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Incorporation of Capillary-Channeled Polymer (C-CP) Fibers Into Micropipette Tips For Solid Phase Extraction With Applications in Bioanalysis

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INCORPORATION OF CAPILLARY-CANNELED POLYMER (C-CP) FIBERS INTO MICROPETTE TIPS FOR SOLID PHASE EXTRACTION WITH APPLICATIONS IN BIOANALYSIS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment of the Requirements for the Degree Master of Science Chemistry

by
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Accepted by:
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Dr. Sarah Harcum
Solid phase extraction (SPE), is a widely used sample preparation method that is currently experiencing a surge in research interest, clever architecture/format development, and stationary phase characterization.\textsuperscript{1-3} SPE devices commonly exist in syringe, cartridge, disk, and micropipette tip formats.\textsuperscript{4} Micropipette tip SPE has enhanced the determination of proteins by electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) mass spectrometry (MS) by handling micro-liter volumes of sample and buffer removal/solvent exchange.\textsuperscript{5-7} Buffers have deleterious effects on ionization taking place in MS sources due to preferential ionization, ion suppression effects, and the formation of adduct species competing for ionization in MS.\textsuperscript{8} Using micropipette tip SPE, proteins can be selectively extracted from biological buffers and exchanged into a solvent more conducive to MS; thereby improving signal and sensitivity. Capillary channel polymer (C-CP) fibers have been used for reversed phase separations of proteins with a number of positive attributes realized.\textsuperscript{9} The highly efficient fluid transport, low material cost, wide range of chemical diversity, and robust fiber materials make the C-CP fibers excellent candidates for various separation formats. Presented here is the introduction of polypropylene (PP) and polyester (PET) C-CP fibers as a stationary/support phases for the SPE of proteins from defined buffered solutions prior to ESI-MS\textsuperscript{10}
and MALDI-MS analysis. Using two benchmark proteins, lysozyme in phosphate buffered saline (PBS) and myoglobin in tris buffer, it was demonstrated by ESI-MS and MALDI-MS that PP C-CP fiber-extracted protein samples exhibited more efficient protein ionization, higher signal-to-noise ratios, and more precise molecular weight determinations when compared to buffered protein samples of the same concentration. The extraction efficiencies of PET and PP fibers have been compared for both lysozyme and bovine serum albumin from PBS. Quantitative as well as qualitative data obtained using ESI-MS, MALDI-MS, and UV-VIS demonstrates the capabilities of C-CP fibers as SPE sorbents in bioanalysis.
DEDICATION

I am dedicating this work to my loved ones. It is through their support, attention, and dedication that I gathered strength and motivation to complete my graduate work at Clemson University.

To my family, thank you for lending a phone call, a letter, a watermelon, or fruitcake in the mail just to remind me that I have unconditional love and support.

To my friends from NSU, thank you for the fond memories and laughter that made the bleak times at Clemson seem short and passable.

To Sebastian, thank you for the future. Often I look ahead to the life and adventure we will experience in the many roads and places we may travel together.
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I thank the entire Marcus Research Group both past and present for their continued support and friendship throughout my graduate career. Our teamwork and group ethics are important and vital to the success of a graduate student. Each person in the Marcus Research Group has lent a supportive and enduring friendship that facilitated my work. I also thank Adrianna Chaurra for her friendship and support.

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CHAPTER 1

INTRODUCTION

The task of sample preparation has challenged analytical laboratories by demanding more reproducible results, accommodating lower technical skills, decreasing the use of organic solvents while providing cleaner extracts, and making the process quicker and more cost effective.\textsuperscript{4} It has been previously shown that more than 60\% of analysis time was spent on preparing the sample as opposed to only 7\% on data acquisition or the actual measurement.\textsuperscript{11} Many techniques exist for sample preparation such as liquid-liquid extraction (LLE) and solid phase extraction (SPE). As a sample preparation technique, LLE has enjoyed popularity, but suffers due to its slow labor-intensive nature and environmental hazards of disposing large amounts of organic solvents.\textsuperscript{4} However, SPE is more easily automated, faster, and generally more efficient than LLE. SPE is a field of methods employed for sample preparation, isolation, concentration, and purification of analytes in complex matrices, defined, and undefined solutions. The technique has become a popular and powerful technique for both chemical isolation and purification.\textsuperscript{12} The mechanism of SPE involves the adsorption of analyte from a liquid or gaseous solution to a solid phase, washing or rinsing to remove interferences, and finally elution in a solvent suitable for further instrumental analysis. The chemistry of the solid phases are
continually developed to meet the needs and applications of SPE in pharmaceutical, fine chemical, biomedical, food analysis, organic synthesis, and environmental laboratories.\textsuperscript{12, 13} The development of SPE sorbent types and architecture formats has experienced considerable growth in the last decade. For example, between 1994 and 1998, manufacturers introduced 84 new SPE products with various designs, applications, and stationary phase formats.\textsuperscript{14}

1.1 Brief History of Solid Phase Extraction

SPE was not recognized as a scientific technique until the 1970s. Considering the three objectives of SPE: 1) concentration, 2) removal of unwanted molecules from the sample (commonly referred to as sample clean-up), and 3) removal of the sample matrix and/or solvent exchange, it is somewhat surprising that the technique did not gain momentum earlier.\textsuperscript{15} The advent of disposable sorbent cartridges, containing porous particles to allow sample preparation and processing by gentle suction, contributed to the initial popularity of SPE devices in the 1970s.\textsuperscript{16} However, prior to the 1970s, solid phases or particles such as carbon and porous polymeric resins were simply known as “sorbents” and were used to removed trace organic solutes from aqueous samples.\textsuperscript{4} Columns filled with this type of solid sorbent were sometimes referred to as “accumulation columns”. As an example, in 1971 the American Public Health Association became quite concerned about the wide variety of chemical pollutants found in water supplies. Of primary concern were organics, more specifically pesticides that had found their way to municipal water
supplies. In 1972, using Rohm and Haas XAD-2, a polymeric resin, Burnham et al. completed a study that demonstrated the need for sample pre-concentration in order to determine pollutants in water samples. The study analyzed pollutants in the water supply of Ames, Iowa which had been noted to have an unpleasant taste and aroma. Preliminary data had identified the contaminants as phenols and cresols. However, a pre-concentration step was necessary to allow analytical detection and identification of the contaminants. To accomplish this, Burnham et al. extracted the unwanted organics by passing 150 liters of water through a polymer resin column (1.5 X 7.0 cm) at 50 mL/min. The adsorbed organics were eluted by passing 15 mL of ethyl ether through the column. The ethyl ether was evaporated into a smaller volume and smaller portions were analyzed by gas chromatography-mass spectrometry (GC-MS). Interestingly, the identified contaminants were related to an abandoned coal gas plant, operated in the 1920s, near the city of Ames. The residues were buried in a pit and had found a way to the water supply. This is an example of how SPE can be used to extract, concentrate, and re-introduce analytes into a solvent ready for instrumental analysis, making analytical detection and identification possible.

Several years prior to 1970 and the work of Burnham et al., activated carbon had been used as an absorptive material for concentrating organics in water. Carbon was used as an adsorbent material because of its high surface area and high affinity for organic isolates in water. The method was called the carbon adsorption method (CAM). Junk et al. studied and reported the recovery
of 58 various compounds ranging from alcohols, alkanes, aromatics, PCB, and phenols. The average percent recovery reported using carbon as a sorbent was 22%, conversely the average percent recovery using the XAD-2 resin for the 58 compounds was 59%. They found that activated carbon suffered low recoveries due to irreversible adsorption when compared to organic polymer resins for isolating organic pollutants from water.

After the 1970s stationary phase development in SPE followed closely with that of high performance chromatography (HPLC). In both techniques, bonded-phase silica, more specifically C18, dominates the field. Early literature using SPE on bonded phase silica was published in 1978 and described the use of the Sep Pak™ C18, a trademark of Waters Corporation, Milford, MA., for the cleanup of histamines from wines and musts. As a stationary phase C18, due to its carbonaceous character, is well suited for non-polar interaction HPLC or SPE. Currently, SPE sorbent types in use are of the bead shape and are commonly packed into a cartridge or a column. Also of current development and use is the incorporation of sorbent particles into disk formats. The disk format has allowed good extraction recoveries at relatively high flow rates due to the use of small porous particles and the rapid mass transport they afford.

Early in the 1990s, rigid porous monoliths made from organic polymers were introduced as alternative stationary phases for liquid chromatography. Shortly after, monolithic polymer stationary phases made their way into the field of SPE. The first monolithic polymer incorporated into a SPE device was poly(styrene-co-divinylbenzene) (PS-DVB). Huck et al. investigated the
recovery of 13 pesticides, using both PS-BVD and octadecyl silica (ODS) phases. It was found that PS-DVB was able to achieve an average recovery of 77% for all thirteen compounds in comparison to 69% achieved by ODS. PS-DVD copolymers proved very effective for the recovery of non-polar compounds due to their inherent hydrophobicity. Monolithic sorbents were also found to be excellent materials for high flow rate SPE experiments. The advantages of monolithic SPE sorbent are the highly interconnected pores, excellent permeability, and enhanced mass transfer due to enabled convectional flow through the channels. Though PS-DVB copolymers were highly effective for extraction of non-polar analytes, polar analytes were most often less well retained. In an effort to expand polymer-based sorbents to achieve hydrophilic interactions, a variety of functionalized polymer beads were introduced to enhance polar analyte retention.

In addition to aiding the analysis of analytes in environmental samples, SPE has also found a niche in the area of proteomics, most notably the extraction of proteins and peptides. New format types, including sorbent phase-packed micropipette tips, have permitted the handling of micro-liter amounts of samples. This has proven especially important in bio-analysis where the quantitative transfer of small amounts of sample is sometimes a necessity.

The evolution of SPE sorbents, formats, and architecture, often parallel and compliment those in HPLC. While an appreciation of historical milestones is equally important in understanding the impact SPE has had on sample
preparation, it is even more important to understand the mechanisms, chemistry, and driving forces that make the technique such as success.

1.2 Basic of Mechanisms of Solid-Phase Extraction

SPE can be well related to LLE, and comparing the two is an exceptional means of understanding SPE.\textsuperscript{33} In a LLE experiment, the analyte and sample matrix is agitated with an extracting solvent, which is not miscible with the sample matrix. After agitation is complete, the sample matrix and the extracting solvent will form two layers with the analyte being partitioned between the layers. The equilibrium formed is governed by the partition coefficient for the analyte, which is the ratio of the concentration of the analyte in the extracting solvent to the concentration in the sample matrix.\textsuperscript{13} A high partition coefficient would indicate that most of the analyte has migrated to the extracting solvent, which is desirable and often the goal of any LLE and also indicates that the extraction solvent provides a more favorable environment for the analyte.\textsuperscript{15} However, LLE suffers many drawbacks as presented in section 1.1. One other difficulty, substances with similar distribution coefficients ($K_D$) require multiple extractions because the number of theoretical plates is low.\textsuperscript{14, 36, 37} All things being considered, SPE can provide fewer steps, less solvent consumption, reduced labor, increased efficiency, and allow easy sample collection and automation.

SPE involves the partition of an analyte between a solvent and a solid phase, with the solid phase having a greater attraction for the analyte than the solvent in which the analyte is dissolved.\textsuperscript{12} As the sample solution passes over a
solid phase, the analyte can be adsorbed and concentrated on the sorbent while the remaining sample components pass through un-retained. In the case that additional sample components interact with the sorbent, they can be later eluted by a rinsing step prior to eluting the analyte of interest. Figure 1.1 illustrates the three simultaneous interactions involved in any SPE experiment. Each interaction affects the retention of the isolate, isolate elution, and competition for adsorbent space by both the isolate and species present in the matrix.

Figure 1.1 Isolate, Sorbent, and Matrix Interaction Triangle. The three interactions acting in concert that affect the efficiency of a SPE experiment.12
1.2.1 Retention/Adsorption

Retention or adsorption can be applied to most natural physical, biological, and chemical systems. Adsorption in SPE occurs when a substance moves from one phase and accumulates on another. The adsorbing phase is commonly referred to as the sorbent, adsorbent, solid surface, or stationary phase. The adsorbing species (analyte) is referred to as the adsorbate, isolate, or solute.\textsuperscript{38} In the case of SPE, an equilibrium concentration of the analyte develops between the matrix solution and the solid surface. This equilibrium can be described as the distribution of the adsorbate between the solid and liquid phase at the point when no additional net adsorption occurs.\textsuperscript{38} The equilibrium can be thermodynamically defined by the distribution coefficient \( K_D \). \( K_D \) is a measure of the amount of analyte that has adsorbed on, interacted with, or the amount that has penetrated the solid phase versus the amount that remains in the sample matrix solution.\textsuperscript{15} \( K_D \) can be visualized by the following equation:

\[
K_D = \frac{[\text{analyte}]_{\text{sorbent}}}{[\text{analyte}]_{\text{sample}}} \quad \text{Eq. 1}
\]

A \( K_D > 1 \) would indicate that the analyte has a stronger affinity or interaction with the sorbent than the sample matrix solution. Whereas a \( K_D < 1 \) would indicate that a greater fraction of the adsorbate remains in the sample solution, thus indicating the sample solution provides an energetically more stable environment for the adsorbate.\textsuperscript{39}
The retention behavior of a given analyte, or the $K_D$, is a function of three factors: the analyte, the solvent, and the sorbent. For the analyte, molecular weight, molecular size, molecular structure, molecular polarity, steric form or configuration, and if present, the nature of competitive analytes present in solution all play important roles affecting the extent of retention. With any analyte, the solvent and sorbent can be altered and thus the retention factor, or the $K_D$, can be manipulated for a specific purpose. For the sorbent, surface area, physicochemical nature of the surface, availability of that surface to the analyte, physical size, morphology, and form all play crucial roles affecting adsorption.

As an example, organic compounds of a particular class become increasingly insoluble in water as molecular weight and size increase. Therefore, as organic compounds in aqueous solution become increasingly insoluble, their respective degree of expulsion from solution onto a non-polar stationary phase increases. As with high performance liquid chromatography, the molecular size of the adsorbate can affect retention where mass transport into porous phases is of concern. Small and large molecules of a given chemical class may have different rates of diffusion or mass transport within porous sorbents. Equally important for the adsorption process are both the pH and temperature of a given system. The pH of a given system can effect the degree of ionization or charge of a given adsorbate and/or adsorbent, thus affecting the types of adsorbate/adsorbent interactions possible. Temperature can affect the equilibrium capacity or sorbent saturation and the rate of adsorption.
Thermodynamically, adsorption processes are, on average, exothermic. The enthalpy change can be expected to be on the same order of magnitude as for condensation and/or crystallization reactions. Also, the capacity of a given system is expected to increase with decreasing temperature; conversely, the rate at which a given system reaches equilibrium capacity is expected to increase with increasing temperature.38

Retention can also be affected by the pretreatment of the sorbent bed. Solvation, a process of “prewetting” the sorbent bed, is a common practice of preparing the sorbent to receive the analyte. It is achieved by passing several bed volumes (volume of liquid phase required to fill the fraction of SPE device not occupied by sorbent) of the sample/matrix solvent through the sorbent bed before introducing the analyte. Solvation has been shown to create an environment that allows analytes to interact more reproducibly with sorbents.12 The reproducibility of adsorption of any SPE experiment benefits from the homogenous treatment of the sorbent, thus the goal of solvating the sorbent bed. However, even though routine treatment of the sorbent increases the likely precision, behavior of adsorbates at the solid-liquid interface is of even greater complexity, especially when macromolecules are of concern.

The retention of macromolecules, such as proteins, often differs drastically from that of small molecules. The difference in retention characteristics is attributed to the conformational changes that macromolecules can attain both in the sample solution and at the sorbent interface.39 Three areas of classification exist to describe adsorption characteristics of proteins and related
macromolecule species. They are: 1) protein characteristics, including protein conformational variability, geography and nature of hydrophobic sites, three-dimensional structure in solution, net charge and charge variance, and isoelectric point; 2) surface characteristics, including heterogeneity and nature of topography, electrical potential, hydrophobicity, water binding, and composition; and 3) medium conditions, including buffer type, pH, flow characteristics, equilibrium concentration, ionic strength, and temperature.40

The adsorption of molecules at the solid-liquid interface is the result of electromagnetic interactions between the individual atoms, ions, molecules, or molecular regions of the adsorbate and the surface.38 The binding forces can be as weak as van der Waals to as strong as covalent bonds in chemisorption.40 In principle, adsorption can be categorized into four regimes; exchange, physical, chemical, and specific. Exchange adsorption, also called ion exchange, is described by the electrostatic interaction of an ionic species with an opposite charge on the surface of a sorbent. Physical adsorption is comprised of van der Waals forces resulting from London dispersion forces and electrostatic forces. Chemical adsorption results in a change in the chemical form of the adsorbate due to the reaction with the adsorbent resulting in a chemisorptive bond. The chemisorptive bond is notably stronger than the adsorbate/adsorbent interaction resulting from physical adsorption. Specific adsorption is a hybrid between both physical and chemical adsorption. It involves the specific attachment of an adsorbate to a functional group present in an adsorbent surface that does not result in transformation of the adsorbate. Specific adsorptions can have a wide
range of binding energies, ranging between the low binding energies of physical adsorptions to the higher energies associated with chemisorption. In most cases, the total net adsorption describing the affinity of an adsorbate for an adsorbent is the combination of all four adsorption regimes.\textsuperscript{38}

The solvent in which the adsorbate is solvated plays a key role in the extent of adsorption. Key attributes of the solution phase that affect adsorption are both the surface tension and solubility. Solvophobicity, related to solubility, describes the insolubility of a substance in the solvent phase. For any given solid phase extraction experiment, it is desirable for an adsorbate to have a relatively high solvophobicity for the initial sample matrix. In that way, the adsorbate can partition from that solvent at interfaces with other phases.\textsuperscript{38} In other words, the bonding that occurs between an adsorbate and the solvent must be broken to allow adsorption from that solvent to the adsorbent. The more soluble an adsorbate is in a given solvent, the smaller the extent of adsorption. Equally important for adsorption to occur is the particular affinity an adsorbate may have for a particular adsorbent. Therefore, two of the primary forces affecting the degree of adsorption are both the solvophobicity of the adsorbate in a given solvent and the affinity of the adsorbate for the given adsorbent. These forces are continually acting in concert and in many ways dictate how effective a SPE experiment can be. The types of interactions will be discussed later in detail.
1.2.2 Sample Purification-Rinsing/Washing

The desired result of retention is to extract the analyte of interest from the sample matrix solution. However, at times, species from the sample matrix can be co-retained with the analyte of interest on the solid phase. To eliminate interferences from the co-retained species during the analysis stage a rinsing or washing step is employed. Wash conditions can be carefully controlled to preserve maximum analyte retention and co-retained species elution. The addition of a rinsing step involves another distribution between the analyte and the interfering species, the sorbent and the liquid wash phase passing over the sorbent. However, retention must be strong enough such that after passing ~20 bed volumes of wash solvent through the SPE apparatus, elution will not occur. As an example, when analyzing proteins by electrospray ionization mass spectrometry (ESI-MS), it has been shown advantageous to perform a wash step to removed any residual buffering species that could compete for ionization during analysis.

1.2.3 Elution

When an analyte is retained on a solid phase, a more desirable environment must be provided to remove or elute the analyte from the solid phase. In most cases, a liquid is employed to elute or desorb the analyte from the stationary phase. If the liquid provides a more desirable environment for the analyte, the analyte will desorb from the sorbent and exist in the liquid, which can be collected. The distinction between traditional chromatography and SPE,
where retention and elution are concerned, is that in SPE the goal is to immobilize the analyte until movement is facilitated by the elution solvent. In some cases, it is desirable for the elution solvent to completely desorb the analyte in the smallest possible volume, thereby concentrating the analyte; in some cases this is not an issue. For example, an adsorbate can be concentrated by carefully selecting extracting conditions such that the adsorbate is retained in a narrow band and then eluted using a smaller volume than it was originally contained. These near perfect conditions are the result of a well understood extraction process where careful attention has been placed on the aforementioned conditions (temperature, pH, sorbent type, etc.) affecting retention and elution.

A complete solid phase extraction involves the careful manipulation, selection, and control of all parameters. Sorbent type, solvent (both wash and elution), and solution matrix can all be manipulated to enhance analyte retention and reintroduce the analyte into a solvent more conducive to further analysis. Figure 2 is an example of what a complete solid phase extraction can resemble. The effective isolation, recovery, and purification of a particular analyte has been achieved.
1.3 Objectives of SPE

1.3.1 Concentration of Analyte

Limits of detection, signal to noise ratios, and sensitivity all play an important role in most analyses. SPE can assist chemical analysis by concentrating the analyte in the following ways: 1) A large volume of the sample can be passed through a minimized volume of sorbent that is optimized to elicit maximum retention of that analyte, 2) Minimization of elution volume. 3) Elution into volatile solvents which can be easily evaporated, further concentrating the
analyte. In the case of analyte concentration, sorbents that have a high capacity for a particular analyte, thus minimizing the amount of sorbent needed or the sorbent bed volume and concentrating the analyte in a smaller volume can be chosen. By changing the elution solvent to provide a more desirable environment for the analyte, the elution volume can be minimized, thus placing the same number of moles of analyte (assuming a highly efficient recovery) in a smaller volume. Also, by elution into volatile solvents the volume of that eluent can be reduced by evaporating the volatile solvent.

1.3.2 Interference Removal/Solvent Exchange

Assuming a successful concentration of analyte is achieved by SPE, analysis can still be quite complex if sufficient sample clean up is not reached. Interfering compounds or matrix effects can co-elute and mask or suppress the signal from the analyte. In a complete and optimized SPE experiment, sorbent type, wash phase, and elution phase are chosen to selectively bind analyte, wash interfering compounds away, and elute the analyte free of interference and suppression. This is the goal of sample clean-up. For example, proteins and peptides often exist in biological buffers of high salt concentration. These small inorganic salts can compete for ionization when the in solution with proteins during mass spectrometry (MS) analysis. It is often necessary to perform SPE on protein samples to allow protein retention, buffer removal, followed by protein elution/ solvent exchange prior to MS analysis.
In keeping with the theme of SPE objectives, it would be unjust not to discuss the benefits of sample matrix removal/solvent exchange. Instruments such as mass spectrometers, gas chromatographs, and nuclear magnetic resonance elicit the need for the analyte to be housed or contained in a specific solvent or solution. SPE can readily achieve solvent exchange by eluting the target analyte in a mobile phase favorable to the instrumental technique being employed. Sorbent type, and elution conditions can be chosen and optimized so that the eluted analyte can be directly analyzed on the instrument of choice with no further sample preparation or pre-analysis treatment. As an example, gas chromatography mass spectrometry (GC-MS) is a common analytical technique employed for samples analysis. However, it requires that analytes be introduced in volatile solvents to permit entrance to the gas phase. This would indicate the careful selection needed for the SPE methodology to allow sample elution by a solvent conducive to the respective GC-MS requirements.

1.4 Column Technology

In addition to SPE being an exceptional methodology for sample preparation, it also offers a multiplicity of architectures and sorbent types. The apparatus technology and sorbent type can be fabricated to fit the specific needs of a particular setting, instrument, or application. SPE architecture and sorbent types will be discussed in detail so that the diversity of available formats is realized.
1.4.1 Architecture

The four basic formats for SPE are cartridges, disks, syringe barrels, and micropipette tips. Each type offers its own set of advantages and has particular applications and settings best suited for its size, shape, and form. A standard SPE cartridge can be made or formatted into a typical syringe barrel or some variation. In most cases, the stationary phase occupies approximately one-third of the volume of the SPE apparatus. Sorbent bed masses in cartridges can range from as little as 10 mg to as much as 10 g. Judgment and selection of cartridge volume and bed mass is dependent on the limit of detection, desired sensitivity, and sample size. Most cartridges consist of a polyethylene body and allow positive pressure as well introduction to a vacuum manifold. The packing material is compressed with frits to hold it in place and improve cartridge flow characteristics. A SPE cartridge in its most simplistic form is illustrated in Figure 3.

Chromatographic media formatted into small objects resembling coins or resembling a very thin, wide SPE cartridge are referred to as disks. Three types of disks are employed as commercial products. The sorbent can be 1) enmeshed into an inert polymer or 2) trapped in a glass fiber or 3) paper filter. Because sorbent material can be trapped or contained by another size/shape retaining compound or material, the same sorbent types found in cartridges are also found in disks. The disk format results in a SPE device much like a cartridge and is commonly housed in a syringe barrel and used in the same manner as a cartridge.
Figure 1.3 The SPE cartridge. The SPE cartridge can take various forms, but always contains an area for sample introduction or sample reservoir, and an area filled with sorbent material.\textsuperscript{4}

Driving the development of the disk format was the need to process sample volumes in less time than the cartridge architecture permitted. Because total length of the sorbent bed traveled by an analyte solution is usually shorter in the disk format, faster flow rates are not as pressure limited as with the cartridge format. The small volume of the disk also allows smaller conditioning, washing, and elution volumes to be used in extraction.\textsuperscript{13}

Syringe barrels are usually the largest of the four types and can have packing volumes and packing masses of 1 to 25 mL and 50 mg to 10 g, respectively. Typically syringe barrels are made of polypropylene. Sample reservoirs come in a range of sizes and are more commonly 50 to 100 mL and
are used for the processing of larger sample volumes such as environmental samples.¹³

New formats for sample preparation have been developed due to the push for miniaturization and the need to handle microliter and sub-microliter amounts of analyte. In the micropipette SPE devices, the stationary phase is packed, embedded or coated on the walls of the pipette tip. Micropipette SPE devices are especially beneficial when dealing with samples such as biological fluids, when purification and concentration are strategic to instrumental analysis. Micropipette SPE devices are heavily involved in the sample preparation techniques in genomics, proteomics, and metabolomics, where quantitative handling and transfer of micro-volumes of sample is crucial.⁸ Matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) and Electrospray ionization mass spectrometry (ESI-MS), two techniques for bioanalysis, both require microliters of sample and thus benefit from analytical SPE on the micro-scale.⁴¹, ⁴² In the 1990's, the first commercially available micro-SPE devices were sold as micropipette tips with C18 chromatographic media incorporated into the tip. The tips were developed by the Millipore Corporation (Bedford, MA) under the trade name of ZipTips™. Present day micro-SPE tips are available from a number of manufacturers, offering a wide range of stationary phase chemistries that may be used in different types of applications.
1.5 Interactions

The sorbents employed in SPE often are parallel to those used in liquid chromatography. Sorbent/support phases can include, but are not limited to, reversed phase, ion exchange, normal phase, hydrophobic interaction, immobilized metal affinity, affinity chromatography sorbents, and polymer-based phases.

1.5.1 Non-polar Interactions

In this type of separation, a non-polar, relatively hydrophobic stationary phase is employed to extract non-polar analytes from polar, often aqueous, sample matrices. In some cases, an aqueous wash is applied to the SPE device prior to elution by an organic solvent as a means of sample clean-up and a matrix removal. The mechanisms for interaction are van der Waal’s forces as well as secondary interactions that can aid in extraction such as hydrogen bonding and dipole-dipole interactions. More specifically, the mode of adsorption for reversed-phase are the interactions between the carbon-hydrogen bonds of the adsorbate and the carbon-hydrogen bonds of the adsorbent. Unbonded silica does not yield non-polar interactions alone, it is only when sorbent functional groups such as C18, C8 or CH are added that non-polar interaction arise. C18 is the most commonly used chemically bonded silica exhibiting non-polar interactions. However, because many adsorbates that are or have non-polar sites can be retained on C18, it is considered a non-selective sorbent.
As a general rule, non-polar extractions are not as selective as ion-exchange or even polar extractions. As such, reversed-phase SPE might not prove highly successful in an attempt to selectively extract an adsorbate from other samples matrix components that are similar in structure. However, in some cases, usually environmental, it is desirable to extract groups of compounds that are dissimilar in structure, but collectively non-polar. Figure 4 demonstrates the problem associated with a non-selective stationary phase when several analytes of similar structure or chemistry are present. In some cases, non-selective sorbents can simultaneously retain unwanted compounds from the sample matrix along with the analyte. Non-selective retention is demonstrated in by the adsorption of all the species present in the solution. Selective retention is demonstrated by the selective adsorption of the analyte and the flow through of the other species present in the solution.

Reversed-phase interactions are usually aided by having the analyte in a very polar, often aqueous, solvent environment. In this way, the polar solvent can help to facilitate the retention of the non-polar analytes. Following retention the adsorbate can then be eluted by a solvent having some degree of non-polar nature strong enough to overcome the adsorbate/sorbent interactions. Depending on the strength of the adsorbate/sorbent interaction, the non-polar character of the eluent can be adjusted to facilitate elution.12
Figure 1.4 Non-selective retention vs. Selective retention. In the SPE experiment on the left non-selective retention of several isolates has occurred due to the extraction chemistry employed. On the right, selective retention of the isolate had been attained extraction chemistries with a strong affinity for that particular isolate.  

1.5.2 Polar Interactions

Opposite to reversed-phase, normal-phase chromatography consist of sorbents constructed of materials more polar than either the solvent and/or the sample matrix being applied to the sorbent. The primary mechanisms for analyte retention in the case of polar interactions are hydrogen bonding, dipole-dipole interactions, induced dipole-dipole, $\pi-\pi$, and a variety of others involving the unequal distribution of electrons. In the case of normal-phase SPE, the more polar analyte is extracted from the organic sample matrix due to retention onto the more polar stationary phase followed by elution by a more polar solvent. Polar interactions can be exhibited by groups such as hydroxyls, amines,
carbonyls, aromatic rings, sulfhydryls, double bonds, and any groups containing heteroatoms such as oxygen, nitrogen, sulfur, and phosphorus.\textsuperscript{12}

One of the more significant types of polar interactions, hydrogen bonding, affects retention when hydrogen bonded to an electronegative atom (oxygen or nitrogen) interacts with another surface bearing a relatively electronegative atom. The interactions that affect polar retention are facilitated by the adsorbate existing in a non-polar solvent, the opposite of non-polar retention. The stronger polar interactions that can occur between the polar adsorbate and the adsorbent cannot be easily overcome by the weaker, non-polar interactions (van der Waals) the adsorbate may have with a non-polar solvent. Polar interactions can be disrupted by the introduction of a polar solvent due to the competition or partition of the polar analyte between the adsorbent and the polar solvent.

1.5.3 Ion-Exchange

SPE extraction applications involving ion exchange sorbents isolate analyte based on the charge of the molecule.\textsuperscript{13} For example, the charge on a protein can be manipulated by changing the pH, thus allowing the use of ion-exchange sorbent formats for extraction.\textsuperscript{8} The ion-exchange interaction can occur when a charged adsorbate in the presence of an oppositely charged adsorbent results in the immobilization of that adsorbate onto the adsorbent due to their attractive forces. Ion-exchange interactions are divided into two categories, cation exchange and anion exchange. Cation exchange refers to positively charged adsorbates while anion exchange refers to negatively charged
adsorbates. Effective ion exchange interactions have two requirements that must be met in order to ensure proper retention. First, the pH of the system must be such that both the adsorbate and the adsorbent are charged. Second, it is not desirable for the matrix/solvent containing the adsorbate to have a charged species that competes with the adsorbate for retention on the oppositely charged adsorbent such as salts and buffers.\textsuperscript{12}

Knowledge of the pKa and pI values of each group present in the system (both for the adsorbate and adsorbent), is required to manipulate the interactions. The pI of a particular functional group or molecule is the pH at which an equal distribution of charged and uncharged groups/molecules exists resulting in an overall net charge of zero.\textsuperscript{43} At pH values below the pI, the net charge on the molecule is positive, conversely, at pH values above the pI the net charge is negative. The pKa of a molecule is the pH at which 50% of the groups carry a charge.\textsuperscript{43}

For ion exchange, it is also important to have the pH two units below or above the pKa value of the group or molecule so that 99% of all groups present are carry a charge. The greater percentage of adsorbate molecules carrying the same charge, more reproducible retention can be expected.

The ionic strength of the solvent/matrix in which the adsorbate is in also plays a crucial role in adsorbate retention. Ionic strength, a reflection of the total ion concentration in the solution, can affect retention by the ionic species present competing with the adsorbate for interaction space with the adsorbent. Thus, it can be said that solutions of low ionic strength may promote retention of the
adsorbate, while solutions of high ionic strength may disrupt adsorbate retention through competitive mechanisms occurring in concert for oppositely charged sites on the adsorbent.

As an example of selectivity and ionic strength competition, a strong anion-exchange adsorbent such as a quaternary amine will exhibit a 250-fold interaction preference with a citrate anion over an acetate anion. Therefore, the addition of an adsorbate in a citrate anion solution would not be a good example of a well executed ion-exchange SPE experiment, due to the competition between the citrate anion and the adsorbate for the sorbent. However, an elution solvent containing the citrate anion would provide excellent elution conditions in the same experiment because of the displacement of the adsorbate anions with the citrate anions, thus facilitating adsorbate elution.12

It is important to note that the three types of interactions previously discussed can coexist in one sorbent format and act in concert to affect analyte retention and elution. In fact, most available adsorbates will exhibit one or more of the three types of interaction mechanisms at any given time. The types of interactions displayed by any given adsorbent at any given time are a function of the system environment that facilitates the dominance of one type of interaction over another. Because of the variability in system conditions required to manipulate retention/elution mechanisms, the search for, development, and characterization of robust sorbent materials continually expands to meet the needs of sample preparation.
1.6 Polymer Stationary/Support Phases

Polymeric materials are employed as sorbent media because of their resistance to pH extremes. Where silica based sorbents degrade when exposed to pH extremes, polymer based sorbents are stable. The range and types of base polymer sorbents are being expanded to functionalized polymer sorbent materials which are increasing the range of applications. PS-DVB-based were the first polymer phases employed for SPE. Polymer based sorbents containing aromatic groups have proven very selective for analytes with aromatic rings due to \(\pi-\pi\) interactions. Extensive reviews on polymeric phases with an emphasis on organic micro-pollutant extractions have been published. Polymer phases in the form of beads have been the most widely used polymer phase in the last decade. A high number of available modifications exist that affect selectivity, retention, and elution. Hundreds of papers were published in the 1970’s and 1980’s describing the use of polymer resins in SPE applications.

The standardization of analytical procedures employing polymeric resins loss momentum due to their major drawback: the necessity of a successful resin cleaning scheme to remove artifact compounds. The aspect of resin cleaning and resin standardization slowed the immediate analytical use of polymer resins as phases in SPE. More recently, hollow polymer fibers have been introduced as solid phases. Organic modifiers have been impregnated in the pores along the inner walls of the polymer fiber to provide chromatographic interaction. Hollow polymer fiber phases have been used for the separation of
proteins and lanthanides.\textsuperscript{55-57} Additionally, polymer fibers serving as both the support and stationary phase have been used for HPLC separations. The available interactions on the surface of the fiber act as the stationary phase, while the fiber itself acts as the support.\textsuperscript{58-60} Similar to the types of interactions provided by polymer beads, polymer fibers have found their way into chromatography applications involving large biomolecules as well.

Capillary channeled polymer (C-CP) fibers were first introduced as a support and stationary phase for liquid chromatography separations in 2003 by Marcus et al.\textsuperscript{50} Polypropylene and polyester fibers were introduced as stationary phase media for the separation of polyaromatic hydrocarbons, organic and inorganic lead compounds, amino acids, and a lipid standard of triglycerides.\textsuperscript{50} Initial success and separation of the various analytes prompted further investigations of the C-CP fibers as stationary phases in reversed-phase LC. Unique to C-CP fibers, as the name implies, are the eight capillary channels surrounding the periphery that run along the fiber axis. As can be seen in Figure 1.5, the eight capillaries can act as channels that facilitate fluid motion through fiber columns. The fibers, created by a spin-melt process, have nominal diameters of around $\sim 50 \, \mu\text{m}$ and have eight capillaries that can range from $\sim 5$ to $20 \, \mu\text{m}$ in diameter. The physical characteristics of the eight channels on the periphery of the fibers allow for the spontaneous wicking along the length of the channels, indicating high efficiency fluid transport. Initially the fibers were manufactured for textile products, where in some cases, the high fluid transport provided by the capillaries was desirable.\textsuperscript{50}
Figure 1.5  SEM image of a single polyester fiber. The eight capillaries surrounding the periphery of the fiber also run along the fiber axis.

The simplistic manufacturing process of the C-CP fibers is applicable to virtually all spin melt polymer systems such as polypropylene, polyester, nylon, and polylactic acid. Chromatographic columns are prepared by pulling a predetermined number of fibers collinearly through stainless steel columns. The non-polar characteristic of the polymer surface and elution order of analytes from polar to non-polar indicated that C-CP fibers could be employed in analytical reversed phase liquid chromatography separations. It was also envisioned that the C-CP fibers would prove applicable in preparatory-scale separations such as SPE.

Since the introduction of the C-CP fibers as stationary phase for analytical separations, the hydrodynamic flow with the C-CP fiber columns have been investigated, C-CP fibers columns have been employed as microbore high-performance liquid chromatography columns, and the C-CP fibers have been
employed for HPLC separations of proteins.\textsuperscript{9, 63, 64} It was found that C-CP fibers facilitated efficient fluid transport in concert with high specific permeability, i.e. low resistance to fluid flow. Additionally, as packing density increased, peak width and distortions also increased, most likely due to the stagnant and restricted zones created by fibers in close proximity, thus suggesting the existence of an optimal packing density for C-CP fiber columns. Peak profiles were also found to be a function of column diameter.\textsuperscript{61} The aforementioned hydrodynamic qualities exhibited by the C-CP fibers were realized by employing an un-retained test compound (uracil). Characterization of fluid flow in C-CP fiber columns employing retained compounds were suggested to determine the complete function of packing density and column diameter on chromatographic quality.\textsuperscript{61}

C-CP fibers employed as microbore HPLC columns, were successful in separating a mixture of uracil, ethylparaben, and propylparaben.\textsuperscript{62} Microbore columns were constructed in varying lengths ranging from 500 to 1000 mm, with the 1000 mm columns consistently outperforming the columns of lesser length. Additionally, it was found that radial compression decreased plate height while maintaining relatively constant specific permeability. Radial compression is a process of applying controlled pressure or mechanical compression to reduce the radius of the column and compress the bed to a controlled volume. Radial compression reduces the void space near the interface between the C-CP fibers and the column walls.\textsuperscript{62}
In addition to chromatographic success and treatment of small molecules, C-CP fibers have also been employed to separate biomolecules, more specifically proteins, under gradient elution conditions. The separation of a four protein mixture (superoxidase, hemoglobin, hemocyanin, and myoglobin) was achieved employing a polypropylene (PP) C-CP fiber column. Advantages of using C-CP fibers as stationary phase for protein separations were suggested due to a range of characteristics. Base polymer materials during the manufacturing/ spin-melt process can be varied, thus changing the chemistry and types of interactions available to C-CP fiber column protein separations. In contrast to derivatized silica beads, the surface of the base fiber materials acts as the stationary phase in C-CP fiber columns, which could indicate enhanced column stability. Derivatization of C-CP fiber surfaces can be employed to achieve various modes of separation mechanisms. The eight capillaries on the periphery of the fiber surface facilitate directed fluid flow, thus reducing system backpressures in comparison to traditional packed bead columns, which should from a hardware standpoint present the possibility of increased flow rate separations. Lastly, the low cost of base fiber materials make C-CP fiber columns a more inexpensive alternative to commercial packed bead technologies. A further study indicated the potential for using the C-CP fiber column format for rapid protein separations.

The aforementioned studies employing C-CP fiber columns in high performance liquid chromatography applications also allude to the fact that the fibers could be used in preparatory scale chromatography. The relative ease
of fiber handling and column construction would make incorporating C-CP fibers into SPE applications and technologies promising. The hydrodynamic capabilities of the fiber geometry could prove advantageous when used on the preparatory scale. Success demonstrated in synthetic protein separations indicate that C-CP fiber formats could be applied to analytical SPE of proteins. As protein analysis by mass spectrometry (MS) continues to gain importance, the need for efficient buffer removal/solvent exchange technologies will follow suit. It is envisioned that chromatographic qualities proven advantageous to HPLC separations of proteins could also apply to SPE of proteins.

Recently capillary-channeled polymers have been introduced as stationary phase in SPE of proteins. Polypropylene fibers have been shown to successfully extracted lysozyme from phosphate buffered saline solutions and myoglobin from tris-buffered solutions. The initial use of C-CP fibers as protein desalting media will be discussed in great detail in the chapters to follow.

1.7 Applications of Solid Phase Extraction

Because a wide range of sorbent chemistries, conditioning solvents, elution solvents, and sample application methodologies exist, a wide range of applications for SPE and thousands of publications vouch for the success of SPE. In addition, the true evaluation of an analytical method is how well it performs for practical day-to-day analysis, where samples can often be quite complex when compared to the clean, defined samples used for initial development. However, the importance of sample preparation has been
recognized and the following sections demonstrate research and areas of development that have realized the benefits of SPE as a technique.\textsuperscript{4}

The Environmental Protection Agency (EPA) has adopted many SPE techniques and devices to perform drinking water analysis replacing former LLE approaches. For example, the standard extraction procedure used by the EPA to extract carbonyl compounds, chlorinated acids, pesticides, herbicides, and organohalides all employ the use of C18 SPE stationary phase device. Initially, the EPA had used LLE to extract oil and grease from water (EPA Method 413.1), but because the process involved Freon (CFC-113), it was banned due to deleterious effects on the ozone layer. EPA Method 1664 is rapidly becoming the dominant method for this extraction and employs SPE.\textsuperscript{14} Environmental applications extend to the analysis of natural water, seawater, wastewater, soil samples, agricultural samples, and countless others.

SPE also plays a vital role in the analysis of food and beverage samples, such as the extraction of caffeine from soft drinks\textsuperscript{65}, isolation of carotenoids from orange juice\textsuperscript{66}, extraction of synthetic colors in beverage alcohol products\textsuperscript{67}, extraction and isolation of lysoalanine from mozzarella cheese\textsuperscript{68}, and the determination of organic acids from honey.\textsuperscript{69} Even more prevalent are applications in the arena of biological analyses. Common biological matrices encountered include plasma, serum, lysed blood, urine, and sputum (saliva).\textsuperscript{13} SPE has recently gained popularity for the extraction and isolation of drugs and substances from bio-samples.\textsuperscript{4}
MS analysis of proteins has increased over the last decade as a result of the valuable structural informational realized and determined by MS. In addition, the use of MS to analyze proteins has also created challenges and opportunities in analytical and bioanalytical chemistry. The Human Proteome Project, related to the Genome Project, inherently has more complexity and challenges due to variable protein structure and modifications (post-translational). While MS provides structural information and detection sensitivity, proteins are often in complex biological buffers and matrices that interfere with MS analysis. Electro spray ionization (ESI) and matrix assisted laser desorption –ionization (MALDI) are two existing MS techniques for the analysis of proteins. ESI ionizes protein molecules that are in solution, and is commonly used following chromatographic separation. Ions are formed in ESI from solutions flowing through a hollow charged needle at 5-10 kV cm\(^{-1}\). Two concentric flows exist in the hollow needle; the innermost flow is the sample, while the outer flow can be a liquid that lends itself to modify the ion content and proper electrical contact with the metal tip. Some ESI designs also incorporate the outermost flow of a carrier gas to assist in droplet formation. The nebulized flow from the needle breaks into droplets \(\sim 1\mu m\) in diameter. The net charge on the droplets attracts them to an electrode some distance from the needle. As solvent continues to evaporate as the charged droplets move across the open volume, ions on the surface are forced into closer proximity, and at some point the repulsion of the ions overcome the surface tension holding the droplet together. At that point, called the Rayleigh limit, small droplets (\(\sim 2\%\) of the original mass) break from the surface.
These newly formed droplets, which maintain ~15% of the original charge, continue to experience solvent evaporation and breakup until the gas phase ions are generated. The ions in ESI contain little energy, thus little fragmentation occurs. This property makes ESI the softest of the soft ionization techniques. ESI gas phase ions are unique in that they favor the formation of multiply charged peaks. Generally these ions are described by the following nomenclature, $(M+nH)^{n+}$, which would indicate a parent molecule with mass M, carrying “n” conjugated protons, with a net charge of “+n” if analyzed in the positive ion mode. An advantage of multiply charged gas phase ions is that the resulting mass spectra of high-molecular weight molecules can be detected using analyzers with a weak nominal mass limit. For example, a molecule having a mass of 20,000 carrying a charge of 20 as a gas phase ion in ESI would appear at m/z=1000. The resulting mass spectra of a pure compound in ESI are readily interpreted as well. In addition to being a soft ionization technique and favoring multiply charged peaks, ionization in ESI is at or near atmospheric pressure.

Though ESI is popular for analysis of proteins and peptides, it is also plagued by low concentrations often found in biological samples and the contaminants found in biological solutions which include buffers, non-volatile salts, and detergents. Common biological buffers are composed of a multiplicity of ionic species and can include sulphates, phosphates, chlorides, as well as cations such as sodium, potassium, and ammonia. Ions from contaminants can reduce the abundance of ions from the analyte of interest and in some cases totally suppress them. Often, contaminant ionic species results
in the formation of adduct ions which further distribute the available ion current, reducing sensitivity. In addition, molecular weight determinations are complicated by the presence of adduct ions.\textsuperscript{42} For example, the presence of sodium and potassium found in the biological buffer phosphate buffered saline (PBS) could result in the formation of adducts such as $[\text{M}+n\text{H}+m\text{Na}]^{(n)+}$, $[\text{M}+n\text{H}+m\text{K}]^{(n)+}$, and $[\text{M}+n\text{H}+m\text{Na}+m\text{K}]^{(n)+}$.\textsuperscript{41} The formation of these adduct species create additional m/z peaks due to the increased adduct mass, thus increasing spectral complexity.\textsuperscript{41} The added complexity afforded by adduct ion formation create complex spectra and sometimes impede molecular weight determinations.\textsuperscript{10}

MALDI is used to sublime and ionize a solid sample that has been co-crystallized with a MALDI matrix. A MALDI matrix, is a small organic molecule having a strong absorption at the wavelength of the laser.\textsuperscript{42} Analyte solutions and matrix solution are intimately mixed and \(~\text{1}\mu\text{L}\) of the resultant solution is spotted onto a target plate. The solvent evaporates and the result is a dried "solid solution" of analyte/matrix crystals. The energy source for MALDI is a laser, for example a nitrogen laser (337 nm), which is pulsed directly onto the sample/matrix crystal. Ion formation is facilitated by the absorption of energy from the laser by the matrix. The rapid heating of matrix crystals causes them to sublime carrying intact analyte molecules in the matrix plume. Very little energy is transferred to the analyte molecule during the expansion process, in fact the analyte may be cooled during the expansion of the matrix plume.\textsuperscript{42} The ionization of analyte molecules can happen at any time during the expansion of
the matrix plume.\textsuperscript{42} The most widely accepted theory involves the gas-phase proton transfer from matrix to analyte in the expanding matrix plume.\textsuperscript{42} The matrix in MALDI serves to absorb the majority of the energy from the laser, thus minimizing analyte molecule damage. Also, one laser wavelength can be used for a number of different analytes because the matrix is absorbing the energy directly not the analyte, making MALDI more universal than other laser ionization techniques.\textsuperscript{42} In contrast to ions formed in ESI, MALDI favors the mono-charged species of the \([\text{M+H}]^+\) nomenclature.

MALDI is generally more tolerant to contaminants such as buffers and salts that ESI.\textsuperscript{41, 42} This can be due to the separation of analyte and contaminant molecules during the crystallization process with the matrix. However, adduct ions may not be resolved from the molecular ions with higher molecular weight analytes, thus the mass accuracy and molecular weigh determinations deteriorates in the presence of contaminants during MALDI analysis.\textsuperscript{41}

Both ESI and MALDI are considered soft ionization techniques. Accurate molecular weights and amino acid sequences can be realized by ESI and MALDI analysis of proteins, upon interpretation of the mass spectra.\textsuperscript{8, 75-77} The sensitivity of both ESI and MALDI is high, at the pico-femtomole level for both techniques. Little or no fragmentation is observed for both. Background signal is usually low providing pure de-contaminated samples for ESI, where background levels are inherently high for MALDI due to the matrix. However, most MALDI spectrometers are equipped with deflection and gating operations that allow the suppression of all molecular ions below a specified region. ESI and MALDI are
well suited for biological samples, having detected biomolecules up to 200,000 and 300,000 Da respectively.\textsuperscript{78} Samples for ESI are in aqueous solution while MALDI samples are in the solid crystalline form. The ionization process for ESI is continuous while for MALDI it occurs in short pulses.

SPE of proteins and peptides from buffered solutions can dramatically benefit both ESI and MALDI analysis.\textsuperscript{8} The removal of salts and buffers allows more complete protein/peptide ionization, increased protein/peptide signals, reduction of adduct ion formation, and more accurate molecular weight determinations.\textsuperscript{8, 79, 80} Presented here, is both the introduction of C-CP fibers as stationary phase for desalting protein solutions, and initial characterization of C-CP fibers, specifically polypropylene (PP) and polyester (PET) for desalting protein solutions. C-CP fibers were evaluated based on recovery, throughput, affect on signal to noise ratios and adduct formation of protein mass spectra, incorporation into micropipette SPE formats, and overall applicability to SPE of proteins. Benchmark proteins, lysozyme, myoglobin, and bovine serum albumin (BSA), were used in the evaluation of C-CP fiber SPE micropipette tips. ESI-MS, MALDI-MS, as well as UV-Vis were used to quantitatively and qualitatively evaluate C-CP fiber protein SPE from buffered solutions.

1.8 Summary

The previous sections described the historical developments of solid phase extraction methodologies, sorbents, and formats. Attention was placed on previous work employing SPE as an analytical preparatory technique for the
isolation, purification, and concentration of analytes. Interaction types, architecture formats, and applications were discussed and related to current sample preparations regimes. Capillary-channeled fibers were discussed which have been shown to have excellent characteristics for chromatographic applications. The research presented here is aimed at introducing capillary-channeled fibers as stationary phases for SPE applications in bio-analysis, related to efficiency and applications.

Chapter 2 describes the initial corporation of C-CP fibers as sorbent material for desalting protein solutions prior to ESI-MS analysis. ESI mass spectra from lysozyme and myoglobin proteins post-extraction using PP fiber-packed micropipette tips exhibited higher signal-to-noise ratios and more spectral clarity that proteins in the original buffer solution. PP fibers were shown to selectively adsorb both myoglobin and lysozyme from tris-buffer and phosphate buffered saline (PBS) respectively.

Chapter 3 describes characterization of PP and PET fiber-packed micropipette tips based on mass balance, recovery, and extraction characteristics. Simple, adaptors were made to allow coupling of fiber-packed micropipette tips to typical 15 mL centrifuge tubs. In this way, protein extractions could be carried out using centrifugal force. Centrifugation allowed more control of flow rate during loading, rinsing, and eluting the protein test solutions. PP and PET fiber tips were compared for the extraction of lysozyme and bovine serum albumin (BSA) from PBS. Both fiber types facilitated protein adsorption from the original buffer solution and elution in a solvent that benefited ESI-MS analysis.
Adsorption of the test protein solutions were similar for both fiber types based on total fiber surface area. Recovery of lysozyme was higher using PP, while recovery for BSA was higher using PET. ESI spectral clarity was compared for post-extraction protein solutions from both fiber types based on signal intensity, signal-to-noise, and signal-to-background ratios.

Chapter 4 describes the use C-CP fibers as sorbent material for desalting protein solutions prior to MALDI-MS analysis. MALDI mass spectra from lysozyme and myoglobin proteins post-extraction using PP fiber-packed micropipette tips exhibited higher signal-to-noise ratios and more spectral clarity that proteins in the original buffer solution. PP fibers were shown to selectively adsorb both myoglobin and lysozyme from tris-buffer and phosphate buffered saline (PBS) respectively. MALDI mass spectra from myoglobin in tris-buffer resulted in low intensity molecular ions and higher intensity adduct species as a result of the tris-buffer. Post-extraction myoglobin MALDI mass spectra were free of adduct peaks and exhibited a high intensity molecular ion peak for myoglobin. The ionization of lysozyme was suppressed due to the presence of low molecular weight inorganic salts in the PBS buffer. MALDI mass spectra of lysozyme post-extraction exhibited a high intensity, accurate molecular ion peak.

Chapter 5 summarizes all experiments employing C-CP fibers as stationary/support phase in micropipette tips. PP and PET fibers have been incorporated in to micropipette tips and shown to desalt and de-contaminate several proteins prior to MALDI and ESI mass spectrometry analysis. It is envisioned that C-CP fibers due to their resistance to pH extremes, low cost of
fiber materials, relative hydrophobicity, and hydrodynamic characteristics could be an attractive alternative to commercial phases for protein sample preparation. The length and diameter of fiber-packed tips has yet to be optimized and could provide higher recoveries than presently attainable under current elution conditions. Spontaneous wicking, a characteristic of the fibers, has yet to be investigated as a means of loading protein solutions. Wicking could prove as an excellent means of quantitatively loading protein solutions. In addition, experiments to extend the work to other benchmark proteins and buffer systems needs to be accomplished to fully realized the potential of the fibers for used in protein sample preparation.
CHAPTER 2

CAPILLARY CHANNELED POLYMER (C-CP) FIBERS AS A STATIONARY PHASE FOR DESALTING OF PROTEIN SOLUTIONS FOR ELECTROSPRAY IONIZATION MASS SPECTROMETRY ANALYSIS

Abstract

Micropipette solid phase extraction (SPE) tips have been used to desalt and purify proteins and peptides from mixtures of buffers and biological solutions. Removing salts and buffers prior to ESI mass spectrometry characterization improves the detection limits and the sensitivity of the protein analyses. Recently, capillary-channeled polymer (C-CP) fibers have been investigated as stationary phases for high performance liquid chromatography (HPLC) separations of proteins. Polypropylene (PP) C-CP fibers incorporated as sorbent materials in micro SPE tips are shown to effectively remove both inorganic and organic buffers from proteins in defined solutions. The architecture of the fibers provides large surface areas in comparison to conventional round fibers and is readily packed into capillaries that can be affixed to micropipette tips. Desalting of protein solutions is demonstrated for electrospray ionization mass spectrometry (ESI-MS) analysis through increased signal to noise ratios and reduced spectral complexity.
2.1 Introduction

Evolving trends toward sample miniaturization in proteomics have driven the need for the development of new sample preparation techniques and methodologies. Various forms of sample preparation are employed for enriching analytes (i.e., proteins and peptides) and increasing sensitivity for minor (but potent) proteins in biological samples. Sample preparation for biocharacterization includes a multiplicity of procedures such as solid phase extraction, dialysis and filtration, selective precipitation and centrifugation, as well as ion-exchange, reverse-phase and normal phase, and affinity chromatographies. All of these methodologies are employed to remove matrix interferences, reduce sample complexity and increase analyte concentration. Efficient combination of these techniques can greatly affect the selectivity and sensitivity of protein detection, particularly by mass spectrometry (MS). Electrospray ionization (ESI) and matrix-assisted laser desorption-ionization (MALDI) are the most common soft ionization sources suitable for protein analysis. Typically, proteins exist in buffer solutions containing salts, small molecular weight organics, and even urea and detergent, depending on the extraction procedures. These buffer solutions could have deleterious effects on ionization taking place in sources due to preferential ionization, ion suppression effects, and the formation of adducts species. In addition, the presence of abundant buffer species can contribute substantial amounts of spectral complexity.
Solid phase extraction (SPE) techniques using hydrophilic, affinity, ion exchange, and hydrophobic interactions, as well as metal chelation, have all been used effectively to desalt, purify, and concentrate proteins prior to MS analysis.\textsuperscript{2, 5, 7, 8, 84-88} In the 1990’s the first commercially available micro-SPE devices were sold as micropipette tips with C18 chromatographic media incorporated into the tip. The tips were developed by the Millipore Corporation (Bedford, MA) under the trade name of ZipTips\textsuperscript{TM}. Present day micro-SPE tips are available from a number of manufacturers, offering a wide range of stationary phase chemistries that may be used in different types of applications.\textsuperscript{8}

Our laboratory has introduced capillary-channeled polymer (C-CP) fibers as stationary phases for the HPLC separation of proteins and peptides.\textsuperscript{9, 63, 64} Some of the advantages to using C-CP fibers as a stationary phase are the wide range of polymer/surface chemistries, robustness of the materials, increased surface area compared to standard-circular cross-section fibers, low back pressure, inexpensive base fiber materials, and the possible application of single fibers on the micro-analytical scale.\textsuperscript{9} Of particular interest to our laboratory is the incorporation of the C-CP fibers as a sorbent in micropipette tip applications. In addition to the above-cited characteristics, the relative ease of fiber packing and the possible use of controlled wicking (as opposed to aspiration) as the loading process are among the useful features. These attributes suggest that the fibers might be an excellent alternative to stationary phases currently in use in commercial micro-SPE devices. We report our initial efforts in the implementation of polypropylene (PP) C-CP fibers as sorbent media for the solid
phase extraction of proteins from inorganic and low molecular weight organic buffers prior to ESI mass spectrometry analysis.

2.2 Experimental Section

2.2.1 Reagents and Chemicals.

Lysozyme, from chicken egg white and myoglobin from equine skeletal muscle, sodium chloride (NaCl), sodium phosphate dibasic (Na$_2$HPO$_4$), potassium phosphate monobasic (KH$_2$PO$_4$), Sigma 7-9° (tris base), and trizma® hydrochloride (tris acid) were obtained from Sigma-Aldrich (Milwaukee, WI). Potassium chloride (KCl) and formic acid were obtained from Fisher Chemicals (Pittsburgh, PA). ACS grade acetonitrile (ACN) was obtained from Mallinckrodt Baker Inc. (Phillipsburg, N.J.) and used as an organic mobile phase. Nanopure Diamond® water (18.2 MΩ/cm) from Barnstead/Thermolyne Water Wystem (Dubuque, IA) was used in solution preparation for buffers and proteins and aqueous mobile phases.

2.2.2 Fiber-Tip Construction.

The C-CP SPE tips were assembled (Fig. 2.1) by a procedure very similar to that employed previously to pack standard format and microbore C-CP fiber chromatography columns.$^{62, 89}$ As in the case of microbore columns, the fibers were pulled through a 0.8 mm i.d. fluorinated ethylene polypropylene (FEP) capillary tubing (Cole Parmer, Vernon Hills, IL). In the studies described here, a fiber loop corresponding to a total of 658, 55 μm diameter PP fibers was pulled
collinearly through a ~300 mm length of the FEP tubing, from which the individual tip structures would be cut. This number of fibers represents an 0.60 interstitial (void) fraction within the capillary.\textsuperscript{62} Compression fittings were placed on the tubing ends, the assembly placed on a high performance liquid chromatography (HPLC) system (Shimadzu LC-10AT, Tokyo, Japan) and the fibers conditioned alternating with acetonitrile and water (twice) for a period of 30 min. at a flow rate of 1 mL/min.

Compression fittings were removed from the conditioned fiber-packed FEP tubing. The tubing was then stretched manually, in the direction of the fiber/capillary axis, past the fiber ends to create ~6 mm of open tubing beyond the end of the fibers to accommodate the micropipette tip. The open end of the tube was slipped over the tip of 1 mL micropipette tip (Redi-Tip\textsuperscript{TM}, Fisher Scientific, Pittsburgh, PA) and was simply held in place by the compression of the tubing around the tip. The opposite end of the tubing was then cut so that 1 cm of fiber-packed tubing was attached to the micropipette tip. Each micropipette tip assembly underwent a final wash step by passing 200 μL of water through the tip prior to use.
Figure 2.1 C-CP fiber micropipette tip assembly procedure. Seven rotations of polypropylene fiber are placed on the circular frame to accumulate 658 fibers that are pulled collinearly through the 0.8 mm i.d. FEP capillary tubing with a monofilament. Pressure is applied in the direction of the fiber/capillary axis to stretch the tubing so that a small amount of depth is created to accommodate the micropipette tip. The capillary is press-fit to the end of the commercial micropipette tip and is ready for use.

2.2.3 Protein Loading Washing and Elution

To reference with existing desalting methods, the same sorts of test solutions and extraction methods were employed in this initial evaluation of the C-CP fiber method. A 40 μg/mL (2.8 μM) lysozyme solution was made in a phosphate-buffered saline (PBS) consisting of 140 mM NaCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ and 2.7 mM KCl @ pH 7.3. A 50 μl aliquot was aspirated onto
the 1 cm fiber tips by drawing up the volume and pushing the volume off five times. The same loading procedure was also performed for myoglobin at 40 μg/mL (2.4 μM) in 100 mM Tris (tris(hydroxymethyl)aminoethane) buffer containing 84 mM tris acid and 15 mM tris base @ pH 7.5.

Protein-loaded tips were washed with a single 100 μL volume of Nanopure water to remove the ionic (salt) species. The proteins (lysozyme and myoglobin) were eluted with a single 100 µL aliquot of 100% ACN through the tip and collecting the eluent in a centrifuge tube. The eluent is diluted to 200 µL with Nanopure water and pH-adjusted with formic acid to a final concentration 0.1% prior to ESI-MS analysis.

2.2.4 Mass Spectrometry

A Waters (Milford, MA) quadrupole-time of flight (Q-ToF micro) mass spectrometer with an ESI source was used for the protein determinations. The protein-containing solutions were injected directly to the ESI source through a Waters (Milford, MA) capillary liquid chromatograph (Cap LC). The flow rate was set at 2 μL/min. The ion source potential was set at 3000 volts in the positive ion mode, with the quadrupole mass filter scanned from 500 to 2500 m/z in 2 seconds. Methanol was injected in between runs to prevent carryover from previous runs. The ESI mass spectra for the proteins in the original buffer media and post-extraction were compared on both a qualitative and quantitative basis. Equal amounts of the proteins were injected throughout the experiment to ensure a non-biased comparison of spectral quality, signal to noise ratios, etc.
2.3 Results and Discussion

2.3.1 Electrospray Ionization of PBS-Extracted Lysozyme

The initial metrics in assessing the ability of the C-CP fiber method for desalting protein solutions include the production of well-distinguished multiply-charged ion species, with high intensities and the ability to deconvolute those spectra to yield high fidelity molecular weights. Shown in Figure 2.2 are the ESI mass spectra obtained from the lysozyme (a fairly hydrophobic protein which should have a high affinity for the polypropylene surface) in the initial PBS matrix and that eluted from the fiber-filled micropipette tip. The mass spectrum of the protein in the buffer solution (top) shows a continuum of signals across the mass range, with only two discernable peaks that can be attributed to ionized proteins. On the other hand, the mass spectrum of the protein post C-CP fiber extraction (bottom) is composed solely of protein related species across mass range. This demonstrates the fact that salts compete for protein ionization and that desalted lysozyme ionizes significantly better. Although the integrated total ion chromatograms for the two solutions are virtually identical, the signal-to-noise ratios of multiply-charged lysozyme ions at m/z = 1590 Da are ~2.5 (TIC) for the buffer solution and ~125 (TIC) for the buffer removed one.

From the raw ESI spectra of the protein solutions, the molecular weight of the protein was deconvoluted using the MaxENT™ 1 algorithm (Waters®). The resultant molecular ion spectra are presented in the inserts. In the case of the buffer solution, a large number of possible molecular ions are identified, including one near the presumed molecular weight. This is a reflection of the weak
lysozyme ionization. As a result, the assignment of this peak as an unique molecular mass unreliable. The spectral purity of the C-CP fiber extracted lysozyme yields an unambiguous deconvoluted spectrum with the identified molecular weight being accurate to that of the neat protein in acidified can, at 35 ppm delta mass from the manufacturer’s certificate.

Figure 2.2 Electrospray ionization mass spectra of lysozyme pre and post-extraction. Lysozyme (40 ppm) from chicken egg white (top) in PBS (140 mM NaCl, 2.7 mM KCl, 10mM Na₂HPO₄, and 1.8 mM KH₂PO₄ @ pH 7.3) and in PBS-removed solution (bottom) via desalting on PP C-CP fibers mounted in a micropipette tip. Spectral insets are the deconvoluted ESI spectra revealing the corresponding molecular ion.
2.3.2 Electrospray Ionization of Tris-Extracted Myoglobin.

Tris (tris(hydroxymethyl)aminoethane) is a commonly used buffer to stabilize proteins in solution. Different from the case of the PBS matrix, tris is an organic buffer that has a modest amount of alkyl character that may have an affinity to the PP fiber surface. In addition, the hydroxide moiety might also have some propensity for hydrogen bonding with the stationary phase. The mass spectra presented in Figure 3 are derived for myoglobin (equine skeletal muscle) at a concentration of 40 μg/ml (2.4 μM) in 100 mM tris, before and after the solid phase extraction on the C-CP fiber phase. The most noticeable feature of the mass spectrum of myoglobin in the buffer-matrix (top) is the presence of multiple cluster peaks that display a mass unit difference of 157 Da between the major peaks; equal to the monoisotopic mass of tris-acid (C$_4$H$_{11}$NO$_3$HCl). This demonstrates the fact that tris-buffer ionizes readily and forms clusters in the ESI source. Abundant tris species compete for availability of protons, and their ionization limits protein ionization. In fact, myoglobin, with a pI = ~7.0 would be expected to be in a predominately anionic form in this pH = 7.5 buffer media.$^{90}$ The addition of the 0.1% formic acid to the extract lowers the pH to ~3.5, and so the myoglobin would be predominately in the cationic stage. The tris present in excess here effectively competes for the available charge, suppressing the protein response. As a result, none of the peaks otherwise present in the fiber-extracted protein spectrum (bottom) appear is the spectrum of the protein-buffer solution. In the tris solution, deconvolution produces no discernable protein information.
Figure 2.3 Electrospray ionization mass spectra of myoglobin pre and post extraction. Myoglobin (40 ppm) from equine skeletal muscle (top) in 100 mM tris buffer and in tris-extracted solution (bottom) via desalting on PP C-CP fibers mounted in a micropipette tip. Spectral inset is the deconvoluted ESI spectrum revealing the corresponding molecular ion.

In the case of the fiber-extracted myoglobin spectrum, the MaxENT™ 1 deconvolution revealed the molecular weight spectrum shown in the inset at 16,951 Da, identical to that obtained for a neat (i.e., without buffer and subsequent extractions), acidified myoglobin solution. A comparison of the ion yields reveals an ~75% reduction in the case of the buffer extract. This level of recovery (25%) is encouraging given the fact that detailed optimization of the procedure has yet to be performed.
2.4 Conclusions

The results from these experiments reveal the potential of polypropylene C-CP fibers as a SPE media for desalting proteins from organic and inorganic buffers. The hydrophobic nature of the fibers binds the protein selectively and allows salts and weakly bound organics to be washed from the fiber tips with aqueous mobile phase. The bound proteins can be eluted with a non-polar solvent such as acetonitrile. This procedure cleans up the protein solutions and improves analyte signal responses in the ESI-TOF-MS spectra in terms of intensity, signal-to-noise ratio and spectral clarity, all of which are critical for characterization of minor protein species in biological samples.

While a number of commercially available SPE media have been adapted to micropipette tip geometries, the C-CP fibers present several unique features. The architecture of the fibers provides enhanced surface area for SPE relative to circular cross section fibers and can be easily fabricated into SPE tips. Different from the case of porous SPE media where voids must be wetted by multiple aspiration steps, the polymer fiber surfaces are amorphous, so there more direct access for binding interactions and likely greater recoveries. The robustness of fiber materials allows for the use of more versatile (and harsher) digestion, extraction, and elution conditions. The inexpensive base fiber materials (e.g., polyester and nylon) provide a wide range of polymer/surface interactions. Fairly straightforward derivatization of the C-CP fiber surfaces opens up a great deal of diversity for different solute-surface interactions. For example, polyester
surfaces treated with simple alkali solutions (e.g., 2 M NaOH), yields a carboxylated surface that can be used for cation exchange extraction.

More rigorous evaluation of the C-CP fibers is currently underway involving other benchmark proteins and peptides, ultimately extending to extracts from biological fluids. The roles of buffer identity, pH, and sample (loading/elution) conditions are being evaluated in a detailed mass balance approach. Accordingly, more direct comparisons with commercially-available phases are in order, including the use of exhaustive wicking as a means of quantitatively exposing test solutions to the fibers as opposed to the use of repetitive aspiration steps. Fiber loading capacity and analytical quantification will be key attributes to be compared. Previous loading studies directed at protein chromatography on C-CP fiber phases, suggest an ~3% (wt protein/wt fiber) capacity. As such, the ~2.3 mg of fibers here should permit extraction of >50 μg of protein. Extension of this present application to the use of C-CP fibers to desalt proteins for analysis by matrix-assisted laser desorption ionization (MALDI) is also in process.
CHAPTER 3

SOLID PHASE EXTRACTION OF PROTEINS FROM BUFFERED SOLUTIONS USING CAPILLARY CHANNELED POLYMER (C-CP) FIBERS: MASS BALANCE, RECOVERY, AND EXTRACTION CHARACTERISTICS.

Abstract

Micropipette solid phase extraction (SPE) tips have been used to desalt and purify proteins and peptides from mixtures of buffers and biological solutions. The presence of residual buffering species can reduce or even sometimes completely suppress protein ionization in the case of electrospray ionization mass spectrometry (ESI-MS) analysis and result in greater complexity in the derived mass spectra. The formation of adduct species can hinder accurate molecular weight determinations. Recently, capillary-channeled polymer (C-CP) fibers have been introduced as stationary/support phases for the SPE of proteins from buffered solutions. In this study polypropylene (PP) and polyester (PET) C-CP fibers were incorporated as sorbent materials in micropipette SPE tips and evaluated in terms of mass balance, recovery, and extraction characteristics. Centrifugation was found to be a viable alternative to aspiration as a means moving fluid and facilitating protein SPE from buffered solutions. Protein quantification in selected fractions for the extraction of lysozyme and bovine
serum albumin (BSA) from phosphate buffered saline (PBS) solutions using both PP and PET fiber-packed tips was performed using UV-VIS absorbance spectrophotometry. ESI mass spectra for the extraction of lysozyme from phosphate-buffered saline (PBS) using both PP and PET fiber-packed tips were compared in terms of the quality of the mass spectra, the signal to noise ratios, and the extraction yields.

3.1 Introduction

It has been shown that ~60% of analysis time is spent on sample preparation as opposed to only ~7% for the actual measurement of sample components. Analytical laboratories are under increasing pressure to provide sample analysis more rapidly and at lower costs. Improvements of existing and exploration of new sample preparation techniques, are aimed at providing more reproducible results, decreasing the use of organic solvents, providing purer extracts for instrumental analysis, and decreasing analysis time, and cost. In the past, liquid-liquid extraction (LLE) played a prominent role in sample preparation. However, LLE suffers from low recovery, labor-intensive procedures, and the use of large amounts of organic solvents. In solid phase extraction (SPE), analytes are adsorbed from a liquid phase by passing the test solution over a sorbent bed. Analytes can then be desorbed by passing an appropriate liquid solvent through the sorbent bed. Judicious selection of the sorbent type and eluting solvent allow for the subsequent instrumental analysis of instrument friendly solutions following SPE.
SPE sorbents also fall into the similar classifications as those employed as stationary phases in high performance liquid chromatography (HPLC). Specifically, sorbents used in reversed-phase, ion exchange, and affinity chromatography are used in protein extractions. Effective SPE sorbents have a variety of desirable properties including reversible adsorption, chemical stability, large surface areas, surface contact with the analyte, and high-percentage recoveries. Large surface areas can affect the uptake of analytes by shifting the distribution of analytes from the liquid phase to the sorbent via large phase ratios. On the other hand, large surface areas are only advantageous when the sample solution achieves effective surface contact with the sorbent. Poor solution contact with sorbent surfaces is often the case when hydrophobic sorbents are employed with aqueous solutions. Good surface contact increases the probability that effective interactions between analyte and sorbent affecting adsorption will occur. Successful SPE not only requires the efficient and reproducible adsorption of analytes; reversible adsorption is necessary to recover analytes. While high recoveries are required when high sensitivity determinations are necessary, precision and accuracy are usually optimum when the recovery is high. Traditionally, bonded-phase silica-type sorbents have been relatively easy to adapt as sorbents for SPE. However, due to pH limitations, the presence of silanol groups, and irreversible adsorption, other sorbent types, particularly polymeric sorbents, are often employed for protein extractions.
The chemical stability of the sorbent is of special consideration when pH conditions are extreme. Chemically bonded silica sorbents are not stable above pH 8 or in highly acidic solutions. In contrast, polymer resins are chemically stable in extreme pH conditions \(^4\). While proteins are generally processed at neutral pH conditions, greater range could permit conditions where the extraction process could be enhanced. Polymer resins employed for SPE during the 1970’s and 1980’s suffered from the presence of leachable impurities trapped in the resin during the polymerization step, which can co-elute with analytes species \(^4\), \(^54\). However, in most cases, pre-rinsing with organic solvents reduces leachable impurities to an acceptable level. High extraction efficiencies for compounds containing aromatic functionality (such as the amino acids tryptophan and tyrosine) are promoted using aromatic polymer sorbents due to \(\pi-\pi\) interactions between the sorbate and sorbent \(^45\), \(^46\). Chemical modification of base polymer supports can provide a variety of interactions and extend applications to more diverse set of analytes than is possible with silica supports. Polystyrene-divinylbenzene (PS-DVB) materials with particle sizes ranging from 10-160 \(\mu\)m have been employed most extensively as SPE sorbents \(^4\), \(^92\). SPE formats packed with particle sizes smaller than 10 \(\mu\)m have to withstand the higher backpressures associated with smaller particle size, which is still a major challenge for polymer phases. To this end, crosslinked polystyrene resins have been the most successful polymer resins for non-polar SPE.

Most sorbent materials exist in the bead shape and are packed into cartridges or columns \(^{14},^{23}\). The intrinsic problem with particulate matter is the
remaining void spaces and the inability to fill the total available volume. This leads to intra-particle channeling and reduced extraction efficiency $^{23}$. The development of new SPE formats such as a disk with embedded sorbent particles has enabled good extraction recoveries at relatively high flow rates $^4$. This is most likely attributed to reduced channeling along the flow axis. This in turn allows for the faster mass transfer and greater specific surface areas afforded by using smaller particles $^{24,26-28}$.

From an application standpoint, SPE has been employed for water samples, pesticides, oil and grease, metal ions, soil samples, agricultural samples, food and beverage, forensic, paper and pulp, and in organic synthesis $^4$. More recently, SPE has taken an important position in the sample pretreatment of biological samples prior to mass spectrometry (MS) analysis $^8$. The specific challenges in the field of proteomics, are due to the complexity of the starting sample matrices, requiring extensive extraction, separation, and purification of proteins prior to instrumental analysis $^8$. The two techniques used most often for the MS analysis of proteins, electrospray ionization (ESI) and matrix-assisted desorption-ionization (MALDI), both require that protein isolates be free of salts, buffers, and detergents $^2,5,7,8,41,42$. SPE can be particularly useful by providing the adsorption of proteins from buffered solutions, followed by elution in a solvent favorable for either MALDI or ESI. The application of SPE for protein purification has been the subject of numerous publications $^4,5,8,84,88$. Fundamental studies of protein adsorption onto SPE sorbents has also been the subject of recent publications $^{93,94}$. 
The adsorptive properties of a protein on a specific sorbent are a function of chemical and physical characteristics of the protein, the sorbent surface properties, and medium conditions. For example, the net charge and charge distribution and hydrophobicity of the protein, the topography, heterogeneity, and surface chemistry of the sorbent, and the pH, temperature, and hydrodynamics of the mobile phase all affect protein adsorption and desorption. In most SPE of proteins on hydrophobic surfaces, the free energy difference between the protein in an aqueous solution and on a hydrophobic sorbent is generally entropically favorable. When hydrophobic regions of a protein interact with hydrophobic sorbents, the free energy of the system is minimized by reducing the contact area between the hydrophobic regions of the protein and the aqueous liquid phase. Adsorptive processes are desirable only to the extent that reversible adsorption or elution is possible under conditions that either preserve the integrity of the analyte and or permit instrumental analysis with the least amount of matrix interference.

This laboratory has introduced capillary-channeled polymer (C-CP) fibers as effective stationary phases for the chromatographic separation of proteins. These fibers have a unique shape in which eight channels run along the axis such that individual fibers are capable of spontaneous movement of fluids. In a column format, the channels of adjacent fibers interdigitate to allow close-packing and very efficient fluid transport. Fibers have been extruded from polypropylene, polyester, and nylon base polymers to provide a diversity of potential surface interactions and modes of separations. While the amorphous fiber surfaces yield
much lower specific surface areas in comparison to porous silica phases, improved mass transfer, greater chemical stability, and lower costs are seen as potential advantages. Extension of that work to sample preparation application has recently been demonstrated in the SPE of proteins from biological buffers prior to ESI-MS analysis. It has been shown that polypropylene (PP) C-CP fibers can effectively bind lysozyme and myoglobin from phosphate buffered saline (PBS) and tris-buffer solutions, respectively. It has also been shown that mass spectra from fiber-extracted protein solutions exhibit higher signal to noise and signal to background ratios; as well as reduce the ESI spectral complexity.

Presented here are the mass balance and recovery characteristics of PP and polyester (PET) C-CP fibers used as sorbent media in micropipette tips to extract and recover lysozyme and bovine serum albumin (BSA) from PBS solutions. The two proteins and fiber types are employed here to illustrate the interplay of surface chemistries that exist in this approach (which are analogous to other SPE sorbents). The C-CP fiber-tip extraction process has been adapted to a centrifugation format, as employed in some commercial SPE cartridge devices, in order to provide greater control of the extraction procedure and more reproducible results than is found in the typical aspiration procedures used in conventional SPE applications as well as the previously described studies. The relative efficiencies of the sequential steps in the protein extraction process were determined using UV-VIS absorbance spectrophotometry. ESI mass spectra of eluted protein solutions were compared to those of the proteins in the
initial buffer media and neat solutions on the basis of analyte signal intensities, signal-to-noise and signal-to-background ratios. It is believed that the C-CP fiber format, incorporating centrifugal loading and elution, holds a number of very promising characteristics for their use in the field of proteomics.

3.2 Experimental Section

3.2.1 Reagents and Chemicals.

Lysozyme (mw=14.3 kDa), from chicken egg white, bovine serum albumin (BSA) (mw ≈ 66 kDa), sodium chloride (NaCl), sodium phosphate dibasic (Na₂HPO₄), and potassium phosphate monobasic (KH₂PO₄) were obtained from Sigma-Aldrich (Milwaukee, WI). Potassium chloride (KCl) and formic acid were obtained from Fisher Chemicals (Pittsburgh, PA). ACS grade acetonitrile (ACN) was obtained from Mallinckrodt Baker Inc. (Phillipsburg, N.J.) and used as an organic mobile phase. De-ionized (DI) water (18.2 MΩ/cm) purified by a Nanopure Diamond® Barnstead/Thermolyne Water System (Dubuque, IA) was used in solution preparation for buffers, proteins, and aqueous mobile phases.

3.2.2 Fiber-Tip Construction.

The construction of fiber-packed micropipette tips is still in the evolution stage. In most manufacturing processes, it is desirable to produce the maximum amount of final product from the minimum amount of starting materials. The number of manufactured tips per ~ 300 mm of fiber packed tubing from the previous method, was limited due to the ability of the FEP tubing to continually
stretch along the fiber axis to create the dead space needed for micropipette insertion \(^\text{10}\). Also, by stretching the FEP tubing along the fiber axis, it is possible that the interstitial fraction (packing density) of the tips changes through radial compression as each tip is formed. Subsequent stretching of FEP tubing to construct the fiber packed tips increased the time and pressure required (an indication that packing density had been increased) for the aspiration of protein solutions when compared to fiber-packed tips made from the first stretch.

The C-CP fiber-packed SPE tips were assembled here using a slightly different methodology than previously described \(^\text{10}\). In the studies described here, fiber loops corresponding to a total of 658 polypropylene (PP) fibers of 55 \(\mu\)m diameter and 320 polyester (PET) fibers of 60 \(\mu\)m diameter were pulled collinearly through \(\sim\)300 mm lengths of the 0.8 mm i.d. fluorinated ethylene polypropylene (FEP) tubing (Cole Parmer, Vernon Hills, IL). A small portion \(\sim\)5 mm at one end of the fiber packed tubing was cut with a surgical grade scalpel (Personna, Mansfield, UK) so the tubing and fiber ends were flush. Different from the previous studies \(^\text{10}\), the fibers from the opposite end were pulled to create \(\sim\)6 mm of space between the tubing end and the fiber end to accommodate the micropipette tip. The end of the tubing with the void space was then slipped over 1 mL micropipette tip (Redi-Tip\textsuperscript{TM}, Fisher Scientific, Pittsburgh, PA). The tubing was cut so that 1 cm of fiber packed tubing was attached to each tip. The process was repeated across the entire length of the \(\sim\)300 mm fiber packed tubing producing \(\sim\)15 tips. The new process of fiber-
packed tip construction increases the number of tips made per unit length of fiber-packed FEP tubing and eliminates radial compression.

3.2.3 C-CP™ Fiber Protein SPE Using Centrifugation to Induce Fluid Movement

Previously, a Transferpette® (Brand Tech, Essex, CT) pipette was used to facilitate the aspiration of the protein solutions through the fiber packed tips. This is the most common approach to SPE extractions, but because it is a manual procedure it is fraught with potential sources of imprecision and is time consuming. Therefore, a new procedure was developed whereby the fluid flow across the sorbent medium was better-controlled and samples could be processed in a parallel fashion. A fiber-packed pipette tip adapter was created to allow the fiber-packed tips to be placed into an accuSPIN™ 1R analytical centrifuge (Fisher Scientific, Pittsburgh, PA). As shown in Fig. 3.1, the conical portion (~10 mm) of a 1.7 mL microtube (Genesse Scientific, San Diego, CA) was cut perpendicular to the microtube axis so that it resembled a hollow cylinder. The fiber-packed micropipette tip (fiber end first) was pressed inside the top of the microtube. The fiber-packed micropipette tip was held in place as the outer wall diameter of the tip approached the inner wall diameter of the microtube. The microtube containing the fiber packed tip was placed into a 15 mL conical centrifuge tube (Nalge Nunc International, Rochester, NY) and held in place by the attached lid of the microtube extending past the outer rim of the conical centrifuge tube. In this manner, the fiber packed tips could be mounted in
the analytical centrifuge by placing the 15 mL conical centrifuge tubes in typical swinging centrifuge buckets.

![Diagram of C-CP fiber-packed tips adaptation to centrifugation](image)

**Figure 3.1** Adaptation of C-CP fiber-packed tips to the centrifugation format. The lower ~10 mm of the microtube is removed, the C-CP fiber-packed tip is inserted and held in place. The microtube is then inserted into the 15 mL centrifuge tube that can be placed in the centrifuge device. Fractions from protein extractions are collected in separate centrifuge tubes.

3.2.4 Comparison of Centrifugation and Aspiration as Fluid Movement Methods in C-CP™ Fiber-Packed Tips.

Lysozyme (40 ppm) was made in a phosphate buffered saline (PBS) matrix consisting of 140 mM NaCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ and 2.7
mM KCl at pH=7.3. A PP fiber-packed tip was attached to a Transferpette® and a 50 μl aliquot of protein solution was manually aspirated five times for a five minute total aspiration time. A rinsing step, consisting of 100 μL of DI-H2O was passed through the fiber-packed tip using positive pressure from the Transferpette® to remove any residual buffering species. Adsorbed protein was eluted and collected in a 1.7 mL microtube, by passing 100 μL of a 50:50 ACN:H2O solution through the tip. In the case of the centrifugation method, a 50 μL aliquot of protein solution was centrifuged through the fiber-packed tip at a rotation rate equating to 300 x G for five minutes total centrifugation time. (This is the time to completely move the solvent through the sorbent phase.) To remove residual buffering salts, 100 μL of water was centrifuged through the tip at 300 x G for 10 minutes. Finally, 100 μL of an 50:50 ACN:H2O solution was centrifuged through the fiber-packed tip at 300 x G for 10 minutes to elute adsorbed lysozyme. The eluted fractions were collected in the 15 mL conical centrifuge tube. Each of the described experiments was performed in triplicate and the results presented as the average values. The eluted fraction was conditioned to 0.1% formic acid and analyzed for lysozyme content using ESI-MS.

3.2.5 Quantification of Lysozyme and BSA in Effluent, Rinse, and Eluted Fractions

The amounts of lysozyme and BSA were determined for the effluent, rinse, and eluted fractions using UV-VIS absorbance at a wavelength of 210 nm.
(corresponding to the peptide bonds in the amino acids). Proteins were loaded onto fiber-packed tips, rinsed, and eluted as described in section 2.4 using the centrifugation method. Additionally, a second, third, and fourth elution was performed by passing 100 μL volumes of the eluting solvent through the fiber-packed tip and collecting each elution in a separate 15 mL centrifuge tube. A total of 24 fiber-tip extractions were performed for each fiber type and protein, 12 fiber-tip extractions employing protein in buffered solution and 12 fiber-tip extractions employing the blank buffer solution. Extraction volumes were combined into sets of three containing a total of 400 μL of eluted protein and/or blank solutions. A total of 400 μL was needed so that the level of solution in the quartz cuvette would encompass the incident light source in the spectrophotometer. In the case of ESI-MS analysis of the lysozyme eluents, the fractions were conditioned to 0.1% formic acid and the quantification was performed using the 9+ and 10+ pseudomolecular ion signals.

3.2.6 Instrumentation and Data Analysis.

A Waters (Milford, MA,) quadropole-time of flight (Q-ToF micro) mass spectrometer equipped with an ESI source was used to obtain mass spectra for eluted protein. For ESI analysis, protein-containing solutions were injected directly to the ESI source through a Waters capillary liquid chromatograph at a solution flow rate of 2 μL/min. The ion source potential was set at 3000 volts in the positive ion mode, with the quadrupole mass filter scanned from 500 to 2500 m/z in 2 seconds. A Genesys 10 (Thermo Electron Corp, Waltham, MA) split beam, dual detector UV-VIS spectrophotometer equipped with a xenon lamp was
used for protein quantification. Absorbance measurements were taken at 210 nm in a 10 mm path length quartz spectrophotometer cell (Starna Cells, Inc., Atascadero, CA). Analytical data from both types of experiments was processed using Microsoft Excel (Seattle, WA).

3.3 Results and Discussion

3.3.1 Comparison of Centrifugation and Aspiration as loading Methods in C-CP Fiber SPE.

In this study, centrifugation was compared to aspiration as the means to move protein solutions through the fiber-packed tips. Polypropylene (PP) fiber-packed tips were used to extract lysozyme (40 ppm) from phosphate buffered saline (PBS) employing both the method of aspirating the solutions using a Transferpette® and by centrifugation. The final eluted fractions from both methods were analyzed using ESI-MS. The speed of centrifugation (300 x G) was experimentally chosen to keep the amount of time required for both loading methods relatively constant at ~5 minutes for loading the 50 μL test solutions. ESI mass spectra can be seen for eluted lysozyme in Fig. 3.2a for the aspiration and Fig. 3.2b for the centrifugation method.
Figure 3.2 Electrospray ionization mass spectrometry comparison of centrifugation and aspiration loading methods. ESI mass spectra obtained from eluted fractions following a) aspiration with a Transferpette® and b) centrifugation. Lysozyme (40 µg/mL) in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄ @ pH 7.3), after extraction by polypropylene C-CP fiber-packed tips and elution in ACN:H₂O.

Significantly higher ion counts, approximately two orders of magnitude, can be seen for the lysozyme spectra obtained from the centrifuged sample. The peak intensity for the ~1590 Da (9+) lysozyme psuedomolecular ion peak was used for the quantitative comparisons, with the region from ~1450 to 1550 Da used to evaluate the spectral background. The signal-to-noise and signal-to-background ratios were ~37 and ~16 respectively for the lysozyme ESI mass
spectra obtained from the aspiration method. For the centrifugation method, the signal-to-noise and signal-to-background ratios were \(~118\) and \(~50\) respectively. Among triplicate extractions, the signal-to-noise and signal-to-background ratios in the ESI lysozyme mass spectra were on average \(3.6\) and \(5.4\) times greater for the centrifugation method. Additionally, better precision (14\% RSD) for the characteristic peak intensities was afforded by the centrifugation method, in comparison to 106\% RSD for the aspiration method among triplicate extractions.

For protein samples centrifuged through the PP fiber-packed tips, directed flow starts in the solvent reservoir (the micropipette tip) and continues in one direction through the fiber-packed portion and is collected in the 15 mL centrifuge tube. In this way, buffered protein solutions are forced through PP fiber-packed tips and allowed to continually interact with “fresh” fiber stationary phase until reaching the end of the tip. Protein solutions traveling through the fiber-packed tip by centrifugal force have a total travel distance of 1 cm, the length of the fiber-packed portion of the tip. However, when protein solutions are aspirated (in and out) of the fiber-packed tips for a total of five times, protein solutions traverse 10 lengths for a total of 10 cm. Linear velocities are an order of magnitude slower for centrifuged samples when total loading time for the two methods is kept relatively constant. The increased ESI peak intensities (i.e., recovery) for centrifugal flow is a function of the relative solution linear velocities for the two methods. By the same token the ESI-MS data clearly show that the centrifugation process is far more controllable/reproducible than manual
aspiration. Finally, the throughput of the centrifugation method has the potential for far greater throughput than manual aspiration.

3.3.2 Protein Quantification Throughout the C-CP Fiber SPE

During the course of a fiber-packed tip protein extraction, the protein has the potential to be found in four well defined areas; 1) the collected fraction from the initial pass through of the test solution (effluent), 2) the aqueous rinse fraction, 3) the mixed-phase elution fraction, and 4) any protein remaining on the fiber/pipette tip surfaces. Analysis of each collected fraction during the protein extraction process can describe the nature of the extraction process in terms of the contributions to the overall efficiency and reproducibility. Polyester (PET) and PP fibers were compared for the extraction of the two proteins, lysozyme and BSA. The two fiber types, PP and PET, offer different interactions based on their base polymer structure. Lysozyme and BSA are used extensively in the characterization of chromatographic stationary phases and have been used previously in this laboratory in reversed-phase separations on C-CP fiber columns.

In each of the following experiments, the test solution contained 2.00 μg of the respective proteins. The amount of protein recovered in each of the solutions was determined through UV-VIS analytical response curves for the proteins performed in the respective solvent systems: PBS (test solution), H$_2$O (rinse), and 50:50 ACN:H$_2$O (elution). Results for both lysozyme and BSA content are presented in Table 3.1 for triplicate extractions. The amount of protein initially
adsorbed was defined as the mass in the influent (2.00 μg) minus the mass in the effluent.

For the extraction of lysozyme using PP fiber-packed tips, an average of 0.43 μg of lysozyme was determined in the effluent, translating to an average of 1.57 μg of lysozyme adsorbed in the PP fiber-packed tip. For PET fiber-packed tips, an average of 0.96 μg of lysozyme was determined in the effluent, translating to an average of 1.04 μg was adsorbed in the PET fiber-packed tips. The fiber surface area of the PP and PET fiber-packed tips are ~11.02 cm² and ~6.58 cm² respectively.

### Table 3.1: Protein Quantification Throughout C-CP Fiber SPE

<table>
<thead>
<tr>
<th>Fiber-Tip Protein Extraction</th>
<th>Influent (μg)</th>
<th>Effluent (μg)</th>
<th>Amount Adsorbed (μg)</th>
<th>Rinse (μg)</th>
<th>Elution 1 (μg)</th>
<th>Elution 2 (μg)</th>
<th>Elution 3 (μg)</th>
<th>Elution 4 (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme extracted by PP</td>
<td>2.00</td>
<td>0.43</td>
<td>1.57</td>
<td>ND</td>
<td>0.69</td>
<td>0.07</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>Lysozyme extracted by PET</td>
<td>2.00</td>
<td>0.96</td>
<td>1.04</td>
<td>ND</td>
<td>0.21</td>
<td>0.04</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BSA extracted by PP</td>
<td>2.00</td>
<td>0.61</td>
<td>1.39</td>
<td>ND</td>
<td>0.17</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BSA extracted by PET</td>
<td>2.00</td>
<td>1.17</td>
<td>0.83</td>
<td>ND</td>
<td>0.19</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND: (not detected) mass below 0.04 μg
Comparing the adsorption of lysozyme on the basis of total fiber surface area, and assuming adsorption on the surface of the micropipette tip and the FEP tubing are negligible, an average of 0.14 μg/cm² and 0.16 μg/cm² were adsorbed on PP and PET fibers respectively. As such, the extent of the adsorptive surface interactions for lysozyme is not significantly different between the two polymer types. The lysozyme concentrations in the aqueous rinse fractions were below the limit of detection for the UV-VIS assay. The average percent recoveries for lysozyme were 44 and 22% from PP and PET fiber-packed tips, respectively. The increased recovery of lysozyme for the PP fiber-packed tips is attributed to the limited types of interaction forces (hydrophobic/London) available for protein adsorption at the PP fiber surface. On the other hand, the PET fiber surface lends itself to additional dipole-induced dipole, hydrogen bonding, and π-π interactions, resulting in stronger adsorption, possibly resulting in lower recoveries under these particular elution conditions. This fact was borne out in chromatographic studies where elution of lysozyme from PP C-CP fiber columns took ~30% ACN:H₂O, while 37% was required to elute from PET ⁹⁶. Lysozyme was also determined in subsequent elution fractions (2-4); however the concentration was either negligible or below the limit of detection for UV-VIS analysis. This suggests that the 100 μL used for the elution volume could be reduced thereby concentrating the protein in the elution step.

For the extraction of BSA using PP fiber-packed tips, an average of 0.61 μg was determined in the effluent, translating to an average of 1.39 μg of BSA.
adsorbed in the PP fiber-packed tip. For PET fiber-packed tips, an average of 1.17 μg was determined in the effluent, translating to an average of 0.83 μg adsorbed in the PET fiber-packed tips. Again, when comparing the adsorption of BSA based on surface area on both PP and PET fibers, an average of 0.13 μg/cm² was adsorbed. BSA concentrations in the aqueous rinse fractions were also below the limits of detection. The recoveries of BSA from the fiber-packed tips were low in comparison to lysozyme, with values of 12 and 23% from PP and PET fibers, respectively. This is not surprising given the previous chromatographic separations on PP and PET C-CP fiber columns, where greater amounts of organic modifier are required for elution of BSA than lysozyme. Low recoveries (24%) for BSA have also been reported from monolithic bonded-phase silica packed tips. In contrast, high recoveries (90%) have been reported for lysozyme from bonded-phase silica packed tips. Because proteins have a specific window of organic composition in which they elute from non-polar surfaces, direct comparison to recoveries of different proteins on different phases and under different eluting solvent conditions are quite tenuous at best. By the same token, optimization of elution phase composition could likely result in higher recoveries of proteins from both the C-CP fiber surfaces.

In order to gain a better sense of the elution efficiencies for the proteins, ESI mass spectra were obtained for lysozyme for the four elution fractions from both PP and PET fiber-packed tips. ESI mass spectra for BSA were not obtained for any of the elution fractions because the concentrations were below the limit of detection.
detection for this ESI-MS instrument. ESI mass spectra for lysozyme elutions 1-4 from PP fiber-packed tips are shown in Figs. 3.3 a-d. Figure 3.3 a (elution 1), exhibited the highest signal intensity for the multiply charged lysozyme peaks among the four elutions.

The peak counts for the ~1590 Da lysozyme 9+ pseudomolecular ion and the background from ~1450 to ~1550 Da were used for signal-to-noise and signal-to-background ratio calculations. The signal-to-noise and signal-to-background ratios for Fig. 3a are 276 and 196 respectively. The ESI mass spectra in Fig. 3.3 show that a majority (~90%) of lysozyme is indeed found in the first elution fraction. Among triplicate extractions of lysozyme from PP fiber-packed tips, the signal-to-noise and signal-to-background ratios for the first elution averaged 228 and 124 respectively, with the peak heights varying by 24% RSD. ESI mass spectra for lysozyme elutions 1-4 from PET fiber-packed tips are shown in Figs. 3.4a-d.
Figure 3.3 Extraction of lysozyme using polypropylene fibers. Electrospray ionization mass spectra of eluted fractions from the extraction of lysozyme (40 µg/mL) from PBS using PP C-CP fiber-packed tips in ACN:H₂O 0.1 % formic acid. Spectra labeled a-d correspond to elutions 1-4 respectively.
Figure 3.4. Extraction of lysozyme using polyester fibers. Electrospray ionization mass spectra of eluted fractions from the extraction of lysozyme (40 µg/mL) from PBS using PET C-CP fiber-packed tips in ACN:H₂O 0.1 % formic acid. Spectra labeled a-d correspond to elutions 1-4 respectively.
Figure 3.4a (elution 1), exhibited the highest intensity for multiply charged lysozyme peaks among the four elutions. The signal-to-noise and signal-to-background ratios were calculated in the same way as with the PP extraction. The signal-to-noise and signal-to-background ratios for Fig. 3.4a are 200 and 136 respectively. Among triplicate extractions of lysozyme from the PET fibers, the signal-to-noise and signal-to-background ratios were on average 115 and 78.8 respectively, with the respective peak heights varying by 89 % RSD due to the much lower recoveries. ESI mass spectra for both the extraction of lysozyme from PP and PET fiber-packed tips qualitatively support the data from the UV-VIS analysis of the eluted fractions.

3.4 Conclusions

Centrifugation has been investigated as an alternative approach for SPE processing of proteins from buffered solutions using C-CP fiber-packed tips. Adaptation of the C-CP fiber-packed tips to the centrifugation format has increased throughput, improved precision, provided better control of extraction processes, and allowed for greater extraction efficiencies for tips of the same surface area when compared to aspiration. Future work will include the investigation of centrifugation speed on protein adsorption SPE using C-CP fiber-packed tips as well as wicking as a means of loading protein solutions. It is envisioned that an optimum time frame and centrifugation speed will exist for protein-fiber interactions to provide maximum protein adsorption and recovery.
The protein extraction process has been evaluated in terms of protein mass balance using both PP and PET C-CP fiber-packed tips. It has been demonstrated that under the current methodology and conditions, PP and PET fiber-packed tips exhibit lower recoveries than typical bonded-phase silica type tips $^{2,8}$. Currently, recovery of adsorbed protein from C-CP fiber-packed tips is $\leq 44\%$, while recovery of protein from bonded-phase silica tips can be nearly 100% $^8$. This is not discouraging given that the use of C-CP fibers in micropipette tips for SPE of proteins from buffered solutions is relatively new <1 year $^{10}$, in comparison to bonded-phase silica in micropipette tips which were introduced in the 1990s $^8$.

Protein mass balance experiments have given insight to protein allocation within an extraction experiment. PP and PET fiber-packed tips have similar performance when compared based on surface areas for the adsorption of lysozyme and BSA from PBS. PP was more efficient (2x) for the recovery of lysozyme from PBS, however PET was more efficient (2x) for the recovery of BSA from PBS. A number of experiments are underway to improve the extraction efficiency of C-CP fiber-packed tips. At present, only one architectural type of C-CP fiber-packed tips has been used for protein SPE, an optimization of fiber shape, fiber-tip length, diameter, and packing density could improve recoveries. Aspiration and centrifugation have been investigated as loading methods, but wicking has yet to be employed for protein loading in C-CP fiber SPE. The geometry of the C-CP fibers with the eight channels running the entire fiber length spontaneously wick fluid. It is envisioned that wicking could provide
an additional quantitative means of loading protein solutions without the need for an external force driving fluid movement. The extension of C-CP fibers to the extraction of peptides from buffered solutions is also underway. Also, C-CP fiber tips are being investigated for applications with matrix assisted laser desorption ionization (MALDI) mass spectrometry analysis of proteins.
CHAPTER 4

CAPILLARY CHANNELED POLYMER (C-CP) FIBERS AS A STATIONARY PHASE FOR DESALTING PROTEIN SOLUTIONS FOR MATRIX ASSISTED LASER DESORPTION IONIZATION MASS SPECTROMETRY ANALYSIS

Abstract

Solid phase extraction (SPE) approaches using micropipette tips have been used to extract, concentrate, and purify proteins from buffered solutions. Desalting of protein extracts improves the signal and sensitivity in both electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS). Recently, capillary-channeled polymer (C-CP) fibers have been incorporated as a stationary/support phase in micropipette tips for the extraction of proteins from buffered solutions prior to ESI-MS analysis. The analogous SPE approach is presented here to demonstrate the applicability of using polypropylene (PP) C-CP fibers as a platform for desalting protein solutions prior to analysis by MALDI-MS. A new method of solvent transport using centrifugal force is employed to yield greater sensitivity and precision than conventional solution aspiration methods. Lysozyme and myoglobin are extracted from representative buffer media, phosphate-buffer saline and Tris, respectively. The MALDI mass spectra from desalted lysozyme and myoglobin
solutions demonstrate that this SPE approach provides enhancement of the signal-to-noise and background ratios, the reduction of spectral complexity, and precise molecular weight determinations.

4.1 Introduction

The characterization of proteins from typical cellular extracts often involves several processes that can complicate further instrumental analysis. The method of removing a protein from a cell depends on the location of the protein within the cell as well as the mechanical characteristics of the source tissue. Proteins located in the cytosol of the cell may be liberated by breaking open the cell (i.e., lysis) by chemical or mechanical means. For example, enzymes can be used to chemically disrupt the cell to free the protein of interest. Mechanical disruption of the cell such as through grinding, the use of a high-speed blender, a French press, or sonication can be used to free proteins from cellular material. In any case, once a protein has been removed from its natural environment it can be damaged by exposure to harmful solvent conditions. The structural integrity of proteins are sensitive to pH, thus cell digestions are routinely performed in buffered solutions at pH ranges for which the protein is stable. Protein-containing buffer solutions can consist of salts, small molecular weight organics, and even urea and detergent, depending on the digestion procedures.

Mass spectrometry is particularly powerful for the identification and characterization of proteins. Soft ionization techniques such as matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI)
mass spectrometry provide intact gas-phase protein ions with little or no 
fragmentation \(^{41, 42}\). MALDI is a soft ionization technique because the matrix 
absorbs most of the incident energy from the laser, thus minimizing damage (i.e. 
fragmentation) of the protein analytes. MALDI favors the formation of singly 
charged molecular ions and has proven effective for protein identification and 
characterization \(^{8, 41, 42, 78}\). MALDI is a fairly universal protein analysis tool 
because the matrix absorbs the laser pulse, thus eliminating the need to adjust 
the laser wavelength to match the absorption frequency of each analyte. 
However, direct analysis of proteins from cellular extracts is usually not feasible, 
because proteins are in buffers that are not compatible with MALDI and ESI 
mass spectrometry \(^{98}\). These buffer solutions can have deleterious effects on the 
ionization taking place in both MALDI and ESI sources due to preferential 
ionization, ion suppression effects, and the formation of adduct species \(^{82, 83}\). In 
addition, the presence of abundant buffer species can contribute substantial 
amounts of spectral complexity, inhibiting protein identification and 
characterization \(^{82}\). With these difficulties noted, MALDI is generally thought to 
be more tolerant of buffers than ESI, due to the separation that occurs during 
crystallization of the protein with the matrix \(^{42}\).

Solid phase extraction (SPE) is a popular technique for the extraction, pre-
concentration, and clean-up of various kinds of analytical samples \(^{33, 54}\). SPE has 
been applied to selectively adsorb proteins from buffered solutions and allow 
elution in solvents conducive to MALDI and ESI-MS analysis of proteins and 
peptides \(^{5, 7, 8, 98-102}\). More recently, micropipette tip-based SPE approaches have
been used for protein desalting prior to MS analysis. Incorporating sorbents into the micropipette tip format, enables the efficient handling of micro-liter volumes of protein extracts, which is often needed in bioanalysis where low concentrations and volumes are commonplace. Historically, polymer phases have proven advantageous due to the chemical robustness of the base polymer material and the resistance to pH extremes. While proteins exist in cells/extracts under relatively neutral pH conditions, there may be some advantages to working outside of the range suitable for silica phases.

Our laboratory has recently introduced capillary channeled polymer (C-CP) fibers as a stationary/support phase for the reversed-phase separation of proteins, with a number of positive attributes realized. C-CP polymer fibers possess a unique geometry with eight capillary channels extending the entire fiber length that permits very efficient fluid flow and mass transfer, while providing greater surface area than available from circular cross section fibers. The highly efficient fluid transport, low material cost, wide range of chemical diversity, and robust fiber materials make the C-CP fibers excellent candidates for various separation formats. More recently, Marcus and c-workers have introduced the concept of mounting C-CP fibers within micropipette tips to affect desalting of protein solutions prior to ESI-MS analysis. We report here initial efforts in the implementation of polypropylene (PP) C-CP™ fibers as sorbent media for the solid phase extraction of proteins from inorganic and low molecular weight organic buffers prior to MALDI-MS analysis. Also described is a new fluid handling approach that employs centrifugation, yielding greater recoveries and
better precision than afforded by conventional aspiration methods. It is believed that the basic characteristics displayed here warrant further developments of this approach to protein sample preparation.

4.2 Experimental Section

4.2.1 Reagents and Chemicals

Lysozyme (chicken egg white), myoglobin (equine skeletal muscle), sodium chloride (NaCl), sodium phosphate dibasic (Na₂HPO₄), potassium phosphate monobasic (KH₂PO₄), Sigma 7-9° (tris base), trizma° hydrochloride (tris acid), sinapinic acid (SA), and trifluoracetic acid (TFA) were all obtained from Sigma-Aldrich (Milwaukee, WI). Potassium chloride (KCl) and formic acid were obtained from Fisher Chemicals (Pittsburgh, PA). ACS grade acetonitrile (ACN) was obtained from Mallinckrodt Baker Inc. (Phillipsburg, N.J.) for use as an organic mobile phase. De-ionized (DI) water (18.2 MΩ/cm) from a Nanopure Diamond® Barnstead/Thermolyne Water System (Dubuque, IA) was used in solution preparation of buffers, proteins, and aqueous mobile phases.

4.2.2 Fiber Tip Construction

The fiber tip construction process differs slightly from that previously described in section 2.2.2.¹⁰ In the studies described here, fiber loops corresponding to a total of 658 PP fibers of 55 μm diameter were pulled collinearly through ~300 mm lengths of 0.8 mm i.d. fluorinated ethylene polypropylene (FEP) tubing from Cole Parmer (Vernon Hills, IL). A small portion
~5 mm at one end of the fiber packed tubing was cut with a surgical grade scalpel from Personna (Mansfield, NG, U.K.) so that the tubing and the fiber ends were flush. Fibers from the opposite end were pulled to create ~6 mm of space between the tubing and the fiber end to accommodate the micropipette tip. The end of the tubing with the ~6 mm of dead space was then slipped over a 1 mL micropipette tip (Redi-Tip™) from Fisher Scientific (Pittsburgh, PA). The tubing was cut so that 1 cm of fiber-packed tubing was attached to each tip. The process was repeated across the entire length of the ~300 mm fiber packed tubing producing ~15 tips.

4.2.3 Adaptation to the Centrifugation Format

Previously, a Transferpette® (Brand Tech, Essex, CT) pipette was used to move (aspirate) the protein solutions through the fiber packed tips. Aspiration, a manual process, allowed little control of flow rate and residence time of protein solutions within the fiber-packed tips. The process of aspiration also has limited throughput as only one sample can be processed at a time. By moving protein samples through fiber-packed tips by centrifugal force, more control of fluid movement can be attained and throughput can be increased to 12 or 24 samples at a time, depending on the capacity of the centrifuge. A pipette tip adapter was created to allow the fiber-packed tips to be placed into an accuSPIN™ 1R analytical centrifuge from Fisher Scientific (Pittsburgh, PA). As shown in Fig. 3.1, the conical portion (~10 mm) of a 1.7 mL microtube from Genesse Scientific (San Diego, CA) was cut perpendicular to the microtube axis
so that it resembled a hollow cylinder. The fiber-packed micropipette tip (fiber end first) was placed inside the top of the microtube. The fiber-packed micropipette tip was held in place as the outer wall diameter of the tip approached the inner wall diameter of the microtube. The microtube containing the fiber-packed tip was placed into a 15 mL conical centrifuge tube from Nalge Nunc International (Rochester, NY) and held in place by the attached lid of the microtube extending past the outer rim of the conical centrifuge tube. The fiber-packed tips are adapted to a typical analytical centrifuge by placing the 15 mL conical tubes in the swinging centrifuge buckets.

4.2.4 Protein Loading, Washing, and Elution

A 40 μg mL⁻¹ (2.8 μM) lysozyme solution was made in phosphate-buffered saline (PBS) consisting of 140 mM NaCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ and 2.7 mM KCl @ pH 7.3. A 50 µL aliquot was centrifuged through the 1 cm fiber tips at 300 x g for 5 minutes. The same loading procedure was also performed for myoglobin at 40 μg mL⁻¹ (2.4 μM) in 100 mM Tris (tris(hydroxymethyl)aminoethane) buffer containing 84 mM tris acid and 15 mM tris base @ pH 7.5. A single 100 µL volume of Nanopure water was centrifuged through the 1 cm tips at 300 x g for 10 minutes to remove the buffer species. The proteins (lysozyme and myoglobin) were eluted from the fiber surfaces by centrifuging a single 100 µL aliquot of 50:50 ACN:H₂O through the tips at 300 x g for 10 minutes and the eluent was collected in the centrifuge tube.
4.2.5 Mass Spectrometry

A Bruker Daltonics Ultraflex II time-of-flight/time-of-flight (ToF/ToF) mass spectrometer (Billerica, MA), with a MALDI source equipped with a Nd: YAG laser (355 nm) was used for protein determinations. The accelerating voltages, 1 and 2, were set at 25.00 and 23.45 kV respectively. The focusing lens voltage used to collimate the ion beam going to the detector was set at 6.00 kV, and the pulsed ion extraction (PIE) delay was set at 100 ns. A 20 mg mL\(^{-1}\) solution of sinapinic acid in 50:50 ACN:H\(_2\)O 0.1% TFA was used as the matrix solution. The MALDI target was prepared by spotting 1 \(\mu\)L of matrix solution on the target followed by 1 \(\mu\)L of protein solution. The spot was allowed to dry before introducing the target into the mass spectrometer. MALDI mass spectra for the proteins in the original buffer media and post-extraction were compared on both a qualitative and quantitative basis. No specific effort was made to optimize the number of laser shots within a data acquisition cycle.

4.3 Results and Discussion

4.3.1 Matrix Assisted Laser Desorption Ionization of Lysozyme Extracted from PBS

The C-CP fiber method for desalting protein solutions was evaluated based on the intensity of the molecular ion species, the precision of protein molecular weight, the signal-to-noise and signal-to-background ratios, and spectral clarity. Shown in Figure 4.1, are the MALDI mass spectra obtained from lysozyme in the initial PBS matrix (top) and post-extraction from the C-CP fiber
tip (bottom). The mass spectrum of lysozyme in the buffer solution shows a continuum of signals across the mass range with no discernable lysozyme molecular ion peak observed after 800 shots of the laser. The absence of a protein-specific spectral signature is likely the result of ion suppression by the small inorganic species in the PBS buffer.

![Mass spectrum of lysozyme](image)

**Figure 4.1.** Matrix assisted laser desorption ionization spectra of lysozyme pre and post-extraction from phosphate buffered saline. 40 µg mL⁻¹ (2.8 µM) lysozyme from chicken egg white in PBS (140 mM NaCl, 2.7 mM KCl, 10mM Na₂HPO₄, and 1.8 mM KH₂PO₄ at pH 7.3) (top), and in PBS-removed solution (bottom) via desalting on PP C-CP fibers mounted in a micropipette tip.

Ionized species from the buffer would show up at much lower mass ranges <300 Da, however when analyzing high molecular weight compounds such as proteins it is common to deflect ions lower than ~2000 Da from reaching the detector. The mass spectrum of lysozyme eluted from the C-CP fiber-
packed tip is composed of a high intensity lysozyme pseudomolecular ion peak at 14309 Da and an adduct peak from the matrix at 14527 Da. The average value and standard deviation of the background was assessed across the mass range of 12000 - 12500 Da. The signal-to-noise and signal-to-background ratios of lysozyme post-extraction are 554 and 186, respectively. Interestingly, the average background signal intensity for the lysozyme in the PBS solution across the same mass range was very similar; with 800 laser firings used in both acquisitions. This reflects the fact that the buffer components, having high proton affinities, compete with protein molecules for ionization in the MALDI process; as such the desalted lysozyme ionizes significantly better. The C-CP fiber-extracted lysozyme sample yielded only a 2 Da (0.01%) difference in the mass-to-charge ratio of the molecular ion compared with the MALDI mass spectrum of a neat (20 μg mL⁻¹ in 50:50 ACN:H₂O) solution of lysozyme. This concentration was chosen for the neat lysozyme sample because it would represent 100% efficiency of the C-CP fiber SPE process. A typical measurement error of ~0.01% can be routinely expected using MALDI-MS. While comparisons of ion intensities in MALDI analysis are complicated by issues related to hitting ‘sweet spots’ within matrix crystals, the signals of the pseudomolecular ions for the neat and post-extraction lysozyme spectra differed by less than 10%. This suggests that the C-CP extraction of lysozyme from PBS was quite efficient.
4.3.2 Matrix Assisted Laser Desorption Ionization of Myoglobin Extracted from Tris Buffer

The use of a hydrophobic surface to immobilize proteins from inorganic buffers such as PBS is far less challenging than the case with the buffer constituents have non-polar character. The mass spectra presented in Fig. 4.2 are for myoglobin in 100 mM tris buffer (top), and after elution from the C-CP fiber phase (bottom).

![Mass spectra of myoglobin pre and post-extraction](image)

**Figure 4.2.** Matrix assisted laser desorption ionization mass spectra of myoglobin pre and post-extraction. 40 μg mL⁻¹ (2.4 μM) myoglobin from equine skeletal muscle in 100 mM tris buffer (top) and in tris-extracted solution (bottom) via desalting on PP C-CP fibers mounted in a micropipette tip.

The most noticeable feature of the mass spectrum of myoglobin in the buffer-matrix is the high intensity envelope of peaks at masses between ~16950 and 17600 Da. A peak at 16959 Da representing the myoglobin pseudo-molecular ion
can be seen, but higher intensity peaks at masses between 17009 and 17574 Da dominate the mass spectrum. The mass difference between the adduct peaks are on average ~56 Da, most likely representing portions of the tris buffer forming multiple adducts with the protein. The mass spectrum of myoglobin after C-CP fiber extraction yields a high intensity peak for the myoglobin pseudomolecular ion at a mass of 16964 Da and a matrix adduct at 17165 Da. The mass observed for myoglobin post-extraction was within 5 Da (0.03%) of a neat solution of myoglobin (20 μg mL⁻¹ in 50:50 ACN:H₂O).

The average and standard deviation of the spectral background were derived between 16000 and 16500 Da. The signal-to-noise and signal-to-background ratio for the myoglobin molecular ion peak in the mass spectra before extraction was 89 and 86 respectively, while the post-extraction signal-to-noise and signal-to-background ratios were 97 and 12 respectively. The diminished signal-to-background ratio for the mass spectra of the post-extraction myoglobin is likely due to the lower concentration of myoglobin found in the eluted solution and the increased number of laser shots (6400 vs 3000), yielding larger noises levels. However, the spectral clarity of myoglobin post-extraction outweighs the loss in signal-to-background ratio.

4.4 Conclusions

Previously, PP C-CP fibers were used to desalt proteins prior to ESI-MS analysis. The results from these experiments extend the potential of PP C-CP fibers as a SPE media for desalting proteins prior to MALDI-MS analysis. PP C-
CP fibers bind protein and allow salts and weakly bound organics to be washed from the fiber tips with aqueous mobile phases. Adsorbed proteins can be eluted from the fiber phase with an organic modifier such as acetonitrile. This procedure purifies protein solutions and improves analyte signal responses in the MALDI mass spectra in terms of reduced ion suppression from buffer species, reduced adduct formation, higher signal-to-noise ratios, and precise molecular weight determinations; all of which are critical for the characterization of biologically active molecules.

Though a number of commercially available SPE media have been adapted to micropipette tip geometries \(^8\), the C-CP fibers present several unique features that could prove advantageous. The robustness of polymer fiber materials allows for the use of more diverse solvent conditions. The wide range of base polymer materials provides several polymer/surface interactions ranging from London dispersion forces, to \(\pi-\pi\) interactions and hydrogen bonding. In addition, the eight capillary channels running the entire fiber length facilitate fluid transport, allowing different means of loading, washing, and elution. Optimization of several parameters including: packing density, fiber-packed tip length, and the linear velocity of fluid flow within fiber-packed tips are underway to improve the efficiency of C-CP fiber extractions. Additionally, the use of wicking as a means of loading protein solutions onto fibers is under investigation. Fiber loading capacity and analytical quantification will be key attributes to be compared with commercial micropipette SPE tips.
5.1 Summary

In Chapter 1, solid phase extraction (SPE) was discussed as an analytical technique for the isolation, purification, and concentration of analytes. Interaction types, architecture formats, and applications have been discussed and related to current sample preparation regimes. The benefits of SPE related to the purification of proteins prior to mass spectrometry analysis have been outlined. The research presented here is aimed at introducing capillary-channeled polymer (C-CP) fibers as stationary phases for SPE of proteins from buffered solutions.

Chapter 2 described the incorporation of C-CP fibers as sorbent material for desalting protein solutions prior to ESI-MS analysis. It was shown that both phosphate buffered saline (PBS) at 154 mM and tris buffer in 100 mM suppressed the formation of multiply charged lysozyme and myoglobin species respectively. ESI mass spectra of lysozyme and myoglobin proteins post-extraction using PP fiber-packed micropipette tips exhibited higher signal-to-noise and signal-to-background ratios and more precise molecular weight determinations compared to the proteins in the original buffer solution.

Chapter 3 described the characterization of PP and PET fiber-packed micropipette tips based on mass balance, recovery, and extraction
characteristics as well as a comparison of two loading methods, centrifugation and aspiration. Aspiration and centrifugation were used to move protein solutions through fiber-packed micropipette tips and compared based on resulting ESI mass spectra quality from eluted fractions and throughput. Simple adaptors have been made to allow coupling of fiber-packed micropipette tips to typical 15 mL centrifuge tubes. Centrifugation of test protein solutions through fiber-packed micropipette tips allowed more control of flow rate, increased throughput, and resulted in extracted samples that exhibited more reproducible ESI mass spectra.

PP and PET fiber tips were compared for the extraction of lysozyme and bovine serum albumin (BSA) from PBS. Adsorption of lysozyme and BSA were similar for both fiber types based on total fiber surface area within the fiber-packed tip. The recovery of lysozyme was higher using PP, likely due to the weaker interactions on the PP fiber surface allowing a greater degree of reversible adsorption under these elution conditions. However, recovery for BSA was higher using PET indicating that recovery of protein from C-CP fiber is both a function of polymer type and protein species. ESI spectral clarity was compared for post-extraction protein solutions from both fiber types based on signal intensity, signal-to-noise, and signal-to-background ratios. ESI mass spectra from lysozyme extracted by PP exhibited more desirable spectral characteristics, most likely due to the increased fiber surface area within the tip allowing for more adsorption and subsequent elution of lysozyme.
Chapter 4 described the use of C-CP fibers as sorbent material for desalting protein solutions prior to MALDI-MS analysis. Parallel experiments initially performed and described in chapter 2 for desalting proteins prior to ESI-MS were completed prior to MALDI-MS. MALDI mass spectra from lysozyme and myoglobin post-extraction using PP fiber-packed micropipette tips exhibited more spectral clarity through reduced ion suppression, reduced adduct formation, and higher signal-to-noise and signal-to-background ratios than proteins in the original buffer solution. PP fibers were shown to adsorb both myoglobin and lysozyme from tris-buffer and phosphate buffered saline (PBS) respectively. MALDI mass spectra from myoglobin in tris-buffer resulted in low intensity molecular ions and higher intensity adduct species as shown previously with ESI-MS. Post-extraction myoglobin MALDI mass spectra were free of tris adduct peaks and exhibited a molecular ion peak for myoglobin precise to ~ 5 Da (0.03%) of a neat sample of myoglobin. The ionization of lysozyme in the MALDI source was suppressed due to the presence of low molecular weight inorganic salts in the PBS buffer. MALDI mass spectra of lysozyme post-extraction exhibited a high intensity molecular ion peak precise to ~2 Da (0.01%) of a neat lysozyme sample.

5.2 Future Work

It is envisioned that C-CP fibers due to their resistance to pH extremes, low cost of fiber materials, relative hydrophobicity, and hydrodynamic characteristics could be an attractive alternative to commercial phases for protein
sample preparation. At present, the use of 1 cm of 0.8 mm I.D. fiber-packed tips has been employed for the removal of proteins from buffering species. An optimization of the length and diameter of the fiber-packed tip could increase protein recoveries. In addition the optimization of fiber packing density (interstitial fraction) needs to be accomplished as done previously with C-CP fiber HPLC columns. Also, spontaneous wicking, a characteristic of the fibers, has yet to be investigated as a means of loading protein solutions. Wicking could prove as an excellent means of efficiently loading protein solutions. In addition, experiments to extend the work to other benchmark proteins and buffer systems are in order to fully realize the potential of the fibers for use in protein sample preparation.
APPENDIX

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Capillary-Channeled Polymer Fibers as a Stationary Phase for Desalting Protein Solutions for Electrospray Ionization Mass Spectrometry Analysis, Fornea, D. S., Wu, Y., Marcus, R. K., Analytical Chemistry; (Small Correspondence); 2006; 78 (15); 5617-5621.

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