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RAPID COMMUNICATION

Ultrasound-based fractional precipitation for the purification of (+)-dihydromyricetin

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Abstract−The purification efficiency of (+)-dihydromyricetin, a bioactive plant flavonoid, was remarkably improved through ultrasound-based fractional precipitation. The precipitation time (up to 30 min) taken to obtain (+)-dihydromyricetin with high purity (87.01-91.66%) and yield (90.63-92.00%) through the ultrasonic fractional precipitation (ultrasonic power: 80-250 W) was shortened by 40-fold as compared to the conventional method (up to 1200 min). In addition, the precipitation rate constant was increased by 12- to 18-fold, but the activation energy was decreased to between −5,787 J/mol and −6,526 J/mol, and thereby the precipitation rate was improved. Furthermore, the size of the precipitate was reduced by 5.1- to 5.5-fold, while the diffusion coefficient was increased by 6.4- to 6.9-fold.

Keywords: (+)-Dihydromyricetin, Fractional Precipitation, Ultrasound, Kinetics, Diffusion Coefficient

INTRODUCTION

Ampelopsis grossedentata is a representative medicinal plant containing a large quantity of flavonoids [1]. In particular, (+)-dihydromyricetin ($C_{15}H_{12}O_8$, molecular weight: 320.25), a representative bioactive component with antioxidant activity, is a major functional flavonoid contained in large amounts in the leaves of *Ampelopsis grossedentata* [2]. It is a functional substance which not only eases hangover and protects the liver, but also shows excellent efficacy in the treatment of inflammation, fatty liver, cancer, diabetes, and hyperlipidemia, and therefore, is widely used as a raw material for foods and medicines [1-3]. Du et al. [4] reported that (+)-dihydromyricetin derived from *Ampelopsis grossedentata* had a significant effect in regulating hypertension. Ye et al. [5] also reported an inhibitory effect on ethanol-induced muscle relaxation, promotion of metabolism in the body, and an antiallergenic effect.

In the commercialization process of the plant-derived biologically active substance (+)-dihydromyricetin for use as a raw material for functional foods or medicines, the active ingredient should be isolated and purified on a bulk scale. However, research on mass production thereof is lacking, and in particular, research on separation and purification of the ingredient which directly benefits commercial mass production is almost nonexistent. Some limited studies were performed in the early 2000 s, finding that (+)-dihydromyricetin of poor purity (<10%) was obtained through solvent extraction and chromatography, or that a crude extract thereof containing terpenoids, lipids, chlorophylls, or phenols could be obtained [6,7]. Furthermore, high-cost chromatography was used in the purification process for high purity, or a crude extract without pre-treatment underwent final purification by high-performance liquid chromatography (HPLC), which was not cost-effective and presented difficulty in scaling up for commercialization [4].

Fractional precipitation is a very simple and effective method that can efficiently purify (+)-dihydromyricetin using differences in solubility. In 2008, an effective purification process was developed that could obtain (+)-dihydromyricetin with high purity (>83.2%) through fractional precipitation, but the fractional precipitation took much time (up to 32 h), which made mass production difficult [8]. In 2014, an improved method was developed that enhanced the precipitation efficiency by increasing the surface area per volume of reactant solution using an ion-exchange resin (Amberlite 200, Amberlite IR 120Na) [9]. Specifically, in the case of Amberlite 200, (+)-dihydromyricetin could be obtained with high yield (>90%) in a relatively short precipitation time (up to 16 h). However, in the precipitation process wherein surface area-increasing materials were introduced, the cost of the ion-exchange resin and recovery of the ion-exchange resin after the precipitation was complete still posed problems. In 2018, a more improved fractional precipitation process using a hydrophilic polymer was reported [3]. In particular, by the addition of hydroxypropyl methylcellulose (HPMC) (0.1%, w/v) during the fractional precipitation, not only was the particle size reduced (32-40%), but also the issues of cost and recovery of the surface area-increasing material were resolved. Despite all of the efforts made, the fractional precipitation of (+)-dihydromyricetin still took such considerable time that mass production was not possible. Therefore, there was a serious demand for a drastic reduction of precipitation time in order for efficient mass production through fractional precipitation to be feasible. Recently, various studies have been conducted to improve the precipitation efficiency so that the nucleation rate could be increased by employing ultrasound waves [10-12]. Such improvement in the precipitation efficiency was due to the acoustic cavitation including formation, growth, and collapse of microbubbles from the solution to which ultrasound was applied. That is, cavitation bubbles formed by ultrasound ultimately collapsed to generate high-speed microjets of the precipitation solution, intense localized heating, and high-pressure shock waves [13]. Building on the ideas of these preceding studies, the present investigators intended to remarkably reduce the precipitation time by the employment of

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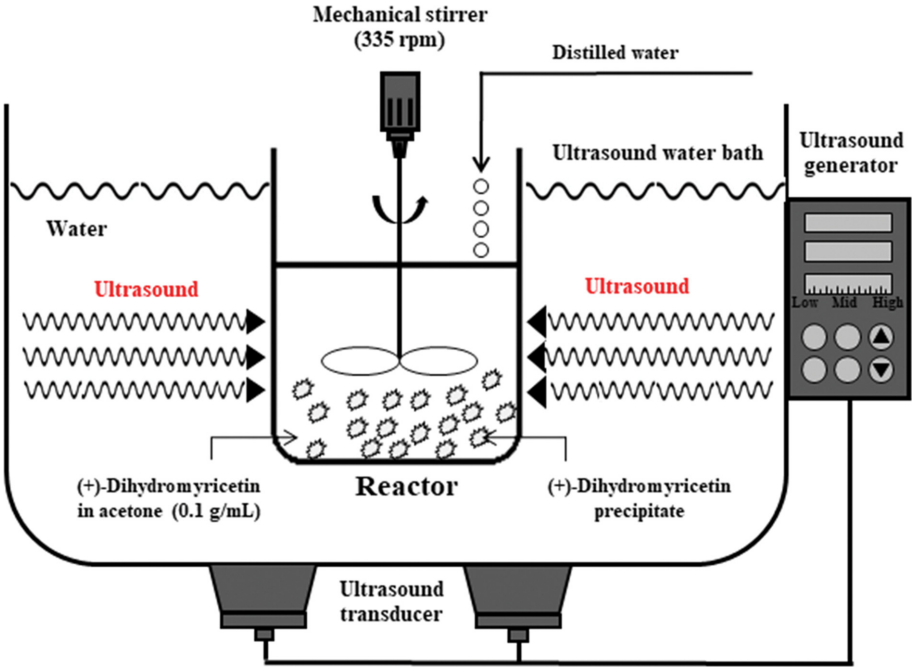

Fig. 1. Schematic diagram of ultrasound-assisted fractional precipitation for purification of (+)-dihydromyricetin from crude extracts.

ultrasound in the fractional precipitation of (+)-dihydromyricetin. In addition, the behavior of fractional precipitation of (+)-dihydromyricetin using ultrasonic waves was investigated in detail through kinetic analysis using the Johnson-Mehl-Avrami-Kolmogorov (JMAK) equation, which can be applied to the precipitation process and the determination of the change in activation energy. Furthermore, the diffusion pattern of molecules was quantitatively identified by determining the diffusion coefficient using the Stokes-Einstein equation.

MATERIALS AND METHODS

1. (+)-Dihydromyricetin Samples

The crude extract (purity: 54.84%) used in the fractional precipitation of (+)-dihydromyricetin in this study was purchased from Guilin Natural Ingredient, Inc. (Guilin, China).

2. (+)-dihydromyricetin Analysis

(+)-Dihydromyricetin content was analyzed using an HPLC system (SCL-10 AVP, Shimadzu, Japan) equipped with a Capcell Pak C18 (250 mm×4.6 mm, 5 μm, Shiseido, Japan) column. Gradient elution was performed using a mixture of distilled water and acetonitrile from 90 : 10 to 30 : 70 over 60 min (flow rate: 1.0 mL/min). The injection volume was 20 μL, and the effluent was monitored at 254 nm using an ultraviolet (UV) detector [3]. An authentic sample (purity: 98%) was purchased from Guilin Natural Ingredient, Inc. (Guilin, China) and used as a standard.

3. Fractional Precipitation

A (+)-dihydromyricetin sample (purity: 54.84%) was dissolved in acetone (0.1 g/mL), and distilled water was added dropwise (distilled water/acetone solution ratio=5/1, v/v) while stirring (335 rpm) to induce precipitation of (+)-dihydromyricetin using the difference in solubility [3,9]. The volume of the batch reactor was 20 mL and the working volume was 12 mL. For the conventional fractional precipitation (control), the precipitation was induced in a thermo-hygrostat (KCL-2000W, EYELA, Japan) at 5 °C while varying the precipitation time (4 h, 12 h, 20 h, and 28 h). Ultrasonic fractional precipitation was performed at varying ultrasonic power (80 W, 180 W, and 250 W) and precipitation time (5 min, 10 min, 20 min, and 30 min) in an ultrasonic bath at 5 °C. The fractional precipitation temperature was set to the optimal conditions determined in the preceding studies [3,8,9]. A schematic diagram of the ultrasonic fractional precipitation process is presented in Fig. 1. The (+)-dihydromyricetin precipitate was obtained through filtration (150 mm, Whatman, Buckinghamshire, UK) after precipitation and was dried in a vacuum oven (UP-2000, EYELA, Japan) at 40 °C for 24 h. The purity and yield of the dried precipitate were analyzed through HPLC.

4. Size Measurement of Precipitate

The shape and size of the precipitate recovered with the fractional precipitation were observed by electron microscope (SV-35 Video Microscope System, Some Tech, Korea) [9]. The precipitate was observed under high magnification (×200) and the size of the precipitate was measured by video image using the IT-Plus System (Some Tech, Korea).

5. Kinetic Analysis

The Johnson-Mehl-Avrami-Kolmogorov (JMAK) equation, which is mainly used in crystallization or precipitation, describes the kinetics of isothermal phase transformation in the process of nucleation and growth. These are expressed as Eq. (1) or Eq. (2) [10,14,15].

$$X(t) = 1 - e^{-kt^n} \tag{1}$$

$$\log\left(\ln\left(\frac{1}{1-X(t)}\right)\right) = n\log t + \log k \tag{2}$$

where X(t) is the precipitation yield of the (+)-dihydromyricetin at

any time t, k is the rate constant of the precipitation, and n is the Avrami index, which describes the characteristics of the crystalline structure and nucleation. The JMAK index (Avrami index) n and the rate constant k can be calculated from the slope and intercept, respectively, through Eq. (2). The suitability of the model was judged through the coefficient of determination (r^2).

6. Calculation of Activation Energy and Diffusion Coefficient

The activation energy (E_a) was calculated using the Arrhenius equation and can be expressed as Eq. (3) or Eq. (4) [10].

$$k = Ae^{-\frac{E_a}{RT}} \quad (3)$$

$$\ln k = \ln A - \frac{E_a}{RT}\cdot\frac{1}{T} \quad (4)$$

where k is the rate constant, A is the frequency factor, E_a is the activation energy (J/mol), R is the gas constant (8.314 J/mol·K), and T is the absolute temperature (K).

The diffusion coefficient of a (+)-dihydromyricetin molecule (B) diffused in the solvent (A) of the fractional precipitation is calculated from the Stokes-Einstein equation and can be expressed as Eq. (5) [12].

$$D_{AB} = \frac{kT}{6\pi r_0 \eta} \quad (5)$$

where k is the Boltzmann constant (1.38×10^{-23} J/K), r_0 is the diameter of the (+)-dihydromyricetin molecule, η is the dynamic viscosity of the solution, and T is the absolute temperature of the solution. The viscosity of the solution was measured using a viscometer (Viscolite 700, Hydromotion, UK). Each sample was analyzed in triplicate.

RESULTS AND DISCUSSION

1. Fractional Precipitation Utilizing Ultrasonic Waves

In this study, ultrasound (power: 80-250 W) was employed to resolve such problems during fractional precipitation of (+)-dihydromyricetin. The fractional precipitation results with and without ultrasound are shown in Fig. 2. In the conventional fractional

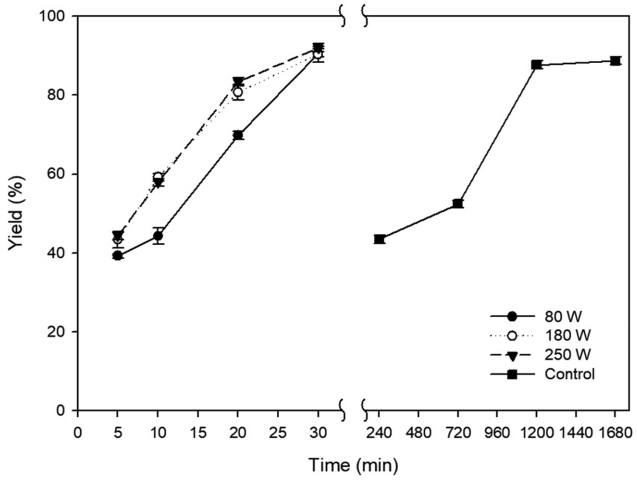

Fig. 2. Effect of ultrasound power on the yield of (+)-dihydromyricetin obtained by fractional precipitation at various ultrasonic powers.

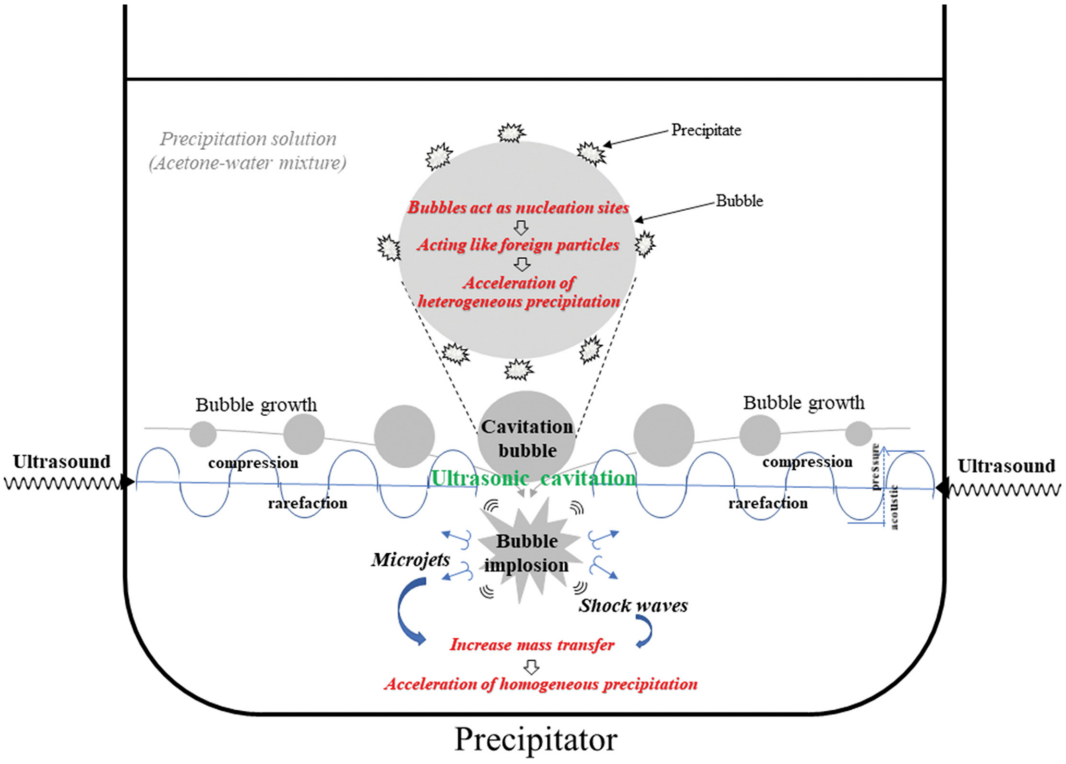

Fig. 3. Principle of ultrasonic fractional precipitation of (+)-dihydromyricetin.

precipitation method without employing ultrasound (control), the (+)-dihydromyricetin yield gradually increased over the precipitation time, and the precipitation was completed at about 1,200 min (yield: 88.7%). Meanwhile, in ultrasonic fractional precipitation, the yield of (+)-dihydromyricetin increased rapidly as the precipitation time elapsed at all ultrasonic powers, and the precipitation was complete in 30 min (yield: >90%). Therefore, the precipitation time could be dramatically shortened; that is, the precipitation time could be shortened about 40-fold compared to the control to obtain a similar yield. This result suggested that the ultrasonic cavitation bubbles themselves not only acted as nucleation sites, but also improved mass transfer due to high-speed microjets of liquid, intense localized heating, and high-pressure shock waves caused by bubble collapse (Fig. 3) [13,16]. This also showed similarity to the experiment in which the precipitation time of paclitaxel was shortened more than 48-fold through the ultrasonic fractional precipitation as compared to the conventional method [10]. The yield according to ultrasonic power at 30 min of the ultrasonic fractional precipitation was 90.63% (80 W), 90.34% (180 W), and 92.00% (250 W). As the ultrasonic power increased, the yield also increased and there was little difference in the yield at powers of 180 W or more. There was no significant difference in the purity, averaging 87.01-91.66% with or without ultrasound (data not shown). These results show that the purity of (+)-dihydromyricetin was almost unchanged during fractionation, which is similar to the results of previous studies [10].

2. Kinetic Analysis of Ultrasonic Fractional Precipitation of (+)-Dihydromyricetin

After plotting $\log\left(\ln\left(\frac{1}{1-X(t)}\right)\right)$ versus logt by applying the experimental data to the JMAK equation for kinetic analysis, the JMAK index (n) and the rate constant (k) were calculated from the slope and intercept as shown in Table 1. The n value was 0.7431 in the control and 0.7966-0.8616 in the ultrasonic fractionated precipitation (80-250 W). In the control, the n value (0.7431 at 5 °C) was slightly smaller than that obtained by fractional precipitation of paclitaxel (1.3190-0.9046 at 5 °C) [10]. In addition, the n value obtained from the ultrasonic fractional precipitation (0.7966-0.8616) was similar to that obtained from the ultrasonic fractional precipitation of paclitaxel (0.4432-1.0140) [10]. The rate constant k was 0.0085 and 0.1034-0.1519 for the control and the ultrasonic fractional precipitation, respectively, and it was increased by 12- to 18-fold in the ultrasonic fractional precipitation as compared to the control. This value was slightly greater than that obtained from the fractional precipitation of paclitaxel (6.310×10^{-5} to 4.355×10^{-3} at 5 °C) [10]. Also, the k values obtained in the ultrasonic fractional precipitation of (+)-dihydromyricetin (0.1034-0.1519) were greater than those obtained in the ultrasonic fractional precipitation of paclitaxel (0.01374-0.50700) [10]. Meanwhile, the activation energy change ($\Delta E_a = E_{a,\,ultrasound} - E_{a,\,control}$) determined through the Arrhenius equation was −5,787 J/mol (80 W), −6,674 J/mol (180 W), and −6,526 J/mol (250 W). By introducing ultrasonic waves, the activation energy decreased, and as the ultrasonic power increased the activation energy decreased further. Similar values were observed at ultrasonic power of 180 W or more. Ultimately, by introducing ultrasonic waves, the activation energy (i.e., the minimum energy required for the reaction) was reduced and the reaction rate increased, and thus the precipitation rate was improved. This was similar to the result of fractional precipitation of paclitaxel with ultrasound [10].

3. Determination of Diffusion Coefficient of (+)-Dihydromyricetin in Solution

To investigate the fractional precipitation behavior more quantitatively, the particle size was measured by electronic microscope for both the conventional fractional precipitation and the ultrasonic fractional precipitation. The results are summarized in Table 2. By the time the precipitation was completed, the mean particle size was 68.587 μm in the control and 13.462 μm (80 W), 12.747 μm (180 W), and 12.500 μm (250 W) in ultrasonic fractional precipitation. The size of particles in the case of the ultrasonic fractional

Table 1. Values of kinetic parameters for the fractional precipitation of (+)-dihydromyricetin at different ultrasound powers

Precipitation type	Ultrasonic power (W)	n[a]	k[a]	ΔE_a (J/mol)[a] ($E_{a,\,ultrasound} - E_{a,\,control}$)	r^2
Control	-	0.743±0.045	0.0085±0.0002	-	0.8272
Ultrasonic precipitation	80	0.862±0.001	0.1034±0.0004	−5,787±85	0.8992
	180	0.797±0.015	0.1519±0.0007	−6,674±97	0.9954
	250	0.837±0.011	0.1423±0.0006	−6,524±94	0.9837

[a]Data are shown as n, k, and ΔE_a±SD.

Table 2. Effect of ultrasound on viscosity, mean particle size, and diffusion coefficient

Fractional precipitation type	Ultrasonic power (W)	Viscosity η (g/cm·s)	Mean particle size r_0 (μm)	Diffusion coefficient D_{AB} (cm^2/s)
Control	-	1.0	68.587	2.9701×10^{-11}
Ultrasonic precipitation	80	0.8	13.462	1.89153×10^{-10}
	180	0.8	12.747	1.99763×10^{-10}
	250	0.8	12.500	2.0371×10^{-10}

precipitation decreased by 5.1-fold (80 W), 5.4-fold (180 W), and 5.5-fold (250 W) as compared to that of the control, and as the ultrasonic power increased, the particle size decreased far more. When ultrasonic waves were introduced, the crystal size decreased due to shock waves and abrasion between particles [12,17]. In general, reduction in particle size of the active pharmaceutical ingredient not only improved the dissolution rate, uniformity of drug dispersion, and oral bioavailability in the formulation, but also enhanced the drying efficiency [18,19]. Therefore, the reduction of (+)-dihydromyricetin particle size is important in the aspect of its use. To quantitatively investigate the diffusion behavior in fractional precipitation, the diffusion coefficient (D_{AB}) was calculated using the Stokes-Einstein equation (Table 2). The viscosity of the solution in the control and the ultrasonic fractional precipitation was measured as 1.0 g/cm·s and 0.8 g/cm·s, respectively. In the case of the control, the D_{AB} value was 2.970×10^{-11} cm^2/s, while in the ultrasonic fractional precipitation it was 1.892×10^{-10} cm^2/s (80 W), 1.998×10^{-10} cm^2/s (180 W), and 2.037×10^{-10} cm^2/s (250 W). When ultrasonic waves were employed, the diffusion coefficient was increased by 6.4- to 6.9-fold as compared to the control, and as the ultrasonic power increased the diffusion coefficient increased slightly. These results are comparable with studies on other APIs (poorly water-soluble drugs) by ultrasonic antisolvent precipitation at 75 W and 20 kHz (i.e., 1.4-fold for itraconazole, 1.82-fold for ibuprofen, 7.9-fold for sulfamethoxazole, and 33.6-fold for griseofulvin) [11]. The increase in the diffusion coefficient in fractional precipitation significantly affects homogeneous nucleation [12,20,21]. Ultimately, ultrasonic waves have a great influence on diffusion during fractional precipitation, which is thought to be due to cavitation phenomena (shock waves and microjets) caused by the collapse of cavitation bubbles [11,12,22]. It is judged that as the diffusion coefficient increases, mass transfer between molecules becomes easier and collisions more frequent, thereby improving the precipitation efficiency (especially the yield) [12].

CONCLUSIONS

The purification efficiency of (+)-dihydromyricetin was remarkably improved by ultrasonic fractional precipitation. In conventional fractional precipitation (control), precipitation was completed around 1,200 min of precipitation (yield: 88.71%), whereas in ultrasonic fractional precipitation, precipitation was completed in 30 min (yield: >90%) at all ultrasonic powers. By introducing ultrasound, the precipitation time of (+)-dihydromyricetin was reduced by 40-fold compared to the control. This may be because the ultrasonic cavitation bubbles themselves acted as nucleation sites and mass transfer was promoted by the high-speed liquid microjets, intense localized heating, and high-pressure shock waves occurring due to bubble collapse. When ultrasound was employed, the rate constant k was increased by 12- to 18-fold compared to the control, and the activation energy was reduced to −5,787 J/mol, −6,674 J/mol, and −6,526 J/mol at powers of 80 W, 180 W, and 250 W, respectively. That is, the introduction of ultrasonic waves increases the reaction rate due to the decrease in activation energy, and thus the precipitation rate is improved. In addition, ultrasound contributed to 5.1-fold (80 W), 5.4-fold (180 W), and 5.5-fold (250 W) reduction in the particle size of (+)-dihydromyricetin as compared to that of the control. Meanwhile, the diffusion coefficient was increased by 6.4- to 6.9-fold as compared to that of the control, and as the ultrasonic power was further increased, the diffusion coefficient increased slightly. In conclusion, the ultrasonic waves greatly affected the diffusion, which might be due to cavitation phenomena (shock waves and microjets) caused by the collapse of the cavitation bubbles.

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