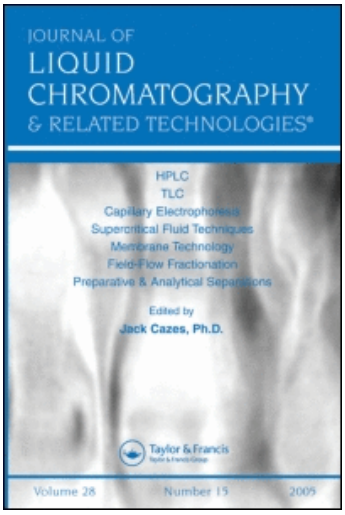

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Quantification of Soybean Phospholipids in Soybean Degummed Oil Residue by HPLC with Evaporative Light Scattering Detection

Yingying Zhang[a]; Yiwen Yang[a]; Qilong Ren[a]; Hailiang Jiang[b]
[a] National Laboratory of Secondary Resources Chemical Engineering, Zhejiang University, Hangzhou, P.R. China [b] Analysis and Measurement Center of Ningbo Institute of Technology, Zhejiang University, Ningbo, P.R. China

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Yingying Zhang, Yiwen Yang, and Qilong Ren
National Laboratory of Secondary Resources Chemical Engineering, Zhejiang University, Hangzhou, P.R. China

Hailiang Jiang
Analysis and Measurement Center of Ningbo Institute of Technology, Zhejiang University, Ningbo, P.R. China

Abstract: A high-performance liquid chromatographic method coupled with evaporative light-scattering detector was used for the accurate quantification of soybean phospholipids. This method is based on normal-phase chromatography with silica gel as stationary phase and a ternary gradient with n-hexane, isopropanol, and water as mobile phase. Major soybean phospholipid classes were separated by optimizing the solvent systems. The ternary solvent of n-hexane:isopropanol:water (53:42:5, by volume) was suitable for the separation of neutral lipids (NL), glycolipids (GL), and phosphatidylethanolamine (PE). By using hexane:isopropanol:water (17:66:17, by volume), phosphatidylcholine (PC), sphingomyelin (SM), and lysophosphatidylcholine (LPC) were separated. PE and phosphatidylinositol (PI), phosphatidic acid (PA), and PC were base line resolved by carefully adjusting the flow rate and switch time of intermediate gradients. The analysis was completed in 32 min, and repeated injections of the samples were possible. The method has good repeatability and accuracy from the view point of high recovery, and low coefficients of variance in retention time and peak area for each phospholipid. Comparison was

Address correspondence to Yiwen Yang, National Laboratory of Secondary Resources Chemical Engineering, Zhejiang University, Hangzhou 310027, P.R. China. E-mail: ceywyang@zju.edu.cn

made between three detectors, i.e., ELSD, UV, and RI. The results show that ELSD is the best in phospholipids analysis.

Keywords: Phospholipid, Quantification, HPLC, ELSD, Soybean degummed oil residue

INTRODUCTION

Phospholipids are found in all biological membranes, and contain extremely complex mixtures of different classes, such as phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidyl choline (PC), sphingomyelin (SM), and lysophosphatidylcholine (LPC).

Phospholipids are widely used as natural emulsifiers, wetting agents, and baking improvers. Moreover, in recent years, numerous applications in dietetics, cosmetics, and pharmaceuticals have been reported. Most phospholipids used in industry have been obtained from a by-product of vegetable oil, i.e., soybean degummed oil residue.

Analysis of phospholipids has been performed by numerous methods, e.g., thin-layer chromatography[1,2] (TLC), and high performance liquid chromatography[3–15] (HPLC). In earlier articles, the quantification of phospholipids had been performed exclusively by TLC, but the method had several disadvantages, e.g., the separation of individual classes was very difficult and time-consuming, and the technique was not always accurate. In the last two decades, HPLC has become the mainstream method for separation and quantitative determination of phospholipid classes.

With few exceptions, silica gel was used as the stationary phase, the mobile phase being either n-hexane-isopropanol-water, or acetonitrile-methanol-water. However, detection of the phospholipids has been a major problem. Although lipids lack specific absorption peaks, UV detection has, nevertheless, been mostly used.[4–7,9,15] The strong absorption in the 200–210 nm is caused by the presence of unsaturated bonds and functional groups such as carbonyl, carboxyl, and amino groups. As the extinction coefficient depends on the unsaturated degree of phospholipids, UV detection does not allow a quantitative estimation. Furthermore, the mobile phase must be UV-transparent, whereas gradients cause baseline drift. Refractive index (RI) detectors are relatively insensitive and incompatible with gradient elution and difficult to stabilize.

In recent years, HPLC with an evaporative light scattering detector (ELSD)[8,11,13] was reported as an effective analysis method for phospholipids, due to better reproducibility and less sensitivity to baseline drift during gradient elution, as compared to UV and RI detectors. On the other hand, the response of some phospholipids, e.g., SM and LPC is higher with ELSD than with UV and RI detectors. Therefore, an HPLC with ELSD procedure was used for the separation and quantitative determination of phospholipids classes in soybean degummed oil residue.

EXPERIMENTAL

Materials

HPLC-grade n-hexane and isopropanol were supplied by Burdick & Jackson International Inc. (Muskegon, MI, USA). Pure water was obtained by a NANOpure ultrapure water system (Barnstead, Iowa, USA). Soybean PE, SM, and LPC were purchased from Sigma Chemical Corp. (St. Louis, MO, USA). Soybean PC and PI were purchased form Lipoid Gmbh (Germany). Soybean degummed oil residue was obtained from Jiusan oils and fats Corp. (Harbin, China). Sample 1 and sample 2 were prepared by us from soybean degummed oil residue.

All lipids were dissolved in n-hexane-isopropanol-water (53:42:5, by volume) and filtered through a 0.45 μm Agilent filter.

Sample Preparation

Soybean degummed oil residue was extracted and separated into phospholipids by the following procedure: 100 g soybean degummed oil residue was mixed with 1000 mL acetone and homogenized at 500 rpm for 2 h with a mechanical stirrer. The slurry was filtered in a Buchner flask. The filter cake was vacuum dried for more than 3 h. The dried acetone-insoluble lecithin was then mixed with 300 mL 95% ethanol at room temperature and homogenized at 400 rpm for 1 h. The slurry was filtered and the filtrate was collected. The filter cake was re-extracted twice with 300 mL 95% ethanol for 1 h as above. The filtrate of the second and third extraction was collected and combined with the first filtrate. The solvent was evaporated in a rotary vacuum evaporator at less than 50°C. The residue was vacuum dried for more than 6 h to obtain sample 1.

Sample 1 was further separated by preparative column chromatography. Sample 1 (8 g) was dissolved in 20 mL hexane-isopropanol-water (1:1:0.175, by volume) and was loaded onto a silica gel preparative chromatographic column. The column was eluted with hexane-isopropanol-water (1:1:0.175, by volume). The eluent, mainly containing phosphatidylcholine, was collected. The solvent was evaporated in a rotary vacuum evaporator at less than 50°C. The residue was completely dried in a vacuum desiccator for more than 4 h to obtain sample 2.

HPLC Procedure and Detection System

HPLC was performed by using an Agilent 1100 Series (including Online-Degasser, QuatPump, Autosampler, and ColCom) with the ELSD 2000 evaporative light scattering detector (Alltech, Deerfield, IL, USA). The data

acquisition system was an HP 35900E (Hewlett, Packard) connected with a Dell PC. Separation was carried out on a 4.6 × 250 mm, 5 μm Waters silica column (Waters Inc., USA) at 35°C.

After optimization of the parameters for the ELSD, the drift tube temperature was set at 63.5°C. The flow rate of the carrier gas (compressed pure nitrogen) was set at 2.0 L/min, and the internal pressure was fixed at 0.4 MPa.

The gradient mode of the mobile phase was applied to adjust the retention time to less than 32 min. Not only NL, PE, PI, and PC, but also PC, SM, and LPC were completely separated. The gradient procedure was listed in Table 1. Briefly, samples were injected at time 0 and were eluted with hexane-isopropanol-water (53:42:5, by volume) for 8 min, at a flow rate of 0.8 mL/min. A linear flow rate gradient up to 1.2 mL/min was performed over the next 0.1 min. Solvent was changed from 53:42:5 to 36:54:10 and the flow rate shifted from 1.2 mL/min to 1 mL/min over the next 16.9 min. After 25.1 min, the elution solvent was changed to 17:66:17 and the flow rate was turned to 0.8 mL/min. In the next 10 min, the whole elution procedure was isocratic. After 40 min the first mobile phase was run again, so that a new sample could be injected after 50 min. To protect the chromatography column, it was flushed with 100 mL of n-hexane at the end of each day. Every week the column was rinsed with 150 mL of hexane-isopropanol-water (17:66:17, by volume) to remove polar contaminants.

RESULTS

Calibration Curves

Although all components in samples can be detected by ELSD, the area unitary method can not be used for quantification. There are two main reasons. One is that the regression equation forms are different for different phospholipids. The other is that the coefficients are different even if they

Table 1. HPLC gradient procedure to separate several soybean phospholipids

Time, min	Flow rate, mL/min	Isopropanol content, vol%	Water content, vol%	Hexane content, vol%
0	0.8	42	5	53
8	0.8	42	5	53
8.1	1.2	42	5	53
25	1	54	10	36
25.1	0.8	66	17	17
35	0.8	66	17	17

are all correlated by linear equations. Therefore, the standard curves method is popular in quantification, while ELSD was used for detection.

To enable direct quantification of phospholipids, calibration curves were elaborated showing the relationship between the peak area and the injection amount of phospholipids. Oppenheimer and Mourey[10] mentioned that the concentration response curves reveal an exponential relationship in a double logarithmic plot. Meeren et al.[11] pointed out that the equations $Y = a\ X^b$ (Y: peak area, X: amount of phospholipids, mg) fit well to the calibration curves of NL, PE, PC, PA, and PI. Vaghela and Kilara[12] mentioned the responses were linear for PE, PS, LPC, CER, PI, PC, and SM from whey protein when the concentrations were below 3 μg. Row et al.[13] viewed the response of PE, which showed linearity when the injection amount was less than 12 μg, while that of PC was the logarithmic function. Caboni et al.[14] used quadratic function $Y = (a + b\ X)^2$ as regression equations.

Depending on the concentration range, the flow rate of the nebulizer gas, the composition, and the flow rate of the mobile phase, most of the calibration curves are linear, except for PC and PE, which agrees well with the previous report. The exponential function of $Y = a\ X^b$ for PC and PE was used, since the linear regression equation had a lower regression coefficient.

The calibration curves for PE, PC, PI, SM, and LPC are shown in Fig. 1. The equations and regression coefficients are listed in Table 2. It can be seen that the regression coefficients of all phospholipids were good. The compositions of soybean degummed oil residue, sample 1 and sample 2, are presented in Table 3. The chromatograms of sample 1 and standard phospholipids are shown in Figs. 2 and 3, respectively.

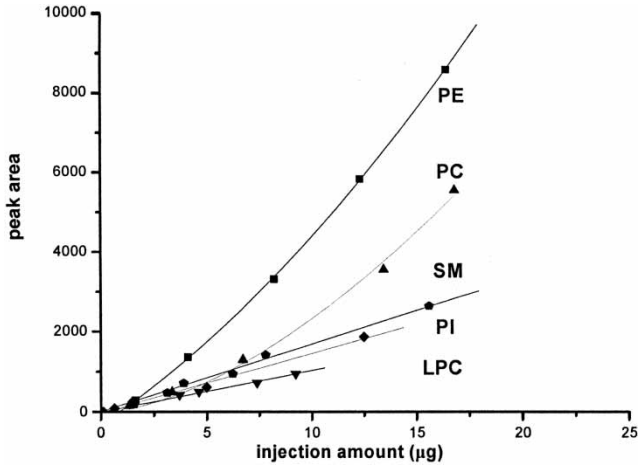

Figure 1. Calibration curves for PE, PI, SM, and LPC.

Table 2. Calibration curve equations of phospholipids

Phospholipids	Calibration curve equation	Regression coefficient R^2
PE	$Y = 189.647\ X^{1.363}$	0.9997
PC	$Y = 54.073\ X^{1.634}$	0.9959
PI	$Y = 146.79\ X$	0.9936
SM	$Y = 168.09\ X$	0.9926
LPC	$Y = 103.47\ X$	0.9906

Accuracy of Analysis

Soybean phospholipids mainly include PE, PI, PC, LPC, and a little SM. Repeatability of the retention time and the peak area was estimated and the results are listed in Table 4. The coefficient of variation of the peak area of LPC was somewhat bigger, probably because it consists of two unresolved peaks. However, it doesn't affect the accuracy of quantification.

Recovery was calculated by adding known amounts of standard phospholipids to Sample 2. The results are listed in Table 5. Although recovery of PC, SM, and LPC is a bit bigger, it is still in the reasonable range. Therefore, the analysis method is comparatively accurate.

DISCUSSION

Selection of Mobile Phase Composition, Gradient Procedure, and ELSD Parameters

The gradient elution mode was employed to separate the phospholipids in soybean lecithin because of their different polarity. A lower water content mobile phase was able to separate neutral lipids, glycolipids (GL), and PE, whereas the higher water content mobile phase might elute the acid phospholipids.

The water content was the most important factor for the resolution of different phospholipids. If less than 3% (by volume) water was present, NL

Table 3. Analytical results of raw material and samples

Sample name	PE	PI	PC	SM	LPC
Soybean degummed oil residue	10.06	6.65	12.82	—	0.65
Sample 1	15.23	10.7	41.15	0.5	1.35
Sample 2	—	15.69	80.57	—	—

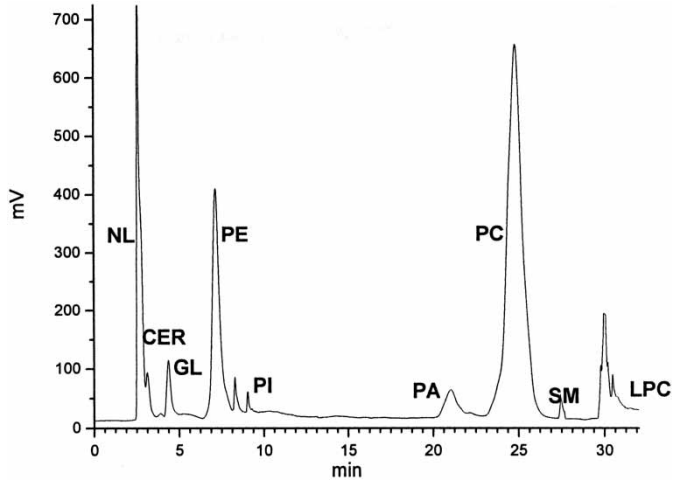

Figure 2. Chromatogram of soybean lecithin sample 1 with ELSD.

and PE were basically separated but PI and PE were co-eluted. When the water content was 5%, it was difficult to elute PC, and the retention time of it was 49.81 min when flow rate was 0.8 mL/min. When the water content was higher than 8%, PE was no longer separated from NL and GL. Therefore, the first gradient mobile phase is hexane-isopropanol-water (53:42:5, by volume). It was observed, that SM and LPC may be separated from PC

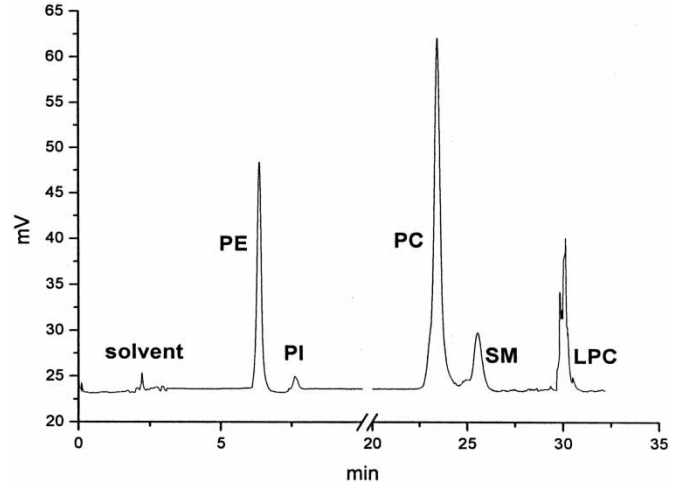

Figure 3. Chromatogram of standard phospholipids with ELSD.

Table 4. The repeatability of the retention time and the peak area for phospholipids[a]

Phospholipids	Retention time, min	Retention time C.V.,[b] %	Peak area, 10^3	Peak area C.V.,[b] %
PE	6.904 ± 0.0066	0.095	11.4121 ± 0.2739	2.40
PC	24.842 ± 0.045	0.181	39.9285 ± 0.8255	2.07
PI	8.281 ± 0.0171	0.207	0.3642 ± 0.0105	2.89
SM	27.375 ± 0.0313	0.114	2.6070 ± 0.0171	1.07
LPC	29.916 ± 0.103	0.346	6.2406 ± 0.0348	5.57

[a] The concentrations of standard solutions were 1.64 mg/ml for PE, 16.8 mg/ml for PC, 0.13 mg/ml for PI, 0.78 mg/ml for SM, and 1.84 mg/ml for LPC. Values given are means ± S.D.

[b] C.V. is coefficient of variation.

when the water content in mobile phase reached 17%. Hence, hexane-isopropanol-water (17:66:17, by volume) was used as the last gradient. PE and PI, PA and PC were separated by carefully adjusting the flow rate and switch time of intermediate gradients.

As for ELSD, the evaporator temperature is the most important. Low temperature will cause baseline drift at gradient elution mode. On the other hand, too high a temperature will also increase noise and, consequently, affects the baseline stability. In this work, the evaporator temperature is set at 65°C, while the temperature of the drift tube is set at 63.5°C.

Comparison with UV and RI Detectors

Since an RI detector is incompatible with gradient elution, the separation was carried out at 35°C in an isocratic mobile phase of hexane-isopropanol-water (17:66:17, by volume), and the flow rate is 0.8 mL/min. A chromatogram of soybean lecithin sample 1 with an RI detector is shown in Figure 4. It is apparent that the detector response of PE, PI, and PC is quite small. Although PE and PC are completely resolved, NL and PE are partly

Table 5. Recovery of HPLC analysis of soybean phospholipids

Phospholipids	Amount added, μg	Amount calculated, μg	Recovery, %
PE	0.82	0.808	98.5
PC	0.672	0.709	105.5
SM	0.78	0.803	102.9
LPC	0.93	0.985	106.0

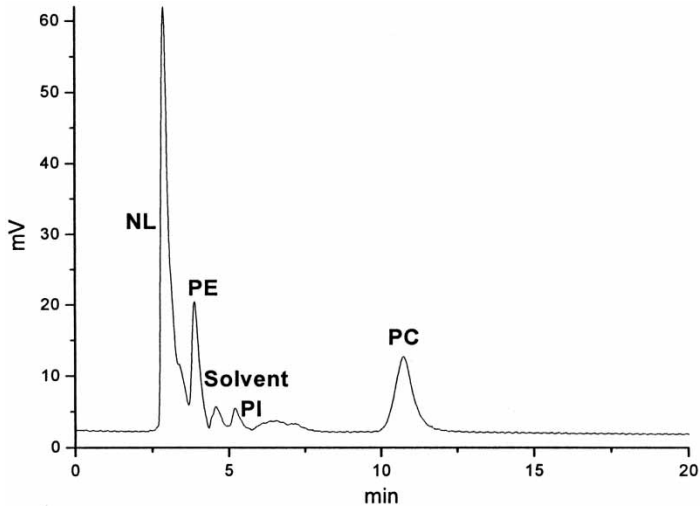

Figure 4. Chromatogram of soybean lecithin sample with RI detector.

resolved. Furthermore, there are no peaks of SM and LPC, which appear in Fig. 3. Similarly, a UV detector may not detect LPC because there is no absorbance in the range of 203–210 nm. The absorbance of PI and SM is so small that their peaks may be neglected. Besides the low absorbance of PI, SM, and LPC, the solvent peak partially overlaps with the PE peak, which makes it difficult to quantify these phospholipids in soybean lecithin.

CONCLUSION

An HPLC method with ELSD is described for the separation and accurate quantification of most important soybean phospholipids. This method is based on normal-phase chromatography with 5 μm silica gel as stationary phase, and a ternary gradient with n-hexane, isopropanol, and water as mobile phase. Not only Neutral lipids (NL), phosphatidylethanolamine (PE), phosphatidylinositol (PI), but also sphingomyelin (SM) and lysophosphatidylcholine (LPC) were well resolved from phosphatidylcholine (PC). The method has good repeatability and accuracy from the view point of high recovery, and low coefficients of variance in retention time and peak area for each phospholipid.

In addition, comparison between three detectors was also made. The hexane-isopropanol-water mobile phase system has obvious absorbance in 203–210 nm, which will decrease the response when used in HPLC with

UV detector. The solvent peak detected by RI partially overlaps with NL and PE, which makes it difficult to quantify PE accurately. The signal response of the same sample is higher with ELSD than with UV and RI detectors. Therefore, ELSD is the best in HPLC analysis of natural phospholipids.

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