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Elemental Detection for Bio-Analytical Applications ____________________________

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Chair: Dr. Joseph A. Caruso

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Interfacing Conventional and Capillary Flow to Argon Plasma: Elemental Detection for Bio-Analytical Applications

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Abstract of Dissertation

Inductively coupled mass spectrometry has, over the years, been a useful tool for the analytical chemist through the capability to qualitatively and quantitatively determine the elemental composition of unknown inorganic substances. Identification has occurred in a large variety of sample matrices such as, water, soil, inks, and industrial wastes. Within the last decade the matrix of interest has expanded into the biological realm. The ability to separate these complex bio-matrices requires reverse phase separation and usually implies the use of an organic modifier in the mobile phase. Due to the nature of the argon plasma, the use of carbon based modifiers destabilizes the plasma at the standard flow rates, decreasing the available ionization energy and depositing carbon on the sampling cone, clogging the entrance into the mass spectrometer interface.

Current research involves evaluating commercially available micro-flow nebulizers which can allow the introduction of organic modifiers for increased chromatographic resolution of sample analytes because of the much lower solvent load to the plasma. Comparisons of various capillary flow nebulizers and spray chamber configurations are presented. An in-house spray chamber designed by the author is also evaluated.

Sensitive and selective techniques for detecting phosphorus- and sulfur- based analytes with an ICPMS equipped with an rf only driven octopole collision/reaction cell (CRC) are investigated. The first technique utilizes Xe gas to reduce and remove polyatomic interferences from $^{31}$P and $^{32}$S. The second uses an oxidation reaction to form new product ions, $^{31}$P$^{16}$O$^+$ and $^{32}$S$^{16}$O$^+$, to move elemental phosphorous and sulfur away from the mass range of elemental interferences.
The remaining focus of this dissertation is to utilize these earlier experiments and data from interfacing capillary flows into the Ar plasma to establish a qualitative and quantitative method for antisense oligonucleotides. This study describes a method for the analytical separation of 21-24-mer oligonucleotides using an RP C\textsubscript{18} capillary column. Spectral data are comprised of element specific $^{31}$P and $^{32}$S, found in the phosphorothioate oligonucleotide backbone. UV (DAD) and elemental $^{31}$P and $^{32}$S data are obtained simultaneously through direct coupling of the capillary HPLC system to the ICPMS.

The scientific significance of the data contained in this dissertation developed a low ppt and reproducible Cr speciation method and lays the foundation for Cr speciation in more complex matrices. The establishment of five interfacing variations of capillary flow nebulizers and four different spray chambers on the 7500ce displayed total consumption sample introduction, without degrading plasma performance. The introduction of O\textsubscript{2} into an octopole collision reaction cell for the first time in ICPMS analysis allows for additional applications in ICPMS ion-molecule chemistry and the possibility of using other reactive gases in the octopole (NH\textsubscript{3}, CH\textsubscript{4}). These studies for the first time, institute the use of element specific synthetic P/S tags in phosphorothioates and natural P tags in biological oligonucleotides to qualitatively and quantitatively detect their presence by ICPMS. The methodology generated an alternative way to quantitate oligonucleotides and assist in the advancement of clinical trials profiling metabolite and pharmacokinetic data for developing anti-sense oligonucleotides.
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1.1 Overview

Many elements within the Periodic Table play essential roles in various biological systems. Most trace metals in the body are found in biological fluids and organs and are bound to a large variety of proteins, called metalloproteins. The characterization of metals and non-metals, with respect to their chemical form, oxidation state and the nature of their ligands has become increasingly important in such fields as biochemistry, pharmacology, medicine, nutrition and agriculture. The area of element specific chemistry has been defined by The International Union for Pure and Applied Chemistry (IUPAC) as it relates to a chemical species, as “a specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure.” There is an increasing demand for chemical speciation information, since the toxicity of some elements depends on their chemical form. Inductively coupled mass spectrometry (ICPMS) is the standard for element specific detection due to its selectivity, sensitivity, large linear dynamic range, diverse interfacing capabilities to separation techniques, and virtually simultaneous multi-element detection Figure 1.1. Elemental speciation analysis occurs through the interfacing of separation techniques such as, gas chromatography (GC), capillary electrophoresis (CE) and liquid chromatography (LC) to the ICPMS. Identification of the species is based upon the isotopic pattern of the element of interest and through retention time matching of standards. However, due to complete sample ionization, limited molecular information can be gained from the hyphenated separation techniques and this is often not sufficient for most element speciation studies. Researchers realize electrospray ionization (ESI) and matrix assisted laser desorption (MALDI) mass spectrometry, can fill the void in the realm of molecular data. However, it
Figure 1.1 Agilent Technologies 7500ce ICPMS with an octopole collision/reaction gas is recognized that these methods often suffer from a lack of sensitivity, ion suppression and the formation of salt adducts. The obvious solution is to combine elemental ICPMS and molecular ESI-MS techniques in order to detect and characterize element labeled organic molecules. In this study, this is called a metallomics approach. This chapter gives a comprehensive account of the analytical techniques involved in ICPMS and the coupling of conventional and capillary liquid chromatography (LC) and the nuances that accompany them.

1.2 Inductively Coupled Plasma Mass Spectrometry (ICPMS)

1.2.1 Sample Introduction

The majority of ICPMS applications involve the analysis of liquid samples, even though there are techniques adapted for gases and solids given throughout the literature. This dissertation research deals completely with liquid sample introduction by coupling conventional and capillary LC flow to the ICPMS.
1.2.1.1 *Liquid Sample Introduction*

There are two main objectives involved in the liquid sample introduction system, generating an aerosol from the liquid sample via a nebulizer and segregating the aerosol droplets by size using a spray chamber. The sample is normally pumped by a peristaltic pump or a LC into the nebulizer. As the sample enters a pneumatic nebulizer it flows through a small capillary tube. Once the liquid exits the capillary tube at the tip of the nebulizer, the liquid is sheared into tiny droplets by a high velocity gas flow (~1 L min\(^{-1}\)).

The concentric is the most common pneumatic nebulizer [Figure 1.2]. Flow rates vary depending on the design. Nebulizers designed for conventional LC flow range from 0.5-1.0 mL min\(^{-1}\), while nebulizers manufactured for capillary and nano LC flow from <100 – 200 µL min\(^{-1}\) and under 500 nL min\(^{-1}\), respectively. Aerosols containing large droplets remove energy from the plasma as the droplets are de-solvated, destabilizing the plasma and reducing the amount of energy available for the ionization process. The spray chamber’s primary function is to allow only small droplets to enter the plasma. The most

![Concentric pneumatic nebulizer](image)
common spray chamber design is a double pass chamber shown in Figure 1.3a. As the droplets exit the nebulizer, droplets > 10 µm in diameter enter the center tube and fall out due to gravity and or collide into the chamber wall. Aerosols from 5 – 10 µm pass between the outer wall and central tube, emerging from the chamber into the torch and plasma.9

Figure 1.3 a) double pass spray chamber, b) cyclonic spray chamber, c) single pass (adapted from reference 9)

Another commonly used spray chamber is the cyclonic chamber Figure 1.3b. In this chamber design the aerosol is tangentially introduced into chamber. A portion of the aerosol is lost as it impacts the chamber wall as the remaining aerosol exits the chamber. The cyclonic chamber is very efficient in removing larger coarse droplets and is becoming more popular among ICPMS analysts.11 A third spray chamber used in this dissertation research is the single pass version, Figure 1.3c. Because the sample flow rate for this chamber is very low (< 10 µL min⁻¹), the solvent totally evaporates inside the chamber. As a result this interface is considered a total consumption system and the
chamber acts as an interface to promote solvent evaporation before the aerosol enters the plasma.\textsuperscript{12}

1.2.2 Sample Ionization

The basic components behind the creation of inductively coupled plasma used in ICPMS are illustrated in Figure 1.4. The plasma torch consists of three concentric tubes; an outer tube, middle tube and sample injector, all are usually made of quartz. Argon gas is usually used to form the plasma and is passed between the outer and middle tubes at \( \sim 15 \text{ L min}^{-1} \). An auxiliary gas flow is passed between the middle tube and sample injector at \( \sim 1 \text{ L min}^{-1} \). A third gas enters the torch from the nebulizer carrier gas at \( \sim 1 \text{ L min}^{-1} \) carrying the fine aerosols from the spray chamber. The injector flow physically creates a channel through the middle of the plasma.\textsuperscript{13} From this location the aerosols undergo desolvation (solid), vaporization (gas), atomization (atom) and ionization (ions) Figure 1.5.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image}
\caption{Components of inductively couple plasma generation for ICPMS\textsuperscript{14}}
\end{figure}
The mechanism for creation of the plasma begins with a spiral (tangential) flow of argon between the outer and middle tubes. A load coil, usually made of copper, surrounds the top end of the quartz torch and is connected to a radio frequency (rf) generator. As rf power is applied to the load coil an alternating current oscillates within the coil. The oscillation causes an electromagnetic field around the area at the top of the torch. A high voltage spark is introduced as argon gas is flowing through the torch. The spark removes electrons from the argon atoms, which are then accelerated in the magnetic field. The accelerated electrons collide with other argon atoms in a cascading chain reaction forming argon ions, and more free electrons creating inductively coupled plasma.\textsuperscript{15}

\textbf{1.2.3 Interface/Ion Focusing}

Movement of analyte ions from the plasma into the mass spectrometer requires an interface to transport ions from the plasma at atmospheric pressure (760 torr) to the mass filter and detector region at $10^{-6}$ torr. Ions are directed through an interface consisting of 2 metallic cones. The first cone known as the sampling cone has a diameter of 1.0 mm and is usually made of nickel on a copper base. The skimmer cone is the second in line, is
made entirely of nickel and has an orifice of 0.4 mm. Both cones can be made of other non-corrosive materials like platinum; however, only nickel cones were used in this research. Ions emerging form the argon plasma will have different kinetic energies based on their mass to charge ratio. If the plasma is held at zero potential, the ion energy spread is 5 – 10 eV. However, if the plasma is not held at a zero potential secondary discharges can occur yielding a wider ion energy spread (20-40 eV), making it increasingly difficult to focus ions into the mass spectrometer.\textsuperscript{16} Figure 1.6 outlines the configuration of the cones and ion optics in an Agilent 7500ce ICPMS system. The ion optics focuses the ion beam and separates the analyte ions from photons and neutral species that are traveling in a straight line. Photons and neutral species cause signal instability and contribute to background noise. Therefore the off-axis lens prevents neutral species and photons from entering the mass analyzer, while positively charged ions are guided by the lens system into the mass analyzer.\textsuperscript{17}

\textbf{Figure 1.6} Ion path of an off-axis lens system (adapted from reference 14)
1.2.4 Collision Reaction Cell Basics

Formation of polyatomic spectral interferences generated from argon, solvent, sample matrix or atmospheric gases can compromise the detection capability of a quadrupole mass analyzer. There are methods to minimize these interferences such as running under cool plasma conditions, matrix separation and utilizing mathematical correction equations for known interferences. A more affective approach has been the use of collision reaction cell technology. The technology was originally designed for organic mass spectrometry to generate daughter ions to assist in the confirmation and identification of the parent molecule.\(^{18}\) The first commercial collision cells for ICPMS were based on a hexapole originally designed for tandem mass spectrometry.\(^{19}\) Current instrumentation continues to use hexapole cells along with quadrupole and octopole configurations. The multi-poled cells operate under rf-only mode. This type of rf field does not separate mass like a traditional mass filter, instead it focuses the ions into a tighter ion beam Figure 1.7. An rf only multi-pole when filled (pressurized) with an inert gas, can focus ions through a process termed collisional focusing. Collisional focusing is dependent on the mass of focused ion and mass of collision gas.\(^{**}\) Once the collision reaction is pressurized with

\[ \text{Small i.d.} = \text{analyte ion} \]

\[ \text{To quadrupole} \]
a gas, ion-molecular gas collisions and ion-molecular gas reactions can occur depending upon the type of multi-poled cell and the gas being introduced. The gas (usually H₂ or He) can interact with the ion beam removing and or reducing polyatomic interferences by either reacting (converting to different species) or colliding (lose of kinetic energy through collisions).²⁰ These processes are accentuated by the negative potential bias on the exit lens of the collision reaction cell relative to the entrance optics of the quadrupole mass filter. The energy barrier created by the more positive voltage on the quadrupole (energy discrimination) capitalizes on the higher kinetic energy loss by the larger polyatomic ions through collisions within the cell, therefore, do not have enough energy to make it over the energy barrier. Allowing primarily the single analyte ions to pass into the quadrupole.¹⁴ In this work an octopole collision reaction cell was utilized and pressurized with xenon as a collisional gas and oxygen as a reaction gas, to contend with spectral interferences Figure 1.8.

Figure 1.8 Collision/reaction cell and octopole assembly depicting the entrance lens (guides ions into octopole), octopole rods (collimate ions into a tight beam) and exit lens (focuses ions into the quadrupole mass filter)
1.2.5 Mass Filtering

Separation of ions according to their mass to charge ratio ($m/z$) in ICPMS, can be accomplished with a variety of mass analyzers. The 3 basic designs are quadrupole, double focusing magnetic sector and time of flight, with the quadrupole being the most common. All of the ICP mass spectral data contained in this dissertation was generated through a quadrupole mass filter. As can be seen in Figure 1.1, the Q-pole mass filter is located between the collision cell and the detector and is maintained under a vacuum of $\sim 10^{-6}$ torr. The quadrupole consists of 4 molybdenum rods of the same length and diameter, each having a hyperbolic surface. The quadrupole by a radio frequency of $3$ MHz and the hyperbolic surface creates an elliptical field, resulting in higher ion transmission at higher resolution. By placing a specific rf on 1 pair of rods and a specific direct current (dc) on the opposing pair, ions of specific mass are electrostatically separated (filtered) and allowed to pass through the quadrupole to the detector. Ions whose $m/z$ ratio does not correspond to the specific rf/dc ratio are ejected from and or collide with the quadrupole or vacuum manifold. By varying the rf and dc fields, but keeping the ratio between them constant, different masses can be selectively filtered through the quadrupole system. Quadrupole scan rates can cover a mass range of 0 – 300 Da (Daltons) in about 0.1 seconds. However performance of a quadrupole is not based solely on its scanning speed but its ability to separate an analyte mass from spectral interferences and or other analyte masses. Traditionally for quadrupoles this is known as resolution and is defined as the width of a mass peak at 10% of its height. Figure 1.9 is a simplified version of a Mathieu stability diagram as it compares the filtering of two different masses entering the quadrupole at the same time. Any rf/dc combinations
Figure 1.9 Simplified Mathieu stability diagram for 2 different masses as modified from reference 22.

shown in the blue region will allow mass A to pass through the quadrupole and rf/dc combinations in the yellow region will allow mass B to pass through the quadrupole. As the slope of the rf/dc scan line increases, so does the degree of separation (resolution) between both masses and as the slope decreases spectral overlap occurs allowing both masses to pass through the quadrupole without being separated. Quadrupoles generally operate at a resolution of 0.3 – 1.0 Da. Another criteria for mass separation is abundance sensitivity; which is the amount of peak tailing that occurs from an adjacent peak 1 Da lower and 1 Da higher than the analyte peak of interest. Figure 1.10 illustrates how the tails of the spectral peak are sharper less pronounced at the high mass side compared to the low mass side. As ions enter the quadrupole they are slowed by the filtering process producing tailing or a shoulder at the low mass end. The peak shape
and degree of tailing is determined by the abundance sensitivity of the quadrupole. The largest impact on abundance sensitivity is the motion and kinetic energy of the ions as they enter and exit the quadrupoles. A combination of factors that can affect ion kinetic energy are the design of the rods, power supply frequency and operating vacuum associated with the “mean free ion path”.

1.2.6 Signal Detection

Signal detection occurs when an ion has traversed the mass filter and is converted into an electrical impulse. The magnitude of the electrical impulse relates to the number of analyze ions. There are a variety of detectors used in ICPMS in conjunction with the various forms of measurement circuitry to perform the ion counting. The signal data from this research was generated by a discrete dynode electron multiplier. As positive analyze ions collide with the surface of a dynode having a negative bias, the collision strips off 1 or more electrons from the dynode’s surface Figure 1.11. The electrons are moved
toward the next dynode by a potential gradient and impact the surface of the second dynode liberating more secondary electrons from the dynode surface. This process continues in a cascading effect resulting in a discrete pulse containing millions of electrons generated from 1 ion hitting the detector.\textsuperscript{24} Pulse counting can normally function within a linear dynamic sample range of 5 orders of magnitude (ppt-100 ppb) generating a signal at the high end of \( \sim 10^6 \) counts per second (cps).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure11.png}
\caption{Schematic diagram of a discrete dynode electron multiplier as modified from reference 14.}
\end{figure}

An ion counting detector is exemplary for analytes of low concentration; however, high concentrations generate such high ion counts the detector becomes saturated and fails to record some of the ions yielding a non-linear response. To overcome the non-linearity the detector design allows for a second mode of ion detection, an analog signal. Instead of using the discrete pulse signal during low count rates counting each individual impact, the analog signal is registered from the current generated by the electron stream as they impact the dynodes. The delay time switching back and forth from pulse and analog modes is critical especially in chromatography for short duration peaks. The lag time or “dead time” as it is known can cause nonlinear responses in a calibration curve.
with a large dynamic range. Figure 1.12a is an exaggerated illustration of what can happen when your analyte generates counts in both response zones. As depicted the analog mode can overlap into the pulse region as do the higher responses of pulse signal overlap into the analog region. To compensate for this “dead time” between pulse and analog, a dead time correction factor can be calculated, generating a larger and linear dynamic range Figure 1.12b. To accomplish this task analyte standards specific to the application are run at two different concentrations; a high concentration producing a signal $> 10^6$ cps and a low concentration producing counts $< 10^4$ cps. The dead time correction factor is a system parameter in the mass spectrometer’s detector circuitry and is specific for each application. Once the dead time has been calculated, the switching process is automatic and can simultaneously collect pulse and analog signals extending the dynamic range to nine orders of magnitude.  

![Figure 1.12](image1.jpg)

**Figure 1.12** a) pulse and analog signal responses of an analyte without dead time correction factor  
  b) pulse and analog signal responses factoring in dead time correction

### 1.3 Separation Techniques

As previously introduced, ICPMS does not yield chemical or structural information from sample analytes (section 1.1). However, it is element specific and easily
interfaces with various chromatographic techniques. Hyphenated techniques involving ICPMS are among the fastest growing application and research areas of atomic spectroscopy. Using chromatographic methods, analytes are separated into their chemical forms and or their oxidation states. Therefore, hyphenated ICPMS can be divided into elemental speciation and molecular speciation. In both instances you are measuring elemental signals; the difference depends on whether the elements being separated are present in the elemental state or as part of a larger molecule. This dissertation research incorporates both techniques; elemental speciation by determining the oxidation state of chromium, Cr(III) or Cr (VI), and molecular speciation by separating and detecting phosphorothioate oligonucleotides based on the phosphorus and sulfur present in their structures.

1.3.1 Conventional/Capillary LC

In LC, a liquid sample, or a solid sample dissolved in a suitable solvent, is carried through a chromatographic column by a liquid mobile phase. Separation is determined by solute/stationary phase interactions including liquid/liquid partitioning, liquid/solid adsorption, ion exchange, size exclusion and solute/mobile phase interactions. In each case, however, the basic instrumentation is essentially the same. Figure 1.13 depicts a typical LC system consisting of a vacuum degasser, binary pump, auto-sampler, thermostatically controlled column compartment and diode array detector (UV/Visible) detector. Connection of HPLC to ICPMS comprises an inert tube inserted directly into the nebulizer form the column or LC detector if running in series. LC flow rates normally range from 0.5 – 1.5 mL min\(^{-1}\). Conventional concentric nebulizers can routinely handle these flows with the proper spray chamber configuration. Nebulizers
have an optimal flow range for fine aerosol production. Fine aerosols are critical for proper desolvation and transport from the spray chamber to the plasma for atomization and ionization of the analyte. Mobile phase can be composed of aqueous buffers for size exclusion (SEC), ion chromatography (IC) and some reverse phase (RP) applications. RP mobile phases when used with standard bore and standard flow columns should not exceed 10% (v/v) organic modifier due to plasma instabilities and the build up of carbon deposits on the sampling and skimmer cones. If greater than 10% (v/v) organic must be used, an optional plasma gas (O\textsubscript{2}) is introduced into the plasma to reduce carbon build up via oxidation and a hotter plasma. If O\textsubscript{2} is used Pt sampler and skimmer cones should be used due to an increase in Ni oxidation. Alternatives when > 10% organic modifier are necessary to use post-column splitting (with some loss of analyte) or preferably reduce

\textbf{Figure 1.13} HPLC system interfaced to ICPMS capillary nebulizer
the organic solvent load to the plasma by using capillary or micro flow LC. ICPMS applications with capillary and nano-flows are rapidly expanding areas of research. Capillary flows require micro-flow nebulizers and have non-standard LC connections and spray chamber configurations. The overall goal is the same as with conventional flow; to match the nebulizer and spray chamber to the flow conditions for optimal aerosol production and transport into the plasma.
1.4 References

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Chapter 2

Determination of Trivalent and Hexavalent Chromium in Pharmaceutical, Nutraceutical and Biological Matrices Using LC-ICPMS
Abstract

This study optimizes parameters to minimize sample preparation and evaluates the resolution, reproducibility and responses of Cr (III) and Cr (VI) in pharmaceutical, nutraceutical, and synthetic saliva samples. The method utilizes an integrated nonmetal pumping system to reduce chromium background that may be present in standard stainless steel LC pumps and tubing. The conventional flow LC pump is coupled to an ICPMS system equipped with a collision cell to diminish polyatomic and isobaric interferences associated with ArC\(^+\), ClO\(^+\), and CrH\(^+\) for \(^{52}\)Cr\(^+\) or \(^{53}\)Cr\(^+\). Separation is achieved via ion exchange chromatography in combination with the formation of a Cr (III)-EDTA complex. Retention time and peak area reproducibility for Cr(III) and Cr(VI) ranged from 0.2 – 1.5% RSD (n=7). Detection limits (3\(\sigma\)) based on sample loop volume and sample matrix, ranged from 19 - 130 ng/L for Cr(III)-EDTA and 22 - 170 ng/L for Cr(VI) anion.

Keywords: Chromium speciation, Anion Exchange Chromatography, ICPMS
2.1 Introduction

After recent studies suggesting ingestion of Cr(VI) may cause chromate induced cancers, there has been a renewed interest in the ability to separate, identify, and quantify trivalent and hexavalent chromium in a broad range of sample matrices.\(^1\)\(^-\)\(^3\) This interest is sparked by the fact that hexavalent chromium can cause many types of DNA damage, while not directly interacting with DNA itself.\(^4\) Cr(VI) complexes, such as chromate \((\text{CrO}_4)^{2-}\) at physiological pH, easily penetrate cell walls through the sulfate and phosphate anion channels. It is accepted that for Cr(VI) to exert its cytotoxic and carcinogenic effects, it has to be reduced inside the cell.\(^2\)

In contrast to Cr(VI), cellular membranes are normally impermeable to Cr(III) cations, which can penetrate the cell wall only under specific conditions. One such condition is through the attachment of low molecular weight organic ligands utilized in dietary supplements, such as picolinic acid, niacin and phenylalanine.\(^5\) Cr(III) is thought to play a key role in carbohydrate metabolism and a deficiency of Cr(III) has been shown to amplify the risk of developing diabetes.\(^6\) This study seeks to develop a qualitative and quantitative method for pharmaceutical preparations. This is relevant in determining oxidative shelf-life of a product [Cr(III) oxidized to Cr(VI)] under long term storage conditions and evaluating if a pharmaceutical preparation only contains Cr(III) after drug synthesis. Both nutraceutical and pharmaceutical samples were analyzed based on this methodology.

There are hundreds of alloys available for orthodontic uses and in recent years there has been a concern about the possible toxic or allergic effects related to these alloys releasing metal ions.\(^7\)\(^-\)\(^9\) Stainless steel alloys that contain 17-22% chromium are generally
used in orthodontic appliances and can corrode over time. Metallic compounds and or ions, released into saliva can pass into the circulatory system from oral cavity tissues.\textsuperscript{10} Lopez-Alias and coworkers, under \textit{in vitro} conditions, reported up to 4 µg/day of chromium could be released in a worst case scenario.\textsuperscript{10} Unfortunately, only total chromium data were collected in this and other literature references.\textsuperscript{8,11} The current study reflects the first speciation of chromium in synthetic saliva.

Atomic spectrometric techniques, inductively coupled plasma mass spectrometry and inductively coupled atomic emission spectroscopy, (ICPMS, ICPAES) directly coupled to chromatographic separations are most widely used for detecting these two forms of chromium. Other ways of detection include flame/graphite furnace AA,\textsuperscript{11-13} anodic stripping voltammetry,\textsuperscript{14,15} UV/Vis,\textsuperscript{16} fluorimetry\textsuperscript{17,18} and chemiluminescence.\textsuperscript{19,20} All modes of detection have their weaknesses and limitations, however, when comparing ICPMS to the other forms of detection, it is the least restricted by sample matrix and background interferences and is element specific and can accommodate simultaneous multi-element multi-isotopic analysis. However, conventional quadrupole ICPMS, although element selective, suffers from spectral and non-spectral overlaps. Polyatomic ion interferences in the form of dimer, oxide and complex polyatomic ions, are typically derived from plasma gas, sample matrix and atmospheric gases surrounding the plasma.\textsuperscript{21} Rowan and Houk in 1989, demonstrated the use of gas phase collisions within a multipole cell under the influence of a linear radio frequency (rf), can reduce spectral interferences produced in an argon plasma.\textsuperscript{22} The commercial development of (rf) driven collision/reaction cells utilizes ion molecule reactions, collisional dissociation, ion energy discrimination and charge transfer mechanisms to defray spectral and non-spectral
The efficiency and selectivity of ion-molecule reactions to reduce spectral interferences depends upon the proper selection of reaction gas, reaction gas flow rates, cell entrance/exit voltages and rf parameters applied to the multipole. Monitoring chromium isotopes $^{50}\text{Cr}$, $^{52}\text{Cr}$, $^{53}\text{Cr}$, and $^{54}\text{Cr}$ allows for unambiguous chromium identification. Some of the polyatomic and isobaric interferences associated with isotopes of chromium include $\text{ArC}^+$, $\text{ClO}^+$, $\text{ClOH}^+$, $\text{CrH}^+$ and $\text{Pd}^{2+}$, and $\text{Fe}^+$ for $^{52}\text{Cr}^+$, $^{53}\text{Cr}^+$, and $^{54}\text{Cr}^+$.

Within the myriad of chromium methods and analytical techniques available, none reference such a diverse sample matrices as saliva, pharmaceutical, and nutraceutical. A greater number of chromium methodologies relate to total chromium concentrations and do not address chromium species. Sample pretreatments are routinely employed for the speciation of chromium and are determined based upon the separation technique and ionic character of the chromium species. The most widely applied sample pre-treatment for chromium speciation is complex formation. $\text{Cr(III)}$ has been complexed with hydroquinolines, trifluoroacetyl acetone, pyridinedicarboxylic acid, and ethylenediaminetetraacetic acid (EDTA). The pH environment of these complexes can range from 2 – 8.5 depending on the complexing ligand. At acidic pH, $\text{Cr(III)}$ exists primarily as a cation, while in alkaline media trivalent chromium easily oxidizes to hexavalent chromium. $\text{Cr(VI)}$ exists as an anion from pH 2 and beyond pH 6. Below pH 2 $\text{Cr(VI)}$ will reduce to $\text{Cr(III)}$ and above pH 7 its ability to oxidize $\text{Cr(III)}$ is enhanced. In a pH neutral aqueous medium $\text{Cr(III)}$ ions form an octahedral cation, surrounded by six water molecules and $\text{Cr(VI)}$ is a stable anion complex. The chelating molecular structure of EDTA has six bonding sites that could be used to coordinate with
Cr(III), but EDTA is not large enough to encompass the entire octahedral complex. Consequently, only five Cr(III)-EDTA bonds are formed, allowing one water molecule to remain in the octahedral complex. Ion-exchange chromatography is common in elemental speciation and is carried out with column packing that contain a charge bearing functional group attached to a polymer matrix. The most common retention mechanism is the simple exchange of sample ions and mobile phase ions with the charged functional group of the stationary phase. In this study the column packing consists of polymethacrylate yielding a positively charged (cation) \( R-NR_3^+ \) functional group stationary phase attracting negatively charged (anions) complexes. Under pH neutral conditions a mono-anionic Cr(III)-EDTA complex is weakly retained on an anion exchange column, amidst a strongly retained chromate anion. Ion exchange chromatography is commonly used in elemental speciation in lieu of ion-pairing reverse phase due to the organic reagents in the mobile phases that can cause interferences resulting in high backgrounds for \(^{52}\text{Cr}\) isotope and blockage of the ICPMS sampling cones after prolonged use. In addition, samples containing a high concentration of anions can overload the column and ion pairing capacity of the mobile phase, resulting in altered retention times, co-elution, and exclusion of the analytes.

This study optimizes experimental parameters to minimize sample prep time and evaluates the resolution, reproducibility and responses of Cr (III) and Cr (VI) by complexing EDTA to Cr(III) and utilizes EDTA as a buffer in diverse sample matrices. This method is similar to the methods reported by Tomlinson et. al. and Byrdy et. al. in 1994 and 1995, respectively. Tomlinson complexed Cr(III) with 2,6-pyridinedicarboxylic acid while Byrdy used EDTA as the chelating agent to complex
with Cr(III) to stabilize the Cr(III) species in solution. The EDTA complexing method was developed further by Sakai and coworkers in 2005 and is the foundation of the current optimized method.\textsuperscript{34-37} The adopted method is applied to pharmaceutical, nutraceutical, and synthetic saliva sample matrices. In this study, the simultaneous determination of Cr(III) and Cr(VI) species by anion exchange ICPMS is described. To date, this is the first work applying a single chromium speciation technique to saliva, pharmaceutical and nutraceutical sample matrices under one methodology.

2.2 Experimental

2.2.1 Instrumentation

The HPLC isocratic pump was a Metrohm 818C (Houston, TX, USA) metal free system. A 1.2 mL min\textsuperscript{-1} flow rate of 5 mM EDTA adjusted to a pH of 7 was maintained throughout the analysis in conjunction with a polymethacrylate anion exchange from Agilent Technologies (G3268-80001) (Santa Clara, CA, USA). Sample introduction consisted of a non-metal 6 port Rheodyne valve made by IDEX Corporation (Rohnert, CA, USA) connecting the sample loop, auto-sampler, and separation column. The auto-sampler, 6 port valve, and external isocratic pump were all controlled by ISIS (Integrated Sampling Introduction System), from Agilent Technologies, Santa Clara, CA, USA

\textbf{Figure 2.1.} This study compared signal responses from 100 and 500 µL PEEK sample loops incorporating an 89 position IAS (Integrated Auto-Sampler) from Agilent Technologies. The ICP mass spectrometer used in this work was a 7500ce quadrupole mass analyzer, equipped with a collision/reaction cell from Agilent Technologies, Santa Clara, CA, USA. H\textsubscript{2} was used as the collision/reaction gas at a flow rate of 3.5 ml min\textsuperscript{-1}. A double pass Scott-type spray chamber was used in conjunction with a MicroMist\textsuperscript{™}
nebulizer from Glass Expansion (Pocasset, MA, USA). Nickel sample and skimmer cones with a 1.0 and 0.4 mm orifice diameter, respectively, were used. All samples and standards were incubated in a NesLab Instruments (Newington, NH USA) RTE-10 temperature controlled circulating water bath.

**2.2.2 Reagents and standards**

Chromium(III) and chromium(VI) standards were produced from Cr(NO$_3$)$_3$ · 9 (H$_2$O) Aldrich Chemical Company, (Milwaukee, WI, USA) and K$_2$Cr$_2$O$_7$ Mallinckrodt Chemicals, (St. Louis, MO, USA). Disodium ethylenediaminetetraacetic acid used for the 5 mM mobile phase and the 15 mM complexing/buffering agent were purchased from Fisher Scientific (Fairlawn, NJ, USA). All solutions were prepared from in-house doubly distilled deionized water (18MΩ, Barnstead, Newton, MA, USA) and pH adjusted to 7 with 50 %(v/v) NaOH solution prepared from reagent grade NaOH supplied by Fisher Scientific. Synthetic saliva was supplied from Dyna-Tek (Lenexa, KS, USA). The pharmaceutical sample used in this study was a proprietary product, while the nutraceutical samples were purchased from General Nutrition Centers (Pittsburg, PA, USA). 10 mL borosilicate glass microwave vials were used for all water bath incubations, were purchased form CEM (Matthews, NC USA). Clear borosilicate screw cap vials (15 mm x 45 mm) functioned as auto-sample vials and were purchased from Fisher Scientific.

**2.2.3 Sample Preparation**

Stock standards were prepared in doubly distilled de-ionized water. Serial dilutions of stock standards and samples were diluted in 15 mM EDTA and incubated for 20 minutes in a 60 °C circulating water bath and allowed to come to room temperature before analysis. Solid samples were dissolved and diluted in 15 mM EDTA before
Figure 2.1 Integrated sample flow diagram for IC-ICPMS system (adapted from reference 35)
incubation. Liquid samples were first quick frozen on dry ice, lyophilized and reconstituted in 15 mM EDTA before analysis. Standards and spikes were added to each sample matrix before any dilutions or lyophilizations were performed.

Pharmaceutical/Nutraceutical

A powdered pharmaceutical sample was analyzed for total chromium content and chromium speciation analysis. Similar determinations were performed on over-the-counter nutraceutical samples consisting of powdered tablets labeled as containing 200 µg of chromium picolinate and over-the-counter gel-caps, also containing 200 µg of chromium picolinate. The powdered tablets were manufactured in 1999, while the gel caps were manufactured in 2006. The pharmaceutical and nutraceutical samples were first dissolved in 200 mL of the 15 mM EDTA solution and 6 mL aliquots were removed from each sample, placed in a borosilicate microwave 10 mL vial, capped and incubated for 20 minutes in a 60 ºC circulating water bath.

Saliva

Synthetic saliva was divided into 1 mL aliquots from the manufacturer and lyophilized as previously described. Samples and spiked samples of saliva were reconstituted in 1 mL of 15 mM EDTA, transferred to 10 mL borosilicate glass vials, capped and incubated for 20 minutes in a 60 ºC circulating water bath. After incubation all samples, spikes and standards were transferred to 15mm x 45 mm screw cap vials and placed in the auto-sampler tray. All samples were incubated and analyzed on the same day.
2.2.4 Chromatographic separation conditions

The anion-exchange column body was constructed of PEEK and packed with a polymethacrylate resin with a particle size of 10 µm (Agilent Technologies, Santa Clara, CA, USA, Santa Clara, CA, G3286-80001), 4.6 x 30 mm. The mobile phase was composed of a 5 mM EDTA solution adjusted to a pH of 7 and maintained at a constant flow rate of 1.2 mL/min throughout the 6 minute runtime. The column was operated at room temperature during the entire study. Data were generated on sample loop volumes of 100 µL and 500 µL for all sample matrices.

2.2.5 Agilent Technologies Separation Kit

The separation kit consisted of a 6 port valve, a Metrohm metal free isocratic pump and 2 high precision peristaltic pumps all controlled by ISIS (Integrated Sampling Introduction System). Also a 100 µL PEEK sample loop incorporated an 89 position Integrated Auto-Sampler while ion separation was performed on the polymethacrylate G3286A column. Mobile phase and sample preparations were perfected and optimized as previously mentioned.

2.1 Results and discussion

2.1.1 EDTA complex optimization

This method exploits the abilities of EDTA to form metal complexes and act as a buffer. The use of EDTA as the complexing agent was chosen because of the stable Cr(III)-EDTA complex and the culmination of studies performed by Caruso and co-workers,32,33,37 Inoue,38 and adapted from Technical Notes from Agilent Technologies.36,37 The complexing stoichiometry between reactants and products is 1:1.28 The rate of reaction is temperature and pH dependent, while independent of the EDTA
concentration. Incubation times and temperatures were optimized for the greatest Cr(III) response while optimizing the concentration of EDTA to act as a sample matrix buffer at pH 7, in an attempt to minimize sample preparation times and the reduction of Cr(VI). Figure 2.2 displays the area responses of a mixture of 50 µg/L Cr(III) and 15 mM EDTA, incubated at room temperature (~25ºC), 40, 50, 60, 70, 80 and 90ºC, with incubation times of 20, 40 and 60 minutes. Using a mobile phase of 5 mM EDTA adjusted to pH 7, the ICPMS area response based on the $^{52}$Cr signal, illustrates the Cr(III)-EDTA complex formation increasing with increasing temperature and incubation times. Considering that higher temperatures favor Cr(III) oxidation to the chromate ion, while at the same time attempting to minimize sample preparation and produce a stable Cr(III)-EDTA complex, an incubation temperature of 60 ºC with an incubation time of 20 minutes was chosen. The selected temperature and incubation time corresponds with the findings of Sakai and coworkers of a stable Cr(III)EDTA complex forming within an hour at 60 ºC. However, the Sakai application incubated at 40 ºC for 3 hours due to the high concentration of competitive metal ions in their sample matrix. Byrdy heated the Cr(III) stock standard with EDTA at 50 ºC for 1 hour at a pH of 4.0 to stabilized the species and guard against oxidation. Tomlinson pre-complexed Cr(III) with 2,6-pyridinedicarboxylic acid by warming to 65 ºC for 2 hours. The data in Figure 2.1 depicts higher responses for the Cr(III)-EDTA complex, but the response at 60 ºC yields the most stable response over the 60 minute period producing a more reproducible and linear response overall. Thereby, markedly reducing the incubation time for Cr(III)EDTA complex formation and therefore reducing the overall analysis time.
2.1.2 Background signal reduction

This method utilizes an integrated pumping system made of non-metallic components to reduce chromium background that exists in standard stainless steel LC pumps and tubing. This is not the first use of a non-metallic LC pump in conjunction with ICPMS; however it is believed to be the first time a direct comparison has been made regarding Cr isotopes. By utilizing the non-metallic pump and sample introduction system, it reduced the $^{52}$Cr background by twenty fold, an important advance when dealing with complex sample matrices due to the need to minimize sample preconcentration which could alter the original chromium species. The ability to integrate the Metrohm 818C into the Agilent ICPMS ChemStation allowed for seamless instrument control and method development. There was not a significant difference in

**Figure 2.2** 50 ppb Cr(III) in 15 mM EDTA 23–90°C for 20, 40 and 60 minutes
LODs between pumping systems. Qualitatively, background from the stainless steel pumping system contributed to disproportionate isotope ratios $^{50}$Cr (2.6%), $^{52}$Cr (75%), $^{53}$Cr (12%) and $^{54}$Cr (11%) for chromium, while the non-metallic pump background resulted in the commonly accepted isotopic ratios of $^{50}$Cr (3.6%), $^{52}$Cr (82.9%), $^{53}$Cr (9.9%) and $^{54}$Cr (2.4%).

2.1.3 Figures of Merit

Limits of detection (LOD), linearity and reproducibility were calculated for each sample matrix relative to sample volume. Table 2.1 lists the figures of merit for the 100 and 500 µL sample loops. The relative standard deviations based on Cr(III) and Cr(VI) peak areas (n=7) for 25 µg/L were 1.5% and 2.2%, respectively for the 100 µL loop and 0.3% and 0.5% for the 500 µL loop. Retention reproducibility for both chromium species on both sample loops ranged 0.2-1.1% (n=7) for 25 µg/L. The limits of detection (3σ) for each chromium species varies slightly for each sample matrix. The LODs generated from the 100 µL sample loop ranged from 110 to 120 ng/L for Cr(III)-EDTA complex and 140 to 170 ng/L for Cr(VI), while the 500 µL loop ranged from 19 to 26 ng/L for Cr(III)-EDTA and 22-34 ng/L for Cr(VI). The LODs are well below the detection limits of Tomlinson and Byrdy and inline with the limits reported by Sakai in hard water samples. Linear range covered 4 orders of magnitude (1 ppm - 100 ppt) for the 100 µL sample loop and 5 orders of magnitude (1 ppm – 10 ppt) utilizing the 500 µL loop. Column recoveries were based upon total chromium peak areas for each species, in each sample matrix, and compared with and without the anion exchange column present during flow injection analysis. Calculated column recoveries were 95% and 98% for the 100 µL and 500 µL sample loops respectively, for all sample matrices.
<table>
<thead>
<tr>
<th></th>
<th>Cr(III) Area</th>
<th>Cr(VI) Area</th>
<th>Cr(III) Retention Time</th>
<th>Cr(VI) Retention Time</th>
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<tbody>
<tr>
<td><strong>Average 100 µL</strong></td>
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<td>1.9 x 10⁶</td>
<td>0.93 min</td>
<td>4.42 min</td>
</tr>
<tr>
<td><strong>Std Deviation</strong></td>
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<td>4.3 x 10⁴</td>
<td>0.01 min</td>
<td>0.02 min</td>
</tr>
<tr>
<td><strong>%RSD</strong></td>
<td>1.5</td>
<td>2.2</td>
<td>1.1</td>
<td>0.5</td>
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<thead>
<tr>
<th></th>
<th>Cr(III) Area</th>
<th>Cr(VI) Area</th>
<th>Cr(III) Retention Time</th>
<th>Cr(VI) Retention Time</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Average 500 µL</strong></td>
<td>7.3 x 10⁶</td>
<td>6.8 x 10⁶</td>
<td>1.08 min</td>
<td>4.45 min</td>
</tr>
<tr>
<td><strong>Std Deviation</strong></td>
<td>2.5 x 10⁴</td>
<td>3.6 x 10⁴</td>
<td>0.01 min</td>
<td>0.01 min</td>
</tr>
<tr>
<td><strong>%RSD</strong></td>
<td>0.3</td>
<td>0.5</td>
<td>0.9</td>
<td>0.2</td>
</tr>
</tbody>
</table>

**Table 2.1** 100 µL and 500 µL Loop Reproducibility

### 2.1.4 Determination of Chromium species in each sample matrix

Synthetic as opposed to human saliva was the specimen of choice due to several experimental conditions and parameters. The current research as it pertains to salvia, utilizes synthetic saliva to minimize the physical and chemical variability found between human subjects based upon their diet, oral hygiene and overall oral health. The optimized EDTA method was applied to each sample matrix and produced baseline separations for both chromium species. Figure 2.3 is an offset chromatogram comparing a blank saliva sample to spiked saliva, depicting baseline resolution of both species utilizing the 100 µL sample loop. Recoveries for spiked solutions (matrix) were defined as the amount that was experimentally determined versus the known amount that spiked into the matrix. Chromium recoveries in each sample matrix were based on the linear slopes ($y = mx + b$) generated from a 5 level spiked matrix calibration curve compared to a duplicate 5 level aqueous calibration curve, generated under identical method conditions. Spiked saliva recoveries were 97% and 95% for Cr(III) and Cr(VI), respectively.
Figure 2.3 5 µg/L spike of Cr(III) and Cr(VI) overlaying a lyophilized saliva blank

Pharmaceutical/Nutraceutical ion exchange chromatograms

The pharmaceutical sample was diluted and dissolved in 15 mM EDTA and contained 5 µg/L of Cr(III) in the sample. A 50 µg/L spike of the sample with both chromium species is overlaid in Figure 2.4. As can be seen by comparing the non-spiked sample to the spiked sample, no Cr(VI) was detected in the pharmaceutical sample. The nutraceutical sample resulted in a similar ion chromatogram, but not displayed. This type of data could aid in the determination of shelf-life and the quality of the final product.
Pharmaceutical and nutraceutical processing equipment are commonly composed of stainless steel and employing this method could determine the presence and species of chromium from the manufacturing process. The recoveries of both Cr(III) and Cr(VI) were 96% in the pharmaceutical sample. However, recoveries varied with the nutraceutical samples and were dependent upon the powdered tablets or gel-caplets. The powdered samples had recoveries of 92% and 95% for Cr(III) and Cr(VI), while the gel caplets recovered 85% and 87% of the Cr(III) and Cr(VI) species, respectively. Based upon other Cr(III)EDTA methodologies in the literature, these recoveries fall well within acceptable limits.

\[27,34,36\]

**Figure 2.4** Pharmaceutical sample overlaid with a 50 µg/L spiked pharmaceutical sample
2.4.1 Conclusions

The evaluation of a commercially available chromium speciation system and how the method can be applied to a variety of sample matrices is presented. Sample pretreatment with EDTA was optimized and standardized to complex with Cr(III) in three diverse sample matrices and formed a stable complex with 20 minutes of incubation at 60 °C. Cr(III) and Cr(VI) were completely separated in under 6 minutes. The mobile phase composition, nonmetallic pump and ICPMS collision reaction cell enabled the monitoring of each chromium isotope. The reproducibility, limits of detection and ruggedness of this procedure facilitates its use in broader and more elaborate sample matrices. Future research will build on this method and incorporate a desalting procedure to enlarge the sample matrix, to samples that contain interfering salts and metals, that could compete with the chromium EDTA complex. Chromium recoveries were acceptable in each matrices based on previously mentioned work, however, matrix matching and sample recoveries should be determined for each sample matrix.

Acknowledgments

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References

Chapter 3

Determination of Sulfur and Phosphorus by Oxygen Reaction Gas in an Octopole Collision/Reaction Cell ICPMS System
Abstract

A sensitive and selective technique for detecting phosphorus- and sulfur- based analytes with an ICPMS equipped with an rf only driven octopole collision reaction cell (CRC) was investigated. High purity oxygen was introduced into an octopole CRC from an auxiliary gas flow manifold installed on an Agilent Technologies 7500ce ICPMS. Optimal oxygen flow rates ranged from 0.2 – 0.4 mL min\(^{-1}\) depending upon the application. The use of oxygen as a reaction gas in this CRC enables thermodynamically allowed oxidation reactions to generate chemical resolution of specific analytes. Nanogram and picogram detection of \(^{31}\text{P}^{16}\text{O}^+\) and \(^{32}\text{S}^{16}\text{O}^+\) was established under continuous flow and time - resolved capillary LC separation, respectively. A potential difference of 7 volts between the octopole (-1V) and quadrupole bias (-8V), enhanced the product ion transmission. The analytical methodology was applied to the qualitative and quantitative determination of sulfur via \(^{32}\text{S}^{16}\text{O}^+\) detection, under continuous flow conditions for sulfur from furfuryl mercaptan, a common food flavoring additive. The detection of phosphorus oxide was derived from a mixture of four synthetic DNA oligonucleotide dT\(_n\) homopolymers separated by capillary LC. Oxygen as a reaction gas is easily implemented on this commercial ICPMS through a 1 mL min\(^{-1}\) flow manifold that is typically used for Xe introduction.

Keywords: Octopole; Collision/reaction cell; ICPMS; Ion-molecule reactions; Oligonucleotides; Food additives
3.1 Introduction

With ICPMS, the selectivity and sensitivity of a specific application can be affected by interfering polyatomic ions originating from the plasma gas, sample matrix, and atmospheric gases surrounding the plasma. The commercial development of linear radio frequency (rf) driven multipole collision cells and reaction cells have been essential in minimizing these limitations. The ion-molecule reactions that occur within the multipole cells were shown in 1989 by Rowan and Houk to drastically reduce some of the spectral interferences produced in an argon plasma by inducing gas phase collisions inside the vacuum chamber.¹

The efficiency and selectivity of ion-molecule reactions to reduce spectral interferences depends upon the proper selection of reaction gas, reaction gas flow rates, cell entrance/exit voltages and rf parameters applied to the multipole. Olesik has observed the overall parameter set points required to achieve ion-molecule reactions using a quadrupole reaction cell to diminish spectral overlap.² A prospectus from Koppenaal and coworkers correlates the historical developments and applications capitalizing on collision/reaction cell techniques, and an in-depth review by Tanner et al. illustrates the theoretical and operational designs of collision cells and reaction cells.³,⁴

The focus on ion-molecule chemistry remains a common theme in the current literature due to the persistent presence of polyatomic interferences in ICPMS analysis. To meet the demand of femtogram and picogram detection in metallomic and pharmaceutical research, new applications involving collision and/or reaction cells are being developed. Over the last decade, a multitude of sample introduction techniques for ICPMS have been evaluated utilizing multipole collision/reaction cells with a variety of
collision/reaction gases. A recent example is the continuous flow analysis of water samples to remove arsenic and selenium interferences via a collision/reaction cell by introducing He and H\textsubscript{2} gases simultaneously into the multipole cell, achieving detection limits as low as 30 ng in a high chloride matrix.\textsuperscript{5}

Hydrogen is one of the most common and effective collision/reaction gases used in ion-molecule chemistry. However, using H\textsubscript{2} can produce interferences with selenium isotopes in samples with a high bromine matrix. Suzuki and coworkers developed a method reacting D\textsubscript{2} in the place of H\textsubscript{2} for total selenium determination and speciation.\textsuperscript{6}

Methods involving various gases, such as H\textsubscript{2}S, Xe, CH\textsubscript{4} and NH\textsubscript{3}, to diminish polyatomic interferences are application specific and are well represented in the literature.\textsuperscript{7-10}

Separation techniques interfaced with ICPMS have also been investigated in conjunction with collision/reaction cells. A gas chromatograph (GC) coupled to an ICPMS equipped with an on-axis octopole collision cell utilizing He was used by Profrock and coworkers to detect the presence of pesticides, in the low ppt and ppb levels, based on their elemental composition.\textsuperscript{11} Ion-pairing reversed phase liquid chromatography (IP-RP-LC) was interfaced to an ICPMS utilizing He in an octopole collision/reaction cell and applied by Richardson \textit{et al}. in the analysis of degradation products from chemical warfare agents.\textsuperscript{12}

The qualitative and quantitative determination of elemental phosphorus and sulfur in a myriad of sample matrices and reaction gases is well documented. Phosphorus is monoisotopic in its natural abundance as \textsuperscript{31}P and has known polyatomic interferences from atmospheric gases surrounding the plasma, which include \textsuperscript{15}N\textsuperscript{16}O\textsuperscript{+} and \textsuperscript{14}N\textsuperscript{16}O\textsuperscript{1}H\textsuperscript{+}. Sulfur isotopes are also difficult to distinguish between sample signal and background
interferences due the large presence and contribution from O, N, and H based polyatomic ions.

Ellis et al. introduced He into an octopole collision/reaction cell to remove polyatomic ions interfering with low abundant and low molecular weight phosphopeptide detection as a biomarker in cerebral spinal fluid.\textsuperscript{13} Schaumloffel and coworkers eliminated sulfur spectral interferences while quantifying sulfur containing peptides using Xe gas in an octopole collision cell.\textsuperscript{14} Mason applied a series of gas mixtures (He, H\textsubscript{2}, and Xe) into a hexapole collision/reaction cell and reduced O\textsubscript{2}\textsuperscript{+}, O\textsubscript{2}H\textsuperscript{+} and NO\textsuperscript{+} interferences to measure sulfur isotope ratios in water samples.\textsuperscript{15}

The introduction of O\textsubscript{2} gas into a hexapole cell by Bandura and coworkers alleviated most of the polyatomic interferences by reacting O\textsubscript{2} with \textsuperscript{31}P\textsuperscript{+} and \textsuperscript{32}S\textsuperscript{+} to produce new product analytes at higher masses, \textsuperscript{31}P\textsuperscript{16}O\textsuperscript{+} and \textsuperscript{32}S\textsuperscript{16}O\textsuperscript{+}, in a mass spectral region of lower interference.\textsuperscript{16} A similar procedure utilizing a hexapole collision/reaction cell was demonstrated by Wang et al to quantify sulfur containing proteins. Bovine serum albumin, superoxidase dismutase, and metallothionein-II were separated by size exclusion chromatography and reacted with oxygen to form \textsuperscript{32}S\textsuperscript{16}O\textsuperscript{+} and \textsuperscript{34}S\textsuperscript{16}O\textsuperscript{+}, representing \textsuperscript{32}S\textsuperscript{+} and \textsuperscript{34}S\textsuperscript{+}, respectively, yielding limits of detection ranging from 8 – 31 pmol for the three proteins investigated.\textsuperscript{17}

In this study, a method has been developed to introduce O\textsubscript{2} gas into a commercially available rf- only driven octopole collision/reaction cell to react with \textsuperscript{31}P\textsuperscript{+} and \textsuperscript{32}S\textsuperscript{+}. Continuous flow analysis was used for the qualitative and quantitative determination of sulfur in furfuryl mercaptan, a common food flavoring additive. The time resolved analysis and detection of phosphorus from a synthetic homopolymer
mixture of dTₙ oligonucleotides, separated by capillary liquid chromatography is also described. To our knowledge this is the first reported use of ICPMS to detect oligonucleotides through their phosphodiester linkages using O₂ as the reaction gas.

3.2 Experimental

3.2.1 Instrumentation, reagents and standards

The ICP mass spectrometer used for this study was an Agilent Technologies 7500ce (Santa Clara, CA, USA). Continuous flow measurements were performed on a Glass Expansion MicroMist™ glass concentric nebulizer (Pocasset, MA, USA), while time-resolved data were generated with a Meinhard Glass Products HEN 90 A.02 capillary concentric nebulizer (Golden, CO, USA) connected to a Meinhard Fit Kit #1-Micro for capillary LC interfacing. This instrument was equipped with an on-axis rf-only octopole collision/reaction cell (CRC) that allowed the octopole chamber to be pressurized under 3 separate gas manifolds. Typically H₂, He and Xe would be introduced separately and/or simultaneously into the Agilent Octopole Reaction System (ORS – Agilent Technologies). The Xe flow manifold has a maximum flow of 1 mL min⁻¹ and is controlled as a percentage setting based on the maximum flow setting. Here, Xe gas was replaced with high purity O₂ (>99.995%) as the reaction gas. All instrument parameters and data analysis were controlled by the ICPMS ChemStation software (Agilent Technologies, Santa Clara, CA, USA). The ICPMS instrumental and acquisition parameters are provided in Table 3.1.

The capillary HPLC system was a modular Agilent 1200 (Agilent Technologies, Santa Clara, Ca, USA) system, which consisted of a vacuum chambered micro-degasser, a binary pump capable of delivering up to 20 µL min⁻¹, a thermostatically controlled well
**ICPMS Parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>spray chamber</td>
<td>Scott double pass(^a); single pass(^b)</td>
</tr>
<tr>
<td>chamber temperature</td>
<td>2 °C(^a); room temperature(^b)</td>
</tr>
<tr>
<td>nebulizer</td>
<td>MicroMist(^\text{TM}) (^a); HEN 90 A.02(^b)</td>
</tr>
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<td>nebulizer carrier</td>
<td>0.98 (L min(^{-1}))(^a); 1 (L min(^{-1}))(^b)</td>
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</tr>
<tr>
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<tr>
<td>plasma gas flow</td>
<td>15 (L min(^{-1}))</td>
</tr>
<tr>
<td>auxiliary gas flow</td>
<td>1 (L min(^{-1}))</td>
</tr>
<tr>
<td>collision/reaction optional gas</td>
<td>45% (^a); 38% (^b) (% flow of 1 mL min(^{-1}) manifold)</td>
</tr>
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<td>quadrupole bias</td>
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</tr>
<tr>
<td>octopole bias</td>
<td>-1 Volts</td>
</tr>
<tr>
<td>octopole RF</td>
<td>190 Volts</td>
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<td>selected masses</td>
<td>47, 48, 50 m/z (^{31})P(^{16})O(^+), (^{32})S(^{16})O(^+), (^{34})S(^{16})O(^+))</td>
</tr>
</tbody>
</table>

\(^a\) continuous flow analysis; \(^b\) time resolved analysis

**HPLC parameters**

<table>
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<td>column temperature</td>
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<tr>
<td></td>
<td>3.00 min: (92)/(8)</td>
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<td>3.01 min (90)/(10)</td>
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<td></td>
<td>15.0 min: (80)/(20)</td>
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<tr>
<td></td>
<td>15.01 min (75)/(25)</td>
</tr>
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<td>runtime</td>
<td>25.0 min</td>
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<tr>
<td>fast composition change</td>
<td>enabled 1.0 min flush 3.0 min equilibration</td>
</tr>
<tr>
<td>DAD wavelength</td>
<td>259 nm, slit 4 nm, reference bandwidth 6 nm</td>
</tr>
</tbody>
</table>

**Table 3.1** Instrument and acquisition parameters for continuous flow of 2-furfuryl mercaptan and LC-ICPMS analysis of polythymidilic acids.

plate sampler with an 8 µL sample loop, a thermostatically controlled column

compartment and a diode array detector equipped with a 80 nL flow cell. An Agilent Zorbax\(^\circledR\) 300 Extend C\(_{18}\) 0.3 x 100 mm capillary column containing a 300Å pore size and 3.5µm particle size was used for oligonucleotide separation. Gradient separation was achieved by 50 mM HFIP (hexafluoro-2-propanol)/8 mM TEA (triethylamine)/ water, pH 8.3 (mobile phase A) and 50 mM HFIP/8 mM TEA/ methanol, pH 8.3 (mobile phase B) under gradient conditions in **Table 3.1**. All LC parameters were controlled and UV data
integrated by the ICPMS computer using co-resident Agilent LC ChemStation software. The HPLC instrumental settings are listed in Table 3.1.

The overall ICP mass spectrometer performance was determined by an aqueous multi-element tune mix (1 ng ml\(^{-1}\)) consisting of \(^7\)Li, \(^{54}\)Co, \(^{89}\)Y, \(^{140}\)Ce, and \(^{205}\)Tl from Agilent Technologies. Capillary nebulizer tuning was achieved by introducing \(^6\)Li, \(^{89}\)Y, \(^{140}\)Ce and \(^{205}\)Tl at 10 ng mL\(^{-1}\) generated from certified stock solutions (1000 µg mL\(^{-1}\)) from SCP Science (Champlain, NY, USA). The ICPMS response was optimized for sulfur and phosphorus by utilizing dilutions of a certified aqueous phosphorus standard (1000 µg mL\(^{-1}\)) from SCP Science into mobile phase A and reagent grade Na\(_2\)SO\(_4\) (20 µg mL\(^{-1}\)) from Fisher Scientific (Pittsburg, PA, USA), diluted in 1% HNO\(_3\). The HFIP (> 99.8%) and TEA (> 99.5%) contained in the HPLC mobile phase were purchased from Sigma-Aldrich (St. Louis, MO, USA). The deionized water for mobile phase A and 1% HNO\(_3\) was from a Milli-Q deionization unit (Millipore, MA, USA). HPLC grade methanol from Fisher Scientific was used for mobile phase B. Polythymidilic acid samples were obtained from the University of Cincinnati DNA Core Facility (Cincinnati, OH, USA) and included one µmol each of \(dT_{10}\), \(dT_{15}\), \(dT_{20}\), and \(dT_{30}\). Synthetic polythymidilic acids are linear polymers made up of a specified number of repeating monomers, each composed of a sugar, a phosphate, and a thymidine base attached to the sugar. Nomenclature for polythymidilic acids \(dT_n\): (d) – deoxyribose, (T) – thymidine base, ( n) – number of repeating monomers.

3.3 Results and Discussion

The optimal parameters for the ICPMS in continuous flow analysis were established by using a tune mix containing \(^7\)Li, \(^{54}\)Co, \(^{89}\)Y, \(^{140}\)Ce, and \(^{205}\)Tl. Element
specific tuning was accomplished using a 1 µg mL⁻¹ sulfur containing solution of Na₂SO₄ in 1% HNO₃. Parameters such as O₂ flow rate, octopole rf power, nebulizer carrier flow and extraction lens voltages were tuned before each sequence of samples and optimized for m/z 48 (³²S¹⁶O⁺). In this study the potential of the collision/reaction cell (OctP Bias, −1V) was set more positive than the quadrupole potential (QP Bias, −8V) to aid in product ion transmission from the CRC to the quadrupole mass filter. The rf only octopole cannot distinguish between the mass of an analyte or that of an interference and so most applications utilize kinetic energy discrimination to further isolate the analyte from the interference. Energy discrimination usually encompasses the CRC octopole bias potential to be slightly more negative than the quadrupole bias, creating a kinetic energy barrier for the larger polyatomic ions. However, this application required the opposite settings to facilitate the transmission of the phosphorus and sulfur oxide product ions into the quadrupole. The standard energy discrimination parameter settings lost most of the 47 and 48 m/z signal. ³¹P¹⁶O⁺ and ³²S¹⁶O⁺ product ions are generated within the collision cell, therefore, the potential of the octopole bias was set more positive (7V) than the quadrupole bias to transmit and draw the product ions more efficiently into the mass filter. Some applications refer to an axial field applied to a hexapole configuration to “herd” the slower ions to the exit aperture. The slowing of the ion beam can occur by a dampening of the axial ion kinetic energy. The octopole configuration in this study was not equipped with an axial field; however, an octopole cell design collimates the ion beam more effectively than a hexapole configuration. This may lessen the need for an axial field in an octopole CRC using oxygen as a reaction gas. The overall LOD is
slightly more than an order of magnitude higher, relative to similar applications with an axial voltage.\textsuperscript{16}

**Figure 3.1** depicts the optimization curves for the aqueous sulfur standard and aqueous matrix blank obtained at various O\textsubscript{2} flow rates. For this application, 30\% O\textsubscript{2} flow (0.3 mL min\textsuperscript{-1}) was the optimal flow rate yielding a LOD (3\(\sigma\)) for sulfur (as $^{32}$S\textsuperscript{16}O\textsuperscript{+}) of 3.5 ng mL\textsuperscript{-1}. Phosphorus sensitivity was adjusted similarly to sulfur but the tuning analyte was introduced via a capillary LC at the methods designated flow rate in mobile phase A containing organic modifier. **Figure 3.2** shows the optimal O\textsubscript{2} flow for $^{31}$P\textsuperscript{16}O\textsuperscript{+} was found to be approximately 20\% (0.2 mL min\textsuperscript{-1}). The optima for phosphorus and sulfur are not identical; however, they are close enough that phosphorus and sulfur determinations can be run simultaneously at an intermediate value.

![Figure 3.1](image-url)  
**Figure 3.1** O\textsubscript{2} flow optimization curves for a 1 \(\mu\)g mL\textsuperscript{-1} sulfur standard in 1\% HNO\textsubscript{3}.
To examine the applicability of the O₂ reaction gas to produce $^{31}\text{P}^{16}\text{O}^+$, a mixture of polythymidilic acids were analyzed by direct capillary LC-ICPMS. **Figure 3.3** depicts a 5 mer polythymidilic acid and illustrates the repeating monomer sequence.

The detection of each oligonucleotide occurs via monitoring of the $^{31}\text{P}^{16}\text{O}^+$ signal. As the number of phosphate atoms in each oligonucleotide varies with length of the oligonucleotide, one will detect an increase in the $^{31}\text{P}^{16}\text{O}^+$ signal which should be proportional to the length of the oligonucleotide when each are analyzed at equimolar concentrations, as shown in **Figure 3.4**. The result is a LOD (3σ) for phosphorus (as $^{32}\text{P}^{16}\text{O}^+$) of 1.4 pg injected on column. Overall, it was found that the detection limits for the synthetic oligonucleotides using $^{31}\text{P}^{16}\text{O}^+$ as the product ion are lower than other hyphenated ion-molecule collision/reaction techniques reported in the literature for monophosphate nucleotides.¹⁷,¹⁸

**Figure 3.2** O₂ flow optimization curves for a 1 µg mL⁻¹ phosphorus standard in mobile phase A.
Figure 3.3 Structure of a 5-mer polythymidilic acid
Figure 3.4 Capillary LC-ICPMS analysis of a mixture of polythymidilic acids (3 pmol mixture on column). The oligonucleotides were detected by monitoring $^{31}\text{P}^{16}\text{O}^+$. 
3.4 Conclusion

The data presented demonstrates the ability of an octopole CRC instrument to treat spectroscopic hindrance of polyatomic interferences through oxygen reaction ion-molecule chemistry. This additional technique on an octopole CRC can qualitatively and quantitatively detect sulfur and phosphorus by oxidation. Further, this is the first report of the ability to separate and quantitatively determine synthetic oligonucleotides through detection of phosphorus in the phosphodiester linkages by an ICPMS collision/reaction cell.

Acknowledgements

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3.5 References

Chapter 4

Interfaces for Capillary LC with ICPMS Detection: A Comparison of Nebulizers/Spray Chambers
Abstract

Three nebulizer and four spray chamber configurations were evaluated to develop a selective and sensitive interface for capillary liquid chromatography with ICPMS. The method was tested using a phosphorothioate oligonucleotide. The influence of nebulizer design and spray chamber volume was determined by peak width measurements through flow injections made with and without the capillary column. This study capitalizes on the element specific detection abilities of ICPMS by detecting phosphorus and sulfur in the phosphorothioate linkage of a 24 mer oligonucleotide by monitoring $^{31}\text{P}^+$ and $^{32}\text{S}^+$. Xenon is utilized in conjunction with an octopole collision/reaction cell to remove common polyatomic interferences $^{15}\text{N}^{16}\text{O}^+$, $^{14}\text{N}^{16}\text{O}^+\text{H}^+$, $^{16}\text{O}_2^+$, $^{14}\text{N}^{18}\text{O}^+$, $^{15}\text{N}^{16}\text{O}^+\text{H}^+$. Micro-flow injection analysis generated absolute detection limits of $^{31}\text{P}$ and $^{32}\text{S}$ of 0.17 pg and 0.16 pg, respectively, corresponding to 16 fmol of 24 mer oligonucleotide injected. This is the first record of a phosphorothioate oligonucleotide being quantitatively detected by ICPMS.

Keywords: Micro-flow nebulizers, capillary LC, ICPMS, Phosphorothioates, Xe collision cell
4.1 Introduction

New analytical techniques continually attempt to narrow analyte selectivity, while simultaneously lowering analyte detection limits. Over the past decade there has been enormous growth in the field of element specific detection by ICPMS in conjunction with the complementary application of electrospray mass spectrometry (ESI-MS).¹ This has been due, in a large part, to the ease of interfacing chromatographic separation techniques to the ICPMS.² There are, however, inherent problems that are associated specifically with conventional LC applications. Two of the most common are; plasma instability when introducing organic modifiers, which often accompany reversed phase separations, and low aerosol (analyte) transport efficiencies from the nebulizer and spray chamber.³,⁴ It has been well documented that by introducing organic solvents into an ICP the plasma temperature, electron density, dynamics of aerosol generation, analyte transport, and the overall ionization processes that occur in the plasma are changed.⁵,⁶ High organic solvent loading can also lead to carbon deposits on the interface cones, causing the cones to eventually clog and, at higher loading, extinguish the plasma.⁶ These issues are addressed in the literature and alternatives are application dependent. An alternative is to simply reduce the solvent load on the plasma by splitting the flow post-column or perform off-line dilution of the separated eluent.⁷ This seems an obvious solution but can cause peak broadening, loss of sensitivity and waste of sample. Other alternatives include cooling the spray chamber to remove organic solvent vapor or utilizing a desolvation membrane. These also can lead to a loss in analyte sensitivity due to trapping the analyte in the spray chamber condensate or in the membrane.⁸,⁹ Carbon deposition on the interface cones has been studied by introducing oxygen into the aerosol flow, which requires a narrower
torch injection tube and the use of platinum interface cones to minimize cone
decomposition due to the harsh environment produced by oxygen addition.\textsuperscript{10, 11}

An ideal interface would allow low level element selective detection in a 100%
organic mobile phase, without a temperature controlled spray chamber, without external
gas supplies needed, and without the need for special torches and cones. In an attempt to
resolve the organic solvent issue where high solvent makes for better chromatography,
but poor plasma performance, there have been extensive advancements with interfacing
micro-flow (< 10 µL min\(^{-1}\)) separation techniques to ICPMS.\textsuperscript{4, 12-15} Capillary
electrophoresis, capillary LC and nano-LC are becoming common techniques in a wide
range of biochemical analyses.\textsuperscript{2, 16, 17} Due to the robust, reproducible, high resolving
power and gradient separation abilities, LC is the most widely applied separation
technique.\textsuperscript{18} However, plasma performance, when operating in the power region of 1000-
2000 Watts, start to degrade if greater than 20-40 mg min\(^{-1}\) of liquid aerosol is introduced.
Therefore, to establish a stable plasma for total aerosol consumption requires flows < 40
µL min\(^{-1}\).\textsuperscript{19} However, conventional nebulizers and spray chambers are inefficient when
down scaling from conventional LC flow rates of 0.5 – 2.0 mL min\(^{-1}\) to capillary flow <
40 µL min\(^{-1}\).\textsuperscript{17} This difficulty is overcome by use of commercially available micro-flow
nebulizers or those constructed in-house. Capillary low flow nebulizers include the high
efficiency nebulizer (HEN),\textsuperscript{20-22} oscillating capillary nebulizer (OCN),\textsuperscript{23, 24} direct
injection high efficiency nebulizer (DIHEN)\textsuperscript{9, 25}, micro-concentric nebulizer (MCN),\textsuperscript{13, 23,}
tetrafluoroethylene-perfluoroalkyl vinyl ether polymer nebulizer (PFA),\textsuperscript{13, 19} and the
parallel path nebulizer (PPN).\textsuperscript{27, 28} Common to the micro-flow nebulizers are the low
volume (< 20 mL) spray chamber designs commercially available and also produced in-
house. Although each nebulizer and spray chamber combination has their own characteristics and properties; determining which combination and design is best suited for a specific application is paramount in generating reproducible and superior figures of merit.

The object of this study was to investigate the capabilities of commercially available micro-flow nebulizers integrated with low dead volume spray chambers for the purpose of phosphorus and sulfur element specific detection with applications to phosphorothioate oligonucleotides. In this study, an analytical method optimizing a capillary LC and ICPMS interface is developed as a selective and low detection level technique. Standard measures, such as oxide and doubly charged ion formation with inorganic standards across the typical mass range, were used to characterize the interface combinations. To illustrate the interface applicability, a 24-mer phosphorothioated oligonucleotide was detected and quantified.

4.2 Experimental

4.2.1 Instrumentation

A quadrupole ICPMS equipped with an octopole collision/reaction cell (7500ce, Agilent Technologies, Santa Clara, CA) generated all mass spectral data. Phosphorus and sulfur determinations were performed simultaneously using xenon gas (>99.995%) at 0.18 mL min\(^{-1}\) in the octopole cell. With Xe a large energy discrimination of +47 volts was applied between the octopole (-48 V) and the quadrupole (-1) to eliminate polyatomic interferences from entering the quadrupole. Optimized tuning parameters are in Table 4.1. An Agilent technologies 1200 capillary LC system consisting of a vacuum degasser, binary capillary pump, thermostatically controlled well plate auto-sampler,
thermostatically regulated column compartment, and a diode array detector (DAD) also was equipped with a 80 nL flow cell. All LC tubing was 50 µm i.d. PEEK coated fused silica. An Agilent Zorbax® 300 Extend C\textsubscript{18} 0.3 x 100 mm capillary column containing a 300Å pore size and 3.5µm particle size was used for nebulizer peak efficiency calculations of the phosphorothioate oligonucleotide. Isocratic flow (50% mobile phase A/50% mobile phase B) was established with a mobile phase composition of 50 mM HFIP (hexafluoro-2-propanol)/8 mM TEA (triethylamine)/ water, pH 8.3 (mobile phase A) and 50 mM HFIP/8 mM TEA/ methanol, pH 8.3 (mobile phase B). All LC parameters were controlled and UV data integrated by the ICPMS computer using co-resident Agilent LC ChemStation software.

<table>
<thead>
<tr>
<th>ICPMS Parameters</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
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<tr>
<td>plasma power</td>
<td>1450 (W)</td>
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</tr>
</tbody>
</table>

**Table 4.1** Optimal ICPMS settings (ion optics adjusted for each specific nebulizer/spray chamber configuration)

In this study, three pneumatic concentric nebulizers with four different spray chamber configurations are compared. Two borosilicate glass high efficiency nebulizers (HEN) from Meinhard\textsuperscript{®} (Golden, CO) consisting of a model 90.A.01 (90-HEN) with an operating carrier pressure of 90 psi and a model 170A.01 (170-HEN), operating at
170 psi. The third nebulizer is the DS-5 micro-concentric nebulizer (MCN) from CETAC (Omaha, NE). Nebulizer specifications and dimensions are listed in Table 4.2. Nebulizer carrier flows for the 90-HEN and the MCN were controlled by the standard carrier flow manifold on the 7500ce, by maintaining instrument argon head pressure of 106 psi (730 kPa). The 170-HEN was supplied by an external argon tank. Instrument carrier gas to nebulizer connection to the 90-HEN was made through an EzyLok connector from Glass Expansion (Pocasset, MA) Figure 4.1a. MCN connections were made using a polypropylene connector designed for 4 mm tubing with two o-rings Figure 4.1b and the 170-HEN connections were made using the Meinhard® Fit Kit#2 for high pressure connections as shown in Figure 4.1c.

<table>
<thead>
<tr>
<th>Nebulizer</th>
<th>HEN 90A.01</th>
<th>HEN 170A.01</th>
<th>DS-5</th>
<th>MicroMist</th>
</tr>
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<td>pneumatic design</td>
<td>concentric</td>
<td>concentric</td>
<td>micro concentric</td>
<td>concentric</td>
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<tr>
<td>annulus design (sample capillary position to carrier gas orifice)</td>
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<td>flush</td>
<td>extended</td>
<td>recessed</td>
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<td>100</td>
<td>100</td>
<td>140</td>
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<tr>
<td>gas exit cross section area (mm²)</td>
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<td>0.011</td>
<td>0.017</td>
<td>0.025</td>
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<td>carrier flow @ 1 L min⁻¹</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>400</td>
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</tbody>
</table>

Table 4.2 Nebulizer specifications and dimensions

Tuning solution was delivered from the capillary pump’s flow sensor outlet directly to each nebulizer’s sample inlet. Pump to nebulizer connections were via 50 µm x 50 cm PEEK coated fused silica tubing. The 90-HEN and 170-HEN coupled to the LC using the Meinhard® Micro Fit Kit #1 to interface with the 50 µm PEEK tubing, while the 50 µm
tubing connected directly to the MCN. Other nebulizers used in this study provided inferior performance for this capLC-ICPMS interface and are not discussed here.

The four spray chambers evaluated in this study were a cinnabar (cyclonic) from Meinhard®, a mini-chamber (single pass) from Burgener Research Inc (Mississauga, Ontario, Canada), an in-house designed chamber (single pass) blown by Hazel Glass (Cincinnati, OH) and the single pass chamber designed specifically for the MCN. Spray chamber details are listed in Table 4.2. The cinnabar chamber was coupled to the ICPMS torch interface through a 90º ball joint union held in place with a spring clamp. The mini-chamber used a 4 mm x 300 mm Tygon tube to attach a ball joint to the torch interface. Both the MCN and in-house chambers utilized a ball joint to couple with the torch. All spray chambers in this work were made of quartz. Each nebulizer/spray chamber configuration was optimized with respect to carrier gas flow rates, rf power, torch alignment, and ion lens settings. All spectra were acquired with nickel sample and skimmer cones under shielded torch (grounded) conditions. A MicroMist (Glass Expansion) concentric nebulizer and Scott double pass spray chamber were used in this evaluation as a reference for tuning and signal responses for conventional nebulizer/spray chamber configuration.

4.2.2 Standards and reagents

An aqueous in-house multi-element tune mix (10 ng mL⁻¹) composed of ⁶Li, ⁸⁹Y, ¹⁴⁰Ce, ²⁰⁵Tl was adapted for optimization of each nebulizer/spray chamber configuration. The tune mix was generated from certified stock solutions (1000 µg mL⁻¹) from SCP Science
Figure 4.1 a) 90-HEN with LC coupling Micro Kit #1; EzyLok carrier gas connection  
b) DS-5 direct LC capillary connection; polypropylene carrier gas connection 
c) 170-HEN with LC coupling Micro Kit#1; high pressure carrier gas fitting

<table>
<thead>
<tr>
<th>Spray Chamber</th>
<th>Scott</th>
<th>Mini-chamber</th>
<th>In-house</th>
<th>MCN</th>
<th>Cinnabar</th>
</tr>
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<td>aerosol path</td>
<td>double pass</td>
<td>single pass</td>
<td>single pass</td>
<td>single pass</td>
<td>cyclonic</td>
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<td>8</td>
<td>8</td>
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<td>8</td>
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<tr>
<td>carrier flow (L min⁻¹)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>inner volume (cm³)</td>
<td>100</td>
<td>9</td>
<td>10</td>
<td>5</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 4.2 Spray chamber specifications and amount of (sample/carrier flow introduced)¹  
¹ flows for each nebulizer/spray chamber individually optimized number reflects the average
Optimization for the phosphorus and sulfur responses came from dilutions of a certified solution of phosphorus (1000 µg mL\(^{-1}\)) from SCP Science and reagent grade Na\(_2\)(SO\(_4\)) (20 µg mL\(^{-1}\)) from Fisher Scientific (Pittsburg, PA). Stock solutions of phosphorus and sulfur were diluted into mobile phase A to a final concentration of 100 ng mL\(^{-1}\) and introduced into the ICPMS via the capillary LC system for maximizing signal responses. The HFIP (≥ 99.8%) and TEA (≥ 99.5%) contained in the LC mobile phases were purchased from Sigma-Aldrich (St. Louis, MO, USA). The high purity deionized (18 M\(\Omega\)) autoclaved water for mobile phase A and standard dilutions were from a Milli-Q deionization unit (Millipore, MA, USA). HPLC grade methanol from Fisher Scientific was used for mobile phase B.

4.2.3 Sample and sample preparation

The synthetic phosphorothioate oligonucleotide was obtained from Biosearch Technologies (Novato, CA). The oligonucleotide sequence was 14 thymidine (T), 4 cysteine (C), and 6 guanine (G) monomers without modifications on the terminal 5’ and 3’ positions. Five calibration standards were prepared by dilution of a 395 pmol/µL aqueous stock solution to 0.1, 1, 10, 50, and 100 pmol µL\(^{-1}\) in high purity deionized (18\(\Omega\)) sterile water.

4.3 Results and Discussion

4.3.1 Nebulizer/spray chamber optimization and response

Maximum ion intensity and minimum formation of oxide and doubly charged species determined the optimum settings for each nebulizer/spray chamber configuration. Tuning solution was introduced under continuous flow from the capillary LC at 8 µL min\(^{-1}\). Comparisons were made between the signal intensity and precision and
production of oxides and doubly charged ions. Figures 4.3 – 4.7 depict some of the doubly charged ion and oxide responses for various configurations. Table 4.3 and 4.4 list the ion responses for all the configurations evaluated in this study. As can be seen from Table 4.3, the 90-HEN/Mini-chamber generated a slight increase in signal intensity over the in-house and cinnabar chambers, and the DS-5 single pass. Some of this variation may be due to chamber designs and chamber volumes, however, in relative to the MCN, DS-5, previous comparisons correlated the observed lower intensity to the annulus design and the kinetic energy available for finer aerosol generation from a flush annulus as opposed to an extended one. The finer the primary aerosol, the higher the mass of aerosol that reaches the plasma. Oxides and doubly charged species are low, necessary for acceptable robust plasma in all configurations. The high pressure 170-HEN/Mini-chamber illustrates a similar trend to the 90-HEN configurations but is not as pronounced as depicted in Table 4.4.

Figure 4.2 DS-5 single pass chamber
Figure 4.3 90-HEN in-house chamber

Figure 4.4 170-HEN Mini-chamber
Figure 4.5 90-HEN cinnabar chamber

Figure 4.6 90-HEN Scott double pass chamber with makeup flow.
Table 4.3 Ion responses (cps) for each configuration 10 µg L⁻¹ tune mix @ 8 µL min⁻¹

<table>
<thead>
<tr>
<th>Scans/Ion</th>
<th>Meinhard® HEN 90A.01 In-House Single Pass cps</th>
<th>%</th>
<th>Meinhard® HEN 90A.01 Mini-Chamber cps</th>
<th>%</th>
<th>Meinhard® HEN 90A.01 Cyclonic Cinnabar cps</th>
<th>%</th>
<th>CETAC DS-5 Micro Concentric Single Pass cps</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li⁺</td>
<td>3.9 x 10⁴</td>
<td>2.7</td>
<td>1.1 x 10⁵</td>
<td>1.8</td>
<td>6.9 x 10⁴</td>
<td>1.6</td>
<td>7.0 x 10⁴</td>
<td>1.4</td>
</tr>
<tr>
<td>²²⁹Y⁺</td>
<td>2.2 x 10⁵</td>
<td>1.4</td>
<td>3.6 x 10⁵</td>
<td>1.6</td>
<td>2.9 x 10⁵</td>
<td>1.2</td>
<td>2.7 x 10⁵</td>
<td>1.3</td>
</tr>
<tr>
<td>¹⁴⁰Ce⁺</td>
<td>2.4 x 10⁵</td>
<td>1.4</td>
<td>3.0 x 10⁵</td>
<td>1.1</td>
<td>2.7 x 10⁵</td>
<td>1.6</td>
<td>2.6 x 10⁵</td>
<td>1.4</td>
</tr>
<tr>
<td>²⁰⁵Tl⁺</td>
<td>1.3 x 10⁵</td>
<td>1.7</td>
<td>1.2 x 10⁵</td>
<td>1.4</td>
<td>1.1 x 10⁵</td>
<td>1.0</td>
<td>1.4 x 10⁵</td>
<td>1.5</td>
</tr>
<tr>
<td>CeO⁺/Ce⁺</td>
<td>1.5%</td>
<td>0.4%</td>
<td>0.3%</td>
<td>1.5%</td>
<td>1.5%</td>
<td>1.5%</td>
<td>1.5%</td>
<td>1.5%</td>
</tr>
<tr>
<td>Ce²⁺/Ce⁺</td>
<td>2.1%</td>
<td>1.7%</td>
<td>1.8%</td>
<td>2.1%</td>
<td>2.1%</td>
<td>2.1%</td>
<td>2.1%</td>
<td>2.1%</td>
</tr>
</tbody>
</table>

Table 4.4 Ion responses (cps) for each configuration 10 µg L⁻¹ tune mix @ 8 µL min⁻¹

<table>
<thead>
<tr>
<th>Scans/Ion</th>
<th>Meinhard® HEN 170A.01 In-House Single Pass cps</th>
<th>%</th>
<th>Meinhard® HEN 170A.01 Mini-Chamber cps</th>
<th>%</th>
<th>Meinhard® HEN 170A.01 Cyclonic Cinnabar cps</th>
<th>%</th>
<th>Meinhard® HEN 90A.01 Scott Double Pass Chamber cps</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li⁺</td>
<td>1.5 x 10⁵</td>
<td>2.4</td>
<td>1.2 x 10⁵</td>
<td>1.9</td>
<td>1.1 x 10⁵</td>
<td>1.8</td>
<td>7.5 x 10⁴</td>
<td>3.5</td>
</tr>
<tr>
<td>²²⁹Y⁺</td>
<td>5.2 x 10⁵</td>
<td>1.2</td>
<td>6.2 x 10⁵</td>
<td>1.7</td>
<td>4.7 x 10⁵</td>
<td>1.6</td>
<td>2.5 x 10⁵</td>
<td>2.5</td>
</tr>
<tr>
<td>¹⁴⁰Ce⁺</td>
<td>5.3 x 10⁵</td>
<td>1.4</td>
<td>6.3 x 10⁵</td>
<td>1.8</td>
<td>4.5 x 10⁵</td>
<td>2.7</td>
<td>2.3 x 10⁵</td>
<td>2.4</td>
</tr>
<tr>
<td>²⁰⁵Tl⁺</td>
<td>2.5 x 10⁵</td>
<td>2.0</td>
<td>2.9 x 10⁵</td>
<td>2.6</td>
<td>2.1 x 10⁵</td>
<td>3.9</td>
<td>1.3 x 10⁵</td>
<td>3.3</td>
</tr>
<tr>
<td>CeO⁺/Ce⁺</td>
<td>0.3%</td>
<td>0.4%</td>
<td>0.3%</td>
<td>1.4%</td>
<td>1.4%</td>
<td>1.4%</td>
<td>1.4%</td>
<td>1.4%</td>
</tr>
<tr>
<td>Ce²⁺/Ce⁺</td>
<td>1.8%</td>
<td>1.4%</td>
<td>1.3%</td>
<td>2.8%</td>
<td>2.8%</td>
<td>2.8%</td>
<td>2.8%</td>
<td>2.8%</td>
</tr>
</tbody>
</table>

All three nebulizers generate small primary aerosols but the 170-HEN generates a finer aerosol due to higher back pressure, therefore, higher gas velocity (more kinetic energy from the nebulizing gas). The 90-HEN connected to the Scott double pass nebulizer produces similar counts to the MCN DS-5 and other 90-HEN configurations, but with
50% higher RDS and practically no change of ion signal intensity at higher nebulizer
carrier gas flow rates. This phenomena is probably due to the double chamber’s large
100 cm\(^3\) volume and micro-flow sample introduction causing fluctuations in total analyte
transport.\(^{11}\) The overall observation of the nebulizer/spray chamber tuning results seems
to indicate that most of the tune solution was in vapor form and the spray chamber had
little to no effect on aerosol transport efficiency, except in the case of the double pass
chamber.\(^{11, 21, 30, 31}\)

### 4.3.2 Analytical performance of the capillary LC-nebulizer interface

As previously stated, all capillary connections utilized 50 µm i.d. PEEK coated
fused silica tubing to minimize dead volume and, thereby, reduce peak broadening. To
evaluate the dispersion effects of the LC interface, capillary column and the spray
chambers, analyte peak width measurements were recorded in the flow injection mode
(without a column). Then 300 nL of a 1 pmoL µL\(^{-1}\) solution containing a 24-mer
phosphorothioate oligonucleotide, was injected (300 fmol, 2 ng, 24-mer), for each
nebulizer/spray chamber configuration. This process was repeated with the capillary LC
column installed and run isocratic with 50% mobile phase B not allowing the 24-mer to
be retained on the column. Figures 4.7 and 4.8 illustrate the peak widths at half
height for the 24-mer by flow injection mode and with the column installed. As shown
by the measurements, there appears to be insignificant peak tailing caused by dispersion
of the sample from the spray chamber dead volume and LC connections. However,
comparing flow injection capillary LC UV (DAD) peak widths to peak widths generated
from nebulizer/spray chamber coupling, the capillary LC UV peaks average 5-8%
narrower. This phenomena can be explained by the low DAD flow cell volume (80 nL)
producing a slight backpressure within the cell as the analyte transverses the cell, generating narrower peak widths in comparison to the nebulizer/spray chamber peak widths. Peak dispersion occurs once it leaves the flow cell via the capillary coupling to the nebulizer. Dispersion can be minimized by shortening the distance from the flow cell exit to the nebulizer, nonetheless, in this work the capillary LC was located on a mobile laboratory cart and the distance was minimized based on practicality. In an attempt to minimize peak dispersion 25 µm id capillary tubing was employed to couple the flow cell exit to the nebulizer; however, this created a large amount of back pressure on the flow cell window and was determined to be too uncertain for routine analysis.

Figure 4.7 $^{31}$P+ and $^{32}$S+ signal from 300 nL flow injection of 24 mer generated from Meinhard 90-HEN/in-house chamber
Figure 4.8 $^{31}$P$^+$ and $^{32}$S$^+$ signal from 300 nL injection with capillary column installed of 24 mer generated from Meinhard 90-HEN/in-house chamber (isocratic, 50% mobile phase B)

Once the capillary column is in place there still appears to be minimal dispersion of the peaks. Notwithstanding, upon comparing the data in Table 4.5, there are measurable differences in peak dispersion based upon the nebulizer spray chamber configurations. This is a different observation than seen in a previous study.\textsuperscript{23} This may be a result of the reduction in sample flow by a factor of 15 from the previous work and the variations of micro-flow nebulizers used in this study. The peak width broadening trend correlates with the increased spray chamber volumes. Taken individually there is not a significant variation, but reviewed as a group one can see how the peak width plays a pivotal role in absolute sensitivity of the 24 mer. The Scott chamber has the largest volume and, therefore, the largest noted peak dispersion. The single pass, total consumption designed chambers (in-house, mini-chamber, DS-5 chamber) have comparable peak widths and generate a narrower peak than the Scott double pass chamber.
4.3.3 Phosphorothioate sensitivity and detection limits

Optimum detection limits for the 24-mer were measured by injecting 300 nL of sample under flow injection conditions. Calibration curves were produced on each configuration by injecting 300 nL of standard solutions of 0.1, 1, 10, 50, 100 pmol µL\(^{-1}\) of the 24-mer phosphorothioate oligonucleotide. Table 4.5 lists the correlation coefficients associated with each configuration and all generated low RSDs and linear responses. For comparison purposes absolute sensitivities based on the total signal counts produced per fmol of analyte are also displayed in Table 4.5. The limits of detection for the 24-mer based on the \(^{31}\)P\(^+\) and \(^{32}\)S\(^+\) responses were determined by injecting 5 blank solutions, calculating their standard deviation, multiplying by 3 and dividing by the slope of the calibration curve (IUPAC). The absolute limit of detection for the 24-mer by flow injection analysis was determined to be 16 fmol (0.12 ng) of the 24-mer, which correlates to 0.17 pg of total \(^{31}\)P and 0.16 pg of total \(^{32}\)S with a linear dynamic range of 4 orders of magnitude. This is the first report of a phosphorothioate oligonucleotide detected and quantified by capLC-ICPMS.
<table>
<thead>
<tr>
<th>Nebulizer/spray chamber configuration (n = 5)</th>
<th>$r^2$</th>
<th>$\frac{y = mx + b}{300}$</th>
<th>% RSD $^{31}$P</th>
<th>% RSD $^{32}$S</th>
<th>peak $\frac{1}{2}$ height 300 fmol on column</th>
<th>% RSD $^{31}$P</th>
<th>% RSD $^{32}$S</th>
<th>24 mer absolute sensitivity $^a$ counts/fmol</th>
<th>% RSD $^{31}$P</th>
<th>% RSD $^{32}$S</th>
</tr>
</thead>
<tbody>
<tr>
<td>90A.01 Meinhard® In-house</td>
<td>0.9981</td>
<td>5.3($^{31}$P) 5.7($^{32}$S)</td>
<td>1.1</td>
<td>1.2</td>
<td>7.7($^{31}$P) 8.0($^{32}$S)</td>
<td>1.8</td>
<td>1.6</td>
<td>202($^{31}$P) 196($^{32}$S)</td>
<td>2.4</td>
<td>2.7</td>
</tr>
<tr>
<td>90A.01 Meinhard® Mini-chamber</td>
<td>0.9998</td>
<td>5.1($^{31}$P) 5.4($^{32}$S)</td>
<td>1.6</td>
<td>1.1</td>
<td>7.7($^{31}$P) 7.4($^{32}$S)</td>
<td>2.0</td>
<td>2.1</td>
<td>228($^{31}$P) 222($^{32}$S)</td>
<td>2.3</td>
<td>2.1</td>
</tr>
<tr>
<td>90A.01 Meinhard® Cinnabar</td>
<td>0.9976</td>
<td>6.5($^{31}$P) 7.3($^{32}$S)</td>
<td>1.8</td>
<td>1.4</td>
<td>8.8($^{31}$P) 9.8($^{32}$S)</td>
<td>2.2</td>
<td>1.9</td>
<td>213($^{31}$P) 200($^{32}$S)</td>
<td>2.0</td>
<td>2.6</td>
</tr>
<tr>
<td>90A.01 Meinhard® Scott double pass</td>
<td>0.9973</td>
<td>9.1($^{31}$P) 9.3($^{32}$S)</td>
<td>2.1</td>
<td>2.2</td>
<td>11.7($^{31}$P) 11.9($^{32}$S)</td>
<td>2.5</td>
<td>2.8</td>
<td>111($^{31}$P) 105($^{32}$S)</td>
<td>2.9</td>
<td>2.8</td>
</tr>
<tr>
<td>CETAC DS-5 Single pass</td>
<td>1.000</td>
<td>5.2($^{31}$P) 5.6($^{32}$S)</td>
<td>1.3</td>
<td>1.0</td>
<td>7.5($^{31}$P) 7.7($^{32}$S)</td>
<td>1.8</td>
<td>1.6</td>
<td>219($^{31}$P) 215($^{32}$S)</td>
<td>1.7</td>
<td>1.3</td>
</tr>
<tr>
<td>170A.01 Meinhard® In-house</td>
<td>0.9985</td>
<td>4.5($^{31}$P) 4.9($^{32}$S)</td>
<td>1.7</td>
<td>2.0</td>
<td>6.9($^{31}$P) 7.5($^{32}$S)</td>
<td>2.4</td>
<td>2.2</td>
<td>246($^{31}$P) 237($^{32}$S)</td>
<td>2.1</td>
<td>2.5</td>
</tr>
<tr>
<td>170A.01 Meinhard® Mini-chamber</td>
<td>0.9997</td>
<td>4.7($^{31}$P) 5.2($^{32}$S)</td>
<td>1.2</td>
<td>1.2</td>
<td>6.4($^{31}$P) 7.3($^{32}$S)</td>
<td>2.0</td>
<td>2.3</td>
<td>254($^{31}$P) 243($^{32}$S)</td>
<td>2.2</td>
<td>2.5</td>
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<tr>
<td>170A.01 Meinhard® Cinnabar</td>
<td>0.9994</td>
<td>5.4($^{31}$P) 6.0($^{32}$S)</td>
<td>1.9</td>
<td>2.1</td>
<td>7.8($^{31}$P) 8.4($^{32}$S)</td>
<td>2.3</td>
<td>2.4</td>
<td>228($^{31}$P) 220($^{32}$S)</td>
<td>3.1</td>
<td>2.8</td>
</tr>
</tbody>
</table>

**Table 4.5** Peak width measurements for nebulizer/spray configurations and absolute sensitivity for the 24 mer by flow injection 300 nL flow injection

$^a$
4.4 Conclusions

Based on the results from this work, good peak width performance can be achieved when interfacing various nebulizer/spray chamber configurations. However, as the peak width data demonstrates, there are variations between configurations. The spray chambers having a larger internal volume have larger peak width variations than the low volume chambers. Absolute sensitivity (cps/fmol) follows the high efficiency nebulizers and lowers with increasing nebulizer flow rate. This would be expected as the higher gas velocity normally translates to more kinetic energy transferred to the aerosol, producing greater sheering and therefore, smaller droplets. In addition, the in-house spray chamber performed well and could be used in future studies. The 170-HEN/mini-chamber will be used in further phosphorothioate research. This is the first reported detection of phosphorothioates by ICPMS and the detection limits for the 24-mer were lower than mononucleotides and DNA adducts previously reported by ICPMS. This research could be used to develop a method to quantitatively and qualitatively detect phosphorothioate oligonucleotides and their metabolites in biological matrices.
Acknowledgements

Financial support of this work was provided by Agilent Technologies (to Kirk E. Lokits and Joseph A. Caruso), the National Science Foundation (CHE 0602413 co-funded by the MPS/CHE and BIO/IDBR Divisions and by the MPS Office of Multidisciplinary Activities, to Patrick A. Limbach) and Pfizer Pharmaceuticals Global Research Development Fund (SRS 00566 to Patrick A. Limbach). The authors would also like to thank Meinhard® Glass Products, CETAC, and Burgener Research Inc for providing the nebulizers, spray chambers and their technical support regarding their optimal use.
4.5 References

Chapter 5

Development of a Selective Technique for Detecting Anti-sense Phosphorothioate Oligonucleotides by IP-RP-ICPMS
Abstract

A method to detect and quantify phosphorothioate oligonucleotides based on phosphorus and sulfur is described. $^{31}\text{P}^+$ and $^{32}\text{S}^+$ were determined by utilizing xenon as a collision gas to remove polyatomic interferences. Oxygen was introduced into the octopole collision/reaction cell (CRC) to oxidize $^{31}\text{P}^+$ and $^{32}\text{S}^+$ and allow the detection of $^{31}\text{P}^{16}\text{O}^+$ and $^{32}\text{S}^{16}\text{O}^+$. Limits of detection were 2 times lower using O$_2$ to determine $^{31}\text{P}^{16}\text{O}^+$ and $^{32}\text{S}^{16}\text{O}^+$ compared to Xe detection of $^{31}\text{P}^+$ and $^{32}\text{S}^+$. Four phosphorothioate oligonucleotides varying from 21 – 24 nucleotides in length were separated and analyzed. Capillary LC mobile phase composition and LC gradient parameters were optimized to achieve maximum resolution between the four oligonucleotides. LODs ranged from 53 – 81 fmol for the 21 – 24-mer, respectively using Xe as a collision cell gas, while LODs when reacting with O$_2$ in the octopole were 35 – 29 fmol, respectively. Elemental $^{31}\text{P}^+$ detection limits were 0.87 pg (21-mer) – 0.57 pg (24-mer) and $^{32}\text{S}^+$ detection from 0.84 pg (21-mer) – 0.55 pg (24-mer) for Xe. $^{31}\text{P}^{16}\text{O}^+$ and $^{32}\text{S}^{16}\text{O}^+$, were 0.41 and 0.39 pg, respectively for the 21-mer and 0.31 and 0.30 pg, respectively, for the 24-mer. This is the first reported detection and quantitative analysis of phosphorothioates by ICPMS and the first reported use of O$_2$ as a reaction gas in an octopole collision/reaction cell.

Keywords: phosphorothioate oligonucleotide, ICPMS, Ion-pairing reverse phase, octopole collision/reaction cell
5.1 Introduction

During drug discovery, pharmaceutical companies perform metabolism studies dealing with absorption, distribution, metabolism and elimination (ADME) of a new drug. Separation, identification and quantification of a new drug and its associated metabolites is known as metabolic profiling. In the realm of ICPMS it is known as elemental speciation. ICPMS has been applied to pharmaceutical research as an element specific detector when a drug contained a particular elemental component (tag) such as, platinum, gold or iodine.

Within the past decade ICPMS has extended further into bio-molecular studies due to the advancement of collision/reaction cell technology (CRC) and micro-flow sample introduction. CRC reduces or eliminates difficulties caused by spectral and non-spectral interferences. Polyatomic interferences may be reduced or eliminated from the analyte m/z of interest through collision, reaction, energy discrimination or some combination. Removal of interferences has been accomplished by a multitude of collision or reaction gases including He, H₂, O₂, Xe and NH₃. For elemental speciation, liquid chromatography separations are often required, but the organic solvent load with a standard bore column destabilizes the plasma. Micro-flow nebulizers (< 10 µLmin⁻¹) produce a much lower organic solvent load to the plasma and couple readily to capillary liquid chromatography (capLC).

In clinical chemistry and toxicology, analysis of DNA and proteins can now benefit from the high sensitivity, linearity and ICPMS matrix robustness with the low solvent flow LC interface as was discussed in Chapter 4. The element selective capabilities of ICPMS can detect phosphorus and sulfur in proteins and peptides, the
presence of phosphorus in DNA adducts and phosphorylated nucleotides.\textsuperscript{16} Methods quantifying peptides and proteins,\textsuperscript{17} determining phosphorylated deoxyribonucleotides,\textsuperscript{18} and quantifying DNA adducts\textsuperscript{19} are given in the literature.\textsuperscript{16,20} These aforementioned methods have used Xe as a means to enable detection of phosphorus or sulfur on octopole and hexapole collision reaction cells, while O\textsubscript{2} has been introduced into quadrupole and hexapole collision reaction cells. This research attempts to demonstrate the ability to simultaneously detect phosphorus and sulfur using either Xe or O\textsubscript{2} on an octopole collision reaction cell.

This study utilizes a novel application for ICPMS by using phosphorus and sulfur as internal target elements to develop analytical methods in an area of pharmaceutical drug design known as antisense technology. The concept of antisense technology employs a synthetic nucleic acid sequence (oligonucleotide), to interact with specific targeted mRNA before it translates amino acid sequences to produce proteins. Translation entails the process of ribosome’s reading mRNA and translating the genetic code into a protein by arranging amino acids in the correct sequence. Many diseases are associated with either inappropriate or inadequate production of proteins. Many traditional drugs deal with disease by focusing on the faulty proteins themselves. Antisense technology pre-empts the production of the faulty proteins. This occurs by synthetically modifying single stranded DNA molecules (oligonucleotides), usually 13-25 nucleotides in length, that hybridize (bind) to a unique sequence of messenger RNA (mRNA) located in targeted cell nuclei.\textsuperscript{21} Figure 5.1 illustrates the binding of the antisense oligonucleotide with the mRNA. The therapeutic effect can occur by the modified DNA:mRNA hybridization or RNase H, an endogenous nuclease, degrading the targeted mRNA, which
in either case, deactivates the mRNA from expressing its genetic code.\textsuperscript{21,22} The synthetic oligonucleotide (DNA) moves on and binds to another target mRNA creating a cascading effect stopping gene expression of the targeted mRNAs and thereby inhibits the production of the inappropriate protein.\textsuperscript{23}

**Figure 5.1** Antisense oligonucleotide mRNA mechanism (used with permission)

Phosphorothioates are the most widely studied antisense oligonucleotides due to their nuclease stability. Replacement of one of the non-bridging oxygens in the phosphodiester linkage by sulfur at each phosphorus position in the oligonucleotide linkage creates expanded antisense activity by increasing its half life inside the body, but is still capable of inducing RNase H activity.\textsuperscript{24} Since the quantification and
characterization of phosphorothioate oligonucleotide (PS-OGN) metabolites are an important part of drug development, there are a variety of techniques utilized for metabolism studies. These include enzyme-linked immuno-sorbent assay (ELISA), conventional HPLC with UV and fluorescence detection, capillary gel electrophoresis and ESIMS detection. \(^{25}\) ELISA based on hybridization is sensitive and allows for high throughput. Minimum sample preparation is required and it is the technique used for quantitative evaluation of the drug’s kinetic pathway through the body. \(^{26}\) The technique does have difficulty distinguishing the parent drug from its metabolites and can over estimate the parent drug. \(^{27}\) Capillary gel electrophoresis separates by the charge on the analytes and; therefore, provides metabolite separations from the parent PS-OGN. Detection by UV/fluorescence in a biological matrix has been reported as low as 20 ng mL\(^{-1}\). However, extensive sample preparation is needed to remove interferences from the sample matrix. \(^{28,29}\) Conventional HPLC with UV/fluorescence detection has problems with sensitivity (40 ng mL\(^{-1}\)) and resolution to separate metabolites from the parent PS-OGN. \(^{30}\) Chromatographic modes for HPLC consist mainly of size exclusion, \(^{31}\) strong anion exchange, \(^{32}\) and ion-pairing reverse phase chromatographies. \(^{33-35}\) The degree of separation by HPLC is determined by the oligonucleotides chain length, charge and base composition. \(^{36}\)

The use of mass spectrometry to characterize, identify and quantify nucleic acids and oligonucleotides is well documented in the literature. \(^{37-39}\) The use of soft ionization techniques, such as electrospray ionization (ESI-MS), is the most common approach. \(^{25,34,40-43}\) **Table 5.1** lists some of the recent mass spectrometry techniques and their respective limits of detection. However, ESI-MS suffers from problems with ion suppression,
adduct formation, multiple charged states (ion envelopes) and matrix affects from biological samples.\textsuperscript{25} Under these circumstances, element selective ICPMS is an attractive compliment to ESI-MS. Unlike compound dependent modes of analysis, the response of an element in ICPMS is independent of its molecular environment.

<table>
<thead>
<tr>
<th>No. of nucleotides in sequence</th>
<th>Mobile Phase</th>
<th>MS detection</th>
<th>Dynamic range (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 mer</td>
<td>A: 10 mM TEAA pH 10 B: ACN</td>
<td>Triple quad MS/MS\textsuperscript{25}</td>
<td>500-10,000 tissue 50-1000 monkey</td>
</tr>
<tr>
<td>18 mer</td>
<td>A: 100 mM HFIP/8.6 mM TEA B: 100 mM HFIP/8.6 mM TEA A: water B: MeOH</td>
<td>Quad Ion Trap MS/MS\textsuperscript{52}</td>
<td>100-10,000</td>
</tr>
<tr>
<td>18 mer</td>
<td>A: 286 mM HFIP/4.0 mM TEA B: 286 mM HFIP/4.0 mM TEA A: water B: MeOH/ACN</td>
<td>Triple quad MS/MS\textsuperscript{43}</td>
<td>125-10,000</td>
</tr>
</tbody>
</table>

\textbf{Table 5.1} Current figures of merit for analysis of oligonucleotides

This study seeks to capitalize on this feature and quantify phosphorothioate oligonucleotides based upon the “covalently bound” phosphorus and sulfur in the phosphorothioate links of the oligonucleotide. With the advances in collision/reaction cell technology detection of phosphorus and sulfur in bio-molecules has become important. Current literature has many ICPMS applications detecting phosphorus in proteins, peptides and DNA adducts,\textsuperscript{13, 18, 44} while sulfur has been investigated in water, proteins, peptides and thio-arsenicals in urine.\textsuperscript{9, 17, 45, 46}

Most ESI-MS applications utilize ion-pairing reverse phase chromatography, specifically HFIP (hexafluoro-2-propanol) and TEA (triethylamine) for PS-OGN determinations.\textsuperscript{25} To enhance the compatibility and complementary nature of ESI-MS and ICPMS, this study describes an ion-pairing gradient method using HFIP/TEA for the
capillary LC separation of four oligonucleotides. The PS-OGNs applied in this work are comprised of a 21 - 24-mers. UV (DAD) and elemental phosphorus and sulfur data were obtained simultaneously through direct coupling of the capillary LC system to the ICPMS.

5.2 Experimental

5.2.1 Instrumentation

The ICP mass spectrometer used for this study was an Agilent Technologies 7500ce (Santa Clara, CA, USA). A Meinhard Glass Products HEN 170 A.01 capillary concentric nebulizer (Golden, CO, USA) sprayed into a single pass mini-chamber from Burgener Research Inc (Mississauga, Ontario, Canada). The nebulizer sample inlet was connected to a capillary LC with the Meinhard Fit Kit #1-Micro for interfacing LC eluent from the DAD. See Chapter 4 of this dissertation for a more complete description. The ICPMS was equipped with an on-axis rf-only octopole collision/reaction cell (CRC) that allowed the octopole chamber to be pressurized via three separate gas manifolds. Typically H₂, He and Xe would be introduced separately and/or simultaneously into the Agilent Octopole Reaction System (ORS – Agilent Technologies, Santa Clara, CA). The Xe flow manifold has a maximum flow of 1 mL min⁻¹ and is controlled as a percentage of the maximum flow setting. Data were acquired with Xe as a collision gas and compared to a second configuration employing high purity O₂ (>99.995%) as a reaction gas. All instrument parameters and data analysis were controlled by the ICPMS ChemStation software. The ICPMS instrumental and acquisition parameters are provided in Table 5.2.

The capillary HPLC system was a modular Agilent Technologies 1200 system, consisting of a vacuum chambered micro-degasser, a binary pump capable of delivering up to 20 µL min⁻¹, a thermostatically controlled well plate sampler with an 8 µL sample
loop, a thermostatically controlled column compartment and a diode array detector equipped with a 80 nL flow cell. All LC connections were made with 50 µm i.d. PEEK.

### ICPMS Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>spray chamber</td>
<td>Mini-chamber</td>
</tr>
<tr>
<td>chamber temperature</td>
<td>room temperature</td>
</tr>
<tr>
<td>nebulizer</td>
<td>HEN 170A.01</td>
</tr>
<tr>
<td>nebulizer carrier flow</td>
<td>180 psi external tank</td>
</tr>
<tr>
<td>interface cones</td>
<td>Ni</td>
</tr>
<tr>
<td>plasma power</td>
<td>1450 (W)</td>
</tr>
<tr>
<td>plasma gas flow</td>
<td>15 (L min(^{-1}))</td>
</tr>
<tr>
<td>auxiliary gas flow</td>
<td>1 (L min(^{-1}))</td>
</tr>
<tr>
<td>collision/reaction optional gas</td>
<td>Xe 18% O(_2) 25%</td>
</tr>
<tr>
<td>quadrupole bias</td>
<td>Xe (-1 Volts) O(_2) (-8 Volts)</td>
</tr>
<tr>
<td>octopole bias</td>
<td>Xe (-48 Volts) O(_2) (-1 Volts)</td>
</tr>
<tr>
<td>octopole RF</td>
<td>160 Volts</td>
</tr>
</tbody>
</table>
| selected masses                  | Xe: 31, 32 m/z \(^{31}\)P\(^+\), \(^{32}\)S\(^+\)\(\))  
|                                 | O\(_2\): 47, 48 m/z \(^{31}\)P\(^{16}\)O\(^+\), \(^{32}\)S\(^{16}\)O\(^+\)\(\)) |

### HPLC parameters

- column flow rate: 8 µL min\(^{-1}\)
- column temperature: 50 °C
- mobile phase A: 150 mM HFIP/4 mM TEA in aqueous pH 7.5
- mobile phase B: 150 mM HFIP/4 mM TEA in methanol pH 7.5
- gradient separation:
  - 0.0 min: (75%) A (25%) B
  - 1.0 min: (75%) A (25%) B
  - 2.2 min: (60%) A (40%) B
- runtime: 11.0 min
- fast composition change: enabled
- DAD wavelength: 259 nm, slit 4 nm, reference bandwidth 6 nm

**Table 5.2** Instrument and acquisition parameters for Xe collision and O\(_2\) reaction gas

coated fused silica tubing. An Agilent Technologies Zorbax® 300 Extend C\(_{18}\) 300 µm x 100 mm capillary column containing a 300Å pore size and 3.5µm particle size was used for oligonucleotide separation. Gradient separation was achieved by 150 mM HFIP (hexafluoro-2-propanol)/ 4 mM TEA (triethylamine)/ water, pH 7.5 (mobile phase A) and
150 mM HFIP/ 4 mM TEA/ methanol, pH 7.5 (mobile phase B) under gradient conditions in Table 5.2. All LC parameters were controlled and UV data integrated by the ICPMS computer using co-resident Agilent LC ChemStation software. The HPLC instrumental settings are listed in Table 5.2.

5.2.2 Standards and reagents

An aqueous in-house multi-element tune mix (10 ng mL\(^{-1}\)) composed of \(^{6}\)Li, \(^{89}\)Y, \(^{140}\)Ce, \(^{205}\)Tl was adapted for optimization of each nebulizer/spray chamber configuration. The tune mix was generated from certified stock solutions (1000 µg mL\(^{-1}\)) from SCP Science (Champlain, NY). Optimization for the phosphorus and sulfur responses came from dilutions of a certified solution of phosphorus (1000 µg mL\(^{-1}\)) from SCP Science and reagent grade \(\text{Na}_2(\text{SO}_4)\) (20 µg mL\(^{-1}\)) from Fisher Scientific (Pittsburg, PA). Stock solutions of phosphorus and sulfur were diluted into mobile phase A to a final concentration of 100 ng mL\(^{-1}\) and introduced into the ICPMS via the capillary LC system for maximizing signal responses. The HFIP (≥ 99.8%) and TEA (≥ 99.5%) contained in the LC mobile phases were purchased from Sigma-Aldrich (St. Louis, MO, USA). The high purity deionized (18 MΩ) autoclaved water for mobile phase A and standard dilutions were from a Milli-Q deionization unit (Millipore, MA, USA). HPLC grade methanol from Fisher Scientific was used for mobile phase B.

5.2.3 Samples and sample preparation

The synthetic phosphorothioated oligonucleotides were obtained from Biosearch Technologies (Novato, CA) and are listed in Table 5.2. Five calibration standards were prepared by dilution of an aqueous stock solution, to 0.1, 1, 10, 50, and 100 pmol µL\(^{-1}\) in high purity deionized (18Ω) autoclaved water. Polythymidilic acid samples were
obtained from Bioneer Inc (Alameda CA) and included one μmol each of dT₁₀, dT₁₅, dT₂₀, and dT₃₀ with nucleotides joined by phosphodiester linkages.

<table>
<thead>
<tr>
<th>PS-OGN</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-mer</td>
<td>5’dT<em>C</em>G<em>T</em>G<em>C</em>T<em>T</em>T<em>T</em>G<em>T</em>T<em>G</em>T<em>T</em>T<em>T</em>C<em>G</em>C<em>G</em>T*T 3’</td>
</tr>
<tr>
<td>23-mer</td>
<td>5’dT<em>C</em>G<em>T</em>G<em>C</em>T<em>T</em>T<em>T</em>G<em>T</em>T<em>G</em>T<em>T</em>T<em>T</em>C<em>G</em>C<em>G</em>T 3’</td>
</tr>
<tr>
<td>22-mer</td>
<td>5’dT<em>C</em>G<em>T</em>G<em>C</em>T<em>T</em>T<em>T</em>G<em>T</em>T<em>G</em>T<em>T</em>T<em>T</em>C<em>G</em>C 3’</td>
</tr>
<tr>
<td>21-mer</td>
<td>5’dT<em>C</em>G<em>T</em>G<em>C</em>T<em>T</em>T<em>T</em>G<em>T</em>T<em>G</em>T<em>T</em>T<em>T</em>C<em>G</em>C 3’</td>
</tr>
</tbody>
</table>

Table 5.3 Phosphorothioate oligonucleotide sequences *denotes phosphorothioate linkage

Synthetic urine was employed in this project (Surine™) from Dyna-Tek Inc (Lenexa, KS) and found to be conducive to the application due to its non-biological matrix. Surine™ contains constituents that mimic human urine without human urine’s biohazard implications and regulations. Bovine serum from Sigma-Aldrich was also used as a sample matrix. Solid phase extraction (SPE) of PS-OGN began in urine or serum with a 1 mL aliquot of synthetic urine placed in 1.5 mL sterile polypropylene conical vial and spiked with a PS-OGN. 200 μL of the spiked matrix was removed and mixed with 500 μL of 5% ammonium hydroxide and 100 μL of chloroform/phenol (1:2, v/v). The sample was mixed thoroughly by vortexing for two minutes and centrifuging at 13,000 rpm for ten minutes. The aqueous layer was removed without disturbing the lower organic layer. 800 μL of 17.2 mM TEA and 200 mM HFIP, pH 8.5, were added to the sample and then loaded onto an Oasis HLB (Waters Corp, Milford, MA) 60 mg cartridge pack. Cartridges were conditioned with 1000 μL of methanol and equilibrated with 1000
μL high purity deionized water. Samples were loaded onto the cartridge and washed with 1000 μL of 5% methanol in water (v/v) and then eluted from the cartridge with 1000 μL of 2% ammonium hydroxide in methanol. Samples were evaporated to dryness and reconstituted in 200 μL of mobile phase A. Chloroform and phenol were bio-ultra grade for molecular biology from Sigma-Aldrich.

A liquid – liquid extraction procedure was also used in this study and was based on an approach by Murphy et al. Standards and sample spikes were prepared as in SPE and then to 200 μL of sample a 100 μL of concentrated ammonium hydroxide from Fisher Scientific was added to each tube. 300 μL of phenol/chloroform/iso-amyl alcohol (25/24/1) bio-ultra grade from Sigma Aldrich were added to each tube. Tubes were vortex-mixed for two minutes and then centrifuged at 13,000 rpm for ten minutes. The aqueous layer from each standard and sample was removed from the tubes and evaporated to dryness. 200 μL of mobile phase A was then added to each residue and vortex-mixed for 30 seconds.

5.3 Results and Discussion

5.3.1 Sample introduction and $^{31}P$ & $^{32}S$ optimization with Xe

The 170-HEN and mini-chamber (Figure 5.2) were chosen due to previous comparative work as detailed in Chapter 4 on nebulizer/spray chamber configurations. The high velocity (180 psi) nebulizer gas flow had an efficient transfer of kinetic energy to the liquid sample, resulting in fine aerosol production, and therefore, high aerosol transport efficiency to the plasma. Optimum Xe flow, ion optics, and collision/reaction cell parameters were established by capillary LC continuous flow of mobile phase A containing 100 ng mL$^{-1}$ of Na$_2$HPO$_4$ and Na$_2$SO$_4$ ($^{31}$P$^+$, $^{32}$S$^+$). To accentuate the kinetic
energy discrimination process (removal of polyatomic interferences) between the octopole and the quadrupole, the octopole bias was set to -48 V and the quadrupole bias set to -1 V for a net energy discrimination voltage of +47 with Xe. A capillary LC gradient from 25-100% mobile phase B at 8 µL min^{-1} was introduced into the ICP for six hours with no visible signs of condensation on the spray chamber walls.

**Figure 5.2** 170-HEN connected with Micro Kit #1 and a mini-chamber Nebulizer carrier gas 180 psi from external Ar tank

Signal stability and signal response (cps) for 100 ng mL^{-1} of $^{31}$P$^+$ and $^{32}$S$^+$, (as Na$_2$HPO$_4$, Na$_2$SO$_4$) were maintained with a RSD of 3.2 and 4.1%, respectively. Based on reports from previous investigators on a different nebulizer design, but similar flow rates, it was concluded the analyte transport efficiency was $\sim$100%. As a result; the configuration was operated in the “total consumption” mode. The limits of detection, calculated
according to IUPAC guidelines (3σ), gave 81 – 53 fmol for the 21 to 24 mer PS–OGN on column, respectively, which correlates to absolute mass limits of 0.54 ng (21 mer) and 0.4 ng (24 mer). Absolute $^{31}$P detection limits ranged from 0.87 pg (21mer) – 0.57 pg (24mer) and absolute $^{32}$S detection from 0.84 pg (21mer) – 0.55 pg (24mer). Lowest limits of detection were reached by flow injection analysis of the 24 mer under non-retained column conditions, (50% B isocratic) of 16 fmol (0.12 ng 24 mer) and 0.17 pg of $^{31}$P and $^{32}$S. Detection limits for phosphorus were in accordance with Profrock et al, who reported $^{31}$P detection of 1.9 pg in tryptic protein digests, and with Yeh and coworkers giving detection limits of $^{31}$P in monophosphate nucleotides of 1.1 – 1.6 pg.

### 5.3.2 Oxygen optimization for phosphorus and sulfur

Parameters such as O$_2$ flow rate, octopole rf power, nebulizer carrier flow and ion optics voltages were tuned before each sequence of samples and optimized for $m/z$ 47 ($^{31}$P$^{16}$O$^+$) and 48 ($^{32}$S$^{16}$O$^+$). In this study the potential of the octopole cell, (OctP Bias, -1V) was set more positive than the quadrupole potential (QP Bias, -8V). Figure 5.3 and Figure 5.4 depict the optimization curves for sulfur and phosphorus at various O$_2$ flow rates. As shown, the optima for phosphorus and sulfur are not identical; however, they are close enough that phosphorus and sulfur determinations can be run simultaneously at an intermediate value. For this study 20% O$_2$ flow (0.20 mL.min$^{-1}$) was chosen. Limits of detection ranged from 35 fmol (21-mer) – 29 fmol (24-mer) on column. The LOD for the phosphorothioates corresponds to absolute limits of 0.25 ng (21-mer) and 0.22 ng (24-mer). Absolute phosphorus and sulfur oxide polyatomic ions gave 0.31 and 0.30 pg, respectively, for the 24-mer and 0.41 and 0.39 pg, respectively, for the 21-mer.
Using O₂ gave somewhat lower limits of detection than Xe. Limits of detection when compared to the Xe collision LOD, O₂ reaction cell has about 2x lower limit of detection. This is mainly due to moving the analytes from an area of high interference to one of less interference. Detection limits were good compared to other studies using an oxygen reaction cell. Wang et al. detected sulfur containing proteins in the pmol range as did Wind and coworkers with sulfur containing peptides and proteins using a sector field ICPMS. 

5.3.3 PS-OGN separations

The principle metabolic pathway of PS-OGNs is cleavage via 3’-exonucleases followed by 5’-exonucleases. The metabolism usually generates a progressive n-1 chain shortened pattern. The metabolites chosen for this study reflect the n-1 metabolic pathway from the 3’ position as shown in Table 5.2. The HFIP/TEA mobile phase was chosen due to its wide acceptance in the literature. However, several mechanisms are involved regarding oligonucleotide separation and retention. When
utilizing ion pair reversed phase LC (IP-RP-LC) retention of the oligonucleotides is driven by charge-charge interactions of the phosphorothioate backbone with the TEA and the hydrophobic interactions of the nucleotide bases and TEA-oligonucleotide complex with the stationary phase. If the concentration of TEA decreases, then the separation efficiency and selectivity also decreases but not exactly proportional with the concentration. HFIP is important to adjust oligonucleotide retention and peak shape. Figure 5.5 depicts the separation achieved for this work optimizing the HFIP concentration to 150 mM and TEA to 4 mM and an operating pH of 7.5. Baseline resolution was not achieved. Control over oligonucleotide separation sacrificed considerable oligonucleotide sensitivity due to a reduction in peak shape (peak height and peak areas).

Figure 5.5 Overlay of 21 – 24 mer UV signal @ 259 nm
Urine samples were spiked with different mixtures of oligonucleotides. Figures 5.6 and 5.7 illustrate the separation of the 21- and 24-mer. Some peak broadening does occur from the DAD flow cell to the nebulizer, however, separation is maintained. The 22- and 24-mer phosphorothioate oligonucleotides were spiked into a bovine serum sample and separation was obtained in the UV signal and the chromatogram as shown in Figures 5.8 and 5.9. The 23- and 24-mer were slightly separated within the UV cell but lost resolution upon reaching the nebulizer.

![UV data of 21/24 mer from extracted urine (80 nL flow cell)](image)

**Figure 5.6** UV data of 21/24 mer from extracted urine (80 nL flow cell)
Figure 5.7 ICPMS data (Xe collision) 21/24 mer urine extract

Figure 5.8 UV data of 22/24 mer from extracted serum (80 nL flow cell)
Retention time reproducibility for all four (21-24-mer) PS-OGNs ranged 0.2-0.7% (n=5) for 500 fmol injected on column. Recoveries for spiked solutions (matrix) were defined as the amount that was experimentally determined versus the known amount that was spiked into the matrix. Oligonucleotide recoveries in each sample matrix were based on the linear slopes \( y = mx + b \) generated from a 5 level spiked matrix calibration curve compared to a duplicate 5 level aqueous calibration curve, generated under identical method conditions. The solid phase extraction recovery was 23%, while the liquid-liquid extraction was 30%. Both of these findings were in line with recoveries limits for similar extraction procedures in the literature. 25, 40, 43
5.4 Conclusions

The data presented represents the first time phosphorothioate oligonucleotides have been qualitatively and quantitatively analyzed by ICPMS. The figures of merit are as good and in most cases better than current methods. This study demonstrates the ability of an octopole collision reaction cell to generate analyte product ions for phosphorus and sulfur ($^{31}\text{P}^{16}\text{O}^+$ and $^{32}\text{S}^{16}\text{O}^+$) using oxygen as a reaction gas in the low picogram level. The collision/reaction cell also has the ability to remove polyatomic interferences for $^{31}\text{P}$ and $^{32}\text{S}$ simultaneously with Xe as a collision gas generating phosphorus and sulfur signals in the picogram range. The nebulizer/spray chamber configuration presented in this study can operate in “total consumption” mode at low (8 µL min$^{-1}$) microliter flows.

Acknowledgements

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5.5 References


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Chapter 6

Conclusions and Future Endeavors
6.1 Conclusion and Future Directions

The data produced in this dissertation research helps to substantiate and establish the pivotal role ICPMS can play in bio-molecule identifications. ICPMS can be used for molecule identification only when combined on-line or off-line with a molecule specific separation technique, due to the harsh ionization process of ICPMS, no molecular information is retained. The presence of an element of interest does not significantly influence the response and therefore generates a species independent response. Stable, covalently bound elemental species can be used for qualitative and quantitative purposes in a series of different molecular species, as illustrated by the phosphorothioate oligonucleotides data. The limitations of sample size, mobile phase composition and micro-flow separation techniques are overcome with a variety of interface choices that exist commercially. The current research has laid the foundation for using on-line analysis as a viable option, when using compatible mobile phases and separation techniques with ESIMS, to simultaneously generate elemental and molecular data. The generation of chromatographic data under the same separation conditions also allows the use of off-line “data mining” applications based on peak patterns and analytes retention times, to correlate molecular and elemental species data.

The use of xenon and oxygen in collision/reaction cells has shown convincingly that non-metals, such as phosphorus and sulfur can be determined with low limits of detection and can be run virtually simultaneously. The use of oxygen in an octopole collision/reaction cell widens the development of analytical methods that can determine the elemental character of a molecule by creating product ions in an area of less interference and
thereby, reaching lower levels of detection. The ability to interface, compare, and
determine application specific nebulizer/spray chamber configuration responses for an
specific analyte will tailor applications based on chromatography requirements and
mobile phase composition to maintain narrow peak widths for optimal sensitivity.

The next challenge is to make nano-flow separations in ICPMS as common place as it is
in ESIMS. Currently, only in-house versions of nano-flow interfaces exist in the literature
for ICPMS. If the resolving power of nano-LC in proteomics and genomics could be
tapped into by ICPMS using only small volumes and providing femtogram sensitivity,
the ultimate goal of elemental speciation analysis in different compartments of a cell
could be met.