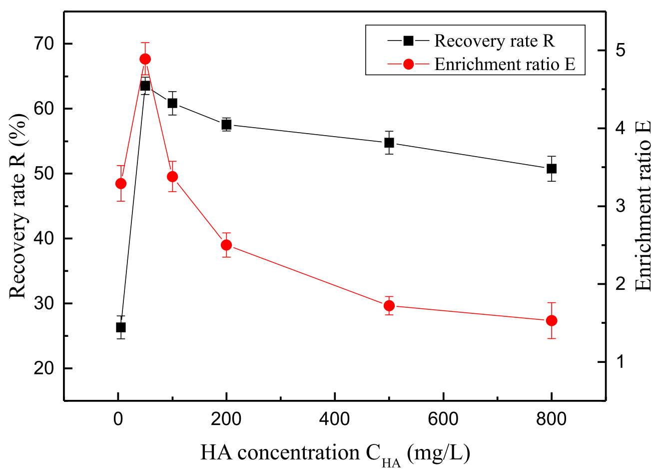

Figure 5. Relationship of recovery rate (R) and enrichment ratio (E) versus HA concentration (C_{HA})

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bubbles in the device is large, and their properties are easy to change. There are many cases where the collision between large momentum bubbles breaks, the water content in the foam phase is less, and there is a large enrichment ratio. However, at the same time, the total specific surface area of adsorption decreases, and HA adsorption is also reduced, and the recovery rate of HA is low. At high concentration, the HA collection rate of the solution increases, which may be related to the nature of HA itself. The hydrophilic and hydrophobic regions of hyaluronic acid molecules are more likely to entangle each other, and the viscosity of the surface of the solution increases, compared with the surface at low concentration. The tension has changed dramatically, and the flotation extraction effect is therefore limited and slowly reduced. The HA content in the solution increases, a large amount of fine and stable foam is formed, more HA adsorbs on the bubbles, the recovery rate increases, but it is not easy to drain, the amount of HA and the amount of liquid carried increase, and the growth rate of water carrying is higher than that. The rate of hyaluronic acid increases and the enrichment ratio decreases The above analysis determined that the optimal concentration of HA in the solution was 50 mg/L. At this point, R = 63.52% and E = 4.89.

Effect of adding liquid volume (V)

The experimental results showed that the liquid loading had a great influence on the HA enrichment ratio, and the enrichment ratio was reduced by one time within the range of the change of the amount of added liquid (Figure 6). Reasons of this phenomenon might be: with the volume increasing, air got more time to sufficiently contact with solution and made mass transfer efficiency larger then led to more HA absorbed to the foam. But therefore, foam height was shorter than the original liquid and it also resulted in a shorter discharging time. These would cause bubbles cracked before they went out and thus made HA back to liquid phase with much water. The adding ratio of water was larger than that of HA that made enrichment ratio decreased. A proper adding liquid volume should ensure that the rate of foam height and original liquid volume is more suitable to liquid eliminating as well as promoting HA entering foam phase. After considering about recovery rate, enrichment ratio and its practical use, the best adding volume was 200 mL. At this volume, R = 50.01%, E = 4.76.

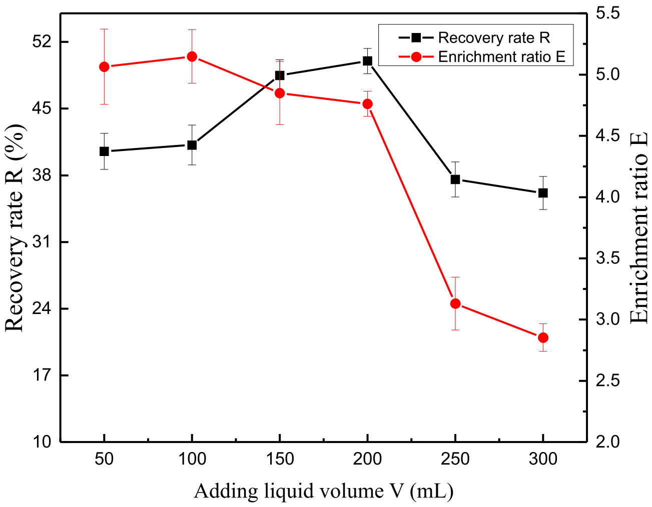

Figure 6. Relationship of recovery rate (R) and enrichment ratio (E) versus adding liquid volume (V)

Effect of collecting time (t_{col})

According to the data analysis in the Figure 7, the collection time beyond a certain value has no significant change in the recovery rate and enrichment ratio. The reason is that as the collection time increases, the foam production and the height of the foam layer gradually increase, the collection rate generally shows a significant increase. When the flotation time is greater than 10 min, the recovery rate changes slowly and slightly decreases, and the removal rate reaches the maximum at 10 min. At about 8–10 min, the separation has reached the equilibrium state, and after 10 min, the foam in the device has basically come out completely, resulting in less change in HA being carried out, the separation effect decreases, and the enrichment ratio will be slightly reduced due to more water in the foam behind. Therefore, it is very important for the balance time of adsorption and the balance of foam removal. Finally, the collection time was determined to be 10 min. At this point, R = 64.24% and E = 4.83.

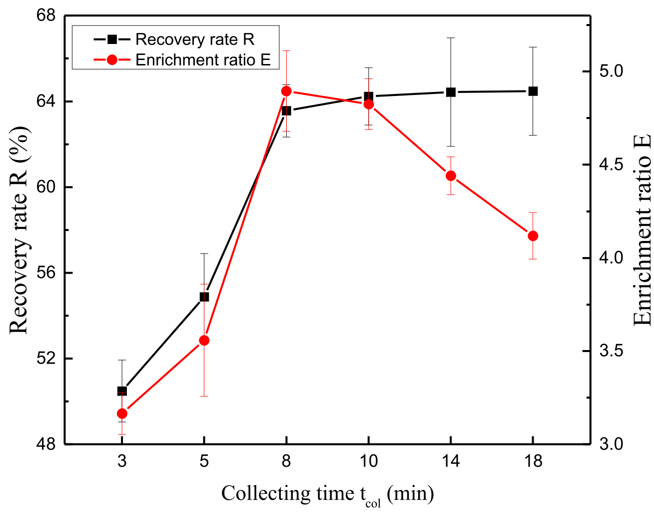

Figure 7. Relationship of recovery rate (R) and enrichment ratio (E) versus collecting time (t_{col})

Analysis of orthogonal experiment result

Values of process parameters in orthogonal experiment are shown in Table 2. After comprehensive analyzing, the effect of collecting time was relatively little because at 10 minutes' collecting time, surface absorb was already finished and made it into an dynamic equilibrium state. HA concentration was finally ensured in 50 mg/L with 10 minutes' collecting time. Get 3 levels on other factors of influence, with the software 'Orthogonal assistant', design and proceed the orthogonal experiment basing on $L_9(3^4)$[18] orthogonal experimental design table of three factors and three levels, which was showed in Table 3.

Table 2. Values of process parameters in orthogonal experiment

C_{HA}, mg/L	C_{CAPB}, g/L	V, ml	pH, -	v_{air}, mL/min	t_{col}, min
50	0.035	200	7	400	10

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Table 3. Levels of orthogonal factors

Level	Factors			
	A, pH	B, v_{air}	C, V	D, C_{CAPB}
1	6	350	180	0.030
2	7	400	200	0.035
3	8	450	220	0.040

Adopting comprehensive scoring method, because the main target of this separating experiment was to purify hyaluronic acid. The comprehensive score y is defined as follows.

$$y = 0.3 \times \frac{Ei\text{-}Emin}{Emax\text{-}Emin} + 0.7 \times \frac{Ri\text{-}Rmin}{Rmax\text{-}Rmin} \quad (3)$$

Based on range and variance analysis of orthogonal experiments (Table 4 and Table 5), effect curve was plotted into Fig. 8 showed, then came to conclusion of the best conditions of extracting HA by foam separating. By comprehensive analyzing from average and range in Table 4, the primary and secondary relationship of various factors of the experiment was: A>D>B>C. According to the variance analysis in Table 5, it can be concluded that the pH value of the solution has a great influence on the foam separation, and the solution CAPB concentration and the air flow rate have a certain influence, and the addition amount has no significant effect on the foam separation. The best condition of foam separating process was $A_2B_1C_2D_1$, meaned that pH = 7, separating air velocity (v) = 350 mL/min, HA concentration (C_{HA}) = 50 mg/L, adding liquid volume (V)=200 mL, collecting time (t_{col})=10 min, CAPB concentration (C_{CAPB}) = 0.035 g/L. Proceed the replication experiment of separating HA in aqueous solution by foam separating for 3 times then average the results. The final result was: R = 66.425%, E = 6.821.

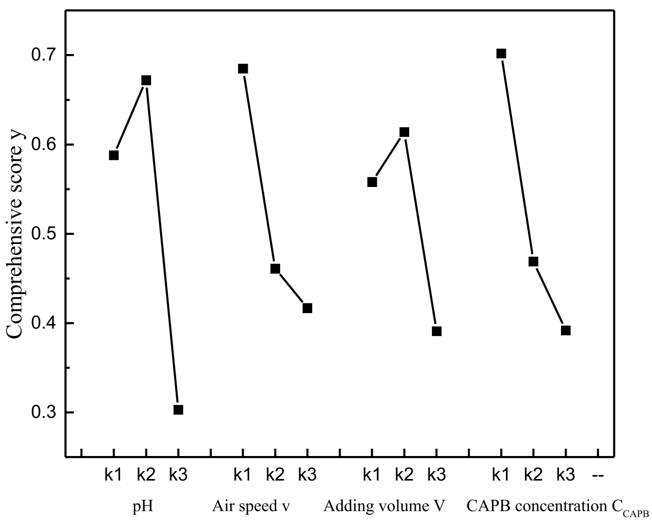

Figure 8. Effect curve

Table 5. Variance analysis

Factors	Sum of square deviation	DOF	F rate	Significance
A	0.225	2	2.778	*
B	0.124	2	1.531	
C	0.081	2	1	
D	0.157	2	1.938	
Error	0.08	2		

CONCLUSION

This paper demonstrates the feasibility of using coconut oil foaming agent (CAPB) as a surfactant for foam separation and recovery of trace hyaluronic acid (HA). We ensure the best condition to extract HA by foam separation is: at room temperature, pH = 7, separating air velocity (v) = 350 mL/min, HA concentration (C_{HA}) = 50 mg/L, adding liquid volume (V) = 200 mL, collecting time (t_{col}) = 10 min, CAPB concentration (C_{CAPB}) = 0.035 g/L. Under these conditions, HA enrichment ratio (E) equals 6.821, HA recovery rate (R) equals 66.425%.

NOMENCLATURE

C_0 – concentration of HA in raw material liquid, g/L
C_{CAPB} – concentration of CAPB, g/L
C_{HA} – concentration of HA, g/L
C_f – concentration of HA in extracted foam liquid, g/L
E – enrichment ratio score (Eq. 2), -
E_i – enrichment ratio, -
E_{max} – enrichment ratio, -

Table 4. The results of orthogonal experiments

No	Factors				Evaluation index		Comprehensive score y
	A	B	C	D	Recovery rate R%	Enrichment ratio E	
1	1	1	1	1	59.862	7.183	0.971
2	1	2	2	2	57.835	7.229	0.570
3	1	3	3	3	56.747	6.242	0.224
4	2	1	2	3	59.773	5.977	0.800
5	2	2	3	1	59.676	5.050	0.663
6	2	3	1	2	57.638	7.411	0.553
7	3	1	3	2	56.378	7.296	0.285
8	3	2	1	3	56.536	5.986	0.151
9	3	3	2	1	57.640	6.781	0.473
k_1	0.588	0.685	0.558	0.702			
k_2	0.672	0.461	0.614	0.469			
k_3	0.303	0.417	0.319	0.392			
Range	0.369	0.268	0.223	0.310			

Addendum

Factors	A	B	C	D
k_1	$(y_1+y_2+y_3)/3$	$(y_1+y_4+y_7)/3$	$(y_1+y_6+y_8)/3$	$(y_1+y_5+y_9)/3$
k_2	$(y_4+y_5+y_6)/3$	$(y_2+y_5+y_8)/3$	$(y_2+y_4+y_9)/3$	$(y_2+y_6+y_7)/3$
k_3	$(y_7+y_8+y_9)/3$	$(y_3+y_6+y_9)/3$	$(y_3+y_5+y_7)/3$	$(y_3+y_4+y_8)/3$
Range	$k_{max} - k_{min}$	$k_{max} - k_{min}$	$k_{max} - k_{min}$	$k_{max} - k_{min}$

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E_{min} – enrichment ratio, -
pH – solution pH, -
R – recovery rate score (Eq. 1), %
R_i – recovery rate, %
R_{max} – recovery rate, %
R_{min} – recovery rate, %
t_{col} – collecting time, min
v_0 – volume of raw material liquid, mL
v_f – volume of extracted foam liquid, mL
v_{air} – separating air volumetric velocity, mL/min
V – volume of adding liquid, mL
y – comprehensive score (Eq. 3), -

Abbreviations

HA – Hyalunoric acid
CAPB – CocamideBetaine

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