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PAPER

Use of ion pairing reagents for sensitive detection and separation of phospholipids in the positive ion mode LC-ESI-MS

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Phospholipids make up one of the more important classes of biological molecules. Because of their amphipathic nature and their charge state (*e.g.*, negatively charged or zwitterionic) detection of trace levels of these compounds can be problematic. Electrospray ionization mass spectrometry (ESI-MS) is used in this study to detect very small amounts of these analytes by using the positive ion mode and pairing them with fifteen different cationic ion pairing reagents. The phospholipids used in this analysis were phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), 1,2-diheptanoyl-*sn*-*glycero*-3-phosphocholine (DHPC), cardiolipin (CA) and sphingosyl phosphoethanolamine (SPE). The analysis of these molecules was carried out in the single ion monitoring (SIM) positive mode. In addition to their detection, a high performance liquid chromatography and mass spectrometry (HPLC-MS) method was developed in which the phospholipids were separated and detected simultaneously within a very short period of time. Separation of phospholipids was developed in the reverse phase mode and in the hydrophilic interaction liquid chromatography (HILIC) mode HPLC. Their differences and impact on the sensitivity of the analytes are compared and discussed further in the paper. With this technique, limits of detection (LODs) were very easily recorded at low ppt (ng L^{-1}) levels with many of the cationic ion pairing reagents used in this study.

Introduction

Phospholipids are well-known and thoroughly studied molecules due to their seminal importance in biological organisms. They are mainly recognized as building blocks of cell membranes.[1] However, they also play a very important role in many other, different cellular signaling events.[1–3] Specifically, phospholipids play a crucial role as second messengers in signal transduction pathways, protein sorting, and apoptosis.[4–9] The basic structure of these molecules includes a hydrophilic head group to which two hydrophobic "tails" are attached. Having such a structure enables these molecules to form lipid bilayers, in which the non-polar tails cluster together in the core of the bilayer.[2] Some common polar head groups found in phospholipids are inositol, glycerol, serine and ethanolamine.[1] These molecules are found as mixtures in biological matrices and are very diverse due to their different degrees of unsaturation, fatty acyl chain lengths and the different polar head groups. A combination of the wide variety of these compounds and the often small differences in their structures can make separating, identifying, and quantifying them challenging.[10–13] Traditional and common methods of analysis of phospholipids include thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC), and HPLC with evaporative light scattering detection (ELSD).[3,4,11,12,14–21] However, these techniques have disadvantages which can become problematic if accurate quantitation, and identification, is needed. Some of these methods also require derivatization (GC), and large sample quantities.[3,4,14–16] Nowadays, mass spectrometry has become one of the main techniques used to accurately detect and identify phospholipids. This technique is very often coupled with HPLC and/or capillary electrophoresis (CE).[11,17–20] For phospholipids, electrospray ionization mass spectrometry (ESI-MS) is the most used technique of mass spectrometry due to its simplicity, soft ionization and capability to accurately identify analytes.[3,9,12,17,21–23]

In this study, we present a new and simple way to detect phospholipids in the positive mode ESI-MS with the aid of multiply charged cationic ion pairing reagents. Previously, many of these analytes could only be detected in the negative ion mode ESI-MS as they mainly carry negative charges.[11,12,22] However, it is well-known that the negative ion mode ESI-MS has some disadvantages when compared to the positive ion mode. Some of these drawbacks include the formation of corona discharge and arcing, which then results in poor spray stability, thus affecting the sensitivity of the analytes.[24,25] It has been shown that these drawbacks can be solved by using halogenated solvents or

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electron scavenging gases, however, these types of solvents are not user friendly in liquid chromatography (LC) analysis in cases where such type of analysis is needed.[26–29] The advantage of the technique used in this study is that it operates in the positive ion mode ESI-MS, therefore eliminating the problems mentioned above and further enhancing detection and the sensitivity of the analytes.[30,31]

The method used herein involves the use of large cationic ion pairing reagents, which upon association with the anions of interest form a new positively charged complex that can now be detected in the positive ion mode rather than the negative ion mode ESI-MS. This method was recently developed by our research group for the detection of the perchlorate ion.[30,31] The successful results lead to an extensive study and the synthesis of many other cationic reagents.[32–38] The major advantages of using this method involve high sensitivity, compatibility with HPLC, and ease of use. Additionally, because of the large positive complexes formed, this method has the advantage of detecting small anions that normally reside below or near the low mass cutoff (LMCO) at a higher mass range where the background noise is lower.

Experimental

The solvents used in this analysis were of HPLC-grade, purchased from Honeywell Burdick and Jackson (Morristown, NJ). The phospholipids were purchased in their sodium form from Avanti Polar Lipids (Alabaster, AL). The predominant species of these phospholipids were as follows: 18 : 2/16 : 0-PE (phosphatidylethanolamine), 18 : 2/16 : 0-PI (phosphatidylinositol), 18 : 2/16 : 0-PS (phosphatidylserine), 18 : 2/16 : 0-PA (phosphatidic acid), 7 : 0/7 : 0-DHPC (1,2-diheptanoyl-*sn-glycero*-3-phosphocholine), 18 : 2/18 : 2-CA (cardiolipin), 18 : 2/16 : 0-PC (phosphatidylcholine), 18 : 1-SPE (sphingosyl phosphoethanolamine). Each cationic reagent was synthesized in the bromide form and prior to the analysis it was exchanged to the fluoride form using an ion-exchange method developed previously.[30]

ESI-MS

ESI-MS analysis was performed on a Thermo Finnigan LXQ (Thermo Fisher Scientific, San Jose, CA) linear ion trap. A Surveyor MS pump (Thermo Fisher Scientific) was used to pump 100% methanol (MeOH) at 300 μL min^{-1}. The different ion pairing reagents used in the analysis were introduced to the mass spectrometer from a Shimadzu LC-6A pump (Shimadzu, Columbia, MD) at a flow rate of 100 μL min^{-1}. Prior to entering the MS, these two solutions, methanol and the ion pairing reagent, were directed to a Y-type mixing tee, resulting at a final flow rate of 400 μL min^{-1} entering the MS. The ESI-MS parameters were set as follows: spray voltage of 3 kV; capillary temperature of 350 °C; capillary voltage of 11 kV; tube lens voltage of 105 V; sheath gas flow was set at 37 arbitrary units (A.U.), and the auxiliary gas flow at 6 A.U. Red PEEK tubing (i.d. 0.005 in.) was used as solvent carrier for the ESI-MS and LC-ESI-MS analyses. The sample analytes were introduced in the MS *via* a six-port injection valve with a 5 μL loop. The concentration of the ion pairing reagent remained constant at 40 μM throughout the study. The analytes were initially dissolved in acetonitrile/methanol (1 : 9) and necessary dilutions were performed only with methanol, until a S/N ratio of three was noted in five replicate injections of each sample. Initial concentration of the analytes was 10 μg mL^{-1}.

LC-ESI-MS

Reverse phase LC was performed on an Ascentis™ C18 column (250 mm × 2.1 mm) obtained from Supelco, Sigma-Aldrich Co. (Bellefonte, PA). The mobile phase used was 60/25/15 isopropanol/acetonitrile/water with 0.1% formic acid. The flow rate was 0.2 mL min^{-1}.

HILIC mode separation was performed on a silica-column (250 mm × 4.6 mm) obtained from Advanced Separation Technologies (Whippany, NJ). The mobile phase used was 70/20/10 acetonitrile/methanol/water with a flow rate of 1 mL min^{-1}. Phospholipids were detected, in both reverse and normal phase LC, at a wavelength of 210 nm. A flow splitter was used in the normal phase separation in which it was adjusted so that 0.7 mL min^{-1} was directed to the waste and 0.3 mL min^{-1} was directed into a mixing tee. Similarly, the ion pairing reagent was directed towards the mixing tee as described earlier on the ESI-MS analysis. Thus, the final flow rate entering the MS remained 0.4 mL min^{-1}. The chromatographic separations for both modes were done by a Thermo Fisher Surveyor autosampler (10 μL injections).

Results and Discussion

In this study nine phospholipids were detected individually with fifteen cationic ion pairing reagents in the positive ion mode ESI-MS. Five of the cationic reagents were doubly charged (Fig. 1) and contained different central cores such as imidazolium, phosphonium, and pyrrolidinium ones. The linear tricationic reagents contained imidazolium core moieties and different terminal functional groups. Their alkyl chain linkages varied from C3 to C12 (Fig. 2). The last group of the pairing reagents, the tetracationic ion pairing reagents, were a little more diverse in their structural configurations when compared to the previous two groups. Four of these tetracationic reagents contained phosphonium based moieties and one consisted of an imidazolium core and phosphonium terminal groups (Fig. 3). Among these, one ion pairing reagent is a cyclic phosphonium based reagent, while all the others are linear. The terminal groups consisted of propyl-, phenyl-, and butyl-functional groups. The alkyl chain linkages varied as well, from a C4 to a C12 linkage (Fig. 3).

The selection of some of these ion pairing reagents was based on our previous study on the ESI-MS mechanisms that produces the enhanced sensitivity of this ion pairing technique.[38] In this study it was revealed that the association/binding of the anions and the ion pairing reagents is achieved in solution and further enhanced *via* ionization in the gas phase. Specific reagents with different alkyl chain linkages and different terminal groups were chosen for comparison purposes and to gain a better understanding of the behavior of these particular analytes.

Table 1 lists the limits of detection for the nine phospholipids in the positive ion mode ESI-MS. The table is set up so that the best pairing agent giving the best sensitivity for each analyte is

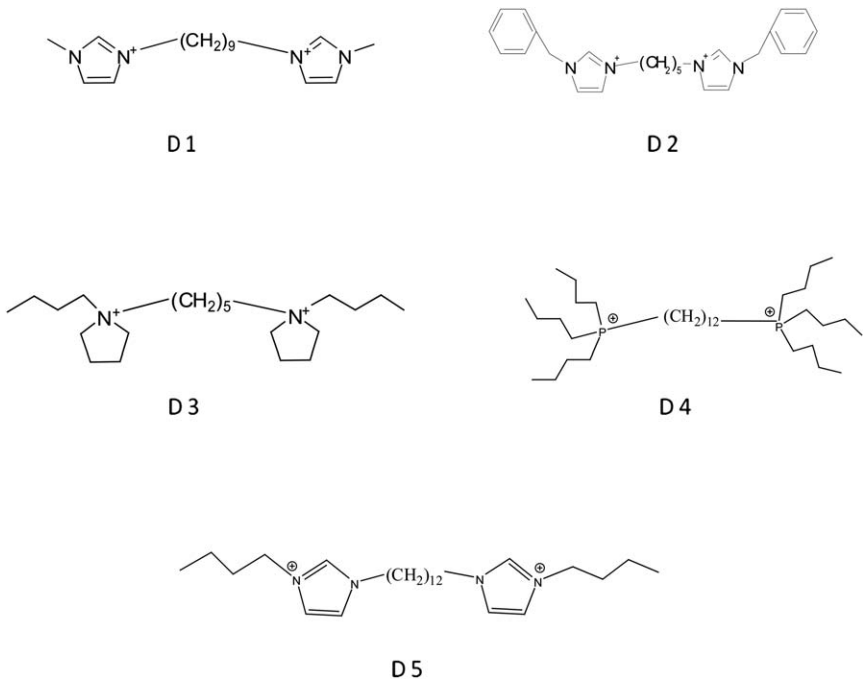

Fig. 1 Structures of the dicationic ion pairing reagents with their corresponding abbreviations used in this study.

placed at the top of the list. Conversely, the pairing agent producing the poorest sensitivity for each analyte is placed at the bottom of the list (Table 1). Based on this data, it is clearly observed that the tetracationic pairing agents consistently produce the best sensitivity for all phospholipids tested. In particular, it can be seen that **Tet 2**, a tetracationic reagent with phosphonium core moiety containing a total of ten phenyl functional groups and C4 alkyl linkages, shows the best

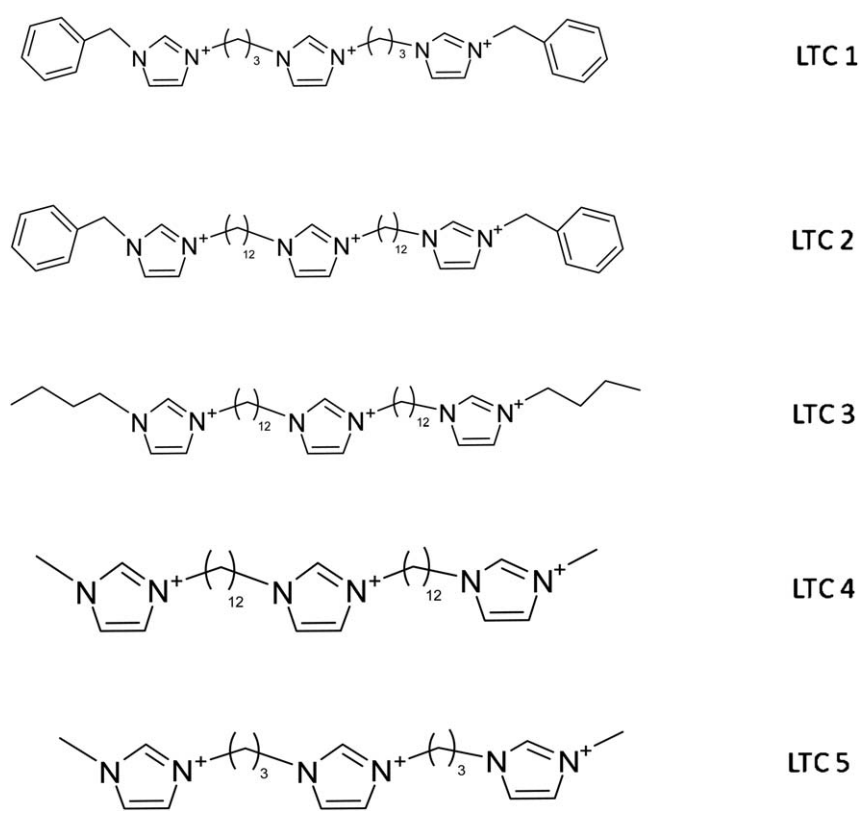

Fig. 2 Structures of the linear tricationic ion pairing reagents with their corresponding abbreviations.

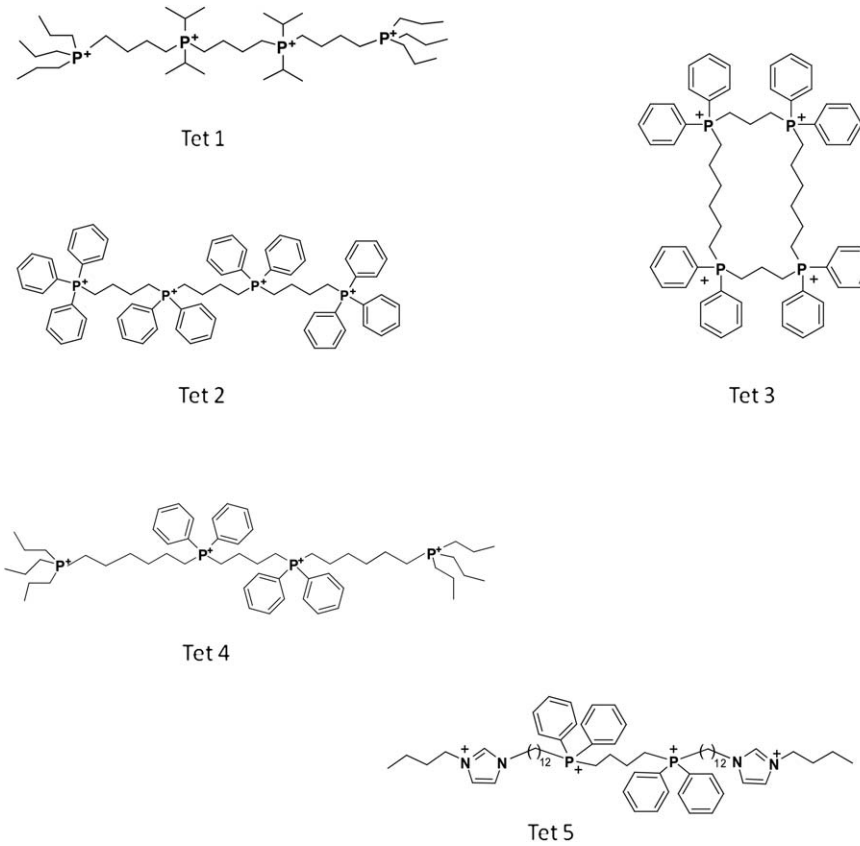

Fig. 3 Structures of the tetracationic ion pairing reagents used in this study with their corresponding abbreviations.

sensitivity (ppq) for **PI**, **PS**, **PC** and **CA**. Out of these four phospholipids, **PI**, **PS**, and **CA** cannot be otherwise detected in the positive ion mode.[11,22]

Under normal conditions they can only be detected in the negative ion mode. For comparison purposes the SIM limits of detection for these analytes were completed in the negative ion mode as well under the same conditions (Table 2). The LODs achieved in the negative ion mode were significantly higher than the ones found in the positive ion mode ESI-MS. For instance, the sensitivity for phosphatidylinositol (**PI**) was found to be 80 times better in the positive ion mode than the negative ion mode (Table 2). Also, cardiolipin (**CA**) has an improved LOD of 40 000 times in the positive ion mode, and even a higher LOD is observed for phosphatidylserine (**PS**) in which the sensitivity is improved by 400 000 times in the positive mode.

Another ion pairing reagent that also performed well in giving low limits of detection for phospholipids was **Tet 4**. This is a tetracationic reagent that is structurally very similar to **Tet 2**. Its structure contains phosphonium based moieties and a mixture of propyl- and phenyl functional groups. This ion pairing reagent showed the lowest sensitivity for phosphatidylglycerol (**PG**) and phosphatidic acid (**PA**). These two phospholipids are usually detected in the negative ion mode as well (Table 2). Our analysis showed that **PA** and **PG** have an improvement in sensitivity of 30 000 times and 590 times, respectively, when detected in the positive ion mode using the ion pairing method (*versus* the detection in the negative ion mode, Table 2).

Tet 4 also performed well as the second best pairing reagent for phosphatidylinositol (**PI**), phosphatidylserine (**PS**), and cardiolipin (**CA**). The rest of the tetracationic reagents that resulted in low sensitivities for our analytes were **Tet 1**, followed by **Tet 3** and **Tet 5**.

These phosphonium based tetracationic reagents, particularly the ones containing phenyl groups, previously have been shown to work very well at lowering the LODs of many anions.[36,37] This could possibly be due to the additional π–π interactions that are present within their structures. Furthermore, having a localized charge on the phosphonium functional group rather than a delocalized charge, such as the imidazolium moiety, might affect the coulombic interactions between the ion pairing reagent and the analyte, therefore affecting the overall sensitivity. Additional mechanistic studies are needed to further understand this behavior of these reagents.[38]

The second group of ion pairing reagents that performed well in detecting low levels of phospholipids were the dicationic reagents. In particular, **D1** (Fig. 1) produced the best sensitivity within this category. **D1** is an imidazolium based reagent containing a C9 linkage chain. Following this reagent, were **D2** and **D3** dications that resulted in adequate sensitivities when coupled with the phospholipids. These cationic reagents include imidazolium and pyrrolidinium moieties respectively. As seen from Table 1, the worst performing reagents in this category were **D4** and **D5**. The common feature of these two ions is the C12 alkyl linkage. The terminal end groups are tripropyl phosphonium and butyl imidazolium for **D4** and **D5** respectively. In this group of ion pairing reagents, it was observed that the length of the alkyl chain seems to be an important feature for sensitive detection of phospholipids. In this case, the chain length varied from C5 to C9

Table 1 Limits of detection for each of the analytes analyzed in this study with the fifteen different ion pairing reagents[a]

PA			PG			PI		
Ion pairing reagent	Mass inj./ng	Charged complex	Ion pairing reagent	Mass inj./ng	Charged complex	Ion pairing reagent	Mass inj./ng	Charged complex
Tet 4	5.00×10^{-5}	3+	**Tet 4**	8.50×10^{-4}	3+	**Tet 2**	5.00×10^{-3}	3+
Tet 2	5.00×10^{-3}	2+	**D1**	1.00×10^{-2}	1+	**Tet 4**	1.00×10^{-2}	3+
D2	2.50×10^{-2}	1+	**Tet 1**	2.50×10^{-2}	3+	**Tet 1**	1.00×10^{-2}	2+
Tet 1	5.00×10^{-2}	2+	**D2**	6.50×10^{-2}	1+	**D3**	1.00×10^{-2}	1+
Tet 3	5.00×10^{-2}	2+	**D3**	1.00×10^{-1}	1+	**LTC 1**	1.00×10^{-2}	2+
LTC 2	7.50×10^{-2}	1+	**LTC 2**	1.00×10^{-1}	1+	**D1**	1.20×10^{-1}	1+
D1	1.00×10^{-1}	1+	**LTC 1**	1.00×10^{-1}	2+	**LTC 5**	1.50×10^{-2}	2+
D3	1.20×10^{-1}	1+	**LTC 4**	1.00×10^{-1}	2+	**LTC 2**	2.00×10^{-1}	1+
LTC 1	1.70×10^{-1}	1+	**LTC 5**	1.50×10^{-1}	2+	**D4**	2.50×10^{-1}	1+
LTC 5	2.00×10^{-1}	2+	**Tet 2**	1.50×10^{-1}	3+	**Tet 5**	3.00×10^{-1}	2+
LTC 3	3.00×10^{-1}	1+	**LTC 3**	2.00×10^{-1}	1+	**LTC 3**	3.50×10^{-1}	1+
LTC 4	5.00×10^{-1}	1+	**D5**	2.00×10^{-1}	1+	**LTC 4**	5.00×10^{-1}	1+
Tet 5	5.00×10^{-1}	1+	**Tet 3**	2.50×10^{-1}	2+	**Tet 3**	5.00×10^{-1}	1+
D5	5.00×10^{-1}	1+	**D4**	2.50×10^{-1}	1+	**D2**	5.00×10^{-1}	1+
D4	5.00×10^{0}	1+	**Tet 5**	5.00×10^{-1}	2+	**D5**	5.00×10^{-1}	1+

PS			PC			PE		
Ion pairing reagent	Mass inj./ng	Charged complex	Ion pairing reagent	Mass inj./ng	Charged complex	Ion pairing reagent	Mass inj./ng	Charged complex
Tet 2	1.00×10^{-5}	3+	**Tet 2**	1.50×10^{-5}	3+	**LTC 1**	3.50×10^{-3}	2+
Tet 4	1.00×10^{-3}	3+	**D1**	5.00×10^{-4}	1+	**Tet 5**	5.00×10^{-3}	1+
D1	1.00×10^{-3}	1+	**Tet 4**	7.50×10^{-4}	3+	**D1**	5.00×10^{-3}	1+
Tet 5	1.50×10^{-3}	3+	**Tet 5**	5.00×10^{-3}	3+	**Tet 2**	1.00×10^{-2}	2+
Tet 1	5.00×10^{-2}	3+	**Tet 1**	4.00×10^{-2}	3+	**D3**	1.50×10^{-2}	1+
LTC 2	1.00×10^{-1}	1+	**D4**	7.50×10^{-2}	1+	**Tet 1**	2.50×10^{-2}	3+
LTC 1	1.00×10^{-1}	2+	**LTC 1**	2.00×10^{-2}	2+	**LTC 2**	3.50×10^{-2}	1+
Tet 3	3.00×10^{-1}	2+	**LTC 5**	1.50×10^{-1}	2+	**LTC 5**	9.50×10^{-2}	2+
D2	3.50×10^{-1}	1+	**LTC 2**	3.00×10^{-1}	1+	**Tet 4**	1.00×10^{-1}	3+
D3	3.50×10^{-1}	1+	**Tet 3**	5.00×10^{-1}	2+	**LTC 3**	1.00×10^{-1}	1+
LTC 5	4.00×10^{-1}	2+	**D3**	5.00×10^{-1}	1+	**LTC 4**	1.00×10^{-1}	1+
D4	5.00×10^{-1}	1+	**D2**	8.50×10^{-1}	1+	**Tet 3**	1.50×10^{-1}	1+
LTC 4	5.00×10^{-1}	1+	**LTC 3**	1.50×10^{0}	2+	**D4**	3.00×10^{-1}	1+
LTC 3	5.50×10^{-1}	1+	**LTC 4**	1.50×10^{0}	1+	**D2**	5.00×10^{-1}	1+
D5	1.50×10^{0}	1+	**D5**	5.00×10^{0}	1+	**D5**	5.00×10^{1}	1+

CA			SPE			DHPC		
Ion pairing reagent	Mass inj./ng	Charged complex	Ion pairing reagent	Mass inj./ng	Charged complex	Ion pairing reagent	Mass inj./ng	Charged complex
Tet 2	5.00×10^{-4}	2+	**Tet 1**	5.00×10^{-6}	2+	**D2**	1.50×10^{-2}	1+
Tet 4	1.50×10^{-2}	2+	**LTC 1**	1.00×10^{-2}	1+	**Tet 1**	4.50×10^{-2}	2+
Tet 1	2.00×10^{-2}	2+	**D2**	1.90×10^{-2}	1+	**Tet 5**	5.00×10^{-2}	3+
LTC 1	1.20×10^{-1}	1+	**Tet 2**	2.50×10^{-2}	2+	**LTC 2**	7.50×10^{-2}	2+
LTC 4	1.20×10^{-1}	1+	**LTC 4**	7.50×10^{-2}	1+	**LTC 3**	1.00×10^{-1}	2+
LTC 5	3.00×10^{-1}	1+	**D4**	7.50×10^{-2}	1+	**LTC 4**	1.00×10^{-1}	2+
LTC 2	5.00×10^{-1}	2+	**LTC 2**	1.20×10^{-1}	1+	**LTC 1**	1.00×10^{-1}	2+
Tet 3	5.00×10^{-1}	2+	**D5**	1.50×10^{-1}	1+	**D3**	1.20×10^{-1}	1+
Tet 5	5.00×10^{-1}	2+	**Tet 4**	1.80×10^{-1}	1+	**D5**	1.50×10^{-1}	1+
D2	1.20	1+	**LTC 5**	1.90×10^{-1}	1+	**Tet 2**	1.50×10^{-1}	2+
D3	1.20×10^{0}	1+	**LTC 3**	3.70×10^{-1}	1+	**Tet 4**	2.50×10^{-1}	1+
D1	1.50×10^{0}	1+	**Tet 3**	4.00×10^{-1}	1+	**LTC 5**	3.00×10^{-1}	2+
D5	2.50×10^{0}	1+	**Tet 5**	5.00×10^{-1}	1+	**Tet 3**	5.00×10^{-1}	2+
LTC 3	2.00×10^{1}	2+	**D1**	7.50×10^{-1}	2+	**D1**	1.50×10^{0}	1+
D4	N/A	N/A	**D3**	1.00×10^{0}	1+	**D4**	1.50×10^{1}	1+

[a] N/A: complex was not able to be detected.

Table 2 Limits of detection for each phospholipid analyzed in the negative ion mode ESI-MS, without the presence of any ion pairing reagent

	Anion mass/g mol^{-1}	SIM LOD/ng
L-Phosphatidic acid (PA)	671.89	1.50×10^{0}
Phosphatidylglycerol (PG)	745.98	5.00×10^{-1}
Phosphatidylinositol (PI)	886.12	4.00×10^{-1}
Phosphatidylserine (PS)	758.97	4.00×10^{0}
Phosphatidylcholine (PC)	758.06	ND[a]
Phosphatidylethanolamine (PE)	746.05	ND
Cardiolipin (CA)	1447.9	2.00×10^{1}
Sphingosyl PE (SPE)	422.29	1.70×10^{-1}
Diheptanoyl-phosphocholine (DHPC)	481.28	ND

[a] ND: not detected at 10 μg mL^{-1}.

and C12, and it was noticed that the dicationic reagent containing C9 chain linkage resulted in the lowest LODs.

The last group of the ion pairing reagents tested were the linear tricationic ion pairing reagents. Overall, this group of reagents did not produce very good sensitivities for the nine phospholipids, as seen in Table 1. All of the tricationic pairing reagents used in this study were linear and contained imidazolium based cores in their structure. The differences among them included the different terminal charged groups and the length of the alkyl chain linkages. Based on our results from the other pairing agents, it was hypothesized that the phosphonium based linear ion pairing reagents might produce lower LODs for the analytes. Thus, a study was completed with a linear ion pairing reagent containing tripropyl phosphonium terminal groups, an imidazolium core, and C12 alkyl linkage. **PG** and **PI** were detected with this ion pairing reagent. However no further improvement

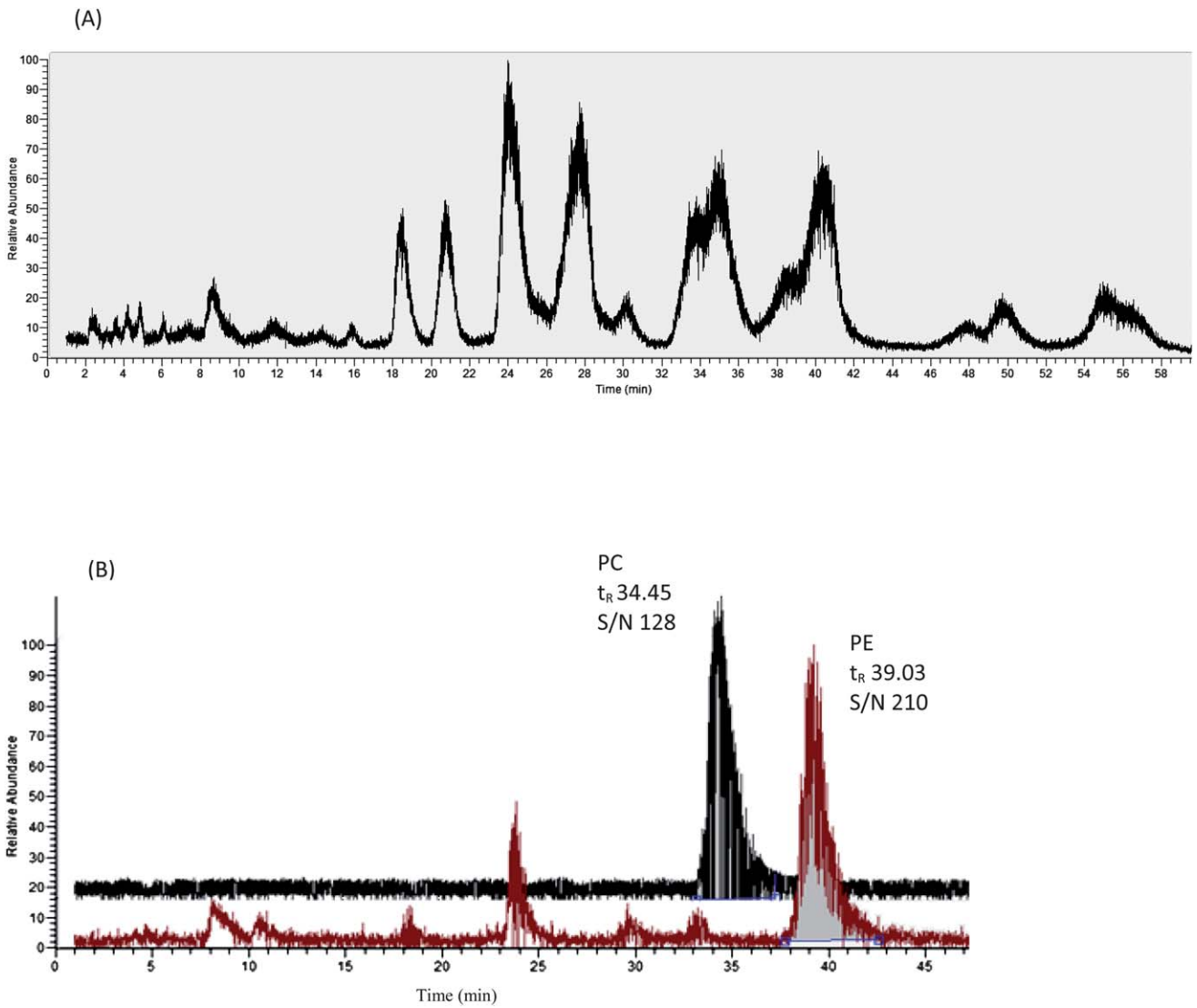

Fig. 4 Chromatographic separation and detection of the PC and PE mixture and their homologues in the SIM positive mode ESI-MS. (A) Represents the total ion chromatogram of this mixture and (B) is the extracted ion chromatogram in which the major species of the phospholipids are detected with tetracation ion pairing reagent **Tet 5**. The separation was performed on an Ascentis™ C18 column (250 mm × 2.1 mm) with a mobile phase of 60/25/15 isopropanol/acetonitrile/water with 0.1% formic acid. The flow rate was set at 0.2 mL min^{-1}.

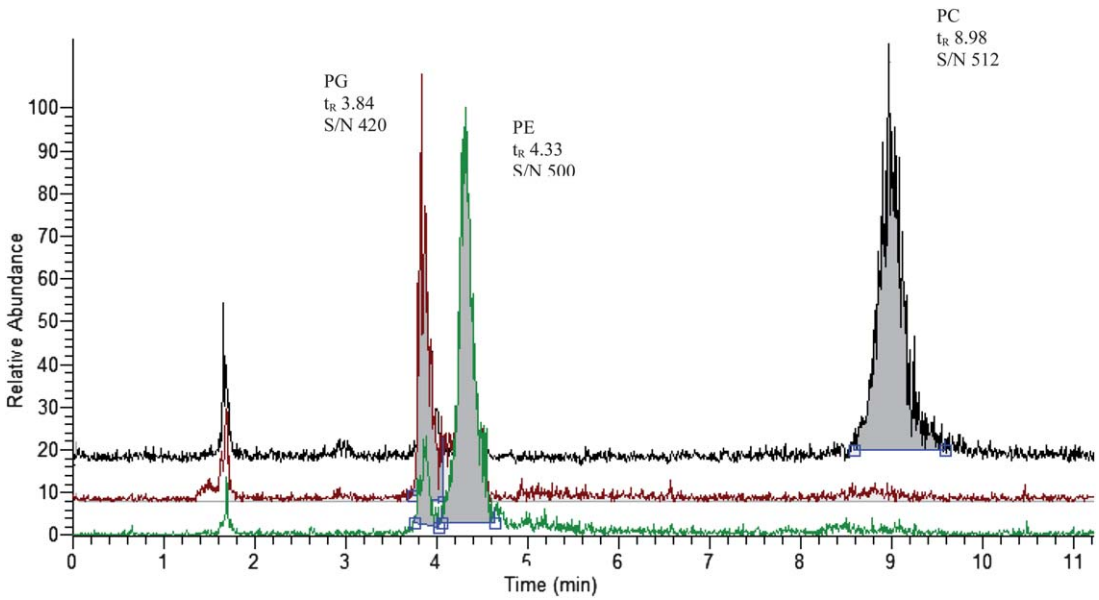

Fig. 5 The extracted ion chromatogram displaying the LC separation of PC, PG, and PE on a silica column in the positive ion mode ESI-MS. Concentration of the analytes was 1 mg mL^{-1} and the mobile phase composition was 70/20/10 acetonitrile/methanol/water with a flow rate of 1 mL min^{-1}. A flow splitter was used, such that only 0.3 mL min^{-1} is mixed *via* a mixing tee with 0.1 mL min^{-1} of the ion pairing reagent, with a final flow rate of 0.4 mL min^{-1} entering the mass spectrometer. The ion pairing reagent used was **Tet 5** (Fig. 3).

was noticed in their LODs. To further understand these results, an extended study (as per ref. 38) would be needed.

In addition to SIM analysis, single reaction monitoring (SRM) experiments were performed as well on these analytes. Previous studies have shown that in many cases SRM analysis further improves the LODs compared to the SIM analysis.[35,37] However, this was not the case for the phospholipids. In this study it was observed that SRM analysis did not improve the sensitivity of the analytes except in a few instances.

For most of the phospholipids, SRM data were not able to be collected because of two main reasons: first, the background noise was very low therefore making it difficult to accurately identify the LODs, and secondly, in many cases a fragment from the parent ion was not observed when energy was applied to the mass of interest. In the instances in which a fragment was detected and enough background noise was available, the LODs monitored for the analytes did not improve when compared to the LODs in the SIM ion mode. Also, the fragments detected were mainly from the ion pairing reagents, in particular the tetracationic reagents.

During the SIM analysis, all possible combinations of ion pairing agents and the analyte were observed and tested. The complex that produced the highest signal was further analyzed and the lowest limit of detection was found for that complex until a signal to nose ratio of three is achieved. For the dicationic reagents the only type of complex formed is a singly charged complex (1+). However due to their multiple charged state, the tricationic and tetracationic reagents create more possibilities of charged complexes to be observed. It was noticed that linear tricationic agents that have short alkyl chain linkages (*i.e.*, **LTC 1** and **LTC 5**, Fig. 2) mainly formed doubly charged complexes (2+). On the contrary, the tricationic agents that contained long alkyl chain linkages within their structure (*i.e.*, **LTC 2**, **LTC 3**, **LTC 4**, Fig. 2) mainly formed singly charged complexes (1+).

The tetracationic ion pairing reagents mainly formed doubly charged complexes (2+). **Tet 2** formed an equal number of 2+ and 3+ complexes, whereas **Tet 4** was the only tetracationic reagent that mainly formed 3+ complexes. During the analysis with **Tet 1** and **Tet 3**, in only a few instances there were singly charged (1+) complexes observed. In every case the complex charge that produced the best LODs is giving in Table 1.

LC analysis was coupled with this technique to further enhance the chromatographic detection of the analytes. Reverse phase LC was first used to separate two phospholipids, **PC** and **PE**. The total ion chromatogram which includes the separation of the analytes and the MS detection of these phospholipids is shown in chromatogram (A) of Fig. 4. This separation was achieved on a C18 stationary phase. Chromatogram (B) of Fig. 4 shows the extracted ion chromatogram in which the total mass of the phospholipids and the ion pairing reagent is monitored. In this chromatographic separation the ion pairing reagent was added post-column at a flow rate of 100 μL min^{-1}. The other peaks observed on chromatogram (A) correspond to other homologous species of **PC** and **PE**. The HPLC chromatogram for the separation of these analytes does not show as many peaks as are seen in the total ion chromatogram (A) in Fig. 4. This is one advantage that the mass spectrometer has over the ultraviolet (UV) detection often used in HPLC. Analytes that do not absorb at a certain wavelength, in this case 210 nm, cannot be detected by the UV detector, however, they can easily be detected by the mass spectrometer as long as they can be ionized.

The extracted ion chromatogram (Fig. 4B) shows increased background noise and not a very high signal to noise (S/N) ratio for these analytes. This signal to noise ratio would result in a much higher LOD than the one reported in Table 1. This decrease in sensitivity is possibly due to the protonation of these analytes by the formic acid present in the mobile phase of this chromatographic separation (see Experimental). Also, another reason contributing

to this decrease in sensitivity could be the mobile phase used in the chromatographic separation, which is not composed of the same solvents that were used in the ESI-MS analysis for the detection of the phospholipids with the ion pairing reagents.

Since this type of LC analysis did not show very high sensitivity, another chromatographic method was developed in which formic acid was omitted and the solvents used were more similar to the ones chosen during the detection of the analytes with just the ion pairing reagent as described earlier. This separation was achieved on a silica column (Fig. 5) with a mobile phase of 70/20/10 acetonitrile/methanol/water. Under these conditions there were three phospholipids that were detected, **PG**, **PC**, and **PE**, where **PG** is a phospholipid that is usually detected in the negative ion mode. The signal to noise ratio in this case remained high and very comparable to the previous results reported in Table 1. Another advantage of using the HILIC phase HPLC in this case is the shorter retention times (approximately 9 minutes).

Conclusions

Fifteen different cationic ion pairing reagents were used in determining the limits of detection of nine phospholipids in the positive mode ESI-MS. The reagents that performed best were the tetracationic pairing reagents, followed by the dicationic and the linear tricationic ion pairing reagents. In particular it was **Tet 2** and **Tet 4**, phosphonium based reagents (Fig. 3), that lowered the limits of detection for most of the phospholipids. The best dicationic reagent in this analysis was **D1**, which also significantly increased the sensitivity of the analytes. The linear tricationic reagents performed equally when compared to each other, but gave poorer results when compared to the other groups of reagents. However as a whole group, based on previous studies, linear tricationic reagents did not perform as well as was expected.[34,35] Thus, in detecting phospholipids tetracationic ion pairing reagents, with phosphonium moieties, phenyl functional groups are recommended in achieving low limits of detection. LC analysis was developed in both reverse and HILIC phase HPLCs. It was also shown in this study that these chromatographic separations were successfully coupled to this ion pairing technique, and a separation and detection of three phospholipids (**PC**, **PG**, and **PE**) were achieved in the HILIC phase mode with satisfactory signal to noise ratios and very short retention times. Other advantages of this technique, besides low limits of detection, and compatibility with HPLC, are ease of use, simplicity, and fast analysis times.

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References

1 R. H. Garret and C. M. Grisham, *Biochemistry*, Thomson, Brooks/Cole, 3rd edn, 2005, pp. 251–277.
2 R. Boyer, *Concepts in Biochemistry*, Wiley & Sons, New York, 3rd edn, 2004, pp. 236–348.
3 M. Pulfer and R. C. Murphy, *Mass Spectrom. Rev.*, 2003, **22**, 332–364.
4 J. Muthing and M. Radloff, *Anal. Biochem.*, 1998, **257**, 67–70.
5 S. J. Singer and G. L. Nicolson, *Science*, 1972, **175**, 720–731.
6 K. Simmons and E. Ikonen, *Nature*, 1997, **387**, 569–572.
7 T. F. Martin, *Annu. Rev. Cell Dev. Biol.*, 1998, **14**, 231–264.
8 E. M. Bevers, P. Comfurius, D. W. Dekkers and R. F. Zwaal, *Biochim. Biophys. Acta*, 1999, **1439**, 317–330.
9 M. Koivusalo, P. Haimi, L. Heikinheimo, R. Kostiainen and P. Somerharju, *J. Lipid Res.*, 2001, **42**, 663–672.
10 M. Petkovic, J. Schiller, M. Muller, S. Benard, S. Reichl, K. Arnold and J. Arnhold, *Anal. Biochem.*, 2001, **289**, 202–216.
11 H. K. Min, G. Kong and M. H. Moon, *Anal. Bioanal. Chem.*, 2009, **396**, 1273–1280.
12 T. Houjou, K. Yamatani, M. Imagawa, T. Shimizu and R. Taguchi, *Rapid Commun. Mass Spectrom.*, 2005, **19**, 654–666.
13 M. Kovacevic, R. Leber, S. D. Kohlwein and W. Goessler, *J. Anal. At. Spectrom.*, 2004, **19**, 80–84.
14 A. M. Descalzo, E. M. Insani and N. A. Pensel, *Lipids*, 2003, **38**, 999–1003.
15 G. M. Patton, J. M. Fasulo and S. J. Robins, *J. Lipid Res.*, 1982, **23**, 190–196.
16 G. A. Arvidson, *J. Lipid Res.*, 1965, **6**, 574–577.
17 H. Y. Kim, T. L. Wang and Y. Ma, *Anal. Chem.*, 1994, **66**, 3977–3982.
18 H. Kim, E. Ahn and M. H. Moon, *Analyst*, 2008, **133**, 1656–1663.
19 C. Wu, Y. Peng, J. Chiu, Y. Ho, C. Chong, Y. Yang and M. Liu, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2009, **877**, 3495–3505.
20 F. Gao, Z. Zhang, X. Fu, W. Li, T. Wang and H. Liu, *Electrophoresis*, 2007, **28**, 1418–1425.
21 C. A. Lytle, Y. D. Gan and D. C. White, *J. Microbiol. Methods*, 2000, **41**, 227–234.
22 D. Y. Bang, E. J. Ahn and M. H. Moon, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2007, **852**, 268–277.
23 H. Nakanishi, H. Ogiso and R. Taguchi, *Lipodomics*, 2009, **579**, 287–313.
24 N. B. Cech and C. G. Enke, *Mass Spectrom. Rev.*, 2001, **20**, 362–387.
25 P. Kebarle, and H. Yeunghaw, in *Electrospray Ionization Mass Spectrometry*, ed. R. B. Cole, Wiley, New York, 1997, p. 14.
26 R. F. Straub and R. D. Voyksner, *J. Am. Soc. Mass Spectrom.*, 1993, **4**, 578–587.
27 R. B. Cole and A. K. Herrata, *Rapid Commun. Mass Spectrom.*, 1992, **6**, 536–539.
28 A. Apffel, J. A. Chakel, S. Fischer, K. Lichtenwalter and W. S. Hancock, *Anal. Chem.*, 1997, **69**, 1320–1325.
29 R. B. Cole and J. Zhu, *Rapid Commun. Mass Spectrom.*, 1999, **13**, 607–611.
30 R. J. Soukup-Hein, J. W. Remsburg, P. K. Dasgupta and D. W. Armstrong, *Anal. Chem.*, 2007, **79**, 7346–7352.
31 P. K. Martinelango, J. L. Anderson, P. K. Dasgupta, D. W. Armstrong, R. S. Al-Horr and R. W. Slingsby, *Anal. Chem.*, 2005, **77**, 4829–4835.
32 J. W. Remsburg, R. J. Soukup-Hein, J. A. Crank, Z. S. Breitbach, T. Payagala and D. W. Armstrong, *J. Am. Soc. Mass Spectrom.*, 2008, **19**, 261–269.
33 M. M. Warnke, Z. S. Breitbach, E. Dodbiba, E. Wanigasekara, X. Zhang, P. S. Sharma and D. W. Armstrong, *J. Am. Soc. Mass Spectrom.*, 2008, **20**, 529–538.
34 M. M. Warnke, Z. S. Breitbach, E. Dodbiba, J. A. Crank, T. Payagala, P. S. Sharma, E. Wanigasekara, X. Zhang and D. W. Armstrong, *Anal. Chim. Acta*, 2009, **633**, 232–237.
35 Z. S. Breitbach, M. M. Warnke, E. Wanigasekara, X. Zhang and D. W. Armstrong, *Anal. Chem.*, 2008, **80**, 8828–8834.
36 X. Zhang, E. Wanigasekara, Z. S. Breitbach, E. Dodbiba and D. W. Armstrong, *Rapid Commun. Mass Spectrom.*, 2010, **24**, 1113–1123.
37 E. Dodbiba, Z. S. Breitbach, E. Wanigasekara, T. Payagala, X. Zhang and D. W. Armstrong, *Anal. Bioanal. Chem.*, 2010, **398**, 367–376.
38 Z. S. Breitbach, E. Wanigasekara, E. Dodbiba, K. A. Schug and D. W. Armstrong, *Anal. Chem.*, 2010, **82**, 9066–9073.