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# Purification of L-alpha glycerylphosphorylcholine by column chromatography

Kangyi Zhang, Xingguo Wang, Jianhua Huang, Yuanfa Liu*

*State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, 1800 Lihu Road, Wuxi 214122, Jiangsu Province, China*

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ABSTRACT

Colorless L-alpha glycerylphosphorylcholine (L-α-GPC) was obtained at 99.8% purity, 69.8% recovery, and with a specific rotation of −2.5° via a five-step procedure. L-α-GPC was first produced by phospholipase $A_1$ hydrolysis of soy lecithin powder. $Ca^{2+}$ and $Cl^-$ were then effectively removed using two successive 001 × 7 cation and D311 anion exchange resin column chromatography procedures. Silica gel column chromatography and decoloration with active carbon were then applied to remove remaining impurities and colorant. Characterization of the L-α-GPC product was well in agreement with the standard. The resin and silica gel showed remarkable ability for L-α-GPC isolation after 10 uses. Thus, this study presents a simple and cost-effective method for preparing L-α-GPC with high yield and purity, low cost, and environmental friendliness, and encourages future investigation into its adaptation for industrial applications.



## 1. Introduction

L-Alpha glycerylphosphorylcholine (L-α-GPC), comprised of choline, glycerol, and phosphate, is well known as the precursor for producing acetylcholine and phosphatidylcholine (PC) in the body [1,2], and for having important medical applications in neurological and psychiatric disorders of the human brain, such as Alzheimer's disease, cerebellar ataxia, schizophrenia, and bipolar affective disorder [3,4]. These potential, valuable uses for L-α-GPC have prompted exploration into its possible role in the brain. Unfortunately, L-α-GPC is scarce in natural sources, and thus research into the production of high-purity L-α-GPC would have important medicinal, social, economic, and research value.

To date, major methods in domestic and international research for preparing and purifying L-α-GPC include isolation from natural organ extracts [5], chemical synthesis and alcoholysis from egg or soy lecithin [6–8], solvent extraction [6], precipitation [9], recrystallization [10], and resin column chromatography [5]. However, these methods possess various difficulties in preparing and purifying L-α-GPC with suitable yield, purity, and environmental effects. For instance, solvent extraction is limited by raw material availability, which thus limits the scale of manufacturing, while chemical hydrolysis and alcoholysis present environmental challenges, and calcium precipitation and recrystallization have poor separation efficiency. Therefore, there is a great demand for a technologically useful and economical method for producing pure, high quality L-α-GPC.

An enzymatic preparation of L-α-GPC would be a very attractive alternative strategy for high efficiency, economic, and environmental friendliness. Phospholipase $A_1$ (Lecitase® Ultra enzyme) as one of the industry scale enzyme, with the lower cost, has been widely utilized in the food and pharmaceutical industries [11], and has potential application for enzymatic preparation of L-α-GPC on scale-up. Soy lecithin powder is a by-product of soy oil refining [12], which has been extensively used in food, nutrition compounding and emulsifiers [13], but application of developing high value-added pharmaceutical grade L-α-GPC is still minor.

However, the purity of enzymatic reaction product is not high and requires further purification. Owing to high adsorption specificities, facile desorption, mechanical strength, its low cost, and successfully application in the industrial refining and purifying of bioactive substances from natural resources [14–16], resin column chromatography is now used to remove ion residue. Moreover, silica gel column chromatography has major advantages such as its simple operation, high yield, reusability, and ability to separate chemicals with similar character and structure, such as isomers [17–19]. But to the best of our knowledge, no data has been reported regarding the performance of resin and silica gel column chromatography in purifying L-α-GPC.

The present work reports a detailed method for preparing and purifying L-α-GPC via five steps. L-α-GPC was first produced by phospholipase $A_1$ hydrolysis of soy lecithin powder, the calcium and chloride ions then removed using resin column

* Corresponding author. Tel.: +86 510 85876799; fax: +86 510 85876799.
*E-mail address:* yuanfa.liu@gmail.com (Y. Liu).

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chromatography, and the eluted product purified using silica gel column chromatography and activated carbon.

## 2. Experimental

### 2.1. Materials

Food-grade soy lecithin powder was kindly provided by East Ocean Oils & Grains Industries Co., Ltd. (Zhangjiagang, China). $CaCl_2$ (ultrahigh purity, certified >99.99%), L-α-GPC standards (specific rotation ($[\alpha]_D^{20}$) of −2.0° to 3.5°) and PC from soybeans and HPLC-grade chloroform and methanol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phospholipase $A_1$ (Lecitase® Ultra, 9500 U $mL^{-1}$) was a generous gift from Novozymes A/S (Bagsvaerd, Denmark). Silica gel for column chromatography (100–200 mesh, 3% moisture content) was provided by Qingdao Haiyang Chemical Co., Ltd. (Qingdao, PR China). Resin 001 × 7 and D311 were provided by Suqing Group (Jiangyin, PR China). All other reagents were analytical grade.

### 2.2. Enzymatic procedure of L-α-GPC

A 50 g weight of soy lecithin powder was placed in a three-necked flask with 500 mL deionized water and stirred to homogeneity at 300 rpm. Then, 2 g of $CaCl_2$ and 9500 U of phospholipase $A_1$ were added to the solution, incubated at 35 °C for 360 min, and the insoluble material removed from the mixture by centrifugation and vacuum filtration. The PC conversion and L-α-GPC yield were calculated by high performance liquid chromatography-evaporative light scattering detector (HPLC-ELSD) analysis.

### 2.3. L-α-GPC purification procedures

#### 2.3.1. Resin column chromatography

Pretreatment of ion exchange resins was conducted as previously reported [20]. In resin column chromatography, pretreated resins were suspended in water and packed individually into columns. The cation exchange resin was used to remove $Ca^{2+}$, and the anion exchange resin was used to remove $Cl^-$. Calcium ions and chloride ions were quantified as previously reported [21,22].

*2.3.1.1. Static adsorption assay.* Static adsorption tests were performed by placing 2 g (dry wt) of pretreated resin in a flask, adding 100 mL of enzyme reaction solution, and capping and shaking the flask in a 25 °C water bath at 120 rpm for 120 min until equilibrium was attained.

*2.3.1.2. Dynamic adsorption.* Based on the results of preceding tests, dynamic adsorption experiments were carried out in glass columns (1.6 cm × 50 cm) wet-packed with 4 g (dry wt) of pretreated resin and equipped with a constant-flux pump to control the eluent flow rate. Reactions solutions were applied to the column in 100 mL lots and pushed through at a flow rate of 1.5 mL $min^{-1}$.

*2.3.1.3. Calculation of adsorption capacity and adsorption ratio.* The following equations were used to quantify the adsorption capacity and adsorption ratio [16,23]. Adsorption capacity (Eq. (1)),

$$q_e = \frac{V_1(C_0 - C_e)}{W} \quad (1)$$

where $C_0$ and $C_e$ are the initial and equilibrium ion concentrations in the reaction solution (μg $mL^{-1}$), respectively; $V_1$ the solution volume (mL), and $W$ the resin weight (g). Adsorption ratio (Eq. (2)),

$$X(\%) = \frac{C_0 - C_e}{C_0} \times 100 \quad (2)$$

where $X$ is the adsorption ratio, defined as the percentage of quantity adsorbed at equilibrium relative to the initial, and $C_0$ and $C_e$ the same parameters as in Eq. (1).

For the next adsorption, the resin columns needed to be regenerated by removing adsorbed $Ca^{2+}$ and $Cl^-$ using HCl and NaOH, respectively.

#### 2.3.2. Silica gel column chromatography

In silica gel chromatography, 100 g silica gel was activated in a drying oven at 120 °C for 60 min, slurried in methanol, and packed into a column (3.0 cm × 50 cm).

L-α-GPC purification experiments were carried out in silica gel columns equipped with a constant-flux pump. The chromatography conditions were methanol as the eluent, loading of 20 mg $g^{-1}$ silica gel, loading concentration of 30 mg $mL^{-1}$, and flow rate at 2 mL $min^{-1}$. Eluate fractions were collected every 30 mL until no response for L-α-GPC was detected by HPLC-ELSD analysis, and fractions of the same peak combined. The combined L-α-GPC fractions were reduced to dryness in a rotary evaporator at 60 °C under vacuum.

The final L-α-GPC fraction was decolorized at 60 °C for 90 min to remove colorant and minor impurities. A weighed sample of the residue was taken and dissolved in methanol for HPLC-ELSD quantitative analysis. The purity and recovery of L-α-GPC were calculated according to the following equations:

$$P = \frac{n \times v}{w} \times 100\% \quad (3)$$

$$R = \frac{W \times P}{m_{GPC}} \times 100\% \quad (4)$$

where $P$ and $R$ represent L-α-GPC purity and recovery, respectively; $n$ the concentration determined by quantitative HPLC-ELSD analyses, $v$ the flask volume, $w$ the sample dry weight, $W$ the residue total weight, and $m_{GPC}$ the theoretical L-α-GPC yield (mg).

After each purification, silica gel was regenerated by heating at 150 °C for 3 h and reused.

### 2.4. HPLC-ELSD analysis

Samples were analyzed by HPLC-ELSD, using a Waters 1525 liquid chromatographic system (Waters Corp., Milford, MA, USA) equipped with a LiChrospher Si column (25 cm × 0.46 cm I.D., 5 μm particle size, Sigma–Aldrich Corp. K.K., Tokyo, Japan) at 35 °C, and eluted with a binary gradient of solvent A (methanol) and solvent B (methanol/water, 8/1, v/v) at 0.97 mL $min^{-1}$. Samples were applied as 5 μL injections and eluted with a linear gradient from 40 to 60% B (v/v) over 10 min, 60% B for 5 min, followed by a linear decrease to 40% B over 3 min. Resolved sample components were identified and quantified by comparison with peak retention times and calibration curves of standard compounds.

## 3. Results and discussion

### 3.1. HPLC-ELSD analysis

HPLC-ELSD profiles (Fig. 1A) of the chemical composition of initial enzymatic reaction solutions showed significant PC peaks and weak lysophosphatidylcholine (LPC) peaks. After 360 min of reaction, new peaks for L-α-GPC and glycerylphosphorylethanolamine (GPE) became clearly visible, while the PC peak intensity became very weak and the LPC peak undetectable (Fig. 1B), resulting overall in a 98% PC conversion and 94.5% L-α-GPC yield, which indicated that phospholipase $A_1$ was a very efficient catalyst for converting PC to L-α-GPC. The main reasons are that the PC *sn*-1

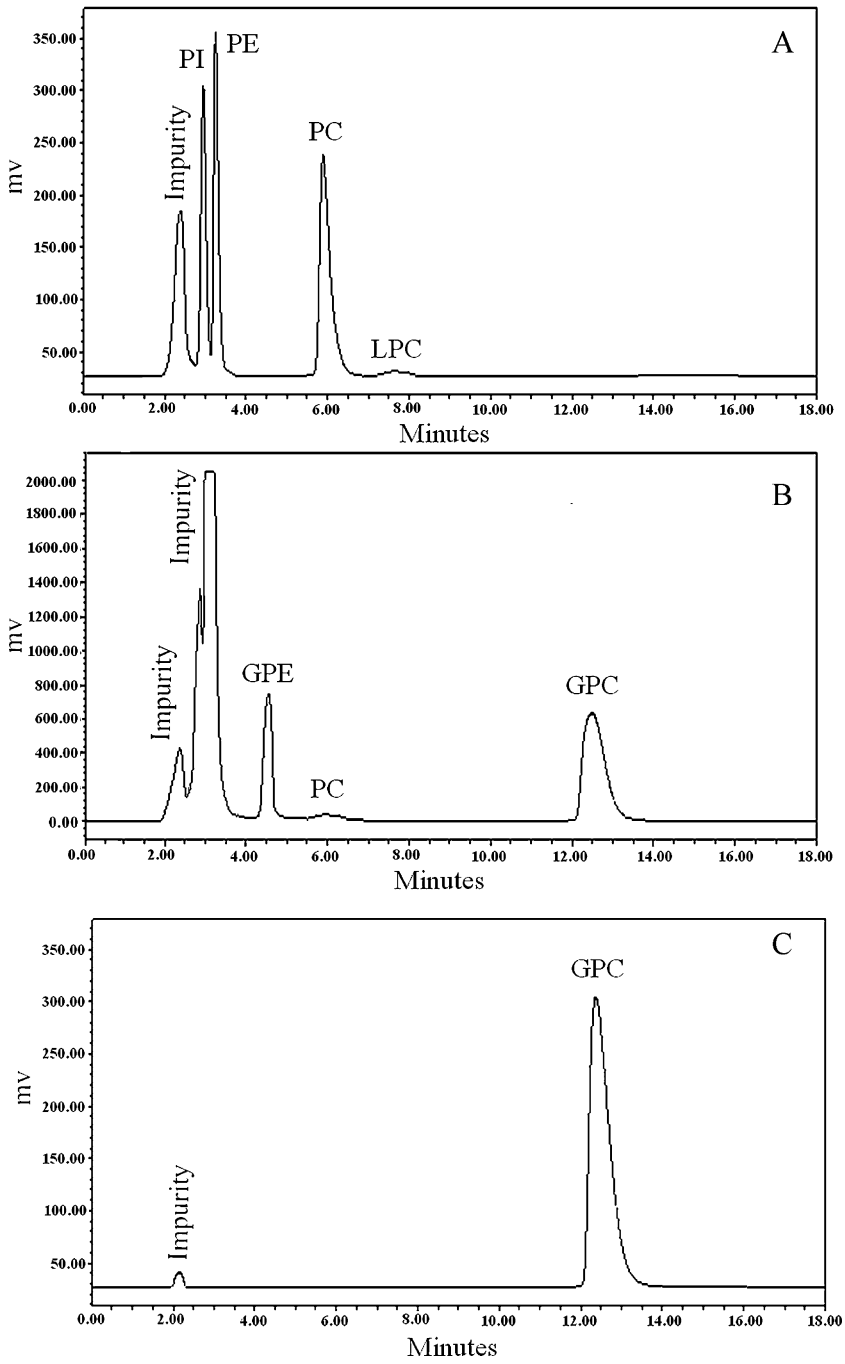


**Fig. 1.** HPLC profiles of (A) initial reaction solution, L-α-GPC (B) before and (C) after purification.

fatty acid was hydrolyzed by phospholipase $A_1$ to produce 2-acyl LPC, then spontaneous acyl migration occurred in which the *sn*-2 acyl moved to the *sn*-1 position to form 1-acyl LPC, and finally the enzyme hydrolyzed the remaining acyl group to produce L-α-GPC. These results were well consistent with previous reports [24,25].

After successive purification procedures, including $Ca^{2+}$ and $Cl^-$ elimination and silica gel packed-column chromatographic purification as described in detail below, the L-α-GPC peak intensity was very prominent, meanwhile the impurity peak intensity was very weak and the GPE peak undetectable (Fig. 1C), which illustrated that L-α-GPC was purified effectively by resin and silica gel column chromatography.

### 3.2. Elimination of $Ca^{2+}$

Phospholipase $A_1$ requires $Ca^{2+}$ as an essential cofactor for the hydrolysis of the fatty acyl ester bonds of phospholipids [26–28]. As a result of this requirement, the 727 μg $mL^{-1}$ $Ca^{2+}$ present in the reaction solution was also present in the product residue, and it was thus necessary to remove it by 001 × 7 cation exchange resin column chromatography.

The static adsorption curve showed that the $Ca^{2+}$ concentration decreased rapidly in the first 10 min, followed by a slower decrease thereafter, and arrival at equilibrium by ~60 min (Fig. 2A). Thus, according to Eq. (1), the adsorption capacity of 001 × 7 resin for $Ca^{2+}$ was 20,550 μg $g^{-1}$. Furthermore, the ability to process enzymatic

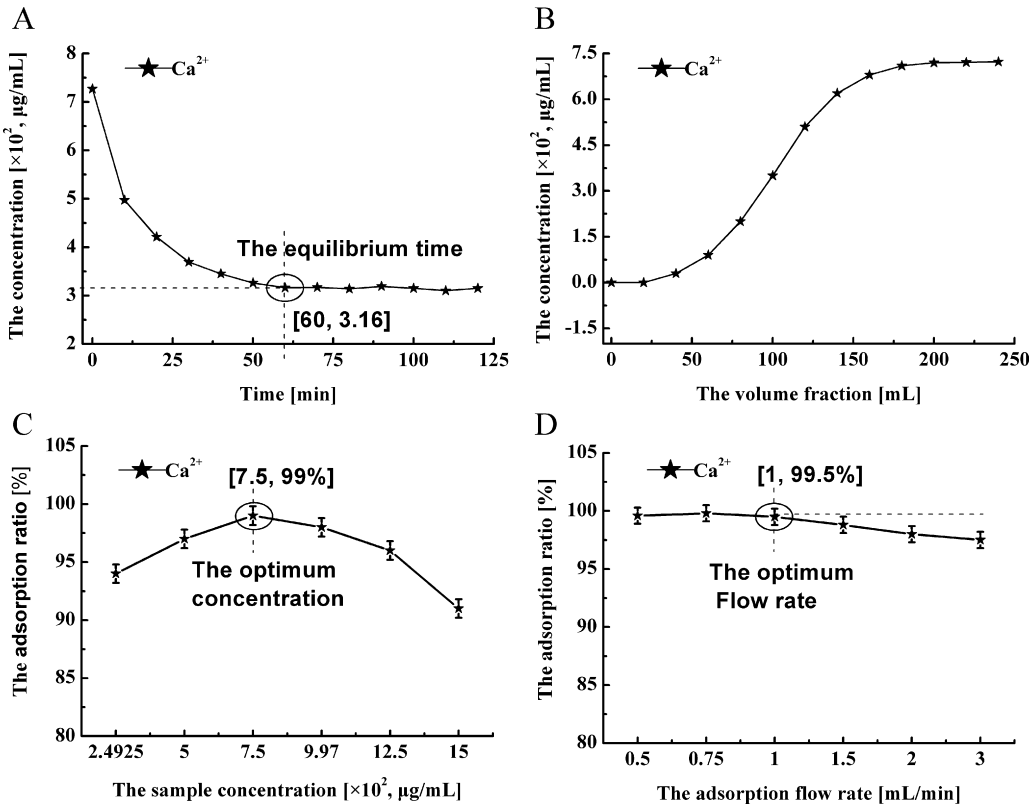


**Fig. 2.** (A) Static adsorption equilibrium curve for $Ca^{2+}$ with 001 × 7 resin; (B) dynamic leakage curve for $Ca^{2+}$ with 001 × 7 Resin; (C) effect of loading concentration on adsorption ratio with 001 × 7 resin; and (D) effect of flow rate on adsorption ratio with 001 × 7 resin.

reaction solutions with this system was 28.3 mL of solution/g of 001 × 7 resin, indicating a very satisfactory adsorption capacity for these purposes.

The dynamic leakage curve for 001 × 7 resin showed that the adsorption equilibrium was reached when the effluent volume treated totaled 160 mL (Fig. 2B). The saturated adsorption capacity was 18, 247.7 $\mu g\,g^{-1}$, and the reaction solution processing ability was 25.1 $mL\,g^{-1}$, very similar to the ability measured above and also quite satisfactory.

The calculated adsorption ratio showed increasing and then decreasing trends with increasing starting $Ca^{2+}$ concentration, showing a satisfactory adsorption ratio of 99% when the loading concentration was 720 $\mu g\,mL^{-1}$ (Fig. 2C). Consequently, considering both adsorption efficiency and effluent $Ca^{2+}$ content, a starting $Ca^{2+}$ concentration of 720 $\mu g\,mL^{-1}$ was used in the following experiments.

Examination of residual $Ca^{2+}$ as a function of flow rate showed that 001 ×7 resin could effectively adsorb $Ca^{2+}$ at flow rates of <1 $mL\,min^{-1}$, with an adsorption ratio of 99.5% (Fig. 2D). However, at >1 $mL\,min^{-1}$, the adsorption ratio decreased with increasing flow rate. Thus, considering the adsorption ratio and adsorption time, 1 $mL\,min^{-1}$ was selected as the most appropriate flow rate for $Ca^{2+}$ adsorption in this system.

The optimal conditions selected for $Ca^{2+}$ removal using this resin comprised a reaction solution processing ability of 25.1 $mL\,g^{-1}$, a $Ca^{2+}$ concentration of 720 $\mu g\,mL^{-1}$, and an adsorption flow rate of 1 $mL\,min^{-1}$. Under these conditions, residual $Ca^{2+}$ at 5.8 ppm was observed in the effluent in confirmative experiments. In view of its low cost, strong acidity, specific surface area etc. [20], D311 resin was considered to be the good matrix of choice for removing $Ca^{2+}$ by packed column chromatography. (Elimination of $Cl^-$ was similar to those of corresponding elimination of $Ca^{2+}$, and was shown in Fig. S1, Supplementary material in the appendices).

Regenerated 001 × 7 and D311 resins were reused 10 reuses without significantly decreasing its adsorption ability to $Ca^{2+}$ and $Cl^-$, respectively.

### 3.3. Eluent selection for silica gel column chromatography

Selection for the mobile phase can not only consider recovery and recycling, or even the amount of eluent required, but must primarily focus on satisfactory chemical separations. Methanol was selected here as the eluting solvent to be tested for L-α-GPC isolation because the polarities of the impurities, GPE, and L-α-GPC were roughly in the same range; furthermore, methanol's relatively low viscosity favored high column efficiency [16,29]. The elution of L-α-GPC here required 2400 mL of methanol at 25 °C and resulted in a product of 99.8% purity and in 67.8% recovery (Fig. 3A). With relatively low recovery, more eluent and a longer desorption time were required or mixed solvent eluents needed to be considered.

L-α-GPC elution profiles using several aqueous methanol solutions showed that 80–100% aqueous methanol completely separated L-α-GPC from the other components (Fig. 3A–C); meanwhile, GPE was also effectively purified, especially at 100% methanol (Fig. 3A); 50–70% aqueous methanol was clearly inadequate for this separation (Fig. 3D–F). This phenomenon might be attributed to increased sample solubility and stronger eluent polarity. Concurrent with improved separation with increased eluent polarity, the amount of eluent and elution time both decreased. But, beyond a certain polarity, at <80% aqueous methanol, the separation efficiency of the column decreased, degrading the separation quality. Thus, as shown in Table 1, 80% aqueous methanol was employed here as the eluent to efficiently separate and recover L-α-GPC in a shorter time with less eluent; the eluent volume required for

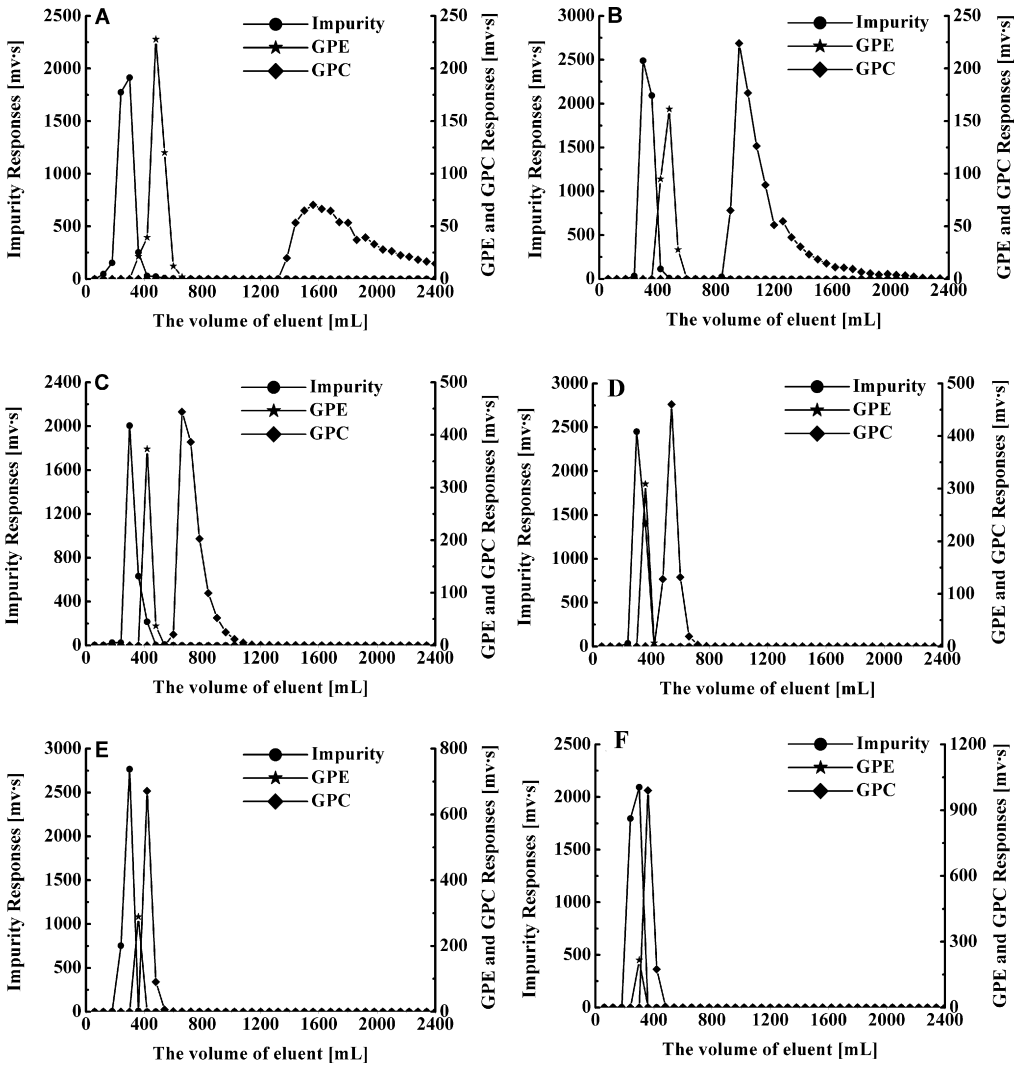


**Fig. 3.** L-α-GPC profiles following elution by various aqueous methanol solutions. (A–F) 100, 90, 80, 70, 60, and 50% methanol, respectively.

eluting L-α-GPC was reduced to 1200 mL, resulting in product of 99.8% purity and a 77.8% recovery.

### 3.4. Effect of the loading amount and loading concentration

With increased loading amounts on silica gel columns (Fig. 4A), purity of L-α-GPC gradually decreased while, concurrently, recovery first increased and then dropped, resulting in a satisfactory 99.5% purity and higher 79% recovery with a loading of 24.4 mg $g^{-1}$ silica gel. The separation efficiency was expected to decrease with increased loading because it would have eventually exceeded column capacity; recovery was not observed to degrade with loading at <24.4 mg $g^{-1}$ silica gel (Fig. 4A). As the total loss of L-α-GPC was nearly constant regardless of the loading amount, the relative loss would have decreased as the load increased. Thus, product recovery was best with loading from 0 to 24.4 mg $g^{-1}$ silica gel, and the decreased recovery with loading >24.4 mg $g^{-1}$ silica gel was surmised to be caused by overloading leading to overlap of neighboring peaks and decreased separation efficiency; these results were consistent with a previous report [15].

The purity and recovery of L-α-GPC on silica gel columns showed increasing and then decreasing trends with increasing starting L-α-GPC concentrations (Fig. 4B), with a satisfactory 98.7% purity and 80% recovery produced with a loading concentration of 16.6 mg $mL^{-1}$. This behavior may be a result of the superposition of various effects, such as the inevitable sample dilution accompanying the process of running samples, in that, with low loading concentrations, band broadening was promoted, the impurities and target L-α-GPC elution peaks overlapped more, and the purity and recovery decreased [30]. However, this phenomenon would disappear with increased loading concentration and, consequently, as observed here with both separation efficiency and recovery, a

**Table 1**
Effects of different concentrations of methanol aqueous solution on the purity and recovery of L-α-GPC.

| The mobile phase (concentrations of methanol) | 100% | 90% | 80% | 70% | 60% | 50% |
|---|---|---|---|---|---|---|
| The purity (%) | 96.5 | 98.6 | 99.8 | 96 | 77.4 | 58.3 |
| The recovery (%) | 70.2 | 76.6 | 79.1 | 72.4 | 56.3 | 45.9 |

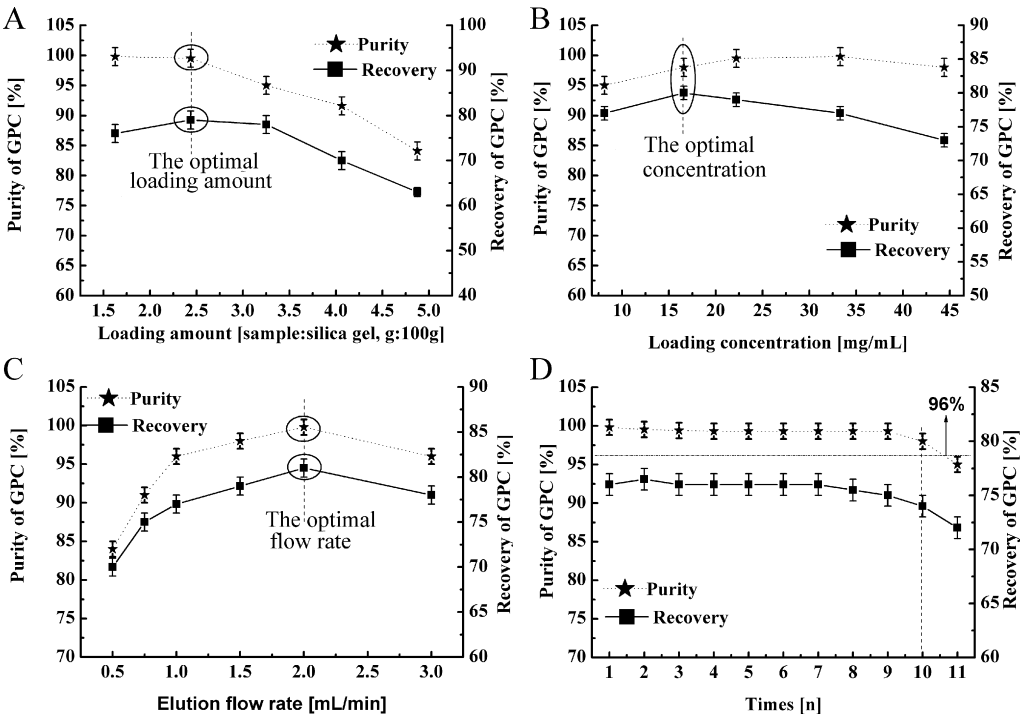


**Fig. 4.** Purity and recovery profiles of silica gel columns evaluated for (A) loading amount; (B) loading concentration; (C) mobile phase flow rate; and (D) reusability of silica gel.

starting L-α-GPC concentration of 16.6 mg mL$^{-1}$ was selected for subsequent experiments.

### 3.5. Effect of the mobile phase flow rate

The dependence of column efficiency on mobile phase flow rate is of great practical importance [17]. Graphing of the L-α-GPC separation efficiency as a function of flow rate showed that these silica gel columns effectively resolved L-α-GPC at 2 mL min$^{-1}$, with the maximum 99.6% purity and 81% recovery (Fig. 4C). At flow rates <2 mL min$^{-1}$, the separation efficiency gradually increased with increasing flow rate, which was attributed to effective diffusion gradually decreasing with increased flow rate, as the flow was too fast to allow time for diffusion to lower separation efficiency. In view of the separation efficiency and time, 2 mL min$^{-1}$ was selected as most appropriate for L-α-GPC separation in subsequent experiments.

The L-α-GPC was obtained with light yellow color (Fig. S2A, Supplementary material) and 99.6% purity after silica gel chromatography purification, and subjected to decolorize with activated carbon. Activated carbon particles have good capacity for molecular adsorption as they offer a large surface area resulting from their porous network structure of interconnected macropores, mesopores, and micropores [31,32]. After decoloration, colorless L-α-GPC (Fig. S2A, Supplementary material) was produced at 99.8% purity, 69.8% recovery, and a specific rotation of −2.5°. These results indicated that activated carbon effectively absorbed colorant and minor impurities from the product, increasing the final product purity.

Characterization of the final L-α-GPC (Supplementary material) by specific rotation ($[\alpha]_D^{20}$, AOAC Official Method 920.142), ultra performance liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS), tandem mass spectrometry (MS/MS), and nuclear magnetic resonance spectroscopy (NMR) were in good agreement with that of the standard.

### 3.6. Reusability of silica gel

During use, silica gel can become saturated with water and organic material that decrease the quality of separation, and thus must be regenerated before reuse (the optimal condition for regenerating silica gel is shown in Fig. S3, Supplementary material). Regenerated silica gel by heating at 150 °C for 3 h was reused 10 times without significantly decreasing its ability to yield good L-α-GPC purity and recovery (Fig. 4D). Compared with the performance of solvent extraction [6], precipitation [9], and recrystallization [10] for L-α-GPC purification, resins and silica gel column chromatography exhibited satisfactory reusability, low cost, and easy availability, which is encouraging for future industrial production of high purity L-α-GPC.

## 4. Conclusions

A novel preparation and purification procedure of L-α-GPC, with colorless, at 99.8% purity and 69.8% recovery, and with a specific rotation of −2.5° was obtained via five-step. Both the resins and silica gel column chromatography showed remarkable separation performance after 10 reuses. The characterization of final L-α-GPC was found to be in good agreement with the standard. Thus, the present work describes a simple and cost-effective method for preparing L-α-GPC with high yield and purity, low cost, and environmental friendliness, and provides encouragement for future exploration and easy adaptation for industrial applications.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.12.003.

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