Optimization of the Column Loadability for the Preparative HPLC Separation of Soybean Phospholipids

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A simple and rapid preparative-scale separation method was investigated in order to obtain pure soybean phospholipids. Because of technical and economical reasons, two coarse, irregular silica gels were selected. Comparing both stationary phases, a ternary mixture of hexane, 2-propanol and water yielded a different elution order of the phospholipids at analytical sample loads, in spite of the chemical similarity of these packing materials. During scale-up, it became obvious that the retention characteristics were largely influenced by the sample load, thus making the 15-35 μm RSiL inappropriate for preparative-scale separations of phospholipids. Moreover, the column loadability could be increased by controlling the flow rate. Hence, a solvent program was elaborated which enabled a column loadability of up to 2% by weight of the stationary phase. Using analytical high performance liquid chromatography, it was shown that the method proposed yielded over 90% pure phospholipids at a recovery of nearly 80%.

KEY WORDS: Mass detector, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, soybean lecithin.

Soybean phospholipids (lecithins) are widely used as natural emulsifiers, wetting agents and baking improvers (1). Moreover, in recent years numerous applications in dietetics, cosmetics and pharmaceuticals have been reported (2). However, in nearly all cases rather crude extracts of lecithins with variable phospholipid compositions are being used. Because the functional properties of specific phospholipids can largely surpass these of lecithins (3-5), simple, rapid and cost-effective preparative-scale methods enabling the purification of the most abundant soybean phospholipids are needed.

To achieve this goal, coarse, irregularly-shaped silica gels have been selected. Volatile solvents were preferred in order to facilitate product recovery. Fager *et al.* (6) separated 10 g of egg yolk phospholipids on a 800 × 1 cm ID silica gel column in which one separation lasted 40-50 hr and consumed about 13 liters of solvents. Recently, more practical separation systems were proposed using standard 25 cm high performance liquid chromatography (HPLC) columns with an internal diameter of several centimeters (7-9). In spite of the application of these wide-bore columns, the sample load was limited to 200 mg, representing only some tenths of a percent by weight of the stationary phase. As a consequence, these methods are very expensive due to the large amounts of solvents necessary.

The method proposed allows a column load up to 2%. Both the purity of the fractions and the recovery of the phospholipids present were quantitatively evaluated using analytical HPLC. Since the mobile phase contains only volatile solvents and water, there is no risk of phospholipid deterioration and product recovery is accomplished by evaporating the solvents in a stream of nitrogen. Moreover, the solvent program includes a stepwise gradient achieved by a single pump in combination with a solvent switcher, so that rather inexpensive equipment can be used. Finally, the method proposed can be completely automated, enabling separations to be run overnight.

EXPERIMENTAL PROCEDURES

HPLC-grade hexane and 2-propanol were supplied by Alltech Associates (Eke, Belgium). Freshly deionized and distilled water was used. Commercial soybean lecithin (Epikuron-100P) was obtained form Lucas Meyer (Hamburg, Germany). Prior to injection, the lecithin was dissolved in hexane/2-propanol (55:44) and passed through a 0.2 μm Dynagard filter (Microgon Inc., Laguna Hills, CA).

Instrumental set-up. A Waters model 590 isocratic HPLC pump controlled the solvent switcher (Waters Associates, Brussels, Belgium). The analytical separations were performed using a 3 μm Spherisorb stationary phase, packed in a 100 × 4.6 mm column (Alltech), whereas for preparative-scale purposes both 15-35 μm RSiL (Alltech) and 15-40 μm silica gel 60 for column chromatography (Merck, Darmstadt, Germany) were considered. These coarse powders were packed in 250 × 4.6 mm columns (Alltech). To avoid particulate contamination of the columns, a 0.2 μm Uptight prefilter (Upchurch Scientific Inc., Oak Harbor, Washington) was inserted. All columns were stored in hexane. A Waters model 441 absorbance detector (214 nm) was used for monitoring preparative separations, whereas an evaporative light-scattering mass detector (Applied Chromatography Systems, Macclesfield, U.K.) was preferred for quantitative analytical HPLC. The latter was operating at an internal air pressure of 1 bar and an evaporator set of 70. Finally, the Frac-100 fraction collector (Pharmacia, Brussels, Belgium) was controlled by the event in/event out box of the pump.

Analytical HPLC. The analytical HPLC method used has been described previously (10). Some minor modifications were needed because a different column was used in the present study.

The initial part of the separation was performed by a mobile phase consisting of hexane, 2-propanol and water (58:39:3.2; v/v/v). After 5 min a second mobile phase containing the same mixture in a 55:44:5 ratio was selected by the solvent switcher. After 18 min the first mobile phase was run again, so that another sample could be injected after 25 min. The flow rate was kept

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constant at 1.8 mL per min. The peak areas were calculated by a Chromatopac C-R1A integrator (Intersmat, Belgium). Using calibration curves, the weight percentage composition was calculated.

Flow injection analysis. The concentration of the collected fractions was estimated using the previously mentioned HPLC system, omitting the column, as a flow injection analysis system. Ten μL of each fraction was injected into a solvent stream consisting of hexane/2-propanol/water (55:44:4) with a flow rate of 0.5 mL/min. Thus, the sample was taken to the mass-detector operating at an internal pressure of 0.8 bar. The evaporator set was fixed at 70. Using a standard series, a calibration curve was established. By curve-fitting, the following equations were derived: $X = \exp\{(\ln(P)+2.07)/1.662\}$ for sample loads smaller than 3 μg and $X = 1.58 + \exp\{(1n(P)+0.36)/0.656\}$ for larger amounts. In these equations P represents the peak area expressed in Volts times seconds, whereas X is the sample load in μg. Using these calibration curves, the experimentally determined peak areas were converted to the corresponding sample loads from which the concentration was calculated.

Van Deemter plot. Twenty μL of a test mixture containing toluene (267 mg/L), diethyl phthalate (167 mg/L) and dimethylphthalate (167 mg/L) was injected at different flow rates. The mobile phase consisted of hexane and 2-propanol in a 95:5 (v/v) ratio. The detection was done by a UV-detector, operating at 254 nm. The height equivalent to a theoretical plate (HETP) was calculated from the peak width and the retention time.

RESULTS

Selection of stationary and mobile phase. As silica gel has frequently been shown to yield very good analytical separations of the major phospholipids (11,12), this rather inexpensive material was thought to be an appropriate stationary phase for preparative separations. Moreover, Mann (13) demonstrated that the cost of a stationary phase providing a given number of theoretical plates was minimal when particles of about 30 μm were used. Besides, the influence of the packing material particle size on the peak broadening becomes less important when increasing the sample load (14). Therefore, a 15-35 μm RSiL (Alltech) and a 15-40 μm silica gel 60 (Merck) were considered.

In most analytical HPLC separation methods either an acetonitrile/water or a hexane/2-propanol/water mobile phase is used (11,12). Because of the limited solubility of phospholipids in acetonitrile, the latter solvent system seemed preferable. Moreover, the published acetonitrile-based mobile phases often include strong acids, which decompose some labile components, such as plasmalogens, during downstream processing (15).

Mobile phase water content. Since the water content of a ternary mixture of hexane, 2-propanol and water is by far the most decisive parameter, the hexane to 2-propanol ratio was kept constant at 55:44 (v/v). The influence of the water content of the mobile phase on resolving power was investigated by separating 150 μg of soybean lecithin, dissolved in 5 μL of hexane/2-propanol (55:44) on both columns (Table 1). The isocratic mobile phase was circulated at 1.8 mL/min and consisted of a ternary mixture of volumetric ratio 55:44:x, with x ranging from 2 to 6. When some phospholipids were still retained after 40 min, the columns were rinsed with a 55:44:6 mixture.

It was shown that the mobile phase should contain at least 3% of water in order to prevent the immobilization of the phospholipids onto the column. Using a 55:44:3 ternary mixture, the retention time of phosphatidylethanolamine (PE) was 20-25 min on both columns, whereas it was eluted nearly ten min earlier if the water content was increased to about 4%. However, these mobile phases did not elute phosphatidylinositol (PI), phosphatidic acid (PA) and phosphatidylcholine (PC). In order to achieve their elution, a water content of at least 5% was necessary. The mobile phase having a 55:44:5 composition caused the phospholipids to elute in the order PE, PI, PA and PC from the Merck silica gel stationary phase. Using the RSiL column, the retention times of PE, PA and PC were quite similar, whereas PI was not eluted within a reasonable time. Finally, a mobile phase containing a 55:44:6 mixture of hexane, 2-propanol and water, eluted all major soybean phospholipids in a short time. Moreover, an additional peak was observed, having a retention time of about 22 min, which

TABLE 1

Influence of the Water Content of the Mobile Phase, Consisting of a Ternary Mixture of Hexane, 2-Propanol and Water at a Flow Rate of 1.8 mL/min[a]

	Mobile phase compostion (hexane/2-propanol/water)							
	55:44:3		55:44:4		55:44:5		55:44:6	
	RSiL	Merck	RSiL	Merck	RSiL	Merck	RSiL	Merck
PE	24.37	20.02	8.70	8.18	4.16	3.76	2.48	2.23
PA				(7.66)	20.88	21.80	6.93	8.01
	(8.69)	(9.51)	(8.65)					
PC				(9.39)	29.06	29.20	8.55	8.88
PI	(13.76)	(7.27)	(13.82)	20.18	(11.70)	13.86	14.75	5.75

[a]The phospholipids which did not appear within 40 min were eluted by hexane/2-propanol/water (55:44:6). The retention times of this second elution are mentioned between brackets.

was thought to be due to the presence of a small amount of lysophosphatidylcholine (LPC).

Scaling-up using the RSiL stationary phase. While the column loadability is mainly determined by the relative retention of the different components of the sample, the RSiL column was selected at first. It was deduced from Table 1 that the retention times of PE, PI and PC would be quite different by eluting the RSiL stationary phase with a stepwise gradient of hexane, 2-propanol and water, in which the original 55:44:4 proportion was changed to 55:44:6. Upon increasing the sample load, however, the relative retention was changed to a large extent; although the three main soybean phospholipids were separated to baseline upon applying 200 μg of soybean lecithin, PI eluted as a broad shoulder of PC when 500 μg was injected. Comparing both chromatograms, it became obvious that the increased retention time of PC was responsible for this unfavorable behavior. In an attempt to improve the separation efficiency for higher loads, the relative retention of PC and PI was increased by selecting an isocratic 55:44:5 mobile phase, but even in this case the excellent resolution achieved at analytical sample loads was completely lost upon increasing the load to 500 μg. Hence, preparative scale separations seemed impossible using the RSiL column.

Scaling-up using the Merck stationary phase. First, the influence of the flow rate was investigated. A Van Deemter plot revealed that the HETP was nearly linearly related to the flow rate between 0.5 and 5.0 mL/min. Initially, a flow rate of 1.0 mL/min was selected as a good compromise between experiment duration and separation efficiency

From Table 1, it was obvious that the main soybean phospholipids were separated by a 55:44:5 ternary mixture of hexane, 2-propanol and water. By increasing the sample load, the resolution between PE and some more apolar impurities such as neutral lipids (NL), glycolipids and diphosphatidylglycerol (DPG) became limiting. Using a stepwise gradient with the mobile phase containing 4% water during the first 30 min, this limitation was largely circumvented, whereas a 5.7% aqueous mobile phase as the second step caused a reduction of the time required for the entire separation from 100 to 80 min. Further decreasing the water content of the first step of the gradient was not advisable, since the improved resolution of PE coincided with the coelution of PA and PI. On the other hand, increasing the water content during the second step of the gradient caused PI and PC to co-elute due to the strong fronting tendency of PC. Approximately 10% of this phospholipid coeluted with PI when a 55:44:6 mobile phase was applied.

Sample load. Using the above-mentioned mobile phase mixture of hexane, 2-propanol and water at 1 mL/min, whose composition was changed after 30 min from 55:44:4 to 55:44:5.7, the influence of the sample load on the retention behavior of the main soybean lecithin components was investigated. Figure 1 reveals that no general rule can be applied. NL and PI were not influenced, whereas PE and especially PC were retained more strongly when the sample load was increased. On the other hand, phosphatidic acid eluted earlier upon increasing the sample load. In addition, the peak shape changed. The NL as well as the PI peak became increasingly broader, whereas the peaks of the remaining phospholipids became broader and asymmetric. The PA peak began tailing and PE as well as PC were characterized by an increasing fronting tendency. From Figure 1 it was concluded that the resolution of PE became most decisive at overload conditions although the separation of PC and PI was limiting at analytical sample loads. As stated before, decreasing the water content of the mobile phase was inappropriate to overcome this problem, since this affected the resolution of PA and PI negatively. Fortunately, the Van Deemter plot of the column indicated that the separation efficiency could be improved by decreasing the flow rate. The HETP was only 70 μm at an optimal flow rate of 0.5 mL/min, whereas it was more than 100 μm at 1.0 mL/min. While at high sample loads, this phenomenon was especially pronounced for the sharper peaks in the initial part of the chromatogram, the flow rate in the last phase of the separation was increased to 2.0 mL/min in order to obtain a nearly-

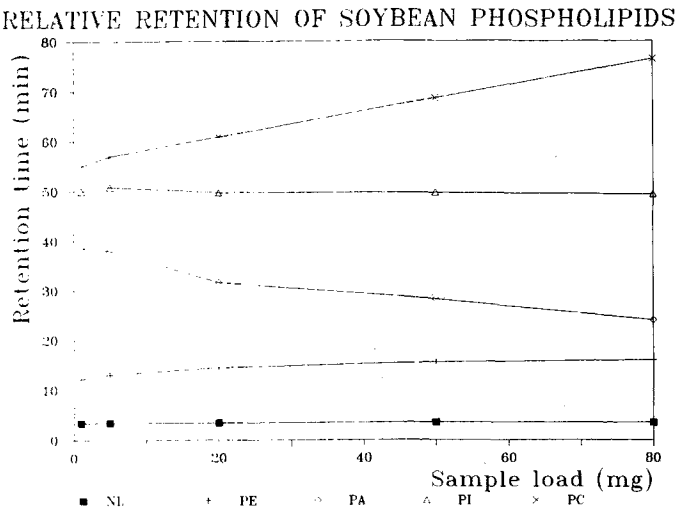

FIG. 1. Influence of the sample load on the retention of the major components of soybean lecithin on a 15-40 μm silica gel 60 stationary phase. The composition of the mobile phase, containing hexane, 2-propanol and water, was changed after 30 min from 55:44:4 to 55:44:5.7; the flow rate amounted to 1 mL per min.

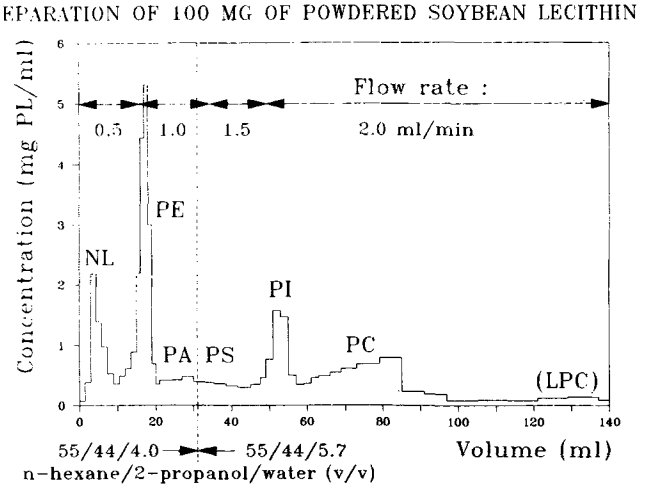

FIG. 2. Off-line chromatogram obtained by flow injection analysis of the fractions collected during the separation of 100 mg of soybean lecithin on a 250 × 4.6 mm ID column packed with 15-40 μm silica gel (Merck). The solvent program is mentioned schematically.

equal duration for the total experiment. The solvent program finally adopted is schematically represented in Figure 2. This program controlled the fraction collector, the flow rate and the composition of the mobile phase.

Evaluation of the method. To enable a quantitative evaluation of the proposed preparative separation method, 1.0 mL of the filtrate of a solution containing 5.0 g of powdered soybean lecithin per 50 mL of hexane/2-propanol (55:44) was injected. A UV-detector with a 1 cm cell path length was used on-line. It has to be noted that the time axis of Figure 3 is not equally spaced. Since a flow rate of 0.5 mL/min was used during the first 32 min, a chart speed of 1 mm/min was selected. The recording chart was fed at 2 mm/min afterwards. Moreover, a slight distortion of the peak shape is observed at 50 and 60 min due to the raising of the flow rate from 1.0 to 1.5 mL/min and from 1.5 to 2.0 mL/min, respectively. Due to the high sample load, the extinction sometimes exceeded the measuring range. Since the UV-detector signal due to phospholipids is known not to be directly related to their mass-concentration, a more reliable off-line chromatogram was elaborated by flow injection analysis using an evaporative light-scattering detector. Thus, the concentration of each fraction was determined very quickly. By comparing Figure 2 to Figure 3, some important differences appeared. First, the very tall PE-peak was striking. Flow injection analysis demonstrated that this phospholipid was well resolved since the various peaks in the UV-chromatogram between NL and PE were shown to represent only trace amounts. Moreover, Figure 2 revealed that phosphatidylserine (PS) and PA were minor components of soybean lecithin in spite of their very large peak area in the UV-chromatogram. Hence, the UV-detector seemed suitable for qualitative evaluations, but was rather inappropriate to obtain quantitative results.

Subsequently, the purity of the fractions was investigated by analytical HPLC (Fig. 4). Using calibration

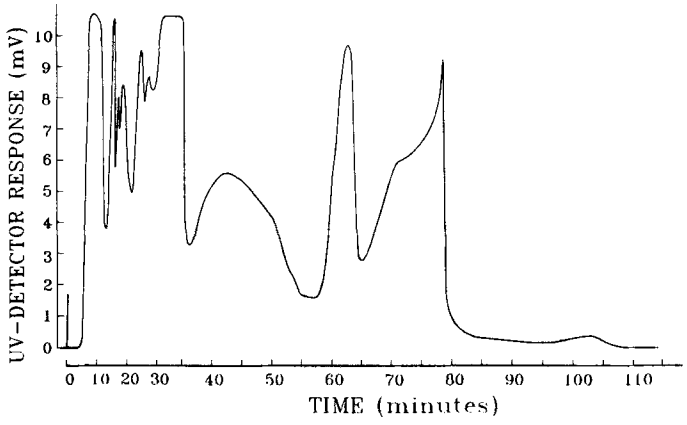

FIG. 3. Separation of 100 mg of soybean lecithin on a 250 × 4.6 mm column, packed with 15-40μm silica gel 60. The mobile phase, whose flow rate was increased from 0.5 to 1.0 mL/min after 32 min, to 1.5 mL/min after 50 min and finally to 2.0 mL/min after 60 min, was a ternary mixture of hexane, 2-propanol and water. After 47 min the composition was changed from 55:44:4.0 to 55:44:5.7. The peaks are identified in Figure 2.

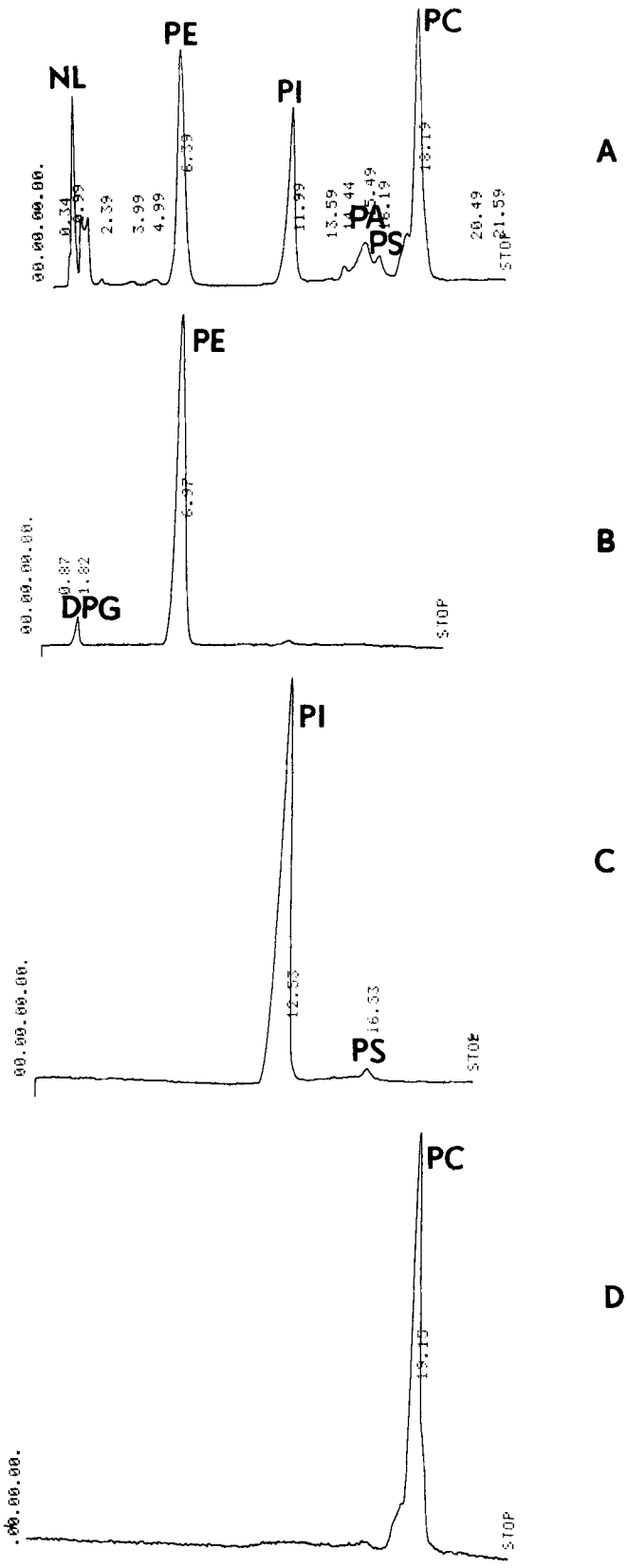

FIG. 4. Analytical HPLC of powdered soybean lecithin (A) and of the fractions number 14 (B), 30 (C) and 39 (D).

TABLE 2

Weight Percentage Composition and Estimated Mass of the Main Fractions Collected During the Preparative Separation of 100 mg of Soybean Lecithin, of which the Composition is Mentioned in the Bottom Line

Fraction number	Weight percentage Composition (%) DPG	PE	PI	PA	PS	PC	Mass (mg)
12	12.5	87.5	0.0	0.0	0.0	0.0	2.20
14	3.1	96.9	0.0	0.0	0.0	0.0	5.31
15	6.7	93.3	0.0	0.0	0.0	0.0	3.00
16	9.1	46.2	15.6	17.0	0.0	0.0	0.70
18	0.0	0.0	17.3	69.2	0.0	0.0	1.25
20	0.0	0.0	23.5	64.9	0.0	0.0	1.44
23	0.0	0.0	24.7	18.1	40.9	0.0	1.07
26	0.0	0.0	37.4	0.0	36.1	0.0	0.87
28	0.0	0.0	82.9	0.0	8.3	0.0	1.53
29	0.0	0.0	97.5	0.0	2.5	0.0	3.16
30							2.94
31	0.0	0.0	80.2	0.0	0.0	19.8	1.00
34	0.0	0.0	0.0	0.0	0.0	99.9	0.92
39	0.0	0.0	0.0	0.0	0.0	99.9	4.72
lecithin	5.4	24.2	16.1	5.6	2.9	36.0	85.20

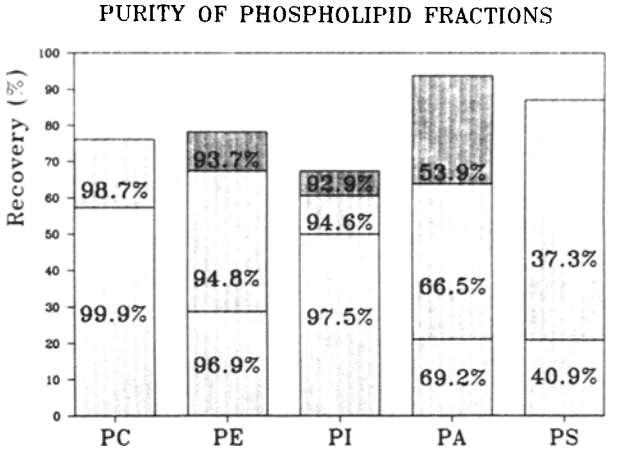

FIG. 5. Graphical representation of the relation between purity and recovery of the main phospholipids originating from the separation of 100 mg of soybean lecithin on a 250 × 4.6 mm column.

curves, the peak areas of the chromatograms were converted to the mass of the components, from which the weight percentage composition of the fractions was calculated. From Table 2, which includes the weight percentage composition of the sample, it is obvious that the three main soybean phospholipids could be obtained more than 97% pure. From the weight percentage composition and the mass of the individual fractions and the injected sample, it was calculated that only 50% of the injected amount of PC and PI was recovered, whereas only 30% of the total amount of PE could be obtained in such purity. By combining fractions, the recovery was increased. Since the purity decreased at the same time, it is obvious that a compromise has to be chosen. The intended application of the purified components determines which of the factors is more decisive. A purity of more than 99% is required for analytical reference samples. Highly-enriched fractions can improve the functional properties of soybean lecithins to a large extent. From Figure 5 it was concluded that the recovery of PC could be increased to more than 75% without any substantial loss in purity; in the case of PI and PE, 70 and 80%, respectively, could be recovered with a purity of about 93%. In addition, this Figure indicated that the PA and PS fractions contained considerably more impurities. One has to be aware, however, of the fact that the injected sample contained only a few percents of these phospholipids. Hence, these compounds were also highly purified.

DISCUSSION

By adjusting the water content of a mobile phase consisting of hexane, 2-propanol and water, all major soybean phospholipids were separated at analytical sample loads on two different brands of coarse, irregular silica gel. By comparing these two rather inexpensive packing materials, it was shown that the use of chemically similar stationary phases can result in a different phospholipid elution order. As the retention time of phospholipids is determined primarily by hydrogen bonding as well as ionic interactions, it was thought that this behavior might be due to differences in the surface charge density, which is strongly dependent on the preparation method of the silica.

Another, even more important difference was observed during scale-up: the sample load of the RSiL column had to be less than 500 μg and up to 100 mg could be fractionated on the Merck stationary phase. This was due to the fact that the separation efficiency of preparative HPLC was not only dependent on the compositions of the stationary and the mobile phases. Both the retention time and the peak shape were also influenced by the sample load as observed in the retention behavior of PI and PC. PI elution was almost independent of the sample load, whereas the retention time of PC increased with the amount of phospholipids injected. The resolution between PI and PC decreased upon increasing the

sample load when PC eluted earlier than PI, as appeared for the RSiL stationary phase. On the other hand, a better resolution was achieved for larger amounts when PC was eluted after PI. Since no general relation can be drawn between the retention behavior and sample load, it was not obvious that a stationary phase yielding a satisfactory analytical separation was suitable for direct scale-up to a preparative scale.

In recent years, the evaporative light-scattering (mass) detector has been shown to be superior to UV-detectors for quantitative, analytical HPLC separations (16,17). However, the UV-detector is inappropriate for either quantitatively or qualitatively evaluating preparative separations since its response is sigmoidally related to the concentration in the solvent stream. A nearly constant signal is generated at high concentrations, causing the peak shape to deviate from a Gaussian distribution curve. In addition, due to this behavior only a very small increase of the peak area is observed for large differences in sample load, making quantitations very inaccurate. Minor components are overestimated, whereas major compounds are underestimated. As a result, resolution seems worse than it actually is. Therefore, on-line monitoring was performed with UV-detection, which is known to provide a linear response over a broad concentration range. Moreover, a very stable baseline is obtained since the high sample load allows selection of a low sensitivity setting. While the extinction coefficients are dependent on the fatty acid composition and the degree of oxidation, quantitative estimations are unreliable. Therefore, an off-line concentration determination of the collected fractions is needed to assess the recovery. Although phosphate determination is mostly preferred, this procedure is quite time-consuming and destructive. In response, an alternative technique was elaborated using the mass detector in a flow injection analysis procedure. This method is very fast, sensitive and reproducible. Moreover, this technique was almost non-destructive. Only 10 μL of each fraction was injected, representing only 1% of the amount of phospholipids collected. Taking into account the volume of each fraction, it was calculated that 73.8 mg was collected. By comparison, weighing the residue after evaporation of 1.0 mL of the clear filtrate yielded 85.2 mg of soybean lecithin. Considering that a small amount was retained on the prefilter of the column and that a part of the sample was eluting after the fractionation was stopped, the accuracy of the proposed flow injection analysis technique becomes evident.

From these results and those of the quantitative analytical separations, it was concluded that the major soybean phospholipids could be obtained at least 93% pure, whereby approximately 80% of the amount of phospholipids injected was recovered. This necessitated the use of a solvent program controlling not only the composition, but also the flow rate of the mobile phase. Two mobile phases, containing hexane, 2-propanol and water, were used. Initially the column was eluted with 30 mL of a 55:44:4.0 ternary mixture, after which a 55:44:5.7 composition was preferred. Since a Van Deemter plot revealed that the HETP was almost linearly related to the flow rate, this parameter was set to only 0.5 mL/min during the first stage of the experiment because the separation efficiency could not be improved by changing the mobile phase composition. As the flow rate was increased afterwards to 2.0 mL/min, the separation was completed within 90 min. The method proposed enabled the fractionation of 100 mg of soybean lecithin on a 250 $\times$ 4.6 mm column, so that the sample load represented almost 2% of the weight of the stationary phase. As a consequence, it is expected that gram quantities could be obtained in a single run using wide-bore columns.

It can be concluded that the method proposed enables the purification of each of the major soybean phospholipids. Using a solvent switcher, the solvent program was achieved with a single pump, avoiding expensive gradient controllers. Moreover, the mobile phase only included volatile solvents. This prevented any deterioration of the collected phospholipids and allowed the fractionated products to be recovered by solvent evaporation. Finally, the column loadability amounted to 2% which presumably would allow gram quantities of pure phospholipids to be obtained using the selected stationary phase in wide-bore, preparative columns.

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