Ultrasonic Effects on Protein Salting-out

X. Ning,[a] H. Pingfang,[b,*] C. Lamei,[c] and OY. Pingkai[b]
[a]College of Environment, Nanjing University of Technology, Nanjing, China, 210009
[b]College of Life Science and Pharmaceutical Engineering, Nanjing University of Technology, Nanjing, China, 210009
[c]College of Science, Nanjing University of Technology, Nanjing, China, 210009

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The addition of inorganic salts to a bovine serum albumin (BSA) colloid to separate protein is a commonly performed method. In this work, we investigate an enhanced ultrasound process for protein separation. The standing time is 4.5 h shorter than that without ultrasound irradiation. The protein can be acquired at a yield of 90.5 % under the conditions of a 20 kHz ultrasound frequency, 60 min of centrifugal separation, and 2 minutes of ultrasound irradiation with a 0.64 W cm^{-2} sound intensity. Influences of sound field factors such as frequency, sound intensity and irradiation time on this process are discussed. The mechanism of ultrasonic salting-out of protein is construed. Results show that the settling velocity of protein can be accelerated by ultrasound irradiation after the salting-out process.

Key words:
Protein, salting-out, orthogonal, ultrasound

Introduction

Ultrasounds are inaudible sound waves in the frequency range of $f = 2 \cdot 10^4$ Hz to 10^9 Hz; thus, they have not been recognized and studied until recently. Ultrasound is widely used due to its distinctive advantages, having become a new field called ultrasonics.[1–2] Ultrasound is applied in chemistry and chemical engineering. Its chemical effect on pyrolysis in cavitation bubbles, chemical bond breaking and free radical generation can be used in organic decomposition synthesis, polymerization, depolymerization and free radical reactions.[3–5]

Ultrasound biotechnology is a new field. Its mechanism includes mass transfer, heating effects and cavitation. Salt separation is a practical method for protein purification.[6] Bovine serum albumin (BSA) was first crystallized by $(NH_4)_2SO_4$ grade separation in 1894.[7] However, the enhancement of salt separation using ultrasound has not yet been reported in the literature.

BSA is a protein secreted from bovine hepatic cells; more than half of the cell weight comes from the serum albumin. Its formula weight is about 67 kDa, and its isoelectric point is 4.7. BSA, one of the first studied proteins, can maintain an osmotic pressure and material carriage. BSA has an important biological function in the blood cycle, detoxification and lipid metabolism. It is widely used in biochemistry, physiology, cytology, immunology, medicine and bioengineering. BSA is thermally stable[8] and easily available. Thus, BSA was chosen as the object for a study of the enhancement effect of ultrasound in salt separation.

This experiment was carried out under a good mass transfer ultrasound reverberation field.

*Correspoding author: Han Pingfang
Email: hpf@njut.edu.cn

Materials and methods

Materials and apparatus

Chemical reagents: BSA, NaCl, $(NH_4)_2SO_4$

Major apparatus: piezoelectric ceramic ultrasound generator (20, 40, 500 kHz), ultraviolet spectrophotometer, centrifuge.

Methods

BSA was dissolved in physiological saline, and an ammonium sulfate saturation (ASS) solution was then slowly added in solution. The solution was maintained at 20 °C for a 10 h standing time or under ultrasound irradiation (at 20, 40 or 500 kHz). After a separation of 1 h at 3000 rpm centrifugation, the precipitate was diluted with distilled water, and then the absorbance was measured at λ = 280 nm.

Results and discussion

BSA concentration standard curve

The BSA concentration was obtained by absorbance measurements at 280 nm. A standard curve of $A = 0.63\ \gamma$ was formulated, where A is the absorbance and γ is the concentration (mg mL^{-1}) of BSA.

BSA solution salting-out

The BSA yield was defined as the mass ratio of BSA in precipitate and that in physiological saline at the beginning. The BSA yield was high in 70 % and 90 % ASS and low below 70% ASS, as shown as Fig. 1. In ASS solutions with more than 70 %, the BSA yield increased slowly. Meanwhile, the solution density increased when the ASS content was higher than 70 %, at which point the BSA precipitated and became suspended at the supernatant surface, which became difficult to separate. This BSA deposition agglomerated easily but was difficult to redissolve.[10–11] Thus, 70 % ASS was chosen as the appropriate salting-out agent due to the resulting high BSA yield and straightforward separation.

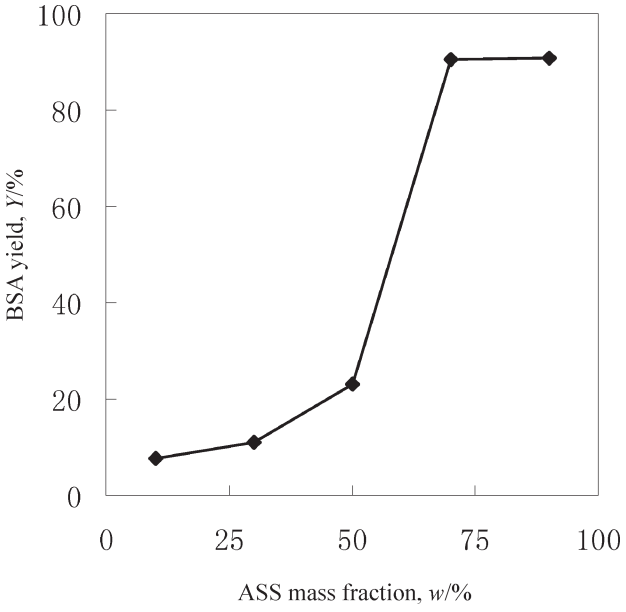

Fig. 1 – *BSA yield vs. ASS mass fraction*

BSA salting-out with different standing times

BSA was difficult to extract in very high concentrations of protein solution. The BSA yield was low when the BSA initial concentration was high. This occurs because when the ASS fraction is fixed, a fixed ion concentration can only act with a fixed protein concentration. The protein was in excess, and the yield was low when the protein concentration was high. When the protein concentration was low, the BSA concentration matched the salting-out agent and the yield was high. Thus, a 0.3 mg mL^{-1} BSA initial concentration was chosen for this work, as shown in Fig. 2. After different standing times, the BSA, whose initial concentration was 0.3 mg mL^{-1} in 70 % ASS, was centrifuged at 3000 rpm for 60 min.

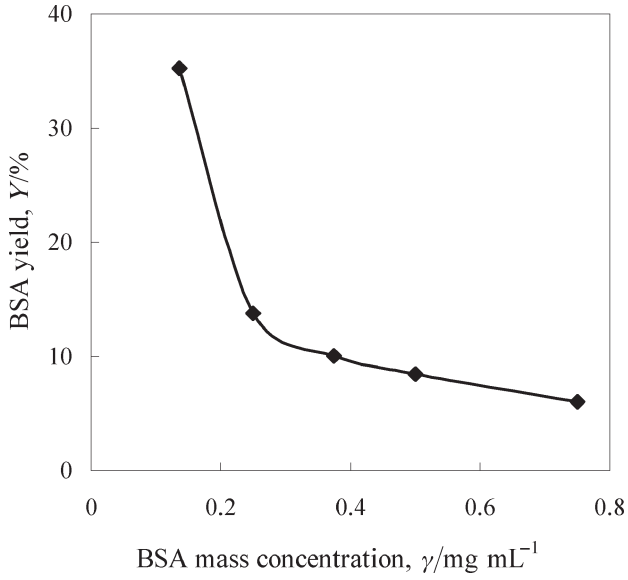

Fig. 2 – *BSA yield vs. BSA concentration*

From Fig. 3, it can be seen that the BSA yield changed slightly when the standing time was longer than 5 h. Thus, a good result can be gained after a 5 h standing time without ultrasound. Salting-out was utilized to extract protein and other large molecule polymers. Following ASS addition, the ASS fraction in some parts of the sample was high and non-uniform, inducing other kinds of protein deposits and co-pre-

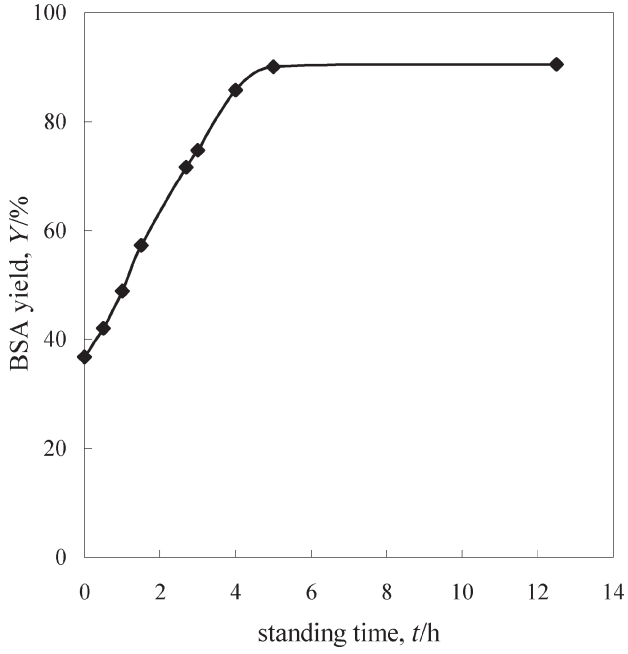

Fig. 3 – *BSA yield vs. standing time*

cipitates. The extraction of pure protein then requires a long time to reach a separation balance. The standing time used in this study was thus 5 h.

Ultrasonic protein salting-out

Ultrasound irritation was imposed during the salting-out process of BSA in ASS solution. The solution was centrifuged for 60 min at 3000 rpm and then measured.

Ultrasound radiation

The experiment was carried out with a BSA initial concentration of 0.3 mg mL^{-1} in 70 % ASS solution, with 20 kHz ultrasound radiation for 2 min and with a sound intensity of 0.64 W cm^{-2}.

From Fig. 4, it can clearly be seen that the BSA yield was 90 % after ultrasonic salting-out and a 30 min standing time. The yield was only 43 % when no ultrasound was applied. Thus, ultrasound has a great influence on BSA yield.

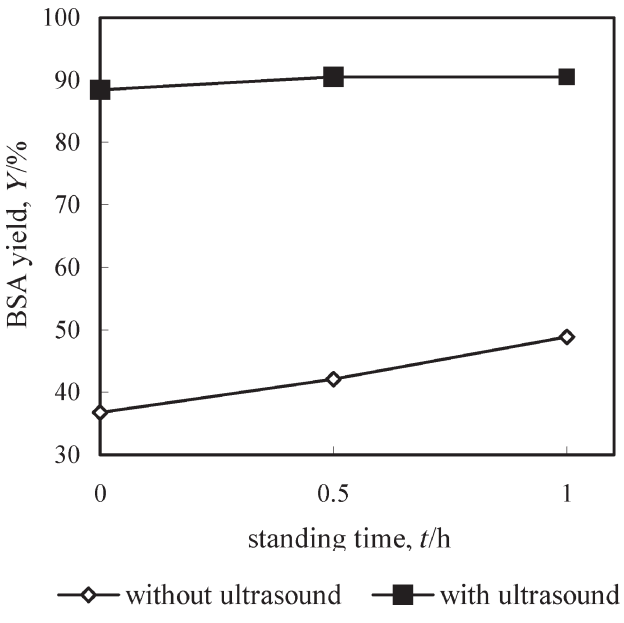

Fig. 4 – *BSA yield after different standing times*

Theoretical analysis of ultrasound intensity and frequency influence on salting-out

Relationship of sound intensity and amplitude[2]

$$I = 2\pi^2 \rho c f^2 A_m^2 \cdot 10^{-7} \quad (1)$$

In which

I – sound intensity, W m^{-2},
ρ –water density, g cm^{-3},
c – sound velocity, cm s^{-1},
f – frequency, Hz,
A_m – amplitude, cm.

Sound intensity is an important controllable parameter in ultrasonic experiments. It can also determine the mass transfer process of the hydration of protein membranes and electrolyte action. When the sound intensity is increased, the protein "particle" amplitude increases as well as the collision frequency. Thus, the protein "particle" agglomeration velocity also increases.

Sound intensity calculation

The sound intensity was determined from the sound pressure.

$$p_a = \frac{U}{M} \quad (2)$$

In which

p_a – sound pressure, Pa,
U – sound inductor output voltage, V,
M – sound inductor sensitivity, 31.6 V MPa^{-1}.

The sound intensity can be calculated according to the relationship between intensity and pressure.

$$I = \frac{p_a^2}{2\rho_0 c_0} \quad (3)$$

In which

p_a – sound pressure, Pa,
ρ_0 – solution density, kg m^{-3},
c_0 – sound velocity in solution, m s^{-1}.

Influence of ultrasound frequency on BSA salting-out

The ultrasound intensity decreases with increasing propagation distance. Its intensity degree can be expressed by the ultrasound decay coefficient α.[11]

$$I = I_q e^{-2\alpha x} \quad (4)$$

In which

I_q – sound intensity at launcher,
I – sound intensity at x distance,
x – propagation distance.

The relationship between the ultrasound decay coefficient α and frequency, according to sound theory, is:[11]

$$C = \frac{\alpha}{f^2} \quad (5)$$

in which C is a constant.

In a fixed temperature system, a lower ultrasound frequency results in a smaller decay constant.

In addition, the sound field was well distributed, which aided in the protein salting-out. Meanwhile, at the same sound intensity, a low frequency can increase the effective protein salting-out irradiation distance. Furthermore, low frequency irradiation could raise the protein "particle" amplitude according to eq. (1), and the protein can easily agglomerate. Thus, low frequency ultrasound should be utilized in the protein salting-out process.

Influence of sound intensity and frequency on BSA yield

Ultrasound irradiation can be introduced into the system in order to move protein relative to the water movement. The sound intensity applied in this case is called the lower intensity. Protein was accelerated to move with the application of sound intensity. During this period, minor protein particles agglomerate and are extracted from the water. However, when the sound intensity is raised above another fixed value, this minor particle movement becomes violent and protein particles may agglomerate in the water. Then, the protein particles disperse and dissolve in water, and the salting-out efficacy is decreased. This value is called the upper intensity. In the protein salting-out process, the ultrasound intensity should be between these two intensities.

Different frequencies of ultrasound irradiation, 20, 40 and 500 kHz, were used in our study. The maximum powers used were 200, 200 and 140 W, respectively. After irradiation, the BSA yield was measured with a zero standing time. In this experiment, the BSA initial concentration was 0.3 mg mL^{-1} in 70 % ASS, the ultrasound irradiation was applied to the samples for 2 min, and the sample was then centrifuged at 3000 rpm for 60 min. The results are shown in Fig. 5.

The maximum protein yield was 88.9 % with 20 kHz ultrasound irradiation, and the corresponding maximum sound pressure was 1.58 kPa. The maximum protein yield was 71.0 % with 40 kHz ultrasound irradiation, and the corresponding sound pressure was 1.58 kPa. The maximum protein yield was 68.9 % with 500 kHz ultrasound irradiation, and the corresponding maximum sound pressure was 1.27 kPa. Thus, the maximum protein yield differed for different ultrasound frequencies. Low frequency irradiation was thus beneficial for salting-out.

Influence of irradiation time on BSA yield

A sound intensity of 0.64 W cm^{-2} at 20 kHz was applied to a BSA sample with a concentration of 0.3 mg mL^{-1} in 70 % ASS. The result was measured with a zero standing time, as shown in Fig. 6. The BSA yield increased as the ultrasonic irradiation time increased. The yield reached a maximum when the irradiation time reached 2 minutes. However, the yield decreased when longer irradiation times were used, and some unexpected reactions happened with a long irradiation time.

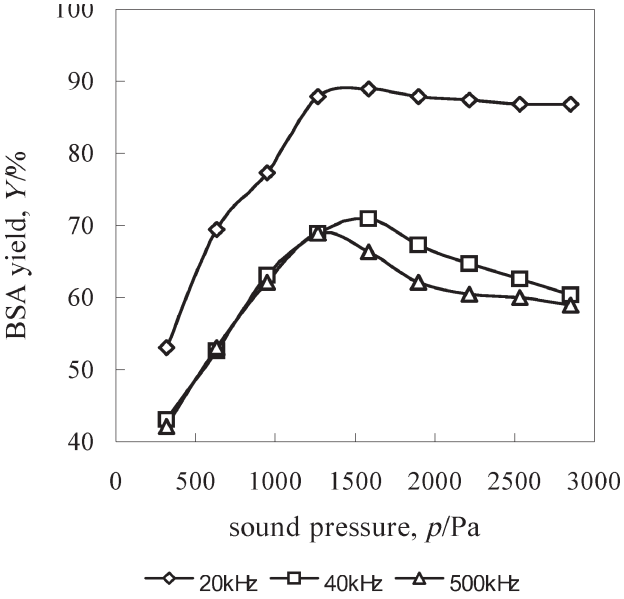

Fig. 5 – *Influence of sound pressure on BSA yield*

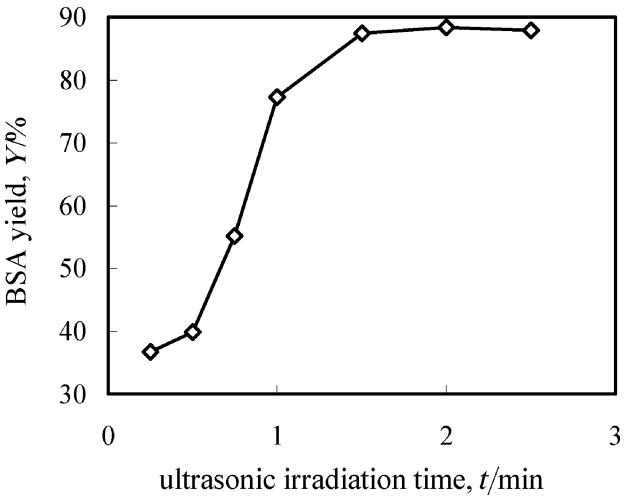

Fig. 6 – *BSA yield after different irradiation times*

Influence of standing time on BSA yield

In order to determine the optimal standing time after ultrasound irradiation and whether the salting-out time can be decreased, different ultrasound irradiation frequencies and standing times were compared.

A 2 min irradiation dose was applied to an 0.3 mg mL^{-1} BSA sample in 70 % ASS under a 20 or 40 kHz ultrasound with 1.58 kPa sound pressure and under a 500 kHz ultrasound with a 1.27 kPa sound pressure. The results are shown in Fig. 7.

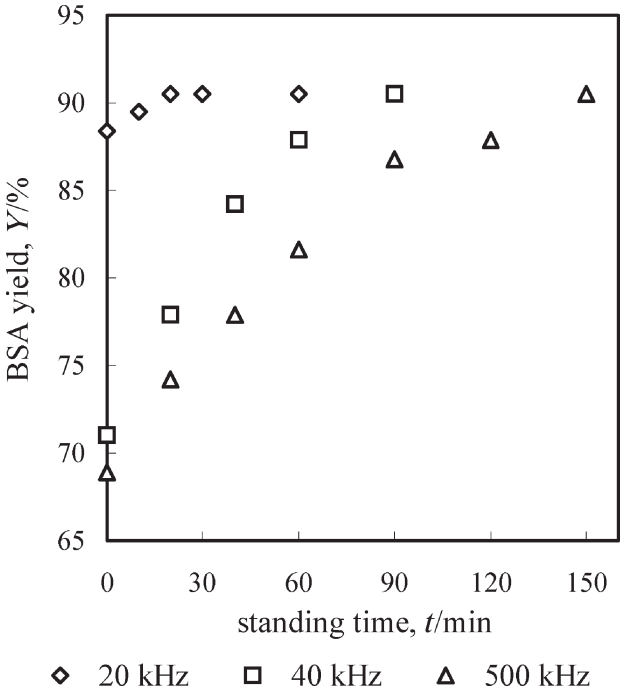

Fig. 7 – *BSA yield under different ultrasound frequencies and standing times*

The BSA yield reached 88.4 % under the 20 kHz ultrasound. When the BSA was centrifuged after a 20 min standing time, its yield reached 90.5 %. However, the yield did not increase for longer standing times. Thus, the maximum BSA yield was achieved with a 20 min standing time.

When the ultrasound frequency was 40 kHz, the BSA yield reached 90.5 % after 1.5 h. Thus, the standing time after ultrasound could be decreased by 3.5 h from that without ultrasound.

When the ultrasound frequency was 500 kHz, the BSA yield reached 90.5 % after 2.5 h. Thus, the standing time after ultrasound could be decreased by 2.5 h from that without ultrasound.

The experimental results show that the process time could be reduced by 4.5 h when an ultrasound frequency of 20 kHz is used. Higher frequencies resulted in lower yields, but the yield with ultrasound was higher than that without ultrasound.

Orthogonal experiment

Orthogonal design

Orthogonal design has some advantages, such as decreasing the number of necessary experiments, adding the ability to analyze the experiment on individual factors and finding the important factor. Thus, 3^3 orthogonal designs are shown in Table 2, and the results are shown in Table 3.

Table 1 – *Configuration of different BSA solutions*

BSA/mg	ASS saturated solution mL	Physiological saline mL	ASS concentration %
15.1	5	45	10
14.9	15	35	30
15.0	25	25	50
15.0	35	15	70
14.7	45	5	90

Table 2 – *Orthogonal design*

Factor / Level	Centrifugation time min	Ultrasonic irradiation time min	Uultrasound ntensity W cm^{-2}
1	20	1	0.52
2	40	1.5	0.64
3	60	2	1.0

Table 3 – *Results of orthogonal experiment*

S.n.	Centrifugation time min	Ultrasonic irradiation time min	Ultrasound intensity W cm^{-2}	BSA yield/%
1	20	1	1	11.08
2	20	1.5	1.5	70.50
3	20	2	2	78.93
4	40	1	0.64	72.61
5	40	1.5	1.0	78.40
6	40	2	0.52	73.13
7	60	1	1.0	75.77
8	60	1.5	0.52	87.89
9	60	2	0.64	89.46

Analysis of results

M_{ij} indicates a data summation in the j-th column and the i-th row; m_{ij} indicates its average value, R_j indicates the average R-control. $R_j = \max\{m_{ij}\} - \min\{m_{ij}\}$ indicates the influence degree on this factor. Thus, the higher the value of R_j, the greater the influence on the factor.

From Table 4, it can clearly be seen that the influence sequence is centrifugation time > ultrasonic irradiation time > ultrasound intensity. The BSA yield of Treatment 9, 89.46 %, was the highest of all of the experiments. That treatment included a 60 min centrifugation and a 2 min ultrasonic irradiation at an 0.64 W cm^{-2} sound intensity.

Table 4 – *Analysis of orthogonal experiment results*

M_{1j}	160.51	159.46	172.10
M_{2j}	224.14	236.79	232.57
M_{3j}	253.12	241.52	233.1
m_{1j}	53.50	53.15	57.37
m_{2j}	74.71	78.93	77.52
m_{3j}	84.37	80.51	77.7
R_j	30.87	27.36	20.33

Mechanism discussion of ultrasonic effect on protein salting-out

A standing period is commonly included in the salting-out process.[10] Thus, it increases the total salting-out time. The ultrasonic flocculation effect was utilized in this work to accelerate the mass transfer between electrolyte ions and the surrounding hydration layer. Then, the partial electric charge on the protein surface was neutralized. Ultrasound accelerates protein collisions, and proteins without hydration layers are easy to flocculate and deposit. Standing is not necessary after an ultrasound-irradiated salting-out period. Thus, the rate of the salting-out process increases.

Conclusions

– The effect of ultrasound on protein salting-out is significant. The process time can be reduced by 4–5 h if ultrasound is introduced into the salting-out process. After 2 min of ultrasonic irradiation, the BSA yield reached 90.0 % and the efficiency was enhanced.

– Ultrasound irradiation does not change the protein salting-out balance, but it can promote mass transfer and increase the BSA flocculation speed.

– Ultrasound has an influence on protein salting-out, and the sound pressure should be maintained under a maximum value.

– When the ultrasound frequency is low, the protein yield is high. Protein yields decrease with increasing frequency.

– The sequence of influence on the protein salting-out yield is centrifugation time > ultrasonic irradiation time > ultrasound intensity. The appropriate process settings are 20 kHz ultrasound with 0.64 W cm^{-2} sound intensity and 60 min centrifugation. The maximum protein yield is 89.64 %.

The ultrasound method is an emerging technology that is gaining attention in the literature. In this work, ultrasound is utilized to decrease the protein salting-out time for economic benefit. Thus, ultrasound applications have significant prospects for protein preparation.

ACKNOWLEDGEMENTS

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List of symbols

A – absorbance, %
A_m – amplitude, cm
α – decay coefficient, m^{-1}
c – sound velocity, cm s^{-1}
f – frequency, Hz
I – sound intensity, W m^{-2}
M – sound inductor sensitivity, V MPa^{-1}
n – centrifugation speed, min^{-1}
P – power, W
p – pressure, Pa
t – standing time, h
U – sound inductor output voltage, V
w – mass fraction, %
x – propagation distance, cm
Y – yield, %
γ – mass concentration, mg mL^{-1}
λ – wavelength, nm
ρ – density, g cm^{-3}

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