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LC-MS analysis of related peptides and anions in the positive mode

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This dissertation is dedicated to:

My parents, who taught me the value of hard work,
My brother and sister, who taught me to enjoy life,
and
My husband, who taught me life is better with a partner by your side

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ABSTRACT

This dissertation focuses on the use of LC-MS for the analysis of related peptides and anions in the positive mode. Separating closely related peptides (those differing by one or two amino acids or the chirality of a single amino acid) can be challenging using reversed-phase liquid chromatography (LC), ion exchange LC, or using ion-pairing agents. Also, the mobile phases that give the best separations in these modes may not be electrospray ionization mass spectrometry (ESI-MS) compatible. Macrocyclic glycopeptide stationary phases were investigated as an alternative to the standard C18 stationary phase for the separation of related peptides. On the macrocyclic glycopeptide stationary phases, high selectivity was observed for single amino acid substitutions (achiral and chiral) regardless of the position of the substitution in the sequence for peptides of thirteen amino acids or less. Selectivity of the macrocyclic glycopeptide stationary phases for a series of diastereomers and larger peptides was also explored and compared to separations achieved on a standard C18 stationary phase. MS compatible mobile phases were used whenever possible.

Negative ion mode is often used for the detection of anions in LC-ESI-MS applications. However, operating in negative ion mode tends to be more problematic than positive ion mode. Singly charged anions can be detected in the positive ion mode if the anions are paired with a dicationic reagent to form a complex with the anion that retains an over all positive charge. This method was also expanded to divalent anions through the use of tricationic reagents. When the anion pairs with the tricationic reagent, an overall positive charge is retained and enables detection by ESI-MS in the positive mode. Different cationic reagents were found to vary tremendously in their ability to pair with anions and produce sensitive ESI-MS signals. The effect of these structural elements on the detection sensitivity

of the complex is examined empirically. A comparision of signal to noise ratios achieved in positive and negative modes also is presented.

CHAPTER 1

INTRODUCTION

1.1. THESIS ORGANIZATION

Liquid chromatography-mass spectrometry (LC-MS) has become one of the most important analysis tools in the analytical laboratory. This dissertation presents new research in two distinct areas: the analysis of structurally related peptides and the ESI-MS analysis of anions in the positive mode. This introduction presents a brief literature overview to both areas. It is followed by five chapters, each one a manuscript either published or submitted for publication. The final chapter presents the general conclusions from both research areas.

1.2. LC-MS BASICS

Because of its simplicity and robustness, UV detection is the standard detection method for most types of liquid chromatography. However, compounds that lack chromophores must be derivatized in order to be detected. Additionally, some applications require greater sensitivity and/or structural information about the analyte. In these types of situations mass spectrometry has been playing an ever increasing role as an LC detector given its sensitivity and specificity.

Interfacing liquid chromatography and mass spectrometry was not as straightforward a process as it might seem given the powerful, dependable instruments available today. Liquid chromatography is used to analyze compounds with various polarities, wide-ranging molecular weights, potentially low thermal stability, and little or no volatility. Thus, a LC-MS interface should ionize this wide variety of analytes. Secondly, in order to detect ions made in the ion source, low pressure must be maintained in the mass spectrometer[1]. Thus,

most of the liquid eluent must be removed before the charged analyte enters the mass spectrometer. This can be particularly challenging as traditional liquid chromatography uses large volumes of eluent, and some solvents have low volatility (e.g., water). Thirdly, the interface should accomplish the first two processes without degrading the analyte.

Various interfaces have been developed for the coupling of liquid chromatography and mass spectrometry[2,3]. However, it wasn't until the development of atmospheric pressure ionization (API) techniques that LC-MS became the widely accepted analytical technique it is today. Electrospray ionization (ESI) is one of the most useful atmospheric pressure ionization techniques. Detailed descriptions of the electrospray ionization process can be found elsewhere[4-6]. However, a brief description is presented here. In ESI, eluent is pumped through a capillary to which a high voltage as been applied. This creates an electric field which nebulizes the LC eluent and simultaneously forms charged droplets at the tip of the capillary. These charged droplets contain the analyte and the remaining solvent must be removed before the analyte enters the mass spectrometer. "Dry" ions from the electrospray process are formed according to one of two generally accepted mechanisms (or a combination of the two)[4,5]: the charge residue model (CRM) and the ion evaporation model (IEM). As originally introduced, ESI interfaces could handle liquid flow rates only in the μL/min range. In order to handle the larger flow rates of liquid chromatography, most commercial ESI interfaces use a sheath gas (usually nitrogen) to further aid in nebulization and drying of the charged droplets. This was originally termed "pneumatically-assisted" ESI, but as this interface has become the standard throughout the industry the adjective "pneumatically-assisted" has largely been dropped.

ESI is usually chosen as the API technique for analytes that already exist as ions in solution, can be easily ionized by gas-phase proton reactions, or for molecules that easily associate with other small ions (e.g., Na^+, NH_4^+, Cl^-). It works very well for large macromolecules such as peptides[7], proteins and polymers[3]. As such, ESI is particularly suited for the LC-MS analysis of peptides and anions.

Even with the progress made in API sources, some difficulties remain when coupling liquid chromatography to mass spectrometry. Even with "pneumatically-assisted" ESI interfaces, flow rates that give the optimimum sensitivity are much lower (100-300 µL/min) than typical flow rates (1 mL/min) used on most analytical size columns[8]. Mobile phase additives should be volatile organic compounds to prevent clogging of the interface and entrance orifice[9]. Additives that form strong ion pairs (like trifluoroacetic acid) can lead to analyte signal suppression[10,11] and other additives (e.g., triethylamine) can deprotonate analytes. Thus a compromise between chromatographic performance and mass spectrometric sensitivity is used. This generally results in a LC-MS method where the most volatile buffer at the lowest concentration is used in order to provide the needed sensitivity and adequate chromatographic peak shapes.

1.3. LC-MS OF PEPTIDES

Understanding the bioactivity of proteins and peptides is essential for the development of future pharmaceuticals. However, more information is needed about the structure of these biomolecules. This is because the activity of such molecules is controlled by the primary amino acid sequence and any post-translational modifications of specific amino acids. Furthermore, any potential pharmaceutical agent must be evaluated for purity,

its metabolites characterized, and the pharmacokinetics and pharmacodynamics determined. As a result, the analysis and characterization of peptides is extremely important.

LC-MS has become a very useful tool for the separation and analysis of peptides. Determining the primary amino acid sequence of a protein by digesting the protein and then using LC-MS to separate and identify the resulting peptide fragments is now a routine procedure[1]. These sequences can then used to design peptide drugs. However, their tendency to be easily hydrolyzed and poor transport across membrane barriers *in vivo* has limited the clinical usefulness of many peptide-based drugs[12]. Medicinal chemistry is developing ways of improving the bioavailability of peptide drug candidates by chemically modifying the peptide sequences. Such strategies involve end modifications, cyclization, N-amide nitrogen alkylation, the use of non-coded amino acids, and changes in the chirality of amino acids[12]. These modifications can produce peptides that are very similar to one another. The most common way of introducing D-amino acids and non-coded amino acids into a peptide sequence is through synthesis. Crude peptides in synthesis mixtures must be purified to remove peptide impurities resulting from synthesis side reactions or incomplete deprotection steps. Peptide impurities leftover from synthesis batches have been known to cause false positives in biological assays[13]. Incomplete removal of protecting groups, amino acid deletions/insertions, incomplete sequences, reactions involving side-chains, and oxidation/reduction of reactive sites[14] create complicated peptide mixtures. Random racemization can also occur during synthesis[15], creating mixtures of peptide diastereomers. The resulting peptides may differ only by a single amino acid in the primary sequence or by a modification to the side chains or N, C-termini of the peptides.

Mixtures of very similar peptides can be particularly difficult to purify. Liquid chromatography is the preferred way to analyze as well as purify these complex peptide mixtures. Ion-exchange[16,17] has been used to purify peptides; however, reverse phase (often using trifluoroacetic acid or other ion-pairing agents)[14,18-23] HPLC is most commonly employed. LC modes with separation mechanisms orthogonal to reverse phase (e.g. ion-exchange and size-exclusion) are combined in series[24-27] or as separate chromatographic steps with alkyl bonded phases (e.g., C18) to provide more resolving power.

Due to the potential complexity and variety of peptide mixtures, there is a continual search for more LC stationary phases that display different selectivities. Recently, molecularly imprinted polymers[28-30] and a mixed-mode reverse phase/weak anion exchange stationary phase[31] have been used for the separation of peptides. Due to the increasing prevalence of D-amino acids in peptides, stationary phases also need to be selective for peptide diastereomers and even enantiomers. While non-chiral stationary phases can separate peptide diastereomers[32-34], only chiral stationary phases (or chiral mobile phase additives) can directly separate enantiomers.

Chiral stationary phases have been used to separate peptide diastereomers of various lengths, but the separation of enantiomers has been generally limited to small di- and tripeptides[35]. This is partly due to the exponential increase in the number of possible stereoisomers with arithmetic increases in the number of amino acid in peptides. This point is illustrated in a study in which a tert-butylcarbamoylquinine based stationary phase lacked the diastereoselectivity to separate all the stereoisomers (both diastereomers and enantiomers) of a series 3,5-dinitrobenzoyl trialanine peptides[36]. All stereoisomers were

separated using two-dimensional LC-MS by using a reverse phase column before the chiral column.

Macrocyclic glycopeptide chiral stationary phases have been used to separate enantiomers of amino acids and small peptides with great success[7, 37-40]. These stationary phases have also shown to be selective for all of the proteinic amino acids[7]. The structures for ristocetin, teicoplanin, and teicoplanin aglycone are shown in Figure 1.1. Teicoplanin aglycone is made by removing the sugar groups from teicoplanin. All three chiral selectors have a peptide backbone and a relatively hydrophobic "basket." All three chiral selectors also contain amine groups. Teicoplanin and teicoplanin aglycone also have a carboxylic acid site; in ristocetin this site is esterified. The functionally diverse macrocyclic glycopeptide stationary phases are capable of interacting through electrostatic, hydrogen bonding, and di-polar interactions. Because of these traits, it seemed possible that the macrocyclic glycopeptide stationary phases could be of use in separating very closely related peptides. Such peptides could include various stereoisomers as well as single amino acid differences in the primary sequence. The separation mechanism for peptides on these stationary phases would likely be different from traditional reverse phase stationary phases due to the additional interactions possible with the various functional groups on the macrocyclic glycopeptides. Furthermore, mobile phases for amino acid/peptide separations on the macrocyclic glycopeptides use aqueous solutions of methanol or acetonitrile with volatile organic buffers as additives (if necessary). Such mobile phases are MS-friendly and facilitate their use in LC-MS applications. Chapters 2 and 3 describe the separation capabilities of the macrocylic glycopeptides for several groups of closely related peptides. Chapter 2 focuses on separating related peptides (up to 13 amino acids long) in different peptide families.

These families consist of peptides that are derived from a common primary amino acid sequence. These peptide sequences differ from peptides within the same family by one or two amino acids (or the chirality of an amino acid). Chapter 3 examines the selectivity for a series of diastereomeric enkephalin peptides and the potential use of the macrocyclic glycopeptide stationary phases for larger peptides (14-28 amino acids). Mobile phases that are fully ESI-MS compatible were used when ever possible to separate the structurally related peptides.

1.4. ANALYSIS OF ANIONS USING MASS SPECTROMETRY

The analysis of anions is very important in many areas of scientific study involving environmental samples (especially water), biological tissues and fluids, and foods and beverages. In such complicated matrices, separation methods are often employed to separate the anions of interest from interfering matrix compounds. Ion chromatography is the most common separation method employed for the analysis of anions. Reverse phase chromatography can be used when an anion has a sufficiently hydrophobic moiety. When most underivatized are analyzed as anions, the anions have little UV absorbance and thus conductivity detection is used as a universal detector[41]. In conductivity detection, analytes are detected when a change in conductivity is measured from the background electrolyte. An increase in conductivity from the background electrolyte level is the direct detection of an analyte while a decrease in conductivity from the background level is considered to be indirect detection. As with most analytical techniques, lowering the background signal (in this case, conductivity) improves detection sensitivity. As a result, most conductivity detection is now performed using background suppression. In suppressed conductivity

detection, eluents such as sodium hydroxide or sodium carbonate undergo cation exchange to produce the corresponding less conductive species (water or H_2CO_3). Suppressor technology has been reviewed and will not be discussed further[42, 43]. Despite its utility, conductivity detection cannot provide structural information about analytes and even lower detection limits are needed in many determinations.

Ion-selective electrodes have also been used as detectors in ion chromatography[44-47]. However, the use of ion-selective electrodes in ion chromatography is not widespread, due in part to the fact that the information provided by the electrode with a separation technique might not be any different than the information determined by flow-injection analysis[41]. Ion-selective electrodes continue to improve in sensitivity and over-all detection limits[48]. Ion selective electrodes are most often used without a separation technique and in applications where the sample size is not limited.

The use of mass spectrometry as a method for the analysis of anions is growing in popularity. This is because the mass spectrometer can act as a second dimension of analysis to discriminate amongst different species, but also to provide more information about the structure and ultimately, the identity of an analyte. Inductively coupled plasma (ICP) and API (usually ESI) are the most common interfaces for ion chromatography. ICP interfaces can handle the 1 mL/min flow rate of traditional ion-exchange columns and can detect several elements with great sensitivity[49,50]. For these reasons, ICP-MS is a very popular method for detecting metallic and halogenated species[51-61]. However, the high temperature of the ICP leaves only the element(s) of interest; thus, no information about the structure of the analyte can be gained from the ICP spectra. This hinders the identification of unknown analytes that contain the target element(s). Identification of the species giving the ICP signal

must still be gained by matching retention times of known analytes. This also requires the complete separation of all analytes containing the target element. Both API techniques (ESI and APCI) can be used to elucidate structural and identity information of analytes from the resulting mass spectrum. Since anions are already negatively charged, ESI operated in the negative mode is the API ionization technique usually employed in the analysis of anions.

Negative mode ESI is the most straightforward method for detecting anions, however it has some drawbacks. Negative ion mode is known to be more prone to corona discharge conditions, which leads to increased chances for arcing[6]. Corona discharge is an electrical discharge that results when solvent droplets near the capillary are ionized, but the electrical field gradient is insufficient for arcing. Both corona discharge and arcing are detrimental to the sensitivity of an analytical method by ESI-MS. Operating under corona discharge conditions increases chemical noise and poor spray stability[62]. The increase in chemical noise comes from ionized solvent molecules[6]. Early on, Yamashita and Fenn noticed that the onset of arcing occurred at much lower applied potentials compared to positive mode[63]. To reduce arcing, they used oxygen at the capillary tip to scavenge excess electrons and solvent components with high electron affinities. Since then, a reduction in corona discharge has been accomplished with the use of electron scavenging gases[64, 65]. Commerical instruments today often use nitrogen (from liquid N_2 dewars or generators) which makes adding high electron affinity gases more difficult. Various halogenated solvents have also been used to lower corona discharge and provide more stable spray[66-68]. Halogenated solvents seem to be more effective at capturing electrons and producing stable halide anions than non-halogenated solvents[66]. Elsewhere, 2-propanol[65] and butanol[9] have been recommended for use as LC-MS solvents when negative mode is used. This is because common solvents used

for the separation of anions such as water and methanol have low gas-phase proton affinities which can result in the protonation of analytes with higher gas-phase proton affinities[68]. None of the solvents listed above are commonly used in either reverse phase chromatography or ion chromatography. For these reasons, it would seem desirable to detect anions in positive mode in which water and methanol, the preferred solvents, work best.

In order to detect anions in the positive mode, the anions would have to be paired in the gas phase with a reagent that has at least one more positive charge than the anion has negative charge(s). The pairing of two oppositely charged species in the gas phase has been known for some time. In fact, such pairing is one source of signal suppression in LC-MS. It is well documented that trifluoroacetic acid added to LC mobile phases to improve the peak shape of positively charged analytes (such as peptides) results in signal suppression of the analytes in the mass spectrometer[11, 69, 70]. Also, ESI-MS and other soft-ionization techniques have been employed to transfer non-covalent complexes formed in solution to the gas phase for various qualitative and quantitative uses[71-85]. Organic bases[86] and cationic surfactants[87-89] have been used for the detection of perchlorate in the negative mode by ESI-MS. The pairing agents pair with two perchlorates (or a perchlorate and another anion) in order to become "visible" to the mass spectrometer. Later Dasgupta, Armstrong, and co-workers used two imidazolium, pyrrolidinium, or alkyl ammonium cations connected by alkyl chains to detect perchlorate at the 25 ng/L level[90]. The dications were introduced into the carrier flow or eluent from an anion exchange separation column via a Y-type tee. Figure 1.2 is a diagram of the instrumental set-up. Sensitivity for perchlorate was maintained even in the presence of sulfate. This is significant as the mass of $H^{34}SO_4^-$ overlaps with that of perchlorate to the extent that quadrupole mass spectrometers cannot resolve the two anions.

Thus, the use of these dications in this manner may help remove interferences from anions with the same mass that bind less strongly to the dicationic reagent. Other benefits include moving the small mass anions out of the low mass region of high chemical noise and increasing the measured mass of anions that reside below the low mass cut-off (LMCO, often m/z 50) of ion traps. This "dicationic reagent" approach has been used along with ion exchange chromatography in order to quantitate perchlorate, iodide, and isocyanate in seawater and seaweed[91], milk[92], and human urine[93].

The final three chapters of this dissertation focus on the expansion of positively charged reagents for the detection of anions in the positive mode. In chapter 3, one dication reagent is used to evaluate the broad applicability of detecting thirty-two anions in the positive mode and determine their limits of detection (LODs). Also in this chapter, the use of MS/MS to increase the detection limits for many of the anions is presented. Six of the anions from chapter 3 are used to explore the how structural differences in dicationic reagents affect the sensitivity of the representative ions and these results are presented in chapter 4. The fifth chapter extends this analytical approach to divalent anions with the use of tricationic reagents.

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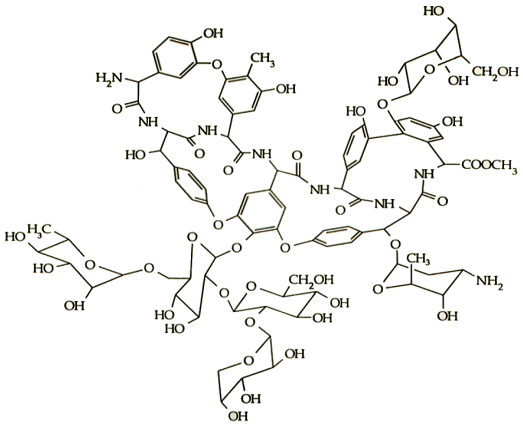
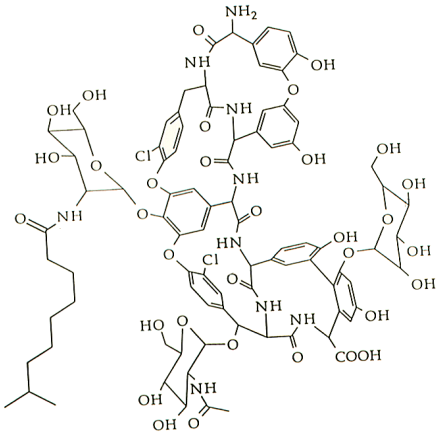
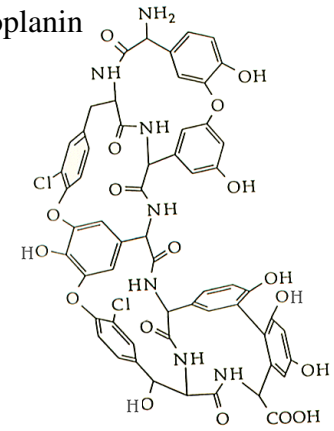




Teicoplanin



Teicoplanin Aglycone

Figure 1.1: Structure of the three macrocyclic glycopeptides used in Chapter 2. Teicoplanin and ristocetin were used in Chapter 3 as well.

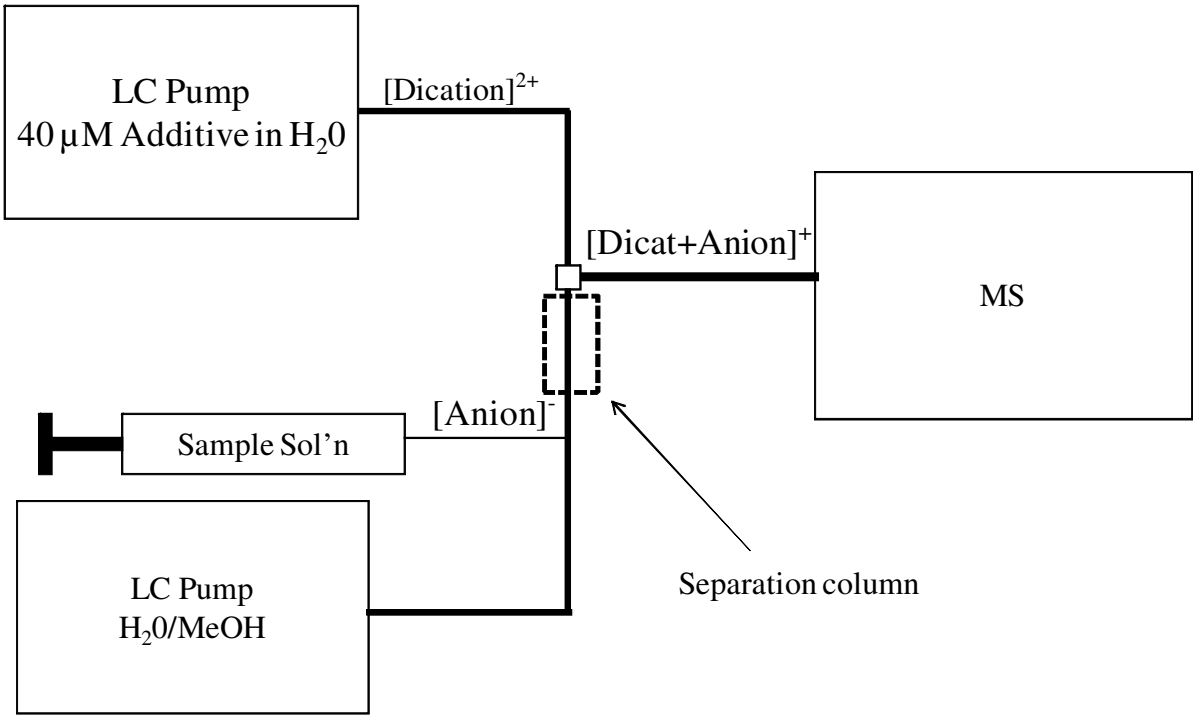


Figure 1.2: Schematic showing the introduction of the dicationic reagent for ESI-MS or LC-ESI-MS analysis of anions in the positive mode.

CHAPTER 2

SELECTIVE SEPARATIONS OF PEPTIDES WITH SEQUENCE DELETIONS, SINGLE AMINO ACID POLYMORPHISMS, AND/OR EPIMERIC CENTERS USING MACROCYCLIC GLYCOPEPTIDE LIQUID CHROMATOGRAPHY STATIONARY PHASES

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ABSTRACT

Separating closely related peptides (those differing by one or two amino acids or the chirality of a single amino acid) can be challenging using reversed-phase liquid chromatography (LC), ion exchange LC, or using ion-pairing agents. Also, the mobile phases that give the best separations in these modes may not be electrospray ionization mass spectrometry (ESI-MS) compatible. Forty-two peptides from 11 peptide families were separated on three macrocyclic glycopeptides stationary phases in reverse-phase mode using ESI-MS-compatible mobile phases. The peptide classes studied were angiotensin, bradykinin,α-bag cell factor, β,γ-cell factor, β-casomorphin, dynorphin, enkephalin, leucokinin, lutinizing hormone releasing hormone, neurotinsin, substance P, and vasopressin. High selectivity was observed for single amino acid substitutions (achiral and chiral) regardless of the position of the substitution in the sequence. Mobile phase optimization, its effect on peptide elution behavior, and chromatographic efficiency is also discussed. Using LC-ESI-MS, a 2 ng limit of detection was obtained, two orders of magnitude lower than the UV detection limit.

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2.1. INTRODUCTION

The separation and analysis of peptides continues to be of paramount importance in many areas of science and technology. Some of these areas include: (a) protein sequencing; (b) analysis, quantitation, and characterization of peptide hormones; (c) synthesis of new peptide drugs; (d) pharmacokinetic and pharmacodynamic studies of pharmacologically active peptides; and (e) other fields involving the environmental, biological, and geochemical sciences.

The separation of complicated peptide mixtures is one of the more important initial steps in protein sequencing. Also, there is increasing interest in detecting single amino acid polymorphisms in proteins [1-4] which would produce the resultant peptide polymorphs after digestion with proteolytic agents. These types of protein alterations emanate from certain single nucleotide polymorphisms [5, 6] and have been linked to diseases by several researchers [7-10].

Low concentrations of peptide hormones are known to elicit a large spectrum of physiological effects [11, 12]. Their identification and quantification in complex biological fluids can be problematic not only because of the complicated matrices, but also due to the large number and higher concentrations of interfering substances [12]. As a consequence of these peptides' profound activity, it is not surprising that pharmaceutical scientists are synthesizing an ever-increasing number of analogues. Frequently, this involves replacing specific amino acids with other natural or more frequently, non-natural amino acid analogues [13-17]. Non-natural amino acids can include: D-amino acids, β-amino acids, unusually substituted α-amino acids, cyclic and bicyclic-amino acids, as well as other useful permutations [14, 15]. In all cases, active potential drug candidates must undergo

pharmacokinetic and pharmacodynamic studies in which they and their metabolites must be distinguished from all other naturally occurring physiological components.

Liquid chromatography (LC) is the predominant separation method used for the analysis of peptides [18-34]. It is often coupled with other separation methods and/or mass spectrometry as part of a two-dimensional (2D) or multidimensional procedure [19, 23, 25, 35]. Reversed-phase LC is the most prevalent method used because of its good resolving power, reproducibility, and ease of use [18, 29, 32, 36]. It has become common practice to use mobile phases consisting of aqueous acetonitrile mixtures containing various ion-pairing agents [26]. Ion-exchange chromatography has also been used widely for the separation of peptides [24-27]. The composition of the mobile phase can be a problem if the separation is interfaced with electrospray ionization mass spectrometry (ESI-MS). Often, this is necessary to enhance both the sensitivity and selectivity of an analysis.

Given the wide variety of peptides, peptide mixtures and complex matrices in which they exist, there is a constant search for different selectivity separation approaches. For example, a porphyrin-based stationary phase was recently proposed for the separation of peptides [37]. When utilizing two-dimensional separations, it is usually desirable to have orthogonal separation methods. Orthogonality is more likely if the separation mechanisms are different from on another. However, the mobile phases have to be sufficiently compatible that the methods can be coupled (if using a continuous automated system).

Macrocyclic glycol-peptide-based (i.e., containing teicoplanin, teicoplanin aglycone, or ristocetin A) chiral stationary phases are widely utilized for enantiomeric separations, including amino acids, dipeptides, and tripeptides [22, 31]. They are known to selectively bind specific amino acids and sequences of amio acids via electrostatic, hydrogen bonding,

and dipolar interactions [14, 31]. It is highly likely that they also are selective for closely related peptides of any chain length. Their separation mechanism, and therefore selectivity, is signicantly different from both C_{18} reversed-phase and ion-exhange LC. Furthermore, the mobile phases that are commonly used with teioplanin-based stationary phases are ESI-MS compatibile. The focus of this work is to evaluate the separation of a variety of closely related peptides on a teicoplanin stationary phase using isocratic elution with ESI-MS-compatible mobile phases.

2.2. EXPERIMENTAL

2.2.1 Materials

Synthetic peptides used in this study were purchased from American Peptie Co. (APC; Sunnyvale, CA, USA) and Sigma (St. Louis, MO, USA). The peptides, their structure, and source are listed in Table 1.

Formic acid, 96% ACS reagent grade (Sigma) was used as mobile phase additive. Acetonitrile (ACN; HPLC grade, Fisher Scientific, Pittsburgh, PA, USA) and deionized water (prepared in the laboratory) were used to make all mobile phases. All samples were dissolved in a water-methanol (50:50) solvent mixture at 1 mg/ml concentration unless mentioned otherwise. Triethylamine (TEA; HPLC grade, Sigma) and acetic acid (ACS grade, Fisher Scientific) were also used as mobile phase additives.

2.2.2. Instrumentation

The chromatographic methods were developed on an HP (Palo Alto, CA, USA) 1050 HPLC system including one auto sampler, one quaternary pump, and one VWD detector operating under ChemStation software. All separations were carried out with analytical

columns from Advanced Separations Technologies (ASTEC; Whippany, NJ, USA) at room temperature. The columns used were Chirobiotic T (250 mm x 4.6 mm), and Chirobiotic R (250 mm x 4.6 mm), Chirobiotic TAG (250 mm x 4.6 mm). LC-MS analyses were carried out on a Thermo Finnegan (San Jose, CA, USA) Surveryor LC system with a photodiode array detection (DAD) system coupled with a Thermo Finnegan LCQ Advantage API ion-trap mass spectrometer with and ESI source. Xcalibur 3.1 was the operating software. Ultra-high purity helium gas (Linweld, Lincoln NE, USA) was used as dampening gas. Praxair (Danbury, CT, USA) nitrogen was used as sheath gas and auxiliary gases

2.2.3. Methods

All HPLC methods are listed in Table 2. Depending on mobile phase conditions, UV-vis detection was performed at wavelengths of 210, 232, or 254 nm. ESI conditions were set to the following: sheath gas = 50 arbitrary units, auxiliary gas = 40 arbitrary units, source voltage = 4.55 kV, capillary voltage = 30.6 V, tube lens offset = -15 V, and capillary temperature = 272°C. LC-MS experiments were carried out using flow rates of 1.0 mL/min, unless noted otherwise.

2.3. RESULTS AND DISCUSSION

2.3.1. Peptide separations

Macrocyclic glycopeptides chiral stationary phases (CSPs) exhibited excellent selectivity in separating closely related peptides. Fig. 2.1 shows the baseline resolution of six enkephalin peptides on the Chirobiotic T (teicoplanin) column in a single isocratic run. The enkephalin peptides are closely related structurally, differing from one another by only one amino acid or the chirality of a single amino acid. In addition, retention times can be reduced

substantially if desired, by utilizing gradient elution (see Section 2.3.3.1). Currie et al. had some success in resolving enekphalin peptides by using a phenyl-bonded column [38]. However, no baseline separation was achieved when four or more enkephalin peptides were present in the mixture. Underberg and co-workers recently coupled size-exclusion chromatography (SEC) and CE to separate large proteins and enkephalin peptides [39]. Although separation was achieved, the system complexity and low chromatographic quality made the separation less desirable. In addition to the good selectivity observed in Fig 2.1., the mobile phase is ESI-MS compatible (as will be shown and discussed for subsequent separations.)

Excellent separations were commonly observed for most of the peptides listed in Table 2.2. Within each family (listed in Table 2.2), the individual peptides are listed in the order of their retention at the elution conditions specified. The selectivity (α) and resolution (R_s) values are reported for adjacent peptides peaks within each family. These values were calculated at optimized isocratic elution conditions for the separation of the entire peptide family. Other elution conditions can be found to further resolve any signle pair of peptides within the family, if desired. Table 3 indicates the macrocyclic glycopeptides column that produced the most effective separations for each class (family) of peptides. The Chirobiotic T column produced the best separations for the largest numbers of families, but all three columns were needed to separate all of the families.

2.3.2. Separation of peptides containing single amino acid polymorphism (SAAP)

As demonstrated in Figure 2.1., the enkephalins were easily baseline separated from each other. Among these separations, enkephalin peaks **2** and **4**, enkephalin peaks **3** and **5**, enkephalin peaks **5** and **6** are different from each other only by a single amino acid. A

particular separation of note is the SAAP represented in peaks **5** and **6**. The glycine in position 2 of one peptide is replaced with an alanine. This difference in the side chains is one of the more subtle substitutions among native amino acids, yet it is easily separated. Enkephalin peaks **1** and **4**, and enkephalin peaks 4 and 6 differ from each other only by the chirality of a single amio acid, making them epimers of one another. Interestingly, these single amino acid chirality polymorphism (SAACP) peptides were not eluted next to each other. At least one other peptide eluted between the epimers. The epimeric position in the peptide chain might play a critical role in determining if the separation is substantial enough to allow another peptide to elute between the epimers, as this behavior was not always observed.

Fig. 2.2 shows several separations of peptides with SAAP. Separation is achieved regardless of whether the amino acid substitution occurs at the N-terminus, middle, or C-terminus of the peptide chain. In each related sequence, the amino acid that is different is highlighted for easier comparison (Fig. 2.2 and Table 2.2). In general, the separation was easier to achieve if the polymorphism occurred at or near the end of the peptide chain. This is because functional group on both ends provide stronger interaction with the stationary phase [31]. It is important to note that these separations were obtained under optimized elution conditions for the entire family of peptides. In the cases where a neutral amino acid is replaced with a positively charged amino acid (Fig. 2.2A, C) there is a tremendous difference in the retention of the peptides. This is largely due to the additional interaction of cationic side chains with the stationary phase. However, differences in electrostatic interactions are not solely responsible for the ultra high selectivities. For example, the substitution of methionine for norleucine (Fig 2.2D) also produces a tremendous change in

the retention behavior of these peptides. Fig. 2.3 shows the separation of peptide epimers (i.e., where the single amino acid polymorphism is due to the opposite chirality of a single amino acid). In these cases, peptides with the chiral SAAP in the middle of the peptide chain were as easy to separate as those with more terminal groups. However, epimers in which the chiral SAAP is α or β to the C-terminal end (Fig 2.1) appear to produce the most facile separations of this class of diastereomers. Interestingly, the epimer containing the D-amino acid always eluted before the other epimer regardless of that observed for monomer native amino acids and some dipeptides [31].

2.3.3 Optimization of peptide separations on Chirobiotic stationary phases

As with most separations of charged analytes in the reverse-phase mode, the percentage and type of organic modifier along with the pH of the mobile phase, must be optimized in order to produce the best separation. Since the macrocyclic glycopepide stationary phases also have ionic sites, the ionic strength of the mobile phase must also be considered.

2.3.3.1 Organic modifier content and retention behavior

In separating small molecules in the reversed-phase mode, most macrocyclic glycopeptides stationary phases have shown the highest selectivity when methanol was used as the organic modifier [13-17, 20-22, 30-34]. While this also was true for the peptides examined here, methanol often produced broad peaks and inefficient separations. Efficiency was greatly improved when acetonitrile was used as the organic modifier. Acetonitrile was used in all the mobile phases reported here, as the increase in efficiency more than compensated for the loss in selectivity.

Regardless of organic modifier type, plots of mobile phase composition (i.e., percent organic modifier) versus retention produced U-shaped retention curve behavior on all macrocyclic glycopeptides stationary phases. Fig. 2.4 shows the elution behavior for two vasopressin peptides on a Chirobiotic TAG stationary phase. The peptides are more strongly retained under high organic content and high aqueous content mobile phases. The strongest eluting mobile phase was generally around half organic and half aqueous content, although the sequence of the peptide determines the exact location of the retention minimum for any retention versus composition curve. Similar U-shape retention behavior of peptides and proteins was commonly observed on alkyl bonded stationary phases [40-48], despite their differences in chemistry from macrocyclic glycopeptide stationary phases. Simpson and Moritz indicated that peptide retention, at high organic modifier concentration, was more like normal phase chromatography (polar stationary phase), which suggests that residual silanol groups also contribute greatly in retention [42]. Bij et al. proposed dual mechanisms, in which the combination of solvophobic and silanophilic interaction was thought to be the reason for retention inversion [48]. Early on, Armstrong and co-workers pointed out that the real reason for the inverse retention behavior at high organic modifier concentration (for many proteins, peptides, and even amino acids) was from the changes in their solubility as the organic concentration in the mobile phase is increased [40, 41]. Under high aqueous mobile phase conditions, the classic reverse-phase mechanism (i.e., hydrophobic association) governs retention, where increased organic modifier amounts decrease retention. Under high organic modifier mobile phase content, peptides become much less soluble in the mobile phase, which means longer retention times. The point of minimum retention (Fig. 2.4) can be approximated by coupling the reverse-phase retention curve and the solubility curve for

any peptide of interest. In some cases, other specific interactions (electrostatic, etc.) can affect the exact location of the retention minimum. More choices in method development are available due to this U-shaped elution curve behavior. For example, the U-shaped elution curve behavior also indicates the possibility to carry out an inverse gradient on this class of stationary phases [40]. Thus, depending on the starting mobile phase composition, a traditional or inverse gradient can be used to decrease retention times, if desired. Most mobile phases in this work use higher organic modifier concentrations due to the increased efficiency observed with such mobile phases without gradient elution.

2.3.3.2 Mobile phase pH

The overall charge on a peptide is determined by the amino acids in the peptide and is a consideration in determining the optimized mobile phase. Under the operating pH range of the macrocyclic glycopeptides stationary phases (pH 2.8-7.5), peptides with basic side chain groups are generally protonated while peptides with acidic side chain groups are mostly deprotonated. The additional positively charged side chains allow for increased interaction of the peptide with the stationary phase through its anionic sites. Thus, cationic peptides can be strongly retained [31]. Adding ammonium salts or acidifying the mobile phase appears to provide competing ions for the anionic sites or protonate them, respectively, thereby decreasing the retention of positively charged peptides. However, for neutral and anionic peptides, the ammonium salts or acid can overwhelm the interaction of the peptide with the stationary phase leading to insufficient retention. Some neutral peptides (e.g., enkephalins) elute near the void volume if salt or acid is added to the mobile phase. Additionally, the specific structure of the stationary phase must be considered as well. For the Chirobiotic T and TAG columns, the mobile phase additive formic acid was required to elute many of the

peptides listed in Table 2.2. However, when the same mobile phase conditions used for separations on the Chirobiotic T or TAG are used on the Chirobiotic R column, the peptides elute near the void volume (data not shown). This change in behavior is due to the presence (or absence) of carboxylic acid sites on the stationary phase. Teicoplanin (Chirobiotic T) and the teicoplanin aglycone (Chirobiotic TAG) have a free carboxylic acid group while the corresponding acid site on the ristocetin (Chirobiotic R) has been esterified.

2.3.3.3 Mobile phase ionic strength

To illustrate the effect of mobile phase additives on retention, peak shape, and resolution, different amounts of ammonium formate were added to the mobile phase while maintaining a constant pH and acetonitrile content. The bradykinin peptides were chosen because this family of peptides contains both neutral and cationic side chains. Peptides with cationic side chains contain up to two arginine residues. Fig. 2.5 shows chromatograms generated using mobile phases containing different ammonium formate concentrations of 2, 5, 15, and 25 mM. At 2 mM ammonium formate, RPGFSPER and RPPGFSPFR did not elute after 100 min (data not shown). Only the first 20 minutes of the chromatogram is shown in order to compare the peak shape for the two peptides that did elute. The basic arginine group in PPGFSPFR produces much more pronounced tailing at this concentration relative to the PPGFSP peptide, which contains no amino acids with cationic side chains. Increasing the ammonium formate concentration to 5 mM drastically shortens the retention times of the peptides with two arginines. However, the more basic peptides still exhibit broad peaks with significant tailing. Raising the ammonium formate concentration to 15 and 25 mM continues to shorten the retention times of the basic peptides while leaving the retention of PPGFSP relatively constant. Greater efficiency is achieved at 15 and 25 mM

compared to 5 mM, though changes tend to be less pronounced. It is expected that higher concentrations of ammonium formate could further enhance peak shape of the more basic peptides. However, the most MS-compatible mobile phase would utilize the lowest salt concentration to give the desired detection sensitivity (as discussed in Section 2.3.4).

2.3.4. Electrospray mass spectrometry detection

Many previous LC methods, developed to separate peptides, used alkyl bonded phases with ion-pairing agents such as: trifluoroethylammonium phosphate (TAAP) [50], or heptafluorobutyric acid (HFBA) [51, 52]. By using these agents in mobile phases under appropriate pH conditions, charged analytes like peptides would form pairs of ions. Instead of eluting in the dead volume, peptides could be retained and separated due to their different hydrophobic interactions with the stationary phase. With the increasing popularity of electrospray ionization mass spectrometry coupled to HPLC, alternatives to the ion pair approach have been sought because of the adverse effects of ion pair reagents on ESI ionization efficiency [53,54]. In this study, all mobile phases developed but the two containing triethylamine were MS compatible. Triethylamine was added to the mobile phase to separate the epimers of the dynorphin family. The large number of basic amino acids present in this family of peptides caused them to interact very strongly with the stationary phase. Triethylamine at pH 2.8 provides stronger competition for the stationary phase than other mobile phase additives.

Fig. 2.6 shows an example of separation of lutinizing hormone releasing hormone peptides using ESI-MS detection. The isocratic HPLC method is simple and ESI-MS compatible. From the mass spectra, the peaks can be easily identified according to their molecular weight. The most abundant ion was usually the $[M+2H]^{2+}$ species, although

sodium adduct products also were observed. This behavior is consistent with ESI spectra of peptides reported elsewhere [55].

In the recent literature, Desai and Armstrong reported the detection limits of amino acids at nanogram and sub nanogram levels by atmosphereric pressure chemical ionization mass spectrometry (APCI-MS) [53]. In this study, APCI-MS gave the best sensitivity for small molecules under M_r 200 and similar sensitivity to ESI-MS for molecules between M_r 200 and 300. Above M_r 300, sensitivity increased for ESI-MS compared to APCI-MS. Fig. 2.7 compares the detection limit of ESI-MS and UV. A 2 ng peptide detection limit was easily achieved by ESI-MS in this study, consistent with the level for single amino acids in Desai and Armstrong's report. This 2 ng detection limit is approximately two orders of magnitude lower than the detection limit obtained using UV detection at 210 nm under identical conditions.

The methods described here exhibit good detection linearity over wide range of peptide concentrations. For two vasopressin peptides, the calibration curve was linear over a concentration reange of 0-1000 µg/mL with R2 values of 0.991 for the first eluting peptide and 0.992 for the second peptide. The methods developed in this study provide not only sensitive detection, but also respectable detection linearity. This sensitive detection with a linear response is necessary for modern peptide assays.

2.4. CONCLUSIONS

Macrocyclic glycopeptides CSPs have great resolving power for closely related peptides separated by HPLC. In general, (1) terminal polymorphisms produced separations of greater resolution than those occurring in the middle of the peptide, (2) substituting a

charged amino acid for an uncharged residue produced a separation of greater resolution than exchanging an uncharged amino acid for another uncharged amino acid or substituting like charged amino acids, and (3) all peptides containing a D-amino acid polymorphism eluted before the corresponding L-amino acid containing peptide. Most of the mobile phase conditions used are MS compatible and good limits of detection can be achieved by using ESI-MS. The peptides on macrocyclic glycopeptides CSPs exhibited U-shaped curves when retention is plotted against the concentration of organic modifier. Mobile phase composition, including the type and amount of organic modifier, mobile phase pH, and ionic strength, plays an important role in peptide elution and peak shape. The selectivity of the macrocyclic glycopeptides stationary pahses for achiral and chiral polymorphisms using ESI-MS-compatible mobile phases should broaden their appeal for use in all areas where peptide separations are important.

2.5. ACKNOWLEDGEMENTS

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Table 2.1: Peptides, sequences, and source

Name	Three-letter sequence	Single-letter sequence[a]	Source
Leu-Enkephalin	Tyr–Gly–Gly–Phe–Leu	YGGFL	Sigma
[D-Ala2,D-Leu5]-Enkephalin	Tyr–D-Ala–Gly–Phe–D-Leu	Y-dA-GF-dL	APC
[Ala2]-Leu-Enkephalin	Tyr–Ala–Gly–Phe–Leu	YAGFL	APC
Met-Enkephalin	Tyr–Gly–Gly–Phe–Met	YGGFM	Sigma
Met-Enkephalin [D-Ala2]	Tyr–D-Ala–Gly–Phe–Met	Y-dA-GFM	APC
[D-Ala2,Leu5]-Enkephalin	Tyr–D-Ala–Gly–Phe–Leu	Y-dA-GFL	APC
[D-Ala2,4,Tyr5]-β-Casomorphin (1–5), amide, bovine	Tyr–D-Ala–Phe–D-Ala–Tyr–NH_2	Y-dA-F-dA-Y-NH_2	APC
[D-Ala2,D-Pro4,Tyr5]-β-Casomorphin (1–5), amide	Tyr–D-Ala–Phe–D-Pro–Tyr–NH_2	Y-dA-F-dP-Y-NH_2	APC
[D-Ala2,Hyp4,Tyr5]-β-Casomorphin (1–5), amide	Tyr–D-Ala–Phe–Hyp–Tyr–NH_2	Y-dA-F-Hyp-Y-NH_2	APC
β-Bag cell factor	Arg–Leu–Arg–Phe–His	RLRFH	APC
γ-Bag cell factor	Arg–Leu–Arg–Phe–Asp	RLRFD	APC
α-Bag cell peptide (1–7)	Ala–Pro–Arg–Leu–Arg–Phe–Tyr	APRLRFY	APC
α-Bag cell peptide (1–8)	Ala–Pro–Arg–Leu–Arg–Phe–Tyr–Ser	APRLRFYS	APC
α-Bag cell peptide (1–9)	Ala–Pro–Arg–Leu–Arg–Phe–Tyr–Ser–Leu	APRLRFYSL	APC
Leucokinin I	Asp–Pro–Ala–Phe–Asn–Ser–Trp–Gly–NH_2	DPAFNSWG-NH_2	APC
Leucokinin II	Asp–Pro–Gly–Phe–Ser–Ser–Trp–Gly–NH_2	DPGFSSWG-NH_2	APC
Leucokinin VII	Asp–Pro–Ala–Phe–Ser–Ser–Trp–Gly–NH_2	DPAFSSWG-NH_2	APC
[Sar1,Thr8]-Angiotensin II	Sar–Arg–Val–Tyr–Ile–His–Pro–Thr	Sar-RVYIHPT	Sigma
Angiotensin II human	Asp–Arg–Val–Tyr–Ile–His–Pro–Phe	DRVYIHPF	Sigma
[Val5]-Angiotensin II	Asp–Arg–Val–Tyr–Val–His–Pro–Phe	DRVYVHPF	Sigma
Angiotensin II antipeptide	Glu–Gly–Val–Tyr–Val–His–Pro–Val	EGVYVHPF	Sigma
[Sar1]-Angiotensin II	Sar–Arg–Val–Tyr–Ile–His–Pro–Phe	Sar-RVYIHPF	Sigma
Bradykinin	Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg	RPPGFSPFR	Sigma
des-Pro2-Bradykinin	Arg–Pro–Gly–Phe–Ser–Pro–Phe–Arg	RPGFSPFR	Sigma
Bradykinin fragment 2–7	Pro–Pro–Gly–Phe–Ser–Pro	PPGFSP	Sigma
Bradykinin fragment 2–9	Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg	PPGFSPFR	Sigma
[Lys8]-Vasopressin	Cys–Tyr–Phe–Gln–Asn–Cys–Pro–Lys–Gly–NH_2	CYFQNCPKG-NH_2	APC
[Arg8]-Vasopressin/AVP	Cys–Tyr–Phe–Gln–Asn–Cys–Pro–Arg–Gly–NH_2	CYFQACPRG-NH_2	APC
Dynorphin A (1–10), porcine	Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–Pro	YGGFLRRIRP	APC
Dynorphin A (1–10), amide, porcine	Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–Pro–NH_2	TGGFLRRIRP-NH_2	APC
[D-Ala6]-LH-RH	pGlu–His–Trp–Ser–Tyr–D-Ala–Leu–Arg–Pro–Gly–NH_2	pEHWSY-dA-LRPG-NH_2	Sigma
[des-pGlu1]-LH-RH	His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2	HWSYGLRPG-NH_2	Sigma
[D-Lys6]-LH-RH	pGlu–His–Trp–Ser–Tyr–D-Lys–Leu–Arg–Pro–Gly–NH_2	pEHWSY-dK-LRPG-NH_2	Sigma
[D-Phe2,D-Ala6]-LH-RH	pGlu–D-Phe–Trp–Ser–Tyr–D-Ala–Leu–Arg–Pro–Gly–NH_2	Pe-dF-WSY-dA-LRPG-NH_2	Sigma
Neurotensin	pGlu–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro–Tyr–Ile–Leu	pELYENKPRRPYIL	Sigma
[Phe11]-Neurotensin	pGlu–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro–Phe–Ile–Leu	pELYENKPRRPFIL	Sigma
[D-Trp11]-Neurotensin	Glp–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro–D-Trp–Ile–Leu	pELYENKPRRP-dW-IL	APC
[D-Tyr11]-Neurotensin	Glp–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro–D-Tyr–Ile–Leu	pELYENKPRRP-dY-IL	APC
[Gln4]-Neurotensin	Glp–Leu–Tyr–Gln–Asn–Lys–Pro–Arg–Arg–Pro–Tyr–Ile–Leu	pELYQNKPRRPYIL	APC
Neurotensin, guinea pig	Glp–Leu–Tyr–Glu–Asn–Lys–Ser–Arg–Arg–Pro–Tyr–Ile–Leu	pELYENKSRRPYIL	APC
[D-Pro10]-Dynorphin A (1–11), porcine	Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–D-Pro–Lys	YGGFLRRIR-dP-K	APC
Dynorphin A (1–11), porcine	Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–Pro–Lys	YGGFLRRIRPK	APC
Dynorphin A (1–13), porcine	Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–Pro–Lys–Leu–Lys	YGGFLRRIRPKLK	APC
[D-Arg6]-Dynorphin A (1–13), porcine	Tyr–Gly–Gly–Phe–Leu–D-Arg–Arg–Ile–Arg–Pro–Lys–Leu–Lys	YGGFL-dR-RIRPKLK	APC
[Nor8]-Substance P	D-Pro–Gln–Gln–D-Trp–Phe–D-Trp–Leu–Nor–NH_2	dP-QQ-dW-F-dW-LN-NH_2	APC
Substance P	D-Pro–Gln–Gln–D-Trp–Phe–D-Trp–Leu–Met–NH_2	dP-QQ-dW-F-dW-LM-NH_2	APC

[a] The "d" denotes the chirality of D-amino acids. A lower case "d" had to be used to distinguish them from aspartic acid, which has the single letter abbreviation "D".

Table 2.2: Selectivity and resolution for peptides on Chirobiotic T, TAG, and R stationary phases

Peptides	Columns											
	Chirobiotic T				Chirobiotic TAG				Chirobiotic R			
	MP[a]	Flow rate (mL/min)	α[b]	R_s[b]	MP[a]	Flow rate (mL/min)	α[b]	R_s[b]	MP[a]	Flow rate (mL/min)	α[b]	R_s[b]
Enkephalins												
Y-**dA**-GF-**dL**	B	1			A	2			C	1		
Y-**dA**-GF**M**			1.09	1.75			1.14	2.31			1.24	2.71
Y**G**GF**M**			1.21	4.07			1.2	3.29			1.18	2.04
Y-**dA**-GF**L**			1.32	5.94			1.3	4.48			1.13	1.39
Y**G**GF**L**			1.26	5			1.24	3.56			1.06	0.06
Y**A**GF**L**			1.18	3.72			1	0			1.09	0.96
β,γ-Bag cell factors												
RLRF**H**	F	2							E	2		
RLRF**D**			2.41	11.58							2.96	2.58
β-Casomorphins												
Y-dA-F-**dA**-Y-NH_2	H	1							I	1		
Y-dA-F-**dP**-Y-NH_2			1.11	2.05							1.19	0.64
Y-dA-F-**Hyp**-Y-NH_2			1.32	5.44							1.95	2.56
α-Bag cell factors 7–9												
APRLRF**Y**					O	1.5			L	1		
APRLRFY**S**							1.32	2.29			1.15	1.3
APRLRFYS**L**							1.29	2.05			1.25	1.72
Leucokinins												
DP**A**FSSWG-NH_2	C	1.2							C	1		
DP**G**FSSWG-NH_2			1.07	1.54							1.11	0.82
DP**A**F**N**SWG-NH_2			1.12	2.81							1.28	1.74
Angiotensins												
EGVY**V**HP**F**	P	0.5							G	2		
DRVY**I**HP**F**			1.93	13.3							1.52	3.26
N-methyl-GRVY**I**HP**T**			1.06	1.25							1.45	2.93
N-methyl-GRVY**I**HP**F**			1.09	1.74							1.46	2.94
DRVY**V**HP**F**			1.07	1.39							1	
Substance P	Q	1							D	1		
dP-QQ-dW-F-dW-L**N**-NH_2											5.00	1.33
dP-QQ-dW-F-dW-L**M**-NH_2			5.93	21.2								
Bradykinins												
PPGFSFR	M	1							K	1.2		
PPGFSPFR			2.2	15.12							1.8	3.86
RPGFSPFR			2.37	15.39							2.16	2.16
RPPGFSPFR			1.1	1.82							1.31	1.31
Vasopressins												
CYFQNCP**K**G-NH_2	O	1			Q	1			J	1		
CYFQACP**R**G-NH_2							1.25	2.31			1.16	1.8
Dynorphins												
YGGFLRRIRP	Q	1.5										
TGGFLRRIRP-**NH_2**			1.26	2.3								
Lutenizing hormone releasing hormone												
pE-**dF**-WSY-**dA**-LRPG-NH_2	P	1							G	1		
pEHWSY-**dA**-LRPG-NH_2			1.38	5.16							2.34	7.68
pEHWSY-**dK**-LRPG-NH_2			1.71	8.56							2.14	3.48
HWSY**G**LRPG-NH_2			1.2	2.92							1.3	1.32
Neurotensins												
pELYENKPRRP-**dW**-IL	N	1										
pELYENKPRRP-**dY**-IL			1.26	1.63					G	1	1.15	1.24
pELYENKPRRP**Y**IL			1.08	0.83							1.28	2.04
pELYENKPRRP**F**IL			1.07	0.82							1.22	1.75

[a] Mobile phases: (A) ACN–water (65:35); (B) ACN–water (75:25); (C) ACN–water (85:15); (D) ACN–5 mM ammonium formate aqueous solution, pH 3 (55:45); (E) ACN–5 mM ammonium formate aqueous solution, pH 3 (60:40); (F) ACN–25 mM ammonium formate, pH 3.0 (30:70); (G) ACN–5 mM ammonium formate aqueous solution, pH 3 (65:35); (H) ACN–16 mM ammonium formate, pH as is (75:25); (I) ACN–16 mM ammonium formate, pH as is (90:10); (J) ACN–20 mM ammonium formate, pH as is (20:80); (K) ACN–32 mM ammonium formate, pH as is (50:50); (L) ACN–40 mM ammonium formate, pH as is (60:40); (M) ACN–0.06% formic acid aqueous solution (35:65); (N) ACN–0.1% formic acid aqueous solution (25:75); (O) ACN–0.1% formic acid aqueous solution (30:70); (P) ACN–0.1% formic acid aqueous solution (35:65); (Q) CAN–0.1% formic acid aqueous solution (40:60); (R) ACN–0.75% triethylamine, pH 2.8 (40:60); (S) ACN–1% triethylamine, pH 2.8 (20:80).

[b] $\alpha = k'_2/k'_1$, $R_s = 2(t_2 - t_1)/(w_1 + w_2)$.

Table 2.2: Continued

Peptides	Columns								
	Chirobiotic T			Chirobiotic TAG			Chirobiotic R		
	MP[a]	Flow rate (mL/min)	α[b]	R_s[b]	MP[a]	Flow rate (mL/min)	α[b]	R_s[b]	MP[a]	Flow rate (mL/min)	α[b]	R_s[b]
pELY**Q**NKPRRP**Y**IL			1.09	0.76							1.25	1.97
pELYENK**S**RRP**Y**IL			0	0							1.48	1.69
Dynophin 1–11												
YGGFLRRIR-**dP**-K	R	0.8										
YGGFLRRIR**P**K			1.12	1.53								
Dynophin 1–13												
YGGFL-**dR**-RIRPKLK	S	1										
YGGFL**R**RIRPK			1.19	1.95								

[a] Mobile phases: (A) ACN–water (65:35); (B) ACN–water (75:25); (C) ACN–water (85:15); (D) ACN–5 mM ammonium formate aqueous solution, pH 3 (55:45); (E) ACN–5 mM ammonium formate aqueous solution, pH 3 (60:40); (F) ACN–25 mM ammonium formate, pH 3.0 (30:70); (G) ACN–5 mM ammonium formate aqueous solution, pH 3 (65:35); (H) ACN–16 mM ammonium formate, pH as is (75:25); (I) ACN–16 mM ammonium formate, pH as is (90:10); (J) ACN–20 mM ammonium formate, pH as is (20:80); (K) ACN–32 mM ammonium formate, pH as is (50:50); (L) ACN–40 mM ammonium formate, pH as is (60:40); (M) ACN–0.06% formic acid aqueous solution (35:65); (N) ACN–0.1% formic acid aqueous solution (25:75); (O) ACN–0.1% formic acid aqueous solution (30:70); (P) ACN–0.1% formic acid aqueous solution (35:65); (Q) CAN–0.1% formic acid aqueous solution (40:60); (R) ACN–0.75% triethylamine, pH 2.8 (40:60); (S) ACN–1% triethylamine, pH 2.8 (20:80).

[b] $\alpha = k'_2/k'_1$, $R_s = 2(t_2 - t_1)/(w_1 + w_2)$.

Table 2.3. Best separations for peptide classes by stationary phase

Chirobiotic T	Chirobiotic TAG	Chirobiotic R
Enkephalins	α-Bag cell factors	Neurotensins
β,γ-Bag cell factors	Vasopressins	
β-Casomorphins		
Bradykinins		
Angiotensins		
Dynorphins		
Leucokinins		
Substance P		

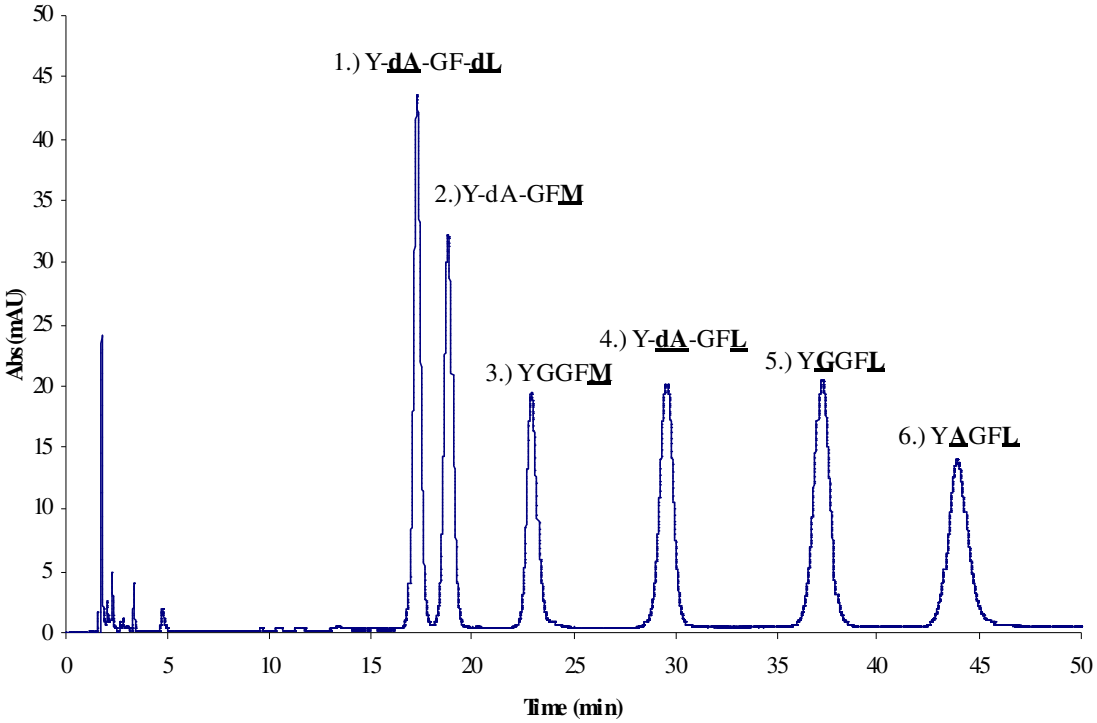

Figure 2.1. Separation of six enkephalin peptides on Chirobiotic T column. Single amino acid polymorphisms (SAAP) occur in: (a) peaks 2 and 4; (b) peaks3 and 5; (c) peaks 5 and 6. Examples of chiral amino acid polymorphisms are: (a) peaks 1 and 4; (b) peaks 4 and 6. Chromatographic conditions are given in Table 2.2.

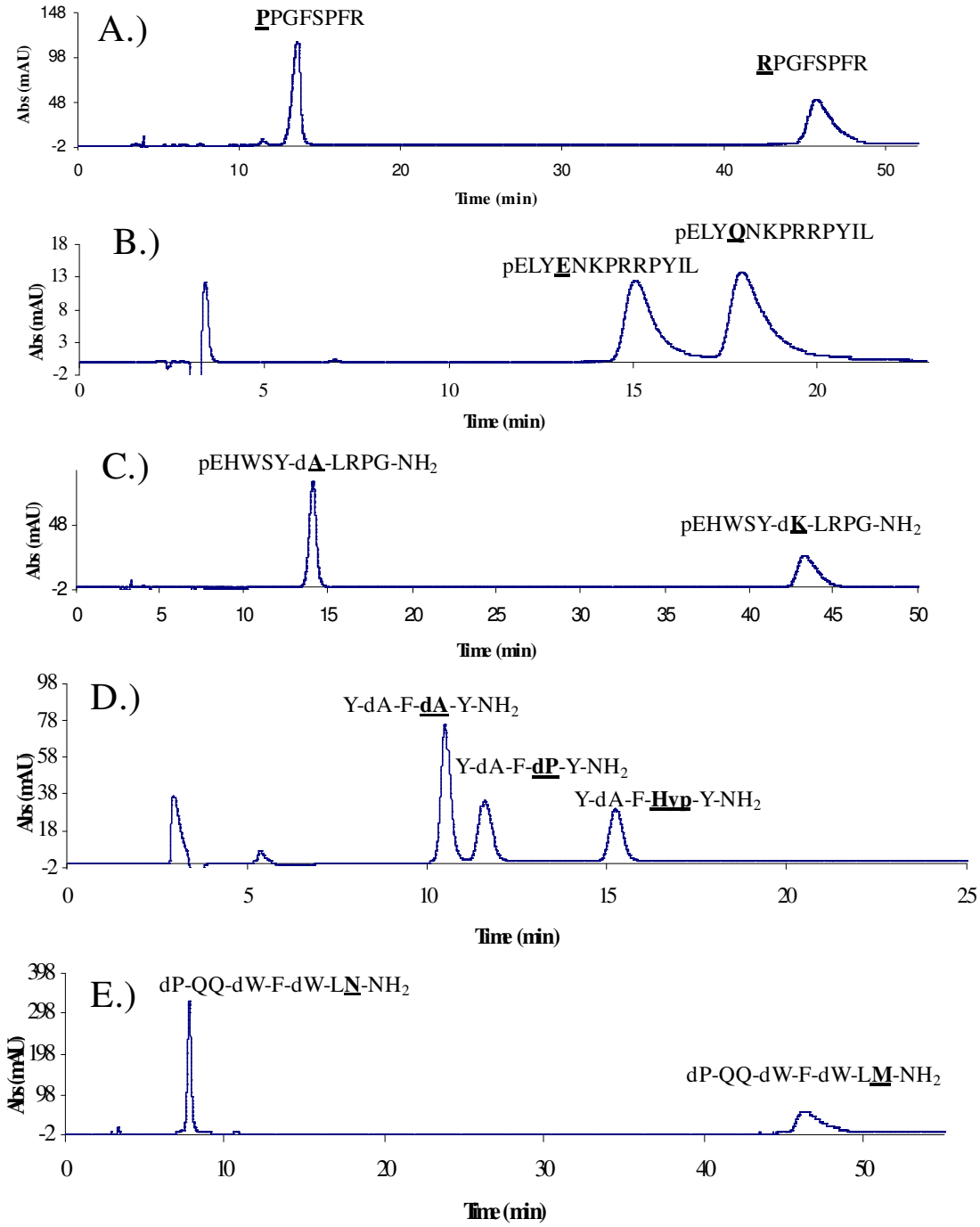

Figure 2.2. Chromatograms showing the effect of the location of a SAAP within the peptide on the separation of the polymorphs. The polymorphism occurs at the: (A) N-terminus (bradykinin family); (B and E) position 4 (neurotensin,β-casomorphin families); (C) position 6 (lutenizing hormone releasing hormone family); or (D) the C-terminus (substance P family) of the peptide. Chromatograms A, C, D and E were generated on a Chirobiotic T column and chromatogram B was generated on a Chirobiotic R column. All chromatographic conditions same as in Table 2.1 using UV detection.

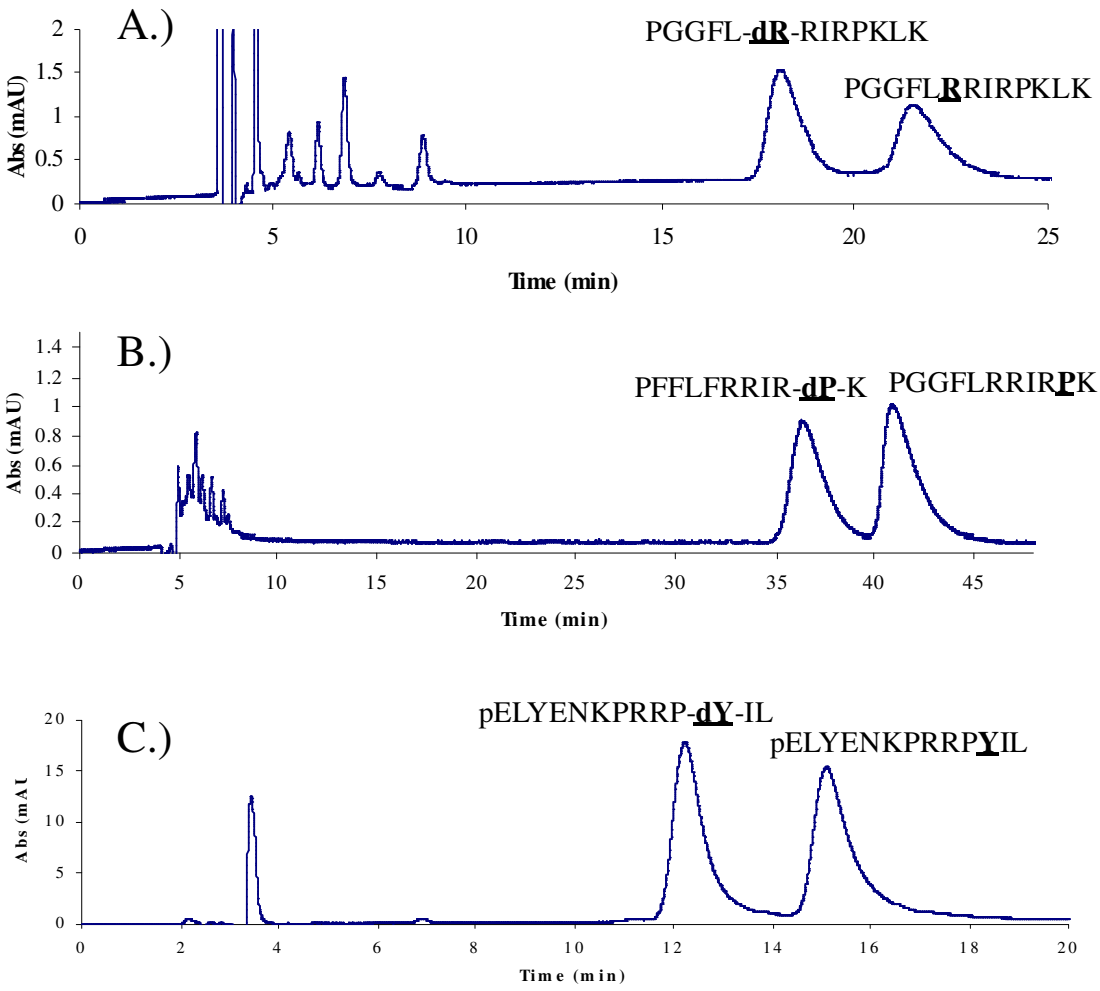

Figure 2.3. Chromatograms showing the effect of the location of a chiral SAAP within the peptide on the separation of the polymorphs. The polymorphism occurs in: (A) position 6 (dynorphin 1–11 family); (B) position 10 (dynorphin 1–13 family); or (C) position 11 (neurotensin family). Chromatograms A and B were produced on a Chirobiotic T column and chromatogram C was produced on a Chirobiotic R column. All chromatographic conditions the same as in Table 2 using UV detection.

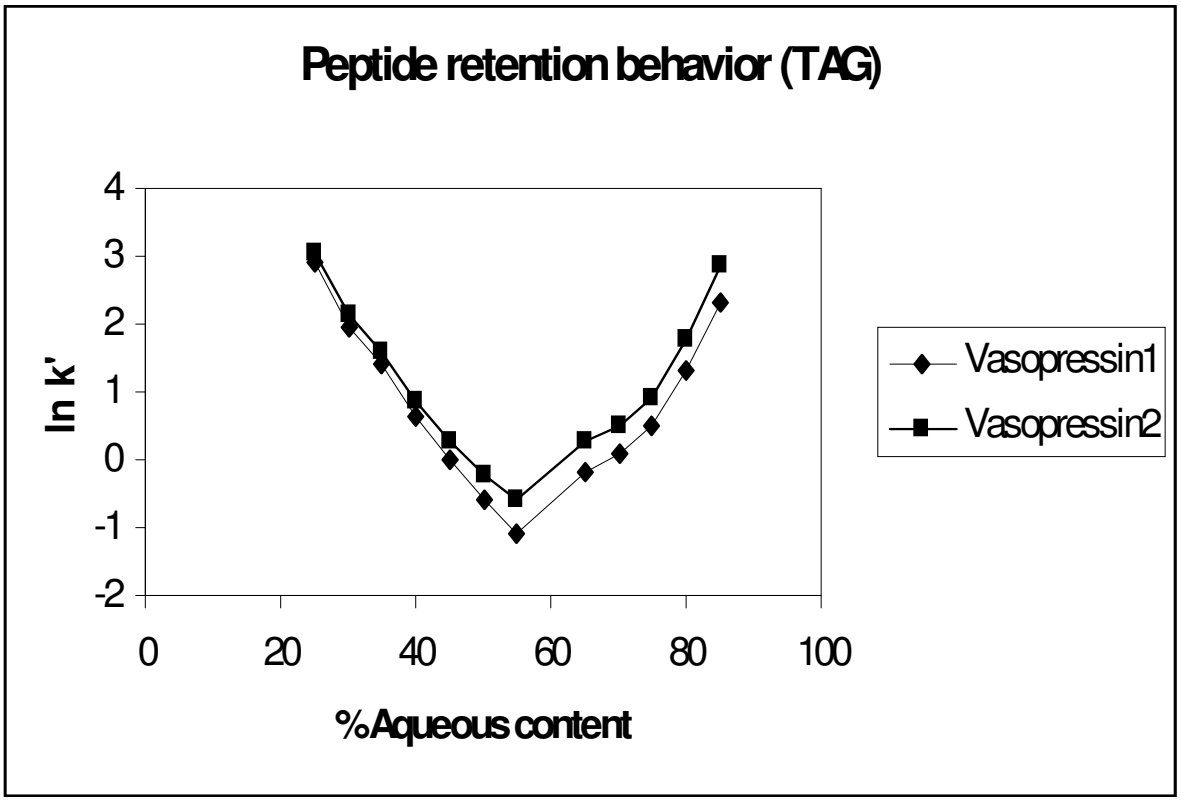

Figure 2.4. Retention of vasopressin peptides on Chirobiotic TAG stationary phase. Increased retention at high organic modifier content is observed due to lower peptide solubility in the mobile phase. Chromatographic conditions: Chirobiotic TAG 250mm × 4.6mm column at a flow rate of 1 mL/min with UV detection at 210 nm. Aqueous solution included 0.1% formic acid.

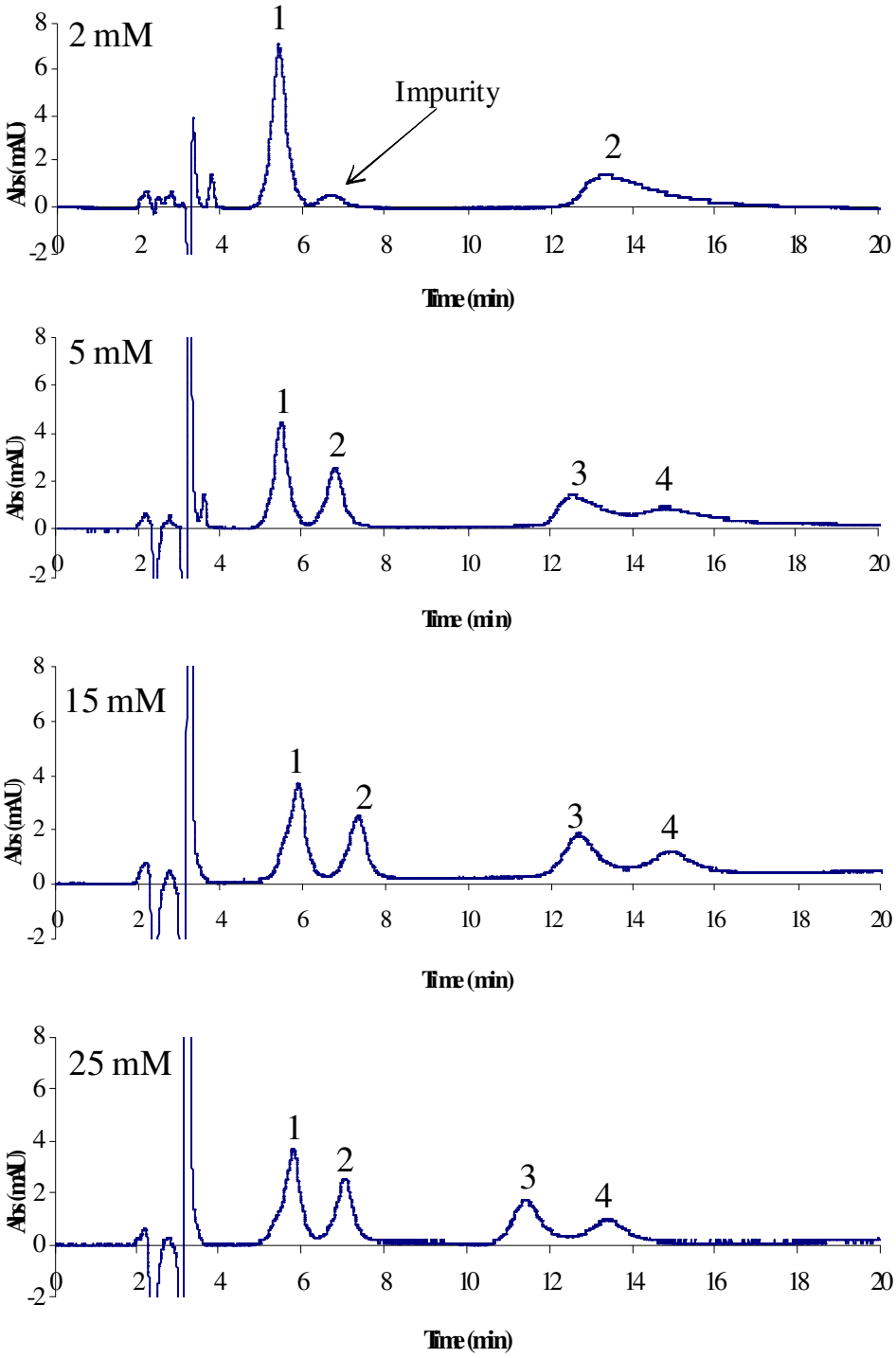

Figure 2.5. The four chromatograms show the effect of ionic strength on the elution of charged peptides. Peptides are from the bradykinin family and the sequence is as follows: (1) PPGFSP; (2) PPGFSPFR; (3) RPGFSPFR; (4) RPPGFSPFR. Chromatographic conditions: Chirobiotic R column, acetonitrile–ammonium formate buffer, pH 3 (60:40), 1.0 mL/min, UV detection at 232 nm.

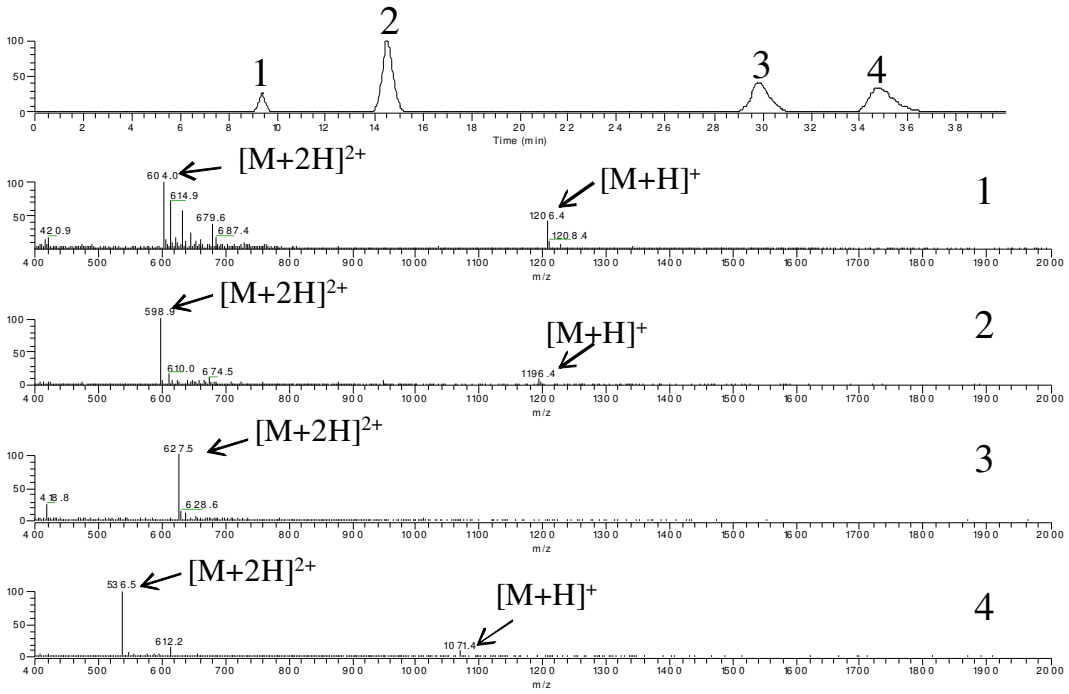

Figure 2.6. LC–ESI-MS of the lutenizing hormone releasing hormone family. Panel A is the base peak chromatogram. Panels B–E are the mass spectra of eachpeak in panel A. HPLC conditions: Chirobiotic T 250mm × 4.6mm column; mobile phase composition: 60% formic acid (0.1%), 40% acetonitrile. Flow rate
1 mL/min.

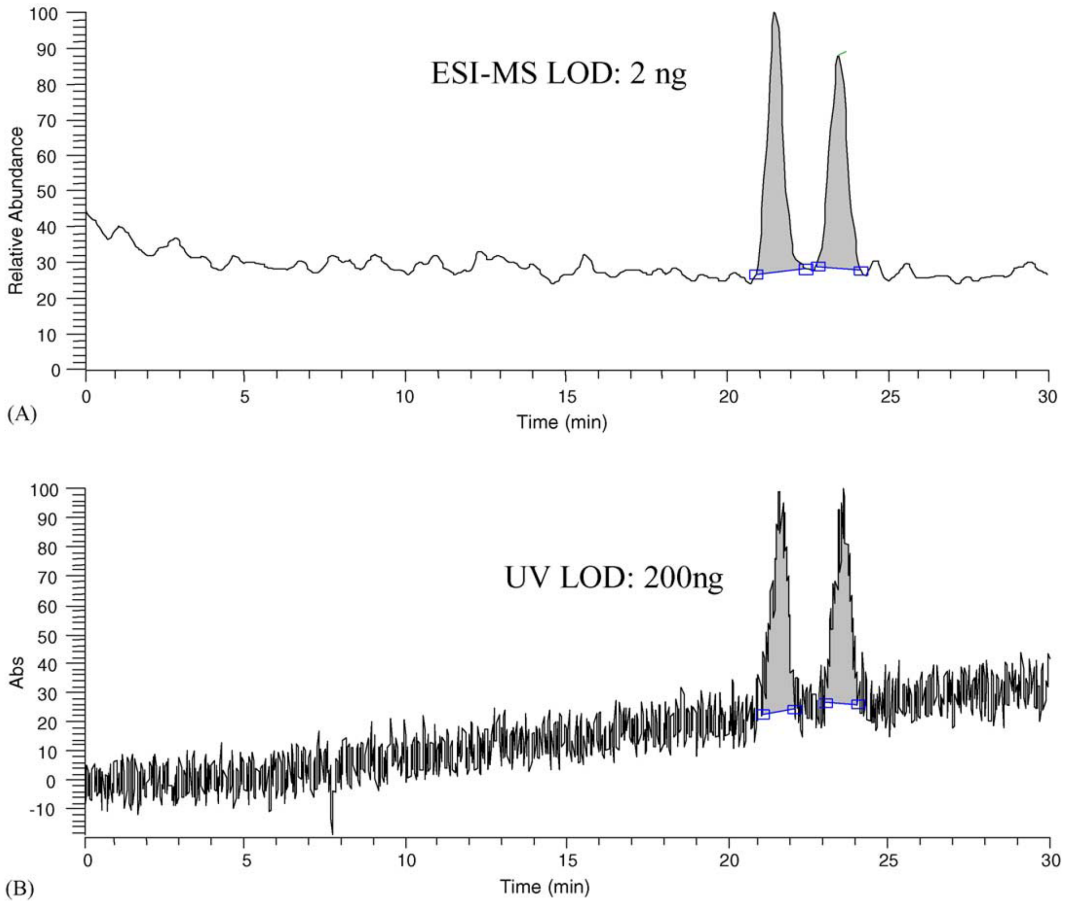

Figure 2.7. The limits of detection for the vasopressin peptides were found for: (A) ESI-MS (selected-ion monitoring mode); (B) UV (210 nm) detection. S/N is the signal to noise ratio of the peaks. Injection volumes (2 _L) were identical for both panels. HPLC conditions: Antibiotic TAG 250mm × 4.6mm column, using a mobile phase composition of 60% formic acid aqueous solution (0.1%), 40% acetonitrile at 0.5 mL/min.

CHAPTER 3

LC AND LC-MS SEPARATION OF PEPTIDES ON MACROCYCLIC GLYCOPEPTIDE STATIONARY PHASES: DIASTEREOMERIC SERIES AND LARGE PEPTIDES

A paper published in Chromatographia[2]

R.J. Soukup-Hein, Jeff Schneiderheinze, Paul Mehelic, D.W. Armstrong

ABSTRACT

Previous work on the LC separation of peptides had shown that macrocyclic glycopeptide stationary phases to be selective for peptides of five to thirteen amino acids in length. In this work, the selectivity of the teicoplanin stationary phase is compared to that of a C18 stationary phase for 7 diastereomeric enkephalin peptides. The teicoplanin stationary phase separated all 7 diastereomeric enkephalin peptides in a single chromatographic run. The insertion of D-amino acids into the primary enkephalin sequence produced areas of hydrophobicity that influenced retention order on the C18 stationary phase. However, analogous trends are not observed on the teicoplanin stationary phase, which is more polar and structurally diverse. Optimization of the mobile phase and the use of a step-gradient for the enkephalin separation on the teicoplanin stationary phase is discussed. Also, the selectivity of macrocyclic glycopeptide stationary phases for peptides of 14, 28, 30, and 36 amino acids also is investigated and compared to separation on a C18 stationary phase. A method for eluting peptides with multiple basic amino acids, which tend to be strongly retained on the macrocyclic glycopeptide stationary phases, is presented.

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3.1. INTRODUCTION

For many years, proteins and peptides were thought to consist only of L-amino acids. While this is true for the majority of these biological compounds, there is growing evidence for the presence of naturally occurring peptides containing D-amino acids. For some time it has been known that microorganisms utilized free and bound amino acids [1], with some microorganisms even incorporating D-amino acids into their cell walls [2]. The discovery of a D-amino acid containing peptide in the venom of a platypus, which is a mammal, is the latest finding of such naturally occurring biologically active peptides in multicellular organisms [3]. The first report of a D-amino acid in a peptide in a multicellular organism was from a frog skin secretion [4]. There are indications that the presence of a D-amino acid in peptides found in biological organisms can be viewed as a very subtle post-translational modification [5,6]. This unusual post-translational modification can be accompanied by other post-translational modifications. Pisarewicz et al. report the recent discovery of D-γ-hydroxyvaline in the venom of *Conus gladiator* in which the D-configuration apparently stabilizes the γ-hydroxylated residue and prevents it from forming a five membered lactone ring [7].

In addition to these naturally occurring peptides, peptides have been synthesized to contain or be made entirely of D-amino acids. The inclusion of a D-amino acid (or acids) into L-amino acid containing peptides creates diastereomeric peptides, while the all D-amino acid peptides are enantiomers of the all L-amino acid peptides. Peptides containing all D-amino acids have been found to be more resistant to proteolysis [8-10]. These all D-amino acid peptides will interact with non-chiral interaction sites in the same way all L-amino acid peptides do, making them very attractive as possible therapeutic agents. For example, a θ-

defensin composed entirely of D-amino acids has been found to provide better protection against HIV-1 than the all L-peptide, most likely due to its increased resistance to proteases [11]. Peptides need not be made of all D-amino acids to exhibit increased resistance to enzymatic activity. Cyclic prodrugs containing diastereomeric peptides have been shown to have different bioconversion rates [12]. Changing the chirality of amino acids in diastereomeric peptides also can influence membrane transport due to changes in their physiochemical properties [13].

For the reasons outlined above, it is important to characterize and analyze peptides both in terms of enantiomeric and diastereomeric compositions. The stereoselective analysis of peptides was recently reviewed by Czerwenka and Lindner [14]. The majority of the analysis of peptide enantiomers and diastereomers has been achieved using HPLC and capillary electrophoresis, although the use of tandem mass spectrometry for tasks involving diastereomers is increasing [14-16]. Peptides with D-amino acids in the primary sequence have displayed different fragmentation patterns from peptides of the same primary sequence made of all L-amino acids [15, 16].

Teicoplanin and its aglycone analogue have been shown to be very selective stationary phases for di- and tri-peptides [17-19]. In 2004, we reported the separation of peptides (up to 13 amino acids in length,containing at most two D-amino acids) with single amino acid polymorphisms, including diastereomeric peptides, on macrocyclic glycopeptide stationary phases [20]. In this work, we explore the selectivity of macrocylic glycopeptide stationary phases in two more areas. First, we investigate the selectivity of teicoplanin for a series of diastereomeric peptides of five amino acids in length. Furthermore, we wanted to examine the selectivity of the macrocyclic glycopeptide stationary phases for peptides of

greater lengths. This report outlines our findings in regards to these issues and how the separations compare to those of a standard C18 stationary phase.

3.2. EXPERIMENTAL

3.2.1 Materials

All solvents (water, acetonitrile (ACN), and methanol) were HPLC grade and purchased from Fisher Scientific (Pittsburg, PA, USA). Ammonium acetate (98%) and formic acid (88% ACS reagent grade) were purchased from Sigma (St. Louis, MO, USA) and J.T. Baker (Phillipsburg, NJ, USA), respectively. Trifluoroacetic acid, 99% spectrophotometric grade, and ammonium trifluoroacetate, 98%, were purchased from Aldrich (Milwaukee, WI, USA). Triethylamine (HPLC reagent grade) was also obtained from Fisher. The commercially available enkephalin peptides were purchased from Sigma (E1a, E2a)and Bachem (E0) (Bachem California, Torrance, CA, USA) and are listed in Table 3.1. Custom synthesized peptides (E1b, E2b, E3, E4) also listed in table 1, were synthesized by both the Iowa State Peptide facility and EZbiolab (Westfield, IN, USA). All peptides listed in Table 3.2 were purchase from American Peptide Co. (APC, Sunnyvale, CA, USA). All separations were carried out on analytical size (250mm x 4.6 mm i.d.) Chirobiotic T, Chirobiotic R, or C18 stationary phases that were obtained from Advanced Separation Technologies (ASTEC, Whippany, NJ, USA).

3.2.2. Instrumentation

A Thermo Finnigan (San Jose, CA, USA) Surveyor LC system was used for all chromatographic methods with a photo diode array detector. Xcalibur version 3.1 software was used for data acquisition and analysis. For LC-MS methods, the same chromatographic

system was coupled to a Thermo Finnigan LCQ ion-trap mass spectrometer fitted with an electrospray ionization (ESI) source. Two separate ESI conditions were used. Conditions for analyzing the enkephalin peptides are as follows: source voltage = +4.5 kV; sheath gas = 25 AU (arbitrary units); auxiliary gas = 10 AU; capillary voltage = +3.3 V; capillary temperature = 270^{o}C; tube lens offset = +15V. The larger peptides were introduced into the mass spectrometer using the following conditions: source voltage = +4.5 kV; sheath gas = 50 AU; auxiliary gas = 40 AU; capillary voltage = +10 V; capillary temperature = 270^{o}C; tube lens offset = 0V.

3.2.3. Chromatographic conditions

Specific chromatographic conditions are reported with the separations. In general, acetonitrile was used as the organic modifier in all separations, with concentrations ranging between 40% and 80%. The aqueous portion of the mobile phase consisted of water with various additives, ammonium acetate buffer, or triethylammonium trifluoroacetate buffer. For the enkephalin peptides, 3mM ammonium acetate pH 3.8 was used as the aqueous portion of the mobile to enhance reproducibility. Flow rates were 0.4-0.6mL/min for LC/MS analysis and 1.0mL/min for UV detection. All peptides were dissolved in a 50/50 mixture of water and methanol. Stock solutions (1 mgmin^{-1}) of dissolved peptides were stored in a freezer. Working solutions were made to contain 10ug/mL of each peptide of interest.

The C18 separations of the enkephalin and bombesin peptides were conducted with the MS compatible mobile phases (aqueous acetic acid or acetate buffers with acetonitrile as the buffer) and similar flow rate conditions (0.5 mL min^{-1} vs. 0.6mL min^{-1})in order to compare the C18 separation to those achieved on the macrocyclic glycopeptides. Slightly lower flowrates were used with the C18 stationary phase since the mobile phases generally

had a higher percentage of aqueous content. Specific chromatographic conditions are reported with the corresponding figures. The separation of galanin, somatostatin, and neuropeptide Y peptides on the C18 stationary phase was based on the method by Kirby et al.[21] with some modifications. Mobile phase A was 0.1% phosphoric acid in water pH 2.25 and mobile phase B was 40% A/60% ACN. Linear gradients were executed as described in the figure captions at a flow rate of 1 $mLmin^{-1}$. UV detection was at 214 nm.

3.3 RESULTS AND DISCUSSION

We report here our findings in two new areas regarding the use of macrocyclic glycopeptide CSPs for the separation of peptides. First, we extend the use of macrocyclic glycopeptides to the separation of a series of diastereomeric enkephalin peptides (with four chiral amino acids). Next, we examined the separation of peptides with more than 13 amino acids. Comparisons of selectivities of the macrocyclic glycopeptide stationary phases with that of a standard C18 stationary phase is reported.

3.3.1 Diastereomeric results

A few publications have examined the elution order of small peptide stereoisomers (two to three amino acids in length) using teicoplanin as the chiral CSP [17,18] in HPLC or the chiral selector in CE[19] . Enantiomeric separations of glycyl dipeptides by teicoplanin in CE have also been reported [19]. These peptides had only one chiral amino acid and thus eluted in the manner consistent with single amino acids. In HPLC studies, only the complete elution order for two peptides (Ala-Ala and Leu-Leu) was determined as all four diastereomers for these two peptides were commercially available. The order of elution for Ala-Ala was (1) D-L (2) L-L (3) D-D (4) L-D [17] while the order for Leu-Leu was (1) D-L

(2) L-L (3) L-D (4)D-D [17,18]. Thus, all peptides with a D-amino acid at the carboxy terminus (which was the primary interaction site) were expected to be more retained than the peptides with an L-amino acid at the carboxy terminus. However, this was not observed for the enkephalin peptide (primary amino acid sequence: Tyr-Ala-Gly-Phe-Leu; YAGFL) family [20], when peptide E2a (Y-dA-GF-dL) eluted as the first of the six enkephalin peptides. Obviously, larger peptides than the di-peptides are needed to more fully understand the role D-amino acids play in the elution order of peptides on the teicoplanin stationary phase. The enkephalin peptide family was chosen to provide the primary sequence as the framework for the additional diastereomeric peptides needed for this purpose.

The peptides were designed to have zero, one, two, three or four D-amino acids (as glycine is not chiral). Two additional peptides containing one and two D-amino acids were included to increase the complexity of the diastereomeric series. The peptides and their sequences are listed in Table 3.1 according to their elution order on the teicoplanin stationary phase; peptides E0, E1a, and E2a have been already analyzed in a previous paper [20]. To the best of our knowledge, this is the first separation of a diastereomeric series of peptides larger than two or three amino acids on this macrocyclic glycopeptide stationary phase.

The optimized separation of all seven enkephalin peptides is shown in Figure 3.1a. The optimization of the mobile phase on the macrocyclic glycopeptide stationary phase will be discussed in a later section. Under these conditions, all peptides are baseline or nearly baseline separated. It is obvious from this chromatogram that the peptides with three and four D-amino acids are the most strongly retained by the stationary phase. In fact, a step gradient was necessary in order to decrease the elution time for peptides E3 and E4. It is important to note that peptides E0 and E4 are enantiomers and are separated with a high

degree of selectivity even using a gradient. E2a elutes first under these conditions as it did in [20] which contained many more peptides with L-amino acids at the carboxy terminus. Also, E1b (with a D-leucine at the carboxy terminus) elutes before the all L-amino acid peptide, though two peptides (E1a, E2b) terminated with an L-leucine at the carboxy end of the peptide do elute before it. It has been shown that single amino acids interact through their carboxylic acid groups with the teicoplanin stationary phase's amino group and that D-amino acids bind more strongly to the stationary phase [17,22]. It is possible that the smaller di- and tri-peptides can still bind to the stationary phase in a mechanism similar to that of the single amino acids. However, once the peptides reach the size of the enkephalin peptides, this may not always be possible as indicated by the elution of E2a and E1b.

Since most of the enkephalin peptides are diastereomers and there are only two enantiomers, a traditional C18 stationary phase could also be used to separate most of the enkephalin peptides. The use of C18 stationary phases to separate enkephalin analogs containing β-methylphenylalanine was reported earlier by Peter et al [23]. The insertion of the β-methylphenylalanine at the fourth position resulted in four diastereomers (two pairs of epimers) from the four isomers of methylphenylalanine. Separation of the epimeric peptides containing the erythro- and threo- isomers of β-methylphenylalanine was much more difficult than separating the epimeric peptides containing the L- and D-isomers[23]. The separation of a diastereomeric enkephalin pair of peptides on a alkylphenyl column was also reported by Currie et al. [24]. The diastereomers resulted from a D-alanine/alanine difference at the second position and a baseline separation was achieved. The diastereomeric set of peptides we have included in this study are more complex than either set in [23] or [24].The separation of the enkephalin diastereomers on a C18 stationary phase is shown in Figure

3.1b. Since the C18 is not a chiral stationary phase it cannot be expected to separate the enantiomers E0 and E4. But, the retention order of the remaining diastereomeric enkephalin peptides is quite different on the C18 stationary phase. E2a is now the longest retained peptide and E3 co-elutes with E1a. E0 and E4 are now the first eluted peptides rather than being among the most retained. Using an isocratic mobile phase of 75% 0.1% (*v/v*) acetic acid in water and 35% acetonitrile, it is possible to separate E1a and E3. But under these conditions, E2a and E2b coelute and neither are well separated from E1b. It should be stated that the elution trends observed in one peptide family may not always hold for other peptide families with different amino acid sequences and/or different numbers of amino acids.

In general, the shape of the peptide dictates how it will be retained by the stationary phase. For an all L-amino acid containing peptide, this shape is determined by the primary amino acid sequence. Also for all L-amino acid containing peptides, the side chains of adjacent amino acids are located on the opposite side of the peptide backbone from the side chains of the adjacent amino acids. Areas of hydrophobicity or hydrophilicity would be determined by the side chains of the amino acids. For a series of diastereomeric peptides, like the enkephalin peptides, the types of side chains are all the same. In this case, the presence of a D-amino acid and its position in the peptide influences its affinity for the stationary phase. Since D-amino acids have the opposite configuration of L-amino acids, replacing an L-amino acid with a D-amino acid places that side chain on the opposite side of the peptide backbone. This different side chain position alters the shape and apparent hydrophobicity of the peptide, and in turn, its affinity for the stationary phase. Kroeff and Pietrzyk showed that the tripeptide A-dA-A displayed longer retention (higher apparent hydrophobicity) than either the AAA or the AA-dA tripeptide on a C8 bonded stationary

phase [25]. The LDL orientation of A-dA-A places all three methyl side groups on the same side of the peptide bond creating a relatively large hydrophobic surface. Using this reasoning, the enkephalin peptide E2b (Y-dA-G-dF-L) which has all the side groups on one side of peptide backbone would be expected to have the longest retention on the C18 stationary phase (see Figure 3.2). Instead, E1b (YAGF-dL) and E2a (Y-dA-GF-dL) are retained longer. One thing all three of these peptides do have in common is that their sequences place the hydrophobic side chains of phenylalanine and leucine on the same side of the peptide backbone. This creates a contigous "zone" or area of hydrophobic groups and this results in these three peptides having the greatest retention on C18 stationary phases. Only partial adherance (of this series of enkephalin peptides on a C18 column) to the retention trend described by Kroeff and Pietrzyk may be due to (1) increased structural complexity of the enkephalin diastereomers, including the different hydrophobicities of the amino acids involved, (2) a more hydrophobic stationary phase, (3) different (less acid, with an organic modifier) chromatographic conditions. However, it is worth repeating that under more acidic conditions E1b, E2a, and E2b are not well separated. Still, it can be said that apparent hydrophobicity plays a large role in determining retention order on a C18 stationary phase.

In contrast, comparing the orientation of the side chains around the peptide backbone with retetion order on the teicoplanin stationary phase yields no obvious correlation. The macrocyclic glycopeptide stationary phases are much more functionally diverse and complex stationary phases than the C18 stationary phase. The macrocyclic glyclopeptides have areas of both hydrophobicity and hydrophilicity in addition to a C-shaped three-dimensional structure [26]. Thus the macrocyclic glycopeptide stationary phases are capable many more

different types of interactions compared to a C18 stationary phase. These multiple interactions result in a selectivity (and retention order) different than C18 stationary phases where retention is more dependent on apparent hydrophobicity.

3.3.2. Optimization of the mobile phase for diastereomeric peptides on the teicoplanin stationary phase:

For the reason mentioned in the experimental section 3mM ammonium acetate buffer pH 3.8 was used as the initial mobile phase of the step gradient. At higher concentrations of buffer or at pH values closer to 7, selectivity for the enkephalins is lost and all peptides elute close to the void volume of the column. At a concentration of 3mM ammonium acetate, the mobile phase provides reproducible retention without losing too much selectivity for the enkephalin peptides, especially E1a and E2b. The acetonitrile concentration was set at 78% as retention increased rapidly at higher concentrations.

Using the aforementioned isocratic mobile phase, the all D-amino acid peptide (E4) retained approximately 120 minutes and E3 was retained approximately 75 minutes. Thus a gradient was needed to reduce the retention times of these two peptides. A step gradient was chosen since linear gradients degraded the separation of the earlier eluting peaks and did not sufficiently decrease the elution of the late eluting peaks. The second mobile phase used in the step gradient needed to be optimized for acetonitrile concentration, additive type and concentration, as well as the initiation time.

All peptides can be strongly retained using mobile phases that are high in organic modifier content or high aqueous content on the macrocyclic glycopeptide stationary phases[17,20] as well as alkyl bonded stationary phases [27,28]. Plots of mobile phase composition versus retention produce U-shaped retention curves, with the retention minimum

usually occurring with mobile phases made of equal amounts organic modifier and aqueous solvent. When peptides exhibit U-shaped retention curves, reverse gradients are a possible way to reduce retention. For the enkephalin peptides, a reverse step gradient from 22% 3mM ammonium acetate buffer/ 78% acetonitrile to 50/50 3mM ammonium acetate buffer pH 3.8 /acetonitrile was tried, but the retention time for peptide E4 was still too long. Even increasing the ionic strength of the buffer did not provide a significant reduction in retention time. The next step was to change the mobile phase additive to formic acid.

Employing a step graident using a second mobile phase of 60/40 acetonitrile/0.1% formic acid in water greatly reduced the retention times of E3 and E4, but the two peptides eluted together. Thus, the acetonitrile concentration was gradually increased to 70% to obtain the chromatograms in Figure 3.1a. Increasing the formic acid concentration to 0.15% or 0.20% also makes E3 and E4 co-elute. The timing of the step gradient also is critical. If the step gradient is started too early, E3 and E4 will co-elute under otherwise optimized conditions (data not shown). The optimized step gradient begins at 35 minutes with the change complete at 36 minutes.

It is also important to note that UV detection with this gradient would be difficult. This step gradient involves a big difference in concentration of two UV-absorbing anions (acetate (3mM) and formate (0.1%*v/v*)). The mobile phase containing 0.1% formic acid causes an increase of several hundred milli-absorbance units (mAU) in the UV baseline (data not shown). This rise makes it difficult to detect E3 and E4. As can be seen in Figure 3.1a, this gradient doesn't interfere with MS detection. In this instance, the MS detector allows for the use of a step gradient that would be very difficult to use with UV detection only.

3.3.3. Separation of peptides containing 14 to 36 amino acids

Previously, the largest peptides separated on the macrocyclic glycopeptide stationary phases contained 13 amino acids [20]. The peptides in this study from the bombesin, galanin, somatostatin, and neuropeptide Y (NPY) families contain up to 36 amino acids. The peptide sequences are shown in Table 2. Relatively few publications on the separation of analogues or related peptides in these families have been published [21, 29-36]. Some of the separations reported are focused on the separation of just two peptides(normally two epimeric peptides) rather than a complex mixture, and the separations were intended for use as a method to determine the purity of a synthesized peptide[28, 29]. Separations were reported involving related somatostatin peptides, but with much shorter sequences [30, 31]. More commonly, separations of a peptide and its related fragments are reported. The separation of peptide fragments from a parent peptide have been published for bombesin [32], galanin [33], and neuropeptide Y [34-36]. In the case of NPY, Racaityte et al. reported difficulty in separating NPY from NPY(3-36) [35], although a separation of NPY, NPY(2-36), and NPY(3-36) was reported using CE [36]. Because of the lack of comparable separations for most of these larger peptides, these peptide families are separated using a method (similar to that developed by Kirby et al. [21]) using phosphate buffer/acetonitrile mobile phase on a C18 stationary phase, except for the Bombesin peptides. The bombesin peptides are separated using an MS-compatible mobile phase in order to compare them under similar conditions to those used for the separation of the other peptides on the macrocyclic glycopeptides.

The bombesin peptides have fourteen amino acids and three of these peptides contain D-amino acids. The optimized separation using isocratic conditions (Figure 3.3a) was achieved using the ristocetin (Chirobiotic R) stationary phase. As shown in Figure 3.3a, there is a high degree of selectivity between the D-amino acid containing peptides and those containing all L-amino acids. This is due in part because the D-amino acids (D-phenylalanines) have replaced amino acids with basic side chains (histidines). Peptides with more basic amino acids are more highly retained on the macrocyclic glycopeptide stationary phases [17, 20]. In the case of the bombisin peptide mixture, ristocetin, rather than teicoplanin, was the more selective and broadly useful macrocyclic glycopeptide stationary phase. Ristocetin also showed a higher selectivity than teicoplanin for the six neurotensin peptides (containing 11 amino acids) previously reported [20]. The separation of the bombesin family on the C18 stationary phase is shown in Figure 3.3b. The selectivity of the C18 stationary phase is similar to the ristocetin stationary phase; however, the retention order is completely reversed. Because a gradient was used, the bombesin peaks are sharper to those on the ristocetin stationary phase. Thus, the column regeneration time must be factored into the overall analysis time for the C18 stationary phase before comparing analysis times of the two methods.

The other peptides in this study are approximately twice as long as the bombesin peptides. While these larger peptides still exhibit U-shaped retention curves, the curve in the mostly organic region of the mobile phase is too steep for useful separations. This is because the peptides rapidly become insoluble in mobile phases containing appreciable quantities of organic modifier. As a result, these peptide separations used mobile phases containing more aqueous buffer than acetonitrile.

The galanin family includes peptides that are thirty amino acids long, with two of the peptides having an amidated C-terminus. Figure 3.4a shows the separation of the galanin peptides on the teicoplanin (Chirobiotic T) stationary phase using UV detection. Formic acid was used as the mobile phase additive. A higher concentration of formic acid was necessary to elute the galanin peptides than was needed to elute the smaller peptides. This mobile phase also is LC-MS compatible, although a slower flow rate must be used for the best sensitivity. The Galanin peptides are not as well separated on the C18 stationary phase using a standard phosphate buffer/acetonitrile gradient (Figure 3.4b). While G2 and G3 are well separated, G1 is not separated from G3. Also, the gradient separation takes longer than the isocratic separation on the teicoplanin stationary phase.

Using the macrocyclic glycopeptide stationary phases, the somatostatin and neuropeptide Y families were very challenging to separate. The peptides in both families contain several amino acids with basic side chains, making them somewhat similar to the previously reported dynorphin family of peptides [20]. The dynorphin family of peptides was separated on the Chirobiotic T column using an acetonitrile/ 1.0% triethylammonium acetate (TEAA) buffer pH 2.8 mobile phase. When this same buffer and acetonitrile concentration was used as the mobile phase, the neuropeptide Y peptides did not elute. The somatostatin peptides did elute, but with extremely broad peaks. To decrease the interaction of the peptides with stationary phase, the acetic acid in the triethylammonium buffer was replaced with trifluoroacetic acid to create a triethylammonium trifluoroacetate buffer. This new buffer system eluted both the somatostatin and neuropeptide Y families, at even lower buffer concentrations (0.25% triethylammonium trifluoroacetate). Thus, it might be possible to use

various step gradients to fractionate peptides according to the number of basic amino acids contained in the larger peptides when using macrocyclic glycopeptide stationary phases.

Isocratic separations using the triethylammonium trifluoroacetate buffer still yielded very broad peaks, so gradient elution was explored. Because of the U-shaped retention curve exhibited by peptides, both traditional and reverse gradients were tried. Reverse gradients caused all of the peptides to elute in a single peak.

Figure 3.5 shows the separation of the somatostatin (a) and neuropeptide Y (b) peptides using a traditional gradient on the Chirobiotic T (teicoplanin) stationary phase. For both peptide families, using a gradient increased the sharpness of the peaks, but did not provide any new separations (peaks). In both Figure 3.5 a and b, the teicoplanin stationary phase shows some selectivity for the separation of diastereomeric peptides. In Figure 3.5a, the first eluting peptide (S1) contained one D-amino acid and was easily separated from S3 which only differed by a leucine or norleucine (Nle) at position 8, the D-tryptophan/tryptophan at position 22, and tyrosine/phenylalanine at position 25. The substitutions in positions 8 and 25 are rather subtle, which suggests the D-tryptophan/tryptophan substitution may be the dominant factor that affects interactions with the stationary phase. Peptides N1-N4 elute in one peak, while peptides N5-N7 elute as the second peak. Here the N6 and N7 contain D-amino acids and elute after most of the all L-amino acid peptides. These are the first peptides over three amino acids in length where a peptide containing a single D-amino acid elutes after peptides containing only L-amino acids. N5 was the only all L-amino acid peptide that co-eluted with the N6 and N7. It is interesting to note that this occurs even with the D-amino acid at a similar distance from the C-terminus as the D-amino acid in the somatostatin peptide S1. This suggests that teicoplanin could still

be selective for epimeric/diastereomeric peptide pairs of this length. The somatostatin peptides were eluted on a C18 stationary phase in the same order as on the teicoplanin stationary phase(data not shown). A better separation of the peptide containing the D-amino acid (S1)was achieved using the C18 stationary phase, but S2 was not well separated from S3 and S4. Neither the teicoplanin or a C18 stationary phase with a phosphate buffer/acetonitrile gradient (data not shown) separate the seven NPY peptides well. However, some differences in selectivity between the two stationary phases are observed. The teico plainin stationary phase separates N7 from N1-3, but these four peptides coelute on the C18 phase. The C18 stationary phase is able to differeniate between N6 and N7 (each with a single D-amino acid in position 32) which co-elute on the teicoplanin stationary phase. This would suggest that for fairly subtle substitutions in peptides the size of NPY are very difficult to separate, regardless of the chosen stationary phase.

3.4 CONCLUSIONS

Seven diastereomeric enkephalin peptides could be separated using the macrocyclic glycopeptide Chirobiotic T (teicoplanin) stationary phase. The teicoplanin stationary phase could separate peptides E1a (Y-dA-GFL) and E3(Y-dA-G-dF-dL) which were not separated on the C18 stationary phase. A step gradient was necessary to reduce the retention of the peptides with three or more D-amino acids on the teicoplanin stationary phase. Peptides with more than 13 amino acids could be separated on the teicoplanin and ristocetin stationary phases with varying degrees of success, with the best separations being achieved for the galanin and bombesin peptide families. A standard C18 stationary phase provided slightly improved separations for the bombesin, somatostatin, and NPY peptide families. A new

approach for eluting very basic peptides from the macrocyclic glycopeptides using triethylamine and trifluoroacetic acid was used to separate the somatostatin and neuropeptide Y families. It may be possible to use this buffer system (at different concentrations) to fractionate large peptides (~30 amino acids or more) based on the number of basic amino acids in the sequence. This will be subject to future studies.

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Table 3.1: Separation of Enkephalin Peptide Diastereomers on Teicoplanin CSP

Peptide ID	Peptide Sequence	k	R_s
E2a[1]	Y-**dA**-GF-**dL**	1.2	
E1a[1]	Y-**dA**-GFL	1.8	5.7
E2b	Y-**dA**-G-**dF**-L	2.1	1.6
E1b	YAGF-**dL**	2.7	4.6
E0[1]	YAGFL	3.3	3.1
E3	Y-**dA**-G-**dF**-**dL**	N/A	20.3
E4	**dY**-**dA**-G-**dF**-**dL**	N/A	2.1

k : defined by the equation $k = (t-t_0)/t_0$ where t_0 is the column void volume. Due to the employed step-gradient, k, is not defined for E3 and E4.

R_s: defined by the equation $Rs = (t_2-t_1)/(w_1+w_2)$ where t_2 is the retention time of the peptide and t_1 is the retention time of the preceding peptide and w_1, w_2 is the baseline width of the peaks. Separation conditions are the same as those in Figure 1a.

[1] Peptides E0, E1a, E2a were previously analyzed on Chrirobiotic T [19]

Table 3.2: Investigated Peptide Families

Peptide ID	Peptide Sequence
	Bombesin Family (14 Amino acids)
B1	pEQRLGNQWAVG**-dF-**L**L**-NH_2
B2	pEQRLGNQWAVG**-dF-**LM-NH_2
B3	pEQR**Y**GNQWAVG**-dF-**LM-NH_2
B4	pEQRLGNQWAVGHLM-NH_2
B5	pEQ**K**LGNQWAVGHLM-NH_2
B6	pEQR**Y**GNQWAVGHLM-NH_2
	Galanin Family (30 Amino acids)
G1	GWTLNSAGYLLGPH**AVG**NHRSFSDKNGLT**S**
G2	GWTLNSAGYLLGPH**AID**NHRSFSDKHGL**T-NH_2**
G3	GWTLNSAGYLLGPH**AID**NHRSF**H**DK**Y**GL**A-NH_2**
	Somatostatin Family (28 Amino acids)
S1	SANSNPA**L**APRERKAGCKNFF-**dW**-KT**Y**TSC
S2	SANSNPA**M**APRERKAGCKNFFWKTFTSC
S3	SANSNPA-**Nle-**APRERKAGCKNFFWKTFTSC
S4	**Y**SANSNPA**M**APRERKAGCKNFFWKTFTSC
	Neuropeptide Y Family (36 Amino acids)
N1	YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY-NH_2
N2	YPSKPDNPGEDAPAEDLARYYSALRHYINL**L**TR**P**RY-NH_2
N3	YPSKPDNPGEDAPAEDLARYYSALRHYINLITR**P**RY-NH_2
N4	YPSKPDNPGEDAPAED**M**ARYYSALRHYINLITRQRY-NH_2
N5	YPSKPDNPGEDAPAED**M**ARY**Y(OME)**SALRHYINL**I**TRQRY-NH_2
N6	YPSKPDNPGEDAPAED**M**ARYYSALRHYINLI-**dW**-RQRY-NH_2
N7	YPSKPDNPGEDAPAEDLARYYSALRHYINLI-**dW**-RQRY-NH_2

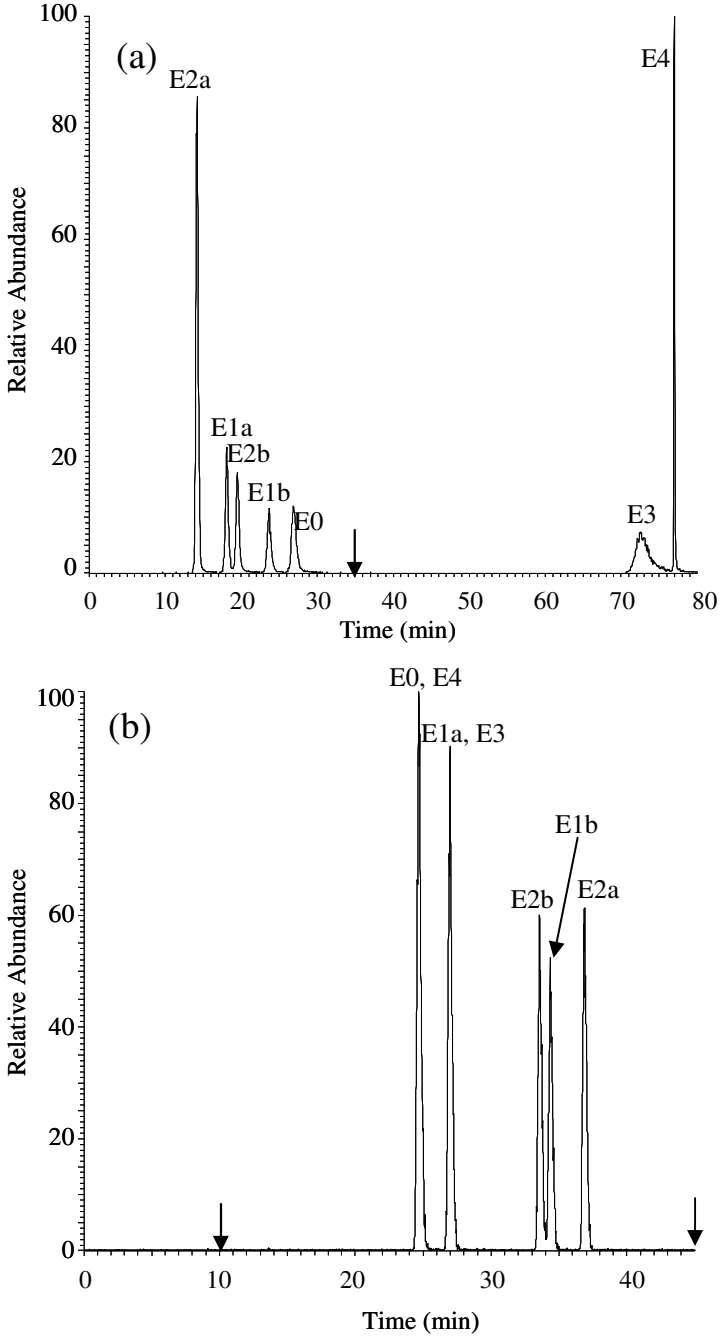


Figure 3.1: Gradient separations of enkephalin diastereomers on (a) teicoplanin and (b) C18 stationary phases. The gradient for (a) was isocratic for 35 minutes with a mobile phase of 78% ACN/22 % 3mM ammonium acetate buffer pH 3.8 then stepped to 70% ACN/ 30% 0.1% (v/v) formic acid in water at 36 minutes. Flow rate was 0.6mL/min. The gradient for (b) was 80% 13mM ammonium acetate (pH as is)/20% ACN for 10 minutes with a linear gradient to 30% ACN complete at 45 minutes. Flowrate was 0.5mL/min. Small arrows indicate the beginning of the step gradient (a) and the beginning and end of the linear gradient in (b).

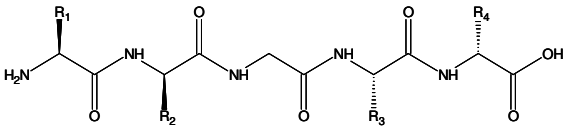
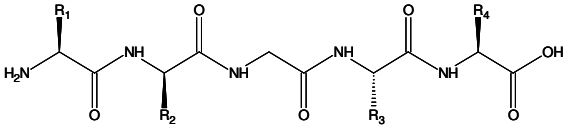
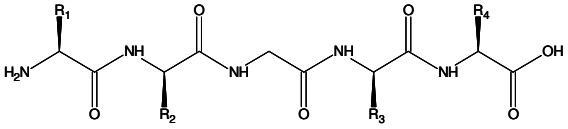
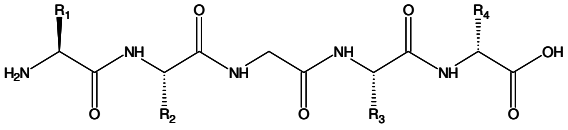
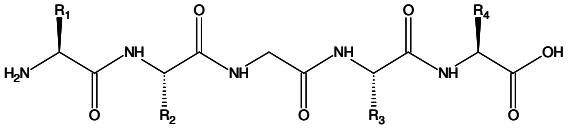
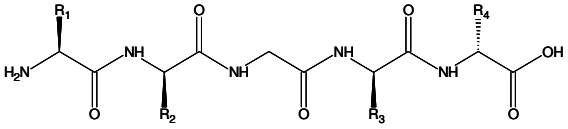
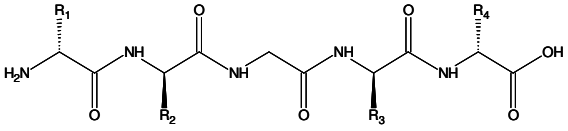
Order of Elution of Enkephalin Peptides on:		Teicoplanin	C18
E2a		1	5
E1a		2	2
E2b		3	3
E1b		4	4
E0		5	1
E3		6	2
E4		7	1

Figure 3.2: The spatial arrangement of the side chains in the enkephalin peptides are shown where: R_1= p-hydroxybenzyl; R_2= methyl; R_3= benzyl; R_4= isobutyl. According to Kroeff and Pietrzyk [25], hydrophobic groups located on the same side of the peptide backbone should increase retention on alkyl bonded stationary phases. The side chains R_3 (phenylalanine) and R_4 (leucine) are the most hydrophobic groups in the enkephalin peptide and peptides with these two groups on the same side of the peptide bond display the most retention.

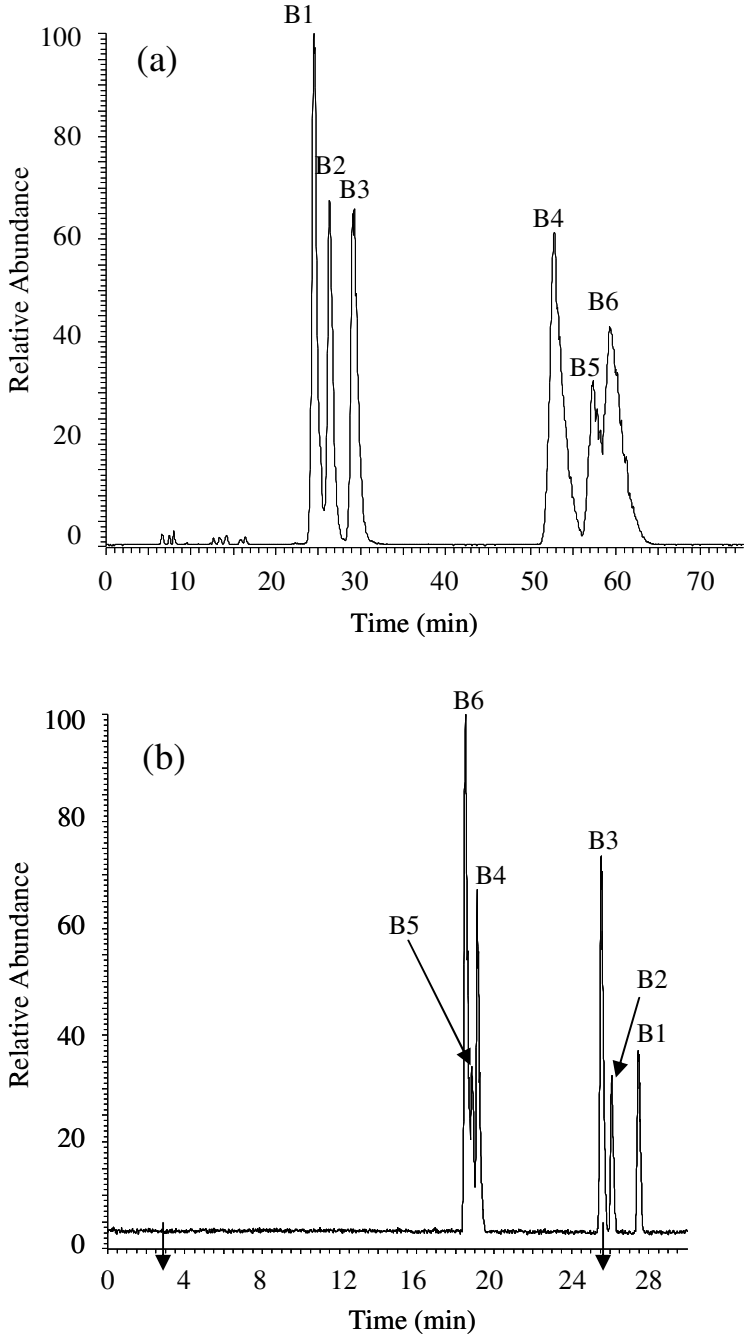


Figure 3.3: The separation on bombesin peptides on (a) Chirobiotic R (ristocetin) and (b) C18 stationary phases. Isocratic separation conditions for (a): 80/20 ACN/Water with 0.1% ammonium trifluoroacetate in both solvents at a flow rate of 0.4mL/min. Gradient separation conditions for (b): 75% A/25% B hold for five minutes to 50%B at 25 minutes at a flow rate of 0.5 mL/min where A is 0.1% Ammonium Acetate in water and B is ACN. *Small arrows* indicate beginning and end of gradient.

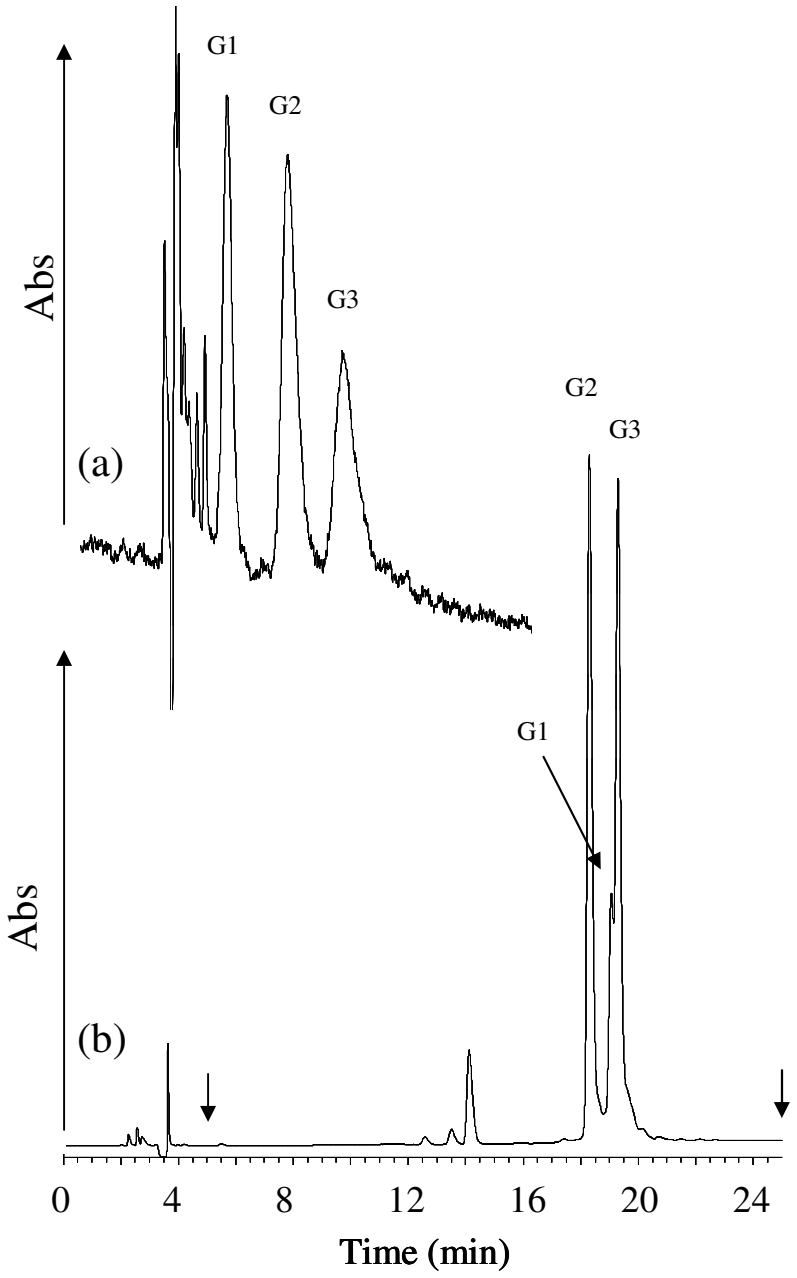


Figure 3.4: Chromatograms showing the separation of the Galanin peptides (which contains 30 amino acids) on the (a) Chirobiotic T (teicoplanin) column and (b) a C18 column. Chromatographic conditions for (a) were 35/65 ACN/Water with 0.2% formic acid in both solvents. Flow rate was 1ml/min with detection at 220 nm. Gradient in (b) was 75%A/25%B hold 5 minutes to 50%A/50%B at 25 minutes where A is 0.1% H3PO4 and B is 40%A/60% ACN. *Small arrows* indicate beginning and end of linear gradient. Flow rate was also 1 mL/min with detection at 214 nm. Galanin sequences are given in Table 3.2.

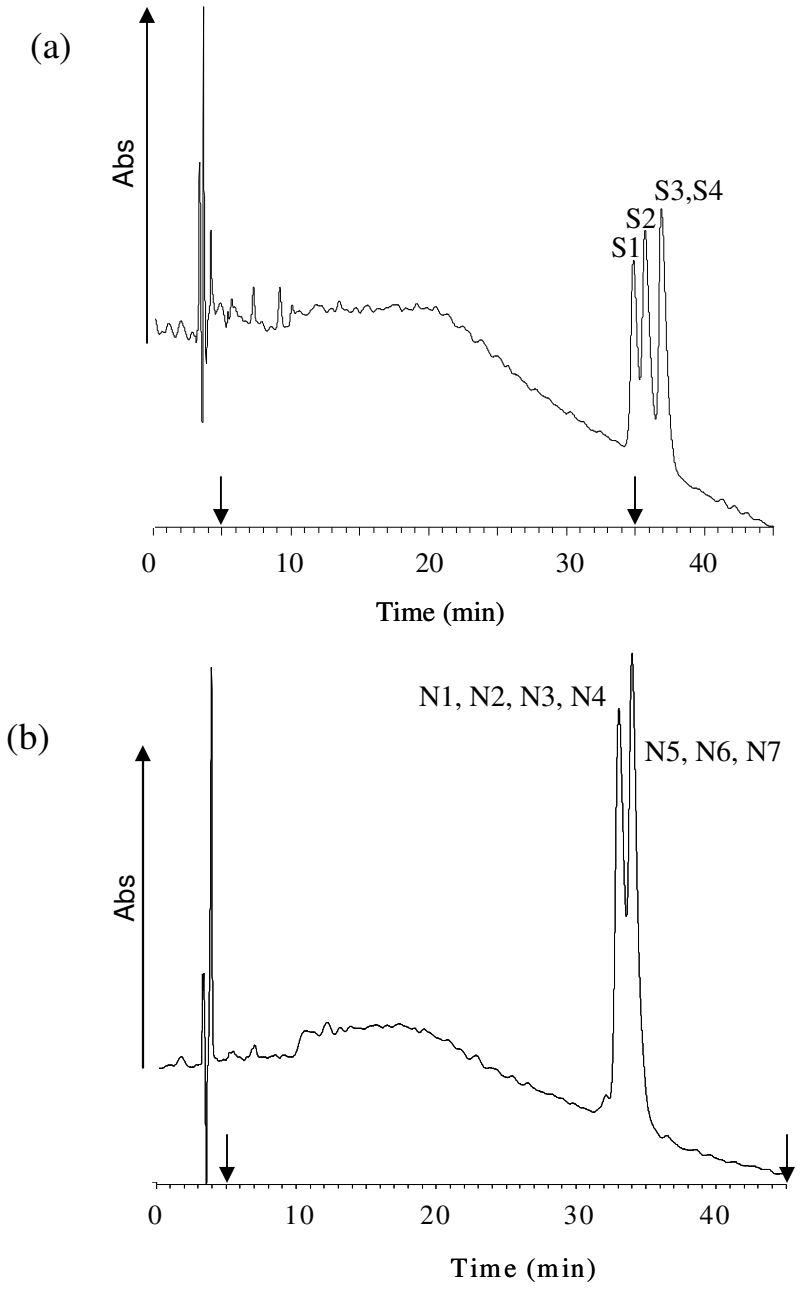


Figure 3.5: Gradient separations of (a) somatostation peptides (28 amino acids) and (b) neuropeptide Y (36 amino acids) on Chirobiotic T stationary phase. Chromatographic conditions for (a) were 10/90 ACN/ 0.25% Triethylammonium trifluoroacetate pH 2.9 hold 5 min to 40/60 at 35min using a flow rate of 1mL/min with detection at 235 nm. For (b), gradient conditions were 10/90 ACN/ 0.25% Triethylammonium trifluoroacetate pH 2.9 hold 5 minutes to 40/60 at 45min. *Small arrows* indicate beginning and end of gradient. Sequences for all peptides can be found in Table 3.2.

CHAPTER 4

A GENERAL, POSITIVE ION MODE ESI-MS APPROACH FOR THE ANALYSIS OF SINGLY CHARGED INORGANIC AND ORGANIC ANIONS USING A DICATIONIC REAGENT

A paper published in *Analytical Chemistry*[3]

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ABSTRACT

Anion analysis continues to be of great importance to many scientific and technical fields. We propose here a general and sensitive method of detecting singly charged anions by ESI-MS and LC-ESI-MS as positive ions. This method utilizes a dicationic reagent to form a complex with the anion that retains an over all positive charge for analysis by MS. Nitrate, thiocyanate, perchlorate, perfluorooctanoic acid (PFOA), halogenated acetic acids, and various other inorganic and organic anions are investigated. The use of tandem mass spectrometry to enhance the detection limits of some of the anions is demonstrated. Chaotropic anions provided the lowest detection limits, with PFOA detected at the hundreds of femtograms level. Indeed, this single approach provides the lowest reported detection limits for a variety of anions, especially PFOA, nitrate, monochloroacetic acid, dichloroacetic acid, and bromochloroacetic acid, among others. The integrated areas and signal to noise ratios for five ions during a chromatographic run in both the positive and negative ion modes are compared. The ability of this method to detect differences in related ions is shown for four arsenic species. Finally, a tap water sample is analyzed for the anions in this study using the dicationic reagent method.

4.1. INTRODUCTION

The analysis of anions is essential in many areas of scientific and technical interest. Most commonly it is utilized in the analysis of environmental samples,[1-15] especially water, human tissues and a variety of other fluids.[16-22] In fact, the entire application area is diverse and includes the characterization of apple juice,[23] marsala wines,[24] and various foods and beverages from around the world.[25] Separation methods are often applied in anion analysis, especially when complex matrices are present. Ion chromatography is the most common separation method used,[4-5,8,11,14-16,20-22,24-33] although reverse-phase chromatography is sometimes an option if ionization of the analyte is suppressed[23] or for ions with sufficient hydrophobicity (e.g., perfluorooctanoic acid, PFOA).[7, 9,17-18,34] Ion-pairing chromatography[19,35]and capillary electrophoresis[36-38] also have been used. GC and GC-MS can provide sensitive and selective analysis methods for anions that have been converted to volatile derivatives before analysis. Such an approach was used in the analysis for trifluoroacetic acid[1-2] and thiocycanate.[39] Direct techniques for the determination of anions that do not involve separation techniques include mass spectrometry,[3, 40-41] spectrophotometry (including ICP),[5, 12, 42-44] and ion-selective potentiometry, and other electrochemical techniques.[45-50]

In the never-ending search for more sensitive and selective methods of analysis, scientists have begun to examine electrospray mass spectrometry as an alternative choice for the analysis of some anions.[3, 7, 9, 11-12, 14-15, 18, 20-22, 25, 28 ,33, 35, 40-41] Given that anions are negatively charged, it is not surprising that most reported results have used the negative ion mode. When analyzing inorganic and organic anions via mass spectrometry, there are some generally acknowledged limitations. For example, small anions with masses below the mass

cut-off of the mass spectrometer (specifically ion traps) cannot be detected. Small, very polar analytes tend to be more hydrated and reside in the more neutral interior of the electrospray droplets, which in turn can lead to lower than expected signals.[51-52] Analytes in the low mass range that are above the cut-off generally reside in the region of high chemical noise.[51] In addition, these anions also can experience some reduced sensitivity compared to larger ions in some mass spectrometers (e.g., ion traps[53]). It is also known that negative mode electrospray conditions are inherently more prone to corona discharge than is the positive mode.[51] Both corona discharge and arcing are more likely in the negative mode due to the high negative voltages (i.e., electrons) being applied to form the electrospray.[51,54] Corona discharge results in a higher background, poor spray stability.[51] In regards to anion analysis, the rather conductive solvents used for reverse phase and especially ion chromatography (water, buffers, methanol, etc.) contribute to corona discharge conditions when high negative voltages are applied to create the electrospray. Analyte signals can be stabilized by using halogenated solvents[55] and/or scavenging gases.[54] There are other factors (pKa, surface activity, etc.) that influence how easily the analyte will form negative ions and how easily these are transferred to the gas phase.[51-52] These are the same factors that affect the ease of ionization in the positive mode, but they can have different effects in the negative mode. For example, small acidic molecules that form negative ions in solution may become neutral in the gas phase due to reactions with weak gas-phase base solvents (i.e., water, methanol).[56] These same weak gas-phase solvents allow for the beneficial protonation of analytes in the positive mode. Consequently, solvents such as propanol, 2-propanol, and butanol have been recommended for negative mode ESI-LC-MS,[57] likely due to their higher gas-phase proton affinities.[56] However, these solvents have much different chromatographic

selectivities and produce higher column operating pressures than methanol and acetonitrile. It makes some sense then that optimum sensitivity in the negative mode isn't always achieved by just applying a negative voltage to the LC column eluent.

Since water, methanol, and acetonitrile are common separation solvents, it would seem desirable to detect anions in these solvents while avoiding the accompanying problems of operating in the negative ion mode. In order to detect the anions in the positive mode, the anions must be paired with another reagent that can produce two (or more) positive charges so that the adduct as a whole retains at least one positive charge. For small anions of very low mass to charge ratios (<100 m/z), the resulting adduct increases the m/z at which the anion is detected. This reduces the low mass bias experienced by these small anions.[53] This is also an effective means for detecting anions whose m/z fall below the low mass cutoff of the mass spectrometer. Even for slightly larger anions, adducts formed with the reagent can move the mass of the adduct to a higher m/z region where there is less background noise. Organic bases[40] and cationic surfactants[3-4] were used to form negatively charged adducts with two perchlorate anions (or a perchlorate and another anion) for detection by ESI-MS in the negative mode. Recently, we first used dications, i.e.,hydrocarbon chains terminated by tetralkylammonium, substituted imidazolium, or substituted pyrrolidinium groups (which were originally synthesized for use as high-stability ionic liquids),[58] to detect perchlorate in the positive mode by ESI-MS.[12] The notable features of this work were (a) its ease of use, (b) ultra high sensitivity, and (c) elimination of background interferences. Indeed this method proved to be nearly as sensitive as any known method for perchlorate[22] and it eliminated the interference from ubiquitous sulfate. This method also is compatible with ion chromatography and has been subsequently been used for the determination of perchlorate,

iodide, and thiocyanate in seawater and seaweed, [15] bovine and human milk, [20] and urine.[21] In this work, we examine the use of geminal organic dications as a general approach for the analysis of a wide variety of singly charged anions by ESI-MS and LC-ESI-MS in the positive mode. We also explore the possibility of using tandem mass spectrometry to further enhance the sensitivity of this method. Further, we compare the anion signals in the negative mode and in the positive mode with this geminal dicationic reagent for five anions separated in a chromatographic run. We also use this ESI-MS method to demonstrate the detection of several environmental arsenic contaminants in an aqueous sample in a single injection and analyze a tap water sample for anions.

4.2. EXPERIMENTAL

4.2.1. Materials

HPLC grade water and methanol were obtained from Burdick and Jackson (Honeywell Burdick and Jackson, Morristown, NJ). Sodium hydroxide and sodium fluoride were of reagent grade. The anions listed in table 1 were purchased as the sodium/potassium/lithium salt or as the free acid and all were of reagent grade or better. The dicationic salt was synthesized according to Anderson et al.[58] in the bromide form.

4.2.2. Methods

The dicationic reagent was exchanged into the fluoride form to maximize the amount available for adduct formation. This was achieved using ion-exchange. Four milliliters of Amberlite IRA-400 in the chloride form was packed into a disposible 10-mL syringe. The column was washed with ten column volumes of 1 M NaOH, ten column volumes of water, seven column volumes of 0.5 M NaF, and ten more column volumes of water to put the resin

in the fluoride form. One milliliter of 0.1 M of the dicationic reagent in the bromide form dissolved in water was passed through the resin and eluted with water into a 10-mL volumetric flask. The resulting stock solution was then used to make up the working solutions in either water or methanol at the desired concentration to give a final dicationic reagent (entering the mass spectrometer) of 10μM.

4.2.3. ESI-MS analysis

The mass spectrometer used in this study was an LXQ (Thermo Fisher Scientific, San Jose, CA) with a six port injection valve used to make injections. The sample loop size was 2 uL. A carrier flow of 300μL/min was provided by a Surveyor MS pump (Thermo Fischer Scientific, San Jose, CA) with a membrane degasser. Introduction of the dicationic solution into the sample stream was accomplished via a Y-type mixing tee. The flow rate of the dicationic solution was 100 uL/min and a Shimadzu LC-6A pump was used for this purpose. ESI ionization conditions for positive mode were as follows: spray voltage: 3 kV; capillary temperature: 350ºC; capillary voltage: 11 V; tube lens: 105 V; Sheath gas 37 arbitrary units (AU); Auxiliary gas: 6 AU. In negative mode the conditions were: spray voltage: 4.7 kV; capillary temperature: 350ºC; capillary voltage: -25 V; tube lens: -6 V; Sheath gas 37 arbitrary units (AU); Auxiliary gas: 6 AU. The MS was operated in either single ion monitoring (SIM) or single reaction monitoring (SRM) acquisition mode. Normalized collision energy for SRM experiments was set at 25 and the activation time was set at 30 ms. Data was collected and analyzed using Xcalibur and Tune Plus software.

The conditions reported here were optimized for the perchlorate adduct and used for all of the anions. This method could likely be further improved by optimizing the MS conditions for the specific anion of interest and to the specific type of mass spectrometer

used. We believe it is likely that other mass spectrometers (e.g., triple quadrupoles) may achieve even lower detection limits when using this technique/reagent.

The precision of this technique is dependent on both the nature and the concentration of the analyte anion (precision decreases as the detection limit is approached). The experimental error for most determinations via this method were less than five percent and can likely be attributed to injection volume variation (± 5%). This would indicate this method is highly reproducible and the association of the anion with the dication is rapid. These results are in accord with previous studies on perchlorate, iodide, and thiocyanate.[12, 15, 20-21]

4.2.4. Chromatography

The instrument configuration from above was modified slightly for chromatographic experiments. A Surveyor autosampler (Thermo Fischer Scientific, San Jose, CA) fitted with a 25 uL injection loop was used for sample introduction. The introduction of the dicationic solution in methanol (positive mode) or pure methanol (negative mode) was located between the column and mass spectrometer. A microbore Cylcobond I (250mm x 2.1mm i.d.) from Advanced Separation Technologies (Whippany, NJ) was used for the separation of anions. Cyclodextrins have been used in the past to selectively bind ions in the absence of organic modifiers.[59] Also it has been shown that most ions, particularly those more chaotropic in nature, can include into the cavity of the cyclodextrin.[59]

4.2.5. Water Analysis

The tap water sample was collected from the cold water tap of a laboratory sink. The water was allowed to run for 15 minutes before collecting the sample in a nalgene bottle. The same configuration used in the MS analysis was used for the determination of anions in

water with one small difference. A five microliter sample loop was used for the determination of bromide and benzenesulfonate. SIM mode was used to identify anions present and SRM used to confirm the association with the dicationic agent. Quantification was performed on the anions individually in either SIM or SRM mode as specified in Table 2.

4.3. RESULTS AND DISCUSSION

In previous work[12] we found that an imidazolium-based dicationic reagent (shown in Figure 4.1A) paired well with perchlorate in the gas phase and produced a very sensitive and interference free analysis for perchlorate. Here, we examine the possibility of using such a dicationic reagent as a general reagent for the sensitive detection of other singly charged anions in the positive ion mode.

Table 4.1 lists the anions included in this study in order of decreasing sensitivity (in the SIM mode). The included anions are of both inorganic and organic types. Also included are anions of broad research interests such perfluorooctanoic acid (PFOA), halogenated acetic acids, and a few environmentally important arsenic species. Additionally, there are several anions (chloride, cyanide, cyanate, thiocyanate, formate, nitrite, nitrate) that would be prone to discrimination by low mass bias or fall below the mass cut-off of certain types of mass spectrometers. Selected ion and selected reaction monitoring acquisition modes were used to find the lowest detectable levels of the anions. Sensitivity in SIM is important for all mass spectrometers, but especially those without the capability to perform MS/MS (single quadrupole mass spectrometers). SRM could provide a way to reduce the noise when anions of interest are in complex matrices. While detecting the anions using SIM is fairly

straightforward, SRM is slightly more complicated. In order for SRM detection to work, there must be a positively charged fragment of the dication-anion adduct remaining after dissociation. Thus, in MS/MS the final ion detected isn't the anion at all, but a remnant of the dication. When the dication-anion adduct is excited and dissociated, the dominant positively charged fragment that results is usually the $[M-H]^+$ cation of the dicationic reagent. This fragment is formed from the dication by the loss of C2 hydrogen on one of the imidazole rings (see Figure 4.1B). The SRM signal results from plotting the intensity of each specific $[dication+anion]^+ \rightarrow [dication-H]^+$ reaction. In most cases, SRM achieved lower detection limits than SIM due to the characteristic reduction in noise. For those anions where the detection limits are the same or higher in the SRM mode, there may be other (unidentified) fragmentation pathways which reduce the abundance of the $[M-H]^+$ cation. Trifluoromethanesulfonate (triflate, TFO) was the only anion for which an alternate fragmentation pathway was identified. The most abundant dication fragment detected in MS/MS for TFO is m/z 207, which corresponds to the dication losing one imidazole group, thereby forming a 1-(non-8-enyl)-3-methyl imidazolium monocation (see Figure 1C). SRM for this transition (m/z 439→207) lowered the detection limit for TFO by two orders of magnitude. While MS/MS has been used to increase the sensitivity of some anions,[7, 9, 17-18, 22, 25, 33] monoatomic anions cannot be fragmented. By using the mass transition of $[dication+anion]^+ \rightarrow [dication-H]^+$, a general MS/MS method can be used for all anions in an analysis. This is the first reported use of MS/MS for the analysis of anions using dicationic reagents.

The ions that show the lowest detection limits in the positive mode (SIM) loosely follow the Hofmeister series. The Hofmeister series orders anions according to their ability to

stabilize or destabilize proteins in solution.[60-61] Anions that stabilize proteins in solutions are termed chaotropic. Here in our study, those anions that are more chaotropic have lower detection limits in the positive SIM mode than those that are less chaotropic. Nitrate and tetrafluoroborate seem to be the ions that do not fall into the order of the Hofmeister series. Nitrate and tetrafluoroborate both exhibit better limits of detection than predicted by the Hofmeister series, while the thiocyante, hexafluorophosphate, iodide, and perchlorate generally performed as predicted by the Hofmeister series. There seems to be two possible reasons for this. First, the dicationic reagent may have slightly different affinity for nitrate and BF_4^- from the much more complex proteins used to determine the Hofmeister series. Secondly, these differences may have more to do with lack of other chemical noise near the m/z of the anion-dication adduct of nitrate and BF_4 than with their place in the Hofmeister series. There also seem to be structural indicators that improve adduct formation with the dicationic reagent. Those anions that contain halogen atom(s) can be detected at lower levels than related anions that lack halogen atoms. The best example of this is the acetate family of anions. Acetate itself is not detected as an adduct (which makes it an ideal, non-interfering buffer component for LC-MS applications), but every acetate with at least one halogen atom can be seen adducted to the dicationic reagent (and with low detection limits, see Table 4.1.) In particular, those anions (PFOA, BF_4, and PF_6) with fluorine atoms usually exhibit exceptional sensitivity. In fact, PFOA was detected at the sub-picogram level, the lowest of the anions tested and lowest ever reported. Both SIM and SRM for PFOA detection limits were twice as low as that of an LC-ESI-MS/MS method with a preconcentration step.[7] Based on these properties, trifluoroacetic acid (TFA) and chloride were expected to have better than observed detection limits. In the case of chloride, its natural occurrence at high levels

increases the noise level at the monitored mass of the dicationic-anion adduct, resulting in a higher detection limit. TFA also suffers from increased background levels, most likely from traces left in the solvent lines of the LC systems. Increasing the oxidation state of the central atom of an anion also seems to be beneficial in some cases. By adding oxygens, the anion increases in size and spreads the negative charge over a larger area. For example, the detection limit for nitrate is over two orders of magnitude lower than for nitrite. The same trend (but to lesser degrees) can be seen for periodiate/iodate/iodide, bromate/bromide, and perchlorate/chloride. Also interesting is the trio of anions: thiocyanate, cyanate, and cyanide. The ability to adduct with the dicationic reagent ranges from very good for thiocyanate to unobservable for cyanide.

For certain ions, the absolute detection limits determined with the dicationic reagent in positive mode by ESI-MS (Table 4.1) compare favorably with absolute detection limits of other methods reported in literature. The literature methods were varied and often included extraction steps that could be used to concentrate the anion of interest before analysis. Using the "dicationic reagent" ESI-MS method, PFOA could be detected at 122 fg (SIM) and 73 fg (SRM) compared to 250 fg by a LC-ESI-MS/MS method that used an eight-fold SPE concentration step.[7] The detection limit for nitrate (1.84 pg for SIM, 1.38 for SRM) was found to be well below that of an IC-ESI-MS/MS technique (1.25 ng),[22] and almost an order of magnitude lower than an ion chromatography method using an PVC membrane anion electrodes as the detector(16 pg).[30] The dicationic reagent ESI-MS method was approximately four times lower than the lowest limits for nitrate of the other methods which was achieved by Hadamard transform CE (~8.70 pg) and required the signal averaging of twenty electropherograms.[38] Even though chloride suffers from a high background signal its

detection limit was just below that of the same ion chromatography method used to determine nitrate.[30] Detection limits achieved using a combination of electrospray ionization and high-field asymmetric waveform ion mobility spectrometry (ESI-FAIM-MS)[41] were lower for trichloroacetic and monobromoacetic acids but higher for monochloroacetic acid, dichlorocetic acid, and bromochloroacetic acid when compared to this method. The only literature values found for BF_4^- and PF_6^- were determined by attenuated total reflectance FTIR on thin film coatings[44] so absolute LODs could not be found. However, the concentration limit given in Hebert et al.[44] was in the same range as the concentration used in this ESI-MS analysis. Perchlorate[22, 25] and thiocyanate[39] are the only two anions located near the top of Table 1 for which the absolute LODs reported here are higher than the lowest literature absolute LODs. However, in the case of thiocyanate in the previous literature reference, the anion had to be converted to the pentafluorobenzyl derivative for analysis by GC-MS. There are of course, some anions listed in Table 1 that were determined at the nanogram level while literature values were at the picogram level. The use of IC-ICP-MS for the speciation of the arsenic species included here is one such example.[16] For a few ions (NTF_2 and bromooctanoic acid) no literature values were found.

In many applications, chromatography is used to remove interferences present in sample matrices and provide a temporal displacement of the analytes of interest. Figure 4.2 is a comparison of five anions chromatographed in both positive (Figure 4.2A) and negative (Figure 4.2B) polarity modes where the masses of the anions or the dicationic adducts were monitored. Even though ten times more thiocyanate (SCN), triflate (TFO), and benzenesulfonate (BZSN) was injected in negative ion mode, these three anions displayed integrated peak areas and signal to noise ratios that were larger in the positive ion mode with

the dicationic reagent than in the negative ion mode (without dicationic reagent). Thiocyanate (m/z 58) is a victim of low-mass bias in the negative mode. It resides so close to the low mass cut-off (m/z 50) that it is not detected at all in the negative ion mode, and shows the largest improvement in the positive ion mode. Despite the fact that five times more PFOA is injected in negative mode, PFOA also shows a marked increase in S/N ratios in positive mode. This is undoubtedly due to its high affinity for the dicationic reagent as discussed above. Trifluoromethanesulfonimide (NTF_2) is the only anion of these five to show comparable signal to noise ratios in the positive and negative ion modes. Part of the reason may lie in the structure of NTF_2. NTF_2 is a large anion and the negative charge is delocalized amongst the nitrogen and sulfur atoms, with the oxygens and trifluoromethyl groups shielding its charge from other charges.[62] Benzenesulfonate and PFOA also are large anions, but in these cases the negative charge is more concentrated at one end of the structure. The more symmetrical NTF_2 anion may be more surface active,[51] which leads to increased sensitivity in the negative ion mode as seen in Figure 4.2. NTF_2 can be detected at low levels in positive ion mode (see Table 4.1) because it would seem to be a fairly chaotropic anion.

The "dicationic reagent" approach to anion analysis uses the anion mass to discriminate against other related anions. Thus, this method may be useful in the detection of several related species without the use of a separation column. This is shown in Figure 4.3 with the detection of four arsenic species using the MS analysis configuration. The three compounds with arsenic in the higher oxidation state form a stronger adduct with the dicationic reagent as discussed above. ICP-MS is very specific for arsenic, but all speciation information is destroyed in the high temperatures of the ICP. Thus, it must be combined with

HPLC for species-specific information. Due to differing toxicities, it is important to know not only total arsenic content, but also the level of the individual arsenic species.[10] This experiment clearly demonstrates the ability of the "dicationic reagent approach" to retain important structural information for related anions.

A tap water sample was analyzed by ESI-MS for the anions contained in Table 4.1. Five anions (chloride, nitrate, bromide, monochloroacetic acid (MCA), and benzenesulfonate) were found at levels higher than the detection limits and confirmed by the production of m/z 289 when SRM was used. Monochloroacetic acid is a known disinfection byproduct and is under regulation by the EPA.[41] Various benzenesulfonates are used in numerous industrial processes.[63] Quantitative results are shown in Table 4.2. The anions in this study were quantified separately (See Experimental and Table 4.2). Since an isotopically labeled internal standard was not available for all of the anions, no internal standard was used for this proof of concept screening. All anions showed good linearity over the calibration range with the common anions chloride and nitrate being the most abundant anions as would be expected.

4.4. CONCLUSIONS

A specific dicationic reagent formed adducts enabling detection of 32 of the 34 anions in the positive mode. For certain chaotropic anions (NO_3^-, BF_4^-, SCN^-, $BZSN^-$) absolute detection limits determined by ESI-MS were in the low picogram range, with PFOA at the femtogram level. Under gradient chromatographic conditions, PFOA adducted to the dicationic reagent gave approximately 30 times higher signal to noise ratios than it did alone in the negative ion mode. In fact, detecting the dication-anion adduct in the positive mode

gave significantly better S/N and higher area counts than negative mode for four out of the five anions. From the determined detection limits, halogenated, oxidized, or other chaotropic anions not included here would also be expected to have low detection limits in the positive mode. Further information is needed about the characteristics of the dicationic reagent that affect adduct formation. This will be the subject of future studies.

4.5. ACKNOWLEDGEMENTS

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Table 4.1: Positive Ion Limits of Detection for Anions Using Dicationic Reagent

Anions	SIM mass	SIM LOD (ng)	SRM mass	SRM LOD (ng)
Perfluorooctanoate (PFOA)	703	1.2E-04	289	7.3E-05
Nitrate (NO_3^-)	352	1.8E-03	289	1.4E-03
Tetrafluoroborate (BF_4^-)	376	2.0E-03	289	3.9E-01
Thiocyanate (SCN^-)	348	2.0E-03	289	2.0E-03
Benzenesuflonate (BZSN)	447	2.1E-03	289	4.1E-04
Trifluoromethanesulfonimide (NTF_2^-)	570	2.3E-03	289	2.3E-03
Hexafluorophosphate (PF_6^-)	435	4.3E-03	289	2.1E-03
Iodide (I^-)	417	6.0E-03	289	2.0E-01
Perchlorate (ClO_4^-)	389	1.0E-02	289	1.0E-02
Dichloroacetate (DCA)	417, 419	1.5E-02	289	2.0E-02
Monochloroacetate (MCA)	383, 385	1.5E-02	289	1.9E+00
Bromochloroacetate (BCA)	461, 463	1.5E-02	289	1.5E-02
Periodate (IO_4^-)	481	4.5E-02	289	1.1E+00
Bromate (BrO_3^-)	417, 419	5.0E-02	289	5.0E-02
Iodate (IO_3^-)	465	6.0E-02	289	1.4E-02
Bromide (Br^-)	369, 371	6.0E-02	289	6.0E-02
Bromooctanoate (BOA)	511, 513	6.0E-02	289	6.0E-02
Trifluoromethanesulfonate (TFO^-)	439	2.0E-01	207	2.0E-03
Trifluoracetate (TFA)	403	2.0E-01	289	2.0E-01
Malate	423	2.1E-01	289	6.4E-02
Bromoacetate (MBA)	427, 429	2.2E-01	289	1.1E-02
Benzoate	411	3.9E-01	289	9.7E-01
Monomethylarsonate acid (MMA^v)	429	6.0E-01	289	4.0E-02
Nitrite (NO_2^-)	336	6.2E-01	289	2.1E-01
Permanganate (MnO_4^-)	409	6.8E-01	N/A	N/A
Arsenate ($H_2AsO_4^-$)	431	1.0E+00	289	4.1E-02
Chloride (Cl^-)	325	1.8E+00	289	1.8E+00
Formate ($HCOO^-$)	335	4.4E+00	289	2.2E+00
Dimethylarsinate (DMA^v)	427	5.6E+00	289	1.0E+02
Trichloroacetate (TCA)	452	6.4E+00	289	2.0E+00
Cyanate (OCN^-)	332	6.4E+01	289	1.9E+01
Arsenite ($H_2AsO_3^-$)	415	1.0E+02	289	2.0E+01
Acetate (CH_3COO^-)	349	>2.0E+03	289	>2.0E+03
Cyanide (CN^-)	316	>2.0E+03	289	>2.0E+03

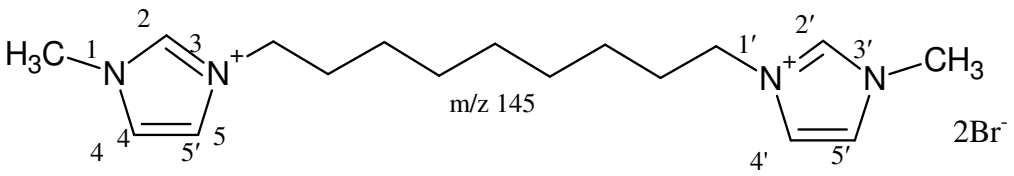
Limits were determined in ESI-MS analysis configuration. Limit of detection (LOD) defined as signal to noise ratio of 3.

Table 4.2: Quantifiable Anions in Arlington Tap Water Sample

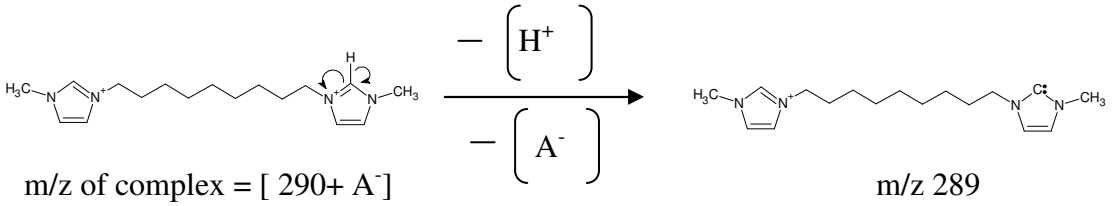
Water Analysis			
Anion	Concentration	Equation	R^2
Chloride	19.0 (± 3.6) ug/mL	$y = (6.50 \times 10^5)x + 1.00 \times 10^5$	0.9913
Nitrate	574 (± 22) ng/mL	$y = (2.68 \times 10^2)x - 2.77 \times 10^3$	0.9972
MCA	49.0 (± 1.6) ng/mL	$y = 86.0x + 5.84$	0.9997
Bromide[1]	57.6 (±2.5) ng/mL	$y = (3.98 \times 10^2)x + 1.37 \times 10^4$	0.9994
BZSN	4.91(± 0.45) ng/mL	$y = 78.1x + 281$	0.9995

[1]: determined in SIM mode. All others determined in SRM mode and quantified separately.

A



B



C

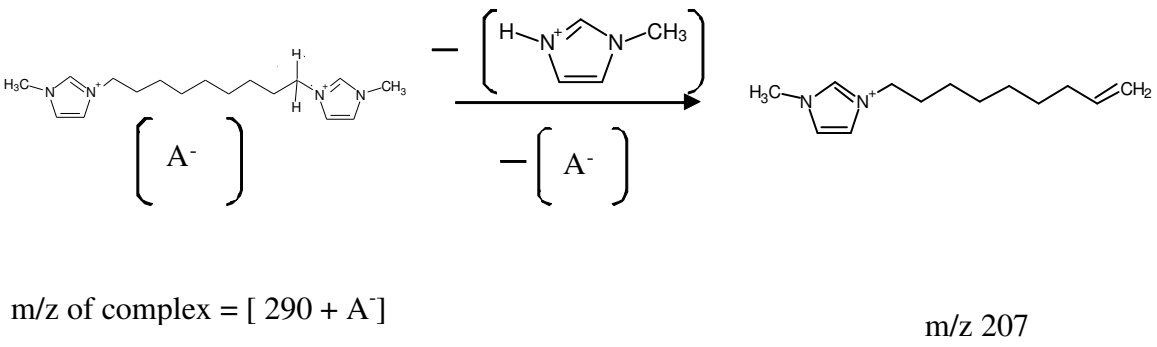


Figure 4.1: Structure of the dicationic reagent in its synthesized form (A) and proposed fragmentation pathways (B, C) for an anion (A^-) of interest.

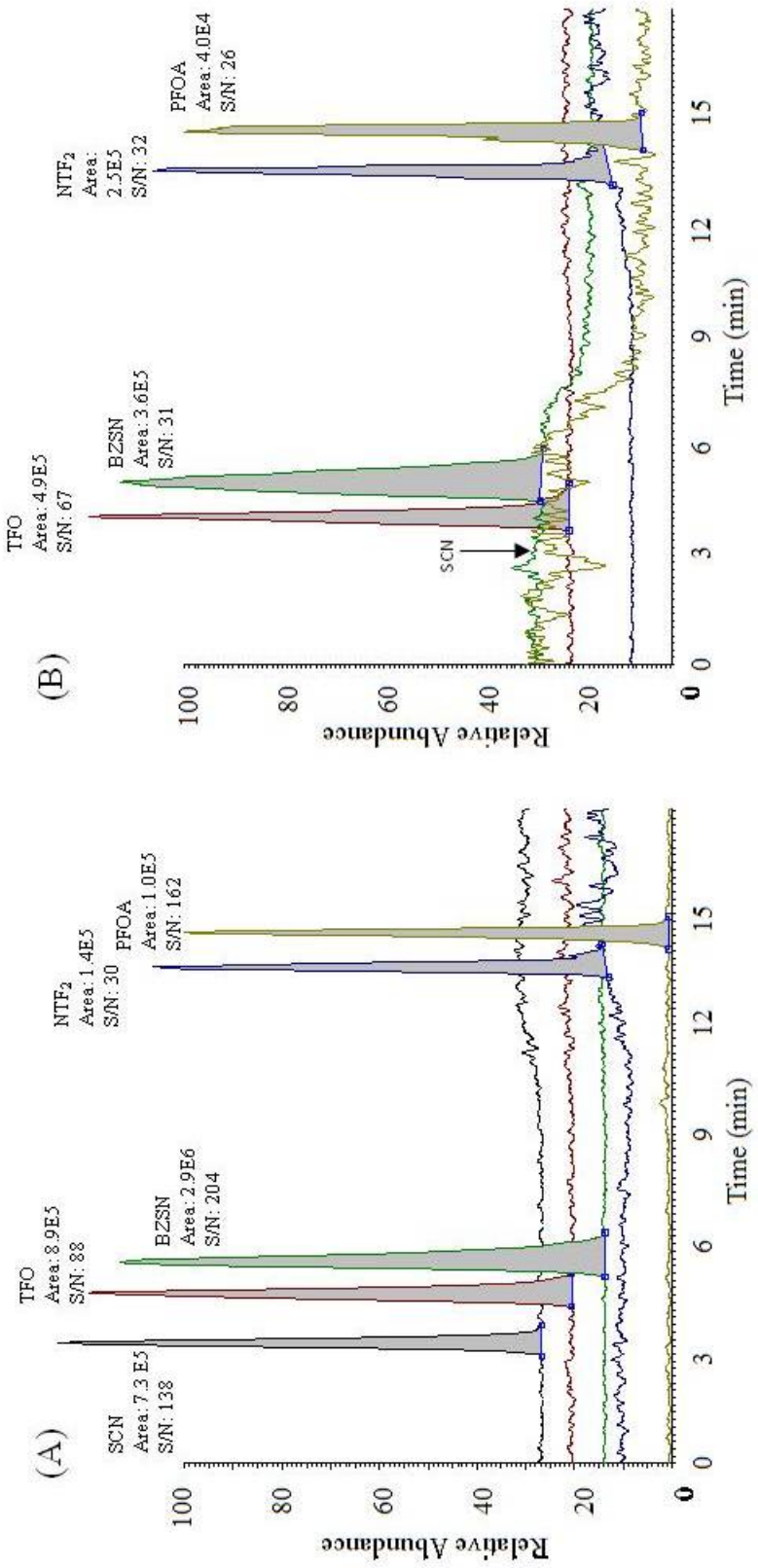


Figure 2: A comparison of the chromatographic separation and sensitivity of 5 anions on a Cyclobond I column detected in the (A) positive and (B) negative SIM modes. The mass injected in (B) is 10x that of (A) for SCN, TFO, and BZSN, 5x for PFOA, and the same for NTF_2. The mass injected in (A) is :1.43 ng SCN, 9.92 ng TFO, 1.16ng BZSN, 0.68 ng NTF_2, and 1.30 ng PFOA. The column was equilibrated for 15 minutes with 100% Water with a linear gradient to 100 % MeOH beginning at 3 minutes and complete at 9 minutes. Flow rate was 300 µL/min. In (A) the dicationic salt solution (40 µM in MeOH) was added post-column at 100 µL/min where as in (B) it is methanol only. SCN: thiocyanate; TFO: triflate; BZSN: benzenesulfonate; PFOA: perfluorooctanoic acid; NTF_2: trifluoromethanesulfonimide.

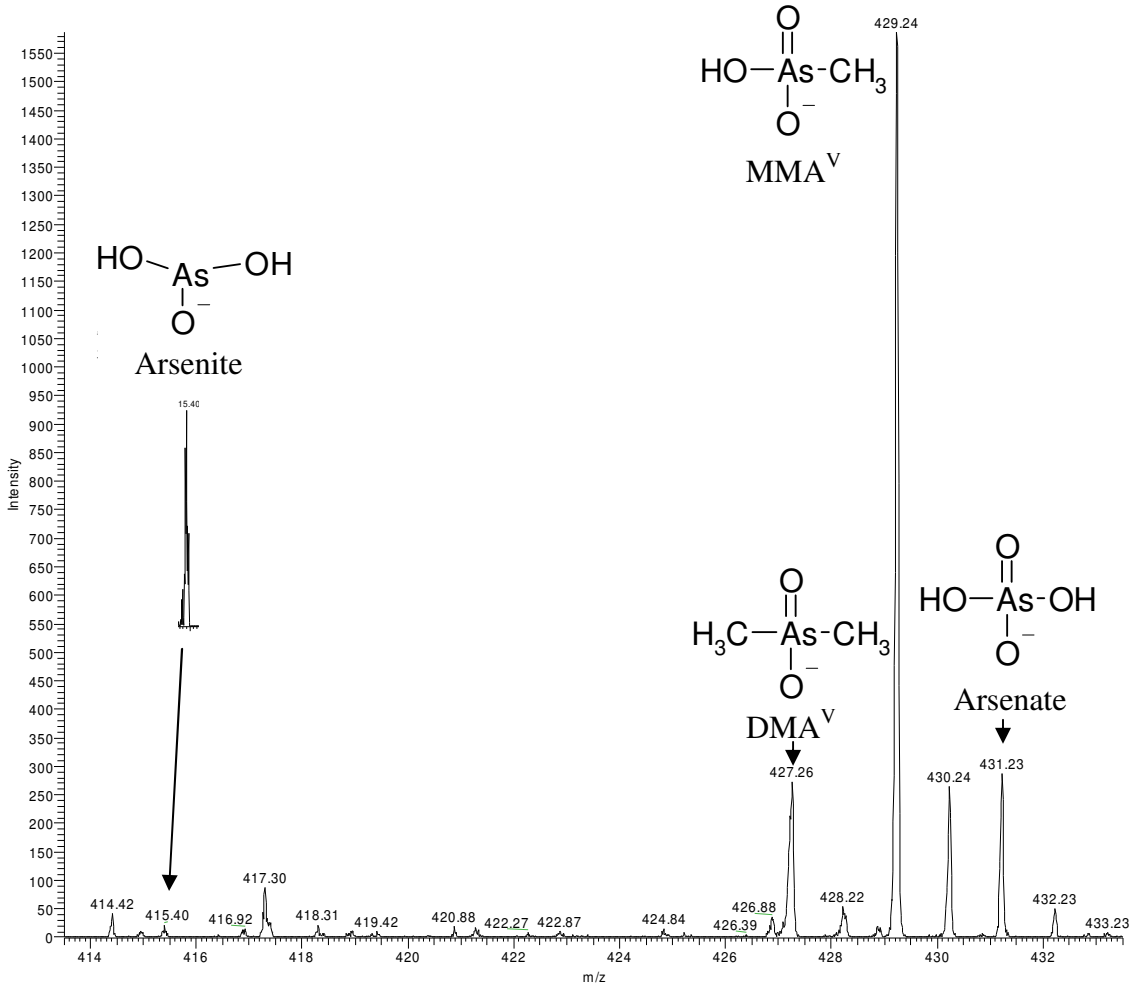


Figure 4.3: ESI-MS mass spectra of a water sample fortified with four arsenic species which are adducted with the dicationic reagent. MMA^V is monomethylarsonic acid and DMA^V is methylarsinic acid where the superscript V denotes the oxidation state of arsenic.

CHAPTER 5

EVALUATION OF DICATIONIC REAGENTS FOR THEIR USE IN DETECTION OF ANIONS USING POSITIVE ION MODE ESI-MS VIA GAS PHASE ION ASSOCIATION

A paper accepted for publication in the *Journal of the American Society for Mass Spectrometry*[4]

Jeffrey W. Remsburg, Renee J. Soukup-Hein, Jeffrey A. Crank, Zachary S. Breitbach, Tharanga Payagala, Daniel W. Armstrong

ABSTRACT

Twenty three different dications were investigated for their effectiveness in pairing with singly charged anions, thereby allowing the electrospray ionization mass spectrometry (ESI-MS) detection of anions as positively charged complexes. Nitrate, iodide, cyanate, monochloroacetate, benzenesulfonate, and perfluorooctanoate were chosen as representative, test-anions as they differ in mass, size-to-charge ratio, chaotropic nature and overall complexity. Detection limits were found using direct injection of the anion into a carrier liquid containing the dication. Detection limits are given for all six anions with each of the twenty-three dications. Each anion was easily detected at the ppb (μg/L) and often the ppt (ng/L) levels using certain dicationic reagents. The ability of dicationic reagents to pair with anions and produce ESI-MS signals varied tremendously. Indeed, only a few dications can be considered broadly useful and able to produce sensitive results. Liquid chromatography (LC)-ESI-MS also was investigated and used to show how varying the dicationic reagent produced significantly different peak intensities. Also, the use of tandem mass spectrometry can lead to even greater sensitivity when using imidazolium based dications.

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[4] Reprinted from *Journal of the American Society for Mass Spectrometry*, **2008**, *19*, 261-269.

5.1. INTRODUCTION

Detection and quantitation of anions is of great importance in a wide variety of scientific fields. Scientists in environmental chemistry, biochemistry, and the food and drug industries all routinely use analytical techniques to study anions. The most common methods for anion analysis include: ion selective electrodes [1, 2], ion chromatography (IC) [3, 4], flow injection analysis (FIA) [5, 6] and a variety of other spectroscopic and electroanalytical approaches. Mass spectrometry is an obvious choice for detection of anions since they are charged species. The advent of electrospray ionization allowed routine analysis of the ionic components in a liquid sample [7]. By coupling ESI-MS with a separation method (i.e. liquid chromatography), a means to separate and detect most compounds is easily accomplished. However, while ESI-MS is widely used in both the positive and negative ion modes, the positive ion mode often is preferred as it can have lower detection limits and higher stability [8-10]. For positive mode analysis, an acidic additive commonly is employed to facilitate protonation of the analyte and to provide a stable electrospray. However, the addition of a basic compound to a water/methanol solvent system does not seem to provide a stable spray for negative mode analysis, resulting in fluctuations of the ion current [8]. It is known that corona discharge is more prevalent in the negative ion mode as opposed to the positive ion mode. Corona discharge can produce a significant rise in background peaks and can also lead to reduced stability for the ion current [11]. Also, undesirable arcing is more prevalent in the negative ion mode. It has been suggested that halogenated solvents such as chloroform [12], hexafluoroisopropanol [13], and 2,2,2,-triflurorethanol [8] be used as opposed to more common solvents. These halogenated solvents produce an abundance of chloride ions at the capillary tip, resulting in a more stable spray formation. To reduce the

occurrence of corona discharge, both electron-scavenging gases [14] and halogenated solvents [15] have proven useful. While carefully choosing amongst the aforementioned solvents may lead to better signals in the negative ion mode, it must be noted that these are not common solvents for use in LC, IC or FIA. Ideally, one would like to be able to use common solvents such as methanol and water and also take advantage of using the positive ion mode, so less optimization is necessary and the problems with negative mode can be avoided.

Recently a method was developed to detect singly charged anions in the positive ion mode, thus eliminating the necessity of using negative ion mode and also eliminating any need for unconventional solvents. This method entails the addition of small amounts of a relatively large, chaotropic, organic dication to the carrier flow solvent which can pair with a single anion to give a positively charged complex of a higher m/z. This approach was first used for the trace analysis of perchlorate [16-19]. Most recently, it was shown to be advantageous for the analysis of over 30 different anions, proving its broad applicability and effectiveness [20]. There are several advantages to this method, among the more important of which are its ease of use and its sensitivity. Indeed, this single method provided the best reported limits of detection (LOD) for a variety of anions, proving to be more sensitive than negative ion mode ESI-MS methods as well as other analytical techniques [20]. Only small amounts of the dication reagent is needed (tens of μM) and it can be added post-column if a separation method is employed so there is no effect on the separation. Finally, there is a key advantage to this method when it is employed with certain quadrupole instruments. By pairing the anion with a large dication, one can eliminate any problems with detection of an anion either below or near the low mass cutoff (LMCO). That is, whereas the anion

previously may either have fallen below the LMCO or so close to it that detection is severely hindered, it can be paired with a dication, thereby moving the detected m/z several hundred mass units higher, to a region of low background interference noise and few interfering peaks.

Apart from the original perchlorate study [16], there has not been any substantial amount of research done on what types of dications provide good or poor results. The dication that was found to provide the best results in the original study (1,1'-(nonane-1,9-diyl) bis(3-methylimidazolium), dication **VIII** in Table 1) was consequently used for multi-anion study of Ref 20. Obviously, differences in the structure and nature of the dication could cause a significant difference in its affinity for different anions, as well as its stability and overall efficacy. The purpose of this study is to examine the effects of using a variety of different types and structures of dication reagents, and to determine whether or not their selectivity, sensitivity and efficacy vary for different anions. Our previous efforts included extensive research in developing dicationic compounds [21-23]. Originally synthesized as ultra-stable ionic liquids [21], this research has led to the development of a wide array of dicationic compounds, including imidazolium [21, 22] and phosphonium based dications, as well as those with differing linkage chains [22, 23] and even unsymmetrical species [23].

In this work, twenty three dication salts are studied for their ability to form a complex with several different anions and be detected by ESI-MS. The salts encompass a wide range of cationic moieties (including imidazolium, pyrrolidinium, pyridinium and phosphonium-based cations) and structures (differing chain lengths, aromaticity, symmetrical and unsymmetrical dications, etc.). Detection limits via direct injection are used to determine efficacy for the complex formed between the dication (dissolved in the carrier stream) and

the anion of interest. The results are evaluated in order to discern which reagents provide the highest selectivity and sensitivity, as well as the structural features that make an effective or ineffective pairing agent. Finally, representative LC-ESI-MS analyses are done to illustrate the effect of using different dicationic reagents for anion analysis in the positive ion mode.

5.2. EXPERIMENTAL

Methanol and water were of HPLC grade and obtained from Burdick and Jackson (Morristown, MJ). Reagent grade sodium hydroxide and sodium fluoride were from Fisher Scientific (Pittsburgh, PA). Anions used were purchased as either the sodium/potassium salt or as the free acid from Sigma-Aldrich (St. Louis, MO). Stock solutions of each anion were made weekly. Chemicals used for the syntheses of the dicationic compounds were also obtained from Sigma-Aldrich.

Dication **I** from Table 5.1 was synthesized by dissolving one molar equivalent of 1,5-dibromo-propane in isopropanol. To this solution, 3 molar equivalents of tripropylphosphine were added. The resulting mixture was stirred and heated to reflux for 48 hours. The solution was then cooled to room temperature and the solvent was removed by roto-evaporation. The crude product was then dissolved in deionized water and washed several times with ethyl acetate to remove any residual starting material. The water was then removed through roto-evaporation, followed by overnight drying in vacuum over phosphorous pentoxide. Dications **II**, **III**, **V-X**, and **XII-XVIII** were made in an analogous manner. Dications **XIX** and **XX** were synthesized by refluxing 1 molar equivalent of (5-bromopentyl)-trimethylammonium bromide in isopropyl alcohol with 3 molar equivalents of 1-methylimidazole and tripropylphosphine, respectively. The resulting product was then

purified as described above. To produce dications **IV** and **XI**, synthesis of the dibromopolyethylene glycol linker chain was first needed. This was accomplished by dissolving tetra(ethylene glycol) was in ether, which was then cooled in an ice bath and reacted with 1.1 molar equivalents of phosphorus tribromide. The reaction was then refluxed for 2hrs. Next, the reaction mixture was pored over ice to react the excess PBr_3. The aqueous layer was discarded and the organic layer was washed four times with an aqueous sodium bicarbonate solution. The organic layer was then dried with sodium sulfate and filtered. Next, the ether was removed by rotary evaporator and the resulting linker was placed under vacuum over night to ensure complete dryness. This linker was then reacted with the appropriate end groups to produce the dication. Dication **XXI** was synthesized by first dissolving one molar equivalent of cinchonidine in *N,N*-dimethylformamide at 80° C. Four molar equivalents of methyl iodide were then added to the mixture and allowed to react for 48 hours. After the solvent was removed by rotary evaporation, the residue was dissolved in methanol. Upon addition of diethyl ether, the product precipitated out of solution, and was collected by filtration and then washed with cold ether. Dications **XXII** and **XXIII** are commercially available compounds (Sigma-Aldrich). All dicationic compounds were anion exchanged to their fluoride form to maximize complex formation between the dication and the injected analyte. This anion exchange procedure is given in Ref.16.

For direct injection analysis, a 40 μM dication-fluoride (DF_2) solution was directed into a Y-type mixing tee at 100 μL/min via a Shimadzu (Columbia, MD) LC-6A pump. Also directed into the mixing tee was a carrier flow consisting of a 2:1 ratio of methanol to water at 300 μL/min from a Surveyor MS pump (Thermo Fisher Scientific, San Jose, CA). After the mixing tee, the final conditions were then 50/50 water/methanol with 10 μM DF_2 at a

flow rate 400 µL/min. Sample introduction was done with the six port injection valve on the mass spectrometer using a 2 µL sample loop. A linear ion trap mass spectrometer (LXQ, Thermo Fisher Scientific, San Jose, CA) was used for this study. The ESI-MS settings were as follows: spray voltage: 3kV, capillary temperature: 350°C, capillary voltage: 11 V, tube lens voltage: 105 V, sheath gas: 37 arbitrary units (AU), auxiliary gas: 6 AU. For the negative ion mode analysis, voltage polarities are reversed, while all other parameter settings were kept. ESI-MS settings for the optimized MCA detection are as follows: spray voltage: 4.5kV, capillary temperature: 350°C, capillary voltage: 35 V, tube lens voltage: 80 V, sheath gas: 25 AU, auxiliary gas: 16 AU. The ion trap was operated using single ion monitoring (SIM).

For the chromatographic experiments, sample introduction was done by a Thermo Fisher Surveyor autosampler (10 µL injections). The stationary phase used was a 10cm C-18 (3 µm particle size) obtained from Advanced Separations Technology (Whippany, NJ). In the chromatograph of the multi-anion sample used for Figure 1, the column was equilibrated with 100% water at 300 µL/min. At one minute, a linear gradient to 100% methanol began and was completed at three minutes. The addition of the DF_2 solution was done post-column at 100 µL/min via the mixing tee. For the chromatographs of the benzenesulfonate samples, the mobile phase consisted of 100% water at 300 µL/min for the entire analysis. To help with spray formation, the DF_2 was prepared as a methanol solution and again added post column. For the negative ion mode runs, pure methanol was introduced into the mixing tee as opposed to the DF_2 in methanol solution. The MS was again operated in SIM mode, monitoring the m/z values of each analyte for the entire run. Where single reaction monitoring was used, the normalized collision energy was set at 25 while the activation time

was for 30 ms. Xcalibur and Tune Plus (Thermo Fisher Scientific) software was used for data collection and analysis.

The experimental parameters described above were adopted from reference 20. The authors strongly recommend further optimization when using a specific dication reagent for use in the detection of (a) specific anion(s). It is believed that these detection limits may be lowered when considerable time is given to optimization or when using a more sensitive mass spectrometer.

5.3. RESULTS AND DISCUSSION

Table 5.1 provides the structure and mass of the wide variety of dications used in this study. Dications **I-V** are phosphonium based while **VI-XIV** contain imidazolium structures (**X** also contains a fluorocarbon linkage chain). Compounds **XV-XXIII** contain other charged moieties including trimethylammonium, pyridinium, and pyrrolidinium. In addition, some "mixed" and non- symmetrical dicationic entities are included (**XIX**, **XX**, **XXI** and **XXIII**).

Table 5.2 lists the limits of detection (LOD) for each of the six representative anions (benzenesulfonate, cyanate, pefluorooctanoic acid, iodide, nitrate, monochloroacetic acid) when successfully paired with the 23 different dicationic reagents. These values were determined by direct injection ESI-MS (see Experimental) and are listed (from top to bottom) in order of sensitivity. Consequently, identifying the dicationic reagents that produce the best results (lowest LOD) as well as those which are ineffective is straight-forward (Table 5.2). The test anions were selected from to provide a cross-section of ions having different sizes and functionalities [20]. Iodide, cyanate and nitrate are relatively common and simple

anions, but vary in size and number of constituent oxygen moieties. Benzenesulfonate (BZSN) was chosen as it is a somewhat larger organic anion and the only test analyte containing a sulfonate group. Monochloroacetate is a representative small haloorganic anion with environmental significance [24]. Perfluorooctanoate (PFOA), a large, anionic fluorocarbon, is unlike any of the other anions. This, along with recent research interest in this compound as an environmental contaminant, make it a good choice for inclusion in this study [25, 26].

It was expected that using different types of positively charged end groups would lead to differing performance. To show this effect, ten different dication reagents that each contain the same pentane linkage can be compared. These ten include dications **II**, **VII**, **XII-XVII**, and **XIX-XX**. Of these, four outperformed the rest. Both dications **XIV** and **XVI** produced good results (low LODs) even when compared to all other dications, while **II** and **VII** did almost as well. While both **VII** and **XIV** are imidazolium based compounds, **II** and **XVI** contain vastly different charged groups (phosphonium and pyrrolidinium). It must also be noted that **XII** produced the worst results of these ten dications. Since **XII** is very close in structure and mass to **XIII**, it seems like the hydroxyl group leads to poorer detection limits. This is possibly due to its increased polarity which would then lead to incomplete desolvation in the gas phase. It is of no surprise that BZSN paired better with the aromatic dications (other than **XII**) which points to pi-pi interactions playing a prominent role in gas phase association. Interestingly, both iodide and cyanate do not seem to pair well with the imidazolium based dications.

The length of the “chain” connecting the cationic moieties is another parameter to consider. There are several analogous dications in this study that differ only by the length of

the hydrocarbon linkage chain. Namely, **I-III** consist of phosphonium based dications, **VI-VIII** are all methyl-imidazolium based, and X**VII** and X**VIII** are alkyl-amine based. Looking at the phosphonium reagents, it can be seen that the C5 linked (**II**) and the C9 linked (**III**) behave similarly. However, the C3 linked (**I**) outperforms these with most of the anions tested, and by a wide margin. The only anions that are not improved upon are MCA (which have similar values) and cyanate. The opposite trend seems to be true for the methyl-imidazolium based reagents (**VI-VIII**), in that the larger C9 linked dication **VIII** produces superior results compared to all of the shorter linked imidazoliums for all anions. The two alkyl-amine dications behaved similarly, apart from PFOA and cyanate. For both of these anions, the C12 linked dication (**XVIII**) produced significantly lower detections limits. However, both the C3 linked phosphonium and the C9 linked imidazolium dications produced lower detection limits than did **XVIII** for all anions except for cyanate.

The effect of using different types of linkage chains was also studied. Three different chain types were studied. A p-xylene linker was used for dications **V** and **IX**, tetraethylene glycol was used for **IV** and **XI**, and a fluorocarbon chain is present on **X**. In general, these more "exotic" linkage chain types were no better and generally worse than their corresponding optimal chain length hydrocarbon counterparts. Since the synthesis of these compounds is generally more complicated, there seems to be no advantage in using these linkage chain types.

A few dications studied did not fit into the categories above and thus, could not be compared in a systematic fashion. These compounds (**XXI-XXIII**) differ significantly from the others in that they do not contain two distinct charged moieties connected by a linkage chain. Some of these are naturally occurring compounds (**XXI**, **XXIII**) while one is a

commercial available "diquat" (**XXII**). These types of compounds were not found to be useful for this method. Most of the anions could not even be detected as a complex with these particular dications. While it is unknown exactly how the dication interacts with the anion, it seems like an appropriate linkage chain that provides some flexibility is very important to ion association. This empirical observation may explain the poor performance of **XXI-XXIII** as well as why dications **V** and **IX** did not perform as well. The p-xylene linked dications (**V, IX**) are the most rigid amongst the symmetrical dications having a linkage chain. Clearly the flexibility of the dication is one factor that is important for complex formation. Ion mobility studies could provide insight into these dication-anion interactions and perhaps indicate how exactly the dication conforms to the anion [27, 28].

From the results described above, a few reagents stand out above the rest. The first is dication **VIII**. This dication performs well for all anions apart from cyanate. The best dication to analyze cyanate was found to be dication **XVI**, which also performs well for the other anions, especially iodide and nitrate. Dication **I** is also a reagent that should be among the first to be evaluated when using this method for any other anion, as it was the top performer for both benzenesulfonate and iodide. Finally, while dication **XIV** was not the best for any particular anion, but it generally was in the top quartile for all of the tested anions, and thus also is considered to be among the most useful dicationic reagents. These four dications (**I**, **VIII**, **XIV**, and **XVI**) encompass a phophonium based dication, a pyrrolidinium based dication, and two imidazolium based dications. Each of these has a different optimum hydrocarbon linkage chain length. It is recommended that these four dications should be evaluated first when determining an anion that has not been previously studied with this gas-phase ion association method.

It should be stated that the interpretation of the empirical results stated thus far has been primarily explained as a consequence of differing binding affinities between the dicationic reagent and the anion. However, it is essential to consider instrumental factors and the role they play in the sensitivity of these measurements. This is particularly true since only a single set of instrumental parameters was used for all dication-anion complex experiments. To demonstrate how instrumental response can significantly alter sensitivity, a complete optimization of instrumental parameters was done for the determination of monochloroacetate (MCA) using dication **XVI**. After optimization of both the electrospray and mass spectrometer parameters (see Experimental), the limit of detection was reduced by a factor of three (from 6.00 pg to 2.00 pg, results not shown). It can clearly be seen that individual optimization will produce increased sensitivity for most of the anions in this study, and that instrument settings/configurations are important.

As an illustration of the pronounced effect of different dicationic reagents on the positive ion ESI-MS signal of anions, three analogous LC-ESI-MS analyses were compared (see Figure 5.1). Two of the recommended dications are used (**VIII,** Figure 5.1A and **XVI,** Figure 5.1B), as well as a moderately successful but not recommended dication **XVII** (Figure 5.1C). Each cation/anion complex was monitored at its appropriate m/z (i.e., the sum of the mass of the dication and the mass of the anion). As can be seen, significant changes in peak area occur for each anion in successive chromatograms. As expected, the recommended dications (chromatograms A and B) outperform dication **XVII**. It should be noted that the worst performing dications (those in the bottom quartile of Table 5.2) would produce peaks that could not be discerned under the conditions of Figure 5.1. Also apparent in Figure 5.1 is that there are great differences even between the two recommended dications. So while the

recommended dications generally perform well across the board, one should always be sure to test at least three or four of the reagents to obtain optimal signal intensity.

Often, this method can achieve significantly lower limits of detection by using tandem MS capabilities. Since this method takes place in the positive mode, the daughter fragment formed after excitation also must be a positive ion, which is a fragment of the dication used. This is another key advantage of using this approach when determining the concentration of structurally-simpler anions (e.g. iodide) that cannot undergo fragmentation under MS/MS analysis in the negative ion mode. In a previous study, it was found that when a dication-anion complex was excited, it lost the anion and either a proton or a methylimidazolium group, resulting in a singly charged fragment that was left for detection. In many cases this reduced the LOD for a variety of anions. This is one distinct advantage of using the imidazolium-based dicationic reagents, as they lend themselves to MS/MS fragmentation more easily than other dications. A typical mass spectrum of the mobile phase under operating conditions (see Experimental) is shown in Figure 5.2. The dication used in this instance is compound **XIV**. Several discernable fragments can be seen in the background even without excitation. The main fragments include the peaks at 227.3 (loss of benzyl imidazolium), 295.3 (loss of $[CH_2\text{-}C_6H_5]^+$), and 385.3 (loss of the acidic proton in the 2-position of imidazolium). Any of these peaks can be monitored after the excitation of the dication-anion complex, usually resulting in a significant decrease in detection limits. This increased sensitivity is illustrated in Figure 5.3, which shows three separate chromatographic runs of 100 ng/mL of benzene sulfonate. While operating under negative ion mode with the addition of methanol post column, a peak can be seen which gives a moderate S/N of 14. By simply using 40 μM of dication **XIV** in methanol and changing to the positive ion mode, an

instant increase in the S/N of almost 10 fold (to 128) is seen. It can easily be seen why this approach is advantageous. This peak can even further be increased by the application of single reaction monitoring (SRM). When the transition of the complex mass (m/z =543.3) to the fragment observed at 227.3 (loss of both the anion and benzyl imidazolium group) is monitored, the S/N increases to 510. This is a 36-fold increase over using the "traditional" negative ion mode to monitor an anion. Since ESI is a "soft" ionization source, the relative abundance of fragments is surprisingly high. The amount of fragmentation seems to be dependent on the capillary temperature. A lower capillary temperature, while decreasing the amount of fragments, did not lead to an increase in sensitivity (possibly due to incomplete desolvation), while a higher capillary temperature (> 400° C) actually led to decreased sensitivity. Interestingly, this fragmentation was only readily seen when using the imidazolium based dications, as other dications (such as phosphonium or pyrrolidinium types) did not lend themselves to significant fragmentation. Consequently, when using the non-imidazolium dications, no increases in sensitivity were seen when performing MS/MS. So while phosphonium or pyrrolidinium based dicationic reagents produce excellent results when using SIM, imidazolium based reagents should be evaluated if MS/MS capabilities are available. The four dications recommended above include two imidazolium based dications that can be used in MS/MS analysis.

5.4. CONCLUSIONS

The use of dicationic reagents to detect singly charged anions via gas phase ion association has been shown to be a highly sensitive method and offers several significant improvements over using the negative ion mode when using tradition solvents. In this work,

twenty-three different dications were evaluated to give insights as to the significant differences in dicationic reagents and which ones were most broadly useful. Four specific dicationic reagents (out of 23) stood out as far as producing superior performance and these are recommended when analyzing other anions. It was shown how this approach can be easily coupled to chromatography to study multiple anions. Also, the importance of choosing the correct dication in order to get significant signals for the anions of interest is demonstrated. Finally, the advantage of using the imidazolium based dications is shown through the application of MS/MS. Further work is needed to determine exactly how the dications interact with anions before any predictive capabilities are possible. Future work will include using this method to lower detection limits of methods that employ the negative ion mode and the possibility of studying doubly charged anions using tricationic species.

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Table 5.1 – Structures and masses of the dications used in this study.

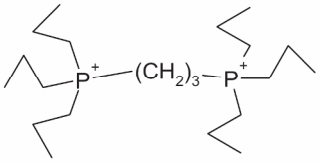
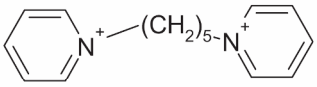
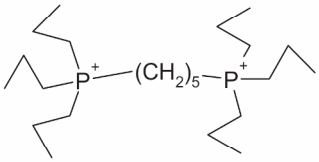
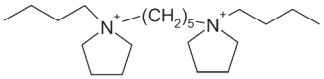
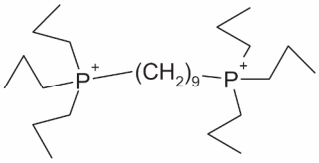
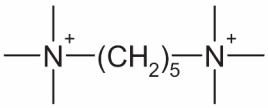
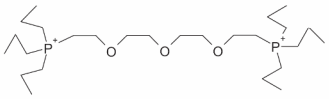
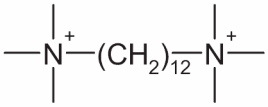
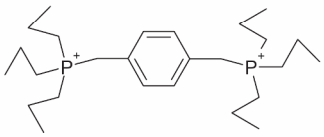
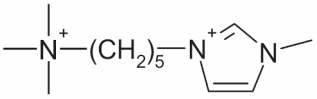
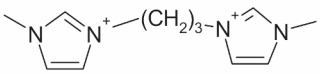
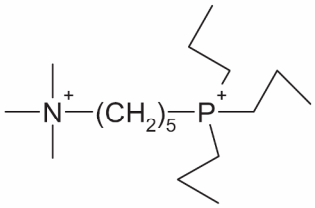
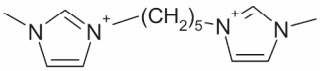
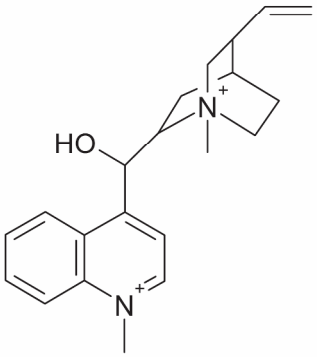
No.	Mass	Structure	No.	Mass	Structure
I	362.6		XV	228.1	
II	390.6		XVI	324.6	
III	446.6		XVII	188.4	
IV	480.6		XVIII	286.6	
V	424.8		XIX	211.2	
VI	206.3		XX	289.4	
VII	234.3		XXI	324.4	

Table 5.1 cont'd.

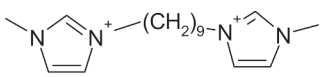
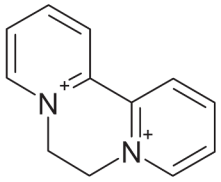
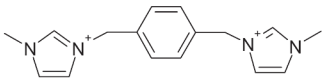
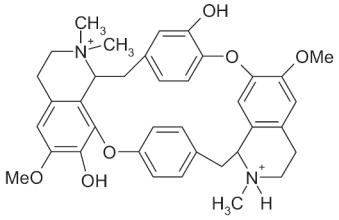
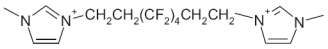
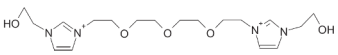
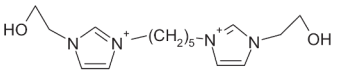
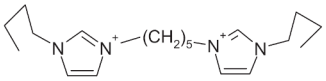
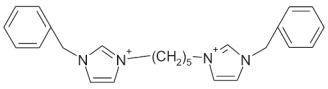
No.	Mass	Structure	No.	Mass	Structure
VIII	290.3		XXII	184.1	
IX	268.3		XXIII	610.6	
X	420.4				
XI	384.4				
XII	294.3				
XIII	318.4				
XIV	386.3				

Table 5.2 – Absolute limits of detection for each anion as detected as a dication-anion complex.

NCO^- LOD		$PFOA^-$ LOD		NO_3^- LOD	
Dication	**Mass Inj (ng)**	**Dication**	**Mass Inj (ng)**	**Dication**	**Mass Inj (ng)**
XVI	6.0E-02	VIII	1.2E-04	VIII	1.8E-03
XVIII	8.0E-02	I	2.5E-04	I	5.0E-03
XXI	2.0E-01	XI	5.0E-04	VII	6.0E-03
III	3.0E-01	IV	2.0E-03	XVI	1.6E-02
IV	6.0E-01	II	3.0E-03	XIII	2.0E-02
II	6.0E-01	V	4.0E-03	XVIII	2.0E-02
XX	8.0E-01	XX	4.0E-03	XIV	2.0E-02
XVII	1.2E+00	XIV	4.5E-03	XVII	2.5E-02
XV	3.0E+00	XVI	6.0E-03	XII	3.0E-02
IX	4.0E+00	XIX	8.0E-03	XIX	4.0E-02
VIII	6.4E+00	VII	8.0E-03	IX	4.0E-02
X	8.0E+00	XVIII	1.0E-02	III	5.0E-02
XXIII	8.0E+00	III	1.0E-02	X	6.0E-02
I	1.5E+01	X	1.0E-02	II	6.5E-02
XIX	2.0E+01	VI	1.4E-02	IV	8.0E-02
V	2.0E+01	IX	1.4E-02	XX	8.0E-02
VI	2.0E+01	XIII	2.0E-02	XI	1.2E-01
VII	2.0E+01	XV	2.0E-02	V	2.0E-01
XIV	1.5E+02	XVII	5.0E-02	XV	2.0E-01
XII	ND	XII	6.0E-02	VI	6.0E-01
XIII	ND	XXI	1.6E+00	XXIII	ND
XI	ND	XXIII	ND	XXI	ND
XXII	ND	XXII	ND	XXII	ND

ND = Not Detected (150 ng highest amount injected)

Table 5.2. Cont'd.

$BZSN^-$ LOD		MCA^- LOD		I^- LOD	
Dication	**Mass Inj (ng)**	**Dication**	**Mass Inj (ng)**	**Dication**	**Mass Inj (ng)**
I	1.0E-03	XVI	6.0E-03	I	1.1E-03
XIV	2.0E-03	II	6.2E-03	V	1.6E-03
V	2.1E-03	IV	6.2E-03	XVI	2.0E-03
VIII	2.1E-03	XIV	1.0E-02	IV	2.2E-03
X	4.0E-03	III	1.2E-02	XIV	4.0E-03
VII	5.0E-03	X	1.2E-02	XVIII	4.0E-03
XIII	5.0E-03	VIII	1.5E-02	II	4.3E-03
IV	6.2E-03	I	1.7E-02	VIII	6.0E-03
IX	7.0E-03	VII	1.8E-02	XX	6.5E-03
VI	8.0E-03	XVII	2.0E-02	III	6.5E-03
XV	8.1E-03	XIII	2.0E-02	VII	8.0E-03
XIX	1.0E-02	XX	2.1E-02	IX	8.1E-03
III	1.6E-02	XVIII	3.0E-02	VI	1.0E-02
XX	1.6E-02	IX	3.0E-02	XIII	1.2E-02
XII	2.0E-02	XV	6.4E-02	XVII	2.0E-02
XVI	2.0E-02	XIX	1.2E-01	X	2.0E-02
II	2.1E-02	XI	3.0E-01	XI	2.0E-02
XVII	4.0E-02	XII	5.0E-01	XII	3.0E-02
XI	5.0E-02	VI	2.0E+01	XIX	5.0E-02
XVIII	1.0E-01	XXI	2.1E+01	XV	1.5E-01
XXI	4.0E+00	XXIII	4.1E+01	XXIII	4.3E+01
XXII	ND	V	5.2E+01	XXII	ND
XXIII	ND	XXII	ND	XXI	ND

ND = Not Detected (150 ng highest amount injected)

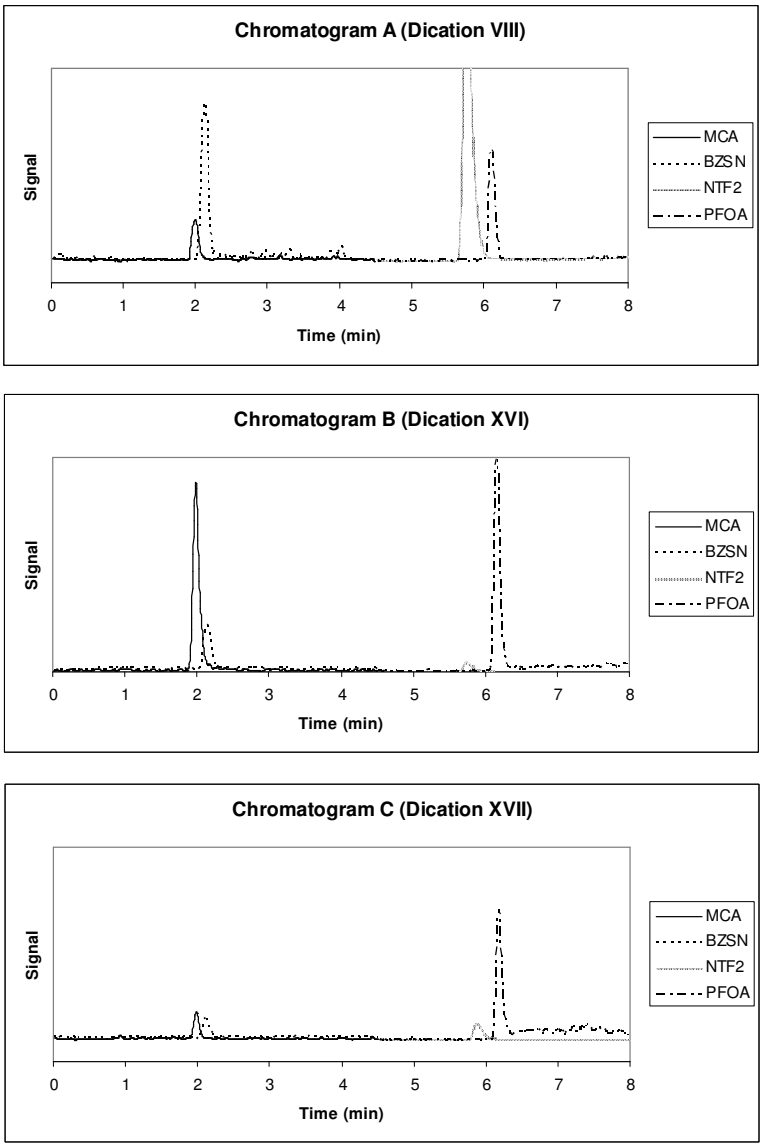


Figure 5.1. Three separate chromatograms showing the separation of a sample containing four anions (150 ng/mL MCA, 50 ng/mL BZSN, 500 ng/mL NTF_2 and 75 ng/mL PFOA). The masses monitored are the sum of the mass of each anion and the mass of the corresponding dicationic reagent. Chromatograms A and B use recommended dications (**VIII** and **XVI**), while chromatogram C does not (**XVII**).

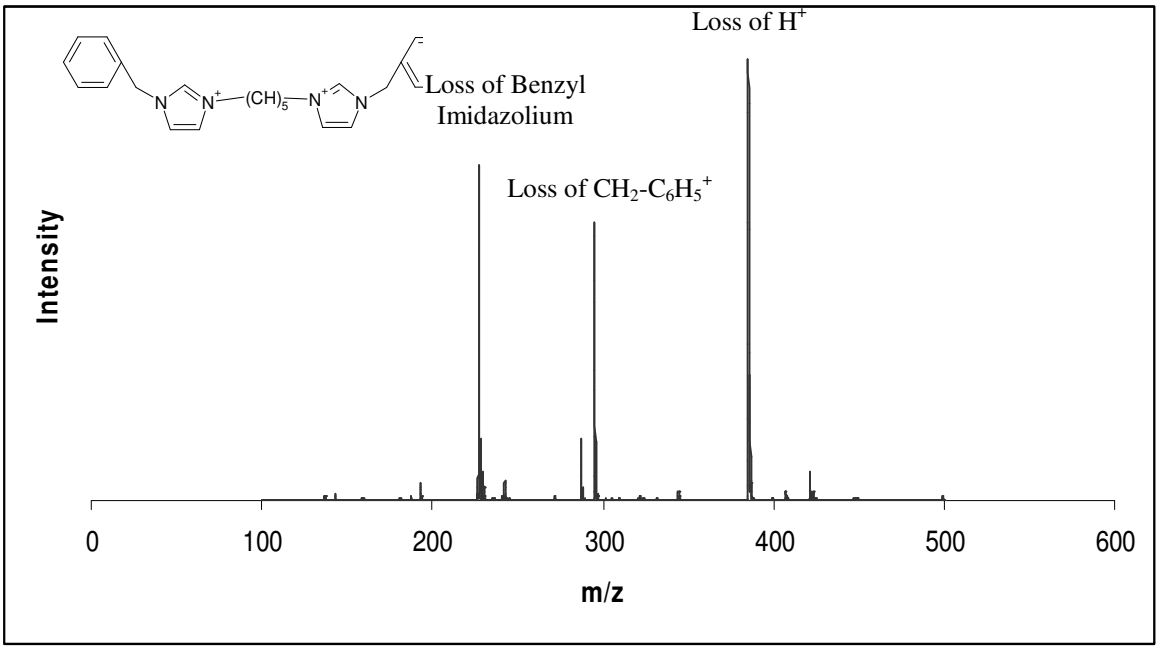


Figure 5.2. Mass spectrum of the mobile phase containing the dicationic reagent under typical operating settings for chromatography. Notice the three most prominent peaks are actually fragments of the dicationic reagent (**XIV**). These fragments can be monitored after excitation of a dication-anion complex to typically lower detection limits.

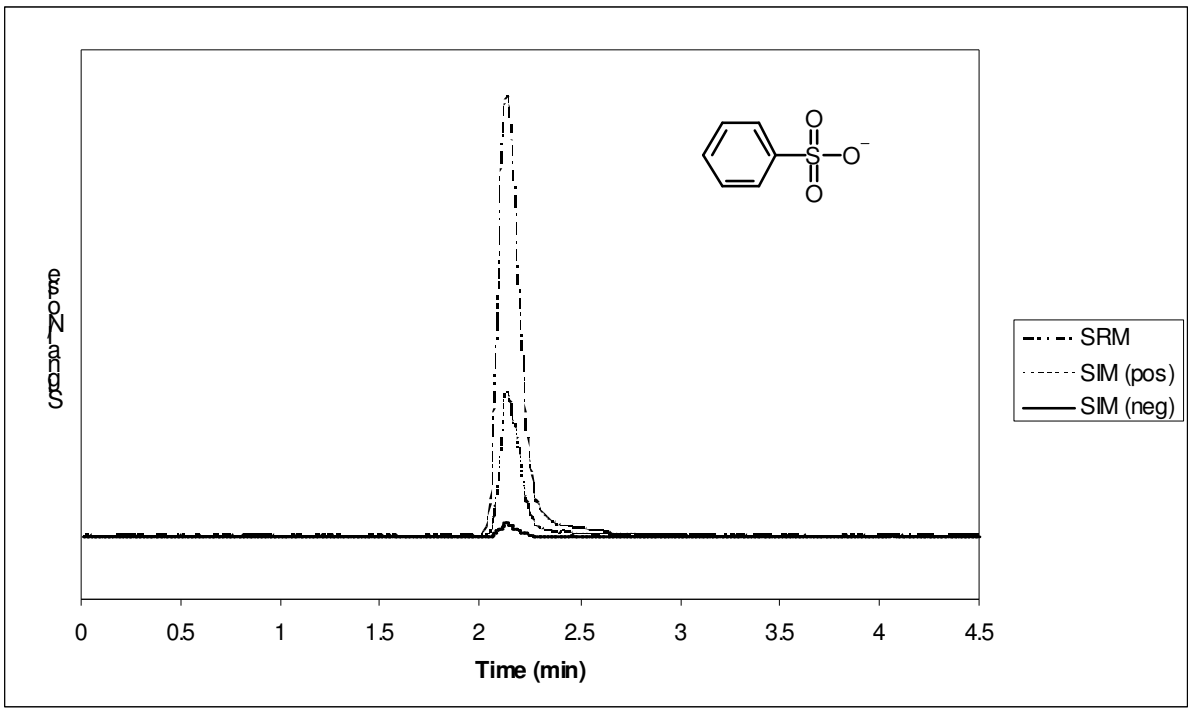


Figure 5.3. Overlapping chromatograms of three separate injections of a 100 ng/mL sample of benzene sulfonate. The solid line represents the use of negative mode, monitoring the mass of the anion (methanol being added post column). When 40 μM of dication **XIV** in methanol is added post column, the mass of the dication-anion complex can be monitored and gives a significant increase in S/N (dotted line). Finally, when single reaction monitoring is used, an even further increase in S/N can be seen, as shown by the alternating line.

CHAPTER 6

EVALUATING THE USE OF TRICATIONIC REAGENTS FOR THE DETECTION OF DIVALENT ANIONS IN THE POSITIVE MODE BY ESI-MS

A paper to be published in *Analytical Chemistry*[5]

Renee J. Soukup-Hein, Jeffrey W. Remsburg, Zachary S. Breitbach, Pritesh S. Sharma, Tharanga Payagala, Eranda Wanigasekara, Junmin Huang, and Daniel W. Armstrong

ABSTRACT

The analysis of anions remains an important task for many areas of science and new sensitive analytical methods continue to be of great interest. In this study we present the use of seventeen tricationic reagents for use as gas phase ion pairing agents for divalent anions. When the anion pairs with the tricationic reagent, an overall positive charge is retained and enables detection by ESI-MS in the positive mode. The seventeen tricationic reagents were made from one of four core structures and seven terminal charged groups. The effect of these structural elements on the detection sensitivity of the complex is examined empirically. A comparison of signal to noise ratios achieved in positive and negative modes also is presented.

6.1. INTRODUCTION

The analysis of anions remains an important task for many areas of science including environmental analysis, the pharmaceutical industry, and the food industry. Flow injection analysis and separation techniques such as ion chromatography have employed ion-selective electrodes[1-5] and spectrophotometric techniques[6-7] to detect anions. However, these detection methods are generally not considered to be universal detectors. Conductivity detection can be used as a universal detector for anions, but the lack of specificity can be a problem for complex samples, even when combined with a separation technique[8]. Mass spectrometry is growing in popularity as a universal detector for anions and it can be used alone[9-10] or in combination with a separation method[8, 11-14].

The negative ion mode is the most common way of detecting anions using ESI-MS. However, operating in negative ion mode with standard solvents found in chromatography (primarily water and methanol) can lead to corona discharge, poor spray stability, and a propensity for arcing[15, 16]. These effects can be suppressed by using electron scavenging gasses[17] or halogenated solvents[16, 18-20]. The substitution of isopropanol or butanol[21] for methanol has also been recommended for operation in negative ion mode. However, these solvents are less commonly used in LC methods involving water and result in higher operating pressures.

Recently, we have successfully used dicationic reagents to detect singly charged anions in the positive mode by ESI-MS[22, 23]. The dicationic reagent paired with the anion in the gas phase and enabled detection in the positive mode using common LC solvents. Additional benefits include (a) moving anions to a higher mass range out of the low mass region dominated by chemical noise (b) increasing sensitivity for anions with masses near the

low mass cutoff of quadrupole instruments (e.g. traps), and (c) help discriminate against interferences with the same mass to charge ratio. This approach has also been used with ion chromatography to determine the levels of perchlorate and two other anions in human urine[24], milk[25], and seawater[26]. The success of dicationic reagents to detect singly charged anions in the positive mode has encouraged us to use a similar approach for the detection of doubly charged anions. When various dicationic reagents were used to detect singly charged anions in the positive mode using ESI-MS, it became clear that some dications were better suited for this type of application than others[27]. Thus, the goal of this note is two fold: (1) to serve as a proof of concept that doubly charged anions can be detected in the positive mode in ESI-MS using tricationic reagents and (2) to begin identification of the structural elements of the tricationic reagents that will enable sensitive detection.

6.2. EXPERIMENTAL

6.2.1 Tricationic Reagent:

The synthesis of the tricationic reagents is presented in the supplemental information. Figure 1 gives the structure of the seventeen cationic reagents used in this study. After purification, the tricationic salts were exchanged to the fluoride form using the procedure reported previously[22, 23] with some modifications. The same amount (4 mL) of anion exchange resin was packed into a disposable 10 mL syringe and put into the fluoride form by washing the column with ten column volumes of 1 M NaOH followed by ten column volumes of water, seven volumes of 0.5 M NaF, and ten volumes of water. The tricationic reagents were dissolved in either water or methanol at a concentration of 0.05M and one milliliter of this solution was passed through the resin and eluted by water into a volumetric

flask. This stock solution was diluted with water to make the working tricationic reagent solution at a concentration so that when it was mixed with the carrier solvent the concentration of the reagent was 10 µM.

6.2.2. ESI-MS:

ESI-MS analysis was carried out on a LXQ (Thermo Fisher Scientific San Jose, CA, USA) linear ion trap. A Surveyor MS pump (Thermo Fisher Scientific) with a vacuum degasser provided the carrier flow (67% MeOH/ 33%Water) at 300 µL/min. The tricationic reagent was introduced to carrier flow using a Y-type tee and a Shimadzu 6A LC pump operated at 100 µL/min was used for this purpose. For analysis in negative mode water replaced the aqueous tricationic reagent solution. The test anions were introduced into the carrier solvent using a six-port injection valve located between the Surveyor MS pump and the Y-type tee. ESI ionization conditions for positive and negative ion modes along with the optimized parameters for fluorophosphate are listed in Table 6.1. Detection limits (defined as S/N=3) for the eleven anions were determined by five replicate injections. The mass spectrometer was operated in single ion monitoring mode for the determination of all limits of detection (LODs). Data analysis was performed in Xcalibur 3.1 software.

6.3. RESULTS AND DISCUSSION

Eleven divalent anions were used to evaluate seventeen different tricationic reagents (see Table 6.2). The anions included both inorganic and organic types and were structurally diverse. Metal-based anions such as dichromate, nitroprusside, and hexachloroplatinate were among the inorganic anions included. Some of the anions were chosen based on the behavior of singly charged anions with dicationic reagents. Singly charged anions with halogen atoms

paired very well with dicationic reagents and so representative divalent anions with bromine or fluorine atoms (bromosuccinate, dibromosuccinate, fluorophosphate) also were included in this study.

The trications synthesized for this study had one of four different "core" structures (Fig.6.1). **A** and **B** have a benzene core while the nitrogen at the middle of core **C** is less hydrophobic. **D** is by far the most flexible of four core structures. Seven different charge carrying groups were used to create the seventeen tricationic reagents. Trications are named by the core used (A, B, C, D) and the type of charged group (1-7). For example, trication **A1** has the benzene core and butyl imidazolium charged groups.

The detection limits for the anions in the positive mode by ESI-MS are given in Table 6.2. Except for dichromate, detection limits for most of the anions were in the hundreds of picograms to nanogram range with the tricationic reagents. The trications are arranged from lowest to highest according to the determined LODs. Using this arrangement, there are a few trends that emerge. From Table 6.2 it becomes obvious that trications **A6** and **B1** provide good sensitivity for a broad range of the representative divalent anions. **A6** (1,3,5-tris-(tripropylphosphonium) methylbenzene trifluoride) performs the best overall since it ranks as one of the top three trication reagents for all of the anions except sulfate and oxalate. Even then, it ranks as the fifth best tricationic reagent for detecting oxalate. Trication **B1** (1,3,5-tris-(1-(3-butylimidazolium)) methyl-2,4,6-trimethylbenzene trifluoride) also does well, but is in the top three less consistently than **A6**. Table 1 also clearly shows that trication **C7** does not pair well with any anion, making it the most ineffective additive tested. **A5** also ranked in the lower half of the trication list for many of the anions. These two tricationic reagents

would be poor choices for developing a sensitive method for the detection of divalent anions by positive ion mode ESI-MS.

When the terminal cationic moieties of the trication are the same, it is possible to compare the effect of the core structure on the performance of the tricationic reagent. While there are exceptions, cores **A** and **B** tend to pair more effectively with the doubly charged anions than those based on core **C** (Fig.6.1). For these eleven anions, a tricationic reagent with a **C** core performs in the top three only four times. Thus, a tricationic reagent with a more rigid aromatic core seems to produce better results. However, the decision whether or not to include methyl groups as substituents on the benzene core is less straightforward. When the charged group is phosphorus-based, the plain benzene core (**A1**) provided lower detection limits compared to the mesitylene (1, 3, 5-trimethylbenzene) core (**B6**). However, the opposite trend was seen in comparing **A1** and **B1**. **A1** seemed to be more susceptible to the loss of one of the butyl imidazole groups under MS conditions (data not shown) than **B1**, which appears to be stabilized by the methyl groups on the mesitylene core. It should also be noted that these cores may have limited flexibility due to the repulsion among their identically charged moieties. Flexibility of the pairing agent was found to be an important factor in the pairing of singly charged anions with dicationic reagents[27]. Trications **D2** and **D6** are more flexible due to their longer chains. However, these trications do not provide good sensitivity for any divalent anions except fluorophosphate. This core structure has several heteroatoms and carbonyl groups which could compromise its effectiveness as a gas phase ion pairing agent that can provide good detection limits. It seems that a more ideal tricationic core would use longer (perhaps solely) hydrocarbon chains to attach the charged groups to a hydrophobic core. This would reduce charge repulsion and increase flexibility.

The nature of the terminal charged groups also influenced the detection limits observed for the anions. For example, the phosphonium based tricationic reagents (**A6**, **B6**, **and C6**) generally paired well with all of the anions. Benzyl imidazolium groups provided the lowest detection limits for nitroprusside and hexachloroplatinate and decent detection limits for o-benzenedisulfonate. This seems to indicate that pi-pi and n-pi interactions play a role in the association of certain specific anions with tricationic reagents. Analogous trends were seen with dicationic reagents[27]. However, two of the charged groups that did well with the dicationic reagents gave lower than expected sensitivities for the representative anions in this study. Reagents with methyl imidazolium and pyrrolidinium groups consistently placed in the middle to lower half of the trications tested regardless of the core structure. Instead, butyl imidazolium groups on the mesitylene core (**B1**) performed better than expected.

It should be noted that the empirical data presented here are the result of several factors in addition to the binding affinity of the anions to the tricationic reagents. A single set of instrumental settings was used for the evaluation of the tricationic reagents. Some variance in instrumental performance between the different complexes is to be expected. The detection limit for oxalate was lowered from 250 pg to 75 pg when conditions were completely optimized (see experimental and Table 6.1) for the oxalate/**A6** complex. This increase in sensitivity is similar to that seen when optimizing dicationic reagents for detecting singly charged anions[27]. Increasing the spray voltage and decreasing the capillary temperature had the biggest impact on the signal intensity.

Figure 6.2 shows is a comparison of signal to noise ratios in the positive and negative ion modes for the two anions hexachloroplatinate and o-benzenedisulfonate. In both cases, using a tricationic reagent in the positive mode produced superior signal to noise ratios even

though ten times less sample was injected. By detecting divalent anions in the positive mode as a complex, the sensitivity for the two anions increases by almost two orders of magnitude. This demonstrates the ability of tricationic reagents to improve the sensitivity of mass spectrometry for divalent anions.

6.4. CONCLUSIONS

Seventeen tricationic reagents have been evaluated as pairing agents for detecting eleven doubly charged anions in the positive mode by ESI-MS. Structural features of the tricationic reagents including the terminal charged groups and the core structure influenced the detection limits for the doubly charged anions. The nature of the optimal charged groups for the tricationic reagents were often different from that found in a previous study for dicationic reagents. The use of tricationic reagents in the positive ion mode increased the S/N ratios of hexachloroplatinate and o-benzenedisulfonate compared to negative mode even though ten times more sample was injected in the negative ion mode.

6.5. ACKNOWLEDGEMENTS

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6.6. REFERENCES

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Table 6.1: MS Parameters

MS Parameters	General Positive Mode	General Negative Mode	Optimized for FPO_3
Spray Voltage (kV)	3	-5	4.7
Capillary temp (°C)	350	250	350
Capillary Voltage (V)	11	28	-21
Tube lens (V)	105	95	-96
Sheath gas (AU)	37	37	37
Auxiliary gas (AU)	6	6	6

(AU): arbitrary units

Table 6.2: Detection limits of doubly charged anions with tricationic reagents

Sulfate		Dichromate		Oxalate		Thiosulfate	
Trication	**LOD (ng)**	**Trication**	**LOD (ng)**	**Trication**	**LOD (ng)**	**Trication**	**LOD (ng)**
B1	1.0×10^{-1}	B1	4.6×10^{-1}	C6	1.5×10^{-2}	A6	1.3×10^{-1}
B4	1.0×10^{-1}	B4	2.0×10^{0}	A1	4.0×10^{-2}	C1	1.3×10^{-1}
A5	1.0×10^{-1}	A6	1.0×10^{1}	B1	4.0×10^{-2}	B2	1.5×10^{-1}
C3	1.3×10^{-1}	C4	1.0×10^{1}	B6	2.3×10^{-1}	C5	1.5×10^{-1}
D6	1.5×10^{-1}	B2	1.0×10^{1}	A6	2.5×10^{-1}	B4	1.6×10^{-1}
C4	2.5×10^{-1}	A1	1.0×10^{1}	C1	3.4×10^{-1}	C4	2.0×10^{-1}
B2	2.5×10^{-1}	A2	1.3×10^{1}	C3	3.8×10^{-1}	B1	2.4×10^{-1}
A1	5.0×10^{-1}	B6	1.5×10^{1}	C4	4.4×10^{-1}	B6	2.6×10^{-1}
A6	5.0×10^{-1}	C2	1.7×10^{1}	A2	5.0×10^{-1}	C6	4.5×10^{-1}
A2	6.3×10^{-1}	C1	1.8×10^{1}	A5	5.0×10^{-1}	C3	5.0×10^{-1}
D2	7.0×10^{-1}	C3	2.0×10^{1}	B2	5.0×10^{-1}	A2	7.5×10^{-1}
C2	7.5×10^{-1}	C5	2.5×10^{1}	C2	7.2×10^{-1}	C2	7.8×10^{-1}
C1	8.8×10^{-1}	C6	4.5×10^{1}	B4	7.5×10^{-1}	A1	1.0×10^{0}
B6	1.5×10^{0}	D6	4.9×10^{1}	D6	8.6×10^{-1}	D2	1.4×10^{0}
C5	1.9×10^{0}	C7	5.0×10^{-1}	C5	1.0×10^{0}	A5	2.1×10^{0}
C6	2.4×10^{0}	A5	1.8×10^{2}	D2	1.5×10^{0}	C7	5.2×10^{0}
C7	2.8×10^{0}	D2	2.5×10^{2}	C7	4.3×10^{0}	D6	1.5×10^{1}

Nitroprusside		Bromosuccinate		*o*-benzenedisulfonate		Hexachloroplatinate	
Trication	**LOD (ng)**	**Trication**	**LOD (ng)**	**Trication**	**LOD (ng)**	**Trication**	**LOD (ng)**
B4	3.2×10^{-3}	A6	7.5×10^{-2}	A6	1.5×10^{-2}	B4	2.6×10^{-2}
A	7.5×10^{-3}	B6	5.0×10^{-1}	C1	2.3×10^{-2}	B1	3.9×10^{-2}
B1	8.6×10^{-3}	C3	5.0×10^{-1}	B1	2.5×10^{-2}	A6	7.5×10^{-2}
B6	1.4×10^{-2}	D6	5.0×10^{-1}	B4	2.5×10^{-2}	C1	1.0×10^{-1}
C4	2.0×10^{-2}	C6	7.5×10^{-1}	C4	3.0×10^{-2}	A1	1.3×10^{-1}
C1	2.7×10^{-2}	A5	1.5×10^{0}	C6	3.8×10^{-2}	B6	1.6×10^{-1}
C5	2.7×10^{-2}	C5	1.6×10^{0}	A1	5.0×10^{-2}	B2	2.0×10^{-1}
C3	4.3×10^{-2}	A2	5.0×10^{0}	C2	5.0×10^{-2}	C4	2.5×10^{-1}
A1	4.3×10^{-2}	C1	5.0×10^{0}	B6	5.0×10^{-2}	D6	5.0×10^{-1}
C2	4.4×10^{-2}	B2	5.0×10^{0}	C5	5.0×10^{-2}	C5	8.8×10^{-1}
A2	4.9×10^{-2}	C2	7.0×10^{0}	A2	7.5×10^{-2}	C3	1.0×10^{0}
C7	6.0×10^{-2}	A1	7.5×10^{0}	C3	1.3×10^{-1}	C2	1.1×10^{0}
B2	1.0×10^{-1}	C4	8.8×10^{0}	D6	1.5×10^{-1}	D6	1.6×10^{0}
D6	1.3×10^{-1}	B4	1.0×10^{1}	A5	2.0×10^{-1}	A5	1.6×10^{0}
C6	2.0×10^{-1}	D2	1.3×10^{1}	C7	3.8×10^{-1}	C7	2.0×10^{0}
A5	3.2×10^{-1}	B1	1.8×10^{1}	B2	1.1×10^{0}	B2	2.2×10^{0}
D2	8.8×10^{-1}	C7	4.5×10^{1}	D2	1.8×10^{0}	D2	2.3×10^{0}

Table 6.2 cont'd.

Dibromosuccinate		Fluorophosphate		Selenate	
Trication	**LOD (ng)**	**Trication**	**LOD (ng)**	**Trication**	**LOD (ng)**
B6	1.5×10^{-1}	A6	3.8×10^{-2}	A6	7.5×10^{-2}
A6	1.8×10^{-1}	D6	7.5×10^{-2}	B4	2.3×10^{-1}
C4	3.8×10^{-1}	B6	1.3×10^{-1}	B1	2.8×10^{-1}
C2	5.0×10^{-1}	D2	1.3×10^{-1}	C4	3.5×10^{-1}
C1	5.0×10^{-1}	B2	1.5×10^{-1}	C6	3.8×10^{-1}
C3	5.0×10^{-1}	C3	2.0×10^{-1}	B6	3.9×10^{-1}
C6	5.0×10^{-1}	B1	2.5×10^{-1}	D6	4.3×10^{-1}
C5	1.1×10^{0}	B4	2.5×10^{-1}	C1	5×10^{-1}
B1	1.3×10^{0}	C6	2.5×10^{-1}	C7	5.6×10^{-1}
B4	1.5×10^{0}	C4	2.8×10^{-1}	C3	7.5×10^{-1}
C7	1.5×10^{0}	C7	2.8×10^{-1}	D2	7.5×10^{-1}
D6	1.5×10^{0}	C1	4.3×10^{-1}	C2	1.1×10^{0}
A1	3.0×10^{0}	C5	5.0×10^{-1}	A2	1.1×10^{0}
A5	5.0×10^{0}	A5	7.5×10^{-1}	B2	1.4×10^{0}
A2	5.0×10^{0}	C2	1.0×10^{0}	A5	2.5×10^{0}
B2	5.0×10^{0}	A1	5.0×10^{1}	A1	4.0×10^{0}
D2	5.0×10^{0}	A2	5.0×10^{1}	C5	1.8×10^{-1}

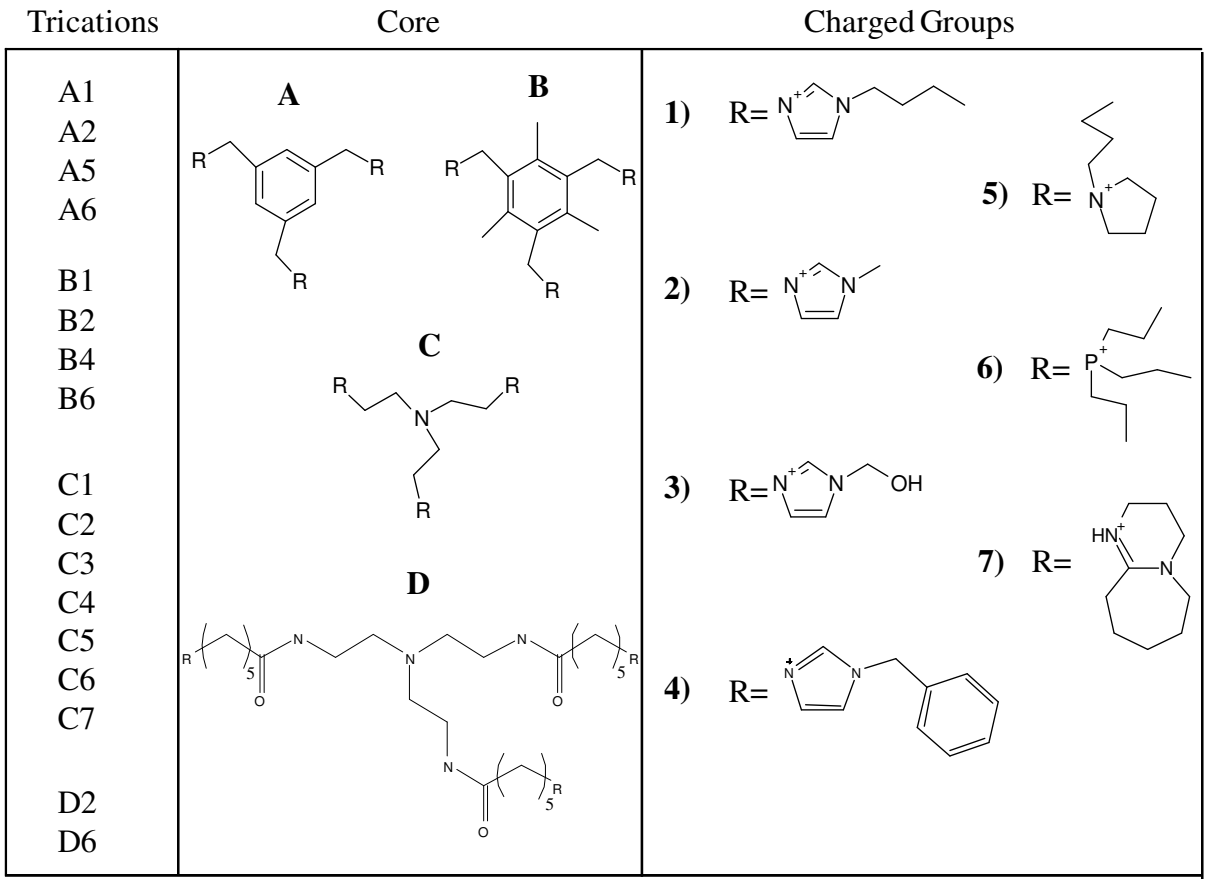

Figure 6.1: Structure and numbering system for the 17 tricationic reagents synthesized and evaluated in this study.

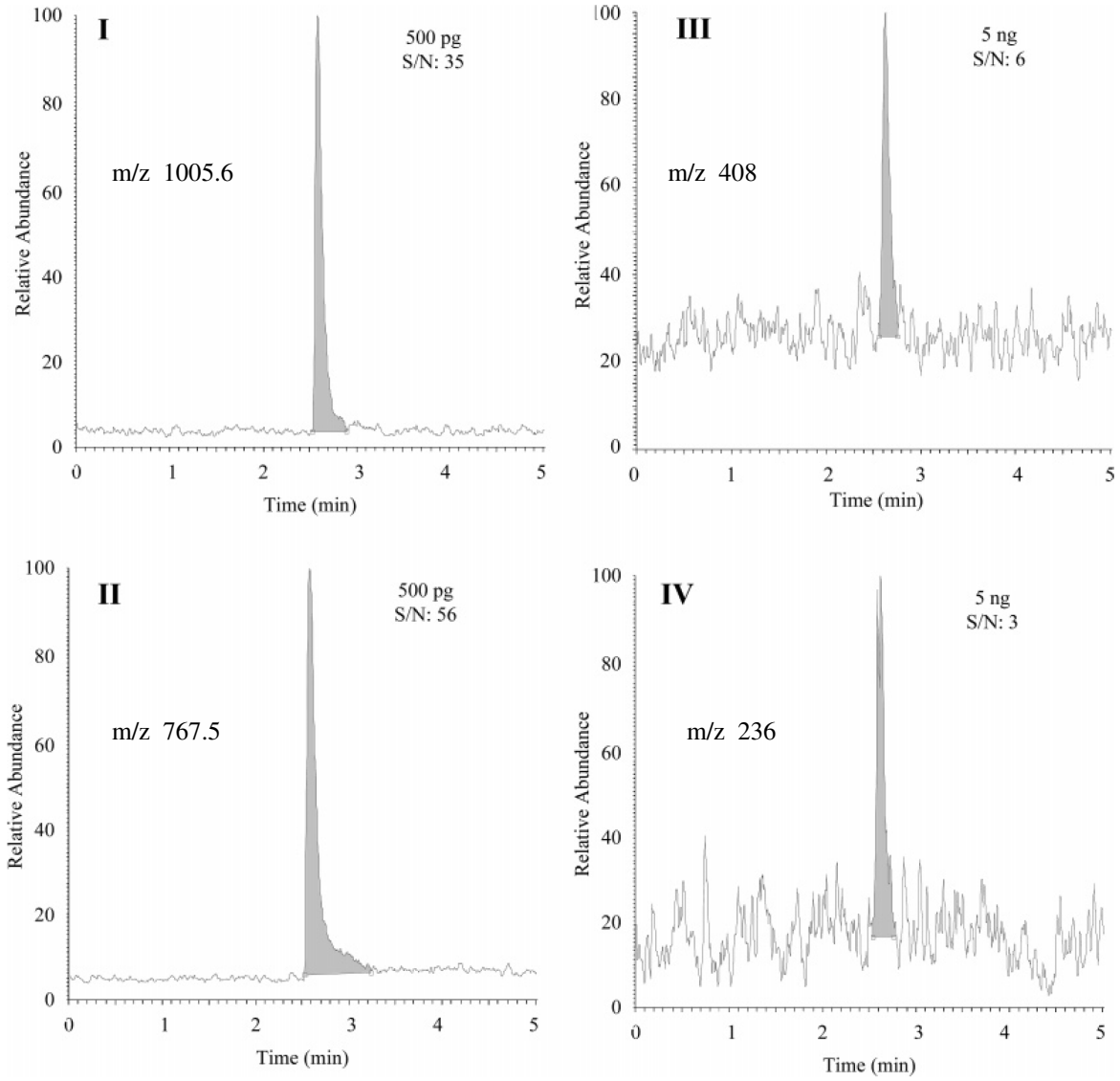

Figure 6.2: A comparison of positive (I, II) and negative modes (III,IV) for hexachloroplatinate (I, III) and o-benzenedisulfonate (II,IV). Tricationic reagents A6 (I) and B1 (II) in water were introduced into the carrier flow after anion injection.in positive ion mode while only water was used in negative ion mode.

CHAPTER 7

GENERAL CONCLUSIONS

The ability of macrocyclic glycopeptide chiral stationary phases to separate structurally related peptides up to thirteen amino acids in length was demonstrated. The fourteen peptide families were involved in this study and some common tendencies were observed. First, amino acid polymorphisms occurring at the terminal ends of a peptide were generally easier to separate than polymorphisms occurring in the middle of a peptide sequence. Second, substitutions of a charged amino acid (positive or negative) for a neutral amino acid produced more pronounced separations than did substitution of another neutral amino acid or an amino acid of the same charge. Third, the presence of a D-amino acid anywhere in the sequence (even at the C-terminus) caused the peptide to elute before the corresponding L-amino acid containing peptide. The teicoplanin stationary phase exhibited the broadest selectivity for separating peptide families. However, the teicoplanin aglycone and ristocetin stationary phases were needed to provide the best separations for some peptide families. The amount and type of organic modifier and mobile phase additives as well as ionic strength were found to influence the retention and peak shape.

Teicoplanin also exhibited good selectivity for a series of seven diastereomeric enkephalin peptides and this selectivity was compared to that achieved on a conventional C18 stationary phase. Included in the seven peptides was one set of enantiomers. On the C18 stationary phase, the retention order was influenced by the orientation of hydrophobic side chains. However, there was no apparent correlation between side chain orientation and retention on the teicoplanin stationary phase. This highlights the difference in separation mechanisms on the C18 and more functionally diverse teicoplanin stationary phases. The

different separation mechanism also resulted in different abilities to separate the diastereomeric peptides. Teicoplanin was able to separate all seven diastereomeric peptides using a step-gradient. Extremely high selectivity was observed for the enantiomeric pair of peptides. It was not possible to completely separate Y-dA-GF-dL, Y-dA-GFL, Y-dA-G-dF-L, YAGF-dL, and Y-dA-G-dF-dL under a single gradient condition on the C18 stationary phase.

Peptides of greater lengths (28-36 amino acids) were challenging to separate with the macrocyclic glycopeptide stationary phases. Peptides with multiple basic amino acids were highly retained and a triethylammonium trifluoroacetate buffer was necessary to elute the peptides from the teicoplanin stationary phase. Only the teicoplanin stationary phase displayed selectivity for these larger peptides. Peptides with subtle substitutions often were very difficult to separate on both the teicoplanin and C18 stationary phases, and both retained some selectivity for pairs of peptide diastereomers.

In the second part of this dissertation, it was demonstrated that the most sensitive detection mode for anions can be the positive ion mode. By pairing singly charged anions with a reagent with two positive charges, the anions could be detected in positive ion mode using ESI-MS. Detection limits were best for chaotropic monovalent anions. The lowest detection limits (hundreds of femtograms) were achieved for perfluorooctanoic acid. Tandem mass spectrometry was used for the first time with this dicationic reagent method to further lower the detection limits for some anions. The monitored transition was from the m/z of the anion-dicationic reagent complex to a fragment of the reagent. This dicationic reagent approach can be used with ESI-MS alone or after a separation column.

For a LC separation of five anions, four of five anions showed higher areas and signal to noise ratios even though 5 to 10 times more sample was injected in negative mode.

The structure of the dicationic reagent was found to be very important for the detection of singly charged anions in the positive ion mode. Vastly different detection limits were observed for a given representative anion with different dicationic reagents. For dicationic reagents, hydrocarbon chains that connected methyl imidazolium, benzyl imidazolium, butyl pyrrolidinium, or tripropyl phosphonium groups were the most broadly useful. However, only imidazolium based dicationic reagents produce fragments useful for analysis in MS/MS. A methylated naturally occurring dicationic compound and two commercially available dicationic compounds did not pair well, if at all, with many of the representative anions. This observation along with other empirical data led to the conclusion that flexibility in the dicationic reagent is an important factor in determining the performance of such reagents.

This approach was also adapted for detecting divalent anions in the positive ion mode by ESI-MS. In order for the anion/cationic reagent complex to be detected by the mass spectrometer, the reagent must now carry at least three positive charges. Tricationic reagents were made from one of four core structures with one of seven different types of terminal charged groups. Tricationic reagents with a hydrophobic benzene core generally provided lower detection limits for the representative anions than reagents with a nitrogen based core. While phosphonium based terminal charged groups still paired well with the divalent anions, different trends were observed with other terminal groups. Higher signal to noise ratios were observed using tricationic reagents in positive mode for

hexachloroplatinate and o-benzenedisulfonate even though ten times more sample was injected in negative mode.

The use of cationic reagents to detection anions in the positive mode is a relatively new research area. Much about how the anions interact with the pairing agents is not yet fully understood. Answering this question will help this application grow into a more mature technique.

APPENDIX

APPENDIX ACCOMPANYING CHAPTER 6
SYNTHESIS PROCEDURES FOR TRICATIONIC REAGENTS

Synthesis of compounds **A1**, **A2**, **A5**, and **A6** involve refluxing 1 molar equiv of 1,3,5-tris(bromomethyl)benzene with 4 molar equiv 1-butylimidazole, 1-methylimidazole, 1-butylpyrrolidine, or tripropylphosphine respectively in isopropanol for 7 days. After removal of isopropanol with a rotary evaporator, the bromide salt was dissolved in water and purified by extraction with ethyl acetate. Water was removed by a rotary evaporator and the remaining salt was dried under vacuum.

Synthesis of compounds **B1**, **B4**, and **B6** involve refluxing 1 molar equiv of 2,4,6-tris(bromomethyl)mesitylene with 4 molar equiv of 1-butylimidazole, 1-benzylimidazole, or tripropylphosphine respectively in isopropanol for 7 days. After the reaction, the products were all purified by extraction with ethyl acetate and dried under vacuum. Compound **B2** was synthesized carrying out the same procedure using 1 molar equiv of 2,4,6-tris(bromomethyl)mesitylene with 4 molar equiv of 1-methylimidazole but using toluene as the solvent.

Compounds **C2**, **C3**, **C4**, and **C5** were synthesized by refluxing 1 molar equivalent of Tris(2-chloroethyl)amine hydrochloride in isopropyl alcohol with 6 molar equivalents of 1-methylimidazole, 1- benzylimidazole, 1-butylpyrrolidine, and 1-(2-hydroxyethyl)imidazole respectively. Rotoevaporation of the solvent yielded the crude hydrochloride salt. This was then dissolved in water with 2 molar equivalents of NaOH. NaOH is used to neutralize the hydrochloride salt. The excess starting material was extracted with ethyl acetate. Final products were synthesized through a metathesis reaction of the chloride salts with lithium trifluorlmethanesulfonimide (NTf_2^-). Specifically, 1 molar equivalent of the chloride salt was dissolved in water and treated with 4.5 molar equivalents of the lithium NTf_2^-. The resulting solution was stirred at room temperature for 24 hrs.

After that, dichloromethane was added to the solution to dissolve the tricationic NTf_2^- salt that has phase separated from the water. The lithium chloride, sodium chloride, excess sodium hydroxide and excess lithium NTf_2^- were removed from the dichloromethane phase with successive washing with water. Removal of dichloromethane through rotoevaportaiton followed by vacuum drying over phosphorous pentoxide at 80 ^{0}C for 24hrs resulted in the pure tricationic ILs with NTf_2^- counter ions.

Compounds **C1** was synthesized by refluxing 1 molar equivalent of Tris(2-chloroethyl)amine hydrochloride in isopropyl alcohol with 6 molar equivalents of 1-butylimidazole for 5 days. Rotoevaporation of the solvent yielded the crude hydrochloride salt. This was then dissolved in water and passed through anion exchange resin - Amberlite IRA-400(Cl) saturated with OH^- anion to obtain the hydroxide salt of the trication. The eluent was then titrated with tetrafluoroboric acid until pH 7. Evaporation of water under vacuum and drying under phosphorous pentoxide at 80^{0}C yield the pure TIL2 as the BF_4^- salt.

Synthesis of compound **C6** involves refluxing 1 molar equiv of tris(2-chloroethyl)amine hydrochloride with 4 equiv of tripropylphosphine in isopropanol. The product was purified by extraction with ethyl acetate and dried under vacuum. Purified chloride salt was converted to hydroxide form using an ion exchange resin. The metathesis of the hydroxide salt was then carried out using fluoroboric acid. Compound 9 was isolated by the subsequent removal of water using rotary evaporator.

Synthesis of compound **C7** involves refluxing 1 molar equivalent of tris(2-chloroethyl)amine hydrochloride with 4 equiv of 1,8-diazabicyclo[5.4.0]undec-7-ene in isopropanol. After removing isopropanol with a rotary evaporator, the salt was dissolved in

water and excess 1,8-diazabicyclo[5.4.0]undec-7-ene was removed by carrying out extractions with ethyl acetate. After addition of sodium hydroxide metathesis process was then carried out using lithium trifluoromethanesulfonimide to isolate compound **C7**.

Core **D** was not commercially available and therefore had to be synthesized. To a solution of tris(2-aminoethyl)amine (5 ml, 33.8 mmol) and triethylamine (23.2 ml, 166.7 mmol) in CH_2Cl_2 (100 ml) at -78 °C was added 6-bromohexanolychloride (16.5 ml, 107.8 mmol) through a syringe under a vigorous stream of N_2. The reaction mixture was stirred for 3h at -78 °C and allowed to stir at temperature for 12h. Then the reaction mixture was poured in to 100 ml of cold water and the aqueous layer was extracted with (3 × 50 ml) of CH_2Cl_2 and the combined organic layer was concentrated in vacuo to give a pale yellow liquid. This was further dried under high vacuum to give **1** in 90% yield as orange color solid.

For the synthesis of **D2**, methylimidazole (1.89 ml, 22.1 mmol) was added to a solution of core **D** (3g, 4.4 mmol) in dry THF and refluxed for 36 hr under N_2. Then the solvent was evaporated under vacuum and resulted thick brown colored liquid was dissolved in 100 ml of water and washed the aqueous layer with ethyl acetate (6 × 100 ml). The aqueous layer was evaporated to dryness and resulted ionic liquid was dried under vacuum for 24 hr to give **2** in 65 % yield.

For the synthesis of **D6**, tripropylphosphene (4.2 ml, 26.4 mmol) was added to a solution of core **D** (3g, 4.4 mmol) in *iso*-propanol and refluxed for 48 hr under a vigorous stream of N_2. Then the solvents were removed in vacuo and resulted light yellow coloured thick liquid was dissolved in 100 ml of water and washed with ethyl acetate (8 × 100 ml).

The aqueous layer was then concentrated in vacuo and further dried under high vacuum to give **3** in 85 % yield as a yellow solid.