

Recovery and purification of cholesterol from cholesterol- β -cyclodextrin inclusion complex using ultrasound-assisted extraction

C₂

complex by using response surface methodology (RMS). A comparison between UAE and other conventional methods (Reflux extraction and Soxhlet extraction) was also conducted. Additionally, the purification of cholesterol by silica gel column chromatography followed by crystallization and the analysis of cholesterol by HPLC were also evaluated.

2. Materials and methods

2.1. Materials

Duck egg and commercial β -CD (purity 97%) were purchased from the Wal-Mart supermarket (Hangzhou, China) and Mengzhou Huaxin Biochemistry Co., Ltd. (Mengzhou, China), respectively. Cholesterol standard (purity 99%) was obtained from Sigma-Aladdin (Shanghai, China). HPLC grade methanol and silica gel (200–300 mesh) were supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All other solvents used were of analytical grade.

2.2. Preparation of C- β -CD inclusion complex

Cholesterol is the components of duck yolk, which was used to prepare C- β -CD inclusion complex in this study. Spray-dried egg yolk powder was mixed with ethyl acetate solution (ratio of solution to egg yolk powder was 12 mL/g) to extract yolk oil. The oil was cooled to 10 °C in a temperature-controlled water bath and added with 0.21 g/mL β -CD solution. After which, the slurry was stirred for 21 min at 950 rpm using a blender (JHS-1, Hangzhou, China), then it was centrifuged at 6000 rpm for 5 min (TG16-WS, Changsha, China). The viscous intermediate C- β -CD layer was recycled and stored at 4 °C for cholesterol recovery studies, which was the by-products of processing low-cholesterol duck yolk oil (the initial cholesterol content in the inclusion complex was 44.25 mg/g). In C- β -CD inclusion complexes molecule, the molar ratio of cholesterol/ β -CD was 1/3 according to Claudy et al. [15]. The type of bond established between included cholesterol and β -CD is no covalent, hydrogen bonding plays an important role in the binding of cholesterol by β -CD.

2.3. Ultrasound-assisted extraction

The experimental procedures were performed by indirect sonication in a temperature controlled ultrasonic cleaning bath (KQ-300KDV, Kunshan, China; 40 kHz, input power 0–300 W, total power consumption 700 W, tank internal dimensions: 30.0 × 24.0 × 15.0 cm). Langevin type piezoelectric ultrasonic transducers of the cleaning bath were placed on the bottom of the extraction vessel. Temperature inside the bath was controlled externally by circulating cold water during extraction, and it was monitored with electronic thermometer (TM-902C, Guangzhou, China) immersed inside of the water (Fig. 1). The absolute ultrasonic power P (W) was calculated by measuring the time-dependent increase in temperature of solvent [16]. Expressed as Eq. (1).

$$P = m \cdot C_p \cdot \frac{dT}{dt} \quad (1)$$

where, C_p is the heat capacity of the solvent ($\text{J g}^{-1} \text{K}^{-1}$), m is the mass of the solvent (g) and dT/dt is temperature rise per second. Then, the level of energy introduced into the system can be expressed as acoustic energy density (AED in W/cm^3) [13,16], which can be determined using Eq. (2).

$$\text{AED} = \frac{P}{V} \quad (2)$$

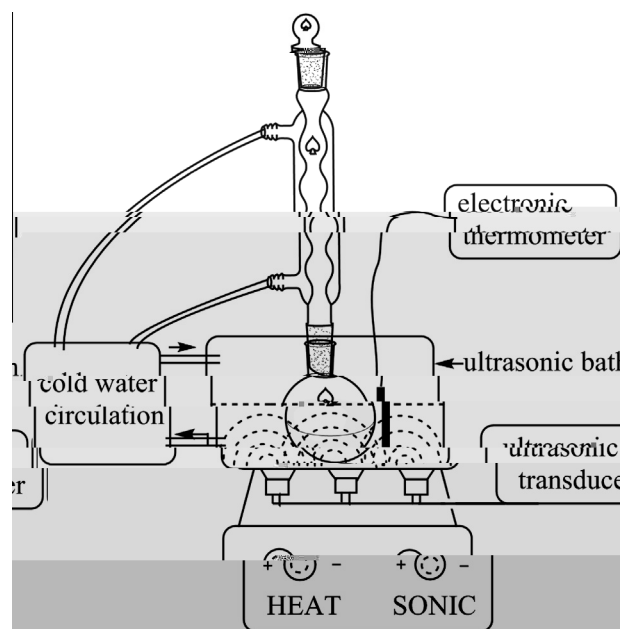


Fig. 1. Schematic illustration of the ultrasonic equipment.

where, V is sample volume (cm^3). The acoustic energy density (AED) were 0.13, 0.17, 0.29 and 0.49 W/cm^3 , respectively, when the power inputs were 100, 150, 200 and 250 W, respectively.

5.0 g of sample was mixed with a certain volume of absolute ethanol (ratio of ethanol to C- β -CD ranging from 8 to 12 mL/g) in 250 mL round-bottom flask, the mixture was adjusted to pH7.0 with 1.0 mol/L KOH. After which, the flask was connected to a condenser and immersed in the middle of the ultrasonic bath each time and at the same depth in the bath water. Extractions were performed under different experimental conditions: ultrasonic power (150–250 W), extraction temperature (50–70 °C) and sonication time (25–45 min). The influence of each parameter was investigated firstly. Each trial was carried out in triplicate. After extraction, the extracts were filtered instantly due to its low solubility in cold ethanol and then concentrated by using rotary evaporator (R-SENCO, Shanghai, China) at 65 °C under 0.08 MPa. The concentrated filtrate was collected and purified in the subsequent studies. The residues were dried at 65 °C for 5 min to remove the residual ethanol in a forced-draft oven (DGX-9143B-1, Shanghai, China) and were used to analyze cholesterol content.

2.4. Conventional extraction

2.4.1. Reflux extraction

5.0 g of sample mixed with 50 mL of absolute ethanol in 250 mL round-bottom flask, the mixture was adjusted to pH7.0 with 1.0 mol/L KOH solution. The flask was then coupled with a condenser and placed in a water bath (201D, Nanjing, China; total power consumption 1500 W), 65 °C, extracted 120 min.

2.4.2. Soxhlet extraction

5.0 g of sample was placed inside a cellulose thimble, extracted with 150 mL of absolute ethanol for 240 min at a temperature of 85 °C in a Soxhlet apparatus (total power consumption 1500 W).

2.5. Measurement of cholesterol

A modified $\text{FeNH}_4(\text{SO}_4)_2$ chromogenic method [17] was used to measure cholesterol content in C- β -CD inclusion complex. The

Table 1

Factors and levels of the central composite rotatable design (CCRD).

Variables	Symbols	Levels					
		Coded	−2	−1	0	1	2
Solvent-solid ratio, A (mL/g)	X_1		8	9	10	11	12
Ultrasonic power, B (W)	X_2		150	175	200	225	250
Extraction temperature, C (°C)	X_3		50	55	60	65	70
Sonication time, D (min)	X_4		25	30	35	40	45

$$X_1 = (A - 10)/1, X_2 = (B - 200)/25, X_3 = (C - 60)/5, X_4 = (D - 35)/5.$$

efficiency of cholesterol extraction from C- β -CD inclusion complex was represented in the form of the extraction yield of cholesterol and was calculated using the following equation Eq. (3).

$$Y(\%) = 1 - \frac{M_2}{M_1 \frac{1}{1-w_1}} \times 100 \quad (3)$$

where Y was the relative extraction yield (%), M_1 was the initial cholesterol content in C- β -CD inclusion complex (mg/g) ($M_1 = 44.25$ mg/g), M_2 was the cholesterol content in the residue (mg/g) and w_1 was the moisture content in the inclusion complex (%) ($w_1 = 38.0\%$ in this study).

2.6. Experimental design and statistical analysis

The optimization of UAE of cholesterol and evaluation main effects, interaction effects and quadratic effects of the formulation were performed by RSM. A four-factor, five-level central composite rotatable design (CCRD) was used to allocate treatment combinations [18]. Table 2 presents experimental design for CCRD and the responses, which consisted of a 2^4 full factorial points, 8 axial points and 7 central points, involving 31 randomized experiments. Cholesterol extraction yield Y (%) was the response, the independent variables were: solvent-solid ratio X_1 (mL/g), ultrasonic power X_2 (W), extraction temperature X_3 (°C) and sonication time X_4 (min). The coded and actual values of the independent variables were presented in Table 1. For each independent variable, the range and central point value was chosen based on the results of preliminary experiments. The actual level (Z_i) can be transformed to a coded value (X_i) by the following equation Eq. (4):

$$X_i = \frac{Z_i - Z_{0i}}{\Delta_i} \quad (i = 1 - 4) \quad (4)$$

where X_i is the coded value, Z_i is the actual value, Z_{0i} is the average of the highest and lowest values for the variable in the design and Δ_i is the distance between the actual value in the central point and the actual value in the high or low level of a variable.

The second-order polynomial Eq. (5) expressed below was used to calculate the extraction yield of cholesterol:

$$Y = b_0 + \sum_{i=1}^k b_i X_i + \sum_{i=1}^k b_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^k b_{ij} X_i X_j \quad (i = 1 - 4, j = 1 - 4) \quad (5)$$

where Y is the predicted response, X_i, X_j are the coded independent variables; b_0 is the intercept term, which is the estimated response at the center point with coded values of X_1, X_2 , and X_3 set at 0. b_i, b_{ii} and b_{ij} are the linear, the quadratic and the interaction regression coefficient of the model, respectively. k is the number of independent variables ($k = 4$ in this study). The experimental design and regression analysis were performed using the Statistical Analysis System software (Version 9.2, SAS Institute Inc., Cary, NC, USA). The predicted model adequacy and suitability were evaluated by analysis of variance (ANOVA).

Verification experiments were conducted under the predicted optimized conditions by the model. The experimental values

Table 2

Central composite rotatable design (CCRD) of factors and responses.

Run	X_1 Solvent-solid ratio (mL/g)	X_2 Ultrasonic power (W)	X_3 Extraction temperature (°C)	X_4 Sonication time (min)	Yield of cholesterol (%)
1	–1	–1	–1	–1	87.94
2	–1	–1	–1	1	90.87
3	–1	–1	1	–1	93.48
4	–1	–1	1	1	94.17
5	–1	1	–1	–1	94.59
6	–1	1	–1	1	95.36
7	–1	1	1	–1	96.31
8	–1	1	1	1	94.34
9	1	–1	–1	–1	93.82
10	1	–1	–1	1	95.71
11	1	–1	1	–1	96.83
12	1	–1	1	1	97.04
13	1	1	–1	–1	95.43
14	1	1	–1	1	96.36
15	1	1	1	–1	97.39
16	1	1	1	1	97.86
17	–2	0	0	0	92.06
18	2	0	0	0	96.35
19	0	–2	0	0	94.76
20	0	2	0	0	98.44
21	0	0	–2	0	95.99
22	0	0	2	0	98.70
23	0	0	0	–2	93.70
24	0	0	0	2	97.39
25	0	0	0	0	96.90
26	0	0	0	0	98.54
27	0	0	0	0	96.58
28	0	0	0	0	97.37
29	0	0	0	0	97.01
30	0	0	0	0	96.98
31	0	0	0	0	97.66

obtained from 3 replications were compared with the predicted value.

2.7. Purification of cholesterol

The purification of cholesterol was carried out by silica gel column chromatography (1.54 cm \times 50 cm i.d.) with subsequent crystallization. The crude extract was saponified and concentrated before loaded onto the silica gel column. n -hexane: isopropanol (98:2, v/v) was used as the eluent. 0.5 g of the concentrated crude extract was dissolved in 5 mL of eluent, which was then loaded onto the silica gel column. The column was eluted at a flow rate of 1 mL/min and the effluent was collected in each 15 mL fraction. These fractions containing cholesterol were further analyzed by HPLC, the fractions with HPLC purity above than 89% was pooled and concentrated. This purified cholesterol was redissolved in 10 mL ethanol at 45 °C, and crystallized by gradually cooling down to 20 °C, stored overnight at 4 °C. The crystallized cholesterol was dried for 12 h to remove the residual ethanol and analyzed by HPLC to determine purity, recovery and yield. The recovery (R) and yield (Y') were calculated according to Cao et al. [19]. The Eqs. (6) and (7) were used:

$$R(\%) = \frac{W_p \times P_p}{W_c \times P_c} \times 100 \quad (6)$$

$$Y'(\%) = \frac{W_p}{W_c} \times 100 \quad (7)$$

where P_p is the HPLC purity of cholesterol in purified product (%), P_c is the HPLC purity of cholesterol in concentrated extract after saponification (%), W_p is the weight of purified product (g), W_c is the weight of the crude concentrated extract after saponification (g).

2.8. HPLC analysis

The samples were previously dissolved with methanol and filtered using 0.45 μm PTFE membranes (Millipore). Then they were analyzed on an Agilent 1100 HPLC system (Agilent Technologies, Inc., Santa Clara, California, USA). The BDS HYPESIL C₁₈ column (4.6 mm \times 250 mm i.d., 5 μm) (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used. The column temperature was 38 $^{\circ}\text{C}$, injection volume was 10 μL . The mobile phase was methanol with a flow rate of 1 mL/min. The detection wavelength was set up at 210 nm.

3. Results and discussions

3.1. Effects of extraction parameters on cholesterol yield

3.1.1. Effect of solvent-solid ratio

Fig. 2A shows the solvent-solid ratio to the yield of cholesterol, which was conducted on the condition of ultrasonic power, extraction temperature and time at 200 W, 60 $^{\circ}\text{C}$ and 30 min, respectively. It is clearly that the yield of cholesterol increased gradually with the solvent-solid ratio rising from 6 mL/g to 10 mL/g. After which, the yield of cholesterol increased slightly as the solvent-solid ratio continue to increase. The reason for this is larger volume of solvent could create a concentration difference, which enhances mass transfer and accelerates diffusion of compounds. But too much solvent would not change much of the driving force. Additionally, the volumetric energy of the ultrasonic wave decreases with the ultrasonic energy propagating in the solvent, as ultrasonic energy is absorbed or scattered by a larger volume of solvent [20]. Considering the solvent consumption and bulky handling in the subsequent processes, a solvent-solid ratio of 10 mL/g was used as the central point in the optimization of process parameters during UAE.

3.1.2. Effect of ultrasonic power

The effect of ultrasonic power (ranging from 0 to 250 W) on the yield of cholesterol was studied when fixed solvent-solid ratio at 10 mL/g, temperature at 60 $^{\circ}\text{C}$ and sonication time at 30 min. It can be seen from Fig. 2B, the yield of cholesterol experienced a considerable increase from 85.95% to 94.94% when the ultrasonic power enhanced from 0 to 150 W. However, after which point, the increasing rate slowed down, the cholesterol yield only increased by 1.67% when the power enhanced from 150 W to 250 W. In fact enlarging the ultrasonic power results in more extensive cavitations, where generate more violent shock wave and high-speed jet [14,21]. Finally, these effects enhance the penetration of the ethanol molecule into the inner areas of C- β -CD inclusion complexes and improve the release of the included cholesterol in a β -CD cavity into solvent [22]. In addition, the ultrasonic energy can reach into the C- β -CD inclusion complexes under the cavitation effects. The non-covalent bond between the included cholesterol and β -CD can be easily broken [15], and a large amount of included cholesterol could be dissociated from C- β -CD. Based on the study, ultrasonic power level of 200 W was selected as the middle levels to apply in RSM optimization.

3.1.3. Effect of temperature

Temperature is another vital factor that would influence the yield of cholesterol, due to the fact that cholesterol has low solubility in cold ethanol. So different temperatures (40, 50, 60, 70 and 80 $^{\circ}\text{C}$) were used to investigate the effect on cholesterol yield with the ultrasonic power 200 W, solvent-solid ratio 10 mL/g and time 30 min. Results indicated that cholesterol yield increased significantly from 47.07% to 95.69% when the temperature enhanced

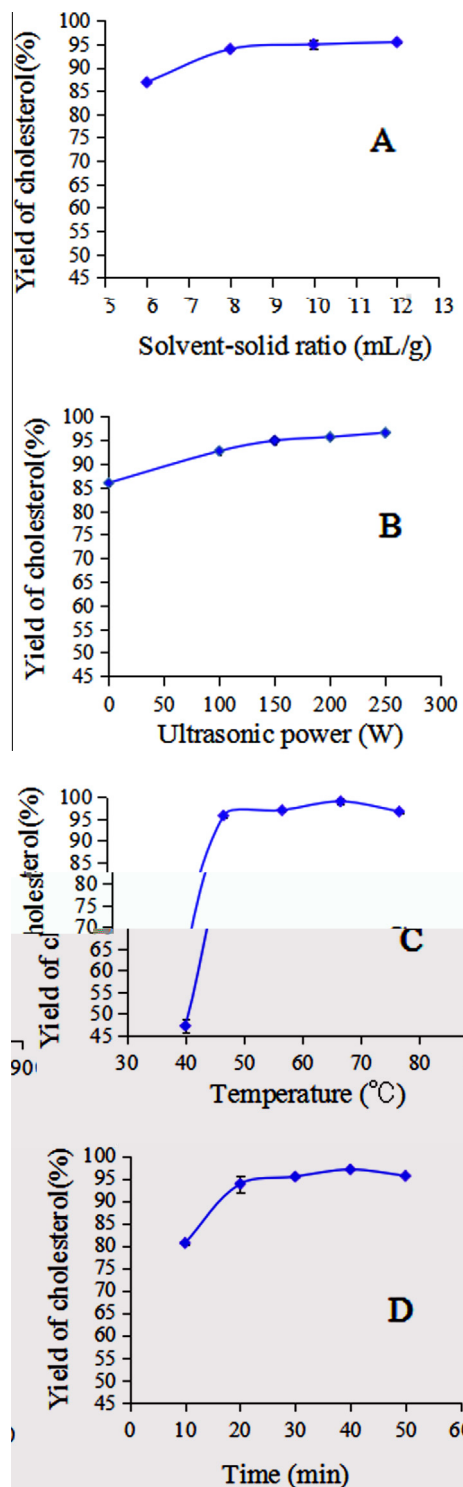


Fig. 2. The effect of solvent-solid ratio (A), ultrasonic power (B), extraction temperature (C) and sonication time (D) on the yield of cholesterol ($n = 3$).

from 40 to 50 $^{\circ}\text{C}$, then the yield remained stable at around this value when the temperature was over 50 $^{\circ}\text{C}$ (Fig. 2C). The formation of C- β -CD inclusion complex formation is exothermic, therefore, increasing temperature could dissociate it. Yamamoto et al. [8] reported the same influence of extraction temperature on cholesterol recovery from C- β -CD inclusion complex. They had found that the maximum cholesterol removal values was observed at 70 $^{\circ}\text{C}$, but the removal of cholesterol was lower than the results

reported in this study. A possible explanation for this effect may be related to ultrasonic cavitation effects and the lower dipole moment of ethanol (1.69 D) compared with water (1.84 D), the latter elevates the interaction between solvent molecules and less polar compounds [23].

High temperature enhances solvent diffusion rates, which facilitate extraction yield. Besides, solvent have greater capacity to solubilize the included cholesterol at higher temperatures. However, Cavitation is reduced at higher extraction temperatures. Because surface tension and viscosity of the extraction solvent is reduced and the vapor pressure of solvent is increased with increasing temperature, so voids are filled with solvent vapors, leading to less violent collapse [14,24]. In fact, the temperature of the crude extracts would decline when they were filtered, while cholesterol has a low solubility in cold ethanol. Therefore, high temperature facilitated the recovery of cholesterol. Based on these results, 60 °C was chosen to be the zero point of the extraction temperature to the subsequent optimization.

3.1.4. Effect of sonication time

The effect of extraction time on the yield of cholesterol was studied with other fixed factors: extraction temperature 60 °C, ultrasonic power 200 W and solvent-solid ratio 10 mL/g. As shown in Fig. 2D, the yield of cholesterol increased steadily with increasing time; up to 20 min. After which time, the yield of cholesterol increased slowly. Thus, a moderate sonication time is necessary. This is due to the fact that extension time may also causes various compounds such as insoluble substances were also suspended in the extraction liquid, resulting in the lower permeability of the solvent. This tendency agrees with reports of other authors in ultrasound-assisted extraction of polysaccharides from pomegranate peel [24]. So we selected 30 min for further experimentation.

3.2. Optimization of extraction parameters and validation

3.2.1. Fitting of second-order polynomial equation

The RSREG procedure of SAS/STAT was used to fitting a second-order polynomial regression equation. The predictive model for the extraction yield of cholesterol (Y) in terms of coded factors is

$$Y = 88.57 + 1.88X_1 + 1.50X_2 + 1.50X_3 + 1.50X_4 + 1.50X_5 + 1.50X_6 + 1.50X_7 + 1.50X_8 + 1.50X_9 + 1.50X_{10} + 1.50X_{11} + 1.50X_{12} + 1.50X_{13} + 1.50X_{14} + 1.50X_{15} + 1.50X_{16} + 1.50X_{17} + 1.50X_{18} + 1.50X_{19} + 1.50X_{20} + 1.50X_{21} + 1.50X_{22} + 1.50X_{23} + 1.50X_{24} + 1.50X_{25} + 1.50X_{26} + 1.50X_{27} + 1.50X_{28} + 1.50X_{29} + 1.50X_{30} + 1.50X_{31} + 1.50X_{32} + 1.50X_{33} + 1.50X_{34} + 1.50X_{35} + 1.50X_{36} + 1.50X_{37} + 1.50X_{38} + 1.50X_{39} + 1.50X_{40} + 1.50X_{41} + 1.50X_{42} + 1.50X_{43} + 1.50X_{44} + 1.50X_{45} + 1.50X_{46} + 1.50X_{47} + 1.50X_{48} + 1.50X_{49} + 1.50X_{50} + 1.50X_{51} + 1.50X_{52} + 1.50X_{53} + 1.50X_{54} + 1.50X_{55} + 1.50X_{56} + 1.50X_{57} + 1.50X_{58} + 1.50X_{59} + 1.50X_{60} + 1.50X_{61} + 1.50X_{62} + 1.50X_{63} + 1.50X_{64} + 1.50X_{65} + 1.50X_{66} + 1.50X_{67} + 1.50X_{68} + 1.50X_{69} + 1.50X_{70} + 1.50X_{71} + 1.50X_{72} + 1.50X_{73} + 1.50X_{74} + 1.50X_{75} + 1.50X_{76} + 1.50X_{77} + 1.50X_{78} + 1.50X_{79} + 1.50X_{80} + 1.50X_{81} + 1.50X_{82} + 1.50X_{83} + 1.50X_{84} + 1.50X_{85} + 1.50X_{86} + 1.50X_{87} + 1.50X_{88} + 1.50X_{89} + 1.50X_{90} + 1.50X_{91} + 1.50X_{92} + 1.50X_{93} + 1.50X_{94} + 1.50X_{95} + 1.50X_{96} + 1.50X_{97} + 1.50X_{98} + 1.50X_{99} + 1.50X_{100}$$

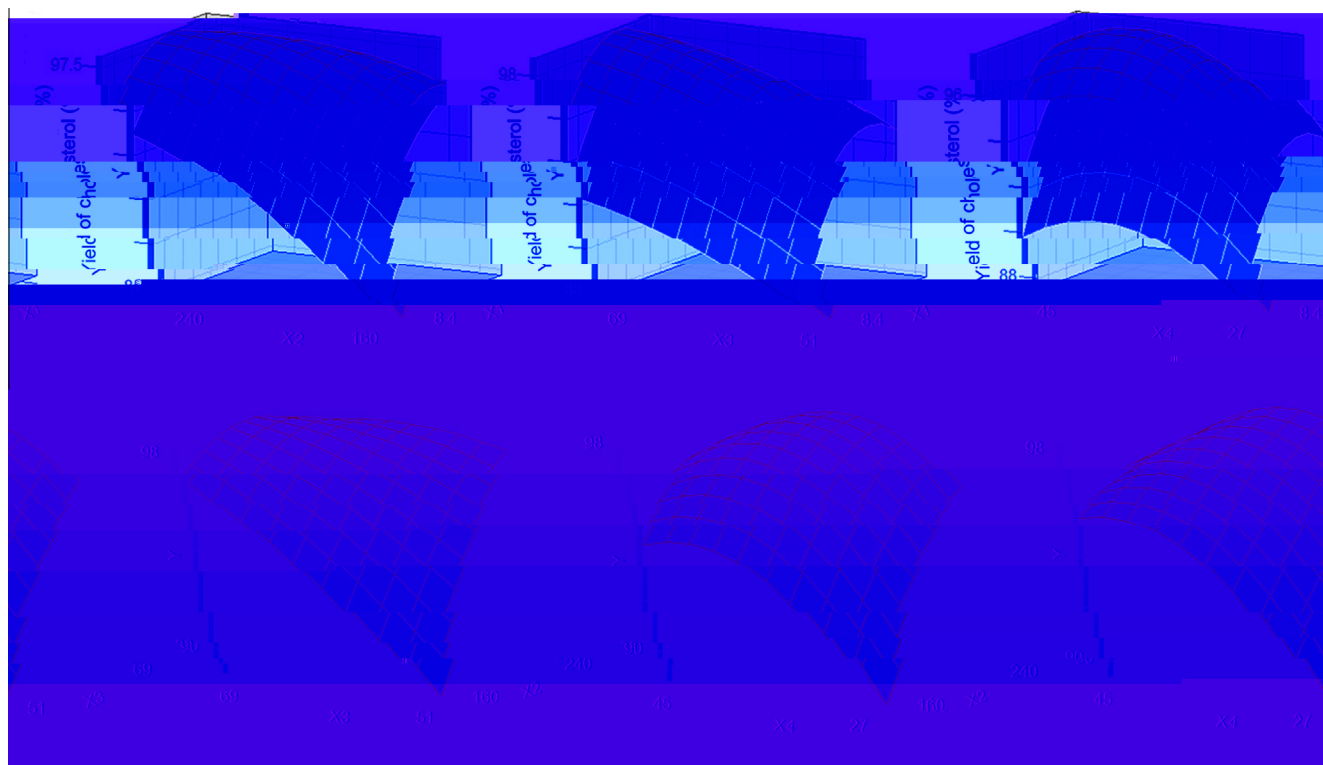


Fig. 3. Response surface plots the effects of interaction for yield of cholesterol: (A) interaction between ultrasonic power and solvent-solid ratio; (B) interaction between extraction temperature and solvent-solid ratio; (C) interaction between sonication time and solvent-solid ratio; (D) interaction between extraction temperature and ultrasonic power; (E) interaction between sonication time and ultrasonic power; (F) interaction between sonication time and extraction temperature.

level, the yield of cholesterol obviously increased with time rose. In addition, when time was fixed, the extraction yield of cholesterol increased as the temperature increased.

The coordinates of the optimized conditions could be calculated through the first derivative of the second-order function (Eq. (8)), the value of which was equal to zero. In this experiment, the optimized values would be $(X_1, X_2, X_3, X_4) = (0.057, 2.028, -0.868, 0.199)$, in terms of natural variables that associated with these coded values were solvent-solid ratio 10.06 mL/g, ultrasonic power 250.69 W, extraction temperature 55.66 °C and sonication time 35.99 min, the predicted yield of cholesterol at optimized point was 98.03%. For operational convenience, the optimal parameters were 10 mL/g, 251 W, 56 °C and 36 min. The verification experiments were conducted at this optimized extraction conditions. The yield of cholesterol of the verification experiments was $98.12 \pm 0.25\%$, which matches well with the predicted value (98.03%) from the second-order polynomial equation, and there are not statistically different at 5% significance level. The cholesterol content extracted was 43.38 ± 0.61 mg/g under the optimized conditions. This suggests that the optimization combination obtained and the predicted results could be valid.

3.3. Comparison of UAE and conventional extractions

Comparison studies were made between UAE of cholesterol at the optimized conditions (10 mL/g, 251 W, 56 °C, 36 min) and other conventional methods (Reflux extraction and Soxhlet extraction). The extraction time, solvent and energy consumption were considered in this comparison. The results in Table 4 indicated that the yields of cholesterol obtained by UAE were the highest but the time and the solvent consumed were significantly lower than other conventional methods applied. The yields of the Soxhlet extraction for 240 min did not achieve these by UAE for 36 min, even the

Table 4

Comparison of general factors for the different extraction methods.

Factors and yield	UAE	Reflux extraction	Soxhlet extraction
Extraction time (min)	36	120	240
Solvent volume (mL/5 g)	50	50	150
Extraction temperature (°C)	56	65	85
Electric energy consumed (W)	700	1500	1500
Yield of cholesterol (%)	98.12 ± 0.25	86.95 ± 0.17	96.34 ± 0.15

Yields are shown as mean \pm SD ($n = 3$).

former worked at a higher temperature (85 °C) and more volume of solvent (150 mL), but the latter was only at 56 °C and 50 mL solvent. By using of UAE, the time was reduced approximately 70% and 85% compared to Reflux extraction and Soxhlet extraction, respectively. In addition, solvent consumption reduction was near to 67% compared to Soxhlet extraction. Compared with these conventional methods when UAE was used the higher extraction yield was obtained and lower energy was consumed (Table 4). As an green extraction technology, the reduction of time, energy, solvent and enhancement of final yield are clearly advantageous for the used UAE. Eh and Teoh [25] also found using ultrasound extraction of lycopene from tomatoes was more energy saving compared with non-ultrasound extraction.

3.4. Ultrasound effects on cholesterol

In order to verify whether cholesterol present in the extracts undergo degradation when ultrasound is used for treatment, the isolated cholesterol without ultrasound treatment was submitted to optimized UAE. The degradation of cholesterol was assessed comparing the initial content to quantified final content after treatment [26]. HPLC analyses show that the cholesterol content

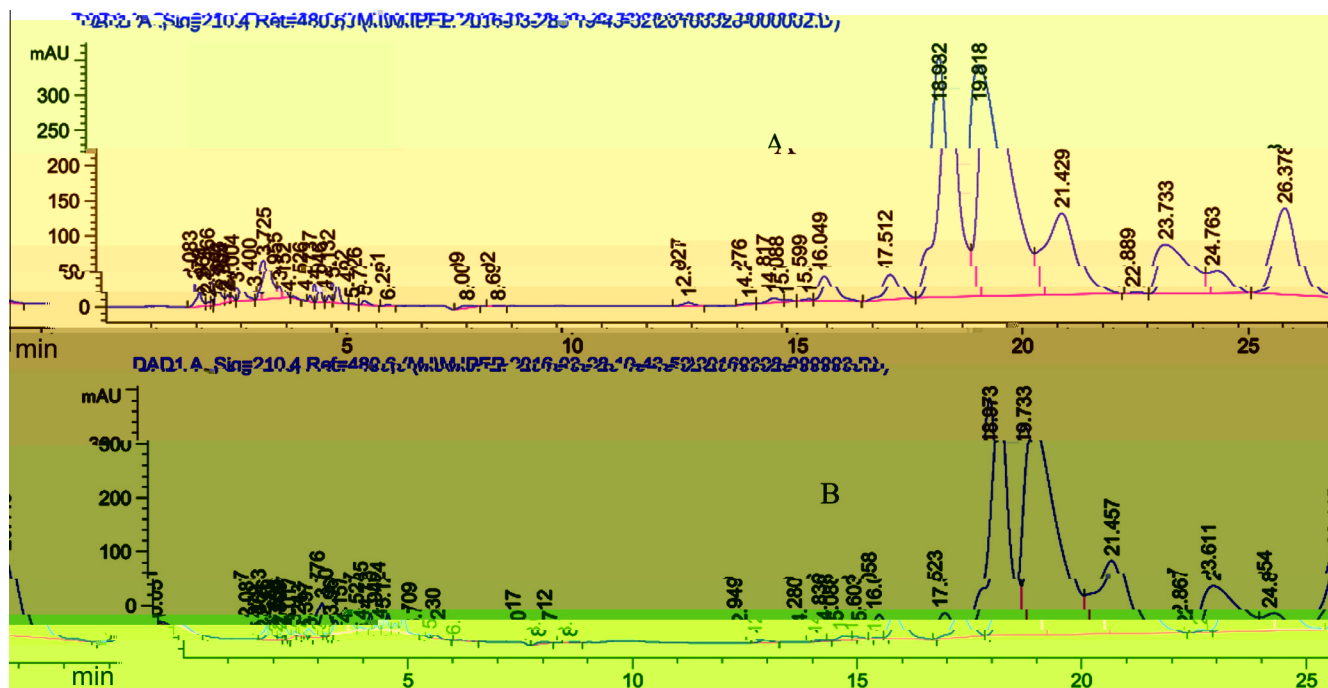


Fig. 4. HPLC of cholesterol (RetTime \approx 18.9 min) from C- β -CD inclusion complex extract before (A) and after sonication (B).

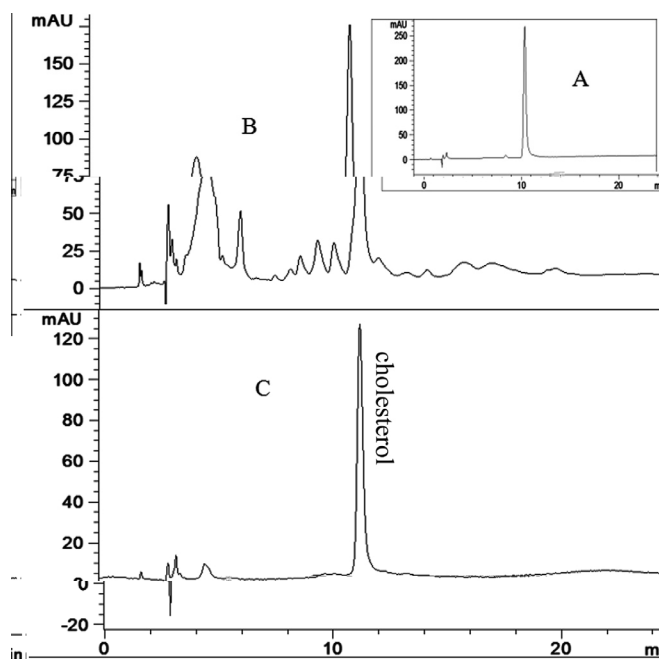


Fig. 5. Characteristic chromatograms of the cholesterol standard (A), extracted samples after saponification (B) and purified cholesterol (C).

was 23.1% and 22.8% before and after ultrasound treatment, respectively (Fig. 4). There is no significant degradation after ultrasound treatment. This change of 0.3% in content can be due to experimental error.

3.5. Purification of cholesterol

Fig. 5 displays the HPLC chromatograms of cholesterol standard, the crude cholesterol extract after saponification, and purification by column chromatography followed by crystallization,

respectively. Fig. 5B reveals that the content of cholesterol in the crude extract was 29.2%. Most of the impurity peaks were before 11.0 min. Fig. 5C shows the cholesterol peak intensity was very prominent, meanwhile most of the impurity peak intensity was very weak, which means that the impurities in the sample with near polarities to cholesterol achieved well separation. The fractions with HPLC purity above than 89.9% (calculated by peak area) was combined, concentrated followed by crystallization and 0.11 g cholesterol was obtained with purity of 95.1% (Fig. 5C), the recovery and the yield was 71.7% and 22.0%, respectively. These results illustrated that cholesterol was purified effectively by silica gel column chromatography followed by crystallization.

4. Conclusions

The results of this study indicated that UAE of cholesterol from C- β -CD inclusion complex is advantageous in increasing the cholesterol yield, shortening extraction time and solvent consumption when compared to conventional Reflux extraction and Soxhlet extraction. From the perspective of removal and recovery of cholesterol from duck yolk oil, the selective inclusion properties of β -CD toward cholesterol and the combination of ultrasound gave an effective method to recover cholesterol. Meanwhile, there was no specific degradation when ultrasound was used for treatment of cholesterol.

The statistical analysis showed that the optimum extraction conditions were: solvent-solid ratio, ultrasonic power, extraction temperature and time at 10 mL/g, 251 W, 56 °C and 36 min, respectively. All these factors showed significant effect on the yield of cholesterol. Under this optimized conditions, the experimental yield of cholesterol was $98.12 \pm 0.25\%$, which was closed with the predicted yield value 98.03%. The purification results showed that silica gel column chromatography followed by crystallization is a simple method of obtaining highly purified cholesterol from crude extract. The cholesterol recovered can be used as a raw material for steroid synthesis. Furthermore, as a green extraction technology, ultrasound can be an efficient way to recover cholesterol from a C- β -CD inclusion complex.

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Appendix A

$$P = m \cdot C_p \cdot \frac{dT}{dt} \quad (1)$$

$$AED = \frac{P}{V} \quad (2)$$

$$Y(\%) = 1 - \frac{M_2}{M_1 \frac{1}{1-w_1}} \times 100 \quad (3)$$

$$X_i = \frac{Z_i - Z_{0i}}{\Delta_i} \quad (i = 1 - 4) \quad (4)$$

$$Y = b_0 + \sum_{i=1}^k b_i X_i + \sum_{i=1}^k b_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^k b_{ij} X_i X_j \quad (i = 1 - 4, j = 1 - 4) \quad (5)$$

$$R(\%) = \frac{W_p \times P_p}{W_c \times P_c} \times 100 \quad (6)$$

$$Y'(\%) = \frac{W_p}{W_c} \times 100 \quad (7)$$

$$Y = 97.29 + 1.33X_1 + 1.05X_2 + 0.95X_3 + 0.55X_4 - 0.66X_1X_2 - 0.11X_1X_3 + 0.068X_1X_4 - 0.56X_2X_3 - 0.35X_2X_4 - 0.45X_3X_4 - 0.95X_1^2 - 0.35X_2^2 - 0.17X_3^2 - 0.62X_4^2 \quad (8)$$

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