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# Synthesis and crystallization purification of phytosterol esters for food industry application

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**Abstract**

This work aimed to investigate the process parameters of phytosterol esters (PE) synthesis catalyzed by copper oxide-nano hydroxyapatite (CuO-NHAP) and the crystallization purification technology. CuO-NHAP was prepared. The synthesis of PE catalyzed by CuO-NHAP was performed. After Response Surface Methodology optimization, the optimal process parameters were as follows: reaction temperature, 180 °C; reaction time, 6.3 h; acid-alcohol molar ratio, 1.9; catalyst amount, 1.05%. Under these conditions, the esterification rate of phytosterols was 99.8%. The crystallization purification technology of esterification product was explored. The optimal crystallization conditions were as follows: crystallization solvent, octanoic acid; crystallization temperature, 0 °C; solvent-product molar ratio, 4: 1; crystallization time, 10 h. Under these conditions, the unreacted fatty acids and other impurities were affectively removed, and the PE purity in purification product was 98.2%. These synthesis and crystallization purification technologies of PE are relatively green, safe, efficient and economic, and have the potential to be applied to the industrial preparation of PE product.

**Keywords:** phytosterol esters; synthesis; CuO-NHAP; crystallization; octanoic acid.

**Practical Application:** This study has provided a basis for preparation of phytosterol esters for food industry application.

## 1 Introduction

Phytosterols are the sterols with similar structure and biochemical characteristics to cholesterols. They have high medicinal value, and can effectively reduce the concentration of neutral triglycerides and cholesterol in blood, thus preventing and treating the cardiovascular diseases (de Jong et al., 2008; Cofán & Ros, 2015; Plat et al., 2019). Due to the high melting point and poor solubility in water and oil, the application of phytosterols in food industry is limited (Brown et al., 2010; Qi et al., 2013). The conversion of phytosterols into fatty acid esters can greatly improve their fat solubility and bioavailability (Yang et al., 2016). The synthesis of phytosterol esters (PE) can be completed by enzymatic catalysis. This method has the advantages of mild conditions and good product quality. However, it still has some problems such as long reaction time, low esterification rate and high cost of biological enzymes, so it is rarely used in practical production (Hellner et al., 2010; Robles-Manuel et al., 2011; Molina-Gutiérrez et al., 2017). At present, the chemical method is still the main technology for synthesis of PE (Valange et al., 2007; Hang, 2012). Previously, we have studied the synthesis technology of PE catalyzed by heteropoly acids and ZnO (Meng et al., 2006; Meng et al., 2010). Recently, in order to improve the catalytic efficiency and stability, we have developed the catalyst copper oxide-nano hydroxyapatite (CuO-NHAP) and preliminarily applied it to the synthesis of PE (Pan et al., 2017), but the preparation process parameters need to be further optimized. The methods of large-scale separation and purification of PE mainly include column chromatography (Chen et al., 2017), Supercritical carbon dioxide extraction (Fornari et al., 2009), etc.. In most of above methods, the n-hexane and acetone are commonly used, which have potential hidden danger and are not environmentally friendly. In this study, the process parameters of PE synthesis catalyzed by CuO-NHAP were optimized by surface methodology. In addition, using the relatively green and safe solvents, the crystallization and separation technology of PE was explored. The objective was to provide a basis for preparation of PE for food industry application.

## 2 Materials and methods

### *2.1 Preparation of CuO-NHAP*

A 0.398 g $Cu(NO_3)_2{\cdot}3H_2O$ was taken to prepare the $Cu(NO_3)_2$ solution with 5% Cu mass fraction. A 2 g of nano hydroxyapatite (NHAP) after reaming treatment was added to the solution. The mixture was stirred vigorously to ensure that the carrier and active components contacted fully, followed by intermittent ultrasonic-assisted immersion for 1 h. Then, the mixture was dried in a vacuum drying oven at 120 °C for 12 h to form the porous honeycomb solid. After calcining at 600 °C for 3 h, the CuO-NHAP product was obtained. It was characterized by specific surface area and pore structure determination, scanning electron microscopy and X-ray diffraction.

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### *2.2 Preparation of PE and optimization of process parameters*

A 10 g of mixed phytosterols (total sterol content > 95%; containing 24% stigmasterol, 45% β-sitosterol, 21% rapeseed sterol and 5% rapeseed sterol; average molar weight of 400; Jiangsu Chunzhigu Biological Products Co., Ltd., Taizhou, China) was put into a 50 ml three-port flask, and then the mixed fatty acids (acid value of 205 mg KOH/g; average molar weight of 180; Jiangsu Chunzhigu Biological Products Co., Ltd., Taizhou, China) were added according to a certain acid-alcohol molar ratio. The mixture was heated in oil bath to a given temperature for complete dissolution, and a certain amount of CuO-NHAP was added to start the esterification reaction. The mixture was sampled at fixed time interval. The reaction process was monitored by thin layer chromatography and acid value determination.

Using reaction temperature, reaction time, acid-alcohol molar ratio and catalyst amount as independent variables and esterification rate as response value, the four-factor and three-level experiment was performed by Response Surface Methodology (RSM) using Design-Expert software to optimize the process parameters of PE preparation.

### *2.3 Determination of crystallization temperature*

The mixture of fatty acid (oleic acid), PE standard and esterification product with certain proportions was dissolved in octanoic acid, methyl caprylate, triglyceride caprylate and ethyl acetate, respectively. The crystallinity of samples in different solvents was analyzed using differential scanning calorimetry (DSC). The crystallization points of samples and solvents were determined by the crystallization curves. DSC analysis conditions were as follows: constant 20 °C for 20 min, temperature increase at 5 °C/min to 65 °C, constant 65 °C for 5 min, temperature decrease at 3 °C/min to -50 °C, constant -50 °C for 30 min, temperature increase at 5 °C/min to 60 °C. This analysis conditions were also suitable for the purity identification of esterification product after crystallization purification.

### *2.4 Determination of crystallization solvent and solvent-esterification product molar ratio*

Using crystallization temperature of PE obtained by DSC analysis as the crystallization temperature, the optimal crystallization solvent and solvent-esterification product molar ratio were determined. PE standard was used as crystallization material, and a certain amount of fatty acid (oleic acid) was added to simulate the esterification product. The solvent (octanoic acid, methyl caprylate, triglyceride caprylate, ethyl acetate, respectively) and PE standard (solvent-PE molar ratio: 1: 1-10: 1) were added to 25 ml graduated glass centrifuge tube for complete dissolution, and then oleic acid with the same mole number as PE standard was added, followed by mixing evenly at 60 °C. The mixture was cooled naturally to room temperature for static crystallization. The crystal volume was record once every 1 h, until it did not increase any more. The centrifuge tube was centrifuged at 8000 r/min and crystallization temperature for 5 min. The optimal crystallization solvent and solvent-esterification product molar ratio was determined according to the separation effect, recovery rate and PE purity in crystals.

The separation effect of crystals was mainly based on whether the crystal and liquid were clearly stratified and whether the interphase interference of crystals was obvious. The recovery rate was calculated as follows: recovery rate (%) = $100 \times\{M_{PE} - [\rho_S \times (V_T - V_C) - M_S]\} / M_{PE}$, where $M_{PE}$ was the mass of PE standard (g), $\rho_s$ was the density of solvent (g/cm$^3$), $V_T$ was the total volume of mixture in centrifuge tube (ml), $V_C$ was the volume of crystals (ml), and $M_S$ was the mass of solvent (g). The PE purity of crystals was analyzed by gas chromatography.

### *2.5 Crystallization purification of esterification product*

According to the optimal crystallization condition obtained in above experiments, the crystallization purification experiment of esterification product was carried out. When the volume of crystals in the centrifuge tube did not increase with time increase, the crystals in the lower layer were collected by rapid suction filtration. The purification product was analyzed by DSC, and was compared with esterification product before crystallization purification. The PE purity in purification product was determined by gas chromatography

## 3 Results and analysis

### *3.1 Optimal process parameters of PE preparation*

In this study, CuO-NHAP was used to catalyze the synthesis of PE. Based on the results determined by single-factor experiments in our previous study [20], the process parameters were further optimized by RSM. The experimental results were shown in Table 1. Taking esterification rate of phytosterols (Y) as the response value, the regression equation was obtained as follows: $Y = 88.00 + 5.00A + 4.79B + 5.12C + 0.58D - 2.75AB + 2.55AC + 6.70AD + 1.92BC - 4.55BD + 0.10CD + 2.80A^2 - 6.51B^2 - 5.28C^2 - 10.17D^2$.

Analysis of variance showed that the F value and P value of the regression equation model were 4.43 and 0.0043, respectively, indicating that this model was extremely significant. The F value and P value of the mismatch term was 2.79 and 0.1676, respectively, indicating that the mismatch term was not significant. The interaction of reaction temperature, reaction time, acid-alcohol molar ratio and catalyst amount had significant effect on the esterification rate, and the order of effect was acid-alcohol molar ratio > reaction temperature > reaction time > catalyst amount (Figure 1).

The regression equation model predicted that, under the condition of reaction temperature 180 °C, reaction time 6.3 h, acid-alcohol molar ratio 1.9 and catalyst amount 1.05%, the esterification rate of phytosterols was 99.7%, which was the highest. Under these conditions, the average esterification rate in three independent repetitive experiments was 99.8%, which was in good agreement with the predicted value. This indicated that, the regression equation model was accurate and reliable.

### *3.2 Optimal crystallization temperature for different crystallization solvents*

The crystallization curves of oleic acid, PE standard and esterification product in different solvents were obtained by DSC. Using octanoic acid as crystallization solvent, in oleic acid with octanoic acid system ($n_{oleic\ acid}$: $n_{octanoic\ acid}$ = 1: 4), the

**Table 1.** RSM design and results for process parameters of PE preparation.

| No. | A | B | C | D | Y |
|---|---|---|---|---|---|
| | Reaction temperature (°C) | Reaction time (h) | Acid-alcohol molar ratio | Catalyst amount (%) | Esterification rate (%) |
| 1 | 160 | 6 | 1.6 | 1.0 | 74.8 |
| 2 | 160 | 7 | 1.8 | 1.0 | 80.5 |
| 3 | 160 | 6 | 1.8 | 0.8 | 85.5 |
| 4 | 170 | 7 | 1.8 | 0.8 | 84.4 |
| 5 | 160 | 5 | 1.8 | 1.0 | 72.8 |
| 6 | 170 | 6 | 1.8 | 1.0 | 95.0 |
| 7 | 180 | 6 | 2.0 | 1.0 | 99.5 |
| 8 | 170 | 6 | 1.8 | 1.0 | 84.9 |
| 9 | 170 | 6 | 1.8 | 1.0 | 85.9 |
| 10 | 170 | 7 | 2.0 | 1.0 | 86.1 |
| 11 | 170 | 6 | 1.6 | 0.8 | 62.9 |
| 12 | 180 | 7 | 1.8 | 1.0 | 86.5 |
| 13 | 160 | 6 | 2.0 | 1.0 | 88.6 |
| 14 | 170 | 7 | 1.8 | 1.2 | 75.5 |
| 15 | 180 | 6 | 1.8 | 1.2 | 94.8 |
| 16 | 170 | 5 | 1.8 | 0.8 | 56.2 |
| 17 | 170 | 6 | 1.8 | 1.0 | 86.3 |
| 18 | 180 | 6 | 1.8 | 0.8 | 84.8 |
| 19 | 180 | 5 | 1.8 | 1.0 | 89.8 |
| 20 | 170 | 7 | 1.6 | 1.0 | 79.4 |
| 21 | 170 | 6 | 1.6 | 1.2 | 69.4 |
| 22 | 170 | 5 | 1.6 | 1.0 | 75.8 |
| 23 | 170 | 6 | 2.0 | 0.8 | 71.7 |
| 24 | 170 | 5 | 1.8 | 1.2 | 65.5 |
| 25 | 170 | 6 | 2.0 | 1.2 | 78.6 |
| 26 | 160 | 6 | 1.8 | 1.2 | 68.7 |
| 27 | 170 | 5 | 2.0 | 1.0 | 74.8 |
| 28 | 170 | 6 | 1.8 | 1.0 | 87.9 |
| 29 | 180 | 6 | 1.6 | 1 | 75.5 |

crystallization point of oleic acid was obvious, which appeared at -4 °C. The signal of octanoic acid was weak, which appeared at -18 °C. When PE standard was dissolved in this system ($n_{oleic\ acid}$: $n_{octanoic\ acid}$: $n_{PE\ standard}$ = 1: 4: 1), the crystallization points of oleic acid and octanoic acid moved to the low-temperature region, which appeared at -13 °C and -28 °C, respectively. The crystallization point of PE was -1 °C. In octanoic acid with esterification product system ($n_{octanoic\ acid}$: $n_{esterification\ product}$ = 4: 1), the crystallization points of PE and oleic acid remained at -1 °C and -4 °C, respectively, but the crystallization point of octanoic acid slightly increased to about -13 °C, which may be related to the dissolution of some linoleic acid in the PE product (Figure 2A).

Using methyl caprylate as crystallization solvent, in oleic acid with methyl caprylate system ($n_{oleic\ acid}$: $n_{methyl\ caprylate}$ = 1: 1), oleic acid and methyl caprylate co-crystallized at -17 °C. When PE standard was dissolved in this system ($n_{oleic\ acid}$: $n_{methyl\ caprylate}$: $n_{PE\ standard}$ = 1: 1: 1), the crystallization point of PE was not affected, while oleic acid and methyl caprylate crystallized at -28 °C and -37 °C, respectively. In methyl caprylate with esterification product system ($n_{methyl\ caprylate}$: $n_{esterification\ product}$ = 1: 1), the crystallization points of methyl caprylate, PE and oleic acid were obviously separated (Figure 2B).

Using triglyceride caprylate as crystallization solvent, in oleic acid and triglyceride caprylate system ($n_{oleic\ acid}$: $n_{triglyceride\ caprylate}$ = 1: 2), oleic acid and triglyceride caprylate crystallized at -13 °C and -42 °C, respectively. The crystallization peak at -23 °C might come from the partial free octanoic acid carried by triglyceride caprylate. When PE standard was dissolved in this system ($n_{oleic\ acid}$: $n_{triglyceride\ caprylate}$: $n_{PE\ standard}$ = 1: 2: 1), oleic acid and triglyceride caprylate co-crystallized at -38 °C, but no obvious crystallization point of PE was found. In triglyceride caprylate with esterification product system ($n_{triglyceride\ caprylate}$: $n_{esterification\ product}$ = 2: 1), the crystallization peak of PE was at -1 °C ((Figure 2C).

Using ethyl acetate as crystallization solvent, in oleic acid and ethyl acetate system ($n_{oleic\ acid}$: $n_{ethyl\ acetate}$ = 1: 9), crystallization peak of oleic acid was at -2 °C, which partially coincided with that of PE. In the presence of PE standard in this system ($n_{oleic\ acid}$: $n_{ethyl\ acetate}$: $n_{PE\ standard}$ = 1: 9: 1), oleic acid and ethyl acetate co-crystallized at -22 °C. In ethyl acetate with esterification product system ($n_{ethyl\ acetate}$: $n_{esterification\ product}$ = 9: 1), the crystallization point of PE had little change (Figure 2D).

Generally speaking, the crystallization point of PE in different solvents was about -1 °C, and the crystallization point

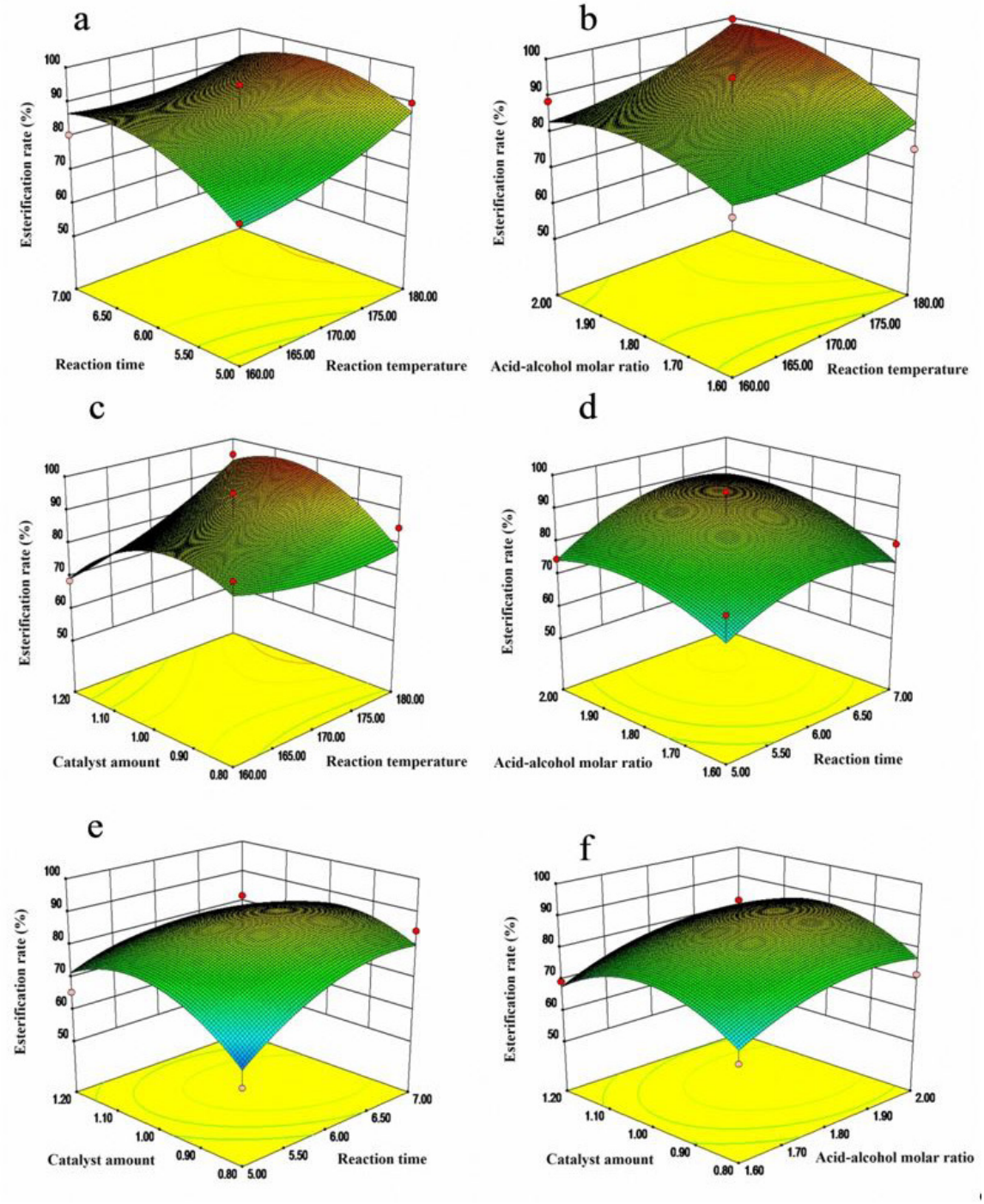


**Figure 1.** Response surface representations for interaction effect of process parameters on esterification rate of phytosterols.

of free fatty acid is below -10 °C. Therefore, the crystallization temperature in subsequent experiments was selected as 0 °C.

### *3.3 Optimal crystallization solvent and solvent-esterification product molar ratio*

Using PE standard with oleic acid (simulating the esterification product) as crystallization material, the crystallization experiment was performed at 0 °C to determine the optimal crystallization solvent and solvent-esterification product molar ratio. Results showed that, when triglyceride caprylate was used as the crystallization solvent, the separation effect and recovery rate were not ideal. The reason may be that the solubility of PE in triglyceride caprylate changes little with temperature fluctuation. When methyl caprylate was used as crystallization solvent, the interphase interference or floating crystals appeared. This may be due to the inclusion of some solvents in crystals for slow decreasing rate of crystallization temperature. When ethyl acetate

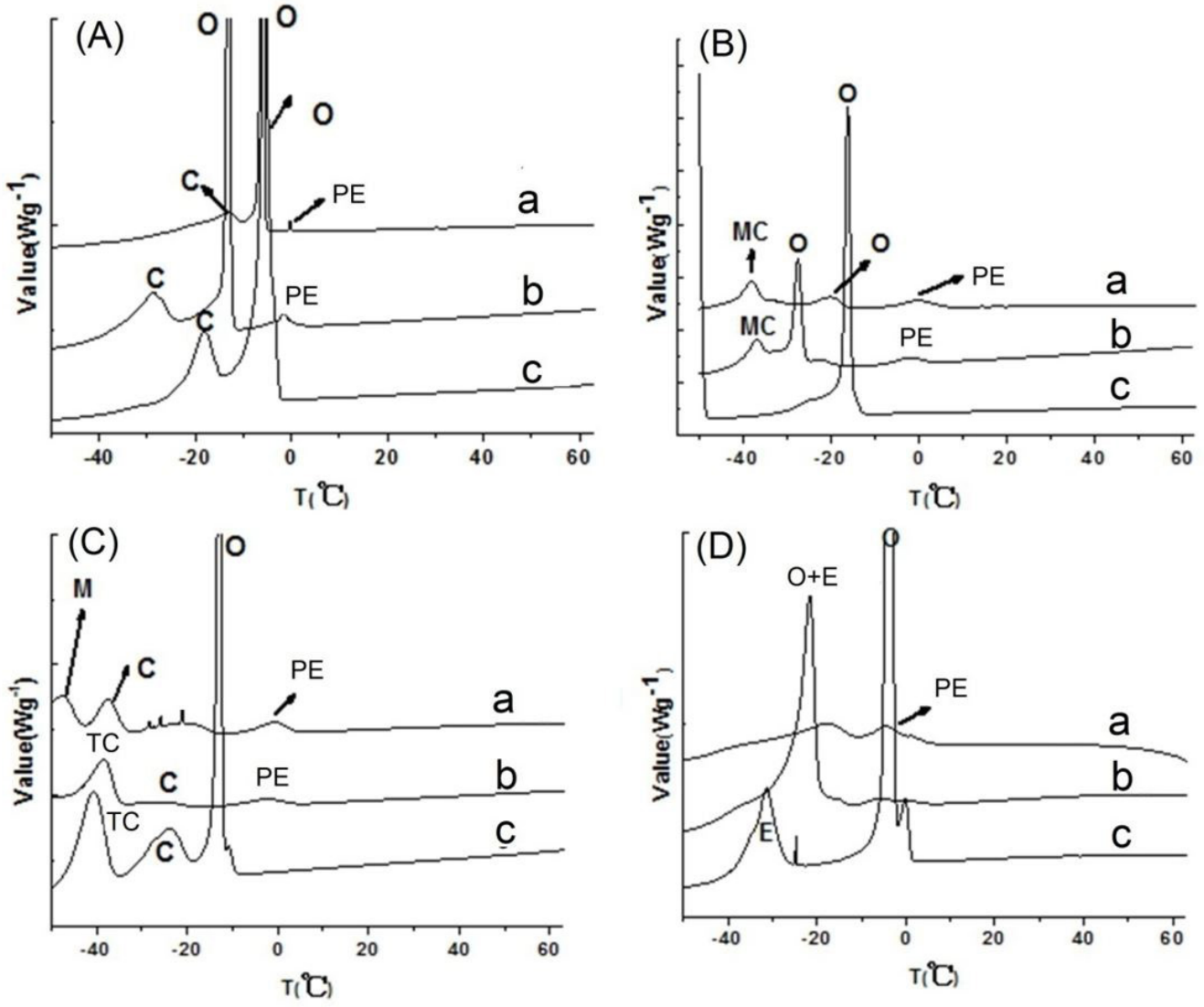


**Figure 2.** Crystallization curves of different components analyzed by DSC. (A) octanoic acid as crystallization solvent (a: $n_{octanoic\ acid}$: $n_{esterification\ product}$ = 4: 1; b: $n_{oleic\ acid}$: $n_{octanoic\ acid}$: $n_{PE\ standard}$ = 1: 4: 1; c: $n_{oleic\ acid}$: $n_{octanoic\ acid}$ = 1: 4); (B) methyl caprylate as crystallization solvent (a: $n_{methyl\ caprylate}$: $n_{esterification\ product}$ = 1: 1; b: $n_{oleic\ acid}$: $n_{methyl\ caprylate}$: $n_{PE\ standard}$ = 1: 1: 1; c: $n_{oleic\ acid}$: $n_{methyl\ caprylate}$ = 1: 1); (C) triglyceride caprylate as crystallization solvent (a: $n_{triglyceride\ caprylate}$: $n_{esterification\ product}$ = 2: 1; b: $n_{triglyceride\ caprylate}$: $n_{PE\ standard}$ = 1: 2: 1; c: $n_{oleic\ acid}$: $n_{triglyceride\ caprylate}$ = 1: 2); (D) ethyl acetate as crystallization solvent (a: $n_{ethyl\ acetate}$: $n_{esterification\ product}$ = 9: 1; b: $n_{oleic\ acid}$: $n_{ethyl\ acetate}$: $n_{PE\ standard}$ = 1: 9: 1; c: $n_{oleic\ acid}$: $n_{ethyl\ acetate}$ = 1: 9). O: oleic acid; PE: phytosterol esters; C: octanoic acid; MC, methyl caprylate; TC, triglyceride caprylate; E: ethyl acetate.

was used as crystallization solvent, the recovery rate was more than 100%, so it was not appropriate.

From the separation effect of crystals, under the same solvent-PE molar ratio, the best separation effect was obtained when octanoic acid was used as crystallization solvent. This indicated that, the solubility of PE in octanoic acid varied greatly with the temperature change. Using octanoic acid as crystallization solvent and 0 °C as crystallization temperature, the specific crystallization conditions, PE recovery rate and PE purity in crystals were shown in Table 2. When the solvent-PE molar ratio was 4: 1 and crystallization time was 10 h, the PE recovery rate was 97.8%, and the PE purity in crystals was 98.6%.

### *3.4 Crystallization purification results of esterification product*

Based on the optimal crystallization conditions (crystallization temperature, 0 °C; solvent-product molar ratio, 4: 1; crystallization time, 10 h) obtained in above experiments, using relatively green and safe octanoic acid as solvent, the crystallization purification of esterification product was performed. The esterification products before and after crystallization purification were analyzed by DSC (Figure 3). Results showed that, compared before crystallization purification, the crystallization peak of free fatty acids disappeared after purification. This further confirmed that, the crystallization using octanoic acid as solvent could

**Table 2.** Crystallization effect of PE in octanoic acid under 0 °C.

| $n_{octanoic\ acid}$: $n_{PE}$ | Crystallization time (h) | PE recovery rate (%) | PE purity (%) |
|---|---|---|---|
| 10: 1 | 13 | 81.0 | 85.2 |
| 9: 1 | 12 | 83.8 | 86.7 |
| 8: 1 | 12 | 87.6 | 90.1 |
| 7: 1 | 11 | 88.9 | 86.3 |
| 6: 1 | 10 | 90.1 | 93.2 |
| 5: 1 | 10 | 90.8 | 95.6 |
| 4: 1 | 10 | 97.8 | 98.6 |
| 3: 1 | 9 | 92.3 | 93.8 |
| 2: 1 | 8 | 88.9 | 95.3 |
| 1: 1 | 8 | 84.9 | 92.7 |

PE, phytosterol esters.

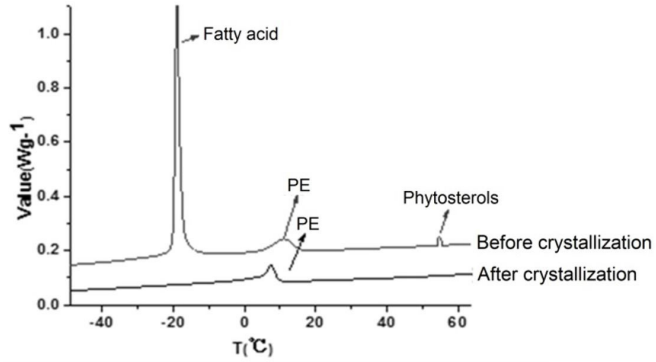


**Figure 3.** DSC analysis of esterification product before and after crystallization purification.

effectively remove the unreacted fatty acids and other impurities in esterification product, and achieve the high-purity recovery of PE. The gas chromatography showed that the PE purity in purification product was 98.2%.

## 4 Conclusion

In conclusion, PE has been successfully synthesized by catalysis with CuO-NHAP. After RSM optimization, the optimal process parameters are as follows: reaction temperature, 180 °C; reaction time, 6.3 h; acid-alcohol molar ratio, 1.9; catalyst amount, 1.05%. Under these conditions, the esterification rate of phytosterols is 99.8%. The crystallization purification of esterification product has been explored. The optimal crystallization conditions are as follows: crystallization solvent, octanoic acid; crystallization temperature, 0 °C; solvent-product molar ratio, 4: 1; crystallization time, 10 h. Under these conditions, the unreacted fatty acids and other impurities are affectively removed, and the PE purity in purification product is 98.2%. These synthesis and crystallization purification technologies of PE are relatively green, safe, efficient and economic, and have the potential to be applied to the industrial preparation of PE product.

## Conflict of interest

The authors declare that there is no conflict of interest.

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