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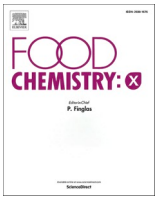

UPLC-MS based lipidomics analysis on optimization of soybean phosphatidylethanolamine extraction

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ABSTRACT

Soybean-derived phosphatidylethanolamine (PE) is a valuable phospholipid component yet its high-purity form is costly and its molecular structure is poorly understood. The present study combined solvent extraction and cryopurification to purify PE. The optimal extraction conditions were as follows: material-liquid ratio 1:15 (g/mL), ethanol base concentration 100:4 ($V_{anhydrous\ ethanol}$ /$V_{25\%\ ammonia}$), extraction temperature 40 °C, time 60 min, extraction twice. The cryopurification conditions were: material-liquid ratio 1:60, ethanol base concentration 100:6 ($V_{anhydrous\ ethanol}$/$V_{25\%\ ammonia}$), freezing temperature − 20 °C, time 20 h. UPLC-QTOF-MS/MS analysis revealed phospholipid composition of raw material, crude product, and purified product. The results showed that the purity of PE in the purified products was 76.74%, and the yield was 72.43% under optimal conditions. 181 phospholipid molecules were quantified. The study successfully explored high-purity PE preparation method and the composition of PE product. It provides a basis for the subsequent exploration of its biofunction and potential applications.

1. Introduction

Phosphatidylethanolamine (PE) is a type of phospholipid that is abundant in both prokaryotic and mammalian cells. It is a crucial component of cell membranes, accounting for 20% to 50% of the total phospholipids in cell membranes (Vance, 2018), and is essential for maintaining cell structure and function. Research has demonstrated that PE is involved in repairing nerve cell membranes (Che et al., 2018), promoting resilience to stress and ameliorating age-related cognitive decline in vulnerable populations (Schverer et al., 2020). Most of the human phosphatidylethanolamine (PE) comes from dietary sources, with a portion also produced through anabolism in the body (Gibellini & Smith, 2010).

Foods high in PE content include soybeans, egg yolks, animal livers, and marine foods. PE from different dietary sources varies in molecular composition and physiological activity (Wang et al., 2022; Xia et al., 2020; Yin et al., 2024; Zhu et al., 2023). Previous study demonstrated that eicosapentaenoic acid in the form of phosphatidylcholine and phosphatidylethanolamine (EPA-PC and EPA-PE) derived from sea cucumbers can reduce chronic inflammation in macrophages and adipocytes induced by a high-sugar and high-fat diet (Tian et al., 2020). Pentaenoic acid may be a promising therapeutic approach for treating chronic inflammatory diseases (Tian et al., 2020). The dietary intake of phosphatidylcholine and phosphatidylethanolamine forms of eicosanoids can act as antagonists against the inflammatory effects of high glycemic and fatty diets (Aldana-Hernández et al., 2021). Additionally, EPA-PE and EPA-PC were reported have the potential to reduce levels of phosphatidylserine (PS) containing docosapentaenoic acid (DPA) and increase levels of phosphatidylethanolamine and PS containing arachidonic acid (AA) in the cerebral cortex (C. Zhang et al., 2021). This could lead to the restoration of lipid homeostasis in demented mice to some extent, providing new therapeutic strategies and directions for dietary interventions in cognitively impaired patients. Furthermore, the impact of various sources of phospholipids, including soybean, egg, dairy products, and marine food, on the hepatic mitochondrial and endoplasmic reticulum of mice with nonalcoholic fatty liver disease induced

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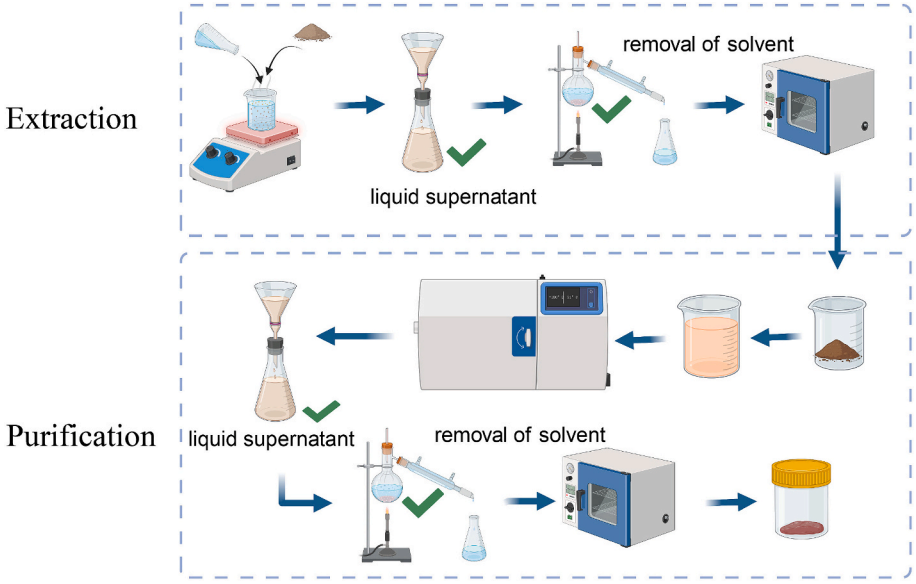

Fig. 1. PE Extraction and Purification Procedure.

by a high-fat and high-sugar diet were different (Zhang et al., 2022). The results showed that the effects of different dietary phospholipids on the phospholipid profile of liver mitochondria and endoplasmic reticulum in mice with non-alcoholic steatohepatitis were differentially increased by various dietary phospholipids, particularly by EPA-PE (Chen, Dong, Zhou, Xu, & Xiang, 2024). The high-sugar, high-fat diet induced structural changes in the membrane, which were counteracted by PE. This may contribute to the improvement of the morphology and function of the mitochondria and endoplasmic reticulum (Fan et al., 2023). PE is currently used in food and pharmaceutical applications, as well as for its high nutritional properties. Soy PE, in particular, has become a popular dietary phospholipid supplement due to its absence of cholesterol esters, lower cost, and richness in unsaturated fatty acids, which are beneficial to health (Nieuwenhuyzen & Tomás, 2010).

Currently, PE is obtained using various methods such as solvent extraction, enzyme catalysis, chemical synthesis, column chromatography, and supercritical extraction. Most phospholipids are soluble in organic solvents such as trichloromethane, ether, and ethanol, but insoluble in acetone. High purity PE can be isolated and purified by using soybean refined phospholipids as raw material and organic solvent extraction (W. Zhang, He, Feng, & Da, 2003). However, the single solvent extraction method yields a poor separation effect and the resulting product has a low PE content, despite being able to obtain naturally occurring PE. Cryocrystallization is a purification method that removes some neutral lipids and less polar inositol phospholipids by freezing crystallization at low temperatures (Nielsen & Shukla, 2004). Although it is more commonly used in industry for the purification of phosphatidylcholine (PC), it can also be used for other purposes. Other methods such as enzyme-catalyzed, column chromatography, and supercritical fluid extraction have high product yields but are costly and limited in production scale. Chemical synthesis, on the other hand, is cumbersome, polluting, and has more by-products. Therefore, to effectively meet market demand, and improve the added value of soybean resources, it is important to explore a simple process method suitable for large amounts of processing.

To enhance the effectiveness of extracting and purifying PE from soybean phospholipids, we optimized the process involving organic solvent extraction and cryopurification. Different PE molecular species can be derived due to the sn-1 and sn-2 of PE linking different fatty acids (Taladrid et al., 2017). Conventional HPLC methods are not suitable for analyzing the molecular composition of purified PE (Kato et al., 2018; Monakhova & Diehl, 2018; Rombaut, Camp, & Dewettinck, 2005). Instead, we used ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS) for further qualitative and quantitative analyses of the extracted PE (Wu et al., 2020). This method is highly sensitive and can accurately quantify trace substances. UPLC-MS-based lipidomics methods have been widely used in food processing and nutrition studies (Wen et al., 2023; J. Zhang et al., 2023; Zhao et al., 2022). These methods have been widely used to analyze the phospholipid composition of egg yolks and marine foods (Wen et al., 2023).

In this study, we optimized the experimental conditions of solvent extraction and cryopurification through completely-randomized designed experiments. We subsequently investigated the phospholipid molecular groups of raw materials, extraction products, and purified products using UPLC-MSMS-based lipidomics methods. This study integrated the extraction and purification protocols of soybean PE and characterized the molecular composition of the purified products. The results provide basic data on the composition of soybean phospholipids and support subsequent nutritional studies of soybean PE.

2. Materials and methods

2.1. Materials

Soybean powder phospholipids (PE 26.8%, PC 4.03%) were from Jiangsu Mann's Bio-technology Co., Ltd. (Kunshan, China). Anhydrous ethanol, 25% ammonia, trichloromethane, dichloromethane, anhydrous acetic acid, and triethylamine, all of which were analytically pure and obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Chromatographically pure reagents, including methanol, n-hexane, isopropanol, and acetonitrile were bought from Tianjin Kemiou Chemical Reagent Co., Ltd. (Tianjin, China). The phosphatidylethanolamine standard, with a purity of at least 98%, was obtained from Sigma-Aldrich Chemical Reagent Co., Ltd. (USA).

2.2. Extraction and purification of PE

The extraction and purification were performed as follows: Soybean powder phospholipids (raw materials) was weighed and alkaline ethanol was added. Among these, alkaline ethanol was prepared by adding different concentration of 25% ammonia (alkalis) into anhydrous ethanol. The mixture was heated at a constant temperature while stirring at a uniform speed for extraction. The resulting mixture was filtered and the supernatant was collected. The solvent was removed

Table 1
Completely randomized design of factors involved in the extraction stage.

Factors	Optimization Interval
material-liquid ratios (g/mL)	1:5, 1:10, 1:15, 1:20, 1:25, 1:30
concentrations of alkaline ethanol (*v*/v)	100:0, 100:1, 100:2, 100:3, 100:4, 100:5
extraction temperatures (°C)	25, 30, 35, 40, 45, 50
extraction time (min)	30, 40, 50, 60, 70, 80
numbers of extractions	1, 2, 3, 4, 5

Table 2
Completely randomized design of factors involved in the cryopurification stage.

Factors	Optimization Interval
Material-liquid ratios (g/mL)	1:20, 1:40, 1:60, 1:80, 1:100
Concentrations of alkaline ethanol (v/v)	100:2, 100:4, 100:6, 100:8, 100:10
Temperature (°C)	−10, -15, -20, -25, -30
Time (h)	8, 12, 16, 20, 24

using a rotary evaporator and the crude PE was obtained by drying the residue in a vacuum drying oven. The PE was added to alkaline ethanol and dissolved with ultrasound. The solution was then placed in a low-temperature freezer to freeze, and the resulting mixture was filtered to obtain the supernatant. The solvent was removed using rotary evaporation, and the remaining product was vacuum-dried at 50 °C to obtain the PE product (Fig. 1). The specific conditions involved in the extraction and cryopurification of PE were subsequently optimized in a completely randomized design. The concentration of PE (*w*/w, %) was determined using high-performance liquid chromatography (HPLC).

$$Yield\ of\ PE\ (\%) = \frac{Mass\ of\ purified\ product \times PE\ concentration\ of\ purified\ product}{Mass\ of\ raw\ materials \times PE\ concentration\ of\ raw\ materials} \times 100$$

2.3. Completely randomized design

For the purpose of facilitating the design and execution of parameter optimization experiments, completely randomized experiment was designed to explore the effect of extraction and purification parameters on PE contents and PE yield. The material-liquid ratios, concentrations of alkaline ethanol, extraction temperatures, extraction time and numbers of extractions were optimized at the extraction stage. The material-liquid ratios, concentrations of alkaline ethanol, temperatures, and times were optimized at the stage of cryopurification. PE contents (%) and yields (%, *w*/w) of purified product were used as test indexes. Tables 1 and 2 showed the experimental design. The detail information about the experimental numbers and parameters was supplemented in Table S1-S3.

2.4. HPLC quantification of PE

The PE concentration was analyzed using HPLC with a GL Sciences Inc. Si-60 column (250 nm × 4.6 nm × 5 μm). The mobile phases were n-hexane/isopropanol/methanol/water/anhydrous acetic acid/triethylamine in a ratio of 30:38:27.2:4.8:0.14:0.016 (*v*/v/v/v/*v*/v) with isocratic elution. The column temperature was maintained at 40 °C, and the flow rate was 1.0 mL/min. The PE product was injected into the HPLC using autosampler. The concentration of PE product was diluted to 0.2 mg/mL before sampling. The injection volume was 10 μL, and the detector was an evaporative light detector with a drift tube temperature of 72 °C and a nitrogen flow rate of 2 L/min. The PE standards were configured with a gradient concentration during injection. The quantification of PE was conducted with the external standard method. The HPLC calibration curve for PE was presented in Fig. S1, and the spike-and recovery for validation of PE extraction was shown in Table S4. As results, the PE quantification was accurate and reliable.

2.5. UPLC-QTOF-MS/MS analysis of phospholipid composition

The UPLC-QTOF-MS/MS based lipidomics analysis referred to reported method (Hu et al., 2021; Yu et al., 2024). 10 mg of the sample was mixed with 10 μL internal standard (10 μg/mL), and then added 2 mL of pre-cooled methanol in a 10 mL glass threaded centrifuge tubes to precipitate the protein at −20 °C for 12 h. After that, 2 mL of dichloromethane was added and vortexed for 1 h. Finally, add another 2 mL of dichloromethane. Mix 6 mL of ultrapure water and centrifuge. Take the lower layer and add 4 mL of dichloromethane for extraction. Repeat this step twice and combine the lower layers. Remove the solvent. The extracted lipids were resuspended in MeOH / $CHCl_3$ (50:50, *v*/v) and diluted to 1 mL. The diluent was then stored at −20 °C until UPLC-MS analysis.

Chromatography System was carried out with a UPLC 30 A system (Shimadzu Corporation, Kyoto, Japan) equipped with a Kinetex C18 column (100 mm × 2.1 mm, 2.6 μm) (Phenomenex) and a Security Guard pre-column of the same material (Phenomenex). The injection volume was 1 μL, column temperature was maintained at 60 °C, temperature of sample chamber at 4 °C, and the flow rate was 400 μL/min. Mobile phase A was composed of water/MeOH/ACN (1:1:1, v/v/v), and mobile phase B was the mixture of IPA/ACN (5:1, v/v), both containing 5 mM ammonium acetate. The gradient elution is as follows: 0–0.5 min, 20% B; 0.5–1.5 min, 40% B; 1.5–3 min, 60% B; 3–13 min, 98% B; 13–13.1 min, 20% B; 13.1–17 min, 20% B.

A TripleTOF 6600 system (AB Sciex, Concord, ON, Canada) equipped with an external calibrant delivery system (CDS) was used for mass spectrometry analysis. Information-dependent acquisition (IDA) was performed for MS/MS analysis in both positive electrospray ionization (ESI+) and negative electrospray ionization (ESI−) modes. In the positive-ion mode, MS parameters are as follows: the declustering potential was set to 80 V, the collision energy was set to 30 V, and the ion spray voltage was set to +5500 V with a mass range from 100 to 1200 *m*/*z*. Gas 1 and gas 2 of ion source were set to 50 psi. Curtain gas was set to 35 psi, and the interface heater temperature was set at 600 °C. In the negative-ion mode, the declustering potential was set to −80 V, the collision energy was set to −30 V, and the ion spray voltage was set to −4500 V with a mass range from 100 to 1200 m/z.

2.6. Statistical analysis

The statistical analysis of the results was conducted using the SPSS version 22.0 software package (IBM software, NY, USA). The differences between the groups were analyzed using one-way ANOVA or Student's *t*-test. One-way ANOVA allows for simultaneous comparison of multiple groups to determine whether significant differences exist. Student's t-test is used for comparing the means of two distinct groups to assess whether the differences between two groups are statistically significant. A *p*-value of <0.05 was considered statistically significant. All measurements were repeated in three times unless otherwise specified.

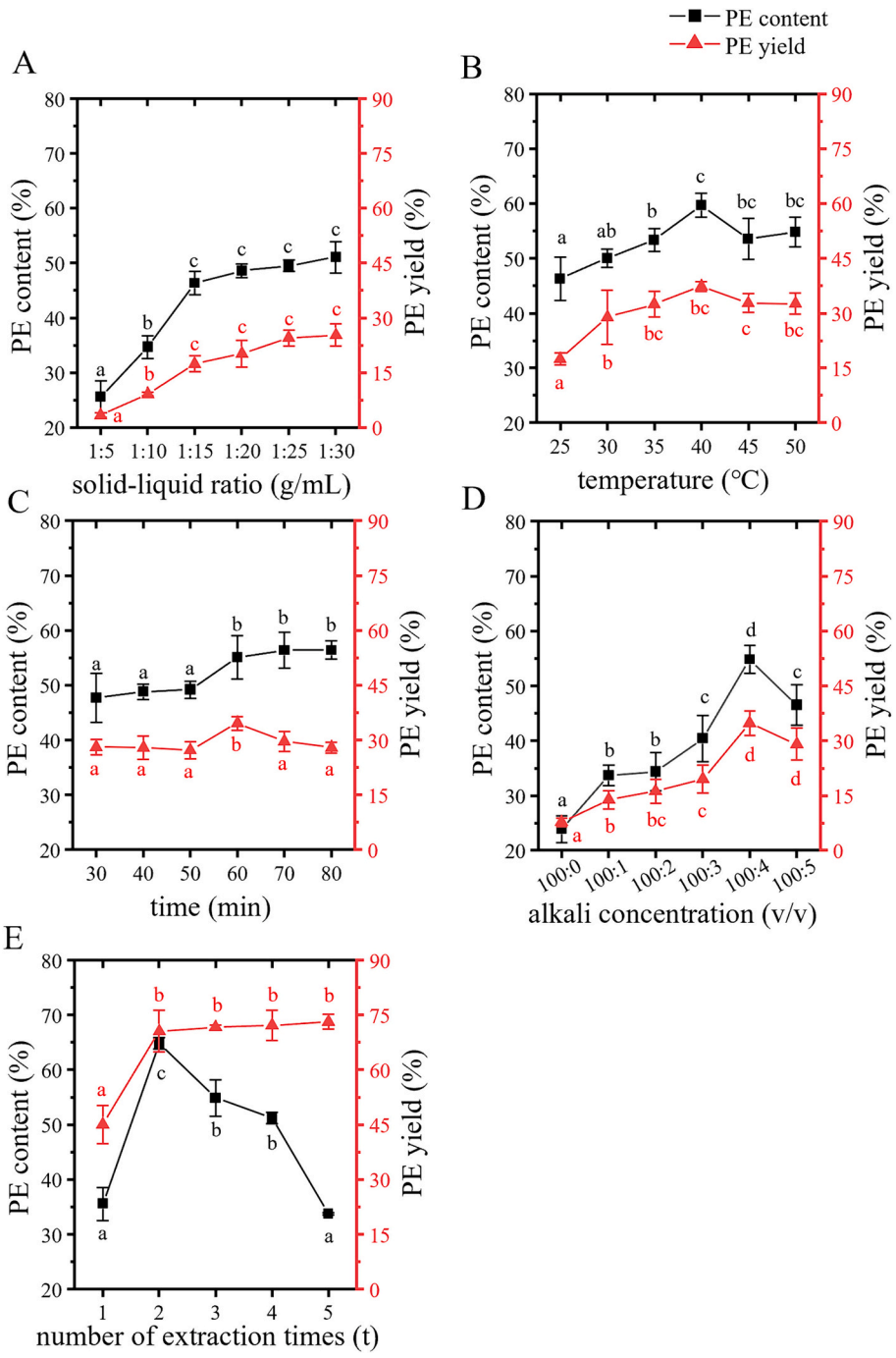

Fig. 2. Content and yield of PE under different extraction conditions. (A)Extraction sold-liquid ratio. (B)Extraction temperature. (C)Extraction time. (D) Alkali concentration ($V_{anhydrous\ ethanol}$: $V_{25\%\ ammonia}$). (E) Number of extractions. Data were expressed as the mean ± standard deviation.

3. Results and discussions

3.1. Effect of different factors on PE content and yield in extracts

It was found that solid-liquid ratio, extraction temperature, extraction time, alkali concentration and times of extraction have an effect on the yield and purity of PE in pre-experiments. We hypothesized that the yield and purity of PE would increase gradually with an increase in the solid-liquid ratio, extraction temperature, extraction time, alkali concentration, and the number of extractions. To validate the speculation, we carried out the completely randomized design. Fig. 2 displayed the impact of various factors on the content and yield of PE in the extract. As shown in Fig. 2A, the extract's PE content and yield raised gradually with the increase of solid-liquid ratio. Specifically, increasing the volume of extraction solvent in the range of 1:5 to 1:15 had a positive effect on the dissolution of phospholipids, as evidenced by a significant increase in PE content. However, the increasing trend of PE content began to slow down when the solid-liquid ratio exceeded 1:15. This might result from that the solubility of PE in the extraction solvent became saturated when a certain amount was reached. As the volume of the solvent further increased, more impurity components dissolved, which relatively reduced the PE content in the extract.

The PE content and yield increased as the extraction temperature rose (Fig. 2B). At 40 °C, the PE content reached its maximum value due

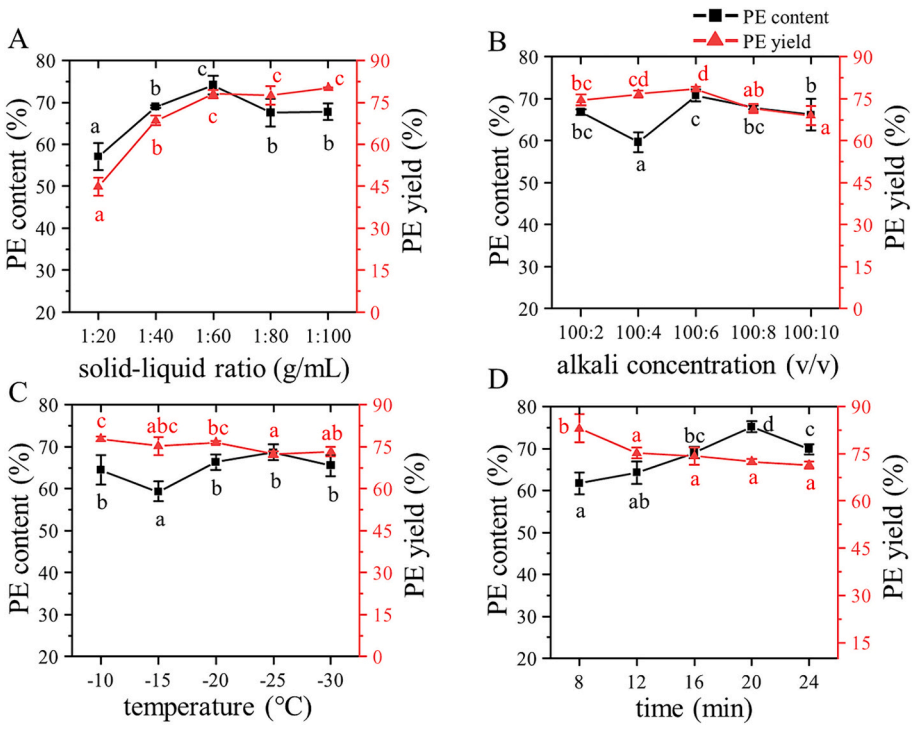

Fig. 3. Content and yield of PE under different freezing conditions. (A) Sold-liquid ratio in freezing. (B) Alkali concentration of frozen solution ($V_{anhydrous\ ethanol}$: $V_{25\%\ ammonia}$). (C) Freezing temperature. (D) Freezing time. Data were expressed as the mean ± standard deviation.

to the improved solubility of PE in the extraction solution. However, further increases in temperature did not result in higher PE content. This was because at higher temperatures, the alkaline alcohol solubles became more difficult to transfer from the agglomerated phospholipids into the extracted liquid phase, which limited further enhancement of PE content. It concluded that 40 °C was the optimal temperature for maximizing the PE content in this extraction process.

The content and yield of PE showed a slow increasing trend with the extension of extraction time (Fig. 2C). During the first 30 min, PE did not reach solubility under the current extraction conditions, resulting in an increasing stage for its content and yield. As time was further extended to 50 min, the content of PE tended to approach saturation under these conditions, indicating that the extraction efficiency was close to the maximum at this time. However, the yield of PE showed a decreasing trend after 60 min. This might be due to the fact that longer extraction times allow more non-PE components to enter the liquid phase, resulting in a decrease in the relative content of PE in the extract. It suggested that extending the extraction time beyond a certain point was not beneficial for the extraction of PE and might introduce more impurities.

The PE content in the extract appeared to increase and then decrease with the growth of concentration of alkaline ethanol (Fig. 2D). The PE content reached the maximum when the concentration of alkaline ethanol reached a ratio of 100:4 ($V_{anhydrous\ ethanol}$: $V_{25\%\ ammonia}$). However, as the base concentration was further increased, both the PE content and yield decreased. This might be due to the enhanced alkalinity of the ethanol solution, which not only facilitates the PE component to dissolve the alkaline ethanol phase but also increases selectivity of other components such as PC and phosphatidylinositol (PI). Therefore, the PE content is relatively lower.

As the number of extractions raised, the content of PE gradually decreased, while the yield of PE showed an increasing trend (Fig. 2E). It could be attributed to the fact that multiple extractions caused more PE to dissolve in the solvent, thus increasing the PE yield. However, as the number of extractions increased, not only PE was dissolved in the solvent, but other components were also gradually dissolved into the alkaline ethanol phase. The removal of these impurities caused a decrease in the relative content of PE in the extracts. Therefore, we determined the optimal extraction process conditions based on the factors of PE content and yield. These conditions were as follows: an extraction solid-liquid ratio of 1:15 (g/mL), an ethanol-base concentration of V (anhydrous ethanol): V (25% ammonia) = 100:4, an extraction temperature of 40 °C, an extraction time of 60 min, and two extractions. These conditions ensured the PE yield while maintaining a high PE content, achieving an optimal balance between extraction efficiency and purity. Since the PE content of crude PE quantified with HPLC was 30% to 60% which did not meet requirements (Fig. 2). The further purification was carried out.

3.2. *Influence of different factors on PE content and yield during cryopurification process*

The effects of various factors on the PE content and yield of products in the process of cryopurification were presented in Fig. 3. The results of a completely randomized design were analyzed. With the increase of the freezing material-liquid ratio, the PE content in the samples showed a tendency of increasing and then decreasing, and the PE yield increased (Fig. 3A). This might be due to the fact that some impurity components first reach saturation concentration at low temperature, and thus begin to precipitate to achieve the purpose of separating the impurity components. So, the PE content in the alkaline ethanol phase increases.

The solubility of other phospholipid components, such as PI, gradually enhanced with increasing alkali concentration in the ethanol solvent (Fig. 3B). This indicated that more of the other phospholipid components dissolved in the supernatant as the alkali concentration increased. At a concentration of 100:4, the solubility of phospholipids (including PE and PI) in the solvent phase was significantly reduced. This might be attributed to the that phospholipids have the lowest solubility at this alkaline concentration. Since these phospholipid components coexisted with PE in the solvent, the increase of other phospholipid led to a decrease in the relative content of PE in the solvent. Therefore, to keep a high PE content, the alkali concentration in the ethanol solvent should be controlled within a suitable range.

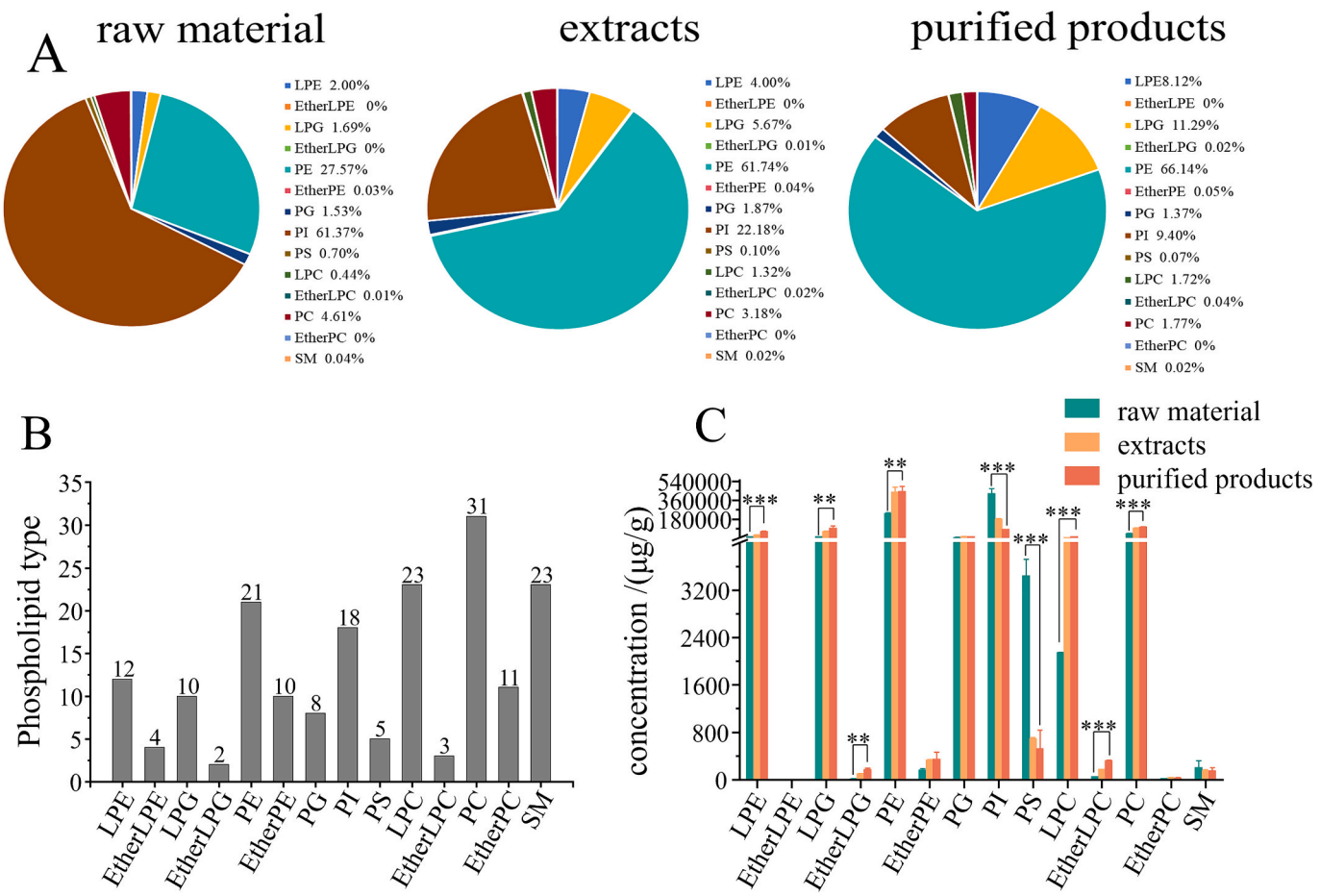

Fig. 4. Distribution of PL species and content in soybean phospholipids. (A) Composition of phospholipids from soybean powder phospholipid raw materials, extracts and purifications. (B) PL composition feature of products. (C) Content of different PLs. Data were presented as the mean ± standard deviation. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the raw material group.

During the cryopurification process, the freezing temperature had a significant effect on the rate of precipitation of PE with impurities (Fig. 3C). When the freezing temperature was reduced to −25 °C, the difference between the precipitation rates of PE and impurities reached the maximum. This indicated that PE and impurities could be separated most effectively at this temperature. However, as the PE content raised, the yield showed a decreasing trend. It might be due to the fact that as the precipitation of PE, some of the PE may not be collected effectively, resulting in a lower yield.

The extension of the freezing time also had an important effect on the separation of PE and impurity fractions (Fig. 3D). As the freezing time increased, the impurity fraction gradually crystallized and precipitated, causing the PE content in the supernatant to increase. When the freezing time reached 20 h, the impurity components might reach phase equilibrium, at which time the separation of PE from the impurity components reaches a maximum. However, if the freezing time is further extended, the PE in the solvent will also begin to precipitate, resulting in a decrease in both PE content and yield.

Considering both PE content and yield, we obtained the following optimal conditions for cryopurification: material-liquid ratio of 1:60, ethanol base concentration of V (anhydrous ethanol): V (25% ammonia) = 100:6, freezing temperature of −20 °C and freezing time of 20 h.

3.3. Validation experiments of optimal conditions for purified PE

The extraction conditions were as follows: material-liquid ratio 1:15 (g/mL), ethanol base concentration $V_{anhydrous\ ethanol}$: $V_{25\%\ ammonia}$ = 100:4, extraction temperature 40 °C, extraction time 60 min, extraction twice. The cryopurification conditions were as follows: material-liquid ratio 1:60, ethanol base concentration $V_{anhydrous\ ethanol}$: $V_{25\%\ ammonia}$ = 100:6, freezing temperature − 20 °C, freezing time 20 h. The extraction and purification of PE were validated under the aforementioned conditions. The purity of PE was 76.74% and the yield was 72.43%, which were close to the predicted results of the completely randomized design. This indicated that it was reasonable and feasible to carry out the extraction and purification of PE under the aforementioned conditions.

The efficacy of different extraction techniques varies. Colum chromatography was associated with the highest rate of PE extraction, although it was also costly and unsuitable for mass production. Our approach, however, did not yield the highest yield, but it offered the advantage of simple operation, minimal costs, and the potential for industrial production.

3.4. UPLC-MS based compositional analysis of phospholipid molecules

To characterize the phospholipid composition of the purified PE products, qualitative and quantitative detection of phospholipid molecules was performed using UPLC-QTOF-MS/MS technique. UPLC-QTOF-MS/MS is a technique that can achieve both highly accurate qualification and highly sensitive quantification simultaneously, particularly well-suited to the qualitative and quantitative analysis of lipid molecules in complex samples. It is a technique that is commonly employed in lipidomics analysis. As shown in Fig. 4A&B, a total of 181 phospholipid

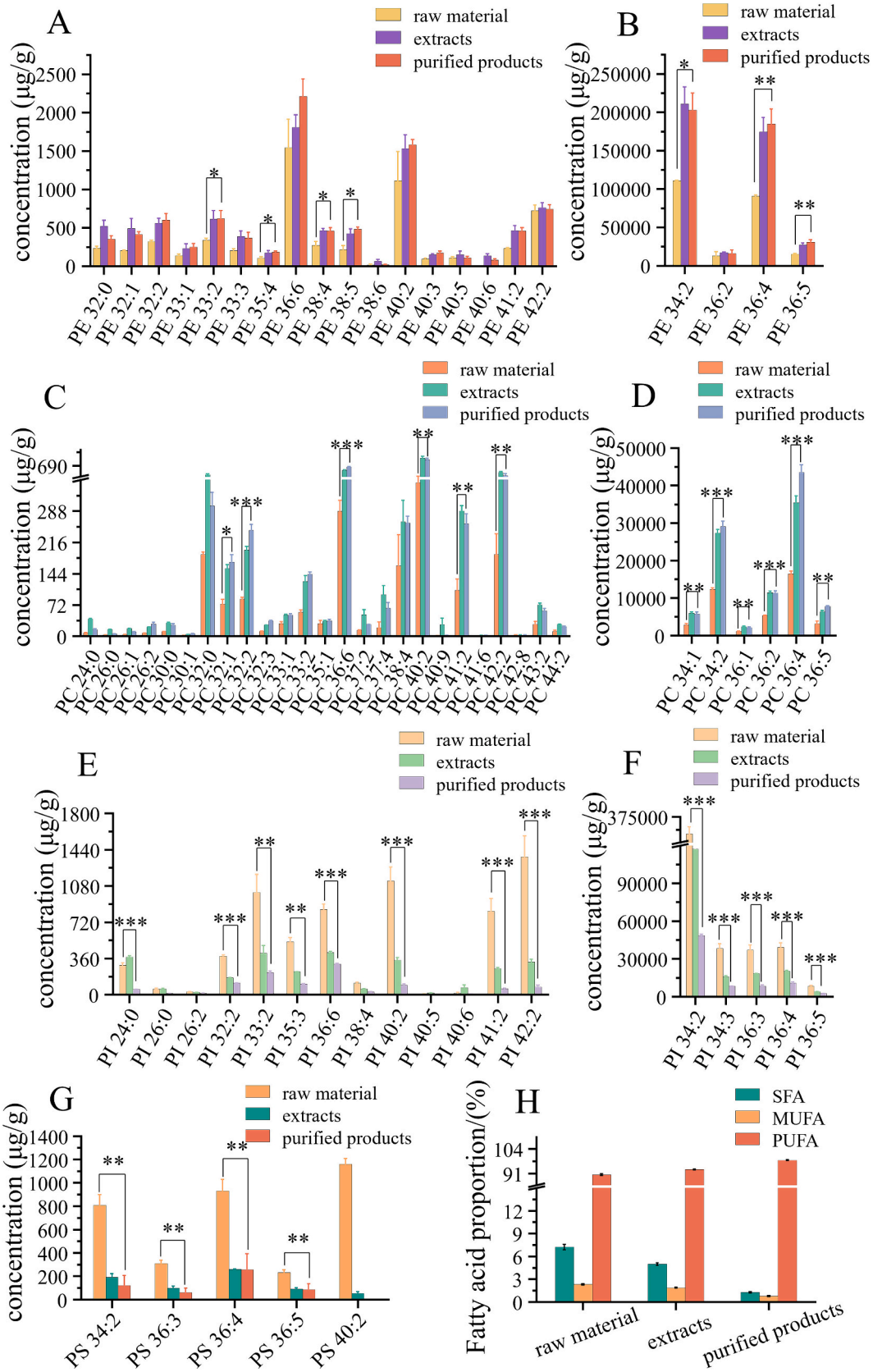

Fig. 5. The contents of PE, PC, PI and PS during the enrichment process (A-G). The composition of fatty acids in raw material, extracts and purified products. Data were presented as the mean ± standard deviation ($n = 3$). Comparative analyses were performed between groups "purified products" and "raw material" only. Significant differences were determined based on *t*-test analysis $p < 0.05$. * $p < 0.05$, ** $p < 0.01$ and, *** $p < 0.001$ compared to the raw material group.

molecules were identified in the raw, extracted, and purified samples of soybean powder phospholipids, including 21 PE, 31 PC, 18 PI, 8 PG, 5 PS, 12 lysophosphatidylethanolamine (LPE), 23 lysophosphatidylcholine (LP-C), 10 lysophosphatidylglycerol (LPG), and 23 sphingomyelins (SM). The results in Fig. 4C showed that the PE content in purified product was significantly enriched along with a significant increase in PC content and a significant decrease in PI and PS content compared to the raw materials and extracts. It suggested that a large.

amount of PI and PS could be selectively separated in the process of alkaline ethanol extraction combined with freeze purification. The PC and PE were mainly retained in the upper layer of the liquid phase, while PI and PS were mainly retained in the lower layer of the precipitate. Thus, it was hypothesized that PI and PS were indirectly enriched in the extraction residue, which also provided a reference for the separation and purification of PI and PS. In the final purified product, the purity of PE reached 76.74%.

The changes in the various types of phospholipids showed significant variability during sample preparation. The detailed qualitative and quantitative information for each phospholipid molecule was summarized in Table S5. According to the results shown in Fig. 5, it was found that the content distribution of PE lipid molecule subclasses had a clear hierarchy in the final purified product. Among them, the content of PE 34:2 (16:0/18:2) dominated as the most predominant component, closely followed by PE 36:4 (18:2/18:2), PE 36:5 (18:2/18:3) and PE 36:2 (18:0/18:2) (Fig. 5A&B). It is noteworthy that the content of PC was also significantly increased during the enrichment of PE, especially the content of PC 36:4 (18:2/18:2), followed by PC 34:2 (16:0/18:2) and PC 36:2 (18:0/18:2) in that order (Fig. 5C&D). On the contrary, the levels of PI and PS showed a significant downward trend, especially the levels of PI 34:2 (16:0/18:2) (Fig. 5E&F) and PS 36:4 (18:2/18:2) (Fig. 5G) were relatively high. The most abundant phospholipid molecules in the LPE, LPG and LPC subclasses were LPE 18:2, LPG 18:2 and LPC 18:2, respectively, whereas ether phospholipids and SM were generally found at low levels.

Wang etc. (Wang et al., 2022) investigated dynamics of the phospholipid profile of breast milk based on lipidomics, found that the number of phospholipid species detected in infant formula was significantly lower compared to breast milk. In particular, some key differential phospholipids, such as PC 36:2, PE 36:2, PE 38:4, PC 34:0, and LPC 18:0, were found at lower levels than in human milk. These differences in the type and content of phospholipids have a profound effect on lipid digestion,

absorption and metabolic processes in infants. Martinez-Ramirez (Martínez-Ramírez et al., 2016) revealed a positive correlation between SM levels and insulin resistance in overweight and obese individuals. This finding was further supported by animal experiments led by Masayuki Sugimoto et al. (Sugimoto et al., 2016) These experiments demonstrated that when mice with the sphingomyelin synthase gene knocked out were fed the same high-fat diet, their SM levels were significantly lower compared to wild mice. Remarkably, these knockout mice also exhibited improved glucose tolerance and insulin sensitivity. Therefore, phospholipids are closely related to health.

The PE enrichment process resulted in a significant increase in the percentage of polyunsaturated fatty acids (PUFA) in the phospholipids, which accounted for 90.48% (Fig. 5H). Meanwhile, monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) accounted for 2.30% and 7.22%, respectively. The proportions of SFA and MUFA also increased during the purification process, which may be attributed to the removal of other impurity fractions, resulting in an increase in their relative proportions. Furthermore, oxidation during the purification process may have impacted some of the PUFA, leading to a reduction in their relative proportions.

Previous report suggested that unsaturated fatty acids (UFA) are the primary components of soybean fat fractions that have functional health effects. Conversely, excessive intake of saturated fatty acids (SFA) is closely linked to an increased risk of cardiovascular diseases. SFA promotes hepatic synthesis of cholesterol in the body (Viñado, Castillejos, & Barroeta, 2019). This, in turn, increases the total cholesterol level in the serum, contributing to the increase in blood cholesterol, triglyceride, and LDL cholesterol levels. This might lead to the formation of atherosclerosis, thereby increasing the risk of cardiovascular and cerebrovascular diseases (Lenighan, McNulty, & Roche, 2019).

Soybean fatty acids vary in content, with linoleic acid highest, followed by oleic acid. Proportion of linolenic acid, palmitic acid, and stearic acid were relatively low (Ali et al., 2019). Research has shown that oleic acid associated with lower blood cholesterol levels (Spencer, Pantalone, Meyer, Landau-Ellis, & Hyten, 2003), linoleic acid was related with atherosclerosis formation, level of blood lipids and thrombosis (Whigham, Watras, & Schoeller, 2007). Linoleic acid and α-linolenic acid are essential fatty acids that cannot be synthesized by the human body and must be obtained through the diet. They play a crucial role in maintaining human health (Röhrig & Schulze, 2016). Scientific studies recommend increasing the intake of unsaturated fatty acids and decreasing the intake of saturated fatty acids to prevent cardiovascular diseases and improve endocrine functions (Patel et al., 2007). Thus, the nutritional value of the purified product is significantly increased through extraction and purification.

4. Conclusion

This study focused on the enrichment method of high-purity phosphatidylethanolamine (PE). The optimization of the PE extraction process was achieved through the combination of solvent extraction and cryopurification methods. We further analyzed the phospholipid composition of the PE product. The results showed that its main phospholipid class included PE 34:2 (16:0/18:2), PC 36:4 (18:2/18:2), LPE 18:2, LPC 18:2, and LPG 18:2. The unsaturated fatty acid proportion of the product was as high as 90.48%, and it was enriched with soy phospholipids containing a high content of essential fatty acids for the human body. These findings provide valuable reference data for further exploring the nutritional absorption and metabolic regulation mechanism of high-purity PE in the organism. They also aid in understanding the extraction and purification process of PE. Our research can provide a basis for in-depth study of the nutritional properties of dietary phospholipids and promote the development and application of related products.

CRediT authorship contribution statement

Boya Wang: Writing – original draft, Investigation, Data curation. **Siqi Wang:** Investigation, Data curation. **Zongyuan Wu:** Data curation. **Junbo He:** Data curation, Conceptualization. **Hong Lin:** Writing – review & editing, Funding acquisition, Conceptualization. **Weinong Zhang:** Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101578.

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