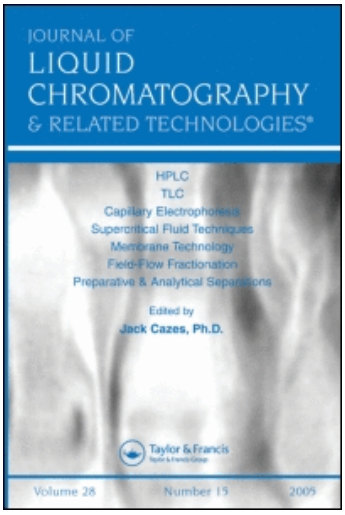

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PURIFICATION OF PHOSPHATIDYLCHOLINE WITH HIGH CONTENT OF DHA FROM SQUID *ILLEX ARGENTINUS* BY COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

A method for purification by countercurrent chromatography (CCC) of phosphatidylcholine with high content of DHA is described. Phospholipids were extracted from squid *Illex argentinus*, characterized by high performance liquid chromatography and their fatty acids profile determined by gas chromatography. Phosphatidylcholine was primarily represented with 48.3 % (w/w) of total phospholipids and contained 39.7 % of DHA (w/w total fatty acids). The analytical separation of phosphatidylcholine, was achieved on a Kromaton II apparatus, in the systems heptane - ethyl acetate - acetonitrile (1:0.65:1, v/v/v) and isooctane - ethyl acetate - acetonitrile (1:0.65:1, v/v/v), at 550 rpm and 1.5 mL/min flow rate using normal ascending and reversed descending elution modes respectively.

The fractions collected were further analysed by high performance thin layer chromatography. Phosphatidylcholine was separated from the other phospholipids of the crude extract and was recovered pure in the lower phase with 95 % yield. Purification at preparative scale was performed on a 1 L column, 550 rpm and 5 mL/min using the same chromatograph. These results prove that CCC constitutes a powerful technique for separation of phospholipids from natural lipidic extracts.

INTRODUCTION

Nutritional importance of polyunsaturated fatty acids (PUFA) has considerably increased during the past decade. In animals, linoleic acid (C18:2 ω6) and α-linolenic acid (C18:3 ω3) found in vegetable food, are metabolized into arachidonic acid (AA, C20:4 ω6) and docosahexaenoic acid (DHA, C22:6 ω3) which are constituents of cellular membranes and play essential role in neuronal and sensorial development.[1] Previous studies have shown, that dietary DHA supply during gestation and feeding is a nutritional factor important for nervous structures development in young mammals.[2]

Recently, Makrides et al.[3] in a comparative study through new-born infants, have found there is a positive correlation between DHA level in erythrocytes and maturation of visual acuity, and Leger et al.[4] have confirmed the high content of phosphatidylcholine (PC) and DHA during constitution of retina in human embryo.

Therefore, in prematures babies who can not be breast-fed or take benefit from human milk, it may be necessary to supply dietary formula with PUFA and especially DHA from fish oil or egg yolk.[5] Here in, is described the use of countercurrent chromatography (CCC) to obtain pure PC with high content of DHA from cephalopod skin, a potentially new source of PUFA.

Some previous studies[6-9] have proposed methodologies to separate various lipid classes including fatty acids esters by centrifugal partition chromatography (CPC). For example, Alvarez et al.[10] succeeded in separating PE and PC standards in heptane-ethanol solvent-system.

Recently, Bousquet et al.[11] have purified PUFA from microalgae by CCC but, no data were collected on separation of phosphatides by this technique. We present, in this paper, the separation of PC from squid phospholipids crude extract by CCC in two solvent-systems.

MATERIALS AND METHODS

Reagents

Solvents of analytical grade were from Carlo Erba (Nanterre, France) or Merck (Nogent sur Marne, France), BHT and phospholipids standards were obtained from Sigma (St Quentin fallavier, France).

Skins from *Illex argentinus* squid were kindly provided by SCA (Société commerciale de l'Adour, Bayonne - France).

Extraction of Phospholipids

Squid skins were cut in small pieces and blended in Stephan grinder at +4°C to give a fresh paste. The phospholipids were extracted directly from the paste by a method adapted from Singleton et al.[12] This method is based on the different solubilities of the various classes of lipids in cold acetone : most of the glycolipids and phosphatides, especially acidic phosphatides in salt form, are insoluble in acetone at -20°C when glycerides and other neutral lipids are soluble in the same conditions.

The solvents used for extraction contained 0.1 % w/v butylated hydroxy toluene (BHT) in order to avoid any oxidation.

In practice, the paste was agitated thorougly with 5 volumes of acetone (v/w) in batch for 2 hrs at 25 °C. The mixture was then filtered and the solids washed three times with one volume of cold acetone. The filtrate was kept at -20°C during 16 hrs to precipitate the phospholipids. The solution was then centrifuged at 2500 rpm for 10 min, acetone supernatant was removed and the pellet was dried under nitrogen.

The acetone-insoluble material contained most of the phospholipids and traces of neutral lipid, whereas, the acetone-soluble fraction contained all the neutral lipids such as glycerides, sterols, sterol esters, hydrocarbons, pigments (carotenoids) and only traces of phosphatides.[13] The crude phosphatides were dissolved in methanol, placed into a rotavory evaporator for the removal of solvent and dried under nitrogen. The crude extract was stored in chloroform at -20°C.

Total lipids Analysis

Total lipids in the crude extract were quantified by extraction with hexane in a Soxhlet apparatus. Fractionation, of the various classes of lipids (neutral lipids, glycolipids and phospholipids), was performed by adsorption chromatography on silica gel (Silica gel 60, particle size 63-200 µm, 70 - 230 mesh, Merck, Nogent sur Marne, France), using a modification of the method described by Borgstom[14] and adapted by Rouser et al.[15] and Soudant et al.[16] 300 mg silica gel in chloroform was used for 10 mg of lipid extract. Neutral lipids were eluted with 10 mL of chloroform, glycolipids with 15 mL acetone - methanol (9:1, v/v) and phospholipids were recovered with 10 mL of methanol. Each class was quantified after solvent evaporation under nitrogen and expressed as % of total lipids.

High Performance Liquid Chromatography

Phospholipids were separated by HPLC as previously described,[17,18] on a column 25 cm long, 7.5 mm internal diameter filled with Lichrosorb Si 60 (5 µm particle size, Merck, Nogent sur Marne, France) using a System Gold 126 chromatograph (Beckman, Gagny, France) equiped with a gradient pump and injection loop of 250 µL. Solvents used were solvent A: hexane, solvent B: isopropanol - chloroform (4:1, v/v), and solvent C: isopropanol - water (1:1, v/v). Crude extract (3 to 5 mg) was injected in solvent D: A - B - C (42:52:6, v/v/v). Elution was performed at room temperature with a linear gradient from 100 % solvent D to 100 % solvent E: A - B - C (32:52:16, v/v/v) in 20 min at 2.5 mL/min flow rate. Detection of the various classes of phospholipids was achieved using a light-scattering detector DDL 11 (Cunow, Cergy-Pontoise, France). Effluents were partially collected from the column to determine the fatty acids profile of each class of phospholipids.

High Performance Thin Layer Chromatography

Lipidic content of fractions collected by countercurrent chromatography was analyzed by HPTLC using an automated multiple development apparatus AMD (Camag, Muttenz, Switzerland). Phospholipids were identified on pre-coated plates (Silica gel 60 F-254, Merck, Nogent sur Marne, France), which were prewashed for 1 hour by diving in isopropanol and then dryed for 30 min at 120°C. Standards and samples application in 6 mm length bands, 4 mm apart, was performed at 4 sec/µl delivery rate by the spray-on technique using a Linomat IV (Camag, Muttenz, Switzerland). The principle of AMD has been previously described.[19,20]

In the present work, we used a 25 steps universal gradient fully automated. The gradient started with the solvent possessing the strongest elution power and in the successive runs the polarity was decreased. Between runs, the solvent previously used was eliminated from the development chamber and TLC plate was dried under nitrogen.

After chromatographic development the plates were dived for 5 seconds in a solution containing 4 mL concentrated sulphuric acid, an 0.4 g manganese chloride tetrahydrate in 120 mL water-methanol (1:1, v/v). The plates were then heated at 120°C for 30 min. Phospholipids appeared as brown zones on a white background and absorption at 550 nm was measured with a TLC Scanner II apparatus (Camag, Muttenz, Switzerland).

Each compound was identified by its Rf and compared with authentic lipid standard.

Gas Chromatography

Total phospholipids (< 5 mg) were dissolved in 5 mL ethanol containing 10% KOH. After 15 hours, released fatty acids were extracted twice with 10 mL hexane, dried under-vacuum and then methylated by refluxing in 10 mL solution HCl - methanol 3 % (v/v) for 1 hour. Fatty acids esters were extracted in hexane, dried under-vacuum and dissolved in chloroform before gas chromatography (GC) analysis.

Phospholipids collected from HPLC, were submitted to methylation with BF3 in 10 % methanolic solution at 90°C for 20 min (PE, PC, PS, PI) or 90 min (SM) according to Morrisson and Smith.[21] Fatty acids methyl esters were collected in hexane and conditioned as described above.

The fatty acids esters were analyzed by GC on a capillary gas column CPWAX 52 CB (50 m long, 0.32 mm diameter, 0.25 μm thickness stationary phase) from Chrompack (Les Ulis, France), using a GC 8000 chromatograph (Fisons, Arcueil, France) fitted with a AS 800 injector and a FID 80 detector. The carrier gas was hydrogen at pressure 1.2 bar and the temperature was programmed from 54°C (for 3 min) to 194°C at 3°C per min.

Samples were injected at 54°C and the detection was carried out at 250°C. Fatty acids esters were identified by their retention times and quantified with reference to standards.

Table 1

Phospholipidic Composition of the Crude Extract Expressed in Percentage of Total Phospholipids

Classes of Phospholipids	% of Total Phospholipids
PE	24.1
PI	17.5
PS	4.9
PC	48.3
SM	5.1

PE: phosphatidylethanolamine; PI: phosphatidylinositol;
PS: phosphatidylserine; PC phosphatidylcholine
SM: Sphingomyellin

Table 2

Fatty Acid Composition of Crude Extract Phospholipids Expressed in Percentage of Fatty Acids

Fatty Acids	Total Phospholipids	PE	PC	PS	PI	SM
Tot. Sat.	35.5	25.1	44.3	34.1	51.5	82.5
Tot. Mon.	8.2	10.0	7.5	12.6	15.4	11.0
20:4n-6	4.3	10.2	1.0	3.3	4.1	0.3
20:5n-3	16.7	34.6	5.0	12.3	20.7	1.0
22:6n-3	33.1	18.5	39.7	35.0	7.1	4.2

Tot. Sat.: Total saturated fatty acids;
Tot. Mon.: Total monounsaturated fatty acids.

Countercurrent Chromatography

The separation of PC from crude extract was carried out with a countercurrent chromatography instrument (Kromaton II SEAB, Villejuif, France). The column was 2.4 mm internal diameter, 5 m long,

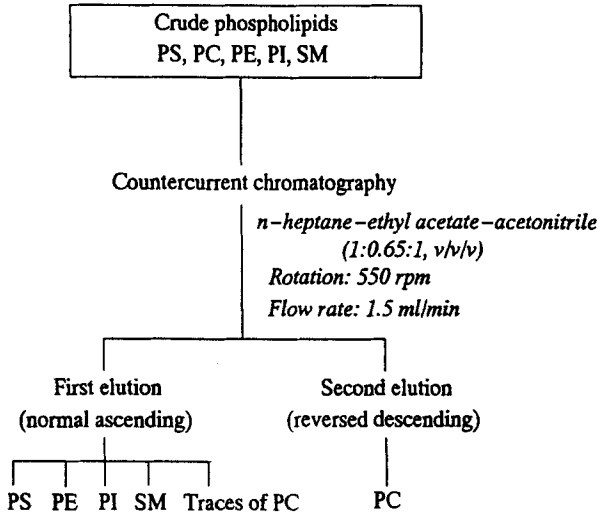

Figure 1. Diagram illustrating the elution modes and separation of phosphatidylcholine by countercurrent chromatography. PS: phosphatidylserine, PE: phosphatidylethanolamine, PI: phosphatidylinositol, SM: sphingomyelin, CP: cholesterol and cholesterol esters.

polytetrafluoroethylene (PTFE) tubing wound on the original holder; its total volume was 75 mL. The rotational speed was 550 rpm.

The chromatographic system was a Kontron 420 isocratic, constant solvent-delivery pump and a fraction collector Frac 300 from Pharmacia.

The partition coefficient of phosphatidylcholine in the binary heptane-acetonitrile and isooctane-acetonitrile two-phase solvent system was favorably modified by addition of ethyl acetate. The two biphasic systems used were heptane - ethyl acetate - acetonitrile (1:0.65:1, v/v/v) and isooctane - ethyl acetate - acetonitrile (1:0.65:1, v/v/v) . The solvents were filtered before used.

Phosphatidylcholine was separated from the other phospholipids at a flow rate of 1.5 mL/min, using the system described as normal ascending and reversed descending elution modes respectively. Fractions were collected every 4 min and were cooled in the dark at -20°C before analysis.

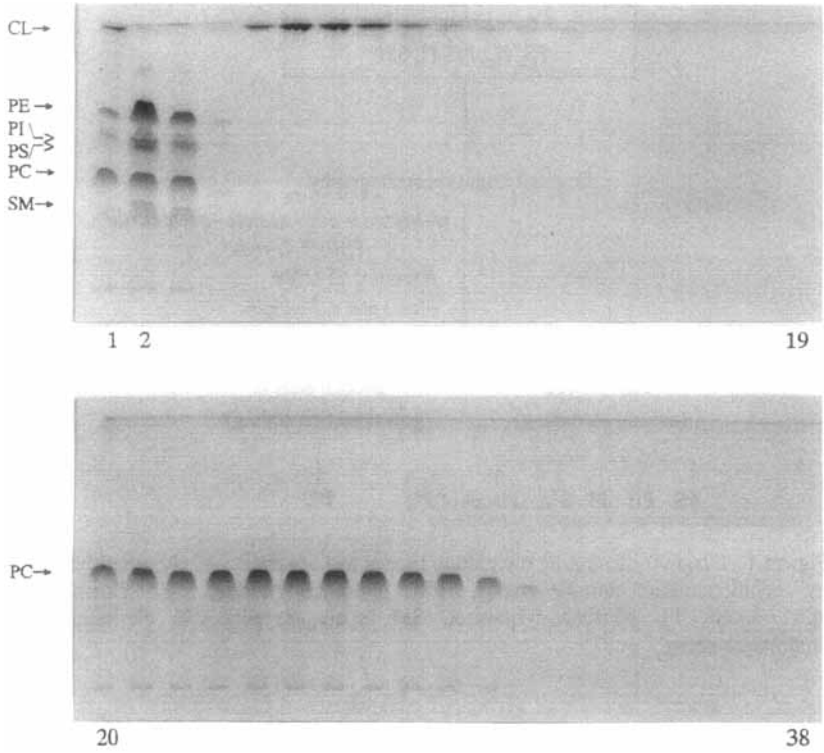

Figure 2. HPTLC analysis of fractions collected by countercurrent chromatography on Kromaton II at 550 rpm and 1.5 mL per mn in solvent system heptane - ethyl acetate - acetonitrile. Plates were developped with a 25 steps gradient in AMD apparatus and revelation of phospholipids was performed with a sulphuric acid and manganese chloride solution in water-methanol 1:1 (v/v). 1, crude extract 50 µg; 2 to 19, upper phase fractions; 20 to 38, lower phase fractions. CL, cholesterol and cholesterol esters; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin.

RESUTS AND DISCUSSION

The crude extract was obtained with 0.72 % yield from rough squid skins and contained 90.3 % of total lipids. The phospholipids represented 97.7 % (w/w) of total lipids by quantification on silica gel. Separation of the various classes of phospholipids was achieved by HPLC and showed that PC was primarily represented with 48.3 % (w/w) of total phospholipids (Table 1). The

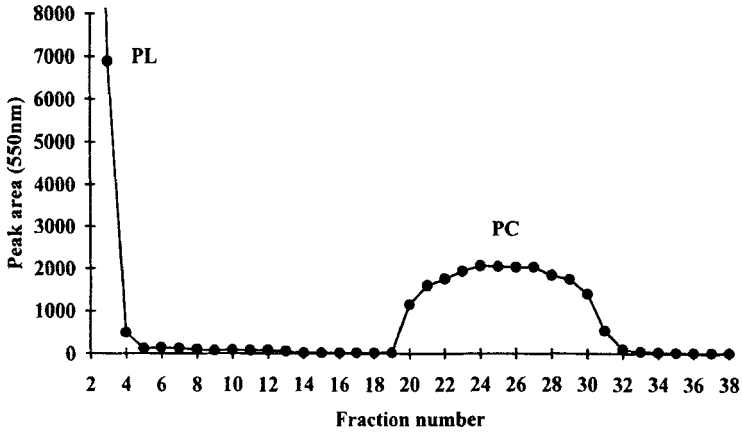

Figure 3. Absorption at 550 nm of phospholipidic content in collected fractions measured on HPTLC plates with TLC scanner II apparatus. Pure PC was collected in fractions 19 to 31 of the lower phase and separated from the other phospholipids of the crude extract found in the upper phase. 2 to 19, upper phase fractions; 20 to 38, lower phase fractions. PL, phospholipids of the crude extract; PC, pure phosphatidylcholine.

final PC content in the crude extract was then 42.6 % (w/w). The crude extract yield was increased to 1.38 % when paste was stored at -20°C for 60 days before acetone treatment, probably because of higher release of lipidic constituents from grinded skins. But, at the same time, PC content was decreased to 36.3 % (w/w total phospholipids).

The fatty acids profile obtained for each class of phospholipids showed that DHA content of PC was 39.7 % and EPA 5.0 % of total fatty acids (Table 2). High DHA content 35.0 % was also exhibited by PS and high EPA content 34.6 % by PE (respectively 4.9 % and 24.1 % of total phospholipids).

Phosphatidylcholine was purified by CCC from the crude extract in the system heptane-ethyl acetate-acetonitrile (Figure 1). Experiments were carried out dissolving 0.32 g to 0.64 g of total phosphatides in 5 mL of mobile phase and injecting into the analytic column. The upper phase (less polar phase) was used as mobile phase. The less polar phospholipids were eluted first, whereas, the most polar remained in the column.

The first three fractions contained phospholipids PE, PI, PS, SM and traces of PC as shown in Figure 2. One contaminant compound was then collected in the next fractions 5 to 12 and analysis showed it was a mixture containing 89

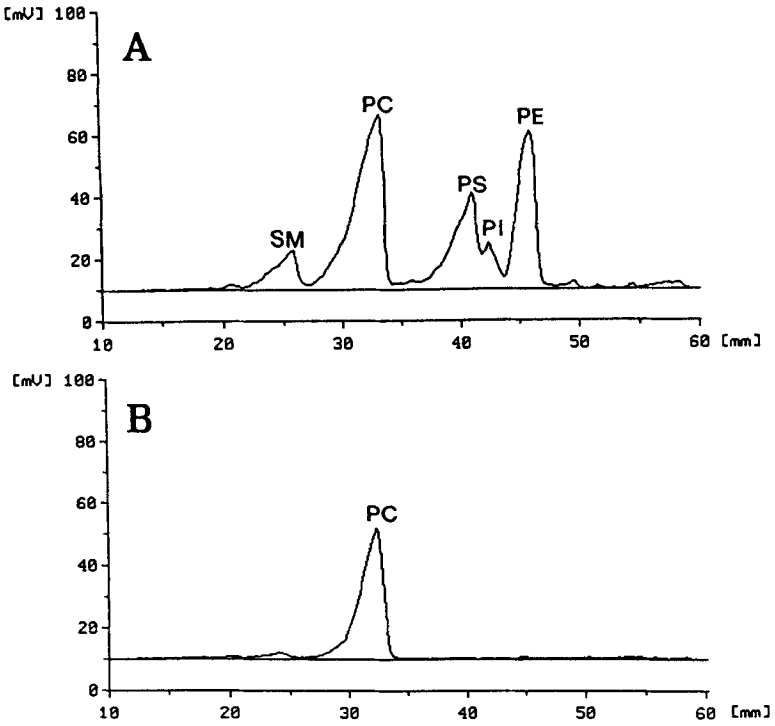

Figure 4. Detection at 550 nm of the various classes of phospholipids on HPTLC plates with TLC scanner II apparatus. A, crude extract; B, fraction containing pure phosphatidylcholine collected by CCC.

% cholesterol, 5 % cholesterol esters and little amount of PC. After running one volume of mobile phase through the column in the normal ascending mode, phosphatidylcholine was eluted in the stationary phase (lower phase) as shown in Figure 3. PC was obtained pure (Figure 4) with an estimated yield of 95 % of total injected PC. Heptane was then replaced by isooctane in the CCC solvent-system for purification of PC from crude extract. Results obtained were identical using heptane - ethyl acetate - acetonitrile (1:0.65:1, v/v/v) or isooctane - ethyl acetate -acetonitrile (1:0.65:1, v/v/v) solvent-system.

One main problem encountered for purification of phosphatides by partition in solvent-systems, is the possibility to induce emulsions that could remain stable. This property reduces the choice of solvents we can use for CCC experiments. The systems hexane - methanol - water (1:0.9:0.1, v/v/v) and hexane - ethanol - water (1:0.9:0.1, v/v/v), previously described by Murayama

et al[6] for purification of PUFA esters by CPC, contained water and were not suitable for separation of phosphatides by CCC. So, we started with the system heptane - acetonitrile (1:1, v/v) exempt of water described by Bousquet et al.[11] for the purification of PUFA from microalgae by CCC. We made it suitable for the separation of polar lipids, such as phosphatides, by adjusting the partition coefficient of phosphatides with ethyl acetate. The value obtained for phosphatidylcholine was 1.01 in the system heptane - ethyl acetate - acetonitrile (1:0.65:1, v/v/v).

This system showed a good behavior in CCC and proved to be well adapted for separation of PC from natural phospholipids. Scale-up experiment was carried out with a 1 L preparative column on the same CCC chromatograph in the system heptane - ethyl acetate - acetonitrile (1:0.65:1, v/v/v) at a flow rate of 5 mL/min and 550 rpm and we succeeded in obtaining pure PC as described above.

The purification of phosphatidylcholine has been the subject of extensive investigations essentially by classic methods such as HPLC. In this study we demonstrate the usefulness of CCC for the same purpose.

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