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Separation of fluorescently labeled phosphoinositides and sphingolipids by capillary electrophoresis

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ABSTRACT

Phosphoinositides (PIs) and sphingolipids regulate many aspects of cell behavior and are often involved in disease processes such as oncogenesis. Capillary electrophoresis with laser induced fluorescence detection (CE-LIF) is emerging as an important tool for enzymatic assays of the metabolism of these lipids, particularly in cell-based formats. Previous separations of phosphoinositide lipids by CE required a complex buffer with polymer additives which had the disadvantages of high cost and/or short shelf life. Further a simultaneous separation of these classes of lipids has not been demonstrated in a robust buffer system. In the current work, a simple separation buffer based on NaH_2PO_4 and 1-propanol was optimized to separate two sphingolipids and multiple phosphoinositides by CE. The NaH_2PO_4 concentration, pH, 1-propanol fraction, and a surfactant additive to the buffer were individually optimized to achieve simultaneous separation of the sphingolipids and phosphoinositides. Fluorescein-labeled sphingosine (SFL) and sphingosine 1-phosphate (S1PFL), fluorescein-labeled phosphatidyl-inositol 4,5-bisphosphate (PIP2) and phosphatidyl-inositol 3,4,5-trisphosphate (PIP3), and bodipy-fluorescein (BFL)-labeled PIP2 and PIP3 were separated pairwise and in combination to demonstrate the generalizability of the method. Theoretical plate numbers achieved were as high as 2×10^5 in separating fluorophore-labeled PIP2 and PIP3. Detection limits for the 6 analytes were in the range of 10^{-18}–10^{-20} mol. The method also showed high reproducibility, as the relative standard deviation of the normalized migration time for each analyte in the simultaneous separation of all 6 compounds was less than 1%. The separation of a mixture composed of diacylglycerol (DAG) and multiple phosphoinositides was also demonstrated. As a final test, fluorescent lipid metabolites formed within cells loaded with BFLPIP2 were separated from a cell lysate as well as a single cell. This simple and robust separation method for SFL and S1PFL and various metabolites of phosphoinositide-related signal transduction is expected to enable improved enzymatic assays for biological and clinical applications.

1. Introduction

Phosphoinositides (PIs) and sphingolipids are important second messenger molecules in lipid signaling networks. PIs are involved in a broad range of biological functions including the regulation of cell growth, proliferation, apoptosis and other cellular activities [1,2]. Disordered PI signaling is common to many diseases, including diabetes, cardiovascular disease, and cancer [3]. For example, the phosphatidylinositol 3-kinase (PI3K) signaling pathway is involved in cancer cell growth, survival, motility, and metabolism and thus is of particular relevance as a target for drug development [4]. In this pathway, PI3K converts PIP2 into PIP3. The phosphatase-and-tensin-homolog-deleted-on-chromosome-ten-(PTEN) protein plays an important role as a tumor suppressor by catalyzing this reaction in the reverse direction. Sphingolipids also play critical roles in a variety of biological processes. Sphingosine-1-phosphate (S1P) is an important second messenger in cell survival and migration [5–7]. S1P is generated by phosphorylation of sphingosine by sphingosine kinase 1 and 2 (SK1 and SK2). The inhibition of the S1P pathway has proven promising in treating cancer and autoimmune diseases [8,9]. By virtue of the emerging roles of PI3K and SK1/2, as important therapeutic targets, direct and validated measurements of the activities of these lipid-modifying enzymes have become vital topics [2,8,10].

The most common techniques for analyzing phospholipids, including TLC and HPLC, are time consuming, labor intensive and

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lack sensitivity and specificity [11,12]. Mass spectrometry has been useful for lipid analysis, including phosphoinositide profiling [13–17]; however, mass spectrometry cannot distinguish the regioisomers of the lipids [17]. Moreover, the mass spectra of phospholipid mixtures containing phosphatidylcholine has been strongly dominated by phosphatidylcholine and lysophosphatidylcholine signals, which has prevented the detection of other phospholipids even if those lipids were present in comparable amounts [18]. Lipid signaling has also been analyzed by fluorescence-based methods. For example, high-throughput assays are based on use of the phosphoinositide-binding pleckstrin homology (PH) domains as detectors in measuring the production or localization of PIP3, but such assays typically require very large numbers of cells (10^5–10^6) [19,20]. GFP-tagged PH domains have been used in microscopy as an indirect assay of the enzymatic activities of PI3K and PTEN, but these molecularly engineered cell-based assays have limited applications, particularly for clinical samples [21–23].

In order to overcome these limitations, chemical separation by CE-LIF has emerged as an effective means for studying a variety of biological analytes including oligonucleotides, amino acids, proteins and lipid products because of the high separation efficiency and excellent detection limits. In order to detect the products of lipid metabolic enzymes by fluorescence-based techniques, a variety of fluorophore-labeled lipid substrates have been developed, often with similar kinetics to the endogenous substrate [24–29]. Metabolites and enzyme activities have been monitored by a variety of investigators using CE-LIF applied to cell lysates and single cells [30–36]. The use of CE in analyzing PI3K and sphingosine kinase activity for *in vitro* and cell-based assays has also been demonstrated [24,34,37]. Electrophoresis in a microfluidic device has likewise been applied to phospholipid separations. Lin et al. reported separation of phospholipids in microfluidic-chip-based micellar electrokinetic chromatography (MEKC) describing a generalized assay format to monitor the activities of lipid-modifying enzymes by on-chip separation of fluorescently labeled substrate and product [38]. To demonstrate feasibility, a mixture of the phosphoinositides phosphatidylinositol (PI), phosphatidylinositol 3-phosphate (PI(3)P) and phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2) were separated in a buffer incorporating 0.1% of the proprietary Coating-3 reagent (Caliper Life Sciences, Hopkinton, MA) with 20 mM sodium deoxycholate, 35% 1-propanol,100 mM Tris (pH 8.5), 1 mM EDTA, 1 mM HEPES (pH 7.0), 1 mM $MgCl_2$, 0.2 mM $MnCl_2$, and 0.4% glycerol; however, the PI(3)P and PI(3,4)P2 peaks were not completely resolved. Another disadvantage of the separation buffer was its complex composition. For their assays of SK and PI3K activity using CE-LIF in a standard glass capillary, the Allbritton group employed a separation buffer containing the proprietary dynamic coating reagent 5% Eotrol LR (Target Discovery, Palo Alto, CA) [24,27]. While baseline separation of all analytes was achieved, shortcomings with buffers utilizing Eotrol included the expense and short shelf life of this reagent. The development of a simple and robust separation method for sphingosine, S1P, PIP2 and PIP3 labeled with fluorophores would contribute to the assay of the corresponding enzymes.

The use of fluorescent lipid substrates or reporters coupled with sensitive and high resolution CE-based analysis has the potential to aid in the unraveling of the complex lipid metabolic pathways at the single-cell level. The method may make possible the simultaneous measurement of the activity of multiple lipid metabolizing enzymes, which would be a boon to lipid signaling pathway investigation. In the current work, a general method for CE-based lipid separation using a simple and low cost separation buffer was sought for performing biochemical assays of lipid modifying enzymes. Three pairs of lipids, sphingosine and S-1P labeled with fluorescein, PIP2 and PIP3 labeled with fluorescein, and PIP2 and PIP3 labeled with bodipy-fluorescein were used to optimize buffer composition and separation conditions, and the results were compared with previous methods. In addition, separation of a mixture composed of DAG and a variety of PIs was evaluated to test the applicability of this method to a broader range of compounds. Finally separation of the lipids from a complex biological matrix, a cell lysate or single cell, was assessed.

2. Materials and methods

2.1. Chemicals and solutions

Sphingosine-fluorescein (SFL), sphingosine 1-phosphate fluorescein (S1PFL, >98%), Bodipy-fluorescein labeled phosphatidyl-inositol (PI, C6, >98%), phosphatidyl-inositol 4-phosphate (PI4P, C6, >98%), phosphatidyl-inositol 5-phosphate (PI5P, C6, >98%), phosphatidyl-inositol 3,4-bisphosphate (PI(3,4)P2, C6, >98%), phosphatidyl-inositol 3,5-bisphosphate (PI(3,5)P2, C6, >98%), phosphatidyl-inositol 4,5-bisphosphate (BFLPIP2, C6, >98%) and phosphatidyl-inositol 3,4,5-trisphosphate (BFLPIP3, C6, >98%) were obtained from Echelon Biosciences Inc. (Salt Lake City, UT, USA). Phosphatidyl-inositol (4,5)-bisphosphate-fluorescein (PIP2FL, >95%) and phosphatidyl-inositol (3,4,5)-trisphosphate-fluorescein (PIP3FL, >95%) were from Cayman Chemical (Ann Arbor, MI, USA). Bodipy-(4,4-difluoro-3a,4a-diaza-s-indacene)-fluorescein (BFL, 99%) was purchased from Invitrogen (Carlsbad, CA). The Bodipy-fluorescein-diacylglycerol (DAG) was prepared by converting BFLPIP2 with phospholipase C (PLC). Sodium deoxycholate (SDC, ≥99.0%), sodium dodecyl sulfate (SDS, ≥98.5%), boric acid (≥99.5%), 1-propanol (≥99.9%) and magnesium chloride (1.00 M ± 0.01 M solution) were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). Tris-(hydroxymethyl)aminomethane (Tris, ≥99.8%), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, ≥99.5%), sodium phosphate monobasic (99.0%) and potassium phosphate monobasic (99.0%) were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Capillary electrophoresis

CE separations were performed using custom-built CE-LIF instruments reported previously or a commercial instrument [27]. To accomplish separations with the custom-built system, a potential difference was applied across the fused-silica capillary (Polymicro Technologies, Phoenix, AZ, ID 50 μm, OD 360 μm, length 41 cm) using a high-voltage power supply (Spellman CZE1000R, Plainview, NY, USA). The excitation light source was an Argon-ion laser (488 nm; JDS Uniphase, Santa Rosa, CA, USA) or a diode-pumped solid-state laser (473 nm; Lasermate, Pomona, CA, USA) coupled to a single-mode optical fiber (Oz Optics, Ottawa, Canada). The fluorescence was collected at a right angle to the capillary and the laser beam using a microscope objective (40×, 0.75 NA, Plan Fluor, Nikon, Melville, NY, USA), and detected by a photomultiplier tube (PMT, R3896; Hamamatsu, Bridgewater, NJ, USA) after spectral filtering with a notch filter (473 or 488 nm; Semrock, Lake Forest, IL) and a band-pass filter (535DF50; Chroma Technology, Rockingham, VT, USA). The PMT signal was amplified (PMT-5, Advanced Research Instruments, Golden, CO) and collected by a data acquisition board (KPCI-3100; Keithley Metrabyte, Cleveland, OH, USA or NI PCI-6229M, National Instruments Corporation, Austin, TX, USA). The sample was loaded by hydrodynamic injection and the injection volume was estimated as described previously [39].

All separations were performed at 22 °C using a voltage of −12 kV at the outlet reservoir with the inlet reservoir held at ground potential unless otherwise stated. The detection window on the capillary was created by removing the polyimide coating either 18.1

or 25.3 cm from the inlet. The separation conditions (Sections 3.1 and 3.2) were optimized using an automated commercial CE system (ProteomeLab™ PA800; Beckman Coulter, Inc., Brea, CA) with a standard capillary (ID 50 μm, length 30 cm, detection window at 20 cm). The separation voltage was 10 kV. Sample loading was performed by pressurizing the inlet (0.5 psi, 5 s) unless otherwise stated.

2.3. Data analysis

The electrophoretic data generated using the custom-built system were plotted and analyzed using Origin 7.5. The data from the PA800 were processed using the 32 Karat software (Beckman Coulter, Inc. Brea, CA). Resolution "*R*" was calculated as: $R = 2(t_{m2} - t_{m1})/(W_1 + W_2)$ where t_m was the migration time and *W* the peak width. For calculating *R* of SFL and S1PFL, the peak width of S1PFL was taken as the combined width of the two isomeric peaks. The migration time of S1PFL was calculated as the average of the isomeric peaks. Theoretical plate number *N* was defined as: $N = 5.54(t_m/W_{1/2})^2$ where t_m was the migration time, and $W_{1/2}$ the peak width at half-height. For S1PFL, *N* was calculated based on the peak of the first isomer.

2.4. Separations utilizing biological samples

PC-3 cells were serum-starved for 4.5 h, trypsinized and incubated with BFLPIP2/histone (200 nM/200 nM) for 5 min at 37 °C. The cells were then washed. The cells were then suspended in lysis buffer (0.2% triton X-100 and 1 mM Na_3VO_4 in the electrophoretic buffer) and frozen in liquid nitrogen until electrophoretically separated. Just prior to separation BFL (0.1 nM) was added as an internal standard. The lysate sample was separated using the optimized electrophoretic buffer. In a second assay, serum starved PC-3 cells were loaded with BFLPIP2/histone for 10 min and rinsed with buffer. Single cells were then loaded into the capillary after laser-based lysis as described previously [32] and the cellular contents separated in 80% NaH_2PO_4 (100 mM, pH 7.3) and 1-propanol (20%). The peaks were identified by comparing the migration time of each with the migration times of standards and the internal marker BFL (when present).

3. Results and discussion

3.1. Method development

3.1.1. Choice of separation buffer

The aim of the current work was to develop a simple, fast, cost-effective and generalizable CE separation method for phospholipid metabolites. To overcome the disadvantages of previous electrophoretic buffers utilized for phospholipid separations, we tested a number of separation buffers followed by optimization of the most promising one. Since PIP2 was readily obtained with a fluorescein or a bodipy-fluorescein label, it was desirable to separate the two labeled forms of PIP2 and PIP3. Additionally, the fluorescent labels impart differing solubility and kinetic parameters for PI3-K, and thus might be used simultaneously in a single assay [2,40]. The separation of PIPs and sphingosines in the same sample was performed with the expectation that the method could be extended to monitor crosstalk between PI3K and sphingosine kinase signaling pathways in future studies and to further demonstrate the generalizability of the method for the separation of phospholipid metabolites.

Four buffers were initially screened using the fluorescein-labeled forms of PIP2 and PIP3: Tris buffer (100 mM, pH 8.6), boric acid buffer (50 mM, pH 7.7), Tris-SDC-propanol (TSP) buffer (100 mM Tris, 10 mM SDC and 15% 1-propanol, pH 8.5), and

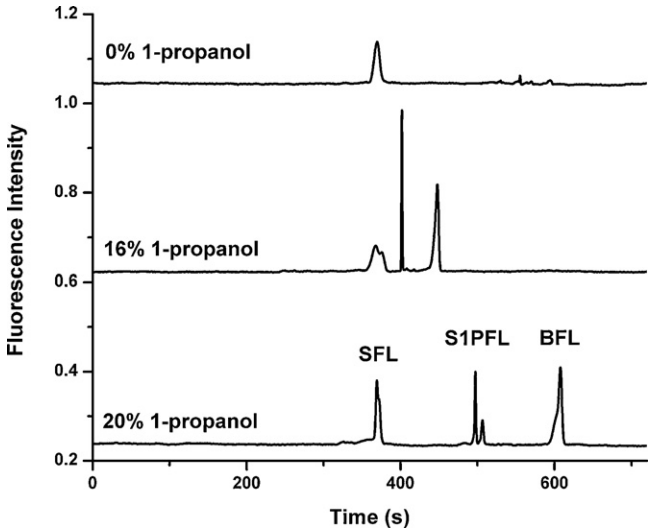

Fig. 1. Separation of BFL, SFL and S1PFL in a buffer composed of different volume fractions of NaH_2PO_4 (40 mM, pH 5.6) mixed with 1-propanol. Curves from the top to the bottom were generated using three different separation buffers: NaH_2PO_4 buffer (40 mM, pH 5.6) – top trace, 84% (v/v) of NaH_2PO_4 buffer (40 mM, pH 5.6) with 16% 1-propanol – middle trace, and 80% (v/v) of NaH_2PO_4 buffer (40 mM, pH 5.6) with 20% 1-propanol – lower trace. The corresponding offsets of the *y* axis are 0.8, 0.4 and 0 for the top, middle and lower traces, respectively.

NaH_2PO_4 buffer (40 mM, pH 5.6). The Tris and boric acid buffers were chosen for initial testing as they are commonly used CE buffers. The TSP and NaH_2PO_4 buffers were chosen based on their prior use for the electrophoretic separation of phospholipids [24,41]. The NaH_2PO_4 buffer demonstrated the most promise for separating PIP2FL and PIP3FL as this buffer yielded two sharp and well-separated peaks. The alternative buffers either did not resolve the two analytes or suffered from poor reproducibility (Figure S1).

3.1.2. Addition of 1-propanol to the NaH_2PO_4 buffer

Since the NaH_2PO_4 buffer (40 mM, pH 5.6) appeared to have potential for the separation of phospholipid analytes, the buffer was also assessed for its ability to separate SFL and S1PFL. BFL was included as an internal standard in these separations. The sample was comprised of BFL (0.5 nM), SFL (5 nM) and S1PFL (5 nM) in TSP buffer. The NaH_2PO_4 buffer alone was unsuitable for separation of the sphingosines (Fig. 1). 1-Propanol has been used as an additive for lipid separations due to its ability to assist in solubilizing the hydrophobic lipids and improve the separation efficiency of lipid analytes [27]. Different volume fractions of the NaH_2PO_4 buffer (40 mM, pH 5.6), 100–80%, were mixed with varying volume fractions of 1-propanol (0–20%), and this buffer system was assessed for the ability to separate BFL, SFL and S1PFL. In the presence of 1-propanol between 0 and 10%, BFL, SFL and S1PFL were not separated (Fig. 1). At a concentration of 16% 1-propanol, separation of these three analytes was achieved. The resolution of SFL and S1PFL was 1.8 ± 0.3 ($n = 5$). The resolution of SFL and S1PFL increased to 7.8 ± 1.3 ($n = 5$) when the 1-propanol concentration was increased to 20% (Fig. 1). The migration times of SFL and S1PFL were 370 ± 4 s and 493 ± 9 s ($n = 5$), respectively. The separation of BFL (1 nM), PIP2FL (10 nM) and PIP3FL (10 nM) dissolved in a sample matrix of TSP buffer was also performed in a buffer of 0% and 20% 1-propanol mixed with the NaH_2PO_4 buffer. With 0% propanol, the migration time of BFL, PIP2FL and PIP3FL was 236 ± 10, 474 ± 35 and 624 ± 63 s ($n = 4$), respectively. The resolution of PIP2FL and PIP3FL was 4.9 ± 1.0. Upon addition of 20% 1-propanol, the migration times for BFL, PIP2FL and PIP3FL changed to 568 ± 3, 1243 ± 28 and 1623 ± 30 s ($n = 5$) and the resolution of PIP2FL and PIP3FL increased to 6.7 ± 0.6 ($n = 5$). Thus the addition of 20% 1-propanol

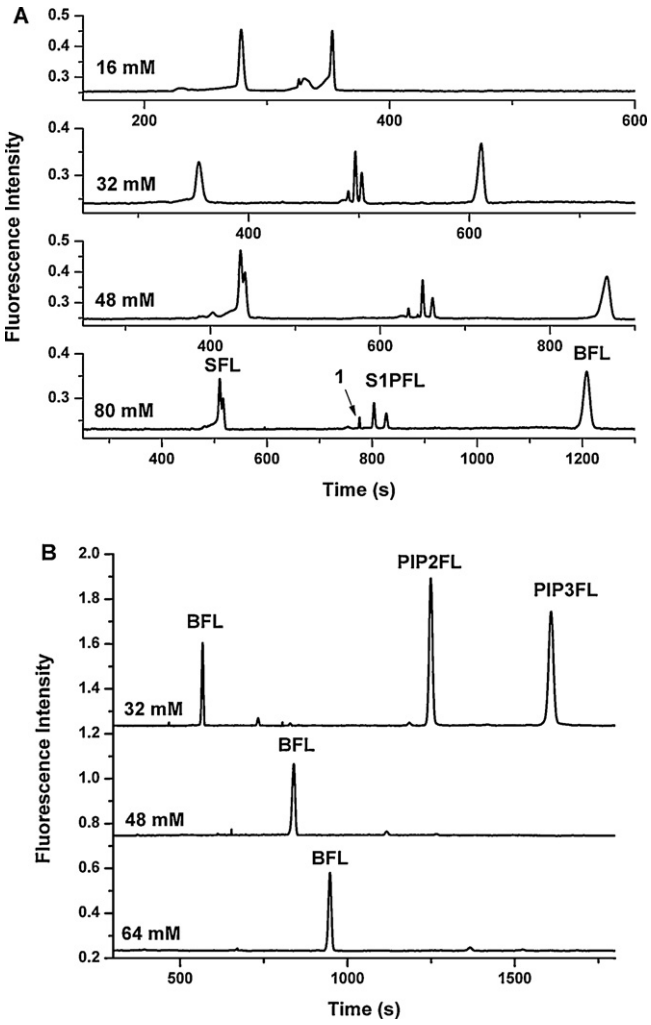

Fig. 2. Impact of NaH_2PO_4 concentration. (A) Separation of BFL, SFL and S1PFL with 16, 32, 48, and 80 mM NaH_2PO_4 (pH 5.6) buffer with 20% 1-propanol (from top to bottom). (B) Separation of PIP2FL and PIP3FL with NaH_2PO_4 buffer (pH 5.6, 20% 1-propanol) at different concentrations: 32, 48 and 64 (from top to bottom). The corresponding offsets of *y* axis are 1.0, 0.5 and 0, respectively. The "1" marks an impurity in the stock S1PFL.

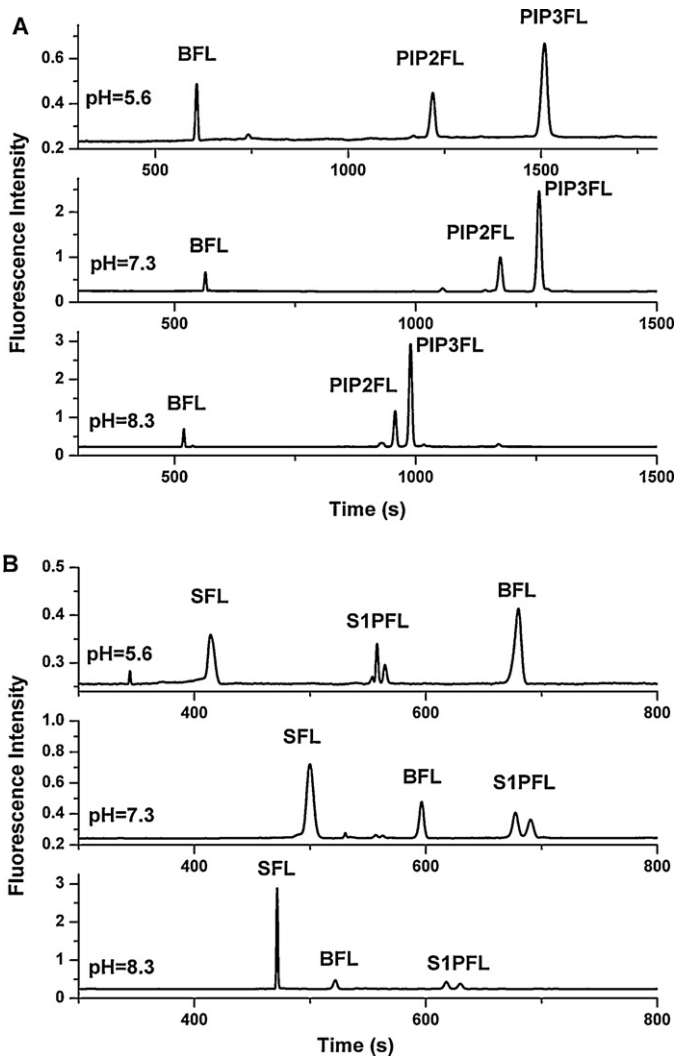

Fig. 3. Influence of pH. (A) Separation of PIP2FL and PIP3FL with NaH_2PO_4 buffer (32 mM NaH_2PO_4, 20% 1-propanol) at pH 5.6, 7.3 and 8.3 (from top to bottom). (B) Separation of SFL and S1PFL with NaH_2PO_4 buffer (80% (v/v) of 40 mM NaH_2PO_4, 20% 1-propanol) at pH 5.6, 7.3 and 8.3 (from top to bottom).

to the NaH_2PO_4 buffer (40 mM, pH 5.6) enhanced the separation of all of the tested lipid analytes.

3.1.3. Optimization of NaH_2PO_4 concentration

To further improve the separation of BFL (0.5 nM), SFL (5 nM) and S1PFL (5 nM) dissolved in TSP buffer matrix, the NaH_2PO_4 concentration was optimized. The stock NaH_2PO_4 buffer pH (pH 5.6) and v/v fraction of the stock NaH_2PO_4 buffer and 1-propanol were kept constant (80/20) while the concentration of NaH_2PO_4 stock buffer was varied from 20 to 100 mM, corresponding to a final NaH_2PO_4 concentration of 16–80 mM, respectively. The concentration of NaH_2PO_4 influenced the migration time of the analytes as well as their resolution (Fig. 2A). The peak splitting of the SFL and S1PFL was most likely due to the presence of two isomeric forms of fluorescein as reported in our previous report [24]. At 16 mM NaH_2PO_4 buffer, S1PF and BFL were not resolved. The resolution improved with increasing NaH_2PO_4 to 6.2 ± 1.8 (40 mM, $n = 5$), 11.4 ± 1.2 (60 mM, $n = 4$), 11.9 ± 0.8 (80 mM, $n = 4$) and 12.5 ± 1.2 (100 mM, $n = 5$). However, the migration times increased from 367 ± 11 s at 32 mM to 513 ± 6 s at 80 mM for SFL. Based on these results, NaH_2PO_4 concentrations of 32, 48 and 64 mM were assessed for the separation of BFL, PIP2FL and PIP3FL (Fig. 2B). At 32 mM NaH_2PO_4, BFL, PIP2FL and PIP3FL were separated and detected within 28 min, whereas at 48 and 64 mM, not all analytes had eluted after 30 min. For this reason, 32 mM was selected as the optimal NaH_2PO_4 concentration.

3.1.4. Assessment of pH

The pH of the stock NaH_2PO_4 buffer (40 mM, pH 5.6, 7.3, or 8.3) was varied prior to addition of the 1-propanol (20%) and the separation of BFL, PIP2FL and PIP3FL was assessed (Fig. 3A). The migration time, peak height and area, theoretical plates, and resolution for PIP2FL and PIP3FL are summarized in Table 1. The migration time of BFL, PIP2FL and PIP3FL decreased with increasing pH from 1490 ± 30 s ($n = 3$) at pH 5.6 to 1006 ± 23 s ($n = 5$) at pH 8.3 for PIP3FL. The peak height of both PIP2FL and PIP3FL increased with pH as expected due to the impact of pH on the quantum efficiency of the fluorophore [42]. The peak height of PIP2FL increased 4.5-fold from 0.21 ± 0.01 ($n = 3$) at pH 5.6 to 0.91 ± 0.04 ($n = 5$) at pH 8.3; the peak height of PIP3FL increased 7-fold from 0.43 ± 0.01 ($n = 3$) at pH 5.6 to 2.66 ± 0.11 ($n = 5$) at pH 8.3. The theoretical plates for PIP2FL and PIP3FL also improved as pH increased. Theoretical plates for PIP2FL rose 2.5-fold from $4.7 \times 10^4 \pm 2.2 \times 10^3$ ($n = 3$) at pH 5.6 to $1.2 \times 10^5 \pm 3.4 \times 10^3$ ($n = 5$) at pH 8.3, and for PIP3FL rose 3-fold from $4.43 \times 10^4 \pm 2.6 \times 10^3$ ($n = 3$) at pH 5.6 to $1.2 \times 10^5 \pm 2.2 \times 10^3$

($n=5$) at pH 8.3. The resolution at pH 5.6 was 5.4 ± 0.1 ($n=3$), but decreased as the pH increased to 2.9 ± 0.1 ($n=5$) at pH 7.3 and 1.3 ± 0.1 ($n=5$) at pH 8.3. For the separation of PIP2FL and PIP3FL, a pH of 7.3 for the NaH_2PO_4 buffer with 1-propanol provided the best compromise between resolution and analyte fluorescence intensity.

The separation of SFL (5 nM) and S1PFL (5 nM) in TSP buffer matrix was also performed in the NaH_2PO_4-propanol buffer system at varying pH (Fig. 3B). BFL, SFL and S1PFL were separated within 12 min at all three pH values. The migration time, peak height, theoretical plates and resolution are summarized in Table S1. The migration time of BFL decreased with increasing pH so that the peak elution sequence was dependent on pH. At pH 5.3, the migration velocity of SFL and S1PFL was greater than BFL, but above this pH the neutral BFL migrated between the sphingosine analytes. As with the PIP2FL and PIP3FL separation, the peak intensities of SFL and S1PFL increased with the pH of the buffer. Again, peak splitting of the S1PFL due to the two fluorescein isomers was seen [24]. The peak height of SFL increased from 0.1 ± 0.01 at pH 5.6 to 0.4 ± 0.1 at pH 7.3 and 2.5 ± 0.5 at pH 8.3 ($n=5$). The sharp increase in the SFL signal from pH 7.3 to 8.3 seen in the figure may be due to stacking as the sample is in a high pH buffer (pH 8.5) while the separation buffer pH is lower [43]. Of the three buffer pH conditions, the pH 8.3 NaH_2PO_4 buffer (80%) with 1-propanol (20%) possessed the best performance with regards to analyte signal intensity and resolution for the sphingosine analytes.

3.1.5. Sample matrix comparison

Prior work demonstrated that efficient dissolution of PIP2 and PIP3 in the sample matrix was an important factor in achieving high separation efficiency and resolution by CE [27]. Thus, the sample matrix for the phospholipid preparation was also optimized in this work. Four sample solvents were tested: TSP buffer (100 mM Tris, 10 mM SDC and 15% 1-propanol, pH 8.5), the NaH_2PO_4 electrophoresis buffer (32 mM NaH_2PO_4, pH 7.3, 20% of 1-propanol), extracellular buffer (ECB, 135 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, and 2 mM $CaCl_2$, pH 7.4), and PBS buffer (137 mM NaCl, 27 mM KCl, 10 mM NaH_2PO_4, 1.75 mM KH_2PO_4, pH 7.4). TSP buffer was tested since SDC is often used to disperse lipids [24,27]. ECB and PBS buffers were chosen as these buffers are cell compatible and are often used as the sample matrix when cells or lysates are separated by CE [29,34]. BFL (1 nM), PIP2FL (10 nM) and PIP3FL (10 nM) were dissolved in each of the sample matrices and then separated in the buffer system optimized for the phosphoinositide analytes (NaH_2PO_4 32 mM, pH 7.3 and 20% 1-propanol). When the analytes were dissolved in ECB and PBS, the migration times, resolution and theoretical plates of the analytes were not reproducible (data not shown). Table S2 summarizes the separation results when TSP or the electrophoresis buffer was used as the sample matrix. TSP buffer was selected as the best sample matrix since it yielded less variation in the analyte migration times and peak areas relative to that of the other buffers.

3.2. Separation of lipids under optimized conditions

3.2.1. Separation of PIP2FL and PIP3FL

A mixture of PIP2FL and PIP3FL (1 nM of each analyte in TSP) was separated in the optimal buffer. The two peaks were well separated in 19 min (Fig. 4A) with $R = 1.8 \pm 0.1$ ($n=3$) and $N = 1.8 \times 10^5 \pm 9 \times 10^2$ for PIP2FL and $N = 1.8 \times 10^5 \pm 2 \times 10^3$ for PIP3FL. The migration times of PIP2FL and PIP3FL were highly reproducible at 1062 ± 4 s (PIP2FL) and 1111 ± 4 s (PIP3FL) (Table 2). The limit of detection (LOD) was 3×10^{-20} mol for PIP2FL and 2×10^{-20} mol for PIP3FL at an S/N of 3.0 ($n=5$).

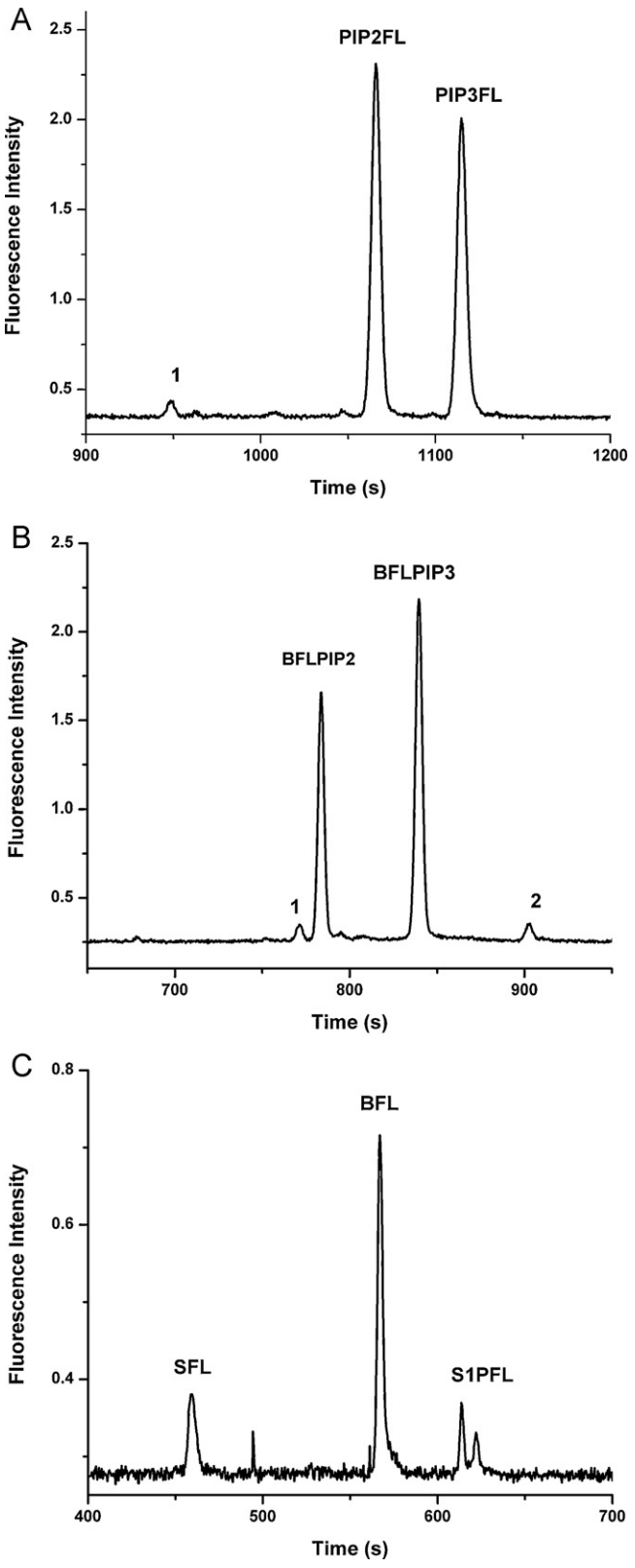

Fig. 4. Separation of lipids in the optimal buffer and sample matrix. (A) Separation of PIP2FL and PIP3FL. (B) Separation of BFLPIP2 and BFLPIP3. (C) Separation of BFL, SFL and S1PFL. In (A) and (B), peaks 1 and 2 were impurities in the lipid stocks.

3.2.2. Separation of BFLPIP2 and BFLPIP3

A mixture of BFLPIP2 and BFLPIP3 (1 nM of each analyte in TSP) was separated in the optimal buffer (Fig. 4B) with $R = 3.7 \pm 0.1$ ($n=3$). The migration times of BFLPIP2 and BFLPIP3 were 779 ± 5

Table 1
Separation of PIP2FL and PIP3FL in NaH_2PO_4 (32 mM) and 1-propanol (20%) at pH 5.6, 7.3 and 8.3.

	Migration time (s)			Peak height		Peak area		Theoretical plates		R^c
	BFL	PIP2FL	PIP3FL	PIP2FL	PIP3FL	PIP2FL	PIP3FL	PIP2FL	PIP3FL	
pH 5.6 ($n=3$)										
Avg[a]	604	1205	1490	0.21	0.44	4.8×10^3	1.0×10^4	4.8×10^4	4.4×10^4	5.4
SD[b]	5	22	30	0.00	0.02	2×10^2	2×10^2	2×10^3	2×10^3	0.1
pH 7.3 ($n=5$)										
Avg	566	1186	1269	0.73	2.19	1.2×10^4	3.4×10^4	9.9×10^4	1.0×10^5	2.9
SD	4	23	27	0.04	0.11	5×10^2	1.5×10^3	4×10^3	3×10^3	0.1
pH 8.3 ($n=5$)										
Avg	523	972	1006	0.91	2.66	1.3×10^4	3.9×10^4	1.2×10^5	1.2×10^5	1.3
SD	5	21	23	0.04	0.11	4×10^2	1.2×10^3	3×10^3	2×10^3	0.1

[a] Average (Avg).
[b] Standard deviation (SD).
[c] R is the separation resolution of PIP2FL and PIP3FL.

and 834 ± 6 s ($n=3$) with theoretical plates of $2.1\times10^5\pm4\times10^3$ and $2.0\times10^5\pm3\times10^3$, respectively (Table 2). The LODs of BFLPIP2 and BFLPIP3 were 4×10^{-20} and 2×10^{-20} mol at an average S/N of 3 ($n=5$). The separation efficiency was improved in comparison to our prior results for these analytes [27]. Likewise, the theoretical plates improved by 54%. These data clearly demonstrate improvements over prior methods that required a complex buffer and high-cost reagents [38].

3.2.3. Separation of SFL and S1PFL

Separation of BFL (0.5 nM), SFL (10 nM), and S1PFL (10 nM) dissolved in TSP matrix was performed in the optimal buffer, since a buffer was sought for simultaneous separation of all of the lipid species (Fig. 4C). The separation of SFL and S1PFL was completed in 11 min with a theoretical plate number and migration time for SFL of $5.2\times10^4\pm4\times10^3$ and 461 ± 4 s. The peak splitting due to the two isomeric forms was again observed for S1PFL [24]. For this separation, $N=2.7\times10^5\pm2.5\times10^4$ for S1PFL ($n=5$), and R was 12.6 ± 0.6. Despite using a simpler buffer system, the resolution for this lipids separation was improved relative to that reported by the Allbritton lab previously [24]. The LOD for SFL was 4×10^{-19} mol and S1PFL was 3×10^{-19} mol with corresponding S/N of 3. Again, these results demonstrate improved detection limits and separation efficiency for these fluorescently labeled sphingosines over prior reports [24,37].

3.3. Separation of mixtures of lipids

The goal of this work was to simultaneously measure multiple lipid metabolites from a biological sample. Thus a mixture of six lipids, including SFL (10.0 nM), S1PFL (2.0 nM), BFLPIP2 (1.0 nM), BFLPIP3 (2.0 nM), PIP2FL (0.5 nM) and PIP3FL (1.5 nM), was separated using the optimal buffer and sample solvent conditions (Fig. 5A). BFL (1.0 nM) was also included as an internal standard. The migration times, peak heights and peak areas for this separation are listed in Table 3. The reproducibility of the migration time, peak height, and peak area was excellent for each analyte. These data show successful and reproducible separation of the six lipid analytes plus an internal standard using a simple buffer system.

Phosphoinositide metabolism is carried out by a complex network of enzymes that can act on a single substrate to create multiple products. For example, PI(4,5)P2 can be converted into: PIP3 by PI3K, to PI4P or PI5P by phosphatases, or to DAG by phospholipase C (PLC). These metabolites can then be converted to additional products, for example, PIP3 can be metabolized to PI(4,5)P2 or PI(3,4)P2 by the phosphatases PTEN and SHIP, respectively [44–46]. In order to determine whether the current buffer would be able to separate these different lipid metabolites simultaneously, a mixture composed of BFL (0.04 nM), and BFL-labeled DAG (4.7 nM), PI (0.04 nM), PI4P (0.2 nM), PI5P (0.4 nM), PI(3,4)P2 (0.4 nM), PIP2 (0.4 nM), PI(3,5)P2 (0.4 nM) and PIP3 (0.4 nM) was separated (Fig. 5B). All analytes were baseline separated (Table S3) except for two sets of lipid pairs which differed only in the phosphate location on the inositol ring: PI4P/PI5P, and PI(3,4)P2/PIP2. Two additional peaks were identified and were due to impurities present in the standard compounds. Efficient separations such as these of multiple lipid metabolites will be valuable in parallel monitoring of the lipid signaling enzymes in cells.

3.4. Separation of lipid mixtures from cell lysates and single cells

PC-3 cells, a prostate carcinoma cell line with upregulated PI3K activity, were loaded with BFLPIP2 by incubation with BFLPIP2 complexed to histone and washed as described previously [34]. The cells were then lysed and the lysate separated electrophoretically using the optimized buffer conditions (Fig. 6A). A variety of fluorescent metabolites were formed in the cell from the starting material, BFLPIP2. Formation of BFLPIP3 by PI3K was readily detected as was formation of BFL-DAG from the action of phospholipase C. BFL-PI was also present as was two unknown fluorescent metabolites. As a greater challenge, a single PC-3 cell was loaded with BFLPIP2, washed, and the contents of a single cell loaded into the capillary

Table 2
Separation of BFLPIP2, BFLPIP3, PIP2FL, PIP3FL, SFL and S1PFL in the optimized buffer.

	BFLPIP2	BFLPIP3	PIP2FL	PIP3FL	SFL	S1PFL
Migration time (s)						
Avg[a]	779	834	1062	1111	461	621[c]
SD[b]	5	6	4	4	4	7
Theoretical plates						
Avg	2.1×10^5	2.0×10^5	1.8×10^5	1.8×10^5	5.3×10^4	2.7×10^5[d]
SD	4×10^3	3×10^3	9×10^2	2×10^3	4×10^3	2.5×10^4

[a] Average (Avg).
[b] Standard deviation (SD).
[c] The listed migration time was the average of the migration times of the two isomer peaks.
[d] The theoretical plate number of the isomer migrating the fastest is shown.

Table 3
Simultaneous separation of six lipids: BFLPIP2, BFLPIP3, PIP2FL, PIP3FL, SFL and S1PFL.

	BFL	SFL	S1PF(1)[c]	S1PF(2)[c]	BFLPIP2	BFLPIP3	PIP2FL	PIP3FL
Migration time (s)								
Avg[a]	551	454	602	610	759	810	924	965
SD[b]	6	4	7	7	10	11	15	16
Peak height								
Avg	0.97	1.48	0.93	0.62	1.45	0.24	1.49	0.41
SD	0.05	0.08	0.05	0.03	0.04	0.00	0.06	0.03
Peak area								
Avg	3.30	10.59	2.91	2.69	6.39	1.05	9.09	2.66
SD	0.18	0.56	0.10	0.13	0.03	0.04	0.30	0.24

[a] Average (Avg).
[b] Standard deviation (SD).
[c] S1PFL(1) and S1PFL(2) represent the first and second isomer peaks of S1PFL.

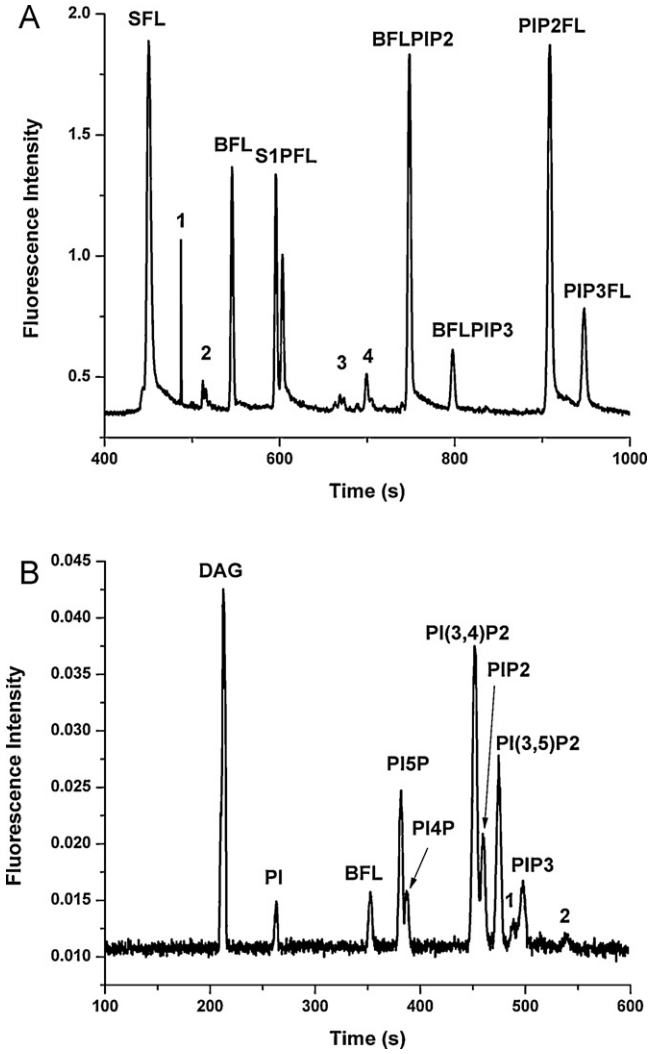

Fig. 5. Simultaneous separation of multiple lipids. (A) Separation of six lipids. A mixture of BFL, SFL, S1PFL, PIP2FL, PIP3FL, BFLPIP2 and BFLPIP3 was separated with the optimal buffer. Peaks numbered 1, 2, 3 and 4 are impurities from the commercial SFL and S1PFL stocks. (B) Separation of a mixture composed of BFL, BFL-labeled DAG, PI, PI4P, PI5P, PI(3,4)P2, PIP2, PI(3,5)P2 and PIP3. Peaks numbered 1 and 2 are impurities in the supplied phosphoinositides.

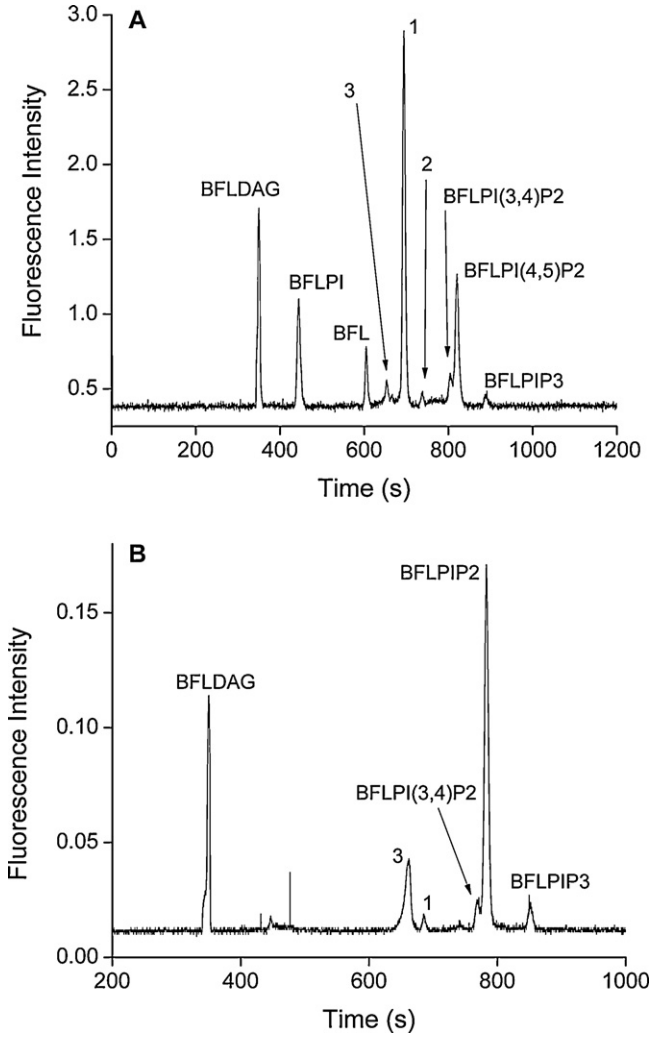

Fig. 6. Separation of lipids from a biological matrix. Separation of fluorescent lipid metabolites from a cell lysate (A) or from a single cell (B). Peaks 1 and 2 are unknown fluorescent metabolites. Peak 3 is an endogenous cell component with fluorescence.

and electrophoresed ($n = 2$ cells). A similar set of metabolites were present in the single cells although in different relative amounts (Fig. 6B). Thus, separation and detection of lipid metabolites in a cell lysate or a single cell was readily performed with this method. The two unidentified metabolite peaks seen in the electropherogram demonstrate a limitation in the use of fluorescently tagged exogenous lipids for these assays. To identify these peaks, electrophoretic standards are needed. These can be generated by *a priori* synthesis of expected metabolic products, or the use of an analytical method such as mass spectrometry coupled to CE-LIF.

4. Conclusion

A simple buffer system was developed to achieve reproducible separation of a variety of phospholipids at room temperature.

The optimized method incorporated a buffer composed of 32 mM NaH_2PO_4, pH 7.3, 20% 1-propanol in a bare capillary. The method provided improved reproducibility and reduced reagent cost compared with separation matrices described in prior reports. In separating PIP2FL and PIP3FL, this new CE separation method provided 54% higher theoretical plate numbers than previous methods. Separations of six fluorescently labeled phospholipids were conducted using this method. The RSD of the migration time for all six phospholipids was less than 1%. In future experiments, this method should enable the separation of fluorescently labeled lipids and their metabolites from complex biological samples. As demonstrated, the fluorescent substrates can be loaded into cells followed by separation of the cellular contents and any fluorescent metabolites formed to better understand cellular metabolism of lipids. The approach is expected to be of value in the assay of phosphoinositides in drug discovery and in small biological samples such as single cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.09.003.

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