ANION DETECTION IN THE POSITIVE ION MODE ELECTROSPRAY IONIZATION MASS SPECTROMETRY BY USE OF CATIONIC ION PAIRING AGENTS: BEHAVIOR AND APPLICATIONS

by

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This dissertation is dedicated to my mother and father, Zana and Ilir Dodbiba, whose selflessness has no limits.

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ABSTRACT

ANION DETECTION IN THE POSITIVE ION MODE ELECTROSPRAY IONIZATION MASS SPECTROMETRY BY USE OF CATIONIC ION PAIRING AGENTS: BEHAVIOR AND APPLICATIONS

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Anion detection and quantitation has long been of great interest to many scientific areas of research in academic and industrial settings. Their importance extends to areas such as water and environmental analysis, biological system analysis, and also the pharmaceutical and food industries.

A variety of sensitive detection methods have been developed for anions. Many of these methods have significant advantages, but also drawbacks. In my dissertation I have described a new method for the detection of anions using electrospray ionization mass spectrometry (ESI-MS) positive ion mode, and cationic ion pairing reagents. Using this method, anions are paired with a plethora of different, di-, tri-, and tetra-cationic ion pairing reagents, thus forming positively charged complexes, which can then be detected in the positive ion mode ESI-MS. The behavior and selectivity of the ion pairing reagents is investigated for the different classes of analytes.

During this study, large classes of small organic and inorganic anions were analyzed. In addition, three unique classes of moderate size molecules were investigated. Two of which

were biological ones: nucleotides and phospholipids. These are very important molecules, which are responsible for the proper functioning of all biological entities. Nucleotides can exist as monomers or constituents of oligomers, thus they can have more than one negative charge. A total of 28 nucleotide and nucleotide based compounds were paired and analyzed with many different cationic ion pairing reagents.

Phospholipids are unique molecules due to their amphipathic character. Phospholipids of different polar groups and different sizes were analyzed in the positive ion mode ESI-MS by using newly synthesized ion pairing reagents. Also, analytical separations of phospholipids were developed using both reverse phase high performance liquid chromatography (HPLC) and hydrophilic interaction liquid chromatography (HILIC)

The last class of analytes studied were metal ions. They are already positively charged and therefore they can be detected directly in the positive ion mode ESI-MS, however most of them have a small mass and several oxidation states which usually makes them fall in the low mass to charge region of the mass spectra where the background noise is significant. In this study metals of different oxidation states were complexed with commercially available chelating agents forming negatively charged complexes. Ion pairing agents were added to these compounds forming overall ternary positively charged complexes.

Analyses for all types of analytes were performed in both single ion monitoring (SIM) and single reaction monitoring (SRM) modes. Limits of detection (LODs) were easily achieved at the parts per billion (ppb) to parts per trillion (ppt) levels, and significant improvements were noticed in the positive versus negative ion mode ESI-MS. This novel method developed herein shows immense potential for sensitive trace studies of many molecules, high throughput and great simplicity. Lastly a mechanism study by ESI-MS was explored to further understand the low limits of detection achieved by this method.

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CHAPTER 1

INTRODUCTION

1.1 Thesis Organization

Mass spectrometry is a technique that in the last two decades has steadily gained importance in analytical chemistry. This dissertation is focused on a novel detection and quantitation method for anions in the positive ion mode electrospray ionization mass spectrometry (ESI-MS). Chromatographic separations by high performance liquid chromatography also are achieved for specific classes of anions.

This dissertation has a total of 8 chapters. The first chapter introduces different separation and detection methods for anions with the main focus being on mass spectrometry. The second and the third chapters introduce newly synthesized ion pairing reagents which were evaluated with small organic and inorganic ions. An application of this method to two main classes of biological molecules, nucleotides and phospholipids, is described and thoroughly discussed in Chapters 4 and 5 respectively. Transition metals also were examined using this method after the addition of chelating agents. This work is described in Chapter 6 of the manuscript. Finally, to better understand this technique, a study on the ESI-MS mechanism of the ion pairing reagents is described in Chapter 7. A general summary of this sensitive method and the results achieved is included in the last chapter, (Chapter 8).

1.2 Separation and Detection Methods of Anions

Anions are atoms or ions that have gained extra electrons. Having a greater number of electrons than protons makes them negatively charged. Anions can be composed of one or more atoms (e.g. polyatoms), therefore forming large organic or inorganic molecules. These anionic molecules are present in many different types of environments. They are found, and play an exceptionally important role, in biological systems. Thus, anions have been thoroughly

studied by many researchers for a very long time. Investigations and different types of anion analysis methods are still undergoing investigation as anions will always be present and will continuously play crucial roles in our lives.

These negatively charged entities also play crucial roles in areas such as, environmental analysis, medicine, food science and many other areas of scientific research. [1-5] Because they are of such great interest, scientists have tried to develop and use a variety of sensitive and accurate methods to detect, separate and quantitate them. Some of the most frequent and most important methods include techniques such as ion chromatography, gas chromatography (GC), flow injection analysis, high performance liquid chromatography and an even more sensitive method involves mass spectrometry (MS).[3-10] Each of these methods and instruments developed throughout time have unique advantages and disadvantages.

1.2.1 Ion Chromatography

Ion chromatography is one of the many techniques used for the separation of ions. This type of chromatography shows high performance and good sensitivity. Unknown analytes or ions can be determined by comparing the chromatograms with the ones provided by standard solutions. Ions can be quantified via this method by measuring the change in conductivity that occurs when each analyte passes through the detector as a function of time. The limits of detection via this method can reach as low as parts per billion (ppb) levels.[11, 12]

Anions and cations have also been able to be separated and detected simultaneously via ion exchange chromatography[13]. This was achieved by coupling two or more columns and conductivity detectors in a series.[14,15] Ultraviolet (UV) detectors are used with ion chromatography for the detection of analytes that have the ability to absorb UV light. Nowadays the ion chromatography systems can be coupled with many different detectors simultaneously for complex sample analytes.

Ion chromatography is used extensively for the monitoring of different water treatments such as wastewater, rainwater, seawater, and tap water.[16-18] Other interesting applications of

ion chromatography are seen in the analysis of potato chips, wine, soil, explosives residues etc.[19-21]

1.2.2 Gas Chromatography

Gas Chromatography (GC) is a well-established analytical technique first introduced by Martin and Synge, whose work was later followed by the first GC experiments performed and published by James and Martin in 1952.[22, 23] Unlike other chromatographic techniques such as ion exchange (IC) or high performance liquid chromatography (HPLC), GC uses gas as a mobile phase.

This is a fast and accurate method used to separate a variety of different compounds in complex mixtures at small analytical scales. Besides its high sensitivity, reproducibility and the very low amounts of analyte that are needed for analysis, gas chromatography is also a rather inexpensive method which has helped increase its use and popularity in academic and industrial settings.

However, the detection of anions or any other species by GC are dependent on their volatility. This is a crucial requirement for an analyte to be detected and quantified by gas chromatography. The analytes of interest need to be volatile and should be able to resist high temperatures without being decomposed. In cases, when the analytes are not volatile there is the possibility that derivatization reactions can be performed to allow further analysis by GC.[24-29] Of course this results in a longer analysis time per sample, and is not always preferred, particularly in industrial settings where the high throughput and efficiency are highly important.

The detectors of gas chromatography are mainly of two distinct types: destructive and non-destructive. Destructive GC detectors typically include flame ionization detector, mass spectrometer and thermionic detectors, while the non-destructive ones include electron capture detector, thermal conductivity detector and gas density detector.[30] Each of these detectors has unique characteristics and they provide different advantages on the detection of an anion or analyte of interest.

1.2.3 Flow Injection Analysis

Flow injection analysis is a continuous flow analyzer system developed in 1974 by Ruzicka and Hansen.[31] In this system a small amount of analyte is injected into a continuous flow, which then mixes with another flowing reagent. The analyte reacts with the reagent and the product enters a detector which provides a response in the form of a Gaussian peak.[31, 32]

This technique has provided applicability to an array of analytical analysis in biochemistry, agriculture, and environmental research.[33-36] The detectors used for flow injection analysis are located downstream from the injection port and can be of various types, such as: biosensor detectors, fluorimeter, calorimeter detectors etc.[37, 37-42]

In general the limits of detection for a flow injection analysis system are in the parts per million and parts per billion ranges for anions and other analytes. Despite the small amounts needed and low reagent consumption, this system has one main drawback which is its discontinuous signal and which is highly affected by the power supply voltage fluctuations.[32]

1.2.4 High Performance Liquid Chromatography

Another very well-known and established analytical technique used for the separation and detection of anions or molecules is high performance liquid chromatography (HPLC). This method has evolved throughout the years and has become one of the most used due to its simplicity, fast analysis time, and great resolving power.

An HPLC instrument relies on high pressure pumps to carry the liquid containing the sample of interest through a column enclosing a stationary phase made of small granulated particles which are covalently bonded to it.[43] The stationary phase is a crucial component of this chromatographic system and together with the mobile phase of choice, different HPLC modes can be created including normal phase, reverse phase, polar organic, hydrophilic interaction liquid chromatography (HILIC), etc. [44-48]

HPLC has found great applicability in many different fields including research in academic and industrial settings, biochemistry, environmental research, pharmaceuticals, and

many others.[49-58] Its use has also increased greatly with the coupling of the different powerful detectors used nowadays. This includes ultraviolet, refractive index detectors, and also the diode array detectors which allow for the simultaneous detection of several analytes at several wavelengths at a time.[32, 59, 60] Other HPLC detectors include fluorescent, electrochemical, light scattering detectors and also mass spectrometers.[61-64]

Highly used, is also ultra high pressure performance liquid chromatography (UHPLC or UPLC), which provides very short analysis time and low sample consumption. UPLC is the same as HPLC, but the stationary phase is made of smaller particles and therefore higher pressures are obtained. Other multidimensional systems have been developed for analyzing complex samples. This includes systems such as one (1D) and second (2D) dimensional UPLC with UV-CAD (collisionally activated dissociation)-MS^n.[61, 65]

1.2.5 Mass Spectrometry

Mass spectrometry is one of the most used techniques for the detection of anions. Its principles date back to the 1800s, however it was not until 1912 that the first mass spectrometer was constructed by Joseph John (J.J) Thomson.[66,67] Initially this instrument was used by physicists to measure the atomic weight of elements and the natural relative abundance of elemental isotopes.[68]

Mass spectrometry detects ions or molecules based on their mass to charge ratio (m/z). [66, 68, 69] The criteria of an analyte to be analyzed by a mass spectrometer are its ability to be ionized and to be dissolved in a liquid. If these two conditions are satisfied, virtually any ion or molecule can be detected, and quantified by mass spectrometry. However, nowadays newer and improved mass spectrometers have been developed which are not limited to just liquid samples, but also solid ones as well.[70, 71]

Mass spectrometers are instruments that have three main components: the ion source, the mass analyzer and the detector (Figure 1.2.5.1).[68] This system operates under vacuum to control the pressure within the instrument and is connected to a computer system. The pressure in these systems is kept very low in order to control the ion collisions which can result in unwanted products and alter the ion path throughout the instrument.[68]

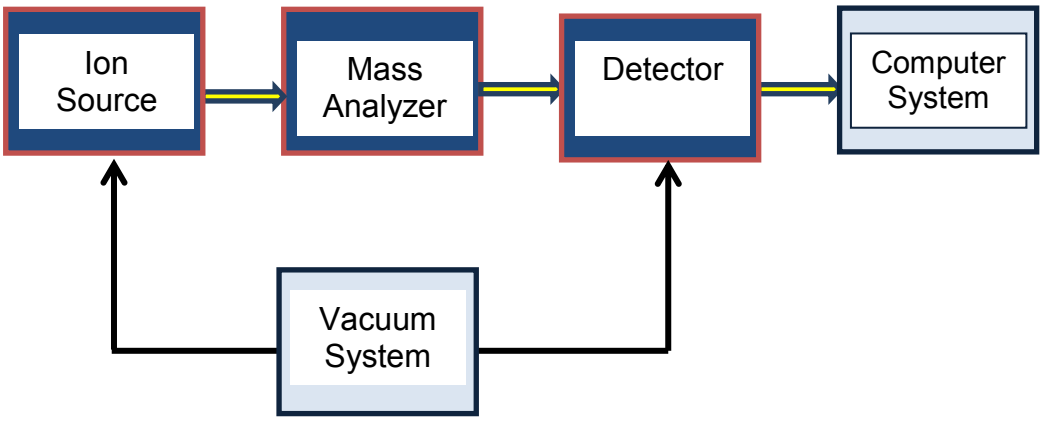


Figure 1.2. Schematic of the main components of a mass spectrometer

1.2.5.1 Ion sources

The ion source is where the analytes get ionized and become charged. Some of the most common ion sources used are spray ionization, ambient ionization, electron and chemical ionization, and gas discharge ion sources.

Spray ionization sources most commonly include electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and thermal ionization.

Electrospray ionization is considered a soft ionization technique as it does not fragment the analyte of interest, and thus has shown to be ideal for the detection and analysis of many different types of analytes including biological molecules such as proteins, nucleotides, phospholipids etc.[72,73-78] Electrospray ionization is also very easy to couple with high

performance liquid chromatography.[79-81] This allows for the analysis of many different complex samples. ESI is widely used on the analysis of polar and ionic analytes and their ionization during this process occurs while in the liquid form.[82]

Atmospheric pressure chemical ionization is a type of chemical ionization that occurs in atmospheric pressure.[83] This technique is used mainly for molecules of low polarity and of low to medium molecular mass. Here the analytes are ionized in the gas phase, contrary to the ESI mode. This occurs by heating the solvent to high temperatures and then dissolving it before subjecting it to corona discharge which then helps to create the necessary ions.[82,84] APCI typically forms singly charged ions by either the addition or the loss of a proton (e.g. $[M+H]^+$ or $[M-H]^-$).[84]

Thermal ionization is a method which ionizes purified molecules by using very high temperatures. The ions are then focused on a beam which passes through electrostatically charged plates. A magnetic field disperses this beam of ions into other ones based on their mass to charge ratios. These beams allow for the precise identification of the different isotopes.[85]

Thermal ionization mass spectrometry has widely been used by nuclear industry for isotopic measurements as it provides high precision levels.[86-88] However, its use has declined throughout the years as this technique requires elaborate sample loading and it necessitates time consuming sample purification.[87] Newer mass spectrometric techniques and instruments have been developed which easily overcome these disadvantages.[89, 90]

Ambient ionization includes ionization sources such as desorption electrospray ionization (DESI), direct analysis in real time (DART) and matrix-assisted laser desorption ionization (MALDI). Desorption electrospray ionization mass spectrometry is a hybrid technique between desorption ionization and electrospray ionization, developed in 2004 by Takats *et al.*[91, 92] DESI is achieved by directing charged droplets of a solvent onto a surface of close proximity

containing the analyte of interest. Upon contact, gaseous ions are formed and enter an atmospheric pressure interface, followed by a mass spectrometer.[91]

This technique was shown to work on a variety of compounds including proteins and peptides present on polymer and metal surfaces.[91, 93-96] In vivo experiments have also been achieved by the use of DESI.[91]

The advantages of this technique involve the ability to directly analyze virtually any type of surface without any sample pretreatment. Another benefit includes the ability to make changes to an experiment as it is taking place. The limits of detection for this technique are in the parts per billion range.[91] Since some of the DESI's uses are aimed at direct biological analysis, this can create significant challenges in performing quantification analysis as it is difficult to always provide the appropriate internal standards.[91]

Direct analysis in real time (DART) is another ambient ionization technique, very similar to DESI. DART was developed by Cody *et al* in 2005.[97] This technique is based on the reactions of exited–state species with reagent molecules and analytes of different polarity. Similarly to DESI, in DART a stream of ionized liquid is pointed at a sample surface in which the analyte of interest is located.

As described by Cody *et al*. the instrumental set up of DART includes a tube divided into several chambers through which a gas such as helium flows through. This gas is then introduced into a discharge chamber containing an anode and cathode. Ions are generated by applying a high voltage to the system.

This beam of ions can be then focused directly at the surface containing the analyte of interest. This type of ion source has found wide applications for biological matrices such as pesticides from plants, waxes, flavors, as well as explosives and illicit drugs from banknotes and other media.[98-104]

Another widely used technique of ambient ionization is matrix-assisted laser desorption ionization (MALDI).[105] This is a soft ionization technique which is mainly used for the analysis of the large biomolecules such as proteins, peptides, biopolymers etc.[106-110]

In MALDI, the analyte of interest requires mixing with a type of matrix which is responsible for the ionization of the sample. This mixture is then placed into a MALDI plate. When the solvent has evaporated, the matrix containing the analyte crystallizes.[105] Once the UV laser is applied to the matrix sample, ions are formed by the addition of a proton to the analyte.[105] Finding the appropriate type of matrix can be challenging as the best matrix is dependent on the type of molecule that is being analyzed.

Electron ionization dates back to the 1920s and it is a type of ionization in which electrons are used to interact with molecules to produce ions.[37, 111-113] During this type of ionization, the electrons are emitted by an electrically heated filament under vacuum conditions.[114] An electric field is then applied to accelerate them. Research has shown that electron energies of 10-70 eV can ionize most organic molecules and significant fragmentation occurs in many of them.[114,115] This excess fragmentation is not always desirable as information is lost on the molecular ion, and this can threaten selectivity and sensitivity. [116, 117]

Chemical ionization is the type of ionization which is based on chemical reactions in the gas phase. In such cases, a reaction gas is produced in an ionization chamber at a pressure of 1 torr.[118] The reaction gas gets ionized and will cause the sample of interest to ionize as well. These types of gas phase collision reactions will create a mass spectrum of the analyte. The reaction gas used in these types of experiments requires that it is nonreactive or slightly reactive with its own gas molecules.[118] Chemical ionization mass spectrometry is a much softer ionization technique than the electron impact. This allows for the collection of more information on unknown complexes.

The most used and well known gas discharge ion source is inductively coupled plasma mass spectrometry (ICP-MS). This type of ionization involves high temperature plasma which

ionizes the analytes and degrades them to their elemental state. They are typically transformed into positively charged compounds. This makes the negatively charged ions difficult to detect by ICP.

ICP-MS has nowadays become a widely used method of choice due its excellent linearity, low detection limit which have reached parts per trillion levels, and the ability to detect different elements, particularly metals in simple and complex biological matrices. However, ICP-MS has a few drawbacks that include the inability to recognize the oxidation state of an element, and also due to the high temperature plasma, it is difficult to obtain molecular information of the comounds that are analyzed.

1.2.5.2 Mass Analyzers

Mass analyzers make up the second most important component of a mass spectrometer. Its purpose is to separate ions based on their mass to charge ratio (m/z). Some of the most frequently used mass analyzers are quadrupole, time of flight (TOF), and ion trap mass analyzers.

Quadruple mass analyzer was first described by Nobel Prize winner Paul Wolfgang in 1950.[119] This type of mass analyzer is composed of four parallel rods. Electric fields connect them together, and also radio frequency (RF) and direct current (DC) voltages are applied to two rods at a time. Specific alternation between these two voltages allows for specific ions to pass through the rods, and for others to collide with them or to be ejected, therefore never reaching the detector.[119]

Some of the main benefits of using a quadrupole mass analyzer include its low cost, good reproducibility, small size and the easy maintenance.[119] Its main disadvantages include the limited resolution it provides and the limited mass range. Also, tandem mass spectrometry experiments can be achieved if additional quadrupoles can be attached in a series.[120, 121] This allows for attaining further molecular information on complex ions.[122]

Time of flight (TOF) mass analyzers are one of the simplest mass analyzers. [123] The ions pass through a tube of 1-2meters (m) and their separation is based solely on the ion kinetic energy and velocity.[119] Unlike the quadrupole, in this type of mass analyzer, all ions will eventually reach the detector, with the smaller and the lighter ones reaching it first.

The benefits of such an analyzer are its low cost, high mass range, speed and its adaptability to MALDI.[124, 125] However, this mass analyzer also has some main drawbacks which include low resolution, limited dynamic range and it is not easily coupled with continuous ion sources (e.g. ESI).[119]

Ion trap mass analyzers are composed of three electrodes which are used to trap the ions in small volumes. By altering the electrode voltage, similar to the quadrupole mass analyzers, ions can be either kept in or ejected from this trap. Ion trap mass analyzers tend to have high ejection efficiencies and high ion storage capacities.[126]Advantages of an ion trap mass analyzer include multiple stages of tandem mass spectrometry experiments and high sensitivity at full scans.[119] The main drawbacks of this mass analyzer are the poor dynamic range and quantitation abilities.[126] Throughout the years linear ion trap analyzers have been coupled with other mass analyzers to further increase their potential to perform tandem mass spectrometry experiments and increase ion-molecule analysis.[127-129]

1.2.5.3 Detectors

Detectors make up the last important unit of a mass spectrometer that ions reach. Once the ions go through the mass analyzer, they are directed towards the detector by which they are electrically detected.[69] There are many detectors used in mass spectrometry, but the ones that are encountered the most are electron multiplier detector, Faraday cup collector, and conversion dynodes.

The electron multiplier detector is mainly used for ion currents less than 10-15 amperes.[69] The basic principle behind an electron multiplier is the formation of secondary electrons. When an electron or particle strikes the detector, it causes the atoms on the surface

layer to generate secondary electrons. The amount of secondary electrons generated depends on factors such as the particle's energy, velocity etc.[130] Electron multipliers feature a special surface material which aids in the formation of extra secondary electrons.[131]

The Faraday cup collector is another well-known detector. It consists of a metal conductive cup which is designed to catch ions in vacuum with suppressor electrode and subsequently producing current which can easily be measured to determine the amount of ions striking the metal cup.[132]The detection limit of the Faraday cup is limited by the quality of the amplifier and the thermal noise found in the resistor.[69] When compared to the electron multipliers, the Faraday cup collector is not as sensitive, however it does have a higher accuracy due to its correlation between the number of ions and the current measured.[69]

Conversion Dynodes are similar to the electron multiplier detector as they are based on the generation of secondary electrons for high mass ions and they reduce the mass discrimination of the detector.[69] A conversion dynode is a simple metal plate which is held at high voltages. Its potential serves to accelerate the ions to efficiently generate secondary particles.

1.3 Ion Pairing Reagents

Ion pairing regents have been widely used in analysis of ions by liquid chromatography-mass spectrometry (LC-MS). They have mainly been used to aid in the separation of ions, or to help with the ionization of molecules. Ion pairing reagents such as dimethylhexyl amine (DMHA), tetrabutylammonium acetate (TBA), or methane sulfonic acid (MSA) are used in liquid chromatography to help with the retention of analytes and the optimization of chromatographic separations.[133-138]

When ion pairing reagents are introduced in LC-MS systems they can be problematic as they contaminate the ion source of the mass spectrometer significantly causing ion suppression, and lowering the analyte sensitivity.[133,139] Efforts on improving the ion pairing methods with mass spectrometry are still ongoing and they include choosing pairing agents that

are very volatile, changing of the flow rate, column diameter and reducing the concentration of ion pairing reagents.[140-143]

In the study presented herein, a successful new detection method of anions is achieved by using cationic ion pairing reagents in the positive ion mode electrospray ionization mass spectrometry. A plethora of di-, tri-, and tetra-cationic ion pairing reagents have been synthesized and evaluated with large and diverse classes of small and large anionic compounds.

In electrospray ionization mass spectrometry, anions and cations can be detected either by negative or positive ion mode respectively. However, detection of anions in the negative ion mode has significant drawbacks. The negative ion mode is prone to corona discharge, which can cause significant arcing.[144] These phenomena can lead to poor spray stability, and higher background noise, therefore resulting in lower sensitivity for the analytes.[145] Halogenated or long chain alcohols type solvents have been known to be used to overcome these problems however they are often not compatible with HPLC which has become extremely useful when paired with mass spectrometry.[144]

To avoid the above concerns it would be ideal for researchers to be able to detect anions in the positive ion mode ESI-MS, which is a much more sensitive ion mode as it does not cause arcing, and it is very compatible with HPLC. To achieve this goal we have paired cationic ion pairing reagents with anions of interest, and as long as the newly formed complex has an overall positive charge, it can now be detected in the positive ion mode. Other advantages include removal of anions from the low mass cut off region, and therefore an overall higher sensitivity can be attanied.

Anion analysis by mass spectrometry can be performed in the full scan, selective ion monitoring (SIM) and selective reaction monitoring (SRM). Since trace analysis is the focus of these studies, only SIM and SRM experiments are performed as small amounts of analyte cannot be detected in the full scan mode.

CHAPTER 2

THE EVALUATION AND COMPARISON OF TRIGONAL AND LINEAR TRICATIONIC ION-PAIRING REAGENTS FOR THE DETECTION OF ANIONS IN POSITIVE MODE ESI-MS

2.1 Abstract

A general and sensitive method for detecting divalent anions by ESI-MS and LC/ESI-MS as positive ions has been developed. The anions are paired with tricationic reagents to form positively charged complexes. In this study, four tricationic reagents, 2 trigonal and 2 linear, were used to study a wide variety of anions, such as disulfonates, dicarboxylates, and inorganic anions. The limits of detection for many of the anions studied were often improved by tandem mass spectrometry. Tricationic pairing agents can also be used with chromatography to enhance the detection of anions. The tricationic reagents were also used to detect monovalent anions by monitoring the doubly charged positive complex. The limits of detection for some of the divalent anions by this method are substantially lower than by other current analytical techniques.

2.2 Introduction

The analysis of anions is of great necessity and interest in many fields of science. Low levels of organic acids have been determined in a variety of samples such as food, environmental, and biological matrices.[14, 62, 147-153] Some dicarboxylic acids, such as glutaric, fumaric, and adipic acids are marker compounds for certain metabolic disorders and have been determined in urine samples.[154] Aromatic sulfonates are used in many industrial processes and consumer products, such as laundry detergents. Many of these sulfonates end up in wastewater and municipal water supplies and have been determined by various methods.[151, 155] Because of the ramifications of low levels of anions in the environment, fast and effective trace methods of analysis are very important.

Complex environmental sample matrices often require a separation technique to isolate the analyte. Common separation methods include ion chromatograph[153, 156-159], ion pair

chromatography,[151, 155, 160] reverse-phase mode chromatography,[161-163] and capillary electrophoresis (CE).[149, 150, 164]To enhance the spectroscopic detection of anions that do not contain a UV chromophore, some CE and high performance liquid chromatography (HPLC) methods utilize sample derivatization[165, 166] or indirect UV or fluorescence detection methods.[167-169] Ions have also been detected by ion selective electrodes and conductivity. Mass spectrometry (MS) provides universal detection for anions and is being used more and more, either alone[157] or paired with a separation technique.[148, 151, 162]

Electrospray ionization (ESI)-MS is a logical choice for ion detection because of the inherent charge state of the analyte. Negative mode ESI-MS is the most common way of detecting anions. Problematically, negative ion mode operation with standard chromatographic solvents, such as methanol and water, can lead to poorer spray stability, corona discharge, and arcing, which ultimately lead to poor detection limits.[144] Halogenated solvents[170,171, 172] or electron scavenging gases[173] can be used to suppress these effects.

Operating in positive mode ESI would help to avoid the stability problems of negative mode ESI-MS and the use of unconventional solvents. A method was developed to detect singly charged anions using positive mode ESI-MS by pairing the anion with a dicationic reagent to create a positively charged complex.[174, 158, 159, 175] There are multiple advantages to this method beyond the use of positive mode ESI-MS. One benefit of monitoring the anion/dication pair is moving the anion to a higher mass region where there is lower background noise. Additonally, anions of low mass are moved well above the low mass cutoff when quadrupole instruments, such as an ion trap, are used. Also, the pairing reagents may be used to differentiate between the analyte of interest and an interference of the same m/z.[174]

Most recently, tricationic reagents were paired with divalent anions, which again could be detected as a singly charged complex.[153, 176] The first group of tricationic reagents used as pairing agents were classified as trigonal trications[177]. These trications have fairly rigid structures and provided detection sensitivity enhancement for many of the anions tested. Past

results have indicated that rigid dicationic pairing agents did not work as well as more flexible dications[177], so a second class of tricationic reagents was developed. The second group of tricationic reagents is linear and more flexible.[178] The limit of detection (LOD) for some of the divalent anions tested was lower for the linear trications than the trigonal cations.[179] In the present study, the best two trigonal and two linear tricationic reagents from these previous studies will be used to determine detection sensitivity for a wide variety of divalent anions. LOD trends for a given tricationic reagent or class and analyte type (e.g. dicarboxylate, disulfonate) would aid in future method development. The use of tricationic reagents in MS-MS and possible dissociation mechanisms are discussed as well. Additionally, these tricationic reagents can be used for the detection of monovalent anions as a doubly charged complex, which has not been previously studied with tricationic reagents. This leads to the possibility of detecting both singly and doubly charged anions using a singular tricationic reagent.

2.3 Experimental

The water and methanol used in these experiments were of HPLC grade and obtained from Burdick and Jackson (Morristown, NJ). Reagent grade sodium hydroxide and sodium fluoride were from Fisher Scientific (Pittsburgh, PA). The anions listed in Tables 2.1 and 2.3 were purchased as either the sodium or potassium salt or in the acid form from Sigma-Aldrich, with the exception of butanedisulfonic acid and 1,5-naphthalenedisulfonic acid which were purchased from TCI America (Portland, OR). Stock solutions (1 mg/mL) were made weekly and diluted serially for analysis.

The tricationic reagents evaluated in this study, as shown in Figure 2.1, were synthesized according to previous reports.[176-180] Before analysis, each trication was anion exchanged to the fluoride form as previously reported.[176]

For direct injection analysis, a 40 μM trication-fluoride solution was pumped into a Y-type mixing tee at 100 μL/min using a Shimadzu LC-6A pump (Shimadzu, Columbia, MD). Also directed into the mixing tee was a 2:1 mixture of methanol: water at a flow rate of 300 μL/min

using the Surveyor MS pump (Thermo Fisher Scientific, San Jose, CA). This set up leads to an overall solvent composition of 50/50 water/methanol with 10 μM tricationic reagent and a total flow rate of 400 μL/min. The six-port injection valve on the mass spectrometer (5 μL loop) was used for sample introduction.

A Finnigan LXQ (Thermo Fisher Scientific) ESI-MS instrument was used for the analysis of anions in this study. The ESI-MS conditions used were: spray voltage 3kV; sheath gas flow, 37 arbitrary units (AU); auxiliary gas flow rate, 6 AU; capillary voltage, 11 V; capillary temperature, 350° C; tube lens voltage, 105 V. The trication-anion complex was monitored in SIM mode with a width of 5 m/z units. This range was chosen to include isotope peaks, and LOD determinations were made from extracted ion chromatograms of the cation-anion complex m/z.

For SRM experiments, the isolation width was 1-5 units with a normalized collision energy of 30 and an activation time of 30 ms. Data was analyzed using the Xcalibur and Tune Plus software. The limits of detection were determined when multiple injections of a given concentration resulted in a signal-to-noise ratio of three.

For the chromatography experiments, sample introduction was made by a Thermo Fisher Surveyor autosampler (5 μL injections). The stationary phase used was a Cyclobond 1 (25 cm x 2.1 mm) obtained from Advanced Separation Technology (Whippany, NJ). The flow rate was 300 μL/min, and the column was equilibrated with 100% methanol and a step gradient to 100% water was applied at 5 minutes. The tricationic reagent (40 μM) was added to the column effluent at 100 μL/min via the mixing tee. The mass spectrometer was operated in SIM mode, monitoring the mass of each di-anion/trication complex throughout the chromatographic run.

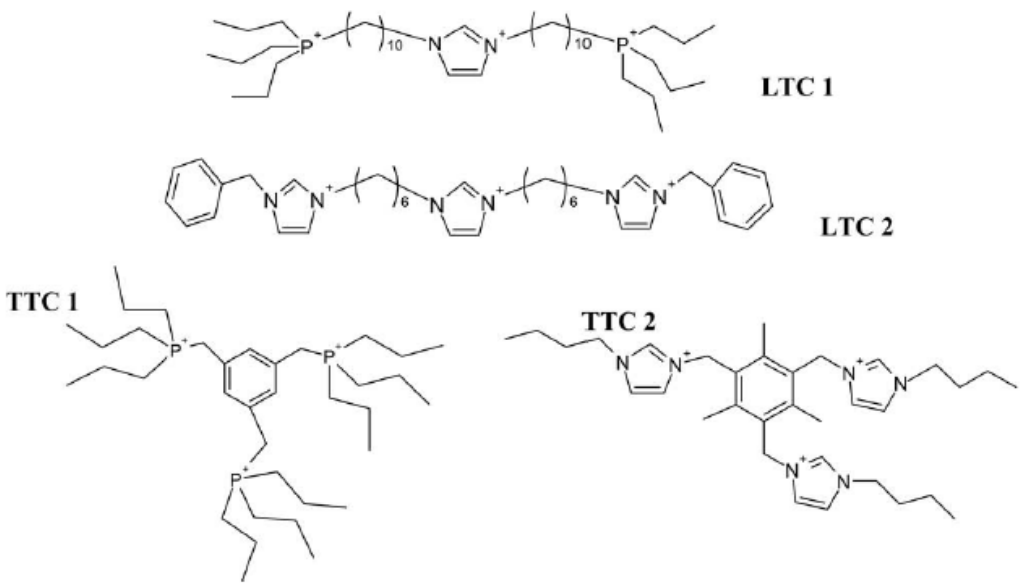


Figure 2.1 Structures of the tricationic ion-pairing reagents.

2.4 Results and Discussion

The tricationic reagents used in this study were chosen to represent the best performing trigonal and linear trications used in previous studies.[176, 179] These four tricationic reagents offer a variety of functional groups as well as differences in rigidity. The linear trications contain both an imidazolium core with different chain lengths and terminal charged groups. Linear trication 1 (LTC 1, Fig 2.1) has C_{10} linkages between the central imidazolium and tripropylphosphonium (TPP) terminal charged groups. Linear trication 2 (LTC 2, Fig 2.1) has benzylimidazolium terminal charge groups with a C_6 linkage chain. Trigonal trication 1 (TTC 1, Fig 2.1) has a benzene core with three TPP charged groups. Trigonal trication 2 (TTC 2, Fig 2.1) consists of a mesitylene core with three n-butylimidazolium groups in the 2,4,6 positions.

A variety of divalent anions were chosen to evaluate the ion-pairing performance of the tricationic reagents. The anions can be divided into categories based on their functional groups. The groups are: disulfonates, dicarboxylates, metal containing compounds, other sulfur containing compounds, and miscellaneous compounds. Within the disulfonate and

dicarboxylate categories, an effort was made to include compounds with varying chain lengths and functional groups to investigate any effect these might have on limits of detection.

Table 2.1 shows the 34 divalent anions used in this study and their limits of detection using each of the 4-tricationic reagents. They are arranged into the anion categories with the lower limits of detection at the top of each category. An examination of the LODs with the bold typeface, which indicate the lowest LOD for each anion, in Table 2.1 indicates that about 2/3 of the lowest LODs are for the linear tricationic reagents. Additionally, LTC 1 and TTC 1, which are the phosphonium containing reagents, (Fig. 2.1), account for 26 (of 34) of the lowest LODs. The exceptional overall performance of the TPP reagents for this set of divalent anions is in agreement with previous studies.[230]

Generally, the disulfonates have lower limits of detection than dicarboxylates. The lowest LODs for the disulfonates are for dihydroxynaphthalenedisulfonate and *m*-benzenedisulfonate using TTC 1. The disulfonates with aromatic groups (dihydroxynaphthalenedisulfonate, *m*-benzenedisulfonate, 4-formyl-*m*-benzenedisulfonate, anthraquinone-2,6-disulfonate) usually had lower LODs than the straight chain disulfonates. Methane, ethane, propane, and butane disulfonic acids were evaluated with each tricationic reagent. There does not appear to be a trend in the detection limit based on the increasing chain length for the disulfonic acids except when using TTC 1, where methane disulfonic acid had a higher LOD than for the longer chain disulfonates. For the disulfonate category as a whole, the trigonal trication reagents performed better than the linear ones.

Two of the other sulfur containing compounds, besides the disulfonates, also showed low LODs. In fact, the LOD for tetrathionate, using LTC 1, is the lowest of all the anions tested when operating in SIM mode (50 femtograms). Tetrathionate and peroxidisulfate were very near the lowest LODs for both LTC 1 and 2, but had LODs higher than most of the disulfonates for TTC 1 and 2.

Table 2.1 LODs for divalent anions using four tricationic pairing reagents in SIM.

	Linear trications		Trigonal trications	
	LTC 1 LOD (ng)	LTC 2 LOD (ng)	TTC 1 LOD (ng)	TTC 2 LOD (ng)
Disulfonates				
Dihydroxynaphthalenedisulfonate	7.50E-02	5.00E-02	**7.50E-03**	1.20E-02
m-Benzenedisulfonate	2.50E-02	5.00E-02	**8.75E-03**	1.00E-02
4-Formyl-m-benzenedisulfonate	1.25E-01	3.75E-02	**1.00E-02**	1.50E-02
Naphthalene-1,5-disulfonate	6.00E-02	**1.25E-02**	2.00E-02	3.00E-02
Butanedisulfonate	1.25E-01	5.00E-02	3.00E-02	**2.00E-02**
Propanedisulfonate	1.00E-01	**2.00E-01**	2.45E-02	7.50E-02
Anthraquinone-2,6-disulfonate	**2.50E-02**	5.00E-02	7.50E-02	5.00E-02
Methanedisulfonate	1.00E-01	**3.00E-02**	6.00E-02	**3.00E-02**
Ethanedisulfonate	**3.50E-02**	2.25E-01	3.60E-02	4.00E-02
Dicarboxylates				
Dipivolyltartarate	1.75E-02	**1.25E-02**	1.50E-02	1.50E-02
Camphorate	**6.00E-02**	1.50E-01	**6.00E-02**	5.00E-01
Phenylsuccinate	1.50E-01	7.50E-02	**5.00E-02**	1.00E-01
Glutarate	**7.00E-02**	2.00E-01	1.00E+00	5.00E-01
Malate	2.60E-01	**5.00E-02**	2.25E-01	5.00E-01
Methylsuccinate	2.00E-01	**7.50E-02**	2.50E-01	1.00E-01
Fumarate	**1.50E-01**	4.00E-01	1.50E+00	7.50E+00
Pimelate	**1.50E-01**	2.00E-01	2.50E+00	7.50E-01
Malonate	2.00E+00	1.38E+00	8.75E-01	**3.00E-01**
Adipate	**5.00E-01**	8.00E-01	2.25E+00	1.50E+00
Dibromomaleate	8.50E-01	1.00E+00	1.00E-01	**1.75E-01**
Chlorosuccinate	3.75E+00	1.88E+00	**2.25E-01**	9.00E-01
Metal containing compounds				
Hexachlororhenate $ReCl_6$	**1.50E-02**	3.00E-02	1.50E-01	2.00E-02
Chromate CrO_4	2.50E-01	7.50E-01	6.25E+00	**7.50E-02**
Molybdate MoO_4	**1.50E-01**	2.50E+00	3.75E-01	7.50E-01
Manganate MnO_4	1.00E+00	—	**3.75E-01**	8.75E-01
Arsenate AsO_4	**7.50E-01**	2.25E+00	2.50E+00	1.00E+00
Other sulfur compounds				
Tetrathionate S_4O_6	**5.00E-04**	2.25E-02	2.50E-02	5.00E-02
Peroxidisulfate S_2O_8	**1.20E-02**	1.65E-02	7.50E-02	2.00E-01
Succinaldehyde bisulfite	1.25E+01	2.50E+01	**1.25E+00**	5.00E+00
Glutaraldehyde bisulfite	3.50E+00	2.50E+00	**1.75E+00**	2.50E+00
Miscellaneous compounds				
Phenylphosphate	**4.00E-02**	1.00E-01	7.50E-02	5.00E-02
Rhodizonate	**1.05E-01**	5.00E-01	3.75E+00	3.75E-01
Hydrogen phosphite	**1.50E-01**	5.00E-01	3.75E-01	2.50E-01
Selenite	1.25E+00	—	**3.50E-01**	3.75E+00

*Limit of detection determined where the amount of analyte used results in S/N = 3.
Bold typeface indicates the lowest limit of detection for each anion.
— Indicates that a dianion/trication complex was not observed.

There appears to be excellent complexation for these sulfur-oxo compounds with the linear trications. Two other sulfur-containing compounds (i.e., the bisulfites) had nearly the highest LODs for all of the tricationic (Table 2.1).

Among the dicarboxylates studied, dipivaloyl-tartrate has the best LOD when pairing with all of the trications studied and for LTC 1 has a lower LOD than all of the disulfonates. For the tricationic reagents with benzene/mesitylene cores or charged groups (LTC 2, TTC 1, and

TTC 2), the dicarboxylates with non-halogen chain substitutions (dipivaloyl-tartrate, phenylsuccinate, methylsuccinate, and malate) have lower limits of detection than the straight chain dicarboxylates (Table 2.1). The halogenated dicarboxylates (chlorosuccinate and dibromomaleate) had lower LODs using the trigonal trications (Table 2.1).

For the straight chain dicarboxylates studied, glutarate, (C_5), had the lowest limit of detection, followed by pimelate, (C_7), and then adipate, (C_6). With LTC 1, the LOD for adipate is about 7 times higher than for glutarate, though they only differ by one carbon in chain length. For the dicarboxylate category in general, the linear trications outperformed the trigonal ones.

The inorganic compounds studied generally had higher LODs than the organic acids and disulfonates. $ReCl_6$ showed the best results of the inorganic compounds studied and had a limit of detection in the top five for LTC 1, LTC 2, and TTC 2. Two phosphorus-containing compounds were also studied. Phenyl phosphate had lower LODs than hydrogen phosphite. This result is in general agreement with earlier work that used dicationic reagents and singly charged anions, which found that more oxidized species had better LODs[174].

The additional application of the tricationic reagent to enhance detection for chromatography is shown in Figure 2.2. Three dianions (camphorate, phenylsuccinate, and naphthalene-1, 5-disulfonate) are separated using a β-cyclodextrin stationary phase. The trication is added post-column. The better peak shape for the late eluting naphthalene-1,5-disulfonate peak is likely due to the step gradient employed. The first two peaks are broadened before the mobile phase is changed, while the third peak is eluted by the strong solvent. Chromatographic retention and separation of dianions could be very useful in cases of complex sample matrixes.

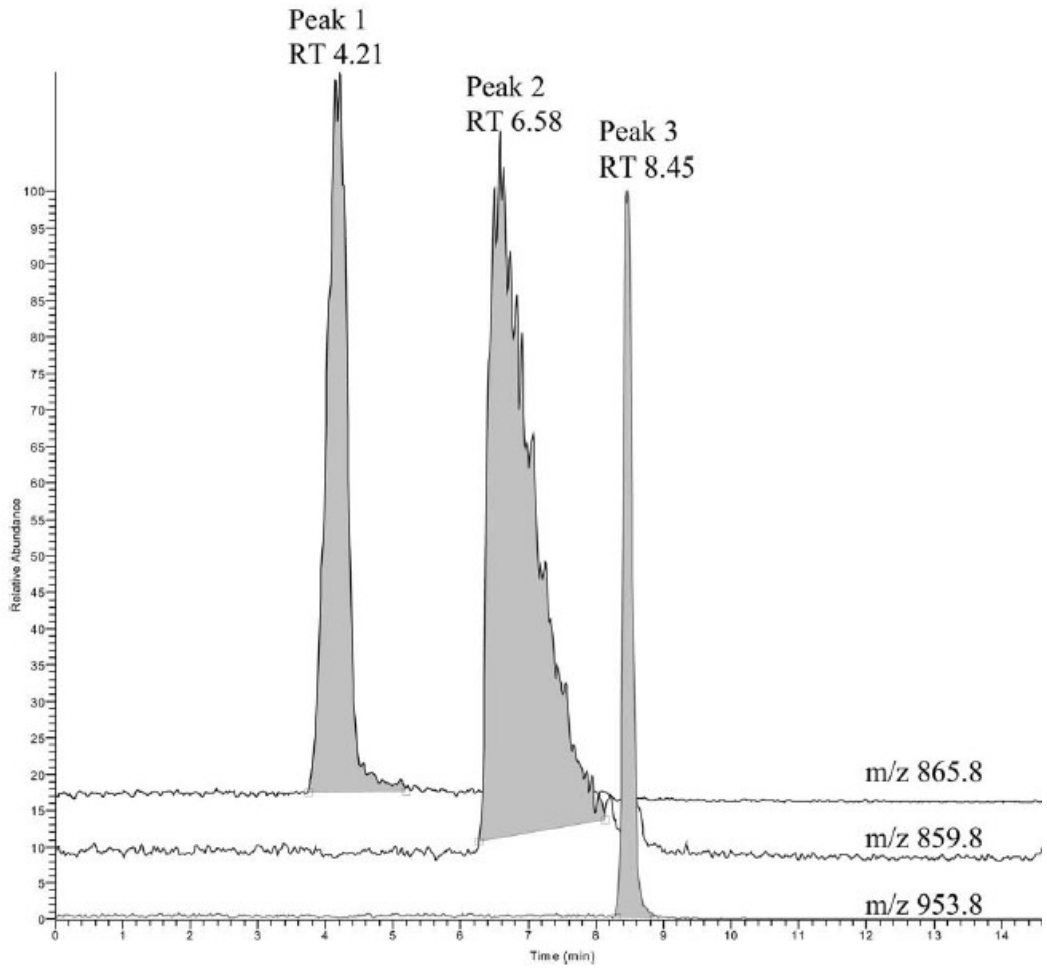


Figure 2.2 An extracted ion chromatogram using tricationic ion-paring agents.

LC separation of camphorate (peak 1), phenylsuccinate (peak 2), and naphthalene-1,5-disulfonate (peak 3) with the retention times (RT) also listed. This separation was performed on a β-cyclodextrin stationary phase (2.1 mm x 25 cm), which was equilibrated with 100% methanol. A step gradient to 100% water was applied at 5 min. The flow rate was 300 μL/min and 40 μM LTC 1 was introduced with a tee-piece to the effluent at a flow rate of 100 μL/min. The three trication–dianion complex masses were monitored simultaneously in SIM mode.

The limits of detection for most of the divalent anions could be reduced by using single-reaction monitoring (SRM). Some advantages of SRM are to improve specificity in analysis, to lower noise in the region being analyzed, and/or to eliminate interference by a background ion in the mass spectrometer. In SRM, the dianion-trication complex is trapped, excited, and the

transition to a resultant fragment is monitored. SRM analysis was peformed for each dianion and the results are shown in Table 2.2.

Table 2.2 LODs for divalent anions using four tricationic pairing reagent in SRM.

	Linear trications		Trigonal trications	
	LTC 1 LOD (ng)	LTC 2 LOD (ng)	TTC 1 LOD (ng)	TTC 2 LOD (ng)
Disulfonates				
Dihydroxynaphthalenedisulfonate	2.75E-03	5.00E-03	7.50E-03	**1.20E-03**
m-Benzenedisulfonate	**5.00E-04**	3.00E-03	6.25E-03	1.25E-03
4-Formyl-m-benzenedisulfonate	5.00E-03	1.00E-02	—	**1.50E-03**
Naphthalene-1,5-disulfonate	**4.61E-04**	4.38E+00	4.50E-03	3.60E-03
Butanedisulfonate	4.50E-03	6.25E-03	**3.50E-03**	**4.50E-03**
Propanedisulfonate	2.00E-02	1.25E-02	7.50E-03	4.50E-03
Anthraquinone-2,6-disulfonate	1.13E-03	**7.50E-04**	3.60E-03	7.90E-03
Methanedisulfonate	3.25E-03	3.15E-03	4.50E-02	**3.00E-03**
Ethanedisulfonate	**1.50E-03**	8.75E-03	1.44E-02	9.80E-03
Dicarboxylates				
Dipivolyltartarate	6.25E-03	**3.75E-03**	1.00E-02	5.50E-03
Camphorate	4.50E-02	4.50E-02	**3.00E-02**	2.00E-01
Phenylsuccinate	1.00E+00	7.50E-02	1.00E-01	**2.50E-02**
Glutarate	**3.75E-02**	6.00E-02	7.50E-01	1.50E-01
Malate	7.00E-02	**1.50E-02**	—	—
Methylsuccinate	**2.40E-02**	3.75E-02	1.05E-01	4.00E-02
Fumarate	**1.00E-02**	2.25E-02	1.50E+00	—
Pimelate	**3.00E-02**	7.50E-02	3.25E+00	7.50E-01
Malonate	**1.00E-01**	1.20E-01	3.00E-01	5.00E-01
Adipate	**1.20E-01**	2.25E-01	2.25E+00	1.50E+00
Dibromomaleate	7.50E-02	3.00E-02	3.50E-02	**2.50E-03**
Chlorosuccinate	1.50E+00	3.75E+00	**4.50E-01**	—
Metal containing compounds				
Hexachlororhenate $ReCl_6$	**2.00E-03**	3.00E-03	1.00E-02	2.00E-02
Chromate CrO_4	7.50E-02	2.25E-01	3.00E-01	**4.00E-02**
Molybdate MoO_4	**2.50E-02**	2.50E+00	5.00E-01	1.58E-01
Manganate MnO_4	3.75E-01	—	**1.25E-01**	7.50E-01
Arsenate AsO_4	**9.00E-02**	2.00E-01	5.75E-01	2.75E-01
Other sulfur compounds				
Tetrathionate S_4O_6	**1.00E-05**	4.00E-04	5.00E-04	5.00E-03
Peroxidisulfate S_2O_8	1.25E-03	**1.15E-03**	6.75E-03	6.00E-03
Succinaldehyde bisulfite	7.50E+00	1.50E+01	**1.50E+00**	5.50E+00
Glutaraldehyde bisulfite	**5.00E-02**	2.50E+00	8.75E-01	3.00E+00
Miscellaneous compounds				
Phenylphosphate	**5.00E-06**	1.00E-03	1.13E-02	1.50E-02
Rhodizonate	**1.05E-01**	5.00E-01	3.75E+00	1.25E-01
Hydrogen phosphite	**3.25E-02**	2.00E-01	1.00E+00	3.50E-02
Selenite	7.50E-02	—	**7.00E-02**	2.63E+00

*Limit of detection determined where the amount of analyte used results in S/N = 3.
Bold typeface indicates the lowest limit of detection for each anion.
— Indicates that a dianion/trication complex was not observed.

For LTC 1, most SRM transitions were to a fragment of the trication. Most of the dianion/trication complexes fragmented to either m/z 665.5 $[LTC1\text{-}2H]^{+1}$ or m/z 367.4 corresponding to the C_{10}TPPImidazole (shown in Fig. 2.3a). The inorganic anions, tetrathionate, peroxidisulfate, fumarate, phenylphosphate, and phenyl succinate did not fragment to m/z 665

or 367.4. For these -2 anions, a portion of the dianion was lost and the +1 complex between the trication and the remainder of the dianion was monitored. An example is tetrathionate where the complex fragment monitored (m/z 811.6) corresponds with the loss of SO_3. The most common fragments for LTC 2 were either the loss of 1 hydrogen each from 2 of the imidazolium rings (m/z 551.3) or the loss of the benzylimidazolium group (Fig 2.3b). For the complexes that lost the benzylimidazolium group, the dianion stayed complexed with the remainder of the trication. This unconventional fragmentation occurred with LTC 2 for the inorganic anions, peroxidisulfate, tetrathionate, rhodizonate, phenylphosphate, and dihydroxynaphthalenedisulfonic acid.

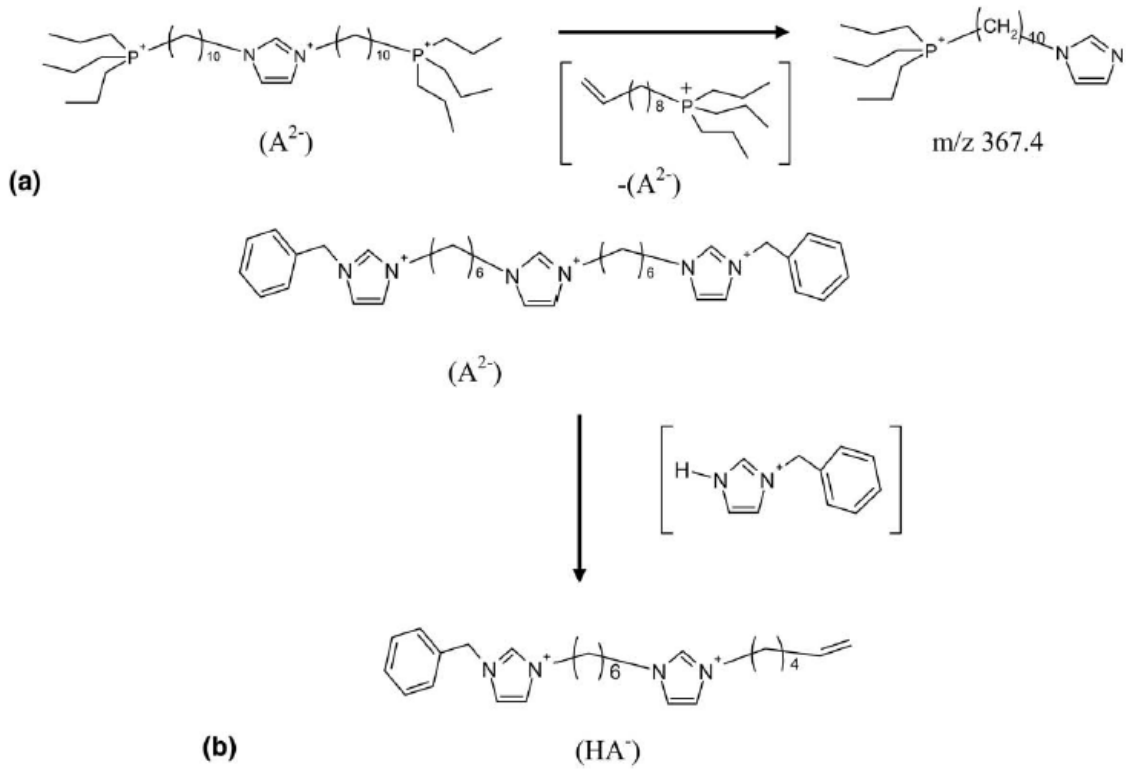


Figure 2.3 Proposed fragmentation pathways for the disulfonates using LTC 1.

The predominant fragment monitored for TTC 1 is the loss of two hydrogens from the methylene carbons between the phosphorus and benzene ring. Only manganate, peroxidisulfate, tetrathionate, hexachlororhenate, and chromate underwent alternate

fragmentation. The major fragmentation pathway for TTC 2 is the loss of the butylimidazolium group from the overall complex, so the dianion remains with the rest of the trication. Arsenate, peroxidisulfate, tetrathionate, rhodizonate, hexachlororhenate, glutaraldehyde bisulfate, dihydroxynaphthalenedisulfonic acid, adipate, and pimelate, succinaldehyde bisulfite, and camphorate followed alternate fragmentation patterns with TTC 2.

The group of compounds that had the largest improvements in LOD between SIM and SRM were the disulfonates. With one or more of the trications studied, each disulfonate had its LOD improved by at least an order of magnitude. The disulfonates were the only analytes to follow fragmentation for LTC 1 and LTC 2 as shown in Figure 2.3a and 2.3b, respectively. While the largest change in LOD was seen for the linear trications, the trigonal trications had the lowest LOD for 5 of the 9 disulfonates studied.

Chlorosuccinate and dibromomaleate also had interesting fragmentation patterns. In the case of these analytes, the halogen is lost from the anion and remains paired with the trication (or a portion of it). This was seen in our previous study on the linear trications[179]. Figure 2.4a illustrates a proposed fragmentation pattern for dibromomaleate using TTC 1. The distinct isotopic pattern for bromine (Fig. 2.4c) is evidence of the gas phase association of the bromine with a +2 fragment of the trication. The improvement in LOD between SIM and SRM was larger for the halogenated dicarboxylates using the trigonal trications.

Phenylphosphate showed an improvement of 2-3 orders of magnitude by SRM for both linear trications. The SRM LODs for the dicarboxylates ranged from just slightly better than SIM LODS to about 8 times better, with the exception of fumarate and malonate, which showed 18-fold (LTC 2) and 20-fold (LTC 1) improvements, respectively. Arsenate (LTC 2), hexachlororhenate (LTC 2, TTC 1), and glutaraldehyde bisulfite (LTC 1) were the only other analytes with improvements of an order of magnitude or more. In general, the linear trications had lower LODs for SRM than the trigonal cations.

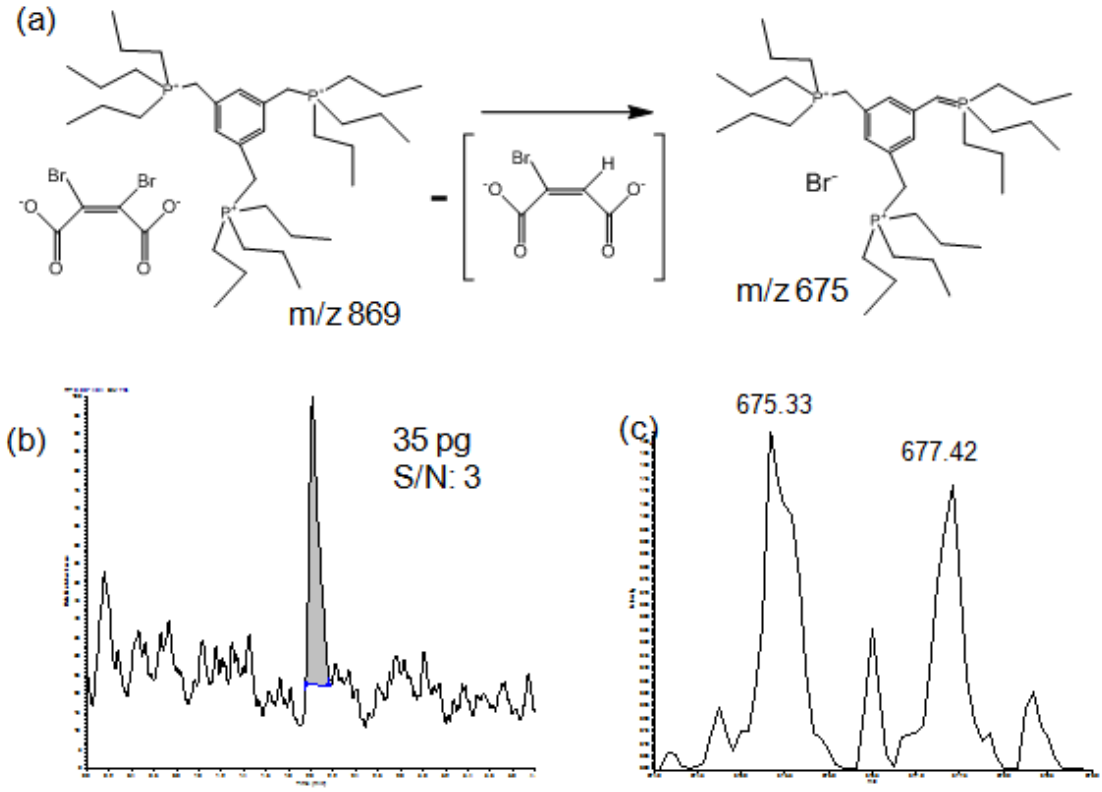


Figure 2.4 Proposed fragmentation pathway of the SRM transition for dibromomaleate.

Panel (**a**) shows the pathway. Panel (**b**) shows an injection monitoring the SRM transition from *m/z* 869à to 675.33 and 677.42. Panel (**c**) is the fragment spectrum observed for the peak shown in (**b**). The main peaks are two mass units apart and nearly the same height, indicative of Br.

The tricationic reagents can pair with doubly charged anions to form complexes with an overall +1 charge, but can also pair with singly charged anions to form +2 complexes. Five "mono-anions" were evaluated using the four tricationic reagents to determine their limits of detection. The data for SIM and SRM for these anions is shown in Table 2.3. The LOD for benzenedisulfonate both by SIM and SRM is the lowest for the five singly charged anions tested. In comparison to the SIM LOD for the dicationic reagents tested in a previous study[185] the LOD for benzenedisulfonate ranks second using LTC 1 as a pairing reagent. The LODs in this study for perfluoroctanate and monochloroacetate are better than 7-8 (of 23) of the

dicationic pairing reagents previously studied.[177] The ability of the tricationic reagents to pair with doubly and singly charged anions shows that the use of a single tricationic pairing reagent could be used to evaluate both monovalent and divalent anions simultaneously.

The LODs in this study compare favorably with those reported for anion analysis by other methods. There are many methods reported for the analysis of biologically releveant organic acids. In our study, the LODs for fumarate and methylsuccinate were 10 and 24 pg, respectively. Lower limits were determined, 0.9 pg fumarate and 0.5 pg methylsuccinate, by an LC method where the analytes were subjected to a long derivatization process to use fluorescence detection.[165].= CE analysis with indirect UV detection was used to determine levels of various organic acids. The LODs under the optimized CE conditions for malonic acid, methylsuccinic acid, glutaric acid, and adipic acid reported are 144 pg, 37.3 pg, 34.9 pg, and 72.2 pg respectively. Our SRM tricationic method showed lower LODs for the malonic and methylsuccinic acids (100 pg and 24 pg), similar results for the glutaric acid (37.5 pg), and higher results for adipic acid (120 pg).[63] A number of the analytes in that study had very similar migration times and without a more specific detection method, might be indistinguishable in that analysis.

Larger improvements over previous methods were seen with the disulfonates. An LOD of 200 pg for benzenedisulfonate by LC-UV was reported.[47] Using our method and TTC 1, the LOD for the same analyte is 8.75 pg using SIM detection and 500 fg using LTC 1 and SRM detection. Other aromatic sulfonates were determined in concentration ranges of 0.1-1 ng/ml by solid phase extraction-ion pair chromatography using UV detection[155] and 100-400 ng/ml by CE/MS.[151] The LOD for napthalene-1, 5-disulfonic acid was determined by ion interaction chromatography both by the direct injection of a large sample volume (100uL) and preconcentration (sample volume of 50 mL).[160] The LODs were 20 ng for the large sample volume and 30 ng for the sample preconcentration. Using the tricationic pairing method and no preconcentration, the LOD for this analyte is 12.5 pg in SIM mode and 461 fg in SRM.

The analysis of inorganic ions is also important, though not always as facile as the detection of organic acids or disulfonates. A coated-wire membrane sensor electrode was used to determine chromate levels in solution.[181] The LOD for this method was determined in a solution that was 116 ng/mL. In our analysis of chromate, the lowest solution concentration we analyzed was 8 ng/mL in SRM mode using TTC 2, for an absolute detection limit of 40 pg. Molybdate levels in various water samples were determined by coprecipitation and neutron activation analysis, a very labor intensive technique which can necessitate the use of a reactor.[182] The limit of detection for this method was 1 pg/mL using a 100 mL sample, for an absolute detection of 100 pg of molybdate. Using LTC 1, the LOD for molybdate in SRM is 25 pg. Another precipitation method was used to preconcentrate $ReCl_6$ followed by detection using selective excitation of probe ion luminescence.[234] In this study, 150 pg of $ReCl_6$ was needed to see an observable signal. In our study, $ReCl_6$ was determined well below 150 pg in both SIM (15 pg) and SRM (2 pg) monitoring modes.

Table 2.3 LODs in SIM and SRM modes for monovalent anions using four tricationic reagents.

	Linear trications		Trigonal trications	
SIM mode	LTC 1 LOD (ng)	LTC 2 LOD (ng)	TTC 1 LOD (ng)	TTC 2 LOD (ng)
Benzenesulfonate	**1.50E-03**	9.00E-03	1.50E-02	3.13E-03
Perfluorooctanoate	5.00E-02	2.75E-02	**1.50E-02**	5.00E-02
Trifluoromethanesulfonimide	**1.05E-02**	7.50E-02	3.00E-02	1.00E-01
Monochloroacetate	7.00E+00	1.25E+01	1.00E-01	**2.00E+00**
Benzoate	6.25E+01	8.75E+00	3.75E+00	**9.65E-02**
SRM mode	LOD (ng)	LOD (ng)	LOD (ng)	LOD (ng)
Benzenesulfonate	**9.50E-05**	2.70E-03	3.50E-03	1.38E-03
Perfluorooctanoate	**3.00E-04**	4.13E-03	3.00E-03	1.63E-03
Trifluoromethanesulfonimide	1.05E-02	6.00E-02	2.50E-02	**3.43E-04**
Monochloroacetate	—	1.00E+01	**1.00E-02**	7.50E-02
Benzoate	—	8.75E+00	3.75E-01	**9.65E-02**

*Limit of detection determined where the amount of analyte used results in S/N = 3.
Bold typeface indicates the lowest limit of detection for each anion.
— Indicates that a dianion/trication complex was not observed.

2.5 Conclusions

Four optimal tricationic pairing reagents were used to determine the limits of detection for 34 divalent anions and 5 monovalent anions. The linear and trigonal tricationic reagents performed about equally as a whole, but the two trications with tripropylphosphonium cationic moieties outperformed trications with imidazolium based charge groups. When evaluating tricationic reagents, our results show that the linear trications provide lower limits of detection for most classes of compounds and should be tested first. The exception to this is the determination of disulfonates, where trigonal trications generally perform better.

The use of tandem MS on the trication/di-anion complex helps to improve the sensitivity of detection for most of the dianions studied. Those complexes that dissociate into fragments not common to the trication showed the lowest limits of detection. Tricationic ion-pairing agents can also be used to determine monovalent anions by monitoring the +2 complexes. Therefore, mixtures of monovalent and divalent anions could be studied using a single tricationic reagent. Many of the LODs in this study are better or similar to those that have been previously reported, however this method is advantageous as it does not involve intricate sample preparation nor preconcentration and may be accessible to more laboratories.

CHAPTER 3

EVALUATION OF TETRACATIONIC SALTS AS GAS-PHASE ION-PAIRING AGENTS FOR THE DETECTION OF TRIVALENT ANIONS IN THE POSITIVE MODE ESI-MS

3.1 Abstract

In previous studies, new ESI-MS approaches were developed for the highly sensitive detection of singly and doubly charged anions in the positive mode ESI-MS by using specially synthesized dicationic and tricationic ion pairing agents respectively. By detecting the positively charged ion complex in the positive mode, LODs for the anions can be lowered by several magnitudes. In this work, we used eighteen newly synthesized tetracationic ion pairing agents, constructed with different geometries, linkages and cation moieties, for the detection of eighteen triply charged anions of different structural motifs. The LODs for these anions were from ten to several thousand times lower in the SIM positive mode than in the negative mode were. These tetracationic agents also were shown to be useful for the detections of -1 and -2 anions. In addition, the LODs for -3 anions can be further lowered by monitoring the daughter fragments of the ion pair complexes in the SRM mode.

3.2 Introduction

New methods of anion analysis are of continual interest provided such methods prove advantageous for analytes of importance in a variety of environmental, biochemical or medicinal applications. Several facile and sensitive methods to detect and quantify anions have been developed to accomplish this task. Currently, ion selective electrodes,[183, 184] conductivity,[185, 186] atomic spectroscopic techniques coupled with flow injection analysis (FIA),[187] and ion chromatography[188, 189] are widely used for the analysis of anion. However, none of these techniques are completely satisfactory because they are either not universal or lack the ability to provide structural information for complex ions.[185] ICP-MS (inductively coupled plasma mass spectrometry) is another common method that is known for its high sensitivity and low limits of detection (LOD). It is now widely used in medical, biological, and forensic fields[190-192].However,

ICP-MS is not applicable for all anions nor does it provide structural information for complex ions because they are destroyed before detection.

Electrospray ionization mass spectrometry (ESI-MS) provides an alternative approach for the analysis of anions and in particular complex ions can be detected in their native forms without decomposition. Coupled with separation methods, ESI-MS is capable of detecting most ionic species. However, as powerful as is ESI-MS in the positive mode, it can suffer from lower sensitivity in the negative mode.[144, 170] One cause for the decrease in sensitivity in the negative mode is the prevalence of corona discharge sometimes leading to arcing events.[173] This phenomenon results in an unstable Taylor cone and higher background noise leading to poorer LODs.[144]

Studies have shown that corona discharge in the negative mode can be suppressed by using halogenated solvents or alcohols with longer alkyl chains such as propanol, 2-propanol, and butanol.[172,173] However, more commonly used solvents such as water, methanol, and acetonitrile are still preferred especially when ESI-MS is coupled with reversed phase liquid chromatography (LC) or ion chromatography. Consequently, it would be highly beneficial to develop methods for sensitive anion detection by ESI-MS using typical LC operating conditions.

Recently, a new approach for anion detection in the positive mode has been developed[158,176,177,179]. This technique uses cationic ion pairing agents to form complexes with anions, which can in turn be detected in the positive mode. The first use of this method was to detect very low levels of perchlorate anions by allowing them to pair with a dicationic reagent in a carrier flow solvent to form a singly positively charged complex detected in the positive mode. This technique was then extended to the detection of a plethora of singly charged anions.[174, 177] This general approach to anion analysis was shown to have many advantages.

First, the LODs achieved with this method in the positive mode are much lower than those possible in the negative mode. Second, only small amounts of the ion pairing agents are needed for any analysis and it can be added pre-column or post-column when LC is employed.

Third, common solvents such as water, methanol and acetonitrile can be used. Finally, many anions which fall below the low-mass-cut-off (LMCO) of trapping MS can now be detected since the complexes are brought to a higher mass range by the pairing reagent. Indeed, moving the lower mass detection of any ion away from a region of higher chemical noise into a higher mass region of less noise (upon complexation with the pairing agent) is usually beneficial. For these reasons, detection of anion/cation complexes in the positive mode has proven to be much more sensitive than detection of the native anion in the negative mode. Also, operation in SRM (single reaction monitoring) mode can further lower LODs. Recently, dicationic ion pairing agents for the LC-ESI-MS of singly charged anion became available commercially.

Following upon the success of using dicationic ion pairing agents to improve the detection limit of singly charged anions, we extended the use of this technique to the detection of dianions through complexation with tricationic pairing agents. It has been determined that benzylimidazolium and tripropylphosphonium are the best cationic moieties and the reagents of flexible linear structure generally work better than rigid trigonal trications.[179] In this work, we move one step further in advancing this technique for anion detection.

Eighteen tetracationic reagents constructed with different cationic moieties connected by different linkages were synthesized. These reagents have been evaluated for their ability to complex 18 trivalent anions for detection in positive mode ESI-MS. SRM experimentation was performed in an attempt to further lower LODs. The best results were compared with the LODs obtained in the negative mode. The four best tricationic reagents identified in previous studies also were tested and compared.[179]

3.3 Experimental

3.3.1 Chemicals

Water and methanol were of HPLC grade and were obtained from Burdick and Jackson (Morristown, NJ, USA). Amberlite IRA-400 ion exchange resin, sodium hydroxide (reagent grade) and sodium fluoride (reagent grade) for the ion exchange were obtained from Sigma-Aldrich (St Louis, MO, USA). The anions listed in Table 3.1 were purchased as the sodium/potassium salt or in the acid form from Sigma-Aldrich (St Louis, MO, USA) and all were of reagent grade or better.

3.3.2 Ion-pairing agents

The structures of the tetracationic reagents are in Fig 3.1. The synthesis of the tetracationic reagents is briefly described by the following steps. Step 1: 1-(3-bromopropyl)-3-methyl-imidazolium bromide was first synthesized by reacting methyl imidazole with excess of 1,6-dibromopropane in DMF at 80°C overnight. It was purified by flash chromatography with 9:1 dichloromethane and methanol. Step 2: 1-(6-(imidazolyl)hexyl)- imidazole was synthesized by reacting excess of sodium imidazole with 1,6-dichlorohexane in DMF at room temperature for 12 hours. It was purified by flash chromatography with 20:1 dichloromethane and methanol. Step 3: Linear tetracation A1 was synthesized by reacting one equivalent of 1-(6-(imidazolyl)hexyl)- imidazole with two equivalent of 1-(3-bromopropyl)-3-methyl-imidazolium bromide in DMF at 80°C for 24 hours. It was purified by flash chromatography with 4:1 dichloromethane and methanol. All the other linear tetracation salts were synthesized in the same fashion.

The four tricationic reagents (Fig 3.2.) used in this analysis was the same as described in earlier studies.[176, 179] All reagents were synthesized in the bromide salt form and exchanged to its fluoride salt form prior to the analysis. The anion exchange was performed with a 10 mL syringe filled with 4 mL of ion exchange resin in the same manner as described in earlier papers.[176, 179]

Table 3.1 Structures of the trivalent anions studied.

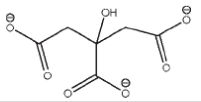
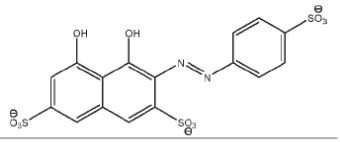
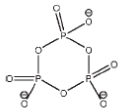
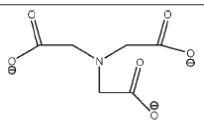
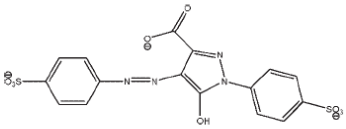
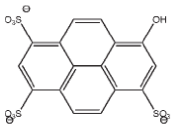
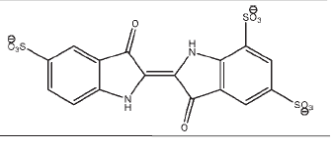
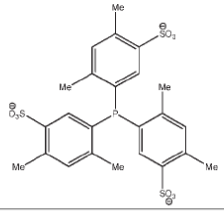
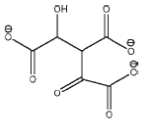
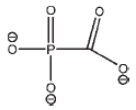
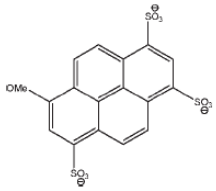
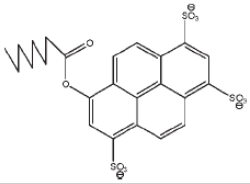
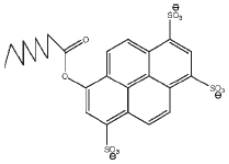
	Name	Mass	Structure
I	citrate	189.0	
II	sulfanilic acid azochromotrop	500.9	
III	trimetaphosphate	236.9	
IV	nitrilotriacetic tricarboxylate	188.0	
V	phosphate	95.0	PO_4^{3-}
VI	tartrazine	465.0	
VII	hexanitrocobaltate	334.9	$Co(NO_2)_6^{3-}$
VIII	pyranine	454.9	
IX	indigotrisulfonate	498.9	
X	hexachlororhodate	315.6	$RhCl_6^{3-}$
XI	tris(2,4-dimethyl-5-sulfophenyl)-phosphine	583.0	
XII	oxalomalic tricarboxylate	203.0	

Table 3.1 *Continued*

	Name	Mass	Structure
XIII	phosphoformate	123.0	
XIV	orthovanadate	114.9	VO_4^{3-}
XV	hexacyanocobaltate	215.0	$Co(CN)_6^{3-}$
XVI	8-methoxypyrene-1,3,6-trisulfonate	468.9	
XVII	8-octanoyloxypyrene-1,3,6-trisulfonate	581.0	
XVIII	8-nonanoyloxypyrene-1,3,6-trisulfonate	595.0	

3.3.3 ESI-MS analysis

A Finnigan LXQ (Thermo Fisher Scientific, San Jose, CA) ESI-MS was used for all of the analyses in this study. An ion pairing reagent aqueous solution (40 μM) was pumped at 100 μL/min using a Shimadzu LC-6A pump (Shimadzu, Columbia, MD) and mixed with 300 μL/min carrier flow (Water/MeOH=2/1, v/v) pumped by a Finnigan Surveyor MS pump. The positive mode ESI-MS conditions were as follows: spray voltage, 3 kV; sheath gas flow, 37 arbitrary units (AU); auxiliary gas flow rate, 6 AU; capillary voltage, 11 V; capillary temperature, 350°C; tube lens voltage, 105 V. When detecting the complex in the positive SIM (Selective Ion Monitoring) mode, the SIM width was set to 5 so as to include the isotope peaks.

For the detection in SRM mode, the isolation widths were between 1 and 5, the normalized collision energy was 30, and the activation time was 30 ms. Xcalibur and Tune Plus

software were used to analyze data. The initial concentrations of anion stock solutions were 1 mg/mL. Serial dilutions were made from the stock solutions, and 5 µl of the anions were directly injected using the six port injector. New stock solutions were prepared every week, and the major error source for this experiment was from the injector (±5%).

The limits of detection were determined to be when a series of five injections at a given concentration resulted in peaks giving a signal to noise ratio of 3. In order to prevent problems from the possible accumulation of the dilute cationic reagents, all the connecting tubing was rinsed with methanol/water 50/50 at 400 µl/min for two hours at the end of each day run. Also, the capillary transfer tube was manually washed with methanol/water 50/50 every week. With this protocol, no problem was ever observed to come from the cationic pairing reagent.

3.4 Results and Discussion

3.4.1 Tested anions

The structures of the 18 trivalent anions used in this study are listed in Table 3.1. Both inorganic and organic species are included. Many of the inorganic trivalent anions are metal complexes, such as hexanitrocobaltate, hexachlororhodate, and hexacyanocobaltate. There are two phosphorous based anions: trimetaphosphate and phosphate. Orthovanadate is a protein-phosphotyrosine phosphatase inhibitor.[193] The organic anions contained either carboxylic and/or sulfonate groups as the anionic moieties. Sodium citrate has three carboxylate groups and is a very common flavor additive in soft drinks. Sulfanilic acid azochromotrop, tartrazine, indigotrisulfonate, 8-methoxypyrene-1, 3,6-trisulfonate, 8-octanoyloxypyrene-1, 3,6-trisulfonate, pyranine and 8-nonanoyloxypyrene-1, 3,6-trisulfonate are dyes. Among them, tartrazine is a commonly used food pigment and also found to be associated with a variety of children's behavioral changes when ingested.[194]

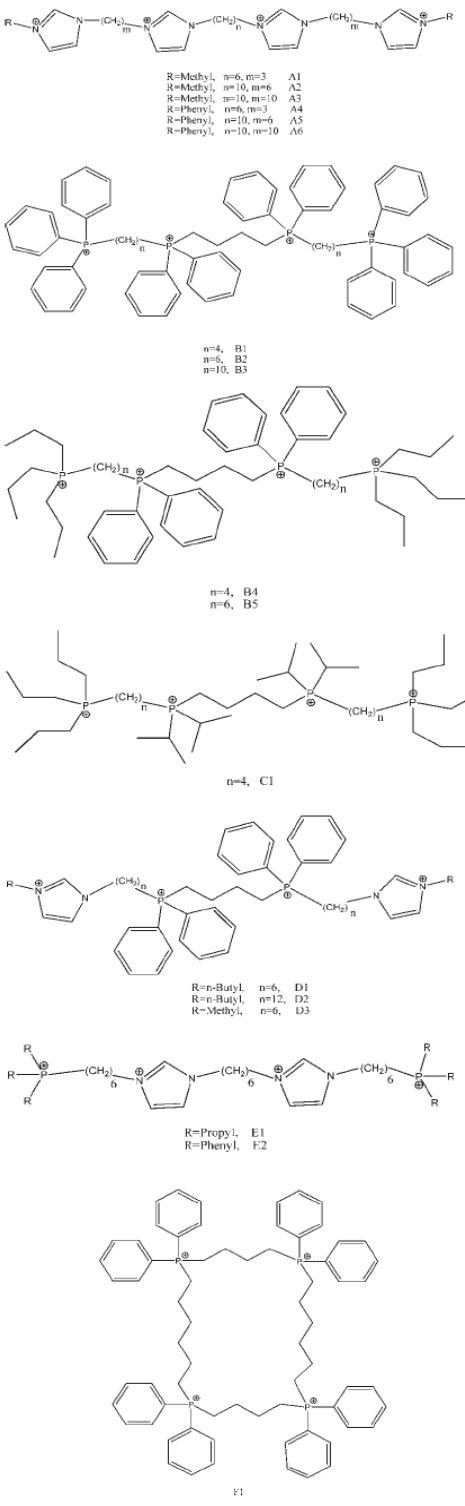
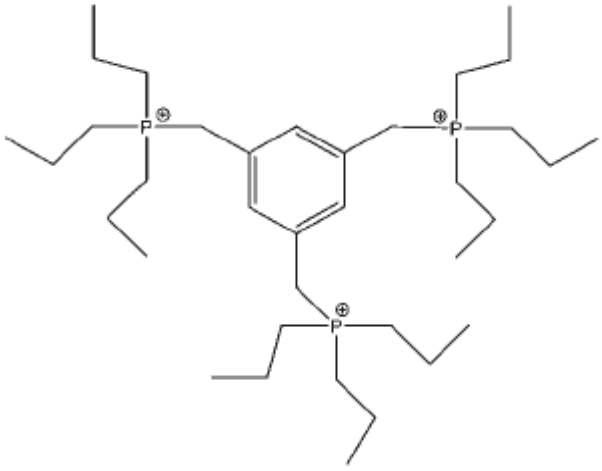
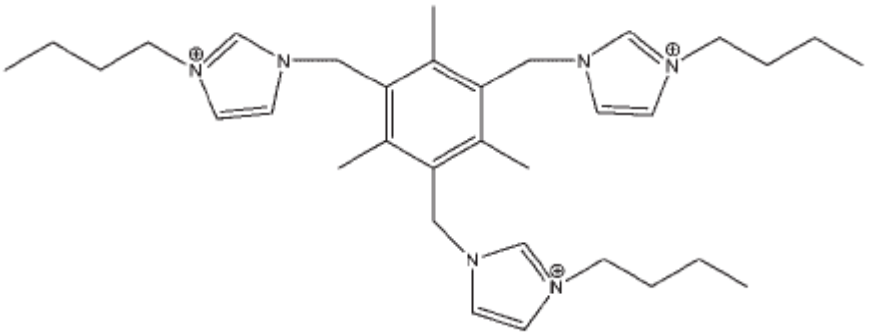


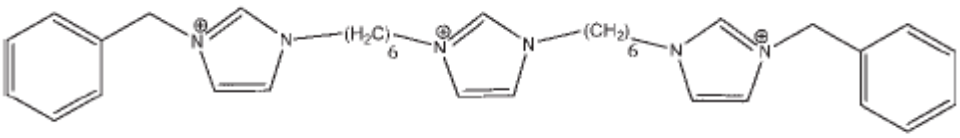
Figure 3.1 Structure of the tetracationic ion-pairing agents.



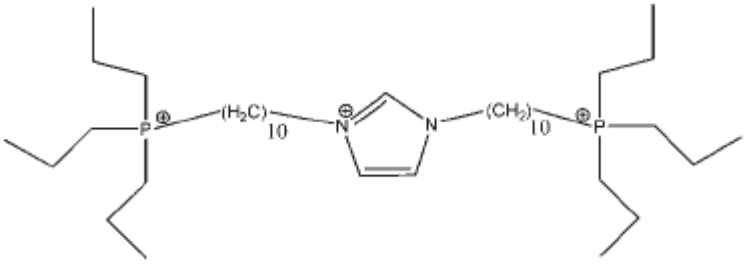
trication 1



trication 2



trication 3



trication 4

Figure 3.2 Structures of the tricationic ion-pairing agents.

3.4.2 Tetracationic ion-pairing reagents

The tetracations synthesized for this study are shown in Figure 3.1. Seventeen of them have the same general linear motif, while one is a cyclic tetracation, which is much more rigid. In previous studies, it was found that linear tricationic ion pairing reagents generally produced better results than trications with a more rigid trigonal geometry.[230] Although it remains interesting to compare the linear tetracations with at least one rigid tetracationic pairing agent in this study, attempts to synthesis more compact rigid tetracations failed due to the repulsion between closely placed cationic moieties. As tetracations have one more charged moiety and one more carbon linkage chain than the linear trications, more variations in the structures can be made, such as the length of the middle and side carbon chain linkages, as well as, the arrangement of different cationic moieties at the middle and end.

All these linear tetracations can be divided to three groups: pure imidazolium based, pure phosphonium based, and imidazolium and phosphonium mixed tetracations. They differ in the center carbon chain length (C_4, C_6, and C_{10}), side carbon chain length (C_3, C_6, and C_{10}), center cation moieties (imidazolium, diisopropyl phosphonium, diphenyl phosphonium), and terminal groups (methyl imidazolium, benzyl imidazolium, triphenylphosphonium and tripropylphosphonium).

3.4.3 LODs in the negative mode

The LODs for the trivalent anions in the negative mode were determined and listed in Table 3.2. It should be noted that most trivalent anions do not exist in their -3 charged state in aqueous solution. They can either be singly protonated to become a divalent anion or doubly protonated to a singly charged ion. Therefore, the LODs in the negative mode were determined from the base peak. For example, all the three charged states (-1, -2, -3) of trimetaphosphate can be seen in the negative mode, and the LOD was obtained by monitoring only the -3 peak as it had the best signal to noise ratio. Three anions (X, XIII, XV) are not detectable at a concentration of 10µg/ml (50ng) which was the highest concentration injected. Signal peaks of

anions XII, VI, and VII can be seen at concentrations of 10 µg/ml (50 ng) but the signal-to-noise-ratios were less than 3, therefore the LODs for these three anions were determined to be greater than 50 ng. The poor LODs are the result of the unstable spray conditions in the negative mode leading to the low ionization efficiency and the fact that some -3 anions (V, XIII) have mass to charge ratios which fall below the low-mass-cut off of the ion trap mass spectrometer. The LODs for the rest of the anions range from 250 pg (XI) to 20 ng (V). Anions containing sulfonate groups generally had lower LODs while metal-containing anions had relatively high LODs. The LODs for eight anions (II, VIII, IX, XI, XIV, XVI, XVI, XVIII) were determined based on their singly protonated form (-2), three anions (I, IV, V) were determined as doubly protonated (-1) species, and only one anion (III) from the unprotonated (-3) species.

Table 3.2 LODs for trivalent anions in the negative mode.

Anion	Mass	LOD (ng)	Base peak*
XI	583.0	0.250	291.9/−2
XVI	491.0	1.50	245.9/−2
XVIII	595.0	1.50	297.7/−2
VIII	454.9	2.50	227.9/−2
XVII	581.0	2.50	291.0/−2
II	500.9	5.00	251.0/−2
I	189.0	5.00	191.0/−1
III	236.9	5.00	79.0/−3
IV	188.0	5.00	190.0/−1
IX	498.9	10.0	249.9/−2
XIV	114.9	15.0	117.0/−2
V	95.0	20.0	97.0/−1
XII	203.0	>50	205.0/−1
VI	465.0	>50	155.0/−3
VII	334.9	>50	111.7/−3
X	314.7	>50	not detected
XIII	123.0	>50	not detected
XV	215.0	>50	not detected

* Base peak indicates the detected mass and charge state of the anion.

3.4.4 LODs in the positive mode (selected ion monitoring)

Table 3.3 lists the LODs for the 18 trivalent anions in the positive mode when complexing with the 18 tetracations. The detected complexes and mass to charge ratios are also listed. The best LODs for the trivalent anions ranged from 7.5 pg to 18 ng. As the anions may exist in three different charge states in solution, it is not surprising that each anion can form three possible complexes (+1, +2, +3) with tetracationic reagents. However, different tetracations pair preferentially with different anionic species. For example, for the detection of phosphate, tetracationic paring reagents C1 and B4 gave the best LODs (380 pg and 400 pg) when the +3 complex was used for detection. Conversely, with other reagents, +1 or +2 complexes gave better signal-to-noise ratios. For some anions, +1 complexes generally gave the best signal to noise ratio compared to +2 and +3 complexes, such as hexacyanocobalte, 8-octanoyloxypyrene-1, 3,6-trisulfonate, 8-nonanoyloxypyrene-1, 3,6-trisulfonate, tris(2,4-dimethyl-5-sulfophenyl)-phosphine and pyranine. Anions typically giving the best LODs for +2 charged complexes were phosphate and 8-methoxypyrene-1, 3,6-trisulfonate.

The prevalence of +2 complexes or +1 complexes can be related to the pKa of the conjugate acid of the trivalent anion. For example, as the pKa of HPO_4^{2-} is 12.76, thus the protonated dianionic form of phosphate will be most abundant in the aqueous solution. This could be the reason why the +2 complexes (tetracation plus HPO_4^{2-}) produce a stronger signal than the +1 complex (tetracation with PO_4^{3-}). However, it should be noted that not all of the complexes are detectable. For example, the complexes of A3, B3, B4, B5 and D2 with hexanitrocobaltate were not observed.

Among the anions studied, tris (2,4-dimethyl-5-sulfophenyl)-phosphine and hexacyanocobaltate gave the lowest LODs overall (7.5 pg with A2 or B5). Compared to the LODs in the negative mode, the greatest improvement was achieved with hexacyanocobaltate when pairing with A2 or B5, The sensitivity was more than 6600 times better in positive mode (7.5pg), as it was undetectable in the negative mode (at 50ng). In general, sensitivity

improvements in the positive mode were in the range of 10-1000 times those in the negative mode. Hexachlororhodate had the highest LOD (18 ng), which is still more than 10 times better than the negative mode (also undetectable). Except for hexacyanocobaltate, metal containing trivalent anions generally have higher LODs than other anions. On the other hand, sulfonate based anions typically had lower LODs than all the other anions. Although 8-octanoyloxypyrene-1, 3,6-trisulfonate and 8-nonanoyloxypyrene-1,3,6-trisulfonate have very similar structures, it is interesting to see that their LODs and best pairing reagents are different. This indicates that even a small change in structure can affect ion pairing. The difference may also be due to the different background noise at the mass to charge ratios of the complexes. For carboxylate based anions, oxalomalic tricarboxylate had the highest LOD (2.5 ng) and nitrilotriacetic tricarboxylate gave the lowest LOD (125 pg). Among the phosphate based anions, trimetaphosphate had the lowest LOD (37 pg) and phosphate (380pg) had the highest.

Among the 18 cationic paring agents tested, phosphonium based tetracations generally produced better results than pure imidazolium based tetracations in the SIM mode. Unsurprisingly, the imidazolium and phosphonium mixed tetracations showed moderate performance. For example, the best eight pairing agents for citrate are all phosphonium based, while the four worst reagents were imidazolium tetracations.

Also, it was found that pairing agents with more aromatic group substitutents worked better for many aromatic anions while alkyl substituted phosphonium agents paired better with alkyl group containing anions. For example, B1 and B4 worked generally better than C1 for anions with aromatic groups. This indicates that π-π interactions can be important for effective ion pairing agents. However, C1 worked better for nonaromatic anions than did B1 and B4. One possible reason is because C1 has relatively less steric bulk about its cationic moieties than do B1 and B4 resulting in stronger cation-anion interactions, as the ionic moieties are closer to one another.

Table 3.3 LODs for trivalent anions using tetracationic pairing agents in SIM mode.

I citrate			II sulfanilic acid azochromotrop			III trimetaphosphate		
cation	LOD (ng)	Base peak*	cation	LOD (ng)	Base peak	cation	LOD (ng)	Base peak
C1	0.150	456.4/+2	B1	0.0500	782.3/+2	C1	0.0368	959.6/+1
B1	0.300	626.5/+2	E2	0.0700	706.3/+2	B4	0.0438	1095.5/+1
B4	0.375	524.5/+2	B4	0.0875	680.3/+2	B1	0.0750	1299.4/+1
tri2	0.400	721.3/+1	D2	0.100	1512.0/+1	F1	0.0875	1230.1/+1
D1	0.500	516.5/+2	C1	0.125	612.4/+2	D1	0.125	1080.0/+1
D2	0.750	600.4/+2	B2	0.150	810.3/+2	B2	0.150	1355.3/+1
F1	1.00	591.3/+2	D1	0.175	1344.0/+1	D2	0.250	1248.0/+1
B5	1.25	552.3/+2	F1	0.200	747.6/+2	B5	0.315	1151.5/+1
D3	1.25	474.3/+2	A3	0.220	1219.7/+1	A6	0.625	1107.7/+1
E2	1.25	550.3/+2	E1	0.225	604.4/+2	A3	0.750	955.6/+1
tri4	1.50	857.8/+1	B3	0.250	1732.7/+1	D3	0.750	498.3/+2
A1	2.00	655.4/+1	D3	0.250	1259.6/+1	A1	1.00	703.3/+1
A3	2.00	907.7/+1	tri1	0.250	1099.5/+1	A4	1.00	855.3/+1
B2	2.00	654.5/+2	A4	0.275	560.2/+2	B3	1.00	1468.6/+1
B3	2.50	710.3/+2	A6	0.300	1371.6/+1	A2	1.25	843.4/+1
E1	2.50	448.4/+2	A1	0.375	1169.8/+1	tri2	1.50	769.3/+1
tri3	2.50	741.4/+1	A5	0.425	1259.7/+1	E1	2.50	472.4/+2
A6	3.00	1059.8/+1	B5	0.500	1415.8/+1	E2	2.50	575.5/+2
A2	3.50	398.3/+2	A2	0.575	1107.5/+1	tri3	2.50	789.4/+1
A4	3.75	404.3/+2	tri2	1.00	1033.3/+1	tri1	4.50	835.4/+1
tri1	5.00	787.3/+1	tri3	2.50	1053.4/+1	tri4	5.00	905.6/+1
A5	>50	No complex	tri4	3.75	1169.8/+1	A5	>50	No complex

IV nitrilotriacetic tricarboxylate			V phosphate			VI tartrazine		
cation	LOD (ng)	Base peak	cation	LOD (ng)	Base peak	cation	LOD (ng)	Base peak
C1	0.125	455.9/+2	C1	0.375	409.4/+2	E2	0.150	688.5/+2
tri2	0.300	720.3/+1	B4	0.400	477.3/+2	B1	0.175	764.3/+2
B4	0.350	524.0/+2	D1	0.560	469.3/+2	A2	0.310	536.3/+2
B1	0.500	625.8/+2	B1	2.00	386.6/+3	A5	0.360	612.4/+2
F1	0.600	590.8/+2	B5	2.00	505.3/+2	D2	0.375	1476.0/+1
B5	0.750	552.8/+2	D3	2.25	427.3/+2	A3	0.500	1183.6/+1
D3	0.750	473.8/+2	F1	2.50	544.3/+2	A4	0.500	542.2/+2
D1	0.875	515.8/+2	tri4	2.50	763.8/+2	tri1	0.500	1063.5/+1
E2	0.875	550.0/+2	D2	3.00	553.4/+2	B4	0.600	662.5/+2
A4	1.00	855.3/+1	E2	3.00	503.3/+2	A1	0.625	931.3/+1
D2	1.00	600.0/+2	A1	3.75	561.3/+1	A6	0.625	1335.8/+1
A1	1.50	654.3/+1	B3	5.00	442.7/+3	B2	0.750	1584.0/+1
A3	1.50	453.8/+2	tri2	5.00	627.3/+1	C1	0.750	1187.7/+1
tri1	1.50	786.3/+1	A2	6.00	351.3/+2	D1	0.750	1308.0/+1
tri4	1.50	856.8/+1	A3	6.00	407.4/+2	D3	0.750	1223.6/+1
tri3	1.75	740.3/+1	A4	6.00	357.2/+2	B3	1.00	1696.5/+1
A6	2.50	529.9/+2	tri1	25.0	693.4/+2	B5	1.00	1380.6/+1
B2	2.50	653.8/+2	E1	35.0	404.4/+2	E1	1.00	586.4/+2
B3	2.50	710.0/+2	A5	50.0	427.4/+2	F1	1.00	1458.2/+1
E1	2.50	447.9/+2	A6	50.0	483.4/+2	tri2	2.50	499.2/+2
A2	3.00	397.8/+2	B2	50.0	405.3/+3	tri3	2.50	590.2/+2
A5	5.00	473.5/+2	tri3	>50	No complex	tri4	5.00	1133.8/+1

VII hexanitrocobaltate			VIII pyranine			IX indigotrisulfonate		
cation	LOD (ng)	Base peak	cation	LOD (ng)	Base peak	cation	LOD (ng)	Base peak
D3	10.0	365.2/+3	B1	0.0750	1517.5/+1	B1	0.150	781.3/+2
E2	18.0	613.8/+2	D1	0.0750	1297.6/+1	D1	0.250	671.3/+1
B1	20.0	1397.5/+1	D2	0.125	1465.5/+1	D2	0.250	1511.0/+1
tri2	20.0	867.0/+2	tri1	0.125	1053.5/+1	A1	0.500	965.4/+1
tri3	25.0	434.5/+2	B4	0.150	1313.5/+1	A3	0.500	1217.7/+1
B2	37.5	1454.5/+1	D3	0.175	1213.4/+1	B2	0.500	809.3/+2
A5	50.0	1093.7/+1	B2	0.200	1574.5/+1	A6	0.625	1370.7/+1
A6	50.0	603.4/+2	B5	0.200	1369.7/+1	A4	0.700	559.7/+2
C1	50.0	1057.7/+1	A1	0.250	921.3/+1	E2	0.750	706.0/+2
D1	50.0	589.5/+2	A5	0.250	1236.2/+1	F1	0.750	1493.5/+1

Table 3.3 *Continued*

VII hexanitrocobaltate			VIII pyranine			IX indigotrisulfonate		
cation	LOD (ng)	Base peak	cation	LOD (ng)	Base peak	cation	LOD (ng)	Base peak
F1	50.0	1309.5/+1	C1	0.250	1177.6/+1	A2	1.00	1106.5/+1
A1	>50	401.2/+2	E2	0.250	1366.0/+1	A5	1.00	1258.7/+1
A2	>50	471.3/+2	A2	0.300	531.3/+2	D3	1.00	1258.6/+1
A3	>50	351.9/+3	B3	0.300	1686.5/+1	B4	1.13	680.0/+2
A4	>50	No complex	F1	0.375	1448.5/+1	tri1	1.50	1097.4/+1
B3	>50	No complex	A3	0.500	1173.7/+1	B3	2.50	1730.7/+1
B4	>50	No complex	A4	0.500	1073.4/+1	B5	2.50	1415.0/+1
B5	>50	No complex	A6	0.625	1325.6/+1	C1	2.50	611.5/+2
D2	>50	No complex	E1	0.625	1161.8/+1	E1	2.50	603.9/+1
E1	>50	No complex	tri2	2.50	987.4/+1	tri2	5.00	1031.4/+1
tri1	>50	No complex	tri3	3.50	1007.4/+1	tri3	20.0	1052.4/+1
tri4	>50	No complex	tri4	50.0	1153.8/+1	tri4	50.0	1168.8/+1

X hexachlororhodate			XI tris(2,4-dimethyl-5-sulfophenyl)-phosphine			XII oxalomalic tricarboxylate		
cation	LOD (ng)	Base peak	cation	LOD (ng)	Base peak	cation	LOD (ng)	Base peak
E2	18.0	613.8/+2	C1	0.00750	1305.8/+1	tri2	2.50	735.0/+1
tri3	25.0	434.5/+2	B4	0.0123	1441.7/+1	A1	5.00	669.2/+1
B1	30.0	688.7/+2	B5	0.0125	1498.8/+1	C1	15.0	925.5/+1
E1	30.0	341.5/+3	B2	0.0150	1701.8/+1	D1	15.0	925.5/+1
A6	42.5	395.9/+3	B1	0.0200	1646.5/+1	tri3	15.0	755.4/+1
A1	50.0	391.5/+2	B3	0.0250	1814.8/+1	F1	20.0	1196.2/+2
B2	50.0	1434.2/+1	F1	0.0250	1576.7/+1	B2	40.0	441.3/+3
C1	50.0	1038.7/+1	D1	0.0300	1425.7/+1	A4	50.0	821.4/+1
D1	50.0	579.8/+2	D2	0.0350	1594.0/+1	A5	50.0	481.3/+2
D3	50.0	537.6/+2	E2	0.0375	747.8/+1	B1	50.0	1266.0/+1
F1	50.0	1309.5/+1	D3	0.0500	1342.5/+1	B3	50.0	717.5/+2
A3	>50	1033.3/+1	A1	0.0750	1049.6/+1	B4	50.0	531.5/+2
B4	>50	1174.6/+1	A2	0.0750	1189.5/+1	B5	50.0	559.5/+3
B3	>50	1546.6/+1	A3	0.0750	1301.7/+1	D3	50.0	481.3/+2
A4	>50	467.5/+2	A4	0.125	1201.4/+1	E2	50.0	557.5/+2
D2	>50	663.8/+2	A6	0.125	1454.3/+1	E1	>50	909.9/+1
tri2	>50	847.9/+1	E1	0.150	645.4/+2	A2	>50	No complex
A2	>50	No complex	tri1	0.150	1181.6/+1	A3	>50	No complex
A5	>50	No complex	A5	0.175	1342.7 /+1	A6	>50	No complex
B5	>50	No complex	tri2	0.250	1115.4/+1	D2	>50	No complex
tri1	>50	No complex	tri3	0.500	1135.9/+1	tri1	>50	No complex
tri4	>50	No complex	tri4	1.50	1049.6/+1	tri4	>50	No complex

XIII phosphoformate			XV orthovanadate			XV hexacyanocobaltate		
cation	LOD (ng)	Base peak	cation	LOD (ng)	Base peak	cation	LOD (ng)	Base peak
C1	0.250	423.4/+2	F1	0.425	554.0/+2	A2	0.00750	821.5/+1
B4	0.300	491.7/+2	B1	0.500	589.3/+2	B5	0.00750	1329.5/+1
B5	0.500	519.3/+2	B2	1.00	1233.3/+1	D1	0.0125	1057.6/+1
tri2	0.750	655.2/+1	B4	1.25	487.5/+2	B1	0.0175	1278.3/+1
B2	1.00	621.3/+2	B5	2.50	515.6/+2	B4	0.0210	1073.6/+1
tri4	1.00	791.6/+2	C1	2.50	419.4/+2	C1	0.0250	938.0/+1
D1	1.25	966.0/+1	B3	3.00	673.7/+2	D3	0.0250	973.5/+1
B1	1.50	593.3/+2	E2	5.00	1025.3/+1	F1	0.0375	1207.4/+1
D2	1.50	1133.6/+1	tri2	5.00	647.3/+1	A1	0.0500	681.3/+1
E2	2.00	517.5/+2	tri4	7.00	392.4/+2	A6	0.0500	1085.7/+1
A1	2.50	589.3/+1	A4	21.0	245.1/+3	B2	0.0500	1334.5/+1
B3	2.50	1354.0/+1	tri1	30.0	713.3/+1	D2	0.0500	1226.5/+1
tri3	3.00	675.4/+1	A6	50.0	329.3/+3	E1	0.0625	921.7/+1
D3	3.20	441.3/+2	D1	50.0	479.5/+2	A3	0.0750	933.5/+1
A4	4.00	371.2/+2	A1	>50	No complex	A4	0.0750	833.7/+1
F1	5.25	558.6/+2	A2	>50	No complex	E2	0.0750	1126.3/+1
A6	6.25	497.4/+2	A3	>50	No complex	B3	0.100	1445.5/+1
A3	10.0	841.7/+1	A5	>50	No complex	A5	0.125	973.6/+1
E1	10.0	415.4/+2	D2	>50	No complex	tri4	0.500	1159.8/+1
A5	10.5	441.3/+2	D3	>50	No complex	tri1	1.25	813.3/+1
tri1	25.0	721.3/+1	E1	>50	No complex	tri3	2.00	767.2/+1
A2	30.0	365.3/+2	tri3	>50	No complex	tri2	5.00	747.3/+1

Table 3.3 *Continued*

XVI 8-methoxypyrene-1,3,6-trisulfonate			XVII 8-octanoyloxypyrene-1,3,6-trisulfonate			XVIII 8-nonanoyloxypyrene-1,3,6-trisulfonate		
cation	LOD (ng)	Base peak	cation	LOD (ng)	Base peak	cation	LOD (ng)	Base peak
B5	0.0500	777.3/+2	B1	0.0500	1496.6/+1	B5	0.0350	821.5/+1
B3	0.0875	675.3/+2	B4	0.0875	1812.7/+1	D2	0.0500	1329.5/+1
B1	0.100	667.3/+2	D1	0.100	1644.5/+1	D3	0.0500	1057.6/+1
A4	0.185	625.3/+2	A5	0.185	1199.9/+1	A5	0.0750	1278.3/+1
B2	0.250	549.3/+2	A2	0.250	1700.7/+1	B1	0.0750	1073.6/+1
C1	0.250	703.3/+2	B5	0.250	1303.8/+1	B4	0.0750	938.0/+1
D1	0.250	701.5/+2	E2	0.250	1424.7/+1	A2	0.100	973.5/+1
E2	0.250	1023.4/+1	tri2	0.250	746.8/+1	D1	0.100	1207.4/+1
B4	0.275	625.2/+2	D3	0.275	1440.7/+1	F1	0.105	681.3/+1
D2	0.375	805.3/+2	B2	0.375	1592.5/+1	A1	0.125	1085.7/+1
D3	0.500	751.7/+2	D2	0.500	1339.6/+1	A6	0.125	1334.5/+1
A1	0.500	1089.5/+1	tri1	0.500	1047.5/+1	C1	0.125	1226.5/+1
A6	0.650	555.2/+2	A4	0.650	1452.3/+1	A3	0.150	921.7/+1
F1	0.750	607.4/+2	C1	0.750	1574.5/+1	B2	0.150	933.5/+1
E1	0.750	742.5/+2	F1	0.750	644.4/+2	E2	0.150	833.7/+1
A5	1.00	861.3/+2	B3	1.00	1342.7/+1	A4	0.250	1126.3/+1
A3	1.25	957.3/+1	A1	1.25	1299.7/+1	B3	0.250	1445.5/+1
A2	1.25	605.4/+2	A3	1.25	1188.0/+1	tri1	0.300	973.6/+1
tri1	1.25	681.4/+2	A6	1.25	1180.5/+1	E1	0.500	1159.8/+1
tri3	2.50	1197.8/+1	E1	2.50	1133.9/+1	tri2	2.50	813.3/+1
tri2	2.75	1133.9/+1	tri3	2.75	1113.5/+1	tri3	2.50	767.2/+1
tri4	5.00	1250.3/+1	tri4	5.00	1250.3/+1	tri4	5.00	747.3/+1

We selected B1, B4 and C1, which are all phosphonium based tetracations, as the ion pairing reagents that outperformed all others for the detection of trivalent anions. These are the first recommended cations to use for the detection of -3 anions. The cyclic phosphonium tetracation worked fairly well, but not as good as the best linear tetracations. This indicates that flexibility also may be an important feature for tetracationic pairing agents. This is analogous to what was found for trication ESI-MS pairing agents.[179]

In addition, since some of the analytes exist mainly in the -2 charge state in solution, it should be possible for them to form +1 complexes with trivalent cations. Consequently, we also used the four best tricationic agents previously found for the detection of -2 anions (Fig. 3.2). The results are also listed in Table 3.3. It is obvious that tetracationic reagents are typically superior to tricationic ones in detecting these anions (except for oxalomalic tricarboxylate, for which trication 2 worked best). In many cases, the tricationic reagents performed worst, thus these agents should not be among the first tested for the detection of -3 anion. Therefore, we

recommend the tetracationic ion pairing agents outlined in this study for detecting trivalent anions in ESI-MS.

Tetracationic pairing agents not only complex trivalent anions but also form cation-anion complexes when paired with singly charged and doubly charged anions. Therefore, we also tested two tetracations (B1 and C1) for the detection of four singly charged and four doubly charged anions. The results are shown in Table 3.4. For monoanions, the results (i.e. sensitivities) found when using tetracations B1 and C1 were not as good as those that found for the less charged pairing agents studied in earlier papers.[63] For example, the best LOD for perfluorooctanate obtained by using a dicationic agent was 0.12 pg while B1 and C1 only gave 150 pg and 90 pg LODs, respectively. For trifluoromethanesulfonimide, the LOD achieved by B1 (4.5 pg) was close to the best LOD achieved by a dicationic ion pairing agent (2.3 pg).[174] Interestingly, B1 and C1 performed well for the detection of dianions. For example, the LODs of *m*-benzenedisulfonate obtained by B1 (12.5 pg) and C1 (12.5 pg) are two times lower than the best LODs obtained with tricationic agents (32 pg).[177] Although further study on the complexation of tetracationic agents with mono- and dianions needs to be done, it has been shown that tetracations can be possibly used as universal ion pairing agents for detecting mono-, di-, and trivalent anions.

Table 3.4 LODs for -1 and -2 anions using tetracationic pairing agents.

		B1		C1	
Anions	Mass	LOD (pg)	Base peak	LOD (pg)	Base peak
perfluorooctanate (−1)	413.1	150	491.8/+3	90.0	424.0/+3
benzenesulfonate (−1)	157.2	15.0	406.6/+3	15.0	338.6/+3
monochloroacetate (−1)	93.5	1000	625.0/+2	2500	523.0/+2
trifluoromethanesulfonimide (−1)	280.0	4.50	447.5/+3	15.0	379.8/+3
hexachloroplatinate (−2)	407.9	25.0	735.2/+2	50.0	633.5/+2
m-benzenedisulfonate (−2)	236.2	12.5	649.4/+2	12.5	547.4/+2
fluorophosphate (−2)	98.0	500	387.3/+3	20.0	477.3/+2
nitroprusside (−2)	216.0	10.0	639.3/+2	25.0	537.6/+2

3.4.5 LODs in the positive mode (Single Reaction Monitoring)

It has been demonstrated in previous papers that using ion pairing agents in the SRM (single reaction monitoring) mode can further reduce the detection limits of anions.[63, 185,208,230] In the SRM mode, anion-cation complexes are first selected, and then disassociated into fragments. The LODs were obtained by monitoring the strongest fragment peak. In this study, we tested the three best tetracations (B1, B4 and C1) for the detection of trianions in the positive SRM mode. The SRM results are listed in Table 3.5. Typically 3 to 10 times better (lower) LODs were achieved.

However, the metal containing anions did not show immense improvements in the SRM mode. For example, the LOD of hexachlororhodate in the SRM mode (10ng) was only slightly better than the LOD in the SIM mode (18ng). The LODs of some anions were lowered to the 100 fg range, for example hexacyanocobalte, 8-methoxypyrene-1, 3,6-trisulfonate and tris(2,4-dimethyl-5-sulfophenyl)-phosphine. When compared to the LODs in the negative mode, the biggest improvements found by using the tetracationic agents are more than four orders of magnitude (as for hexacyanocobaltate).

3.5 Conclusions

Eighteen newly synthesized tetracationic ion pairing agents with diverse structures have been evaluated for the detection of trivalent anions in both positive SIM and SRM modes of ESI-MS. The best LODs obtained in the positive mode were compared with the LODs for the negative mode. Improvements from 10 to greater than 6600 times were found in the SIM positive mode. It has been determined that the phosphonium based reagents generally gave lower LODs than the imidazolium based tetracations.

The pairing agents overall geometry plays as an important role as the nature of the cationic moieties in its effectiveness. The three best tetravalent reagents were selected for SRM mode experiments. Furthermore, the utility of these tetracations was demonstrated by also using them to successfully complex mono- and dianions. The LODs of most anions were lower

in the SRM mode and up to four orders of magnitude of improvement was seen for the SRM mode as compared to the negative mode. Finally, it needs to be noted that fluorescence spectroscopy also would be highly sensitive for the pyranine type trivalent anions. However, ESI-MS maintains certain other distinct advantages in that it is universal (i.e., also does nonfluorescent samples), can analyze several ions simultaneously and provide structural information.

Table 3.5 LODs for trivalent anions in the SRM positive mode.

	Tetracation B1		
Anion	Precursor ion	Base fragment peak	LOD (ng)[b]
I	626.5/+2	530.3/+2	**0.0625**[a]
II	782.3/+2	773.3/+2	**0.0150**
III	1299.4/+1	1037.4/+1	0.0150
IV	625.8/+2	530.3/+2	0.150
V	386.3/+3	353.9/+3	50.0
VI	764.3/+2	742.3/+2	0.0188
VII	1397.5/+1	1185.3/+1	10.0
VIII	1518.5/+1	1255.4/+1	**0.0125**
IX	781.5/+2	819.2/+1	**0.500**
X	688.7/+2	652.3/+2	250
XI	1646.5/+1	1385.3/+1	0.00250
XII	1266.0/+1	1133.5/+1	75.0
XIII	593.3/+2	571.3/+2	0.50
XIV	589.3/+2	500.3/+2	0.0150
XV	1278.3/+1	503.3/+1	0.0125
XVI	777.3/+2	769.8/+2	**0.00113**
XVII	1644.0/+1	1381.5+1	**0.0125**
XVIII	1658.5/+1	1395.5+1	**0.0200**
	Tetracation B4		
Anion	Precursor ion	Base fragment peak	LOD (ng)
I	524.5/+2	428.3/+2	**0.0625**
II	782.3/+2	655.8/+2	**0.0150**
III	1095.5/+1	401.3/+1	0.0200
IV	524/+2	215.2/+1	0.0650
V	477.33/+2	428.3/+2	**0.250**
VI	662.5/+2	640.3/+2	**0.0150**
VII	N[a]	N	N
VIII	1313.5/+1	401.3/+1	0.125
IX	680.0/+2	241.1/+1	1.00
X	N	N	N
XI	1441.7/+1	1281.5/+1	0.0250
XII	531.5/+2	295.2/+1	**0.750**
XII	491.7/+2	469.3/+2	**0.0175**
XIV	487.5/+2	448.3/+2	**0.0125**
XV	1073.6/+1	401.3/+1	**0.00200**
XVI	675.3/+2	595.3/+2	0.0750
XVII	1440.7/+1	1279.6/+1	0.125
XVIII	1454.5/+1	1293.6/+1	0.200
	Tetracation C1		
Anion	Precursor ion	Base fragment peak	LOD (ng)
I	456.4/+2	425.4/+2	0.0250
II	612.4/+2	485.8/+2	0.0250
III	959.6/+1	917.6/+1	**0.00375**
IV	455.9/+2	215.3/+2	**0.0500**
V	409.4/+2	280.3/+2	**0.250**
VI	1187.0/+1	1143.8/+1	0.125
VII	1057.7/+1	845.6/+1	**5.00**
VIII	1177.6/+1	1135.6/+1	**0.0125**
IX	611.5/+2	590.3/+2	**0.500**
X	1037.8/+1	541.4/+2	**25.0**
XI	1305.8/+1	1263.8/+1	**0.000600**
XII	925.5/+1	792.6/+2	25.0
XIII	423.4/+2	397.3/+2	0.25
XIV	419.4/+2	621.4/+2	0.0188
XV	938.0/+1	333.3/+2	0.00220
XVI	607.4/+2	546.3/+2	0.00375
XVII	1303.8/+1	1261.7/+1	0.0250
XIII	1317.8/+1	1275.8/+1	0.0250

[a] Complex is not detected. [b] Numbers in bold indicate the best LODs.

CHAPTER 4

DETECTION OF NUCLEOTIDES IN POSITIVE MODE ELECTROSPRAY IONIZATION MASS SPECTROMETRY USING MULTIPLY CHARGED CATIONIC ION PAIRING REAGENTS

4.1 Abstract

Nucleotides are a class of molecules that play an essential role in biological systems. A new method has been developed in the detection of nucleotides. These molecules can exist as monomers or constituents of oligomers and polymers. As such they carry from one to several negative charges. In this study different cationic ion pairing reagents were used to complex with each of the twenty-eight nucleotide monomers and nucleotide containing compounds. By using this method, these discrete set of anions were able to be detected in the positive mode electrospray ionization mass spectrometry (ESI-MS), as positive charged complexes. Tandem mass spectrometry experiments were also completed on the ion pairing reagents that performed the best in the single ion monitoring (SIM) ion mode, and the sensitivity was lowered even further for most of the anions. Limits of detection (LODs) for compounds such as thymidine diphosphate were improved as much as 100 times compared to the SIM mode, and 750 times when compared to the negative mode. A few nucleotides did not show a significant increase in sensitivity when analyzed in the positive ion mode, but in general the new method developed here in resulted in a much greater sensitivity than traditional detection in the negative ion mode.

4.2 Introduction

Nucleotides are compounds that carry and store genetic information in all living systems.[195] These important molecules consist of three main components, a ribose sugar, a nitrogenous base and one or more phosphate groups. Nucleotides also play very important roles in different metabolic pathways. They are used as main energy sources, such as adenosine triphosphate (ATP) and guanosine triphosphate (GTP), and the single nucleotide units are present in many important biochemical compounds such as coenzyme A, flavin

adenine dinucleotide (FAD), nicotinamide adenine dinucleotide (NAD), and many others.[195,196] Since these molecules are so important in understanding many enzymatic pathways, it becomes of great importance to also detect them often at low levels and in complex biological matrices. In deoxyribonucleic acid **(**DNA) and ribonucleic acid **(**RNA) sequencing, when sample is limited, effective and sensitive methods for detecting all fragments are beneficial. In these cases methods that can be interfaced with a separation method such as liquid chromatography (LC) or capillary electrophoresis (CE) are particularly desirable. Thus, researchers have developed many useful techniques and methodologies for nucleotide detection.[197-201]

Since nucleotides and nucleotide analogs possess between one and three phosphate groups, they are negatively charged. Traditionally, these compounds have been detected by electrochemical detection, fluorescence and many times by ultraviolet (UV) absorbance as they all contain UV chromophores.[201-208] Other quantitative methods include mass spectrometry (MS) and other hyphenated systems coupled with it such as, capillary electrophoresis-MS, capillary electrophoresis-micro high performance liquid chromatography inductively coupled plasma-MS, matrix assisted laser desorption ionization time of flight-MS and many others.[201, 209-212]

However, most of these methods require specific steps prior to the analysis of the compounds. This includes the derivatization of analytes, solubilization of samples in certain matrixes or coating of capillaries with certain polymers (in the case of CE). Furthermore some of these methods require very close attention to the conditions of the experiments as slight changes that occur can significantly alter the quantification of the analytes.[21, 209, 212]

In this paper we use a simple method that was previously developed by our group, in which negatively charged analytes associate with cationic ion pairing reagents resulting in an overall positively charged complex.[174] This allows for the detection of negative charged analytes in the positive mode ESI-MS. In comparison to other anion detection techniques, including negative mode ESI-MS, this new methodology has many advantages, such as ease of use, compatibility with HPLC and CE experiments, and ultrahigh sensitivity.[174, 213] The overwhelming

success of this technique lead to the synthesis of additional task specific ion pairing reagents.[175-177, 179, 214-216] The main advantage of this method is the ability to avoid using the negative ion mode ESI-MS, with its drawbacks, which is typically the mode of choice for anion detection. One shortcoming is the formation of corona discharge and as a consequence the development of arcing in the negative mode. As a result the spray stability becomes poor and the background noise is much higher as the solvent molecules are ionized due to this electric discharge.[144, 145] Furthermore, solvent selection when operating in the negative mode is more critical and often requires the use of less common solvents such as longer chained alcohols or halogenated solvents. In contrast, sensitive detection in the positive mode can be attained by using more common protic solvents like water and methanol.[144]

In this work the selected nucleotides and nucleotide based compounds were analyzed in the SIM positive ion mode with several ion pairing reagents.[216] Since nucleotides can carry more than one negative charge, dicationic, tricationic, and tetracationic ion pairing reagents were tested with each analyte. Lastly, tandem MS experiments were performed on selected ion pairing reagents so that further improvements in sensitivity could be observed.

4.3 Experimental

The solvents used for the analysis were HPLC grade and were purchased from Honeywell Burdick and Jackson (Morristown, NJ). Each cationic reagent was synthesized in the bromide form and subsequently exchanged to the fluoride form by using an ion-exchange method developed by Hein et al.[174] The anions used in this experiment were purchased in the sodium, potassium or as the free acid form from Sigma-Aldrich (St. Louis MO). All were of reagent grade and were used with no further purification. Solutions of anions were prepared fresh daily. The concentration of each ion pairing reagent was 40 μM and the serial dilution started at a maximum concentration of 10 μg/mL for each analyte. Different parameters such as temperature flow rate and ion pairing reagent concentrations have been previously optimized[175].

The synthetic pathways of the cationic ion pairing reagents used in this paper are discussed in previous reports.[145, 176, 177, 179, 180, 214, 217]

The ion pairing reagent was introduced to the mass spectrometer from a Shimadzu LC-6A pump (Shimadzu, Columbia, MD) at a flow rate of 100 µL/min. The mass spectrometer used was a Finnigan LXQ (Thermo Fisher Scientific, San Jose, CA). The ESI-MS conditions were set as follows: capillary temperature of 350°C, spray voltage of 3 kV, capillary voltage of 11 kV, sheath gas flow at 37 arbitrary units (AU), and the auxiliary gas flow at 6 AU. The solvent mixture and the ion pairing reagent were introduced into a mixing tee before entering the mass spectrometer resulting in a 50/50 mixture of water/methanol and a total flow rate of 0.4 mL/min.

The samples were injected through a six port injector and a sample loop of 5 µL was used. Five replicates of each prepared dilution were injected into the mass spectrometer for analysis, and subsequent dilutions were prepared until a signal to noise ratio as low as three was reached. The mass spectrometer used in this study uses Xcalibur Tune Plus software and the limits of detection in this analysis were determined based on Genesis Peak Detection Algorithm. The samples were analyzed in the single ion monitoring (SIM) and single reaction monitoring (SRM) positive ion modes.

4.4 Results and Discussion

Twenty-eight different nucleotide and nucleotide based compounds were detected in the positive and negative ion mode ESI-MS. These compounds were chosen for comparison purposes based on their different structural elements. Table1 lists all of the compounds used, along with the abbreviations used throughout this study. The cationic ion pairing reagents used in this study (Figure 4.1) were chosen as the ones that performed best, giving the lowest limits of detection for anions in our previous studies.[175, 179, 214, 215] The selected linear dicationic reagents include phosphonium, pyrrolidinium, and imidazolium charged moieties. The abbreviations used to denote each pairing agent are given in Figure 1 as well.

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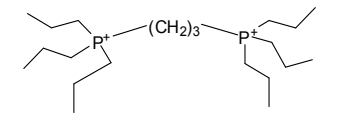
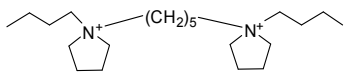
Table 4.1. Nucleotide compounds used in this study with their corresponding abbreviations.

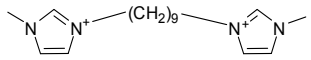
Anion	**Abbreviations**
Adenosine Monophosphate	AMP
Inosine Monophosphate	IMP
Uridine Monophosphate	UMP
Thymidine Monophosphate	TMP
Guanosine Monophosphate	GMP
Cytidine Monophosphate	CMP
Adenosine Monophosphoramidate	AMPP
Bromoadenosine Monophosphate	BAMP
cyclic Guanosine Monophosphate	cGMP
cyclic Adenosine Monophosphate	cAMP
cyclic Cytidine Monophosphate	cCMP
cyclic Thymidine Monophosphate	cTMP
Adenosine Diphosphate	ADP
Thymidine Diphosphate	TDP
Cytidine Diphosphate	CDP
P1P3-Diadenosine Triphosphate	P1P3-DATP
Adenosine Triphosphate	ATP
Cytidine Triphosphate	CTP
α-Nicotinamide Adenine Dinucleotide	α-NAD
β-Nicotinamide Adenine Dinucleotide	β-NAD
Flavin Adenine Dinucleotide	FAD
Fluoro-deoxyUridine Monophosphate	FdUMP
Deoxy-Guanylyl-Guanosine	DGG
Cytidyl-Uridine	CU
Guanylyl-Adenosine	GA
Thymidylyl-Thymidylyl deoxyCytidine	TTdC
S-Adenosyl-C-Homocysteine	SACH
Uridyl Uridine	UU



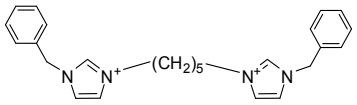
D 1



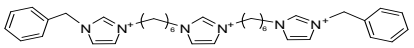
D 2



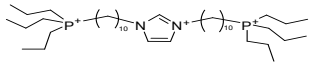
D 3



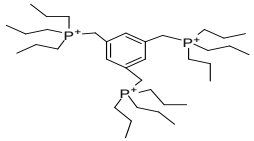
D 4



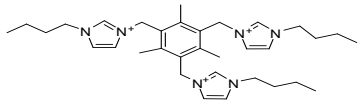
LTC 2



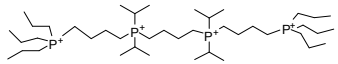
LTC 1



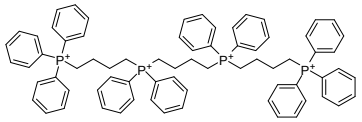
TTC 1



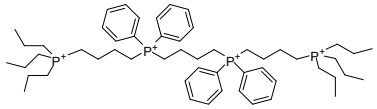
TTC 2



Tet 1



Tet 2



Tet 3

Figure 4.1 Structures of the different ion pairing reagents used in this study with their corresponding abbreviations.

The linear tricationic ion pairing reagents contain an imidazolium core with a variety of alkyl chain linkages and different terminal functional groups (LTC1, LTC2). The trigonal tricationic reagents have a more rigid configuration and contain a mesitylene core (TTC2), and a benzene core (TTC1). The linear tetracationic reagents selected are all alkyl or aryl phosphonium salts (Figure 4.1).

Each of the eleven ion pairing reagents were separately tested for their efficacy to complex with each of the twenty-eight nucleotide compounds. Table 4.2 lists the SIM limits of detection for these analytes. For each nucleotide sample, the limits of detection are listed with the corresponding cationic ion pairing reagent with the best sensitivity placed at the top of the list. The overall best cationic ion pairing reagent showing the lowest limit of detection (LOD) for most nucleotide compounds was the trigonal trication with a mesitylene moiety and three butylimidazolium groups (i.e., TTC2 in Figure 4.1). Other ion pairing reagents that performed well were Tet 2 and Tet 3, with Tet 2 producing slightly better sensitivities for these anions.

In previous studies it was shown that the linear cationic reagents were better candidates as complexing agents possibly due to their flexibility.[214] However, as the data in Table 2 indicates, one trigonal tricationic reagent (TTC2) outperformed all others, including the flexible linear ones. In contrast, one ion pairing reagent that performed poorly when paired with nucleotide analytes was LTC 1, a linear trication with an imidazolium core, C_{10} alkyl chains, and propyl phosphonium terminal groups; which previously was shown to be one of the superior ion pairing reagents for other -2 charged anions.[215]

As mentioned earlier, the cationic ion pairing reagents Tet2 and Tet3 (Figure. 4.1) also provided very good results by substantially lowering the limits of detection for these nucleotide anions as compared to the linear tricationic reagents and some of the dicationic ion pairing reagents. Both of these cationic reagents are aromatic substituted phosphonium salts. Thus π-π-ELECTRON-interactions may be an important structural feature in an ion pairing reagent that

produces low LODs. Of the four dicationic ion pairing reagents analyzed, D4 was the only one that showed low limits of detection for a few of the nucleotide analytes. The other dicationic reagents unfortunately did not perform well. Within its class D4 was the only ion pairing reagent that contained additional π-π interactions due to the two benzyl functional groups present at the terminal ends of the structure (Figure 4.1). Among the tricationic ion pairing reagents, the two trigonal tricationic ones, TTC1 and TTC2, performed significantly better than the linear reagents, LTC1 and LTC2 (Figure.4.1). In general, LTC2 ion pairing reagent performed better when compared to LTC1. In particular, LTC2 gave moderate results when complexed with nucleotide diphosphate analytes.

Since many of the nucleotides tested were multiply charged, it was the trication and tetracation pairing reagents that produced the lowest LODs for most of the analytes. It is important to note that for the nucleotide triphosphate type compounds, low LODs were more difficult to attain. For example, P_1P_3- diadenosine triphosphate, was able to be detected only by four of the ion pairing reagents out of eleven that were tested. A similar trend was also noticed with adenosine triphosphate and cytidine triphosphate. This could be due to the fact that the phosphate bonds can hydrolyze very quickly in aqueous solutions. Additionally, the increase in number of the phosphate groups increases the polarity of the compound, and as such complete desolvation of the ion becomes more difficult to achieve, therefore resulting in partial ionization.

Having multiple phosphate groups also increases the total number of potential charged species present in the mass spectrum as these groups can be protonated to a different degree. As a result, the background noise can increase resulting in higher limits of detection. The combination of these three possible phenomena could be the cause of achieving higher LODs for these particular nucleotides.

Another interesting observation was the significant difference in sensitivity obtained for two nucleotide isomers of nicotinamide adenine dinucleotide (NAD), α-NAD and β-NAD. Regardless of conditions, the limits of detection for β-NAD were lower than those for α-NAD

throughout the analysis. These compounds were purchased as a dipotassium salt and monosodium salt for β-NAD and α-NAD, respectively.

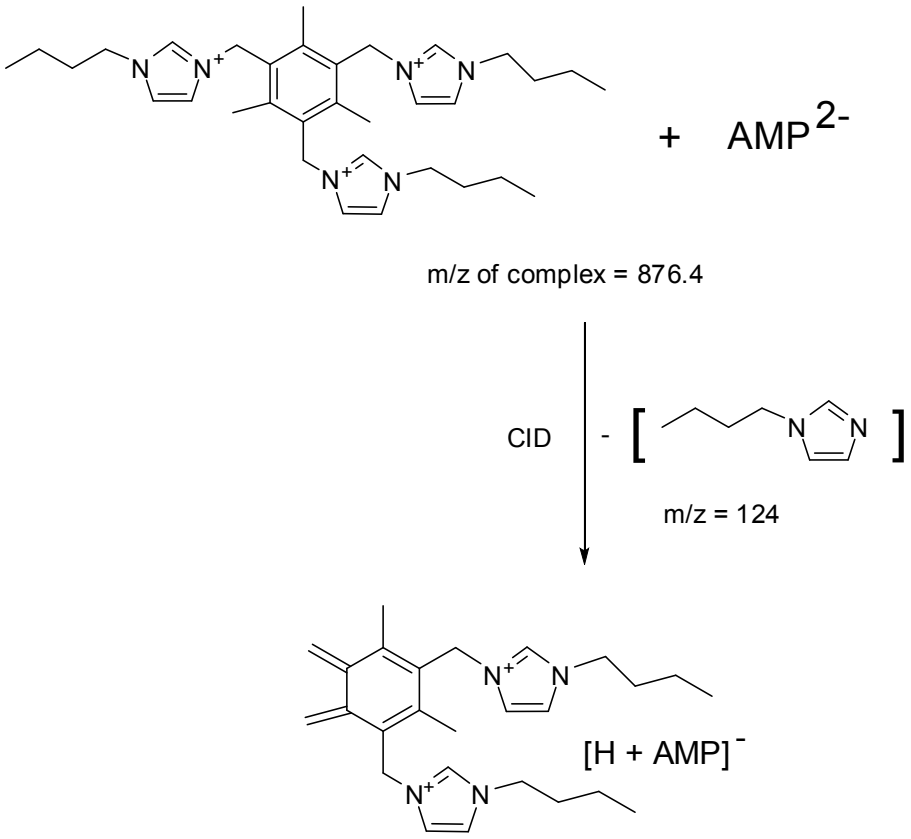


Figure 4.2. A possible mechanism pathway for the fragmentation of AMP/TTC2 complex in SRM experiments. Ion pairing reagents and analyte abbreviations can be found in Table 4.1 and Figure 4.1.

In particular, α-NAD showed the best sensitivity when associated with D1, D2 and D3 (Table 4.2). Since this compound carries one negative charge it was no surprise that the lowest limits of detection were achieved by using dicationic ion pairing reagents. In contrast to α-NAD,

β-NAD showed its worst limits of detection with dicationic reagents, D1, D2, and D3. The lowest limits of detection for β-NAD, were obtained when tricationic and tetracationic ion pairing reagents were used. This was an expected finding due to its inherent dianionic state. The sensitivity in this case improved significantly when using TTC2, Tet1 and Tet3 ion pairing reagents.

To investigate the interesting behavior of these two isomers, further experiments were performed. The α-NAD compound produced a lower pH value than β-NAD in aqueous solution, therefore the pH for α-NAD was increased to match the β-NAD sample, and conversely the pH for β-NAD was lowered to match the pH of α-NAD. Surprisingly, this change in pH did not produce any improvements in the limits of detection for this compound. Based on these results we could hypothesize that it is the geometry of the isomer that is affecting the sensitivity of this complex ion in the gas phase. Currently work is being undertaken to study this behavior and how these and other complexes bind and ionize in the gas phase environment compared to solution.

The three best ion pairing agents for SIM are TTC 2, Tet 2, Tet 3, followed by D 4 which performed quite well in the detection of few nucleotides. Based on these results, these four ion pairing reagents were further tested in the SRM mode (Table 4.3). Additionally, an ion pairing reagent that did not perform as well as the others, LTC 1, was added to the analysis by SRM in order to ascertain whether it was capable of improvements in lowering the sensitivity of the compounds. In the SRM mode, the parent ion monitored previously in the SIM mode, is isolated and energy is applied to fragment it. The daughter ions are monitored and because background noise is now lowered, sensitivity is increased.

Table 4.2. Limits of detection for each compound in the SIM ion mode. Final LOD were determined when a S/N ratio of 3 was achieved. All abbreviations are given in Figure 4.1 and Table 4.1.

AMP		IMP		UMP		TMP		GMP		CMP	
Mass inj.(ng)	IPR	Mass inj.(ng)	IPR	Mass inj.(ng)	IPR	Mass inj.(ng)	IPR	Mass inj.(ng)	IPR	Mass inj.(ng)	IPR
7.5E-02	Tet 2	1.5E-01	Tet 3	6.0E-02	TTC 2	1.0E-01	D 4	1.8E-01	TTC 2	8.0E-02	TTC 2
7.5E-02	Tet 3	1.9E-01	Tet 2	8.0E-02	Tet 3	1.0E-01	Tet 3	2.5E-01	Tet 2	1.0E-01	Tet 3
1.2E-01	D 4	1.9E-01	TTC 2	2.0E-01	D 4	1.0E-01	TTC 2	2.5E-01	D 4	1.4E-01	D 4
1.7E-01	Tet 1	2.3E01	Tet 1	2.5E-01	D 2	1.5E-01	Tet 2	3.2E-01	Tet 3	1.8E-01	Tet 2
1.8E-01	TTC 2	3.8E-01	LTC 2	2.5E-01	Tet 2	3.0E-01	TTC 1	7.5E-01	LTC 2	2.5E-01	D 2
2.5E-01	LTC 2	4.0E-01	D 4	3.5E-01	D 1	4.5E-01	Tet 1	1.0E+00	TTC 1	5.0E01	D 3
3.0E-01	D 2	4.5E-01	D 1	6.0E-01	TTC 1	5.0E-01	D 2	1.3E+00	Tet 1	5.0E-01	D 1
6.0E-01	TTC 1	6.3E-01	D 2	6.0E-01	D 3	7.5E-01	D 3	2.0E+00	D 3	6.0E-01	Tet 1
1.0E+00	D 1	1.5E+00	TTC 1	8.4E-01	Tet 1	1.0E+00	D 1	2.2E+00	D 1	7.5E-01	LTC 2
1.3E+00	LTC 1	2.0E+00	D 3	1.0E+00	LTC 2	1.3E+00	LTC 2	3.5E+00	LTC 1	9.7E-01	TTC 1
2.3E+00	D 3	2.3E+00	LTC 1	3.0E+00	LTC 1	3.5E+00	LTC 1	4.5E+00	D 2	2.5E+00	LTC 1

AMPP		BAMP		cGMP		cAMP		cCMP		cTMP	
Mass inj.(ng)	IPR	Mass inj.(ng)	IPR	Mass inj.(ng)	IPR	Mass inj.(ng)	IPR	Mass inj.(ng)	IPR	Mass inj.(ng)	IPR
1.7E-01	Tet 3	1.3E-01	TTC 2	3.0E-02	Tet 2	3.0E-02	Tet 2	3.0E-02	Tet 2	3.5E-02	Tet 2
1.8E-01	Tet 2	3.0E-01	D 4	1.0E-01	Tet 3	4.0E-02	D 1	5.0E-02	Tet 3	4.0E-02	D 4
1.8E-01	D 4	3.5E-01	TTC 1	1.0E-01	TTC 1	4.0E-02	D 2	6.0E-02	D 3	5.0E-02	Tet 1
2.2E-01	Tet 1	4.0E-01	D 1	1.1E-01	D 1	4.0E-02	D 4	7.0E-02	Tet 1	5.0E-02	Tet 3
2.5E-01	TTC 1	4.0E-01	LTC 2	1.3E-01	Tet 1	6.0E-02	TTC 1	1.0E-01	D 4	5.0E02	TTC 1
3.0E-01	D 1	5.0E-01	D 2	1.8E-01	D 4	1.2E-01	D 3	1.3E-01	TTC 1	7.5E-02	D 2
3.7E-01	D 2	8.7E-01	Tet 1	2.5E-01	TTC 2	1.5E-01	Tet 3	2.5E-01	TTC 2	1.5E-01	D 1
1.2E+00	LTC 1	1.0E+00	D 3	3.7E-01	LTC 2	1.5E-01	LTC 2	3.0E-01	D 2	1.8E-01	D 3
1.2E+00	LTC 2	2.5E+00	LTC 1	6.0E-01	D 2	1.6E-01	Tet 1	5.0E-01	D 1	2.5E-01	LTC 2
1.5E+00	D 3	5.0E+00	Tet 2	7.5E-01	LTC 1	5.0E-01	TTC 2	7.5E-01	LTC 2	3.0E-01	TTC 2
5.0E+00	TTC 2	7.5E+00	Tet 3	1.2E00	D 3	8.5E-01	LTC 1	1.0E+00	LTC 1	7.5E-01	LTC 1

ADP		TDP		CDP		P1P3-DATP		ATP		CTP	
Mass inj.(ng)	IPR	Mass inj.(ng)	IPR	Mass inj.(ng)	IPR	Mass inj.(ng)	IPR	Mass inj.(ng)	IPR	Mass inj.(ng)	IPR
3.0E-01	TTC 2	1.0E+00	Tet 2	1.3E+00	TTC 2	2.5E+00	Tet 3	0.8E-01	TTC 2	2.5E+00	TTC 2
1.0E+00	LTC 2	1.0E+00	Tet 3	1.8E+00	Tet 3	7.5E+00	Tet 2	1.5E+00	Tet 3	3.0E+00	Tet 3
1.2E+00	Tet 3	1.6E+00	LTC 2	2.5E+00	Tet 2	3.7E+01	D 1	5.0E+00	Tet 1	6.5E+00	Tet 1
2.5E+00	Tet 1	1.8E+00	TTC 2	3.7E+00	LTC 2	5.0E+01	Tet 1	7.5E+00	Tet 2	1.0E+01	Tet 2
3.7E+00	D 4	3.0E+00	D 4	5.0E+00	D 4	> 50	LTC 1	1.0E+01	LTC 2	1.0E+01	LTC 1
5.0E+00	Tet 2	4.5E+00	Tet 1	1.5E+01	TTC 1	> 50	D 2	2.5E+01	TTC 1	3.5E+01	TTC 1
7.0E+00	D 1	1.0E+01	LTC 1	3.0E+01	D 3	> 50	D 3	5.0E+01	D 1	5.0E+01	D 3
1.2E+01	TTC 1	1.7E+01	D 3	3.7E+01	D 1	> 50	D 4	>50	LTC 1	5.0E+01	D 1
1.5E+01	D 2	2.0E+01	TTC 1	5.0E+01	Tet 1	> 50	TTC 1	>50	D 2	>50	D 2
2.5E+01	D 3	3.0E+01	D 2	5.0E+01	LTC 1	> 50	TTC 2	>50	D 3	>50	D 4
5.0E+01	LTC 1	5.0E+01	D 1	5.0E+01	D 2	> 50	LTC 2	>50	D 4	>50	LTC 2

Table 4.2 *Continued*

a-NAD		b-NAD		FAD		F-dUMP		DGG	
Mass inj.(ng)	IPR	Mass inj.(ng)	IPR	Mass inj.(ng)	IPR	Mass inj.(ng)	IPR	Mass inj.(ng)	IPR
3.5E+00	D 3	2.5E-01	TTC 2	2.0E-02	D 1	2.0E-01	D 2	2.5E-01	Tet 3
4.3E+00	D 1	4.0E-01	Tet 3	2.0E-01	TTC 2	2.5E-01	D 4	4.2E-01	Tet 2
5.0E+00	D 2	5.0E-01	Tet 1	6.5E-01	Tet 3	3.0E-01	TTC 2	5.0E-01	TTC 1
7.5E+00	TTC 3	1.2E+00	TTC 1	1.2E+00	LTC 2	3.5E-01	Tet 2	1.0E+00	TTC 2
1.5E+01	Tet 3	1.7E+00	Tet 2	2.0E+00	Tet 1	4.0E-01	Tet 1	1.0E+00	D 4
1.5E+01	TTC 2	3.0E+00	LTC 1	2.5E+00	Tet 2	4.0E-01	TTC 1	2.5E+00	LTC 2
1.7E+01	Tet 1	3.7E+00	LTC 2	3.0E+00	TTC 1	5.0E-01	Tet 3	4.0E+00	D 1
2.5E+01	LTC 2	1.0E+01	D 3	5.0E+00	LTC 1	7.5E-01	D 3	4.0E+00	D 3
4.0E+01	Tet 2	1.5E+01	D 1	5.0E+00	D 4	1.2E+00	LTC 1	5.0E+00	D 2
5.0E+01	LTC 1	2.0E+01	D 2	> 50	D 2	1.5E+00	LTC 2	2.5E+01	LTC 1
>50	D 4	5.0E+01	D 4	> 50	D 3	N/A	D 1	5.0E+01	Tet 1

CU		GA		TTDC		SACH		UU	
Mass inj.(ng)	IPR	Mass inj.(ng)	IPR	Mass inj.(ng)	IPR	Mass inj.(ng)	IPR	Mass inj.(ng)	IPR
1.1E-01	TTC 2	2.0E-01	Tet 2	3.0E-01	TTC 2	2.4E-01	Tet 1	2.5E-01	TTC 2
3.0E-01	Tet 2	2.5E-01	Tet 1	4.0E-01	Tet 1	3.0E-01	Tet 3	3.5E-01	TTC 1
5.0E-01	D 4	3.0E-01	Tet 3	5.0E-01	Tet 2	5.0E-01	TTC 2	6.0E-01	Tet 1
6.0E-01	LTC 1	3.0E-01	TTC 1	6.0E-01	Tet 3	5.0E-01	Tet 2	9.0E-01	D 4
6.0E-01	Tet 1	9.0E-01	TTC 2	9.0E-01	TTC 1	6.0E-01	D 2	1.0E+00	Tet 2
1.0E+00	TTC 1	1.0E+00	D 4	1.5E+00	LTC 2	2.5E+00	D 4	1.2E+00	D 3
1.0E+00	D 3	1.2E+00	LTC 2	1.5E+00	LTC 1	2.5E+00	LTC 2	1.5E+00	LTC 2
1.5E+00	Tet 3	1.5E+00	D 3	6.0E+00	D 4	3.5E+00	LTC 1	2.0E+00	Tet 3
3.7E+00	LTC 2	1.5E+00	LTC 1	5.0E+01	D 1	5.0E+00	TTC 1	1.0E+01	LTC 1
3.9E+00	D 2	> 50	D 1	5.0E+01	D 2	5.0E+01	D 3	1.5E+01	D 2
5.0E+00	D 1	> 50	D 2	5.0E+01	D 3	>50	D 1	5.0E+01	D 1

The sensitivity of the compounds in the SRM mode was further lowered mainly by Tet 3 and TTC 2 followed by Tet 2 ion pairing reagents. LODs for all but one compound, flavin adenine dinucleotide, improved in the SRM mode. Compounds such as TDP and α-NAD improved their sensitivity by 100 times compared to the SIM mode. Unfortunately, LTC 1 was not one of the pairing reagents that gave the lowest sensitivities both in SIM and SRM experiments. However, compared to the results in SIM mode, this tricationic reagent showed the greatest improvement in sensitivity for most of the compounds in the SRM mode when compared to other ion pairing reagents. For example, the limits of detection for thymidine

diphosphate were lowered 100 times in the SRM mode when compared to the SIM mode, and an overall improvement by 750 times when compared to the negative mode (Table 4.4).

During this study, +1 and +2 fragments were monitored from the fragmentation of different anion-cationic ion pairing reagent complexes. The fragmentation patterns for all anion-ion pairing reagent complexes were unique for the different ion pairing reagents. For example, when the linear trication LTC 1 complexed with some analytes, the resulting fragmentation pattern resulted in a doubly charged ion that corresponded to the ion pairing reagent dissociated from the anion with the additional loss of one proton (i.e. a 2+ fragment with a resulting mass to charge ratio of 333.4) For twenty four out of twenty eight analytes that were analyzed using TTC 2, the fragment monitored in SRM was identical.

Figure 4.2 shows a proposed mechanism pathway for the fragmentation of this anion/ion pairing reagent complex when undergoing gas phase dissociation. The fragment monitored was the result of the collision-induced dissociation (CID) of this trigonal cationic ion reagent in the SRM experiment. These fragments represented a complex consisting of the pairing reagent associated to the anion, with the loss of a neutral butyl imidazolium side group, which equates to a mass loss of 124. The similar fragmentation pattern seen in this analysis shows again the stability of this trigonal trication ion pairing reagent once it complexes with these nucleotide compounds. The anion in this case remains associated with the ion pairing reagent when undergoing collision induced dissociation (CID). Conversely, in the LTC1 experiments, the ion pairing reagent completely dissociates from the anion.

It was also noticed that in the SRM experiments the cyclic nucleotides containing purine nitrogenous bases (cAMP and cGMP) responded similarly to the same ion pairing reagents throughout the analysis. For example, Tet 2 that showed the lowest limits of detection for both compounds, while TTC 2 and D 4 showed the second and third best SRM results respectively (Table 4.3).

Table 4.3. Limits of detection for the SRM experiments. All abbreviations are given in Figure 4.1 and Table 4.1.

AMP		IMP		UMP		TMP		GMP		CMP	
Mass Inj (ng)	IPR	Mass Inj (ng)	IPR	Mass Inj (ng)	IPR	Mass Inj (ng)	IPR	Mass Inj (ng)	IPR	Mass Inj (ng)	IPR
7.5E-03	D 4	1.0E-02	TTC 2	2.5E-03	TTC 2	1.5E-02	Tet 3	2.5E-02	TTC 2	5.0E-02	TTC 2
1.2E-02	TTC 2	3.0E-02	Tet 2	2.0E-02	Tet 3	2.5E-02	Tet 2	6.5E-02	LTC 1	5.0E-02	Tet 3
4.5E-02	Tet 2	1.3E-01	LTC 1	7.5E-02	Tet 2	5.0E-02	TTC 2	7.5E-02	Tet 2	9.0E-02	Tet 2
5.0E-02	LTC 1	1.7E-01	Tet 3	1.0E-01	D 4	7.0E-02	LTC 1	1.0E-01	D 4	1.0E-01	LTC 1
1.0E-01	Tet 3	5.0E-01	D 4	3.0E-01	LTC 1	1.0E-01	D 4	1.5E-01	Tet 3	1.0E-01	D 4

AMPP		BAMP		cGMP		cAMP		cCMP		cTMP	
Mass Inj (ng)	IPR	Mass Inj (ng)	IPR	Mass Inj (ng)	IPR	Mass Inj (ng)	IPR	Mass Inj (ng)	IPR	Mass Inj (ng)	IPR
1.5E-01	Tet 3	3.5E-03	Tet 2	2.5E-02	Tet 2	7.0E-03	Tet 2	2.5E-02	D 4	2.0E-02	D 4
1.5E-01	LTC 1	2.0E-02	D 4	2.5E-02	TTC 2	1.0E-02	TTC 2	5.0E-02	Tet 3	2.5E-02	TTC 2
2.5E-01	Tet 2	2.2E-02	LTC 1	5.0E-02	D 4	1.2E-02	D 4	5.0E-02	TTC 2	5.0E-02	Tet 3
5.0E-01	D 4	5.0E-02	Tet 3	7.5E-02	Tet 3	7.5E-02	LTC 1	1.0E-01	Tet 2	6.0E-02	Tet 2
1.7E+00	TTC 2	5.0E-02	TTC 2	9.5E-02	LTC 1	1.0E-01	Tet 3	1.2E-01	LTC 1	1.1E-01	LTC 1

ADP		TDP		CDP		P1P3DATP		ATP		CTP	
Mass Inj (ng)	IPR	Mass Inj (ng)	IPR	Mass Inj (ng)	IPR	Mass Inj (ng)	IPR	Mass Inj (ng)	IPR	Mass Inj (ng)	IPR
5.0E-02	TTC 2	1.0E-02	Tet 3	7.5E-02	Tet 3	7.5E-01	Tet 3	3.0E-01	Tet 3	2.5E-01	Tet 3
3.7E-01	LTC 1	1.0E-01	TTC 2	5.0E-01	Tet 2	4.0E+01	Tet 2	7.5E-01	TTC 2	5.0E-01	TTC 3
5.0E-01	Tet 2	5.4E-01	LTC 1	5.0E-01	TTC 2	N/A	D 4	2.5E+00	Tet 2	3.0E+00	LTC 1
1.2E+00	Tet 3	8.5E-01	D 4	5.0E-01	D 4	N/A	TTC 2	N/A	D 4	5.0E+00	Tet 2
1.8E+00	D 4	1.0E+00	Tet 2	3.0E+1	LTC 1	N/A	LTC 1	N/A	LTC 1	N/A	D 4

Table 4.3 - *Continued*

a-NAD		b-NAD		FAD		FdUMP		DGG	
Mass Inj (ng)	IPR	Mass Inj (ng)	IPR	Mass Inj (ng)	IPR	Mass Inj (ng)	IPR	Mass Inj (ng)	IPR
3.5E-02	TTC 2	5.0E-02	TTC 2	4.5E-02	Tet 2	3.0E-02	Tet 3	1.0E-01	Tet 3
1.5E+01	LTC 1	1.5E-01	Tet 2	7.5E-02	Tet 3	4.0E-02	Tet 2	1.5E-01	Tet 2
2.5E+01	Tet 2	5.0E-01	Tet 3	1.2E-01	TTC 2	4.5E-02	LTC 1	1.5E-01	TTC 2
N/A	Tet 3	5.0E+00	D 4	4.0E-01	D 4	1.0E-01	TTC 2	2.5E-01	LTC 1
N/A	D 4	1.5E+01	LTC 1	5.0E-01	LTC 1	2.0E-01	D 4	3.0E+00	D 4

CU		GA		TTDC		SACH		UU	
Mass Inj (ng)	IPR	Mass Inj (ng)	IPR	Mass Inj (ng)	IPR	Mass Inj (ng)	IPR	Mass Inj (ng)	IPR
5.0E-02	Tet 2	5.0E-02	Tet 2	5.0E-02	TTC 2	1.0E-01	LTC 1	2.0E-01	Tet 2
5.0E-02	TTC 2	5.0E-02	Tet 3	1.0E-01	Tet 2	1.5E-01	TTC 2	2.5E-01	TTC 2
1.0E-01	D 4	6.0E-02	LTC 1	1.0E-01	Tet 3	5.0E-01	Tet 3	6.0E-01	Tet 3
3.5E-01	Tet 3	4.5E-01	TTC 2	3.0E-01	D 4	2.5E+00	Tet 2	1.0E+00	LTC 1
3.6E-01	LTC 1	1.0E+00	D 4	1.5E+00	LTC 1	5.0E+00	D 4	2.5E+00	D 4

*

N/A- A daughter ion was not able to be detected.

A similar trend was also seen for the pyrimidine compounds (cCMP and cTMP). Since both pyrimidines (thymine and cytosine) are structurally similar (i.e. only differing by one methyl group, such behavior was expected. Likewise, the purines (guanosine and adenine) are also very similar to each other, thus they also behaved alike.[195, 218]

Another important observation involved the behavior of halogenated compounds such as bromo-adenosine monophosphate (BAMP) and fluoro-deoxyuridine monophosphate (F-dUMP). In the SIM ion mode these compounds were detected at low sensitivities when paired with the dications and the trications, specifically the trigonal tricationic reagents. However, in the SRM experiments it was the tetracationic ion pairing reagents that showed the best sensitivity for both compounds. This shows how diverse these nucleotide compounds are in their behavior with different ion pairing reagents when analyzed in different detection modes.

For comparison purposes all of these anions were tested in the negative mode as well. Table 4.4 is a summary of the best limits of detection found in the negative mode, SIM positive ion mode and SRM positive ion mode respectively. Attempts to perform SRM experiments in the negative mode were not successful. Compared to the negative mode, the SIM results show an increase in sensitivity for all compounds except one, thymidylyl- thymidylyl-deoxycytidine, which showed no improvement in the SIM mode; however its sensitivity was lowered in the SRM mode.

This technique worked extremely well for a few compounds such as α-NAD, which was not detectable in the negative mode. With the optimum ion pairing approach we were able to detect it at very low sensitivities (35 pg when using TTC 2). Also, the nucleotide triphosphate compounds were detected only at high concentrations when using the negative mode, however their sensitivity was lowered over two orders of magnitude when complexed with appropriate ion pairing reagents. In a living cell, the concentration of AMP can vary between 345 ng to 3452 ng,[219] and our ion pairing method is able to detect this analyte at a detection limit as low as 0.0075 ng.

When analyzing nucleotides by this method it is strongly recommended that the samples are made fresh daily as the phosphate bonds are very susceptible to hydrolysis from the aqueous environment. When analyzing any nucleotide based compound, to achieve low sensitivities, more than one ion pairing reagent must be tested.

Also, as we have shown in our previous studies, additional instrumental parameters can be optimized for each sample to achieve even higher sensitivity.[175, 176] In this study a single set of instrumental and ionization conditions were used (see Experimental).

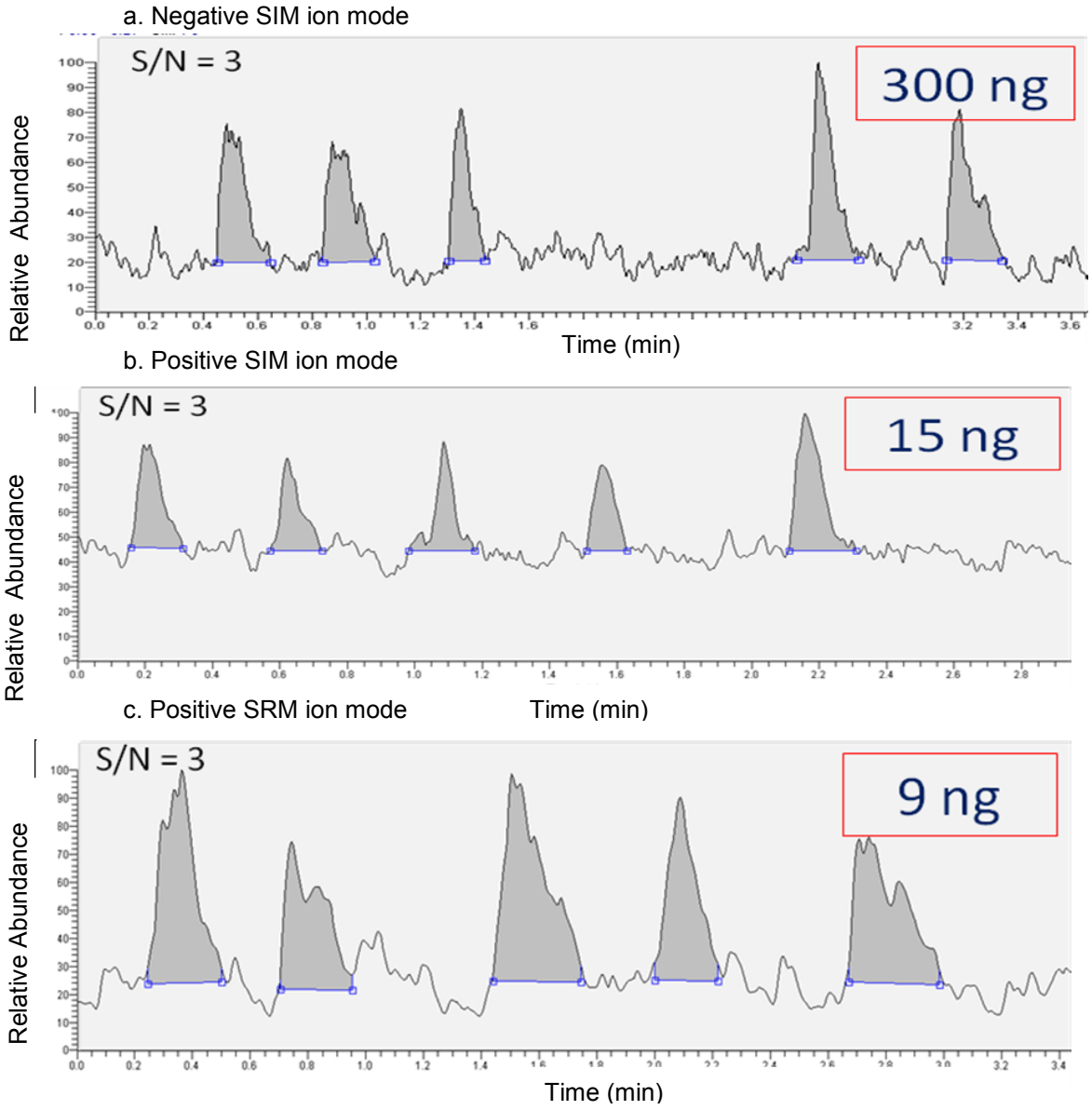


Figure 4.3. Limits of detection of AMP found in the SIM negative ion mode where no ion pairing reagent was added. (a), SIM positive ion mode where ion pairing reagent is present (b) and SRM positive ion mode where ion pairing reagent is present(c). This spectra shows five replicate injections of the same sample analyte in the different ion modes. The m/z monitored in the negative SIM ion mode was 345.2, in the SIM ion mode was 703.6 and in the SRM ion mode 439.2.

Table 4.4 Summarized LODs for nucleotide compounds found in negative and positive ion modes.

Anion	Anion mass	LOD in Negative Mode(ng)	Best LOD in Positive Mode SIM (ng)	Best LOD in Positive Mode SRM (ng)
Adenosine Monophosphate	345.2 (-2)	1.5E+00(-1)	7.5E-02(+2)	7.5E-03(+1)
Inosine Monophosphate	346.2 (-2)	1.5E+00(-1)	1.5E-01(+2)	1.0E-02(+1)
Uridine Monophosphate	322.2 (-2)	7.5E-01(-1)	6.0E-02(+1)	2.5E-03(+1)
Thymidine Monophosphate	320.2 (-2)	1.1E+00(-1)	1.0E-01(+1)	1.5E-02(+1)
Guanosine Monophosphate	361.2 (-2)	1.5E+00(-1)	1.8E-01(+1)	2.5E-02(+1)
Cytidine Monophosphate	321.2 (-2)	1.5E+00(-1)	8.0E-02(+1)	5.0E-02(+1)
Adenosine Monophosphoramidate	345.2 (-1)	7.5E-01(-1)	1.7E-01(+2)	1.5E-01(+2)
Bromoadenosine Monophosphate	424.1 (-2)	1.7E+00(-1)	1.3E-01(+1)	3.5E-03(+2)
cyclic Guanosine Monophosphate	344.2 (-1)	7.5E-01(-1)	3.0E-02(+2)	2.5E-02(+1)
cyclic Adenosine Monophosphate	328.2 (-1)	5.0E-01(-1)	3.0E-02(+2)	7.0E-03(+1)
cyclic Cytidine Monophosphate	304.2 (-1)	1.2E+00(-1)	3.0E-02(+3)	2.5E-02(+1)
cyclic Thymidine Monophosphate	303.2 (-1)	2.0E-01(-1)	3.0E-02(+2)	2.0E-02(+1)
Adenosine Diphosphate	425.3 (-2)	2.0E+00(-1)	3.0E-01(+1)	5.0E-02(+2)
Thymidine Diphosphate	399.1 (-2)	7.5E+00(-1)	1.0E+00(+2)	1.0E-02(+2)
Cytidine Diphosphate	400.1 (-2)	3.0E+00(-1)	1.3E+00(+1)	7.5E-02(+2)
P1P3-Diadenosine Triphosphate	736.4 (-3)	5.0E+01(-2)	2.5E+00(+1)	7.5E-01(+1)
Adenosine Triphosphate	505.1 (-2)	5.0E+01(-1)	8.0E-01(+1)	3.0E-01(+1)
Cytidine Triphosphate	481.3 (-2)	5.0E+01(-1)	2.5E+00(+1)	2.5E-01(+1)
α-Nicotinamide Adenine Dinucleotide	662.4 (-1)	> 50	3.5E+00(+1)	3.5E-02(+1)
β-Nicotinamide Adenine Dinucleotide	661.4 (-2)	3.0E+01(-1)	2.5E-01(+1)	5.0E-02(+1)
Flavin Adenine Dinucleotide	783.7 (-2)	5.0E-01(-1)	2.0E-02(+1)	4.5E-02(+1)
Fluoro-deoxyUridine Monophosphate	324.1 (-2)	3.0E+00(-1)	2.0E-01(+1)	3.0E-02(+1)
Deoxy-Guanylyl-Guanosine	595.4 (-1)	9.0E-01(-1)	2.5E-01(+2)	1.0E-01(+2)
Cytidyl-Uridine	549.4 (-1)	2.5E+00(-1)	1.0E-01(+2)	5.0E-02(+1)
Guanylyl-Adenosine	611.5 (-1)	5.0E-01(-1)	2.0E-01(+2)	5.0E-02(+1)
Thymidylyl-Thymidylyl deoxyCytidine	833.8 (-2)	3.0E-01(-2)	3.0E-01(+1)	5.0E-02(+1)
S-adenosyl-C-Homocysteine	383.4 (-1)	5.0E-01(-1)	2.4E-01(+2)	1.0E-01(+2)
Uridyl Uridine	548.4 (-1)	5.0E-01(-1)	2.5E-01(+2)	2.0E-01(+1)

4.5 Conclusion

Eleven different multiply charged cationic ionic liquids have been used as ion pairing reagents to study nucleotides in the positive mode ESI-MS. The overall best ion pairing reagents were found to be the trications and tetracations, with one trigonal trication (TTC 2) containing a mesitylene core and three butyl imidazolium functional groups, being the superior one. It appears that aromatic moieties are an essential feature in a good nucleotide ion pairing reagent. This approach was shown to enhance the detection and analysis of this very important class of negatively charged biomolecules. Furthermore this technique could be coupled to CE and HPLC thereby producing a more effective and sensitive way to analyze nucleotide monomers and nucleotide containing compounds. This method has virtues of great simplicity, fast analysis time and enhanced limits of detection when compared to traditional methods for the analysis of nucleotide and nucleotide analogues.

CHAPTER 5

USE OF ION PAIRING REAGENTS FOR SENSITIVE DETECTION AND SEPARATION OF PHOSPHOLIPIDS IN THE POSITIVE ION MODE LC-ESI-MS.

5.1 Abstract

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 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 (LOD) were very easily recorded at low ppt (ng/L) levels with many of the cationic ion pairing reagents used in this study.

5.2 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.[218] However, they also play a very important role in many other, different cellular signaling events.[195, 218, 220] Specifically, phospholipids play a crucial role as second messengers in signal transduction pathways, protein sorting, and apoptosis.[221-224, 226]

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 nonpolar tails cluster together in the core of the bilayer.[195] Some common polar head groups found in phospholipids are inositol, glycerol, serine and ethanolamine.[218] 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.[227-229]

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).[7, 64, 220, 228-236] 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.[7, 220, 230-232] 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).[64, 228, 233-235] 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.[220, 226, 229, 233, 236-238]

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.[228, 229, 237] 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, arcing, which then results in poor spray stability, thus affecting the sensitivity of the analytes.[144, 145]

It has been shown that these drawbacks can be solved by using halogenated solvents or 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.[171-173, 239] 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.[174, 175]

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.[174, 175] The successful results lead to an extensive study and the synthesis of many other cationic reagents.[177, 179, 214-216, 240, 241]

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.

5.3 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.[175]

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 300μL/min. 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. 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 400μL/min entering the MS.

The ESI-MS parameters were set as follows: spray voltage of 3 kV; capillary temperature of 350ºC; capillary voltage of 11 kV; tube lens voltage of 105 V; sheath gas flow was set at 37 arbitrary units (AU), and the auxiliary gas flow at 6 AU. Red PEEK tubing (i.d.0.005 in.) was used as solvent carrier for the ESI-MS and LC-ESI-MS analysis. The sample analytes were introduced in the MS via a six port injection valve with a 5μL loop. The concentration of the ion pairing reagent remained constant at 40μM 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 10μg/mL.

LC-ESI-MS *Reverse phase LC* was performed on an Ascentis^{Tm} 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 (250mm x 4.6mm) obtained from Advanced Separation Technologies (Whippany, NJ). The mobile phase used was 70/20/10 acetonitrile/methanol/water with a flow rate of 1mL min^{-1}. Phospholipids were detected, in both reverse and HILIC phase LC, at a wavelength of 210 nm. A flow splitter was used in the normal phase separation 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. The chromatographic separations for both modes were done by a Thermo Fisher Surveyor autosampler (10µL injections).

5.4 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. 5.1) 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
(Figure 5. 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 moities and one consisted of an imidazolium core and phosphonium terminal groups (Figure 5.3).

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 (Figure 5.3).

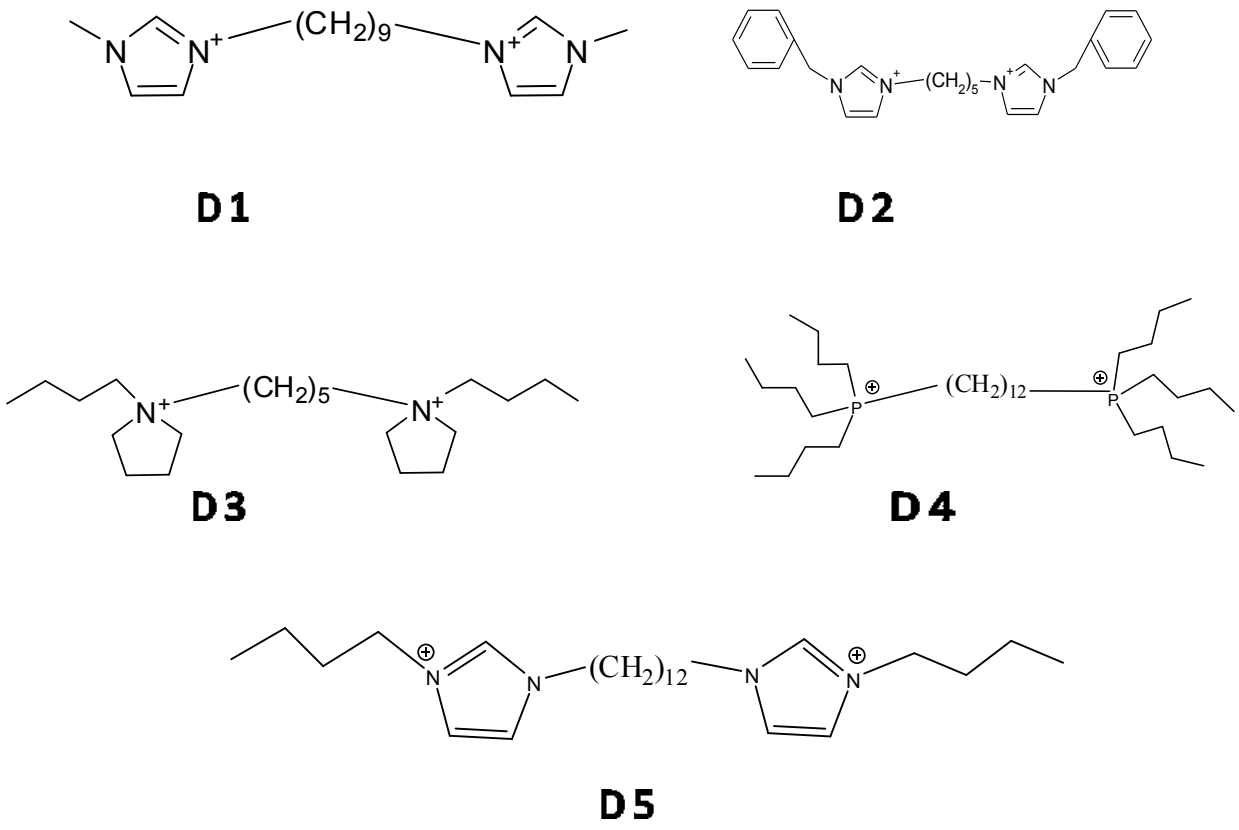


Figure 5.1. Structures of the dicationic ion pairing reagents.

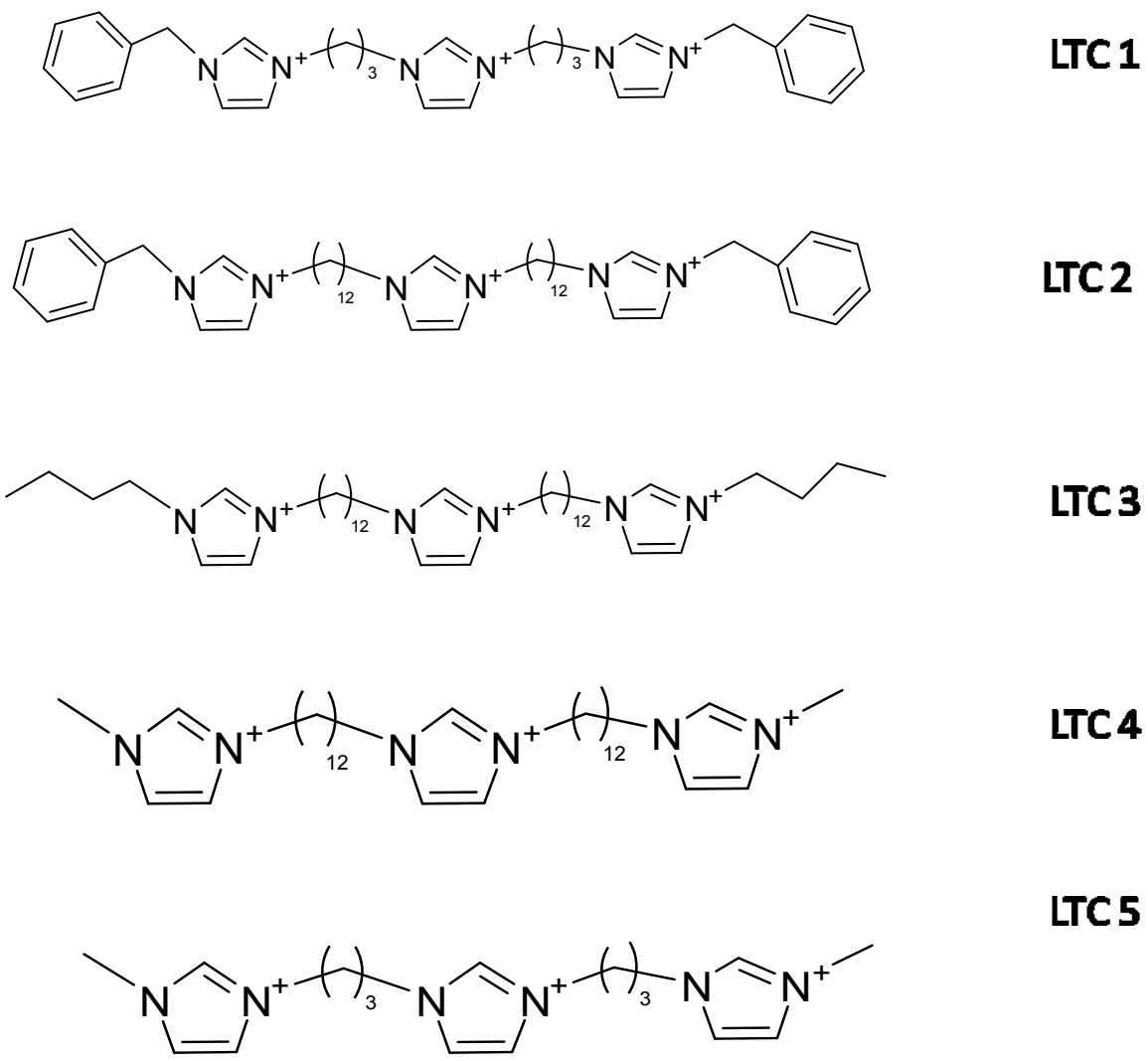
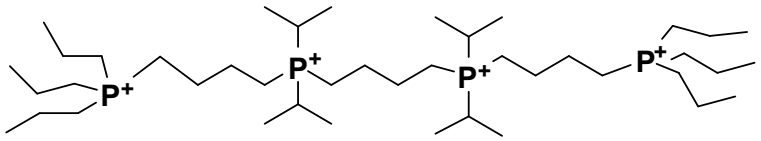
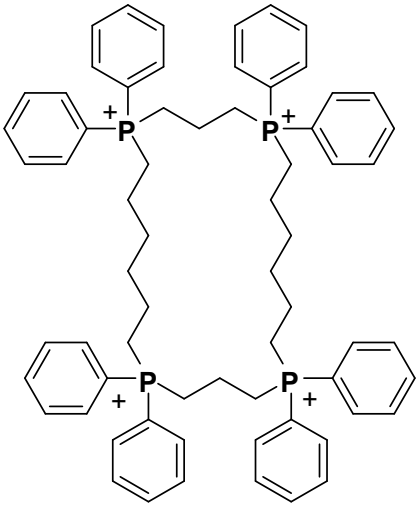


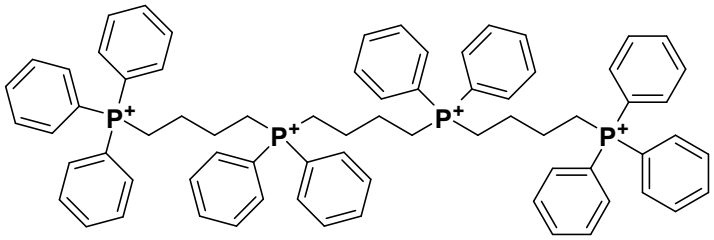
Figure 5.2. Structures of the linear tricationic ion pairing reagents.



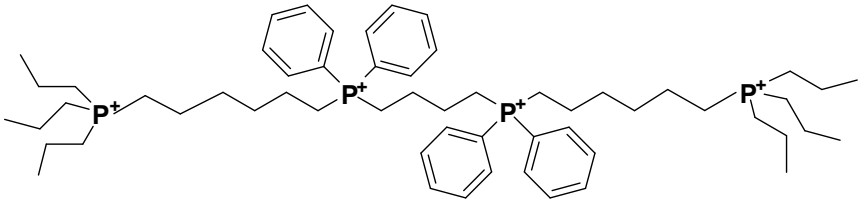
Tet 1



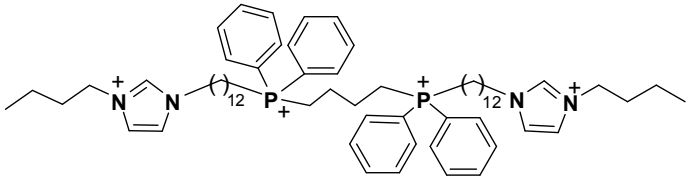
Tet 3



Tet 2



Tet 4



Tet 5

Figure 5.3. Structures of the tetracationic ion pairing reagents.

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.[240] 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 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 5.1). 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 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.[228, 237]

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 5.2). 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 5.2). 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.

Table 5.1. Limits of detection of phospholipids analyzed with fifteen different ion pairing reagents.

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^{0}	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.

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 5.2). 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 5.2). **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.[216, 241] 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 overall sensitivity. Additional mechanistic studies are needed to further understand this behavior of these reagents.[240]

The second group of ion pairing reagents that performed well in detecting low levels of phospholipids were the dicationic reagents. In particular, **D 1** (Fig. 5.1) produced the best sensitivity within this category. **D 1** is an imidazolium based reagent containing a C9 linkage chain. Following this reagent, were **D 2** and **D 3** dications that resulted in adequate sensitivities when coupled with the phospholipids. These cationic reagents include imidazolium and pyrrolidinium moieties respectively.

Table 5.2. LODs for each phospholipid analyzed in the negative ion mode ESI-MS.

Phospholipid	Anion Mass (g/mol)	SIM LOD (ng)
L-Phosphatidic Acid (PA)	671.89	1.50E+00
Phosphatidylglycerol (PG)	745.98	5.00E-01
Phosphatidylinositol (PI)	886.12	4.00E-01
Phosphatidylserine (PS)	758.97	4.00E+00
Phosphatidylcholine (PC)	758.06	ND*
Phosphatidylethanolamine (PE)	746.05	ND
Cardiolipin (CA)	1447.9	2.00E+01
Sphingosyl PE (SPE)	422.29	1.70E-01
Diheptanoyl-phosphocholine (DHPC)	481.28	ND

*ND - Not Detected at 10µg/mL

As seen from Table 5.1, the worst performing reagents in this category were **D 4** and **D 5**. The common feature of these two ions is the C12 alkyl linkage. The terminal end groups are tripropyl phosphonium and butyl imidazolium for **D 4** and **D 5** 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 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 5.1. 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 was noticed in their LODs. To further understand these results, an extended study 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.[240, 241] 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+).

Table 5.3. Limits of detection of the analytes in the single ion monitoring (SRM) mode with three dicationic reagents.

	D 1		**D 2**		**Tet 1**	
	SIM LOD (ng)	SRM LOD (ng)	SIM LOD (ng)	SRM LOD (ng)	SIM LOD (ng)	SRM LOD (ng)
Phosphatidic acid (PA)	1.0E-01	3.0E-01	2.5E-02	N/A	5.0E-02	N/A
Phosphatidylglycerol (PG)	1.0E-02	2.3E-01	6.5E-02	1.0E+00	2.5E-02	2.5E-02
Phosphatidylinositol (PI)	1.2E-01	N/A	5.0E-01	N/A	5.0E-01	N/A
Phosphatidylserine (PS)	1.0E-03	N/A	3.5E-01	2.5E-01	1.0E-02	1.5E-02
Phosphatidylcholine (PC)	5.0E-05	N/A	8.5E-01	N/A	4.0E-02	5.0E-02
Phosphatidylethanolamine(PE)	5.0E-03	N/A	5.0E-01	N/A	2.5E-02	2.5E-02
Cardiolipin (CA)	1.5E+00	N/A	1.2E+00	N/A	2.0E-02	1.5E-03
Sphingosyl PE (SPE)	7.5E-01	N/A	1.9E-02	2.5E-02	5.0E-06	2.5E-05
1,2-diheptanoyl-sn-glycero-3-phosphocholine (DHPC)	1.5E+00	N/A	1.5E-02	9.0E-03	4.5E-02	1.5E-01

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. 5.2) 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. 5.2) 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 5.1.

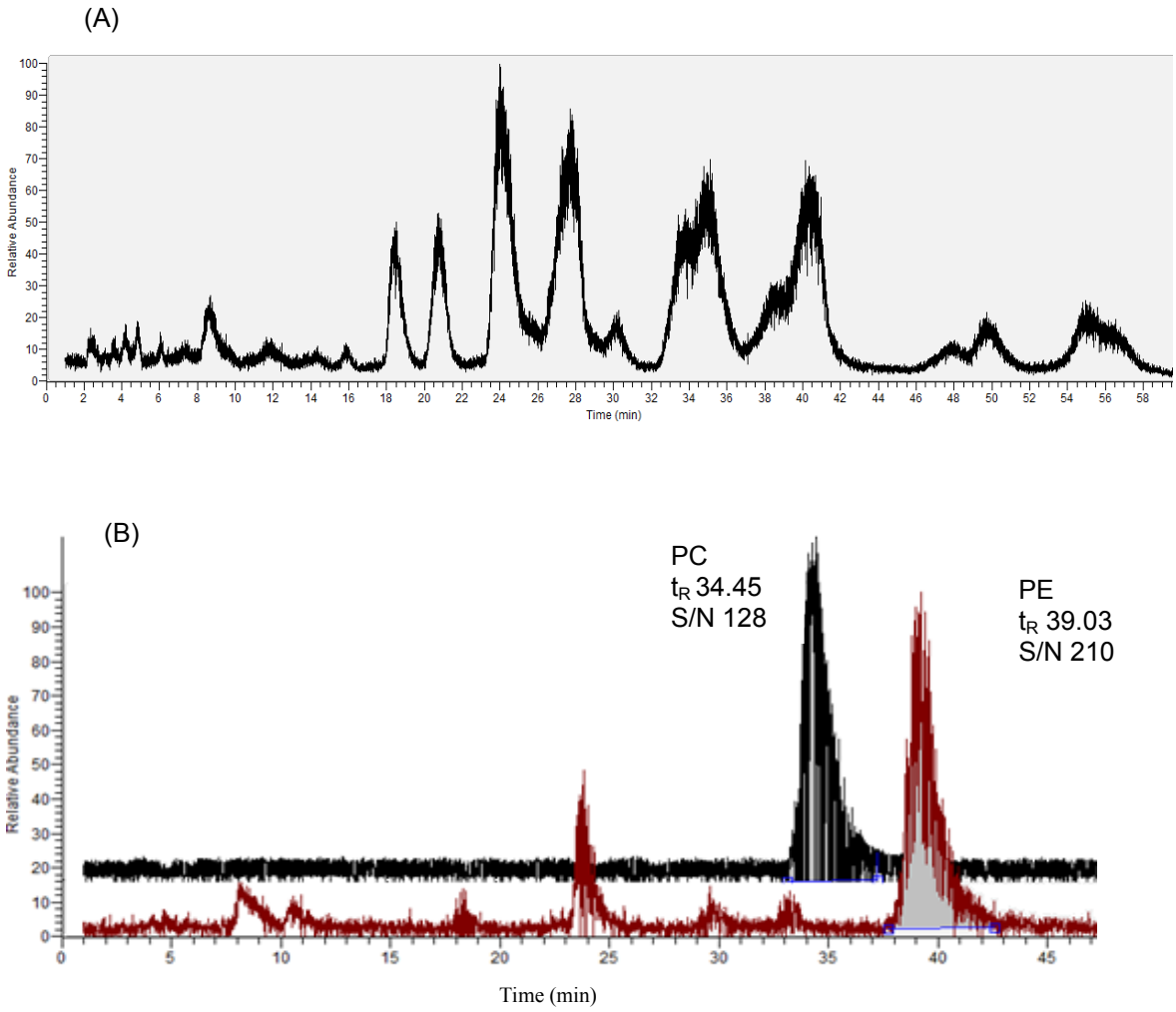


Figure 5.4. Chromatographic separation and detection of the PC and PE mixture and their homologues in the SIM positive mode ESI-MS. (A) represents the total ion chromatogram of this mixture, and (B) is the extracted ion chromatogram in which the major species of the phospholipids are detected with tetracation ion pairing reagent Tet 5. The separation was performed on an Ascentis^{Tm} 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}.

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 Figure 5.4. This separation was achieved on a C18 stationary phase. Chromatogram (B) of Figure 5.4 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. 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 Figure 5.4. 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. 5.4, B) 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 5.1. This decrease in sensitivity is possibly due to the protonation of these anlaytes 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.

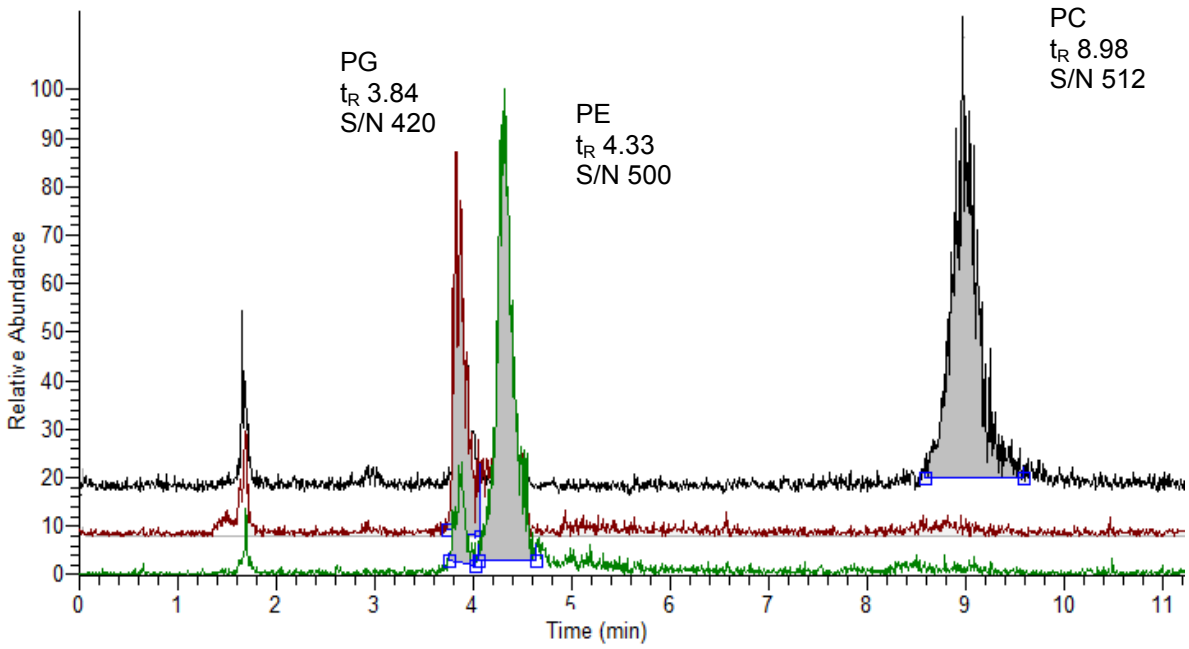


Figure 5.5. The extracted ion chromatogram displaying the LC separation of PC, PG, and PE on a silica column in the positive ion mode ESI-MS. Concentration of the analytes was 1mg/mL and the mobile phase composition was 70/20/10 acetonitrile/methanol/water with a flow rate of 1mL min^{-1}. A flow splitter was used, such that only 0.3 mL min^{-1} is mixed via a mixing tee with 0.1mL min^{-1} of the ion pairing reagent, with a final flow rate of 0.4mL min^{-1} entering the mass spectrometer. The ion pairing reagent used was Tet 5 (Fig. 3).

This separation was achieved on a silica column (Figure 5.5) 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 5.1. Another advantage of using the HILIC phase HPLC in this case is the shorter retention times (approximately 9 minutes).

5.5 Conclusion

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.5. 3) that lowered the limits of detection for most of the phospholipids.

The best dicationic reagent in this analysis was **D 1**, 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, and phenyl functional groups are recommended in achieving low limits of detection.

LC analysis was developed in both reverse and HILIC phase HPLC. 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**) was 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.

CHAPTER 6

SENSITIVE ANALYSIS OF METAL CATIONS IN THE POSITIVE ION MODE ESI-MS, USING COMMERCIAL CHELATING AGENTS AND CATIONIC ION PAIRING REAGENTS

6.1 Abstract

Metals play a very important role in many scientific and environmental fields, and thus their detection and analysis is of great necessity. Traditionally, when using electrospray ionization mass spectrometry (ESI-MS) metals are detected in the negative ion mode after pairing with negatively charged chelating agents. A simple and very sensitive method has been developed herein for the detection of metals in the *positive* ion mode ESI-MS. In this study it is shown that the detection of chelated metal ions is much more sensitive in the positive ion mode rather than the more commonly utilized negative ion mode ESI-MS.

Metal ions are positively charged, and as such they can potentially be detected in the positive ion mode ESI-MS, however their small mass to charge (m/z) ratio makes them fall in the low region of the mass spectrum, which has the largest background noise. As such, their detection can become extremely difficult. A better and well known way to detect metals by ESI-MS is by chelating them with complexation agents.

Currently, there are many commercially available chelating agents that are used effectively in the detection of metals in the negative ion mode ESI-MS. In this study eleven different metals, (Fe (II), Fe (III), Mg (II), Cu (II), Ru (III), Co (II), Ca (II), Ni (II), Mn (II), Sn (II), and Ag (I)), were paired with two commercially available chelating agents: ethylenediamine-tetra acetic acid (EDTA) and ethylenediamine-disuccinic acid (EDDS). Since negative ion mode ESI-MS has many disadvantages compared to the positive ion mode ESI-MS, it would be very beneficial if these negatively charged complex ions could be detected in the positive mode. Such a method is described in this paper and it is shown to achieve much lower sensitivities. Each of the negatively charged metal complexes is paired with two linear imidazolium based tricationic ion-pairing reagents and four phosphonium based tetracationic ion pairing reagents.

The new positively charged ternary complexes are then analyzed in the positive single ion monitoring (SIM) mode and single reaction monitoring (SRM) mode ESI-MS. The results clearly revealed that the presence of the cationic reagents significantly improved the sensitivity for these analytes, often by several orders of magnitude.

These metal complexes were also analyzed in the negative ion mode for comparison purposes. Very low limits of detection (LODs) were achieved for all the metals in this study in the positive ion mode. The LODs for most metals were in the ppt (ng L^{-1}) levels, and in a few cases ppq (pg L^{-1}) levels were reached. This novel method developed herein for the detection of metals improved the LODs significantly when compared to the negative ion mode ESI-MS and shows great potential in future trace studies of these and many other species.

6.2 Introduction

Ionized metal species are present in many different biological, ecological and industrial environments; as such they play a very important role in our lives. In many instances metals are found associated with many different organic ligands. For example, about one quarter of all existing proteins require a specific metal to help not only fulfill their precise functions in biochemical reactions, but also to maintain their stable state.[196, 242]

Also, different oxidation states alter the metal's specific role in a particular environment. For example, the oxide form of ruthenium (Ru), such as ruthenium (VIII) tetraoxide (RuO_4) is considered highly toxic, however ruthenium complexes containing Ru(II) and Ru(III) have been studied extensively and have shown great potential as anticancer agents when bound to certain ligands.[243-245] Knowing the correct oxidation state of Ru is also very important as Ru(II) is much more stable than Ru(III), and this does not only affect biological environments, but photochemical systems as well.[245]

The metals examined in this study can have different oxidation states and are crucial to many different ecological and industrial systems. They are cobalt (Co), calcium (Ca), nickel (Ni), manganese (Mn), tin (Sn), silver (Ag), iron (Fe), magnesium (Mg), and copper (Cu).

A number of methods have been developed for the accurate detection and quantification of these metals. Among the most used methods to detect metals are atomic absorption spectrometry (AAS), emission spectroscopies (ES), and inductively coupled plasma mass spectrometry (ICP-MS). [246, 247] Of course, due to the high temperatures of these methods, speciation and the ability to determine oxidation state of the metals can be problematic.[82, 247-250]

Another technique used to detect metals and their organic complexes is electrospray ionization mass spectrometry (ESI-MS) which is a softer ionization technique, and can further provide complete characterization of a metal-organic sample. [82, 249-254] The goal of this study is to detect anionic analytes (chelating agent + metal) in the positive ion mode at a higher sensitivity than the traditional negative ion mode ESI-MS, and to find the best ion pairing reagent suitable for this task. In order to use this approach, the metal ions have to first associate with an anionic chelating agent.

Chelating agents are organic molecules that complex with metals with different coordination geometries and strengths. Currently, there is a large selection of commercially available chelating agents. These complexes have been studied extensively by ESI-MS.[254-257]

In this study, two well-known chelating agents were chosen and used for metal analysis. They are: ethylenediamine-N,N,N',N'- tetraaceticacid (EDTA) and ethylenediamine-N,N'-disuccinic acid (EDDS). These multidentate ligands are aminocarboxylic acid compounds and as such they form negatively charged metal ion complexes[254, 249, 258, 259]. Thus, they are detected in the negative ion mode ESI-MS. However the negative ion mode tends to be unstable due to the fact that corona discharge is more prevalent, therefore creating chances for arcing, higher background noise and often resulting in an overall unstable Taylor cone and unstable signals.[144, 249] These phenomena can result in a higher overall limit of detection (LOD). These problems can be avoided if the positive ion mode is used.

In this study we have created a method in which we take these negatively charged metal complexes and associate them with multiply charged cationic pairing reagents, creating

an overall positive charged ternary complex that can be easily detected in the positive ion mode ESI-MS. Many cationic ion pairing reagents have been synthesized and evaluated previously.[175, 176, 178, 179, 214-216, 241] In this study six cationic ion-pairing reagents are examined as they were found to be effective (giving the lowest LODs) based on previous studies.[179, 213, 214, 216, 240, 241, 260]

In addition to the very low limits of detection, other advantages of this method include great simplicity, very fast analysis times, and compatibility with high performance liquid chromatography (HPLC) and capillary electrophoresis (CE).[174, 213, 260] The large mass of these cationic ion pairing reagents adds another major benefit to this technique which is the ability to bring small metal ions out of the low mass cut off (LMCO) to a higher mass range where the background noise is lower. This study was performed in the selected ion monitoring (SIM) and selected reaction monitoring (SRM) mode.

6.3 Experimental

HPLC grade solvents were purchased from Honeywell Burdick and Jackson (Morristown, NJ). The metals and the chelating agents were purchased from Sigma-Aldrich (St. Louis, MO). The cationic ion pairing reagents were initially synthesized in the bromide form and then exchanged to the fluoride form prior to analysis. This ion-exchange method has been previously described by Soukup-Hein *et al.* [176] The synthesis of the ion pairing reagents has been previously discussed.[178, 179, 215]

Each solution (metal and chelating agent) was prepared daily in situ and the serial dilutions started at 1μM. The ion pairing reagents are in aqueous solutions and their concentrations were maintained constant at 40 μM. An external Shimadzu LC-6A pump (Shimadzu, Columbia, MD) was used to introduce the ion-pairing reagent to a Y-type mixing tee at a flow rate of 100 μL/min (Figure 1). A 67:33 methanol/water mixture was also directed into the mixing tee at a flow rate of 300μL/min, creating an overall flow of 50/50 water/methanol entering the mass spectrometer with a total flow rate of 400μL/min.

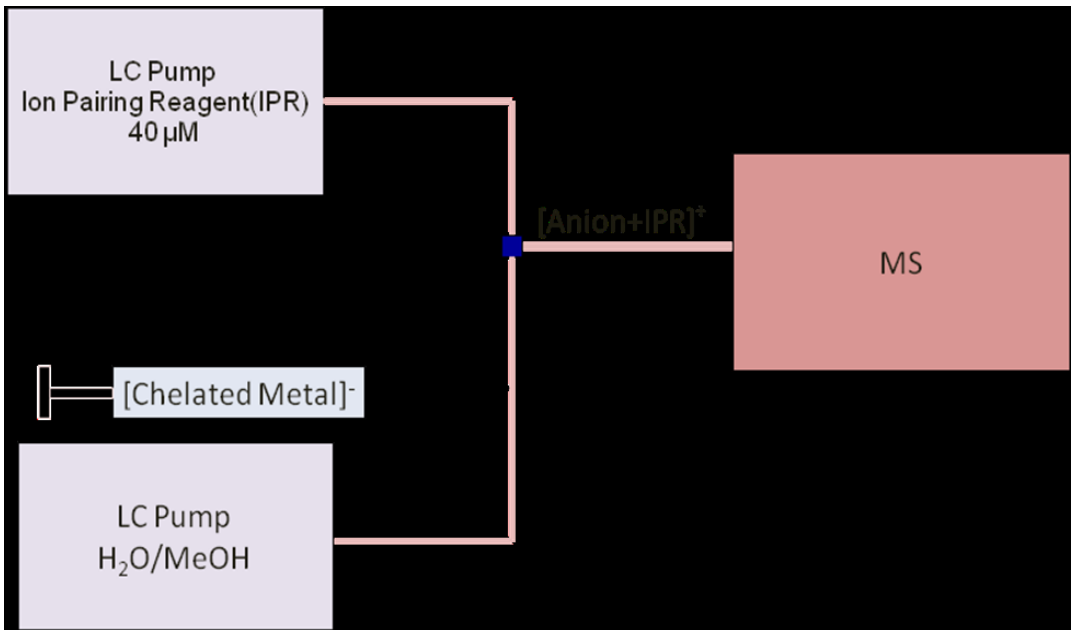


Figure 6.1. A schematic for the set-up used to perform the ion association experiments. The setup requires two LC pumps whose effluent is mixed in a low volume mixing Y-shaped tee, prior to entering the ESI-MS. One pump is used to continually supply the cationic reagent, were as the other pump offers the solvent flow in which the anions (chelated metals) are injected. The two flows meet in the mixing tee and the ion association occurs, and then the flow enters the MS. The metals are mixed with the chelating agents in situ, prior to this analysis.

A Surveyor MS pump (Thermo Fisher Scientific, San Jose, CA) was used to pump the methanol/water mixture prior to entering the mass spectrometer. To introduce the sample, a six port injection valve with a 5µL injection loop was used. Red PEEK tubing (i.d.0.005 in./125 µm) was used as solvent carrier for the ESI-MS analysis. In this study the LODs are reported as exact masses instead of concentrations to avoid confusion caused by different sample injection loops used in different studies.

The mass spectrometer used was a Thermo Finnigan LXQ (Thermo Fisher Scientific, San Jose, CA). The ESI-MS parameters were set as the following: spray voltage of 3 kV; capillary temperature of 350ºC; capillary voltage of 11 kV; tube lens voltage of 105 V; sheath gas flow was set at 37 arbitrary units (AU), and the auxiliary gas flow at 6 AU.

Necessary dilutions were prepared for each sample until five replicates of signal to noise (S/N) ratio of 3 was observed for each analyte. The data was analyzed using Xcalibur and Tune Plus software. The limits of detection in this analysis were determined based on Genesis Peak Detection Algorithm. The MS parameters were fixed to achieve a good sensitivity for the analyzed ternary and binary complexes. The parameters were kept the same in the negative and positive ion mode.

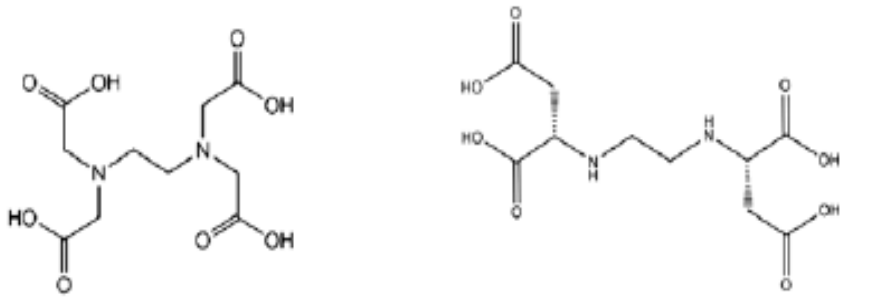
6.4 Results and Discussion

In this study eleven metals of different oxidation states were paired individually with two commercially available chelating agents: ethylenediamine-tetraacetic acid (EDTA) and ethylenediamine-disuccinic acid (EDDS) (Figure 6.2). These negatively charged complexes were then analyzed sequentially by positive mode ESI-MS with six cationic ion pairing reagents (Figure 3). Thus, new and larger ternary complexes detected were all positively charged. A large pool of multiply charged cationic ion pairing reagents had been previously synthesized and evaluated.[176, 179, 215, 241, 260, 261]

A recent study revealed that there are three main factors that contribute to the sensitivity these ion pairing agents achieve. They are: ionization efficiency, surface activity, and redox reactions occurring at the tip of the electrospray.[240] Based on these prior studies two linear tricationic and four tetracationic ion-pairing agents were selected for this study. Linear trication 1 has an imidazolium core with two C_6 alkyl chains linking the imidazolium core to the terminal groups (LTC 1, Figure 3). The two end groups of this reagent are two tripropyl phosphonium groups. The second linear trication has an imidazolium core as well, and it contains C_3 alkyl chain linkages and benzyl imidazolium terminal functional groups (LTC 2, Figure 3).

The second set of reagents are the tetracationic ion pairing agents. Three of these are entirely phosphonium based salts (Tet 1, Tet 3, and Tet 4 in Figure 3) with a mixture of benzyl-,

isopropyl and/or propyl functional groups. The alkyl linkages vary from C4 to C6. Tet 2 is the only ion pairing regent containg a mixture of phosphonium, and imidazolium based moieties.



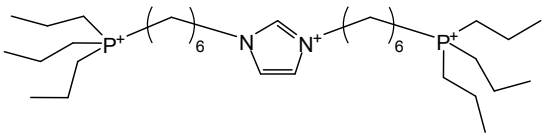
Ethylenediamine-*N,N,N',N'*-tetraacetic acid (EDTA) Ethylenediamine-*N,N'*-disuccinic acid (EDDS)

Figure 6.2. The structures of the two metal chelating agents used in this analysis.

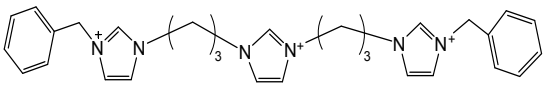
The diphenyl phosphonium groups are positioned in the center of the structure separated by C4 linkages (Figure 6.3). Table 1 shows the limits of detection (LOD) achieved for each $[M^{n+}$+ EDTA$]^-$ complex in the SIM and SRM mode when paired with each of the ion pairing reagents (Fig. 6.3). All results are reported as exact masses (ng) of the metal, rather than concentrations to avoid any confusion caused by variations in the size of the sample injection loop which varies in different studies (a 5µL loop was used in this work, see *Experimental*).

The bold-cursive typeface indicates the lowest limit of detection achieved for each metal. It is clear that the best ion-pairing reagent that resulted in the lowest LODs is Tet 1. In fact, for both SIM and SRM modes, Tet 1 produced the lowest LODs. It is also important to note that the limits of detection were further lowered, by varying degrees, for each metal when the analysis was carried out in the SRM mode. Indeed, Table 6.1 shows that the lowest limit of detection was achieved in the SRM mode for each metal complex (typically femtogram to picogram levels). Other ion pairing reagents that performed well were LTC 1 and Tet 3. The ion pairing reagents that produced poorer results were Tet 2, Tet 4, and LTC 2.

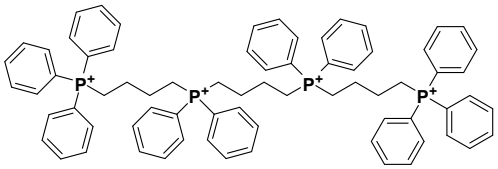
Table 6.2 shows the limits of detection when EDDS was used as a chelating agent. Similarly, every $[M^{n+}$+ EDDS$]^-$ complex was individually paired with every ion pairing reagent.



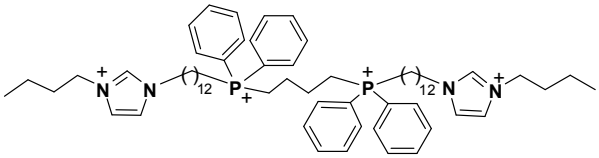
LTC 1



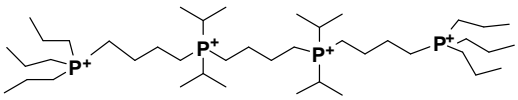
LTC 2



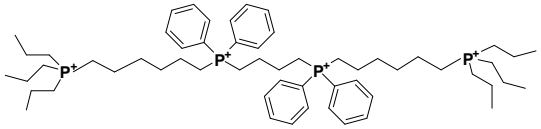
Tet 1



Tet 2



Tet 3



Tet 4

Figure 6.3. The structures of the linear tri-, and tetra-cationic ion pairing reagents.

Again, the results show that, the lowest limits of detection were achieved when Tet 1 was used as the ion pairing reagent. Interestingly, in the case of EDDS the lowest limit of detection for each metal was achieved by Tet 1 in the SRM mode, except for one metal, Ag^+. The lowest limit of detection for silver (Ag^+) was reached when LTC 2 was used as a pairing

agent (10 x improvements when compared to Tet 1, Table 6.2). Also, the lowest LOD for nickel (2.9E-04 ng, Ni^{2+}) was achieved by two pairing agents: LTC 1 and Tet 1 (see Table 6.2).

As found for the EDTA metal complexes, both LTC 1 and Tet 3 produced good LODs as well. The worst LODs for each metal-EDDS complex were again attained when LTC 2, Tet 2 and Tet 4 were used. One exception to this trend was LTC 2 and Tet 4 which produced the lowest LOD for only one metal (silver, Ag^{+}).

Upon examination of the data, it can be seen that the ion pairing reagent that produced the best LOD in the SIM mode for a specific metal, was not necessarily the same one that produced the lowest LOD in SRM experiments (Table 6.2). For example, the best LOD in the SIM mode for $[Ag^{+}+EDDS]^{-}$ complex was achieved by Tet 3, however the best LOD in the SRM mode was produced by LTC 2. This held true for all metals except one. The same ion pairing reagents produced the lowest, average and highest LODs for both $[Fe^{2+}+EDDS]^{-}$ and $[Fe^{3+}+EDDS]^{-}$ complexes. For example the lowest LODs for both complexes were achieved by Tet 1, the second lowest were achieved by Tet 4, the third lowest limits of detection were achieved by Tet 3 and so on. This trend was unique only to Fe(II) and Fe(III) in the SRM mode and only with EDDS. Also, oxidation/reduction reactions were not observed when analyzing Fe (II) and Fe (III) standards.

Table 6.4 shows the summarized results of the best LODs achieved in the positive and negative ion mode ESI-MS. The data in this table also shows the best chelating agent and ion pairing reagents that were responsible in providing the lowest LODs. The final column of this table summarizes the improvements in sensitivity achieved in the positive mode. Based on these results it was concluded that the best overall ion-pairing reagent in detecting metals as ternary cationic complexes was Tet 1. These low limits of detection could be due to the presence of additional π interaction moities present in Tet 1, which seems to be an important feature in some ion pairing reagents. Dicationic ion pairing reagents were attempted for this analysis, however the LODs were significantly higher (data not shown).

Table 6.1. Limits of detection (ng) and the corresponding charge of each metal complex. Complexes were detected in SIM and SRM positive ion modes ESI-MS. LODs reported were achieved for each metal when complexed with *EDTA* as a chelating agent and with the entire corresponding cationic ion pairing reagents used in this study.

	LTC 1		LTC 2		Tet 1		Tet 2		Tet 3		Tet 4	
	SIM LOD	SRM LOD	SIM LOD	SRM LOD	SIM LOD	SRM LOD	SIM LOD	SRM LOD	SIM LOD	SRM LOD	SIM LOD	SRM LOD
Cu^{2+}	2.3E-03/1+	1.5E-03/2+	4.7E-03/1+	1.4E-02/2+	2.2E-04/2+	***5.3E-05***/2+	3.1E-02/2+	9.4E-03/2+	1.5E-03/1+	5.3E-03/1+	3.1E-02/1+	3.7E-02/1+
Mg^{2+}	1.8E-02/1+	4.5E-03/3+	3.6E-03/1+	1.8E-03/3+	7.2E-04/2+	***7.2E-04***/2+	8.4E-03/3+	7.8E-03/3+	8.4E-03/2+	1.6E-03/2+	4.8E-02/2+	3.6E-02/2+
Ca^{2+}	1.9E-04/1+	***1.8E-05***/3+	1.0E-02/1+	4.0E-03/3+	4.0E-04/2+	6.0E-05/2+	1.6E-03/1+	4.0E-04/1+	2.0E-03/2+	1.5E-04/2+	5.0E-03/2+	2.0E-03/2+
Co^{2+}	1.0E-01/1+	1.3E-03/+2	1.4E-02/1+	N/D	1.7E-03/2+	***2.9E-04***/2+	9.4E-03/1+	N/D	3.6E-03/1+	3.6E-03/1+	5.9E-03/1+	1.4E-01/2+
Fe^{2+}	2.8E-02/2+	***3.3E-04***/2+	1.4E-02/2+	1.1E-02/2+	2.8E-03/2+	1.1E-03/2+	1.6E-01/1+	N/D	1.2E-02/2+	3.6E-04/2+	1.1E-02/3+	7.0E-03/2+
Fe^{3+}	2.8E-02/1+	2.5E-0/1+3	7.8E-02/1+	8.4E-03/3+	2.8E-03/2+	***2.8E-04***/2+	1.6E-01/1+	1.6E-01/1+	4.2E-03/2+	4.2E-03/2+	5.6E-02/4+	5.6E-02/1+
Ni^{2+}	2.9E-02/1+	8.7E-04/3+	8.1E-02/1+	1.7E-02/2+	7.2E-04/2+	***2.9E-04***/2+	6.9E-02/1+	1.8E-02/1+	1.1E-02/2+	1.1E-03/2+	1.4E-02/1+	2.3E-03/1+
Mn^{2+}	4.1E-02/1+	1.2E-03/3+	1.0E-01/1+	2.4E-02/2+	1.6E-03/2+	***6.8E-05***/2+	6.3E-02/2+	8.2E-03/2+	2.4E-02/2+	2.4E-02/2+	3.0E-02/2+	1.9E-03/1+
Ag^{+}	5.3E-02/2+	5.3E-02/3+	2.6E-02/1+	N/D	4.0E-02/2+	9.6E-03/2+	2.6E-01/2+	N/D	2.4E-02/1+	2.4E-02/1+	1.6E-01/2+	***1.2E-03***/2+
Ru^{3+}	5.1E-01/4+	N/D*	1.9E-01/2+	N/D	7.6E-03/1+	***1.2E-03***/1+	1.7E-02/1+	2.0E-03/1+	2.0E-02/1+	5.1E-03/1+	1.0E-02/1+	7.6E-03/1+
Sn^{2+}	1.8E-01/1+	7.2E-02/1+	2.1E-01/1+	6.0E-01/2+	1.5E-02/2+	***4.5E-03***/1+	1.8E-01/2+	1.5E-02/2+	1.2E-02/1+	7.2E-02/1+	4.2E-01/2+	N/D

*N/D – Indicates that a product ion was not able to be detected

Bold and cursive typeface indicates the lowest limit of detection achieved for each analyzed anionic complex $[M^{n+}+EDTA]^{-}$.

Table 6.2. Limits of detection (ng) and the corresponding charge of each complex. Complexes were detected in SIM and SRM positive ion modes ESI-MS. LODs reported were achieved for each metal when complexed with ***EDDS*** as a chelating agent with the entire corresponding cationic ion pairing reagents used in this study.

	LTC 1		**LTC 2**		**Tet 1**		**Tet 2**		**Tet 3**		**Tet 4**	
	SIM LOD	**SRM LOD**	**SIM LOD**	**SRM LOD**	**SIM LOD**	**SRM LOD**	**SIM LOD**	**SRM LOD**	**SIM LOD**	**SRM LOD**	**SIM LOD**	**SRM LOD**
Cu^{2+}	1.5E-02/1+	1.2E-02/2+	2.5E-02/1+	2.3E-02/2+	1.8E-04/2+	***3.1E-05***/2+	3.7E-03/1+	2.9E-04/1+	1.7E-03/2+	3.1E-04/2+	3.1E-01/1+	2.5E-03/1+
Mg^{2+}	1.8E-03/2+	4.9E-05/3+	1.2E-03/2+	1.4E-03/3+	2.1E-04/2+	***1.8E-05***/2+	9.6E-04/2+	3.0E-04/2+	2.4E-03/2+	2.6E-04/2+	4.8E-05/2+	7.5E-03/2+
Ca^{2+}	4.8E-04/2+	1.5E-03/3+	5.0E-03/2+	4.4E-03/3+	6.0E-03/3+	***1.5E-04***/3+	1.5E-01/2+	6.3E-03/2+	1.2E-02/2+	3.7E-03/2+	1.3E-02/2+	4.0E-03/2+
Co^{2+}	5.9E-03/2+	1.8E-03/3+	1.7E-01/1+	1.1E-01/1+	4.4E-04/2+	***1.4E-04***/2+	1.4E-01/1+	1.3E-02/1+	1.4E-03/1+	1.8E-03/2+	2.9E-01/1+	9.2E-02/2+
Fe^{2+}	1.4E-03/2+	7.4E-04/3+	2.8E-02/2+	4.2E-03/2+	8.4E-04/2+	***8.8E-05***/2+	8.4E-02/1+	2.1E-03/1+	8.4E-03/3+	1.2E-04/3+	2.5E-02/3+	9.2E-05/3+
Fe^{3+}	3.3E-03/2+	1.0E-02/2+	2.8E-01/1+	1.4E-01/3+	2.8E-03/2+	***8.3E-05***/2+	8.4E-03/1+	N/D	1.4E-03/3+	9.3E-03/1+	7.5E-03/3+	1.1E-03/3+
Ni^{2+}	2.3E-02/1+	***2.9E-04***/3+	5.8E-02/2+	5.8E-02/3+	1.1E-03/2+	***2.9E-04***/2+	1.4E-01/1+	7.2E-02/1+	8.7E-03/2+	5.8E-03/1+	6.9E-02/3+	1.4E-03/3+
Mn^{2+}	2.7E-02/2+	5.5E-02/3+	5.5E-02/2+	N/D	8.2E-03/2+	***2.7E-03***/2+	6.0E-02/1+	6.3E-02/1+	2.7E-02/2+	4.1E-03/2+	2.7E-02/3+	2.2E-02/3+
Ag^{+}	1.0E-01/2+	N/D	1.0E-01/1+	***1.0E-03***/2+	5.3E-02/2+	1.0E-02/2+	6.4E-02/2+	N/D	2.9E-02/1+	3.7E-03/1+	2.1E-01/1+	N/D
Ru^{3+}	2.5E-01/4+	N/D*	N/D	N/D	6.1E-03/2+	***7.6E-04***/2+	3.0E-01/1+	8.1E-02/1+	5.1E-02/1+	N/D	2.5E-01/1+	1.0E-02/1+
Sn^{2+}	2.8E-01/1+	N/D	3.6E-01/1+	N/D	7.2E-02/2+	***1.2E-02***/2+	6.0E-01/2+	2.1E-02/2+	1.2E-01/1+	6.0E-02/1+	6.0E-01/1+	N/D

*N/D – Indicates that a product ion was not able to be detected

Bold and cursive typeface indicates the lowest limit of detection achieved for each analyzed anionic complex $[M^{n+}+EDDS]^{-}$.

This was expected since a doubly charged ion pairing reagent could not make an overall positively charged complex because the chelating agents can carry multiple negative charges under normal conditions. Other factors that can contribute to the overall sensitivity of this method are the ability of the ions to ionize efficiently, and solution phase binding[240] .

The goal of this study was to create a sensitive method in which anionic ions can be detected at a higher sensitivity in the positive ion mode rather than the traditional negative ionization mode ESI-MS. The sensitivity of the cationic pairing approach is even more apparent when compared to LODs achieved in the negative ion mode where no cationic ion pairing reagent is present. These results are shown in Table 6.3. Analysis of each complex was performed in both SIM and SRM modes. The SRM analysis was not successful for most complexes (Table 6.3). The limits of detection were significantly higher in the negative ion mode. For example, Mg^{2+} and Cu^{2+} showed an improved LOD of more than 5000x in the positive ion mode.

Other metals such as Ca^{2+}, Fe^{2+}, Ru^{3+}, were not detected at all in the negative ion mode at our starting concentration when using EDDS as the chelating agent. In the positive mode ESI-MS these three metals were easily detected at ppb/ppt levels. Other metals were detected with a few hundred to a thousand times greater sensitivity in the positive ion mode.

Comparisons of the MS flow injection profiles for Ca^{2+} in negative and positive ion mode ESI-MS are shown in Figure 6.4. Panel (A) shows the lowest LOD (20 pg) of $[Ca^{2+}+EDTA]^{1-}$ detected in the negative ion mode. Figure 6.4 (B) shows an injection of 400 fg of $[Ca^{2+}+EDTA]^{1-}$ complex also in the SIM negative ion mode and a signal was not observed. Panel (C) of Figure 6.4 shows the same 400 fg sample now analyzed in the SIM positive ion mode ESI-MS (using Tet 1). This is a >50x improvement in LOD from negative to SIM positive ion mode. This limit of detection was further lowered in the SRM positive ion mode also using Tet 1 (Fig. 6.4, D).

Table 6.3. The best limits of detection (pg) of the metal complexes, achieved during this analysis in the negative and positive ion mode ESI-MS. The first column of the table shows the lowest LODs achieved in the negative ion mode and the corresponding chelater. The second and third column shows the LODs (pg) in positive ion mode and the chelater and ion pairing reagents that was responsible for these LODs. The last column of the table represents the improvements in LODs from negative to positive ion mode ESI-MS.

	Negative Ion Mode LODs	**Positive ion mode LODs**	**Chelater/Ion Pairing Reagents responsible for lowest LODs**	**Improvement**
Cu 2+	9.4 /EDDS	0.031	EDDS /Tet 1	303x*
Mg 2+	14 /EDTA	0.018	EDDS /Tet 1	778x
Ca 2+	20 /EDTA	0.018	EDTA /LTC1	1111x
Co 2+	140 /EDDS	0.14	EDDS /Tet 1	1000x
Fe 2+	28 /EDTA	0.088	EDDS /Tet 1	318x
Fe 3+	28 /EDTA	0.083	EDDS /Tet 1	337x
Ni 2+	34 /EDDS	0.29	EDDS /Tet 1	117x
Mn 2+	24 /EDTA	0.068	EDTA /Tet 1	353x
Ag +	100 /EDDS	1	EDDS /LTC 2	100x
Ru 3+	450 /EDTA	0.76	EDDS /Tet 1	592x
Sn 2+	300 /EDTA	4.5	EDTA /Tet 1	67x

The flow injection analysis displayed in (D) (Figure 6.4), represents $[Ca^{2+}+EDTA]^{1-}$ complex in the SRM positive ion mode when the same ion-pairing reagent (Tet 1) was used. In this case, to achieve a S/N of 3 the concentration was lowered to 60 fg (~7x and 340x improvement in comparison to the SIM positive and SIM negative ion mode respectively). The low background noise contributes to the low LODs achieved in this mode. SRM analysis was not successful for this complex in the negative ion mode.

Table 6.4. The limits of detection (pg) of all metals when complexed with each of the chelating agents in both SIM and SRM analysis.

	EDTA		EDDS	
	SIM LOD (pg)	SRM LOD (pg)	SIM LOD (pg)	SRM LOD (pg)
Cu^{2+}	280	N/A	9.4	N/A
Mg^{2+}	14	N/A	18	24
Ca^{2+}	20	N/A	N/A	N/A
Co^{2+}	290	N/A	590	140
Fe^{2+}	28	28	N/A	N/A
Fe^{3+}	28	N/A	30	N/A
Ni^{2+}	290	N/A	34	140
Mn^{2+}	24	N/A	30	140
Ag^{+}	480	N/A	100	N/A
Ru^{3+}	450	N/A	N/A	N/A
Sn^{2+}	300	N/A	1500	N/A

When comparing the two chelating agents, EDTA complexes had better LODs for four out of the 11 metals analyzed in this study (Ca^{2+}, Mn^{2+}, Ag^{+}, Sn^{2+}). These improvements varied from 3 times (Ag^{+}) to 40 times (Mn^{2+}) when compared to EDDS complexes. There was only one metal, Ni^{2+}, for which the lowest LOD (2.9E-04 ng) was achieved by both chelating agents. Currently, we are examining a much broader range of chelating agents to see if further improvements in the limits of detection can be achieved, and to better understand which chelating agents would be optimal for this type of analysis.

When analyzing the large ternary complexes in this study, all possible mass to charge (m/z) formations were monitored. In most cases, +1 and +2 complexes were observed in both SIM and SRM positive ion mode analysis. In the SRM experiments, these ions undergo collision-induced dissociation (CID), which causes the precursor ion (monitored in the SIM mode) to fragment into many product ions. Fragmentation patterns varied with the pairing reagents. In most cases, the new ions monitored in the SRM mode were just from the ion pairing reagents. For example, the ions monitored from LTC 2 resulted in a loss of one benzylimidazolium group or the loss of two hydrogen atoms from the imidazolium moieties. The main fragment monitored from LTC 1 was a loss of tripropyl-phosphonium terminal group.

In cases where the fragment represented the loss of a terminal charged group, the rest of the ion pairing reagent remained associated with the chelated metal. An example of this is shown in Figure 5, which represents a proposed fragmentation pathway for the tandem MS analysis of the $[Mn^{2+}+EDTA]^{2-}$ complex in the positive ion mode ESI-MS.

When Tet 1 is used as an ion pairing reagent, the overall complex monitored in the SIM positive ion mode has a m/z of 1405.6 (2+). In this mechanism (Figure 6.5) the new fragment monitored is a complex representing the tetracationic reagent (which loses one terminal end group, triphenyl phosphonium group, m/z 262 and becomes triply charged) associated in the gas phase with the $[Mn^{2+}+EDTA]^{1-}$ complex.

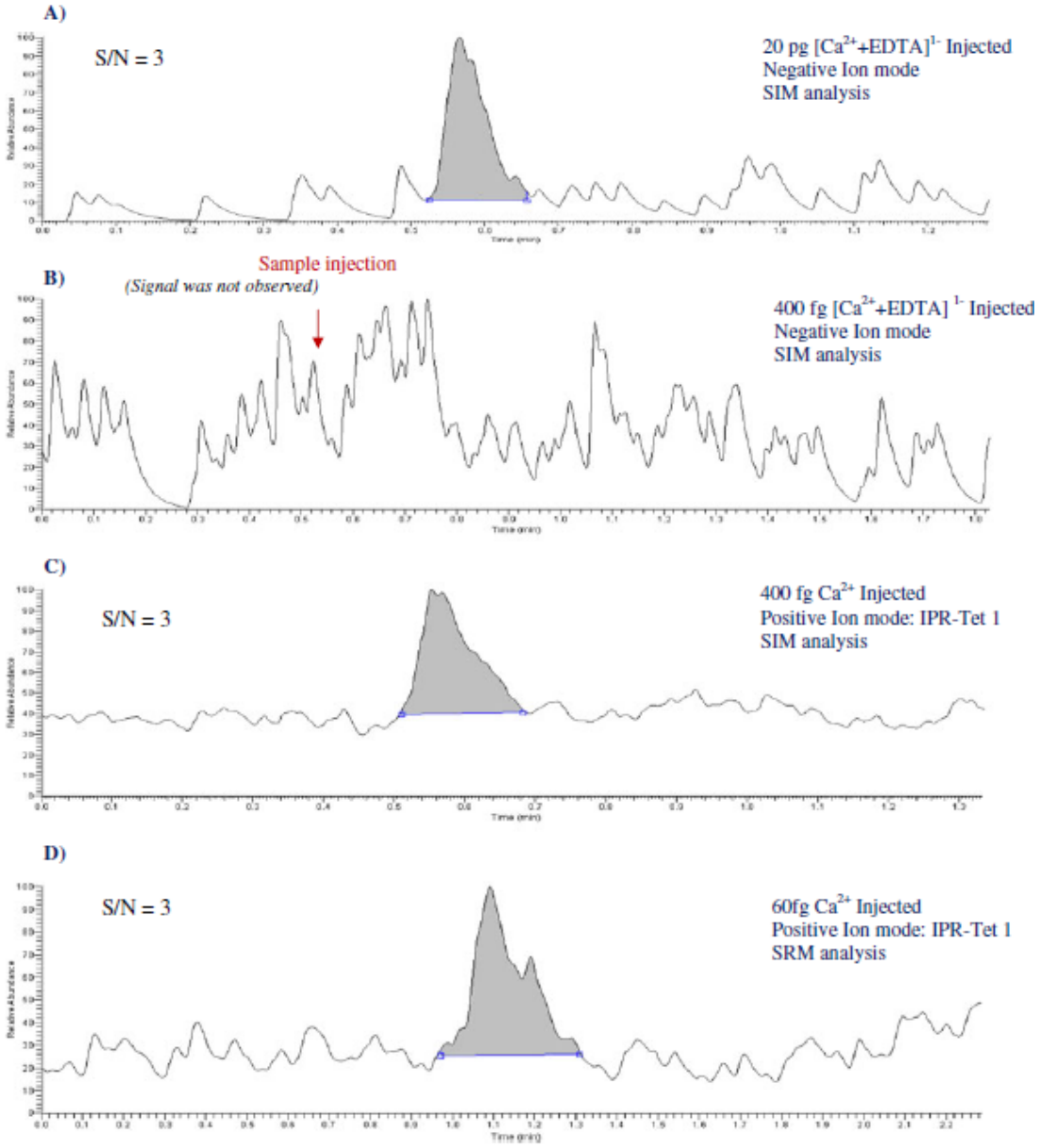


Figure 6.4. A comparison of limits of detection of Ca^{2+} achieved in the negative and positive ion mode ESI-MS. The panels shown in (A) and (B) demonstrate an injection of the $[Ca^{2+}+EDTA]^{1-}$ complex monitored in the SIM negative ion mode at two different concentrations. Panels (C) and (D) display the results achieved when the $[Ca^{2+}+EDTA]^{1-}$ complex was monitored in the SIM and SRM positive ion mode with the addition of the ion pairing reagent Tet 1. The m/z monitored in the negative ion mode was 329.1(1-), in the positive SIM ion mode was 695.7(2+), and in the positive SRM ion mode was 564.3(2+).

During CID, $[Mn^{2+}+EDTA]^{2-}$ complex gains a proton becoming singly charged, thus forming an overall new complex with a mass to charge ratio of 571.7 (+2). A further study on the effect of pH and other important variables on these types of analytes and analysis has been completed and is submitted elsewhere.[261] It was shown that the best detection limits were obtained when the analyzed solutions were at $5 \geq pH \leq 7$.The method developed herein shows comparable results with other, widely and commonly used methods such as ICP-MS and AAS. A comparison of the reported LODs of the metals by more conventional techniques with the results of this study are given in Table 6.5.[63, 262-269]

Table 6.5. Comparison of detection limits (µg/L) for five metals measured by three different instruments and methodologies.

Metals	ICP-MS	AAS	ESI-MS
Mn (II)	0.00026*	0.13	0.0136
Cu **	0.002	0.05	0.0062
Fe	0.00117	1.06	0.0166
Ni (II)	0.02	1.9	0.058
Ag (I)	0.15	0.6	0.2

* Since not all the references reported the amount of sample injected to find the LOD of these metals, for comparison purposes we converted our LODs to concentration units.
** The oxidation cannot be distinguished by ICP-MS and AAS, however it can by ESI-MS. In this table Cu(II) and Fe (II) were monitored by ESI-MS

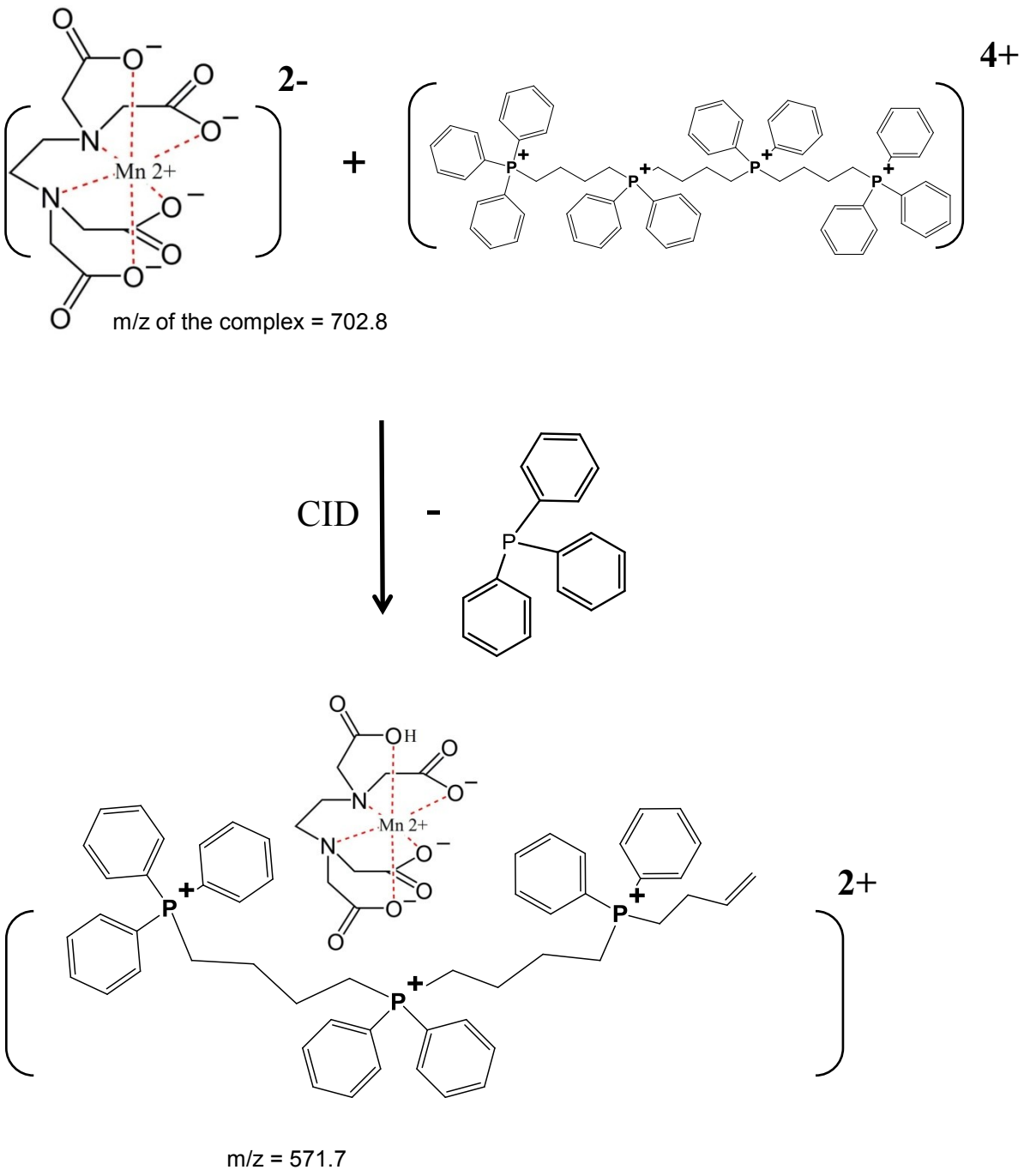


Figure 6.5. A proposed fragmentation pathway for the tandem MS analysis of Mn^{2+} when complexed with EDTA and Tet pairing reagent in the positive ion mode ESI-MS.

6.5 Conclusion

In this study two common chelating agents have been used to form anionic complexes with eleven different metals. These compounds were then paired with tri-, and tetra-cationic ion pairing reagents, which were synthesized in our laboratories. These new ternary complexes were positively charged, and were monitored in the SIM and SRM positive ion mode ESI-MS, while the LODs were reported and compared to the ones achieved in the negative ion mode. The LODs in the positive mode were significantly better (often several orders of magnitude) than in the negative ion mode.

The best ion-pairing reagent for the detection of metals is the perphenylphosphonium tetracation Tet 1 (Figure 6.3) followed by LTC 1 and Tet 3. It appears that phosphonium based cationic reagents with aromatic moieties and short alkyl chain linkages are ideal ion pairing reagents for the detection of chelated metals. These limits of detection can be further improved with further optimization of ESI parameters.

Also, different, more sensitive mass analyzers such as a quadrupole mass analyzer would further lower the limits of detection especially in the SRM mode. The new detection method described herein was applied successfully to free metals in aqueous solution. Further investigations of the different uses of this method are under considerations. This experimental approach is very simple, with short analysis times, and most importantly, very low limits of detection. The flow rates used with this technique are compatible with HPLC and CE. Other advantages of this method include the detection of low mass cations at higher mass ranges where there is less background noise, sample preparation or pre-concentration step are often not needed and sample speciation is straight forward.

CHAPTER 7

DICATIONIC ION-PARING AGENTS USED FOR THE DETECTION OF ANIONS IN POSITIVE ION MODE ESI: MECHANISTIC DETERMINATIONS AND CONSIDERATIONS

7.1 Abstract

Recently, we have shown that dilute multivalent cationic reagents can be paired with analyte anions in ESI-MS, thereby allowing them to be detected in the positive mode at very low limits of detection. However, there can be differences in the efficiency of this technique depending on the nature of the cationic pairing agent and the anion being analyzed. In this study, three dicationic ion-pairing agents and four singly charged anionic species were examined in a series of experiments to elucidate the mechanism of action that allows for such sensitive detection and the profound differences in the selectivity of this ion-pairing method. The binding constants for the dication/anion complexes were determined by NMR and ESI-MS. The results indicated that the binding of these species is greatly enhanced as they move from the solution phase to the gas phase. Furthermore, surface tension measurements for the complexes were performed. This test revealed that, as the dication pairs with the anion, it creates a surface-active species within the ESI droplet. This is determined to be one of the major factors that leads to the overall sensitivity enhancement. This has led to a better understanding of how this ion-pairing technique produces unprecedented limits of detection for anions and why there are selectivity differences in pairing agents of different structures.

7.2 Introduction

Throughout the preceding chapters a new technique for the detection of anions in the positive ion mode of electrospray ionization mass spectrometry (ESI-MS) has been described. This has been achieved through complexation of the anions with multiply charged cationic ion-paring reagents. In each of these experiments, many limits of detection (LOD) have been reported. Nearly all the LODs determined were far lower than those found using the negative

ion mode with no cationic ion-pairing reagent. Furthermore, several of these LODs were found to be lower than any reported by any analytical technique.[174]

All of the LODs were determined empirically with little disscusion about the mechanism by which these ion-paring reagents produce such sensitive results. In fact, it is very interesting that complexation can produce such high sensitivity considering the formation of complexes is generally regarded as a source for ionization suppression. For example, it has been documented that the addition of trifluoracetate to a spray solution will suppress the signal of positively charged analytes (such as peptides).[270, 271] Nonetheless, the complexation described herein has proven to enhance the signal for most anionic species.

In previous studies, many empirical observations were made concerning the structural motifs of both the anions and the ion-pairing reagents, which seemed to enhance detection. First, it was generally observed that more chaotropic anions yielded lower LODs. Soukup-Hein *et al*. showed that the relative order of sensitivity for some anions loosely followed the Hofmeister series (a series that describes the ability for an anion to change the ordering of water molecules).[174] Also, it was observed that anions containing halogen atoms were more sensitive than non-halogen containing analogues. This again was attributed to the fact that these anions were more chaotropic. Lastly, it was observed that anions of a higher oxidation state had lower detection limits than those of a lower oxidation state.

It was observed that flexible dicationic agents performed better than rigid reagents. This was also found to be true for tricationic and tetracationic ion-paring reagents. Other trends that were observed are as follows: i) reagents having hydroxyl groups worked more poorly than those that did not; ii) the presence of aromatic groups in the ion-paring agents was found to be advantageous when detecting aromatic anions, indicating that π-π interactions are important; iii) it was found that reagents containing alkyl linkage chains between the cationic moieties performed as well as polyethylene or perfluoro-alkyl linked dications (thus the extra synthetic work required for the later could be avoided); iv) phosphonium based tetracationic ion-pairing

reagents outperformed imidazolium based agents. From these observations, four dicationic, four tricationic, and three tetracationic ion-pairing agents have been identified as most successful. Their success has lead to the commercialization of one dicationic and one tricationic ion-pairing reagent.[272]

Though these empirical observations have been made, there is still a lack of understanding as to the exact mechanism(s) that allow this ion-pairing method to be so successful. It appears there are at least three factors. First, there must be some consideration as to the role of the solution phase binding between the ion-pairing agent and the anion. The second factor is the ionization efficiency of the complexes. A third point to be considered is the potential for the anion (in its complexed form) to undergo oxidation during the electrospray process. The later is one source for the observation that anions in higher oxidation states were detected more sensitively. This factor will not be examined in this study.

In this work three dicationic ion-pairing reagents and four mono-valent anions are studied. The solution phase binding constants, as determined by Nuclear Magnetic Resonance spectroscopy (NMR) are evaluated. For comparison, an online dynamic titration technique is used to determine the binding constants by ESI-MS.[273] The ionization efficiency of these systems are correlated to their surface activity and measured using surface tensiometry. A comparison of the LODs produced by these systems to the results found herein will be made. From these results, a clearer understanding of the factors that affect these systems will be revealed and additional mechanistic considerations will be presented.

7.3 Experimental

7.3.1 Materials

Sodium bromide (NaBr), sodium iodide (NaI), sodium benzoate (NaBzO), and sodium benzenesulfonate (NaBZSN) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The deuterated methanol and deuterated water used in the NMR titrations, as well as, the sodium hydroxide, sodium fluoride, and Amberlite IRA-400 (chloride form) used for the ion exchange,

were also purchased from Sigma-Aldrich. The deionized water used for the surface tension experiments was obtained from our in-house Milli-Q (Millipore Corporation, Billerica, MA, USA) system. The water and methanol used in the ESI studies were purchased from Burdick and Jackson (Morristown, NJ, USA). The dicationic reagents were synthesized in their bromide form as outlined in Chapter 5 and were subsequently ion exchanged to their fluoride form following a previously outlined procedure.[174]

7.3.2 ESI-MS analyses

The MS used in this study was a Thermo Finnigan LXQ (Thermo Electron Corporation, San Jose, CA, USA). The general set-up for both the LOD determinations and the dynamic titrations was the same. In short, a Thermo Finnigan Surveyor MS pump (Thermo Electron Corporation, San Jose, CA, USA) was used to provide a 300 µL/min solvent stream (67/33 methanol/water) which was mixed (via a low volume mixing tee) with a 100 µL/min solvent stream containing 8 µM dicationic ion-pairing reagent. The second solvent stream was applied using a Shimadzu LC-6A pump. The resulting solution was a 2 µM dicationic ion-pairing reagent solution in 50% water 50% methanol. This solution was pumped directly into the ESI interface at 400 µL/min without any flow manipulation. The flow from the Surveyor pump provided the solvent for which the anions were added. Red PEEK tubing (i.d. 0.005 in) and Blue PEEK tubing (i.d. 0.010 in) were used for all the solvent streams in the LOD studies and dynamic titration experiments, respectively.

The anions were added using direct injection via a 6-port injector equipped with 5 and 10 µL injection loops for the LOD measurements and dynamic titrations, respectively. The MS conditions were as follows: spray voltage, 3 kV and 5 kV (for the LOD and dynamic titration studies, respectively); capillary temperature, 350 °C and 275 °C (for the LOD and dynamic titration studies, respectively); capillary voltage, 11 V; tube lens voltage, 105 V; sheath gas flow, 37 arbitrary units (AU); auxillary gas flow, 6 AU.

For the LOD measurements, stock solutions of the anions were made at a concentration of 1 mg/mL. From the stock solutions, serial dilutions were made to minimize error. To find the LODs, the solutions were injected five times and successively diluted until a set of five injections at a given concentration resulted in a signal-to-noise (S/N) ratio of 3. Selected Ion Monitoring (SIM) was applied to find the LODs.

For the dynamic titration measurements, solutions containing either 10 µg/mL (for NaI and NaBZSN) or 20 µg/mL (for NaBr and NaBzO) were injected. The only difference in the general set-up was the addition of a long length of tubing (≈6 m) after the mixing tee and before the ESI interface. This tubing is essential for the dynamic titration technique, such that the complex will diffuse longitudinally and create a Gaussian distribution.[273] The titration for each system was repeated six times while spectra was taken from a m/z range of 100 to 1000. The data was treated using an in-house software tool that was written in Microsoft Visual C # 2005 Express Editions.[273] To run this program, the relative response ratio of complex to free host was assumed to be 1. The software automatically generated dissociation/binding constants for the systems.

7.3.3 NMR analyses

The instrument for the NMR binding determinations was a JEOL ECX 300 MHz (Tokyo, Japan). The solvent used was 50% deuterated methanol 50% deuterated water. Methanol was used as the lock solvent. All experiments were completed at 25 °C. The shifts were determined in reference to a tetramethylsilane (TMS) standard.

The dicationic ion-pairing reagent solutions were prepared by weighing 10 mg of the dicationic salt and dissolving them in 0.8 mL of 50% deuterated methanol 50% deuterated water. The anion stock solutions were prepared in deuterated water such that 2 µL of the stock contained 0.1 molar equivalent of the ion-pairing reagent that was being titrated. A zero measurement was first made for just the free dicationic solution. Next, successive additions of the anions and measurements of the chemical shift changes were recorded. From 0.1 to 1.0

molar equivalents, measurements were made at every 0.1 equivalents. From 1.0 to 2.0 molar equivalents, measurements were made at every 0.2 equivalents. Finally, 2.5 and 3.0 molar equivalents were measured. In each case, protons alpha to the positive charge in the dications were monitored as the titration was performed. The change in the chemical shifts and the concentration of the titrant were then used to determine the binding constants through non-linear least squares regression using the WinEQNMR2 (Galway, Ireland) computer software.[274] The error was estimated using a biased linear regression estimate treating the partial derivatives as constant coefficients of the parameters.

It is important to note, that the systems were also titrated with sodium fluoride to observe in changes in chemical shift from the change in ionic strength. In doing so, it was determined that changes in ionic strength did not cause measurable changes in chemical shifts.

7.3.4 Surface tension analyses

The instrument used for the surface tension measurements was a Fisher Model 20 Surface Tensiometer. The platinum ring used had a mean circumference of 5.94 cm and a ring/wire radius ratio of 53.2113942. The surface tensiometer was calibrated using an object of known mass, such that the readings obtained were directly in Dynes/cm. All measurements were made at 25 °C.

The surface tension based titrations were performed by the successive addition of anions to a 0.1 M dication bulk solution. The increments for the addition of titrant (anions) were 0.1 molar equivalents up to 2.0 molar equivalents, then 0.5 molar equivalents up to 5.0 molar equivalents. Measurements were made in triplicate. Also, pure water was titrated with the anions an identical fashion to serve as a blank.

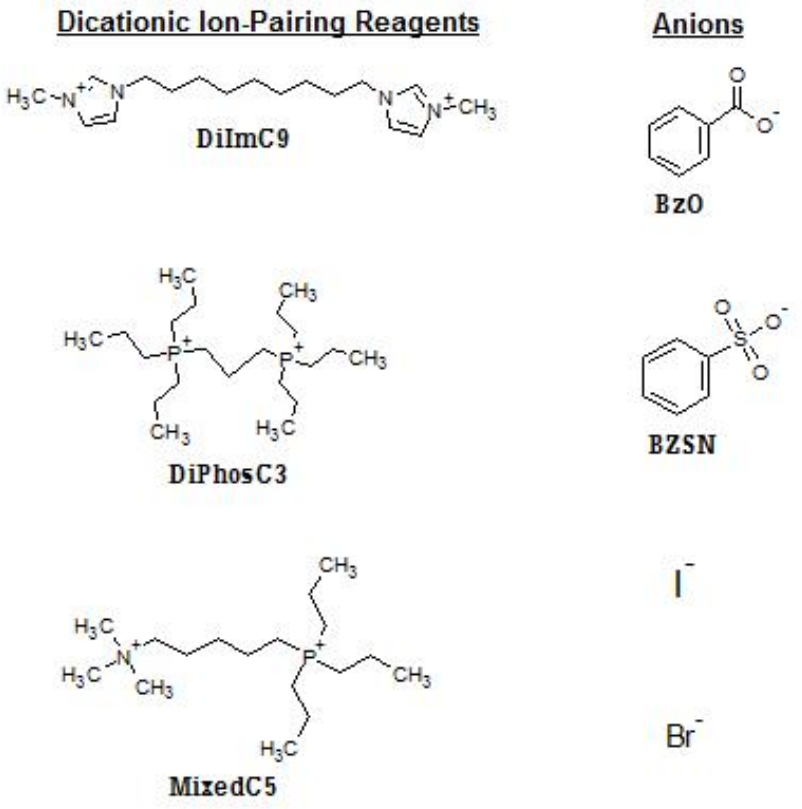


Figure 7.1 Structures of the pairing agents and anions.

7.4 Results and Discussion

7.4.1 LODs in the SIM positive mode

Many of the ion-pairing systems studied herein have been reported previously[174], while others have not. All anions used in this study were in their sodium salt form, which in some cases (sodium benzoate) differed from the original analysis. For this reason, it was first necessary to determine the limits of detection (LODs) for all the systems used in this study. Table 7.1 lists the LODs found for four anions (Figure 7.1) in conjuction with three dicationic ion-pairing agents (Figure 7.1).

As can be seen in Table 7.1, the LODs spanned a range of approximately two orders of magnitude.The DiPhosC3/iodide complex was detected at 1.08 x 10^{-3} ng, while the MixedC5/benzoate complex was only detected at 1.68 x 10^{-1} ng. The data was consistent with previous studies[174,177], with the exception of the detection of benzoate (BzO^-). Here the detection limits for BzO^- are lower. This is because the initial studies used benzoic acid as the source for BzO^-, whereas, sodium benzoate was used in this study. Due to the weak acidity of benzoic acid, it is sensible that the use of the sodium salt would yield lowed LODs.

However, the overall trends in sensitivity, as reported earlier, have been preserved. In short, benzenesulfonate ($BZSN^-$) and iodide (I^-) produced lower LODs than bromide (Br^-) and BzO^-. Also, DiPhosC3 and DiImC9 generally produce lower LODs than MixedC5.

Table 7.1 LODs for the dication/anion complexes.

Dication	$BZSN^-$	BzO^-	I^-	Br^-
DiImC9	2.06 x 10^{-3}	1.68 x 10^{-2}	6.00 x 10^{-3}	6.00 x 10^{-2}
DiPhosC3	1.03 x 10^{-3}	1.26 x 10^{-1}	1.08 x 10^{-3}	1.17 x 10^{-1}
MixedC5	1.55 x 10^{-2}	1.68 x 10^{-1}	3.88 x 10^{-2}	7.77 x 10^{-2}

LODs reported as the abosolute mass in ng.

7.4.2 Solution phase binding constants determined by NMR

The first factor that may lead to the low LODs (and the differences from complex to complex) obtained through this method is the ability for the dicationic ion-pairing reagent to associate with the anions of interest in solution. It is important to note that this association must take part in the solution phase. If it did not take part in solution, large dication/fluoride complex signals should be observed. This is because the dicationic ion-pairing agent is added in its fluoride form, and if the binding were occurring exclusively in the gas phase, fluoride complexes would dominate, due the small size-to-charge ratio of fluoride and its higher concentrations.

These complexes were never observed in the background. Knowing this, it is of up-most importance to determine the solution phase binding constants for these systems. Our first attempt to determine the association constants for the complexes was done using CE. Binding constants for some monocationic and monoanionic salts, as determined by CE, have been reported previously.[275, 276] However, severe wall interaction of the dicationic ion-pairing agents, among other reasons, hindered the success of this approach. Binding constant determinations using NMR also is a well-established method. In fact, NMR based evaluations of cation-anion associations have already been reported.[277]

In these reports, NMR titrations were performed and the binding constants were determined through a non-linear regression-fitting program WinEQNMR2. Association constants for all of the dication-anion pairs in this study were obtained by this approach (see Experimental). Figure 7.2 shows an example of a non-linear NMR titration performed for this study. As can be seen, the titration data exhibits a good fit to the curve and thus could be used to obtain the association constant.

Figure 7.3 illustrates the solution phase binding constants as determined by NMR. The determined binding values ranged from 53 M^{-1} to 128 M^{-1} for the DiImC9/BzO^- complex and the DiPhosC3/I complex, respectively. Literature values for ion interactions is aqueous media were just slightly less than this range, while other reports for ion interactions in organic media (ACN) were much higher (due to the absence of waters of hydration)[277]. Since the solvent used in these experiments was 50% methanol and 50% water (by volume), the magnitude of these constants are quite reasonable.

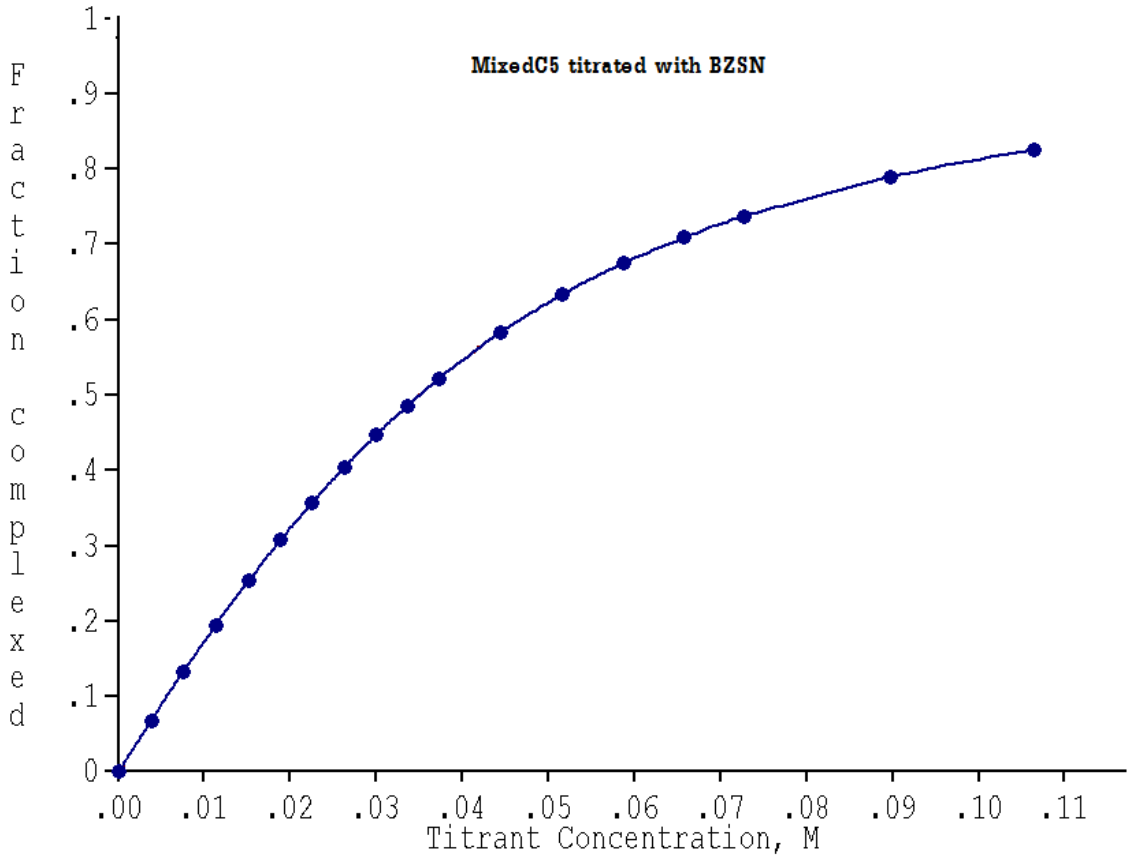


Figure 7.2 Non-linear curve used to determine binding constants by NMR.
For the conditions used to obtain this curve, see the Experimental section
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Though the binding between dication and anion is fairly weak (compared to gas phase interactions), some notable differences were observed. The most interesting observation was that DiPhosC3 typically produced larger association constants. As reported previously, phosphonium based ion-pairing agents tend to work quite well for the ESI-MS detection of anions. It seems that at least one of the reasons for their success is their ability to bind anions in solution more strongly than other pairing agents.

Next, it was observed that iodide associates most strongly to the non-aromatic dications (DiPhosC3 and MixedC5), while the aromatic DiImC9 dication preferred to bind benzenesulfonate ($BZSN^-$). This indicates that π-π interactions are important for the association

of the DiImC9 dication to pair with anions. Lastly, it was observed that in all cases, bromide had smaller association constants than iodide. This result indicates that binding takes part in the solution phase, as it is known that bromide will be more hydrated than iodide, and thus bind cations less strongly.

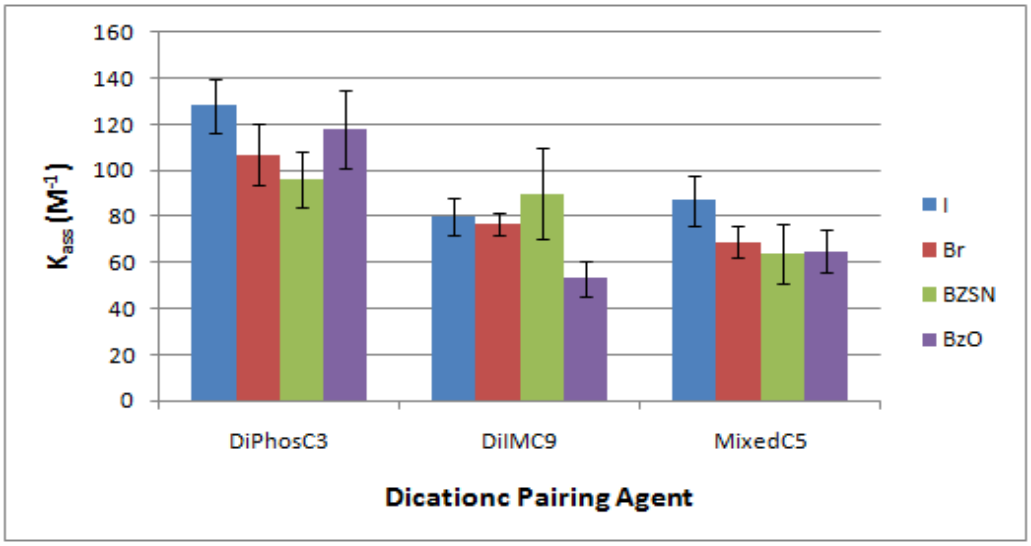

Figure 7.3 Solution phase binding constants determined by NMR.
For the experimental parameters used and the estimates of the errors, see the Experimental section.

However, a direct correlation between the solution phase binding constants and the LODs in ESI-MS was not always found. However, some trends can be explained. First, the binding constants for iodide are always higher than those for bromide. This directly follows the LOD trend. Second, iodide and $BZSN^-$ typically have larger association constants than bromide or BzO^-. This again is reflected in the LODs. In contrast, the large range in LODs (two orders of magnitude or more) cannot be fully explained by looking at the solution binding alone. For example, MixedC5 always generated LODs about an order of magnitude less than DiImC9.

This is not reflected in the binding data, which suggests these two paring agents have similar binding efficacies for these anions.

7.4.3 Association constants determined by ESI-MS

Considering that these LODs are obtained using ESI, a measure of the association behavior using ESI would be useful. To accomplish this, on-line dynamic titrations were performed. This technique is explained in detail elsewhere.[273] In short, a constant amount of host (dication) is introduced to the ESI-MS, while and injection of guest (anion) is made.

The host-guest complex is then allowed to diffuse in a length of tubing to yield a Gaussian distribution of the complex. By treating each scan of the MS as an individual titration step, a large number of steps can be recorded. Plotting the ratio of complex intensity over free dication intensity versus the time, and using a Gaussian peak-fitting program, a binding constant can be generated. An example of the Gaussian peak fit is shown in Figure 7.4 and the resulting binding constants are shown in Figure 7.5.

As can be seen in Figure 7.5, most of the trends observed in the NMR experiments were not maintained. One observation was that iodide and $BZSN^-$ yielded larger association constants than did bromide or BzO^-. This followed the LOD trends. The NMR results indicated that DiPhosC3 generally yielded the largest binding constants, whereas the ESI-MS results indicate that MixedC5 binds anions best. This is not in correlation with the LOD results, where MixedC5 was the least effective ion-pairing agent.

It should be noted that in the determination of these binding constants, the ratio of the response factors for the complex and the dication was assumed to be unity. This introduces a large potential error in these measurements. Ways to determine the actual response factor of the complex continue to be investigated.

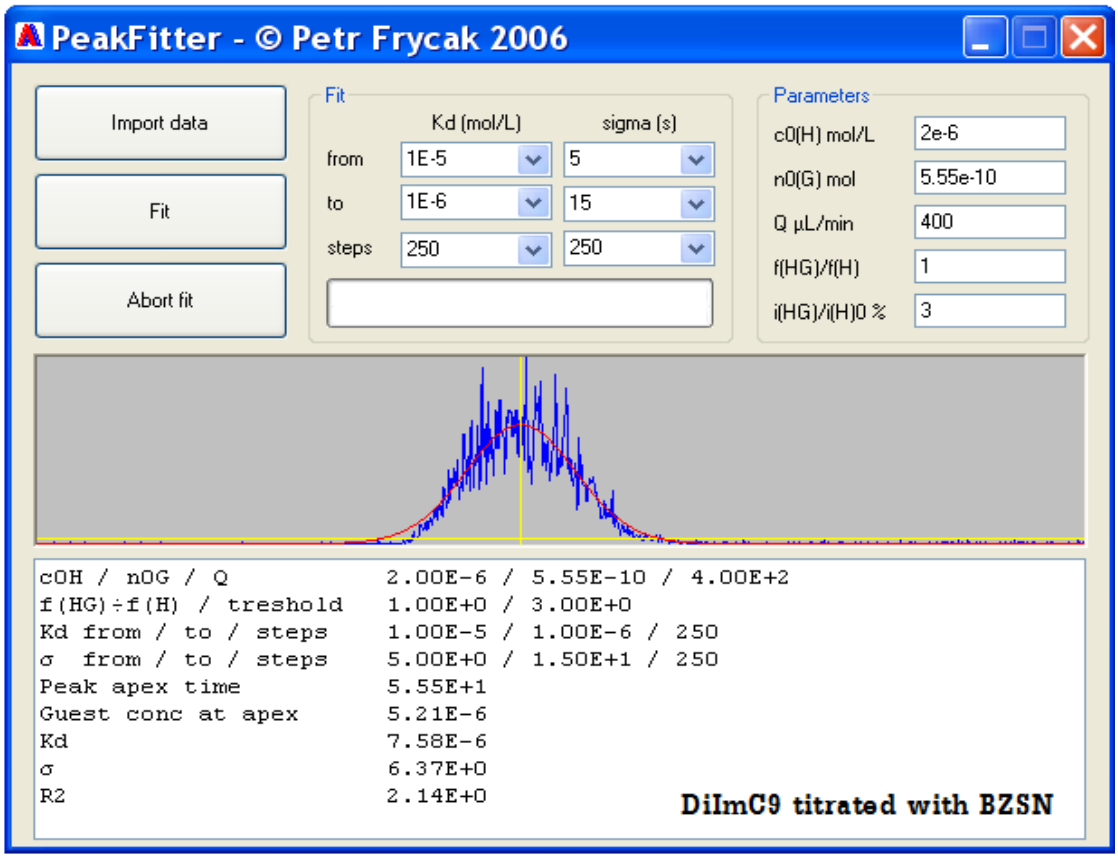

Figure 7.4 Example of Gaussion peak fitting.

This is the output file from the in-house peak fitting program. The system used to produce this figure was DiImC9/BZSN. For ther parameters, see the Experimental section.

Perhaps the most profound discrepancy between the binding constants determined by ESI and those found using NMR is simply the amount (i.e. orders of magnitude) by which they differ. All the solution phase binding constants were found to be approximately 10^2 M^{-1}, whereas the values obtained by ESI were approximately 10^3-10^5. This means their values differ by about 2 orders of magnitude. This indicates that a considerable enhancement in the binding occurs when these complexes are desolvated. Though the desolvation process is complicated, there have been reports of enhancement of host-guest binding systems when they undergo electrospray ionization.[278-281, 257]

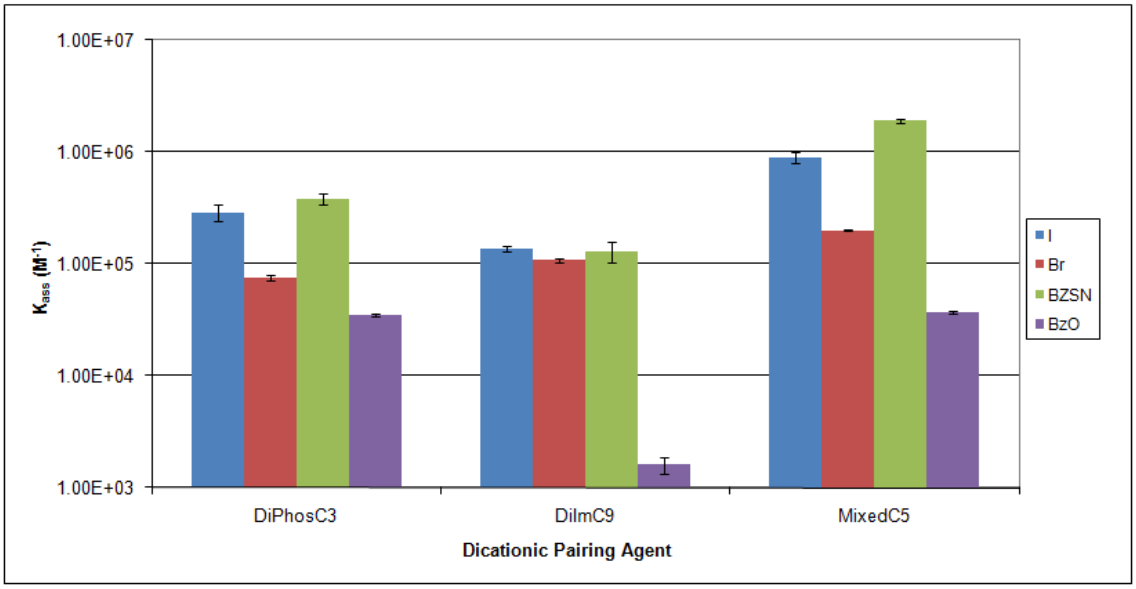

Figure 7.5 Solution phase binding constants determined by ESI-MS.

One such study showed that the "shrinking of the droplet" and consequent increase in concentration can lead to enhanced complex formation.[280] Since the dication/anion complexes observed in the ESI-MS are 1:1 binding systems (1:2 would be neutral and not observed), their rate constant would be second order. If this rate (the rate at which the dication, anion, and complex equilibrate) can occur faster than the desolvation process, the concentration of the complex will increase exponentially.

A second model which further describes the enhancement of binding systems during the ESI process is referred to as the equilibrium partitioning model (EPM).[278, 281] Other similar models have been reported earlier for the enhancement of analyte signals in charged droplets containing surfactants.[282, 283] This model was referred to as modified aerosol ionic redistribution. In short, the EPM describes the ESI droplets as biphasic, in which the outer phase is hydrophobic and the inner phase is hydrophilic. Figure 7.6 represents this model. If there are

two phases, there will be a partitioning of the host, guest, and complex between the inner phase to the outer phase. Thus, there are far more equilibria (3 partitioning and 2 binding) to be considered than just that of the complex formation in the bulk solution. These different equilibria are also represented in Figure 7.6. Thus, this model suggests that the ion intensities observed in the mass spectra are not only a result of the ion binding, but also the partitioning equilibria of the species. The following section will investigate the affinity for the anions, dications, and the complexes for the more "hydrophobic" outer phase.

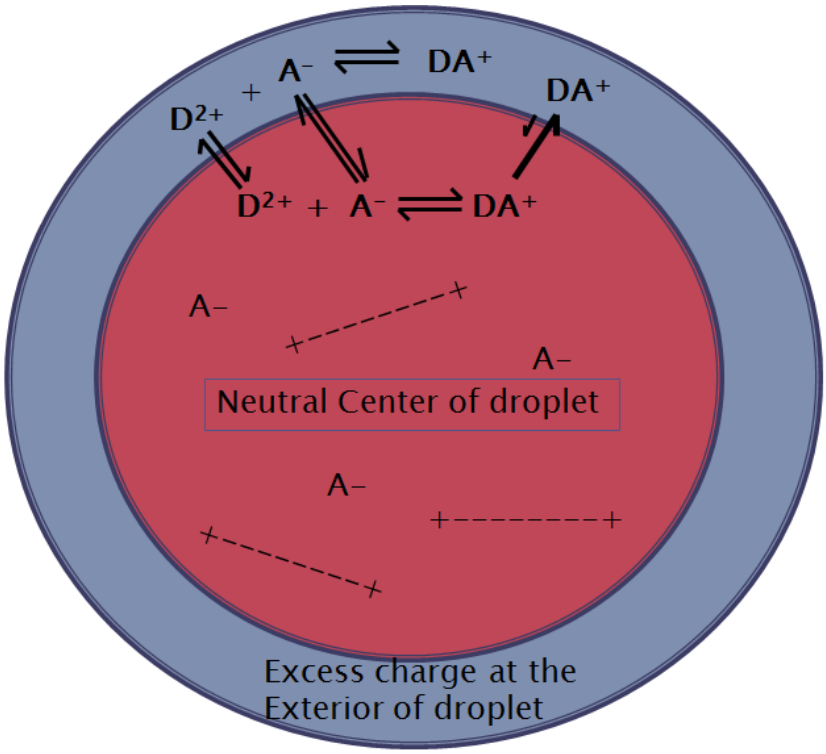

Figure 7.6 Equilibrium partitioning model.

7.4.4 Surface tension measurements

To better understand the relative ability for the dications, the anions, and the complexes to partition to the outer phase of the electrospray droplet, surface tension experiments were performed. These experiments will help show that the 1:1 complexes have a much greater surface activity than the free dication or the anion.

For a compound to have high surface activity it should possess a lipophilic portion, which will prefer to reside in or near the droplet-air interface, which is more hydrophobic. The hypothesis for this experiment is that the dicationic ion-pairing reagents themselves are not very surface active, as they possess two symmetrically spaced cationic moieties. However, when the complex forms, one of the charged moieties is neutralized, thus creating a surfactant-like compound.

Figure 7.7 shows the results of titrating 0.1 M dication (DiImC9) bulk solution with an anion ($BZSN^-$) and taking several surface tension measurements over the course of the titration. Also, provided in Figure 7.7 is result of adding the anion just to water rather than the dication bulk solution. What should first be discussed is the surface activity of the anion itself. As can be seen by Figure 7.7, the addition of $BZSN^-$ only slightly decreases the surface tension of water, meaning it is not very surface active. It should be noted that when NaBr and NaI were used, the surface tension of water increased. This is a well-documented phenomenon.[284,285] Thus, when the droplets first form, the anions will surely reside in the center of the droplet.

The next observation that was made from these experiments is that the dications alone are just slightly more surface active than the anions, lowering the surface tension of water by no more than 7 dynes/cm. All three dications had similar surface activity as determined by surface tensiometry. For reference, the surface tensions of 0.1 M DiPhosC3, DiImC9, and MixedC5 were 64.8, 66.3, and 67.2 dynes/cm, respectively. This supports the hypothesis that the dicationic reagents themselves are not tremendously surface active.

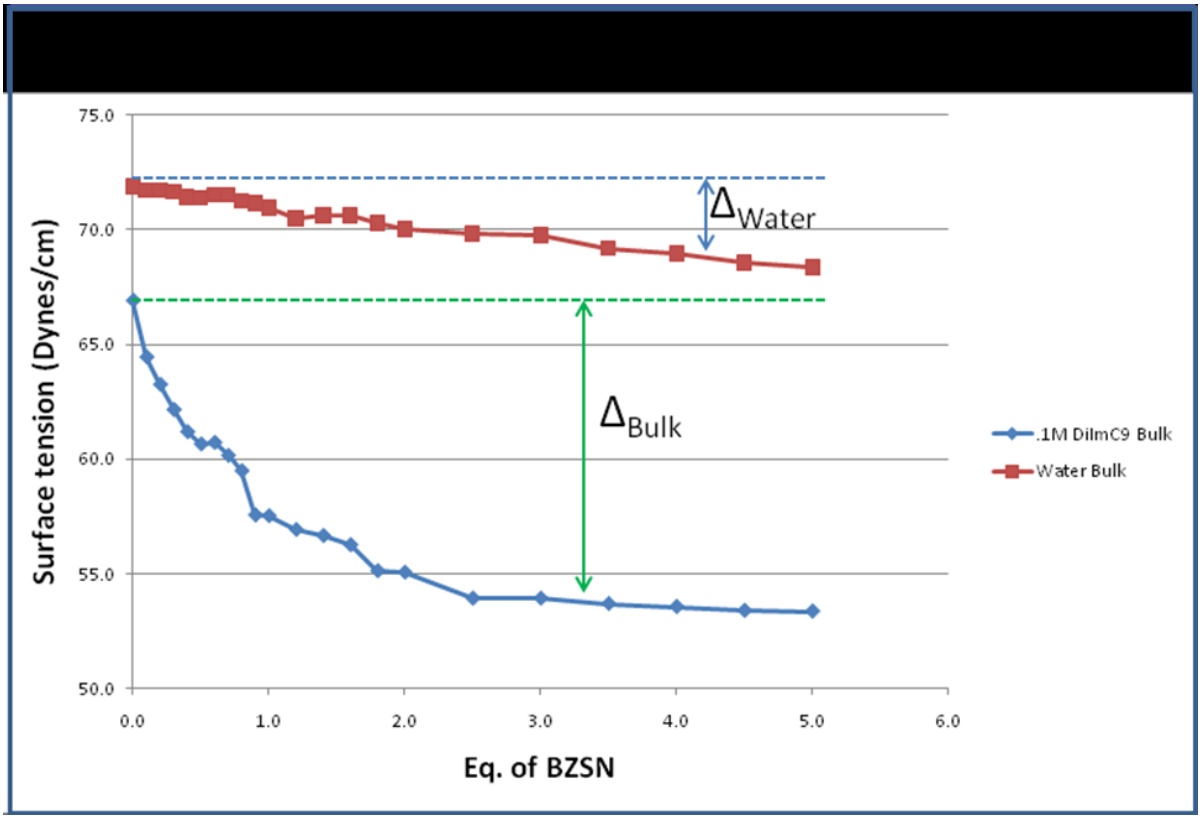

Figure 7.7 Surface tension measurements for titrating DiImC9 with $BZSN^-$.

This plot represents surface tension vs. benzenesulfonate (BZSN) concentration in neat water and a 0.1M aq. solution of the dication DiImC9. For more details on the procedure used to obtain this plot, see the Experimental section.
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The most interesting result that was observed throughout this experiment was the dramatic decrease in the surface tension of dication bulk solutions when titrated with anions. This effect is illustrated in Figure 7.7. At two molar equivalents of $BZSN^-$ added, the surface tension of the dication bulk solution was lowered by 12 dynes/cm, whereas when an equivalent amount of $BZSN^-$ was added to water, the surface tension only decreased by 2 dynes/cm. As a result, the existence of the DiImC9/$BZSN^-$ complex lowered the surface tension of water by nearly 20 dynes/cm. To make the data comparison for each dication/anion system simpler, the

$\Delta\Delta\gamma_{x\ eq}$ values will be compared (where $_{x\ eq}$ denotes the number of molar equivalents of anion that was added). $\Delta\Delta\gamma_{x\ eq}$ was determined through the following relationship:

$$\Delta\Delta\gamma = \Delta_{bulk} - \Delta_{water}$$

where Δ_{bulk} and Δ_{water} are the differences in surface tension from the point where no anion was added to the point where the described number of molar equivalents ($_{x\ eq}$) of anion is added. These values also are indicated in Figure 7.7. Subtraction of Δ_{water} serves as a normalization factor and was a slightly negative number for the inorganic salts which increased the surface tension of water and a slightly positive number for the organic salts (as shown in Fig. 7.7).

Table 7.2 lists the $\Delta\Delta\gamma$ values for all the systems in this study. Several key observations can be made from Table 7.2. First, the $\Delta\Delta\gamma$ values for bromide are usually quite low. This directly correlates to the finding that bromide detection by positive mode ESI-MS was not as sensitive as for the other anions. However, BzO^-, which also was not particularly sensitive, had $\Delta\Delta\gamma$ values that were fairly comparable to BZSN and iodide.

Next, it was observed that the DiPhosC3/I complex resulted in very high $\Delta\Delta\gamma$ values. In fact, after the addition of 3 molar equivalents of iodide, precipitation occurred. This means that this complex is very surface active and eventually becomes not only chaotropic but completely insoluble in water at higher concentrations. Clearly this is a factor that led to the very low LOD found for DiPhosC3 and iodide.

Another system that yielded large $\Delta\Delta\gamma$ values was the DiImC9/$BZSN^-$ complex. Again this was one of the more successful systems tested in terms of LODs. Yet, there is not a direct correlation between all of these results and the LOD values. For example, $BZSN^-$ had lower detection limits with DiPhosC3 than with DiImC9 or MixedC5, yet the $\Delta\Delta\gamma$ values for DiPhosC3 and $BZSN^-$ are slightly lower than the $\Delta\Delta\gamma$ with the other systems.

Table 7.2 ΔΔγ values for all the systems studied.*

Complex	$\Delta\Delta\gamma_{5eq}$	$\Delta\Delta\gamma_{1eq}$	$\Delta\Delta\gamma_{2eq}$	$\Delta\Delta\gamma_{3eq}$	$\Delta\Delta\gamma_{4eq}$	$\Delta\Delta\gamma_{5eq}$
DiImC9 + BZSN	5.8	8.5	10.0	10.9	10.5	10.1
DiImC9 + BzO	3.8	5.6	5.0	6.5	6.7	7.0
DiImC9 + I	2.5	4.6	5.9	7.6	8.4	8.6
DiImC9 + Br	0.7	1.4	2.9	4	5.4	5.6
DiPhosC3 + BZSN	3.7	5.4	6.8	7.7	7.7	7.5
DiPhosC3 + BzO	4.4	6.3	7.5	8.5	8.7	9.3
DiPhosC3 + I	6.1	9.5	12.3	precip	precip	precip
DiPhosC3 + Br	1	1.5	2.6	3.4	4.1	5.4
MixedC5 + BZSN	4.3	5.9	6.7	7.6	7.6	7.2
MixedC5 + BzO	3.7	5.6	6.3	7.1	6.7	6.7
MixedC5 + I	3	4.9	6.7	8.7	9.2	9.7
MixedC5 + Br	0.2	1.1	2.1	2.1	3.7	4.5

*For a detailed descripton of how ΔΔγ was calculated, see the preceeding text and Fig 7.7. For other parameters used to obtain these values, see the Experimental section.

Most importantly, there is clearly an increase in the surface activity of all these systems when the complex forms. This will cause a significant partitioning of the complex to the outter region (surface) of the droplet. This increase in surface activity is identified as a major factor that leads to the low LODs seen with these systems. Furthermore, the partitioning of the complex to the outer phase of the droplet will cause depletion in the concentration of the complex in the inner phase.

If the binding kinetics are faster than the desolvation process, more complex will be formed to satisfy the association constant. Due to the high surface activity of these complexes, this process may occur several times during the desolvation process, thus enhancing the degree of association of these systems.

Lastly, it can be concluded that the formation of a surface-active complex may be part of the reason why it was observed previously that flexible dications yield lower LODs than rigid agents. It was hypothesized that the ability of the ion-paring agents to “wrap around” the anions could stabilize the gas phase complex. Here we have shown that another possible reason for the success of flexible ion-paring reagents is their ability to form surface-active complexes. A scenario that would be less likely with more rigid dications.

7.5 Conclusions

In this study, three dicationic ion-pairing agents and four mono-valent anions were used as models to further understand the mechanism by which dication-anion complexes lead to ultra-high sensitivity in ESI-MS. First, the solution phase binding constants were evaluated using NMR. This resulted in the confirmation that these ions indeed bind in solution.

The binding constants were also evaluated using ESI-MS. These results did not follow the same trend as the NMR data. More importantly, it was observed that the association constants obtained via ESI-MS were approximately 2 orders of magnitude greater than the solution values determined by NMR. This leads to the conclusion that there is a enhancement in the binding which occurs during the desolvation process.

Both the shrinking droplet and the EPM models have been used to better describe the process that occurs during desolvation. Surface tension measurements were completed to better understand the relative surface activity of the complexes. The results showed that the complexes greatly decrease the surface tension of water. This leads to the conclusion that as the complexes form they will have a greater affinity for the surface of the droplet, thus they have greater ionization efficiencies. Indeed this may be the driving force for the low LODs reported for these systems. However, neither the binding constants nor the surface tension alone can be directly correlated with the LODs. It is a combination of these effects that controls the obsereved sensitivities.

Additional information concerning the rate at which these binding system equilibrate and the rate at which they partition to the outer phase of the droplet will be needed for a more complete explanation of the mechanism. Also, the response factors were assumed to be unity. Future work should be done to develop a method by which the actual response factors can be determined. Nevertheless, a better understanding as to how these dicationic ion-paring reagents achieve their “sensitivity enhancement effect” has been gained.

CHAPTER 8

GENERAL SUMMARY

Detection of anions is of great importance in many research areas including biochemical, pharmaceutical, and environmental research. Mass spectrometry has become the method of choice for trace analysis. In this study we show a new and simple method for the detection of anions in the positive ion mode electrospray ionization mass spectrometry.

A large set of di-, tri- and tetra-cationic molecules are used as ion pairing reagents for the detection of anions. Chapters two and three show the investigation and evaluation of a set of linear and trigonal tricationic and tetracationic ion pairing reagents with different divalent and trivalent anions. This sensitive method was applied to two classes of biological molecules, nucleotides and phospholipids. Limits of detection for all were lowered significantly when compared to the traditional negative ion mode detection.

It was also shown that this method could easily be coupled with HPLC to perform chromatographic separations. In this case the ion-pairing reagent was added postcolumn without interfering with the analytical separation, in both reverse phase mode and HILIC mode HPLC.

This sensitive detection method was also applied to metal ions. A set of transition metals were chelated with commercially available chelating agents and then paired with ion pairing reagents. These ternary complexes were easily detected in the parts per billion to parts per trillion levels in the positive ion mode ESI-MS.

To better understand the high sensitivity achieved by this method, a mechanistic investigation was performed (Chapter 7), in which it was discovered that the surface activity plays an important role in the low limits of detection achieved with all anions tested, when using our ion pairing reagents.

Through this study we have been able to identify the ideal ion pairing reagents for several classes of anionic molecules. This technique has shown pronounced low limits of

detection, great simplicity, fast analysis times, and adaptability to different chromatographic techniques (e.g. HPLC and CE). Sigma-Aldrich Co. has now commercialized four of our cationic agents as ion pairing reagents for mass spectrometry. As the field of mass spectrometry will continue to thrive, we hope the method developed herein will contribute to its great success.

APPENDIX A

PUBLICATION INFORMATION AND CONTRIBUTING AUTHORS

CHAPTER 2: “The evaluation and comparison of trigonal and linear tricationic ion-pairing reagents for the detection of anions in positive mode ESI-MS.” Warnke, Molly M.; Breitbach, Zachary S.; Dodbiba, Edra; Wanigasekara, Eranda; Zhang, Xiaotong; Sharma, Pritesh; Armstrong, Daniel W. Journal of the American Society for Mass Spectrometry (2009), 20(3), 529-538

CHAPTER 3: “Evaluation of tetracationic salts as gas-phase ion-pairing agents for the detection of trivalent anions in positive mode electrospray ionization mass spectrometry.” Zhang, Xiaotong; Wanigasekara, Eranda; Breitbach, Zachary S.; Dodbiba, Edra; Armstrong, Daniel W. Rapid Commun. Mass Spectrom. (2010), 24(8), 1113-1123.

CHAPTER 4:“Detection of Nucleotides in Positive Mode Electrospray Ionization Mass Spectrometry Using Multiply Charged Cationic Ion Pairing Reagents” Dodbiba, Edra; Breitbach, Zachary S.; Wanigasekara, Eranda; Zhang, Xiaotong; Armstrong, Daniel W. Analytical and Bioanalytical Chemistry (2010), 398(1), 367-376.

CHAPTER 5:“Use of ion pairing reagents for sensitive detection and separation of phospholipids in the positive ion mode LC-ESI-MS.” Dodbiba, Edra; Xu, Chengdong; Wanigasekara, Eranda; Moon, Myeong H; Armstrong, Daniel W. Analyst (2011), 136, 1586.

CHAPTER 6: “Sensitive analysis of metal cations in positive ion mode electrospray ionization mass spectrometry using commercial chelating agents and cationic ion-pairing reagents.” Dodbiba, Edra; Xu, Chengdong; Wanigasekara, Eranda; Armstrong, Daniel W. Rapid Communicatios of Mass Spectrometry (2012), 26, 1005-1013.

CHAPTER 7:“Mechanisms of ESI-MS Selectivity and Sensitivity Enhancements When Detecting Anions in the Positive Mode Using Cationic Pairing Agents.” Breitbach, Zachary S.; Wanigasekara, Eranda; Dodbiba, Edra; Schug, Kevin A; Armstrong, Daniel W. Analytical Chemistry (2010), 82, 9066-9073.

APPENDIX B

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BIOGRAPHICAL INFORMATION

Edra Dodbiba has obtained a Bachelor of Science in Biochemistry from The University of Texas at Arlington in May 2008. As a senior undergraduate she entered Dr. Daniel W. Armstrong's research group where she worked for one year. In Fall 2008 Edra joined Dr. Armstrong's research group as a graduate student and obtained a Doctor of Philosophy Degree in Analytical Chemistry at the University of Texas at Arlington in May 2013. Her research interests include gas phase ion chemistry, analytical method development for biomolecules and exploring new avenues and applications of mass spectrometry. Edra's interests also involve separations of small chiral and achiral molecules by different chromatographic techniques.