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Original Paper

Metal cations for the determination of fluorescent phosphoinositides by capillary electrophoresis

Phosphatidylinositol (PI) and its phosphorylated derivatives known as phosphoinositides (PIPs), are essential regulators of cell signaling and membrane trafficking, cytoskeletal dynamics, and nuclear functions. Disruption of PI metabolism is associated with disorders such as immune dysfunction, cardiovascular disease, and cancer; therefore, there is currently great interest in studying PIPs and their metabolic enzymes. Here, we describe a method for the separation of fluorescent PI and its seven fluorescent phosphorylated derivatives by CE-LIF. The CE method utilizes a Tris buffer and sodium deoxycholate in the presence of 30% 1-propanol and 5% of a dynamic coating reagent, EOTrol™ low reverse (EOTrol LR). It is simple, fast, highly sensitive, and it offers LODs in the order of 1.5 amol. The effect of cations such as lithium, sodium, potassium, cesium, barium, manganese, zinc, magnesium, calcium, spermine, and gentamicin were evaluated. Calcium and magnesium provided the best selectivity and resolution for the separation of the analytes while magnesium offered the best data reproducibility. The developed CE method would be useful in the studies of enzymatic activity in the PI and PIPs metabolic pathways using CE-based *in vitro* and CE cell-based assays, and/or for drug screening.

Keywords: CE / Fluorescent phosphoinositides / Glycerophospholipids / Lipids / Metal cations

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1 Introduction

The binding of monovalent and divalent cations to cell membrane lipids plays a key role in a number of important physiological processes. For example Ca^{2+} plays an important role in blood coagulation by bridging enzymes and substrates to acidic glycerophospholipids (GPLs) of endothelial or other membranes [1 – 3]. Tatulian *et al*. evaluated the binding of Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+} to phosphatidylserine [4] while Roux *et al*. investigated the binding of Mg^{2+}, Ca^{2+}, Li^{+}, K^{+}, and Na^{+} to membrane bilayers of 1-palmitoyl-2-oleoylphosphatidylcholine and 1-palmitoyl-2-oleoylphosphatidylserine by ^{2}H NMR [5]. In addition, Toner *et al*. investigated the binding of K^{+}, Mg^{2+}, Ca^{2+}, spermine, and gentamicin to phosphoinositides (PIPs) by measuring the effect of these cations on the electrophoretic mobility of multilamella vesicles formed from mixtures of phosphatidylcholine (PC) and either phosphatidylinositol (PI), phosphatidylinositol 4-phosphate, or phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$). In this study, it was concluded that a negligible fraction of the $PI(4,5)P_2$ molecules in a cell binds to calcium ions, but a significant fraction may bind to the magnesium and spermine ions [6]. Spermine is a linear polyamine molecule involved in cellular metabolism found in all eukaryotic cells, and it exists as a polycation at physiological pH. On the other hand, gentamicin is an aminoglycoside. It is an antibiotic frequently used against bacterial infection, and is ionized above pH 7.0. At pH 7.4, gentamicin and spermine are thought to possess charges of +3.5 and +3.6, respectively [7]. Despite this knowledge on binding of GPLs to metal ions and polycations, to the best of our knowledge, there are no studies that have explored the use of these ions for the separation of membrane lipids.

PI and PIPs are cell membrane lipids that have been implicated in many aspects of cell physiology. These aspects include cell growth and proliferation, apoptosis, ion channel regulation, vesicle trafficking, and cytoskele-

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Abbreviations: BODIPY FL, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid; **EOTrol LR,** EOTrol™ low reverse; **GloPI or GloPIP$_n$,** PI or PIP$_n$ attached to BODIPY FL via an acyl chain; **GPLs,** glycerophospholipids; **HyPIP$_n$,** PIP$_n$ attached to BODIPY FL via an accessory phosphatidylethanolamine head group; **MEKC,** micellar EKC; **PC,** phosphatidylcholine; **PI,** phosphatidylinositol; **PIPs,** phosphoinositides; **SDC**, sodium deoxycholate

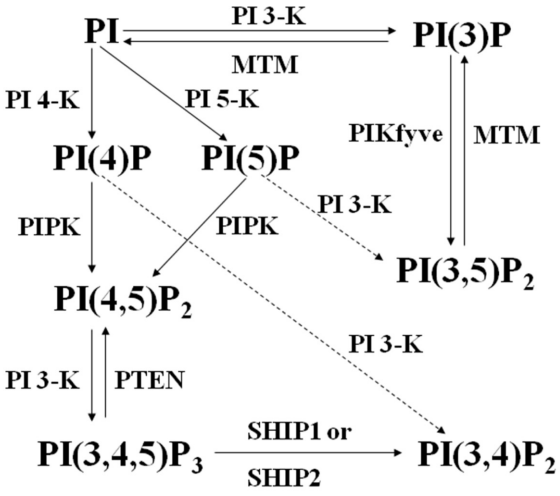


Figure 1. Chemistry of phosphoinositide metabolism; interconversions of PI and PIPs by lipid kinases and lipid phosphatases.

tal changes, as indicated by recent reviews [8 – 12]. PI, the precursor of PIPs, is synthesized primarily in the endoplasmic reticulum and is then delivered to other cell membranes. Reversible phosphorylation of the PI ring at the 3, 4, and 5 positions results in the generation of 7 PIP species (Fig. 1). The versatility of these lipids stems from their ability to function either as substrates for the generation of 2nd messengers, as membrane anchoring sites for cytosolic proteins, or as regulators of the actin cytoskeleton [13, 14]. The cellular levels of PIPs are mostly regulated by kinase or phosphatase enzymes. Apparently, the disruption of PIPs signaling in cells is a precursor to many disease states, including diabetes, cardiovascular disease, and cancer [15]. These lipid-modifying enzymes play an important role in the regulation of PI and its metabolites; thus, they are currently being considered as therapeutic targets for the treatment of cancer, inflammation, atherosclerosis, and other diseases [8 – 12, 15, 16]. Therefore, there is an increased need for the development of highly sensitive techniques for the determination of enzymatic activity and quantitative determination of these signaling GPLs in mammalian cells, and for drug screening.

GPLs are difficult to separate because they tend to aggregate in aqueous media at concentrations above their critical micellar concentrations, and most GPLs lack chromophores. The most common techniques for the determination of GPLs are HPLC [17 – 22] and TLC [23, 24]. However, these chromatographic techniques lack the sensitivity required to detect GPLs in a single cell or in a small cell population. In order to improve the sensitivity and selectivity for the above mentioned techniques, indirect photometric, indirect UV, and fluorescence detection methods have been developed with at best LODs of 0.03 – 0.1 pmol [25 – 28]. However, separation and detection of cell signaling GPLs at the single cell level remains difficult because the concentrations of many of these signaling lipids is nanomolar to micromolar in a 1 pL cell volume. Therefore, technologies with LODs of attomole to zeptomole are needed for the analyses of trace amounts of GPLs in single cells or cell lysates [16].

CE is well suited for the analysis of GPLs due to its high resolution, excellent separation efficiency, minimal sample volume, and short analysis time. However, only a few research articles have reported the use of CE for the determination of GPLs [27 – 36]. The limitations of CE for the determination of GPLs were discussed in our previous study [37].

In that study, we separated a mixture of PI(4,5)P_2 and phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P_3) labeled with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (BODIPY FL) at the hydrophobic tail (GloPI(4,5)P_2, GloPI(3,4,5)P_3) or near the hydrophilic head group (HyPI(4,5)P_2, and HyPI(3,4,5)P_3) by CE-LIF. We developed a capillary-based separation for these fluorescent GPLs, and also investigated the mechanism of separation. The existing school of thought is that GPLs separation in an aqueous media in a capillary requires micellar EKC (MEKC) [32, 33]. In addition, many of the MEKC methods developed for the separation of GPLs are carried out in the 40 – 55°C temperature range [30 – 34, 36]. Our results were quite surprising. We separated the above mentioned phospholipids at room temperature and showed that these fluorescent GPLs did not separate by MEKC, and indeed that surfactants were not even required for resolution of these GPLs.

In this work, we investigated the effect of several cations for the determination of PI and its seven PIP derivatives labeled with BODIPY FL on the acyl chain by CE-LIF. The names and structures of these fluorescent analytes are shown in Fig. 2. Of the eight analytes, there are two sets of lipids that are quite challenging to separate because they differ only by the position of the phosphate group on the inositol ring. These are GloPI(3)P, GloPI(4)P and GloPI(5)P, and GloPI(3,4)P_2, GloPI(3,5)P_2 and GloPI(4,5)P_2. To the best of our knowledge, this is the first time that all of these eight analytes have been separated by CE in a single run. To develop the separation method, the concentration of each of the buffer constituents was optimized. In addition, the role of the buffer additives was evaluated as well as the influence of the metal cations on the sample matrix. The optimized method separated the mixtures of the eight analytes in less than 14 min and was efficient and reproducible. We also recorded LODs in the order of 1.5 amol. The fluorescent

PI and fluorescent PIPs used as analytes are valuable in both *in vitro* and *in vivo* assays of GPLs metabolism. For instance, the use of these lipids with the optimized capillary-based separation method will be of utility in cell-based assays.

2 Materials and methods

2.1 Reagents

The fluorescent PI and PIPs were purchased from Echelon Biosciences (Salt Lake City, UT, USA). Tris, sodium deoxycholate (SDC), 1-propanol, ACN, methanol, $MgCl_2$, NaCl, NaH_2PO_4, Na_2HPO_4, Triton X-100, gentamicin, and spermine were purchased from Sigma–Aldrich (St. Louis, MO, USA). $CaCl_2$, $BaCl_2$, $MnCl_2$, $ZnCl_2$, KCl, and LiCl, were purchased from Fisher Scientific (Fair Lawn, NJ, USA). EOTrol LR was purchased from Target Discovery (Palo Alto, CA, USA). All the reagents were of analytical reagent grade and used as received.

2.2 CE

CE was performed on a Beckman ProteomeLab PA800 CE system (Fullerton, CA). The machine was equipped with LIF detector using an air-cooled 3.5 mW argon laser (Beckman Instruments), with excitation at 488 nm and emission at 520 nm, and was installed with a 32 Karat 7.0 Software (Beckman Instruments) for instrument control and data analysis. Separations were performed with 30 cm fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) having 50 μm id, 375 μm od (20 cm effective length). New capillaries were preconditioned by successively flushing with 1 M NaOH, for 30 min and rinsed with distilled deionized H_2O, for 20 min at 20 psi. Prior to each run, the capillary was flushed with 0.1 M NaOH and distilled deionized H_2O for 2 min each and the separation buffer for 3 min. Hydrodynamic injection was carried out at 0.5 psi for 10 s. During the separation, the capillary column was thermostated at 25°C and an electric field strength of 667 V/cm was applied in reverse polarity mode (anode at the detector) unless stated otherwise.

2.3 Preparation of solutions

BODIPY FL was attached to the PIPs *via* the acyl chain. These lipids were dissolved in methanol and were dispersed in lipid buffer (0.1% Triton X-100, 5 mM Tris, pH 7.0) before injection. For the separation of the mixtures of the eight fluorescent GPLs, the identity and elution order of the peaks were determined by sequentially spiking an additional fluorescent GPL into the solution of the corresponding analyte mixture and repeating the separation. The lipid samples were diluted to 10–50 nM prior to injection into the capillary.

2.4 Data analysis software

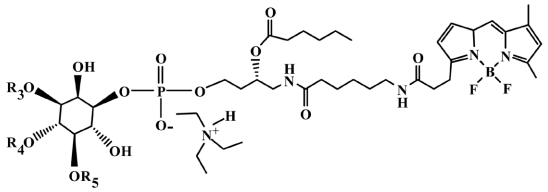
The CE system was installed with a 32 Karat 7.0 Software (Beckman Instruments) capable of performing a wide range of data analyses including calculation of migration times, peak heights, peak areas, resolution (R_s), and the number of theoretical plates (N). The RSD of the migration times, peak heights, and peak areas were calculated using equations from [38]. At least nine measurements were made for each experimental condition for which RSD values were calculated.

3 Results and discussion

3.1 Method development

The aim of this work was to develop an optimized method to separate fluorescent PI and seven fluorescent PIPs quickly and efficiently. The method used in this work was similar to the method we developed for the separation of GloPI(4,5)P_2, GloPI(3,4,5)P_3, HyPI(4,5)P_2, and HyPI(3,4,5)P_3 by CE-LIF [37]. The separation buffer was composed of 100 mM Tris (pH 8.5), 5 mM SDC, 1 mM $MgCl_2$, 30% 1-propanol. In addition, 5% EOTrol LR, a dynamic coating based on a substituted polyacrylamide, was used to suppress the adsorption of the analytes on the capillary wall, as well as reverse and decrease the velocity of the EOF (EOTrol™ Product Technical Guide, T. D., Inc., Palo Alto, CA 2004; www.targetdiscovery.com.) [39, 40]. The separation was entirely dependent on the presence of divalent metal cations in the buffer. Under reverse polarity mode, when 667 V/cm was applied across a capillary filled with the optimized buffer containing (A) 1 mM $MgCl_2$ or (B) 1 mM $CaCl_2$, near baseline separation of all eight analytes in Fig. 2 (analyte mixture) in about 14 min was observed (see Fig. 3A and B). Notably, we observed that with a concentration of 1 mM $CaCl_2$ in the buffer, the order of elution of GloPI and GloPI(3,4,5)P_3 was reversed compared to the elution order when 1 mM $MgCl_2$ was used, (see Fig. 3). This reversal trend was investigated further by evaluating the effect of each metal cation at different concentrations in the buffer. The results will be discussed below in the section on the effect of metal cation concentration.

An electric field strength of 1000 V/cm combined with the optimal buffer yielded excellent theoretical plates for all analytes without compromising the analyte resolution, and the separation was completed in less than 8 min using (i) 1 mM $MgCl_2$ or (ii) 1 mM $CaCl_2$ in reverse polarity mode, Fig. 4A and B. However, the migration times were not reproducible at this high electric field strength. Therefore, the effect of voltage on the separa-



Order of Elution	Name (BODIPY FL)#	R3	R4	R5
1	GloPI(3)P	$PO_3H(HNEt_3)^*$	H	H
2	GloPI(4)P	H	$PO_3H(HNEt_3)$	H
3	GloPI(5)P	H	H	$PO_3H(HNEt_3)$
4	GloPI(3,4)P_2	$PO_3H(HNEt_3)$	$PO_3H(HNEt_3)$	H
5	GloPI(3,5)P_2	$PO_3H(HNEt_3)$	H	$PO_3H(HNEt_3)$
6	GloPI(4,5)P_2	H	$PO_3H(HNEt_3)$	$PO_3H(HNEt_3)$
7	GloPI(3,4,5)P_3	$PO_3H(HNEt_3)$	$PO_3H(HNEt_3)$	$PO_3H(HNEt_3)$
8	GloPI	H	H	H

All the structure names have BODIPY FL as the prefix

* Triethylammonium ($HNEt_3$) is the counter ion

Figure 2. Chemical structures and names of the fluorescent GPLs separated. The word "Glo" indicates the fluorescent tag (BODIPY FL) is attached to the GPL *via* an acyl chain. The numbers (1–8) are used to identify and indicate the order of elution of these analytes in the CE electropherograms.

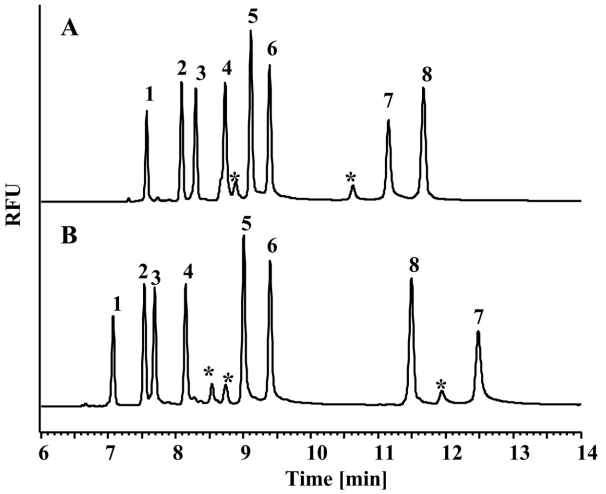


Figure 3. Separation of mixture of GloPI and seven GloPIPs (analyte mixture) at 667 V/cm in a buffer containing 100 mM Tris (pH 8.5), 5 mM SDC, 30% 1-propanol, 5% EOTrol LR, and (A) 1 mM $MgCl_2$ or (B) 1 mM $CaCl_2$. The injection size was 0.5 psi for 10 s. The y-axis is the relative fluorescence units (RFU). The peaks labeled * are impurities.

tion of the eight analytes was investigated by varying the electric field strength applied across the 30 cm capillary. The electric field strengths applied were 500, 667, 833, and 1000 V/cm. The total elution time of the analytes decreased from 20 min at 500 V/cm to less than 8 min at 1000 V/cm. It was noted that the resolution of the analytes slightly decreased when the voltage was increased;

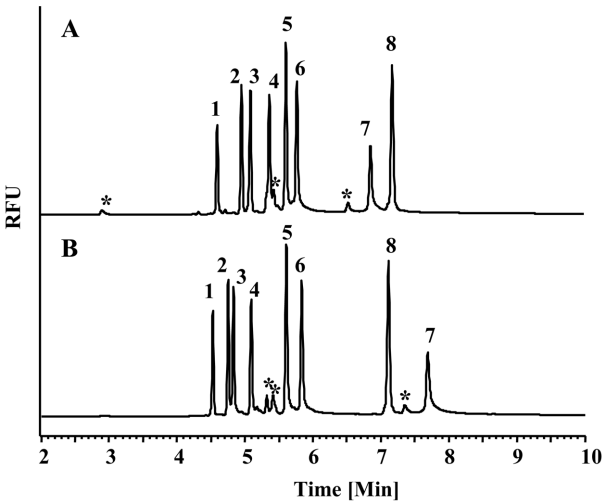


Figure 4. Separation of the analyte mixture using the buffer and separation conditions in Fig. 3 except the CE run was performed at 1000 V/cm (A) 1 mM $MgCl_2$, and (B) 1 mM $CaCl_2$.

however, this was accompanied by higher theoretical plates. Unfortunately, at 1000 V/cm the migration times were not reproducible and only six to seven runs could be performed per capillary at this high voltage. This was probably due to joule heating. The optimum electric field strength was 667 V/cm, selected based on an ohms plot, and data reproducibility. Using this electric field strength each capillary column was able to withstand at least 50 runs.

Two other buffer systems were optimized for the separation of these lipid analytes. However, these buffer systems were unable to resolve the analyte mixture efficiently. One of the buffers was 25 mM sodium borate at pH 9.3 containing 20 mM SDC, and 20% ACN, while the other buffer was 30 mM NaH_2PO_4 + 20 mM Na_2HPO_4 at pH 7 containing 20% 1-propanol, 5 mM SDC, and 5% EOTrol LR. After several attempts to optimize SDC, organic solvent additive (ACN, methanol, and/or 1-propanol), and the pH, the resolution remained poor (data not shown).

3.2 Mechanism of separation

When the optimized method is used, every buffer additive plays a specific role in the separation of the analytes. EOTrol LR played a significant role in preventing the analytes from adsorbing to the capillary walls as well as reducing the EOF. This is because smaller, broader peaks were observed which migrated faster when EOTrol LR was omitted in the buffer compared to buffer with EOTrol LR. This observation was consistent with what we had observed in our previous study using different species of fluorescent PI(4,5)P_2 and PI(3,4,5)P_3 [37]. The 1-

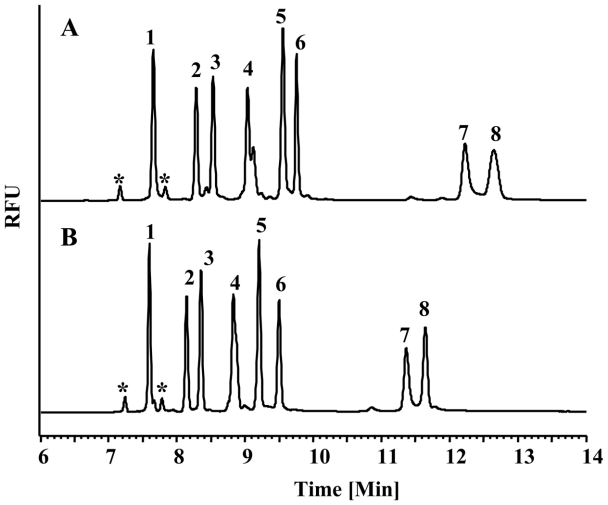


Figure 5. Separation of the analyte mixture using separation conditions in Fig. 3 A except (A) without SDC and (B) with 5 mM SDC.

propanol was used to disperse the lipids in the run buffer while the divalent cations accentuated the resolution of the analytes. As can be seen from Fig. 5, it is patently clear that the effect of SDC is at best, minimal in separating the analytes. These data further buttress the notion that the mode of separation does not depend on the differential partitioning of the analytes between the buffer and SDC micelles; therefore, the mode of separation is CZE and not MEKC. Significant reduction in the elution time and an increment in peak sharpness were observed when 5 mM SDC were added to the buffer solution. Thus, it can be suggested that SDC plays a role in preventing the negatively charged species of the analytes from adsorbing to the capillary wall. When the SDC concentration was varied from 5 to 20 mM the elution time increased with a minimal change in the resolution of the analytes (data not shown). It is likely that higher SDC concentration elevates the ionic strength of the buffer causing a marginal increase in the analyte migration time. Results from this work indicate that the key to the separation of these fluorescent GPLs is the metal cation in the buffer. The differential binding of each analyte to the metal cations which are thought to align on the capillary wall has the effect of reducing the net charge to mass ratio relative to the other analytes causing differential migration and hence the separation.

3.3 Effect of magnesium and calcium

The optimized buffer requires a divalent metal cation such as Mg^{2+} or Ca^{2+} for baseline separation to be achieved. This was confirmed by investigating the role of different metal ions on the separation of fluorescent PI and its seven fluorescent PIP derivatives. It was concluded that Mg^{2+} and Ca^{2+} ions were the only metal cations that brought about baseline separation of all eight analytes in a mixture. Figure 6 illustrates the separation of the analyte mixture with (A) no metal ions in the buffer, (B) 1 mM $MgCl_2$, and (C) 1 mM $CaCl_2$ when the electric field strength of 500 V/cm was applied across the 30 cm capillary. GloPI above pH 7.0 is ionized and has one negative charge which can be neutralized by the presence of dications in the buffer. At pH 8.5 GloPI (peak labeled number 8 in Fig. 6) is thought to be neutralized by the presence of Mg^{2+} or Ca^{2+} ions; thus, it was expected it would elute at the same time as the EOF. Based on the above assumption, it can be deduced from Fig. 6 that the EOF was not altered by the presence of 1 mM concentration of either $MgCl_2$ or $CaCl_2$.

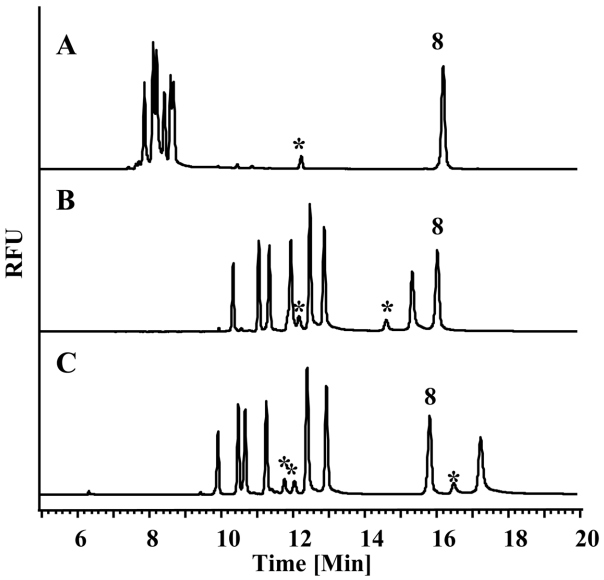


Figure 6. Separation of the analyte mixture using separation conditions in Fig. 3 except (A) no metal ions in the buffer, (B) 1 mM $MgCl_2$, and (C) 1 mM $CaCl_2$ added to the buffer, and the CE run was performed at an electric field strength of 500 V/cm.

However, it is obvious that the negatively charged fluorescent PIPs bind differentially with either Mg^{2+} ions (Fig. 6B) or Ca^{2+} ions (Fig. 6C) present in the buffer or aligned on the capillary wall; thus, altering their electrophoretic mobility and allowing baseline separation of the analytes.

3.3.1 Effect of magnesium and calcium cation concentration in the buffer

The effect of increasing the concentration of Mg^{2+} and Ca^{2+} ions in the optimized buffer was investigated. The optimized buffer containing 0.1, 0.5, 1, 2, 3, or 5 mM of either $MgCl_2$ or $CaCl_2$ was used to separate the analyte mixture each at a time. The analytes eluted in less than 10 min when a concentration of 0.1 or 0.5 of either

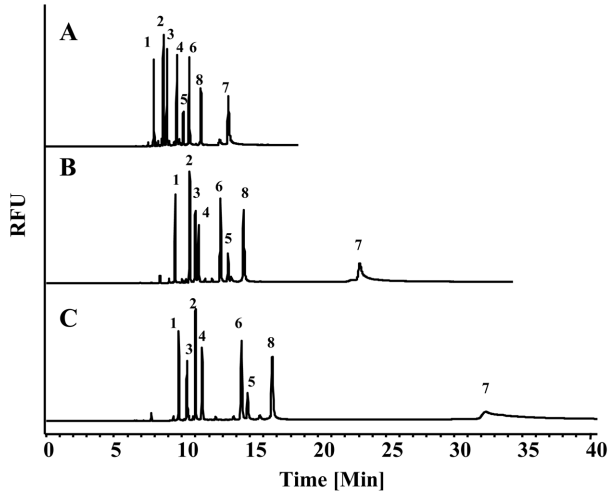


Figure 7. Separation of the analyte mixture using separation conditions in Fig. 3 except (A) 2 mM $MgCl_2$, (B) 3 mM $MgCl_2$, and (C) 5 mM $MgCl_2$ added to the buffer instead of 1 mM $MgCl_2$ or $CaCl_2$, and the CE run was performed at an electric field strength of 833 V/cm.

$MgCl_2$ or $CaCl_2$ was used but most of the GloPIPs coeluted. Near baseline separation of all the eight analytes in a mixture was observed when 1 mM of either $MgCl_2$ or $CaCl_2$ was added to the buffer (Fig. 3). At the optimum electric field strength of 667 V/cm the separation using 5 mM $MgCl_2$ took over 60 min and the PIP_3 peak was very broad (data not shown). Therefore, an electric field strength of 833 V/cm was used for this study. The resolution of the analytes increased as the concentration of either $MgCl_2$ or $CaCl_2$ in the buffer was raised and the migration times increased from less than 15 min for 2 mM concentration to over 30 min for the 5 mM concentration. It was observed that concentrations of $MgCl_2$ above 1 mM resulted in a change in the elution order between GloPI(3,4,5)P_3 and GloPI *i.e.*, peak 7 and 8 in Fig. 3A and B. For 2 mM $MgCl_2$ and above, GloPI(3,4,5)P_3 spent more time in the capillary compared to the other analytes and eluted after GloPI (see Fig. 7). In addition, the elution order of the other analytes also varied with the increase in $MgCl_2$ concentration. For example GloPI(4,5)P_2 (peak # 5) eluted after GloPI(3,5)P_2 (peak # 6) when 3 and 5 mM $MgCl_2$ was used, and GloPI(5)P (peak # 2) eluted after GloPI(4)P (peak # 3) when 5 mM $MgCl_2$ was used, as shown in Fig. 7B and C. This change in elution order was only observed when the concentration of $MgCl_2$ but not $CaCl_2$ was varied in the optimized buffer. These analytes that change their elution order are very similar; thus, the switch in their elution order is not surprising. However, it is also possible that as the concentration of $MgCl_2$ is increased in the buffer, more Mg^{2+} ions become available to bind to these analytes; thus, slightly lowering their electrophoretic mobility and increasing their migration time.

Divalent cations have been effectively employed for the suppression of the EOF [41 – 43]. Addition of 5 mM of any of the alkaline earth metal ions reduces the EOF to approximately 55% of its normal value at pH 6 [41]. However, Mg^{2+} is mostly preferred because of its greater solubility in buffer solutions [41, 43]. Pietrzyk and colleagues observed a dramatically stronger suppression of the EOF at higher pH. For example 2 mM Mg^{2+} yielded an electroosmotic mobility (μ_{eof}) of only 2.0×10^{-4} $cm^2/(V \cdot s)$ in a pH 8 Tris buffer compared to μ_{eof} of 4.5×10^{-4} $cm^2/(V \cdot s)$ over the pH range of 4.5 – 7.5 [43]. Melanson *et al.* proposed that the stronger suppression of EOF at higher pH may be due to a sufficiently high silanol population on the inner capillary surface; thus, multiple silanol groups are simultaneously involved in the retention of a metal ion [44]. Based on these studies, we would have expected to observe shorter migration times as the concentration of $MgCl_2$ is increased in the buffer. However, we observed exactly the opposite, the migration time of the analytes increased. It is possible that the Mg^{2+} suppressing power is minimized by EOTrol LR which is used to suppress the EOF and minimize the analyte wall interactions. On the other hand, it seems that by increasing the Mg^{2+} concentration in the buffer, more ions become available to bind to the charged PIPs especially GloPI(3,4,5)P_3 (peak # 7) in Fig. 7. This data also suggests that Mg^{2+} bind more tightly to GloPI(3,4,5)P_3 than the other GloPIPs.

3.3.2 Effect of magnesium and calcium cation concentration in the sample

The effect of adding Mg^{2+} or Ca^{2+} cations on the sample was also evaluated. This was done by adding 0.1, 1, and 5 mM and excess of either $MgCl_2$ or $CaCl_2$ in the sample each at a time and the separation was done using the optimum buffer with and without the metal cations. The study performed without the metal cation in the buffer was aimed at investigating whether the metal cations remained preferably bound to the fluorescent GLPs during the separation. For the separations where either $MgCl_2$ or $CaCl_2$ was only added to the sample, apart from GloPI all the other analytes co-eluted (data similar to that shown in Fig. 6A was observed). When the separation was done with either $MgCl_2$ or $CaCl_2$ in both the sample and the buffer there was no major difference compared to when the separation was performed with these ions in the buffer only (data similar to that shown on Fig. 6B and C for Mg^{2+} and Ca^{2+} respectively was observed). This observation suggested that the separation was based on differential binding of the PIPs to the metal cation in the buffer and further indicated that the metal cation played a more important role when it was in excess in the buffer rather than in the sample. The differential binding of each analyte to the metal cations (some of which are thought to align on the capillary wall) has the effect of reducing the net charge to mass ratio relative to the

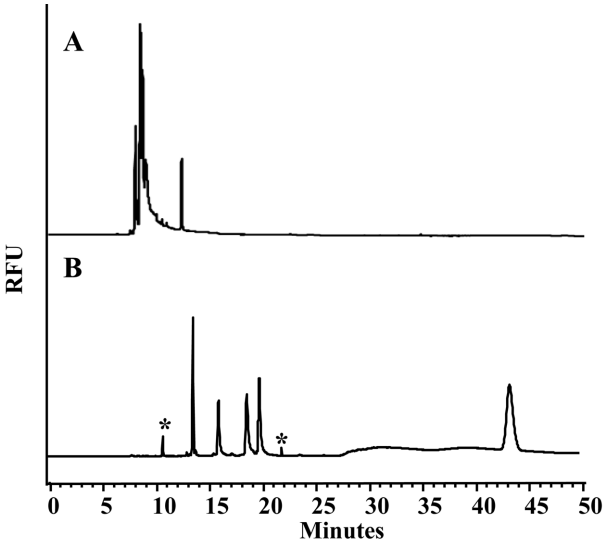


Figure 8. Separation of the analyte mixture using separation conditions in Fig. 3 except (A) 1 mM gentamycin, and (B) 1 mM spermine were added to the buffer instead of 1 mM $MgCl_2$ or $CaCl_2$.

other analytes causing differential migration and hence the separation.

3.4 Effect of other metal ions and polycations

Other metal ions like Ba^{2+}, Zn^{2+}, Mn^{2+}, Li^+, Na^+, K^+, and Cs^+ did not show any appreciable effect in separating the phosphinositides. At best only four peaks were resolved (data not shown). Gentamicin and spermine were also tried as buffer additives. Different concentrations of gentamicin ranging from 0.1 to 5 mM were tested. However, the separations obtained at all these concentrations were poor compared to separations obtained using the other cations investigated in this study. Spermine was better at separating the analyte mixture compared to gentamicin. However, only five out of the eight analytes were baseline resolved and migration times of 40 min or more were observed for the last eluting peak as shown in Fig. 8. Toner *et al.* showed by biophysical means that both spermine and gentamicin bind to PIPs such as PI and PI(4,5)P_2 using PC/PI and PC/PI(4,5)P_2 vesicles [6]. They observed that the intrinsic binding constant of spermine to PI was similar to that of gentamicin to PI. In addition, the effect of spermine on the zeta potential of PC/PI(4,5)P_2 vesicles was similar to that of gentamicin. Our results indicate that the fluorescent PIPs associated more with spermine than with gentamicin. These observations may be due to the structural differences between spermine and gentamicin. It is possible that the positive charges on the gentamicin molecule become sterically hindered based on its orientation on the capillary, thus minimizing analyte interactions compared to spermine.

Table 1. %RSD of the migration times, peak heights, and peak areas, and the average number of theoretical pates (*N*) for nine sequential runs of the BODIPY FL-labeled PI and PIPs separated using the conditions described in Fig. 3.

Additive	Analyte	Elution time	Peak area	Peak height	Average (*N*)
1 mM $MgCl_2$	GloPI(3)P	0.4	7.3	9.8	140 000
	GloPI(5)P	0.4	7.1	6.7	137 000
	GloPI(4)P	0.5	7.3	6.3	132 000
	GloPI(3,4)P_2	0.5	7.9	7.5	127 000
	GloPI(4,5)P_2	0.5	7.7	8.2	145 000
	GloPI(3,5)P_2	0.5	6.7	6.5	134 000
	GloPI(3,4,5)P_3	0.6	10.7	8.8	156 000
	GloPI	0.5	7.5	7.9	160 000
1 mM $CaCl_2$	GloPI(3)P	1.6	12.2	12.3	170 000
	GloPI(5)P	1.8	11.3	10.5	174 000
	GloPI(4)P	1.8	12.4	14.7	154 000
	GloPI(3,4)P_2	1.9	10.5	12.0	155 000
	GloPI(4,5)P_2	1.9	14.2	14.4	156 000
	GloPI(3,5)P_2	2.0	13.3	13.8	158 000
	GloPI(3,4,5)P_3	2.6	16.7	23.3	115 000
	GloPI	2.5	13.2	11.8	175 000

3.5 Effect of pH and temperature

To investigate the effect of pH on the separation of the fluorescent analytes, the buffer was adjusted to a range of different pHs and the effect on the separation evaluated. Poor separations were observed for pH 7 and 10. The buffers at pH 8 and 9 provided near baseline separations of the analytes but the optimal separation was at pH 8.5 for the separation performed with a buffer containing either $MgCl_2$ or $CaCl_2$.

The effect of varying the separation temperature was also evaluated. The separation at the optimum buffer conditions containing 1 mM $MgCl_2$ or 1 mM $CaCl_2$ was performed at 15, 20, 25, 30, 40, and 50°C. The electric field strength applied for this temperature study was 500 V/cm. The separation time decreased from 30 min for the separation performed at 15°C to 12 min at 50°C; however this was accompanied by a loss in resolution. At 30°C and above, the analyte peaks started to co-elute (data not shown). An optimum separation temperature of 25°C was chosen because all analytes were baseline separated and the elution time was reasonably short as shown in the Fig. 3 at the optimum electric field strength of 667 V/cm.

3.6 Reproducibility and separation efficiency

Reproducibility of the migration times, peak areas, and peak heights of the optimal separation buffer with either Mg^{2+} and Ca^{2+} cations were established by ten consecutive runs over a number of days. These results can be seen in Table 1. The best reproducibility was observed when the capillary was rinsed with 0.1 M NaOH, water, and the separation buffer sequentially between runs. Table 1 lists the

%RSD of the migration times, peak heights, and peak areas, and the average number of theoretical plates (*N*) for nine sequential runs of the analytes using the optimized conditions of Fig. 3 for 1 mM $MgCl_2$ and 1 mM $CaCl_2$. It was shown that the optimal buffer with 1 mM $MgCl_2$ provided more stable and reproducible results compared to the buffer with 1 mM $CaCl_2$. The migration times obtained when $CaCl_2$ was used were stable, but the peak heights and the peak areas showed a SD that was higher than those obtained with the buffer containing $MgCl_2$.

4 Concluding remarks

A CZE method was optimized for the separation of fluorescent labeled PI and its seven PIPs derivatives. Different types of cations were used as buffer additives and it was found that Mg^{2+} and Ca^{2+} were the only cations that gave good separation. The buffer system was optimized at 100 mM Tris buffer, 30% 1-propanol, 5 mM SDC at pH 8.5. It was also found that SDC was not vital for the separation to occur. Therefore, the mode of the separation was CZE and not MEKC. Separation of all eight fluorescent analytes was achieved in less than 7 min, with very good resolution and efficiency at the optimized electric field strength and capillary temperature of 667 V/cm and 25°C, respectively. In addition, the detection limit determined using the optimized CE separation method was 1.5 amol. Due to the sensitivity of the CE-LIF system, even trace differences can be picked up in biological samples. The developed method has great potential for the analysis of trace amounts of PI and PIPs in biological microenvironments such as with purified enzymes, single cells, or cell lysates.

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