

# Capillary Electrophoresis of Phospholipids with Indirect Photometric Detection

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## Abstract

The capillary electrophoresis (CE) separation of different anionic phospholipid classes including phosphatidic acids (PA), phosphatidylserine, phosphatidylinositol, phosphatidylglycerol (PG), and cardiolipin using indirect detection with adenosine monophosphate (AMP) is described. A standard mixture of PAs ( $C_{14}$ ,  $C_{16}$ , and  $C_{18}$ ) can be separated in 10 min by CE using 5mM AMP and 100mM boric acid in 10% water–80% methanol–10% acetonitrile. Although nonionic surfactants such as Brij 35 can improve the CE resolution of PAs, the separation time and the baseline noise are both increased. Optimization of the organic solvent in the running electrolyte is important. Methanol provides faster electroosmotic flow than propanol, and 10% acetonitrile effectively reduces migration time further by a factor of 1.4–2.2, depending on the phospholipid. The concentration limit of detection ranges from approximately 2 to 6 mg/L, and the mass limit of detection is as low as 21 pg. Linearity from 19 to 100 mg/L is established for cardiolipin and  $C_{16}$ -PG. Phospholipids in soybean and brain extract samples could be profiled.

## Introduction

The determination of phospholipids, the basic structural components of biological membranes, is important in the study of cell activities. Commercially, phospholipids are obtained from raw soybean oil and used in a variety of foods as emulsifiers, wetting agents, and antioxidants. Phosphatidic acids (PAs), important metabolic intermediates of phospholipid biosynthesis, consist of two nonpolar aliphatic ester functionalities and a polar phosphoric acid head group. The three major anionic derivatives, with functional groups esterified to the phosphoric acid group PA, are phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylglycerol (PG) (Figure 1). Phosphatidyl-

ethanolamine (PE) and the PG class, including the dimer cardiolipin, are particularly important components of the *E. coli* membrane.

High-performance liquid chromatography (HPLC) in both the normal and reversed-phase modes has been reviewed as the primary method (1) for the separation of these major phospholipids and also phosphatidylcholine (PC). Normal-phase HPLC, using silica and hexane–alcohol mixtures with small amounts of dilute acid or base as the mobile phase, has been an important approach (2). Diol-modified silica has been shown to be advantageous for improving peak shapes of phospholipids and has been used for separating platelet activity factor (3). Recently, soybean phospholipids have been separated using a  $\beta$ -cyclodextrin bonded silica column in the normal-phase mode (4). Abidi and Mounts (5) reported the separation of phospholipids by ion-pair HPLC using quaternary ammonium ion-pairing agents in an acetonitrile–water mobile phase and detection at 208 nm. By employing a  $C_{18}$  column, they have separated the PAs of varying length at the percent level in animal and plant samples. There are some additional reports on the separation of phospholipids by reversed-phase HPLC using a  $C_{18}$  column and hexane–alcohol mobile phase (6,7). If only qualitative information was required, a sample size of 10 mg was enough, but the analysis time in these reports was typically 2 to 3 h.

There have been few reports using capillary electrophoresis (CE) for the separation of phospholipids. Two such papers described the utilization of micellar electrokinetic chromatography (MEKC) with sodium cholate (8) or sodium deoxycholate (9) in 30% *n*-propanol and direct ultraviolet (UV) detection at 200 nm. Zwitterionic PC and PE, as well as PI, PS, and PA, in a 200 mg/L lecithin sample were separated in 10 min (9). Although no detection limits were provided, linearity of PC, PE, PI, and PS standards was shown from 5 to 100% (8). A comparison of MEKC with HPLC (9) showed that the former technique provided a much higher peak capacity, resulting in an improved resolution for PS in particular.

Previously, we have shown that ribonucleotides, particularly

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adenosine monophosphate (AMP), are useful UV-absorbing chromophores for CE with indirect photometric detection (IPD) of polyphosphates and polyphosphonates (10). The same CE approach using AMP in the run buffer has been an effective means for the separation and quantitation of aliphatic phosphorylated esters (11) and sugars (12). The objective of this work is to separate different anionic classes of phospholipids by CE using AMP as the IPD reagent and to explore the effect of organic modifiers and neutral surfactants on the separation. To the best of our knowledge, the CE separation of PG compounds is demonstrated for the first time. Detection limits as low as 2 mg/L are possible.

## Experimental

### Instrumentation

The CE instrument was an Applied Biosystems 270A (Foster City, CA) with a Macintosh SE+ computer equipped with a Rainin (Woburn, MA) Mac Integrator data acquisition system. The polyimide-coated fused-silica capillary (50- $\mu\text{m}$  i.d., 320- $\mu\text{m}$  o.d.) with various effective lengths ranging from 40 to 55 cm was obtained from Polymicro (Phoenix, AZ).

### Reagents and Chemicals

The monosodium salt of AMP (99% purity), soybean lipid extract, bovine brain extract, sodium salt of cardiolipin (R = primarily octadecadiene) from bovine heart, PG (C<sub>16</sub>:0), L- $\alpha$ -PI from crude soybean extract, L- $\alpha$ -PS from porcine brain extract, synthetic L- $\alpha$ -PA (C<sub>14</sub>, C<sub>16</sub>, and C<sub>18</sub>), Brij-35, and polyethylene glycol were purchased from the Sigma Chemical Company (St. Louis, MO). Methanol, acetonitrile, 2-propanol, *n*-hexane (all HPLC grade) and boric acid (99.5%) were obtained from Fisher Scientific (Fair Lawn, NJ).

### Preparation of Solutions

All stock solutions were prepared from water (greater than 17 MW resistance) taken from a Barnstead (Dubuque, IA) purification unit. The stock solution containing 50mM AMP and 1M boric acid was adjusted to pH 5.4 by addition of 1N NaOH, and then it was diluted to 5mM AMP–100mM boric acid with the desired organic solvent. All of these buffers were filtered through a 0.2-mm Gelman (Ann Arbor, MI) filter prior to use. All phospholipid samples were dissolved in a mixture of 10% water–90% methanol.

### Procedure

A new capillary was subjected to a standard wash cycle for 3 h using 1M NaOH and 1 h using water at 30°C. As a daily routine procedure, the capillary was flushed with 1M NaOH for 10 min, water for 2 min, and equilibrated

with the running electrolyte for 10 min. Between injections, the capillary was flushed for 2 min with water, 2 min with 1M NaOH, and then 3 min with the running electrolyte. All separations were carried out by applying a voltage of +30 kV (normal polarity with a positive injection end and negative detector end). Sample injection time was most commonly 6 s, but 3 and 10 s were also used. The capillary temperature was set at 30°C. IPD was at 259 nm. The effective electrophoretic mobility ( $u_{\text{ef}}$ ) was found by subtracting the electrophoretic flow (EOF) from the apparent electrophoretic mobility ( $u_{\text{ap}}$ ). The parameters EOF or  $u_{\text{ap}}$  were calculated from

$$\frac{L_{\text{eff}}L}{(t_mV)} \text{ (cm}^2\text{V}^{-1}\text{s}^{-1}\text{)} \quad \text{Eq 1}$$

where  $L_{\text{eff}}$  and  $L$  are the length of the capillary to the detector and the total length of the capillary, respectively;  $V$  is the voltage; and  $t_m$  is the migration of the neutral marker or phospholipid.

## Results and Discussion

As noted in the structures of phospholipids shown in Figure 1, these compounds can have a permanent  $-1$  charge because of deprotonation of the P–OH group with an absorbance band in the low UV wavelength range from the carbonyl groups. Several attempts to monitor PAs at less than 50 ppm via direct detection at 205 or 200 nm were unsuccessful. Based on a UV-visible spec-

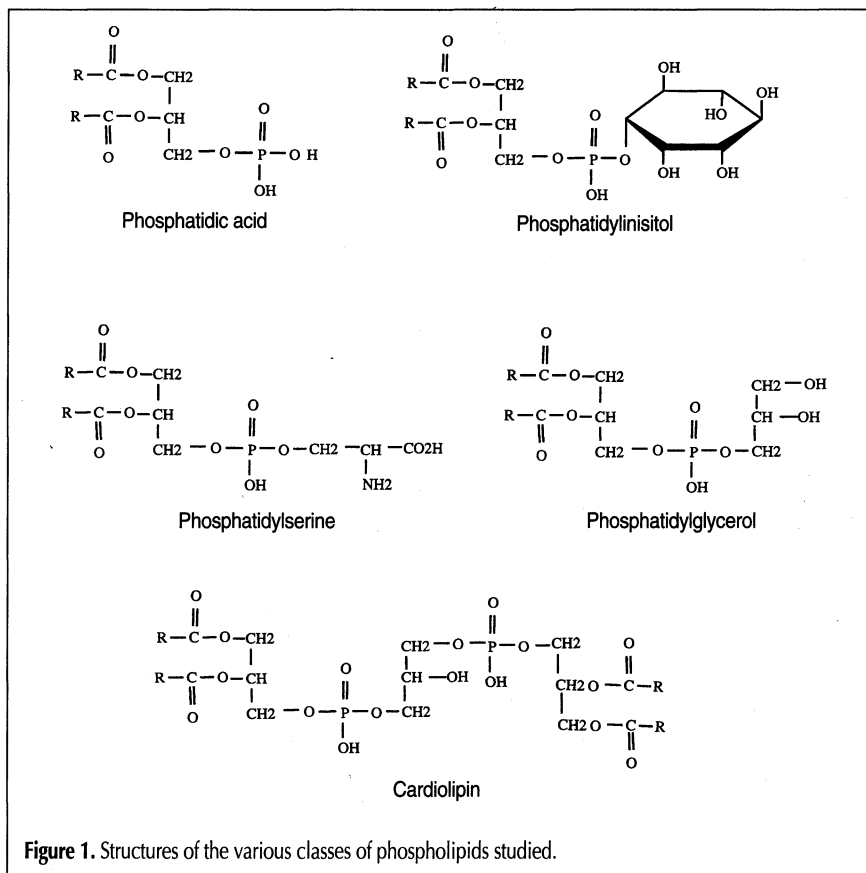


Figure 1. Structures of the various classes of phospholipids studied.

trum of C<sub>14</sub>-PA, and considering the short capillary pathlength of  $50 \times 10^{-4}$  cm, a 100-ppm solution would only give an absorbance of about  $1 \times 10^{-3}$ . Therefore, an indirect detection approach using AMP at 259 nm and normal-polarity CE was considered.

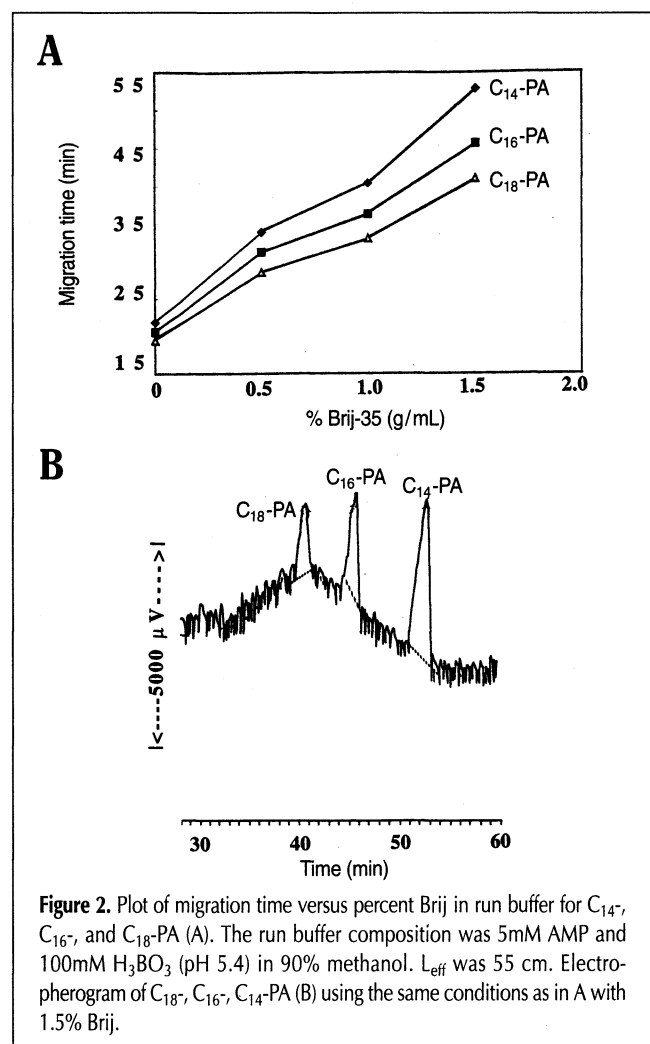
The effect of the running electrolyte pH on the separation of PAs (C<sub>14</sub>, C<sub>16</sub>, and C<sub>18</sub>) was of interest. The three pH values used in this work were 5.4, 6.0, and 7.0. For these studies, the running electrolyte was always 90% methanol–10% water with 5 mM AMP and 100 mM boric acid. As the pH of the running electrolyte was increased, the migration times became longer because of the increasing analyte charge (deprotonation of the second P–OH group) despite the faster EOF. At pH 5.4, the separation was completed in less than 16 min, but 26 min was required at pH 6.0 and 38 min was required at pH 7.0. Separations with a pH below 5.4 were not attempted in this study; although the stock solution containing 50mM AMP and 1M boric acid can be prepared at pH 5, the boric acid and AMP precipitated out of solution within approximately 2 min. All subsequent runs were done at pH 5.4 to minimize the analysis time.

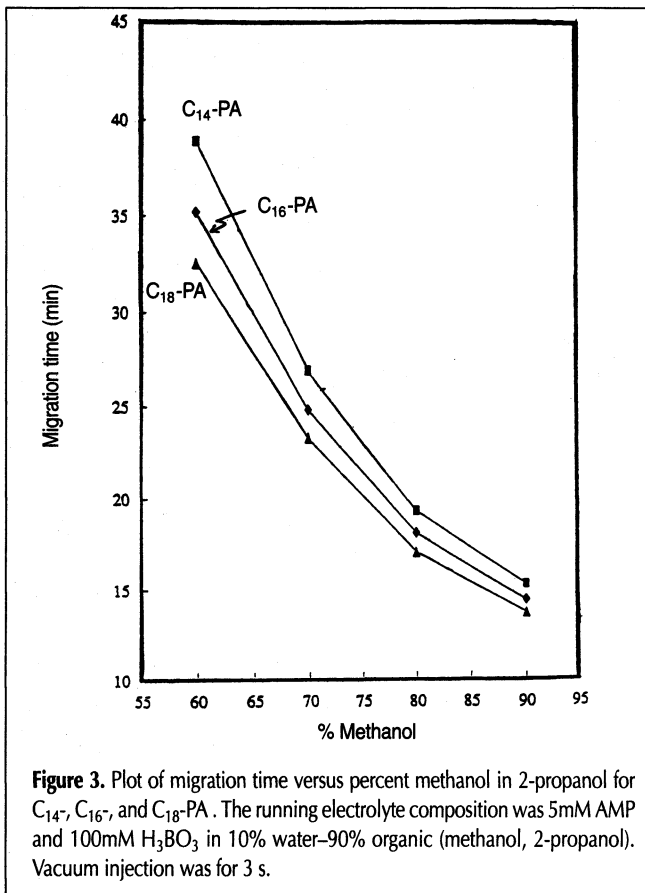
Nonionic surfactants such as Brij 35 and polyethylene glycol (PEG) were briefly studied for phospholipid separations. Use of these surfactants below the critical micelle concentration was intended to improve the solubility of phospholipids and possibly still be able to introduce a partitioning mechanism. Addition of the surfactant could affect the EOF by way of a change in viscosity and/or capillary surface charge. By increasing the Brij content from 0.5 to 1.5%, the migration times of PAs were found to increase by about 10 min (Figure 2). Because the neutral marker only increased from 9.45 to 13.45 min, it was possible that interaction of the PAs with the Brij may have occurred. Although good resolution of the PAs was possible (Figure 2), this concentration of Brij caused an increase in baseline noise. Brij could be useful in facilitating the resolution of PAs which differ by only one carbon atom in the alkyl group. However, the use of organic solvents was explored to gain faster analysis times (or better detectability) without a significant loss in resolution.

Organic solvents added to a buffer electrolyte alter the polarity and the viscosity of the running buffer. As a consequence, both the EOF and  $\mu_{ef}$  of the electrolyte and analyte are affected. In this work, the use of organic solvents was essential for both solubility and selectivity between different nonpolar phospholipid chains. Several organic modifiers with different percentages were tried. Nonpolar solvents such as hexane (6%) showed a significant decrease in EOF, but no separation improvement was obtained. It must be pointed out that nonpolar solvents are not very miscible, and as a result, the allowable percentage of these solvents in the running electrolyte is limited. Previously, propanol and/or methanol have been used as mobile phase components for HPLC and CE. It was found that a 90%-organic solvent primarily composed of an alcohol is required to achieve the best solubility and selectivity. A plot of migration times for PA (C<sub>14</sub>, C<sub>16</sub>, and C<sub>18</sub>) versus percent methanol in 2-propanol used in the running electrolyte is shown in Figure 3. The migration order C<sub>18</sub> < C<sub>16</sub> < C<sub>14</sub> is consistent with the faster mobility of long-chain compounds when normal polarity voltage is selected. The viscosity of the buffer decreased, resulting in increased EOF and  $\mu_{ef}$  as the propanol was replaced by methanol in the running electrolyte. Methanol also has a higher dielectric constant than propanol; this

resulted in a faster EOF and shorter analysis times as the methanol percentage increases (Figure 3). The magnitude of EOF is inversely related to the alkyl chain length of the alcohols (13).

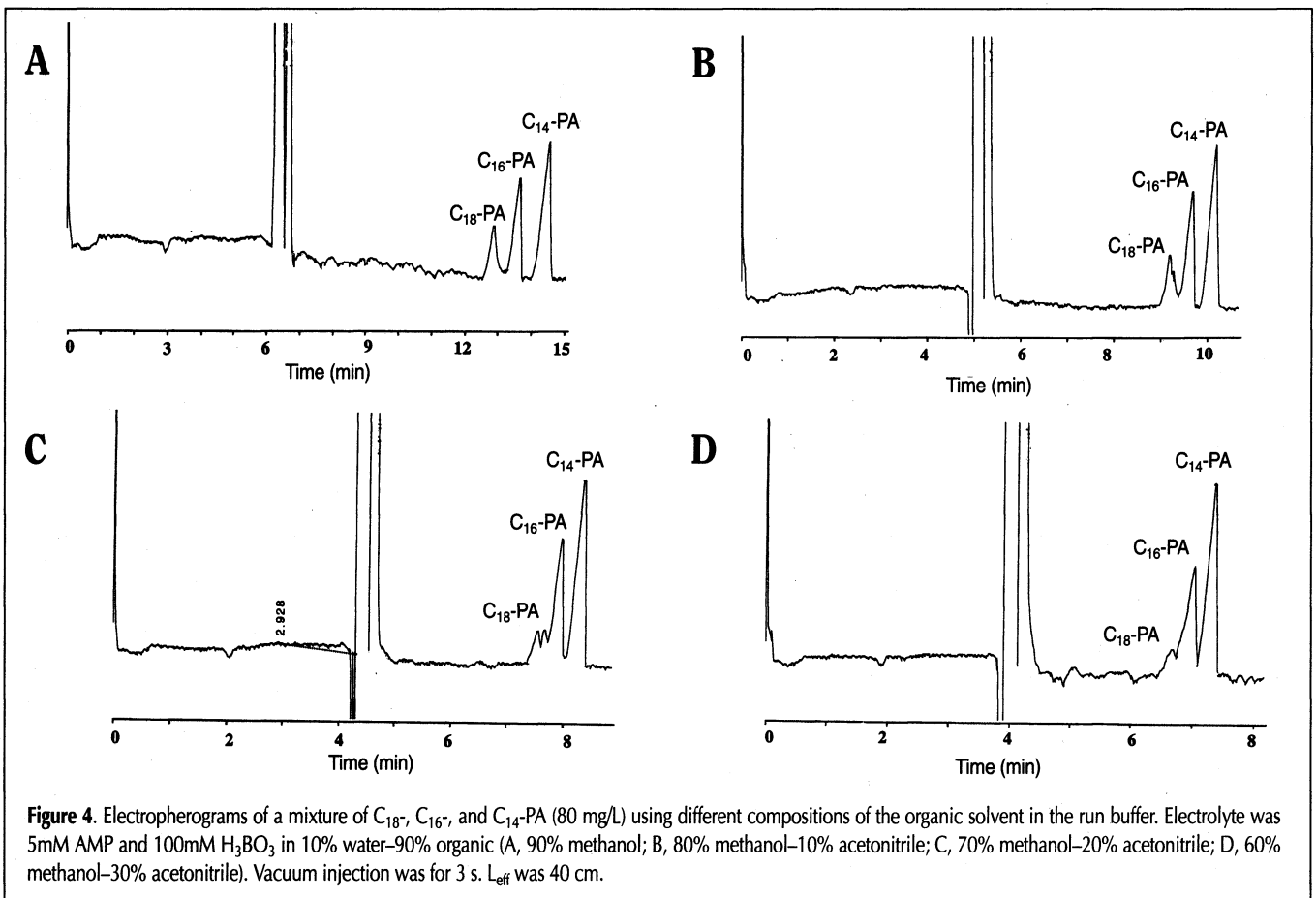
Fujiwara and Honda have investigated the influence of acetonitrile and methanol on EOF (14), demonstrating that a combination of these solvents was effective in separating positional isomers. A comparison of separation time for PAs (C<sub>14</sub>, C<sub>16</sub>, and C<sub>18</sub>) in the presence of acetonitrile is shown in Figure 4. A change from 0 to 10% acetonitrile showed a decrease in the EOF from 6.6 to 5.3 min, but the migration time for C<sub>14</sub>-PA decreased from 14.5 to 10.2 min. Buffer solubility restricted the percentage of acetonitrile to a maximum of 30%. As shown in Figure 4, the higher percentage of acetonitrile contributed to the loss of resolution when a capillary with a 40-cm effective length was used. To achieve a good resolution at 30% acetonitrile, a longer capillary (effective length of 55 cm) must be used, but analysis times remain around 11 min (data not shown). In general, the addition of acetonitrile (up to 20% of the running electrolyte) does increase the  $\mu_{ef}$  of some PAs from about  $-1.2 \times 10^{-4}$  to approximately  $-1.4 \times 10^{-4}$  before leveling out (at approximately 30%). Because of the improvement in analysis time without loss of peak resolution, further work was performed using the shorter capillary with 10% acetonitrile in the running electrolyte. Table I shows a migration time comparison of different phospholipid





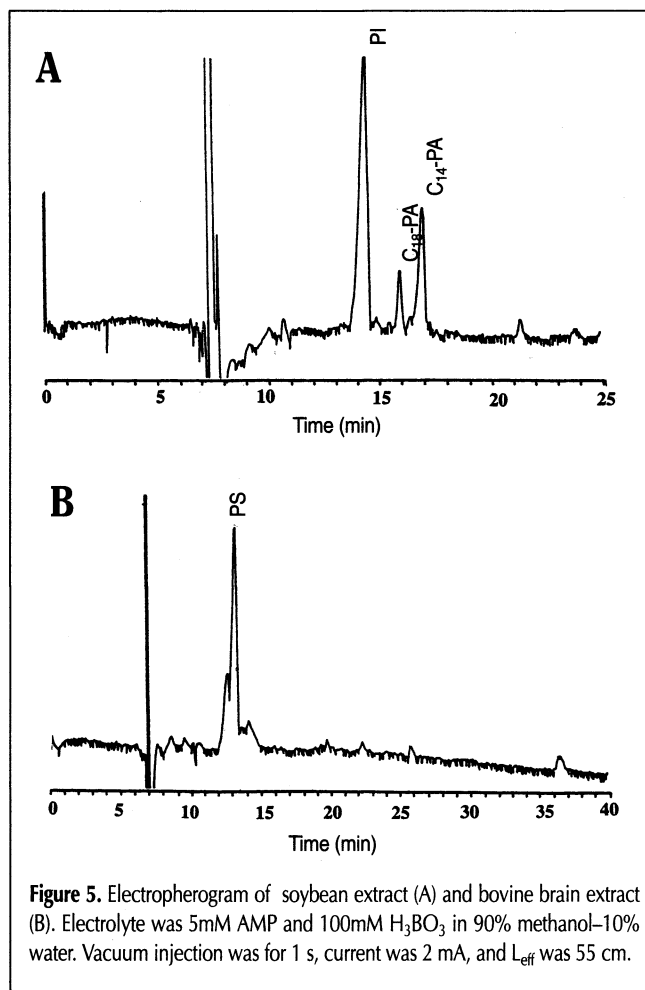
classes in the absence and presence of acetonitrile. The separation time of the PAs was decreased by a factor of approximately 1.4, but that for the other phospholipids was decreased a factor of approximately 2. It should be noted that in most cases, the presence of acetonitrile showed an improvement in peak sharpness as well as reduced retention. For example, the  $C_{14}$ -PA peak height increased from 4000  $\mu V$  at 0% acetonitrile to 5000 and 6000  $\mu V$  at 10 and 20% acetonitrile, respectively. Areas of all peaks were approximately the same because all the analytes were injected at the same injection size and concentration in different runs. The migration time for cardiolipin is the longest, as expected, because it has an additional negative charge from the second P–OH group. The fast mobility of PI is expected, based on the bulky inositol substituent.

The applicability of the developed CE separation for other classes of phospholipids is illustrated in Figure 5, which shows the electropherograms of soybean and bovine brain extract. These separations were done earlier in this research study using only methanol as the organic co-solvent; therefore, the migration times are longer. The soybean lipid sample was found to contain PI,  $C_{18}$ -PA, and  $C_{14}$ -PA, based on samples spiked with standards. These results are consistent with the reported contents, indicating that the preparation was primarily PI with the possible presence of PA and PE. Any PE would be difficult to distinguish from the offscale neutral marker peak under these CE conditions. The electropherogram of 0.2% bovine extract (Figure 5) shows one major peak corresponding to PS. The shoulder on the PS peak may be caused by PI, as was indicated



by the possible presence of this compound in this extract sample. A separation of cardiolipin and C<sub>16</sub>-PG standards is displayed in Figure 6. The linearity results for cardiolipin and C<sub>16</sub>-PG were found to be in the range of 19 to 100 ppm (4 points,  $n = 2$ ) and 19 to 57 ppm (4 points,  $n = 4$ ), with correlation coefficients of 0.999 and 0.998 and the least square equations of  $y = 717x - 1501$  and  $y = 398x - 288$ , respectively. The relative standard deviation (RSD) for replicate cardiolipin standards ranged from 4 to 8%.

Table I shows the concentration and mass limits of detection of different classes of phospholipids using prepared standard solutions. These detection limits in the 2–6 mg/L range are similar to those previously found for phosphorylated surfactants (11) after CE with AMP indirect detection. A mass detection limit as low as 21 pg was noted; picomole detection limits were in the 0.03–0.1 range and were not tabulated because of uncertainty of some molecular weights. It must be mentioned that the intensity of the PS and PI peaks decreased gradually during replicate runs; capillary adsorption of these compounds, noticeable at low levels, cannot be discounted. However, we believe these IPD limits of detection are significantly better than those possible using direct detection at low UV wavelengths. Similar compounds having only a C=O functional group, such as sulfonated ricinoleic acid, have been reported to be detectable only at the 25 ppm level at 200 nm after CE separation (15).



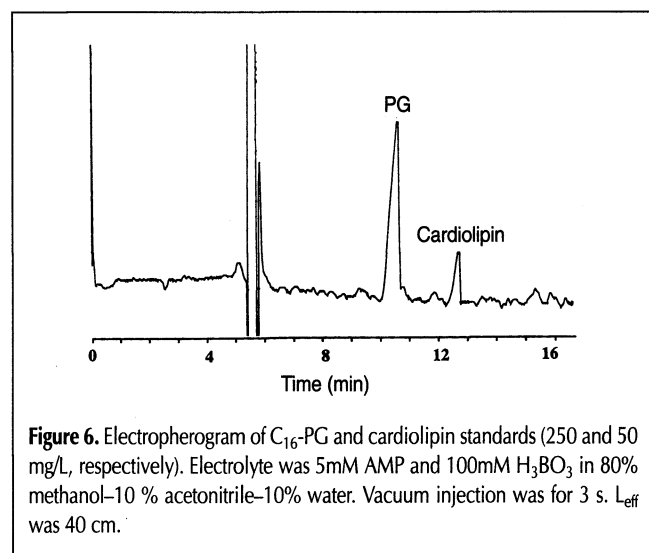
**Figure 5.** Electropherogram of soybean extract (A) and bovine brain extract (B). Electrolyte was 5mM AMP and 100mM H<sub>3</sub>BO<sub>3</sub> in 90% methanol–10% water. Vacuum injection was for 1 s, current was 2 mA, and  $L_{\text{eff}}$  was 55 cm.

## Conclusion

The CE separation and indirect detection of phospholipids is a very viable approach, particularly if the sample is fairly well characterized. Good detection limits for trace impurities may be necessary. Zwitterionic phospholipids, such as PC and PE, cannot be detected indirectly with this method; this can be considered both a selectivity advantage as well as an obvious disadvantage. Future work is dedicated to developing CE at low pH with indirect detection of these positively charged surfactants using a cationic chromophore.

## Acknowledgments

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**Figure 6.** Electropherogram of C<sub>16</sub>-PG and cardiolipin standards (250 and 50 mg/L, respectively). Electrolyte was 5mM AMP and 100mM H<sub>3</sub>BO<sub>3</sub> in 80% methanol–10% acetonitrile–10% water. Vacuum injection was for 3 s.  $L_{\text{eff}}$  was 40 cm.

**Table I. Comparison of Phospholipid Detection Limits and Migration Times in Presence of 0% and 10% Acetonitrile\***

Compound (standard)	Migration time (min) <sup>†</sup>		Detection limit	
	0% ACN	10% ACN	Milligrams per liter <sup>‡</sup>	picograms <sup>§</sup>
C <sub>14</sub> -PA	14.5	10.2	2	34
C <sub>16</sub> -PA	13.6	9.7	2	34
C <sub>18</sub> -PA	12.9	9.2	2	34
PI	16.2	8.5	5	145
C <sub>16</sub> -PG	19.8	10.4	3	21
Cardiolipin	24.7	12.1	5	145
PS	16.6	9.5	6	104

\* CE conditions: voltage, +30 kV applied for the separation; IPD, 259 nm; 5mM AMP and 100mM H<sub>3</sub>BO<sub>3</sub> in 10% water–90% methanol or in 10% water–80% methanol–10% acetonitrile. The running electrolyte with acetonitrile was used for the detection limits.

<sup>†</sup> Difference in neutral marker peaks at the two ACN percentages was only approximately 1 min.

<sup>‡</sup> Calculated from peaks representing a signal-to-noise ratio of 3.

<sup>§</sup> Calculated as described in reference 15.

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