Optimization of \( n \)-Hexane-Acetone System for Extraction of Phosphatidylcholine and Phosphatidylethanolamine by Response Surface Methodology

Chunling Huang and Dong Cao* 

School of Food Science and Technology, Jiangnan University, 1800 Lihu Road, Wuxi 214122, Jiangsu province, P.R.CHINA

Abstract: Eggs are nutritious and cheap and easily available. Egg yolk is one of the sources of phosphatidylcholine (PC) and phosphatidylethanolamine (PE). PC and PE have good emulsifying properties, and they are widely used and in high demand for pharmaceutical, feed and cosmetic applications. Red cordyceps egg yolk powder (RCEYP) was selected as the raw material to obtain high content of PC and PE by ethanol extraction and low temperature cryoprecipitation in \( n \)-hexane-acetone system (HAS), in which the process conditions of PC and PE extraction by HAS process were optimized. The phospholipids were quantified by high performance liquid chromatography (HPLC) with evaporative light scattering detector (ELSD). The effects of freezing time, material-liquid ratio, acetone washing times, solvent ratio of \( n \)-hexane to acetone and freezing temperature on the PC and PE contents and the phospholipid yield were investigated. The optimal conditions for the extraction of PC and PE from RCEYP by HAS were determined by Box-Behnken design (BBD) as follows: the solvent ratio of \( n \)-hexane to acetone was 1:6, the freezing time was 11.31 h, and the freezing temperature was \(-19^\circ C\). The total content of (PC+PE) in the phospholipids precipitated under these conditions amounted to 96.16%, of which 81.12% was PC and 15.04% was PE.

Key words: phosphatidylcholine, phosphatidylethanolamine, extraction, optimization, Box-Behnken design

1 Introduction 

Lecithin is a negatively charged mixture of phospholipids (PLs). It is an attractive research material because of its low cost, wide source and green safety, and it is widely used in food processing, medicine and health care, feed industry and cosmetics, etc. Poultry eggs are one of the sources of PLs. There are differences in the phospholipid composition of different types of egg yolks. The phospholipid composition of hen eggs, pigeon eggs and goose eggs have advantages compared to other types of eggs. Hen eggs, in particular, are a good choice in terms of taste and price. Similarly, the chemical composition of hen eggs differs between genotypes, and the composition and total content of PLs, sterols and fatty acids in their yolks can vary. The protein content of the egg yolk is 16.28% to 17.85%, the moisture content is 45.87% to 46.82%, and the fat content is 22.9% to 34.0%. The fat composition of egg yolk is mainly composed of triglycerides, PLs and sterols.

The PLs contained in egg yolk include phosphatidylcholine (PC), phosphatidylethanolamine (PE), lysophosphatidylcholine (LPE), lysophosphatidylcholine (LPC), sphingomyelin (SM), and phosphatidylglycerol (PG). Of these, PC and PE are the main PLs in egg yolk. PC and PE are also the material basis for the emulsion membrane of fat milk, and changing the composition of the lipid membrane can have an effect on its properties. Changes in the PC/PE ratio of egg yolk PLs can have an effect on the sterilization stability of fat emulsions. In addition, high purity PC and PE are used as emulsifiers, excipients, fillers and drug carriers. In addition to their basic functions as nutritional additives in food, PC and PE are useful for liver damage, cardiovascular diseases, cancer prevention, immune regulation, and signaling. Based on the above properties and functions, the extraction and application of PC and PE have been studied by a wide range of scholars.

The extraction methods of PC and PE mainly include organic solvent extraction, supercritical extraction, column chromatography, membrane separation and other methods. The organic solvents commonly used in organic solvent extraction include hexane, acetone, chloroform, ethyl acetate, and methanol.
traction are ethanol, ether, chloroform, n-hexane, acetone, etc. Generally, there will be two or more solvents of different polarities or a combination of different methods for extraction\(^{21,28,29}\). These methods are usually based on fresh eggs, since dried egg yolk preparations contain both LPC and the corresponding free fatty acids (FFA). It is much easier to purify PC and PE from fresh eggs than dried eggs of different purity\(^{30}\). The extraction of PC and PE by organic solvent extraction is a classical and commonly used method in which the lipids are first extracted with ethanol and then precipitated by a mixture of n-hexane and acetone at low temperature to obtain high contents of PC and PE. However, there are few reports on the extraction of phosphatidylcholine and phosphatidylethanolamine using n-hexane and acetone two-solvent systems, and the influencing factors and the interaction between factors are not clear, requiring further study.

In this study, the above method was improved and named as “n-hexane-acetone system (HAS)”. The influencing factors of HAS were studied in detail for the first time, and the process conditions (solvent ratio of n-hexane to acetone, freezing time, and freezing temperature) were optimized by response surface analysis (RSM). A theoretical basis was provided for the variation of PC and PE contents under different conditions to obtain lecithin with high PC and PE contents.

2 Materials and Methods

2.1 Materials

E70 egg yolk powder (E70EYP) was purchased from Guangzhou Baiyunshan Hanfang Modern Pharmaceutical Co. Ltd (Guangzhou, China) and stored at \(-20^\circ\text{C}\). Grass eggs and red cordyceps eggs were purchased from a local supermarket in Wuxi City, Jiangsu, China. Acetone, anhydrous ethanol and other common reagents were of analytical grade, purchased from Sinopharm Group (Beijing, China). Methanol, isopropanol and n-hexane were of HPLC grade, and purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China).

2.2 Extraction of PC and PE from three egg yolk powders

In the first step, the egg yolk ingredients were screened. The egg shells of grass eggs and red cordyceps eggs were carefully broken, and the egg whites and yolks were separated with an egg separator, keeping the yolks. The egg yolks were stirred well and dried in a vacuum drying oven, thus obtaining grass egg yolk powder (GEYP) and red cordyceps egg yolk powder (RCEYP). The method was improved here on the basis of the method described by Gladkowska et al.\(^{10}\). PC and PE were extracted from three egg yolk powders using the improved method. Briefly, Dissolve E70/Grass/Cordyceps egg yolk powder (5 g) fully in anhydrous ethanol and mix with an electric mixer for 20 min with shear stirring. After stirring, the mixture was filtered and the precipitate was extracted again with anhydrous ethanol. The ethanol extracts were combined and the solvent was evaporated in a rotary evaporator at 50°C. The remaining solute was dissolved with n-hexane, followed by the addition of room temperature acetone. The mixed solution was frozen at \(-20^\circ\text{C}\) for 20 h to precipitate the phospholipids. The solvent was removed and the precipitate was washed repeatedly with acetone (\(-20^\circ\text{C}\) until the solvent became clear and transparent. The phospholipids obtained after the above steps were dried in vacuum and analyzed for PC and PE content in phospholipids by high performance liquid chromatography (HPLC). The yield of the final egg yolk phospholipid sample was determined using equation (1):

\[
\text{Yield}(\%) = \frac{W_1}{W_2} \times 100
\]

Where \(W_1\) is the weight of the dried egg yolk phospholipids obtained after removal of acetone and \(W_2\) is the weight of egg yolk powder before extraction\(^{31}\).

2.3 SFE optimization

After the screening of egg yolk raw materials, further single-factor experiments were conducted on the HAS. The effect of freezing time (5, 10, 15, 20, 25 h), material-liquid ratio (1:2, 1:3, 1:4, 1:5, 1:6), acetone washing times (1, 2, 3, 4, 5, 6), solvent ratio of n-hexane to acetone (1:3, 1:4, 1:5, 1:6, 1:7), and freezing temperature (4, \(-7\), \(-15\), \(-20\), \(-25^\circ\text{C}\)) on simultaneous extraction of PC and PE from egg yolk powder were studied.

2.4 Quantitative analysis of PC and PE by HPLC-ELSD

The PC and PE contents of the sample were quantified by HPLC equipped with 1525 Binary system (Waters, Milford, MA, USA) and a 3300 evaporative light scattering detector (ELSD, Alltech, Lexington city, Kentucky state, USA), the nitrogen flow rate and drift tube temperature of the ELSD were separately set as 1.8 L/min and 72°C, respectively. Nitrogen was the nebulizing gas. The column used as stationary phase was a silica column (250 nm × 4.6 mm, 5 μm, Yueze, Huai’an City, Zhejiang Province, China). The column was kept at 40°C. Samples and standards were eluted under a gradient program using two mobile phases. Phase A was methanol/water/glacial acetic acid/triethylamine (85:15:0.45:0.05, by vol), and phase B was n-hexane/isopropanol/phase A (20:48:32, by vol). The gradient elution program was designed as follows: 90-70% B for 0-20 min; 70-5% B for 20-35 min; 5-90% B for 35-36 min; 90-90% B for 36-40 min. The total run time was 40 min at a flow rate of 1 mL/min. Each sample was fully dissolved in chloroform/methanol (2:1, v/v), filtered through a 0.22 μm filter to eliminate particles, and injected at a volume of 10 μL. Standard curves were plotted using known standard...
PC and PE concentrations and the resulting curves were used for the analysis of PC and PE fractions.

2.5 Response surface design
Based on the experimental results of single factor, the phospholipid extraction conditions of the HAS were further optimized using RSM to obtain the highest total (PC + PE) content. Three independent variables, including solvent ratio of $n$-hexane to acetone ($X_1$), freezing time ($X_2$), freezing temperature ($X_3$), were investigated. The coding levels and value ranges of the three variables are shown in Table 1. A total of 17 runs (Table 2) of the three variables, including five center points, were designed by Box-Behnken design using Design-Expert software 8.0.6 (Stat-Ease, Inc., USA). Total (PC + PE) content was specified as the response value. According to the experimental design, each experiment was executed and then the observed values of the output response [i.e., (PC + PE) total content] were input in the software for fitting a statistical model. The statistical model for the three independent variables can be written as $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \epsilon$, where $Y$ was the total content of (PC + PE), $X_1$ was the solvent ratio of $n$-hexane to acetone, $X_2$ was the freezing time, and $X_3$ was the freezing temperature. The coefficients of the equations were determined by Design-Expert 8.0.6 using analysis of variance (ANOVA).

2.6 Statistical analysis
All experiments were performed in triplicate to ensure

| Table 1 | Factors and levels of response surface methodology (RSM). |
|---------|----------------------------------------------------------|
| Independent variable | Levels |
| $X_1$: Solvent ratio of $n$-hexane to acetone | $-1$ | $0$ | $+1$ |
| $X_2$: Freezing time | $1:6$ | $1:5$ | $1:4$ |
| $X_3$: Freezing temperature | $5$ | $10$ | $15$ |

| Table 2 | Experimental design and the results of the Box-Behnken Design (BBD). |
|---------|----------------------------------------------------------|
| Run | Solvent ratio of $n$-hexane to acetone ($X_1$) | Freezing time ($X_2$, h) | Freezing temperature ($X_3$, °C) | Total content of (PC+PE) (%) | Predicted response |
| 1 | $1:6$ (−1) | $5$ (−1) | $−20$ (0) | $92.15 \pm 1.91$ | $91.93$ |
| 2 | $1:5$ (0) | $10$ (0) | $−20$ (0) | $94.98 \pm 1.38$ | $95.22$ |
| 3 | $1:5$ (0) | $10$ (0) | $−20$ (0) | $95.06 \pm 0.59$ | $95.22$ |
| 4 | $1:4$ (+1) | $15$ (+1) | $−20$ (0) | $93.59 \pm 0.13$ | $93.81$ |
| 5 | $1:5$ (0) | $15$ (+1) | $−25$ (−1) | $91.27 \pm 1.19$ | $91.56$ |
| 6 | $1:5$ (0) | $15$ (+1) | $−15$ (+1) | $94.26 \pm 0.46$ | $93.75$ |
| 7 | $1:4$ (+1) | $10$ (0) | $−15$ (+1) | $94.31 \pm 2.05$ | $94.61$ |
| 8 | $1:6$ (−1) | $10$ (0) | $−25$ (−1) | $94.17 \pm 0.52$ | $93.87$ |
| 9 | $1:5$ (0) | $5$ (−1) | $−15$ (+1) | $90.72 \pm 1.75$ | $90.43$ |
| 10 | $1:5$ (0) | $10$ (0) | $−20$ (0) | $95.15 \pm 0.26$ | $95.22$ |
| 11 | $1:5$ (0) | $10$ (0) | $−20$ (0) | $96.03 \pm 1.65$ | $95.22$ |
| 12 | $1:6$ (−1) | $10$ (0) | $−15$ (+1) | $94.95 \pm 1.12$ | $95.46$ |
| 13 | $1:4$ (+1) | $10$ (0) | $−25$ (−1) | $90.75 \pm 0.42$ | $90.24$ |
| 14 | $1:4$ (+1) | $5$ (−1) | $−20$ (0) | $88.77 \pm 0.33$ | $88.76$ |
| 15 | $1:5$ (0) | $10$ (0) | $−20$ (0) | $94.86 \pm 1.96$ | $95.22$ |
| 16 | $1:6$ (−1) | $15$ (+1) | $−20$ (0) | $95.11 \pm 1.72$ | $95.22$ |
| 17 | $1:5$ (0) | $5$ (−1) | $−25$ (−1) | $86.14 \pm 1.32$ | $86.66$ |
the accuracy of the data. The results were expressed as means ± standard deviations. Significant differences were considered at $p<0.05$. Drawing software OriginPro 2019b (Origin Lab, USA) and Excel 2016 (Microsoft, USA) were used for data analysis.

3 Results and Discussion

3.1 Selection of raw materials

The percentages of PC and PE in the three egg yolk powders, E70EYP, GEYP and RCEYP, were shown in Fig. 1. The contents of PC and PE were different in all three ingredients. The results were in agreement with Ginka A Antova et al. Among the three raw materials, E70EYP had the highest PC content of 23.74%, but at the same time, it also had the lowest PE content of 4.22%. The PC (12.78%) and PE (4.8%) contents of RCEYP were higher than the PC (10.44%) and PE (4.35%) contents of GEYP. E70EYP was sourced from the supplier’s egg yolk powder, which was de-oiled at the factory and had a lower neutral lipid content than the laboratory’s homemade RCEYP and GEYP that had not been de-oiled. The PC and PE contents of the three raw materials after extraction by HAS were shown in Fig. 2. RCEYP presented one of the better experimental results with a PC content of 77.41% and a PE content of 14.45%, and the total content of (PC + PE) was higher than the other two raw materials at 91.86%. The PE (10.11%) content of E70EYP, on the other hand, is significantly lower than that of GEYP and RCEYP. The E70EYP was from the supplier, while the GEYP and RCEYP were both homemade yolk powder from fresh eggs made in the laboratory. This showed that the PC and PE content of the homemade RCEYP and GEYP were superior to that of E70EYP from the manufacturer. This agreed with Marcia A. da Silva et al. Therefore, RCEYP was the better choice. RCEYP was chosen as the raw material to continue the following experiments.

![Fig. 1](image1)

The three pie charts from left to right indicate the content of each component in E70EYP, GEYP, and RCEYP respectively. The “slash” area indicates the content of PE; the “white” area indicates the content of PC; and the “gray” area indicates the content of other components.

![Fig. 2](image2)

The three pie charts from left to right indicate the contents of each component in the samples extracted by the n-hexane-acetone system for E70EYP, GEYP, and RCEYP, respectively. The “slash” area indicates the content of PE; the “white” area indicates the content of PC; and the “gray” area indicates the content of other components.
3.2 SFE optimization

3.2.1 Effect of freezing time on simultaneous extraction of PC and PE

Figure 3A described the effect of freezing time on the yolk phospholipid yield and PC and PE contents during the egg yolk phospholipid precipitation extraction under the material-liquid ratio of 1:4, freezing temperature of $-20^\circ\text{C}$, the number of acetone washes of 3 times and the $n$-hexane to acetone solvent ratio of 1:5. The yield of egg yolk phospholipids gradually increased in the first 15 h with the extension of freezing time; after the freezing time exceeded 15 h, the yield tended to be stable. The content of both PC and PE fractions in the phospholipids obtained by precipitation extraction was lowest at a freezing time of 5 h, with 10.45% and 69.44%, respectively. The PE content reached a maximum at 15 h with 15.05%. The PC content increased significantly from 5 to 10 h, and the highest content was 81.3% at 10 h, when the total amount of (PC + PE) reached the maximum value of 92.77%. After that, the PC content and the total amount of (PC + PE) decreased slightly with the extension of freezing time. Interestingly, the ratio of PC/PE increased from 6.6 to 7.1 from 5 h to 10 h, which indicates that the relative increment of PC is greater than that of PE. This may be due to the fact that the sedimentation rate of PC is greater than that of PE during this time period. The content of each component of the precipitated extracts obtained at different freezing times varied, probably due to the different precipitation rate and precipitation order of the components such as PC, PE, LPC, LPE, SM. These substances have complex interactions with the HAS.

Fig. 3 In the figures (A), (B), (C), (D) and (E), the effects of freezing time, material-liquid ratio, acetone washing times, solvent ratio of $n$-hexane to acetone, and freezing temperature on the yolk phospholipid yield and PC and PE contents during the extraction of egg phospholipids from $n$-hexane-acetone system are shown, respectively. The “slash” bar indicates PE content; the “white” bar indicates PC content; the “black” bar indicates the total content of (PC + PE) fraction; and the broken line indicates the yield of yolk phospholipids.
3.2.2 Effect of material-liquid ratio on simultaneous extraction of PC and PE

The material-liquid ratio can often be an important influencing factor as well. Therefore, this study investigated the effect of the material-liquid ratio on the yield of egg yolk phospholipids and the content of PC and PE under the freezing temperature of \(-20^\circ\text{C}\), three acetone washes, 1:5 solvent ratio of \(n\)-hexane to acetone, and freezing time of 20 h. According to Fig. 3B, it can be seen that the yield of egg yolk phospholipids increased significantly with the increase of \(n\)-hexane and then leveled off. The lowest phospholipid yield of 5% was obtained when the ratio of ethanol extract mass to \(n\)-hexane dosage was 1:2. When the ratio was 1:4, the highest yield of 14.2% was obtained. When the ratio was from 1:2 to 1:6, both PC and PE contents increased and then leveled off. The total amount of \((\text{PC} + \text{PE})\) reached a maximum at 1:4 with 91.86%. Therefore, the amount of solvent was too small and the content of PC and PE in the precipitated phospholipids was low. The reason may be that when there is little solvent, the solution concentration is large, the viscosity increases, the diffusion coefficient is small, and the mass transfer resistance is large, thus not being in full contact with acetone, resulting in low yield and low PC and PE content. If the amount of solvent used is too large, the PC and PE content will not increase after reaching a certain value, leading to the waste of solvent. Therefore, 1:4 is a better choice for PC and PE extraction.

3.2.3 Effect of acetone washing times on the simultaneous extraction of PC and PE

The effects of acetone washing times on the yield of egg yolk phospholipids and the content of PC and PE were studied under the material-liquid ratio of 1:4, freezing temperature of \(-20^\circ\text{C}\), freezing time of 20 h and the \(n\)-hexane to acetone solvent ratio of 1:5. As shown in Fig. 3C, the yolk phospholipid yield gradually decreased with increasing the number of acetone washes. The PE content increased from 12.63% to 14.45% and the PC content increased from 67.74% to 77.41% when the number of acetone washes was from 1 to 3. The content of both increased slowly when the number of washes was from 3-6. When washed 6 times, the total amount of \((\text{PC} + \text{PE})\) reached the maximum value of 93.41% with a yield of 13.2%. During the experiment, we observed that the colour of the acetone solution gradually became lighter as the number of washes increased. The colour of the solution gradually changed from yellow to clarified and clear as it went from 1-4 times. This is due to the fact that the neutral lipids are decreasing, and therefore, the relative content of PC and PE in the yolk phospholipids obtained by precipitation increases. Considering the economic saving, the number of acetone washes of 5 times is more appropriate.

3.2.4 Effect of \(n\)-hexane to acetone solvent ratio on the simultaneous extraction of PC and PE

The effects of solvent ratio of \(n\)-hexane to acetone on the PC and PE contents were investigated under the material-liquid ratio of 1:4, freezing temperature of \(-20^\circ\text{C}\), freezing time of 20 h, and three times of acetone washing. The results showed that when the solvent ratios of \(n\)-hexane to acetone were 1:1 and 1:2, no precipitation was produced and the phospholipids remained dissolved in \(n\)-hexane. When the solvent ratio reached 1:3, the phospholipids started to precipitate. As shown in Fig. 3D, when the solvent ratio of \(n\)-hexane to acetone was changed from 1:3 to 1:7, the yolk phospholipid yield and PE content both increased and then decreased slightly. The PE content reached a maximum of 14.45% at 1:5; the PC content increased from 68.12% to 78.3%; and the total \((\text{PC} + \text{PE})\) content was highest at 1:6 (92.1%). Phospholipids such as PC and PE have different head groups, and they have different solubilities and different polarities in organic solvents. The precipitation rate and the precipitation mass of the solute were changed due to the increase in the volume of polar solvent and the polarity of the co-solvent during the solvent ratio from 1:3 to 1:7, thus affecting the precipitation of PC, PE, and other phospholipids. At the same time, the volume of \(n\)-hexane-acetone solvent system becomes larger, and the area contact between solute and solvent increases, which facilitates the dissolution of neutral lipids and phospholipid precipitation.

3.2.5 Effect of freezing temperature on the simultaneous extraction of PC and PE

In general, the temperature affects the solubility of the solute in the solvent. Thus, this study explored the influence of freezing temperature on the simultaneous extraction of PC and PE under the material-liquid ratio of 1:4, freezing time of 20 h, three acetone washes, and 1:5 solvent ratio of \(n\)-hexane to acetone. During the experiments, it was found that no precipitation was produced at temperatures of 10\(^\circ\text{C}\) and 15\(^\circ\text{C}\). Only when the temperature was lowered to a certain value, precipitation precipitated in the mixed solution. As can be seen from Fig. 3E, the phospholipid yield decreased with the increase of freezing temperature in the interval from \(-25^\circ\text{C}\) to \(-4^\circ\text{C}\). When the temperature was higher than \(-20^\circ\text{C}\), the yield decreased rapidly, and the lowest yield was obtained at \(-4^\circ\text{C}\). The total amount of \((\text{PC} + \text{PE})\) increased and then decreased significantly with the increase of temperature, and both PC and PE contents reached the highest value at \(-20^\circ\text{C}\). It can be seen that the solubility of free fatty acids, PC, PE, SM, LPC, LPE and other solutes in the \(n\)-hexane-acetone solvent system at low temperatures are different from each other, and they may affect and interact with each other, thus causing changes in phospholipid yields and PC and PE contents. It is noteworthy that the PC/PE ratio was gradually decreased during the increase of
−25°C to 4°C, which indicates that the temperature has a greater effect on the PC content. It may be possible to regulate the PC/PE ratio in the production of egg yolk phospholipids by controlling the temperature.

3.3 RSM optimization

The single-factor experiment could not reveal the interaction between the factors for the extraction of PC and PE, nor the optimal operating conditions for the extraction of phospholipids from the HAS, so the experimental conditions were further optimized using a Box-Behnken design model to obtain the maximum value of the total content of (PC + PE). Table 2 demonstrates the coded and actual values for three levels of three factors (solvent ratio of n-hexane to acetone, freezing time, and freezing temperature). Through multiple regression analysis, the prediction equation of the total content of (PC + PE) was obtained as follows:

\[
Y = 95.22 - 1.12X_1 + 2.06X_2 + 1.49X_3 + 0.46X_1X_2 + 0.7X_1X_3 - 0.4X_1X_2 + 0.068X_1^2 - 2.88X_2^2 - 1.74X_3^2 \tag{2}
\]

Where Y is the predicted response value for the total content of (PC + PE); \(X_1\), \(X_2\), and \(X_3\) denote the coded values for solvent ratio of n-hexane to acetone, freezing time, and freezing temperature, respectively.

Table 3 showed the results of the BBD analysis of variance (ANOVA) for the response surface model. The results revealed that the \(p\)-values of the model terms \(X_1\), \(X_2\), \(X_3\), \(X_1X_2\), \(X_1X_3\), \(X_2X_3\) and \(X_1^2\) were significant (i.e. \(p < 0.05\), while the \(p\)-values of \(X_1X_2\), \(X_1X_3\) and \(X_2^2\) were not significant. The \(p\)-value of the model was lower than 0.0001, with F-value of 37.88. This indicates that the model was highly significant. The \(p\)-value of lack-of-fit of the model was higher than 0.05, indicating that the lack-of-fit was not significant for the error, the experimental error and other factors had little effect on the response value, and the regression equation was a good fit. The coefficient of determination (R² = 0.9799) of the model indicated that the predicted value of the model had 97.99% reliability. The predicted coefficient of variation (Pre-R² = 0.7854) and the adjusted coefficient of variation (Adj-R² = 0.9540) of the model were in good agreement. The value of 19.748 for Adeq Precision indicated that the model could be used to navigate the design space. From Table 2, it was observed that the results of the predicted and observed values were very similar, so the model was applicable to the prediction of the total content of (PC + PE) in the phospholipids obtained by precipitation. Furthermore, according to the ANOVA results, freezing time had the greatest effect on the total content of (PC + PE), followed by freezing temperature and solvent ratio of n-hexane to acetone.

The interactions of solvent ratio of n-hexane to acetone (\(X_1\)) and freezing time (\(X_3\)) on the total (PC + PE) content were shown in Fig. 4A. The total (PC + PE) content gradually decreased when the solvent ratio gradually increased. Figure 4B depicted the interaction of solvent ratio of n-hexane to acetone (\(X_1\)) and freezing temperature (\(X_3\)) when the freezing time (\(X_3\)) was 10 h. The total (PC + PE) content increased and then decreased in the range of −25°C to −15°C. From Fig. 4C, it is known that the total content of (PC + PE) increased and then decreased during the extension of the freezing time from 5 h to 15 h. The optimal process conditions were obtained by Box-Behnken software analysis for a solvent ratio of n-hexane to acetone of 1:6, a freezing time of 11.31 h, and a freezing temperature of −19°C. Under these conditions, the total content of (PC + PE) was obtained as 34.91%.

Table 3 Analysis of variance (ANOVA) of response surface model.

| Source        | Sum of Squares | Df | Mean Square | F-value | p-value |
|---------------|----------------|----|-------------|---------|---------|
| Model         | 115.17         | 9  | 12.80       | 37.88   | <0.0001 |
| \(X_1\)       | 10.04          | 1  | 10.04       | 29.70   | 0.0010  |
| \(X_2\)       | 33.83          | 1  | 33.83       | 100.12  | <0.0001 |
| \(X_3\)       | 17.73          | 1  | 17.73       | 52.48   | 0.0002  |
| \(X_1X_2\)    | 0.86           | 1  | 0.86        | 2.56    | 0.1536  |
| \(X_1X_3\)    | 1.93           | 1  | 1.93        | 5.72    | 0.0481  |
| \(X_2X_3\)    | 0.63           | 1  | 0.63        | 1.87    | 0.2137  |
| \(X_1^2\)     | 0.020          | 1  | 0.020       | 0.058   | 0.8165  |
| \(X_2^2\)     | 34.91          | 1  | 34.91       | 103.31  | <0.0001 |
| \(X_3^2\)     | 12.74          | 1  | 12.74       | 37.70   | 0.0005  |
| Lack of Fit   | 1.49           | 3  | 0.50        | 2.28    | 0.2218  |
| Pure Error    | 0.87           | 4  | 0.22        |         |         |
| Cor Total     | 117.54         | 16 |             |         |         |

\(R^2 = 0.9799\), \(\text{Adj } R^2 = 0.9540\), \(\text{Adeq Precisior} = 19.748\)
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Fig. 4  Response surface plots of (PC + PE) fraction content in egg yolk. (A), (B) and (C) show the effects of solvent ratio of n-hexane to acetone and freezing time, solvent ratio of n-hexane to acetone and freezing temperature, and freezing time and freezing temperature on the content of (PC + PE) in egg yolk powder, respectively.

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Fig. 5  (A) indicates the high performance liquid chromatogram (HPLC) of RCEYP raw materials. Peaks: 1, PE (Rt = 4.424 min); 2, PC (Rt = 18.236 min); (B) indicates the experimentally obtained HPLC of RCEYP after response surface optimization. Peaks: 1, PE (Rt = 4.216 min); 2, PC (Rt = 16.874 min).

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(PE) was 96.69%. As shown in Fig. 5B (the retention times of PE and PC were 4.216 min and 16.874 min, respectively), the final experimental value of the total content of (PC + PE) in egg yolk phospholipids was 96.16%, with 81.12% of PC and 15.04% of PE. Figure 5A showed the high performance liquid chromatogram of RCEYP raw material. Comparing with Fig. 5B, it can be seen that after purification by HAS, the components in front of PE (triglycerides, free fatty acids, etc.) have been completely removed, and only PE and PC components and a little bit of other unknown substances remain in the figure, which are probably lysophospholipids and sphingolipids.

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4 Conclusion

Different PC and PE contents were observed in all three egg yolk powders. The homemade yolk powder from the laboratory (GEYP and RCEYP) was superior to the yolk powder provided by the manufacturer (E70EYP). Single-factor experiments and response surface optimization were performed for RCEYP. The results showed that freezing
time had the greatest effect on the total content of (PC + PE), followed by freezing temperature and solvent ratio of n-hexane to acetone. The HAS can achieve the extraction of high-purity lecithin with only a few simple organic solvents. The process is simple, easy to recover solvents, low cost, and has a high potential for industrialization. This study provides a basis for the preparation of high content PC and PE lecithin.

Author Contributions
The design and conduct of the study was the responsibility of all authors. Chunling Huang was responsible for conducting the experiments, data analysis and processing, and writing the paper. Dong Cao was responsible for guidance.

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Conflicts of Interests
All authors have no conflict of interest.

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