

IONIZATION COMPETITORS EXTEND THE LINEAR RANGE OF ELECTROSPRAY
IONIZATION MASS SPECTROMETRY

by

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Dedication

This thesis is dedicated to my father, who taught me that the largest task can be accomplished if it is done one-step at a time. It is also dedicated to my mother for her Dua', love, and care.

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My utmost gratitude goes to my thesis advisor, Dr. Kevin Schug for allowing me to join his team, for his expertise, kindness, and most of all for his support and guidance. My thanks and appreciation goes to my committee members, Dr. Richard Timmons, and Dr. Purnendu Dasgupta for their invaluable time and advice. I also like to thank my colleagues in the Schug group for their great friendship and support through out my career.

Finally, I want to thank my father who is my role model and my mother whose love is endless. My brothers and sister whose words made me manage through hard times.

November 25, 2009

ABSTRACT

IONIZATION COMPETITORS EXTEND THE LINEAR RANGE OF ELECTROSPRAY IONIZATION MASS SPECTROMETRY

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ESI-MS has proven its applicability in quantitative analysis for a wide range of analytes. Its effectiveness relies on achieving sensitivity and linear range, although the latter is known to be limited. ESI-MS response varies among charged species due to differences in analyte structure. The working curve associated with quantitative analysis has a limit of linearity. At high analyte concentration, instrument response plateaus because the surface of the charged droplet reaches saturation. Several studies have proposed to enhance the linear range by enhancing gas-phase analyte charging, by facilitating droplet evaporation, or by improving ion transmission through the mass spectrometer. This thesis focuses on examining the usefulness of surface-active ionization competitor co-analytes to extend the upper LR range of ESI-MS.

The introduction of ionization competitors, such as tetraalkyl ammonium acetates and poly glycols allow for an enhancement in the working curve associated with quantitative analysis at higher analyte concentrations, through an increase in the linear range with minimal sacrifice in sensitivity. Discrete measurements reveal an increase in LOL from $<100\mu\text{M}$ (without competitor) to $\sim 350\mu\text{M}$ (with competitor).

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CHAPTER 1

INTRODUCTION

Electrospray ionization mass spectrometry (ESI-MS) has become a widely used analytical technique in modern chemical and biological research due to its high sensitivity and broad applicability [1, 2]. Because of the extreme complexity of many biological samples (e.g., proteomics analyses), the effectiveness of ESI-MS depends substantially on both its achievable sensitivity and dynamic range. In this context, it has been recognized that optimization of the electrospray ionization (ESI) process has a large effect on achievable sensitivity and limit of detection [3]. Furthermore, the analyte ion signal detected with the mass spectrometer depends on the concentration of the analyte ion in the solution and how this signal is affected by the presence of other electrolytes. Other electrolytes are practically always present as impurities in the solvent as other co-analytes [4]. In addition, one important factor can determine sensitivity in ESI-MS, the efficiency by which molecules that are converted into gas-phase ions are transferred through the various stages of mass spectrometer and detected. Another source of effectiveness lies in achieving a wide linear range, however the latter is known to be limited.

In ESI-MS, compared to other analytical methods, deviation from linearity occurs at high analyte concentration as response becomes saturated, and at low concentration due to background interference. While operating in the saturation region of ESI calibration curve, the response of some analytes may be limited. This is due to a combination of instrumental factors and some fundamental limitation in the ability to produce a charged analyte in the ESI process. As such, the benefit of having a wide linear dynamic range is especially important for small molecule quantitation during, for example drug research, in environmental analysis, and in a variety of other fields. In small molecule quantitation during drug research, the dosage form can

cause an order of magnitude difference in quantitative results. In proteomics, the dynamic range of proteins in real samples can be in excess of 10 orders of magnitude with further complications arising from varying ionization efficiencies for the representative enzymatic peptide fragments. Thus having a wide dynamic range makes experimentation simpler by avoiding repeat assays and dilution issues. Conclusively, extension in the high end of the linear dynamic range could be highly advantageous for improved quantitative analysis with ESI-MS. Thus, possible ways to extend the linear dynamic range to higher concentration might include a more efficient charging of gas-phase analyte molecules, evaporating ESI droplets more effectively, or improving ion transmission through the mass spectrometer.

For this work, "ionization competitors" have been chosen, to study their effectiveness in increasing the linear range of ESI-MS, and with a concurrent goal of minimal sacrifice in sensitivity. Ionization competitors are surface active co-analytes, known for lowering the surface tension of a liquid (i.e. the interfacial tension at a phase or interface boundary). Surfactants are usually organic compounds that are amphiphilic, containing both hydrophobic and hydrophilic groups. They can be generally classified as nonionic, cationic, and anionic species [5]. Some types of surfactants (e.g. sodium dodecyl sulfate or Triton-X) are known to effectively compete for ionization against analytes of interest, and lower their ESI-MS response. Highly surface-active components tend to be enriched at the surface of charged droplets produced during ESI. However, only a few reports have appeared that describe the ESI-MS detection of analytes in the presence of surfactants. In this work, we show that the analyte ion signal can be detected under conditions capable of elucidating the analyte concentration in the charged droplet in the presence of surfactants. In this work, two classes of competitors, cationic tetraalkyl ammonium acetates and nonionic poly glycols, are investigated to study their effect on the ESI response character of two representative analytes, *tert*-butylcarbamoyl quinine and Bradykinin peptide.

The focus of this work is to investigate the amenability of ESI-MS for evaluating the response of *tert*-butylcarbamoyl quinine (tBuCQN) and Bradykinin peptide motif in the presence

and absence of ionization competitors. Specifically, we show that ionization competitors can be used to extend the linear range in the ESI-MS analysis of some standard analytes without an appreciable decrease (and in some cases, with an increase) in sensitivity. Such work is fundamental to increasing the applicability of ESI-MS in quantitative determinations. We also envision this work to increase the applicability of ESI-MS in the determination of binding constants for noncovalent systems. For example, a wide linear range would allow the investigation of weaker interaction systems, where a significant degree of association requires that higher concentration of interactants be evaluated in a linear response regime [6].

CHAPTER 2

GENERAL BACKGROUND

2.1 Introduction

Mass spectrometry (MS) has progressed to become a powerful analytical tool for both quantitative and qualitative applications. The first mass spectrometer was constructed in 1912 [7] and since then it has been developed for analysis of virtually all chemical species, from inorganic atoms to biological macromolecules, with practically no mass limitations. Proteomics research, in particular, has become increasingly dependent on MS technologies. This is due to the advances gained through the development of soft ionization techniques, such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), which can transform nonvolatile and thermally labile biomolecules into gas phase ions, for subsequent mass analysis. Among other advantages, ESI can be interfaced with high efficiency separation techniques, which enhances its role in the life and health sciences where complex analyte mixtures are regularly encountered. On the other hand, MALDI has the advantage of producing singly charged ions of peptides and proteins, minimizing spectral complexity.

Electrospray ionization - mass spectrometry (ESI-MS) has developed at a tremendous pace since the end of the 1980s [1, 8]. Its uniqueness from other analytical techniques lies in providing detailed information regarding molecular weights and structures from extremely small quantities of material. In this context, three features of ESI-MS set it apart from other mass spectrometric techniques. First, is the unique ability to produce multiply charged ions. This allows the production of highly-charged forms of very large molecular weight compounds, which can then be visualized using a broad range of mass analyzers. Second, it can be interfaced with

many types of separation techniques, including high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). This allows for the separation of complex mixture and the subsequent introduction of analyte components into the ESI source in a flowing solvent stream. Third, is the extreme softness of the ESI process, which permits the preservation of intact analyte ions in the gas phase, as well as even noncovalent interactions between molecules. As such, ESI-MS is a venerable technique in the field of analytical chemistry. Today, laboratories across a wide range of research fields routinely use ESI-MS for quantitative and qualitative analysis.

2.2 The Mechanism of Electrospray Ionization Mass Spectrometry

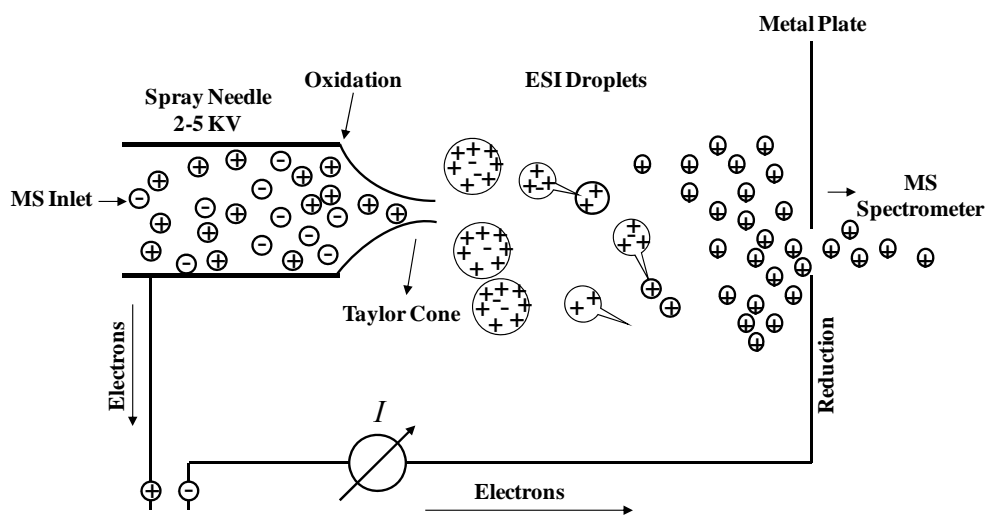


Figure 2.1: Schematic of the electro spray ionization process (positive mode) [11].

ESI is a process in which polar and ionizable molecules, present in solution, can be transferred to the gas phase, where they are analyzed in the mass spectrometer based on their mass-to-charge (m/z) ratio. This is of great importance because close to half of all chemical and biochemical processes involve ionizable compounds in solution.

The process involved in this transfer (for conventional ESI) is initiated by applying a high electric field of $\pm 3\text{--}5\text{ kV}$ (depending on the ionization mode, $+3\text{--}5\text{ kV}$ for positive ionization mode) to the tip of a capillary containing a solution of electrolyte ions at a concentration between $10\ \mu\text{M}$ to $500\ \mu\text{M}$ [1, 8]. The presence of a high electric field causes the formation of a dipolar layer in the liquid at the end of the capillary tip. This is due to the spatial separation of the electrolyte ions. This layer causes the formation of a cone at the tip of the capillary called a Taylor cone, through which a fine jet of solution extends until it is dispersed into highly charged droplets [9]. During their trip from the tip of the capillary to the counter electrode, located $\sim 1\text{ cm}$ apart and driven by a potential gradient, charged droplets undergo a series of desolvation and fissioning steps. Droplets subdivide as the coulombic repulsion at the surface of the droplet (which is increasing due to solvent evaporation) overcomes the surface tension holding the droplet together. This limit is known as the Rayleigh stability limit [10].

The precise mechanism of formation of gas phase ions by ESI has been an active debate, and has been the topic of many reviews [11, 12]. There are two predominant theories for the formation of gas phase ions from small, charged droplets. The charge residue model (CRM), proposed by Dole and coworkers, states that the increased charge density due to solvent evaporation causes large droplets to break down into smaller and smaller droplets, which eventually consist of only one single ion [13, 14]. On the contrary, the ion evaporation model (IEM) proposed by Iribarne and Thomson assumes that at some point during the droplet subdivision process, conditions become favorable for solvated ions to “evaporate” from the surface of the charged droplet, resulting in a release of solvated ions from droplet surface [15, 16]. Closer examination of the two models reveals that many of the observed spectral features can be explained with either model; preference for ionization by the CRM is generally attributed to macromolecular ions, whereas small molecules are believed to ionize through ion evaporation. Both theories predict that ions that interact weakly with the solvent will be expressed preferentially in the gas phase. Solvated ions, once released into the gas phase, are

stripped of their solvent molecules by passage through a heated capillary which leads into the high vacuum region of the mass spectrometer.

2.3 Application of ESI-MS to quantitative analysis

ESI-MS has greatly simplified the application of quantitative HPLC and CE methods for analysis of highly polar molecules in the pharmaceutical industry [17, 18]. It provides a detection method which is much more specific, and in many cases more sensitive, than common spectroscopic detection techniques. In ESI-MS, the ion signal is proportional to analyte concentration and largely independent of flow rate and injection volume used for sample introduction [19]. The signal is linear from the limit of detection (usually nM) to around 10 μ M of analyte concentration.

For quantitative measurement, it is important to incorporate an internal standard in the procedure to compensate for losses during sample preparation and variable detection sensitivity of the MS system. The analyte-dependence of ionization response in ESI-MS can not be overstated [20]. The internal standard should have a structure similar to that of the analyte and the ideal practice is to synthesize an internal standard by incorporating stable isotopes on the molecules of interest. For example, for the quantification of free carnitine (162 Da), an internal standard containing 3 deuterium atoms to replace 3 hydrogen atoms was used (165 Da) [21]. The 3 Da mass difference can be easily resolved in the mass spectrometer, and the internal standard can be expected to behave identically to the analyte of interest through the sample preparation and analysis procedure. When an ideal internal standard is not available, molecules with similar structure can also be used. For example, ascomycin has been used as an internal standard for ESI-MS/MS analysis of the immunosuppressant tacrolimus [22].

Another critical issue in quantitative ESI-MS is suppression of ionization due to matrix interferences. A biological sample would give significantly lower ionization signals compared to pure standard solutions with similar analyte concentrations. This phenomenon is the result of

high concentrations of both non-volatile materials and electrolytes from the biological sample being present in the spray with the analyte [23]. Possible non-volatile interfering solutes are salts and lipids in the biological samples. To overcome the matrix interference, extensive sample purification processes are required, for example, liquid-liquid extraction or solid phase extraction using disposable columns. However, many sample preparation procedures are time-consuming and can have poor recovery characteristics. A recent development is to use short LC columns (or guard columns) and apply a fast HPLC purification (e.g. for 2–5 minutes) prior to MS analysis [24]. The HPLC serves to separate the non-volatile compounds from the analyte. For HPLC systems with column-switching capability, the analyte in the biological sample can be purified and concentrated on separate columns prior to MS analysis. Such an automated sample purification system utilizing affinity chromatography is best illustrated by a recently published rapid quantification method for transferrin isoforms in serum [25].

Conventional ESI-MS is not typically characterized by an extensive linear range for quantitative analysis. This can be a significant disadvantage. Several strategies for expanding the linear working curve in bioanalysis, using quantitative HPLC / tandem mass spectrometry (HPLC-MS/MS) have been reported. Curtis et al. reported the tracking and analysis of multiple ions in a rat plasma assay for a proprietary experimental drug where the linear range was expanded from 2 to 4 orders of magnitude, depending on which ion was monitored [26]. Moreover, Shi reported the use of stable isotope labeled internal standards (SIL-IS) for the same task in HPLC-ESI-MS, an approach which significantly expanded the linear range by 4 orders of magnitude. Because the signal of the analyte is normalized to that for the internal standard, a linear response relationship can be maintained over a great range of concentrations. Unfortunately, stable isotope internal standards can be difficult to obtain or expensive to synthesize for some applications [27]. To provide a general sense of the upper limit of the linear range in typical analyses, several groups have reported near 10 μM upper

limits in the ESI-MS linear dynamic range (LDR) [28-30], though other groups have reported slightly higher (up to 100 μ M) LDR upper limits [31-33].

The nature of ESI as a soft ionization technique makes it applicable for quantitative speciation of non-covalent interactions. This is due to the low energy imparted to the ions during the electrospray process, compared to other mass spectrometric techniques. ESI-MS can be used to determine the binding affinity between a host and a guest analyte pair. Different solution phase spectroscopic methods like nuclear magnetic resonance spectroscopy (NMR) [34, 35], calorimetry [36, 37], potentiometry [38, 39], and ultraviolet visible spectroscopy (UV-VIS) [40, 41] are commonly used to investigate and quantify these host-guest noncovalent interactions. However, the above-mentioned techniques require relatively large quantities of material (milligram scale) and are slow. An appropriate quantity of the interactants of interest may be difficult to obtain or produce, limiting the application of these traditional approaches. In the case of using ESI-MS to study noncovalent complexes, several important features of MS can be exploited over the other more traditional techniques. Low sample consumption and high speed of analysis are the most obvious merits of an ESI-MS-based method to study noncovalently bound complexes. ESI-MS measurements can be performed with less than micrograms of materials in a matter of minutes. Additionally, compounds do not require labeling or binding to a solid support, and the stoichiometry of interactions can be readily determined based on regular mass intervals. The nature of ESI as a "soft" ionization method can be tuned to preserve specific interactions in solution and transfer them into the gas phase as ions [42]. Furthermore, the ionic complex can be isolated in the gas phase to study its properties (e.g., using the kinetic method, collision-induced dissociation, or other gas phase ion manipulation methods).

A wide range of mass spectrometry-based methods have been established for determining binding affinities of host-guest complexes. Both solution phase measurements (where ESI-MS is used as a detector) and gas phase measurements (where the complex is isolated in the gas phase and dissociated by one of several available methods) have been

reported [43, 44]. Solution phase methods include MS-based titration techniques [45-49], melting curves measurements [50], competitive binding analyses [51], and the newly introduced dynamic titration (a more efficient variant of MS titration). Schug et al. have specifically focused on the development of high throughput dynamic titration techniques for studying small-molecule binding systems. As an example, they have reported the binding affinity of cinchona alkaloid chiral selectors with chiral N-blocked amino acids using ESI-MS and a high throughput band-broadening dispersion method [52]. All of these methods rely on monitoring the ion abundances of host, guest, and/or complex ions in the mass spectrum, and seek to correlate these ion abundances with the solution phase equilibrium concentrations of the species involved. Ion abundances of relevant species are then used in place of the equilibrium concentrations in the equilibrium expression to obtain either the association constant or the dissociation constant. It is important to note, however, that it can often be difficult to deconvolute influences from the ESI process on solution phase equilibria. Ideally, the instrumental parameters should be optimized so that the equilibria of interest are preserved and transferred into the gas phase undisturbed. In practice, the ability to accomplish this can be system dependent.

2.4 Correlation between gas-phase ion abundances and solution phase concentrations

It is pertinent to mention a few of the most important aspects that affect the response of ESI-MS for routine analyses. A key question that is often asked is whether the ion abundances, as observed in the mass spectrum, are indeed representative of the solution phase concentrations of the analytes. For the most part, and in the absence of competing ions in solution, experimental evidences point to the fact that there is reasonable correlation between electrospray mass spectral ion abundances and the solution phase concentration of analytes. Enke and Cech have extensively studied the importance of various solution-phase factors, such as solvent composition and analyte characteristics, on electrospray ionization response [53]. However, Kebarle and Reschke have recognized that gas phase reactions can also have a

significant effect on ESI response. For instance, the proton transfer reaction is a fundamental chemical reaction that has been investigated in both solution and gas phase [54]. Kebarle and Godbole were early pioneers in the study of gas-phase ion-molecule reactions under equilibrium using ESI-MS. They investigated ion-clustering reactions involving solvation of H_3O^+ and protonated methanol clusters and concluded that solvent with highest proton affinity is expected to suppress the response of all other solvents and analytes [55, 56].

One of the most important points to consider while using ESI-MS is the choice of solvent. Solvent properties such as polarity, viscosity, and dielectric constant, may evoke marked effects on the electrospray process and alter the charging as well as charge state distribution of analytes. Organic solvents (generally with low dielectric constant) have the capacity to hinder charge separation, since high rate of evaporation will shorten the period of time for which a low surface charge density is maintained on the droplet surface. Hence, this leads to a decrease in the proportion of low charge state ions that are able to get desorbed from the droplet surface (via ion evaporation) as solvent evaporates. In addition, droplets of smaller size are produced when spraying solvents that have less surface tension, thus leading to better sensitivity. As such, Zhou et al. investigated the molecular ion response characteristics for a set of structurally diverse natural products under different organic solvent composition in LC-MS analyses [57]. Highly volatile solvents will exhibit higher rates of evaporation and will allow the droplets to reduce in size faster during the electrospray process. This phenomenon results in higher ion abundance in the case of small ions where ion evaporation is considered the dominant ion formation mechanism [31]. This emphasizes the dependence of the nature of the analyte on the choice of organic solvent, such as methanol or acetonitrile, in enhancing or suppressing the signal.

Another factor may also occur when solutions containing multiple species are sprayed. Multiplicity of species in the sprayed solution often leads to competitive ionization during the electrospray process or ion suppression (in the case where certain ions have greater surface

activities than the ions of interest). Competition for ionization, as well as ion suppression, may be due to impurities in the solvent or electrolytes and buffer solutions introduced deliberately (or otherwise) from other processes such as chromatographic separations. Accordingly, Raffaelli and Bruins found that post-column addition of quaternary ammonium compounds could improve MS response [58]. Similarly, Mcluckey and Pan reported the affect of small cations (metal ions and quaternary ammonium cation) on the identity of observed protein ions. They observed an influence on the charge state distribution from the nature of the cationizing reagent, and found that these additives also had marked effects on the magnitude of ion signals [59].

Overall, compounds that have different proton affinities and surface activities in electrosprayed droplets will have different ionization efficiencies. Consequently, they can also have different ranges of linear response. Due to the importance of a consistent and reproducible linear range for making quantitative measurements in ESI-MS, and the different views apparent in the literature, we report here studies to extend the linear dynamic range by introducing ionization competitors as surface active co-analytes. This work comprises a concise, systematic study of the ESI-MS response of two representative analyte ions in the presence and absence of surface active agents that have a significant variation in the physicochemical properties. It is our hope that this study can add new insight into the mechanism of the ESI process, as well as provide a starting point for further investigation of concepts, which are fundamentally important to the use of ESI-MS for quantitative analyses.

CHAPTER 3

EXPERIMENTAL DETAILS

3.1 Introduction

The experiments defined in this thesis can be broadly categorized into two classes based on the aims and objectives they serve to achieve. The first category of experiments addresses the study of response factor determination for *tert*-butyl carbamoyl quinine (**1**) as a model analyte, in the presence and absence of cationic quaternary ammonium acetate (**A1**, **A2**) and nonionic poly glycols (**B1**, **B2**) surface active ionization competitors using ESI-MS.

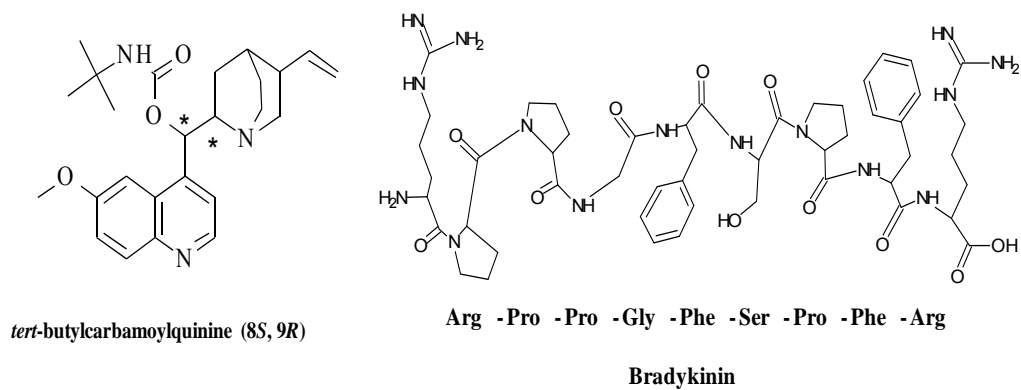
The experiments in this category include:

- Experiment 1: ESI-MS-based response factor determination for *tert*-butyl carbamoyl quinine in the presence and absence of cationic ionization competitors.
- Experiment 2: ESI-MS-based response factor determination for *tert*-butyl carbamoyl quinine in the presence and absence of nonionic ionization competitors.

The second category of the experiment pertains to the study of the effect of the ionization competitors on bradykinin (**2**) response during ESI-MS analysis. Part of this study includes examination of the partitioning between cationic ionization competitors and Bradykinin in the electrospray droplet and the influence of this process on suppressing the response of the peptide analyte.

The experiments in this category include:

- Experiment 3: ESI-MS-based response factor determination for bradykinin in the presence and absence of cationic ionization competitors.
- Experiment 4: ESI-MS-based response factor determination for bradykinin in the presence and absence of nonionic ionization competitors.



1 **2**
Figure 3.1: Structures of tBuCQN chiral selector **1**, BK peptide **2**.

Table 3.1: List of Ionization Competitors employed.

Ionization competitor	Structure	Class
TMAA ^{A1}		cationic
TBAA ^{A2}		cationic
PEG 400 ^{B1}		nonionic
PPG 2700 ^{B2}		nonionic

3.2 Instrumentation and Experimental Setup

All measurements were performed on an LCQ-DECA-XP-ESI- quadrupole ion trap mass spectrometer from Thermo Electron Corporation (West Palm Beach, FL). The mass spectrometer is interfaced to a Surveyor HPLC system with a built in autosampler and pump. This technique benefits from the capacity of automation in every step, allowing the preparation, mixing, and injection of samples using an HPLC auto-sampler that is directly interfaced to ESI-MS. The conventional ESI source was operated at a spray voltage of 5 kV in the positive ionization mode using a nitrogen sheath gas flow rate of 20 arbitrary units. Method optimization for voltages was carried out by direct injection of 10 μM of the analyte of interest (**1**, **2**) into the ESI source at a flow rate of 15 $\mu\text{L}/\text{min}$. The capillary temperature set to 200 $^{\circ}\text{C}$, capillary voltage was set to 30 V, and the tube lens offset voltage was set to 15 V.

Cationic (Tetramethyl ammonium acetate (TMAA), tetra butyl ammonium acetate (TBAA)) and nonionic (Polyethylene glycol (PEG 400), Polypropylene glycol (PPG 2700)) employed in this experiment were purchased from Sigma-Aldrich Inc. (St. Louis, MO)). Enantiomerically-pure **1** was synthesized and purified at the Institute for Analytical Chemistry and Food Chemistry at the University of Vienna (courtesy of Professor Wolfgang Lindner and Dr. Norbert Maier), and was used without further purification. Bradykinin **2** and ammonium acetate were purchased from Sigma-Aldrich Inc. All bulk solvents (water, methanol (MeOH)) used were LC-MS grade and supplied by J.T Baker (Phillipsburg, NJ).

For the response factor experiment, a set of different concentration (1-500 μM) of analytes **1** and **2** were prepared separately in a set of 1 mL sample solutions in 50/50 methanol/water and 500- μM ammonium acetate buffer along with a fixed concentration of the ionization competitor at each run. The samples were introduced by an autosampler using the above specified pump flow rate for each system, so that a plateau signal, lasting for approximately 2 minutes for each sample injection, was obtained. Each data point was measured in a triplicate to obtain mass spectra, each of which were represented by an average

of 50 scans. A convenient flushing method was incorporated between each triplicate in order to alleviate carry-over effects. Each complete measurement was repeated three times with fresh solutions. Calibration curves were constructed for each measurement in order to study the effects of the ionization competitor on the analyte response simply by applying a linear regression model, from the lowest concentration up to the “critical concentration”, defined as the upper limit of linearity for the calibration points tested.

3.3 Experiments: ESI-MS based Response Factor Determination

The aim of these experiments were to study the influence of quaternary ammonium acetates (tetramethyl ammonium acetate **A1**, tetrabutyl ammonium acetate **A2**) present at a fixed concentration (0, 10, 75 μM) on the sensitivity of two representative analytes. Cationic surfactants are highly responsive in ESI-MS. This feature of the cationic surfactants is due to the presence of a permanent positive charge, surrounded by hydrophobic side chains. On the contrary, the nonionic surfactants (PEG 400, PPG 2700; 0, 50, 200 μM) tend to be less responsive due to the presence of hydrophilic side chains. The analytes of interest have been chosen to represent small molecules and peptide molecules, which are responsive in the positive ionization mode of ESI-MS. *Tert*-butyl carbamoyl quinine, tBuCQN, is a chiral selector used for the construction of enantioselective HPLC media [60, 61]. It has been reported in several binding affinity studies to be highly responsive during electrospray process due to the presence of a positive charge on the protonated tertiary amine on the quinuclidine group [45, 52]. Bradykinin, a basic peptide, was investigated to provide a comparable study between species that possess different response. In this context, it will be an interesting study to evaluate the critical concentration with and without the presence of such competitors on the response of tBuCQN and bradykinin, as illustrated in figure 3.

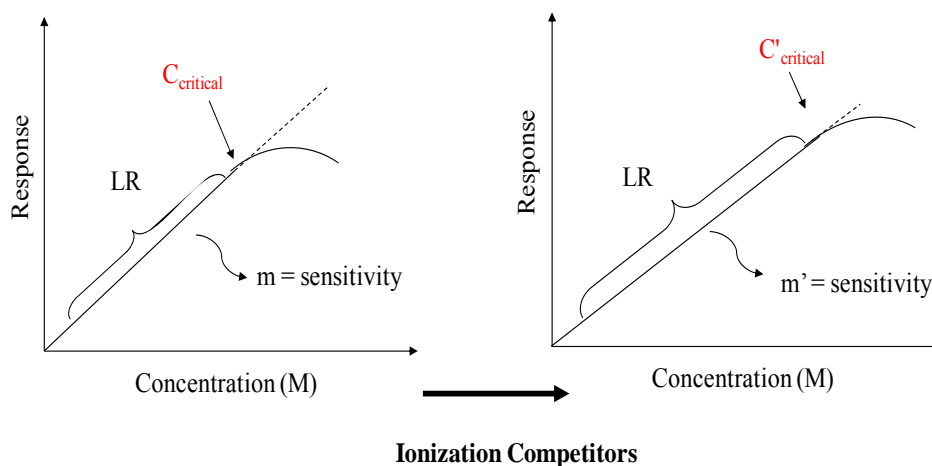


Figure 3.2: Schematic representation reveals the effect of ionization competitors in extending linear range. LR: linear range; $C_{critical}$: higher concentration within linear range; m : sensitivity corresponds to the slope of the best-fit straight line.

In the same experimental setup as described above, a family of nonionic ionization competitors' polyethylene glycol **B1** and polypropylene glycol **B2** were incorporated separately in the sample mixture. This case study introduces two different classes of surfactants, cationic and nonionic, distinctive in their activity on the surface of the charged droplet during electrospray process.

Data Collection:

The signal intensity of a species X is assumed to be directly proportional to its equilibrium concentration $[X]$ in solution:

$$I_x = F_x [X],$$

Where F_x is the response factor. When X exists primarily in a singular defined equilibrium state, the equilibrium concentration is the same as its total concentration C_x :

$$I_x = F_x C_x.$$

Each injection corresponds to a fixed concentration of ionization competitor along with a fixed analyte concentration. The analysis is repeated, holding the ionization competitor concentration constant and varying the analyte concentration. In essence, this analysis amounts to the

construction of calibration plots, incorporating 10 data points, in the presence and absence of each ionization competitor.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

In this work, ionization competitors were used to extend the linear range of ESI-MS. Two classes of surface-active co-analytes (cationic and nonionic) were studied to examine their effect on the response of conventional analytes in the positive ionization mode. As mentioned previously, the effectiveness of ESI-MS in quantitative analysis relies on achieving high sensitivity and, ideally, a wide linear dynamic range. The working curve associated with quantitative analysis has a certain limit of linearity that has been reported to be approximately 100 μM for most moderately responsive analytes [31-33]. However, at higher analyte concentration, a deviation from linearity will occur due to saturation on the surface of the charged droplets produced during the ESI process. A limited amount of “excess charge” exists on the surface of the droplet [62]; thus, a linear correlation between response and solution concentration ceases to exist as the excess charge is used up at high analyte concentration. The analyte ion signal saturates at a sample concentration defined as C_{critical} , as depicted in Figure 4.1. At sample concentrations above the defined critical concentration, the ion signal ceases to rise in direct proportion to analyte signal.

The presence of cationic or nonionic surfactants will have an impact on the ionization of the selected analyte. Surfactants are expected to modulate the surface tension of the charged droplets as they traverse the source region and release gas-phase ions. As such, two aspects are taken into consideration when proceeding with this study to identify beneficial effects imparted by the ionization competitor: a) Extension of linearity with minimal concentration of ionization competitor; and b) minimum sacrifice in analyte sensitivity. These observed effects have been recorded and compared. Further, an understanding of the manifestation of the

effects can be gained by considering the notion of excess charge, as it relates to the equilibrium-partitioning model, first described by Enke and coworkers [62, 29].

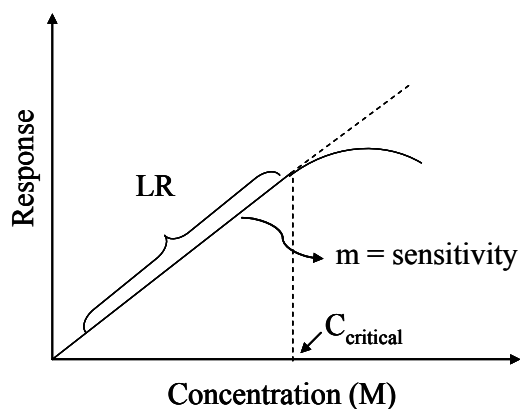


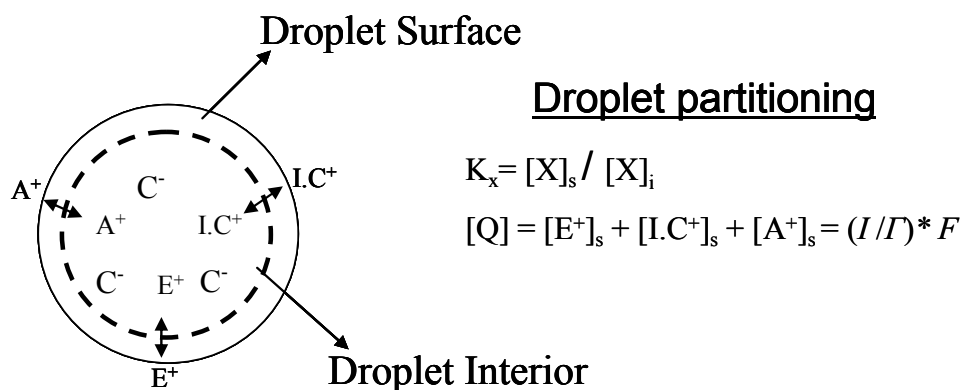
Figure 4.1 Schematic of analyte ion signal at the detector of the mass spectrometer as a function of analyte concentration in solution. 'LR' denotes linear range, 'm' denotes the slope of the linear portion of the curve (sensitivity), and ' $C_{critical}$ ' is the concentration at which the curve departs from a linear response.

4.2 Limited Amount of Excess Charge

Several theories have been proposed to explain why ESI response is limited at high concentrations. Kebarle and Tang originally suggested a limitation in ESI response at high concentrations to be a result of an upper limit in the amount of analyte that can be charged in the ESI process [29]. Enke expanded on this assumption, pointing out that, because a fixed amount of excess charge is available on ESI droplets, at the point at which the analyte concentration exceeds the excess charge concentration, its ESI response should level off [62]. However, only in situations where the charging of analyte is completely efficient will the upper limit in the calibration curve correspond exactly with the point at which the analyte concentration exceeds the concentration of excess charge. If all of the analyte is not being charged, then presumably the ESI response can increase even after the analytical concentration of analyte exceeds the concentration of excess charge. Nonetheless, if no other factors were involved,

then one would expect to see saturation in ESI response, due to charge limitation, to occur at some point after the analyte concentration becomes greater than the excess charge concentration.

4.3 Partitioning Model



A: Analyte; I.C: Ionization Competitor
 C: Counter Ion; E: Electrolyte; Q: sum of the surface concentrations; *I*: Circuit Current; *I'*: Flow Rate; *F*: Faraday Constant

Figure 4.2 Schematic representation of equilibrium partitioning model.

To better understand the process involving transfer of charged species from solution to gas phase during ESI, an equilibrium partitioning model (EPM) has been developed by Enke for simple charged analytes [62], and was later expanded by Brodbelt and coworkers for application to host-guest complexes [63, 64]. The model describes the droplet as having two different regions, as shown in Figure 4.2: An electro-neutral droplet interior and a charged droplet surface. Analyte species in the droplet compete for the limited number of charged sites at the droplet surface and partition between the phases depending on the nature of the species. The charged sites, or excess charge [Q], is defined as the sum of the concentrations of cationic species (in the positive ionization mode) that have migrated to the droplet surface $[X]_s$. Polar

and highly hydrophilic species tend to prefer the core of the droplet, whereas less polar species tend to migrate to the surface of the droplet. Ion response is recorded for those species released from the droplet surface into the gas phase. In a particular droplet, multiple species compete for surface sites on the droplet, and thus a competitive partitioning process controls the relative abundance of ions at the droplet surface, and consequently, in the gas phase. In this work, surface active co-analytes were shown to be amenable for improving the upper linear dynamic range of ESI-MS, even though, in many cases, they compete for droplet sites and decrease analyte ion sensitivity [63].

4.4 Ionization competitors versus *tert*-butyl carbamoyl quinine and Bradykinin

The main goal of this thesis was to study the effect of “ionization competitors,” as surface-active co-analytes, on the response of a cinchona-type analyte and bradykinin peptide. The novelty lies in comparing the effects of two classes of competitors (cationic and nonionic) on analyte ion response. Nonionic surfactants, being more hydrophilic and highly solvated, are expected to evaporate more slowly from the ESI droplet than the positively-charged quaternary ammonium acetates. A comparison of the effects of the ionization competitors is made based on their different physiochemical attributes and their concentration.

As shown in Figure 4.3, the ion abundances corresponding to the analytes **1** and **2** are easily distinguishable in the ESI-MS spectrum, making it possible to construct calibration curves by monitoring the intensity of the analytes at different concentration levels, in the presence and absence of the different ionization competitors. In ESI-MS, we can define the response factor, which relates the ion intensity of a species to its solution concentration as $F_x = i_x / [X]$, where $[X]$ is the equilibrium concentration of species X and i_x is its absolute ion intensity taken from the mass spectrum). The value of “i” can be obtained from the spectrum for each concentration point as the sum of the observed ion signals for that analyte.

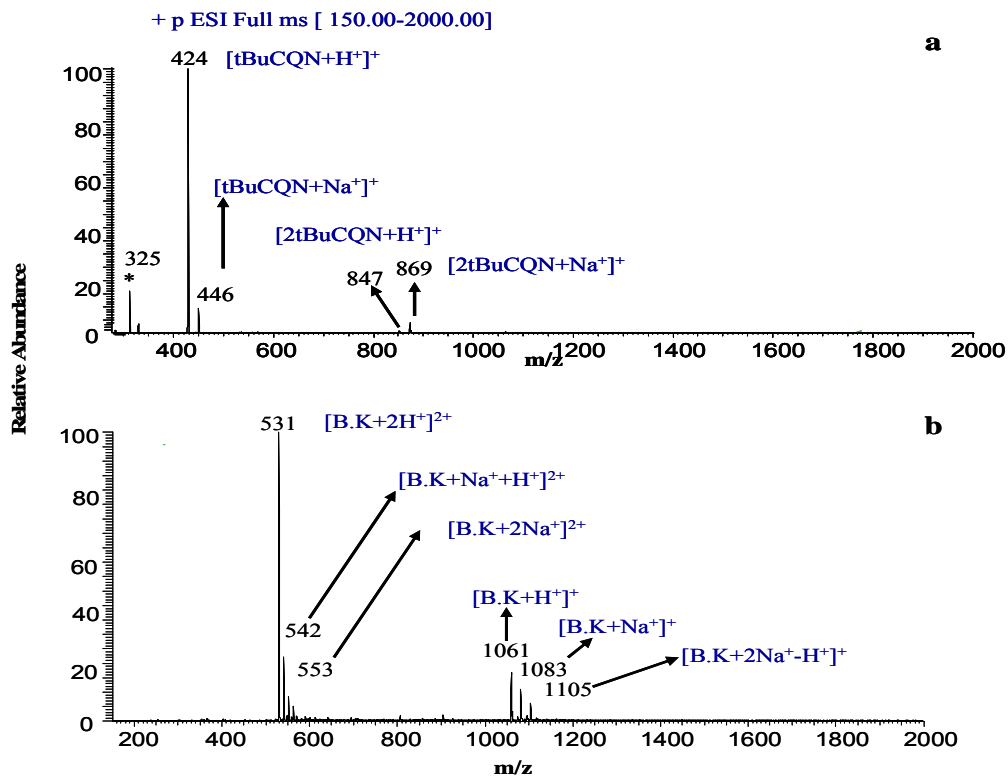


Figure 4.3 Representative full-scan mass spectra for the analytes (10 μM) used to examine the cationic and nonionic surface-active ionization competitors in extending LR of ESI-MS. a) tBuCQN, b) B.K.

In the case of single component system, made up of analyte 1 in 50:50 methanol water, it has been shown that the tBuCQN signal saturates at approximately $50\mu\text{M}$ with a correlation coefficient > 0.99 ; the calibration curve was linear over the concentration ranges of $1.0 - 50 \mu\text{M}$. The critical concentration shifts to $100 \mu\text{M}$ for bradykinin with a correlation coefficient >0.96 . Thus, the spray current keeps rising with increasing sample concentration until C_{critical} is reached, at which point, droplet saturation processes begin to be observed.

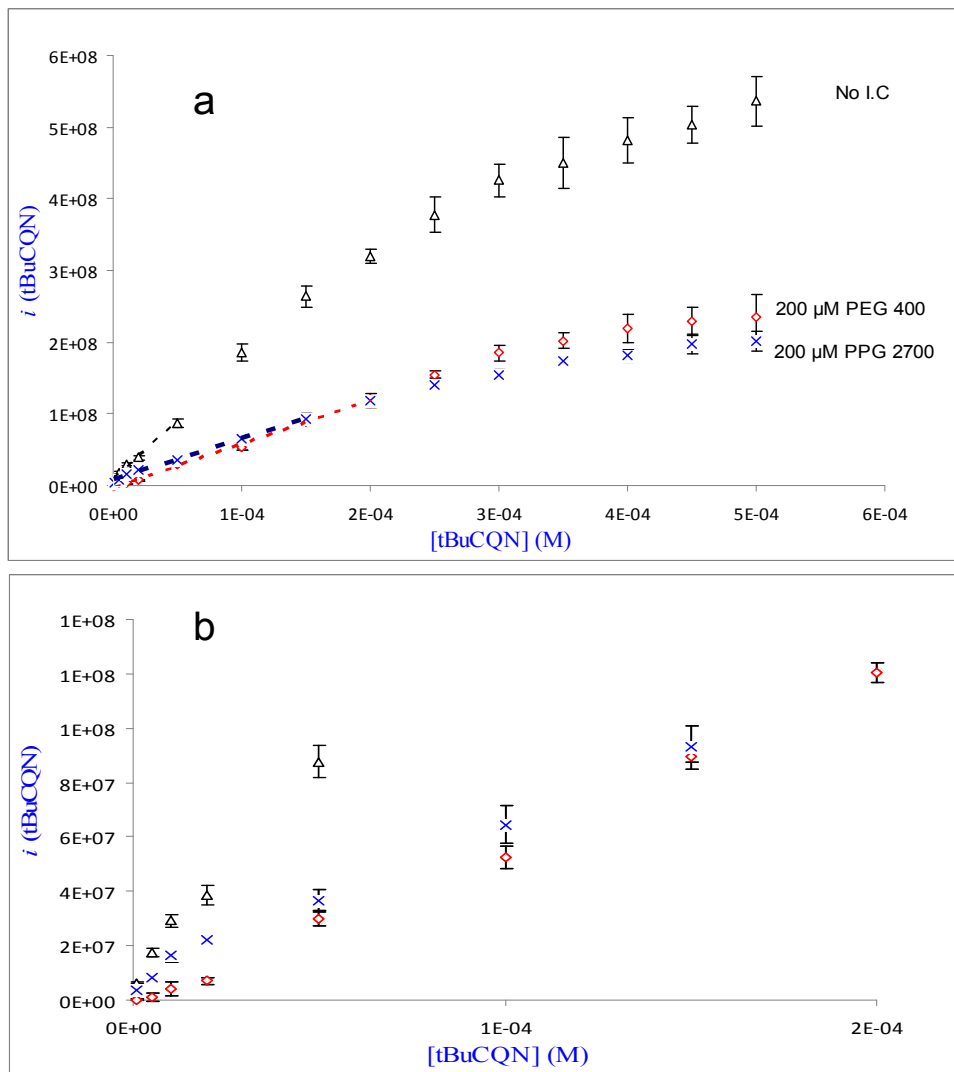


Figure 4.4 ESI -MS response for a single solution of tBuCQN and solution mixtures containing PEG 400 and PPG 2700. a) 1 μM - 500 μM ; b) 1 μM - C_{critical}

Figure 4.4 shows the response profile for tBuCQN in the presence and absence of poly glycol nonionic surfactants. The presence of poly glycols in solution at a fixed concentration of 200 μM increased the critical concentration in the calibration curve by a factor of 4 for PEG 400, and a factor of 3 for PPG 2700 with correlation coefficients >0.99 , in comparison to the critical concentration of analyte in a single component solution. In the absence of ionization competitor, and as analyte concentration was increased, the response curve began exhibiting

saturation behavior, since fewer ions could reach the droplet surface. As the ionization competitor was introduced, the sensitivity of the analyte signal decreased due to competitive partitioning effects. The nonionic surfactant is hydrophilic, and likely resides to a large degree in the bulk interior of the droplet. At these concentrations, some of the surfactant does migrate to the droplet surface to reduce ionization efficiency of the analyte. However, the most striking result is that the calibration curves showed higher critical concentration values, and were linear up to 200 μM for PEG 400 and 150 μM for PPG 2700.

Delineated in Table 4.1, the slopes of the linear regression lines indicate a sacrifice in sensitivity as the poly glycols compete for the surface of the droplet. A plausible explanation for extension of the linear range is that the presence of nonionic surfactants lowers the surface tension of the droplets, causing them to subdivide earlier. As such, the analyte is distributed among more droplets, which limits the onset of saturation and pushes the limit of linearity to higher concentrations. Unfortunately, the poly glycols also take up some of the sites on the droplet surface and reduce sensitivity. This is illustrated in Figure 4.5, which reflects the response of poly glycols competing on the surface of the droplet, which reduces the sensitivity of tBuCQN.

Table 4.1 Data obtained for tBuCQN (1 μM -500 μM) in 50/50 MeOH/H₂O solvent conditions. (a) R² and slope for points from 1 μM to C_{critical}. (b) R² for all data points from 1 μM to 500 μM .

Ionization Competitor	C _{crit} (μM)	Slope ^(a) \pm S.D *10 ¹²	R ^{2(a)}	R ^{2(b)}
No competitor	50 μM	1.59 \pm 0.09	0.990	0.958
50 μM PEG 400	100 μM	0.62 \pm 0.02	0.995	0.973
200 μM PEG 400	200 μM	0.60 \pm 0.02	0.996	0.997
50 μM PPG 2700	100 μM	0.55 \pm 0.02	0.992	0.974
200 μM PPG 2700	150 μM	0.57 \pm 0.02	0.993	0.970
10 μM TMAA	100 μM	1.40 \pm 0.08	0.993	0.950
75 μM TMAA	300 μM	0.74 \pm 0.01	0.999	0.992
10 μM TBAA	100 μM	14.62 \pm 0.04	0.996	0.925
75 μM TBAA	150 μM	11.12 \pm 0.02	0.998	0.980

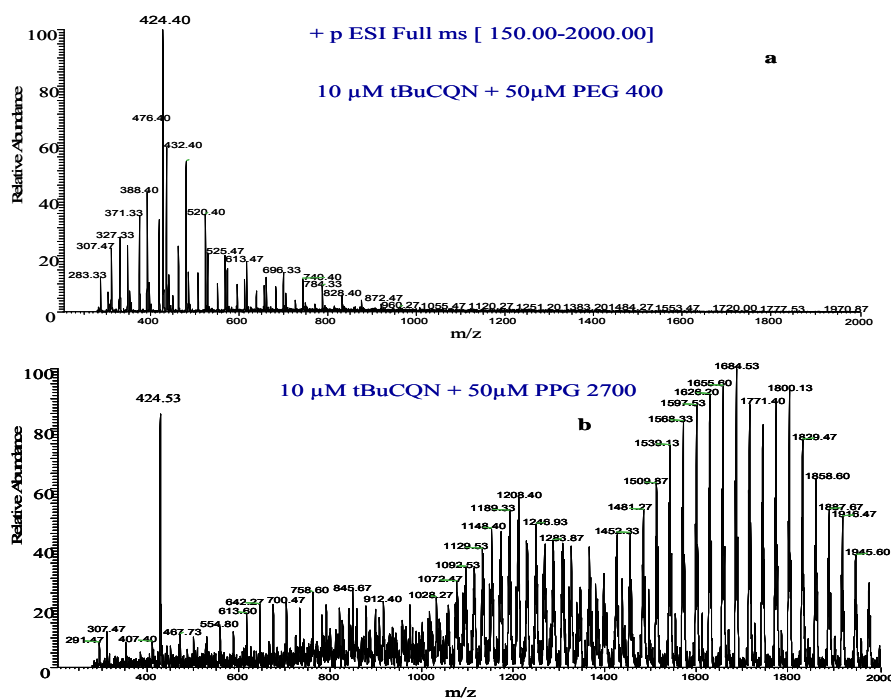


Figure 4.5 Representative full-scan mass spectra for tBuCQN (10 μM) with a) 50 μM PEG 400, b) 50 μM PPG 2700 revealing the response of nonionic surfactants. The protonated ion response of tBuCQN can still be observed at $m/z = 424$.

To evaluate the effects of the different classes of surfactants on the response of tBuCQN, calibration curves were generated in the presence of quaternary ammonium acetates. Curves showing the effects of varying the ionization competitor concentration from 0 to 10 to 75 μM reveal an extension of the upper limit of linearity by a factor of 3 for tetramethyl ammonium acetate (TMAA), and by a factor of 6 for tetrabutyl ammonium acetate (TBAA), relative to an absence of the competitor. These data are also shown in Table 4.1. Essentially, by varying the concentration of TMAA, signal intensity can be traded for a greater dynamic range, as illustrated in Figure 4.6. This would not be surprising, in light of the previous discussion for the poly glycols, except that in the presence of TBAA, the slope of the linear regression line showed a significant increase in sensitivity, compared to the absence of competitor. In contrast, TMAA reduced sensitivity in a manner similar to the poly glycol competitors. The difference in the length of the alkyl groups of the quaternary ammonium surfactants is expected to change their surface activities, as well as their solvation energies. Since the cationic tetraalkyl ammonium ions are inherently similar, the solvation energy is determined primarily by the degree of non-polarity of the alkyl groups. Tetraalkyl ammonium ions migrate to the droplet surface extremely efficiently, accounting for their extremely high signal response in ESI-MS. However, ions with shorter alkyl groups, such as Me_4N^+ , have more polar character, which allows them to be better solvated by methanol and water [63].

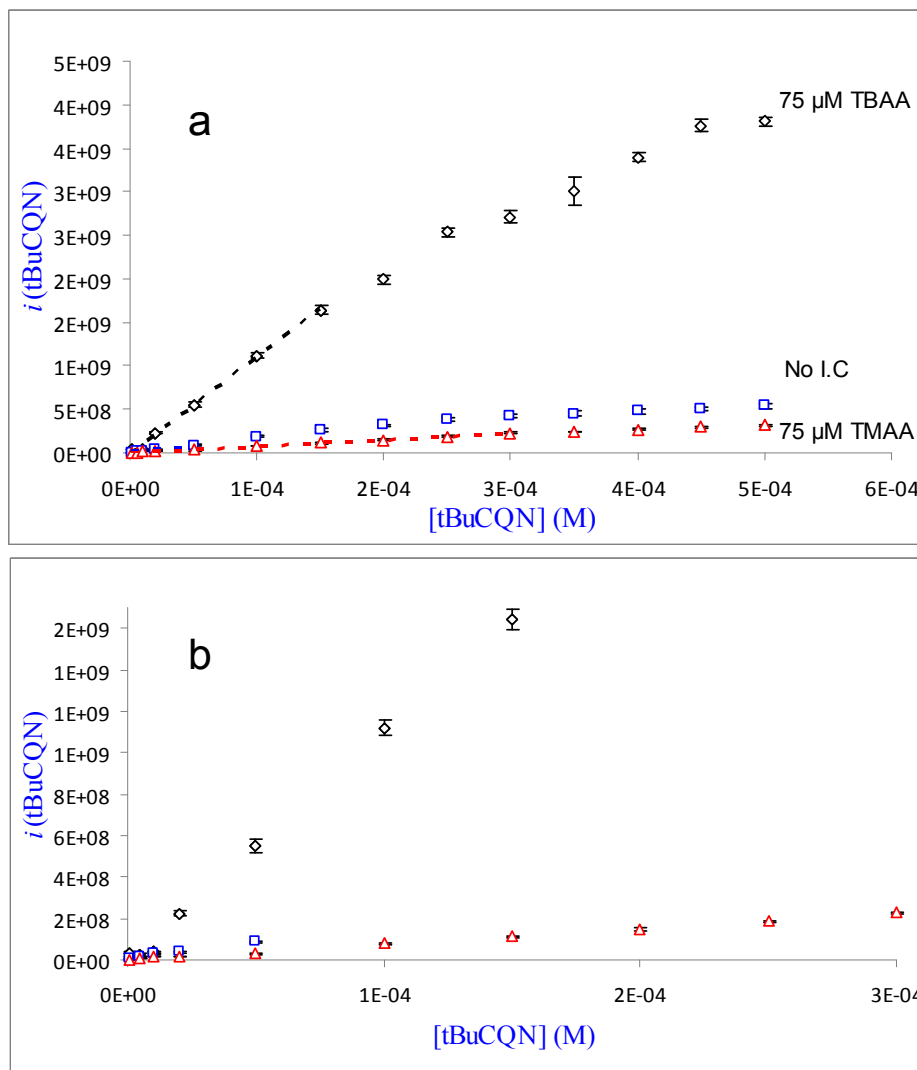


Figure 4.6 ESI -MS response for a single solution of tBuCQN and solution mixtures containing TMAA and TBAA. a) 1 μ M- 500 μ M; b) 1 μ M- $C_{critical}$

The increase in the linear range of tBuCQN in the presence of nonionic surfactants is associated with a decrease in sensitivity. Accordingly, the most solvophilic surfactants tend to suppress the analyte signal significantly. Quaternary ammonium acetates turn out to be the best competitors for extending the linear dynamic range without sacrifice in sensitivity. In the presence of TBAA, the sensitivity was enhanced by a factor of 5. The reasoning behind this effect is difficult to explain, however, it may be due to the capacity of the four hydrophobic butyl

groups to facilitate the movement of more tBuCQN molecules on to the surface of the droplets. This reasoning is partly made due to the remoteness of the nitrogen group on the protonated quinuclidine ring of tBuCQN from the t-butyl carbamate pendant group. Van der Waals forces between the tertiary butyl groups of tBuCQN with the butyl alkyl groups on TBAA can occur. As such, this may cause a higher partitioning for the analyte in the presence of TBAA by transporting more analytes ions to the surface and hence increase the analyte response. Coupled with the decreased surface tension, this “cooperativity” between TBAA and tBuCQN partitioning into the droplet leads to an increase in sensitivity. This reasoning could be experimentally tested by measuring the response of quinine in the presence of TBAA, as we assume that the response of quinine will not be enhanced due to the absence of tert-butyl group.

Studies of the effect of poly glycols and quaternary ammonium salts on the response of bradykinin were also performed. The presence of poly glycols in solution at a fixed concentration of 200 μM increases the critical concentration in the calibration curve by a factor of 2 for PEG 400, and a factor of 5 for PPG 2700 with correlation coefficients (R^2) > 0.99, compared to the critical concentration of analyte observed in the absence of ionization competitor (Figure 4.7). Beyond these points, signal saturation and deviation from linearity were clearly evident from the graphical examination of the data. The sensitivity seems to be sacrificed in the presence of poly glycols; this can again be ascribed to the poly glycols taking up some of the sites on the droplet and suppressing the analytes signal, thus decreasing its response (Table 4.2).

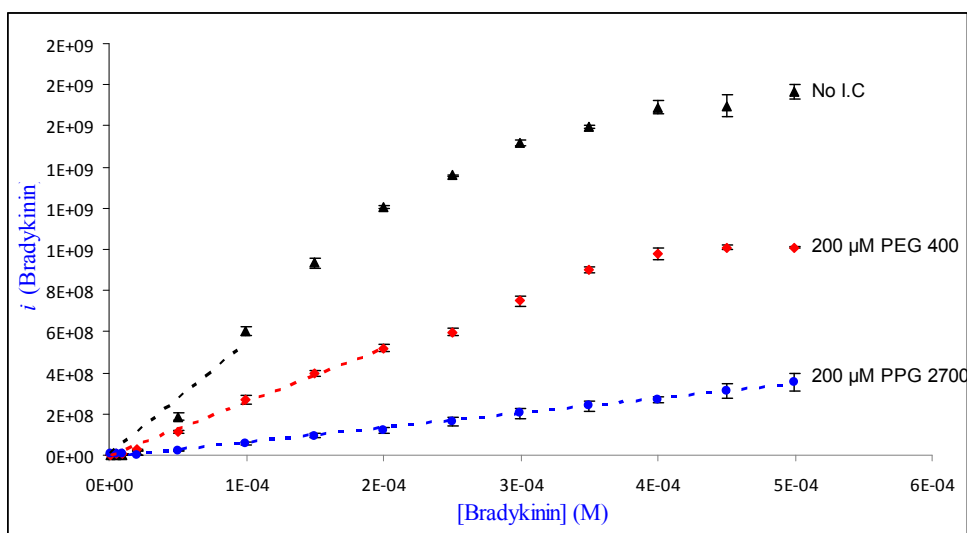


Figure 4.7 ESI -MS response for a single solution of Bradykinin (No I.C.: Ionization Competitor) and solution mixtures containing PEG 400 and PPG 2700.

Table 4.2 ESI-MS analysis of Bradykinin (1 μM -500 μM) in 50/50 MeOH/H₂O solvent conditions. (a) R² and slope for points from 1 μM to C_{critical}. (b) R² for all data points from 1 μM to 500 μM .

Ionization Competitor	C _{crit} (μM)	Slope ^(a) \pm S.D *10 ¹²	R ^{2(a)}	R ^{2(b)}
No competitor	100 μM	6.2 \pm 0.6	0.990	0.932
50 μM PEG 400	150 μM	5.13 \pm 0.02	0.998	0.957
200 μM PEG 400	200 μM	2.98 \pm 0.07	0.997	0.979
50 μM PPG 2700	200 μM	4.67 \pm 0.03	0.989	0.924
200 μM PPG 2700	500 μM	0.69 \pm 0.02	0.997	0.997
10 μM TMAA	200 μM	6.01 \pm 0.02	0.990	0.922
75 μM TMAA	250 μM	5.11 \pm 0.02	0.997	0.950
10 μM TBAA	50 μM	1.21 \pm 0.03	0.955	0.121
75 μM TBAA	100 μM	2.31 \pm 0.06	0.970	0.811

Interestingly, the presence of Me₄N⁺ does not significantly suppress the analyte signal of Bradykinin. Additionally, an increase in critical concentration by a factor \sim 3 was observed, relative to the limit recorded in the absence of the competitor (Table 4.2). However, the

presence of TBAA seems to initially increase and then drastically decrease the response of bradykinin as the concentration of the latter exceeds 100 μM , as shown in Figure 4.8. These observations are slightly different from what has been observed for *tert*-butyl carbamoyl quinine.

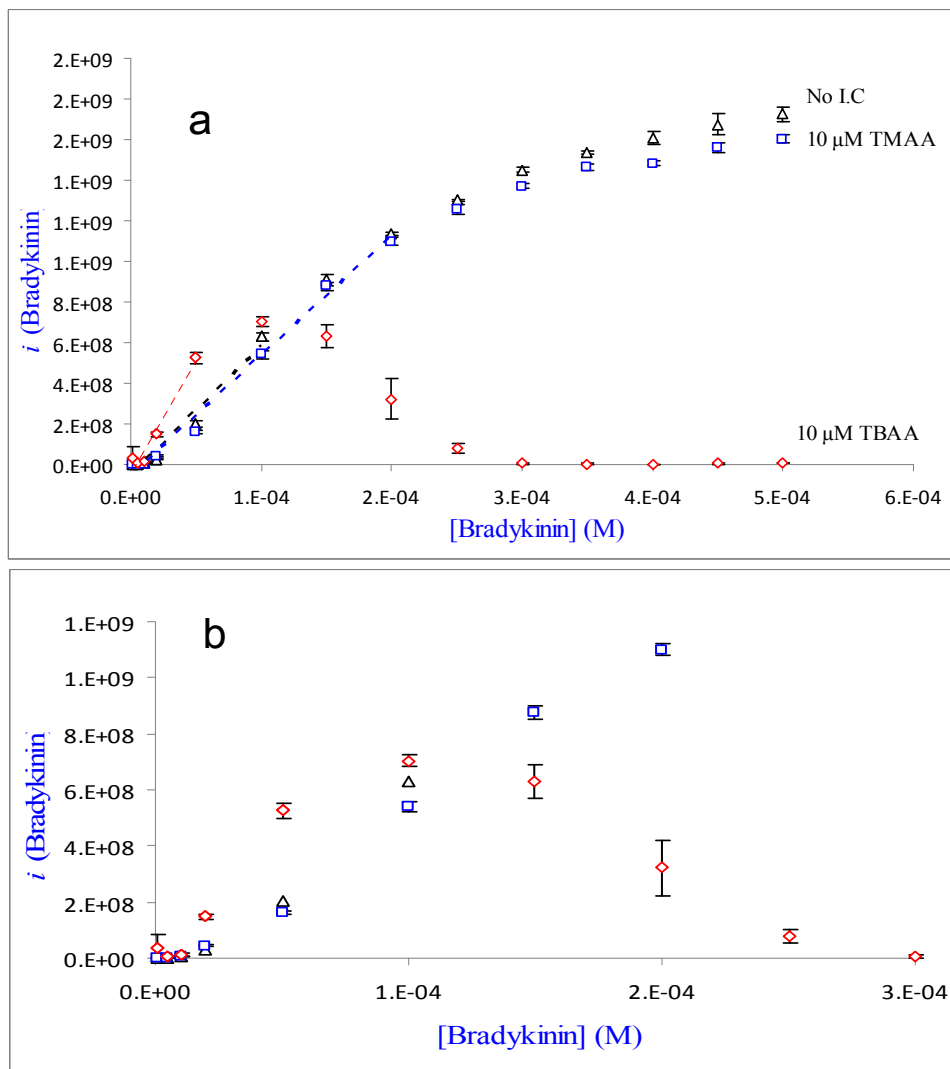


Figure 4.8 ESI -MS response for a single solution of Bradykinin (No I.C: Ionization Competitor) and solution mixtures containing TMAA and TBAA. a) 1 μM - 500 μM ; b) 1 μM - C_{critical}

There can be two speculations drawn out of these results. First, bradykinin is more responsive than tBuCQN, and hence migrates toward the surface of the droplet more efficiently.

In this context, TMAA with smaller less hydrophobic side chains seems to partition with the singly and doubly protonated guanidine moieties of the arginine amino acid residues present at the side chains of bradykinin peptide, minimizing the loss of sensitivity to a certain extent. Again, here we ascribe the maintenance of bradykinin sensitivity in the presence of TMAA to be due to a cooperative partitioning process. However, TBAA with larger more hydrophobic side chains seems to compete with bradykinin in getting to the surface of the droplet thus suppressing the response of bradykinin at higher analyte concentrations. Importantly, doubly-charged bradykinin overrides the signal of singly-charged by 2 orders of magnitude at low concentrations (Figure 4.3-b). As the analyte concentration is increased, more of the singly-charged ion is observed. This can be expected. In general, as the analyte concentration is increased there are fewer protons available in the droplet to enable to formation of the doubly-charged ion. The presence of TMAA does not seem to suppress the ratio of doubly to singly charged intensities and partition with bradykinin thus allowing enough analyte ions to reach the gas phase without loss in sensitivity (Figure 4.9). In fact, initially, the ratio of doubly-to-singly-charged ions increases initially with TMAA present. Moreover, in the presence of TBAA, the ratio of doubly to singly charged intensities reaches unity and the signal of the analyte is suppressed. This can be explained because at the surface of the droplet, the TBAA are effectively out-competing the singly and doubly charged species that are trying to migrate towards the surface. In addition, the decrease in surface tension is not aiding the escape of the analyte into the gas phase, but instead is suppressing its signal. The same behavior is seen with poly glycols through which a suppression of the analyte signal in the presence of PPG 2700 due to the fact that as ratio of doubly to singly charged intensities of bradykinin is decreased. Consequently, the singly and doubly charged ions are competing with each near the surface droplet, where TBAA is residing on the surface area that is being reduced due to solvent evaporation.

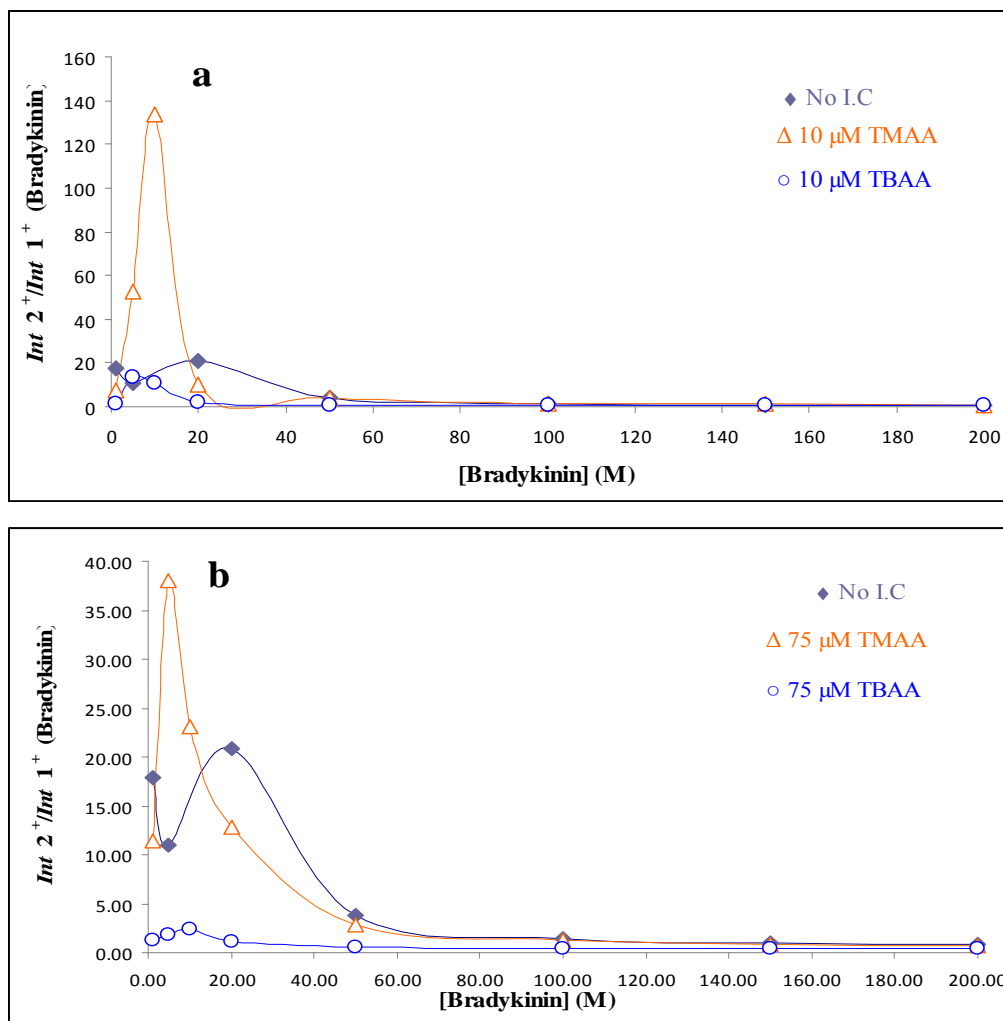


Figure 4.9 Plot of the ratio of doubly charged to singly charged bradykinin intensities as a function of the analyte concentration and the presence of ionization competitor ((a) cationic 10 μM; (b) cationic 75 μM).

Quaternary ammonium acetates turn out to be the best competitors for extending the linear dynamic range without a large sacrifice in sensitivity. Moreover, TBAA extends the LR and enhances the sensitivity of singly charged analyte (tBuCQN) with the increased hydrophobic side chains of the quaternary salt. It is plausible explanation that the reason behind the increase in sensitivity is due to higher partitioning for the analyte in the presence of TBAA by transporting more analytes ions to the surface and hence increases the analyte response. On the contrary, the same quaternary salt (TBAA) seems to suppress the bradykinin signal due to;

a) its nature of residing on the surface of the droplet, and b) effectively out-competing the singly and doubly charged bradykinin species that are trying to migrate towards the surface of the droplet. Poly glycols enhance the linear range with a minimal sacrifice in sensitivity.

CHAPTER 5

CONCLUSION

Electrospray ionization mass spectrometry (ESI-MS) is a widely used analytical technique in modern chemical and biological research due to its high sensitivity and broad applicability. ESI-MS has greatly simplified the application of quantitative HPLC and CE methods for analysis of highly polar molecules in the pharmaceutical industry. Conventional ESI-MS is not typically characterized by an extensive linear range for quantitative analysis. This can be a significant disadvantage. The work presented in this thesis reports this use of “ionization competitors” to extend the linear dynamic range of ESI-MS. With further development, such an approach could be useful for enhancing quantitative analysis in biological systems. Here, it is assumed that the mass spectrum peak intensities represent the solution phase equilibrium concentration of appropriate species. Speculations are made about the usefulness of cationic surfactants (TMAA and TBAA) for extending the linear dynamic range as the former differs from the latter in affecting the sensitivity of tBuCQN and bradykinin when present in electrosprayed solution mixtures.

The emphasis of this work was to explore the fundamental relationship between cationic/ nonionic surfactants and conventional analytes in extending the upper limit of linearity with minimal concentration of surfactants used and minimal sacrifice in sensitivity. Hereby, surfactants with the highest solvophilic characteristics such as PEG 400 and PPG 2700 were shown to extend the linear range, but also suppress the analyte response significantly. This, in part, is believed to be related to the nature of surfactants which reduce the surface tension of the droplets, causing them to subdivide earlier. However, nonionic surfactants seem to take up some of the sites on the droplet surface and reduce sensitivity.

On the contrary, surfactants with low solvophilic characteristics and higher response factor such as quaternary ammonium acetate salts tend to enhance the analyte signals more rapidly in comparison with nonionic surfactants. For example, TBAA tends to enhance the sensitivity of tBuCQN when incorporated in the solution mixtures. Moreover, TMAA does not suppress the response of bradykinin. This is conceivable in accordance to the presence of highly hydrophobic side chains on TBAA. Hence, the increase in sensitivity is due to higher partitioning for the analyte in the presence of TBAA by transporting more analyte ions to the surface and hence increases the analyte response. In conclusion, TBAA show promising results in extending the limit of linearity at low concentrations, and even provides an enhancement in sensitivity for some analyte species. More work is needed to understand the true generality of these effects.

Important quantitative information was obtained about the ability of ionization competitors in extending the linear dynamic range of ESI-MS using a discrete measurements method. The main advantage of ionization competitors is their potential in reducing the surface tension of droplets thus allowing more distribution of analytes among more droplets prior to reaching Rayleigh's limit. Future studies will be conducted to further examine the reliability of ionization competitors in extending the limit of linearity without sacrifice in sensitivity. First, we could apply a new dynamic response factor determination method developed by Schug et. al to collect data on a wider range of analytes and competitors more quickly [52]. Second, the use of ionization competitors can be used to study weak binding systems. Extending the linear range allows the use of higher guest concentrations, to reach a higher degree of complex formation. However, an important aspect that has to be closely monitored is the interference of the IC in the binding equilibria.

Application of this dynamic method can be studied further to assess the different parameters of response factor for different analytes in the presence of ionization competitors [20]. Results of dynamic method can be compared with the current results obtained by discrete

measurements method. Subsequently, a simulation can be performed to model the temporal compositional gradient produced by flow injection and to assess the effects of linearity and ionization response on the shape of the distribution. The presence of surface-active modifiers reveals a promising result in expanding the upper linear dynamic range by a significant amount with minimal sacrifice in sensitivity. To demonstrate the utility of this approach, a series of weak affinity binding systems could also be studied (e.g. tBuCQN binding amino acid enantiomers) where a significant degree of association requires that higher concentrations of interactants be evaluated in a linear response regime.

APPENDIX A
CHAPTER 3 CITATION

Manuscript in preparation

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BIOGRAPHICAL INFORMATION

Bilal Hani Bazzi was born in Beirut, Lebanon. He attended *La Source*' School during his elementary studies. He graduated from Beirut Community School (BCS) in Haddath, where he earned his Senior School Diploma as an honor student. He traveled to United States and attended Richland College, Dallas, TX, where he obtained an Associate Degree in Science in one of the most outstanding Dallas County Community College Districts. Following his first degree, he joined University of Texas at Arlington in Spring semester of 2006 and achieved his Bachelor of Science in Chemistry. While in this program at UTA, he worked for 6 months as a teaching assistant in the Chemistry Department. In addition, he worked as a research assistant, conducted research under the supervision of Dr Kevin Schug, and received the American Chemical Society / Department of Analytical Chemistry Award for outstanding research as an undergraduate student as well as the Chemistry Department Award at UTA for outstanding research. During his graduate studies, he worked under the supervision of Dr Kevin Schug on investigating the effects of Ionization Competitors in extending the linear dynamic range of ESI-MS. He later went on to FRITZ Industries, Inc. and worked as an Analytical Chemist.