SEPARATION, DETECTION, AND QUANTITATION OF UNDERIVATIZED CARBOHYDRATES USING LIQUID CHROMATOGRAPHY-ELECTROSPRAY IONIZATION-TRIPLE QUADRUPOLE-TANDEM MASS SPECTROMETRY

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Abstract

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Carbohydrate analysis has many important implications in health, consumer products, and industrial processes. Among many possible analytical techniques for their analysis, mass spectrometry (MS) is an attractive approach, offering high sensitivity and specificity. In particular, electrospray ionization (ESI)-MS affords several advantages over other ionization techniques: the ability for direct coupling with upstream chemical separation techniques, its suitability for quantitation, and its compatibility with relatively inexpensive quadrupole mass analyzers for the analysis of high molecular weight compounds. However, the sensitivity of ESI-MS towards carbohydrates tends to be much less than for other classes of compounds, such as peptides. This is due to their lack of easily protonable or deprotonable chemical groups as well as their high hydrophilicities.

Increasing an analyte's sensitivity in ESI-MS can be achieved by optimizing detection strategies and solvent parameters. Flow injection analysis-ESI-tandem MS (MS/MS) was used to investigate the influence of solvent parameters on the signal intensity of glucose. Solvent parameters explored included the organic solvent and its ratio to water, the additive and its concentration, and solution pH. The use of ammonium

trifluoroacetate in 80:20 methanol:water in the positive ionization mode resulted in the highest signal intensities. It was also found that acetonitrile suppressed ionization in the positive ionization mode.

The effects of solvent parameters on the relationship between response factors and concentrations was also studied for glucose, sucrose, and raffinose using a continuous stirred tank reactor coupled to ESI-MS/MS. This technique is able to generate a continuum of response factors across a wide concentration range from a single injection. The profiles and magnitudes of the response factor vs. concentration profiles varied widely, and were dependent upon the ionic species monitored and the solvent parameters used. Monitoring acetate, chloride, and formate adducts resulted in the best linearities, useful for quantitative analysis.

Lastly, liquid chromatography-ESI-MS/MS was used to determine the concentrations of fructose, glucose, and sucrose in hard cider and apple juice samples. The method was validated with respect to selectivity, linearity, the limit of detection, the limit of quantitation, accuracy, and precision. Notably, a correlation was found between the fructose and glucose concentrations in hard cider samples that did not contain detectable amounts of sucrose ($n=9$; $R^2=0.98$).

Table of Contents

List of Illustrations

List of Tables

Chapter 1

Introduction to Dissertation

Saccharides are Earth's most abundant biomolecules and include carbohydrates and their derivatives. Their biological functions include energy storage, structural support, and biological recognition. Aside from supplying calories, carbohydrates also influence the taste of foods and beverages. Carbohydrates also play important roles in brewing and papermaking and are used industrially as adhesives, binders, and thickening agents.

The analysis of carbohydrates has many important implications in health, the quality of consumer products, and industrial processes. Among many possible approaches for their analysis, mass spectrometric (MS) techniques are particularly attractive due to their potentials for high sensitivity and selectivity. The use of electrospray ionization (ESI) for the MS analysis of carbohydrates offers several advantages over other ionization techniques, including the ability for direct coupling with upstream chemical separation techniques, the ease of quantitation, and the ability to use relatively inexpensive quadrupole mass analyzers for the analysis of high molecular weight analytes. Tandem MS (MS/MS) can further improve selectivity, reduce chemical noise, and provide additional structural information. Chapter 2 provides background information on the development of MS leading up to the introduction of ESI and the theory and practice of ESI as well as that of quadrupole and triple quadrupole mass analyzers.

There are a number of challenges that must be addressed when developing ESI-MS methods for carbohydrate analysis. Due to their high hydrophilicities, ESI-MS tends to be less sensitive towards carbohydrates than it does towards other classes of compounds, such as peptides. Additionally, carbohydrates lack easily protonable or deprotonable chemical groups, so the choice of the detection strategy is crucial. Chapter

3 reviews the literature on the ESI-MS analysis of carbohydrates, including detection strategies, derivatization procedures, the influence of solvent parameters on sensitivity, and MS/MS fragmentation.

As previously mentioned, the relatively low sensitivity of carbohydrates towards ESI-MS is an important issue. Derivatization can address this challenge, but it is often accompanied with increased labor, lower throughput, and a more complex sample. Optimizing solvent parameters for achieving high signal intensities is a more attractive alternative. The influence of solvent parameters, including additives and their concentrations, solution pH, and the organic co-solvent and its ratio to water on the ESI-MS/MS signal intensity of glucose, a model carbohydrate analyte, in both the positive and negative ionization modes was investigated using flow injection analysis. The highest signal intensities were achieved using 80:20 methanol:water solutions containing ammonium trifluoroacetate in the positive ionization mode, monitoring an ammonium-glucose adduct selected reaction monitoring (SRM) transition. Acetonitrile was found to suppress the ionization of glucose in the positive ionization mode through competition with glucose for cation adduction. Method details and results for this study are included in Chapter 4.

Response factors for ESI-MS are often concentration-dependent, and relatively constant response factors across a wide concentration range is highly desirable for quantitative analysis. A continuous stirred tank reactor (CSTR), which dilutes its contents exponentially over time, was coupled with ESI-MS/MS to yield continua of response factors across wide concentration ranges for SRM transitions of glucose, sucrose, and raffinose under a variety of solvent parameters in both the positive and negative ionization modes. The profiles and magnitudes of the response factor *vs.* concentration plots varied widely, and were dependent upon the analyte, the monitored ionic species,

and solvent parameters. Notably, acetate-, chloride-, and formate-carbohydrate adducts in 80:20 acetonitrile:water resulted in the most constant response factors across wide concentration ranges. Chapter 5 covers the details and results of this study.

Carbohydrates not only provide nutrients and the substrate for fermentation for yeast during brewing, but also influence the taste and other qualities of alcoholic beverages. Carbohydrates were analyzed in hard cider and apple juice samples using hydrophilic interaction liquid chromatography-ESI-MS/MS. Fructose, glucose, and sucrose were quantified using this method. The method was validated with respect to selectivity, linearity, the limit of detection, the limit of quantitation, accuracy, and precision. Chapter 6 provides details on the background, methodology, and results of this study.

There are many more opportunities for carbohydrate and saccharide analysis by ESI-MS as well as for the extension of the methodologies developed in this dissertation to more applications and analyte sets. Chapter 7 provides a summary for this dissertation and discusses details of possible related future work.

Chapter 2

Background on ESI-QqQ-MS/MS

2.1 A Brief History of Mass Spectrometry

The potential for the practical application of mass spectrometry (MS) for chemical analysis was recognized in its infancy by its inventor, J. J. Thomson. His vision did eventually come to fruition, but unfortunately, he did not live long enough to witness this himself. The components of Thomson's invention had all of the necessary functionalities that all mass spectrometers require: a glow discharge tube as an ionization source, charged plates and a magnet that separated ions based on their mass-to-charge ratios (m/z) as a mass analyzer, and a phosphorescent surface or a photographic plate as a detector.[1] Notably, this instrument was used in the discovery of the isotopic composition of neon.[1] A device of a similar design was used by Thomson in his research on the nature of cathode rays for which he was credited with the discovery of the electron and was awarded the 1906 Nobel Prize in Physics for this work.[2] Since its inception, advances in instrumentation and operation have improved the sensitivity and resolving power while extending the applicability of MS beyond permanent gases to inorganic salts, metals, volatile organic compounds, and large biomolecules. Discussed below is a brief historical perspective highlighting these advances as they pertain to the expansion of the sphere of influence of MS into organic compounds and biomolecules, with a focus on ionization techniques leading up to the development of electrospray ionization (ESI).

During the 1910s through the 1930s, investigators began using their own built in-house mass spectrometers to study the isotopic composition of various elements and ionization processes. In 1918, Arthur J. Dempster reported the first use of electron ionization (EI) in mass spectrometry, in which he analyzed solid samples.[3] Following the

first report of the application of EI-MS to gases,[4] ionization processes of volatile organic compounds were studied using this technique.[5–7]

The first industrial application of MS was for petroleum analysis, with the first preliminary report on its potential published in 1941.[8] The demand from this immense market led to the introduction of the first commercially available mass spectrometers later that decade. This was, in turn, followed by an explosion in the use and application of MS. The development of gas chromatography (GC)-MS, beginning in 1959, tremendously improved analytical capabilities for complex volatile samples.[9]

EI was extraordinarily useful for ionizing volatile compounds and generating structurally diagnostic fragment ions, but the commonly absent molecular ions from its resulting mass spectra was a serious drawback for qualitative analysis. Field ionization, first reported in 1954, was the first “soft” ionization technique, which generated high abundances of molecular ions from volatile samples.[10] Chemical ionization (CI), introduced in 1966, was another soft ionization technique that also allowed for the selective ionization of components of a gaseous sample through the judicious selection of the reagent gas.[11]

The ionization techniques mentioned thus far had only limited success in their applications to nonvolatile compounds. Derivatizing chemical groups to increase the volatility of otherwise nonvolatile compounds allowed for their analysis by MS using gas-phase ionization techniques,[12,13] though this was still limited to relatively low molecular weight compounds (~<600 Da). Pyrolysis has also been used to generate volatile compounds derived from nonvolatile substrates, though at the cost of a more complex analysis and a loss of molecular weight information.[14,15] Alternatively, nonvolatile samples can be directly inserted into a modified CI or EI source, but the presence of molecular ions from resulting mass spectra are usually only transient, as the timescale for pyrolysis

to take place is approached.[16] The detection of the molecular ion of echinomycin (1100 Da) in 1975 using EI with a direct insertion probe was a remarkable achievement at the time.[17]

Field desorption ionization, first reported in 1969, was one of the first soft ionization techniques developed for nonvolatile samples.[18] The introduction of other soft desorption ionization techniques for the analysis of nonvolatile organic compounds rapidly followed: plasma desorption (1974)[19]; laser desorption (1978)[20]; secondary ion MS (1978)[21]; fast atom bombardment (FAB; 1981)[22]; matrix-assisted laser desorption/ ionization (MALDI; 1985)[23,24]. MALDI, capable of generating intact ions from molecules with masses in the MDa range, has since nearly completely supplanted all other desorption ionization techniques mentioned above for biological MS.

The maturation of GC-MS demonstrated the tremendous analytical potential of the coupling of online chemical separation and MS and inspired efforts to develop an analog for liquid chromatography (LC). Generating a continuous and stable stream of desolvated ions and maintaining low pressures in the mass analyzer were particularly challenging for liquid samples. The development of atmospheric pressure chemical ionization for liquid samples by corona discharge began in the 1970s,[25] and this technique is still in use today for nonpolar analytes in liquid streams. Other ionization techniques for continuously flowing liquid samples were developed in the 1980s, including thermospray (1983)[26], ESI (1984)[27,28], and continuous flow-FAB (1986)[29]. ESI has since then utterly dominated other ionization techniques for applications involving nonvolatile charged, ionizable, and polar analytes in liquids.

Today, ESI and MALDI are the ionization techniques of choice for biological MS, each with advantages and disadvantages over the other. Samples can be prepared for and analyzed by MALDI-MS relatively quickly, but quantitation can be difficult to achieve.

MALDI-MS tends to generate mostly singly-charged ions, simplifying mass spectral interpretation and making it more appropriate for the analysis of complex samples without prior chemical separation. ESI-MS is generally more sensitive and better for quantitation than MALDI-MS and can be directly coupled with upstream chemical separation techniques, including LC and capillary electrophoresis. Molecules with multiple ionizable chemical groups tend to be multiply-charged when analyzed by ESI-MS, and their ion current may be split into an "envelope" of multiple charge states. This obviously can complicate mass spectral interpretation and reduce sensitivity relative to the hypothetical situation where all of an analyte's ion current is aggregated into one m/z channel, but this can be advantageous in allowing the use of relatively inexpensive quadrupole mass analyzers for detecting and accurately determining the molecular masses of high molecular weight biomolecules. These competitive techniques have led to numerous breakthroughs in the biological sciences, clinical diagnostics, environmental analysis, and many other important fields. In recognition of their important contributions to biological analysis, part of the 2002 Nobel Prize in Chemistry was awarded to John B. Fenn and Koichi Tanaka for the development of ESI and MALDI, respectively.

2.2 Electrospray Ionization

2.2.1 Historical Development of Electrospray Ionization

The process of electrospray was known long before its application for MS and had been used industrially to apply an even coating of paint onto the exterior of automobiles. The idea of utilizing electrospray as an ionization source for MS was conceived by Malcolm Dole in late 1960s. In 1968, Dole *et al.* published the first ESI-MS paper, though some of the results were almost certainly misinterpreted due to misconceptions and operational flaws.[30] The potential of ESI-MS, along with the generation of its first actual mass spectra, was demonstrated independently by John B.

Fenn of Yale University and M. L. Aleksandrov of the University of Leningrad in 1984.[27,28] ESI-MS has since then matured into an indispensable instrument for biological MS.

2.2.2 ESI Instrumentation and Operation

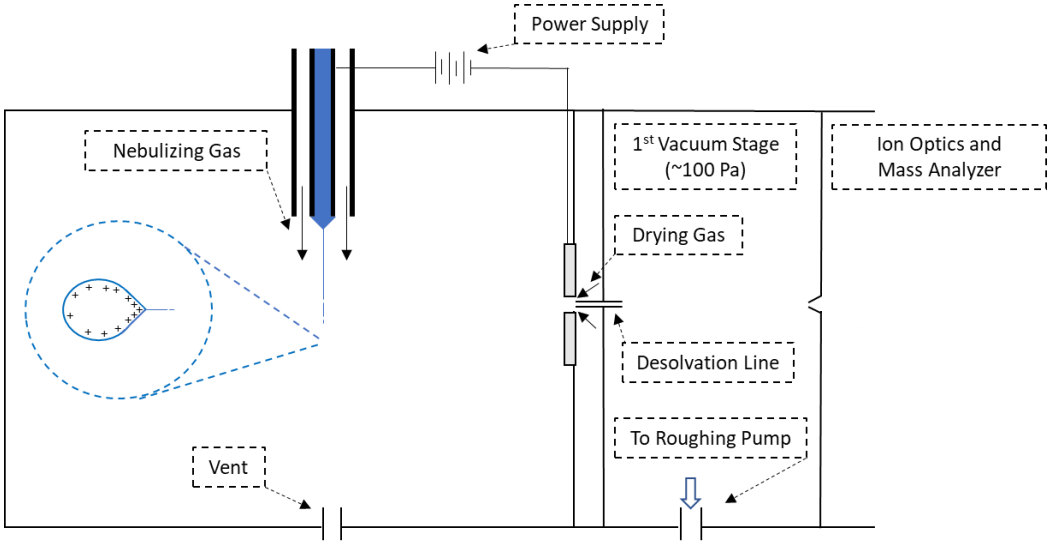

Figure 2-1. Diagram of a typical electrospray ionization source and a cartoon of Coloumbic fission

A diagram of a typical ESI source is displayed in Figure 2-1. A liquid sample is either loaded into a metal needle or forced to flow through it. A high voltage power supply is connected to the needle and the orifice separating the atmospheric pressure region and the first vacuum stage, causing oxidation to occur in the solution within the needle (in the positive ionization mode) and positive ions to migrate to the meniscus on the tip of the needle by electrophoresis.[31,32] Electrostatic repulsion from the buildup of charge distorts the meniscus into a “Taylor cone” which ejects a stream of solution that breaks up into small charged droplets.[32] Droplet formation is often aided by nebulizing gas (usually nitrogen) flowing from a tube concentric to the spray needle.[33,34] The droplets shrink in

the bath gas of hot nitrogen contained in the atmospheric pressure region as solvent molecules evaporate, increasing their surface charge densities. When the repulsive electrostatic forces from the excess charge exceed the surface tension of the droplet, the droplet becomes unstable, and smaller highly-charged second generation droplets are emitted from a Taylor cone projecting from the droplet surface towards the orifice. This process is repeated and results in the production of charged, solvated analytes by several mechanisms (*vide infra*).

Electrostatic potentials maintained by the instrument drive the migration of ions from the atmospheric pressure region to the first vacuum stage. Desolvation of the ions is promoted by a countercurrent flow of drying gas (usually nitrogen) from the orifice and a heated desolvation line leading into the first vacuum region.[27] Additionally, the recondensation of solvent molecules on ions due to cooling caused by the adiabatic expansion of ions and gas into the first vacuum stage is prevented by maintaining an electrostatic potential between the desolvation line and the skimmer leading into the ion optics region, causing collisional heating.[28]

2.2.3 Ion Formation and Analyte Selectivity in ESI

Several mechanisms have been proposed for the production of gas-phase ions by ESI, the two most prominent being the charge residue model (CRM) and the ion evaporation model (IEM). In the CRM, charged droplets undergo many Coulombic fissions until the final solvent molecules associated with the ion evaporate.[30] This mechanism likely holds true for polymers, proteins, and other large molecules.[35] In the IEM, ions evaporate from the surfaces of small charged droplets, driven by the electrostatic repulsion of the excess charge.[27,36] Small ions such as alkali metal cations likely follow this model.

For either model, ionization is expected to be selective for relatively nonpolar analytes that reside on the surface of charged droplets.[37,38] These analytes are more likely to be incorporated into the small, highly charged droplets that are emitted from a parent droplet during Coulombic fission. Charged additives and impurities can suppress the ionization of the analyte of interest by competition for the occupation of the surface layer of the droplet and, thus, reduce sensitivity. In the case of adduct formation, ionization can also be suppressed due to competition for association with the limited number of ions in the droplet.[39]

Indeed, many of the behaviors observed in ESI-MS, including higher response factors for relatively nonpolar analytes, the relationship between signal intensity and analyte concentration (*vide infra)*, and ionization suppression in the presence of co-analytes, impurities, and electrolytes, are predicted by a simple partitioning model, proposed by Christie G. Enke.[40] In this model, the surface and interior of the electrospray droplets are treated as separate “phases” in which charged chemical species exist in equilibrium.[40] The surface phase contains all of the excess charge, while the interior phase is electrically neutral.[40] It is assumed that the relative abundances of ions observed in mass spectra, not accounting for m/z-dependent differences in transmission efficiencies, are proportional to their concentrations in the surface phase, and selectivity in ESI-MS results from competition for limited “excess charge sites”.[40] It is therefore predicted that ESI-MS response factors increase with increasing surface activities, and the degree to which the ionization of an analyte is suppressed depends upon both the concentrations of other charged species, as well as their surface activities.[40]

The acidic or basic properties of an analyte can also affect its ESI response. For protonated species monitored in the positive ionization mode, the signal intensity of an analyte at a given concentration tends to increase with increasing pK_a of its conjugate

acid.[41] In other words, more basic analytes tend to have greater response factors in ESI-MS than less basic analytes. In addition to an analyte's acid-base properties in solution, these properties in the gas-phase can also affect selectivity, especially as the concentration of a competing analyte with a higher gas-phase acidity/ basicity increases.[41]

2.2.4 The Relationship Between Signal Intensity and Analyte Concentration in ESI

The relationship between signal intensity and the concentration of an analyte is important for quantitative analysis in general. In the case of ESI-MS, the majority of the research effort for this topic has been focused on permanently-charged and basic analytes in the positive ionization mode. Generally, at relatively low analyte concentrations (eg. ≤ 1 μM), this relationship is linear.[34,41] At relatively high concentrations, analyte response factors tend to decrease, causing signal intensity *vs.* concentration traces to "flatten out".[34,37,41] This can be due to either the saturation of the droplet surface sites by the analyte or competition with other species of similar or greater surface activities.

2.2.5 Effects of Solvent Parameters on ESI Response

Solvent parameters can greatly influence the ESI response of an analyte. For example, increasing the ratio of the organic solvent:water in methanol:water or acetonitrile:water solvent systems often increases the signal intensities of all monitored analytes, due to their relatively low dielectric constants, volatilities, heats of vaporization, and surface tensions.[27,34,42,43]

In some cases, especially for relatively weak acids and bases, the signal intensities of protonated or deprotonated analyte species can be increased by the addition of an acid or a base, recpectively.[41,44,45] For example, Yamashita and Fenn found that decreasing the pH of a solution containing alanine increased the signal

intensity of its protonated species in the positive ionization mode, but increasing the solution pH did not affect the signal intensity of the deprotonated species in the negative ionization mode.[44] There have also been reports of decreases in signal intensities of basic analytes following to the addition of a dilute strong acid into its sample solution.[41] It is likely that ionization suppression is competitive with favorable thermodynamic effects.

Salts are often present in solutions analyzed by ESI-MS, especially for biological samples and effluent from upstream chemical separation processes. Salts often suppress ionization[41] to higher degrees at higher concentrations by competing with charged analytes for the occupation of sites on the surface of the electrospray droplet and reducing their vapor pressures.

2.3 Triple Quadrupole Mass Spectrometry

2.3.1 Overview of Quadrupole Mass Analyzers

Quadrupole mass analyzers (QMAs) were conceived of and developed by Wolfgang Paul and coworkers, beginning in 1953.[46] QMAs are relatively inexpensive, highly reproducible, robust, small, and probably the most commonly used mass analyzer for MS. In addition, their fast scan speeds make them well-suited for quantitation and as detectors for chemical separation techniques involving flow. QMAs function as m/z filters, allowing for the transmission of ions at a specified m/z, while discarding the rest. Mass spectra are generated by shifting or “scanning” the m/z transmission window while monitoring the ion currents for each m/z channel. QMAs are usually operated at unit mass resolution. Operating at a wider m/z scan range or higher resolution decreases the duty cycle for each m/z channel and results in lower sensitivities. For untargeted analyses, QMAs can be operated in scan mode, while for more sensitive targeted analyses, selected ion monitoring (SIM), where only specified m/z channels are monitored, is used.

2.3.2 Theory and Operation of Quadrupole Mass Analyzers

QMAs consist of four parallel cylindrical or nearly cylindrical electrodes arranged in a square array. Each electrode is electrically connected to the electrode diagonal to it, while also electrically insulated from its two nearer neighboring electrodes. A power supply is used to apply voltage waveforms to the electrodes, consisting of a time-independent direct current (DC) voltage and a superimposed time-dependent radio-frequency (RF) voltage. A diagram displaying the arrangements of the electrodes and their electrical connections is displayed in Figure 2-2. The DC voltage applied to one pair of electrodes is positive, while a negative DC voltage of the same magnitude is applied to the other pair. Additionally, the RF voltages applied to the two pairs of electrodes are 180° out of phase with the other. The amplitude of the RF voltage waveform is of a greater magnitude than that of the DC voltage, so the pair of electrodes with the applied positive DC voltage has a negative applied voltage during part of the RF cycle and *vice versa*.

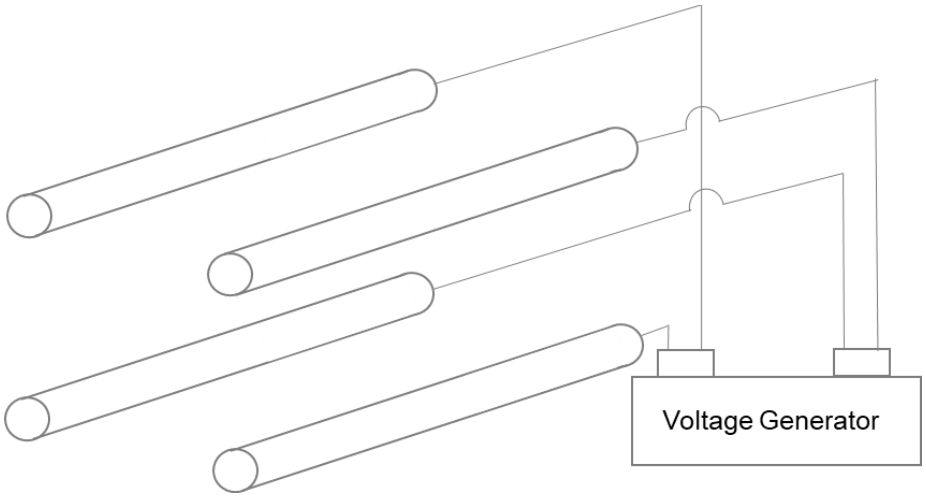

Figure 2-2. Diagram of a Quadrupole Mass Analyzer

Qualitatively, the mechanism of the operation of QMAs is often explained by describing the pairs of electrodes with positive and negative applied DC voltages as high-pass and low-pass m/z filters, respectively, in the case of the analysis of positive ions.[47] For the pair of electrodes with an applied positive DC voltage, ions are directed to and oscillate about the central axis during most of the cycle. During the part of the RF cycle where the electrode voltage is negative, ions are accelerated towards the electrodes. Ions of higher m/z have greater momenta, and thus, accelerate towards the electrodes to a lesser extent than ions of lower m/z. Ions below a certain m/z strike the electrodes and are neutralized during this time in the RF cycle. Similarly, for the electrode pair with an applied negative DC voltage, ions are directed towards the electrodes most of the time during a cycle. During the part of the RF cycle where the electrode voltages are positive, lower m/z ions accelerate away from the electrodes to a greater extent than ions of higher m/z, and ions above a certain m/z continue to move towards the electrode where they collide and are neutralized by virtue of their higher momenta.

An exact quantitative description of the trajectories of ions in a quadrupole field as a function of their m/z involves a system of differential equations that is rather unintuitive. Importantly, an ion's stable trajectory in a QMA depends on the DC and RF voltages, the RF frequency, and its m/z. QMAs are typically operated at constant RF frequency and RF:DC voltage ratio, and mass spectral scans are performed by changing the magnitudes of the RF and DC voltages by the same factor, shifting the m/z transmission window.

2.3.3 Tandem Mass Spectrometry

Tandem MS (MS/MS) involves the selection of a "precursor" ion of a certain m/z, the fragmentation of that ion, and the selection of a "product" ion of a certain m/z resulting

from the precursor ion's fragmentation. MS/MS offers several advantages over single stage MS, including better selectivity, reduced chemical noise, more detailed structural information, and, in some cases, lower limits of detection. The first and second MS stages can be operated under different combinations of scan and SIM modes. Operating both stages in the SIM mode is referred to as selected reaction monitoring (SRM) and can be highly selective for an analyte of interest. Neutral loss scanning, where both MS stages are operated in the scan mode and the m/z channel monitored in the second MS stage is less than that of the first MS stage by a constant value, as well as precursor ion scanning, where the first and second MS stages are operated in the scan and SIM mode, respectively, are useful for targeting structurally related compounds. Lastly, product ion scanning, where the first and second MS stages are operated in the SIM and scan mode, respectively, is useful for structural elucidation.

2.3.4 Triple Quadrupole Mass Spectrometry

Triple quadrupole (QqQ) mass spectrometers, developed in the late 1970s by Yost and Enke, consist of three sets of quadrupole electrodes arranged in tandem.[48] The first set functions as the first MS stage. The second set is operated using only RF voltages, allowing ions of a wide m/z range to be transmitted, and contains the collision cell for collision-induced dissociation (CID). The third set functions as the second MS stage. QqQ-MS/MS enjoys the same advantages as single stage QMAs, while having the capability for MS/MS experiments.

2.3.4 Electron Multiplier Detectors

After ions have been generated and mass analyzed, they must be detected by some means in order to obtain useful data. For QqQ-MS, electron multipliers are the most commonly used detectors. Electron multipliers can either consist of discrete electrodes (dynodes) held at successively less negative voltages or as a single,

continuous unit held at a voltage gradient. In the case of the detection of positive ions, the ion's impact on the dynode causes the emission of electrons. The electrons are then accelerated to the next dynode in discrete electron multipliers or region in continuous electron multipliers in which their impact causes the emission of a greater number of electrons. This process is repeated until the electrons reach the final electrode, the anode, where the current from the electrons is converted into a voltage difference by an amplifier, and this voltage difference is measured and processed by a computer. Electron multipliers are very sensitive detectors, capable of producing in excess of 10^6 electrons per ion striking the detector.[49] In addition, their response times are fast, making them ideal for coupling MS to upstream chemical separation techniques. Continuous electron multipliers are less expensive than discrete electron multipliers and are most commonly used.

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Chapter 3

The Analysis of Carbohydrates by ESI-MS: A Review

3.1 Abstract

Carbohydrate analysis has many important implications in health, the quality of consumer products, industrial processes, and other areas of fundamental and practical significance. For their analysis in complex samples, the remarkably high sensitivity and selectivity offered by mass spectrometry (MS) makes it an attractive approach. Electrospray ionization (ESI)-MS for carbohydrate analysis has received considerably less attention than matrix-assisted laser desorption/ionization-MS, though it does provide several advantages including the ability for direct coupling with upstream chemical separation techniques, ease of quantitation, and better sensitivity. In this review, prior work on detection strategies, derivatization techniques, the influence of solvent parameters on the ESI-MS response and the MS/MS fragmentation pathways for carbohydrates are discussed.

3.2 Introduction

Saccharides are Earth's most abundant class of biomolecules. They are involved in numerous biological functions including energy storage, structural support, biological recognition, and cell-cell adhesion.[1] Monosaccharides are the fundamental chemical units of saccharides and include nonhydrolyzable carbohydrates and their derivatives. In this review, carbohydrates are defined as molecules composed of monosaccharides with the empirical formula of CH_2O.

The analysis of carbohydrates has many important implications in biology, nutrition, health, food quality, and commercial and industrial processes. The choice among numerous possible approaches for their analysis depends upon the desired chemical information and the sample complexity, a common theme in analytical

chemistry. If the analysis requires the determination of the concentrations of individual carbohydrate species in a sample, separation prior to or during analysis and/ or the use of a selective detector is required. The analyst presented with this task will need to address at least some of the following challenges during method development: The similar chemical and physical properties among carbohydrates; the presence of multiple forms of reducing carbohydrates in solution by mutarotation; the presence of stereocenters (except for glyceraldehyde); the monosaccharide composition and their sequence for oligo- and polysaccharides; variable linkage positions; and branching.

The remarkably high sensitivity and specificity offered by mass spectrometry (MS) and tandem MS (MS/MS), especially when coupled with online chemical separation techniques, make them attractive approaches for carbohydrate analysis. The historical development of carbohydrate analysis by MS closely follows the evolution and maturation of MS, in general. Beginning in the 1960s, the hurdle of applying MS to nonvolatile carbohydrates was overcome through the use of chemical reagents to derivatize their hydroxyl groups, resulting in an increased volatility.[2–4] Derivatization also allowed for carbohydrate analysis by gas chromatography-MS,[5] a powerful technique still widely used today. The introduction of “soft” ionization techniques for nonvolatile samples, beginning in 1969 with field desorption ionization,[6] was a significant breakthrough for the qualitative analysis of carbohydrates. Today, electrospray ionization (ESI) and matrix-assisted laser desorption/ ionization (MALDI) have nearly completely dominated other soft ionization techniques for most applications, though many of the detection strategies and MS/MS fragmentation studies still in use with and applicable to ESI-MS today were developed and elucidated with earlier ionization techniques, especially fast atom bombardment (FAB).[7] ESI-MS is particularly advantageous in its ability to be directly coupled with online liquid chromatography (LC), capillary electrophoresis (CE), and other

separation techniques involving liquid flow. Additionally, quantitative analysis is more easily achieved in ESI-MS than MALDI-MS, low molecular weight carbohydrates do not suffer from interferences from matrix-related ions, and charge states tend to increase with increasing molecular weight, allowing the use of relatively inexpensive quadrupole mass analyzers for their analysis.

The use of ESI-MS for the detection of carbohydrates was first reported by Aleksandrov and coworkers in 1984.[8] Since then, ESI-MS has been applied to carbohydrate analysis for numerous samples, including seawater and sediment pore water,[9] foods and beverages,[10–17] whole bacterial cell hydrolysates,[18] human serum[19] and plasma[20–22], plant tissues,[23–30] atmospheric aerosols,[31,32] paper,[33] tobacco products,[34] human tears,[35] marine mucilage,[36] plant gums,[37] and watercolor paint.[37].

This review first covers detection strategies, including derivatization techniques, used for the ESI-MS analysis of carbohydrates, a crucial topic due to the lack of easily protonatable or deprotonatable chemical groups in carbohydrates. Next, the influence of solvent parameters on the ESI-MS response of carbohydrates is described. Lastly, MS/MS fragmentation pathways for carbohydrates are discussed.

3.3 Detection Strategies for Carbohydrates by ESI-MS

3.3.1. ESI-MS Analysis of Underivatized Carbohydrates

The strategy for the detection of carbohydrates by ESI-MS requires careful consideration, as they are not permanently charged nor do they possess easily protonatable or deprotonatable chemical groups. In addition, carbohydrates are hydrophilic, and their responses in conventional µL/ minute flow rate ESI-MS tend to be less than that of other classes of compounds, such as peptides.[38] Sensitivity, linearity, the quality of information yielded from MS/MS experiments, the desired isotopic features, interferences with isotopically labeled internal standards, and compatibility with upstream

chemical separation (if applicable) are all factors to be considered when choosing a detection strategy.

In the positive ionization mode, carbohydrates are most commonly monitored as adducts of metal ions or other cations, especially as lithiated,[27] sodiated,[24,33,35,36] potassiated,[10,12] and ammoniated[31] adducts. Cluster ions, such as $[nM+cat]^+$, can also be monitored and tend to increase in abundance at higher analyte concentrations.[14,37,39] Multiply cationized adducts have been detected for higher molecular weight carbohydrates (e.g., maltooligosaccharides), and these tend to accommodate more charges as their degree of polymerization (DP) increases.[39,40] Adducts with neutral salt attachments may also be observed in certain combinations of additives and solvents, with this propensity tending to increase with increasing DP.[41] Other alkali metals,[20,21] alkaline earth metals,[42] and post-transition metals[43] have been used for carbohydrate cationization in ESI-MS. Cationized adducts containing solvent molecules have also been detected.[44]

Carbohydrates are rarely monitored as protonated species. Indeed, sodiated and/ or potassiated adducts often dominate ESI mass spectra of sugars, even when using LC-MS grade solvents. Low molecular weight carbohydrates (e.g. mono- and disaccharides) may not be detectable as protonated species under certain solvent parameters.[9,12,44] Higher molecular weight sugars (e.g., cyclodextrins)[45] can yield abundant singly and multiply protonated species, with higher charge states achieved at higher DP. It is worth mentioning that use of acidic conditions to promote protonation of oligosaccharides during ESI-MS may lead to significant glycosidic bond cleavages.[46]

In the negative ionization mode, deprotonated sugars[15,34,47] and sugars adducted to anions are most commonly monitored. Charge states of deprotonated species tend to increase with increasing DP.[48] Anions used as adducts include acetate, chloride,[17]

formate,[16,28–30] and iodide[22]. Chloride adducts are usually generated by the addition of chloride salts into the sample solution, though the addition of hydrochloric acid[17] and the electrochemical reduction of chloroform in the spray needle in the negative ionization mode have also been used to generate chloride adducts.[32] Iodide adducts have also been generated in this way using iodoform.[22]

Aside from choosing a detection strategy, which will lead to high sensitivities, other factors may require consideration. Ionic species that possess multiple isotopes at significant abundances, such as chloride adducts, will split the ion current across multiple m/z channels, resulting in reduced sensitivity. Additionally, isotopic mass spectral peaks originating from adduct-forming ions can interfere with the analysis of isotopically labeled internal standards.[20,22] However, the isotopic structure of an ion can be helpful for increasing confidence in qualitative confirmations.[32] High molecular weight ions may be desirable for obtaining high signal-to-noise ratios (S/N) due to the lower chemical noise at higher m/z channels.[20]

3.3.2 Derivatization of Carbohydrates for ESI-MS

Derivatization of carbohydrates can be used to enhance their ESI-MS sensitivity, improve their upstream separations, eliminate the need for additives, and provide higher quality structural information. For reducing sugars, the attachment of hydrophobic, permanently charged, and/ or highly basic chemical groups via reductive amination[49–51] or other reactions involving their carbonyl groups[52–55] is a common approach for increasing their ESI-MS sensitivities. Improvements of the limits of detection of over three orders of magnitude have been reported using this approach,[49,50] though its applicability is obviously limited to reducing sugars.

Derivatization can also increase the diagnostic value of MS/MS data. For example, permethylation is useful for assessing branching patterns by distinguishing

product ions resulting from single and multiple glycosydic bond cleavages based on their m/z shifts.[25,56] The ratios of product ion abundances in MS/MS and MS^3 spectra for cyclic ferrocenyl boronate esters of carbohydrates have been demonstrated to differentiate stereoisomers of mono- and disaccharides.[57] The reduction of reducing sugars to alditols can be used to distinguish product ions formed from reducing and non-reducing ends.[26]

Lastly, derivatization can improve carbohydrate separation or allow for their separation by techniques whose performances are generally poor towards underivatized carbohydrates. Appending charged chemical groups to carbohydrates has been demonstrated for their analysis using CE-ESI-MS.[51] Derivatization with nonpolar groups can allow for satisfactory separation of carbohydrates using LC-ESI-MS on reversed-phase LC columns.[55,58]

Although derivatization offers several important advantages for the ESI-MS analysis of carbohydrates, it comes with drawbacks. Derivatization can be costly in terms of labor and time, often requiring multiple steps along with sample clean-up. Analysis may be complicated by the presence of reagents, the formation of multiple products, and incomplete product formation[50]. The choice of its implementation will depend upon the sensitivity, MS selectivity, cost, and separation requirements for a given application.

3.4 Effects of Solvent Parameters on ESI-MS Response

3.4.1 Effect of the Additive

The careful selection of additives can lead to increased signal intensities, better linearities, and higher quality MS/MS data for structural elucidation or stereoisomer differentiation (*vide infra*). Relative affinities to ionic species, which contribute to their relative signal intensities, is dependent, in part, by the spatial orientation of their hydroxyl groups.[59] The ionic radius of the coordinating cation has a critical influence on the

stability of complexes formed with carbohydrates; its careful consideration can lead to high efficiency of adduct formation and high abundances of informative product ions.[60]

Several studies on the influence of additives on the ESI-MS response of carbohydrates have been reported in the literature. Kohler and Leary investigated the effects of metal chloride additives, introduced through a triaxial probe, on the signal intensity of various saccharides.[61] They reported an increase in the signal intensity of cellobiose by a factor of 70 using 1 mM LiCl, monitoring $[Cellobiose+Li]^+$, relative to using 1% acetic acid where $[Cellobiose+H]^+$ was monitored.[61] Mallet *et al.* studied the effects of several additives at various concentrations on the signal intensity of deprotonated raffinose, among other analytes, by separately pumping sample and additive solutions through a tee leading into to the mass spectrometer.[62] Ammonium hydroxide was found to enhance signal intensity, while perfluorinated carboxylic acids severely decreased signal intensity.[62] Other investigators have also used ammonium hydroxide to increase sensitivity for deprotonated carbohydrate species.[15] $[M-H]^-$ signal intensity can also be enhanced by the addition of an anion forming species, such as ammonium fluoride, which can decompose in the intermediate vacuum region to produce additional deprotonated species.[63] In cases where adducts are formed from trace contaminants, such as sodium, the addition of additives containing these ions to standardize their concentrations sometimes results in reduced sensitivities.[19,64]

In a recent publication, Thacker and Schug used flow injection analysis (FIA)-ESI-MS/MS to study the effects of various solvent parameters, including the additive and its concentration, the organic co-solvent and its ratio to water, and solution pH, on the signal intensity of glucose in both the positive and negative ionization mode.[65] It was found that monitoring the ammonium adduct selected reaction monitoring (SRM) transition of glucose in 80:20 methanol:water with ammonium trifluoroacetate resulted in

the highest signal intensity.[65] In 80:20 acetonitrile:water solutions, important for hydrophilic interaction LC (HILIC), the highest signal intensities were achieved with ammonium formate and lithium fluoride, monitoring deprotonated SRM transitions.[65] In another study by Thacker and Schug, a continuous stirred tank reactor (CSTR) was coupled to ESI-MS/MS to determine response factors for glucose, sucrose, and raffinose across a wide concentration range from a single injection under a variety of solvent parameter combinations.[64] For sucrose and raffinose, the highest response factors were obtained by monitoring their sodium adduct SRM transitions in 80:20 methanol:water, without any additive. Interestingly, adding 100 µM NaCl decreased their response factors by over an order of magnitude. Ammonium trifluoroacetate resulted in the highest response factor for glucose in both 80:20 methanol water and 80:20 acetonitrile:water, monitoring its ammonium and trifluoroacetate adduct SRM transitions, respectively. In addition, the best linearities for all three analytes were obtained by monitoring acetate, chloride, and formate adducts in 80:20 acetonitrile:water with ammonium acetate, sodium chloride, and ammonium formate additives, respectively.

The identity of the additive can affect the signal intensity of carbohydrate ions, even when they are not involved in adduction. For example, the substitution of ammonium chloride for sodium chloride reduced the signal intensities for deprotonated maltooligosaccharides, due to the acidity of ammonium.[39] The addition of formic acid to 1 mM NaOAc led to higher sensitivity for $[M+Na]^+$.[46] Other investigators have also reported increased signal intensities for carbohydrate-sodium adducts upon the addition of low concentrations of formic or acetic acid.[35,66]

The counterion of the ionic species that forms an adduct with carbohydrates can also affect its signal intensity. For example, Striegel *et al.* reported the detection of lithiated maltopentaose adducts using LiCl and LiBr as additives. The adduct for LiF was

not detected.[41] This was attributed to the unusually high lattice energy of LiF.[41] In the aforementioned FIA-ESI-MS/MS study, dramatic differences in signal intensity were also found upon exchanging the counterion.[65] Most strikingly, the signal intensity for the glucose-ammonium SRM transition for 0.05% ammonium trifluoroacetate, pH 2.9, in 80:20 methanol:water was higher by a factor of over 24 than for its ammonium acetate analog.[65]

3.4.2 Effect of Additive Concentration

Often, there exists an optimum concentration of additives where signal intensity is most enhanced and ionization suppression is low. Mauri *et al.* reported optimal concentrations between 1-3 mM for sodium, potassium, and cesium for the FIA-ESI-MS analysis of maltooligosaccharides in solvents and diluted beer samples.[12] As an example from the FIA-ESI-MS/MS study, the glucose-lithium adduct SRM signal intensity for 80:20 methanol:water was highest in the presence of 1 mM lithium fluoride; signal intensities obtained from the addition of 5 mM and 100 µM lithium fluoride were only 70% and 33%, respectively, for that of the 1 mM solution.[65] Klampfl and Buchberger reported the optimal concentration of diethylamine in coaxial sheath fluid for the CE-ESI-MS of various carbohydrates as 0.25%.[11] In Mallet *et al.*'s FIA-ESI-MS study, the optimal concentration of ammonium hydroxide for deprotonated raffinose was 0.50%.[62]

3.4.3 Effect of Solvent

In the positive ionization mode, solutions based on acetonitrile:water tend to result in lower signal intensities than those based on methanol:water.[16,31,65] This is likely due to competition with acetonitrile for cation adduction.[65] In the FIA-ESI-MS/MS study, a general increase in signal intensity was found with increasing concentration of methanol in methanol:water solvents, in both the positive and negative ionization modes.[65] For acetonitrile:water solvents, a sharp drop in signal intensity was observed when changing

from 0% to 20% acetonitrile in the positive ionization mode; in the negative ionization mode, a general increase in signal intensity was seen with increasing acetonitrile content, up to about 80% for most additives, likely due to the limited solubility of glucose and the additives used in solvents of a high acetonitrile content.[65] Cheng *et al.* reported increases in signal intensities of over a factor of 10 upon the post-column addition of ethanol through a tee for the analysis of paper cellulose using LC-ESI-MS.[33]

3.5 MS/MS for Carbohydrates

3.5.1 General Fragmentation Patterns for ESI-MS/MS

MS/MS can be used to decrease chemical noise, provide structural information, and differentiate between isobars. Typically, collision-induced dissociation (CID) is used to fragment carbohydrates in ESI-MS/MS, and, thus, the following discussion will be limited to CID. Fragmentation can also be performed in the intermediate vacuum region between the nozzle and skimmer, though, in this case, there is no isolation or selection of a precursor ion. The reader is directed to the following reference on the nomenclature for carbohydrate MS/MS fragmentation nomenclature.[7]

MS/MS fragmentation pathways for carbohydrates usually involve the loss of water molecules, cross-ring cleavages, and glycosidic bond cleavages.[7] Product ions resulting from cross-ring cleavages are useful for differentiating linkage isomers,[67] and those resulting from glycosidic bond cleavages are useful for determining the sequence of monosaccharide residues.

3.5.2 MS/MS of Positively Charged Species

Protonation of carbohydrates occurs unselectively at their hydroxyl groups, producing mostly fragments as a result of glycosydic bond cleavages.[68] Linear carbohydrates cationized to charge-dense cations (e.g., lithium[69]) often undergo cross-ring cleavages under CID, while those cationized to less charge-dense cations (e.g.,

potassium and cesium) tend to only produce the adducted cation during MS/MS. The ionic radius of the cation also influences coordination and, thus, MS/MS spectra. For example, Fura and Leary demonstrated that MS/MS spectra of calcium-coordinated fucosyllactose isomers yielded unambiguous linkage position information, while those coordinated with magnesium did not. This was proposed to be caused by the more selective coordination of calcium due to its larger ionic radius. Dicationized cluster ions, such as $[M+2Li-H]^+$, have also been demonstrated to yield MS/MS spectra with fragment ions resulting from cross-ring cleavages.[70]

3.5.3 MS/MS of Negatively Charged Species

In contrast to the MS/MS spectra of protonated carbohydrates, those of deprotonated species are accompanied by fragment ions resulting from cross-ring cleavages.[68] This has been explained by the selective deprotonation of carbohydrates for 1-OH, while protonation is unselective.[9,67,68] There has been far less research efforts focused on the use of MS/MS for anionic adducts than for cationic adducts for the characterization of carbohydrates. Product ions in MS/MS spectra of anionic carbohydrate adducts can include the deprotonated carbohydrate, the anion, carbohydrate fragment ions, or combinations thereof.[71] The extent to which carbohydrate-containing product ions are formed depends upon the gas-phase basicities of the carbohydrate and the adduct-forming anion.[72] Anionic adducts composed of anions of relatively low gas-phase basicities, such as bromide, tend to produce MS/MS spectra dominated by the adduct-forming anion.[72] The ability to distinguish linkage isomers based on MS/MS spectra has been demonstrated for chloride-adducts of various disaccharide species.[71]

3.6 Conclusions

ESI-MS offers numerous advantages for the analysis of carbohydrates, including high selectivity, ease of quantitation, the ability to provide structural information, and the ability for direct coupling to upstream chemical separation techniques. The relatively low sensitivity of ESI-MS towards carbohydrates due to their high hydrophilicities and their lack of easily protonable or deprotonable chemical groups make the choice of detection strategies, the implementation of derivatization techniques, and solvent parameters crucial. MS/MS is useful for providing additional structural information, enhancing selectivity, reducing chemical noise, and differentiating isomers. Much less research efforts in biological MS has been focused on carbohydrate and saccharide analysis relative to other classes of biomolecules, such as peptides and proteins, and there remains many fundamental and practical research opportunities for future investigations.

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Chapter 4

Effects of Solvent Parameters on the Electrospray Ionization – Tandem Mass Spectrometry Response of Glucose

4.1 Abstract

The importance of saccharides, the most abundant biomolecules on Earth, extends beyond their biological roles and to consumer products and industrial processes. Electrospray ionization-tandem mass spectrometry (ESI-MS/MS) is an attractive tool for the analysis of underivatized saccharides (US), but they tend to have relatively low sensitivities due to their low surface activities and lack of easily protonable or deprotonable chemical groups. An understanding of the influences that solvent parameters have on their signal intensities would enhance the usefulness of ESI-MS/MS for their analysis. Solutions of glucose, a model analyte for US, in various combinations of solvent, additive, additive concentration, and pH were analyzed by flow injection analysis (FIA)-ESI-MS/MS in both the positive and negative ionization modes. The blank-corrected signal intensities of the solvent parameter combinations were then compared. The addition of acetonitrile led to severe ionization suppression in the positive ionization mode through its competition with glucose for cation adduction. High signal intensities were achieved under wide pH and concentration ranges for methanol:water solutions containing ammonium trifluoroacetate in the positive ionization mode. The highest signal intensities for acetonitrile:water solutions were from those containing ammonium formate or lithium fluoride in the negative ionization mode. An understanding of the influence of solvent parameters on the signal intensity of a given analyte is useful for guiding the selection process of mobile phases/ flow solvents that lead to low limits of detection or the minimization of matrix effects by allowing its detection at high dilution factors.

4.2 Introduction

Saccharides are the most abundant class of biomolecules on Earth and are involved in numerous biological functions including energy storage, structural support, and biological recognition. The analysis of saccharides has important implications in food science, clinical diagnostics, and other biological, commercial, and industrial applications.

The potentials for high specificity and a low limit of detection imparted by electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) make it an attractive tool for the analysis of saccharides in complex matrices. However, the lack of easily protonable or deprotonable chemical groups in, and the low surface activity of, underivatzed saccharides (US) result in lower ESI-MS/MS sensitivity relative to compound classes that have these properties, such as peptides.[1] Interestingly, it has been demonstrated that US can be detected as sensitively as peptides using nano-ESI.[2] However, systematic studies regarding the optimization of solvent parameters for the ESI-MS/MS analysis of US have been limited to date. Alternatively, the enhancement of ESI-MS sensitivity of saccharides can be achieved through derivatization. For example, Muddiman and coworkers have demonstrated this by tagging *N*-linked glycans as well as maltoheptaose with hydrophobic hydrazides.[3,4]

In the positive ionization mode, metal adducts of US are often monitored.[5-7] The addition of ammonium salts to the sample solvent to generate ammonium-US adducts with stereochemically diagnostic MS/MS spectra has also been reported.[8] Volatile ammonium salts are more ideally suited as additives in sample and flow solvents for ESI-MS/MS than nonvolatile metal salts. In the negative ionization mode, US can be detected as deprotonated species[9] or by their anion-adducts.[10] Several halogenated solvents including chloroform[11] and iodoform[12] have also been reported to generate halide-US adducts in ESI-MS.

Investigating the influence of solvent parameters on the ESI-MS/MS signal intensity of US would be very useful for devoloping methods with low limits of detection and minimizing matrix effects, by allowing their detection at high dilution factors, but to date there have been few studies on this subject. Using postcolumn addition of several metal chlorides to various saccharides from the eluent of a liquid chromatography (LC) column, Kohler and Leary achieved the highest signal intensities of metal-saccharide adducts with lithium chloride and sodium chloride.[5] Mallet *et al.* studied the effects of several acid and salt additives at various concentrations, as well as solid phase extracts of plasma, on the signal intensity of raffinose (detected as a deprotonated species) among other test analytes, by separately pumping sample solutions and additive solutions in a tee leading to the MS.[13] Ionization suppression was particularly severe in the presence of perfluorinated carboxylic acids, and the signal intensity was most enhanced with the addition of 0.50% ammonium hydroxide. Discussions of method development in LC-ESI-MS chromatographic or application studies are another information source. Several groups have reported poor signal intensities of US in the positive ionization mode in acetonitrile (ACN):water mobile phases.[14,15] Liu *et al.* reported an increase of the peak area of sodiated maltotriose in 60:40 ACN:water by more than a factor of 10 by the addition of 0.1% acetic acid or formic acid.[16]

The aim of this study was to investigate the effects of various solvent parameters, including the addition of volatile ammonium salts (ammonium acetate, ammonium bicarbonate, ammonium formate, and ammonium trifluoroacetate (ammonium TFA)) at various concentrations, pH (except for ammonium bicarbonate), and the ratio of the organic cosolvent to water (methanol (MeOH) and ACN) on the ESI-MS/MS signal intensity of glucose, a model analyte for US. Values chosen for solvent parameters were those commonly used in LC-MS analysis of US. In addition, the comparison of the

glucose ESI-MS/MS signal intensity in sample solvents containing lithium fluoride and ammonium fluoride salts, and that of the volatile ammonium salts was also of interest; the influence of those aforementioned solvent parameters, except for pH, were also assessed for these salts.

4.3 Experimental

4.3.1 Chemicals

All chemicals except for glucose were of LC-MS grade. *D*-(+)-glucose (≥99.5%) was purchased from Sigma-Aldrich (Steinheim, Germany). Trifluoroacetic acid was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Formic acid (98-100%) was purchased from EMD Millipore (Billerica, MA, USA). Lithium fluoride (99.99%) was purchased from Alfa Aesar (Haverhill, MA, USA). Acetic acid, ACN, ammonium bicarbonate, ammonium fluoride (≥98.0%), ammonium hydroxide (≥25% in water), MeOH, and water were purchased from Honeywell (Morris Plains, NJ, USA).

4.3.2 Sample Preparation

5% (v/v) aqueous stock solutions of acetic acid, formic acid, and trifluoroacetic acid, each adjusted to either pH 2.9, 5.9, 6.9, and 7.9 by an aqueous solution of ammonium hydroxide, were prepared. A 5% (w/v solvent) aqueous stock solution of ammonium bicarbonate was shaken vigorously for a few minutes until the pH rose to 8.1, presumably due to the outgassing of carbon dioxide. 25 mM aqueous stock solutions of lithium fluoride and ammonium fluoride were prepared. All additive stock solutions except for ammonium formate were used within three days of preparation; the ammonium formate solutions were used up to five days after preparation. An aqueous 1000 ppm glucose stock solution was prepared and forced through a 0.45 µm PTFE syringe filter. Aliquots from stock solutions and solvents were mixed in LC autosampler vials (1.8 mL, borosilicate glass). Both a 1 ppm glucose solution and a blank containing no glucose

were prepared for each combination of solvent parameters investigated. The pH of the sample solvent was reported as the pH of the aqueous stock solution of the salt from which it was comprised.

4.3.3 Flow Injection Analysis – Electrospray Ionization – Tandem Mass Spectrometry

All measurements were obtained on a Shimadzu LCMS-8040 (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) triple quadrupole mass spectrometer with an ESI source and the following LC modules (all Prominence series from Shimadzu): CBM-20A communications bus module; DGU-20A5 online degasser; LC-20AD XR LC pump; and SIL-20AC XR autosampler with a 50 µL injection loop. The autosampler was connected directly to the ionization source with PEEK tubing. The instrumental parameters, set for analyzing a wide range of analytes, were as follows: Positive ionization mode interface voltage, 4.5 kV; negative ionization mode interface voltage, -3.5 kV; desolvation line temperature, 250 °C; heat block temperature, 400 °C ; nebulizing gas (N_2) flow, 3 L/min.; drying gas (N_2) flow, 5 L/min.; dwell time, 100 msec; loop time, 236 msec; CID gas (Ar) pressure, 230 kPa; flow injection solvent, LC-MS water; injection volume, 50 µL; flow rate, 20 µL/min. Selected reaction monitoring (SRM) parameters, chosen for their high signal intensities in samples with concomitantly low signal intensities in blanks, are displayed in Table 4-1. Each sample and blank were analyzed in triplicate. Mass spectral scans in the positive ionization mode were taken under the same flow injection and ionization source conditions as above, with a scan range of 10-1000 *m/z* and a scan speed of 1000 *m/z*/ sec.

The signal intensity data for both the positive and negative ionization mode SRMs were exported from *LabSolutions* (v.5.80, Shimadzu) to Microsoft (Redmond, WA, USA) Excel (v.15.0.4963.1000). For each data file, the average signal intensity and its standard deviation from 1.00 to 1.50 min, a temporal region with relatively stable signal

intensities for most samples, was determined. The signal intensity for each solvent parameter combination was reported as the difference between the average of the aforementioned averaged time windows of triplicate measurements and that of its blank. The standard deviation of the signal intensity determined for each solvent parameter combination took into account both the standard deviations of each individual measurement and the standard deviation of the average amongst triplicate measurements, with the assumption that these contributed independently to the overall standard deviation.

Table 4-1. SRM precursors, transitions, and collision energy (CE) voltages for glucose with various sample solvent additives

Additive	(+)		(-)	
	Precursor	SRM	Precursor	SRM
None	$[M+H]^+$	181 → 69; CE: - 30 V	$[M-H]^-$	179 → 101; CE: 10 V
Ammonium TFA	$[M+NH_4]^+$	198 → 85; CE: - 18 V	$[M+TFA]^-$	293 → 113; CE: 10 V
Ammonium Acetate	$[M+NH_4]^+$	198 → 85; CE: - 18 V	$[M+OAc]^-$	239 → 119; CE: 12 V
Ammonium Formate	$[M+NH_4]^+$	198 → 85; CE: - 18 V	$[M-H]^-$	179 → 101; CE: 10 V
Ammonium Bicarbonate	$[M+NH_4]^+$	198 → 85; CE: - 18 V	$[M-H]^-$	179 → 101; CE: 10 V
Ammonium Fluoride	$[M+NH_4]^+$	198 → 85; CE: - 18 V	$[M-H]^-$	179 → 101; CE: 10 V
Lithium Fluoride	$[M+Li]^+$	187 → 127; CE: - 18 V	$[M-H]^-$	179 → 101; CE: 10 V

4.4 Results and Discussion

4.4.1 Effect of Additives on Signal Intensity

Every additive investigated had at least certain combinations of solvent parameters that resulted in higher signal intensities than solutions without additives, in both the positive and negative ionization modes for both 80:20 MeOH:water and 80:20 ACN:water solvent compositions. These data are summarized in Table 4-2. Notably, the protonated glucose SRM transition monitored for solutions containing no additives was observed in 10 ppm glucose in 80:20 MeOH:water solutions (data not shown) but not in

Table 4-2 Influence of solvent parameters on the ESI-MS/MS signal intensity of glucose

Additive	Concentration	pH	80:20 Methanol:Water				80:20 Acetonitrile:Water			
			(+) SRM	%RSD	(-) SRM	%RSD	(+) SRM	%RSD	(-) SRM	%RSD
None	-	-	-30	116	2530	5.7	-320	51	1650	5.9
Ammonium TFA	1.00%	2.9	53600	0.9	1620	3.4	2630	4.0	990	7.1
		5.9	80200	3.2	1750	7.2	3350	7.2	900	5.8
		6.9	54200	1.3	2200	3.1	2970	5.1	940	6.5
		7.9	42900	1.1	2980	2.5	2430	5.4	1240	4.2
	0.50%	2.9	68900	2.0	3010	4.1	2560	5.9	1760	5.6
		5.9	66800	1.4	4270	3.5	3040	3.7	1680	4.5
		6.9	63000	1.1	4290	4.0	3090	3.1	1800	4.2
		7.9	50000	2.9	4500	5.5	2670	5.5	2040	7.1
	0.10%	2.9	81500	0.8	7640	1.7	1410	5.3	4140	3.4
		5.9	53000	1.1	9410	1.8	1390	4.7	4090	4.0
		6.9	54600	1.0	10300	2.3	1720	5.6	4730	3.2
		7.9	49700	0.9	11000	2.8	2160	3.8	5520	4.7
	0.05%	2.9	92500	4.9	10700	1.8	960	4.3	5060	19
		5.9	47700	2.1	10000	20	950	7.5	4930	23
		6.9	47800	1.7	10100	21	970	4.7	5440	24
		7.9	46600	1.4	14100	1.6	1710	19	10800	12
Ammonium Acetate	1.00%	2.9	14200	1.2	8830	3.1	0	5500	7130	3.3
		5.9	5170	3.5	13200	1.9	Immiscible			
		6.9	5380	2.3	13600	1.6	Immiscible			
		7.9	5400	2.7	9400	2.1	Immiscible			
	0.50%	2.9	12800	2.9	10500	1.7	1	460	12100	3.5
		5.9	4330	2.4	9340	2.7	Immiscible			
		6.9	5160	2.3	10200	1.6	Immiscible			
		7.9	4770	2.1	11900	2.0	Immiscible			
	0.10%	2.9	5120	2.1	13800	1.5	0	420	13600	2.1
		5.9	7450	2.4	13600	1.4	1	710	9830	8.5
		6.9	7840	1.4	14700	1.3	1	290	11700	9.6
		7.9	6660	2.0	14400	1.3	1	330	7180	5.0
	0.05%	2.9	3810	2.3	14300	2.3	0	860	16800	2.6
		5.9	9810	3.1	15300	1.8	-1	650	9680	4.7
		6.9	11400	1.9	16200	1.9	1	630	9050	5.0
		7.9	9900	2.4	16500	1.5	1	240	12000	6.7
Ammonium Formate	1.00%	2.9	2150	3.2	4480	2.7	12	73	3750	3.9
		5.9	1430	4.1	3500	2.2	Immiscible			
		6.9	1440	4.7	3870	3.2	Immiscible			
		7.9	1470	3.9	3070	4.3	Immiscible			
	0.50%	2.9	3180	3.3	7480	3.1	4	300	7250	6.4
		5.9	1870	3.2	5790	2.1	Immiscible			
		6.9	2010	3.5	6910	4.2	Immiscible			
		7.9	2000	3.8	5140	4.8	Immiscible			
	0.10%	2.9	6660	2.0	22200	2.0	15	78	18200	1.6
		5.9	5430	2.2	22300	1.6	16	64	18900	1.8
		6.9	5230	2.0	19200	2.6	20	58	17900	2.0
		7.9	4730	6.3	14600	8.1	16	68	20600	1.9
	0.05%	2.9	7430	1.6	28300	2.0	3	420	19300	14
		5.9	8000	2.2	34800	1.6	21	48	26300	1.6
		6.9	8320	1.6	37800	1.2	17	54	25300	2.0
		7.9	8560	2.6	41000	1.8	17	68	29300	1.8
Ammonium Bicarb.	1.00%	8.1	36800	1.3	2680	3.6	Immiscible			
	0.50%	8.1	19000	1.8	1640	4.5	120	25	350	7.1
	0.10%	8.1	9750	2.3	1830	3.5	4	420	2030	5.6
	0.05%	8.1	11100	2.7	2980	2.5	-3	630	3690	3.6
Ammonium Fluoride	5 mM	-	56900	3.3	2980	3.3	500	33	2170	3.4
	1 mM	-	44400	3.6	5000	2.0	440	11	3780	4.2
	500 µM	-	19900	3.2	5590	1.9	360	15	3240	2.8
	100 µM	-	4390	5.4	4970	7.5	26	120	5150	3.4
Lithium Fluoride	5 mM	-	28600	3.3	11700	4.1	530	5.9	21200	4.4
	1 mM	-	41000	10	21500	13	670	6.3	23200	1.7
	500 µM	-	39400	2.5	27400	2.6	600	8.7	22100	2.6
	100 µM	-	13400	2.2	13400	1.7	160	15	12900	3.1
Color Map Scale			0	12500	25000	37500	50000	62500	75000	87500	100000

1 ppm solutions. It was possible to monitor the sodium adduct in the 1 ppm glucose in 80:20 MeOH:water solution, but because this is derived from sodium contaminants at unknown concentrations, it was not desirable for this study.

Based upon the solvent parameter combination resulting in the highest signal intensity for each additive, the additive resulting in the highest signal intensity overall was ammonium TFA followed by ammonium fluoride, ammonium formate, lithium fluoride, ammonium bicarbonate, ammonium acetate, and no additive (Table 4-2). High signal intensity (up to 36 times that of solutions without an additive) was achieved over the entire range of pH values and concentrations investigated for ammonium TFA in 80:20 MeOH:water in the positive ionization mode.

4.4.2 Effect of Additive Concentration on Signal Intensity

Increasing the concentration of salt additives increases the conductivity and, thus, the concentration of the excess charge emitted from the ESI source.[17] It is expected to result in a higher concentration of ion-analyte complexes in solution, but it can also suppress ionization at higher concentrations. No general relationship between additive concentration and signal intensity was found under the ranges investigated, but it was shown to play a critical role in signal intensity optimization (Table 4-2). For example, the solvent parameter combination that resulted in the highest signal intensity among 80:20 organic:water solvent combinations, 0.05% ammonium TFA (at pH 2.9) in 80:20 MeOH:water in the positive ionization mode, was about 1.7 times higher than the solution at 1.00%. It was also found that optimal additive concentrations for a given ionization mode in either 80:20 MeOH:water or 80:20 ACN:water did not generally correspond to the optimal additive concentrations under different combinations of ionization mode and base solvent.

4.4.3 Effect of Solution pH on Signal Intensity

No general relationship between solution pH and signal intensity was found in solutions in which this was investigated (i.e., with ammonium TFA, ammonium acetate, and ammonium formate), although the solution pH had a dramatic effect on the signal intensity for some of these solvent parameter combinations, as shown in Table 4-2. Indeed, the highest signal intensity achieved for any 80:20 organic:water solvent combination was with 0.05% ammonium TFA at pH 2.9 in 80:20 MeOH:water in the positive ionization mode, which was more than 1.9 times greater than that of solutions at higher pH values with other parameters being the same.

4.4.4 Effect of Organic Cosolvent on Signal Intensity

MeOH and ACN differ in several properties relevant to ESI, including vapor pressure, surface tension, and gas-phase thermochemistry. In the positive ionization mode, the signal intensities for all solutions based on 80:20 ACN:water were lower than those based on 80:20 MeOH:water by more than an order of magnitude (Table 4-2). This phenomenon had been reported previously for ammoniated adducts of various monosaccharides,[14] sodiated adducts and protonated species of mono- di- and trisaccharides,[15] and ammoniated adducts and protonated species of a proprietary compound containing an unsaturated lactone and a methyl sulfone group.[18] In the negative ionization mode, most solutions based on 80:20 MeOH:water also had higher signal intensities than their 80:20 ACN:water counterparts. Because solutions with a high ACN content are useful in hydrophilic interaction liquid chromatography (HILIC) separations, it is notable the highest signal intensities obtained in 80:20 ACN:water were with 0.05% and 0.10% ammonium formate solutions at all pH values investigated, as well as with lithium fluoride at concentrations of 500 µM, 1 mM, and 5 mM.

MS scans of solutions containing 0.10% ammonium formate (at pH 7.9) in 80:20 MeOH:water and 80:20 ACN:water with and without 1 ppm glucose provided evidence that ACN suppresses the ionization of glucose ionized by ammonium adduction in the positive ionization mode by competing with glucose for ammonium adduction (Figure 4-1). For the samples that did not contain glucose, the MeOH-based solution had much less ion current resulting from ammonium-MeOH adducts (*m/z* 50, $[MeOH+NH_4]^+$; Figure 4-1A) than the ACN-based solution (*m/z* 59, $[ACN+NH_4]^+$ and *m/z* 100, $[2ACN+NH_4]^+$; Figure 4-1B). The ammonium-glucose adduct (*m/z* 198) was only detected in the MeOH-based solution (Figures 4-1C and 4-1D). An analogous experiment was performed with 1 mM lithium fluoride but was of limited value due to both lithium-MeOH and lithium-ACN adducts saturating the detector (data not shown).

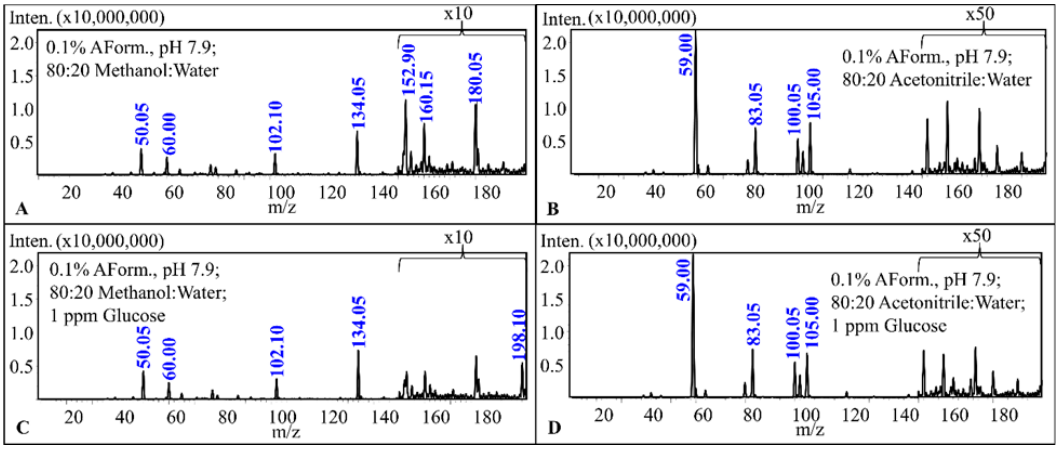

Figure 4-1. Average MS scan in the positive ionization mode from 1.00-1.50 min. A) 0.1% ammonium formate, pH 7.9, in 80:20 MeOH:water, B) 0.1% ammonium formate, pH 7.9, in 80:20 ACN:water, C) 1 ppm glucose in 0.1% ammonium formate, pH 7.9, in 80:20 MeOH:water, D) 1 ppm glucose in 0.1% ammonium formate, pH 7.9, in 80:20 ACN:water

4.4.5 Effect of Organic:Water Solvent Ratio on Signal Intensity

An increase in signal intensity was expected to accompany an increase in the organic cosolvent composition in organic:water solvents due to an increase in vapor pressures and a decrease in surface tension.[19] Increasing the MeOH:water ratio (up to 96% for lithium fluoride and ammonium fluoride and up to 98% for the other solutions) in the positive ionization mode led to a general increase in signal intensity, as shown in Figure 4-2A. However, in the case of ammonium fluoride there were values of MeOH:water ratios optimal for signal intensity at 40% and 50% MeOH. There was also a general increase of signal intensity with increasing MeOH content in the negative ionization mode (Figure 4-2B).

Increasing the ACN:water ratio in the positive ionization mode resulted in a severe decrease of the signal intensity, as shown in Figure 4-2C. In the negative ionization mode, a general increase of signal intensity with increasing ACN:water content was also observed (Figure 4-2D). However, the three additives that resulted in the highest signal intensities (ammonium formate, lithium fluoride, and ammonium acetate) had optimal ACN concentrations between 50% and 80%.

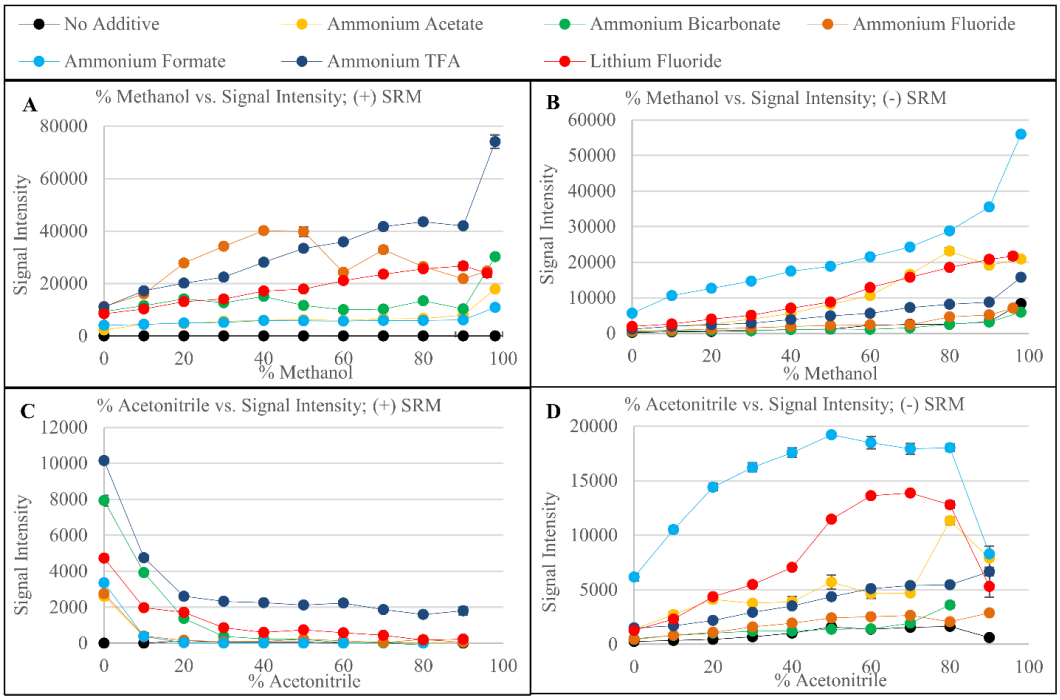

Figure 4-2. SRM signal intensity vs. % organic solvent for: A) methanol-based solvent systems in the positive ionization mode; B) acetonitrile-based solvent systems in the positive ionization mode; C) methanol-based solvent systems in the negative ionization mode; and D) acetonitrile-based solvent systems in the negative ionization mode

4.4.6 Day-to-Day Signal Intensity Reproducibility

The repeated analysis of certain sample solution combinations in 80:20 organic:water solvent for the organic:water solvent ratio experiments (Figure 4-2), and the pH/ additive concentrations/ MeOH:water vs. ACN:water experiments (Table 4-2), as well as those in 100% water measured separately in the MeOH and ACN sample sets in Figure 4-2, provided an opportunity to assess the day-to-day signal intensity reproducibility. This was determined as the % difference between the signal intensities of solutions measured separately in two different data sets. Only combinations of solvent

and ionization mode that had a signal intensity greater than 1000 were considered, and these results can be seen in Figure 4-3. In the positive ionization mode, it was found that

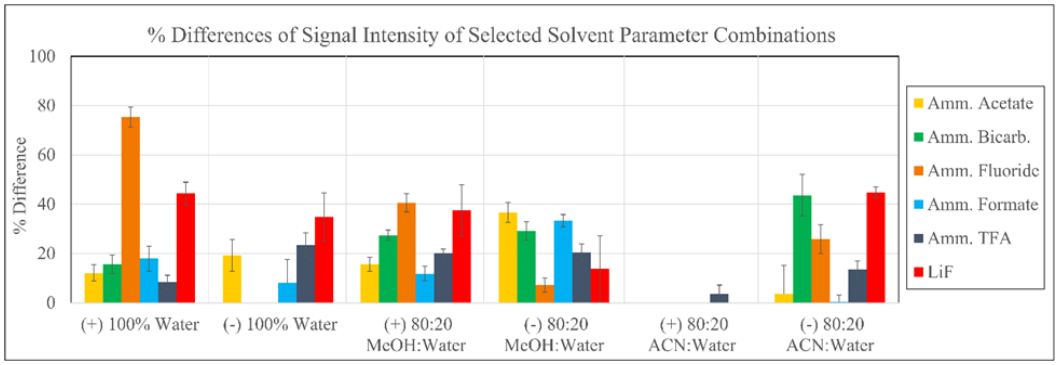

Figure 4-3. Percent differences of signal intensities of selected solvent parameter combinations for which signal intensity was at least 1000 measured on different days

Lithium fluoride and ammonium fluoride solutions in 100% water and 80:20 MeOH:water had the highest % difference. For both of these additives and for both 100% water and 80:20 MeOH:water, the experiments were performed within 1-2 days for each pair of data sets. Percent differences for these data sets ranged from 38% (lithium fluoride in 80:20 MeOH:water) to 75% (ammonium fluoride in 100% water), and their signal intensities invariably decreased from the first to second experiment for these experiments. This was thought to be caused by the adsorption of the relatively low concentrations of the salts in these solutions to the interior of the glass vials containing them. To explore this possibility, an additional FIA-ESI-MS/MS experiment was performed under the same instrumental parameters for a 1 ppm glucose solution in 1 mM ammonium fluoride in 80:20 MeOH:water along with its blank in separate glass vials after 8 and 24 hours after preparation as well as in a polypropylene tube 24 hours after preparation. For the glass vials, there was an increase of 6% and 8% in signal intensities from 8 to 24 hours in the

positive and negative ionization modes, respectively (data not shown). The signal intensities for the sample stored in the polypropylene tube were 84% and 105% greater than that in the glass vial 24 hours after preparation in the positive and negative ionization modes, respectively (data not shown). These data suggest that adsorption of the contents of a sample to the interior of its container can affect the signal intensity for an ESI-MS/MS experiment, but it is doubtful that the this explains the discrepancies illustrated in Figure 4-3 given that all samples were contained in glass vials. The cause of these differences is not known. The additives resulting in the highest percent differences in the negative ionization mode were ammonium bicarbonate (44%) and lithium fluoride (45%) in 80:20 ACN:water.

4.5 Conclusions

Exploring the influence of solvent parameters on the ESI-MS/MS signal intensity of a given analyte not only allows for the selection of the optimal solvent parameter combination resulting in high sensitivity and a low limit of detection, but it is also useful for assessing appropriate ranges of these parameters for upstream separation by LC without compromising sensitivity. In this study, the solvent was found to greatly influence the signal intensity of glucose; the most striking example of which was ACN:water solutions suppressing ionization in the positive ionization mode. Higher concentrations of the organic cosolvent generally enhanced the signal intensity in the negative ionization mode. Additionally, the choice of additive and its concentration and the solution pH also influenced its signal intensity. The highest signal intensities were obtained with ammonium TFA in MeOH:water solutions in the positive ionization mode for the entire ranges of concentrations and pH values investigated. For solutions composed of ACN:water, important for HILIC separations, the highest signal intensities resulted from ammonium formate at 0.05% and 0.10% for all pH values investigated as well as 500 µM

to 5 mM lithium fluoride solutions, all in the negative ionization mode. Admittedly, the use of more than one compound as model analytes for this study was considered as much more desirable after the conclusion of these experiments. Indeed, glucose-cation complexes may be particularly weak due to its lack of axial hydroxyl groups.[20] The mechanisms through which solvent parameters affect analyte signal intensity in ESI-MS are incredibly complex and involve changes in chemical equilibria in both solution and in the gas phase, including effects arising from changes in conductivity, vapor pressure, and surface tension. Clearly, more studies are needed to decouple these contributions. Ongoing research efforts include the investigation of response factors as a function of US concentration and solvent parameters, as well as the development of sensitive HILIC-ESI-MS methods for analyzing US in highly diluted real-world samples, such as beer and honey.

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Chapter 5

Use of a Continuous Stirred Tank Reactor for the Determination of Electrospray Response Factors and its Application to Underivatized Sugars Under Various Solvent Parameters

5.1 Abstract

The relationship between the electrospray ionization (ESI)-mass spectrometric (MS) response of an analyte and its concentration has been well studied for permanently-charged and basic analytes in the positive ionization mode, but there has been a lack of research effort for other analytes, and for the negative ionization mode, in general. In this study, this relationship was investigated for various adducts and deprotonated species of glucose, sucrose, and raffinose using a continuous stirred tank reactor coupled with ESI-tandem MS to obtain a continuum of response factors across a wide concentration range in both the positive and negative ionization mode with a single injection under 18 different combinations of solvents and additives. Profiles of response factors vs. concentrations varied widely and were dependent upon the analyte and solvent parameters. The use of ammonium trifluoroacetate resulted in the highest response factors for methanol-based and acetonitrile-based solvents in the positive and negative ionization mode, respectively. Ammonium acetate, ammonium formate, and lithium chloride in 80:20 acetonitrile:water in the negative ionization mode resulted in good linearities, useful for quantitative analysis. In the positive ionization mode, response factors tended to increase with an increase in the molecular weight of the analyte, and acetonitrile was generally found to decrease response factors. We have also demonstrated the ability of CSTR-ESI-MS to visualize ionization suppression in the presence of co-analytes. These data should be useful for liquid chromatography-ESI-MS method development for sugar analysis, to help guide the choice of mobile phase that will result in high sensitivity and linearity.

5.2 Introduction

The development of electrospray ionization (ESI)- mass spectrometry (MS), along with matrix-assisted laser desorption/ ionization-MS, in the 1980s revolutionized analytical chemistry, especially in its ability to analyze thermolabile biomolecules. ESI-MS is a remarkably sensitive and specific technique, but its response for a given analyte is dependent upon the analyte's physicochemical properties, instrumental and solvent parameters, and sample matrix components. [1–10] Increasing an analyte's response factor may be achieved through derivatization, [11,12] but that process adds labor and can complicate the analysis if the reaction is incomplete, or if multiple products are formed. Optimizing the instrumental and solvent parameters for high response factors across a wide concentration range is a more attractive option in terms of simplicity and throughput for the quantitative analysis of an analyte at an unknown concentration.

The relationship between the ESI-MS signal intensity of an analyte and its concentration has been studied for metals, [2,13] protonated basic compounds, [2,13] and quaternary amines, [2,14] but there is a particular lack of this research for adducts of neutral compounds and in the negative ionization mode. In general, an analyte's signal intensity increases linearly with its concentration up to a point where it levels off.[2,13,14] Indeed, this behavior has been successfully modeled on the assumption that an equilibrium exists between charged analyte molecules in the surface and the interior "phases" of the electrospray droplet.[1] According to this model, the excess charge resides at the droplet surface where analyte molecules are able to enter the gas phase as solvated ions, whereas analyte molecules in the interior "phase" of the droplet, where their charges are balanced by counterions, are unable to enter the gas phase.[1] Thus, the decrease of analyte response factors at higher concentrations are due to either the saturation of

analyte species at the droplet surface or competition with other charged species in the droplet for the occupation of surface sites.

Given the important roles that underivatized sugars (US) play in nutrition, food quality, and other biological and commercial applications, they are an attractive analyte set for studying the ESI-MS response factor as a function of concentration for neutral compounds. Literature on ESI-MS response factors or signal intensities under different solvent parameters for US have been primarily limited to application studies, as well as some fundamental studies that include few variables.[15–20] Mallet *et al.* investigated the effects of various additives at several concentrations on the ESI-MS signal intensity of raffinose (detected as a deprotonated species), along with other analytes, by separately pumping analyte and additive solutions into a tee leading to the ESI source.[3] Relative to solutions containing only 50:50 methanol (MeOH):water, those containing perfluorinated carboxylic acids were found to dramatically decrease signal intensity relative to the pure solvents, while those containing ammonium hydroxide led to the highest gain in signal intensity.[3] Recently, we have studied ESI- tandem MS (MS/MS) signal intensities of glucose under a variety of solvent parameters including the additive and its concentration, pH, and the organic cosolvent and its ratio to water in both the positive and negative ionization mode using flow injection analysis (FIA).[21] Our most notable findings included 1) evidence for ionization suppression by acetonitrile (ACN) in the positive ionization mode, 2) high signal intensities for MeOH-water-based solutions containing ammonium trifluoroacetate (ammonium TFA) under a wide range of additive concentrations and pH values in the positive ionization mode, and 3) high signal intensities for ACN-water-based solutions containing ammonium formate as well as lithium fluoride (LiF) in the negative ionization mode.[21]

In this study, the relationship between the ESI-MS/MS response factors and concentrations for glucose, sucrose, and raffinose (model analytes for US), under various solvent parameters was investigated using a continuous stirred tank reactor (CSTR). The CSTR allowed for the determination of a continuum of ESI-MS/MS response factors within a wide concentration range from a single injection. Solvent parameters explored included the organic cosolvent (80:20 MeOH:water and 80:20 ACN:water) and the additive (ammonium acetate, ammonium formate, ammonium hydroxide, ammonium TFA, lithium chloride (LiCl), lithium fluoride (LiF), and sodium chloride (NaCl)). In addition, for solutions containing NaCl and LiCl, two sets of response factor vs. concentration profiles were separately generated from two different multiple reaction monitoring (MRM) transitions for each analyte in both the positive and negative ionization modes.

5.3 Experimental

5.3.1 CSTR Theory

A CSTR consists of a chamber with at least one inlet and one outlet and is operated with a continuous flow of a fluid through the device. When a small volume of sample is injected into the CSTR flowpath, a uniform, though continuously diluted, chemical composition throughout the chamber is ensured by mechanical agitation through the incorporation of a stir bar in the device. When a plug of a solution of analyte of a small volume relative to that of the CSTR (5 and 661 µL for the plug and CSTR, respectively, in this study) is introduced into the chamber from the inlet flow stream, it can be assumed that the analyte is immediately dispersed throughout the chamber and diluted by a factor equal to the quotient of the volume of the analyte solution plug and that of the CSTR. The concentration of the analyte in the CSTR, and, thus, the concentration of the analyte in the CSTR effluent, is then diluted exponentially over time, as described quantitatively in Equation 1**.**

$$c(t) = c_i * \frac{V_{plug}}{V_{CSTR}} * e^{-\frac{F*t}{V_{CSTR}}}, (1)$$

Where c_i is the initial concentration of the analyte in the plug, V_{plug} is the volume of the plug injected, V_{CSTR} is the volume of the CSTR chamber, e is Euler's constant, F is the volumetric flow rate, and t is time elapsed after the introduction of the sample plug into the CSTR.

5.3.2 Chemicals

D-(+)-glucose (≥99.5%), sucrose (≥99.5%), *D*-(+)-raffinose pentahydrate (≥98.0%), LiCl (≥99%), and NaCl were purchased from Sigma-Aldrich (Steinheim, Germany). Trifluoroacetic acid (LC-MS; ≥99.5%) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Formic acid (LC-MS; 98-100%) was purchased from EMD Millipore (Billerica, MA, USA). LiF (99.99%) was purchased from Alfa Aesar (Haverhill, MA, USA). Acetic acid (LC-MS), ACN (LC-MS), ammonium hydroxide (LC-MS; ≥25% in water), MeOH (LC-MS), and water (LC-MS) were purchased from Honeywell (Morris Plains, NJ, USA).

5.3.3 Sample Preparation

Aqueous stock solutions of 250 mM glucose, sucrose, and raffinose were separately prepared and forced through a 0.45 µm PTFE syringe filter. These were kept frozen when not in use. Aqueous additive solutions were used within 2 days after preparation. For ammonium acetate, ammonium formate, and ammonium TFA, 10 mM aqueous solutions of their acids at a pH of 6.8, adjusted by ammonium hydroxide, were prepared; the pH of the organic-water solutions containing these salts are reported as the pH of their aqueous stock solutions. Sample solutions were prepared from the analyte stock solutions, pure organic solvents, and the appropriate volume of the additive solution under investigation for a final composition of 80:20 organic:water solvent. Sample

solutions in 80:20 MeOH:water contained 13.1 mM of each glucose, sucrose, and raffinose, and, due to solubility, those in 80:20 ACN:water contained 658 µM of each analyte. Upon the initial dilution of samples in the CSTR, the concentrations of the analytes (c_0) in the MeOH-based and ACN-based sample solutions were 100 µM and 5 µM, respectively.

5.3.4 CSTR-ESI-MS/MS

All measurements were taken on a Shimadzu LCMS-8040 (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) triple quadrupole mass spectrometer with an ESI source and the following LC modules (all Prominence series from Shimadzu): DGU-20A5 online degasser; CBM-20A communications bus module; LC-20AD XR LC pumps; and an SIL-20AC XR autosampler with a 50 µL injection loop. For all experiments, one MRM transition in both the positive and negative ionization mode was monitored for each analyte. These were chosen for their high signal intensities in samples and low signal intensities in blanks. For each solvent parameter combination, instrumental parameters were optimized to maximize the signal intensity of the MRM transition which had the lowest signal intensity, except for glucose in the positive ionization mode in 80:20 ACN:water solutions; glucose was not detected in any of these solutions. MRM transition selection and the optimization of instrumental parameters were performed by FIA of 50 µL plugs containing 100 µM of each analyte; both the sample solvent and flow solvent were composed of the solvent parameter combination under investigation. MRMs along with their precursor ions and collision energies for each analyte and solvent parameter combination can be found in Table 5-1. The following instrumental parameters were the same for all experiments: Organic solvent flow rate, 160 µL/ min; aqueous additive solution flow rate, 40 µL/ min; injection volume, 5 µL; dwell time, 100 msec; loop time,

648 msec; and CID gas (Ar) pressure, 230 kPa. Other instrumental parameters optimized for each sample set are displayed in Table 5-2.

The CSTR (described previously)[22] was equipped with a 3 x 6.4 mm Teflon-coated magnetic stir bar (Bel-Art Products, Wayne, NJ, USA) and stirred with a magnetic stir plate (Fisher Scientific International, Inc., Hampton, NH, USA, catalog number 11-520-16SH) at a “Stir” setting of 1. The interior volume of the CSTR, measured gravimetrically with the stir bar inside, was 661 ± 7 µL. For the CSTR experiments, PEEK tubing was used to connect the autosampler to the inlet of the CSTR and the ESI source to the outlet of the CSTR. Analysis times ranged from 30 to 60 minutes, until all MRM signal intensities decayed into stable baselines. Samples were analyzed in triplicate.

The signal intensity data for all MRMs were exported from *LabSolutions* (v.5.80, Shimadzu) to Microsoft (Redmond, WA, USA) Excel (v.15.0.4963.1000). Signal intensities with respect to time were smoothed by a 9-cell rolling average. Blank measurements for each MRM transition were taken as the average of the final minute of each run from the rolling averaged signal intensities. The time domain was converted to a concentration domain using Equation 1. The response factors were determined as the quotient of the blank-corrected rolling averaged signal intensities and the concentration.

5.4 Results and Discussion

5.4.1 Experimental Optimization

In our previous study, we had compared blank-corrected signal intensities of 1 ppm glucose in various sample solvent parameter combinations using the same generic instrumental parameters for each sample with the rationale that the data would be useful for ESI-MS/MS method development for underivatized saccharide analysis, in general.[21] However, it is not clear that the results would remain valid for other underivatized saccharides/ sugars or under different instrumental parameters. For this reason, we

Table 5-1. SRMs monitored and their collision energies and precursor ions for glucose, sucrose, and raffinose

Additive Solution	Organic Cosolvent	(+, Positive Ionization Mode)			
			MRM		
		Precursor	Glucose	Sucrose	Raffinose
Water	Methanol	$[M+Na]^+$	203 > 23; -30 V	365 > 203; -27 V	527 > 365; -30 V
Water	Acetonitrile	$[M+Na]^+$	203 > 23; -30 V	365 > 203; -27 V	527 > 365; -30 V
500 µM NaCl (1)	Methanol	$[M+Na]^+$	203 > 23; -30 V	365 > 203; -27 V	527 > 365; -30 V
500 µM NaCl (1)	Acetonitrile	$[M+Na]^+$	203 > 23; -30 V	365 > 203; -27 V	527 > 365; -30 V
500 µM NaCl (2)	Methanol	$[2M+Na]^+$	383 > 203; -13 V	707 > 365; -15 V	1031 > 527; -25 V
500 µM NaCl (2)	Acetonitrile	$[2M+Na]^+$	383 > 203; -13 V	707 > 365; -15 V	1031 > 527; -25 V
500 µM LiCl (1)	Methanol	$[M+Li]^+$	187 > 127; -18 V	349 > 187; -30 V	511 > 349; -36 V
500 µM LiCl (1)	Acetonitrile	$[M+Li]^+$	187 > 127; -18 V	349 > 187; -30 V	511 > 349; -36 V
500 µM LiCl (2)	Methanol	$[2M+Li]^+$	367 > 187; -19 V	691 > 349; -19 V	1015 > 511; -25 V
500 µM LiCl (2)	Acetonitrile	$[2M+Li]^+$	367 > 187; -19 V	691 > 349; -19 V	1015 > 511; -25 V
500 µM LiF	Methanol	$[M+Li]^+$	187 > 127; -18 V	349 > 187; -30 V	511 > 349; -36 V
500 µM LiF	Acetonitrile	$[M+Li]^+$	187 > 127; -18 V	349 > 187; -30 V	511 > 349; -36 V
NH_4OH, pH 11	Methanol	$[M+NH_4]^+$	198 > 85; -18 V	360 > 163; -14 V	522 > 163; -22 V
NH_4OH, pH 11	Acetonitrile	$[M+NH_4]^+$	198 > 85; -18 V	360 > 163; -14 V	522 > 163; -22 V
10 mM NH_4OAc, pH 6.8	Methanol	$[M+NH_4]^+$	198 > 85; -18 V	360 > 163; -14 V	522 > 163; -22 V
10 mM NH_4OAc, pH 6.8	Acetonitrile	$[M+NH_4]^+$	198 > 85; -18 V	360 > 163; -14 V	522 > 163; -22 V
10 mM NH_4Form, pH 6.8	Methanol	$[M+NH_4]^+$	198 > 85; -18 V	360 > 163; -14 V	522 > 163; -22 V
10 mM NH_4Form, pH 6.8	Acetonitrile	$[M+NH_4]^+$	198 > 85; -18 V	360 > 163; -14 V	522 > 163; -22 V
10 mM NH_4TFA, pH 6.8	Methanol	$[M+NH_4]^+$	198 > 85; -18 V	360 > 163; -14 V	522 > 163; -22 V
10 mM NH_4TFA, pH 6.8	Acetonitrile	$[M+NH_4]^+$	198 > 85; -18 V	360 > 163; -14 V	522 > 163; -22 V
Additive Solution	Organic Cosolvent	(-, Negative Ionization Mode)			
			MRM		
		Precursor	Glucose	Sucrose	Raffinose
Water	Methanol	$[M-H]^-$	179 > 101; 10 V	341 > 179; 14 V	503 > 179; 24 V
Water	Acetonitrile	$[M-H]^-$	179 > 101; 10 V	341 > 179; 14 V	503 > 179; 24 V
500 µM NaCl (1)	Methanol	$[M-H]^-$	179 > 101; 10 V	341 > 179; 14 V	503 > 179; 24 V
500 µM NaCl (1)	Acetonitrile	$[M-H]^-$	179 > 101; 10 V	341 > 179; 14 V	503 > 179; 24 V
500 µM NaCl (2)	Methanol	$[M+Cl]^-$	215 > 35; 10 V	377 > 341; 15 V	539 > 503; 20 V
500 µM NaCl (2)	Acetonitrile	$[M+Cl]^-$	215 > 35; 10 V	377 > 341; 15 V	539 > 503; 20 V
500 µM LiCl (1)	Methanol	$[M-H]^-$	179 > 101; 10 V	341 > 179; 14 V	503 > 179; 24 V
500 µM LiCl (1)	Acetonitrile	$[M-H]^-$	179 > 101; 10 V	341 > 179; 14 V	503 > 179; 24 V
500 µM LiCl (2)	Methanol	$[M+Cl]^-$	215 > 35; 10 V	377 > 341; 15 V	539 > 503; 20 V
500 µM LiCl (2)	Acetonitrile	$[M+Cl]^-$	215 > 35; 10 V	377 > 341; 15 V	539 > 503; 20 V
500 µM LiF	Methanol	$[M-H]^-$	179 > 101; 10 V	341 > 179; 14 V	503 > 179; 24 V
500 µM LiF	Acetonitrile	$[M-H]^-$	179 > 101; 10 V	341 > 179; 14 V	503 > 179; 24 V
NH_4OH, pH 11	Methanol	$[M-H]^-$	179 > 101; 10 V	341 > 179; 14 V	503 > 179; 24 V
NH_4OH, pH 11	Acetonitrile	$[M-H]^-$	179 > 101; 10 V	341 > 179; 14 V	503 > 179; 24 V
10 mM NH_4OAc, pH 6.8	Methanol	$[M+OAc]^-$	239 > 179; 7 V	401 > 341; 13 V	563 > 503; 14 V
10 mM NH_4OAc, pH 6.8	Acetonitrile	$[M+OAc]^-$	239 > 179; 7 V	401 > 341; 13 V	563 > 503; 14 V
10 mM NH_4Form, pH 6.8	Methanol	$[M+Form]^-$	225 > 179; 8 V	387 > 341; 12 V	549 > 503; 15 V
10 mM NH_4Form, pH 6.8	Acetonitrile	$[M+Form]^-$	225 > 179; 8 V	387 > 341; 12 V	549 > 503; 15 V
10 mM NH_4TFA, pH 6.8	Methanol	$[M+TFA]^-$	293 > 113; 10 V	455 > 113; 16 V	617 > 113; 22 V
10 mM NH_4TFA, pH 6.8	Acetonitrile	$[M+TFA]^-$	293 > 113; 10 V	455 > 113; 16 V	617 > 113; 22 V

Table 5-2. Optimized instrumental parameters for SRM transitions

Additive Solution	Organic Cosolvent	Positive Voltage (kV)	Negative Voltage (kV)	Nebulizing Gas (L/min)	Drying Gas (L/min)	Desolvation Line Temp. (°C)	Heat Block Temp. (°C)
Water	Methanol	5	-4.5	1.5	5	250	250
Water	Acetonitrile	3	-4.5	1	5	200	250
500 µM NaCl (1)	Methanol	5	-4.5	1.5	5	250	200
500 µM NaCl (1)	Acetonitrile	5	-4.5	1	5	200	200
500 µM NaCl (2)	Methanol	5	-4.5	1.5	5	250	200
500 µM NaCl (2)	Acetonitrile	5	-4.5	1.5	5	250	250
500 µM LiCl (1)	Methanol	5	-4.5	2.5	15	250	250
500 µM LiCl (1)	Acetonitrile	5	-4.5	0.5	3	200	200
500 µM LiCl (2)	Methanol	5	-4.5	2.5	13	250	200
500 µM LiCl (2)	Acetonitrile	5	-4.5	0.5	13	250	250
500 µM LiF	Methanol	5	-4.5	1.5	15	250	300
500 µM LiF	Acetonitrile	5	-4.5	1.5	13	250	200
NH_4OH, pH 11	Methanol	5	-4.5	1.5	15	200	200
NH_4OH, pH 11	Acetonitrile	5	-4.5	2	15	200	200
0.10% NH_4OAc, pH 6.8	Methanol	5	-4.5	1.5	9	200	250
0.10% NH_4OAc, pH 6.8	Acetonitrile	5	-4.5	1.5	9	250	200
0.10% NH_4Form, pH 6.8	Methanol	5	-4.5	1.5	15	200	250
0.10% NH_4Form, pH 6.8	Acetonitrile	5	-4.5	2	15	200	200
0.10% NH_4TFA, pH 6.8	Methanol	5	-4.5	3	5	250	250
0.10% NH_4TFA, pH 6.8	Acetonitrile	5	-4.5	3	5	250	250

included three model analytes and chose to optimize instrumental parameters for each solvent parameter combination in this study.

Signal intensities generally increased with increased needle voltages in both the positive and negative ionization modes, often linearly from at least 2 – 4.5 kV in both ionization modes. However, signal intensities were much less affected by needle voltages for sample solutions that did not contain additives, likely due to their low concentrations of electrolytes. Voltages exceeding -4.5 kV in the negative ionization mode were accompanied by an unstable baseline, presumably due to corona discharge [4], and, thus, were not used. Desolvation line bias voltages were also explored, but altering these from a default setting of 0 V did not result in any increase of signal intensity.

Desolvation line and heat block temperatures significantly affected signal intensities. The MRM with the lowest signal intensity was found for glucose (either in the

positive or negative ionization mode) in most sample solvents, so these temperatures were usually optimized for it. Optimal temperatures for glucose were usually lower than those for sucrose and raffinose. For example, the 100 µM NaCl in 80:20 ACN:water (1) solution had a desolvation line temperature of 200 °C, optimized for $[Glc\text{-}H]^-$, but the signal intensities for $[Suc+Na]^+$ and $[Raf+Na]^+$ MRM transitions were less than 30% of their values at a desolvation line temperature of 300 °C (data not shown). Differences in optimal temperatures amongst the analytes were likely due to a combination of higher temperatures required to desolvate higher molecular weight ions, as well as a lower thermal stability of lower molecular weight adducts (in the case of $[Glc\text{-}H]^-$, it may be formed by the loss of a neutral molecule from an anionic adduct, such as HCl from $[Glc+Cl]^-$, in the intermediate pressure region).[23]

5.4.2 Ionization Suppression by Co-Analytes

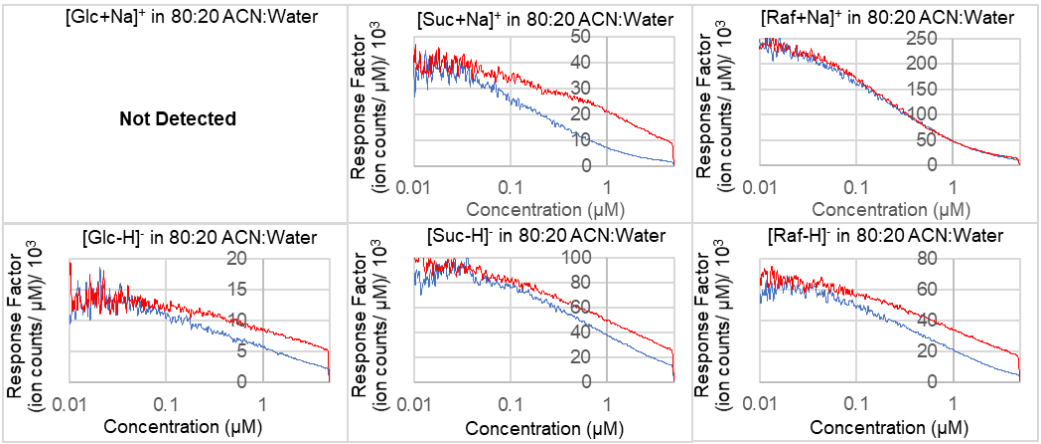

Figure 5-1. Response factor vs. concentration for [M+Na]+ and [M-H]- in 80:20 ACN:Water for solutions containing glucose, sucrose, and raffinose (blue) and solutions containing individual analytes (red)

Analyzing samples as mixtures of analytes in solution was highly desired, given the amount of time involved for each analysis, but their response factors may be decreased relative to those in solution without co-analytes due to ionization suppression. To assess ionization suppression by co-analytes, response factor vs. concentration profiles were generated for mixtures of glucose, sucrose, and raffinose as well as for the individual analytes in 80:20 ACN:water, 80:20 MeOH:water, and 100 µM NaCl in 80:20 MeOH:water (1). These profiles can be found in Figures 5-1, A-1, and A-2. Generally, the response factor profiles for solutions containing a single analyte and those containing a mixture of the three converged around 10 to 100 nM as their concentrations decreased. At relatively high concentrations, solutions containing individual analytes usually had higher response factors than those containing all three analytes. This was attributed to ionization suppression in the solutions containing all three analytes due to the presence of co-analytes.

5.4.3 General Characterization of Response Factor vs. Concentration Profiles

Response factor vs. concentration profiles in their reproducible concentration ranges for mixtures of glucose, sucrose, and raffinose in various solvent and additive combinations are displayed in Figures 5-2 through 5-5 and A-3 through A-18. Most of these profiles can be broadly classified into the following groups: (A) response factors increase continuously with decreasing analyte concentration (e.g., $[M-H]^-$ for 80:20 ACN:water (Figure 5-2)); (B) response factors increase with decreasing analyte concentration, flattening out at a certain point (e.g., $[M-H]^-$ for 100 µM LiF in 80:20 ACN:water (Figure 5-3)); (C) response factors are nearly constant throughout the analyte concentration range (e.g., $[M-H]^-$ for 100 µM LiCl in 80:20 ACN:water (Figure 5-4)); or (D) response factors reach a maximum at a certain concentration (e.g., $[2M+Na]^+$ for 100 µM NaCl in 80:20 MeOH:water (Figure 5-5)).

Groups A-C are all compatible with different concentration regimes in Enke's partition model,[1] but D is not. Group D consists of $[2M+Na]^+$ and $[2M+Li]^+$; and their behavior can readily be explained on the basis of thermodynamic equilibrium. The presence and absence of the $[2M+Na]^+$ adduct at relatively high and low analyte concentrations, respectively, has also been reported by Kruve *et al.* for glyceryl triacetate and glycerol tributyrate.[24] Solvent parameters yielding profiles, that fall into groups B and C would be the most useful for quantitative analysis. The most promising combinations of solvent parameters and adducts monitored found in this study for quantitative analysis, chosen for their high response factors and being in groups B or C, include $[M+Cl]^-$ in 100 µM NaCl in 80:20 ACN:water (Figure A-6), $[M+OAc]^-$ in 2 mM AA in 80:20 ACN:water (Figure A-14), and $[M+Form]^-$ in 2 mM AF in 80:20 ACN:water (Figure A-16).

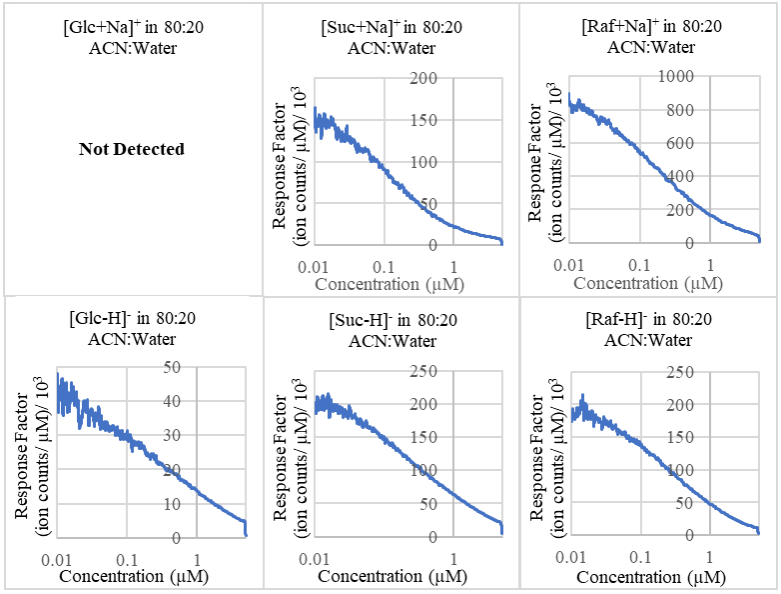

Figure 5-2. Response factor vs. concentration for $[M+Na]^+$ and $[M-H]^-$ in 80:20 ACN:water

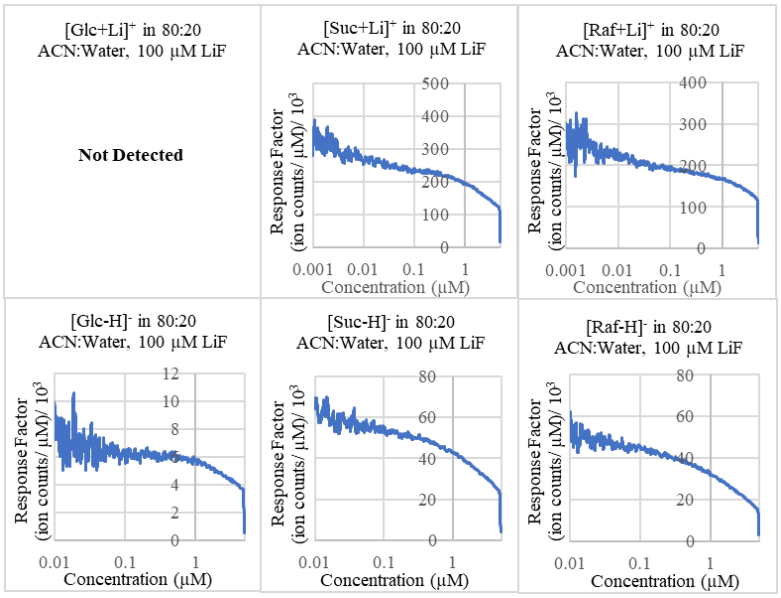

Figure 5-3. Response factor vs. concentration for $[M+Li]^+$ and $[M-H]^-$ in 100 µM LiF in 80:20 ACN:water

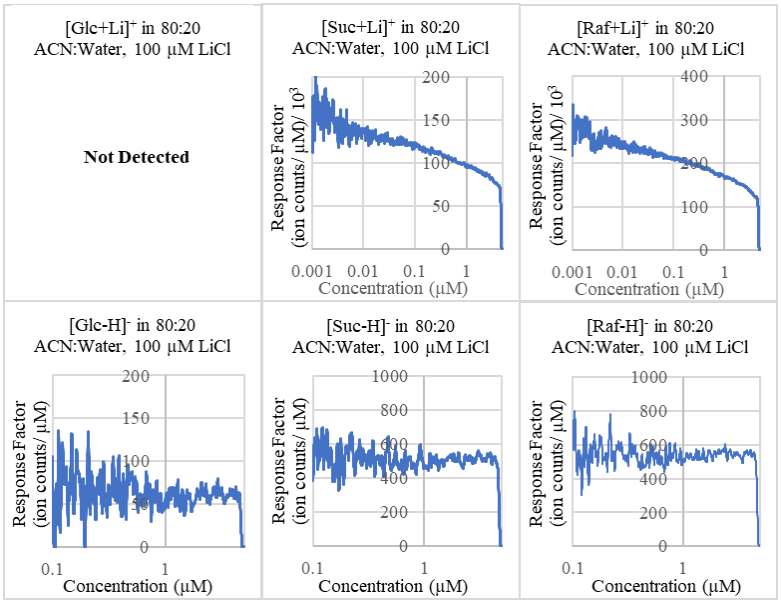

Figure 5-4. Response factor vs. concentration for $[M+Li]^+$ and $[M-H]^-$ in 100 µM LiCl in 80:20 ACN:water

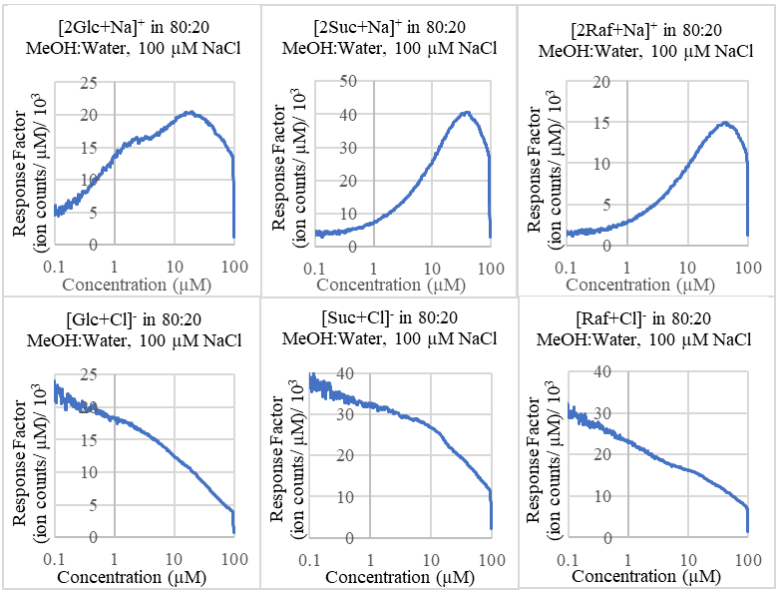

Figure 5-5. Response factor vs. concentration for $[2M+Na]^+$ and $[M+Cl]^-$ in 100 µM NaCl in 80:20 MeOH:water

5.4.4 Effects of Analyte on Response Factors

Response factors for all combinations of analytes, solvent parameters, and ionization modes at 100 nM are displayed in Table 5-3; all further discussion on response factors in this paper will be referencing the values in this table. This concentration was chosen for having response factors in the reproducible range for most sample solutions and having minimal ionization suppression by co-analytes. The relative standard deviations (RSD)s of the values displayed in Table 5-3 were less than 15% for response factors greater than 1,000; for response factors less than 1,000, RSDs were less than 20%, except for $[2Suc+Na]^+$ (34%) and $[2Raf+Na]^+$ (35%) for 100 µM NaCl in 80:20 ACN:water, $[2Suc+Li]^+$ (23%) for 100 µM LiCl in 80:20 MeOH:water, and $[Glc-H]^-$ (24%) for 100 µM LiCl in 80:20 ACN:water.

Table 5-3. Response factors at 100 nM

Additive Solution	Organic Cosolvent	(+, Positive Ionization Mode) MRM			
		Precursor	Glucose	Sucrose	Raffinose
Water	Methanol	$[M+Na]^+$	31,000	1,800,000	5,900,000
Water	Acetonitrile	$[M+Na]^+$	-	91,000	540,000
500 µM NaCl (1)	Methanol	$[M+Na]^+$	48,000	860,000	980,000
500 µM NaCl (1)	Acetonitrile	$[M+Na]^+$	-	57,000	390,000
500 µM NaCl (2)	Methanol	$[2M+Na]^+$	4,800	3,700	1,300
500 µM NaCl (2)	Acetonitrile	$[2M+Na]^+$	-	380	540
500 µM LiCl (1)	Methanol	$[M+Li]^+$	2,100	180,000	90,000
500 µM LiCl (1)	Acetonitrile	$[M+Li]^+$	-	120,000	210,000
500 µM LiCl (2)	Methanol	$[2M+Li]^+$	2,500	700	50
500 µM LiCl (2)	Acetonitrile	$[2M+Li]^+$	-	300	300
500 µM LiF	Methanol	$[M+Li]^+$	400	210,000	210,000
500 µM LiF	Acetonitrile	$[M+Li]^+$	-	230,000	190,000
NH_4OH, pH 11	Methanol	$[M+NH_4]^+$	39,000	570,000	800,000
NH_4OH, pH 11	Acetonitrile	$[M+NH_4]^+$	-	320,000	1,100,000
10 mM NH_4OAc, pH 6.8	Methanol	$[M+NH_4]^+$	45,000	520,000	850,000
10 mM NH_4OAc, pH 6.8	Acetonitrile	$[M+NH_4]^+$	-	45,000	450,000
10 mM NH_4Form, pH 6.8	Methanol	$[M+NH_4]^+$	12,000	280,000	420,000
10 mM NH_4Form, pH 6.8	Acetonitrile	$[M+NH_4]^+$	-	130,000	520,000
10 mM NH_4TFA, pH 6.8	Methanol	$[M+NH_4]^+$	110,000	310,000	190,000
10 mM NH_4TFA, pH 6.8	Acetonitrile	$[M+NH_4]^+$	-	23,000	190,000
Additive Solution	Organic Cosolvent	(-, Negative Ionization Mode) MRM			
		Precursor	Glucose	Sucrose	Raffinose
Water	Methanol	$[M-H]^-$	25,000	210,000	260,000
Water	Acetonitrile	$[M-H]^-$	29,000	150,000	140,000
500 µM NaCl (1)	Methanol	$[M-H]^-$	500	6,000	3,400
500 µM NaCl (1)	Acetonitrile	$[M-H]^-$	-	400	400
500 µM NaCl (2)	Methanol	$[M+Cl]^-$	22,000	38,000	30,000
500 µM NaCl (2)	Acetonitrile	$[M+Cl]^-$	43,000	79,000	70,000
500 µM LiCl (1)	Methanol	$[M-H]^-$	-	-	-
500 µM LiCl (1)	Acetonitrile	$[M-H]^-$	-	500	500
500 µM LiCl (2)	Methanol	$[M+Cl]^-$	1,700	2,800	2,700
500 µM LiCl (2)	Acetonitrile	$[M+Cl]^-$	600	2,100	2,600
500 µM LiF	Methanol	$[M-H]^-$	5,400	28,000	21,000
500 µM LiF	Acetonitrile	$[M-H]^-$	6,000	53,000	44,000
NH_4OH, pH 11	Methanol	$[M-H]^-$	6,500	35,000	34,000
NH_4OH, pH 11	Acetonitrile	$[M-H]^-$	5,700	44,000	40,000
10 mM NH_4OAc, pH 6.8	Methanol	$[M+OAc]^-$	73,000	130,000	150,000
10 mM NH_4OAc, pH 6.8	Acetonitrile	$[M+OAc]^-$	19,000	73,000	88,000
10 mM NH_4Form, pH 6.8	Methanol	$[M+Form]^-$	18,000	60,000	57,000
10 mM NH_4Form, pH 6.8	Acetonitrile	$[M+Form]^-$	14,000	62,000	59,000
10 mM NH_4TFA, pH 6.8	Methanol	$[M+TFA]^-$	90,000	95,000	54,000
10 mM NH_4TFA, pH 6.8	Acetonitrile	$[M+TFA]^-$	120,000	130,000	57,000

Glucose had the lowest response factor for most of the solvent parameter and ionization mode combinations, lower than that of sucrose and raffinose by a factor of more than 200 in the case of $[M+Li]^+$ for 100 µM LiF in 80:20 MeOH:water. Glucose was not detected in the positive ionization mode in any solvents based on 80:20 ACN:water (see discussion below in *Effects of Solvent on Response Factors*). Exceptions in which glucose did not have the lowest response factor included $[2M+Na]^+$ and $[2M+Li]^+$ for 100 µM NaCl and 100 µM LiCl, respectively, in 80:20 MeOH:water in the positive ionization mode and $[M+TFA]^-$ for 2 mM AT in both 80:20 MeOH:water and 80:20 ACN:water in the negative ionization mode.

Raffinose had the highest response factor for most solvent parameters in the positive ionization mode. Sucrose and raffinose had similar response factors in the negative ionization mode with $[M+TFA]^-$ for 2 mM ammonium TFA in both 80:20 MeOH:water and 80:20 ACN:water being the most dramatic exception. For these solutions, the sucrose MRM transition response factors were greater than those of raffinose by 73% and 120% for 80:20 MeOH:water and 80:20 ACN:water solutions, respectively.

It was expected for response factors to increase with molecular weight, given the increase in the number of hydroxyl groups available for binding to ions and deprotonation. This trend was generally observed in the positive ionization mode with the exceptions of $[2M+Na]^+$ and $[2M+Li]^+$ MRM transitions. In the negative ionization mode, response factors invariably increased from glucose to sucrose but no generalization could be made from sucrose to raffinose. Along with the number of hydroxyl groups, their arrangement (e.g. axial vs. equatorial) can also affect the stability of adducts, and therefore, their response factors.[25] Indeed, Kruve *et al.* reported that the ability of oxygen- and nitrogen-containing bases to chelate to sodium ions greatly affected their ionization

efficiencies.[24] It is also possible that the relatively low desolvation line and heat block temperatures used for optimizing glucose MRM transitions may not adequately desolvate raffinose, nor sucrose. Lastly, the differential efficiency of the production of product ions from precursor ions in the collision cell may also play a role.

5.4.5 Effects of Solvent on Response Factors

Glucose was not detected in the positive ionization mode for any solution based on 80:20 ACN:water, in agreement with our previous study where we provided evidence that ACN directly suppresses ionization by competing with glucose for cation adduction.[21] Low ESI-MS signal intensity for underivatized sugars in the positive ionization mode in ACN-water solvents has also been reported by other research groups.[19,26] $[M+Na]^+$ and $[2M+Na]^+$ response factors for sucrose and raffinose both decreased from 80:20 MeOH:water to 80:20 ACN:water solutions, though this decrease was less dramatic for raffinose (Table 5-3.). This decrease in the degree of ionization suppression with increasing molecular weight is likely due to an increase in the number of hydroxyl groups, which may result in more points of interaction and hence, stronger binding of the analytes to the adduct-forming ions. In 100 µM LiCl solutions for $[M+Li]^+$ and $[2M+Li]^+$, response factors for sucrose decreased to a much lesser extent from 80:20 MeOH:water to 80:20 ACN:water than for $[M+Na]^+$ and $[2M+Na]^+$ in solutions where these MRM transitions were monitored, though for raffinose the response factors were actually enhanced. The response factors for $[M+Li]^+$ in 100 µM LiF in 80:20 ACN:water and 80:20 MeOH:water were similar for both sucrose and raffinose. The $[M+NH_4]^+$ response factors for sucrose decreased from 80:20 MeOH:water to 80:20 ACN:water, although the extent of which depended upon the counterion; no general trend was observed for raffinose.

The influence of the solvent on response factors in the negative ionization mode was generally less dramatic than that in the positive ionization mode, although it did

influence the response factor in many cases. The most striking example was found for $[M-H]^-$ in 100 µM NaCl where response factors in the ACN-based solutions were lower by a factor of more than 10 for all analytes relative to the MeOH-based solution.

5.4.6 Effects of Additives on Response Factors

The highest overall response factors were obtained by monitoring $[M+TFA]^-$ for 2 mM ammonium TFA in 80:20 ACN:water for glucose and $[M+Na]^+$ in 80:20 MeOH:water without an additive for sucrose and raffinose. No additive in any solvent or ionization mode increased the response factor for each analyte relative to solutions that did not contain an additive, though some did increase the response factor for the analyte with the lowest response factor. The additive that led to the highest response factor of the analyte with the lowest response factor for that additive was ammonium TFA for both MeOH- and ACN-based solvents, monitoring $[M+NH_4]^+$ and $[M+TFA]^-$ in the positive and negative ionization mode, respectively. Monitoring $[M+NH_4]^+$ for ammonium TFA solutions in 80:20 MeOH:water was also found to result in the highest signal intensity for glucose in our previous study.[21]

It was unexpected that the $[M+Na]^+$ response factors for sucrose and raffinose in pure solvents were higher than those in 100 µM NaCl, although it has been reported that the addition of low concentrations of sodium acetate to an ACN:water mobile phase in an LC-MS method resulted in lower signal intensities for glucose and glycerol.[27] Kruve and Kaupmees also reported higher formation efficiencies of sodium adducts for 17 different nitrogen- and oxygen-containing bases for 80:20 ACN:water without any additives relative to solutions containing additives, although this was accompanied by a decrease in repeatability.[28] For these cases, it is likely that there are competitive effects of ionization suppression and higher concentrations of $[M+Na]^+$ complexes in solution from the addition of sodium ions. It is also notable that a maxima at around 100 nM for

raffinose and a more faint hump near 50 nM for sucrose in the $[M+Na]^+$ response factor-concentration plots are present in pure 80:20 MeOH:water solvents, but not those with 100 µM NaCl (Figures A-3 and A-4)). The cause of this is not known.

5.4.7 Effects of Counterions on Response Factors

Monitoring some of the same adducts with different additives provided an opportunity to assess the effect of counterions on their response factors. Response factors for $[M+Cl]^-$ for 100 µM NaCl solutions in both 80:20 MeOH:water and 80:20 ACN:water were more than 10 times higher than those in 100 µM LiCl for all analytes. In the response factor vs. concentration plots, there were also maxima between 10 and 100 µM for $[M+Cl]^-$ for 100 µM LiCl in 80:20 MeOH:water for glucose and sucrose that were absent in their 100 µM NaCl counterparts (Figures A-6 and A-8). No other general trends were observed, although the identity of the counterion can greatly affect response factors and other characteristics of response factor vs. concentration profiles.

5.5 Conclusions

CSTR-ESI-MS is capable of generating a continuum of response factors across a wide range of analyte concentrations with a single injection, providing details that would be difficult or tedious to obtain by sequential injections of various concentrations. It is also theoretically possible to obtain limits of detection using this technique, though signal intensities at low analyte concentrations lacked the reproducibility to do so in this study. This has also shown to be a convenient technique for visualizing ionization suppression in the presence of co-analytes. A CSTR of lower volume would be preferable to the one used in this study, generating the same information in a shorter time frame.

In addition to demonstrating the fundamental capabilities of this technique, practical information in the analysis of US by ESI-MS was yielded as well. ACN-water-based solvents, important for hydrophilic interaction LC, were found to suppress the

ionization of US in the positive ionization mode, and ammonium acetate, ammonium formate, and LiCl were all found to be good candidates for mobile phase additives in LC-ESI-MS, resulting in high sensitivities and good linearities in the negative ionization mode. Currently, we are developing LC-ESI-MS/MS methods for the quantitative analysis of US in beer.

5.6 Acknowledgement

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5.7 References

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Chapter 6

Determination of the Concentrations of Fructose, Glucose, and Sucrose in Hard Ciders and Apple Juice by Hydrophilic Interaction Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry

6.1 Abstract:

The annual sales of hard ciders in the United States is approaching half a billion dollars. Methods for determining their carbohydrate content would have important implications in their quality assurance and nutritional value. We developed a hydrophilic interaction liquid chromatography-electrospray ionization-tandem mass spectrometry-based method to determine the concentrations of fructose, glucose, and sucrose in commercial hard cider and apple juice samples. This method was validated with respect to selectivity, linearity, limit of detection (LOD), limit of quantitation, accuracy, and precision. In addition, we investigated six different sample preparation methods on two cider samples. LODs ranged from 0.0100 to 0.100 mg/ mL. We applied the method to 11 different cider samples, 1 apple ale sample, and 3 different apple juice samples. Among our significant findings, we found a correlation between the fructose and glucose concentrations in hard cider samples that did not contain detectable amounts of sucrose ($n=9$; $R^2=0.98$).

6.2 Introduction

Sales of hard cider in the United States in 2015 were reported to be 436 million dollars, a 15% increase from that of the previous year.[1] Ciders are produced from the fermentation of apple juice, and their taste and quality is dependent upon a number of factors, including the species of apples used and their degree of ripeness, the species of yeast used along with other brewing parameters, and the substances added to the cider at any point in the production process.[2] In particular, the carbohydrate content of ciders

has a critical influence on their sweetness and overall taste.[3] Levels of sugar can be as high as 25 g for a 355 mL bottle, and, therefore, their analysis has important implications for their quality assurance and nutritional value.

Methods for determining the concentrations of individual carbohydrates in ciders would be the most useful for their characterization, though the similar chemical and physical properties of carbohydrates, the presence of isomers, and the mutarotation of reducing sugars present formidable challenges. Liquid chromatography (LC)-based methods are attractive in their ability to separate carbohydrates that would otherwise not be easily distinguished by convenient analytical techniques. Underivatized carbohydrates are most commonly detected using LC with refractive index detection,[4–6] evaporative light scattering detection, [7–10] amperometry, [11–13] and mass spectrometry (MS). [12,14,15] MS detection is particularly advantageous, because it provides high sensitivity and specificity, while also yielding qualitative information. Electrospray ionization (ESI) is the most commonly used ionization source for the LC-MS analysis of carbohydrates. [12,14,15]

The most common LC modes used for underivatized carbohydrates are anion exchange,[12–14] cation exchange,[5,11] and hydrophilic interaction LC (HILIC).[4,7,9,10] The eluents used for ion exchange chromatography contain high concentrations of salts, which must be removed prior to ESI to minimize ionization suppression.[12,14] In contrast, the high content of polar organic solvents used in the mobile phases in HILIC are well-suited for ESI-MS.[16] Alkylamino-bonded silica is perhaps the most common HILIC stationary phase for carbohydrate analysis.[4,7,9,10] However, the bonded primary amine can react with reducing sugars, forming a Schiff base, which results in the loss of analyte, as well as poor column stability and reproducibility.[17] Other commonly used HILIC stationary phases for carbohydrate analysis include those based on amide,[18,19] β-cyclodextrin,[15] and zwitterionic[20] functionalities. Alternatively, carbohydrates can be

derivatized to allow for their satisfactory separation on reversed-phase columns and/ or increase their sensitivities towards ESI-MS,[21,22] though derivatization is often accompanied by increased labor and can complicate the analysis if multiple products are formed or if the reaction is incomplete.

The aim of this work was to develop, validate, and apply a HILIC-ESI-MS/MS method to quantitate fructose, glucose, and sucrose in hard cider and apple juice samples using HILIC-ESI-MS/MS. The method was validated with respect to selectivity, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, and precision. The method was applied to 11 different cider samples, 1 apple ale sample, and 3 different apple juice samples. To the best of our knowledge, this is the first report of a validated LC-MS method for the determination of carbohydrates in cider.

6.3 Materials and Methods

6.3.1 Materials

D-fructose (≥99%), *D*-glucose (≥99.5%), sucrose (≥99%), and formic acid (98-100%; LC-MS grade) were purchased from Sigma (St. Louis, MO, USA). Acetic acid, acetonitrile (ACN), ammonium hydroxide (≥25% in water), and water (all LC-MS grade) were purchased from Honeywell (Morris Plains, NJ, USA). Cider and apple juice samples were purchased from local retailers. Table 1 contains a brief description of each sample, provided by manufacturers of the products tested.

Standard solutions for fructose, glucose, and sucrose were prepared separately by dissolution in LC-MS water to a concentration of 1,000. mg/ mL, followed by filtration through a polytetrafluoroethylene (PTFE) syringe filter of a pore size of 0.2 μm. These solutions were used during method development and to confirm the identities of analytes detected in samples based on retention times. In addition, a solution containing 10,000 mg/ mL of each fructose, glucose, and sucrose in LC-MS water was used for LOD, LOQ,

and spike-and-recovery experiments as well as in the preparation of standards used to construct the calibration curves. The 10,000 mg/ mL solution was diluted to 2,000. mg/ mL and 20.0 mg/ mL to prepare calibration standards ranging from 5.00 – 250. mg/ mL and 0.050 – 2.50 mg/ mL, respectively. The 20.0 mg/ mL solution was further diluted to 0.200 mg/ mL to prepare standard solutions of concentrations ranging from 0.00050 – 0.025 mg/ mL for LOD and LOQ experiments.

Table 6-1. Brief description and determined concentrations of fructose, glucose, and sucrose for all samples.

Sample	Description	Fructose		Glucose		Sucrose	
		Conc. (%)	RSD (%)	Conc. (%)	RSD (%)	Conc. (%)	RSD (%)
C1	Crisp	0.669	1.2	0.556	0.64	2.77	0.54
C2	Crisp	2.06	0.63	2.23	0.058	0.0849	1.1
C3	Sweet	0.991	6.4	0.987	4.2	2.86	0.89
C4	Not Sweet, Not Dry	1.08	3.5	0.448	3.0	<LOD	
C5	Dry	0.182	1.6	0.172	0.87	0.997	1.8
C6	Dry	0.554	0.57	0.423	0.44	1.18	0.77
C7	All Natural	1.38	2.6	0.753	3.1	<LOD	
C8	Rose	0.897	0.87	0.768	0.74	0.847	0.72
C9	Rose	0.414	0.92	0.291	2.9	1.86	1.4
C10	Green Apple	0.598	0.20	0.526	0.64	3.34	0.81
C11	Easy-to-Drink	0.622	0.80	0.323	0.62	2.01	1.1
AA1	Apple Ale	0.211	2.3	0.214	1.1	2.70	0.69
AJ1	Organic	4.96	0.73	1.47	0.74	1.97	0.049
AJ2	100% Pure	5.77	0.34	2.25	0.78	1.56	1.0
AJ3	From Concentrate	4.61	2.0	2.24	2.5	1.04	2.3

6.3.2 Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry

All experiments were performed on a Shimadzu LCMS-8040 (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) liquid chromatograph - electrospray ionization - triple quadrupole mass spectrometer equipped with the following modules (all

Prominence series from Shimadzu): DGU-20A5 online degasser; SIL-20AC XR temperature-controlled autosampler; two LC-20AD XR pumps; CTO-20AC column oven; and CBM-20A communications bus module. The column used was an XBridge BEH amide column (2.1x100 mm; 2.5 μm) equipped with a VanGuard XBridge BEH amide guard column (2.1x5 mm; 2.5 μm) (all from Waters Corporation, Milford, MA, USA).

Mobile phase A was prepared by adjusting the pH of a 1.00% (v/v) solution of formic acid in LC-MS water to pH 9.00 with ammonium hydroxide, followed by filtration through a 0.2 μm PTFE filter and dilution by a factor of 20 in LC-MS water. Mobile phase A was prepared fresh every 48 hours. Acetonitrile was used as mobile phase B. The following chromatographic parameters were used: Flow rate, 0.200 mL/ min.; %B, 80% (isocratic); oven temperature, 60 °C; run time, 12 min.; autosampler tray holder temperature, 15 °C; injection volume, 5 μL.

The following MS parameters were used: Needle voltage, -4.5 kV; desolvation line temperature, 250 °C; heat block temperature, 250 °C; nebulizing gas flow (N_2), 1.5 L/ min; drying gas flow (N_2), 5 L/ min; dwell time, 50 msec; collision-induced dissociation gas pressure (Ar), 230 kPa; loop time, 106 msec. The selected reaction monitoring (SRM) transition used to monitor fructose and glucose had a precursor m/z of 225.10 and a product ion m/z of 179.10 at a collision energy of 8 V; the SRM parameters used to monitor sucrose were 387.15, 341.15, and 8 V, respectively.

6.3.3 Sample Preparation

Approximately 10 mL of apple juice and cider samples were collected in 15 mL polypropylene centrifuge tubes. The tubes were then shaken vigorously and opened to vent out carbon dioxide. This was repeated until the samples no longer appeared to be outgassing carbon dioxide. The samples were then sonicated for 1 minute to drive out the remaining dissolved carbon dioxide.

Six different sample preparation techniques were applied to two cider samples, and the analyte peak areas and their standard deviations were compared. For sample preparation technique 1 (SPT 1), the sample was diluted by a factor of 100 to a solvent composition of 80:20 ACN:water, followed by centrifugation and syringe filtration directly into an autosampler vial. For SPT 2 - 6, the samples were diluted by a factor of 10 to a solvent composition of 100% water, 60:40 ACN:water, 70:30 ACN:water, 80:20 ACN:water, and 90:10 ACN:water, respectively. These solutions were then centrifuged, syringe filtered, and further diluted by a factor of 10 directly into autosampler vials to a final solvent composition of 80:20 ACN:water. SPT 4 was ultimately chosen, though diluted by a factor of 100 instead of by 10 into the autosampler vials in order to ensure that all analyte concentrations in all samples fell into the range of the calibration curve.

6.3.4 Validation

The selectivity of the method was determined by comparing the retention times of fructose, glucose, and sucrose in standard solutions and samples. Blanks consisting of only 80:20 ACN:water were also periodically analyzed.

Standard solutions of fructose, glucose, and sucrose used to assess linear working ranges, LODs, and LOQs as well as to construct the calibration curves were prepared at concentrations of 0.0500, 0.100, 0.250, 0.500, 1.00, 2.50, 5.00, 10.0, 25.0, 50.0, 100., and 250. mg/ mL. Linearities were assessed based on their R^2 values. The linear range is defined here as the maximum concentration range leading to a linearity of at least 0.999. LODs and LOQs were determined as the minimum concentrations required to consistently achieve signal-to-noise ratios of at least 3 and 10, respectively, and additional standard solutions for these experiments were prepared at concentrations of 0.000500, 0.00100, 0.002500, 0.0100, and 0.0250 mg/ mL.

To assess the accuracy of the method, two cider samples and two apple juice samples were spiked with a standard solution of 10,000 ppm fructose, glucose, and sucrose to increase their concentrations in the analyzed sample by 10 ppm. This experiment was repeated with the same two cider samples and four additional cider samples after being stored in a freezer for two days, the latter of which were analyzed using freshly prepared mobile phases and a newly constructed calibration curve.

The precision of each measurement was determined by its percent relative standard deviation (% RSDs) from triplicate measurements. Intraday precision was assessed by comparing the peak areas for 2 cider samples and 2 apple juice samples analyzed 9 hours apart.

Each sample and standard was analyzed in triplicate. Paired *t*-tests were performed to assess any significant statistical differences (two-tailed P value < 0.05) between peak areas resulting from different sample preparation techniques applied to the same sample. Statistical differences in analyte concentrations in different samples were determined with unpaired *t*-tests (two-tailed P value < 0.05). Analyte concentrations were related to their peak areas by weighted least squares regression using the solver add-in in Microsoft Excel (v. 16.0.8431.2270; Redmond, WA, USA). The concentration range of the calibration curve was from 1.00 - 100. mg/ mL for fructose and glucose and from 0.500 - 50.0 mg/ mL for sucrose; all calibration curves were constructed with 8 standards of differing concentrations.

6.4 Results and Discussion

6.4.1 Method Optimization

6.4.1.1 Optimization of LC-MS/MS

In our previous work,[23] we found that monitoring the SRM transitions for acetate- and formate-adducts of carbohydrates in solutions containing their ammonium salts

resulted in good linearities, so we decided to use these as additives for this study. During method development, in brief, we found that: a combination of a relatively high temperature and high solution pH in mobile phases containing ammonium acetate or ammonium formate was required to collapse the two anomeric peaks of glucose into one; 80% ACN offered the best compromise between high fructose-glucose resolution and short retention times; and monitoring the SRM transitions of formate adducts resulted in higher peak areas than for acetate adducts, especially for fructose. For solutions containing fructose, glucose, and sucrose, an isocratic method of 80% B was able separate and elute all analytes in under 7 minutes, though the run time was increased to 12 minutes due to the presence of unidentified, very low intensity disaccharide peaks lasting until 10.50 minutes for many of the samples. Isocratic methods for HILIC are highly desirable, as the time required for reequilibration using gradient methods can be on the order of 10-30 minutes.

Admittedly, our original intention was to quantitate fructose, glucose, and maltooligosaccharides in beer samples by HILIC-ESI-MS/MS, but we were unable to chromatographically separate most of these analytes from interfering isobaric compounds in all beer samples analyzed. We later found that our developed method does not suffer from isobaric interferences when applied to ciders, so we pursued that. For both the beer and cider analyte sets, the separation of fructose and glucose and the peak shape of glucose were found to be the most critical parameters to optimize during method development, and we believe that it is worth mentioning here.

6.4.1.2 Optimization of Sample Preparation

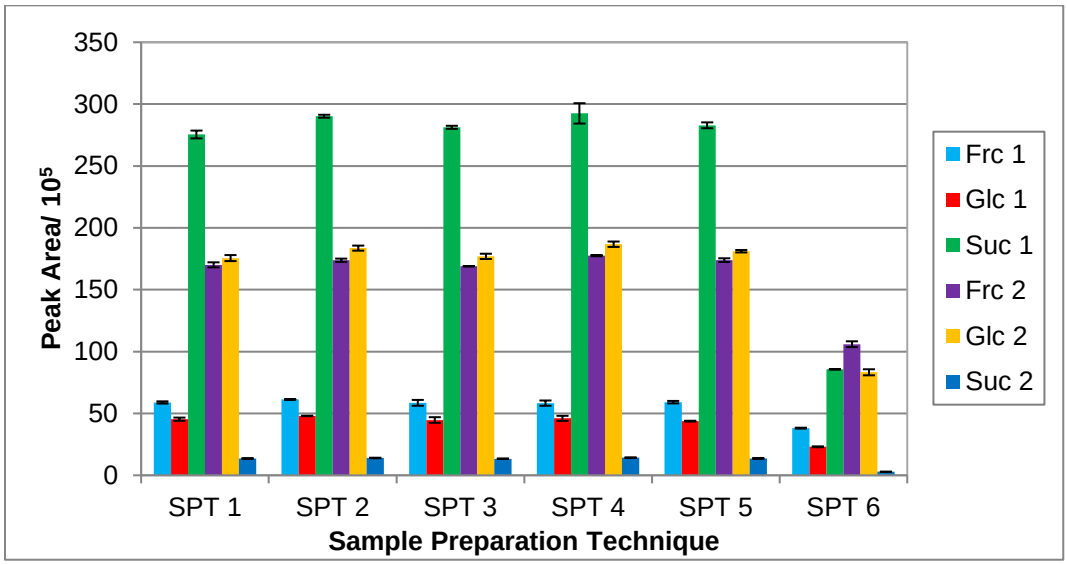

Figure 6-1. Chromatographic SRM peak areas for fructose, glucose, and raffinose in cider samples 1 and 2 obtained under different sample preparation techniques.

Prior to analysis, it was not known whether the same dilution factor would be appropriate for all cider samples, so each SPT for the sample preparation optimization experiments had a relatively low final dilution factor of 100. Results from the sample preparation experiment are displayed in Figure 6-1. Most strikingly, peak areas for all analytes in both cider samples for SPT 6, where the samples were initially diluted by a factor of 10 in 90:10 ACN:water, were lower than those of SPT 4, where the samples were initially diluted by a factor of 10 in 70:30 ACN:water, by between 35 and 79%. Notably, emulsions on the bottom of the centrifuge tubes were observed upon the initial dilution for SPT 6 and, to a lesser extent, SPT 5. Loss of analytes by their partitioning between the aqueous-rich emulsion and organic-rich supernatant likely explains the relatively low peak areas for samples prepared by SPT 6. Peak areas for SPT 2 and SPT

4 were significantly higher than the other SPTs for at least one analyte in at least one sample, but they were not statistically different from each other. Solutions prepared under SPT 4 were much easier to filter than SPT 2, so SPT 4 was ultimately chosen for this method. After constructing the calibration curve and analyzing more samples, it was realized that a final dilution factor of 100 was too low for all of the analytes in all of the samples to fall within the linear range. SPT 4 was then modified to result in a final dilution factor of 1,000 by changing the dilution factor from the filtrate to the autosampler vial from 10 to 100.

6.4.2 Method Validation

The retention times of the analytes in standards matched closely with those in samples. No analytes were detected above their limits of detection in any analyzed blanks.

LODs, LOQs, linear ranges, and linearities for fructose, glucose, and sucrose are displayed in Table 6-2. LODs and LOQs ranged from 0.0100 – 0.100 and 0.0500 – 0.500 mg/ mL, respectively. Linear ranges spanned between 2 and 3 orders of magnitude.

Table 6-2. LODs, LOQs, linear ranges, and linearities for fructose, glucose, and sucrose

Parameter	Fructose	Glucose	Sucrose
LOD (mg/ mL)	0.0250	0.100	0.0100
LOQ (mg/ mL)	0.250	0.500	0.0500
Linear Range (mg/ mL)	0.250-250.	0.500-250.	0.0500-50.0
Linearity (1-100 mg/ mL)	0.9994	0.9998	0.9967
Linearity (0.5-50 mg/ mL)	0.9996	0.9992	0.9993

Results from the % recovery experiments are displayed in Table 6-3. During the first set of experiments, all analytes in all samples had % recoveries between 81.8 and 101% except for sucrose in C1, which was 72.0%. Upon repeating these experiments

under fresh mobile phases and a newly constructed calibration curve for the two cider samples in the original set, prepared separately, along with four additional cider samples in Set 2, % recoveries for all analytes in all samples were between 81.7 and 107%. It is not known why the % recovery for sucrose in C1 fell outside of the 80-120% range in Set 1 but not Set 2.

Table 6-3. Results from % recovery experiments

Sample	Fructose		Glucose		Sucrose	
	% Recovery	% RSD	% Recovery	% RSD	% Recovery	% RSD
			Set 1			
C1	87.9	1.3	92.8	1.3	72.0	3.9
C2	81.8	8.3	88.7	5.2	96.7	0.84
AJ1	86.1	10	96.1	4.9	90.4	3.5
AJ2	92.1	5.9	101	6.3	94.2	4.8
			Set 2			
C1	93.7	3.0	93.3	2.9	83.1	3.7
C2	92.4	15	85.2	22	105	1.4
C4	97.9	5.2	90.8	3.6	99.1	0.60
C6	103	11	98.8	8.7	107	2.4
C8	98.9	3.0	94.5	3.1	98.2	4.7
C10	99.6	0.42	95.0	2.1	81.7	16

The % RSDs for all measurements were less than 7%. For intraday precision, the % differences between peak areas for samples analyzed 9 hours apart were less than 6% for all analytes.

6.4.3 Application to Cider and Apple Juice

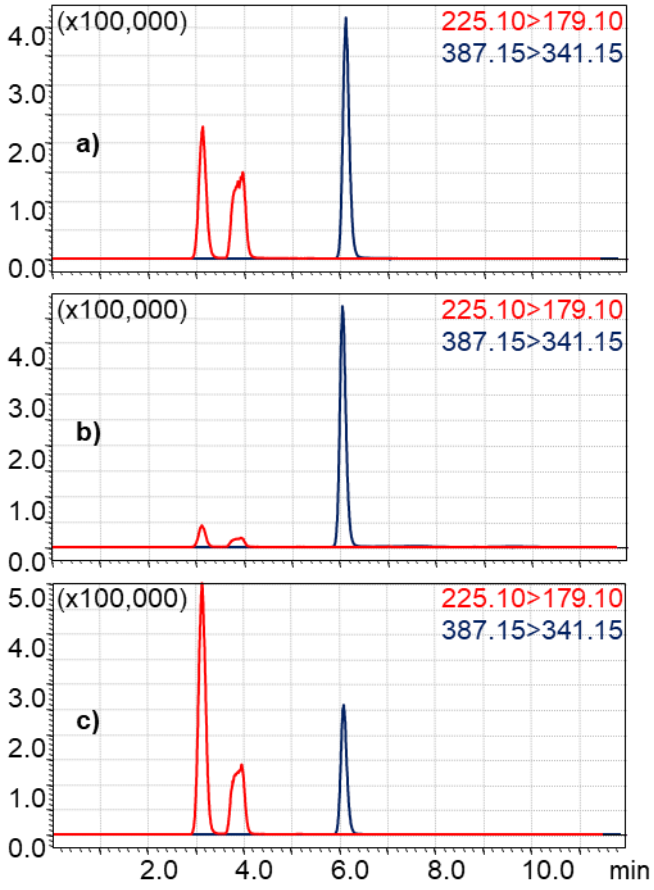

Figure 6-2. Representative SRM chromatograms for samples of. a) 25.0 mg/ mL fructose, sucrose, and sucrose standard mix; b) C9; and c) AJ3

Table 6-1 displays the fructose, glucose, and sucrose concentrations along with their % RSDs for all samples, and Figure 6-2 displays representative chromatograms for a standard containing 25.0 mg/ mL each of fructose, glucose, and sucrose as well as for sample C9 and sample AJ3. It was expected that the apple juice samples would have higher concentrations of fructose, glucose, and sucrose than all of the cider samples, unless they were added after fermentation. The concentration of fructose was higher in the apple juice samples than all of the other samples by at least a factor of 2; neither

fructose nor corn syrup were listed in the ingredients of any of the samples. The concentration of glucose was higher in the apple juice samples than all of the other samples by at least a factor of 1.5, except for C2; C2 and C9 list glucose syrup as one of their ingredients. The apple ale sample as well as 4 of the cider samples (C1, C3, C10, C11) had significantly higher sucrose concentrations than all of the apple juice samples, and only 4 cider samples (C2, C4, C7, C8) had sucrose concentrations significantly lower than those of the apple juice samples. C1, C6, C10, and C11 list cane sugar or sucrose syrup as one of their ingredients. C3 contains “natural flavor”, and no list of ingredients could be found for the apple ale sample. Apple juice is listed as an ingredient for C1 and C8-C11. The manufacturers of C4 and C7, the only samples in which no sucrose was detected, as well as that of C7 claim that no additional sugars are used.

Apple juice is known to have a relatively high ratio of fructose-to-glucose, and it has even been suggested to use this information, along with the sucrose concentration, to assess adulteration in apple juice samples.[2] It is notable that the two cider samples containing undetectable amounts of sucrose, C4 and C7, had the highest and third highest fructose-to-glucose ratios among the cider samples and apple ale sample, respectively. It was thought plausible that the fructose-to-glucose ratio would negatively correlate with sucrose concentration, but this was not the case ($R^2 < 0.15$). The only correlation among the data found for the cider samples was between fructose and glucose concentration ($R^2 = 0.81$). When the two samples with no detectable sucrose were removed (C4 and C7), the correlation dramatically improved ($R^2 = 0.98$). Given the wide variety of sweetening agents listed in the ingredients among the samples, this was surprising and warrants further investigation into the reason and applicability of this correlation.

6.5 Conclusions

We have developed, validated, and applied a HILIC-ESI-MS/MS-based method for the determination of the concentrations of fructose, glucose, and sucrose in cider and apple juice samples. The isocratic nature of the method eliminates the need for column reequilibration, allowing for rapid analyses, although it may not be applicable to samples containing high concentrations of higher molecular weight carbohydrates, such as beer, which may require unacceptable amounts of time for their elution. These measurements have important implications for quality assurance and nutritional value, and similar methodology would be expected to be applicable to other similar samples, such as the juice of other fruits. In the future, we would like to assess the ability of this method to differentiate between natural and artificial apple juice.

6.6 Acknowledgement:

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Chapter 7

Summary and Future Work

The development of electrospray ionization (ESI), along with matrix-assisted laser desorption/ionization, in the 1980's were revolutionary for allowing high molecular weight and thermolabile biomolecules to be analyzed by mass spectrometry (MS). Since then, biological MS research has been much more focused on the analysis of peptides and proteins than other classes of biomolecules, leaving many fundamental and practical research opportunities. The analysis of carbohydrates is particularly challenging due to their similar physical and physical properties, low ionization efficiencies, the presence of isomers that are often not easily distinguished by tandem MS (MS/MS), and the mutarotation of reducing sugars which is often detrimental to their separation. Although the advances in chemical separation technologies and mass spectrometry over the past few decades have greatly improved carbohydrate analysis, there still exists many unresolved challenges and opportunities.

Derivatization has often been used to increase the ESI-MS response factors of carbohydrates, but this is often accompanied by increased labor and can complicate the analysis is the reaction is incomplete or multiple products are formed. Optimizing detection strategies and solvent parameters is a more attractive alternative in terms of simplicity and throughput. The influence of solvent parameters, including the additive and its concentration, pH, and the organic solvent and its ratio to water, on the ESI-MS/MS signal intensity of glucose was studied using flow injection analysis. Notable findings included: the direct suppression of the ionization of glucose by acetonitrile in the positive ionization mode through its competition with glucose for cation adduction; the dramatic influence of the identity of the counterion of ammonium on the signal intensity of glucose in the positive ionization mode, where ammonium-glucose SRM (selected reaction

monitoring) transitions were monitored; and an increase in the organic solvent content generally leading to an increase in signal intensity with the exception of acetonitrile in the positive ionization mode, which led to a decrease in signal intensity. Although this study likely represents one of the most comprehensive investigations of the effects of multiple solvent parameters on an analyte's ESI-MS response, there remains many opportunities for future work.

It would be highly desirable to assess if other carbohydrate species behave similarly to glucose under the same solvent parameters, as the number and orientation of a carbohydrate's hydroxyl groups may influence their interaction with ions involved in adduction. This may also give insight into the nature of ion-carbohydrate binding. It would also be useful to compare analyte signal intensities under different solvent parameters under individually optimized instrumental settings. Applying this methodology to glycans, glycoconjugates, and other classes of analytes would also be of fundamental and practical significance. As previously mentioned, carbohydrates isomers are often difficult to differentiate based on their MS/MS spectra and their similar chemical and physical properties often make their separation challenging. The ratios of SRM signal intensities in the positive and negative ionization mode may be useful for their deconvolution, and a judicious choice of detection strategies along with solvent and instrumental parameters may be helpful for this. Lastly, the observation of solutions containing ammonium trifluoroacetate (TFA) having much higher signal intensities for ammonium-glucose SRM transitions than other ammonium salts warrants further investigation. It is believed that this is due to the relatively high surface activity of TFA, which decreases the surface tension of the electrospray droplets and allows coulombic fission events to occur at lower surface charge densities. Ionic surfactants are known to suppress ionization in the ionization mode corresponding to their charge, but it is worth investigating if, in general,

the presence of surfactants enhance signal intensities of analytes monitored in the ionization mode opposite of the surfactant's charge. This effect would also be interesting to study for nonionic and zwitterionic surfactants as well.

A continuous stirred tank reactor (CSTR) coupled to ESI-MS/MS was used to determine the response factors of glucose, sucrose, and raffinose across wide concentration ranges from a single injection under a variety of detection strategies and solvent parameters. The response factor vs. concentration profiles varied widely, and were dependent upon the identity of the monitored ionic species and the solvent parameters used. Monitoring acetate, chloride, and formate adduct SRM transitions led to the best linearities, useful for quantitative analysis. This methodology should also be useful for determining limits-of-detection and limits-of-quantitation, but these experiments lacked the reproducibility at lower concentrations for their determinations. A smaller volume CSTR chamber would be desirable to decrease run times and possibly improve reproducibility at lower concentrations. In the CSTR study, each additive was only investigated at one concentration and only under 80:20 acetonitrile:water and 80:20 methanol:water. For future work, the effects of additive concentrations and the organic solvent-to-water ratio on an analyte's linear range as well as its ability to measure ion-carbohdyrate binding affinities would be worth pursuing. This methodology should also be extended to other classes of compounds, including glycans and glycoconjugates.

Lastly, we applied a hydrophilic interaction liquid chromatography (HILIC)-ESI-MS/MS method to determine the concentration of fructose, glucose, and sucrose in cider and apple juice samples. Among our most notable findings, the fructose-to-glucose ratio was invariably higher in apple juice samples, and the concentrations of fructose and glucose correlated with each other for the cider samples. The carbohydrate profile of ciders is much less complex than other samples, such as beer, and separating and

distinguishing isobaric carbohydrates with current analytical technologies is extremely challenging. This would perhaps be best addressed with the introduction of more selective LC stationary phases or other separation techniques. Two-dimensional LC seems to be particularly promising for this challenge, and would be worth pursuing.

Appendix A

CSTR Generated Response Factor vs. Concentration Profiles

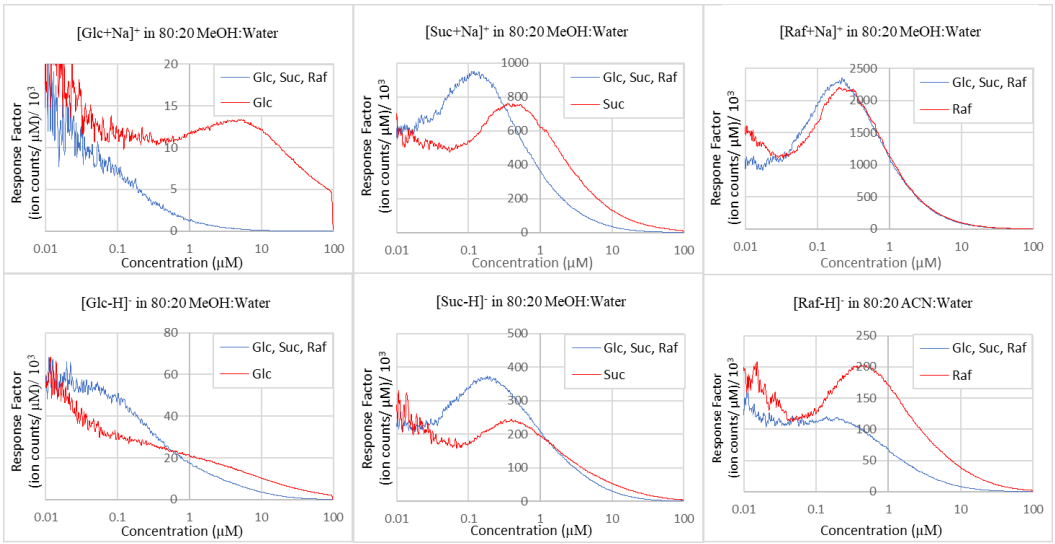

Figure A-1. Response factor vs. concentration for $[M+Na]^+$ and $[M-H]^-$ in 80:20 MeOH:Water for solutions containing glucose, sucrose, and raffinose (blue) and solutions containing individual analytes (red)

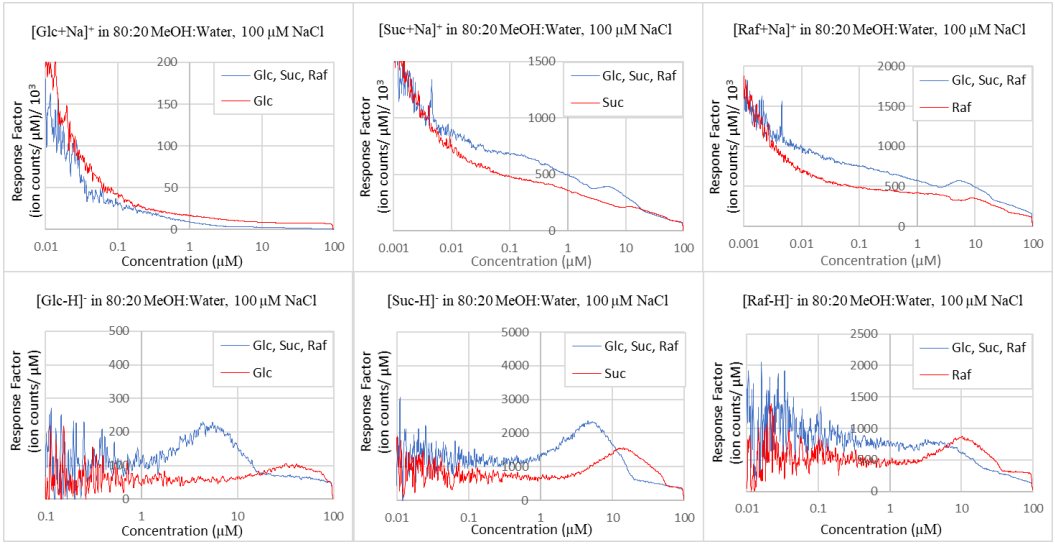

Figure A-2. Response factor vs. concentration for $[M+Na]^+$ and $[M-H]^-$ in 80:20 MeOH:Water, 100 µM NaCl for solutions containing glucose, sucrose, and raffinose (blue) and solutions containing individual analytes (red)

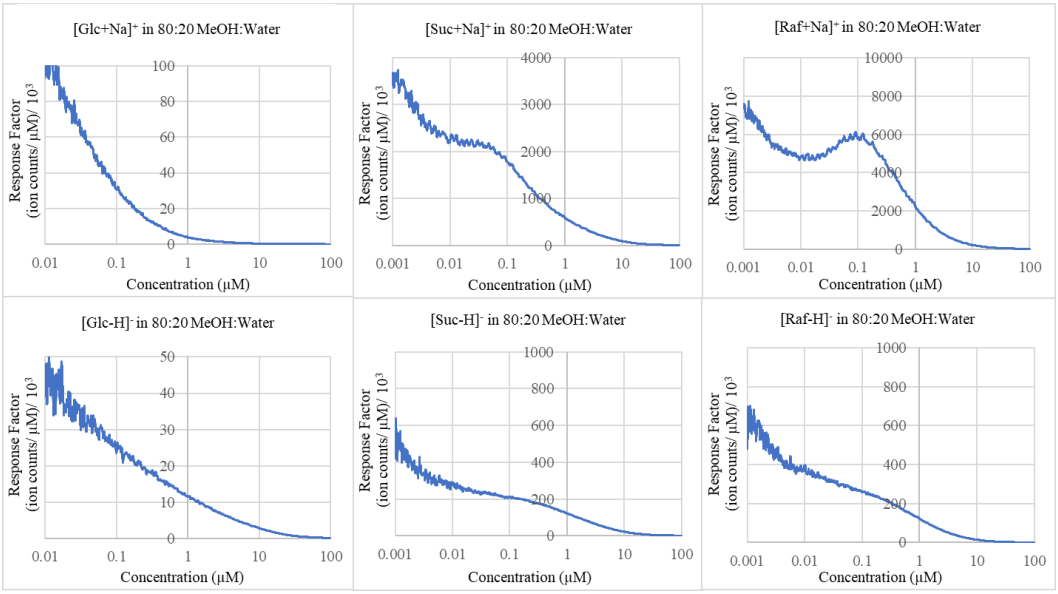

Figure A-3. Response factor vs. concentration for $[M+Na]^+$ and $[M-H]^-$ in 80:20 MeOH:water

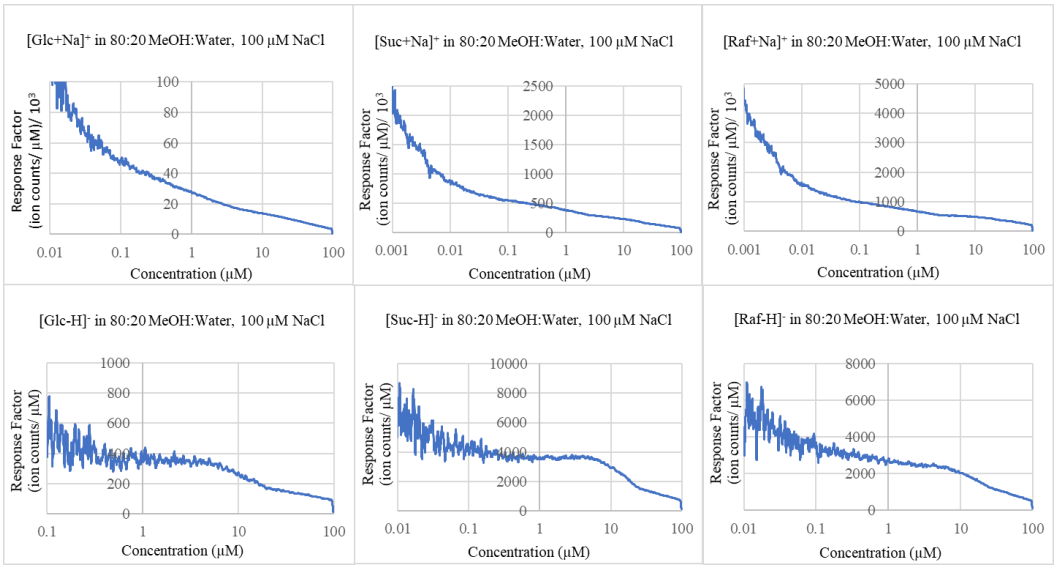

Figure A-4. Response factor vs. concentration for $[M+Na]^+$ and $[M-H]^-$ in 100 µM NaCl in 80:20 MeOH:water

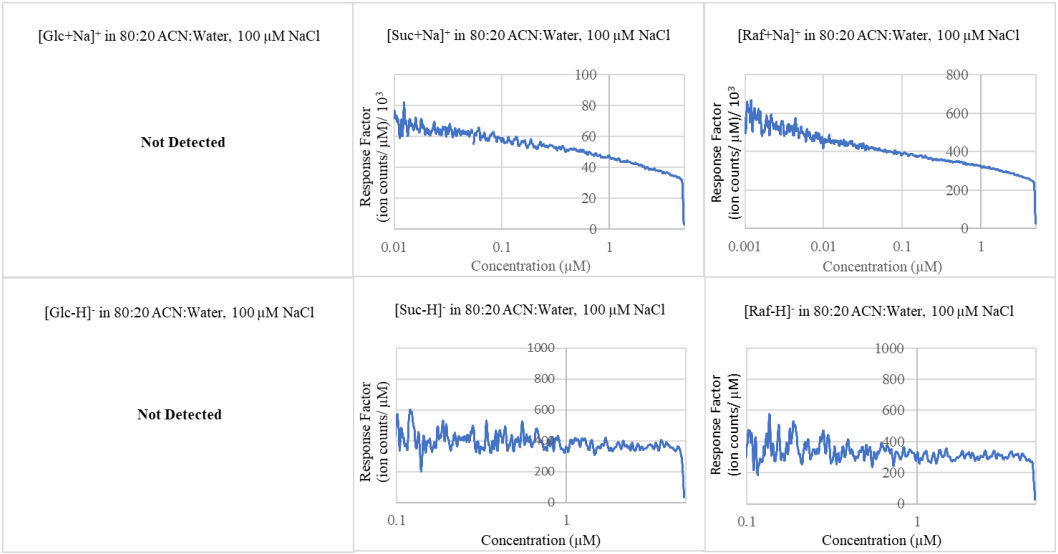

Figure A-5. Response factor vs. concentration for $[M+Na]^+$ and $[M-H]^-$ in 100 µM NaCl in 80:20 ACN:water

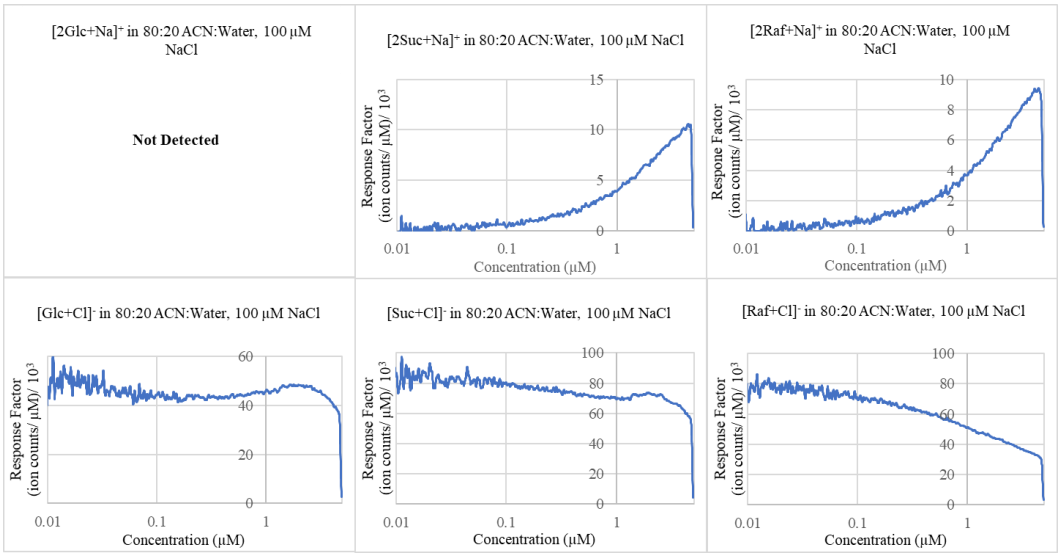

Figure A-6. Response factor vs. concentration for $[2M+Na]^+$ and $[M+Cl]^-$ in 100 µM NaCl in 80:20 ACN:water

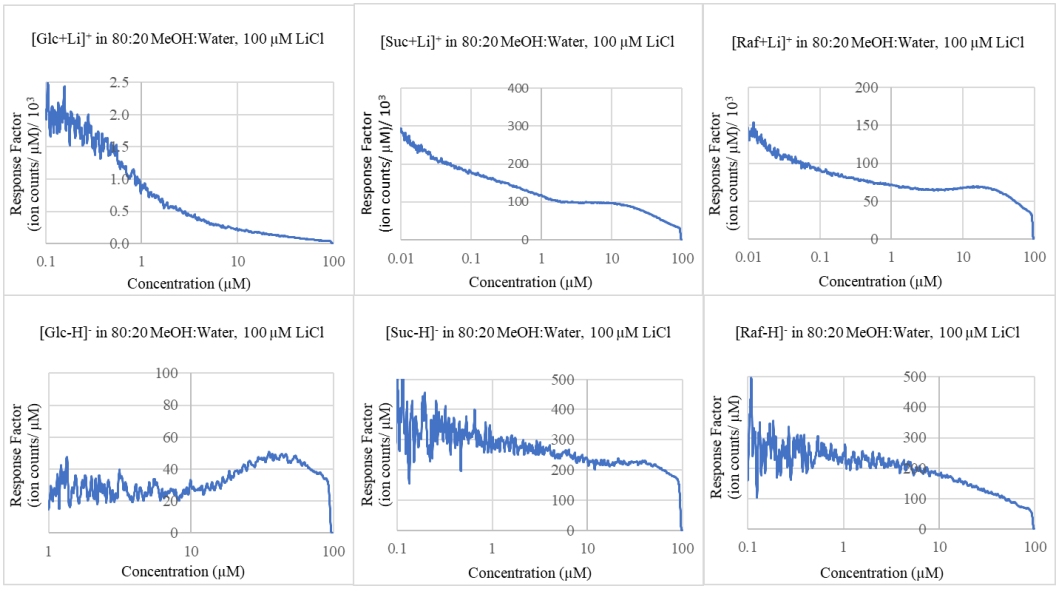

Figure A-7. Response factor vs. concentration for $[M+Li]^+$ and $[M-H]^-$ in 100 µM LiCl in 80:20 MeOH:water

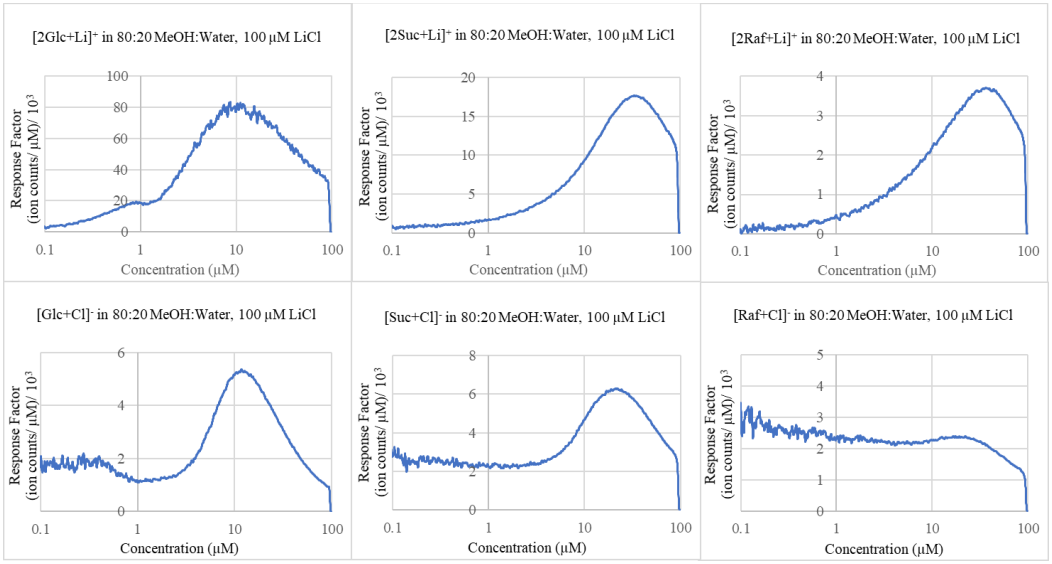

Figure A-8. Response factor vs. concentration for $[2M+Li]^+$ and $[M+Cl]^-$ in 100 µM LiCl in 80:20 MeOH:water

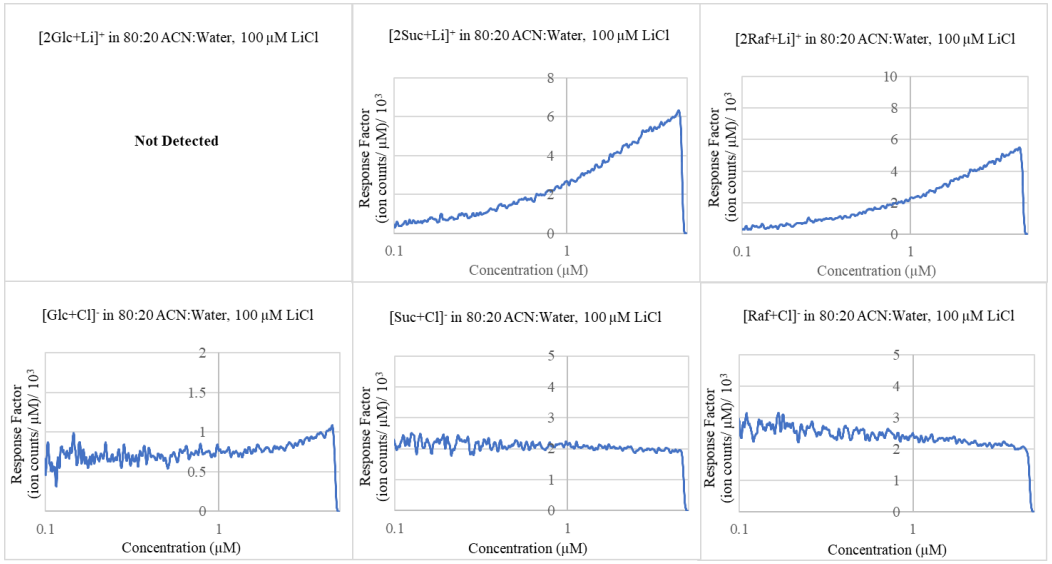

Figure A-9. Response factor vs. concentration for $[2M+Li]^+$ and $[M+Cl]^-$ in 100 µM LiCl in 80:20 ACN:water

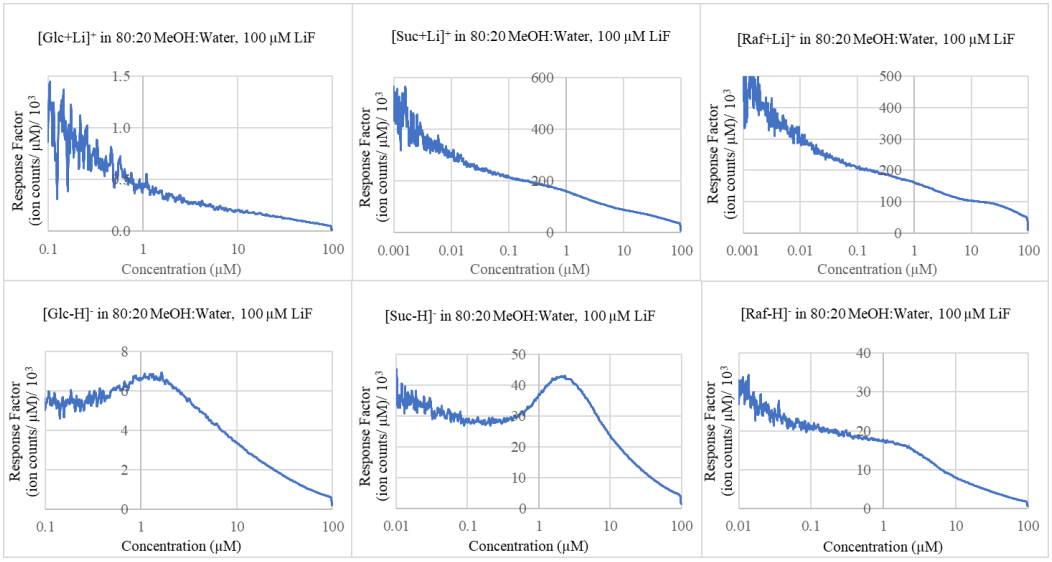

Figure A-10. Response factor vs. concentration for $[M+Li]^+$ and $[M-H]^-$ in 100 µM LiF in 80:20 MeOH:water

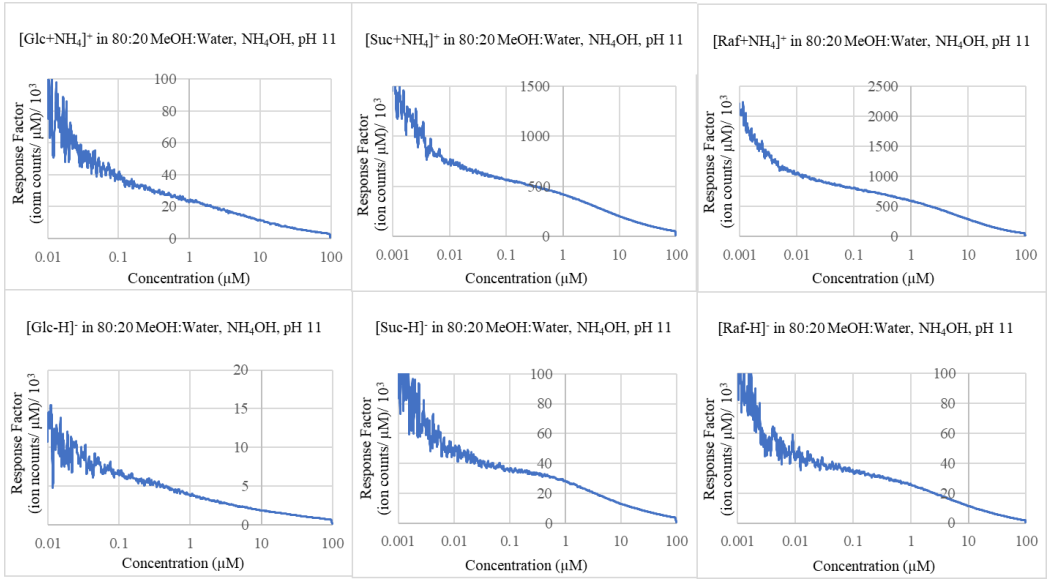

Figure A-11. Response factor vs. concentration for $[M+NH_4]^+$ and $[M-H]^-$ in NH_4OH, pH 11, in 80:20 MeOH:water

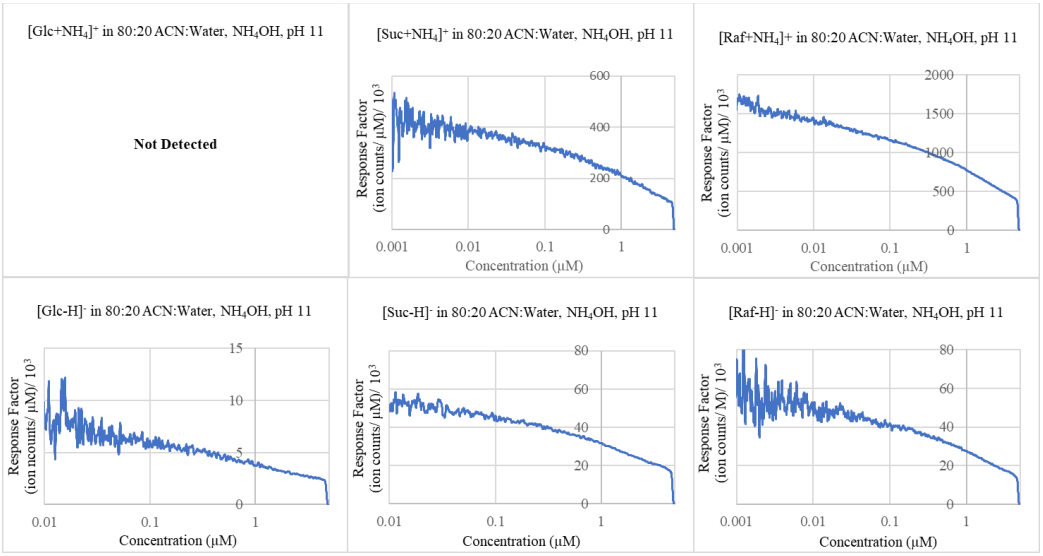

Figure A-12. Response factor vs. concentration for $[M+NH_4]^+$ and $[M-H]^-$ in NH_4OH, pH 11, in 80:20 ACN:water

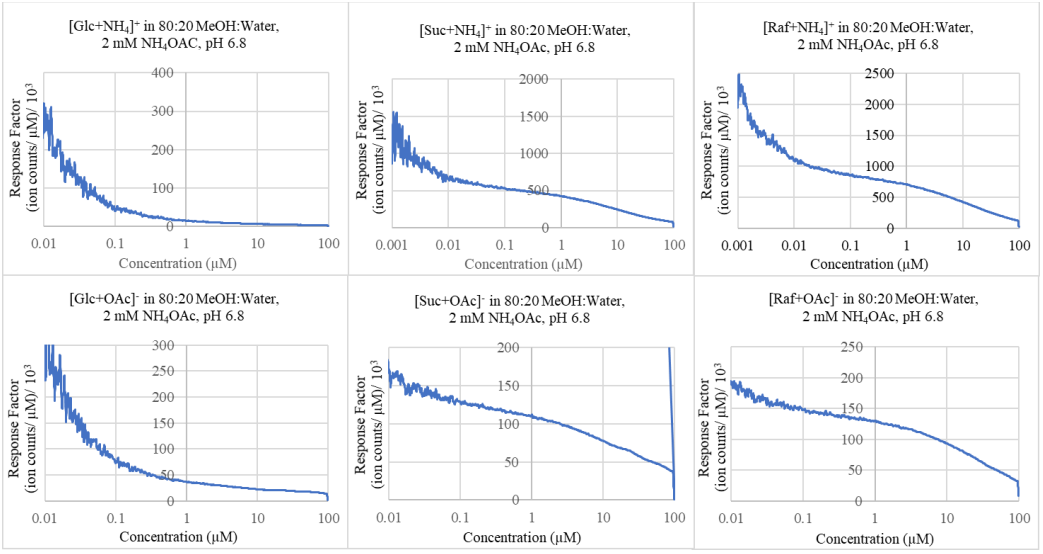

Figure A-13. Response factor vs. concentration for $[M+NH_4]^+$ and $[M+OAc]^-$ in 2 mM NH_4OAc, pH 6.8, in 80:20 MeOH:water

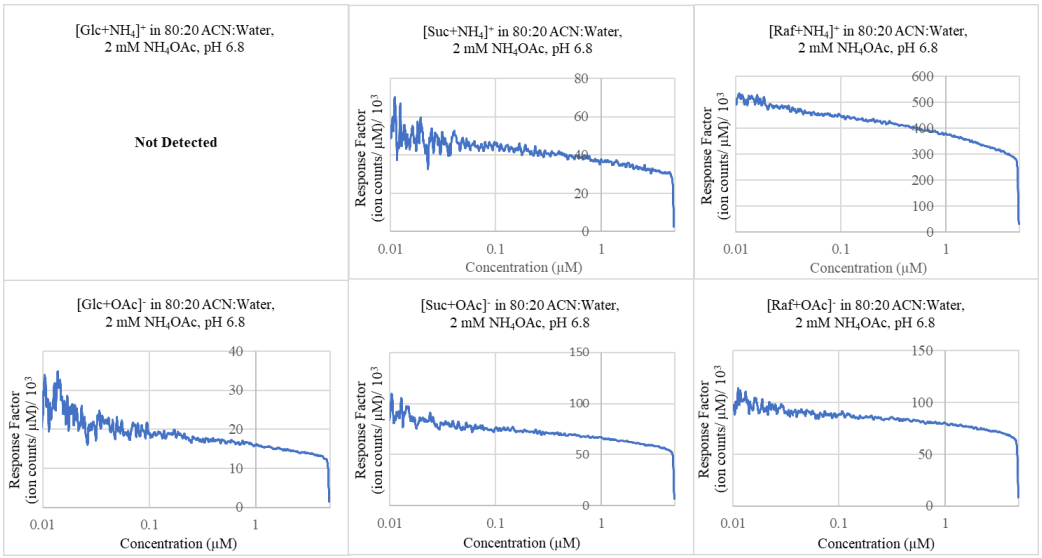

Figure A-14. Response factor vs. concentration for $[M+NH_4]^+$ and $[M+OAc]^-$ in 2 mM NH_4OAc, pH 6.8, in 80:20 ACN:water

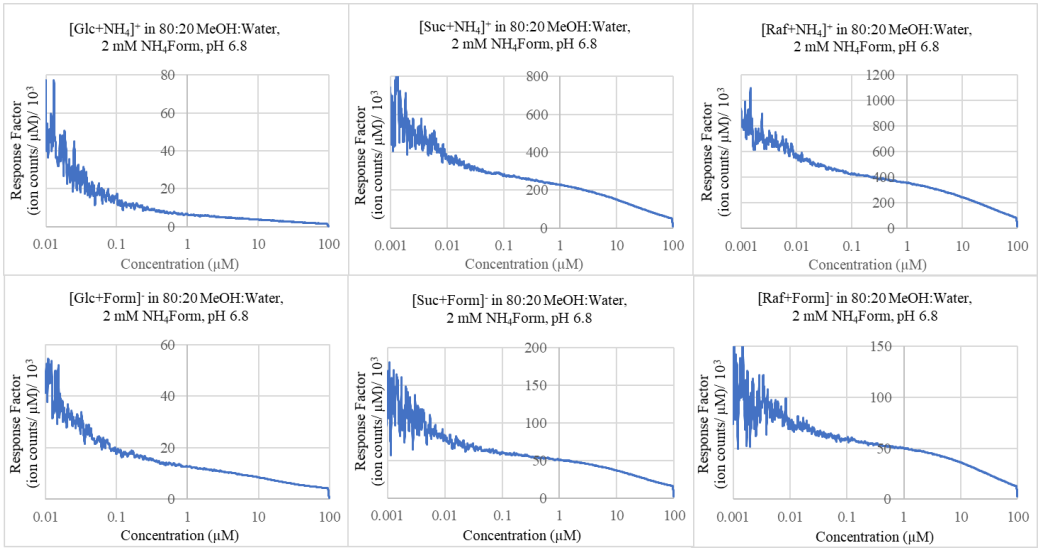

Figure A-15. Response factor vs. concentration for $[M+NH_4]^+$ and $[M+Form]^-$ in 2 mM NH_4OAc, pH 6.8, in 80:20 MeOH:water

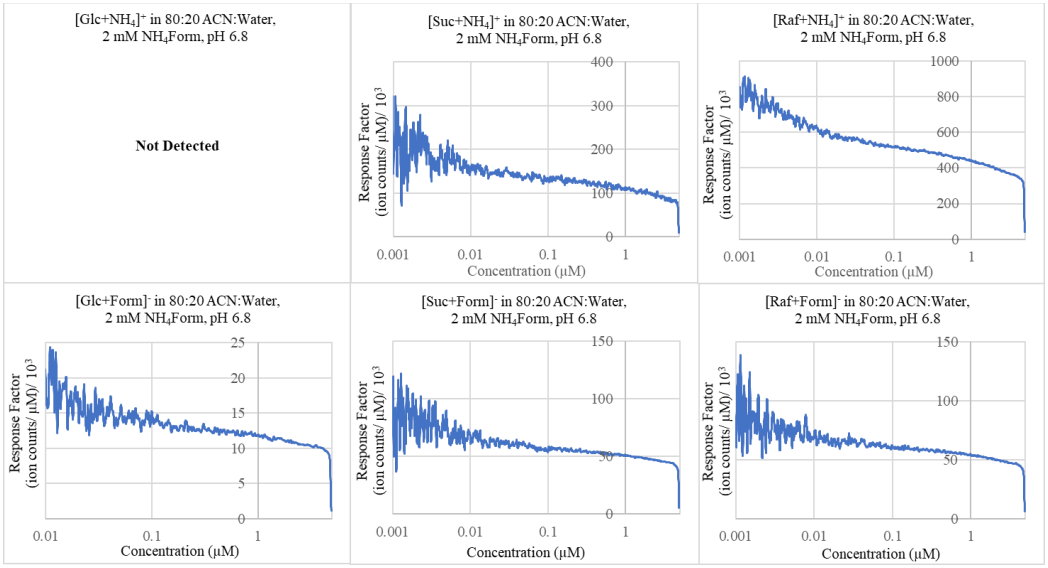

Figure A-16. Response factor vs. concentration for $[M+NH_4]^+$ and $[M+Form]^-$ in 2 mM NH_4OAc, pH 6.8, in 80:20 ACN:water

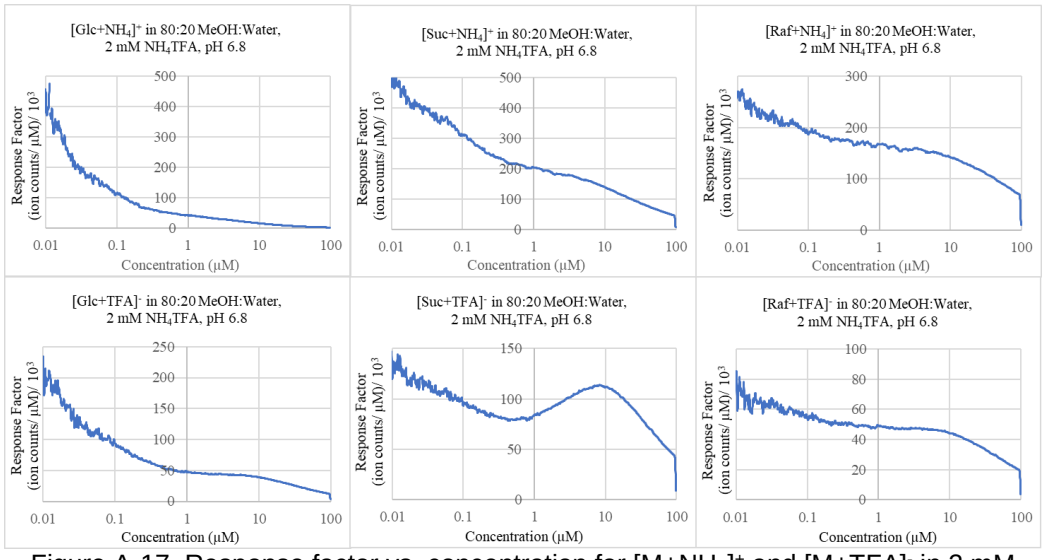

Figure A-17. Response factor vs. concentration for $[M+NH_4]^+$ and $[M+TFA]^-$ in 2 mM NH_4TFA, pH 6.8, in 80:20 MeOH:water

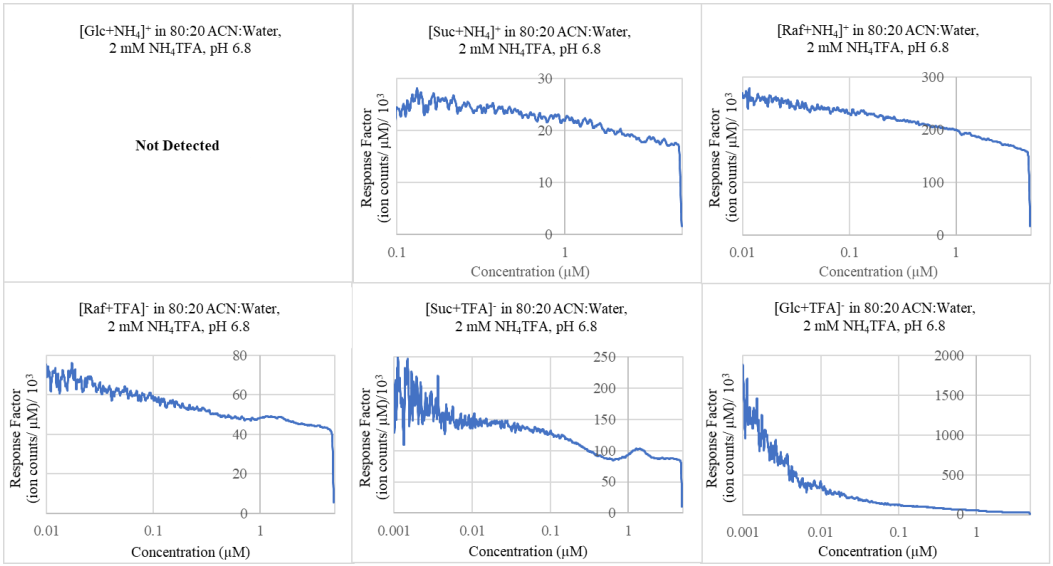

Figure A-18. Response factor vs. concentration for $[M+NH_4]^+$ and $[M+TFA]^-$ in 2 mM NH_4TFA, pH 6.8, in 80:20 ACN:water

Biographical Information

Jonathan began his studies at the University of Texas at Arlington in the spring of 2011 after spending his first semester of college at Texas Tech University. During his undergraduate years, his academic performance was recognized with the R. L. Hoyle Award for Outstanding Junior in 2012 and the Sharon and Donald L. Jernigan Chemistry Scholarship in 2013. He also worked in Dr. H. V. Rasika Dias's inorganic chemistry research lab synthesizing organometallic compounds with novel fluorescent properties. Jonathan graduated *magna cum laude* with a BS in Chemistry in the fall of 2013.

Jonathan then continued his education at UTA under the supervision of Dr. Kevin A. Schug in the spring of 2014. His first three semesters of research involved the chemical analysis of groundwater in areas potentially impacted by hydraulic fracturing and wastewater from oil and gas development. Later, he worked on developing methods for detecting proteins imparting antibiotic resistance in bacteria and preparing polyvinyl alcohol-coated silica as a stationary phase for liquid chromatography. Though these projects did not ultimately bare tangible fruits, the experience gained in these efforts were invaluable. He spent the spring of 2017 in the Czech Republic on an academic internship at Palacký University in Olomouc under Dr. Karel Lemr and at the Laboratory of Molecular Structure Characterization at the Czech Academy of Sciences in Prague under Dr. Vladimír Havlíček. That summer, he took an industrial internship at Restek Corporation in Bellefonte, PA working in the LC Solutions division. Over the next year, he began and completed research related to his dissertation topic, fundamental and applied ESI-MS/MS and LC-ESI-MS/MS for the analysis of underivatized carbohydrates.

He graduates with his PhD from UTA in July 2018. He plans to join the lab of Dr. Subramaniam Pennathur of the University of Michigan as a post-doctoral research fellow in the fall of 2018 and pursue a career in academia.