Miniaturization of Chemical Analysis Tools: Micro-Solid Phase Extraction Tips for Protein Extractions and Development of a Miniaturized Glow Discharge Source for Elemental Analysis

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MINIATURIZATION OF CHEMICAL ANALYSIS TOOLS:
MICRO-SOLID PHASE EXTRACTION TIPS FOR PROTEIN
EXTRACTIONS AND DEVELOPMENT OF A MINIATURIZED
GLOW DISCHARGE SOURCE FOR ELEMENTAL ANALYSIS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Chemistry

by
Benjamin T. Manard
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Accepted by:
R. Kenneth Marcus, Committee Chair
Kenneth A. Christensen
Jeffrey Anker
Philip Brown
ABSTRACT

An increased interest has been placed on the miniaturization of chemical processes. Focus is placed on rapid chemical analysis, decreased operation cost, minimal generation of chemical waste, and reduced sample size. Presented here is an approached the goal of “miniaturization” through two distinct areas of analytical chemistry: 1) employing capillary-channeled polymer (C-CP) fibers as a stationary phase in micro-SPE (solid phase extraction) tips for the extraction of proteins from buffered solutions (i.e. biofluids) with analysis by mass spectrometry (MS) and 2) the development of the liquid sampling-atmospheric pressure glow discharge (LS-APGD) as an excitation source for elemental analysis.

Determination of proteins in biological matrices is vital for the identification of disease-specific biomarkers. With this in mind, research efforts have grown tremendously for protein analysis in biofluids. More importantly, research has focused on development of extraction/separation techniques necessary for performance on miniaturized levels (i.e. low volume aliquots/concentrations) prior to analysis with mass spectrometry (MS). MS is commonly used for detection due to its sensitivity and the abundant chemical information it provides on analysis. However, buffered species (i.e. salts and biofluids) can be detrimental to MS analysis due to mechanisms such as co-ionization. Presented here, is the use of C-CP fibers as a stationary phase for the extraction of proteins from these matrices. These C-CP mirco-SPE tips have shown to be effective for extraction
of nanogram quantities of proteins from buffered species. More importantly, extractions have been optimized to be performed with samples sizes of ~1 µL compared to commercially available tips which require up to 100 µL of sample. Investigations of fiber packing density, tip length, loading capacities, and load/elution volumes were performed to improve the efficiency of C-CP tips for the extraction of proteins. After optimization, C-CP tips were utilized for extracting nanogram quantities of proteins (<5 µL aliquots) from urine matrices.

Additionally, there is a need to design spectrochemical instruments with lower power consumption, reduced sample sizes, compact footprint, and the ability to be operated under ambient conditions. In this dissertation the liquid sampling-atmospheric pressure glow discharge (LS-APGD) is employed as a miniaturized approach towards elemental analysis focusing on the determination of plasma characteristics (i.e. plasma temperatures) under parametric evaluations for elemental analysis of solutions and the use of the LS-APGD source as a secondary excitation/ionization source following laser ablation of solid materials. Excitation, rotation, and ionization temperatures along with electron number density and robustness characteristics of the LS-APGD were studied under parametric evaluation. It was determined that the LS-APGD show great promise in terms of handling complex samples (liquid and laser ablated particles) without presenting matrix effects. Both directions demonstrate promising results with regards to miniaturization of chemical analysis techniques.
in hopes of developing rapid procedures that require small quantities of sample while operating at low costs and producing little to zero chemical waste.
DEDICATION

This dissertation is dedicated to everyone who believed in me. It is the love and support that each individual provided that assisted me in obtaining all of the goals set in my path.

To my magnificent fiancée, JoAnna Brown, you furnished me with such drive to be the absolute best and accomplish all ambitions. You have always been my supporter and have supplied an endless amount of love and patience throughout this journey.

To my family (Mom, Dad, and Ryan) and my newly acquired family (Mr. and Mrs. Brown, Seth, and Iris). I sincerely thank-you for the abundance of love and comfort that I felt each and every day.

To my close friends Chris Cox, William Peters, Jonathan Kops, and Ryan Harwell. For each and every one helped in their own way. I cherish the memories together as your presence is what kept me sane in this journey.

Finally, to ALL of my friends and family who have impacted my life tremendously through the years. I would like to thank my Clemson family, friends, and colleagues for making this experience the best years of my life.

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CHAPTER ONE

INTRODUCTION

MINIATURIZATION IN CHEMICAL ANALYSIS

The improvement of chemical analysis methods and instrumentation has progressed exponentially over the last two decades\(^1\), \(^2\). Incentives have been centered on developing miniaturized chemical processing sources with significant reduction in carbon-footprint, sample size requirements, chemical waste/operating consumables, and cost. Such sources must be physically and experimentally robust and perform rapid analysis with high analyte efficiency. Advantages of these processes are not limited to the mentioned characteristics, but extend in a greater detail as miniaturized systems allow for higher analysis throughput as time-of-analysis and sample requirements are greatly reduced\(^1\)-\(^3\). Also, the “lab” can be brought to the sample, where prolonged sample handling times and the necessity for large quantities of sample are significantly reduced. One could imagine, especially in terms of nuclear chemistry, how the reduction of handling hazardous materials could benefit by bringing miniaturized instrumentation to the sample, thus nearly eliminating sample handling. Lastly, not only are operating/experimentation costs greatly reduced with improved instrumentation, but sample analysis costs are also greatly reduced. Employing
instrumentation in which chemical analysis can be performed on minute sample sizes greatly decreases both analysis costs and the sample requirements⁴.

These advancements have extended to many processes in analytical chemistry such as separations, mass spectrometry, optical spectroscopy, electrochemistry, sample preparation, and sensor technology. The combination of said processes (i.e., separation + detection) can be classified as “micro total analysis systems” (μTAS) and can be seen in devices such as lab-on-a-chip and even the implementation of optical spectrometers on a chip employed for the detection of chemical species⁵,⁶. The terminology μTAS, was first used in 1989 in hopes of developing methods that encompass miniaturization in sampling acquisition, sample preparation, instrumentation (e.g., pumps, power, and reagents) and detection¹,³. These lab-on-a-chip based instruments are excellent examples of instrument miniaturization that provide capabilities of great versatility with reduced operating costs and sampling that allows for instrumentation portability⁷.

This dissertation will discuss the miniaturization of chemical analysis methods and instrumentation in two distinct fashions: 1) utilizing micro solid phase extraction (μSPE) tips for the extraction of proteins from buffers/salts extending to biofluids (e.g. urine) prior to analysis by matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) and 2) employing a liquid sampling-atmospheric pressure glow discharge (LS-APGD) as a miniaturized excitation/ionization source for elemental analysis.
SAMPLE PREPARATION IN PROTEOMICS

Clinical chemistry is a research area in which interest has rapidly increased over the past two decades, particularly the rapid screening of biofluids (e.g. plasma, urine, saliva) for potential biomarkers\textsuperscript{8-15}. Biomarkers are biomolecules (e.g. proteins) that can be readily found in bodily fluids and utilized for the investigation of bodily functions and the presence/absence of specific diseases\textsuperscript{8}. Identification of such biomarkers are extremely advantageous as they are universally found in biofluids that typically correspond to the area of interest such as diagnostics of heart diseases through plasma analysis\textsuperscript{16}. Of the biofluids, urine analysis has grown tremendously over the last decade. Unlike other biofluids such as plasma and cerebrospinal fluid (CSF), urine sample acquisition is noninvasive and for the most part, easily obtained\textsuperscript{8, 17}. Unfortunately, while easily obtainable, protein levels in urine are typically low\textsuperscript{8}. An abundance of protein usually stems from diseases/illness or a form of medicinal treatment as small proteins (<40 kDa) can enter the urine by means of glomerular filtration in which the smaller proteins can freely filtrate thought the glomerular barrier\textsuperscript{8, 18}. There are numerous detection methods employed for protein analysis of biofluids ranging from spectroscopic (UV-vis, fluorescence) to mass spectrometric (MS) techniques. MS techniques are employed for complex samples containing multiple biomolecules, as they can be detected with high
spectral resolution. However, problems arise upon analyzing biomolecules that are in native, buffered environments as co-ionization between concomitants and target species can hinder instrumentation sensitivity. Presented in this dissertation, matrix-assisted laser desorption/ionization spectrometry (MALDI-MS) will be employed for the detection of proteins.

MALDI-MS for protein analysis

There are two commercially available ionization sources for mass spectrometric analysis of biomolecules, electrospray ionization (ESI) and MALDI\textsuperscript{19-22}. For analysis of biomolecules, a “soft” ionization method must be employed, primarily to reduce fragmentation in comparison to “hard” ionization. MALDI was originally introduced by Karas and Hillenkamp in 1988 at the University of Frankfurt and was further developed by Tanaka (who would be awarded a part of the Nobel Prize in chemistry in 2002)\textsuperscript{22}. Prior to 1970, the idea of performing mass spectrometry on large molecules was unheard of; however in the 1970’s, desorption/ionization (DI) techniques were being investigated and showed promising results. Early DI techniques in MS would bombard samples with laser pulses inducing ionization due to energy transfer to the analyte, and were successful for small molecules extending to aromatic compounds; however larger molecules were limited by severe fragmentation. MALDI-MS takes on a very similar approach employing direct laser bombardment inducing DI. The
difference is seen in the sample preparation prior to laser bombardment in which the target species is saturated with an organic matrix (matrix:analyte >1000:1)\textsuperscript{19, 22, 23} as loosely demonstrated in Fig. 1.1. MALDI-MS is simply described as bombarding the analyte saturated with matrix with a pulsed laser in which sample is ejected in a gas plume as seen in Fig. 1.1. Laser ablation (10 Hz) typically employs a pulsed UV (337 nm) over a short duration (nanosecond), while infrared (IR) laser can be employed for specific applications\textsuperscript{23}. Typical laser spot sizes range from \(\sim 5 \, \mu\text{M}\) to \(\sim 300 \, \mu\text{M}\) which can be important especially when utilizing MALDI-MS for imaging of sample surfaces.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure1.png}
\caption{Illustration of matrix assisted laser desorption/ionization in which 1) laser bombardment induces the 2) desorption/ionization of the target species prior to transport into the mass analyzer. The analyte species is saturated in a MALDI-matrix.}
\end{figure}
The sample preparation in regards to the matrix can take on a variety of formats. The most common “dried-droplet” method consists of premixing of the analyte and a matrix solution prior to deposition (1-2 µL) onto a MALDI target\textsuperscript{22, 24}. Other methods include depositing (1-2 µL) matrix onto the target allowing to dry, then applying 1-2 µL of the analyte\textsuperscript{25}. In all methods, analytes are co-crystalized on the MALDI target plate upon solvent evaporation. The organic matrix functions in several roles during the desorption/ionization process and is not completely understood in terms of ionization mechanisms\textsuperscript{22, 23}. Firstly, the matrix physically saturates the analyte as it serves as a cushion to protect the analyte from a pulsed laser, which induces analyte fragmentation. It is well known that the fragmentation occurs from direct laser DI bombardment, however, employing a matrix prior to laser bombardment allows for the ionized molecules to remain intact as the matrix absorbs the photons (energy) from the laser instead of the analyte\textsuperscript{19, 26}. MALDI matrices also plays a role in “diluting” the analyte so that analyte-analyte interactions are decreased upon laser ablation\textsuperscript{22}. Secondly, it is proposed that the (typically organic acid) matrix serves as the primary means for inducing sample ionization. While there are numerous reviews that discuss potential ionization mechanisms, all include observations of highly excited matrix molecules\textsuperscript{22, 23}. It has been illustrated that employing sinapinic acid for the ionization of proteins yields singly protonated molecules, proposed by proton transfer. However, studies with 2,5-dihydroxybenzoic acid (DHB) demonstrated \([M+Na]^+\) (in principle) adduct peaks even with no sodium
present in the analyte or matrix. R. Brown proposed that since DHB has high solubility in water, the alkali ions tend to co-crystallize and upon solvent vaporization, salts of DHB can affect the ionization\textsuperscript{22}. While many factors contribute to ionization (laser energy, matrix), no clear-cut mechanism can be explained for all MALDI-MS analyses; however, proton transfer in either the solid or gas phase is the most widely accepted mechanism.

While MALDI is an excellent candidate for biomolecule ionization in MS, there are some shortcomings in terms of quantitative analysis and sample pretreatment\textsuperscript{27, 28}. Quantitative analysis can be difficult as shot-to-shot reproducibility is limited. Not only is there error in the laser ablation (such as the reproducibility in mass ejected) but crystallization of the sample is typically inhomogeneous. Matrix-analyte crystallization can be improved by an increased organic solvent composition in the matrix solution as evaporation is faster, or by the incorporation of a MALDI matrix sprayer in which nebulized droplets of matrix are deposited onto the sample surface. In this process, evaporation time and small droplet volumes compared to the 1 µL deposition are improved. Lastly, sample pretreatment is a priority, especially for the analysis of biomolecules. High concentrations of buffer and other concomitants which are normally present in biomolecule analysis, hinder the desorption/ionization process\textsuperscript{17, 20, 28}. Buffers/organic concomitants tend to easily co-ionize with the target sample, inducing ion suppression to the point of undetectable target ion peaks in complex samples (biofluids)\textsuperscript{28, 29}. In general, in MALDI-MS a sample clean-up must be
performed when buffer/concomitant exceeds 100 µM in concentration\textsuperscript{27, 28}. Especially in biomarker identification, sample clean-up is always performed, not only due to the large quantities of contaminants, but also due to the low abundance of proteins in which pre-concentration is warranted.

\textit{Separations/extractions of proteins from biofluids}

Gel electrophoresis (GE)\textsuperscript{10, 30}, high performance liquid chromatography (HPLC)\textsuperscript{17, 31}, and solid phase extraction (SPE)\textsuperscript{8, 32} are three of the most commonly employed techniques for protein separations/extractions prior to analysis with MS. While many methods provide advantages/disadvantages for separating out analytes of interest, GE would be viewed as the best for being able to separate the largest quantities of biomolecules due to its ability perform multi-dimension separations. GE works by loading up to 1000 µL sample volumes containing up to 1 mg of each protein in a specific loading/separation lane\textsuperscript{8}. GE commonly employs separations in two dimensions in which molecules can be separated by their isoelectric point (pI) and molecular weight\textsuperscript{33}. Advantages are clearly seen in which complex samples, even up to 1400 distinct protein spots in urine, can be separated with high resolution as demonstrated by Pieper\textsuperscript{30}. Yet, GE separations are laborious and timely, taking up to 48 hours, pending sample complexity. GE separation disadvantages can also extend to poor resolution (protein smears/streaks) making further analysis/detection
difficult\textsuperscript{33}. If MS is required for the detection of bands, analysis can be performed directly on the band itself (by MALDI-MS) or bands must be excised followed by digestions prior to MS analysis. These methods (e.g. band excision) can be laborious and typically not reproducible as band smearing can be an issue. While many methods have been employed to reduce hindrances such as depletions, pre-chromatographic separations, or gel staining, GE is indeed a laborious and time-consuming method. Another method, HPLC, has seen increased interest since the development of MALDI/ESI-MS as faster separations (in comparison to GE) and automation can be employed for complex samples with MS analysis\textsuperscript{17,34}. Much effort has been dedicated to employing HPLC for not only the separation of proteins and other targets of interest, but also for desalting/purification so that MS can be employed\textsuperscript{12,35}. As previously mentioned, MS is unable to directly detect biomolecules in their native environments due to the ion suppression induced from the buffers, salts, and contaminants; therefore, HPLC is commonly employed to remove these hindrances. A downside to the employment of HPLC as a means of separating/extracting proteins includes low sample throughput (one injection/separation per run) and large quantities of waste, especially when organics or acids are employed at high flow rates (>1 mL min\textsuperscript{-1}).

When considering miniaturized separation/extraction techniques, solid phase extraction (SPE) methods are the most widely used and excel in performing rapid extractions on small volumes (<1 mL) with minimal solvent
waste (<1 mL). SPE works by adsorbing target species onto a solid sorbent material while allowing contaminants (buffers, salts, and organics) to pass through, or to be removed in an aqueous wash step as seen in Figure 1.2 a-b\textsuperscript{36-38}. Finally, the target species is eluted in a solvent which corresponds to subsequent analysis (Figure 1.2 c) as a greater affinity for the elution solvent is achieved compared to the solid stationary phase. Most commonly, SPE is employed for one or more of three specific reasons: 1) sample concentration, 2) removal of contaminants from the sample (clean-up), and 3) removal of a matrix by a solvent exchange\textsuperscript{36}. While all are equally important pending the application,

![Diagram](image)

**Figure 1.2** Demonstration of the solid phase process in which the first step involves the (a) loading of sample (protein and salt/buffer) followed by an (b) aqueous wash step. Finally, the remaining desired analyte is (c) eluted in a MS-friendly solvent.
in regards to MS, the removal of contaminants is the most vital due to the suppression induced during the ionization process. While SPE has indeed served as an ideal means of miniaturized sample clean-up since the early 1970’s, research expanded upon the introduction of ESI and MALDI-MS in the mid 1980’s and early 1990’s as sample preparation is a necessity for successful MS analysis of biomolecules\textsuperscript{36, 38, 39}. Trends then shifted from SPE “cartridges” in which milliliters of sample were required to even more miniaturized methods with SPE stationary phases (typically silica-based C\textsubscript{18}) being mounted onto micropipette tips in the late 1990’s with the first commercially available micropipette tip-based SPE platform by Millipore\textsuperscript{40}. Commonly employed in biofluids analysis, these Zip Tips™ are incorporated in initial sample pretreatment to remove low molecular weight compounds for protein purification prior to HPLC-MS\textsuperscript{41, 42}.

In the 2000’s SPE stationary phases saw a shift in stationary phase material with the implementation of polymer based stationary phases\textsuperscript{43}. Polymer based phases were sought out to correct for the disadvantages of the traditional bonded silica-C\textsubscript{18} sorbents for extraction of biomolecules. Typically, the silica based sorbents demonstrate poor surface contact with the solutions/solvent as silica is hydrophilic and the hydrocarbon chains of the stationary phase are hydrophobic resulting in the poor surface interactions. Other disadvantages include robustness and sensitivity issues as irreversible binding can occur due to the disruption of the stationary phase as the sorbents are limited in pH range\textsuperscript{43, 44}. 
Polymer stationary phases offer a plethora of advantages as they exhibit high mass transfer rates due to the lack of relative porosity which results in rapid adsorption/desorption of analytes, thus increasing analyte recoveries. Other advantages include robustness to solvents and the ease surface modifications to the polymer surface. Polymer SPE phases allow for the ease of modification, particularly employing molecularly imprinted polymers for extractions in biofluids prior to further HPLC separations\textsuperscript{45}. SPE stationary phases employed for biofluids extractions have also taken other identities such as cotton\textsuperscript{46}, nanoparticles\textsuperscript{47}, and magnetic beads\textsuperscript{9} all having specific advantages with the overall goal of achieving rapid and efficient extractions on small volumes/concentrations of proteins and other biomolecules prior to further analysis. The work presented here will focus on employing polymer fibers as the stationary platform for SPE of biomolecules from salts/buffers extending to the extreme conditions of biofluids.

\textit{Capillary-channeled polymer fibers}

Marcus and co-workers have placed a great deal of effort employing capillary-channeled polymer (C-CP) fibers as stationary phases for separations (HPLC columns) and extractions (micro-columns and SPE tips) of biomolecules\textsuperscript{48-51}. C-CP fibers are produced by melt extruding specific polymer beads (e.g., polypropylene (PP), polyester (PET), or nylon-6) which can be
beneficial when particular interactions are sought out such as hydrophobic, hydrophilic, or ionic. C-CP fibers are unique in that their structure involves eight capillary channels (~10 µm width) that run collinearly along the length of each fiber (length of column) which enable more surface area compared to circular polymer fibers. Fibers (~ 40 µm nominal diameters) are pulled through column supports (e.g., fluorinated ethylene propylene, stainless steel) in which fibers interdigitate yielding an increased surface area with the ability to pack more fibers. The capillary channels on the fibers allow for capillary-induced wicking of liquids which benefits fluid transport (even of biofluids). In its implementation as HPLC columns, linearity and lack of pores decreases backpressures typically associated with silica-based and standard polymer phases. This phenomenon allows for separations to occur at increased linear velocities (75 mm s⁻¹), which in turn can improve separation efficiency, especially for the separation of biomolecules⁵²,⁵³.

Presented here, PP C-CP fibers will be utilized for the extraction of biomolecules. Polypropylene allows for the rapid adsorption/desorption of proteins through hydrophobic interactions as the “hydrophobic foot” of each protein will interact with the hydrophobic surface of PP. In terms of SPE, extractions involve the partitioning of analyte species onto the stationary phase and then to the elution solvent (mobile phase). For successful extractions, target species must demonstrate a greater affinity to the stationary phase than the
solution which it resides, and then a greater affinity for the elution solvent. This process is demonstrated by equation 1.1 where the distribution coefficient, $K_D$

$$K_D = \frac{S_e}{C_s} \quad \text{E.q. 1.1}$$

is equal to the ratio of analyte concentration on the stationary phase ($S_e$) to the concentration of analyte in the elution solvent ($C_s$). When more of the $S_e$ is present, the analyte has a stronger affinity for the stationary phase. When the distribution coefficient is smaller ($<1$), the analyte is successfully removed in the elution solvent. In the implementation here, a semi-non-polar elution solvent (acetonitrile (ACN)) is employed to remove the adsorbed species. The hydrophobic biomolecules are eluted as the hydrophobic interactions are reduced upon addition of elution solvent. The first part of this dissertation studies the processes of adsorbing proteins (more specifically urinary proteins) in complex mixtures (biofluids) to C-CP micro SPE tips followed by the removal (aqueous wash) of contaminants. The eluted “clean” proteins are then detected by MALDI-MS analysis. Studies demonstrated very promising results for successful extractions from minute sample aliquots ($<10 \mu$L) at low concentrations ($<10$ ng).
The need for compact field-deployable instrumentation for atomic analysis is of great interest\textsuperscript{54-58}. Forensics and national security are just a few areas in which the focus of developing miniaturized instrumentation has been placed. Particularly when dealing with radioactive chemicals, instrument portability is a necessity to limit sample handling and transport back to laboratory environments. Currently, the most commonly employed technique in elemental analysis instrumentations is the inductively coupled plasma (ICP) employing optical emission spectroscopy (OES) or MS detection\textsuperscript{59-61}. ICP-OES/MS is a highly robust and sensitive chemical analysis instrument for elemental determinations. Unfortunately, this commercially available and widely used instrument has seen insignificant advancements in terms of miniaturization. This high-power (1-2 kW) source possesses large operating costs as requirements include large gas volumes (up to 16 L min\textsuperscript{-1}) and solvent volumes. Size restrictions (multiple vacuum pumps, exhausts requirements, etc.) also prevent portability applications, in terms of MS detection. Ideally, portable instruments are user friendly and can provide rapid chemical analysis of samples in the field. Portable instrumentation would save time and prevent sample loss and hazardous handling in transporting back to the lab. This dissertation will discuss an approach towards rapid atomic analysis utilizing a miniaturized plasma with a very small footprint and low operating costs.
Elemental analysis instrumentation

In terms of atomic instrumentation, analysis of liquid samples has been one of the primary areas of research. In order to maintain sample homogeneity, sample digestions and separations (removing interferences) are typically incorporated prior to analysis, making liquid introduction one of the key research topics of elemental instrumentation. The two most widely utilized techniques for elemental analysis for liquid analysis are atomic absorption spectrometry (AAS) and the ICP OES/MS. In both cases, the liquid sample is introduced and atomized in the flame or plasma. For ICP, detection can either be performed by measuring the photons emitted from the excited species in the plasma by optical emission spectroscopy (OES) or analyzing the ions by mass spectrometry (MS). For the fundamental operating principles in the ICP, a liquid sample is introduced (typically utilizing a peristaltic pump at mL min\(^{-1}\)) into a nebulizer, which converts the liquid phase sample into an aerosol of droplets (by utilizing a gas such as Ar at L min\(^{-1}\)). The droplets are then separated by a spray chamber in which the larger droplets are removed and all of the fine droplets, which become dry particles upon solvent evaporation, are transported into the plasma torch for atomization/ionization/excitation. Many studies pertaining to the ICP have been dedicated towards determining/monitoring plasma characteristics. There are many plasma characteristics which are vital for efficient atomization, excitation, and ionization such as excitation temperature \(T_{\text{exe}}\), ionization...
temperature ($T_{\text{ion}}$), gas rotational temperatures ($T_{\text{rot}}$) and electron number density ($n_e$). All of these characteristics have been studied in great detail in parametric evaluations (looking at the effects of gas flow rate, sample introduction, etc.) as well as characterization of the ICP spatially$^{61,65,66}$.

Once the particles are in the plasma, there are two distinct ways of detecting the excited/ionized species: 1) optical emission spectroscopy (OES) and mass spectrometry (MS). In OES detection, the excitation source is utilized to generate photons of light (from the emission of excited introduced species returning to ground-state). In MS-detection, the ICP generates positively charged ions which are directed to the mass analyzer, typically by means of an applied voltage$^{59}$. In most cases, the ions are directed at a different path (towards a detector) so that photons are not directed alongside the ions as they can overload the analyzer, ideally only ions are detected for enhanced sensitivity. In terms of MS, a large production of ions allows for 3-4x lower detection limits compared to OES analysis; however, OES instrumentation is cheaper in terms of production and operating costs.

**Miniaturized liquid sampling sources**

Development of miniaturized plasmas for elemental analysis is foreseen as the future for the development of atomic portable instruments$^{55,56,58,67,68}$. In the early 1990’s Cserfalvi et al. developed a new and innovative source for the
analysis of liquid solutions with OES detection\textsuperscript{69, 70}. The electrolyte-cathode discharge (ELCAD) is designed so that an electrolytic solution is employed as the cathode. The ELCAD source was the first low-power, DC source to be operated at atmospheric pressure for elemental analysis of liquid solutions. The ELCAD advantageously utilizes an electrolytic solution as the cathode, which enables ease of introducing aqueous samples into the plasma. The electrolytic solution/sample flows (>1 mL min\textsuperscript{-1}) upward as a “waterfall” from an inlet tube as seen in Figure 1.3. A counter electrode (anode), typically tungsten (W), is placed ~3 mm from the cathode in which a discharge is formed.

\begin{center}
\textbf{Figure 1.3} The electrolyte cathode glow discharge source set-up
\end{center}
after application of DC voltage (<1 kV) and discharge current of ~80 mA\textsuperscript{70}. Much research has been dedicated towards the development of the ELCAD in terms of plasma characterizations (i.e. plasma temperature)\textsuperscript{71, 72}, analytical performance\textsuperscript{69}, spatially resolved plasma characteristics\textsuperscript{71}, and operating parameters\textsuperscript{70}.

The introduction of liquid electrolytic glow discharges sparked great interest in terms of portable elemental instrumentation, and in turn, several designs have been investigated. Marcus and co-workers investigated this phenomenon by developing a liquid sampling-atmospheric pressure glow discharge (LS-APGD)\textsuperscript{73}. With the same mindset as Cserfalvi, the LS-APGD was initially investigated in terms of elemental analysis of liquid solutions. The aim was to develop a low power source utilizing smaller solution/sample flow rates so that interfacing with chromatographic separations could be achieved and chemical waste was reduced. As mentioned earlier in regards to the ELCAD, higher flow rates were required, up to 10 mL min\textsuperscript{-1} and required a reservoir for waste containments, which increases the potential for safety hazards. The LS-APGD was initially developed utilizing electrolytic flow rates less than 100 µL min\textsuperscript{-1} and in a “total consumption” mode in which all solvents/sample were completely vaporized in the plasma, leaving no chemical waste. The LS-APGD works by forming a glow discharge between an electrolytic solution (10-100 µL min\textsuperscript{-1}) and a counter electrode (nickel, stainless steel, etc.) upon the application of a dc
Voltage (<1 kV) and a discharge current (<100 mA) as seen in Figure 1.4. Versatility can be seen in the LS-APGD in terms of detection sources employed (OES and MS analysis)\textsuperscript{73, 74}, chemical analysis information (atomic and molecular)\textsuperscript{75}, and means of sample introduction (liquid, solid surfaces\textsuperscript{75}, gases\textsuperscript{75}, and laser ablated particles\textsuperscript{76-78}). This dissertation will describe the utilization of the LS-APGD as an excitation/ionization source for the mentioned samples as well as detailed studies of plasma characteristics (temperatures, electron number density, and robustness) upon the introduction of species.

\textbf{Figure 1.4} LS-APGD set-up for OES detection
This chapter described an overview of miniaturization of chemical analysis procedures/instrumentation. The first half discussed in detail the sample preparation required for biofluids/biomolecules prior to MS detection, specifically MALDI-MS. The second half described current methods for elemental analysis of solutions while illustrating the trends in miniaturization of this instrumentation. Chapters two and three illustrate in detail the utilization of C-CP fiber packed μ-SPE tips for the extraction of proteins prior to analysis by MALDI-MS. Chapter two discusses the potential of employing this method as a rapid extraction technique for small aliquots (<10 μL) while chapter three goes into further detail describing the procedural optimization studies. Chapter three studies elution solvent, aqueous washing steps and fiber tip length to give further information regarding the extraction procedures. These two chapters were published in *Journal of American Society for Mass Spectrometry* (Benjamin T. Manard, R. K. Marcus, 2012, vol. 23, pp. 1419-1423) and *Analytical Methods* (Benjamin T. Manard, R. K. Marcus, 2013, vol. 5, pp 8171-8184), respectively. Chapter four demonstrates an application area in which the C-CP fiber tips will be employed for the extraction of urinary proteins from a complex biofluids (urine) followed by subsequent analysis with MALDI-MS and is submitted for publication as a special issue on “Urine Proteomics” in the journal of *Proteomics – Clinical Applications*. In regards to miniaturization of elemental analysis instrumentations chapters five,
six, and seven all demonstrate studies pertaining to the LS-APGD. Chapter five investigates the plasma characterization of temperatures, electron number density, and robustness under a parametric evaluation of the LS-APGD. Said characteristics were studied upon introducing both liquid and laser ablated particles. Chapter five was published in *Spectrochimica Acta Part B* (Benjamin T. Manard, J.J. Gonzalez, Arnab Sarkar, Meirong Dong, Jose Chirinos, Xianglei Mao, Richard E. Russo, and R. K Marcus). Chapter six describes in detail the robustness in terms of spectrochemical analysis upon the introduction of liquid matricies and was submitted in a special journal issue dedicated to Nicolo Omenetto in *Spectrochimica Acta Part B* (Benjamin T. Manard, J.J. Gonzalez, Arnab Sarkar, Xianglei Mao, Lynn X. Zhang, R. K. Marcus, and Richard E. Russo). Chapter seven illustrates the LS-APGD as a secondary excitation source for laser ablated particles and describes parameter effects as well as robustness in terms of ablating particles into the microplasma and will be submitted for publication in *Spectrochimica Acta Part B*. 
REFERENCES


CHAPTER TWO

CAPILLARY CHANNELED POLYMER (C-CP) FIBERS AS A STATIONARY PHASE FOR SAMPLE CLEAN-UP OF PROTEIN SOLUTIONS FOR MATRIX-ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY

INTRODUCTION

Proteomics is centered on the ability to identify and characterize proteins while maintaining their structural integrity. With this in mind, proteolysis and cell digestions must be performed in buffered solutions to ensure stability, perforce biological functions, or to maintain nontoxic cellular surroundings. Unfortunately, buffers are detrimental in protein analysis by mass spectrometry because they suppress the signal from the desired proteins\(^1\). Matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) is a widely used technique for the analysis of biomacromolecules because of its ability to softly-ionize analytes with little or no fragmentation; however, studies have shown samples containing buffer concentrations of 100 mM or greater must be desalted before analysis with MALDI-MS\(^2\), \(^3\). Because of the importance of the problem of buffer/matrix suppression, various approaches have been explored to remove these contaminants prior to MALDI-MS\(^1\)-\(^5\).
High-performance liquid chromatography (HPLC), 2-D electrophoresis, and centrifugation are common techniques employed for the clean-up of protein samples prior to MALDI-MS\(^1\). In the absence of needing a chemical separation of proteins, solid phase extraction (SPE) is the most popular technique for extraction and clean-up of biological samples. SPE works by adsorbing target molecules onto the surface of the stationary phase while buffers and surfactants are washed away\(^6\). Proteins are eluted from the stationary phase in a solvent that is generally compatible with the direct deposition on the MALDI target. Primary advantages of SPE over other techniques include: reduced analysis time, small amounts of elution solvent, and small volumes of (typically) low-cost sorbent materials\(^7\). More recently, SPE platforms has been miniaturized and used with micropipette tip-based fluid manipulations for protein desalting, allowing for effective handling of microliter-volume samples\(^8\), \(^9\). Many approaches use a C\(_{18}\) derivatized porous silica media for the purification of peptides from buffers/salts as described above\(^10\). Problems encountered in various micropipette tip platforms include robustness and sensitivity issues for small amounts of protein because of inefficient interactions and low recoveries\(^11\).

This laboratory has developed capillary-channeled polymer (C-CP) fiber stationary phases for the liquid chromatography separation and solid phase extraction (SPE) of proteins\(^12\)-\(^15\). C-CP fibers are advantageous when compared to other stationary phases because of the wide range of polymer/surface chemistries available, robustness, low cost, and the ability for microanalytical
separations. A subtle, but very important, characteristic is the lack of porosity of the C-CP fibers, resulting in rapid adsorption/ionization kinetics and high recoveries\textsuperscript{13}. In addition to chromatographic column, polypropylene (PP) C-CP fibers housed in micropipette tips have been employed for the SPE/desalting of protein solutions prior to ESI-MS\textsuperscript{15}. Here, proteins adsorb onto the hydrophobic PP C-CP fiber surfaces while allowing salts to directly pass or be washed off in with a simple aqueous rinse step. Bound proteins are eluted with ESI-friendly solvents, such as acetonitrile:water (ACN:H\textsubscript{2}O) mixtures. Intensity, signal-to-noise ratios (S/N), and spectral clarity of analytes were improved dramatically in 150 mM buffer due to the clean-up with C-CP fibers prior to ESI-MS analysis.

Polypropylene C-CP fibers are employed here as a stationary phase for SPE of proteins from buffer solutions prior to MALDI-MS analysis. A new protocol for the SPE processing, employing a simple centrifugation method, provides far greater sample throughput and overall superior precision than manual aspiration\textsuperscript{15, 16}. Efficient desalting is performed for proteins (cytochrome c and lysozyme) on the sub-micromolar level (nanogram absolute mass) in the presence of up to 1 M Tris-HCL. The practicality of the method is demonstrated by the successful desalting of single-micromolar concentrations of protein from a mock-urine sample. It is believed that C-CP fibers in a micropipette tip format present a very promising approach to protein sample clean-up prior to MALDI-MS analysis.
**EXPERIMENTAL**

*Reagents and Chemicals*

Cytochrome c (bovine heart, MW = 12.2 kDa), lysozyme (chicken egg white, MW = 14.3), and sinapinic acid (SA) were each obtained from Sigma-Aldrich (Milwaukee, WI). Tris-HCl (Teknova, Hollister, CA, USA) and ACS grade acetonitrile (ACN) were obtained from Fisher Scientific (Pittsburg, PA). MilliQ water (18.2 MΩ/cm) derived from a NANOpure Diamond Barnstead/Thermolyne Water System (Dubuque, IA, USA) was used in solution preparation of buffer species, proteins, and aqueous mobile phases. A synthetic urine matrix was prepared in house, using the typical constituent concentrations of 233 mM KHCO₃, 110 mM KCl, 13 mM K₂SO₄, 65 mM glysine, and 5 mM urea.

*Fiber-Tip Construction*

C-CP SPE tip preparation methodology is identical to the procedure previously used in constructing C-CP fiber microbore HPLC columns\(^{14}\). The PP C-CP fibers are pulled through 0.8-mm-i.d. fluorinated ethylene polypropylene (FEP) capillary tubing (Cole Parmer, Vernon Hills, IL). In the present studies, a total of 658 PP fibers of ~55 µm diameter were pulled collinearly through ~300 mm of the tubing, yielding an interstitial (void) fraction of \( \epsilon_i \approx 0.6 \). Approximately 10 mm was cut off an end of the packed tubing with a surgical grade scalpel so
that the fiber and tubing ends were flush. On the opposite end of the tubing, a ~6 mm free space was left between the tubing and the fiber ends. The end of the tubing with the dead space was slipped over a 1 mL micropipette tip (Redi-Tip™ Fisher Scientific, Pittsburg, PA). The tubing was then cut so that 1 cm of packed fibers was attached to each tip. The tip construction process yielded ~20 tips from the C-CP fiber column.

**Adaptation to the Centrifugation Format**

A centrifugation-based approach has been implemented, resulting in much higher sample throughput through parallel processing and far greater levels of experimental control\(^\text{16}\). A pipette tip adapter was made so the fiber-packed tips could be placed into a six-bucket benchtop centrifuge (Clinical 50, VWR, West Chester, PA). The conical portion (~10mm) of a 1.7 mL microtube (Genessee Scientific, San Diego, CA) was cut perpendicular to the tube axis. The fiber-packed micropipette tip was placed inside the top of the microtube. The microtube was then placed into a 15 mL conical centrifuge tube (VWR) as depicted in Fig. 2.1, for placement in the centrifuge.
Figure 2.1 Adaptation of the C-CP fiber-packed tips format for centrifugation-driven SPE. The bottom of the microtube is removed and the C-CP fiber-packed tip is inserted. The microtube with the fiber tip is then inserted into a 15 mL centrifuge tube so that it can be placed into a centrifuge device. The fractions are then collected in separate centrifuge tubes.

**Protein Loading, Washing, and Elution**

Solutions of cytochrome c were made in Tris-HCl buffer with buffer concentrations of (100-1000 mM). A 2.5 µM lysozyme solution was also made in 100 mM Tris-HCl buffer. Loading aliquots ranging from 3-10 µL of the protein solutions were centrifuged through the fiber tips at 4000 RPM for 2 min. The washing step included a 20 µL aliquot of MilliQ water, centrifuged at 4000 RPM for 2 min. The proteins were then eluted from the fiber surfaces by an aliquot
equal to the loading volume of 50:50 ACN:H₂O through the tips at 4000 RPM for 2 minutes. The total processing time for the procedure (for multiple tips in parallel) is approximately 10 minutes.

**Mass Spectrometry**

A Bruker Dalonics (Billerica, MA) Microflex LRF, MALDI-TOF mass spectrometer was used in the positive ion, linear mode for the protein analysis. The Bruker Dalonics Compass software was used for control and data processing. The MALDI-TOF is equipped with a nitrogen laser (337 nm) operating at a pulse rate of 60 Hz. 200 laser shots at 79% laser power were used to acquire the spectra. A 20 mg mL⁻¹ solution of sinapinic acid in 50:50 ACN:H₂O 0.1% TFA was used as the matrix solution. The MALDI target was prepared by spotting 1µL of the matrix solution followed by 1 µL of the protein solutions, with time for drying between each step. Signal-to-noise ratios are based on the standard deviation of the signals in a 100 Da mass window centered 2000 Da below the protein molecular ions.

**RESULTS AND DISCUSSION**

*MALDI-MS of Cytochrome c and Lysozyme from 100 mM Tris Buffer*
The SPE of protein solutions was accessed by comparing analyte signal intensities and signal-to-noise ratios of protein samples before and after extractions with the C-CP fiber micropipette tips. Separate 10 µL aliquots of each protein were loaded onto the C-CP fiber tips. The mass spectra presented in Fig. 2.2 illustrate the relationship between the signals of 3.1 µM cytochrome c and 2.8 µM lysozyme in 100 mM Tris buffer before and after SPE desalting. The mass spectra show no discernible signals representative of the proteins in the presence of 100 mM tris buffer. On the other hand, proteins extracted from the buffer using the C-CP fibers yielded high fidelity spectra. Comparisons with the protein signal intensities for the SPE extracted proteins to neat protein solutions (same solvent and concentration, without buffer) provide a level of assessment of the degree of overall recovery. There is a <5% loss in signal intensity through the course of the SPE process, perhaps due to irreversible binding to the fibers or the micropipette tip components themselves. In any case, such performance is testament to the overall efficiency of the methodology.
Another very important aspect in proteomic applications is the ability to clean up samples having low concentrations of protein in the presence of high matrix concentrations. A serial dilution (0.25 – 3.1 µM) of cytochrome c in the 100 mM Tris buffer was prepared and analyzed in triplicate. A 1 µL volume of the lowest protein concentration (representing a protein mass of 3 ng) was still detected with spectral clarity. Extrapolation to a limit of detection based on the

Figure 2.2 MALDI-MS spectra of (top) 2.8 µM lysozyme and (bottom) 3.1 µM cytochrome c in 100 mM Tris buffer before (in red) and after (in blue) extraction on PP C-CP fiber-based micropipette tips
S/N=3 yields a value of ~0.5 ng, or ~40 nM in the original buffer solution. This ability to perform buffer removal on nanogram quantities of protein in a high abundance of buffer (buffer:protein ratio of 400,000:1) illustrate the effectiveness of miniaturizing the process.

*Effect of Tris Buffer Concentration on MALDI-MS Recoveries*

The concentration of buffer employed can differ substantially in bioanalysis applications, with varying effects in any MS analysis. Additionally, salt/matrix concentrations vary tremendously across sample types, such a blood, cell extracts, or urine. With this in mind, desalting techniques should be effective at not only buffer concentrations (100 mM) but perhaps concentrations up to 1. Solutions of 3.1 µM cytochrome c solutions were prepared in buffer concentrations of buffer, ranging from 100 mM to 1 M (including a neat solution), to quantify the overall signal recoveries as well as the resultant S/N characteristics. Both quantities decrease monotonically as the buffer concentration increases, but exhibiting only 50-60% depressions versus the neat protein. Important to note here is that each of the samples was subjected to the same wash/elute procedure. So the results obtained may be improved further by simple use of higher-volume DI-H2O washing procedure.

The relative immunity to high salt/buffer concentrations begs the question as to the direct applicability of the C-CP fiber SPE method. To test this point,
cytochrome c was spiked into a synthetic urine matrix at a concentration of 3.1 µM and analyzed directly by MALDI-MS. As seen in Fig. 2.3a, the cytochrome c molecular ion is not at all detectable at the 3.1 µM level in the urine matrix. Following extraction on the PP C-CP fiber tips, the cytochrome c was detected with high spectral clarity depicted in Fig. 2.3b, illustrating the effectiveness of protein extraction from a urine matrix. Consistent with the results described previously, the LOD for the protein in the mock urine is ~67 nM for this 1 µL sampling.

![Figure 2.3 MALDI-MS spectra of (a) 3.1 µM cytochrome c in synthetic urine and (b) 3.1 µM cytochrome c in synthetic urine following SPE on PP C-CP fiber based micropipette tips](image)
CONCLUSION

The results from these experiments extend the potential of PP C-CP fibers at stationary phase for SPE methods for sample clean-up prior to MALDI-MS analysis. The C-CP fiber tips can effectively retain protein and allow weakly bound organics and buffers to be washed away, with the proteins eluted in MALDI-friendly solvents. This purification procedure improves analyte signal response and S/N characteristics which are critical for biomolecule analysis. The ability to perform SPE for samples across these matrix conditions supports the potential use of the C-CP fiber SPE tips for various biofluids (e.g. blood, saliva) prior to MALDI-MS. Optimization of several parameters including fiber packing density, tip length, volumes of washing and elution steps, and elution solvents are under investigation with the potential to improve the capabilities demonstrated here.

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CHAPTER THREE

OPTIMIZATION OF CAPILLARY-CHANNELED POLYMER (C-CP)
FIBER STATIONARY PHASE EXTRACTIONS OF PROTEINS FROM MALDI-MS SUPPRESSING MEDIA

INTRODUCTION

Mass spectrometry (MS) methods are ubiquitous in their role in identifying and characterizing proteins and other biomolecules\textsuperscript{1-3}. MS is an essential element in the proteomics arsenal, for example identifying specific biomarkers for the investigation of diseases, because it allows for comprehensive profiling across diverse biomolecules, chemical forms, concentration ranges, and sample types. The two most common forms of ionization source used in these sorts of analyses are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI)\textsuperscript{4}. MALDI is frequently used in proteomics analyses due to its ability to softly generate analyte ions, yielding spectra that are principally composed of singly-charged analyte ions. In most instances, MALDI occurs via proton transfer from an acidic matrix component or possibly cation (e.g. Na\textsuperscript{+}, Ag\textsuperscript{+}) attachment through the addition of those species to the matrix cocktail. On the other hand, ESI generates multiply charged species, lessening the required mass range of the instrument, but can greatly complicate the
spectral interpretation. One could argue that ESI has certain throughput advantages due to its ready interfacing with liquid chromatography (LC) and capillary electrophoresis (CE) separation methods.

A common challenge to MALDI and ESI sources are the matrix effects imposed by complex sample solution. Buffers, salts, and surfactants are constituent of many biological samples in order to retain stability, preserve biological function, or mimic cellular surroundings\textsuperscript{5-7}. Unfortunately, these various species act as contaminants in the ionization processes of ESI and MALDI, suppressing analyte (protein, peptide, etc.) ionization as well as generating large amounts of background spectral signals and complicated spectral interpretation through the formation of analyte adduct (e.g., protein-salt) ions. Ion suppression (principally by a competitive process) can affect the situation to the point of eliminating signals in favor of buffer species in both ESI and MALDI. For the case of MALDI-MS employed here, a general rule of thumb is that samples having matrix concentrations of over 100 mM need to be desalted prior to analysis in order achieve acceptable sensitivity and spectral fidelity\textsuperscript{8, 9}.

Over the past two decades a great deal of effort has been directed at developing high efficiency sample clean-up procedures for proteomics applications. In addition to matrix removal, biomarkers and other important analytes are commonly found in low concentrations, thus extraction/concentration techniques must be employed to improve their detectability. This is not just true for MS analysis, but also for any further
chemical separations or spectrometric determinations\textsuperscript{6, 10-14}. Methods of determining biomarkers from biofluids (e.g. urine) generally require a three-step process that begins with centrifugation and two additional separation methods\textsuperscript{15}. Protein isolation/separation practices have been explored through methods such as gel electrophoresis (GE), high performance liquid chromatography (HPLC), capillary electrophoresis (CE), and centrifugation\textsuperscript{14}. Trends have moved towards the miniaturization of these processes as analyses on a microanalytical scale require minute quantities of sample, separation times on the order of seconds, and sample waste is nearly eliminated\textsuperscript{5, 11}. Typical sample clean-up and extraction procedures are accomplished with aliquots of biofluid volumes ranging from 50-100 μL, with approximately 1-3 μg of protein extracted prior to mass spectrometry\textsuperscript{6}. Recently, techniques allowing for extraction of single-μL (1-3 μL) aliquots and/or single-ng (3-10 ng) amounts of total protein have been particularly attractive in the case of biomarkers\textsuperscript{10, 16, 17}.

Solid phase extraction (SPE) is perhaps the most common and widely accepted technique for extraction and clean-up of proteins from complex media (buffers, urine, etc.). SPE works by adsorbing target analytes onto the surface of the stationary phase while allowing buffer components and other unwanted species to be washed away, leaving the analytes of interest adsorbed to the stationary phase\textsuperscript{13}. Target proteins are then eluted in a MS friendly solvent that releases the target from the stationary phase. SPE stationary phase materials are comparable to those applied in HPLC columns; however, the working
volumes utilized during SPE are generally <100 µL\textsuperscript{12}. SPE has rapidly developed and improved over the past decade, while trending towards miniaturization by utilizing micropipette tip-based fluid manipulations for protein desalting prior to MS analysis\textsuperscript{5}. Other advantages in micro-SPE type devices include the ability to incorporate automated liquid-handling instrumentation\textsuperscript{7, 11}. Potential problems encountered with micro-SPE tips include robustness especially when utilizing C\textsubscript{18} stationary phase as variable pHs can induce irreversible binding, resulting in low recoveries that are detrimental when extracting nanogram amounts of protein\textsuperscript{5, 13, 18}. Beyond polymer bead micro-SPE approaches, Wuhrer and co-workers used cotton fibers packed into micropipette tips. While cheap, higher quantities are required (up to 5 µg) and the SPE tips have low recovery characteristics (~40 % average recovery)\textsuperscript{19}.

This laboratory has developed capillary-channeled polymer (C-CP) fibers as stationary phases for HPLC protein separations\textsuperscript{20-22}. Analytical protein separations have been performed in reversed phase, ion exchange, and hydrophobic interaction modes on polypropylene, polyester, and nylon 6 C-CP fiber columns. C-CP fibers are advantageous when compared to other stationary phases due to low cost, chemical robustness, and wide range of polymer chemistries. Another advantage to C-CP fiber phases is the high mass transfer rates, provided by virtue of the lack of fiber porosity (pore radii <1.5 nm) in comparison to the size of proteins\textsuperscript{21-23}. As such, very high mass transfer rates and high recoveries are realized\textsuperscript{21, 22}. In chromatographic separations, very high
linear velocities (>50 mm s\(^{-1}\)) can be used without penalty in terms of the van Deemter C-term broadening\(^{22-24}\). Another advantage is that C-CP fibers do not suffer from the irreversible binding that typically effect derivatized silica or carbohydrate stationary phases.

The combination of high fluidic permeability and high mass transfer efficiencies make C-CP fiber platforms excellent candidates for SPE applications \(^{25-28}\). Most relevant to the work here, polypropylene (PP) C-CP fiber stationary phases have been mounted on micropipette tips for SPE/desalting of protein solutions prior to ESI and MALDI-MS analysis\(^{25, 27}\). In this application, proteins adsorb onto the PP surface due to hydrophobic interactions while allowing the concomitant solution constituents to pass through, or to be washed off with a rinse step. Adsorbed proteins are then eluted with an MS-friendly solvent consisting of an aqueous acetonitrile (ACN) mixture. C-CP fiber SPE has been affected in both a microtip format as well as an in-line method convenient for ESI-MS. Mounting fibers within polymeric microbore tubing allows them to be press-fit to conventional micropipette tips for fluidic processing by manual aspiration\(^{25}\) or simple centrifugation\(^{27}\).

A previous communication described the basic concept of using C-CP fibers in the micropipette format prior to MALDI-MS analysis\(^{27}\). Reported here are the results from a study of various sample manipulation parameters pertaining to the utilization of PP C-CP fiber tips for the extraction of proteins from buffered species for subsequent analysis by MALDI-MS. The processing
steps of load, wash, and elute were all evaluated with regards to fiber tip length, solvent volumes, and solution composition, respectively. In this application, a simple benchtop centrifuge was used to impart solution flow through the fiber-filled tips. The optimized procedure was then employed to extract a three-protein suite of cytochrome c, lysozyme, and myoglobin from a synthetic urine solution. The C-CP fiber micro-SPE tips allow for effective extraction of the three proteins, at low concentrations (< 5 µM) from the urine mixture with detection of each protein obtained with high spectral clarity. It is believed that the general methodology is now poised for applications in biomarker identification with the positive attributes of allowing small sample volumes to be processes in a rapid fashion with very high levels of sample/signal recovery and a very inexpensive and easily implemented platform.

**EXPERIMENTAL**

*Reagents and Chemicals*

The test proteins cytochrome c (bovine heart, MW = 12.2 kDa), lysozyme (chicken egg white, MW = 14.3 kDa), and myoglobin (MW = 16.3 kDa), and MALDI matrix sinapinic acid (SA) were all obtained from Sigma-Aldrich (Milwaukee, WI, USA). Proteins were prepared in MilliQ water (18.2 MΩ/cm) derived from a NANOpure Diamond Barnstead/Thermolyne Water System (Duboque, IA, USA), Tris-HCl was purchased from Teknova (Hollister, CA, USA),
and synthetic urine (194 g L\(^{-1}\) urea, 6 g L\(^{-1}\) CaCl\(_2\), 11 g L\(^{-1}\) MgSO\(_4\), and 80 g L\(^{-1}\) NaCl) from Ricca Chemical Company (Arlington, TX, USA). Acetonitrile (ACS grade) from Fisher Scientific (Pittsburgh, PA, USA) and trifluoroacetic acid (TFA) from Sigma-Aldrich were used in preparing the elution solvents.

**C-CP Fiber Column/Tip Construction**

The general methodology for preparing C-CP fiber micropipette tips has been described in detail previously\(^\text{25}\). Based on detailed studies regarding chromatographic efficiencies\(^\text{24, 29}\), the fibers were packed in the FEP tubing with an interstitial (void) fraction of \(\varepsilon_i \approx 0.6\). Specifically, 658 PP C-CP fibers (~30 µm in diameter) were pulled collinearly through ~300 mm of 0.8-mm i.d. fluorinated ethylene polypropylene (FEP) tubing (Cole Parmer, Vernon Hills, IL, USA). The ends of the column were cut with a surgical grade scalpel so the fiber and tubing ends were flush. End fittings (Valco Instruments, Houston, TX, USA) and column unions (VWR International, West Chester, PA, USA) were attached to the column for direct connections to the HPLC unit. The assembled column was subjected to sequential cleaning steps (100% ACN and 100% DI-H\(_2\)O) to remove residual post-extraction spin-coating or surface contamination prior to tip preparation. Following cleaning, fibers were pulled towards one end of the column to generate 6 mm of void space (which is press-fit to the commercial 1 mL micropipette tips (Redi-Tip\textsuperscript{TM} Fisher Scientific, Pittsburgh, PA)) and the desired length of fiber-filled
capillary cut as needed. This tip assembly process yields ~20 tips from a single C-CP fiber column.

**Adaptation for Centrifugation Processing**

The vast majority of MALDI-MS instruments have the capacity of applying multiple samples on a single target. In order to achieve higher sample throughput and gain higher levels of experimental control, sample processing in parallel using centrifugation processing has been implemented\(^{26-28}\). In this way, the number of samples processed is limited only by the bucket capacity of the centrifuge. A pipette tip adaptor was made in order to secure the fiber-packed tips into a six-bucket benchtop centrifuge (Clinical 50; VWR, West Chester, PA, USA). The fiber-packed micropipette tip was placed inside the top of a 1.7 mL microtube (Genesse Scientific, San Diego, CA, USA) that was cut perpendicular to the tube axis leaving ~10 mm of the conical portion and is then placed in a 15 mL centrifuge tube (VWR). A photograph of a C-CP fiber micropipette assembly (following passage of a green food coloring) is shown in Fig. 3.1.
Figure 3.1 Photograph of a C-CP fiber-packed micropipette tip adapted into an apparatus for centrifugation after processing of a green food dye

Chromatographic System and Operations

The chromatographic system consisted of a Dionex (Sunnyvale, CA, USA) Ultimate 3000 with a LPG-3400SD pump, WPS-3000TSL autosampler, and a VWD-3400 RS variable wavelength UV-Vis absorbance detector monitoring at 216 nm. Dionex Chromeleon software was utilized for data analysis of the chromatograms with further analysis performed with Microsoft (Seattle, WA, USA) Excel. A gradient separation was performed in order to determine the
optimal conditions to elute various proteins from the C-CP fiber surface. An injection of 10 µL of the three protein suite (cytochrome c, lysozyme, and myoglobin) with a mobile phase flow rate of 0.8 mL min\(^{-1}\) MilliQ water with 0.1% TFA was used from 0-3 minutes to ensure full adsorption of the protein to the fiber surface. A gradient was initiated at 3 minutes with ACN containing 0.07% TFA until it reached 100% at 12 minutes.

Protein Extraction Utilizing PP C-CP Tip

Various volumes and concentrations of the test proteins/matrices were loaded by centrifugation through the PP C-CP tips at 4000 RPM for 2 min. Next, an aqueous step was incorporated to remove the undesirable buffer/contaminates with an aliquot (10-100 µL of MilliQ water) centrifuged (1900 RCF) for 2 min. The desired proteins were then eluted with an ACN:H\(_2\)O containing 0.07% TFA by centrifuging (4000 RPM) for 2 min. The eluent was collected for further analysis in the centrifuge tube. The total processing time for this procedure is approximately 8 min, and it allowed for multiple extractions (6 here) to occur simultaneously, only limited by the capacity of the centrifuge used.

Mass Spectrometry
A Bruker Daltonics (Billerica, MA, USA) Microflex LRF, MALDI-TOF mass spectrometer was utilized for the protein analysis. The mass spectrometer was used in the linear, positive ion mode. The Bruker Compass software was used for instrument control as well as data processing. The Microflex LRF was furnished with a nitrogen (337 nm) laser operating at a pulse rate of 60 Hz. An average of 200 laser shots at 79% laser power was utilized to ablate the sample. The MALDI matrix was composed of 20 mg mL\(^{-1}\) sinapinic acid in 50:50 ACN:H\(_2\)O with 0.1 % TFA. Proteins samples were applied to the target by placing 1 µL of the matrix solution onto the plate, followed by 1 µL of the extracted solution; allowing the aliquots to dry between steps. The reported mass spectrometric responses (intensities) are the average of triplicate analyses. Reported signal-to-noise ratios were based on the standard deviation of the signals in a 100 Da mass range centered 2000 Da below the molecular ion peaks.

**RESULTS AND DISCUSSION**

*Optimization of Elution Solvent Strength*

Based on sample preparation norms for MALDI-MS, previous C-CP fiber protein SPE studies have employed an elution solvent of 50:50 ACN:H\(_2\)O for releasing proteins\(^{27}\); however, the effectiveness of this step has never been evaluated. Elution solvent composition might be expect to differ among proteins
as each protein contains a different “hydrophobic foot”. Proteins are more or less hydrophobic depending on the amino acid composition on the outside surface of the conformation; hence there may be different levels of solvent strength required among a protein mixture. In order to determine the requisite elution solvents for extracting cytochrome c (cyto c), lysozyme (lyso), and myoglobin (myo), a gradient elution, reversed phase separation was performed using a microbore PP C-CP fiber column. A 10 µL injection of sample was introduced with a mobile phase consisting of 100% milliQ water and held constant for 3 min to simulate the protein loading on the C-CP tip. After 3 minutes, a 0 – 100% gradient of ACN (with 0.07% TFA) was initiated over a 10 minute period to represent the elution step. The flow rate of the mobile phase was 0.8 mL min\(^{-1}\) in order to mimic the velocity of solvent through the tip when using centrifugation. Based on the chromatographic retention times, the elution percentages were calculated to be 41, 43, and 50% ACN for cyto, lyso, and myo, respectively. Once this range was determined, isocratic elution experiments were performed to assess the total protein recoveries. As presented in Table 3.1, the solvent composition of 60:40 ACN:H\(_2\)O (with 0.07% TFA) yielded the highest total recoveries and high precision (n=3) (while using modest organic content), and so was subsequently use as the SPE elution solvent for the remainder of the described studies.
Table 3.1 Total protein recoveries (in units of integrated area) for isocratic elution of the three-protein suite from a microbore PP C-CP fiber column

<table>
<thead>
<tr>
<th>Mobile Phase Composition</th>
<th>Average Area of Eluted Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>55:45 ACN:H₂O with 0.07% TFA</td>
<td>65.49 ± 0.88</td>
</tr>
<tr>
<td>60:40 ACN:H₂O with 0.07% TFA</td>
<td>84.06 ± 0.40</td>
</tr>
<tr>
<td>65:35 ACN:H₂O with 0.07% TFA</td>
<td>80.02 ± 3.28</td>
</tr>
<tr>
<td>70:30 ACN:H₂O with 0.07% TFA</td>
<td>82.92 ± 0.39</td>
</tr>
</tbody>
</table>

Optimization of Wash Volume

While the typical low molecular weight components of buffers (and urine for that matter) have a very low affinity for the PP C-CP fiber surfaces, there must be an efficient wash step to remove held-up salts following the passage of test solutions. Without effective washing, salt remnants are swept off in the protein elution step. Previous studies using C-CP fiber packed tips for protein clean-up/extraction employed a wash step (20-100 µL) of water in order to remove undesired buffer species\(^{27}\). Even though results depicted successful removal of the contaminant species (as judged by the MALDI-MS spectral quality), no systematic evaluation has been performed. As previously stated, samples in the presence of 100 mM buffer or greater need to be extracted in order to be effectively determined by MALDI-MS. The effectiveness of the wash step is even more important when proteins of low abundance are present\(^{8}\). The amount of buffer employed in solutions can differ substantially in bioanalysis applications, with varying effects in any MS analysis. Taken a step farther, salt/matrix
concentrations vary even more across types of samples such as blood, cell extracts, or urine. With all of this considered, desalting techniques should be effective at not only typical buffer concentrations, but perhaps as high as 1 M as typical in many biofluids.

![Figure 3.2](image)

**Figure 3.2** Effects of wash volumes on MALDI-MS responses for 10 µL applications of 5 µM cytochrome c in various concentrations of Tris-buffer; 10 µL 60:40 ACN-H₂O w/ 0.07% TFA elution solvent

The effectiveness of the sample desalting procedure was investigated by observing the effects of the initial buffer concentrations and wash volumes on the
MALDI-MS responses for 5 µM cytochrome c solutions in up to 1M Tris-HCl buffer. The data plotted in Fig. 3.2 suggest that aqueous wash volumes of 100 µL are effective in gaining high signal recoveries. The cyto c signals for each buffer concentration approach the same level above this point. Importantly, the signal intensities and variability seen here are on par with those of the protein simply dissolved in the neat elution solvent, with the latter reflecting the uncertainties of the MALDI-MS sampling\textsuperscript{27}. Indeed, studies of protein SPE on C-CP fibers with UV-vis absorbance detection reflect sample-to-sample precision of 10-15\% RSD, with ~90\% recoveries\textsuperscript{28}. Even at the highest buffer concentration, the PP C-CP fiber tips were able to provide high spectral clarity and intensities. Closer inspection of the responses at the lowest wash volume (5 µL) reveals the expected result that the signal recoveries are inversely related to the buffer concentrations as remnants are not effectively eluted. Based on the responses depicted in Fig. 3.2, a 100 µL aqueous wash was employed for the remainder of the studies in both the Tris buffer and urine matrix protein extractions.

Roles of Load/Elution Volumes and Fiber Tip Length on Protein Loading

As discussed previously, potential for miniaturization is a key attribute in SPE phase development for proteomics, specifically towards the identification of biomarkers. Certainly, there are tradeoffs between bed volumes and total binding capacities, particularly under dynamic conditions. There are also
considerations in terms of the ability to completely wet the sorptive phases in each step of the SPE process (load, wash, and elute). Earlier studies utilizing C-CP fiber micropipette tips employed load/elution volumes of 100 µL, which are typical of commercial SPE micropipette systems on 1 cm long fiber-packed tips. More recently, lower volumes (10 µL) were explored for C-CP fiber protein extractions prior to MALDI-MS. While successful in extracting nanogram levels of proteins, no optimization was performed. Being able to extract target species from such minute aliquots could be beneficial for areas ranging from analyzing limited amounts of biofluids in forensics or obtaining samples for neonatal studies.

**Figure 3.3** Effects of load/elution volumes on MALDI-MS responses for applications of 5 µM cytochrome c in 750 mM Tris-Buffer to various C-CP fiber tip lengths; 60:40 ACN-H$_2$O w/ 0.07% TFA elution solvent
With an emphasis to utilizing smaller sample volumes, PP C-CP fiber micropipette tips of 2.5 – 10 mm in length were evaluated relative to the MALDI-MS signal recoveries for a range of sample load/elution volumes (same volumes used in both steps). It is important to note that the actual bed volumes of these tips range from ~0.8 – 3 µL, respectively. The signal responses for the loading of a constant cytochrome c concentration of 5 µM in 750 mM Tris-HCl were assessed as a measure of the phase wetting characteristics, binding capacities, and recoveries. Once eluted, 1 µL of that solution was analyzed by MALDI-MS. The tradeoffs between easily wetting a short tip format and its limited binding capacity are seen in Fig. 3.3. In practice, because the total amount of exposed protein is the same at each load/elute volume, the signals should be within the experimental error. Indeed, the responses should be the same for every combination of tip size and processing volume. What is easily seen at the low sample volumes is the fact that the small bed volume tips are more effectively wet/loaded. This trend can be attributed to the void volume that each tip possesses. In the cases where the load/elution volumes are less than the bed volumes, solution passage though the tips (and thus analyte interactions with the fiber surface) will not be expected to be homogenous in the time frame of the test solution passing through the tips. More importantly, the same portions of the fiber beds are not likely to be wet in both the load and elution steps. A second important point to note here is the fact that small bed volumes have lower binding capacities. For example, it is readily seen that the loading of the 2.5 mm long
tips are maximized and invariant beyond the 3 \( \mu \text{L} \) load volume. A response trending towards a limiting case is also seen for the 5.0 mm tip. Higher signals for the longer tips at increased tip lengths reflect their higher binding capacities.

Separate loading/recovery studies performed on the 2.5 and 10 mm C-CP fiber tips (Fig. 3.4) showed that the MALDI-MS signals increase fairly linearly for the 2.5 mm tips to a loading value of \(~200\) ng, while they continued above \(750\) ng of protein for the 10 mm tips before saturation begins to be evident. This latter value is completely in line with previous protein loading values of \(~1.0 - 1.5\ \mu \text{g cm}^{-1}\) of C-CP fiber tips as determined by UV-VIS absorbance spectrophotometry\(^{28}\). Interesting responses are apparent at the lower end of the concentration ranges for each tip, as seen in Fig 3.4. In the case of the small bed volumes, the response is linear from the lowest applied mass of 19 ng of cyto c up to \(~200\) ng, while the recoveries are not quantitative for the larger tips until \(250\) ng is applied. It is believed that the modest load/elution volume (10 \( \mu \text{L} \)) here fails to adequately wet the fiber surfaces in the longer tip, limiting the recoveries at the low mass loadings. For example, a 1 \( \mu \text{L} \) load/elution volume for \(5 \ \mu \text{M}\) cyto c in Tris buffer, the recovery from a 2.5 mm tip is \(> 5x\) that of the 10 mm-long tip, with the sample-to-sample variations of the peak heights for that concentration being \(~5\ \%\text{RSD}\). The smaller tips allow for better bed saturation during the elution step, while such a small volume of solvent does not access the large tip bed space. Based on the signal-to-noise characteristics observed in the 19 ng cytochrome c samples, the limits of detection in this 750 mM Tris-buffer
matrix are ~0.2 ng. Thus, small format tips are preferred when processing small solution volumes, but obviously at the expense of total binding capacity.

**Figure 3.4** MALDI-MS responses as a function of cytochrome c mass loadings (10 µL volume in 750 mM Tris-buffer) extracted with C-CP fiber tips (10 mm and 2.5 mm); 10 µL 60:40 ACN-H$_2$O w/ 0.07% TFA elution solvent

On the high end of the mass loading range, the situation exists wherein there must be sufficient solvent volume to elute the adsorbed protein (regardless of the tip size), while not overly diluting the recovered protein. This trade-off is dictated by the solubility of the protein in the elution solvent and the residence time of the solvent through the tip. For example, increases in elution volume on a 2.5 mm tip loaded with 250 ng of cyto c (presumed saturated) revealed that
while the MALDI-MS signals derived from 1 µL aliquots of the respective eluates decreased as expected by dilution, there were increased proteins recoveries. Specifically, a 10 µL elution volume yielded an MS response that was only a factor of 2 less than the 1 µL elution, where it should have been 10x less. Thus, the mass of eluted protein elution was far higher in the larger volume. The use of higher elution volumes also resulted in much improved sample-to-sample precision, decreasing from ~20 to 10% RSD. Here again, use of higher elution volumes results in more uniform wetting of the fiber tip (as seen as well in the loading characteristics). Therefore, to the extent that samples are diluted, larger elution volumes are preferable, with the actual size being dictated by the total amount of protein adsorbed and the bed size.

Analysis of a Protein Suite in Synthetic Urine

The advantages of employing urine as the sample matrix for proteomic screening for biomarkers include its non-invasive sampling, relatively large sample volumes, and relatively high stability\textsuperscript{15, 30, 31}. While it is obvious that the majority of the protein content in urine is kidney-originating, and thus presents biomarkers for kidney ailments, 30% of urine protein content is plasma-originating. As mentioned previously, as with most proteomic approaches, some form of chromatography is typically applied before protein identification by MS. Even so, urine samples must be remediated of low molecular weight, ionic
compounds\textsuperscript{15, 17, 32, 33}. As such, filtration, dialysis, and ultrafiltration methods are commonly employed for this process. The initial proof-of-concept of the C-CP fiber SPE methodology demonstrated excellent protein recovery at the single micromolar level from a urine matrix\textsuperscript{27}. Furthermore, very recent work demonstrated that myoglobin recoveries from urine using this same methodology were \(~90\%\)\textsuperscript{28}. Based on the method optimization described herein, a three-protein suite was prepared in a synthetic urine matrix. The protein concentrations were 4.8 \(\mu\)M cytochrome, 4.2 \(\mu\)M lysozyme, and 3.5 \(\mu\)M myoglobin, representing matrix-to-protein mole ratios of \(>1,000,000:1\). The MALDI-MS spectra derived from the proteins within the synthetic urine matrix are totally non-descript, with an average signal background across the relevant mass range of \(~15\) counts, with a standard deviation of \(~4\) counts. As can be seen in Fig. 3.5, the post-SPE spectrum clearly reflects the respective protein signatures. Scale expansion of that spectrum reveals no appreciable changes in the background level or the spectral noise. A simple assessment of the attainable limits of detection using a \(3\sigma_{\text{background}}\) definition yields concentration values on the order of 30 nM, which translate to absolute masses of \(~0.5\) ng for the 1 \(\mu\)L deposited sample. Note that this value is in excellent agreement with the value for cyto c obtained in the 750 mM Tris-buffer presented with respect to Fig. 3.4.
Importantly the average variability observed for the responses for the three proteins, across 4 replicates was 24.3%, again very much in line with the Tris-buffer data. While there is room for improving this performance, it is very encouraging given the protein levels and general variability in the MALDI-MS experiment. Finally, the optimized procedure has resulted in a ~2x improvement in LOD over the previous work, principally by increasing the protein signal recoveries by almost 3x.
CONCLUSIONS

The results from these experiments reveal the use of PP C-CP fibers as a stationary phase for solid phase extraction of proteins from salt-laden matrices (1M Tris-HCl and a synthetic urine) for MALDI-MS analysis. The hydrophobic nature of the PP C-CP fibers allow for the adsorption of proteins while residual buffer components can be removed by a simple aqueous wash. Immobilized proteins can then be eluted in MALDI-friendly solvents, yielding protein mass spectra of high clarity. Optimization of the various processing parameters allows for a greater understanding on how the C-CP fiber packed tips function, especially trade-offs between the size of the fiber bed, solution volumes, and protein binding capacity. The ability to easily detect sub-nanogram levels of protein from 1 µL sample aliquots shows high promise for the utilization of C-CP fiber packed SPE tips for micro-bioanalytics. To be clear, this level of performance has been obtained on what is a departmental service instrument, and so is not particularly sensitive. It is believed that the C-CP fiber micropipette tip platform can be readily implemented as a processing step before other chromatographic separations to affect greater sensitivity and practical peak capacities. This work also complements work on the use of an in-line SPE approach coupled to ESI-MS. Future work will now be dedicated into modifying C-CP fiber tips for removal of specific proteins for specific applications and creating depletion columns for removal of unwanted proteins.
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CHAPTER FOUR

CAPILLARY-CHANNELED POLYMER (C-CP) FIBERS FOR THE RAPID EXTRACATION OF PROTEINS FROM URINE MATRICIES PRIOR TO DETECTION WITH MALDI-MS

INTRODUCTION

Clinical chemistry has shown a rapid increase over the past decade in terms of biofluids analysis with mass spectrometry (MS). Analysis of biofluids (i.e. plasma, urine, saliva, etc.) for specific proteins and other biomolecules is necessary for the investigation of bodily functions and the presence or absence of disease. Of the biofluids, urine specimens are considered the most easily obtained and most commonly used samples in clinical analysis\textsuperscript{1, 2}. Dating back to ancient screenings, glucose detection was determined by observing whether or not ants were attracted to urine\textsuperscript{1}. However, due to its low protein concentrations and matrix complexity, urine is considered a difficult biofluid to analyze. Typically, MS is employed to analyze urine for biomolecules due to its ability to determine individual components in complex mixtures with excellent sensitivity and specificity when compared to spectroscopic techniques (i.e. UV-Vis)\textsuperscript{3, 4}. MS analysis of biological compounds commonly employs one of two soft ionization methods: 1) electrospray ionization (ESI) and 2) matrix-assisted laser/desorption ionization (MALDI). In ESI, the sample is ionized in a
nebulization process in which multiply-charged ions are produced\textsuperscript{5-7}. While multiply-charged ions does decrease the mass analyzer range, having multiply charged species is detrimental to the analysis of complex mixtures as spectral overlap of different proteins' charged states complicates interpretation. To correct for the complication, separation techniques must be employed to isolate/detect the specific proteins. MALDI is an excellent ionization source for more complex samples as predominantly singly charged ions are generated via proton transfer from the added matrix, making MALDI-MS an excellent candidate for a rapid top-down approach for early detection of disease specific biomarkers\textsuperscript{2, 5, 8}.

Unfortunately, for both ionization sources, the analytes of interest (i.e. proteins) must be extracted from their native environments, as salts and small organic molecules can be detrimental to analysis, particularly in biofluid analysis\textsuperscript{9-12}. With MALDI-MS salts/organics are co-ionized with the target proteins upon laser bombardment and can result in signal suppression up to the point of being non-detectable depending on the matrix\textsuperscript{9}. Problems ensue therein when analyzing biofluids such as urine that are not only rich with salts/organics, while target proteins are of such low abundance, detection by MS is near impossible. In order to prevent this issue, sample preparation is utilized to remove the target species prior to analysis by MALDI-MS, as described by Zerefos and Vlahou\textsuperscript{13}. Methods or the combination of methods that are typically employed for the extraction of proteins from biofluids for MS detection include high performance
liquid chromatography (HPLC)\textsuperscript{4}, solid phase extraction (SPE)\textsuperscript{14-16}, ultracentrifugation\textsuperscript{17}, ultrafiltration\textsuperscript{2}, and gel electrophoresis\textsuperscript{18-21}. Protein concentrations in urine are very low in comparison to other biofluids (e.g. plasma), which places greater emphasis on sample preparation in order to prevent protein loss and denaturing\textsuperscript{22}.

For complex situations, gel electrophoresis (GE)\textsuperscript{23} is commonly employed as some studies have illustrated up to 1500 distinct protein spots in urine\textsuperscript{20}. While this technique is beneficial in complex separations, large analyte mass is required (<100 µg), methods are time consuming (up to 24 hours), and resolution is low making the analysis of fragmented bands difficult. Another limitation to employing GE is excision of the protein bands. While a common approach in regards to GE-MALDI-MS for biomarker analysis\textsuperscript{19}, excision processes are timely and can lead to sample loss as band-overlapping is common with complicated gels, hence, large quantities of analyte are required. A top-down approach utilizing an SPE extraction procedure prior to analysis with MS could offer a rapid, reproducible, and robust technique for biomarker profiling in comparison to two-dimensional GE. In terms of automation, reproducibility, high throughput, and rapid extraction procedures HPLC and SPE are excellent candidates. Zerefos has also demonstrated minimizing sample preparation procedures by implementing ultrafiltration devices in which masses below 5-10 kDa can be effectively removed from the urine specimen (1 mL) prior to analysis with MALDI-MS. While the results yielded an abundance of information for mass ranges up
to 20 kDa with high throughput, protein identification (>20 kDa) was compromised².

Current sample preparation trends have shifted towards miniaturization of techniques in which rapid extractions can be performed on small sample volumes¹², 24, 25. More specifically, significant research has been dedicated to µ-SPE techniques²⁶-²⁸. SPE works on a basic principle in which target species are adsorbed onto a stationary phase, similar to HPLC, while contaminants are washed away. Target analytes are then eluted in an MS-friendly solvent for subsequent analysis. SPE methods differ from HPLC methods with lower volumes necessary (<1 mL), faster extraction times, and decreased solvent waste. SPE methods also extend to biofluids analysis such as urine and plasma protein profiling¹⁰-¹², 16, 29. Both SPE and HPLC methods have been studied in terms employing unique stationary phases such as mesoporous silica beads¹⁰, magnetic beads²⁹, and even implementing magnetic nanoparticles³⁰ for extractions from biofluids. In order to further enhance miniaturization processes, SPE formats have been implemented onto micropipette tips to further reduce sample and solvent volumes and to increase throughput potential for automation¹⁴, 24. Much research has been dedicated to this implementation ranging from C₁₈ “Zip Tips” from Millipore³¹ to gold nano-particle stationary phases¹¹, ³² and even employing cotton packed tips for hydrophilic interaction liquid chromatography based extractions¹⁵. Presented here, micro-SPE tips
packed with capillary-channeled polymer (C-CP) fibers is extended towards clinical applications in a top-down approach towards biomarker identification.

Marcus and co-workers have been investigating capillary-channeled polymer (C-CP) fibers as stationary phases for biomolecule separations/extractions. C-CP fibers are advantageous in comparison to other stationary phases due to their chemical robustness, high mass transfer rates, and wide range of polymer chemistries (e.g. polypropylene (PP), polyester (PET), nylon-6). High chemical robustness allows for immunity to the irreversible binding typically found in silica-based stationary phases, thus increasing extraction recoveries. High mass transfer rates allow for high linear velocities (> 50 mm s\(^{-1}\)) that negate penalties typically found with van Deemter C-term broadening. For these reasons, C-CP fibers have been used, not only for HPLC protein separations, but also as in-line desalting columns for protein analysis by ESI-MS, and as micro-SPE tips for protein extractions prior to MALDI-MS. A C-CP fiber in-line desalting column proved to be an effective and rapid method for removing salts, buffers, and organics to improve the spectral clarity of ESI-MS, while reducing time and eliminating potential sample loss. C-CP fiber packed micro-columns (< 10 cm in length) attached to micropipette tips were able to demonstrate efficient extractions of nanogram quantities of protein from minute sample sizes (< 10 µL) prior to analysis by MALDI-MS. Studies revealed that sub-microliter sampling could be implemented, and for the extraction of small
mass quantities (<10 ng), shorter tips (2.5 mm) could advantageously employed to increase recoveries\textsuperscript{36}.

In this study, mock urine and human urine matrices were spiked with typical urinary proteins (β2-microglobulin, retinol binding protein, and transferrin) at biologically relevant concentrations. Method development was investigated in terms of aqueous washing volumes and elution solvents, as variance is commonly seen with different contaminants and proteins. Proteins possess various amino acid configurations and a “hydrophobic foot” in which the outer surface configuration will be more or less hydrophobic pending on the composition. Each slightly different configuration between proteins will require a potentially different organic solvent strength in the elution step. In terms of contaminant removal, a varied level of aqueous wash was employed as harsh matrices will require more wash in order to effectively remove all contaminants. After a systematic evaluation of the method, PP C-CP fiber packed micro-SPE tips proved to be an efficient method for extraction for micro-sale extractions from urine matrices. Prior to extraction, the analytes of interest were virtually undetectable in the urine matrix. It is believed the proposed methodology illustrates great promise in terms of micro-scale biofluids analysis for biomarker determinations. Applications are foreseen to extend from neonatal subjects to forensics analysis as high quantities of biofluids may not be available in those instances.
EXPERIMENTAL

Reagents and chemicals

Three test proteins β2-microglobulin (human urine), retinol binding protein (human urine), and transferrin (human) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and prepared (0.4-10 µM each) in mock urine and a certified, drug-free human urine control from UTAK laboratories (Valencia, CA, USA) and MilliQ water (18.2 MΩ•cm) derived from a NANOpure Diamond Barnstead/Thermolyne Water System (Dubuque, IA, USA) for comparison to ideal conditions. The elution solvent was prepared with MilliQ water, various percentages of ACS grade acetonitrile and 0.07% trifluoroacetic acid (TFA) both from EMD Millipore (Billerica, MA, USA). Sinapinic acid (Sigma-Aldrich), prepared as 20 mg mL⁻¹ in 50:50 ACN:H₂O with 0.1% TFA, was employed as the matrix required for MALDI-MS analysis.

C-CP Fiber Tip Construction

The construction of the C-CP fiber packed tip remains relatively unchanged from previous publications. C-CP fibers are packed with an interstitial fraction of $\epsilon_i = 0.6$ in 0.8 mm i.d. fluorinated ethylene polypropylene (FEP) tubing (Cole Parmer, Vernon Hills, IL, USA), mimicking the studies previously performed with regards to separation efficiency. C-CP fibers are
pulled collinearly through the FEP tubing such that the fiber legs interdigitate to form collinear channels. In this study, a total of 658 PP C-CP fibers (~55 µm in diameter) were packed in each tip. The tips were cut with a surgical-grade scalpel with one end being flush with the fibers and the other end leaving ~5 mm void space for interfacing with the 200 µL pipette tip from Neptune (San Diego, CA, USA).

**Adaptation for Centrifugation Fluidic Processing**

In order to increase reproducibility, sample throughput, and run parallel experiments, centrifugation was employed for fluid manipulation. To adapt to a centrifugation format, the C-CP fiber packed tips were configured so they could be inserted into 15 mL centrifuge tubes (VWR, West Chester, PA, USA). The micro-SPE tip was placed inside of a 1.7 mL microtube (Genesse Scientific, San Diego, CA, USA) that was cut leaving ~10 mm of the bottom removed. This apparatus was then placed in the 15 mL centrifuge as depicted in previous publications. The finished product was placed in a Symphony 4417 (VWR) centrifuge operating at 1900 x g relative centrifugal force (RCF).

*Extraction procedure employing C-CP micro-SPE tips*
The extraction procedure is illustrated in Fig. 4.1. After sample collection (step 1), protein spiked urine samples (10 µL) were loaded (step 2) onto the C-CP micro-SPE tips and centrifuged for 30 seconds at 1900 x g RCF. Following the loading step, an aqueous wash (step 3) was performed where in water (50-150 µL) was centrifuged through the tips for 60 seconds to remove contaminants (i.e. salts). To elute the proteins from the C-CP fiber surface (step 4), 10 µL of the elution solvent (ACN:H₂O with 0.07 % TFA) was centrifuged through the tips for 30 seconds. The eluent containing the purified proteins was then spotted onto the MALDI target plate with sinapinic acid. The total sample extraction time, including spotting matrix/eluent onto the target, is approximately 5 minutes for 12 extractions (only limited by centrifuge capacity).

Figure 4.1 Illustration of the procedure employing C-CP fiber packed µ-SPE tips for the extraction of urinary proteins from urine matrices prior to analysis by MALDI-MS
MALDI-MS

A Bruker Daltonics (Billerica, MA, USA) Microflex LRF, MALDI-TOF-MS was employed in positive ion, linear mode for the detection of extracted species. For instrumentation control and data processing, Bruker Compass software was employed. Further data analysis was performed with Microsoft Excel (Redmond, WA, USA). The MALDI-MS employed a nitrogen (337 nm) laser with a pulse rate of 60 Hz for the sample ionization. Sample spectra were acquired with 200 laser shots at 60% power. Sinapinic acid (20 mg mL\(^{-1}\)) in 50:50 ACN:H\(_2\)O with 0.1% TFA was utilized as the MALDI matrix. In terms of foreseeable automation, 1 µL of matrix was spotted onto the MALDI target, allowed to dry, and then a 1 µL aliquot of eluted sample was spotted on the matrix. Reported signal-to-noise ratios were described as the standard deviation of the background signal across a 100 Da window, centered 2000 Da below the molecular ion peaks of the target proteins.

RESULTS AND DISCUSSION

Urinary proteins have potential to serve as excellent biomarkers not only for kidney and urological diseases, but also many other small proteins that can freely filter through the glomerular barrier as widely described\(^1\),\(^{39}\). With the recent advancements in mass spectrometry (MS), urinary biomarkers can be
screened rapidly with excellent mass resolution for complex samples\textsuperscript{4}. Presented here, C-CP fibers are investigated as a stationary phase for micro-SPE tips for the extraction of urinary proteins from urine matrices. Urinary proteomics is rapidly growing in terms of clinical studies for potential biomarker analysis. Here, β2-microglobulin, retinol binding protein, and transferrin were investigated as the “target” analytes as they are all universally studied as potential biomarkers in urine. These target proteins were utilized due to their common presence in biofluids as well as being commonly cited for potential biomarkers\textsuperscript{19,40,41}. β2-microglobulin levels in cerebrospinal fluid (CSF) has been studied extensively for human African trypanosomiasis (HAT) biomarkers as increased levels detected after Western blot and ELISA test have demonstrated positive tests for HAT\textsuperscript{19}. Other employments of β2-microglobulin as a biomarker are currently being investigated for acute rejection for renal transplantation\textsuperscript{42}. Studies have suggest RBP as a potential biomarker as the increased excretion of RBP correlates with a weakened uptake of RBP which could indicate proximal tubular dysfunction\textsuperscript{40}. Recent studies have suggested RBP levels in plasma could be employed for the early detection of coronary heart disease\textsuperscript{41}. While the purpose of this paper does not necessarily pertain to the profiling of urine for biomarker determinations, the results illustrate the utilization of an innovative stationary phase for the extraction of target species from microliter-size aliquots of urine.
**Investigation of elution solvent**

Solid phase extraction techniques utilize an isocratic approach in terms of elution solvent such that a single concentration/solution is employed to simultaneously remove the analytes from the stationary phase surface. Various modes of chromatography (i.e. ion exchange, reversed phase) and stationary phases (i.e. polymer, C$_{18}$) have been employed for SPE of various compounds$^{27,43}$. Polymer phases have been recently investigated due to its chemical robustness and immunity to harsh elution solvents$^{43,44}$. Presented here, polypropylene polymer fibers are employed for reversed phase extractions of proteins. Proteins are composed of amino acids and pending the composition, they are more/less hydrophobic due to the presence hydrophobic amino acids (e.g. alanine, valine, isoleucine, etc.). In order to achieve efficient recovery, organic-based elution solvents are typically employed for the removal of adsorbed species. These solvents are also typically MS friendly, making interfacing/automation more accessible as solvent exchange is not necessary. SPE techniques utilize an isocratic approach in terms of elution solvent, such that a single concentration/solution is employed to simultaneously remove the analytes from the stationary phase surface. With elution solvent strength being dependent on the target species, methods must be performed on a case by case basis. The study was performed by monitoring MALDI-MS signal intensity of β2-microglobulin and retinol binding protein in both mock and certified human urine
matrices as the amount of ACN in the elution solvent was increased (Fig. 4.2). While transferrin was also in the protein mix, only β2-microglobulin and retinol binding protein were plotted as they had the response for all conditions (50-75% ACN in water).

![Graph](image)

**Figure 4.2** Investigation of elution solvent in regards to protein recovery by monitoring signal

Present here, 10 μL of the protein suite (in synthetic and natural urine) was loaded onto the C-CP fiber packed μ-SPE. A single aqueous wash (100 μL) was next performed to remove the unwanted species (e.g. salt). Finally, the proteins were eluted in a solvent composing of a given percentage of ACN (50-75%) in water with 0.07% TFA. While indeed, other elution solvents should be explored when pertaining to extraction procedures, this manuscript is only meant
to serve as a proof-of-concept so an elution solvent that has typically been employed in other C-CP fiber reversed phase separations/extractions\textsuperscript{14, 36}. The highest recoveries were found when employing an elution solvent of 55:45% ACN:H$_2$O with 0.07% TFA, and higher percentages were not effective in removing the adsorbed protein. This result varied slightly from previous reports utilizing PP C-CP tips (60:40% ACN:H$_2$O), as different proteins were utilized\textsuperscript{36}. As a reference, identical concentrations of the three proteins prepared in water (as a “clean” sample) were also studied to monitor analyte recoveries. Proteins recovered from water and mock urine gave nearly identical spectra. However, proteins recovered from natural urine gave slightly lower intensities and larger background signal (5x), implying the extraction procedure should be optimized in terms of buffer removal with increased contaminants such as a more vigorous wash step. The following section will discuss efficient buffer removal from complicated matrices such as natural urine.

\textit{Investigation of wash volume for contaminant removal}

It has been illustrated that compounds found in buffers have a weak affinity for the C-CP fiber surface. In low concentrations, buffers can be readily removed with minute aqueous wash steps (5 µL). However, highly contaminated species, up to 1M salts/buffer or biofluids, require a more vigorous washing step. Seen in Fig. 4.2, the analyte response for the extracted protein is approximately
1.3x greater for the extraction from mock urine compared to natural urine for β2-microglobulin and 1.5x greater for retinol binding protein, implying that the wash step could be different for the two matrices as the amount of contaminants are in different concentration.

**Figure 4.3** Investigation of volume of aqueous wash step for optimal removal of urine matrices

Figure 4.3 demonstrates how protein clean-up/extraction is affected by varying the aqueous wash volume. In these studies, 10 µL of the protein suit in mock and human urine was loaded onto the PP C-CP fiber packed micro-SPE tips. Various aqueous wash volumes were employed with a constant elution solvent (10 µL) optimized in the first section (550:45 ACN:H₂O with 0.07% TFA). Similar to previous studies, matrix removal is most efficient when the aqueous
wash is 10x the sample load volume for highly contaminated (1 M) solutions. It is known that target proteins are virtually non-detectable by MALDI-MS in the presence of 100 mM salts and organics, even more so for biofluids which are termed as 1M contaminants. Not surprising, the mock urine can be efficiently eluted at lower wash volumes (50-75 µL) in comparison to the volume required for the complicated matrix of human urine (100 µL). Pertaining to the lower wash volumes, it can be seen that the aqueous wash does not effectively remove the matrix interferences as analyte signal from MALDI-MS suffers from ion suppression. Without proper extractions/sample clean-up, analysis is virtually impossible with regards to mass spectrometry as there is a direct relationship between contamination/matrix interferences to analyte signal. Proteins in less contaminated solutions (i.e. buffers and mock urine) require smaller volumes of wash step compared to proteins in highly concentration harsh matrices including biofluids which are considered to be of 1M concentrations in theory. Based on the results from Fig. 4.3, 100 µL of water was employed to remove the urine matrix in the following experiments.

Analysis of a three protein suite in human urine before/after SPE

Figure 4.4 presents spectra taken with and without an optimized extraction procedure from human urine in which 10 µL of the three protein suite is loaded onto a PP C-CP micro-SPE tip, followed by a 100 µL aqueous wash, then elution
with 10 µL of 55:45 ACN:H₂O with 0.07% TFA. Figures 4.4a and b illustrate spectra acquired without employing SPE. It is vividly clear which matrix is inducing the harshest effects on ionization. Seen in fig. 4.4c, β2-microglobulin (11716 Da) from the protein mixture can be seen in the mock urine mixture to some extent while retinol binding protein (20581 Da) has hardly detectable signal and transferrin (81791 Da) is undetectable. None of the proteins in the mixture are detectable in the complicated human urine matrix. Also, in comparison of background signal, the mock urine has a background signal deviation of ~7 Da versus to the human urine deviation of ~40 Da without any sample clean up, as expected due to the induced matrix interferences on the ionization process of MALDI cause by the urine contaminants. After SPE of the human urine protein spiked solution, the background signal levels decreased by ~50%, illustrating the removal of contaminating species as seen in Fig. 4.4d. The three proteins that were spiked in the human urine matrix, β2-microglobulin (11716 Da), retinol binding protein (20581 Da), and transferrin (81791 Da) are all detectable and can be seen with high spectral clarity. Additional to the pseudo molecular ion, M+H⁺, the doubly-charged species is also detectable for transferrin (M+2H⁺ = 40078 Da) and retinol binding protein (M+2H⁺ = 10253 Da). The lower response for retinol binding protein could be explained by lack of purity, while transferrin is close to the instrumentation mass limit of 100,000 Da hindering detection. Also, the objective of this manuscript is not necessarily focused on high spectral clarity/response as experiments were evaluated on a departmental MALDI-MS
instrument; therefore, sensitivity is not optimal for detection of such biomarkers.
Figure 4.4 MALDI-MS spectra of urinary proteins in a) human urine matrix and b) mock urine matrix prior to extraction with C-CP fiber packed µ-SPE tips and c) after extraction. d) is a zoomed in spectra of the background before and after extraction.
With the optimized procedure, detection limits were assessed to determine if the PP C-CP fiber packed micro-SPE tips would be successful for extraction of low mass quantities. The above experiments were performed on “stock” solutions containing 7.1, 7.9, and 10.4 µM of each protein (β2-microglobulin, retinol binding protein, and transferrin) in either mock or human urine for method optimization. Different from commercially available SPE micropipette tips, C-CP fiber packed micropipette tips have illustrated efficient extractions of low volumes (10µL) proteins, correlating to 0.83, 1.66, and 8.30 µg of protein each for these tests. The method was then carried out for lower protein concentrations/mass quantities ranging down to 1.14, 1.26, and 1.66 µM for each protein respectively correlating to 0.13, 0.24, and 1.33 µg total mass. In terms of detection limits, transferrin (~80 kDa) signal became undetectable when loading ~1.6 µg, primarily due to the instrumentation mass limitation to 100 kDa. Detection limits were determined by the “SBR-RSDB” method\textsuperscript{46} for each protein (β2-microglobulin, retinol binding protein, and transferrin) was calculated to be 5.3, 30.3, and 184 nM, respectively, and seen in figure 4.5. More importantly, these concentrations were for 1 µL deposits on the MALDI-MS target. When discussing total mass analyzed, the LODs were 58.3 and 600 picograms for β2-microglobulin and retinol binding protein and 14.7 ng for transferrin. Additionally, as described earlier, the mass spectra are taken from the deposit of 1 µL onto
the MALDI target. Therefore, the spectra is only accounting for $1/10^{th}$ fraction of the total mass that is eluted, and that is assuming 100% recovery. So MALDI-MS analysis is actually only being performed on sub-nanogram levels. Again, the analysis performed on the C-CP fiber $\mu$-SPE tips extractions were performed on a departmental bottom tier MALDI-MS.

![Figure 4.5 Spectra of protein suite at the lowest concentration with calculated limits of detection](image)

**CONCLUSIONS**

PP C-CP fibers have illustrated promising results as a stationary phase for micro-SPE micropipette tips. Studies illustrated efficient extraction of a urinary 3-protein suite in which extractions were carried out from mock urine and a certified
human urine solution. The optimal elution solvent was determined to be 55:45% ACN:H$_2$O with 0.07% TFA and employing a 100 µL aqueous wash to remove contaminants that typically induce co-ionization with the MALDI ionization process, thus preventing direct analysis of biofluids. While actual instrumentation (MALDI-MS) could be improved in terms of sensitivity, the illustrated results are meant to serve as a fundamental study illustrating the potential to employ PP C-CP fibers to extract analyte from biofluids. The presented work demonstrated effectiveness of extracting low mass quantities of protein (ng levels) in small volumes (10 µL), from highly contaminated species. This work was also centered on the ability for automation, to reduce sample preparation time. Future studies are intended to focus on depletion columns to remove abundant proteins (albumin) and fiber modification for the specific capture of target species (affinity chromatography).

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CHAPTER 5

LIQUID SAMPLING-ATMOSPHERIC PRESSURE GLOW DISCHARGE (LS-APGD) AS A SECONDARY EXCITATION SOURCE: ASSESSMENT OF PLASMA CHARACTERISTICS

INTRODUCTION

As the trend of miniaturization in analytical chemistry instrumentation has progressed, the development of new atmospheric pressure plasma sources has seen increased interest. Currently, there is a need to design spectrochemical instruments with lower power consumption, reduced sample sizes, compact footprint, low operating costs, and the ability to be operated under ambient conditions\textsuperscript{1-3}. Reductions in sampling size have been addressed over the past two decades by laser-ablation (LA), allowing for chemical analysis of solids without sample preparation\textsuperscript{4-6}. LA utilizes a short-pulsed, high-power laser beam to remove material for either a direct analysis of the plasma volume by optical emission spectroscopy (OES) or to be transported into a secondary excitation/ionization source such as the inductively coupled plasma (ICP)\textsuperscript{7, 8}. The former, termed laser-induced breakdown spectroscopy (LIBS), is easily miniaturized for portability with applications expanding to exploration on the surface of Mars\textsuperscript{9, 10}. By utilizing a secondary source, higher power densities and longer residence times can be utilized for enhanced sensitivity and lower limits of
detection (LODs) when compared to LIBS$^6$, $^{11}$. To this end, atto-/femtogram detections limits are readily achieved in ICP-MS$^{12}$.

Unfortunately, the conventional ICP is not ideal as a secondary source as minute amounts of ablated mass are introduced into the relatively large plasma volume ($\sim 125 \text{ mm}^3$), not to mention the high operating cost and large footprint of the base ICP-OES/MS instrument$^6$. Few efforts have addressed miniaturizing the ICP or reducing the operational overhead of LA-ICP instrumentation. Improving designs by incorporating alternative plasmas as the secondary excitation/ionization source of particulates should be explored, especially regarding the miniaturization of the spectrochemical instrumentation. In recent years, a few secondary sources have been proposed for the detection of laser ablated particles such as microwave induced plasmas (MIP) and the flowing atmospheric pressure afterglow (FAPA)$^4$, $^{13}$.

Marcus and co-workers developed a liquid sampling-atmospheric pressure glow discharge (LS-APGD), which is sustained between an electrolytic liquid ($\text{HNO}_3$) flowing from a glass capillary and a metallic counter electrode placed $\sim 2$ mm away$^{14-16}$. The operational space of this excitation/ionization source fits well in terms of the miniaturization characteristics mentioned above. While early LS-APGD works focused on OES monitoring, recent efforts have demonstrated the ability of the source to effectively ionize solution-introduced species by mass spectrometry (MS)$^{17, 18}$. The scope of application has been expanded through the introduction of LA-generated particles$^{19, 20}$. This combination, LA-LS-APGD,
has been shown to be qualitatively similar to LA-ICP, with efficient vaporization/excitation/ionization of particles produced from nanosecond and femtosecond pulsed lasers. Particularly relevant for LA sampling, the LS-APGD operates at much higher power densities (~10 W mm\(^{-3}\) versus 0.1 W mm\(^{-3}\)) and a much smaller plasma volume (~1 mm\(^{3}\) versus ~125 mm\(^{3}\)) than the ICP (i.e., less dilution). Finally, simple gas dynamics calculations suggest plasma transit times of 5-30 ms vs. ~1 ms for the ICP, though these must be verified experimentally.

When assessing plasma performance, fundamental properties must be characterized to understand excitation conditions, which may vary with operation parameters or the introduction of sample material\(^{21, 22}\). Optical emission spectroscopy (OES) is a versatile means of characterization of atmospheric pressure flames and plasmas, typically by the fitting of molecular bands (e.g., N\(_2\), OH) to obtain rotational temperatures (T\(_{\text{rot}}\)), determinations of excitation temperatures (T\(_{\text{exc}}\)) by monitoring excited states of various species such as plasma gases or sample species, and measurement of the broadening of emission lines (H, He) to yield plasma electron number densities (n\(_e\))\(^{23-25}\). Finally, the ability of an spectrochemical source to be immune to sample-induced perturbations can be assessed through changes in the “robustness” factor determined by the Mg II/Mg I emission intensity ratios\(^{26, 27}\).

The research presented here utilizes OES to evaluate the properties of the LS-APGD to gain fundamental knowledge regarding the source as a means for secondary excitation/ionization of LA-introduced particles. T\(_{\text{rot}}\), T\(_{\text{exc}}\), and n\(_e\) were
evaluated across a matrix of plasma operation conditions generated through a central composite, design of experiments (DOE) approach, studying the roles of electrode separation distance, discharge current, and carrier gas (He) flow rate. Molecular bands, OH and the N$_2$ second positive system$^{28}$, were utilized for rotational/vibrational temperatures while the emission of He and Mg lines was utilized for the determination of excitation temperatures and plasma robustness. In addition to baseline values for these quantities determined for aqueous HNO$_3$ blanks, the plasma was stressed by introduction of large amounts of magnesium, to determine potential deleterious effects due to sample matrix. In one case, Mg$^{2+}$ was added in the nitric acid feed at a concentration of 1000 µg mL$^{-1}$, and in the other case metal shards were compacted in a paraffin matrix and introduced via laser ablation. Such studies set the stage for applications in the analysis of matrix-laden solutions and LA sampling.

**EXPERIMENTAL**

*LS-APGD Source*

The LS-APGD source is relatively unchanged from previous publications, incorporating the flow of laser-ablated particles into the microplasma through the *hollow* counter electrode$^{19, 20}$. The microplasma is sustained between an electrolytic solution (5% HNO$_3$) and the counter electrode (nickel, 0.3 cm o.d., 0.1 cm i.d.) through which the He carrier gas flows, transporting ablated particles
from the ablation cell into the plasma volume. A syringe pump (New Era Pump Systems Inc., model NE-1000 Multi-Phaser, Farmingdale, NY) was used to deliver the electrolytic solution through a fused silica capillary (360 µm o.d., 100 µm i.d. Idex Health and Science, Oak Harbor, WA) which is housed within a metal capillary (nickel, 0.16 cm o.d., 0.06 cm i.d.). Helium was employed as a sheath gas (0.2 L min\(^{-1}\)) flowing between the capillaries, as optimized in a previous publication\(^{19}\). Power for the microplasma was delivered by a Glassman High Voltage Inc. power supply (0-100 mA, 0-2 kV, High Bridge, NJ) operating positive polarity with a 10 kΩ, 225 W ballast resistor (Ohmite, Arlington Heights, IL) placed in-line with the powered solution electrode (the counter electrode was held at ground potential). It is important to point out that the entirety of the LS-APGD plasma components, power supply, gas metering, and the ablating laser are mounted on a single 30.5 x 30.5 cm\(^2\) optical bread board. Potential plasma perturbations were assessed by introducing 1000 µg mL\(^{-1}\) magnesium (CPI International, Santa Rosa, CA) in the electrolytic feed solution and by the ablation of a 2% Mg pellet (2% in paraffin) that was made in-house (LBNL).

*Laser ablation apparatus*

For the introduction of laser ablated particles a commercial laser ablation system (J100 Applied Spectra, Inc, Freemont, CA, USA) consisting of a nanosecond laser (Nd:YAG) with a 5-ns pulse duration was used and operated at
its fundamental wavelength of 1064 nm, and variable energy (max 50mJ) and repetition rate (1-10Hz). Laser ablation was performed in a helium atmosphere. The J-100 ablation system is equipped with an ablation chamber that could accommodate samples up to 100 mm diameter with flexibility in volume and wash-out time.

**Optical emission measurements**

Broad wavelength range measurements were performed using an optical fiber-based spectrometer (Aurora, Applied Spectra, Fremont, CA). This spectrometer consists of six channels, each composed of a 2048 pixel CCD detector dedicated to different spectra regions. A fused silica biconvex lens (35 mm focal length, 25.4 mm diameter) was used to focus the entire microplasma image onto the input optic of the fiber bundle connected to the spectrometer. While there are known inhomogeneities in the LS-APGD, this approach is the most pragmatic way of sampling the ~1 mm³ plasma volume, analogous to the case with LIBS analyses. A composite (simultaneous) spectrum is acquired using a 1.05 ms gate over each of the 500 laser shots, with spectral resolution of 0.05–0.12 nm across the 190-1040 nm wavelength range. For the calculation of $n_e$, Stark broadening of the H (I) 656.3 nm and He (I) 587.6 nm lines was measured employing a 1.25 m focal length, 2400 gr mm⁻¹ grating, Czerny-Turner spectrometer, (Horiba-JY, Model 1250M, Longjumeau, France), with an
intensified charged-coupled device (ICCD) detector (Princeton Instruments, PI MAX 1024 Gen II, Trenton, NJ 08619 USA). This detection system yields a spectral window of ~13 nm with 0.04 nm resolution. The same optical coupling was used in this case as the analytical (array) spectrometer system. Experimentally-determined line widths were processed under Lorenz fitting to isolate Stark effects from Doppler broadening contributions. Corrections were employed by subtracting the FWHM from the corresponding Hg lines to correct for instrument broadening. The ICCD acquisition was set at a 1 µs delay, a gate width of 150 µs, and a gain set at 200 out of a maximum setting of 256. Due to experimental constraints, laser ablation sample introduction could not be performed in conjunction with the high-resolution optical measurements.

**Experimental Design**

The various plasma species were optically monitored during the course of varying the electrode gap, He carrier gas flow rate, and applied current of the LS-APGD, with the specific parameters listed in Table 1. A central composite DOE, with three experimental factors, was used to study the effects of the operating parameters on the plasma properties. The experimental design and the surface responses were generated using Statgraphics (Warrenton, VA). The central composite design generates a random sequence of experiments and parameter combinations across a range of parameter values centered among typical
operating conditions. A total of n=16 parameter combinations was used throughout this work; chosen by the algorithm. In each set of plasma characteristic determinations, estimated response surfaces are generated to illustrate cooperative effects of two parameters, keeping the third constant. The determined values (i.e. plasma temperatures, etc.) were processed using a multiple linear regression approach to determine the significance of parameter effects. The analysis is presented in the form of standard Pareto plots \(^{32}\), indicating the magnitude of each parametric dependency (including cross dependencies) and their statistical significance relative to the overall experimental variability. A statistical significance value of 2.57 was used here (as designated by the vertical line on each plot), based on the 5 degrees of experimental freedom and a 95% confidence level. Throughout this work, the electrode gap is indicated as parameter A, the He carrier gas flow rate B, and the discharge current C, with the cross-dependencies indicated as the products of those letters.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range</th>
<th>Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current (mA)</td>
<td>45 - 65</td>
<td>5</td>
</tr>
<tr>
<td>Carrier Gas (He) Flow Rate (L min⁻¹)</td>
<td>0.15 - 0.75</td>
<td>0.2</td>
</tr>
<tr>
<td>Electrode Gap (mm)</td>
<td>0.5 - 3.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Solution Flow Rate (µL min⁻¹)</td>
<td>300</td>
<td>Constant</td>
</tr>
<tr>
<td>Sheath Gas (He) Flow Rate (L min⁻¹)</td>
<td>0.2</td>
<td>Constant</td>
</tr>
</tbody>
</table>

Table 5.1 LS-APGD microplasma operation parameters employed throughout this work

**RESULTS AND DISCUSSION**

*Spectroscopic characteristics and identification of species in the LS-APGD*

Optical emission spectroscopy (OES) is an excellent tool for performing plasma diagnostics. In the case of the LS-APGD, the various probe species enter the plasma from the electrolytic solution, the sheath/carrier gas (He), and as ambient species. The spectral breadth of the spectrometer (190-1040 nm) allows for comprehensive sampling in a simultaneous fashion. Figure 5.1 shows a broadband spectrum collected from LS-APGD with the simple introduction of the plasma-sustaining electrolytic HNO₃ solution. Molecular bands such as OH and N₂ are present and are used to provide rotational temperatures (T_rot). OH molecular emission (A^2Σ⁺→X^2Π) can be seen from 306 nm extending to ~316
nm where an interference exists from N\textsubscript{2} band structure. The N\textsubscript{2} second positive molecular system (c\textsuperscript{3}\Pi\textsubscript{u}→B\textsuperscript{3}\Pi\textsubscript{g}) is seen as prominent bands across the range of 316 nm to 405 nm. There also is strong emission from atomic species including H I, He I, O I, and N I. The hydrogen Balmer lines (H\textalpha (656.3 nm) and H\textbeta (486.1 nm)) and He I lines at 587.6 and 706.5 nm are routinely employed for the determination of electron number density (n\textsubscript{e}) through Stark broadening. Beyond these features, the spectra of analytes introduced into the plasma are dominated by atomic transitions, more in line with those of flame spectroscopy\textsuperscript{23} rather than the ionic transitions common to ICP sources\textsuperscript{33}. This spectral simplicity improves the potential for development of portable instrumentation using compact, array-based spectrometers.

Figure 5.1 Broadband spectra (190-1010 nm) collected from Aurora Spectrometer illustrating the presence of molecular and atomic species.
Plasma rotational temperatures were investigated in order to understand how each parameter affected the kinetic energy input. Rotational temperatures are important when characterizing plasmas due to the assumption that $T_{rot}$ is the same magnitude as gas-kinetic temperatures, implied from lower energies involved in the excitation process and the rapid interchange between the rotational and kinetic energy of a molecule\textsuperscript{34, 35}. The fitting of experimental emission spectra for the determination of rotational and vibrational temperatures is frequently employed\textsuperscript{36}, as demonstrated for LIBS analysis\textsuperscript{10, 37}. The calculations, simulation information, and band fitting parameters utilized in these studies are expressed thoroughly with a detailed design in those publications\textsuperscript{10, 37, 38}. The spectra were fit to the $\Delta v = -1$ and $\Delta v = -2$ bands for N$_2$ as seen in Fig. 5.2a for each experimental condition (n=16). The average $T_{rot}$ across all conditions was determined to be 831 for the fitting of the $\Delta v = -1$, and 742 K for each condition for the $\Delta v = -2$ bands; ranging from 730-1100 K over the totality of parameters. Importantly, the values obtained at any one set of conditions is reproducible to better than 1% RSD.
Figure 5.2 Experimental fitting of the (a) N$_2$ second positive system (c$^3\Pi_u \rightarrow B^3\Pi_g$) and (b) OH A$_2\Sigma^+ (v=0) \rightarrow X_2\Pi (v=0)$ transition region
Figure 5.3a depicts a typical N$_2$-based T$_{rot}$ response surface generated through the central composite DOE parameters, changing the He carrier gas flow rate and the discharge current, and holding the electrode spacing constant. Statistical analysis of the interrelated (standardized) effects of the three control parameters are displayed in the Pareto plot (Fig. 5.3b). The standardized effects, seen in Fig. 5.3b, reflect the fact that carrier gas flow rate (B) has the largest effect on rotational temperatures, that being a negative effect. In literal terms, increases in the He flow rate lower the kinetic temperature within the plasma region. This is not surprising as He has a very high thermal conductivity, effectively carrying kinetic energy from the plasma. As indicated by the vertical line in the plot, the other interactions have no statistical significance, though the magnitudes (positive and negative) provide insights into plasma processes.
In order to test for any perturbations in plasma energetics by sample loading, \( T_{\text{rot}} \) values were re-determined with the addition of the 1000 \( \mu \text{g mL}^{-1} \) Mg test solution. In this case, changes in \( T_{\text{rot}} \) are expected to reflect energetic loads due to the initial solution vaporization and droplet desolvation conditions. Introduction of Mg in the solution feed increased \( T_{\text{rot}} \) values by <100 K on average, which is within the experimental error. In the case of the LA-introduced Mg particles, one might expect a thermal load on the plasma for the vaporization of particles is more energy costly than creation of free atoms from solution. Here
again, the average rotational temperature was not statistically different from the neat electrolyte solution. Importantly, the parametric response trends for the $T_{rot}$ across the n=16 combinations with sample introduction are essentially the same as projected in Figs. 5.3a and b.

The second spectrochemical probe for assessing $T_{rot}$ is the OH band system. The high-resolution spectrometer was employed here due to the interference from the $N_2$ band ($\Delta v=1$) seen in Fig. 5.1. A typical emission spectrum and corresponding fitting for the OH band is shown in Fig. 5.2b. The quality of the fittings are similar to those for $N_2$ above $^{10, 37}$ using calculations $^{39}$ and constants $^{40}$ from the literature. The average $T_{rot}$ across the n=16 conditions was calculated to be 1311 K, with <1 %RSD variability at any given set of conditions. While the temperatures derived for the two spectrochemical probes differ by ~500 K, the use of different spectrometers and detectors, and different levels of accuracy in the fitting and spectroscopic constants suggest that the values are in reasonable agreement.

Greater insight is revealed by evaluating the variation of OH $T_{rot}$ as a function of the discharge parameters. While the response surface in Supplementary Data Fig. S.1 is qualitatively similar to that of Fig. 5.3a, quantitatively it is seen that the OH-derived temperatures differ by a factor-of-2, while for the $N_2$-derived values differ by only ~40% as He flow rate is changed. This greater level of influence on $T_{rot}$ is easily seen in the corresponding Pareto plot, as the standardized effect for He flow (factor B) has a value of ~23 negative
in this case. Also seen in the OH-derived $T_{\text{rot}}$ trends, discharge current now has a significant positive effect with a quadratic positive effect also seen for gas flow rate (BB). The final significant effect is a negative quadratic effect of electrode spacing (CC). In general, the OH-derive $T_{\text{rot}}$ values show greater parametric sensitivities than the $N_2$ values, but the directionality (positive or negative) is the same for each set of discharge parameters for both probe systems.

Rotational temperatures were accessed for the OH band emission relative the introduction of the Mg test solution and LA particles. Here it was also seen that the differences in the $T_{\text{rot}}$ values (and their experimental variability) showed no statistical difference from the case of the neat electrolyte solution. This overall lack of perturbation of the gas kinetic temperatures implies that the power density within the microplasma (>10 W mm$^{-3}$) is sufficient in affecting the vaporization of solutions at a flow rate of 300 $\mu$L min$^{-1}$ and the digestion of ns-LA particles. The extended residence times within the microplasma surely work to this end as well. The $T_{\text{rot}}$ values reported here are typical of propane-air combustion flames$^{25}$ and low power atmospheric pressure plasmas$^{41, 42}$. They are lower as well in comparison to the SC-GD described by Hieftje and co-workers$^{43}$ and the miniaturized and dc-$\mu$APGDs of Jamroz et al.$^{44, 45}$.

_Determination of excitation temperatures by He and Mg atomic lines_
The excitation temperature ($T_{\text{exc}}$) is one of the central parameters for plasma characterization, describing the relative populations of atomic species in the ground and excited states under the assumption of a Boltzmann distribution. Many factors are incorporated into this type of distribution including statistical weights of specific levels, population density of the atom in the ground state, the atom’s statistical weight, and excitation potential of the level$^{24, 25, 46}$. As such, elements of very different atomic structure are prone to yield incongruent $T_{\text{exc}}$ values, particularly in the absence of local thermodynamic equilibrium (LTE). To address this specific point, He I and Mg I transitions were used in this evaluation.

This work takes a similar approach used by Quintero to calculate $T_{\text{exc}}$ from a high pressure microwave-induced plasma (MIP)$^{47}$, employing Boltzmann plots of four He I lines, using transition constants provided therein. While greater numbers of transitions is generally preferable, the correlation coefficients ($R^2$) for all of the derived Boltzmann plots (16 parameter combinations, each in triplicate) were all >0.900. The average $T_{\text{exc}}$ values (for all parameters) calculated from the He I lines, was determined to be 2672 K, with temperatures ranging from ~2100 - 2900 K across the 16 different conditions. This ~3x increase between $T_{\text{rot}}$ and $T_{\text{exc}}$ is fairly common across the spectrum of spectrochemical devices, as the energy transfer between plasma electrons and atoms is more efficient than the heavy body collisions that distribute thermal energy. The response surface of Fig. 5.4a reflects the roles of He flow rate and discharge current on $T_{\text{exc}}$ for a constant electrode gap value. The Pareto plot presented in Fig. 5.4b suggests that the He
flow rate has a small positive correlation. While current and electrode distance individually have little effect, the combination of increasing the two (AC) does increase \( T_{\text{exc}} \) values to a small extent. This is an interesting relationship as increasing the electrode gap decreases the microplasma power density, while increases in current have the opposite effect. This cooperative effect will be discussed in a latter section pertaining to electron number density. Overall, the lack of significant effects suggests that the kinetic energy (temperature) of electrons in the plasma remains fairly constant across the parameters.

![Figure 5.4](image)

**Figure 5.4** a) Response surface for excitation temperatures determined by Boltzmann distribution of the He (I) lines as a function of He flow rate and discharge current and b) Pareto plot illustrating parametric significance across the test parameter matrix

For comparison purposes, a set of four Mg I lines (from solution introduction) was used to calculate \( T_{\text{exc}} \), yielding an average value of 2643 K.
Figure 5.5 illustrates the derived $T_{\text{exc}}$ values for the 16 different plasma conditions calculated from both He I and Mg I lines. The He I transitions originate at excitation energies of $\sim 23$ eV, while those of Mg I involve relatively low energies of $4.3 - 5.1$ eV. Under the simple assumption that conditions of LTE do not exist, very different values would have been expected$^{24, 25}$. The average values for these two probes are statistically the same, suggesting that the excitation environment/processes are very similar for these very different excited states. Introduction of Mg particulates via LA yielded an average $T_{\text{exc}}$ value He I of 2564 K across the plasma parameter set, indicating that the particle load has no appreciable effect on the microplasma excitation conditions. Thus, a high level of robustness exists as heavy matrix solutions or LA particles of Mg are digested without perturbation of plasma excitation conditions.

![Figure 5.5 Illustration of the two methods of calculating atomic excitation temperatures (He, Mg) and how they were related in regards to condition number](image-url)
Determination of electron number density by He and H line broadening

Previous works by Venzie and Marcus investigated potential matrix effects in the LS-APGD-OES source upon addition of EIEs\textsuperscript{15}. The lack of any perturbations was attributed to a very high electron number density as milliamps of dc-current pass through the microplasma. The electron number density ($n_e$) is commonly determined through the Stark broadening of spectral lines emitted by argon (Ar), helium (He), and the hydrogen Balmer series ($H_\alpha$ and more commonly $H_\beta$)\textsuperscript{21, 22, 35, 46}. In the present work, two probes were employed to identify/circumvent potential biases. Firstly, the Balmer alpha line ($H_\alpha$) was utilized for the calculation; however, instead of FWHM, the full width half area (FWHA) method was employed. Gigosos determined that when using FWHA, excitation temperatures play little role in effecting the number density and derived the equation for calculating $n_e$ from the FWHA of the $H_\alpha$ line\textsuperscript{48}.

\[
H_\alpha: \text{FWHA} = 0.549 \text{ nm} \times (\frac{n_e}{10^{23} \text{ m}^3})^{0.67965} \]  

[5.1]

Based on this methodology, the average $n_e$ was calculated to be $2.8 \times 10^{15} \text{ cm}^{-3}$ across the 16 plasma parameters. As seen in Fig. 5.6, the particular plasma parameters do not substantially change the derived number densities (with a range of 14%, relative) for the HNO\textsubscript{3} blank. Introduction of the 1000 µg
mL⁻¹ Mg²⁺ solution into the plasma has little effect on \( n_e \), with the responses of the two solution types paralleling each other throughout the parametric matrix.

![Figure 5.6](image)

**Figure 5.6** Illustration of how electron number density was related to condition number with (Red) and without (Black) the introduction of Mg calculated from the H\(_\alpha\) line (a) and the He 587 nm line (b).

The second method for calculating \( n_e \) incorporated the He I 587.3 nm line, utilizing a different mathematical approach to limit potential bias. For this case, the FWHM was used for the Stark-broadened He line, with the electron impact parameter (W) required to complete this determination. The electron impact parameter is a coefficient that is related to specific Stark broadened lines and specific excitation temperatures\(^{49}\). While it is temperature dependent, temperature plays
an insignificant role in the determination and only loosely effects the calculation. Other parameters that play a role in obtaining Eq. 5.2 are the ion broadening parameter and population of particles in the Debye sphere.

\[ \Delta \lambda \frac{1}{2} = 2W \times \frac{n_e}{10^{16}} \quad [5.2] \]

Based on the measured He I FWHM data, the average \( n_e \) was calculated to be \( 9.7 \times 10^{15} \) cm\(^{-3} \) across the parameter space. While the magnitude of the number densities are on the same scale (\( \sim 10^{15} \)), the He I-derived number density is \( \sim 3 \) times higher, which is likely due to the origins and concentrations of the two species in the microplasma environment. This increase could be due to pressure broadening of the He I line as the concentration of He species in and around the plasma is very high due to the flowing of He gas through both electrodes (0.2 L min\(^{-1} \) and \( \sim 0.5 \) L min\(^{-1} \)), and is reflected in Fig. 5.1 as He I intensities exceed those of H I. Self-absorption by He species in the cooler periphery regions of the plasma would contribute to line broadening. Spatially-resolved measurements, likely with Abel inversion, will be needed to further evaluate this situation. Figure 5.6 illustrates the same trends throughout the parametric evaluation with the He I-based \( n_e \) values, with and without the introduction of the Mg solution. In fact, the parametric dependencies seen in both means of calculating \( n_e \) parallel each other in the parameter space, serving to validate the two experimental approaches.
The spread of $n_e$ values in Fig. 5.6 is not appreciable, though the response surface generated as a function of flow rate and discharge current, does show a slight $n_e$ dependence on the carrier gas flow rate, as shown in Fig. 5.7a. The slight, but statistically significant, effect of carrier gas flow rates is borne out in the Pareto plot of Fig. 5.7b. Very interestingly, no other parameters, including the discharge current, have appreciable influences. The fact that $n_e$ does not increase, even though the current is increased by a factor of 50% across the experiments, reflects the situation where the discharge volume is increasing; thus keeping the density relatively constant. By the same token, it is likely true that the counter flow of the He carrier gas may compress the plasma, thus increasing the density. This phenomenon would also explain the reasoning for the strong positive relationship between $T_{exc}$ and gas flow rate.
Figure 5.7  a) Response surface for electron number densities determined by Stark broadening of the H\textsubscript{α} line of the Balmer series as a function of He flow rate and discharge current and b) Pareto plot illustrating parametric significance across the test parameter matrix

Monitoring plasma robustness characteristics through Mg II:Mg I ratios

The Mg II:Mg I ratio is widely used to benchmark spectrochemical robustness and analytical performance of plasma emission sources. Typically, this ionic:atomic ratio is monitored through changes in plasma conditions (parameter studies) or the introduction of other species that would potentially induce matrix effects\textsuperscript{26,50-52}. There are essentially two aspects of the robustness parameter; the magnitude that reflects the ionization temperature (T\textsubscript{ion}) of the source, and the measured value that one would want to be unchanged as conditions are changed (i.e., a robust plasma). In principle, any emitting ion:atom
pair could be employed to assess changes in excitation/ionization conditions. The Mg (280.27 nm:285.21 nm) line pair is used here to allow comparisons with other sources and magnesium was easily introduced in the solution and particulate phases. Previous studies by Venzie and Marcus suggested a immunity to changes in the ratio with the addition of EIEs to the LS-APGD\textsuperscript{15}. Figure 5.8 illustrates the fact that both carrier gas flow rate and discharge current have minimal positive effects on the ratio. As might be expected from trends determined for $T_{\text{exc}}$ and $n_{e}$, carrier gas is the parameter that affects the ratio to a larger extent. The magnitude of the ratio (ranging from 0.5-1.3) with an average of 0.7, suggests that the extent of forming excited stated Mg ions is low versus the ICP (ranging from 7-11\textsuperscript{26}). By the same token, the lack of large changes across the parametric matrix suggests that the LS-APGD should be relatively immune to fluctuations in excitation conditions due to minor imprecisions in operational control; an asset in field-based applications.
The liquid sampling-atmospheric pressure glow discharge (LS-APGD) has been characterized relative to its rotational ($T_{rot}$) and excitation ($T_{exc}$) temperatures, electron number densities ($n_e$) and robustness, with an eye towards applications as a secondary excitation source for LA particle analysis and field deployment applications. Carrier gas flow rate, electrode distance, and applied current were incorporated in the parametric overview in the cases of blank solutions (1 % HNO$_3$), heavy solution matrices (1000 $\mu$g mL$^{-1}$ Mg$^{2+}$), and Mg.
particles introduced via LA. The high power density (>10 W mm$^{-3}$) microplasma exhibits rotational and excitation temperatures that are in line with propane-air combustion flames and low power ICP and MIP sources. Excitation temperatures are on par with other glow discharge sources. On the other hand, the electron number densities were on par with analytical ICPs. In no case of sample loading (solution or LA particles) were there statistically significant changes in the determined plasma characteristics.

Further experimentation is needed in terms of introducing other solution matrices (composition and concentration) to generate a more in-depth profile of the susceptibility to analytical matrix effects. Understanding these plasma fundamentals allows the plasma to be modified for specific needs. Such fundamental studies will be extended to the recent demonstration of the LS-APGD as a source for ambient desorption ionization-mass spectrometry (ADI-MS) $^{53}$. At this point, the analytical performance, low-overhead operational space, diversity in sample forms, and high levels of robustness of the microplasma suggest applications in areas where current laboratory-scale instrumentation is not appropriate.

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REFERENCES


CHAPTER SIX

INVESTIGATION OF SPECTROCHEMICAL MATRIX EFFECTS IN THE LIQUID SAMPLING-ATMOSPHERIC PRESSURE GLOW DISCHARGE (LS-APGD) SOURCE

INTRODUCTION

There recently have been appreciable efforts dedicated towards instrument miniaturization relative to spectrochemical analysis methodologies\textsuperscript{1-4}. Decreases in footprint, power requirements, solvent waste, and sample size are all characteristics targeted with the miniaturization of plasma excitation/ionization sources. The driving forces for these investigations involve both the economics of performing an analysis, but also the potential for field deployment applications. The low operating powers (<100 W) employed in many of these small-volume devices suggest that they may not have the ability to effectively vaporize/dissociate/excite analyte species in solution for determinations by optical emission spectroscopy (OES). Thus, analogous to the Beenakker-cavity MIPs\textsuperscript{5, 6}, the majority of these sources are dedicated to vapor phase samples. Beyond solvent-loading difficulties, there would seem to be a propensity for matrix effect in the case of complex samples wherein the introduction of easily ionizable elements (EI\Es) or the formation of refractory species could cause plasma disruption and alter the spectrochemical characteristics (e.g., excitation
Changes in excitation conditions and altered energy partitioning are some of the mechanisms contributing to matrix effects in sources employed for elemental analysis. As shown in inductively-coupled and microwave-induced plasmas (ICPs and MIPs), increases in the power delivered to plasmas improves their tolerance to solvent loading, while also reducing the extent of matrix effects\textsuperscript{13,14}. However, the operational space for such high power devices is contrary to the driving forces for miniaturized instruments.

A great deal of research has been dedicated to studying the comparatively minor spectrochemical matrix effects in the ICP\textsuperscript{10-13,15-18}. Similar detailed studies are scarce in regards to miniaturized plasmas, where the vast majority of fundamental spectrochemical studies are performed under conditions where there is no appreciable sample burden\textsuperscript{19-24}. Perturbations of excitation/ionization conditions (i.e., potential matrix effects) must be considered for practical analyses, particularly for the analysis of complex samples with target analytes in low concentrations. In the case of field-deployable instrumentation, there is certainly a need to do as little sample manipulation as possible, and so matrix effects should be well characterized. This would apply to all spectrochemical sources. Based on the projected fields of use for microplasmas, matrix-effect studies are important for the analysis of natural materials such as biosamples (e.g. biofluids) and geological samples (e.g. rocks) that are rich in EIEs. A low power source that is not significantly afflicted by matrix effects would be a
valuable part of the spectrochemical instrumentation portfolio, especially in field deployment situations.

The term “robustness” is used generically in analytical chemistry to describe the ability of a method/instrumentation to tolerate the loading of a specific sample matrix or inaccuracies that are the result of poor instrument control. Clearly, there are many steps in the spectrochemical analysis process that could result in matrix effects, including interspecies reactions, changes in sample introduction efficiency, and perturbations of excitation/ionization conditions. The latter we refer to here as “spectrochemical robustness”. Many metrics can be employed for characterizing spectrochemical robustness, including gas rotational temperatures ($T_{rot}$), excitation temperatures ($T_{exc}$), ionization temperature ($T_{ion}$) and the ionic:atomic line ratios of specific elements, most commonly magnesium\textsuperscript{10, 11, 17, 25, 26.} Potential changes in the first three “temperatures” can reflect whether or not the source can withstand the addition of different sample/matrix/solvent species without affecting the plasma thermal (kinetic), excitation, and ionization conditions, respectively.

The last metric, ionic:atomic line ratios, is particularly employed in ICP spectrochemical analysis as ionic transitions of the “monitor element” should be more sensitive to plasma changes than atomic transitions, with the ratio being independent of the absolute photon yield of analytes under those conditions\textsuperscript{26, 27}. The Mg II 279.5 nm and Mg I 285.2 nm lines are most commonly used in ICP-OES due to the proximity of the ionic and atomic lines and the similar excited-
state energy levels\textsuperscript{17, 18}. The Mg II:I ratio is a reflection of the ionization
temperature of the source on a fundamental level, but the robustness defined by
the simple line ratio serves as a convenient monitor of plasma
excitation/ionization conditions. There are two ways in which plasma robustness
can be used as a metric; 1) the absolute value which reflects the overall
ionization conditions and 2) its immunity to change upon matrix introduction.
Under robust plasma conditions (ratios for the ICP in the range of 9-11), the Mg
II:Mg I ratio remains relatively constant with the introduction of concomitants,
implying no change in the plasma excitation/ionization characteristics.
Magnesium line ratios allow for real-time assessment of source
excitation/ionization conditions with commercial optical spectrometers\textsuperscript{28}.

Marcus and co-workers have developed the liquid sampling-atmospheric
pressure glow discharge (LS-APGD) microplasma as a low power (<50W), small
plasma volume (~1 mm\textsuperscript{3}), and small instrument footprint (<125 cm\textsuperscript{2}) source for
detection by OES\textsuperscript{29, 30}. Differentiating aspects of the LS-APGD versus other
liquid electrode devices\textsuperscript{3, 4, 31} are the very high power densities (>10 W mm\textsuperscript{3}) and
ability to run in “total consumption” modes at flow rates up to 0.5 mL min\textsuperscript{-1}. The
LS-APGD has demonstrated very promising initial performance as an elemental
MS source\textsuperscript{32, 33}. The LS-APGD also is effective for OES and MS analysis of
laser-ablated particles\textsuperscript{34-36} and direct surface probing for ambient desorption
ionization mass spectrometry (ADI-MS)\textsuperscript{33}. A parametric evaluation of the LA-LS-
APGD-OES implementation was performed with respect to discharge current,
gas flow rate, and electrode gap\textsuperscript{35}, and their effects on rotational temperatures, excitation temperatures, and electron number densities. Those studies yielded average values of $T_{\text{rot}} \approx 1100$ K, $T_{\text{exc}} \approx 2700$ K, and $n_e \approx 3 \times 10^{15}$ cm$^{-3}$, in line with earlier studies\textsuperscript{37}, but importantly remaining relatively unperturbed across the range of discharge parameters or by the introduction of LA particulates.

Presented here is an in-depth investigation of the spectrochemical robustness of the LS-APGD microplasma. The influence of 11 different elements at a relatively high concentration of 500 µg mL$^{-1}$ was investigated as reflected in changes in the fundamental plasma metrics of excitation temperature, Mg II: Mg I ratio, and ionization temperature. Attention was paid to the potential dependences on the first and second ionization potentials, heats of vaporization, boiling points, and metal-oxide bond dissociation energies. The extent of spectrochemical perturbations and absolute analyte (Mg) responses was evaluated for the case of Ba added at concentrations of 50 – 1000 µg mL$^{-1}$. Such studies set the groundwork in moving forward in the development of the LS-APGD as a low cost, low power, small footprint spectrochemical source.

**EXPERIMENTAL**

*Sample Preparation*

Elemental stock solutions (1000 µg mL$^{-1}$) of Ba, Ca, Cu, Ga, In, Mg, Sr and Zn prepared in 1 M nitric acid were obtained from CPI International (Santa Rosa,
CA). Other stock solutions (Rb, Na, and Li) were prepared from analytical grade salts (Fisher Scientific, Waltham, MA) at a concentration of 1000 µg mL$^{-1}$ in 1 M nitric acid, each. The test elements, as seen in Table 6.1, were chosen to achieve a range of atomic masses, first and second ionization potentials, and thermodynamic properties. Magnesium was spiked into each test solution at a concentration of 100 µg mL$^{-1}$ to each solution as the constant probe element.

<table>
<thead>
<tr>
<th>Element</th>
<th>Atomic Mass (Da)</th>
<th>1$^{st}$ Ionization Potential (eV)</th>
<th>2nd Ionization Potential (eV)</th>
<th>Heat of Vaporization (kJ mol$^{-1}$)</th>
<th>Boiling Point (K)</th>
<th>Metal-Oxide Bond Dissociation Energy (kJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li</td>
<td>6.94</td>
<td>5.39</td>
<td>75.64</td>
<td>147.1</td>
<td>1342</td>
<td>284</td>
</tr>
<tr>
<td>Na</td>
<td>22.99</td>
<td>5.14</td>
<td>47.35</td>
<td>97.4</td>
<td>883</td>
<td>257</td>
</tr>
<tr>
<td>Mg</td>
<td>24.31</td>
<td>7.65</td>
<td>15.04</td>
<td>127.4</td>
<td>1090</td>
<td>394</td>
</tr>
<tr>
<td>Ca</td>
<td>40.08</td>
<td>6.11</td>
<td>11.87</td>
<td>154.7</td>
<td>1484</td>
<td>464</td>
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<tr>
<td>Cu</td>
<td>63.54</td>
<td>7.73</td>
<td>20.29</td>
<td>300.4</td>
<td>2562</td>
<td>343</td>
</tr>
<tr>
<td>Zn</td>
<td>65.39</td>
<td>9.39</td>
<td>17.96</td>
<td>123.6</td>
<td>907</td>
<td>284</td>
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<tr>
<td>Ga</td>
<td>69.73</td>
<td>5.99</td>
<td>20.51</td>
<td>254.2</td>
<td>2204</td>
<td>285</td>
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<td>Rb</td>
<td>85.47</td>
<td>4.18</td>
<td>27.28</td>
<td>75.7</td>
<td>688</td>
<td>255</td>
</tr>
<tr>
<td>Sr</td>
<td>87.62</td>
<td>5.70</td>
<td>11.03</td>
<td>136.9</td>
<td>1382</td>
<td>454</td>
</tr>
<tr>
<td>In</td>
<td>114.82</td>
<td>5.79</td>
<td>18.87</td>
<td>231.8</td>
<td>2072</td>
<td>360</td>
</tr>
<tr>
<td>Ba</td>
<td>137.33</td>
<td>5.21</td>
<td>10.0</td>
<td>140.3</td>
<td>1897</td>
<td>563</td>
</tr>
</tbody>
</table>

Table 6.1 Physicochemical characteristics of spectrochemical probe and concomitant matrix elements.

**LS-APGD-OES apparatus**

The LS-APGD set-up remained relatively unchanged from previous works$^{34, 35}$. As depicted in Fig. 6.1, the microplasma is sustained between the electrolytic liquid (HNO$_3$) and the counter electrode upon the application of a d.c.
potential (Glassman High Voltage Inc.; 0-100 mA, 0-2 kV, High Bridge, NJ). The positive supply output is coupled to the counter electrode through a 10 kΩ, 225 W ballast resistor (Ohmite, Arlington Heights, IL) and the solution electrode is held at ground potential. Throughout this work, the power supply was operated in a constant current mode, set at 60 mA as this provides high OES sensitivity. The electrolytic solution (5% HNO₃) is delivered through the fused silica capillary at a constant flow rate of 200 µL min⁻¹ using a New Era (Farmingdale, NY) Model NE-1000 syringe pump. The glass capillary (360 µm o.d., 100 µm i.d.) is housed within a Ni capillary (0.16 cm o.d., 0.06 cm i.d.), between which the helium sheath gas flows (0.2 L min⁻¹) to assist in plasma stability. The Ni counter electrode (0.3 cm o.d., 0.1 cm i.d.) is placed ~1 mm opposite the electrolytic solution. The footprint of the plasma apparatus is <125 cm².
Figure 6.1 Components of the liquid sampling-atmospheric pressure glow discharge optical emission spectroscopy (LS-APGD-OES) source with a photograph of the microplasma taken through the optical coupling lens.

Plasma optical emission was collected with a fused silica biconvex lens (35 mm focal length, 25.4 mm diameter) focusing the plasma image onto the entrance aperture of an optical fiber coupled to the spectrometer (Aurora, Applied Spectra, Fremont, CA). The insert in Fig. 6.1 is a photographic image of the microplasma taken through the collection lens. This spectrometer consists of six channels, each composed of a 2048 pixel CCD detector dedicated to different spectral regions. A composite (simultaneous) spectrum is acquired using a 1.05 ms gate time, with spectral resolution of 0.05–0.12 nm across the 190-1040 nm
wavelength range. Data collection and spectral manipulation were performed with the Aurora system software, with calculations and plotting functions performed in Microsoft Excel (Redmond, WA). Statistical analysis was performed employing Statgraphics software (Warrenton, VA). In each analysis data was modeled by the fitting of regression equations. Equations utilized were linear, exponential, reciprocal-Y, reciprocal-X, double reciprocal, logarithmic-X, multiplicative, square root-X, square root-Y, and S-curve in order to determine statistical relevance per relationship of spectrochemical metrics (e.g. $T_{\text{exe}}$) to physicochemical characteristics of probe elements. P-values were determined though the Statgraphics software and statistical significance was demonstrated to be within the 95% confidence level when the P-value was less than 0.05 and 90% when the P-value was less than 0.10.

**RESULTS AND DISCUSSION**

The efficiency of successive processes occurring in spectrochemical sources (i.e., nebulization, desolvation, vaporization, dissociation, and excitation/ionization) is a function of the solvent/solute (analyte and matrix) composition and the available energy to affect the aforementioned processes. To a first approximation, the ability to sustain the ICP at 1-2 kW powers (high thermal capacity) with high electron densities differentiates its efficiency and lowered susceptibility to matrix effects versus combustion flames. Until recently,
it was the inability to operate MIPs at sufficiently high powers that limited their use to OES detection following gas chromatography separations\textsuperscript{38}.

In the LS-APGD the feed solution composition, dictated by the sustaining electrolyte and sample make-up, could change the aqueous phase conductivity, changing the discharge i-V characteristics. As such, the heat generated at the solution/air interface would affect the rate of solution evaporation and sample introduction. The viscosity, surface tension, and vapor pressure of the solution would affect vaporization rates, evaporating droplet sizes, and latent internal energy. This is a different situation than combustion flames and ICP/MIPs, where nebulization, and to a great extent desolvation, occurs prior to entering the plasma. The physical characteristics of solutes (e.g., Table 6.1) might be expected to play more complex roles in the LS-APGD as the sample solution is an integral part of the functioning of the device. The feed solution composition, dictated by the sustaining electrolyte and the sample make-up, could change the aqueous phase conductivity, thus changing the discharge i-V characteristics. As such, the heat generated at the solution/air interface would affect the rate of solution evaporation and sample introduction rate. The viscosity, surface tension, vapor pressure of the solution would affect vaporization rates, evaporating droplet sizes, and latent internal energy. While all uses of the LS-APGD involve a total-consumption (i.e., total sample vaporization) mode of introduction, any differences in the amounts of energy remaining for the gas phase process could induce spectrochemical matrix effects. Once in the gas
phase, potential matrix effects in the LS-APGD would be similar to direct current plasmas (DCPs) and arc/spark sources, as changes in electron number densities could effect the current flow through the plasma. The range of elemental characteristics presented in Table 6.1 represents a variety of conditions to assess the spectrochemical robustness characteristics of the LS-APGD microplasma.

**Influence of concomitant elements on the plasma excitation temperatures**

The most common first-line assessment of a spectrochemical source is the excitation temperature ($T_{\text{exc}}$). $T_{\text{exc}}$ reflects the partitioning of energy amongst the excited states of the probe species. Previous studies in this laboratory have used an Fe spike as the spectrochemical probe for different powering modes and sheath gas identities, yielding values from ~2500 – 3600 K. To best monitor the excitation conditions across the range of concomitant elements, He I emission was monitored with the $T_{\text{exc}}$ values extracted based on the assumption of a Boltzman distribution of states. The approach was similar to that employed by Quintero et al. where excitation temperatures were calculated for a microwave induced plasma (MIP). While only four He I lines (501.6, 587.6, 706.5, and 728.1 nm) were utilized in this determination, the accuracy was sufficient to identify differences between matrix probes, as specific temperatures are not necessarily the overall goal. The majority of the correlation coefficients ($R^2$) for
the derived Boltzmann plots were greater than 0.900, with triplicate determinations under each experimental condition varying by <5 %RSD.

Figures 6.2 a-e depict the derived He I excitation temperatures plotted as a function of the concomitant element characteristics of first and second ionization potentials, heat of vaporization, boiling point, and metal-oxide bond energy, respectively. In the first case, the dashed horizontal lines depict a single standard deviation (plus and minus) for all of the measurements. As can be seen, the $T_{\text{exc}}$ values remained relatively unchanged across the breadth of concomitant test elements; there were virtually no changes in He I line intensities as the different concomitant elements were introduced into the plasma. Statistical analysis across each of the elemental constituent parameters reveals no level of correlation for the mathematical fits at the 90% confidence level, with all P-values being greater than 0.125. The average excitation temperature across the various species was determined to be 2769 ± 79 K for the LS-APGD; the average temperature of 2672 K was presented in previous work using 5% HNO$_3$ as the electrolytic solution$^{35}$. The consistency of these values across various elemental species reflects little-to-no perturbations of the excitation environment. Studies in MIPs have illustrated a temperature change (some up to 300 K) upon the introduction of EIEs$^{14}$, with the extent of variation significantly decreasing upon increasing the power of the plasma up to 1.5 kW, to eliminate matrix effects. While the LS-APGD is operated at total powers of 36 W, the power density is ~30 times higher than the conventional ICP, thus it is suggested that this microplasma
is highly robust (immune to perturbations of excitation temperature) with respect to moderately-high (500 µg mL\(^{-1}\)) sample loadings.
Figure 6.2 Correlations of He I-based excitation temperatures with the introduction of 11 matrix elements (500 µg mL⁻¹ each) in terms of a) first ionization potential, b) second ionization potential, c) heat of vaporization, d) boiling point, and e) metal-oxide bond dissociation energy

Influence of concomitant elements on the plasma ionization temperature

The excitation temperature of a spectrochemical source depicts only a portion of the overall kinetic processes. Beyond the extent of populating atomic states, bulk ionization rates and further population of excited atomic ions must be concerned. Under the assumption of local thermodynamic equilibrium (LTE), the ionization temperature ($T_{ion}$) is the fundamental measure of the latter processes. The Saha equation, Eq. 6.1, is commonly used in deriving the ionization temperature assuming the existence of LTE.
As described previously, the ionic-to-atomic line ratio of Mg was used to monitor plasma-related matrix effects by changes in the excitation/ionization conditions within the plasma. This ratio can be utilized under the assumption that the electron number density is known. The ionic:atomic ratio is related in this equation where I is the emission intensity, $n_e$ is the electron number density, $g_p$ and $g_q$ are the statistical weights of the upper and lower state (respectively), $A_{pq}$ is the transition probability, $\lambda_{pq}$ is the wavelength of the specific transition, $k$ is the Boltzmann constant, $E_{i^+}$ is the first ionization potential of magnesium, $E_{q^+}$ and $E_q$ are the energy levels of the upper state for the ionic and atomic emissions (respectively), and $T$ is the ionization temperature of the plasma. An electron number density of $3 \times 10^{15}$ cm$^{-3}$ was utilized for calculating ionization temperature based on Stark broadening of the hydrogen Balmer alpha line$^{35}$. Calculations of the ionization temperature were performed using Microsoft Excel as well as by a predictive modeling program developed in LabView, with both yielding similar results.

In comparison to the excitation temperatures presented in Fig. 6.2 (~2800 K), the derived ionization temperatures averaged 6665 ± 151 K across the concomitant additive elements. The general difference between ionization and excitation temperatures in the LS-APGD ($T_{\text{ion}} > T_{\text{exe}}$) follows the observation in non-thermal ICPs, though the two characteristic temperatures are in much closer

$$\frac{I_{\text{ionic}}}{I_{\text{atomic}}} = \frac{4.83 \times 10^{15}}{n_e} \times \left( \frac{g_q A_{pq}}{\lambda_{pq}} \right)_{\text{ionic}} \times \left( \frac{\lambda_{pq}}{g_q A_{pq}} \right)_{\text{atomic}} \times T^{3/2} e^{-\frac{(E_{i^+}+E_{q^+}-E_q)}{kT}}$$  [6.1]
proximity for the ICP with values of $T_{\text{ion}} \approx 6000-8000$ K and $T_{\text{exc}} \approx 5000-7000$ being typical\textsuperscript{42-44}. Large differences between ionization and excitation temperatures have been cited for other microplasmas such as the solution-cathode glow discharge (SCGD)\textsuperscript{22, 23}. The discrepancy between ionization temperature and excitation temptation does not change the fact that the LS-APGD might be considered as being at LTE, as a “single” temperature is not a necessarily requirement\textsuperscript{40}. More correctly, the individual $T_{\text{ion}}$, $T_{\text{exc}}$, etc. must be uniform across various spectrometric species\textsuperscript{45}, as has been found in previous LS-APGD works\textsuperscript{35, 37, 46}.

Statistical evaluation of significant factors across the physical parameter space of Table 6.1 showed a virtual lack of meaningful correlations. The only parameters effecting the ionization temperature were the 2\textsuperscript{nd} IP and metal-oxide bond strengths of the added elements, showing slight correlations at the 90% confidence levels; a reciprocal trend in the former and a linear dependence in the latter. There is indication of outliers from the norm in Fig. 6.3, where the derived ionization temperatures are plotted as a function of the 2\textsuperscript{nd} IP of the test elements (would be helpful to me if I knew which elements were related to each point on the graph without having to go to the table of element IP. Here, it is seen that 500 $\mu$g mL\textsuperscript{-1} additions of Ba and Ca impart slight increases in the ionization temperature. Kitagawa and Takeuchi proposed that the introduction of the EIEs into a microwave plasma increased the overall collision rate in the plasma (i.e., more electrons)\textsuperscript{47}, with the increased collisions correlating with the increased
ionization temperatures. In Fig. 6.3, Sr stands out as an outlier as the $T_{\text{ion}}$ determined upon its addition is on the lower end of the range of measured values. As a general measure of potential spectrochemical matrix effects, there are no appreciable perturbations of $T_{\text{ion}}$ seen across these test elements.

Figure 6.3 Correlation between the measured ionization temperatures and the second ionization potential for each of the 11 matrix elements (500 $\mu$g mL$^{-1}$ each)

**Influence of concomitant elements on the plasma robustness parameter**

While the ionization temperature is a fundamental parameter of a spectrochemical device, the robustness parameter as described by Mermet$^{17,26}$ provides both a reflection of source excitation/ionization conditions as well as a
facile means of assessing changes in those conditions. While measured Mg II/Mg I values are part of the calculation of $T_{\text{ion}}$, Eq. 6.1 is composed of many factors, each of which has a level of uncertainty. For example, a single value of $n_e$ was used in those calculations, which may not be an accurate assumption. Indeed, this further argues toward the practical utility of the robustness parameter. In general, miniaturized plasmas typically have lower ionic:atomic ratios than an ICP, mainly due to the lower power and decreased plasma temperatures. The ICP’s high excitation/ionization temperatures (<7000 K) generate ionized species more effectively than that of miniaturized plasmas (~3000 K). As such, robustness parameters for ICPs are much higher (10x) than the LS-APGD and other miniaturized plasmas$^{46,48}$.

As might be expected based on the parametric dependences seen for ionization temperatures, only the 2$^{\text{nd}}$ IP and metal-oxide bond strengths of the concomitant elements have any influence on the derived robustness parameters. In fact, both quantities display significant effects at the 95% confidence level, though the overall impact in terms of the range of derived values is not appreciable. It is important to emphasize that the raw intensity values for the Mg II and Mg I transitions vary little across the different concomitant elements; suggestive of minimal analytical matrix effects for the 500 $\mu$g mL$^{-1}$ additives. Neither the Mg II or Mg I show a statistical correlation to either factors, but the ratios do. Figure 5.4a depicts the dependence of the Mg II/Mg I values on the 2$^{\text{nd}}$ IP of the test elements, revealing a reciprocal dependence with a P-value of
0.034. I like seeing the elements labeled in this figure! The baseline value (i.e. absence of matrix elements) of the ratio was measured by the introduction of the 100 \( \mu \text{g mL}^{-1} \) Mg solution in 5% HNO\(_3\), yielding a value of \( \sim 1.2 \), which is in agreement with previous LS-APGD studies\(^{46}\). In this case, Ba and Ca show a stronger (positive) influence on the ratio than seen in Fig. 6.3, lying farther outside the \( \pm 1 \) standard deviation boundaries. As in the case of the ionization temperatures, Sr yields a ratio that reflects much lower excitation/ionization efficiency than would be expected based on its 2\(^{nd}\) IP. Exclusion of the Sr response from the data set results in an improvement in the statistical P-value to 0.002. While there is a high level of mathematical correlation, again, it must be pointed out that overall, the variation across the suite of elements is predominately within \( \pm 1 \) standard deviation of the complete experiment set. It must also be pointed out that, the variability in the Mg II:Mg I ratios is driven almost exclusively by the lower-energy Mg I responses, as the Mg II responses are virtually independent of the concomitant element identity. One would expect that the ionic transition, having a much higher total excitation energy, would be more prone to changes in plasma conditions.

Given the relative low total power and low excitation temperatures of the LS-APGD, one might have expected some contribution of the concomitant element’s 1\(^{st}\) IPs in terms of spectrochemical matrix effects, as in the classical case of EIEs in combustion flames\(^7,9\). This is not the case. The lack of impact here may lie in the relatively high electron number densities of the LS-APGD; on
the order of $10^{15} \text{ cm}^{-3}$. As such, enhanced electron densities by virtue of EIEs are inconsequential. Russo et al. demonstrated that elements with low 2\textsuperscript{nd} IP (<15 eV) prompt extreme matrix effects in the ICP\textsuperscript{11}, more so than low first ionization potentials. Further works by Chan and Hieftje reinforce this relationship, but also point to secondary factors affecting matrix effects across a wide range of elements\textsuperscript{18}. Elements of low secondary IP are mainly of the group two elements (Ca, Sr, and Ba), with Ba having the lowest second IP (10.0 eV) of all elements. The introduction of Ba and Ca to the LS-APGD yields increased Mg II:I ratios (and to a lesser extent $T_{\text{ion}}$) implying a more energetic plasma. This trend is opposite that found in ICPs, where elements with low 2\textsuperscript{nd} IPs lead to lower ionic:atomic ratios\textsuperscript{11, 18}. In general, low 2\textsuperscript{nd} IP elements have been suggested to be readily ionized via Penning collisions, removing important argon metastables from the overall plasma energy pool and suppressing measured ionic:atomic emission ratios. Chan and Hieftje noted Ca, Sr, and Ba as being anomalies in their relative matrix effects, having less suppressive effects than elements of comparable 2\textsuperscript{nd} IPs\textsuperscript{18} due to the lack of a Penning pathway for ionic state population. Russo et al. had found that use of He as the carrier gas in their experiments virtually eliminated the 2\textsuperscript{nd} IP-based suppression effects\textsuperscript{11}, pointing to the fact that metastable depletion was active in the Ar ICP.

Mechanistic explanations for the correlations with 2\textsuperscript{nd} IPs here, which in overall magnitude are rather small, suggest different effects than the ICP. This is reasonable as the LS-APGD is primarily composed of “reactive” water-originating
species, versus the ICP that is predominately water vapor diluted in Ar. Differences exist also as He is used as the sheath/cooling gas here. As such, primary energetic species which will affect the overall distribution of energy will be electrons, metastable He atoms and ions, and ionized water molecules and water fragments (e.g., H, H⁺, etc.). If treating Ba, Ca, and Sr as a group, and acknowledging that Sr is somewhat of an anomaly, one must consider the total energy to create the doubly-charged ions. As listed in Table 6.1, only these three elements can be created via a Penning collision with 19.8 He metastable atom. Decreasing He*ₘ populations via this path would not likely effect the Mg II emission which originates from ~12.05 eV state above the ground Mg atom level. On the other hand, if one supposes the that doubly-charged ions are formed from M+ precursors, then these may be formed by a charge transfer with H₂O⁺ which has an IP (and thus electron affinity) of ~12.7 eV. This process would lessen the energy drain on the plasma as the group II species are more easily formed, and thus leave more energy for the ionization/excitation of the Mg probe species. The relative contributions of sheath gas and water-based species need much further investigation. The use of other ion:atom pairs, such as Zn may be more instructive, as greater sensitivity to changes in plasma conditions have been noted in ICP diagnostics¹¹,¹⁸.
Figure 6.4 Resultant Mg II:Mg I emission intensity ratios correlated with a) second ionization potential and b) metal-oxide bond dissociation energy of the 11 matrix elements (500 µg mL⁻¹ each)
The second significant factor affecting the plasma robustness parameter is the metal-oxide bond strength of the concomitant element. Metal-oxide ions have been noted in LS-APGD mass spectra\textsuperscript{32}, though it cannot be said whether or not those ions are plasma-originating or formed in transit from the plasma to the MS sampling orifice. As shown in Fig. 6.4b, Mg II:I ratios show an overall slight positive trend as a function of the metal-oxide bond energies, yielding a P-value of 0.024 for a linear fit, indicative of a correlation at the 95% confidence. As in the case of the 2\textsuperscript{nd} IP correlations, neither of the individual transitions shows a correlation, but the ratio does. More clearly seen here than in Fig. 6.4a, Sr seems to be an anomaly among the group II elements. Here again, removal of Sr from the test data results in greatly improved correlations, reducing the P-value to 0.005. This overall trend seems counterintuitive as the need to dissociate metal oxides to yield free atoms and ions would be an energy drain on the plasma.

\textit{Influence of concomitant element concentration on the plasma robustness parameter}

It is not only the identity of concomitant elements, but also their concentration, that contributes to spectrochemical matrix effects. In fact large concentrations of almost any element can “overload” spectroscopic flames and plasmas. In
addition to EIE-type effects, problems such as disruption of normal operating conditions exist wherein the energy of the system is not sufficient to affect the gas-phase processes of desolvation, vaporization, and dissociation to create free atoms. While the previously-discussed studies of plasma excitation conditions were undertaken at relatively-high loadings (500 µg mL⁻¹), it is important to gain an understanding how the concomitant element loading can effect this microplasma. Because Ba showed the largest effects on the ionization temperature and plasma robustness parameters, it was chosen as the test element to identify potential concentration-based spectrochemical matrix effects. The data in Fig. 6.4 illustrates the effect of Ba concentration to the Mg II:Mg I emission ratio across a range of 66 – 1000 µg mL⁻¹. As seen in the derived robustness parameter values, there is essentially no change in the excitation/ionization characteristics from the case of no Ba addition to the point of a 0.1 % loading. The variability seen in the emission ratios, as noted previously, is predominately in the denominator of the calculation (Mg I emission), as the Mg II responses across the range Ba loadings varied by less than 6% RSD, independent of the Ba content. From the point of view of Ba being an analyte, its emission response across this concentration range increased linearly with a slope close to unity, with no indication of self-absorption. Thus, to this level of loading, with what is the most perturbing element, the plasma exhibits what would classified and little-to-no spectrochemical matrix effects.
Figure 6.5 Resultant Mg II:Mg I emission intensity ratios as a function of the added barium concentration

CONCLUSIONS

The liquid sampling-atmospheric pressure glow discharge (LS-APGD) has been evaluated for spectrochemical matrix effects using excitation temperature, ionization temperature and the plasma robustness parameter. Ten elements of diverse atomic weight, 1st and 2nd ionization potential, heat of vaporization, boiling point, and meta-oxide bond energy were added at concentrations of 500 µg mL⁻¹. Plasma temperatures ($T_{exc}$ and $T_{ion}$) remained relatively unchanged throughout the introduction of the various elemental probes, with variations
across the entire probe element suite of <3 %RSD. Studies suggest that the high power density (>10 W mm$^{-3}$) and high electron density (>10$^{15}$ cm$^{-3}$) microplasma can effectively tolerate a wide range of elements without disruption of the plasma, demonstrating a spectrochemically robust plasma. Only in the case of the plasma robustness parameter, the Mg II:Mg I emission intensity ratio are any element-specific differences seen. Overall, the robustness parameter varied by 0.95 ± 0.3 across the concomitant element suite. The absolute values would be considered “non-robust” in comparison to the ICP, but the actual range across many matrices suggests a high level of tolerance not reflected in the absolute value. The only physicochemical aspects of the added elements that had statistically significant correlations were the 2$^{\text{nd}}$ ionization potential (a reciprocal dependence) and the metal-oxide bond strengths (a linear dependence). These effects were minimal, making mechanistic assignments difficult. When looking at the robustness parameter as a function of the total amount of concomitant element introduced, the Mg II:Mg I ratios did not significantly change upon changing the concentration of Ba from 66 - 1000 µg mL$^{-1}$, with the Mg II response changing by less than 3.5 %RSD.

Consistent with previous research, the LS-APGD demonstrates high promise as an elemental excitation/ionization source due to its low operating costs and minimal matrix effects. Questions remain to be answered regarding practical matrix effects across different sample types. Reported here was the extent of spectrochemical perturbations. There may be physical matrix effects
related to the ability to generate consistent atom populations from solutions of diverse bulk compositions. As one looks to field-deployable applications, samples of different ionic strength, viscosities, and heats of vaporization will surely be encountered. These effects must be quantified, but the lack of appreciable spectrochemical matrix effects bodes well for development towards these applications.

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REFERENCES


CHAPTER SEVEN

LIQUID SAMPLING-ATMOSPHERIC PRESSURE GLOW DISCHARGE AS A SECONDARY EXCITATION SOURCE FOR LASER ABLATION GENERATED AEROSOL: INVESTIGATION OF PLASMA ROBUSTNESS AND STABILITY

INTRODUCTION

The realm of analytical chemistry instrumentation for e.g. separation and mass spectrometric analyses has progressed considerably over the last decade in terms of miniaturization\(^1\),\(^2\). However, little research has been dedicated to instrument miniaturization in atomic spectroscopy. The availability of miniaturized instrumentation in general and in atomic spectroscopy in particular is of crucial importance for prompt field analyses. Field-deployable instruments for elemental, isotopic, and/or molecular characterizations of environmental samples allow for rapid screening analyses of e.g. post-detonation debris in forensic analyses and the fast assessments of samples of high interest to be collected for a more thorough analysis in the lab.

Considering excitation/ionization sources, the ICP is beyond doubt the source of choice when it comes to inorganic, elemental analysis\(^3\),\(^4\), not at least
due to its superior excitation/ionization efficiencies. However, the applicability of the ICP with regard to its implementation in portable analytical instrumentation is limited, mainly because of its large footprint, high operating costs due to relatively high gas (~15 L min\(^{-1}\)) and power requirements (1-1.5 kW). Recently, the usefulness of glow discharge plasmas as excitation/ionization sources in atomic spectroscopy has been more and more recognized, which is also highlighted by several reviews dedicated to this technique\(^{5,7}\). Low power, compactness, and need for only small sample amounts (i.e. low µL min\(^{-1}\) range) result in the glow discharge plasmas’ attractiveness for implementing them as excitation/ionization sources in miniaturized elemental analysis instrumentation\(^{5,8,9}\).

Regarding sample introduction, laser ablation (LA)\(^{10,11}\) opened new doors in terms of direct solid sampling for elemental and isotopic analysis, particularly in ICP-MS\(^{10,12}\). In LA typically a short-pulsed (fs-ns) high-power (mJ) laser beam is focused onto a solid sample. A laser-induced plasma is formed at the sample surface upon laser bombardment due to the ionization of vaporized, ejected mass by absorption of incident laser light. Thermal vaporization, comprising of melting and vaporization of the sample surface, is the dominant mechanism for nanosecond (ns) laser pulses with irradiances less than \(10^8\) W cm\(^{-2}\). Finally, the condensation of vapor results in the generation of nanoparticles\(^{12}\). Generally, following the ejection of material, there are two means of determining the sample’s elemental composition: 1) direct analysis of the laser-induced plume by optical emission spectrometry (OES), which is
commonly referred to as laser induced breakdown spectroscopy (LIBS) and 2) analysis by optical emission (OES) or mass spectrometry (MS) after transportation of nanoparticles into a secondary excitation/ionization source, which is in most cases an ICP\textsuperscript{12, 13}. Because of its small footprint, compared to ICP-OES/MS, and the fact that only minimal or even no sample preparation is needed, LIBS is highly appreciated in case of field-deployable instruments. The use of LIBS has even expanded to planetary\textsuperscript{14} and under water elemental analyses\textsuperscript{15, 16} as there is no need for a secondary excitation/ionization source. However, aerosol generated by laser ablation may be subject to secondary ionization/excitation for increasing the sensitivity for elemental analysis. Typically, secondary excitation sources such as the ICP, are exhibiting higher power densities (1 W mm\textsuperscript{-3}) and longer analyte plasma residence times (~15 ms) than the laser-induced LIBS plasma, with both parameters typically yielding higher sensitivity for elemental analysis\textsuperscript{13}. Disadvantageous of employing an ICP, which is the most commonly utilized secondary excitation source\textsuperscript{11, 17, 18}, is that its relatively large volume (i.e. \textasciitilde125 mm\textsuperscript{3}), compared to microplasmas may lead to a “dilution” of the LA-generated aerosol. Thus, in order to circumvent this “dilution” effect in case of direct solid sample analysis, one would envision the availability of a source miniaturized with respect to its volume. The usefulness of coupling LA with a sequential discharge technique, i.e. LA-spark induced breakdown spectroscopy (LA-SIBS), was for example demonstrated by Li et al.\textsuperscript{19}. In case of LA-SIBS, two discharge processes are obtained by 1) a low current arc formed
between two electrodes and 2) a pulsed laser directed between the electrodes onto the sample surface. Compared to conventional LIBS, improved emission signals and signal-to-noise ratios were obtained without the need for analyte transport. However, high voltages (~10 kV) were required for the discharge to be sustained. Moreover, glow discharge plasmas using a hollow-cathode discharge have also been employed as secondary excitation sources for LA generated aerosol dating back to Iida and co-workers. More recently, Tereszchuk et al. demonstrated the use of a pulsed direct current (DC) glow discharge as a secondary excitation source for copper samples with OES detection. Studies revealed a up to 75 fold signal enhancement compared to a traditional LIBS set-up.

Marcus and co-workers have recently employed an in-house built liquid sampling-atmospheric pressure glow discharge (LS-APGD) for the secondary excitation/ionization of LA produced particles. Up to now, it has been employed, together with both OES and MS, for the analysis of liquids, gases, and aerosol particles, generated by both nanosecond and femtosecond laser ablation. The LS-APGD, a truly miniaturized source, exhibits a small plasma volume (<1 mm$^3$), but with high power densities in the 10 W mm$^{-3}$ range. The plasma, needing only low operating power (less than 10 W), is formed between an electrolytic solution acting as one electrode and a hollow (for transporting the LA-generated particles) counter electrode. The footprint of the plasma stage is less than 12 cm$^2$ and it has minute operating costs, mainly due to reduced gas
consumption (<1 L min\(^{-1}\) compared to 15 L min\(^{-1}\)). In recent publications\(^{24, 27}\) plasma characteristics (i.e., plasma temperatures, electron number density, and robustness) were thoroughly investigated in hopes of a better understanding of the LS-APGD’s potential as an excitation/ionization source for liquid samples. While the plasma excitation temperature (~2600 K) may not be as high as that of a typical ICP (up to 8000 K), the electron number density \((3 \times 10^{15} \text{ cm}^{-3})\) is in the same order of magnitude, particularly due to the high plasma power density (~10 W mm\(^{-3}\)), making it an excellent miniaturized source for atomic spectroscopy.

The aim of this paper is a thorough investigation of the LS-APGD’s plasma robustness and stability for direct solid sampling by means of laser ablation. While much effort has been put into the investigation of plasma characteristics in case of liquid sampling\(^{24}\), a detailed study of how aerosol particles affect the plasma environment has not yet been approached. The knowledge and understanding of such plasma characteristics is of crucial importance for further method developments and the full assessment of the source’s usefulness in atomic spectroscopy, employing both liquid and solid sampling. LA-LS-APGD operating parameters such as glow discharge current, carrier gas flow rate, and distance between the electrodes were optimized by ablating a brass sample and measuring Cu emission lines. Plasma robustness and spectroscopic matrix effects were assessed by determining magnesium (Mg) ionic:atomic ratios and plasma rotational temperatures after introducing LA-generated aerosol containing easily ionized elements (i.e. Ca, Sr) into the plasma.
EXPERIMENTAL

Reagents and Chemicals

Test strontium and calcium pellets were homogenously made in-house at Lawrence Berkeley National Lab (LBNL). Each test pellet was made in paraffin and contained a constant amount of magnesium (3.43%) in order to monitor Mg ionic:atomic ratios. Sr and Ca concentrations in each pellet were varied and can be seen in Table 1. To test for homogeneity calibration curves were generated based on laser ablation (in replicates of 4) for each sample. Each ablation generated a spectra containing an accumulation of 150 shots. Each calibration curve (Sr and Ca) had correlation greater than $R^2 = 0.90$. The small error could be deduced to the slight inhomogeneity or LS-APGD performance. Finally, a raster of 25 different locations was performed on the Sr pellet yielding %RSD of 8%.
<table>
<thead>
<tr>
<th>#</th>
<th>Mass CaCO(_3) (g)</th>
<th>Mass MgCO(_3) (g)</th>
<th>Mass Paraffin (g)</th>
</tr>
</thead>
<tbody>
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<td>0.062</td>
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<td>4.764</td>
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<td>2</td>
<td>0.125</td>
<td>0.173</td>
<td>4.702</td>
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<td>3</td>
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<td>0.173</td>
<td>4.577</td>
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<tr>
<td>4</td>
<td>0.5</td>
<td>0.173</td>
<td>4.327</td>
</tr>
<tr>
<td>5</td>
<td>0.749</td>
<td>0.173</td>
<td>4.077</td>
</tr>
<tr>
<td>6</td>
<td>0.999</td>
<td>0.173</td>
<td>3.828</td>
</tr>
<tr>
<td>7</td>
<td>1.124</td>
<td>0.173</td>
<td>3.703</td>
</tr>
<tr>
<td>8</td>
<td>1.2487</td>
<td>0.174</td>
<td>3.5755</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>#</th>
<th>Mass of SrCO(_3) (g)</th>
<th>Mass of MgCO(_3) (g)</th>
<th>Mass of Paraffin (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>0.173</td>
<td>4.819</td>
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<tr>
<td>2</td>
<td>0.017</td>
<td>0.173</td>
<td>4.81</td>
</tr>
<tr>
<td>3</td>
<td>0.042</td>
<td>0.173</td>
<td>4.785</td>
</tr>
<tr>
<td>4</td>
<td>0.084</td>
<td>0.173</td>
<td>4.743</td>
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<tr>
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<td>0.173</td>
<td>4.658</td>
</tr>
<tr>
<td>6</td>
<td>0.337</td>
<td>0.173</td>
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<tr>
<td>7</td>
<td>0.505</td>
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<td>4.322</td>
</tr>
<tr>
<td>8</td>
<td>0.697</td>
<td>0.173</td>
<td>4.13</td>
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<td>9</td>
<td>0.753</td>
<td>0.173</td>
<td>4.074</td>
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<tr>
<td>10</td>
<td>0.842</td>
<td>0.173</td>
<td>3.985</td>
</tr>
</tbody>
</table>

Table 7.1 Test pellet composition

**Laser Ablation Set-up**

Laser ablation was accomplished by means of the “J100” nanosecond laser ablation system (J100 Applied Spectra, Inc, Freemont, CA, USA). The system was operated at this fundamental IR wavelength (i.e. 1064 nm) and as well as in the UV region (i.e. 213 nm). Instrumental parameters are given in Table 2.
<table>
<thead>
<tr>
<th><strong>LA &quot;J100&quot; system</strong></th>
<th>Static point ablation</th>
<th>Static point ablation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ablation mode</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wavelength / nm</td>
<td>1064</td>
<td>213</td>
</tr>
<tr>
<td>Pulse width / ns</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Energy / mJ</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>Repetition rate / Hz</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Number of shots</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Number of replicates</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>He carrier gas flow rate / L min⁻¹</td>
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<td>0.1</td>
</tr>
<tr>
<td><strong>LS-APGD microplasma</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheat gas flow rate / L min⁻¹</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Electrolytic solution (5 % HNO3) / µL min⁻¹</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Electrode distance / mm</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Glow discharge current / mA</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td><strong>&quot;Aurora&quot; optical emission spectrometer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delay time / µs</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Integration time / ms</td>
<td>1.05</td>
<td>1.05</td>
</tr>
<tr>
<td>spectral resolution / nm</td>
<td>0.05–0.12*</td>
<td>0.05–0.12*</td>
</tr>
<tr>
<td>Spectrometer</td>
<td>6-Channel CCD, Aurora</td>
<td></td>
</tr>
<tr>
<td>Detector</td>
<td>2048 pixel charged coupled device (CCD)</td>
<td></td>
</tr>
<tr>
<td>* across the 190-1040 nm wavelength range</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7.2 Instrumental parameters

**LS-APGD**

The LS-APGD design used in this study was slightly modified to allow for the introduction of laser ablated particles by employing a hollow counter electrode.
see in Figure 1 and discussed in previous publications $^{23, 24}$. The microplasma was sustained in an electrical circuit between two electrodes. An electrolytic solution (1 mol L$^{-1}$ HNO$_3$) flowing by means of a syringe pump (New Era Pump Systems Inc., model NE-1000 Multi-Phaser, Farmingdale, NY, USA) in a fused silica capillary (i.e. 360 µm o.d., 100 µm i.d., Idex Health and Science, Oak Harbor, WA, USA) served as the anode while the counter electrode (nickel) served as the cathode. The aforementioned silica capillary was entrapped within a metal capillary (nickel, 0.16 cm o.d., 0.06 cm i.d.), and a helium sheath gas flows in the inter-capillary gap for plasma stability. Power applied to the microplasma was delivered by a Glassman High Voltage Inc. power supply (0-100 mA, 2 kV, High Bridge, NJ, USA) while a 10 kΩ, 225 W ballast resistor (Ohmite, Arlington Heights, IL, USA) was placed in-line prior to connection with the anode. A hollow cathode (nickel, 0.3 cm o.d., 0.1 cm i.d.), which was held at ground potential to complete the electrical circuit, was employed in order to allow for the transport of the LA generated particles. Optimized instrumental parameters employed for plasma robustness studies are given in Table 2.
Figure 7.1 LS-APGD source set-up for the introduction of laser ablated particles with optical emission spectroscopy detection

Optical Emission Spectrometer

Emission from the LS-APGD microplasma was collected employing an optical fiber-based spectrometer (Aurora, Applied Spectra, Freemont, CA, USA) which allows performing broad wavelength (190-1040 nm) measurements. Focusing of the entire microplasma image; i.e. emission, onto the fiber optic bundle attached to the “Aurora” spectrometer was accomplished using a fused silica biconvex lens (35 mm focal length, 25.4 mm diameter, Thorlabs Inc., Newton, NJ, USA). The “Aurora” spectrometer was equipped with six channels,
whereat each channel employed a 2048 pixel charged coupled device (CCD) detector, enabling the acquisition of composite (simultaneous) spectra. Accumulated spectra were generated from individual sample locations by applying 150 shots per spectrum. Peak and data processing were performed using the Aurora software (Applied Spectra, Freemont, CA, USA) and Microsoft Excel (Redmond, WA, USA), respectively. Instrumental parameters are summarized in Table 2.

Optimization of LA-LS-APGD-OES Set-Up

Optimization of instrumental parameters (i.e., glow discharge current, He laser ablation carrier gas flow rate and electrode gap) of the LA-LS-APGD set-up was accomplished by monitoring copper (Cu) emission lines (i.e., 325, 327, 510, and 521 nm) after ablation of a brass sample employing a laser wavelength of 1064 nm. A thorough parametric evaluation of the LA-LS-APGD set-up, addressing parameters such as microplasma sheath gas flow rate, electrolytic solution flow rate, He laser ablation carrier gas, discharge current and laser energy, has been previously published by Marcus and co-workers. However, as a slightly modified set-up was used in this study, parameters such as glow discharge current, He laser ablation carrier gas and electrode distance, were re-addressed in this study.
RESULTS AND DISCUSSION

*Influence of glow discharge current, electrode gap, He laser ablation carrier gas flow rate on sensitivity and plasma performance*

Generally, higher glow discharge currents are favorable in terms of higher plasma temperatures and a more energetic plasma, leading to more excited species compared to lower currents and plasma temperatures. Optimization of the glow discharge current yielded a ~2-fold Cu (I) 327 nm emission line signal intensity at 60 mA (current at which the plateau was reached) compared to 45 mA, with the intensity linearly increasing with the applied current. This observation is also in accordance with 23, and a study 20 in which a hollow-cathode discharge was employed as a secondary excitation source for LA-generated particles. Reaching a plateau at 60 mA suggests that all species are already excited at this current and that a further increase does not increase the number of excited states. In addition, at a certain threshold (i.e. 70 mA) higher currents lead to plasma overheating and electrode damage. However, compared to the LS-APGD set-up for liquid sample introduction 28, a slightly higher current is applied for direct solid sample introduction; i.e. 60 mA compared to 40 mA, as also already observed by Marcus and co-workers in a previous study 23. The higher discharge current required for best sensitivity could possibly result from an increased cooling of the plasma, as previously illustrated 24, due to an additional
He laser ablation carrier gas flow that is introduced into the plasma through the counter electrode. In case of liquid sampling a He sheath gas is solely flowing between the anode and the fused silica capillary transporting the electrolytic solution.

Considering the electrode gap between the anode and the hollow counter electrode, the optimum distance in terms of highest sensitivity was determined to be 1 mm as seen in Figure 2a. Increasing the electrode gap, and thus increasing the plasma volume (i.e. dilution of the plasma by expansion), yielded a loss in sensitivity, which is also in good accordance with a previous publication $^{24}$. The increased plasma volume leads to a decrease in plasma temperature and electron number density. As a result, less energy is available for excitation processes, which is reflected in the decrease of sensitivity $^{24}$. However, improved precisions are observed with increasing electrode gaps, and thus larger plasma volumes. A potential explanation for this observation are longer plasma residence times, allowing plasma processes to take place in a more homogenous way compared to shorter plasma residence times. Indeed actual plasma residence times must be determined in future investigations as done by Aeschliman et al $^{29}$. 
Figure 7.2 Optimization of operating parameter of a) electrode gap distance and b) He carrier gas flow rate of the LA-LS-APGD-OES set-up

The optimum He laser ablation carrier gas flow rate was determined to be 0.1 L min⁻¹. Lower flow rates caused overheating of the hollow counter electrode,
whereas higher flow rates led to a decrease in the Cu (I) 327 nm emission line responses, as depicted in Figure 2b. In addition, lower flow rates yield more precise measurements compared to higher flow rates; e.g. 9 % and 36 % RSD for 0.1 L min\(^{-1}\) (signal intensity: 250000 a.u.) and 0.8 L min\(^{-1}\) (signal intensity: 10000 a.u.), respectively. At higher flow rates only a fraction of LA-generated particles may be directly entering the plasma as some particles may be swept around the plasma, and thus not being prone to digestion and excitation at all. In addition, higher flow rates are yielding shorter plasma residence times, thus shortening the time available for digestion and excitation.

Optimized LA-LS-APGD instrumental parameters (i.e. current: 60 mA, electrode gap: 1 mm, He gas flow rate: 0.1 L min\(^{-1}\)), were employed for assessing the plasma stability over an hour. Precisions of five individual single spot ablations (number of shots: 150, repetition rate: 10 Hz) ranged from 7 % to 21 %, whereas a short-term reproducibility (i.e. RSD of five individual single spot ablations over 60 minutes) of 8 % was observed for measuring the Cu (I) 327 nm emission line in brass. This short-term reproducibility indicates good plasma stability even upon the introduction of relatively large particles, resulting from the use of a relatively high laser energy (i.e. 50 mJ) employed for the ablation of brass \(^{30}\). In addition, the LS-APGD’s capability for effectively digesting and exciting the introduced species is demonstrated again, see also \(^{23}\).
Robustness of LS-APGD microplasma upon introducing LA-generated particle aerosol

Recently, the LS-APGD microplasma’s robustness with regard to spectroscopic matrix effects was successfully demonstrated for liquid sampling. Determining the robustness of excitation/ionization sources upon the introduction of different matrices is of importance for assessing its usefulness as its ability to effectively digest (i.e. vaporize/atomize/excite/ionize) matrix species introduced into the plasma impacts the accuracy of measurement results. Robustness is usually assessed by means of Mg and/or Zn ionic:atomic emission line ratios and plasma rotational temperatures upon the introduction of easily ionized elements (EIEs), respectively, which can easily lead to plasma disruption; therefore introducing such elements (e.g., Sr, Ca) at varying concentrations allows to gaining a greater understanding of a plasma’s robustness characteristics. Thus, in order to investigate spectroscopic matrix effects and to determine the robustness of the LS-APGD secondary excitation source for LA-generated particle aerosol, magnesium ionic:atomic ratios and plasma rotational temperatures were monitored after ablating calcium (Ca) and/or strontium Sr pellets and the introduction of the respective aerosol into the LS-APGD. Mg ionic:atomic ratios were employed for serving as a “robustness parameter” in this study. The usefulness of this parameter for monitoring excitation/ionization efficiencies is described in detail by Mermet.
The ablated pellets contained varying amounts of Ca and Sr (Table 1), respectively, as matrix elements, while the Mg amount was held constant. Laser ablation was accomplished using a 213 nm laser ablation system, applying LA-optimized LS-APGD instrumental parameters (Table 2). Illustrated in Fig. 3, no significant differences could be observed in the Mg ionic:atomic (279 nm : 285 nm) ratios for increasing amounts of (a) Ca (Mg II:I = 1.25 ± 0.25 (1 x SD)) and (b) Sr (Mg II:I = 0.66 ± 0.04 (1 x SD)). Generally, the plasma is considered as robust if a change in concentration of the matrix inducing species does not affect the ionic:atomic ratios of the Mg emission. The observed Mg ionic:atomic ratios upon introduction of LA generated aerosol containing Ca and Sr are in good accordance with data observed for liquid sample introduction (Ca Mg II:I = 1.46 ± 0.17 (1 x SD) and Sr Mg II:I = 0.71 ± 0.14 (1 x SD)). The lower Mg ionic:atomic ratios upon the introduction of Sr compared to that of Ca can be possibly explain by the lower thermal properties.
Figure 7.3 Monitoring the Mg ionic:atomic line ratios to determine plasma robustness when ablating a) calcium and b) strontium into the LS-APGD
Next, the magnesium analyte signal was analyzed in comparison to the percent of Ca and Sr in the pellet. In the Ca pellet, the Mg ionic and atomic signal illustrated a slight decrease under increase Ca concentrations as seen in Figure 4a. In the case of Sr, the Mg signals seem hardly effected and seen in Figure 4b. When comparing % RSD of the data, Mg in the Ca pellet deviated by ~20 percent while only ~5 percent in the Sr pellet. Reasons can be deduced to the similar properties mentioned earlier in that Sr has lower heat of vaporization, melting/boiling points, and metal-oxide bond dissociation energy indicating relative ease of ionization. With Ca’s thermal properties being higher, more work must be implemented for efficient excitation, more particularly ionization.
Figure 7.4 Monitoring of the Mg atomic and ionic line intensity while introducing a) calcium and b) strontium. 

Plasma rotational temperatures were determined by fitting the nitrogen molecular bands as done in previous LS-APGD rotational temperature measurement’s. Again no significant differences could be observed by introducing easily ionized elements, i.e., Ca and Sr and can be seen in Figure 5. The plasma rotational temperatures were determined to be ~1100 K, which also is in good accordance with a previous study demonstrating plasma robustness/stability using a LS-APGD source for the digestion of laser ablated particles along with previously mentioned liquid introduction. Another note, the %RSD determined across the range concentration range of Sr and Cr were each
under 2% further illustrating plasma stability, even upon the introduction of typical elements that induce matrix effects.

![Graph of LS-APGD rotational temperatures (K) in reference to the addition of calcium and strontium.]

**Figure 7.5** Monitoring LS-APGD rotational temperatures (K) in reference to the addition of calcium and strontium.

**CONCLUSIONS**

The knowledge and understanding of the LS-APGD’s plasma characteristics is essential for a full assessment of this source’s potential and usefulness as a secondary excitation source in case of direct solid sampling by means of laser ablation. The within this work performed studies demonstrate well that the LS-APGD has the capabilities, due its high plasma power densities (~10 W mm\(^{-3}\)), to
effectively digest even relatively large particle aerosol generated by high energy ns laser pulses. The influence of instrumental parameters (i.e. LA carrier gas flow rate, electrode gap, and glow discharge current) on the sensitivity demonstrated the necessity of a thorough optimization. Low He carrier gas flow rates (i.e. 0.1 L min\(^{-1}\)) and large electrode distances are suggested to be favorable in terms of longer plasma residence times. However, in case of larger electrode gap distances and larger plasma volumes, less energy is available per mm\(^3\) for excitation processes, which results in an overall loss of sensitivity. The determination of actual plasma residence times have to be addressed in future investigations, using e.g. a camera [ref Aeschlimann]. Plasma robustness investigations yielded no significant changes in plasma rotational temperatures as well as in ionic:atomic Mg emission lines upon the introduction of easily ionized elements into the plasma. This effect has to be further addressed in future investigation will focus on non-spectroscopic matrix effects, investigating parameters influencing signal suppression/enhancement.

**ACKNOWLEDGEMENTS**

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REFERENCES


CHAPTER 8

SUMMARY

The research presented here demonstrated the utilization of two chemical analysis methods both aiming to improve the miniaturization of current techniques. Firstly, capillary-channeled polymer (C-CP) fibers were employed as the stationary phase of a micro-solid phase extraction (µSPE) tip for the extraction of proteins from complex mixtures prior to analysis by matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). Secondly, a miniaturized plasma was employed as an excitation source for elemental analysis. Chapter 1 introduced the need for miniaturization on both these fields (clinical and elemental analysis) alongside the fundamental theory required in each application such as MALDI-MS and SPE for clinical analysis and atomic analysis instrumentation and newer developed sources such as the electrolyte cathode discharge (ELCAD) for elemental analysis.

Chapter 2 provided the preliminary results of employing C-CP fiber based µSPE tips for the extraction of proteins (cytochrome c and lysozyme) from buffered species (Tris-buffer and synthetic urine). The C-CP fibers effectively retain proteins by adsorption onto the hydrophobic stationary phase (polypropylene) allowing concomitants to be washed away with an aqueous
rinse. This process allows for improved analyte detection by MALDI-MS with increased signal-to-noise characteristics and spectral clarity. Importantly, preliminary results demonstrated the usefulness of performing extractions on low quantity of sample down to 1 µL, even in potential biofluids (synthetic urine).

Chapter 3 extended on chapter 2 by further investigation of each step in the SPE process including the elution process and washing step as well as the SPE tip lengths. It was seen that with more contaminated matrices, larger wash volumes were required. For the tip length and load volume presented in this chapter, 100 µL proved to efficiently remove all unwanted species. The load and elution volumes were also investigated in respect to tip length. It was found that the lower aliquots for the load/elute step favored smaller tip lengths as an increased efficiency of interactions on the fiber surface. Having such small aliquots on larger tips prohibit effectively obtaining the necessary interactions such as saturating the tip with elution solvent to adequately remove the adsorbed protein. This chapter also determined that a function of protein concentration is related to tip length similar to the aliquot volume in that lower quantities of protein are more efficiently extracted from smaller tips (2.5 mm) compared to larger (10 mm).

Chapter 4 presented the employment of C-CP fiber µSPE tips for clinical applications such as urine analysis for biomarkers. Three urinary proteins that have previously been sought out for biomarker identification were effectively extracted from a certified human urine matrix. Studies such as elution solvent
characteristics and aqueous wash were performed to effectively extract the urine proteins from its matrix. Resultant spectra showed excellent spectral clarity of the proteins as well as significant decrease in the background noise of the analysis by removal of the ion suppressing species (urine matrix). Limits of detection from this process of extraction and MALID-MS detection were determined to be 58 and 600 pico-grams for β2-Microglobulin and retinol binding protein, respectively.

Chapter 5 presented information regarding the liquid sampling-atmospheric pressure glow discharge as a miniaturized source for elemental analysis. The aim of this chapter was to determine the plasma characteristics such as rotational temperature, excitation temperature, and electron number density in regards to the operating parametric evaluation. The studies presented here give insight to the LS-APGD plasma fundamental processes. These characteristics were also studied when introducing typical matrix-inducing species. It was found that even upon addition of large concentrations of said species, temperatures and plasma electron number density remained relatively unchanged. All experiments were performed with the goals of developing a miniaturized elemental analysis source with sights set on portability.

Chapter 6 demonstrated the effectiveness of the LS-APGD in terms of being “immune” to spectrochemical matrix effects. Studies were performed by introducing 11 test elements to monitor plasma robustness such as how the plasma temperature is affected by the introduction of said species as well as
monitoring the excitation/ionization characteristics of the Mg (II/I) lines. It was found there was a correlation between plasma changes and the second ionization potential (IP) of the test elements as lower second (IP) induced the greatest effect. These results were very similar to studies performed with the inductively coupled plasma (ICP). Even though correlation was slight, the effect was minimal due to the high plasma power densities being able to effectively digest analyte and resisting change in its characteristics.

Chapter 7 presented in more detail the LS-APGD as a secondary excitation source following laser ablation. Studies here described some parametric evaluation in regards to applied current, gas flow rate of particles into the plasma, and electrode gap. Two different lasers were employed, 1064 and 213 nm with two different energies, 50 and 5 mJ respectively. Plasma characteristics were also monitored in regard to the addition of species that typically induce the largest matrix effects (strontium and calcium). It was shown that the ionization/excitation conditions remained relatively unchanged; however, in the case of calcium, there was signal suppression with increased amounts of calcium entering the plasma.

The research presented here established an innovative method for the extraction of proteins prior to MALDI-MS analysis. Further studies will be implemented especially in terms of fiber modification for the capture of specific proteins that would be beneficial, especially in clinical analysis. Research also provided a greater insight to plasma characteristics of the LS-APGD in hopes of
further improving the source as a miniaturized elemental excitation/ionization source. In both instances, miniaturization can be seen as a key aspect in which both projects are progressing towards. Over all, analysis methods in which sample size, waste, and operating costs can be decreased will allow for more efficient and timely analysis.

Future goals pertaining to C-CP fiber packed µSPE tips need to be addressed in multiple fashions. Firstly, studies must be investigated pertaining biofluid standards in regards to effectively extracting desired proteins. The studies demonstrated in this dissertation pertained to spiked samples; therefore the transition must be made for standard solutions. Secondly, C-CP fibers modified for SPE illustrate great promise for the capturing of specific proteins, such as bovine serum albumin (BSA). Much research is being dedicated towards eliminating abundant proteins (i.e. SA) in order to improve detection of low quantity proteins (potential biomarkers). Lastly, it is foreseen that C-CP fiber packed µSPE tips could be implemented for metal extractions. Functionalizing C-CP fibers for capture/release of metal species, such as uranium could be beneficial in terms of trace metal analysis as sample preparation is a vital step.

In regards to the LS-APGD, future work can be seen with multiple directions. In its implementation for excitation for OES analysis, more optimization and research must be placed on the source components such as electrode make-up, sheath gas composition, and source structure (i.e. geometries). While indeed fundamental studies on the plasma characteristics
were employed here, other experiments are warranted such as determining particle residence time, plasma composition (is helium inside or surrounding the plasma), and more investigation of matrix effects. Overall, these studies must be performed as the LS-APGD is demonstrating promising results for a miniaturized ionization/excitation source in elemental analysis along with potential for a field deployable instrument.