STUDYING AND MODIFYING PAPER TO LOWER DETECTION LIMITS FOR PAPER SPRAY MASS SPECTROMETRY

by

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A Dissertation

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Doctor of Philosophy

Department of Chemistry

Indianapolis, Indiana

August 2019

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In loving memory of Ester Voglund and Ellen Bills; two wonderful grandmothers who provided love and encouragement throughout my career. I couldn't have asked for better role models of people dedicated to their family and community.

ACKNOWLEDGMENTS

To succeed in life, you must figure out that you can and be given the freedom to do so. My parents Ron and Barb Bills have provided love and support in all my endeavors as far back as I can remember. Words can't capture everything they have done for me and I can't thank them enough. A special thanks to my brother Ryan Bills who has been my truest friend.

My graduate career has been productive and enjoyable due to my mentor Nick Manicke. His level headedness and kind disposition make him approachable; which, was a terrific boon while I found my feet as a scientist. I wouldn't have chosen anyone else on earth as an advisor.

I would also like to thank my undergraduate mentors Rebecca and Sean Peebles and Douglas Klarup at Eastern Illinois University for giving me my first experiences with research. It gave me an opportunity to try research early on, which was a great help when starting grad school.

Finally, I owe a debt of gratitude to all the people at IUPUI who made my graduate career memorable. A thank you to Frédérique Deiss, Greta Ren, and Christine Skaggs for cofounding the chemistry graduate student society Mole with me and to Partha Basu for encouraging us to do so. To the people who made all the bureaucracy of grad school make sense, Kitty O'Doherty and Beveryl Hewitt, their unshakable professionalism was a constant help. I would also like to thank Shelley Rae Spicuzza for all of her help with 3D printing and laser cutting in the Think it Make it lab. Last, but not least, a thank you to Chengsen Zhang for being a friend and colleague when Nick's lab was first starting.

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ABSTRACT

Author: Bills, Brandon, J. PhD
Institution: Purdue University
Degree Received: August 2019
Title: Studying and Modifying Paper to Lower Detection Limits for Paper Spray Mass Spectrometry
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In this work we developed paper spray mass spectrometry methods to obtain lower detection limits for pharmaceuticals and drugs of abuse. The second chapter investigates blood fractionation membranes for their ability to obtain lysis free plasma from whole blood without changing the drug concentration relative to centrifugation. We presented a device capable of obtaining and analyzing plasma samples from whole blood and obtaining quantitative results similar to traditional methods. In the third chapter the properties of the paper substrate are investigated systematically for their impacts on ionization efficiency and recovery in combination with the solvent choice. The fourth and fifth chapters detail a simple method for lowering detection limits using a method called paper strip extraction. In this method biofluids are wicked through either sesame seed oil or solid phase extraction powder on a paper strip to concentrate and preserve (in the case of THC) analytes out of biofluids. The use of 3D printing for rapid prototyping and how it potentially impacts paper spray MS sensitivity is outlined in the final chapter.

CHAPTER 1. INTRODUCTION

1.1 Mass Spectrometry and Ionization

Mass spectrometry depends on two components: ionization, where molecules are evolved into the gas phase and charged into ions; and detection, where ions are separated and detected based on their mass and charge. The technique dates back over one hundred years to the work of J. J. Thomson and his apparatus for deflecting gas phase ions using electric and magnetic fields based on their charge to mass ratio[1]. Using this apparatus Thomson was able to detect multiple charge states of hydrogen, and proved the existence of isotopes by detecting ^{20}Ne and ^{22}Ne [1]. A more efficient mass spectrometer, which uses a magnetic field to deflect ions in a parabolic path came about several years later in the lab of F. W. Aston, who was Thomson's student[2]. Aston used this instrument to investigate over 50 elements and found that isotopes tended differ in mass to charge by nearly whole number increments[2]. Concurrently, in the lab of A. J. Dempster a similar mass spectrometer was built, but, with a gas discharge ion-source that could generate ions in a more reproducible fasion[3].

Modern mass spectrometers employ a variety of techniques for separating and detecting ions. These range from low resolution instruments, such as the linear ion trap developed by Church in 1969[4], to high resolution instruments like the orbitrap invented by A. Makarov in 2005. The orbitrap is capable of 5 part per million mass accuracy (how close the mass to charge measurement is to the true value; 5 part per million is within 0.0005% of the true value) and up to 150,000 resolution (how well the instrument is capable of differentiating molecules with similar masses; 150,000 resolution means that the instrument can distinguish between molecules with a 0.001 amu mass difference at 150 amu molecular mass)[5]. Mass spectrometers analyze ions by separating them using magnetic or electric fields, then measuring them by either sending the ions to a

detector[6], employing a sensor to detect the frequency of oscillation of trapped ions[5], or both. For example, ion trap mass spectrometers use electric fields to trap ions then eject them to mass analyzers for detection. In contrast, orbitraps trap ions in a chamber with specialized geometry and establish a pattern of movement for the ions. Ions separate into distinct regions, so that the frequency of the ions can be measured by the induced current. These modern spectrometers use different methods, but ultimately, they all measure mass to charge.

At the interface, where molecules enter the mass spectrometer, is the ionization source. The purpose of the ionization source is to get molecules into the gas phase and charged. In early mass spectrometers, the elements being analyzed were already a gas[1], and they were ionized by a beam of electrons through a process called electron ionization(EI)[3]. As the high energy electrons interact with the gas phase molecules, a thermal electron is removed from the molecule. This results in positively charged radical cations, which often have excess internal energy[2]. The excess imparted energy frequently causes the molecule to break apart into smaller pieces, a process called fragmentation. Positively charged intact and fragmented ions enter the mass spectrometer and are measured based on both their mass to charge and their relative abundance. Modern mass spectrometers often contain means to fragment the ions further, often through the impact of the molecules with chemically inert gas molecules such as nitrogen or helium. The measured mass to charge and relative abundance of all the ions and fragments is called a mass spectrum. Because molecules tend to fragment reproducibly when using the same type of ionization and fragmentation, the mass spectrum of fragment ions can be used to identify the original molecule. This is useful for fields that require identification of unknown molecules such as medicine for toxicology screening, or in forensic science for drug of abuse screening[7-8].

Since the original experiments in mass spectrometry, a variety of methods for ionizing molecules have been developed to accommodate samples in different physical states, and to vary how much the ions are fragmented. In Thomson's early work, solid phase samples were initially ionized by hitting the surface with a beam of electrons to evolve gas phase ions[1]. One of the major drawbacks of electron ionization is its tendency to heavily fragment ions, also known as hard ionization. Softer techniques came about later; Munson and Field in 1966 developed chemical ionization (CI), where a reagent gas is ionized which then ionizes the gas phase molecules without heavy fragmentation[9]. Other ionization methods were developed for solid state samples such as secondary ion mass spectrometry (SIMS) in the 1960's by Liebl[10] and Castaing[11], fast atom bombardment (FAB) by Barber in 1980[12], or liquid SIMS (LSIMS) by Hass in 1984[13]. Both SIMS and FAB have been largely replaced by matrix assisted laser desorption ionization (MALDI). Developed in 1985 by Hillenkamp and Karas[14], MALDI involves a solid matrix consisting of a reactive compound mixed with the sample that is excited with a laser to generate gas phase ions.

Modern day mass spectrometers are often paired with either liquid or gas chromatography to separate the components of a sample prior to introduction to the mass spectrometer. The type of chromatography often determines what type of ionization method is used. For example, gas chromatography, which already utilizes gas phase molecules, often uses EI because of its ease of use and reproducible fragmentation, which enables mass spectral libraries (spectral databases). Initially, liquid chromatography posed problems because it introduced a large volume of solvent that had to be removed to generate gas phase ions at ambient atmospheric pressure. Atmospheric pressure ionization (API) using corona discharge was developed by Horning and Carroll in the 1970's[15-16], which incorporated a stream of gas to help remove solvent from molecules. Atmospheric pressure techniques were further refined in the 1980's and lead to some of the most

prevalent techniques used today, including atmospheric pressure chemical ionization (APCI) developed in 1987[17] and electrospray ionization (ESI) by Fenn in 1989[18]. APCI, similar to CI, is a soft ionization method where a gas such as N_2 is charged and reacted with solvent molecules that further react with analytes in the gas phase to produce ions. ESI involves charging the solvent with a high voltage relative to the inlet of the mass spectrometer. The large difference in potential causes the solvent to spray in a cone of droplets, known as a Taylor cone, toward the inlet. The charged droplets become smaller as the solvent evaporates; when they reach the point where there is too much charge for its volume, called the Rayleigh limit, further fission occurs. Eventually, either through fission or evaporation, molecules are left in the gas phase with a charge from a chemical agent that either adds or removes a proton. Using these techniques, a sample can be separated into its individual components which can be analyzed separately. Chemical specificity is achieved through both the retention time (how long it takes for a component to go through the chromatography column) as well as its mass spectrum.

1.2 Ambient Ionization

Chromatography is a useful technique but increases the analysis time per sample and requires a significant investment of resources to maintain. Ambient ionization, direct analysis of an unmodified or minimally modified surface or sample under atmospheric conditions, forgoes chromatography to directly analyze a sample. The first two examples were desorption electrospray ionization (DESI) in 2004[19] and Direct analysis in real time (DART) in 2005[20]. In both cases, a sample is placed near an inlet of a mass spectrometer for direct analysis using either a spray of charged solvent for DESI or a stream of charged gas for DART to generate ions. Since the introduction of DESI and DART, a variety of new techniques have emerged[21]. These techniques primarily differ in how they generate gas phase ions. For example, both laser ablation electrospray

ionization (LAESI)[22] and electrospray-assisted laser desorption/ionization (ELDI)[23] utilize lasers to impart energy and generate gas phase ions while surface acoustic wave nebulization (SAWN)[24] uses acoustic waves. Another option includes applying solvent and voltage to a porous surface to generate electrospray as in paper spray[25-26]. The field of ambient ionization has grown substantially in 15 years, with over 40 methods for directly analyzing surfaces and samples in the literature since the advent of DESI in 2004.

Ambient ionization can rapidly acquire information; however, the lack of sample preparation presents problems to analysis. Often the surface or sample being analyzed is complex, with non-analyte species present at high concentrations. For example, blood contains proteins, lipids and salts present at concentrations that are much higher than the analyte of interest. These matrix components can lead to a complicated mass spectrum, and can cause matrix effects. Since the analytes of interest must be freed from the matrix and compete for ionization during analysis[27], poor recovery and ionization efficiency are common problems to overcome. Tandem mass spectrometry (MS/MS) can improve selectivity by fragmenting specific parent ions and measuring diagnostic fragments unique to the analytes of interest, but matrix effects still increase detection limits.

1.3 Paper Spray Mass Spectrometry

Paper spray mass spectrometry (MS), developed in the Cooks lab in 2010, involves spotting a biofluid on a triangle of paper and applying solvent and voltage to generate a plume of charged solvent droplets, known as a Taylor cone, similar to electrospray ionization (Figure 1.1)[25-26]. The solvent wicks through the biofluid spot and extracts analytes, so they can go from the biofluid to the gas phase in minimal time. Paper spray-MS has been used to obtain rapid results for monitoring therapeutic and recreational drugs, lipids, proteins, explosives, and biomarkers[27]. The technique

has also been expanded to spray off a variety of porous non-paper matrices like wood[28], leaves[29] and threads[30] and has been combined with other techniques such as Raman[31], so that one sample can be used to obtain a Raman spectrum in addition to a mass spectrum. Quantitation down to ng/mL and sub-ng/mL ranges has been achieved for many analytes through use of stable isotopic labels (SIL) of analytes as internal standards[32-35]. In recent years efforts to improve detection limits of paper spray-MS have included using a solid phase extraction step integrated in a paper spray cartridge[36] or conducting a liquid-liquid extraction on top of hydrophobic paper[37]. Through these techniques the detection limits for paper spray-MS can be regularly lowered to sub-ng/mL detection limits.

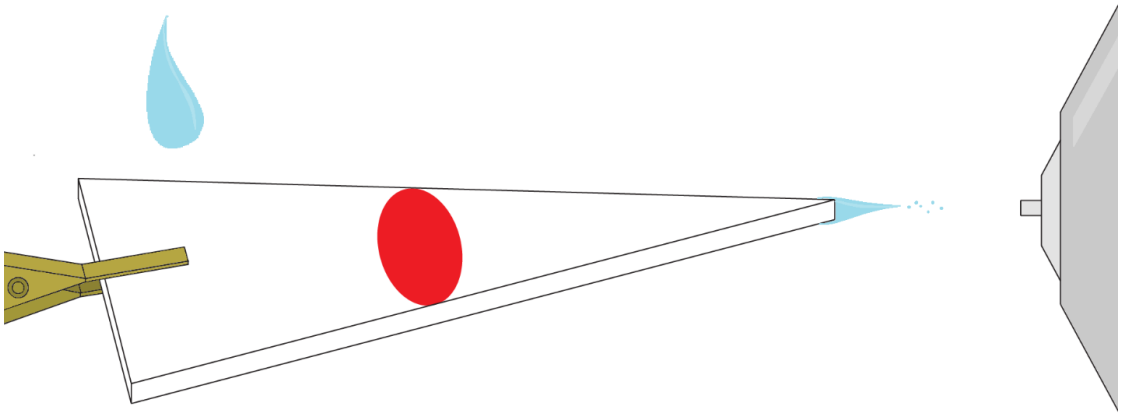

Figure 1.1: Paper Spray Mass Spectrometry. Solvent and voltage applied to a wedge of paper containing a dried biofluid to generate a Taylor cone in front of a mass spectrometer inlet.

Paper spray MS has advantages over traditional drug screening methods such as immunoassays. Developed by Berson and Yalow in 1959[38], IAs employ an antibody to a specific analyte and an indicator that has a distinct color change when antibody binds the target molecule. However, these tests have several drawbacks, such as poor selectivity due to the antibody binding molecules with a similar structure[39], poor sensitivity for low concentration analytes in complex matrices, and limited ability to multiplex or detect multiple analytes simultaneously[40]. Paper spray-MS has been shown to have detection limits at biologically relevant concentrations in a variety of biofluids[26]. Multiplexing is also easier; paper spray MS has been shown capable of detecting over 130 drugs and their metabolites simultaneously during a single 2.5 minute analysis[41].

Rapid analysis through paper spray-MS is useful for applications such as therapeutic drug monitoring (measuring a therapeutic drug to ensure effective and non-toxic dosage) and the newer problem of detecting new psychoactive substances (NPS) (new drugs that mimic more well-known drugs in function but have distinct molecular structures). Monitoring drug concentrations in a patient is important, as each patient will metabolize the drug at a different rates[42]. Tacrolimus and cyclosporin, immunosuppressive drugs used to prevent rejection of transplanted organs, are routinely monitored in clinical settings because there is a narrow window between the effective concentration of the drug and the concentration at which the drug is toxic. A method has been developed with paper spray-MS, and it has been shown measurable using paper spray-MS with accuracy comparable to traditional HPLC-MS methods[32, 43]. Paper spray-MS also shows promise for dealing with NPS (Figure 1.2). These compounds, often developed during pharmaceutical research, are similar to normal drugs of abuse in structure or function[44]. Since NPS' are new with limited available data and can have extremely high potency with unknown side effects, they pose an acute health risk to their users. For example, opioids like fentanyl and carfentanil have

potencies approximately one hundred and one thousand times respectively than that of the historically more common heroin[45]. This wide range of potency has played a major role in the recent surge of opioid related deaths in the United States[46]. Another problem associated with NPS is the large number of possible target analytes. Synthetic cannabinoids, come from an extensive selection of compounds and are sprayed on dried plant matter that is marketed as incense "not for human consumption[47]." With a poor understanding of potency, dosage, and side effects it is not uncommon for people to end up in the emergency room without knowing the exact compound that they took. High potency at lower than normal concentrations and large number of potential targets are a challenge for traditional immunoassays but can be dealt with through paper spray-MS. Paper spray allows samples to be spotted and ionized within minutes, while mass spectrometry has the capability to differentiate between a wide variety of analytes simultaneously based on their unique spectrum.

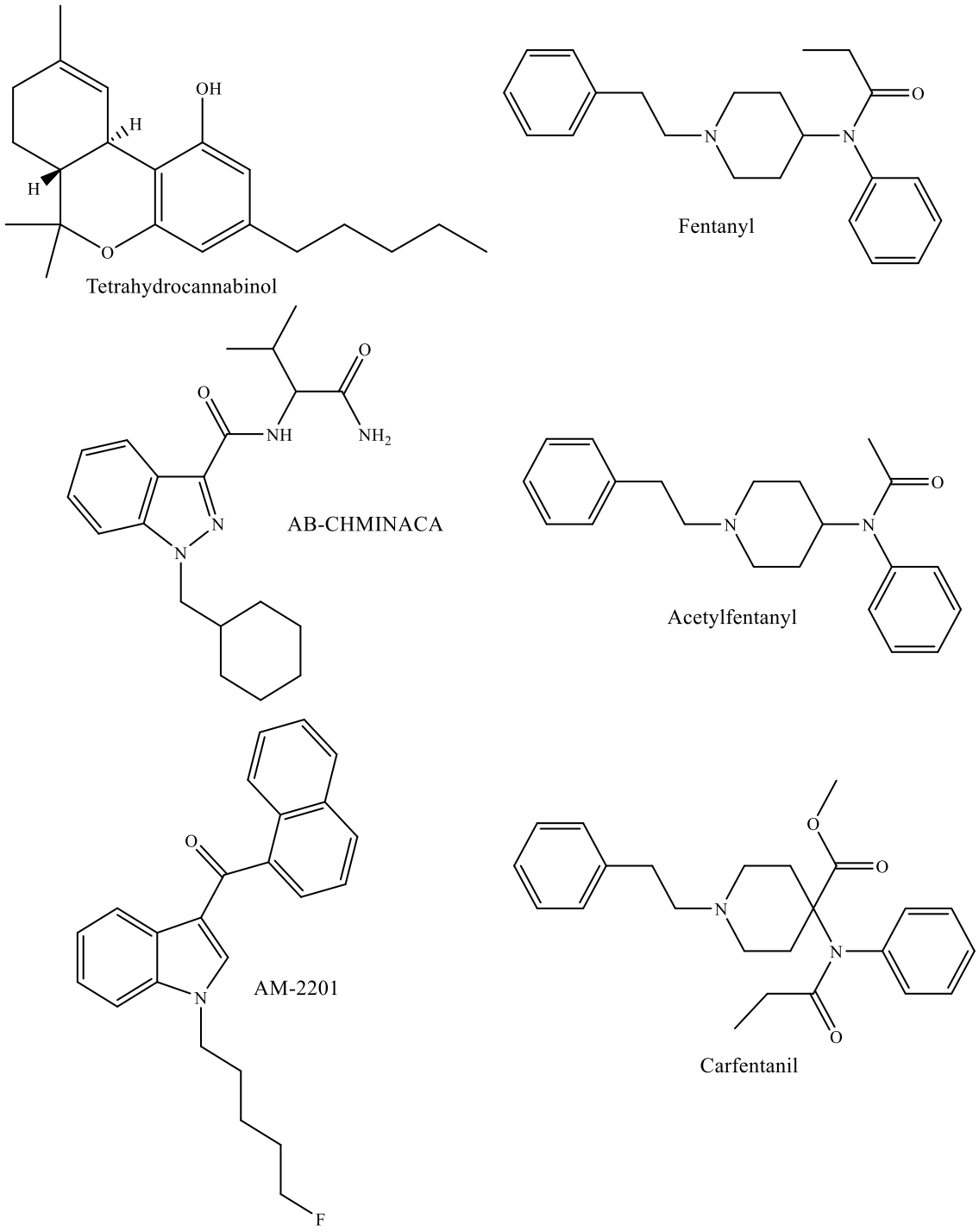

Figure 1.2: Examples of New Psychoactive Substances: AB-CHMINACA and AM-2201 are synthetic cannabinoids that mimic tetrahydrocannabinol in function. Acetylfentanyl and carfentanil are analogues of fentanyl.

The focus of this dissertation is to improve paper spray-MS, particularly its ease-of-use. Several problems still stand in the way of adoption of paper spray-MS for clinical applications. A major issue is that current protocols for monitoring drugs in biofluids focus on the concentration in plasma[48], which adds an extra centrifugation step for paper spray analysis. The first chapter details work on a paper spray cartridge that can obtain plasma from whole blood, with minimal red blood cell lysis and without changing the concentration in plasma relative to plasma obtained by centrifugation. Separating out plasma from whole blood only addresses one concern for one biofluid. A problem for analysis of any biofluid remains the presence of matrix components, which can lead to poor recovery and ionization efficiency[27]. In the second chapter a variety of papers with varying physical properties are investigated to determine what impact those properties have on matrix effects during paper spray-MS, and whether paper can be selected for its impacts on matrix effects to lower detection limits. Detecting NPS' is covered in the third and fourth chapters. For example, tetrahydrocannabinol (THC) is the active ingredient in marijuana and can significantly impact driving capabilities[49-51] and it represents a very labile and difficult analytical target[52] for paper spray-MS. The third chapter focuses on preserving and concentrating natural cannabinoids for detection along with synthetic cannabinoids in urine and oral fluid. The fourth chapter is dedicated to finding more facile methods of using solid phase extraction powder to concentrate a wider selection of designer drugs from urine, oral fluid, blood and plasma. This is accomplished both by coating the powder onto the end of a strip of paper and by immobilizing the powder in an autosampler cartridge. The final chapter focuses on the use of 3D printing in research on paper spray-MS with a focus on how the plastic potentially impacts detection limits. A brief discussion on how to select the proper plastic when printing a device concludes the final chapter.

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CHAPTER 2. ON-CARTRIDGE BLOOD FRACTIONATION FOR DRIED PLASMA ANALYSIS BY PAPER SPRAY MASS SPECTROMETRY

2.1 Abstract

Drug monitoring of biofluids is often time consuming and prohibitively expensive. Analysis of dried blood spots offers advantages, such as reduced sample volume, but can require extensive sample preparation and trained lab technicians. Alternatively, paper spray mass spectrometry allows for rapid analysis of small molecules from blood spots with minimal sample preparation. Plasma is often the preferred matrix rather than blood for bioanalysis; however, centrifugation is required to remove red blood cells which adds time and cost. We demonstrate here a paper spray cartridge containing a plasma fractionation membrane to obtain plasma from whole blood samples. Three commercially available blood fractionation membranes were evaluated based on the accuracy of the drug concentrations in the plasma and the extent of cell lysis/penetration. The accuracy of the plasma concentrations in membrane fractionated plasma was evaluated using high performance liquid chromatography – mass spectrometry. While the fractionation membranes were capable of yielding plasma samples with low levels of cell lysis, the membranes exhibited drug binding to varying degrees relative to plasma obtained by centrifugation. Using the membrane exhibiting the lowest binding we developed a simple paper spray cartridge capable of obtaining a reproducible volume of plasma from whole blood. Quantitative analysis of the plasma samples by paper spray MS yielded similar results to HPLC-MS, but without the need for an offline extraction step or chromatography.

2.2 Introduction

For certain drugs, it is critical to monitor concentrations within the body. There is a therapeutic window at which the drug is most effective without causing adverse side effects such as organ toxicity, neurological effects, and hepatoxicity[1-2]. However, every patient metabolizes drugs at different rates, making dosing difficult[3]. Monitoring drugs in blood can be expensive due to extra sample preparation steps such as centrifugation and protein precipitation as well as the extra cost in shipping refrigerated samples. This has led to a constant push towards analytical techniques that are both rapid and cost effective with minimal sample preparation and analysis requirements.

Because of its simplicity and low cost, paper spray mass spectrometry (MS) shows potential for point of care analysis[4-6]. Paper spray MS has been shown to be useful in the quantitation of a wide variety of pharmaceuticals including immunosuppressive drugs such as tacrolimus and cyclosporine[7-8] and illicit drugs[9-11]. One limitation for paper spray is that, while whole blood is easier to obtain, it is the plasma concentration that is often desired[12]. In general, the use of plasma instead of blood is more readily accepted in pharmacokinetic studies[13]. This is especially true for antipsychotic drugs where drug plasma concentration correlates to blocked receptors and efficacy[14]. Another concern is inaccuracy caused by variable hematocrit or red blood cell percentage of the whole blood[15]. Adding a centrifugation step to obtain plasma, however, would increase the method's complexity which adds time and cost to each sample. Several methods for obtaining plasma from whole blood without resorting to centrifugation have been reported using methods ranging from acoustics[16] to labyrinth like mazes[17]. These methods are able to provide plasma with minimal effect on the matrix, but, are too complex for an inexpensive disposable collection device. Blood fractionation membranes are a potential low cost solution.

These membranes can be used to obtain plasma from whole blood while still yielding accurate drug concentrations in specific cases[18,19].

Here we investigate the use of three plasma separation membranes for small molecule drug analysis. Using HPLC-MS, the membranes were evaluated based on their ability to obtain hemolysis free plasma and not interfere with small molecule drug concentrations. The membranes were then incorporated into a simple paper spray cartridge to enable direct plasma analysis from a drop of whole blood. Agglutinating agents, compounds that induce blood cell clumping, were also added to the membranes to determine if blood fractionation could be improved without contributing to drug binding. Finally, the results from the plasma fractionation cartridge using paper spray MS were compared to HPLC-MS to determine whether accurate results could be obtained in a shorter amount of time.

2.3 Materials

Atenolol, carbamazepine, fentanyl, methadone, nortriptyline, selegiline, chlorpheniramine, cotinine, alprazolam, diazepam and their stable isotopic labels (SIL), except atenolol D7, were obtained from Cerilliant (Round Rock, TX, U.S.A.). Atenolol D7 was obtained from CDN isotopes (Pointe-Claire, QC, Canada) as a powder (≥98% purity). HPLC grade methanol, acetonitrile, formic acid and acetic acid came from Fisher Scientific (Waltham, MA, U.S.A). Five individual donor human blood samples came from Innovative Research in K_2EDTA treated vials (Novi, MI, USA). NoviPlex cards were obtained from Novilytic (West Lafayette, IN, U.S.A.), while CytoSep and Vivid blood fractionation membranes were obtained from the Pall Corporation (Port Washington, NY, U.S.A.). The holder that contained the Vivid membrane was 3D printed using a Stratasys Objet 30 pro using VeroBlue photopolymer (Eden Prairie, MN, U.S.A). The paper spray cartridges and other sample holders were machined out of Delrin and HDPE plastic

respectively (McMaster-Carr) (Elmuhurst, IL, U.S.A.) on a bench-top mini milling machine (Sherline - Vista, CA, U.S.A.). Whatman grade 31 ET chromatography paper was used for the spray substrate and sample storage (GE Healthcare Life Sciences - Pittsburgh, PA, U.S.A.). Human fibrinogen (50-70% protein), alum (aluminum potassium sulfate dodecahydrate ≥98% purity), carbamazepine powder (≥98% purity), and NaCl were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.).

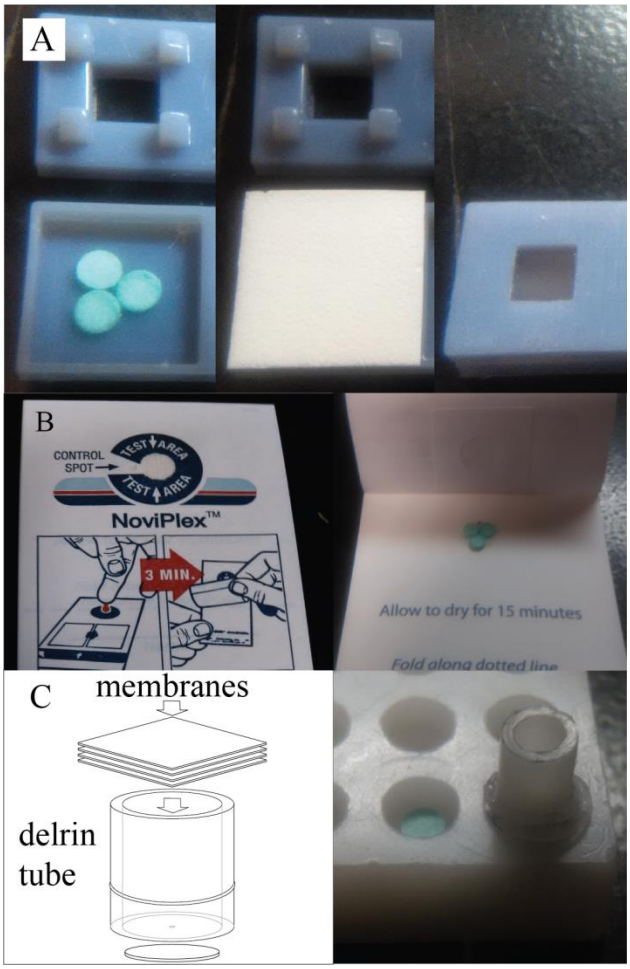

Figure 2.1: Examples of Different Methods of Blood Fractionation. Sample punches dyed green for contrast. (A) Sample holder for the vivid membrane (left to right): holder with the sample punches, 13 mm square of vivid membrane, and closed holder with membrane inside (B) Noviplex extraction card with sample punches. (C) Left: diagram of delrin tube used to contain the 4 squares of CytoSep membrane. Right: picture of the Delrin tube inside the holder with the CytoSep membranes in the tube.

2.4 Methods

2.4.1 Plasma Separation

Each type of membrane required a different set up to extract plasma from whole blood (Figure 2.1). Extracted plasma was collected on 3 mm punches of Whatman 31ET filter paper. The Vivid GR membrane (Figure 2.1A) was prone to ripping so a special holder was 3D printed to contain the punches and a 13 mm x 13 mm membrane. When the top piece of the holder was put into position, it forced the membrane into a bowl shape. Extraction involved adding 40 µL of whole blood to the membrane bowl and allowing 3 minutes for the plasma to wick through. The NoviPlex plasma extraction card (Figure 2.1B) was already designed to be a self-contained sampling device and was used without modification except the sample disk was replaced with three Whatman 31ET punches. A 30 µL aliquot of whole blood was added to the top layer followed by 3 minutes of sitting to allow the plasma to wick through to the sample punches. The CytoSep membrane was assembled inside of a disposable tube by inserting four 5 mm x 5 mm squares of grade 1660 membrane one at a time (Figure 2.1C). Four stacked membranes were required to improve plasma fractionation. The disposable insert was made of a 9 mm length of Delrin tube (3.175 mm inner diameter) with a piece of wax paper with a small hole in it glued over the bottom. To obtain plasma, the insert containing the membranes and wax paper was placed in a tightly fitted plastic holder with a sample punch beneath (Figure 2.1C). Whole blood was applied to the top of the insert and plasma wicked through over 15 minutes. Each sample punch was weighed before and after sample was applied to obtain the mass of the plasma.

2.4.2 Preparation of Bio-fluids, Calibrators, and Controls

The samples for HPLC-MS/MS were prepared using five different lots of blood at two concentrations: 1.5 µg/mL and 0.3 µg/mL for each drug. Each blood sample was taken through the three different membranes in triplicate. Drug stock solutions were diluted to working solutions in 0.6 mg/mL aqueous saline before being spiked into whole blood to minimize solvent based cell lysis. All spiked blood samples had a maximum methanol content of 1.5% (v/v). Samples were incubated at least 1 hour at 37°C prior to fractionation and were applied to the membrane while still warm. Samples were allowed to dry for 1 hour at room temperature in open air to dry. Plasma control samples were prepared by centrifuging an aliquot of the blood at 1500 rpm for 30 minutes.

Calibrators and controls were prepared in pooled drug free plasma from the five blood donors. The calibration curve was prepared by spiking the pooled plasma with of atenolol, carbamazepine, fentanyl, nortriptyline and methadone at concentrations of 0.03 µg/mL, 0.1 µg/mL, 0.3 µg/mL, 1 µg/mL and 3 µg/mL for each compound. A control sample was prepared separately at 1 µg/mL for each drug to verify the validity of the calibration curve. Calibrators and controls were spotted on paper punches at 2.3 mg, the average mass of plasma obtained during fractionation. Calibrators were run in duplicate, and a control was run every 10 samples.

2.4.3 HPLC-MS/MS

Dried sample punches were transferred to a 500 µL centrifuge tube containing 100 µL of extraction solvent (15% methanol by volume in water containing 0.1 µg/mL each of atenolol D7, carbamazepine D10, fentanyl D5, nortriptyline D3 and methadone D3) and were vortexed for 30 minutes. The sample punches were then removed, and the solutions centrifuged at 3000 rpm for 5 minutes. An 80 µL aliquot was transferred to an HPLC vial from each sample for analysis. Analysis was carried out using an Agilent 1100 liquid chromatography system (Agilent

Technologies, Santa Clara, CA.) coupled to a Thermo Scientific LTQ XL mass spectrometer using a hypersil gold C18 column (Thermo Scientific, San Jose). The sample volume injected was 15 µL. The HPLC separation was a gradient elution (Solvent A was water with 0.01% formic acid, solvent B was methanol with 0.01% formic acid) at 200 µL/min. The gradient was as follows: 15% Solvent B for 2 minutes, followed by a linear increase to 80% Solvent B over 2 minutes, then to 95% Solvent B over 1.2 minutes. Solvent B was held at 95% for 4.5 minutes before being dropped and held at 15% for 5 minutes with a flow rate of 400 µL/minute for re-equilibration. MS was conducted in positive ion mode using electrospray ionization (ESI). The sheath and auxiliary gas flow rates were set to 20 and 5 arbitrary units respectively. The instrument was operated in MS/MS mode with a spray voltage of 4.5 kV. Measurements were obtained for a unique fragment of the $[M+H]^+$ ion for each analyte and ratios were determined by dividing the peak area for the analyte by the peak area for the SIL. The slope and y intercept for the curve for each drug was found using 1/x weighted least squares[20]. Because each of the three membrane types yielded a different mass of plasma, final concentrations obtained from the calibration curve were normalized based on the average mass of plasma obtained for each membrane type using equation 1:

$$C_f = C_i\left(\frac{M_{calibration}}{M_{samples}}\right) \quad (1)$$

where C_f is the final concentration, C_i is the concentration calculated obtained without normalizing the mass of the plasma calibrators, $M_{calibration}$ is the average mass of plasma spotted for the calibrators, and $M_{samples}$ is the average mass of plasma obtained for a particular plasma fractionation method. $M_{calibration}$ was 2.3 mg. $M_{samples}$ was 2.2mg, 2.8 mg, and 1.5 mg for the cytosep, vivid, and the noviplex membranes, respectively.

2.4.4 Paper Spray

Plasma fractionation and paper spray MS was carried out on a custom-built spray cartridge involving the CytoSep membrane (Figure 2.2). The blood sample was applied to the removable tube, which contained the fractionation membranes in contact with a sample punch below the tube. When the punch was saturated after 15 minutes, additional plasma could not wick through and the tube insert was discarded. An aliquot of 0.5 µg/mL SIL in methanol, with a volume equivalent to the average mass of extracted plasma (2.2 µL for CytoSep membrane), was spotted onto the dried plasma punch. After drying, the paper disc containing the plasma sample was pushed down onto the spray substrate.

Analysis was performed by positioning the cartridge in front of an LTQ XL mass spectrometer with the paper tip 2.5 mm away from the MS inlet. A 30 µL aliquot of spray solvent (95:5:0.01 methanol:water:acetic acid) was pipetted on top of the sample disc, where it wicked through the sample and onto the spray substrate. Methanol based solvent gave low signal for cotinine so a spray solvent of 90:10:0.01 acetonitrile:water:acetic acid was used for those samples. A voltage of 4.5 kV was applied to initiate the plume of ions and remained on for 60 seconds. Analysis was carried out in MS/MS mode using the base peak fragment of the $[M+H]^+$ for quantitation. For experimental purposes, one spray cartridge was re-used for each analysis and sample preparation was conducted in a block of plastic with holes (Figure 2.1C). Between runs the cartridge was cleaned with methanol and the spray substrate was replaced to reduce carryover.

2.4.5 Determining Hematocrit and Membrane Induced Hemolysis

The five lots of blood were prepared by filling a 25 mm segment of a borosilicate glass capillary (0.86 mm inner diameter Sutter Instrument, Novato, CA) with blood followed by sealing the tube with paraffin wax and centrifuging at 1500 rpm for 30 minutes. Hematocrit was calculated

by measuring the packed cell volume relative to the total sample volume. A calibration curve was created by mixing whole blood (a mix of equal parts of all 5 lots of blood) with centrifuged plasma to make calibrants ranging from 0.45% to 9% (v/v) cellular material in plasma. Blood cell content calibrators were dried and extracted in solvent as described for the HPLC samples. The extent of red blood cell lysis and penetration in the membrane fractionated plasma samples was measured by measuring the absorbance of the sample from the extracts at 413 nm using a Thermo Scientific NanoDrop 2000 spectrophotometer.

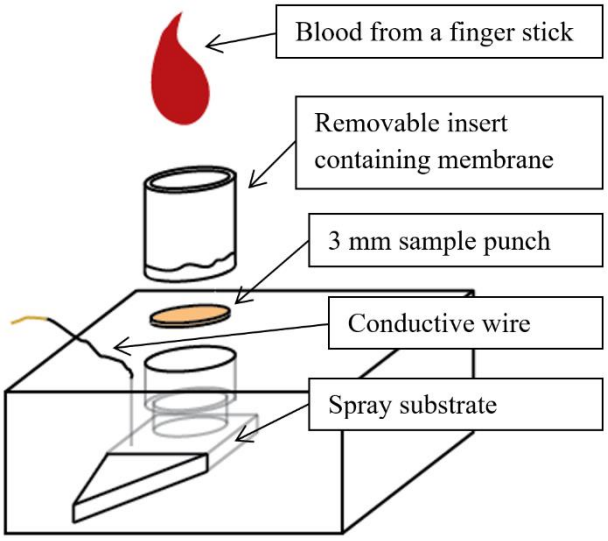

Figure 2.2: Schematic for Paper Spray Cartridge with Removable Blood Fractionation Insert. The plastic is Delrin and the sample punch and spray substrate are Whatman grade 31 ET filter paper.

2.4.6 Membrane Treatment to Improve Plasma Separation

Two different blood agglutination agents were investigated as membrane treatments to improve separation. CytoSep membranes, treated with the protein fibrinogen at concentrations ranging from 20 to 1000 µg/mL, were tested for separation efficiency and potential drug binding. Fibrinogen solutions were spotted onto each membrane in four aliquots of 5 µL allowing roughly an hour between aliquots for drying at room temperature. The non-protein based agglutinating agent alum was also tested at concentrations ranging from 20 to 60 µg/mL. In the final configuration, only the top membrane in the stack was treated with 5 µL of 40 µg/mL fibrinogen. To determine if paper spray MS could be used to obtain quantitative results similar to that of HPLC-MS/MS, plasma that was extracted using membranes treated with human fibrinogen was compared to centrifuged plasma samples. The curve and samples were prepared as described for the HPLC-MS/MS experiments. The samples came from a single lot of blood with 7 replicates for the membrane separated samples (n=6 for the 1.5µg/mL samples due to an insert failure) and 5 replicates for the centrifuged control samples.

2.5 Results and Discussion

2.5.1 Evaluation of Hematocrit, Plasma Mass and Lysis

Three commercially available plasma separation membranes (Vivid GR asymmetric polysulphone membrane, Noviplex blood fractionation card and Cytosep grade 1660 lateral blood fractionation paper) were evaluated for their ability to obtain reproducible amounts of red blood cell free plasma (Table 2.1). Five lots of human blood with hematocrits ranging from 45 to 54% were evaluated. The average mass of plasma was taken from 6 replicates of each blood lot and the percent volume of red blood cells was determined based off 1 replicate from each lot compared to a calibration curve. Both the Vivid GR and Noviplex card showed good plasma fractionation

with a total red blood cell content below 1% (v/v). The Cytosep grade 1660 membrane stacked 4 high showed noticeably poorer separation at 6% red blood cells, most likely due to being a lateral flow membrane.

The CytoSep membrane was selected for further testing in combination with a cartridge that could obtain plasma from whole blood. Ideally, the paper spray cartridge would be able to deliver a reproducible volume of plasma from poorly controlled blood sample volumes. Volumes of blood ranging from 30 to 50 µL were applied to the treated membranes and it was found that the volume of blood had no effect on the extracted volume of plasma (Table 2.2). This is likely due to the sample punch becoming saturated and preventing excess plasma from wicking through.

Table 2.1: Mass and Red Blood Cell Content of plasma Obtained by Three Membranes. Average and 95% confidence interval for the wet plasma mass obtained for each membrane and average and 95% confidence interval red blood cell content in the plasma.

Extraction method	Mass of plasma (mg)	Red blood cell % (v/v)
CytoSep grade 1660	2.2±0.4	6±2
Vivid GR	2.82±0.06	0.22±0.09
Noviplex	1.53±0.04	0.8±0.2

Table 2.2: Mass of Plasma Obtained from Variable Blood Volumes Using Cytosep Membrane. The 95% confidence intervals for mass of plasma extracted from whole blood using fibrinogen treated CytoSep membranes from different volumes of whole blood (N=5).

Volume of whole blood	Mass of plasma extracted (mg)
30 µL	2.7 ± 0.2
40 µL	2.7 ± 0.2
50 µL	2.7 ± 0.5

Table 2.3: Drug Concentrations in Plasma Obtained by Three Blood Fractionation Membranes. Averaged plasma concentrations in µg/mL from whole blood fractionated by three different membranes.

	Original Concentration	CytoSep	Vivid	Noviplex	Centrifuge
Atenolol	0.3 µg/mL	0.32	0.24	0.25	0.37
	1.5 µg/mL	1.66	1.08	1.17	1.67
Carbamazepine	0.3 µg/mL	0.26	0.11	0.16	0.32
	1.5 µg/mL	1.41	0.44	0.59	1.53
Fentanyl	0.3 µg/mL	0.24	0.08	0.24	0.35
	1.5 µg/mL	1.28	0.37	1.22	1.77
Methadone	0.3 µg/mL	0.21	0.16	0.23	0.47
	1.5 µg/mL	1.04	0.99	1.15	1.79
Nortriptyline	0.3 µg/mL	0.06	0.13	0.20	0.29
	1.5 µg/mL	0.36	0.54	1.06	1.51

2.5.2 Assessment of Drug Binding

Drug loss due to binding to the membrane was evaluated by measuring the drug concentration (Table 2.3) of the plasma obtained by membrane filtration and the percent decrease relative to centrifuged plasma (Table 2.4). Calibrators and controls were prepared directly in plasma to ensure a known plasma concentration. The experimental samples were prepared in whole blood as opposed to plasma to allow the drugs to distribute between the blood cells and the plasma to account for any changes in concentration caused by unequal distribution of drug.

Table 2.4: Decrease in Drug Concentration in Plasma Obtained Using Three Membranes. The average % decrease in drug concentration relative to the concentration found in centrifuged plasma for three membranes at two concentrations.

	Original Concentration	CytoSep	Vivid	Noviplex
Atenolol	0.3 µg/mL	-13%	-36%	-33%
	1.5 µg/mL	0%	-36%	-30%
Carbamazepine	0.3 µg/mL	-17%	-65%	-51%
	1.5 µg/mL	-8%	-71%	-62%
Fentanyl	0.3 µg/mL	-31%	-76%	-31%
	1.5 µg/mL	-28%	-79%	-31%
Methadone	0.3 µg/mL	-55%	-66%	-50%
	1.5 µg/mL	-42%	-45%	-36%
Nortriptyline	0.3 µg/mL	-78%	-57%	-29%
	1.5 µg/mL	-76%	-64%	-30%

Table 2.5: Change in Drug Concentration in Plasma Obtained Using Treated Membranes. Change in concentration of drugs in plasma extracted using CytoSep grade 1660 membrane (both untreated and treated with alum or fibrinogen) relative to plasma obtained via centrifuge. NS indicates that the difference between the results for the control and experimental were not statistically significant ($p>0.05$).

Drug	logP	$K_{B/P}$	Untreated (plasma)	Untreated (blood)	Fibrinogen (blood)	Alum (blood)
selegiline	3.08	1.7	-5% ^{NS}	-26%	-43%	-21%
chlorpheniramine	3.74	1.34	-5% ^{NS}	-31%	-46%	-18%
atenolol	0.57	1.07	8% ^{NS}	-2% ^{NS}	-8% ^{NS}	-4% ^{NS}
carbamazepine	2.1	1.06	13% ^{NS}	5% ^{NS}	-3% ^{NS}	-10% ^{NS}
fentanyl	4.02	0.97	-3% ^{NS}	-31%	-24%	-31%
cotinine	0.39	0.88	-4% ^{NS}	-15%	-19%	-0% ^{NS}
alprazolam	2.23	0.78	5% ^{NS}	-14%	-11%	-37%
methadone	4.14	0.75	-10% ^{NS}	-51%	-45%	-13%
diazepam	2.63	0.58	15% ^{NS}	-4% ^{NS}	17%	-6% ^{NS}

Both the Vivid GR and the NoviPlex membranes caused a measurable decrease in the concentration for all five drugs while the CytoSep membrane showed a noticeable decrease for three drugs, but not for atenolol and carbamazepine. The presumed cause of the decrease in drug concentration in membrane fractionated plasma is that the drug is binding to the membrane. It has been shown in several studies that polysulfone based membranes, like the Vivid GR, cause a decrease in drug concentration in blood[21,22]. This means that membranes like the Vivid GR will be poor choices for obtaining plasma for therapeutic drug monitoring purposes.

2.5.3 Membrane Treatment to Improve Plasma Separation

The CytoSep membrane showed the lowest amount of drug binding, but also resulted in the highest level of red blood cell content in the collected plasma sample. Methods were investigated for improving the plasma fractionation efficiency from this membrane. Previous work showed that causing cells to agglutinate allowed for plasma to be separated from blood cells in paper based devices[23]. This work employed anti-A and anti-B antibodies and thus would not cause type O blood (which lack these antigens) to agglutinate[23]. The alternative options of fibrinogen, a key protein in the coagulation cascade[24], and alum[25] were used instead. Fibrinogen and alum were spotted directly onto the membranes and allowed to dry before assembly of the insert.

Fibrinogen concentrations ranging from 20 µg/mL to 1000 µg/mL were tested and concentrations of 75 µg/mL or greater showed measurable drug binding for the initial test set of drugs. However, concentrations less than 50 µg/mL showed minimal improvement in cell lysis/penetration. The 50 µg/mL fibrinogen treated membranes showed cell lysis/penetration of approximately 3% compared to approximately 6% for the untreated membrane. This was an improvement to the plasma separation efficiency, so the treated membrane was selected for further testing.

Similarly, different concentrations of alum were evaluated for their improvements to separation efficiency. However, it was found that the amount of alum required to improve the plasma separation also yielded irreproducible amounts of plasma on the collection disc. A lower concentration (40 µg/mL instead of 60 µg/mL) of alum was chosen for further testing even though the separation efficiency was similar to that of the untreated membrane. This testing was performed to evaluate if a non-protein agglutination agent would have a noticeable impact on the binding properties of the drugs.

2.5.4 Evaluation of Errors from Drug Binding and Cell Lysis

A selection of drugs with varying logP and $K_{B/P}$ (a measurement of the distribution between red blood cells and plasma) values were tested to better understand the binding characteristics of the treated and untreated CytoSep membrane as well as the errors caused by blood cell lysis. Plasma samples were tested along with blood to determine if plasma samples could be used to evaluate membrane drug binding without the complicating effects of red blood cell lysis/penetration (Table 2.5).

None of the nine drugs tested showed significant binding to the membrane from plasma samples. When whole blood was applied to the membrane rather than plasma, six of the drugs showed a measurable decrease in analyte concentration. If cell lysis/penetration was the principal source of error in blood, then the error after passing through the membrane should be negative for drugs with a $K_{B/P} < 1$ (drug is more concentrated in plasma so lysing cells would dilute the plasma concentration), positive for drugs with a $K_{B/P} > 1$ (drug more concentrated in the red blood cells so lysing cells increases plasma concentration), and minimal for drugs with a $K_{B/P}$ close to 1 (drug evenly distributed between cells and plasma). The change in drug amount in the collection disc did not correlate with the $K_{B/P}$. For example, selegiline and chlorpheniramine have a $K_{B/P}$ above

1, but showed a significant decrease in concentration after passing through the membrane. Methadone had a negative error, but it was much greater in magnitude than would be expected based off the measured levels of cell lysis/penetration and its $K_{B/P}$. This error is likely caused by higher levels of drug binding to the membrane in blood samples compared to plasma. One possible reason for the higher levels of membrane drug binding in blood samples compared to plasma samples is the slower passage of blood samples through the membrane. Plasma samples pass through the membrane considerably faster due to lower viscosity and because the membrane is not clogged by blood cells. The decreased extraction time (roughly 5 times less than whole blood) could lead to less apparent binding due to the shorter interaction time with the membrane. This indicates that plasma is not a suitable matrix for evaluating drug binding by the fractionation membranes.

Table 2.5 also shows the logP (octanol-water partitioning) for each analyte. All of the drugs with a logP of 3 or greater exhibited negative deviations of 20% or more against the untreated membrane in whole blood samples. All the drugs with a logP below 3, on the other hand, deviated by 15% or less. This suggests that drugs with a $K_{B/P}$ close to 1 and a logP below 3 are the least likely to be biased by membrane fractionation.

Plasma drug concentrations obtained using the membranes treated with agglutination agents were also evaluated. Although fibrinogen significantly decreased the amount of cell lysis/penetration, it did not improve the analyte error. For the alum treated membranes, the amount of cell lysis/penetration was not significantly different than the untreated membrane for most analytes. This suggests that while agglutinating agents may reduce cell lysis, the amount of error from membrane interactions has a more significant impact.

2.5.5 Quantitative Analysis by Paper Spray MS/MS

We built a prototype paper spray cartridge incorporating a plasma fractionation membrane (Figure 2.2). A drop of blood applied to the cartridge yields a plasma spot that can be analyzed directly from the cartridge via paper spray MS without any additional sample preparation. To evaluate the ability of the method to quantify plasma concentrations, blood samples were prepared with concentrations of 0.3 and 1.5 µg/mL. The plasma was then fractionated using the fibrinogen treated membrane inserts and compared to centrifuged plasma from the same blood sample using calibration curves (Figure 2.3). The results for the calibration lines and differences between the membrane fractionated plasma samples and centrifuged plasma control samples are shown in table 2.6. Concentration data for both the paper spray MS and HPLC-MS results can be found in table 2.7. While the slopes of the lines for HPLC-MS experiment were different than paper spray-MS, likely due to the difference in how the samples were prepared, the atenolol and carbamazepine concentrations and detection limits were similar for both the membrane fractionated plasma and centrifuged plasma using both methods. However, the paper spray results were obtained without an extraction step (30-35 minutes) or a HPLC run (around 14 minutes per sample). This significantly reduced the amount of time and effort required to obtain the result.

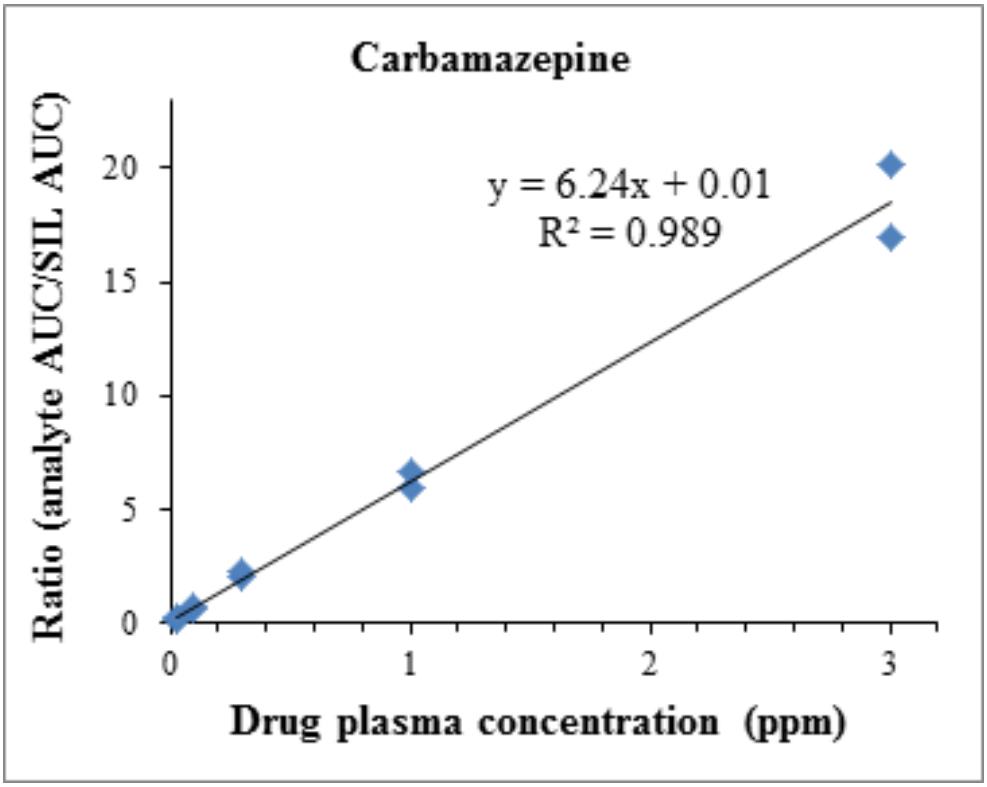

Figure 2.3: Paper Spray MS Calibration Curve for Carbamazepine. Calibration curve for carbamazepine in plasma obtained by paper spray mass spectrometry.

Table 2.6: Comparing Centrifuged Plasma and Membrane Fractionated Plasma Using HPLC and Paper Spray. HPLC-MS and paper spray MS results for the calibration curves and the deviation between plasma obtained by centrifugation to the plasma fractionated using a fibrinogen treated membrane. NS indicates that the difference between the results for the control and experimental were not statistically significant ($p>0.05$).

Drug	Method	Slope	Intercept	r2	LOD µg/mL	% deviation, 0.3 µg/mL	% deviation, 1.5 µg/mL
Atenolol	HPLC	0.405	0.002	0.982	0.03	-2% NS	-0.4% NS
Carbamazepine		4.18	0.1	0.983	0.04	10% NS	-9% NS
Atenolol	paper spray	0.185	0.0007	0.995	0.02	-9% NS	-8% NS
Carbamazepine		6.24	0.01	0.989	0.03	-4% NS	-10% NS

Table 2.7: Comparing Drug Concentrations in Centrifuged Plasma and Membrane Fractionated Plasma Using HPLC and Paper. Spray Average plasma concentrations with standard deviation using HPLC-MS and paper spray MS.

Drug	Method	Cytosep 0.3 µg/mL	Cytosep 1.5 µg/mL	Centrifuge 0.3 µg/mL	Centrifuge 1.5 µg/mL
Atenolol	HPLC	0.32±0.06	1.7±0.2	0.33±0.03	1.7±0.2
Carbamazepine		0.26±0.06	1.4±0.2	0.29±0.03	1.5±0.2
Atenolol	paper spray	0.24±0.02	1.6±0.3	0.22±0.02	1.7±0.1
Carbamazepine		0.23±0.02	1.5±0.3	0.24±0.03	1.6±0.3

2.6 Conclusion

In conclusion, 3 different membranes were evaluated for lysis and drug binding. These membranes were made from a variety of materials ranging from polymers to natural and synthetic fibers. Two of the three membranes, the Vivid GR polysulfone membrane and the NoviPlex plasma extraction card, exhibited noticeable amounts of drug binding for each of the five drugs tested. The third membrane, the CytoSep membrane, exhibited no significant binding for atenolol and carbamazepine. While the CytoSep membrane had the most favorable drug binding characteristics, it showed the poorest fractionation efficiency as measured by the red blood cell content in the fractionated plasma. Overall, this membrane could be used for quantitative analysis for drugs with a logP less than three and a $K_{B/P}$ near unity, but is not a universal solution.

A proof-of-concept design for a simple paper spray cartridge for performing direct plasma analysis from a drop of blood was built and tested. The results were found to be comparable to extraction followed by HPLC-MS/MS. Unbiased results are only obtainable for atenolol and carbamazepine, however, due to drug binding and cell lysis. Further investigations are required to identify membrane materials that are capable of maintaining good plasma fractionation efficiency but without nonspecific drug binding.

2.7 References

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CHAPTER 3. THE IMPACTS OF PAPER PROPERTIES ON MATRIX EFFECTS DURING PAPER SPRAY MASS SPECTROMETRY ANALYSIS OF COMMON AND EMERGING DRUGS OF ABUSE

3.1 Abstract

Designer drugs, drugs synthesized in a lab that mimic other drugs of abuse, have become a major cause of death in the United States. Limitations in either speed, sensitivity or selectivity of current analytical techniques hinders rapid detection. Paper spray mass spectrometry (MS) is a rapid ambient pressure technique capable of detecting analytes in complex matrices. However, due to a lack of sample cleanup and chromatography, matrix effects can have a significant impact on the detection limits. Previous work has shown that the paper used has an impact on matrix effects, but the current literature lacks a systematic approach to studying different properties of paper with regards to matrix effects. In this work, the effect of pore size, flow rate, and thickness on ionization efficiency and recovery was assessed. Cellulose thin layer chromatography (TLC) plates were made along with a universal spray cartridge to provide a porous spray substrate similar to paper but with easily changed thickness. It was found that substrates with the highest filtration properties (thicker, slower flow rate, or smaller pore sizes) had lower analyte recovery but improved ionization efficiency. This was verified with an offline extraction conducted with a 3D printed centrifuge extractor. Paper/solvent combinations were tested with urine samples to determine if selecting a paper and solvent with better ionization efficiency could improve detection limits. While minimizing ionization suppression improved detection limits for some drugs, those charged at physiological pH were largely unaffected. For analytes that showed improvement, both the paper and the solvent had an impact; but, solvent had the larger impact.

3.2 Introduction

In recent years, designer drugs (drugs produced in a lab to mimic the effects of other drugs of abuse) have become more prevalent[1-3]. Manufacturers take advantage of these drugs' unique structures to avoid detection and regulation[1]. Synthetic cannabinoids, for example, are sprayed on dried plant matter and often marketed as herbal incense while being marked as "not for human consumption"[4]. However, the active ingredient may be one or more of any molecule from a library of compounds developed for research purposes[4-5]. This can have disastrous consequences as the dosage is poorly controlled, and the potency of any individual compound can vary wildly[2, 5]. As a result, consumers can experience side effects and can end up overdosing[2, 4]. Another alarming trend is that fentanyl and its derivatives are being found with increasing regularity as both adulterants in heroin and as drugs of abuse by themselves[2-3]. Coupled with a highly variable range in potency of fentanyl analogues (ranging from less potent to one hundred times more potent than fentanyl[6]) has contributed to a spike in overdose fatalities[2-3, 6]. Due in part to these emerging drug trends the number one cause of injury death in the united states has become drug poisoning (ahead of suicide, motor vehicle crashes, and firearms)[2].

With the rise in number and potency of illicit drugs comes a need for rapid, selective and sensitive analytical techniques. A quick screening method for samples could give law enforcement an early warning about what type of drug is showing up locally and help emergency rooms be prepared for further overdose cases. The most common methods used for drug screening are often either immunoassays (IAs) or mass spectrometry (MS) paired with gas chromatography (GC) or high performance liquid chromatography (HPLC)[7-10]. While IAs are simple and fast, they have problems with sensitivity and selectivity making them poorly suited for the current crisis[11]. GC-MS and LC-MS have better selectivity and sensitivity, but require sample preparation and the chromatography step adds analysis time to each sample. Ideally a screening technique would be

simple and direct with minimal required training and fast analysis times making paper spray MS a viable option.

Many designer drugs require low detection limits due to their high potency. A limitation to detection limits for paper spray-MS is the presence of matrix effects. Without a separation or sample cleanup step the detection limits can be increased by poor analyte recovery and ion suppression[12]. Recent work on improving detection limits has involved integrating a solid phase extraction step with a paper spray cartridge[13], performing a solvent extraction from the sample on top of hydrophobic paper[14], and utilizing a membrane to filter out the red blood cell component of blood[15]. An alternate approach involves changing the porous spray substrate to optimize detection limits. Studies have included paper treated with various coatings (such as silica or zirconia)[16-19], comparing different types of paper[20-21] and non-paper porous spray substrates[22-27]. These studies give insight on the role the porous substrate takes in paper spray-MS. However, few studies of the spray substrate take a systematic approach to try to elucidate what specific properties of the paper have what impacts on the matrix effects. Previous work has shown that by increasing the distance between the sample and spray tip lowers the impact of ion suppression while hurting recovery[28]. This suggests that the paper itself could play a role in mitigating matrix effects.

Here we describe a systematic approach to comparing spray substrates to better determine what properties of the paper impact ionization efficiency and recovery. Initially a subset of drugs was selected that included drugs with varying physical properties as well as fentanyl and synthetic cannabinoids. To study a single property, such as thickness or pore size, papers were selected with as many similar properties as possible with one property significantly different. Cellulose based thin layer chromatography (TLC) plates were made to provide a cellulose based substrate with easily controllable dimensions. To accommodate the wide variety of spray substrates and

minimize any differences in experimental procedure a universal paper spray cartridge capable of housing any type of porous spray substrate was fabricated using a combination of 3D printing and a bench top milling machine. The ionization efficiency results were further tested using a 3D printed modification to a centrifuge tube that allowed for solvent to be pulled through a dried biofluid and spray substrate and subsequently analyzed by syringe elution using ESI-MS.

3.3 Materials

All the pharmaceuticals and their stable isotopic labels (SIL), except atenolol D7, were obtained from Cerilliant (Round Rock, TX, U.S.A.). Atenolol D7 was obtained from CDN isotopes (Pointe-Claire, QC, Canada) as a powder (≥98% purity). AB-CHMINACA and AM-2201 and there SIL's were obtained from Cayman Chemical (Ann Arbor, Michigan, U.S.A). HPLC grade methanol, acetonitrile, formic acid and acetic acid came from Fisher Scientific (Waltham, MA, U.S.A). Blood and urine were obtained from a single volunteer. The papers tested included Whatman grade 31 ET and 3MM chromatography paper and grade 4 and 5 filter paper from GE Healthcare Life Sciences (Pittsburgh, PA, U.S.A.). Cellulose powder (20 µm and 50 µm particle size) and starch (from rice) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). The clamp to hold the universal cartridge together was 3D printed in VeroBlue photopolymer using an Objet 30 pro from Stratasys (Eden Prairie, MN, U.S.A). The universal cartridge and the base of the TLC plates were Delrin plastic from McMaster-Carr (Elmuhurst, IL, U.S.A.). The cartridge was manufactured using a bench-top mini milling machine from Sherline (Vista, CA, U.S.A.). The centrifuge modification was 3D printed using a polypropylene filament using an Ultimaker 2+ extended (Geldermalsen, Gelderland, Netherlands). The TLC plates were cut using a Speedy 300 laser engraver from Trotec (Wels, Austria).

3.4 Methods

3.4.1 Paper Selection

Papers were selected in pairs with the criteria that most physical properties were as similar as possible and one property was significantly different. Ultimately two pairs of paper were selected based on their different pore sizes (filter paper) and flow rates (chromatography paper) given on the manufacturer website (Table 3.1).

The flow rate during paper spray was determined experimentally using a glass capillary with 1 µL gradients marked on the side. The capillary was placed nearly horizontal against the back edge of the paper and the rate of solvent consumption was measured using a stopwatch to time how long it took for 15 µL of solvent to be consumed. The flow rate due to paper spray was determined by finding the difference between the average rate of solvent consumption for three runs without voltage (evaporation only) and with voltage (paper spray and evaporation).

3.4.2 Analyte Selection

To better understand if and how the different physical properties of small molecules interact with the selected paper properties a selection of seven pharmaceuticals were selected in addition to two synthetic cannabinoids and fentanyl (Table 3.2). In order to study the wide variability of analyte physical properties that can be encountered, analytes were selected from pharmaceuticals with documented properties to include a wide range of logP, pK_a (acid and base), and physiological charge values. The remaining three analytes were included as targets of specific interest for the current crisis.

Table 3.1: Properties of Whatman Paper. Physical properties of chromatography and filter paper. (*) Indicates properties not listed on the manufacturer website that were measured using a caliper and scale.

Paper	Pore Size (µm)	Thickness (µm)	Weight (g/m^2)	Flow Rate (mm/30 min.)
Whatman Grade 4 Filter	25	210	92	-
Whatman Grade 5 Filter	2.5	200	100	-
Grade 3MM Chromatography	-	340	186*	130
Grade 31 ET Chromatography	-	500	183*	225

Table 3.2: Drug Properties. Physical properties of the pharmaceuticals from drugbank.

Analyte	Molecular Weight	logP	pKa (acid)	pKa (base)	Physiological Charge
Alprazolam	308.77	2.23	18.3	5.08	0
Atenolol	266.336	0.57	14.08	9.67	1
Carbamazepine	236.269	2.1	15.96	-3.8	0
Diazepam	284.7	2.63	NA	2.92	0
Fentanyl	336.471	4.12	NA	8.77	1
Gabapentin	171.237	-1.9	4.63	9.1	0
Hydrocodone	299.368	2.13	18	8.61	1
Phenylephrine	167.205	-0.69	9.07	9.69	1

3.4.3 TLC Plate Fabrication

Ideally, spray substrates could be fabricated with controllable properties for targeted studies of how different physical aspects of the spray substrate impact the overall analysis. The problem is that paper manufacture is a complex process with many subtle aspects involved in producing a uniform and reproducible product. A simpler alternative is to manufacture TLC plates using cellulose powder as simulacrum of paper. TLC plates can be manufactured from a wide variety of stationary phases and binders[29]. Black Delrin plastic cut into two-inch squares was used as a backing for the plates. Because 0.3 mm Delrin plastic is sold in rolls instead of flat sheets it was baked under a brick of aluminum at approximately 150°C for four hours to flatten the squares and cooled under the brick until room temperature. The surface was scored with sand paper prior to baking and washed prior to addition of the stationary phase. Starch was used as a binder at three percent by mass to improve ruggedness of the plate. A slurry was prepared by mixing the cellulose-starch mixture in a 1:3 ratio with boiling water to make a thicker stationary phase or 1:6 with boiling water to make a thinner stationary phase. One aliquot of 3 mL of the slurry was spread evenly on the scored surface of the Delrin plastic square. The plates were dried on the benchtop at room temperature. The plates were cut with the stationary phase face down into uniform shapes (Figure 3.1) using a Speedy 300 Trotec laser engraver at approximately eighteen percent power. Thick stationary phase TLC plates had 770±90 µm of cellulose powder adhered while thin stationary phase TLC plates had 140±10 µm.

3.4.4 Universal Spray Cartridge

To minimize the differences between how the spray substrates were analyzed, a spray cartridge was designed that could accommodate substrates of vastly different thickness and fragility. The cartridge consists of three parts (Figure 3.2). The first part of the cartridge, milled

out of delrin plastic, consisted of a top and bottom that fit together to hold the spray substrate. The top portion had a hole to contain a 3-millimeter sample punch directly on top of the spray substrate with a larger hole above it to serve as a solvent reservoir. A small hole behind the solvent well contained a wire that contacted the spray substrate and allowed the application of voltage. The second part, 3D printed out of photopolymer, was a clamp that fit around the substrate holder with a nut and bolt to tighten the two pieces together.

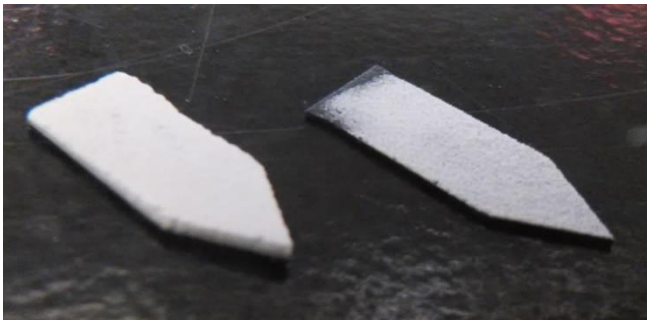

Figure 3.1: TLC Spray Substrates. Cut TLC spray substrates with a thick (left) and thin (right) stationary phase.

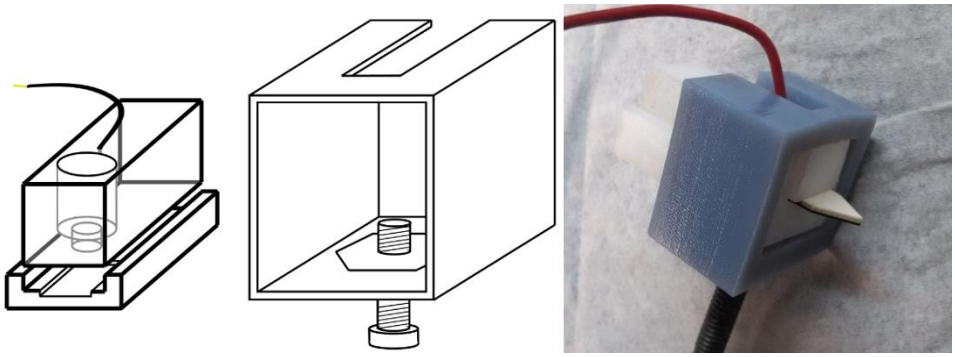

Figure 3.2: Universal Spray Cartridge. Delrin substrate holder with solvent well and conductive wire (left), 3D printed clamp to hold parts together (middle), and cartridge containing thick TLC spray substrate on right.

3.4.5 Analytical Run

Analysis was carried out on a Thermo Fisher Scientific Q Exactive Focus Orbitrap mass spectrometer in parallel reaction monitoring mode (PRM). Measurements were made of the most intense fragment of the $[M+H]^+$ precursor for each analyte. An analytical run consisted of two minutes of 4.5 kV applied voltage with six seconds of 0 V at the beginning and end of the analytical run. Samples consisted of 2.5 µL of either blood or methanol containing 2.5 µg/mL of each analyte spotted on a 3 millimeter punch of 31 ET chromatography paper. Samples were dried at room temperature on the benchtop. The spray solvent was spiked with 250 ng/mL of stable isotopic labels (SIL) of the ten analytes. The spray solvent consisted of either 95:5 methanol:water with 100 ppm acetic acid for filter paper or 90:10 acetonitrile:water with 100 ppm formic acid for the TLC plates and chromatography paper. When comparing solvents to optimize detection limits, both solvents used formic acid.

3.4.6 Comparing Recovery and Ionization Efficiency

Recovery was measured as a ratio between the area under the curve (AUC) for the analyte, which should decrease with poorer recovery, divided by the AUC for the SIL in the spray solvent which should be unaffected by recovery. Relative recovery is the ratio of the recovery from a biofluid and the recovery from neat as shown in equation 1.

1. $$Relative\ Recovery = \frac{\left(\frac{AUC_{Analyte\ from\ sample}}{AUC_{SIL\ in\ solvent}}\right)_{biofluid}}{\left(\frac{AUC_{Analyte\ from\ sample}}{AUC_{SIL\ in\ solvent}}\right)_{neat}}$$

The signal of the SIL contained in the spray solvent should primarily be decreased by the matrix components coeluting from the sample so measuring the ratio of the SIL using a biofluid sample and a matrix free sample measures the relative ionization efficiency as shown in equation 2.

2. $$Relative\ Ionization\ Efficiency = \frac{(AUC_{SIL\ in\ solvent})_{biofluid}}{(AUC_{SIL\ in\ solvent})_{neat}}$$

When comparing similar spray substrates (i.e. comparing two kinds of filter paper or two thicknesses of TLC plate) the change in relative recovery or relative ionization efficiency was calculated as an absolute difference of the two percentages. The results for each pair of spray substrates were evaluated for statistical significance ($p<0.05$) using a student T test ($n=5$ for each spray substrate).

3.4.7 Sample Extractor

There is an inherent amount of imprecision when the SIL is dissolved in the spray solvent due to the variability in the absolute signal (30% relative standard deviation is common). This makes it difficult to confirm trends in the ionization efficiency which is based solely on the absolute signal as shown in equation 2. To verify the trends observed with paper spray-MS using a secondary method an extractor was designed to pull solvent through the spray substrates so that it could be sprayed using a syringe pump and electrospray ionization (ESI) spray source. The extractor was 3D printed in polypropylene plastic, to be solvent resistant, and designed to be inserted into a 2 mL centrifuge tube using the cap to seal the extractor (Figure 3.3).

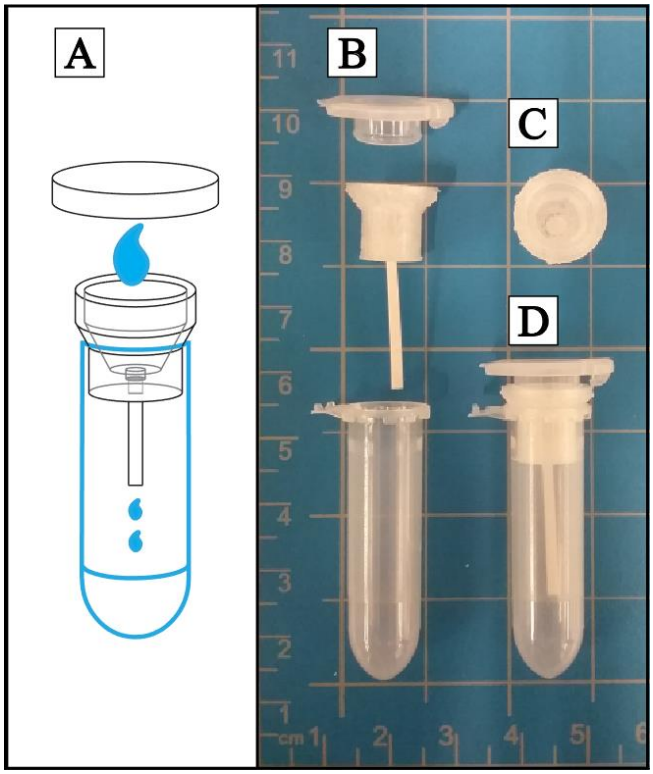

Figure 3.3: Centrifuge Extractor. A: diagram of extractor, B: individual components of extractor, C: top down view, D: assembled extractor.

Changes in ionization efficiency were measured by comparing the absolute signal of the SIL spiked into a urine extract, solvent that had been passed through dried urine samples, and the SIL spiked into the same urine extract after it had been passed through the different papers. The urine extract was generated by stacking three 3 mm punches of 31ET paper with dried urine in the extractor and adding spray solvent before it was centrifuged at 2500 RPM for 10 minutes. Three extracts were pooled then processed a second time through the four different papers to assess the ability of different papers to remove matrix components. Within the extractor a strip of paper was fed through a square slot and bent over in the bottom of a 3 mm diameter well. On top of the paper multiple punches of the same paper were inserted to hold the paper strip in place and prevent leaks. Six punches had to be used for the filter papers while four and three punches were used for 3MM and 31ET respectively to account for the differences in paper thickness. Three replicate extractions were tested for each paper and compared with three extracts that hadn't been passed through additional paper. The resulting solutions were spiked with 250 ng/mL of the ten drug's SIL and analyzed by syringe pump infusion to the Thermo H-ESI source at 4.5 kV, a sheath gas flow rate of 15, an auxiliary gas flow of 5 arbitrary units, and flow rate of 10 µL per minute.

The percent signal increase was calculated using equation 3 where $AUC_{SIL\ Paper\ Extracted}$ is the area under the curve for the SIL spiked into urine extract that had been passed through additional paper and $AUC_{SIL\ Urine\ Extract}$ is the area under the curve for the SIL spiked into urine extract that hadn't been passed through any additional paper. Papers were compared by finding the absolute difference between the percent signal increase.

3. $$Percent\ Signal\ Increase = \left(\frac{AUC_{SIL\ Paper\ Extracted}}{AUC_{SIL\ Urine\ Extract}} - 1\right) * 100\%$$

3.4.8 Optimizing Detection Limits

To determine the impact of paper properties on detection limits, calibration curves were generated using combinations of two spray solvents, methanol and acetonitrile, and the two most drastically different papers, grade 4 filter paper (which is thin with large pores) and 3MM chromatography paper (which is thicker with a slower flow rate). Urine was selected as a biofluid due to past work showing that it often has poor ionization efficiency with minimal problems with recovery[28]. To ensure that analyte binding to a plastic cartridge did not influence results at low concentrations, a positioning device that only contacted the spray substrate via an alligator clip was 3D printed. A pipette tip in contact with the paper acted as a solvent reservoir (Figure 3.4).

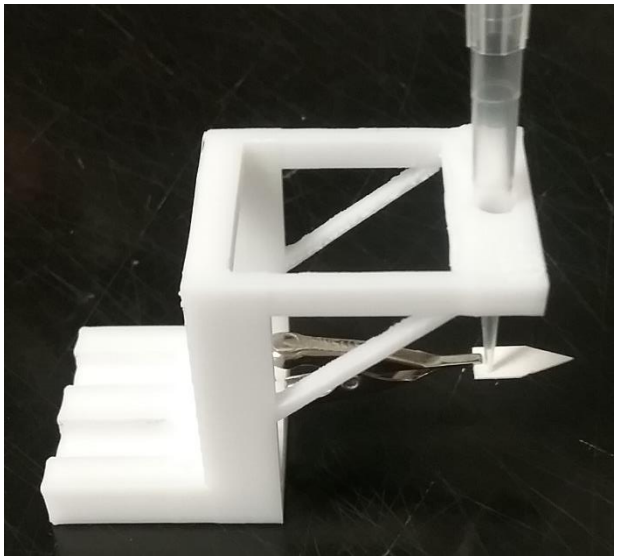

Figure 3.4: Minimal Contact Cartridge. 3D printed cartridge to minimize contact between the paper and the cartridge.

Calibrators were prepared at 5, 10, 50, 100, 500, and 1000 ng/mL in urine. The SIL internal standard was spiked into each sample at 50 ng/mL. Two replicates were run at each concentration with data points yielding no signal being omitted. Slope and Y-intercept were determined by linear regression[30] with a weighting factor of $1/X^2$. Detection limits were calculated by three times the standard error of the Y-intercept divided by the slope. Changes in absolute signal intensity were calculated by averaging the signal for the SIL for ten runs and finding the factor by which the signal increased when changing grade 4 filter paper to 3MM paper without changing the solvent (acetonitrile), changing from methanol to acetonitrile solvent without changing the paper (3MM) and changing from conditions for higher theoretical recovery (grade 4 filter paper with methanol) to higher theoretical ionization efficiency (3MM with acetonitrile solvent).

3.5 Results and Discussion

3.5.1 Flow Rate During Paper Spray

The average flow rate of solvent due to paper spray (Table 3.3) was calculated in µL/minute by finding the difference between the rate of solvent consumption without voltage and the rate of solvent consumption during paper spray. It was found that when comparing filter papers or chromatography papers, a smaller pore size or slower chromatographic flow rate corresponds to a slower flow rate during paper spray mass spectrometry. The flow rate also seemed to be similar between the two solvents tested.

3.5.2 Comparing relative Ionization Efficiency and Relative Recovery

Relative ionization efficiency and relative recovery were determined for each pair of papers and the differences evaluated using a T test ($p \leq 0.05$) to determine if the results were statistically different. An absolute difference was calculated for papers that had statistically different results

for the majority of the analytes. A consistent trend emerged when comparing grade 4 to grade 5 filter paper, 31ET to 3MM chromatography paper, thin stationary phase TLC plates to thick stationary phase TLC plates: an increase in the relative ionization efficiency (Figure 3.5) accompanies a decrease in the relative recovery (Figure 3.6).

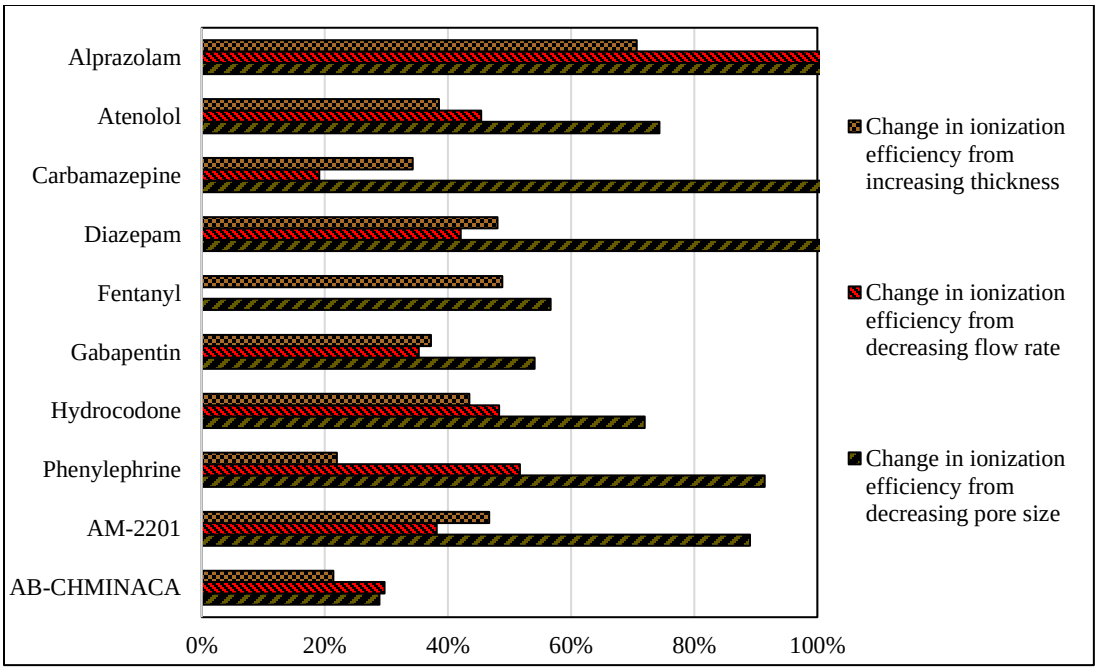

Figure 3.5: Changes in Ionization Efficiency. The absolute change in relative ionization efficiency when comparing increased substrate thickness (thin cellulose powder TLC plate to thick TLC plate), decreased flow rate (grade 31 ET chromatography paper to 3MM chromatography paper), and decreased pore size (grade 4 filter paper to grade 5 filter paper). Bars showing a change in ionization efficiency over 100% are due to higher relative standard deviation from the SIL being in the spray solvent.

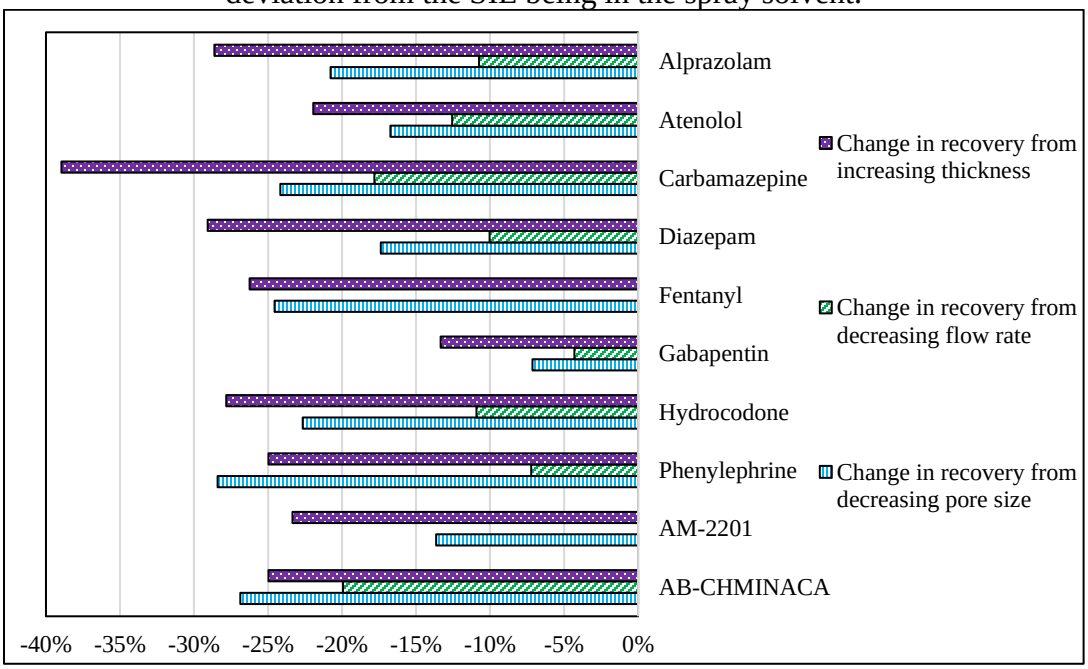

Figure 3.6: Changes in Recovery. The absolute change in relative recovery when comparing increased substrate thickness (thin cellulose powder TLC plate to thick TLC plate), decreased flow rate (grade 31 ET chromatography paper to 3MM chromatography paper), and decreased pore size (grade 4 filter paper to grade 5 filter paper).

The common factor for the trends shown in figures 3.5 and 3.6 is that the decrease in recovery and increase in ionization efficiency correlates with the filtration effectiveness of the papers. This is shown with the decreasing pore size (the filter paper), decreasing flow rate (chromatography paper) and with substrate thickness (the TLC plates). In table 3.3 it was shown that smaller pore size also led to a decrease in flow rate so it could also be a factor of rate of flow through the paper. Another factor is that smaller pore size and increased thickness increases the interaction surface area between the paper fibers and the solvent, which in turn allows greater interaction of the analyte and matrix components with the cellulose. This result is similar to previous work where an extended path length originally showed this tradeoff between decreased recovery and improved ion suppression[28]. This means that properties of the spray substrate that improve the spray substrates ability to act as a filter, including longer path length, thicker paper, slower flow rate or smaller pore size, will decrease recovery while improving ionization efficiency.

3.5.3 Using Centrifuge Extractor to Determine Ionization Efficiency

To verify trends in ionization efficiency (Figure 3.5) a centrifuge was used to pull spray solvent with matrix components through different papers. The solvent was then pooled and sprayed using a syringe pump and ESI source to measure signal change. The percent signal increase was calculated for each paper using equation 3; the effect of decreasing pore size (comparing grade 4 filter paper to grade 5 filter paper), decreasing flow rate (comparing 31ET chromatography paper to 3MM chromatography paper), and the two most significantly different papers (3MM chromatography paper and grade 4 filter paper) are reported in figure 3.7.

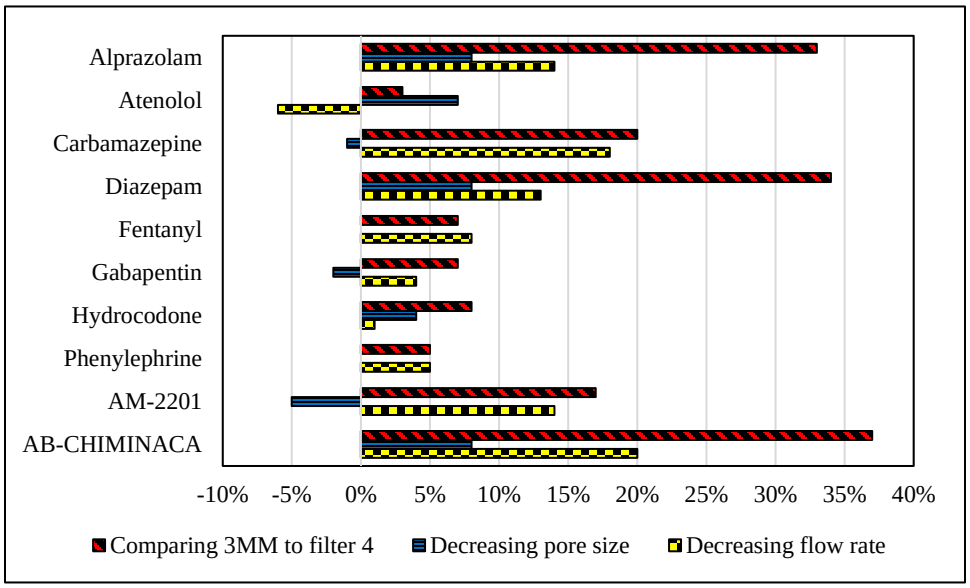

Figure 3.7: Comparing Signal Increases from Extracting Through Different Papers. The change in percent signal increase for the SIL spiked in urine extract when compared to the same urine extract that was passed through various papers. The papers compared have decreasing pore size (comparing grade 4 filter paper to grade 5 filter paper) and decreasing flow rate (comparing 31ET chromatography paper to 3MM chromatography paper).

Looking at the results in figure 3.7, several of the trends pointed out earlier repeated. Alprazolam, carbamazepine, diazepam, AM-2201 and AB-CHMINACA showed a consistently higher signal with the slower flow rate 3MM being better than 31ET. The differences also showed up between grades 5 and 4 filter paper, but were less extreme. This could have been due to the thinness of the filter paper offsetting the change in pore size. Comparing two dramatically different papers, 3MM which is thick with a slow flow rate and grade 4 filter paper which is thin with a large pore size, the changes in ionization efficiency were more pronounced. Other trends emerged that were not obvious with the less precise paper spray measurements. Atenolol, fentanyl, gabapentin, hydrocodone and phenylephrine all showed minimal improvement compared to the other analytes tested. Looking at the properties of these analytes in table 3.2, most of these analytes are charged at physiological pH except for gabapentin which is zwitterionic. A few outliers exhibited trends opposite to expected behavior (specifically a decrease in ionization efficiency for carbamazepine, gabapentin and AM-2201 with decreasing pore size and atenolol with decreasing flow rate). These outliers are most likely due to higher imprecision from working with thinner paper or an analyte that shows minimal improvement from having the matrix cleaned up using paper (in the case of atenolol).

3.5.4 Optimizing Detection Limits

The trends discussed above showed a consistent tradeoff between ionization efficiency and recovery. This was also found in the two solvent systems investigated, with acetonitrile showing higher ionization efficiency and methanol showing better recovery (data not shown). This makes predicting an ideal combination of substrate and solvent to improve detection limits difficult. In theory, if the primary limitation to detection limits for a biofluid is one matrix effect and not the other it should just be a matter of selecting a solvent and paper to minimize one matrix effect. For

example, urine has been shown to have minimal problems with recovery and is an ideal choice for determining if selecting the paper and solvent to maximize ionization efficiency improves the detection limits to a significant extent. Four combinations of solvent and paper (the two solvents acetonitrile and methanol with the two papers grade 4 filter paper and 3MM chromatography paper) were evaluated for their detection limits based on estimates from a calibration curve (Table 3.4).

Table 3.3: Paper Spray Flow Rates. Flow rates ± the standard deviation (N=3) for each of the commercially available papers with each solvent in µL/minute.

	90:10 acetonitrile:water	95:5 methanol:water
31ET	5.8±0.5	5±1
3MM	2.4±0.2	2.5±0.3
filter 4	3.1±0.2	2.5±0.3
filter 5	1.1±0.1	1.0±0.3

Table 3.4: Detection Limits Using Two Different Papers and Two Different Solvents. Detection limits estimated from calibration curves in urine using four different combinations of paper and solvent. The solvents contained 10% and 5% water for acetonitrile and methanol respectively. *The SIL for phenylephrine was a non-detect for these runs and is omitted.

	3MM		Filter 4	
Drug	acetonitrile	MeOH	acetonitrile	MeOH
AB-CHMINACA	0.94	7.6	1.7	37
Alprazolam	0.66	5.3	1.2	3.8
AM-2201	1.4	3.1	1.5	1.9
Atenolol	2.3	2.5	1.5	2.8
Carbamazepine	1.0	1.7	1.0	2.2
Diazepam	1.8	2.0	2.5	4.9
Fentanyl	0.86	1.2	1.1	1.0
Gabapentin	11	11	9.4	15
Hydrocodone	2.6	8.6	2.7	2.5

If optimizing the solvent and paper combination for ionization efficiency was all that was required to lower detection limits in urine, then the combination of 3MM chromatography paper and acetonitrile would have given the lowest detection limits. For several analytes this is true, but the difference is minimal when comparing 3MM and grade 4 filter paper both using an acetonitrile solvent. To get an idea of the absolute change in signal for the SIL the change in signal intensity was calculated (Figure 3.8) when comparing different papers with the same solvent (acetonitrile), different solvents but the same paper (3MM) and when changing both as a factor difference.

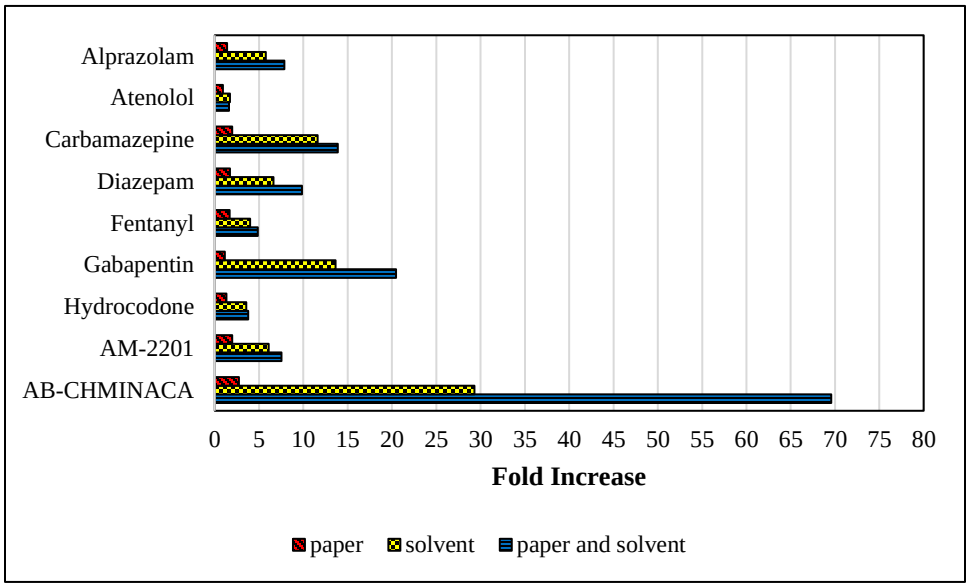

Figure 3.8: Increases in Signal from Changing Paper or Solvent. The factor changes in absolute SIL signal when changing the paper (grade 4 filter paper to 3MM), when changing the solvent (methanol to acetonitrile) and when changing both. *The SIL for phenylephrine was a non-detect for these runs.

As shown in figure 3.8 increases in absolute signal were greater when changing the solvent than the paper, but the increase was greatest when both were changed. Looking at the data in terms of the properties of the analytes from table 3.2 the analytes charged at physiological pH (atenolol, fentanyl and hydrocodone) all showed increases in signal under a factor of five. This suggests that analytes that tend to have lower overall ionization efficiencies, namely those that aren't charged at physiological pH, are more likely to show more significant changes in absolute signal when using papers that act as a more efficient filter (like 3MM paper) and acetonitrile for the spray solvent. This is reflected in table 3.4 in that atenolol and fentanyl (both of which are charged at physiological pH) had very similar detection limits for all four combinations while alprazolam and AB-CHMINACA showed a noticeably lower detection limit for 3MM paired with acetonitrile. However, this did not hold true for every analyte and in many cases the improvements in detection limits were minimal. With detection limits being a factor of both ionization efficiency and recovery and with each analyte behaving differently it is difficult to predict whether optimizing for one matrix effect will drastically improve detection limits. In general, it has been shown here that using a paper that acts as a more effective filter in combination with a solvent with better ionization efficiency such as acetonitrile improved absolute signal intensity and sometimes the detection limits for uncharged analytes.

3.6 Conclusion

In conclusion, a systematic approach was used to evaluate the impact of specific paper properties on matrix effects during paper spray-MS. Two pairs of paper were selected with similar properties except for one property being significantly different. Cellulose TLC plates were manufactured to test the impact of thickness. A selection of ten analytes were used to gauge the

differences associated with differing physical properties. An offline extractor was 3D printed to allow for the study of paper properties without using paper spray.

It was found that there is a consistent tradeoff between ionization efficiency and recovery with spray substrates that act as a more efficient filter due to smaller pore size, slower flow rate or thicker spray substrate lowering recovery while improving ionization efficiency. Experiments using an offline extractor verify the trends associated with pore size, flow rate and substrate thickness with regards to ionization efficiency and reveal that analytes with a positive charge around physiological pH tend to be less sensitive to paper properties with regards to ionization efficiency. An evaluation of the detection limits for the ten analytes using combinations of acetonitrile and methanol with 3MM chromatography paper and grade 4 filter paper showed that lowering detection limits using paper spray-MS is more complicated than minimizing a specific matrix effect and that different analytes will have different optimal detection limits. However, the optimizing the paper and solvent for ionization efficiency when using a urine matrix did improve signal intensity for most analytes uncharged at physiologic pH and noticeably improved detection limits for AB-CHMINACA and alprazolam. The solvent choice appeared to cause a much more significant change than the paper with acetonitrile being better for ionization efficiency than methanol.

3.7 Acknowledgments

Funding for this research was provided by a grant from the National Institute of Justice, Office of Justice Programs under award number **2016-DN-BX-007**.

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CHAPTER 4. USING SESAME SEED OIL TO PRESERVE AND CONCENTRATE CANNABINOIDS FOR PAPER SPRAY MASS SPECTROMETRY

4.1 Abstract

Cannabinoids present a unique set of analytical challenges. An increasing number of states have voted to decriminalize recreational marijuana use, creating a need for new kinds of rapid testing. At the same time, synthetic compounds with activity similar to THC, termed synthetic cannabinoids, have become more prevalent; posing a separate set of health risks. A rapid method capable of detecting both natural and synthetic cannabinoids would be useful in cases of driving under the influence of drugs, where it might not be obvious whether the suspect consumed marijuana, a synthetic cannabinoid, or both. Paper spray mass spectrometry is an ambient ionization technique which allows for the direct ionization of analyte from a biofluid spot on a piece of paper. Natural cannabinoids like THC, however, are labile and rapidly disappear from dried sample spots, making it difficult to detect them at clinically relevant levels. Presented here is the development of a protocol to concentrate and preserve cannabinoids in urine and oral fluid on paper that can subsequently be analyzed using paper spray mass spectrometry to detect THC and synthetic cannabinoids simultaneously. Sesame seed oil is investigated both as a means of preserving THC and as part of a technique, termed paper strip extraction, wherein urine or oral fluid is flowed through an oil spot on a strip of paper to preconcentrate cannabinoids. This technique has been shown to preserve THC in a spot for at least 27 days at room temperature, and is capable of detecting THC and synthetic cannabinoids at low ng/mL concentrations, making it suitable as a rapid screening technique. The technique has been adapted to be used with a commercially available autosampler.

4.2 Introduction

Cannabinoids, both natural and synthetic, have become increasingly important analytical targets. Marijuana and cannabidiol oil for recreational use in the United States has been decriminalized in an increasing number of states[1-2]. Concurrently the use of synthetic cannabinoids (SCs) has increased. Synthetic cannabinoids are synthetically manufactured compounds that mimic the active ingredient of marijuana tetrahydrocannabinol (THC) in structure or function[3-4]. Both types of cannabinoids raise a host of legal and health issues with their increased use. Cannabis has been shown to impact psychomotor skills associated with driving, and the effects are amplified when combined with alcohol. States must find effective methods to detect THC and its metabolites in suspected Driving Under the Influence of Drugs (DUID) incidents[5-7]. In addition, rapid and sensitive testing is needed for a variety of other applications such as employment testing. As of 2015, drug abuse violations made up the highest number of arrests in the United States with marijuana possession making up the largest percent of these arrests[8]. SCs represent a different set of challenges. These compounds are applied to dried plant material marketed as incense "not for human consumption[9]." The dosage and potency are poorly understood, as the compounds come from a library generated during research into cannabinoid receptor agonists for pharmaceutical purposes[9-10]. As a result, the risk of unpredictable side effects and overdose are higher, and users cannot know the exact compound they consumed.

Historically, drug testing for marijuana use has been done in urine, and targets THC metabolites; commonly 11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH). Detection typically starts with an immunoassay for screening followed by gas chromatography-mass spectrometry (GC-MS) for confirmation[11-12]. Liquid chromatography (LC)-MS is another potential technique, while alternate matrices for detection include blood, plasma, hair or oral fluid[1, 13-14]. Synthetic cannabinoids pose a problem for these traditional methods. Due to the large

number of potential compounds and the wide variation in potency, immunoassays are not sensitive or selective enough[15]. While chromatography methods combined with mass spectrometry are sensitive and selective, they often require extensive sample preparation, special training, and lengthy analysis times. A faster and easier technique would be beneficial as cannabinoid use increases.

Paper spray MS is a potential solution, but, cannabinoids pose problems for several reasons. For both natural and synthetic cannabinoids, the concentration in biofluids can be low and detection limits for paper spray are affected by matrix effects[5, 13, 16-17]. This is especially problematic for THC and similar compounds, which are labile and degrade rapidly in dried spots through a number of pathways including photo and thermal degradation[18]. Attempts to improve detection limits of paper spray have included adding a solid phase extraction component to a paper spray cartridge[19], performing a solvent extraction from the biofluid on top of hydrophobic paper[20] and utilizing a membrane to filter out red blood cells from whole blood[21]. These methods can help lower detection limits, but can also increase the cost or complexity of the analysis. Ideally, the detection limits of paper spray for cannabinoids could be improved by concentrating and preserving the analytes without drastically increasing the cost of the analysis or difficulty to analyze the sample.

Herein we present a simple method to preserve and concentrate certain analytes for paper spray MS. Sesame seed oil, the vehicle used for the synthetic THC dronabinol[22], was evaluated for its ability to preserve cannabinoids in urine and oral fluid dried spots. Theoretically, hydrophobic THC would concentrate in the oil. To this end paper strips with and without sesame seed oil at one end were evaluated the ability to concentrate analytes as urine wicked through the oil coated paper. A subset of analytes was selected to include both natural and synthetic

cannabinoids as well as other pharmaceuticals with varying physical properties. After the sample flowed through the oil spot, the paper strip was cut into pieces and analyzed for drug distribution throughout the strip. Long term preservation was evaluated by finding the detection limits of analytes after 1, 7, and 27 days of storage at room temperature in the dark. Paper strip extraction was also compared to normal paper spray to determine if extraction improved detection limits. Finally, paper strip extraction was incorporated into a disposable cartridge and analyzed on a commercial autosampler to show the potential of the technique for rapid analysis.

4.3 Materials

Analytes and their stable isotopic labels (SIL), except atenolol D7 and the synthetic cannabinoids, were obtained from Cerilliant (Round Rock, TX, U.S.A.). Atenolol D7 was obtained from CDN isotopes (Pointe-Claire, QC, Canada) as a powder (≥98% purity). AB-CHMINACA, AB-CHMINACA metabolite 4, 5-fluoro ADB, 5-fluoro ADB metabolite 2 and AM-2201 and the SIL's of AB-CHMINACA and AM-2201 were obtained from Cayman Chemical (Ann Arbor, MI, U.S.A). HPLC grade methanol, acetonitrile, palmitic acid, linoleic acid and oleic acid came from Fisher Scientific (Waltham, MA, U.S.A.). Concentrated sulfuric acid came from EMD Millipore (Burlington, MA, U.S.A.). Whatman grade 31 ET and 3MM chromatography paper came from GE Healthcare Life Sciences (Pittsburgh, PA, U.S.A.). Sesame seed oil, sesamolin, mineral oil and butylated hydroxytoluene came from Sigma-Aldrich (St. Louis, MO, U.S.A.). Sesamin came from ApexBio (Taiwan). The top half of the autosampler cartridge was 3D printed using a polypropylene filament using an Ultimaker 2+ extended from Ultimaker (Geldermalsen, Gelderland, Netherlands). The bottom half of the autosampler cartridge was produced by Prosolia (Indianapolis, IN, U.S.A.). Urine and saliva were provided by a single volunteer.

4.4 Methods

4.4.1 Sesame Oil Preservation

The ability of sesame oil to preserve different cannabinoids overnight was evaluated relative to oleic acid, a major component of sesame seed oil[23], and mineral oil. Fentanyl was included as a non-cannabinoid analyte comparison. Urine was spiked at 1 µg/mL for (−)-*trans*-Δ^9-tetrahydrocannabinol (THC), 11-Hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC), 11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH), 11-Nor-9-carboxy-Δ^9-THC glucuronide (THC glucuronide), AB-CHMINACA, AM-2201 and fentanyl in a glass vial (structures in Figure 4.1).

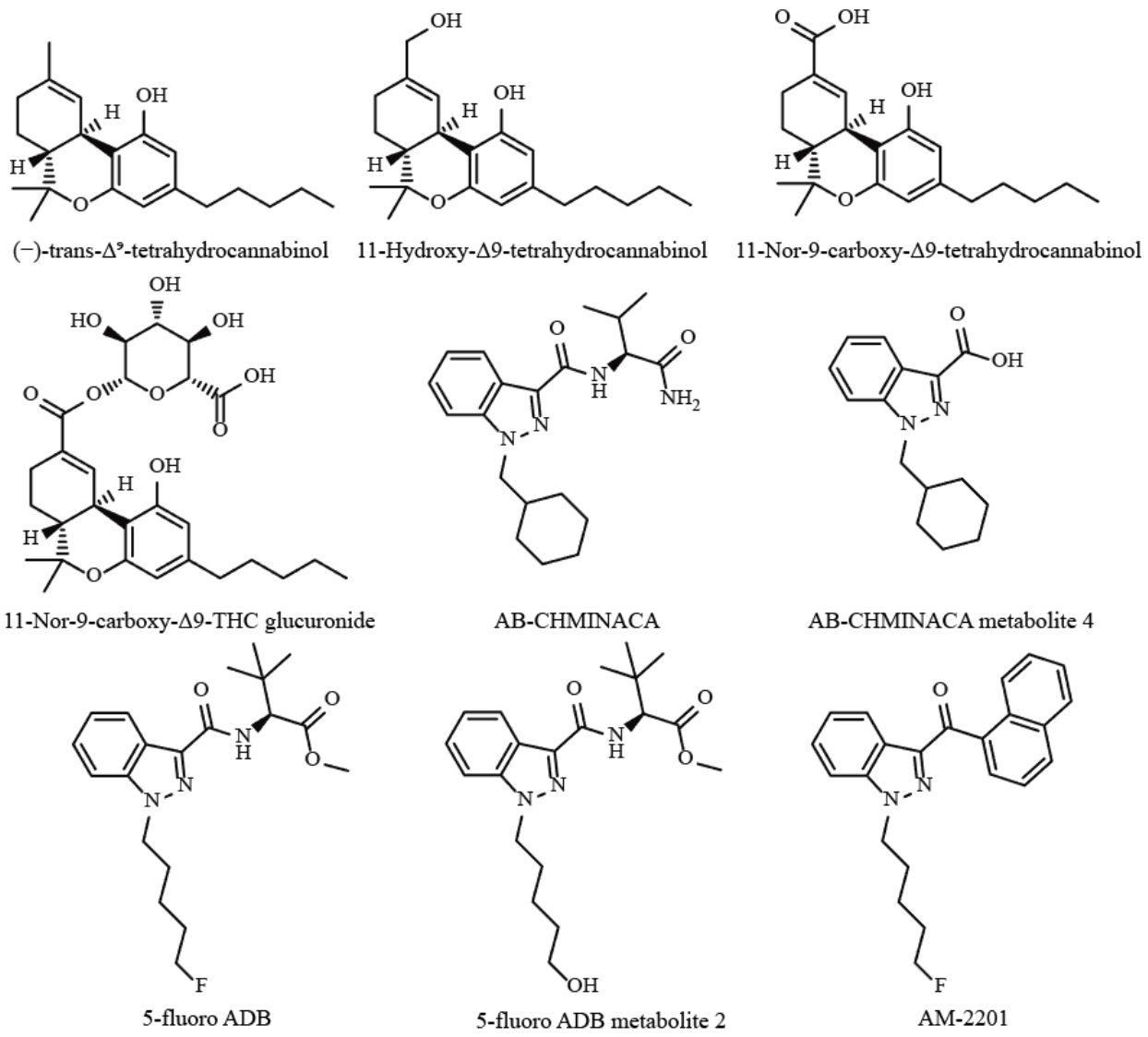

Figure 4.1: Cannabinoid Structures. Structures for THC, THC metabolites and synthetic cannabinoids.

Samples were spotted in 5 µL aliquots using a glass capillary onto 5 mm x 5 mm squares of 31ET chromatography paper, with and without 5 µL of the different oils, and allowed to dry for 1 hour or 1 day at room temperature on the counter. After drying, sample squares were spotted with 2.5 µL of methanol containing 500 ng/mL of the stable isotopic labels (SILs) THC D3, THC-COOH D3, AB-CHMINACA D4, AM-2201 D5 and fentanyl D5 in methanol. THC glucuronide and 11-OH-THC used THC D3 for an internal standard.

Analysis was carried out using a holder to minimize contact between plastic and paper as described in previous work (Figure 3.4)[17]. The holder supports an Eppendorf tip that holds solvent and an alligator clip that holds the sample square and paper tip. The paper tip was positioned in front of the inlet of a Thermo Fisher Scientific Q-Exactive Focus orbitrap mass spectrometer and 60 µL of solvent, 80:20 acetonitrile: methanol with 25 mM sulfuric acid[22], was added to the Eppendorf tip. After the solvent flowed through the sample square to the paper spray tip clipped beneath it, 4.5 kV of voltage was applied via a secondary alligator clip for 1 minute. The instrument was run in parallel reaction monitoring (PRM) mode and the area under the curve for the most abundant fragment for each compound was integrated. The ratios of the area under the curve for the analyte divided by its SIL were found and compared between samples dried for different lengths of time and with different oils.

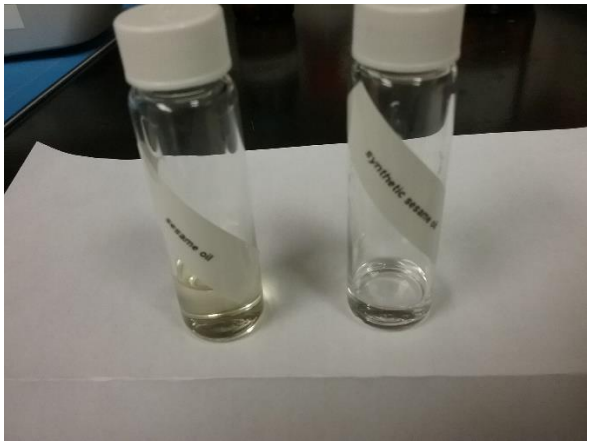

Figure 4.2: Natural and Synthetic Sesame Seed Oil. Sesame seed oil (left) and synthetic sesame seed oil (right).

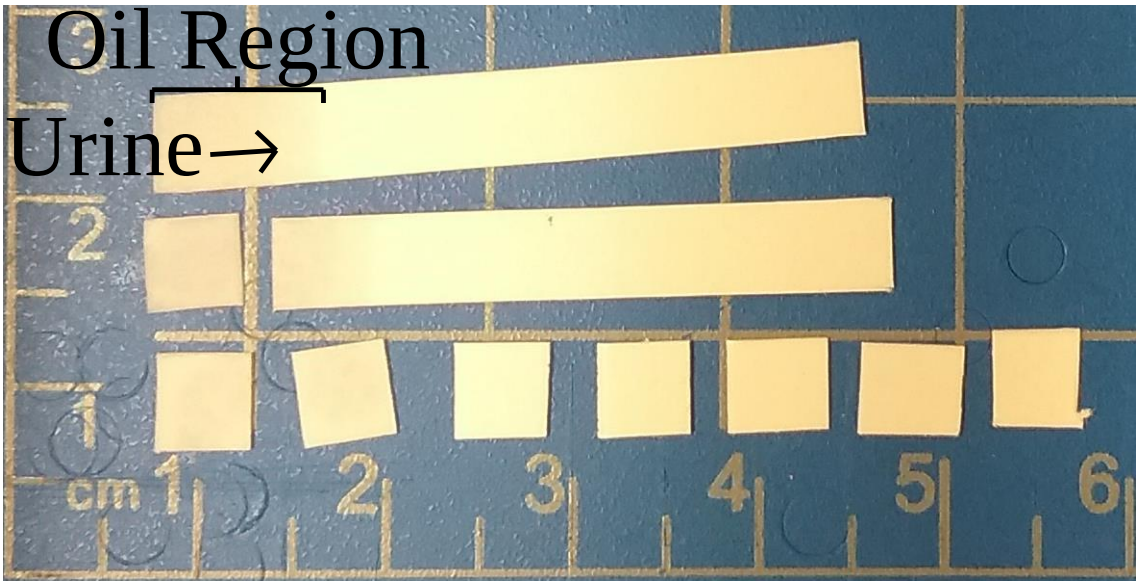

Figure 4.3: Paper Strip Extraction. Top: Urine was flowed throw an oil spot on a strip of paper. Middle: After drying the first 5 mm of strip was cut off and analyzed. Bottom: After drying the extraction strip was cut into 5 mm increments and each segment analyzed.

To determine if the antioxidants in sesame seed oil were the primary source of preservation of THC, a synthetic sesame seed oil was prepared by mixing the fatty acids that make up over 90% of sesame seed oil: oleic, linoleic and palmitic together in a 48:37:8 ratio. This oil, (Figure 4.2), was tested for its ability to preserve (−)-*trans*-Δ^9-tetrahydrocannabinol (THC) and its metabolites 11-Hydroxy-Δ^9-tetrahydrocannabinol (OH-THC) and 11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH). The experiment was carried out as described in the methods section "Sesame Oil Preservation" except instead of sesame seed oil, synthetic sesame seed oil with a relevant % by weight of antioxidant present. The naturally occurring antioxidants were spiked in at concentrations similar to natural sesame seed oil: 0.6% sesamin, 0.5% sesamolin or both sesamin and sesamolin (same %)[23]. The food preservative butylated hydroxytoluene (BHT) was included as a synthetic alternative.

To analyze larger volumes of urine, samples were prepared using a technique termed paper strip extraction. Strips of 3MM chromatography paper were cut into 5 mm x 40 mm strips, and 2.5 µL of sesame seed oil was spotted at one end of each strip. Using a glass capillary, 50 µL of spiked urine was applied to the end of the strip containing the sesame oil and allowed to dry. After drying the first 5 mm segment of the strip was removed for analysis (Figure 4.3).

The ability for sesame seed oil to preconcentrate THC and other hydrophobic analytes was evaluated. Urine was spiked at 100 ng/mL for atenolol, alprazolam, carbamazepine, diazepam, gabapentin, fentanyl, AB-CHMINACA, AM-2201, THC glucuronide and THC. Paper strip extraction was carried out using 50 µL aliquots of the urine followed. After drying, the strips were then cut into 5 mm increments (Figure 4.3), and each segment was spiked with 5µL of a 500 ng/mL solution of the SILs: THC D3, THC-COOH D3, AB-CHMINACA D4, AM-2201 D5 and fentanyl D5 in methanol. The individual squares were analyzed by paper spray and the ratio between the

analyte and the SIL was plotted as a function of distance traveled through the paper strip. Three strips were analyzed with and without sesame seed oil and the results were averaged. The list of analytes for this experiment included drugs with a range of logP values to determine if it was a factor in determining whether an analyte preconcentrated.

The preservation capabilities of sesame oil were evaluated over prolonged storage. A calibration curve was generated in urine spiked at 0.5, 5, 10, 100, 500 and 1000 ng/mL for THC, 11-OH-THC, THC-COOH, AB-CHMINACA, AM-2201, and fentanyl. The internal standard was a SIL spiked into each calibrator at 100 ng/mL. Two replicates were extracted and analyzed for each concentration as well as three blanks. Calibration curves with or without sesame oil were analyzed after 1, 7, and 27 days. Samples were stored in the dark at room temperature in a loosely covered plastic dish. The slope and Y-intercept were determined by linear regression[24] with a weighting factor of $1/X^2$. Samples showing no signal or signal lower than three standard deviations above the average blank signal were omitted. Detection limits were calculated as three times the standard error of the Y-intercept divided by the slope.

4.4.2 Preconcentration Effects on Detection Limits

Samples were prepared in oral fluid and urine by either using paper strip extraction or direct spotting on paper for a total of four combinations to determine whether this method made a noticeable impact on detection limits. Urine was spiked at 0.5, 5, 25, 100, 500 and 1000 ng/mL of THC, 11-OH-THC, THC-COOH, AB-CHMINACA, AM-2201, 5F-ADB and AB-CHMINACA metabolite 4 (structures in Figure 4.1) and 100 ng/mL SIL for each analyte except 11-OH-THC and THC-COOH which used the SIL for THC. Paper strip extraction was carried out as described earlier and direct spotting was done by spotting 5 µL directly onto 5 mm segments of paper. For samples prepared with oil but without paper strip extraction, a strip of paper with oil

was prepared as normal, after the oil distributed on the paper the first 5 mm segment was removed and spiked with urine. Two replicates at each concentration and three blanks were analyzed after 1 and 28 days to evaluate the effects of preconcentration and preservation.

4.4.3 Automated Analysis

To prove the technique could be used for rapid screening, a prototype disposable cartridge was designed to be analyzed using a commercially available autosampler. The cartridge consisted of two parts that snapped together around the sample square and paper spray tip. The bottom half and steel ball bearing, used as an electrical contact, from the original autosampler cartridge were used with a 3D printed top modeled in Sketchup and printed using polypropylene filament on an Ultimaker 2 extended+. 5-fluoro ADB metabolite 2 was analyzed in addition to the analytes mentioned earlier (structure in Figure 4.1).

4.5 Results and Discussion

4.5.1 Sesame Oil Preservation

(−)-*trans*-Δ^9-tetrahydrocannabinol (THC) and its metabolites can be difficult to analyze because they are unstable. Additionally, due to their hydrophobicity, a significant portion of THC can be lost from aqueous matrices like urine to plastic containers[18]. Dronabinol, the synthetic version of THC sold for pharmaceutical purposes, uses sesame seed oil to preserve THC for extended periods of time at room temperature[22]. To determine whether this preservation could be extended to urine spots, sesame seed oil was added to the paper used to store the sample. Two additional oils were also tested as a comparison to determine whether sesame seed oil is specifically required, or if the major component of sesame seed oil, oleic acid, or a non-fatty acid oil, mineral oil, would also preserve THC. The amount of analyte remaining was measured as a

relative amount compared to the SIL spotted shortly before analysis. Preservation was measured by determining the percent decrease between samples that were dried for an hour and samples that were dried for a day (Table 4.1).

Table 4.1: Decrease in Drug Concentrations Using Three Oils for Preservation. Percent change in analyte signal (relative to freshly spotted internal standard) for dried urine samples after 24 hours storage versus one hour.

Analyte	No Preservative	Sesame Oil	Oleic Acid	Mineral Oil
THC	-92%	-7%	-100%	-95%
11-OH-THC	-89%	-32%	-100%	-71%
THC-COOH	-88%	-6%	-96%	-53%
AB-CHIMINACA	-16%	-12%	-41%	-19%
AM-2201	-29%	-16%	-23%	-42%
Fentanyl	-34%	7%	-25%	-29%

Table 4.2: Decrease in Drug Concentrations Using Different Antioxidants in Synthetic Sesame Seed Oil for Preservation. Decrease in sample present measured by the decrease in ratio between the analyte and stable isotopic label (SIL) when the SIL is spotted immediately after the sample compared to a when the SIL is spotted a day after the sample.

	THC	OH THC	COOH THC
No Oil	-100%	-100%	-100%
Synthetic Sesame Oil (SSO)	-93%	-89%	-99%
SSO+Sesamin	-92%	-86%	-100%
SSO+Sesamolin	-93%	-80%	-100%
SSO+Sesamin+Sesamolin	-98%	-93%	-100%
SSO+BHT	-93%	-26%	-97%

Without oil, THC and its metabolites showed a significant drop over the course of a day while the two synthetic cannabinoids and fentanyl did not, indicating that the synthetic drugs were more stable than the natural cannabinoids in the dried urine spot. Of the three oils tested, only sesame seed oil effectively preserved THC. Sesame seed oil is a mixture of fatty acids, antioxidants and other natural products[23, 25]. A previous study on dronabinol indicated that the antioxidants within the oil likely helped THC preservation[22].

To test whether antioxidants were the reason THC was preserved in dried urine spots, a mixture of oleic, linoleic and palmitic acid in a similar ratio as sesame seed oil was tested. The synthetic sesame seed oil was spiked either with the naturally occurring antioxidants sesamin and sesamolin or with the food preservative butylated hydroxytoluene and compared to the same oil without antioxidants (Table 4.2). As shown in table 4.2, no combination of the naturally occurring antioxidants combined with synthetic sesame seed oil preserved THC or its metabolites to any significant amount. This could mean that there are other components of sesame seed oil that help preserve THC. There is a distinct color to natural sesame seed oil that is not present in the synthetic (Figure 4.2) which suggests that the synthetic sesame seed oil is missing components to make it as similar as possible to natural sesame seed oil. An alternate option is that there could be an impurity in the oils that is preventing the antioxidants from functioning. What is out of the ordinary is that OH-THC was preserved, at least partially, in synthetic sesame seed oil with BHT. Given the complex nature of natural oils it would be a labor intensive process to narrow down the exact source of sesame seed oils preservation of THC which is beyond the scope of the current work.

The fact that THC adsorbs onto plastic container walls from urine prompted the idea that it could be extracted from urine as it flows through a hydrophobic matrix, such as sesame seed oil on a strip of paper. This process is referred to here as paper strip extraction (Figure 4.3). This

method of extraction was evaluated with a variety of analytes with varying logP values to determine how they would distribute (Figure 4.4).

The cannabinoids (THC, THC glucuronide, and both synthetic cannabinoids) decreased significantly by the third segment (10-15 mm) regardless of the presence of sesame seed oil on the paper. All the other analytes except for diazepam had a more uniform distribution throughout the paper strip regardless of the presence of oil. Diazepam showed an even distribution without oil but was more concentrated in the first 10 mm with oil. The distribution of the drugs was not strictly determined by hydrophobicity. While all the molecules that concentrated at the head of the strip are hydrophobic, the fact that the more hydrophilic THC glucuronide also concentrated suggests additional interactions played a role. This meant that there were two potential mechanisms for improving detection limits using paper strip extraction; preservation from sesame seed oil and preconcentration of hydrophobic analytes. To determine the extent that preservation plays on detection limits over time sets of calibration curves were prepared by paper strip extraction with and without sesame seed oil. Extraction was carried out and the 5 mm sample segments were stored at room temperature in a drawer for 1, 7 and 27 days then analyzed to determine the change in detection limits over time. The preservation effects of sesame seed oil are best illustrated by looking at the calibration curves for THC without sesame seed oil before and after 27 days of storage (Figure 4.5: A1 and A2 respectively) compared to with sesame seed oil over the same length of time (Figure 4.5: B1 and B2 respectively).

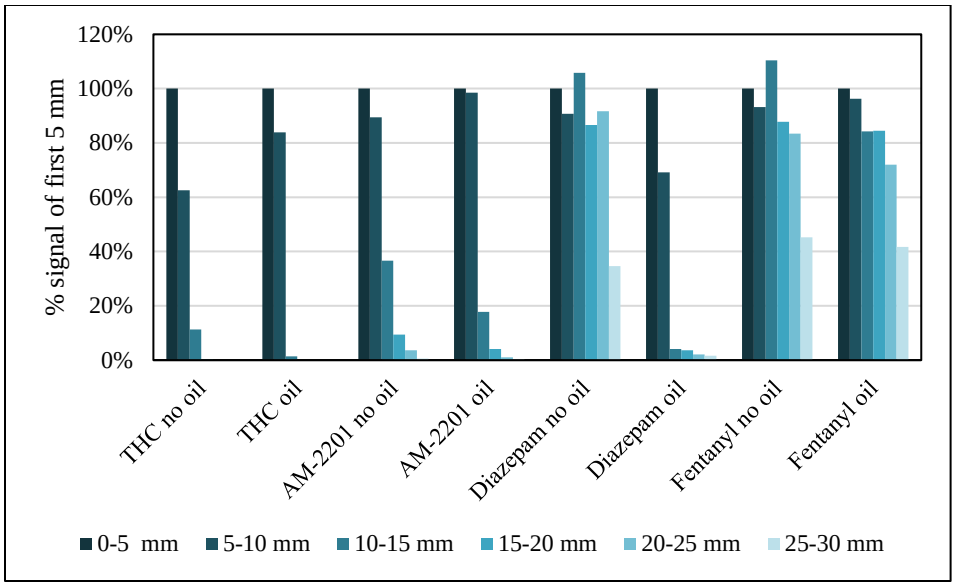

Figure 4.4: Preconcentration During Paper Strip Extraction with and without Sesame Seed Oil. Decrease in analyte concentration throughout a strip of paper with or without sesame seed oil at the end. Measurements expressed as a percentage of the analyte/SIL ratio for that segment relative to the ratio in the first 5 mm segment.

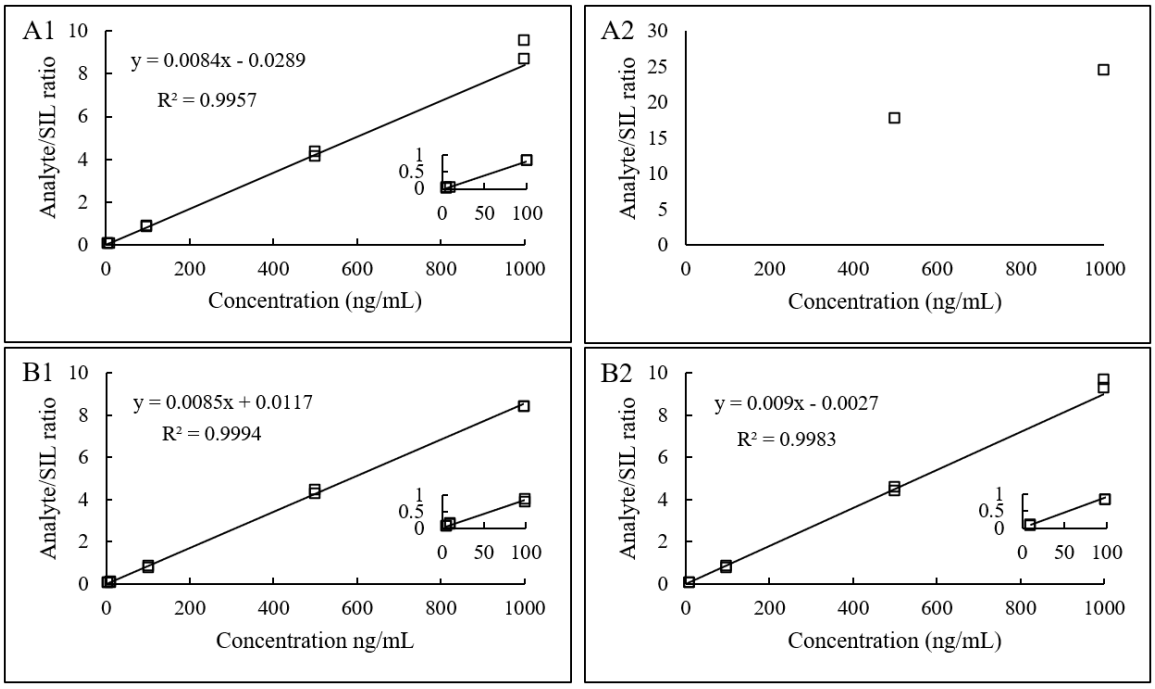

Figure 4.5: Calibration Curves for THC Before and After 27 Days of Storage With and Without Sesame Seed Oil. Calibration curves for THC after 1 day (A1 and B1) and after 27 days (A2 and B2) using paper strip extraction (A1 and A2) and paper strip extraction with sesame seed oil (B1 and B2).

By day 27 the calibration curve without oil (Figure 4.5A2) shows only two detectable concentrations, while the curve with sesame seed oil (Figure 4.5B2) still shows signal down to 10 ng/mL, demonstrating a marked improvement in stability for THC. The detection limits for the analytes were found at different time intervals (Table 4.3). Fentanyl was included as a non-cannabinoid comparison.

The synthetic cannabinoids showed minimal change over time, with or without the addition of oil. THC and 11-OH-THC show a measurable increase in the lowest detectable concentration over time without sesame seed oil. THC-COOH showed significant degradation over time even with sesame seed oil, albeit still improved over no preservative.

4.5.2 Preconcentration Effects on Detection Limits

In the previous section it was shown that sesame seed oil preserves THC, but doesn't investigate the effects of preconcentration. The fact that in table 4.3, THC still had a low ng/mL detection limit with extraction but without sesame seed oil after 24 hours, while in table 4.1 over 90% of THC directly spotted on paper was gone in the same amount of time suggests that preconcentration could significantly improve detection limits. To test the effects of preconcentration on detection limits and preservation, biofluids were stored on paper with sesame seed oil with and without preconcentration and analyzed after 1 day and 28 days (Table 4.4). Oral fluid was added as a second non-invasive biofluid (Table 4.5).

Table 4.3: Detection Limits Over Time in Urine Using Paper Strip Extraction. Lowest detectable urine concentration (ng/mL) after paper strip extraction with and without sesame seed oil after storage at room temperature. *Detection limits significantly above the 100 ng/mL internal standard are rough estimates.

Analyte	Day 1		Day 7		Day 27	
	No Oil	Oil	No Oil	Oil	No Oil	Oil
THC	2	1	30	1	≥500*	3
11-OH-THC	20	4	30	4	≥1000*	4
THC-COOH	10	5	130	30	≥1000*	120
AM-2201	0.8	1	1	0.6	4	0.8
AB-CHMINACA	1	3	1	1	3	2
Fentanyl	2	3	0.8	3	2	7

Table 4.4: Urine Detection Limits Over Time With and Without Paper Strip Extraction. Lowest detectable urine concentration (ng/mL) with and without paper strip extraction after storage at room temperature with sesame seed oil. *Detection limits well above the 100 ng/mL internal standard are rough estimates.

Urine	Day 1		Day 28	
	Without Concentration	With Concentration	Without Concentration	With Concentration
THC	20	2	10	2
11-OH-THC	9	4	70	20
THC-COOH	200	10	≥500*	40
AM-2201	0.2	0.2	0.2	0.2
AB-CHMINACA	1	0.2	1	2
AB-CHMINACA M4	130	60	70	80
5F-ADB	2	0.3	0.3	0.3

Table 4.5: Oral Fluid Detection Limits Over Time With and Without Paper Strip Extraction. Lowest detectable oral fluid concentration (ng/mL) with and without paper strip extraction after storage at room temperature with sesame seed oil. *Detection limits well above the 100 ng/mL internal standard are rough estimates.

Oral Fluid	Day 1		Day 28	
	Without Concentration	With Concentration	Without Concentration	With Concentration
THC	20	1	30	1
11-OH-THC	4	3	70	5
THC-COOH	10	3	≥1000*	60
AM-2201	0.06	0.08	0.2	0.08
AB-CHMINACA	4	0.6	3	2
AB-CHMINACA M4	50	100	100	100
5F-ADB	2	0.2	2	0.2

Table 4.6: Autosampler Detection Limits. Detection limits in ng/mL in oral fluid using sesame seed oil and paper strip extraction in a half 3D printed autosampler cartridge.

Analyte	LOD
THC	4
5F-ADB	0.1
5F-ADB M2	0.5
AB-CHMINACA	6
AM-2201	0.1
AB-CHMINACA M4	20

Tables 4.4 and 4.5 show a noticeable improvement to detection limits from preconcentration for THC. THC-COOH behaved as in table 4.3; showing heightened detection limits after 28 days, even with oil. However, for both THC-COOH and 11-OH-THC without preconcentration, there was an increase in detection limits after 28 days for both biofluids. This suggests that there is a secondary mechanism for the elimination of the two THC metabolites that isn't abated by preconcentration or sesame seed oil. The metabolite AB-CHMINACA M4, which is more hydrophilic than AB-CHMINACA, showed worse detection limits than AB-CHMINACA under all conditions. The fact that the three metabolites studied show worse results than the original analytes suggest that paper strip extraction with sesame seed oil is less effective for metabolites. This could be due to metabolites typically being less hydrophobic than the original analyte. However, the detection limits for THC without preconcentration ranged from 10-30 ng/mL while with preconcentration the limit ranges between 1-2 ng/mL; showing that preconcentration does lower detection limits, for certain analytes. AM-2201 and AB-CHMINACA had consistent results regardless of the biofluid, preconcentration, or storage time. 5F-ADB in most cases showed about an order of magnitude improvement in its detection limit when performing paper strip extraction. These results suggest that paper strip extraction has the potential to improve the detection limits for synthetic cannabinoids relative to directly spotting the analyte on paper, but it is not consistent and is analyte dependent. More importantly, the detection limits for the synthetic cannabinoids did not get worse with the addition of oil, meaning that both natural and synthetic cannabinoids can be detected with the same method.

4.5.3 Automated Analysis

The main attractions of paper spray mass spectrometry are its ease of use and short analysis times. Any modifications to the technique should maintain the speed and simplicity of the

technique; otherwise those modifications defeat the purpose of paper spray. Ideally, the analysis would also compatible with automation to enable high throughput analysis. A prototype paper spray cartridge was developed to demonstrate the potential for simple, fast, and automated implementation of paper strip extraction. The top half of the cartridge was 3D printed in polypropylene (top part of Figure 4.6:A) and the bottom half came from an injection molded paper spray autosampler cartridge (bottom part of Figure 4.6:A). Polypropylene was selected for the top half due to its resistance to organic solvents. When the top half was snapped onto the bottom half, it made a seal around the sample square such that solvent flowed through the sample to the paper spray substrate.

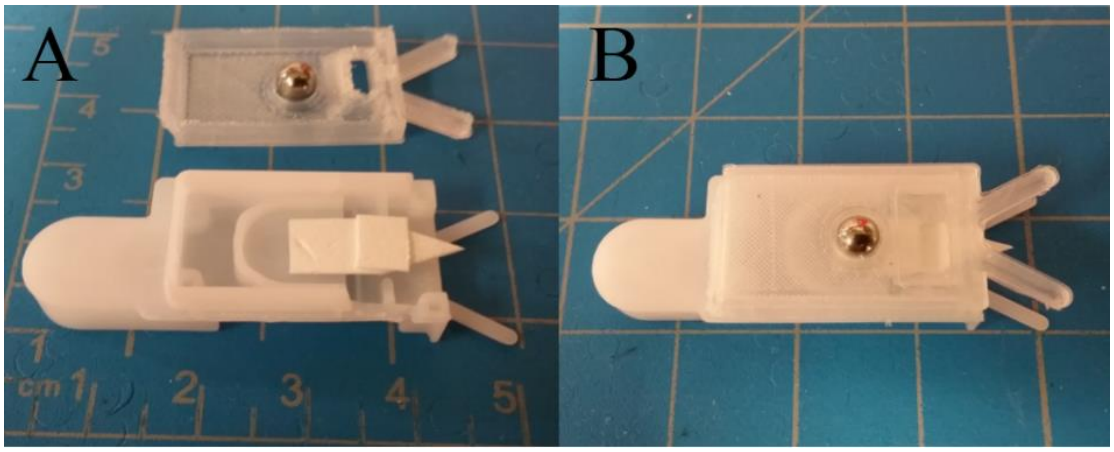

Figure 4.6: Prototype Paper Strip Extraction Cartridge. A. 3D printed polypropylene top half and injection molded plastic bottom half, B. assembled cartridge.

A calibration curve was generated using the prepared cartridges to determine; the LOD calculated from the calibration curves are shown in table 4.6. THC-COOH was also run in this experiment, but there were insufficient data points for a calibration curve. This was unexpected because THC analysis performed similarly to previous experiments. The three synthetic cannabinoids and two metabolites likewise behaved similarly. It's possible that some interferent eluting from the 3D printed plastic specifically affected THC-COOH ionization. Beyond this anomaly the autosampler cartridge results showed that the paper strip extraction technique is compatible with automation.

Paper strip extraction was evaluated here as a means of analyzing natural and synthetic cannabinoids. As the detection limits for synthetic cannabinoids were not significantly worse when comparing paper strip extraction to direct spotting (Tables 4.4 and 4.5), nor were they significantly worse after extended storage (Tables 4.3-4.5), the usefulness of paper strip extraction lies in having a technique capable of simultaneous and rapid detection of both synthetic and natural cannabinoids. To be a valid option for routine analysis for applications such as determining driving under the influence of drugs (DUID) or workplace drug screening, the technique must be sensitive enough to at least detect levels indicative of recent use in that specific biofluid. Current workplace drug testing methods typically focus on THC-COOH as a biomarker in urine with cut offs at 50 ng/mL for the initial screen and 15 ng/mL for the subsequent confirmation[11]. Looking at the paper strip extraction results for THC-COOH, there is a discrepancy between the detection limits for multiple experiments under similar experimental conditions. Also, the results were significantly worse in oral fluid and when using an automated cartridge. Considering the results in table 4.3, THC-COOH detection limits got noticeably worse over time relative to THC, indicating that THC-COOH was unstable in the urine spot even with sesame seed oil (even if the degradation was

slower than without oil). This result casts doubts on using paper strip extraction for routine detection of THC-COOH. However, THC-COOH is a metabolite that can be present for days in biofluids at variable levels over time depending on the frequency of use[26], and thus is not a good marker on its own for determination of recent use in cases of DUID[5]. For recent use, it has been proposed that THC itself is a better biomarker, as its presence in biofluid spikes within a few hours of use and drops off quickly[27-29]. For urine samples, the low ng/mL detection limits obtained by paper strip extraction are close to the 1.5 ng/mL cut-off proposed as an indication of recent use[28]. For oral fluid, the concentration of THC can vary quite a bit after recent use[27], but the cut-off concentration for THC from Substance Abuse and Mental Health Services Administration (SAMHSA) is 2 ng/mL[30] and the cut-off for DUID from the European Union's Driving Under the Influences of Drugs, Alcohol, and Medicines (DRUID) are 1 ng/mL[31]. This also lies within the range detectable by paper strip extraction, although some further refinement of the technique and improvement in the detection limits are needed to decrease false negatives for samples near the cut-off value. Still, paper spray coupled to paper strip extraction represents a new opportunity to rapidly screen for both synthetic and natural cannabinoids from urine or oral fluid samples.

4.6 Conclusion

A method of concentrating and preserving THC and its metabolites in urine or oral fluid spots was demonstrated and integrated into cartridge compatible with a paper spray autosampler. By flowing urine or oral fluid through paper, synthetic and natural cannabinoids were found concentrate at the head of the paper. THC, which is normally labile and difficult to analyze from a biofluid spot, can be preserved for at least 27 days at room temperature with the addition of sesame seed oil to the paper. Combining these two techniques improved detection limits for THC to ng/mL levels in urine and oral fluid, close to current cut-off values for detection in cases of

DUID. This method was also able to simultaneously detect natural and synthetic cannabinoids from a single sample.

4.7 Acknowledgments

Funding for this research was provided by a grant from the National Institute of Justice, Office of Justice Programs under award number **2016-DN-BX-007**, by the National Institute on Drug Abuse of the National Institutes of Health under award number R21DA043037, and by Indiana University's Responding to the Addictions Crisis Grand Challenges initiative.

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CHAPTER 5. NOVEL SOLID PHASE EXTRACTION PAPER SPRAY TECHNIQUES FOR DETECTING NEW PSYCHOACTIVE SUBSTANCES IN BLOOD, PLASMA, ORAL FLUID AND URINE.

5.1 Abstract

Synthetic drugs that mimic more traditional drugs, termed designer drugs or new psychoactive substances, have led to an epidemic of overdose cases in recent years. Fentanyl and fentanyl analogues continue to cause deaths due to their high potency. At the same time synthetic cannabinoids skirt regulation and detection due to the large number of possible targets available from pharmaceutical research. Traditional drug assays are either slow and elaborate, or not specific or sensitive enough. Paper spray mass spectrometry (MS) offers a rapid and simple means to detect these different types of analytes in a variety of biofluids. The limits of detection for paper spray MS, however, are often inadequate for synthetic drugs due to the higher potency of these molecules. Previous work has shown that integrating solid phase extraction (SPE) into a paper spray cartridge significantly improves limits of detection. The addition, however, increases the complexity and the cost of disposable cartridges, and is incapable of analyzing more viscous biofluids like whole blood. In this work, solid phase extraction powder was mixed with corn starch to make a porous and rugged solid that can be used with paper spray. This solid was coated on the end of a strip of paper and used to extract drugs from plasma, oral fluid and urine to improve detection limits for new psychoactive substances and a selection of prescription drugs with known physical properties. To analyze whole blood, the SPE solid was combined with a commercially available autosampler cartridge and a lateral blood fractionation paper was used to pull the blood through the solid. Certain analytes showed improvements to limits of detection ranging from a factor of 5 to over a factor of 50.

5.2 Introduction

New psychoactive substances (NPS), also called synthetic or designer drugs, are similar to historically more common drugs in structure or function and have been causing problems in the United States and around the world in recent years[1-3]. These drugs have unique structures from their more well-known counterparts, making them more difficult to detect and regulate[1]. A majority of the deaths are caused by opioid overdoses from compounds such as fentanyl and fentanyl analogues[3-4]. These analogues can have potencies that range over several orders of magnitude[4-5], making it is easy to overdose if the dosage isn't well controlled or if heroin is cut with fentanyl[6]. This has led to drug poisoning being the number one cause of injury death in the United States in recent years[6]. Another class of NPS, synthetic cannabinoids, present a different problem in that there are libraries of compounds available left over from pharmaceutical research into cannabinoids[7]. These compounds are sprayed on dried plant material and marketed as incense "not for human consumption[8]." The dosage of these compounds is often poorly controlled, the potency of each compound has not been studied, and there are unknown side effects. All of these factors can cause patients to end up in the emergency room with an overdose on an unknown substance[8].

Traditionally, analysis of drugs of abuse involves an immunoassay screening step, followed by either gas or liquid chromatography paired with a detector[9-12]. However, immunoassays are insufficient for screening in the new crisis. The lethal concentrations of more potent NPS are often below what immunoassays can detect in complex matrices like blood[13]. Immunoassays are also not selective enough to tell different analogs apart from each other, nor are they able to easily implement multiplexing to determine whether multiple drugs are present[13]. Chromatography paired with different detectors can have the required sensitivity and specificity, but is problematic for high throughput screening because it requires extensive sample preparation followed by a time-

consuming chromatography run. This adds time and training requirements to the procedure as well as cost. There is a clear need for methods that are fast, cheap, simple, sensitive, and have high specificity.

Paper spray mass spectrometry (MS) is fast, cheap and simple, but, a major concern is increased limits of detection due to the presence of matrix effects[14]. Biofluids are complex matrices containing high concentrations of salts and biomolecules which are still present when the biofluid is directly analyzed. In previous work, the properties of the paper were studied to determine if these matrix effects could be mitigated, but careful selection of the paper only had a small improvement on detection limits for certain biofluids[15-16]. Alternatively, extracting analytes either through a liquid extractions of the biofluid on hydrophobic paper[17] or solid phase extractions (SPE) using a cartridge with a built in SPE component[18] has been shown to noticeably lower detection limits. Rapid ambient ionization techniques that utilize solid phase extractions aren't limited to paper spray either. Coated blade spray techniques use a bound stationary phase on metal blades to extract analytes from biofluid to achieve pg/mL detection limits for certain analytes[19-20]. Preparing these blades, however, requires an offline extraction step which also increase method complexity. In addition, solid phase extraction techniques have difficulties with viscous biofluids like blood. This is because SPE extraction of whole blood normally requires extensive work such as centrifugation, protein precipitation or dilution prior to the extraction[21-22]. In addition, adding SPE to paper spray increases the complexity of the autosampler cartridge and the analysis.

In this work, a new and easier means of concentrating analytes out of biofluids using SPE powder is investigated and combined with paper spray-MS. SPE powder is mixed with a corn starch binder to make a porous solid and coated on the end of a strip of chromatography paper. Plasma, oral fluid and urine are passed through the SPE region to concentrate analytes out of the

biofluid, a subsequent rinse helps mitigate matrix effects. Limits of detection were determined and compared to unmodified paper spray-MS analysis of each biofluid. Whole blood was found to be incompatible with this SPE paper strip extraction approach, so a different technique was developed for whole blood. Undiluted whole blood was extracted vertically through a SPE solid contained in an autosampler cartridge to a waste pad designed to help wick blood. Extracted whole blood was rinsed both while wet and after drying and the limits of detection determined relative to directly spotting in the autosampler cartridge. Finally, a design for a paper spray autosampler cartridge with built in SPE strip extraction was assembled as a proof of concept using parts from a commercially available cartridge combined with 3D printed components.

5.3 Materials

Analytes and their stable isotopic labels (SIL), except atenolol D7 and the synthetic cannabinoids, were obtained from Cerilliant (Round Rock, TX, U.S.A.). Atenolol D7 was obtained from CDN isotopes (Pointe-Claire, QC, Canada) as a powder (≥98% purity). AB-CHMINACA and AM-2201 and their SIL's were obtained from Cayman Chemical (Ann Arbor, MI, U.S.A). Pooled human plasma, HPLC grade methanol, and acetonitrile came from Fisher Scientific (Waltham, MA, U.S.A.). Whatman grade 31 ET, 3MM chromatography paper and grade 4 filter paper were bought from GE Healthcare Life Sciences (Pittsburgh, PA, U.S.A.). Corn starch was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Strata-XL SPE tubes were purchased from Phenomenex (Torrance, CA, U.S.A.). CytoSep blood fractionation paper was donated by the Pall Corporation (Port Washington, NY, U.S.A.). The autosampler cartridge was produced by Prosolia (Indianapolis, IN, U.S.A.). The top half of the autosampler cartridge was 3D printed using a polypropylene filament using an Ultimaker 2+ extended (Geldermalsen, Gelderland, Netherlands). Delrin plastic sheets (0.35 mm thick, black) were ordered from

McMaster-Carr (Elmhurst, IL, U.S.A.). Urine and saliva were provided by a single donor. Whole blood was drawn from a separate donor.

5.4 Methods

5.4.1 Making SPE Extraction Strips and Cartridges

SPE extraction strips were made by coating a slurry of SPE powder and a binder onto the end of a strip of chromatography paper then letting it harden into a porous solid. The slurry was prepared by adding boiling water in a 2:1 ratio (volume:mass) to SPE powder containing cornstarch at 3% by mass. The slurry was heated by submerging the container in a boiling water bath for 5 minutes. The slurry was spotted in 10 µL aliquots for oral fluid experiments and 20 µL aliquots for urine or plasma on the end of wet strips of 31ET chromatography paper. The strips were 5x40 mm for oral fluid experiments and 15x35 mm with a 5x5mm square segment at one end for urine and plasma. Adding a 5x5 mm square of grade 4 filter paper to the top of the slurry on the strip added another surface for the slurry to adhere to and increased ruggedness of the device once the slurry dried. For whole blood extraction the top half of an autosampler cartridge was removed and the rear solvent well was sealed on the bottom with aluminum foil. The well was filled with 10 µL of slurry and allowed to dry before removing the foil. After drying the mixture of starch and SPE powder was rugged enough to be handled without fracturing. The finished extration devices for oral fluid, urine or plasma, and whole blood are shown in Figure 5.1A-C respectively.

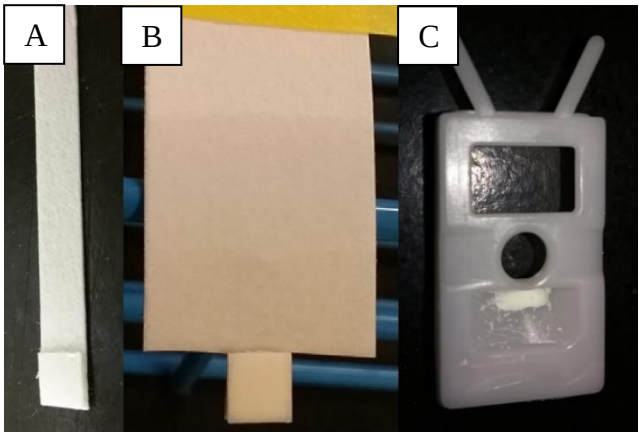

Figure 5.1: Three Different SPE Extraction Devices for Paper Spray-MS. A) SPE strip for oral fluid extraction, B) SPE strip urine and plasma extraction, C) SPE in an autosampler cartridge for whole blood extraction. SPE material contained under square of filter paper at the bottom of the strip in A and B and in the rear solvent well in C.

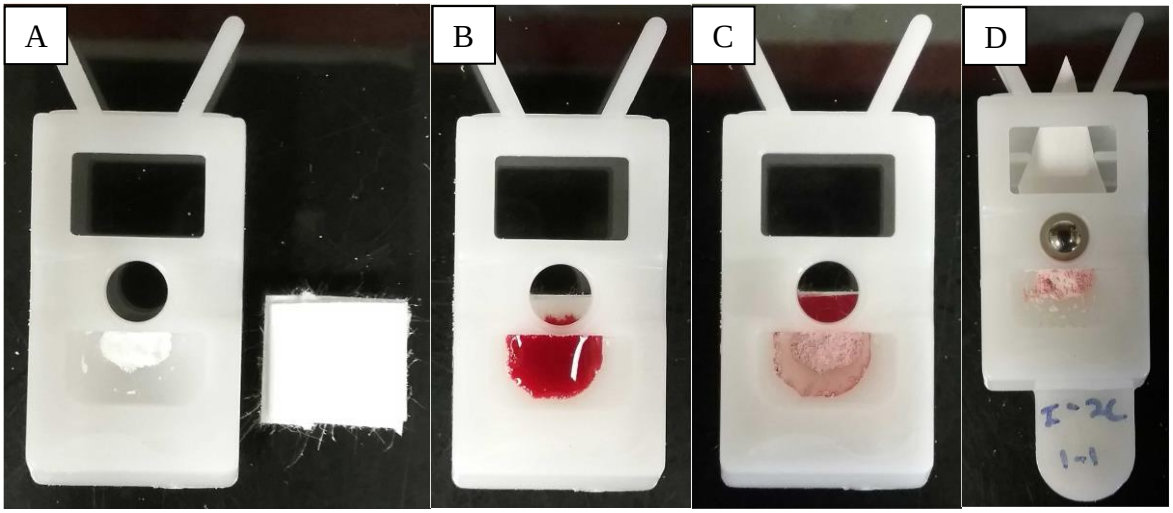

Figure 5.2: Extraction of Whole Blood on an Autosampler Cartridge. A) Top half of an autosampler cartridge with SPE solid contained in rear solvent well and waste pad. B) Whole blood flowing through SPE to waste pad. C) SPE after sample dried. D) Assembled cartridge after water wash step.

5.4.2 Analyte Retention

Plasma, oral fluid and urine were extracted using the SPE strips on 3MM paper and analyzed to determine whether analytes were being concentrated. Biofluids were spiked at 500 ng/mL of the prescription drugs atenolol, carbamazepine, fentanyl and diazepam and the NPSs carfentanil, AB-CHMINACA and AM-2201 as well as the metabolite norfentanyl. Extraction was carried out by flowing 50 µL of the biofluid through the SPE region at the end of the strip. Following extraction and drying the strip was cut into 5 mm increments and spiked with 5 µL of 500 ng/mL stable isotope labeled (SIL) analogs of the analytes except for norfentanyl and carfentanil which used fentanyl-d5. Paper spray-MS was carried out by using an alligator clip to attach the sample square onto a paper tip and positioning it in front of the inlet of a Thermo Fisher Scientific Q-Exactive Focus orbitrap mass spectrometer. Two 30 µL aliquots of 9:1 acetonitrile: water with 100 ppm formic acid were added to a solvent well consisting of an Eppendorf tip contacting the sample square. After adding the solvent, ionization was carried out by applying 4.5 kV of voltage to the paper for 1.7 minutes. The MS was run in parallel reaction monitoring mode (PRM), measuring the MS/MS spectrum for each analyte and its SIL. Each 5 mm increment was analyzed separately and the area under the curve during each run was calculated for each analyte and its SIL. Drug retention was evaluated by calculating the ratio between the analyte in the square divided by the SIL and normalizing the value to the ratio from the first 5 mm of the strip.

5.4.3 SPE Strip Extraction of Plasma, Oral Fluid and Urine

Limits of detection in different biofluids were evaluated by analyzing calibration curves with and without SPE extraction. Calibration curves were prepared in plasma, oral fluid and urine at 0.1, 1, 10, 25, 100 and 1000 ng/mL each with a 100 ng/mL internal standard consisting of SILs. For each biofluid, two measurements were made at each concentration as well as three blank

measurements. Extraction was carried out as described before except that 100 µL was extracted for plasma and urine using the paper strip with a larger width (Figure 5.1B). After drying, the same volume of milli-Q water (50 µL for oral fluid, 100 µL for plasma and urine) was flowed through the SPE region as a clean-up step. Direct spotting was conducted by spotting 5 µL of biofluid directly on a 5x5 mm square of 31ET. After extraction and drying the first 5 mm segment containing the SPE material was cut from the rest of the strip and attached to a wedge of 31ET chromatography paper with a point using an alligator clip, and the same was done with the directly spotted squares. Paper spray-MS was carried out as described earlier except that the spray solvent volume was increased for the SPE samples to two aliquots of 40 µL of solvent. Ratios between the analyte and SIL were determined for each calibration level, and a calibration curve was constructed. Any data points which were lower than three times the standard deviation of the blanks were excluded. Slopes and Y-intercepts were calculated using linear regression[23] with a weighting factor of $1/X^2$. Limits of detection were set at three times the standard error in the Y-intercept divided by the slope.

5.4.4 SPE Cartridge Extraction of Whole Blood

A calibration curve was prepared as described earlier but in whole blood using an autosampler cartridge with SPE material in the solvent slot (Figure 5.2A). Two aliquots of 35 µL of whole blood were flowed through the SPE to a waste pad consisting of three 1x1 cm squares of grade 1660 cytosep paper (Figure 5.2B).

Two aliquots of 75 µL of milli-Q water was applied to the SPE, where it flowed through the SPE material to 31ET chromatography paper waste pads. Cartridges were washed either while the blood was still wet or after two hours of drying at room temperature (Figure 5.2C). For direct spotting 10 µL of blood was spotted directly on the paper inside the autosampler cartridge. The

cartridges were then assembled (Figure 5.2D) and analyzed as described earlier except the solvent was 1:1 acetonitrile: methanol with 100 ppm formic acid. Direct whole blood analysis was carried out with 60 µL of spray solvent where as SPE whole blood samples used 80 µL. Calibration curves and limits of detection were calculated as described previously.

5.4.5 Prototype Cartridge for Implementation of SPE Strip Extraction

A cartridge with a built-in SPE strip extraction was assembled as a proof of concept. An autosampler cartridge had the top half removed and replaced with a 3D printed version, (Figure 5.3A), designed to flow biofluid and solvent through the SPE region of an extraction strip. The top was modeled in Sketchup and printed using polypropylene filament on an Ultimaker 2 extended+. The SPE extraction strip was adhered to a 0.35 mm thick piece of Delrin plastic, (Figure 5.3A), and positioned on top of a paper spray tip within the cartridge (Figure 5.3B). The strip was perforated with a razor blade between the SPE region and the waste strip. The bottom half of the cartridge, (Figure 5.3A), had plastic cut away from one side with a razor blade to make room for the extraction strip. After extraction the excess paper was removed along with the Delrin (Figure 5.3C).

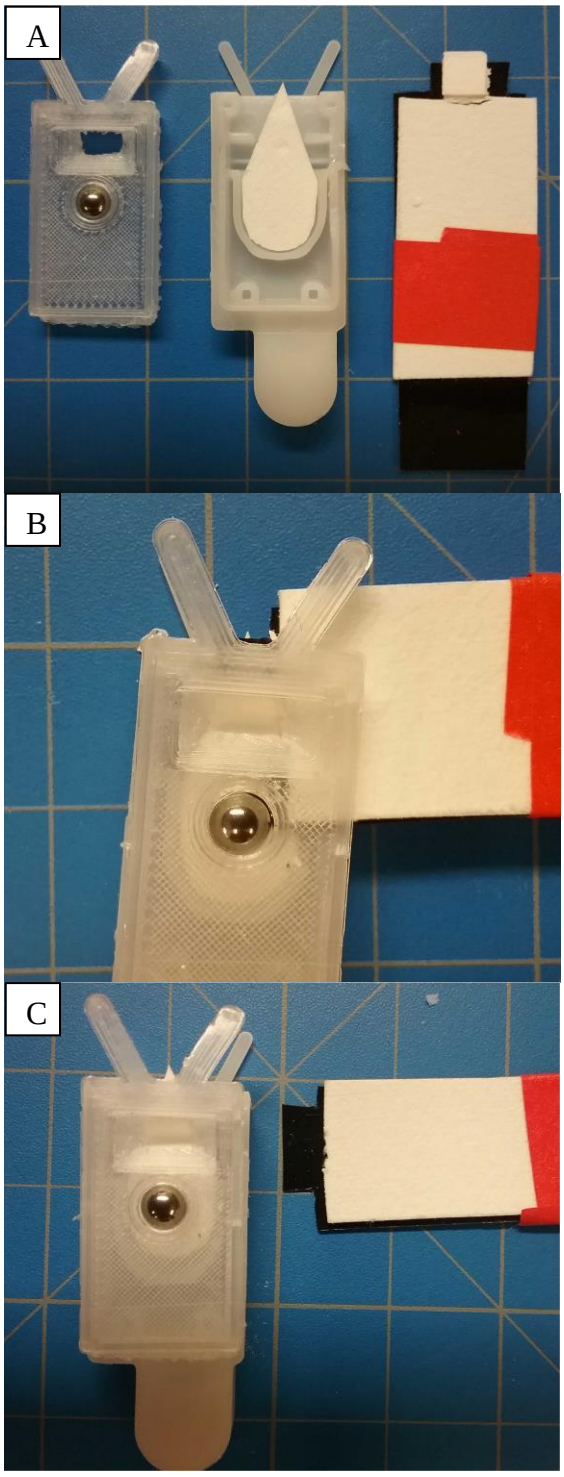

Figure 5.3: SPE Strip Autosampler Cartridge. A) The components of an autosampler cartridge with built in SPE strip extraction. B) The assembled cartridge. C) The assembled cartridge after removing the waste strip.

5.5 Results and Discussion

5.5.1 Analyte Retention

The first test of the SPE extractions strips was to confirm that passing biofluid through the SPE region retained the analytes. To this end, three different biofluids were selected and spiked with fentanyl, carfentanil, and norfentanyl (a fentanyl analogue and metabolite), AB-CHMINACA and AM-2201 (synthetic cannabinoids) as well as the pharmaceuticals diazepam, atenolol, and carbamazepine. Pharmaceuticals were included because physical and chemical properties such as logP and pKa are not known for many NPS; including analytes with known physical properties helps determine whether any observed trends are related to those properties. After extraction and dividing the strip into 5 mm increments, each segment was spiked with an internal standard. The ratio between the analyte and the SIL IS was used to determine if the analytes were concentrated. A similar experiment was carried out with a strip of 3MM chromatography paper without SPE material as a comparison. The 3MM paper was used instead of 31ET because 3MM was found to concentrate certain analytes even without SPE material, and that property needed to be considered when evaluating the effectiveness of the SPE coated strip. The results were normalized to the first 5 mm increment and the results for carbamazepine, fentanyl, and AB-CHMINACA (Figure 5.4A-C, respectively).

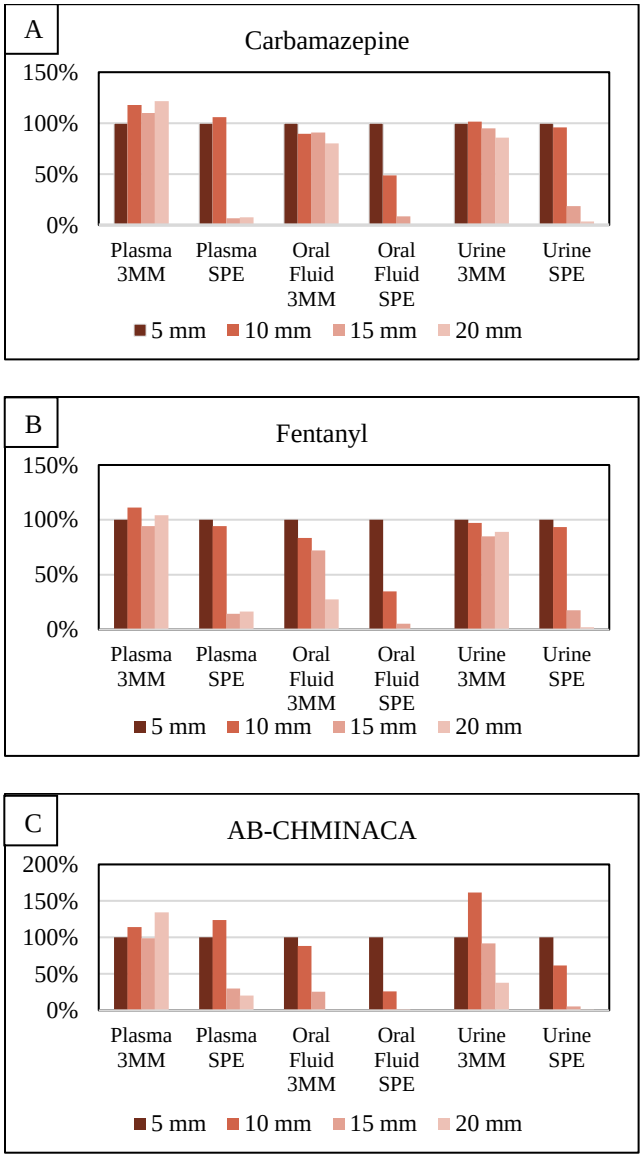

Figure 5.4: Preconcentration Using SPE Strip Extraction. Change in relative concentration of A) carbamazepine, B) fentanyl and C) AB-CHMINACA in three biofluids over the first 20 mm of a strip of 3MM chromatography paper with and without SPE on the first 5 mm. All measurements are expressed as a percentage of the analyte amount in the first 5 mm.

For all three biofluids, most of the analytes showed the same trends as carbamazepine (Figure 5.4A) and fentanyl (Figure 5.4B); significant analyte retention was observed when the SPE material was present but not for plain paper. This result indicated that, in general, the SPE material will preconcentrate drugs from larger sample volumes much more effectively than plain paper. AB-CHMINACA (Figure 5.4C) and AM-2201 showed a different trend in oral fluid and urine samples. In these two matrices, the synthetic cannabinoids tended to concentrate on chromatography paper even without SPE material. This is most likely due to the synthetic cannabinoids' high hydrophobicity. The concentration effect was still more pronounced with SPE present, however. In plasma samples, the synthetic cannabinoids did not show any concentration effects for plain paper. This is likely because of the high protein content in plasma samples, which would have bound the drugs non-specifically and carried them along the strip.

5.5.2 SPE Strip Extraction of Plasma, Oral Fluid and Urine

SPE extraction can lead to lower detection limits if there isn't also an increase in matrix effects. Plasma, oral fluid, and urine were extracted using the SPE paper strip and washed with milli-Q water to minimize matrix effects caused by water soluble components like salts. Limits of detection from the SPE strip and from directly spotting the biofluids were calculated based on their calibration curves (Table 5.1).

Table 5.1: Limits of Detection in Plasma, Urine, and Oral Fluid Using SPE Strip Extraction. Lowest detectable concentration (ng/mL) in plasma, urine, and oral fluid when extracted using SPE strip and when directly spotting the biofluid on paper (no SPE). Oral fluid SPE strip extraction was carried out with half the amount of SPE and half the amount of biofluid volume as plasma or urine.

	Plasma		Urine		Oral Fluid	
	SPE	No SPE	SPE	No SPE	SPE	No SPE
AB-CHMINACA	0.2	5	2	5	0.4	0.5
Acetyl Fentanyl	0.1	2	0.01	0.1	0.01	0.02
AM-2201	2	3	0.3	0.9	0.6	0.07
Atenolol	0.2	10	1	4	0.5	2
carbamazepine	0.2	3	0.2	2	0.04	0.04
carfentanil	0.02	0.5	0.01	0.4	0.04	0.4
Diazepam	0.2	2	2	2	0.03	0.4
Fentanyl	0.2	1	0.02	0.2	0.03	0.03
Norfentanyl	0.4	3	4	80	0.03	0.4

Table 5.2: Detection Limits in Whole Blood after Extraction. Limits of detection (ng/mL) for analytes in whole blood when directly spotted on paper (no SPE), extracted using SPE then washed while dry, and extracted using SPE then washed while still wet.

	No SPE	Dry wash	Wet Wash
AB-CHMINACA	1	0.3	0.2
Acetyl Fentanyl	0.08	0.01	0.01
AM-2201	2	0.3	0.2
Atenolol	3	0.4	0.3
Carbamazepine	2	0.03	0.2
Carfentanil	0.5	0.1	0.09
Diazepam	20	0.3	0.3
Fentanyl	0.3	0.03	0.2
Norfentanyl	0.7	0.7	0.4

Of the 27 drug-matrix combinations, 14 showed a factor of ten or more improvement for SPE strip extraction relative to direct paper spray MS. For oral fluid samples, significant improvement was obtained for carfentanil, diazepam and norfentanyl. Plasma and urine samples showed large improvements in detection limits for 11 of the drug-matrix combinations, ranging from around a factor 10 for acetyl fentanyl in urine and diazepam in plasma to upwards of 50 for atenolol in plasma.

Sample volume is an important consideration during method development. For plasma and urine, 100 µL was found to give better detection limits than used because extracting only 50 µL of biofluid (results not shown). There is a limit to the volume of biofluid that can be extracted by this method, however. The rate of flow through the SPE material slows at higher volumes because the wicking rate decreases as the sample wicking distance increases[24]. It is also possible that components of the biofluid clog the SPE material, which would have a cumulative effect at higher volumes. Also, a lower sample volume was necessary for oral fluid because the enzymes in oral fluid degraded the starch binder; the SPE material was no longer held together and had to be analyzed with care not to spill the powder. To improve robustness of oral fluid analysis by this method, additional research is needed to identify a water wettable binder that is not degraded by enzymes found in oral fluid. Despite the lower extraction volume and the difficulty of working with the samples, oral fluid still showed marked improvement for three of the nine analytes.

5.5.3 SPE Cartridge Extraction of Whole Blood

SPE extraction of biofluids like plasma has been shown to be an effective way to improve limits of detection[18]. Analyzing whole blood would be faster as it wouldn't require the added step of removing red blood cells. However, whole blood is viscous and therefore difficult to flow appreciable quantities through SPE material. Furthermore, whole blood does not flow laterally

easily because it tends to clog as it travels through normal chromatography paper. Both of these factor makes it incompatible with SPE strip extraction. To overcome these problems, a setup to accommodate whole blood was devised (Figure 5.2). Here, whole blood was flowed vertically through the SPE solid contained within the solvent well of a paper spray autosampler cartridge. The blood sample flowed through SPE material onto a waste pad consisting of three layers of cytosep lateral blood fractionation paper (Figure 5.2). This fractionation paper is a material designed to wick whole blood and to separate out plasma from the slower eluting red blood cells in the process. The dry SPE-starch solid was found to firmly adhere to the acetal plastic cartridge throughout the extraction process without adhesives. After applying blood sample, the SPE material was washed with milli-Q water either when the whole blood was dry or while the blood was still wet. Washing removed most of the coloration of the SPE material (Figure 5.2D) and the limits of detection were compared to direct spotting of the whole blood as shown in table 5.2. Extraction of whole blood showed a consistent improvement of detection limits for most analytes. The washing procedure didn't depend on whether the blood was dry or wet prior; except in the case of fentanyl and carbamazepine. Washing after the whole blood was dry performed better relative to wet.

5.5.4 Method Usefulness

Together, these methods demonstrate the ability of paper spray with integrated SPE to improve detection limits for synthetic cannabinoids, opioids, and pharmaceuticals in a variety of matrices. To be useful, the detection limits must be close to biologically relevant concentrations. For synthetic cannabinoids in general this is difficult as there are many potential targets and the potency of each is often unknown[7-8]. In 2014, two studies on AB-CHMINACA exposure found ranges of 0.4-14.3 ng/mL in plasma of patients experiencing acute delirium and seizures[25] and

whole blood concentrations of 0.6-10 ng/mL in suspected impaired drivers[26]. In another study looking at synthetic cannabinoids similar to AM-2201 in postmortem plasma samples, concentrations were found at 16.3, 140 and 0.86 ng/mL for MAM-2201, AM-1220 and AM-2232 respectively[27]. These studies give a rough idea of the concentration range expected for these specific analytes, which is in the low to sub-ng/mL range. Looking at the results in tables 5.1 and 5.2, the detection limits for the synthetic cannabinoids AB-CHMINACA and AM-2201 were in the low to sub ng/mL range for the three biofluids. This is close to the biological concentrations, and the method may be suitable as a screening technique. The method needs further validation to be used for quantitation. For fentanyl and fentanyl analogues, the biologically relevant concentration range depends on the potency[5]. Acetylfentanyl, for example, has been found at over 100 ng/mL in blood samples from several intoxications and fatalities, while carfentanil has been found in the 0.01-0.617 ng/mL ranges[5]. Looking at the results in table 5.2, the method is adequate for detection of acetylfentanyl with detection limits at 0.01 ng/mL, but would fail to detect carfentanil in cases of intoxication where whole blood concentrations are below 0.1 ng/mL. If the matrix were plasma or urine, however, the detection limit would be significantly lower. Beyond the two illicit drug classes of synthetic cannabinoids and fentanyl analogues a number of pharmaceuticals were also evaluated. They included the beta blocker atenolol, the anticonvulsant carbamazepine and the benzodiazepine diazepam and were included as examples of non-illicit targets with documented physical properties. The fact that the three pharmaceuticals showed measurable improvements to their detection limits in various biofluids suggests a universal improvement for hydrophobic drugs using these methods. This could be useful for therapeutic drug monitoring of more potent drugs where chromatographic techniques are too time consuming and expensive.

5.5.5 Prototype Cartridge for Implementation of SPE Strip Extraction

The aim of this work is to develop a SPE method that can easily be integrated with paper spray-MS. Of the two means of doing this, SPE strip extraction of plasma, urine and oral fluid are the simpler option for ease of use. Due to the viscosity of whole blood multiple waste pads are required for extraction and washing making it a more complicated method to implement with paper spray-MS than the SPE strip. SPE strip extraction can be added to a commercially available autosampler cartridge with a few modifications (Figure 5.3A-C). First, the top half of the autosampler cartridge can be replaced with a 3D printed version that can snap onto the original bottom half of the cartridge (Figure 5A) and fits around the SPE region of the strip when assembled (Figure 5B) to channel biofluid and solvent. The strip is adhered to a thin strip of Delrin plastic (Figure 5A) to prevent biofluid from saturating the spray tip beneath during extraction. After extraction, washing, and drying, the waste portion of the strip can be torn along perforation leaving behind the SPE region sitting on top of the spray tip ready for analysis (Figure 5C). This design is relatively simple requiring minimal effort to conduct a SPE extraction prior to analysis. This design would work best for plasma or urine extraction as oral fluid has been shown to degrade the starch binder.

5.6 Conclusion

Two simple methods to implement solid phase extraction with paper spray mass spectrometry were investigated. A solid consisting of SPE powder and starch was coated onto paper or contained in the solvent well of an autosampler cartridge. The strip was tested to prove that analytes would concentrate significantly in the SPE region and then modified for the extraction of larger volumes of plasma and urine. A second method was developed for the direct analysis of whole blood which is more viscous and difficult to extract. Whole blood, plasma, oral fluid and

urine were extracted looking at a selection of synthetic cannabinoids, fentanyl analogues and pharmaceuticals. Depending on the analyte and matrix detection limits were improved by a factor of five to over a factor of fifty. Finally, SPE strip extraction was integrated into an autosampler cartridge for easy and fast sample extraction of plasma or urine. This could help facilitate rapid screening for NPS from biological fluids.

5.7 Acknowledgments

Funding for this research was provided by a grant from the National Institute of Justice, Office of Justice Programs under award number **2016-DN-BX-007**, by the National Institute on Drug Abuse of the National Institutes of Health under award number R21DA043037, and by Indiana University's Responding to the Addictions Crisis Grand Challenges initiative.

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CHAPTER 6. SELECTION OF 3D PRINTING PLASTICS FOR FABRICATION OF MASS SPECTROMETRY DEVICES

6.1 Abstract

Recently, 3D printing has become a cost-effective means for rapid prototyping devices for mass spectrometry. However, the plastic could bind analytes or leach contaminants, leading to bias or ionization suppression. This is a concern for paper spray mass spectrometry (MS) which benefits from rapid prototyping but the organic solvents used will make direct contact with the 3D printed plastic. In this work, seven types of commercially available 3D printing plastics were evaluated for their impacts on ionization efficiency and non-specific binding of seven pharmaceuticals and the protein cytochrome C. Using those results, plastics for each solvent and analyte type were selected to evaluate whether observed changes in ionization efficiency and recovery cause changes in detection limits during paper spray mass spectrometry. These experiments determined that a using a paper spray cartridge made from polyethylene terephthalate with acetonitrile causes an order of magnitude decrease in detection limits for several analytes relative to those with no plastic present. In all other cases, if no visible change (discoloration or deformation) to the plastic occurred, there was a minimal impact on paper spray detection limits. The work is concluded with a brief editorial on how to select a 3D printing material based on its physical and chemical properties.

6.2 Introduction

3D printing, in which objects are created from digital models layer-by-layer in an additive process, has become a useful tool to hobbyists and professionals alike. Detailed plastic parts and prototype devices can now be custom made in hours using machines that are rapidly becoming

more sophisticated and affordable[1]. This is useful in academia to create low cost and custom parts for use during research. 3D printed devices have found use as custom and low cost containers for reactions[2-3], as microfluidic devices to conduct experiments in a single device[4-8], and paired with detectors such as mass spectrometers and optical sensors to produce custom parts and modifications[9-11]. The speed and low cost of 3D printing allows for rapid prototyping and iterative design. This is useful in paper spray mass spectrometry (paper spray MS) which focuses on low cost solutions to analytical problems.

When selecting a material for use with mass spectrometry, it is important to consider if the material will impact the final results. In paper spray MS and other experiments where the printed material makes direct contact with organic solvents, the plastic can deform or dissolve based on chemical interactions[2]. These dissolved components could contribute to decreased ionization efficiency and detection limits during paper spray MS[12]. In this work, the commercially available fused deposition modeling (FDM) 3D printing materials polyethylene terephthalate, polypropylene, nylon, acrylonitrile butadiene styrene, polylactic acid, and polycarbonate were investigated for solvent compatibility, effects on ionization efficiency, and non-specific binding of analytes for both small molecule pharmaceuticals and the protein cytochrome C. These results were used to determine a potential worst and best material for analysis which were then used to make a cartridge to contain solvent and paper during paper spray MS. These cartridges were used in a set of experiments to determine the limits of detection to evaluate the impact of the 3D printing material. Finally, these results are discussed with reference to the ease of printing and useful properties of these materials.

6.3 Materials

All the pharmaceuticals and their stable isotopic labels (SIL), except atenolol D7, were obtained from Cerilliant (Round Rock, TX, U.S.A.). Atenolol D7 powder was obtained from CDN isotopes (Pointe-Claire, QC, Canada). HPLC grade methanol, acetonitrile, and formic acid were obtained from Fisher Scientific (Waltham, MA, U.S.A). Bovine and equine cytochrome C came from Sigma Aldrich (St, Louis, MO, U.S.A.). Whatman grade 31 ET chromatography paper was obtained from GE Healthcare Life Sciences (Pittsburgh, PA, U.S.A.). Porous polyethylene 0.6 mm thick was purchased from Porex (Fairburn, GA, U.S.A.). Carbon rods for sputter coating were purchased from SPI Supplies (West Chester, PA, U.S.A.). 3D models were generated in Sketchup from Trimble (Synnyvale, CA, U.S.A.). Test cartridges were printed using an Ender 3 3D printer from Creality (Shenzhen, China). Polyethylene terephthalate filament (PET), polypropylene (PP) filament and white polylactic acid (PLA) filament came from Verbatim (Chiyoda, Tokyo, Japan). Nylon filament (brand name is ePA) came from eSun (Shenzhen, China), while acrylonitrile butadiene styrene (ABS) filament, Colorless PLA filament and polycarbonate plastic was purchased from Afinia 3D (Chanhassen, MN, U.S.A.). Makerbot (New York, NY, U.S.A. and Hatchbox (Pomona, CA, U.S.A.), respectively. All filaments had a diameter of 1.75 mm.

6.4 Methods

6.4.1 Evaluating Ionization Suppression and Recovery for Small Molecules

Various 3D printing plastics are available depending on the printer being used. A selection of plastics with different polymeric subunits and printing temperatures for FDM 3D printing were purchased (properties in table 6.1 structures in figure 6.1).

Table 6.1: 3D Printing Plastics. Printable plastics evaluated for impact on analytical device detection limits. Acetonitrile compatible means that the plastic doesn't significantly change shape when submersed in acetonitrile. (*Brand name of filament).

Plastic	Abbreviation	Polymeric subunit	Printing Temp (°C)	Acetonitrile compatible
Polyethylene terephthalate	PET	$OCO(C_6H_4)COOCH_2CH_2$	210-230	Yes
Polypropylene	PP	$CH(CH_3)CH_2$	220	Yes
ePA* (nylon)	PA6	$NH(CH_2)_5CO$	230-260	Yes
Acrylonitrile butadiene styrene	ABS	$(C_3H_3N)_x\,(C_4H_6)_y\,(C_8H_8)_z$	260-270	No
Polylactic acid-colorless	PLA	$CH(CH_3)COO$	215	No
Polylactic acid-white	W-PLA	$CH(CH_3)COO$	200-220	No
Polycarbonate	PC	$O(C_6H_4)C(CH_3)_2(C_6H_4)OCO$	240-250	No

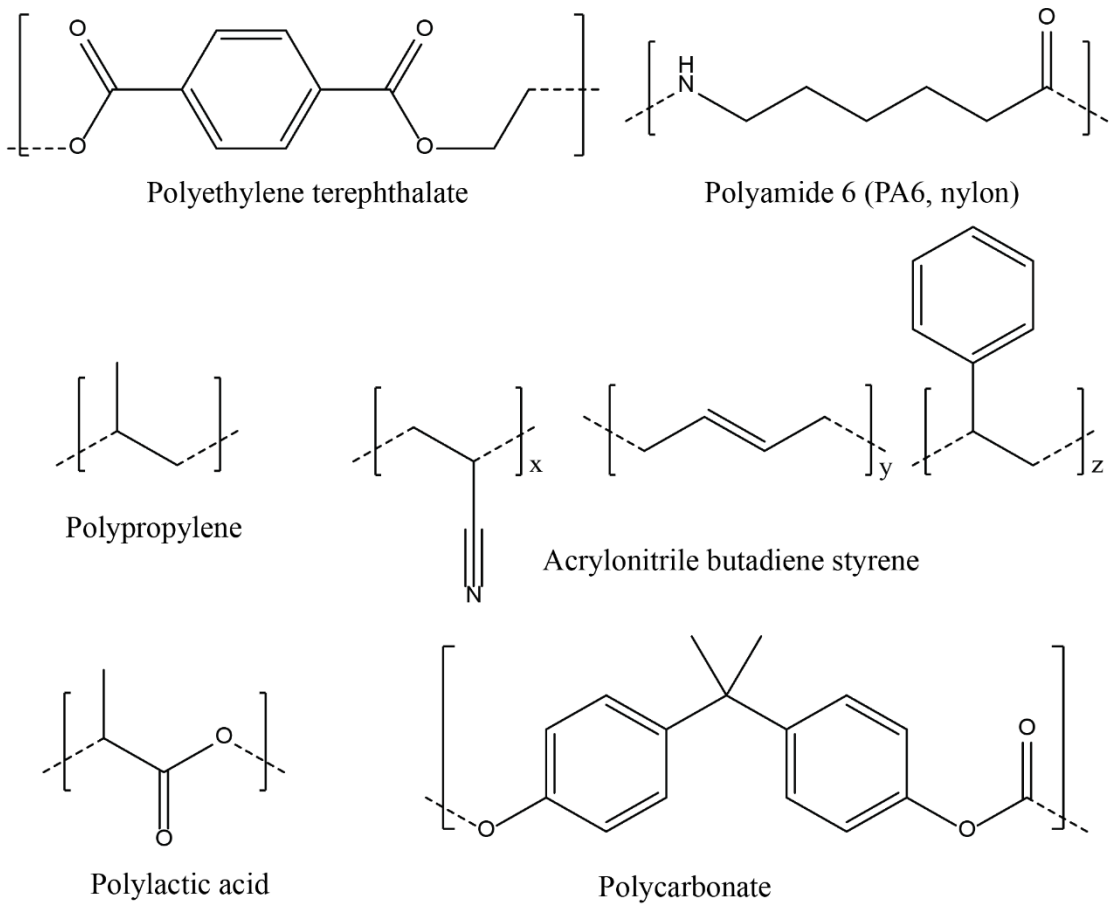

Figure 6.1: 3D Printing Plastic's Structures. Molecular structure for each polymer analyzed.

These plastics were cut into 5-10 mm segments then washed three times in milliQ water and methanol. After drying the filament pieces were measured out in 100 mg samples into glass vials. A 500 µL aliquot of a methanol or acetonitrile solution containing atenolol, carbamazepine, diazepam, fentanyl, gabapentin, hydromorphone and phenylephrine at 100 ng/mL was added to each sample. Samples were vortexed for one hour and left to soak until analysis. To measure the loss of analyte through non-specific binding and potentially reactions with the plastic, hereafter collectively referred to as lowered recovery, a 100 µL aliquot was removed from the sample and spiked with 5 µL of a 2000 ng/mL internal standard solution consisting of stable isotopic labels (SIL) for each of the pharmaceuticals dissolved in methanol. Controls consisted of aliquots of the original solution without any added plastic. Samples were also spiked at 100 ppm formic acid before analysis to aid ionization. Analysis was carried out by direct injection electrospray ionization (ESI) on a Thermo Fisher Scientific Q Exactive Focus Orbitrap mass spectrometer (San Jose, CA, U.S.A.) in parallel reaction monitoring mode (PRM). Samples were analyzed for 1 min at 4.5 kV with 10 arbitrary units of sheath gas and a sample flow rate of 10 µL/min. Ratios were calculated based on the area under the curve for the most intense fragment for each analyte and its SIL. Changes in recovery were determined as the average percent change between each sample and the controls (N=3). To evaluate changes in ionization efficiency, aliquots of the plastic extract without SIL were injected by autosampler into a stream of solvent, either methanol or acetonitrile at 100 ppm formic acid, with SIL being infused at a T-junction by syringe pump. Solvent flow was maintained at 50 µL/min by a G2226A nanopump and G1379A degasser in combination with a G1329A autosampler all from Agilent (Santa Clara, CA, U.S.A.). The syringe pump infused a 1000 ng/mL SIL solution at 5 µL/min. MS/MS signal of the SIL for each pharmaceutical was measured for 30 seconds before and during the infusion of plastic extract. Three replicates

analyzed for each material. Ion suppression/enhancement was calculated as a ratio between the total area under the curve during the infusion to before the infusion. Changes in ionization efficiency were calculated as the average percent decrease between controls and plastic extract.

6.4.2 Evaluating Ionization Efficiency and Recovery for Cytochrome C

Experiments to evaluate of the impact of 3D printed plastic on recovery and ionization efficiency of the protein equine cytochrome C were carried out in the same way as the pharmaceuticals with a few changes. When measuring recovery, plastics were soaked in 3:1 methanol:water at 1% formic acid and 100 µg/mL of equine cytochrome C. Ionization efficiency was evaluated by injecting aliquots of extracts by autosampler into a stream of solvent with 1 mg/mL of equine cytochrome C flowed in by T junction. Measurements for both experiments were made by integrating the area under the curve for the most intense peak of the intact protein.

6.4.3 Changes in Detection Limits

The impact of 3D printed plastic on detection limits was investigated for the seven pharmaceuticals and cytochrome C. The plastics with the largest and smallest combined detriment to signal for each solvent (acetonitrile, methanol and methanol: water) and analyte type (small molecule pharmaceutical or the protein cytochrome C) were selected and used to print paper spray cartridges. These cartridges, shown in figure 6.2, were used to hold a pentagonal wedge of 31ET chromatography paper for pharmaceutical analysis or carbon sputtered porous polyethylene for protein during paper spray MS[13].

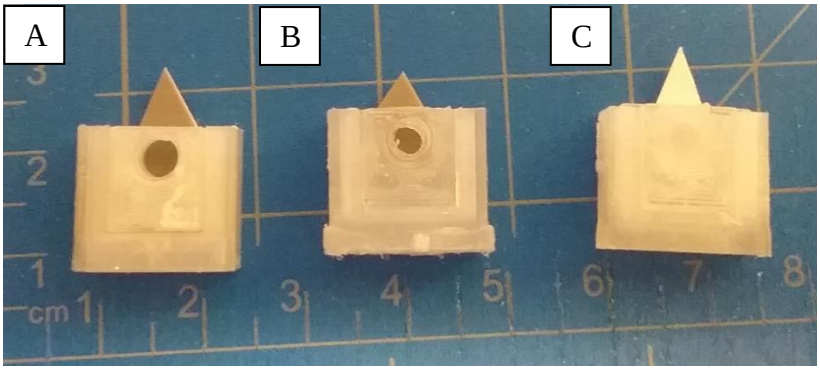

Figure 6.2: 3D Printed Paper Spray Cartridges. A) colorless PLA, B) PP and C) PET. A and B contain carbon sputtered porous polyethylene and C) contained Whatman 31ET.

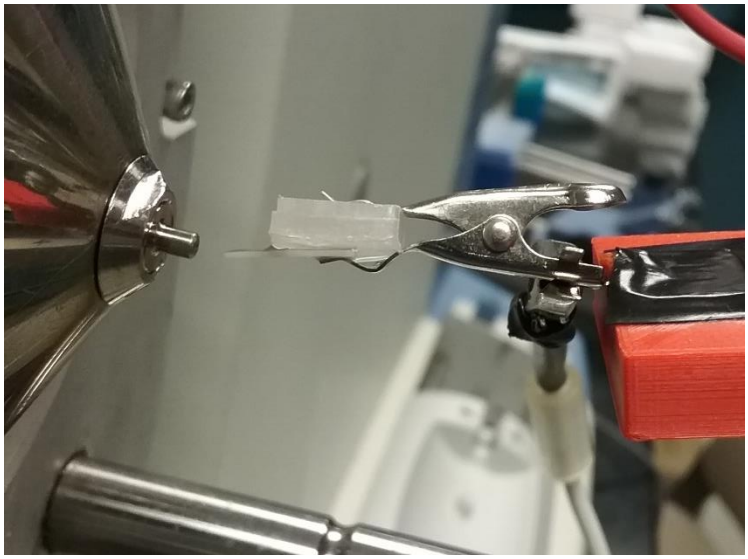

Figure 6.3: Paper Spray Analysis of Cytochrome C. Paper spray MS analysis of cytochrome C using a 3D printed solvent well clipped onto porous polyethylene positioned in front of a mass spec inlet.

Porous polyethylene was sputter coated with carbon using a Desk V sputtering system (Denton Vacuum, Moorestown, NJ, U.S.A.) by pumping down to 10^{-4} torr and applying 15-30 A to the carbon rods[13]. Deposition was repeated until a coating of at least 100 nm was reached. During analysis of proteins, only the top part of the cartridges shown in figure 6.2 was clipped to the polyethylene to act as a solvent well when positioned in front of the mass spec inlet as shown in figure 3. The assembly was angled downward to aid the flow of solvent along the coated polyethylene.

Paper spray was carried out for 90 seconds at 4.5 kV on an LTQ-XL linear ion trap mass spectrometer. Voltage was applied either by an alligator clip directly attached to the paper or a wire inserted into the cartridge. A holder with minimal plastic contact to the solvent and paper as shown in earlier chapters (figure 3.4) was used as a comparison for both small molecules and cytochrome C.

Calibration curves were prepared and analyzed using the different cartridges to evaluate changes in detection limits. For pharmaceuticals, calibration standards were prepared at 0.5, 1, 10, 25, 100, and 500 ng/mL with 100 ng/mL SIL. Equine cytochrome C was prepared at 5, 10, 50, 75, 100, and 150 µg/mL with 100 µg/mL bovine cytochrome C as an internal standard. Ratios between the analytes and internal standards were found for each calibration level and a calibration curve constructed. Any data points which were lower than three times the standard deviation of the blanks were excluded. Slopes and Y-intercepts were calculated using linear regression[14] with a weighting factor of $1/X^2$. Limits of detection were set at three times the standard error in the Y-intercept divided by the slope.

6.5 Results and Discussion

6.5.1 Evaluating Ionization Efficiency and Recovery for Small Molecules

3D printing offers rapid prototyping for development of analytical devices. When designing devices that make direct contact with organic solvent, e.g. paper spray MS, it is important to ensure that the plastic being used doesn't negatively impact the detection limits of the analysis. This could be caused by lowered recovery of analytes through non-specific binding, reactions with the plastic, or by lower ionization efficiency from leached monomers or other contaminants. To study whether these factors, a variety of commercially available 3D printing filaments (the feedstock fed to 3D printers) were cut, washed, and soaked in either acetonitrile or methanol. Two types of PLA, white and colorless, were selected to measure whether coloring agents have a noticeable effect. Acetonitrile was obviously incompatible with certain plastics (PLA, ABS and PC) as they visibly swelled, fractured or otherwise deformed when fully submersed in the solvent. These plastic-solvent combinations were not studied further. PET discolored slightly in acetonitrile but appeared otherwise unchanged after 24 hours of submersion, so it was further evaluated.

To evaluate changes in recovery for small molecules the solvents were spiked with seven pharmaceuticals prior to soaking the plastic. After 24 hours of exposure to the plastic extract aliquots were drawn off, spiked with SIL versions of the analytes and analyzed for changes in the ratio relative to the original acetonitrile and methanol solutions (figures 6.4A and 6.5A respectively).

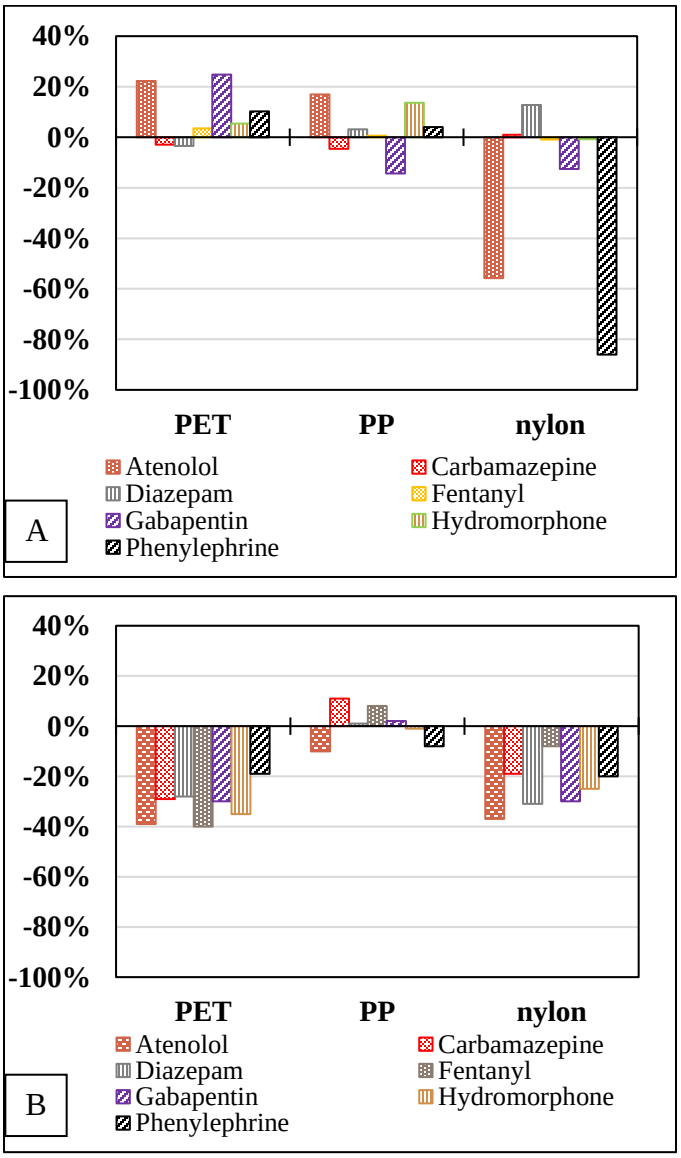

Figure 6.4: Matrix Effects on Pharmaceuticals Caused by 3D Printed Plastics in Acetonitrile. Changes in A) recovery and B) ionization efficiency for pharmaceuticals in acetonitrile with 3D plastic filament.

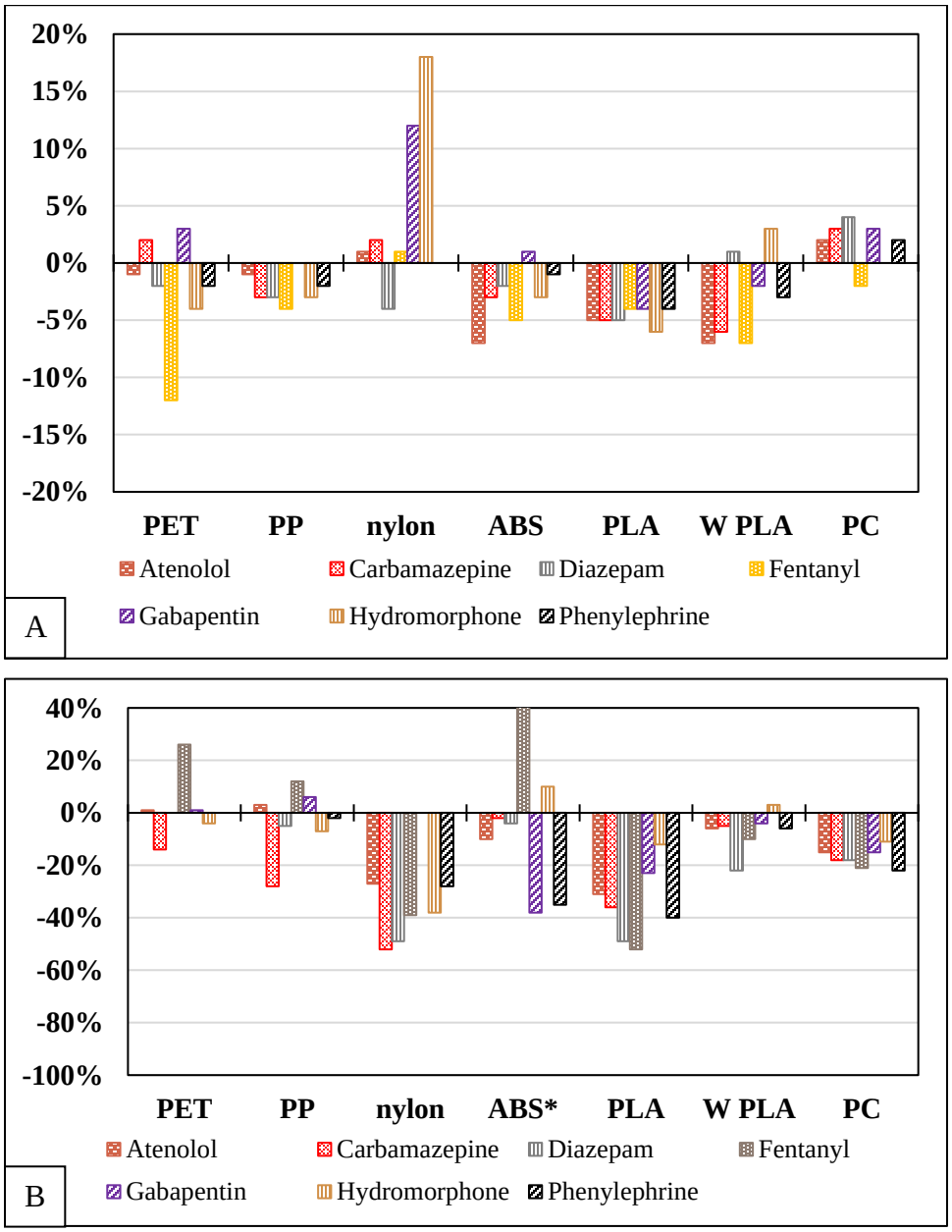

Figure 6.5: Matrix Effects on Pharmaceuticals Caused by 3D Printed Plastics in Methanol. Changes in A) recovery and B) ionization efficiency for pharmaceuticals in methanol with 3D plastic filament. *Fentanyl with ABS plastic showed ~120% ionization enhancement, but, the Y-axis wasn't scaled to prevent visual bias relative to the other results.

Looking at the results in figure 6.4, most analytes showed changes in recovery less than 25%. Two specific analytes, atenolol and phenylephrine, showed significant loss when exposed to nylon suggesting some kind of interaction with the plastic. Figure 6.5A demonstrates that no analyte showed a greater than 20% change in recovery indicating that methanol likely kept all the analytes well solvated in the presence of the different plastics.

To analyze effects of plastic on ionization efficiency, extracts of the plastics were introduced by autosampler into a stream of solvent which was subsequently mixed with a solution of SIL analogs via a T-junction at a constant flow rate. Changes in SIL signal were measured as percent decreases relative to the baseline before the addition of extract, and these ratios were compared to injections of plastic free solvent (results for acetonitrile and methanol shown in figures 6.4B and 6.5B respectively). Looking at the results for acetonitrile extracts in figure 6.4B, there seems to be a general trend of PP having lower impact on ionization efficiency relative to the other two plastics. This result suggests that both PET and nylon leached compounds into the solvent that impact ionization to a greater extent than PP. The contaminants were likely either unreacted monomers or some additives used in the manufacture of the filament (added to give the plastic useful properties) such as plasticizers, flame retardants and fillers. Looking at the methanol results in figure 6.5B, PP along with white PLA and PET show smaller impacts on ionization efficiency for most analytes, while colorless PLA and nylon both appear to have a larger detriment. White PLA and uncolored PLA had measurably different results which was unusual especially because the white PLA caused less ion suppression even though it had added coloring agents. The white and colorless PLA were from different manufactures, however, suggesting that manufacturing methods, raw material sourcing, and additives can have a significant impact on

ionization efficiency. Another anomaly occurred with fentanyl showing measurable ionization enhancement from ABS plastic, which none of the other six drug exhibited.

6.5.2 Evaluating Ionization Suppression and Recovery for Cytochrome C

Evaluation of plastic effects on the analysis of cytochrome C was carried out similar to the small molecules except that bovine cytochrome C was used as an internal standard instead of a stable isotopic label. In addition, instead of using either pure acetonitrile or methanol, a solvent mixture more amenable to protein analysis was used. The changes in ionization efficiency and recovery are shown in figure 6.6. White PLA, PET, and PP resulted in greater than 20% ion suppression. None of the plastics showed a significant decrease in recovery. Oddly, nylon exhibited an increase in apparent recovery, suggesting something may have leached out of the plastic and caused either ionization enhancement for equine cytochrome C or ionization suppression for the internal standard. The mass spectrum for the cytochrome c recovery experiment with nylon (figure 6.7A) shows multiple extraneous peaks relative to the same mass spectrum for PP (figure 6.7B) or any other plastic tested. Several of the extraneous peaks (453, 679 and 701) are known adducts of nylon[15].

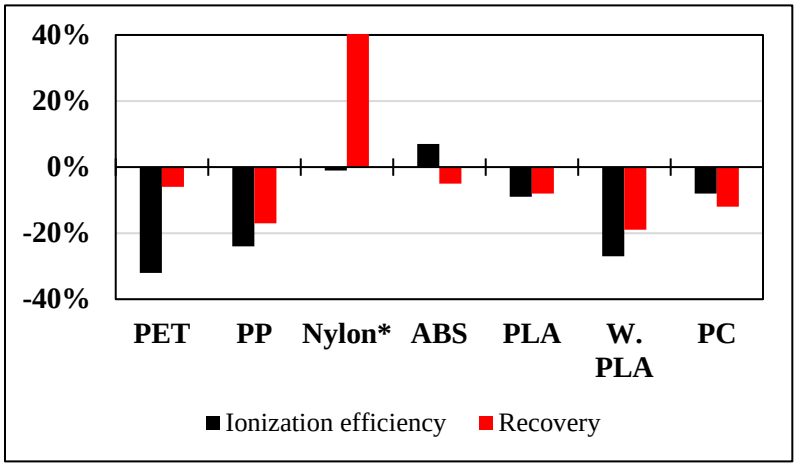

Figure 6.6: Matrix Effects on Cytochrome C Caused by 3D Printed Plastics in Methanol. Changes in ionization efficiency and recovery when analyzing cytochrome C solutions with added 3D printer filament. *Equine cytochrome C with nylon showed ~65% recovery, but, the Y-axis was not scaled to prevent visual bias relative to the other results.

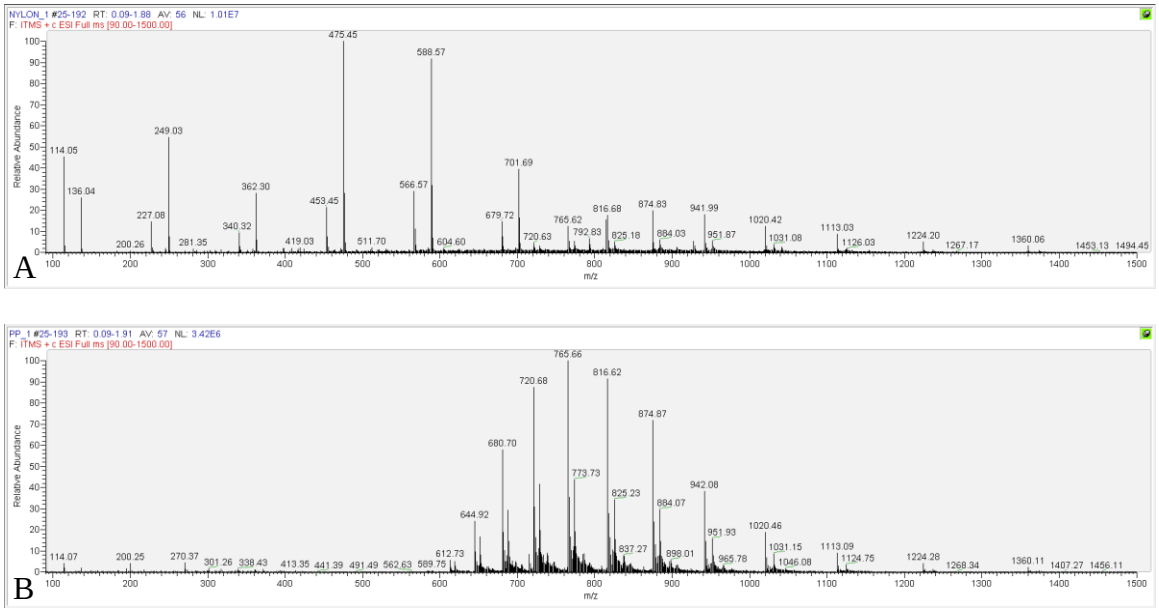

Figure 6.7: Mass Spectra of Cytochrome C in Solutions Containing 3D Printing Plastic. Mass spectra of equine and bovine cytochrome C in solutions containing A) nylon and B) PP.

6.5.3 Changes in Detection Limits

The results indicated there are instances of measurable changes in ionization efficiency and recovery with specific combinations of analytes, solvents, and plastics. The extraction times were long, however, and may not reflect the impact in the analytical device where the interaction times will be much shorter. To explore this possibility, paper spray MS cartridges were printed from some of the plastics studied here. Two plastics were selected for each spray solvent based on the largest and smallest combined decrease in ionization efficiency and recovery from figures 6.4-6. PP showed the least impact on signal for small molecules in both acetonitrile and methanol. The most deleterious plastics were nylon and PLA for acetonitrile and methanol respectively. A PET cartridge was also tested with acetonitrile because it discolored after exposure. For cytochrome C analysis, PP was selected as most likely to cause the largest change and colorless PLA as the plastic with the smallest change. Each solvent was also analyzed just using an alligator clip to hold the paper as shown in figure 3.4. Limits of detection were determined based on linear regression of calibration curves and the results for the seven pharmaceuticals are shown in table 6.2 for acetonitrile and table 6.3 for methanol.

Table 6.2: Paper Spray Detection Limits of Pharmaceuticals Using 3D Printed Cartridges and Acetonitrile. Limits of detection (ng/mL) of pharmaceuticals in acetonitrile analyzed by paper spray MS in cartridges made of different 3D printable plastic or with just an alligator clip.

	Acetonitrile			
	PET	PP	Nylon	Clip
Atenolol	7	0.2	0.2	0.06
Carbamazepine	0.8	0.2	0.3	0.3
Hydromorphone	3	0.2	0.2	0.2
Gabapentin	6	3	13	5
Phenylephrine	8	10	8	10
Fentanyl	0.4	0.7	1	2
Diazepam	4	0.3	0.3	0.3

Table 6.3: Paper Spray Detection Limits of Pharmaceuticals Using 3D Printed Cartridges and Methanol. Limits of detection (ng/mL) of pharmaceuticals in methanol analyzed by paper spray MS in cartridges made of different 3D printable plastic or with just an alligator clip.

	Methanol		
	PLA	PP	Clip
Atenolol	0.09	0.1	0.1
Carbamazepine	0.2	0.3	0.3
Hydromorphone	0.1	0.1	4
Gabapentin	5	5	5
Phenylephrine	3	3	7
Fentanyl	0.4	0.2	0.4
Diazepam	0.5	0.4	0.2

Cytochrome C showed little change in detection limits with respect to cartridge material; the limit of detection was 2 µg/mL for both colorless PLA and PP and 3 µg/mL when just using an alligator clip. For small molecules, acetonitrile with PET showed at least an order of magnitude increase in the detection limits for atenolol, hydromorphone and diazepam. There was discoloration of PET in acetonitrile which could have been the reason for the decrease in ionization efficiency for all analytes in figure 6.5A for PET. Nylon however showed comparable decreases in ionization efficiency but didn't change the detection limits meaning discoloration isn't the only indicator of ionization suppression. The changes in detection limits for small molecules in acetonitrile did not show a consistent trend for all analytes. Atenolol and phenylephrine both showed decreases in recovery of 50-80% from acetonitrile with nylon in figure 6.4A, but only atenolol showed an increase in detection limits using the cartridge. In addition, several analytes showed changes in ionization efficiency with PLA plastic and methanol in figure 6.5B, but didn't show a change in detection limits. The fact that the experiments measured changes in ionization efficiency and recovery that didn't reflect the changes in detection limits is most likely due to the differences in how the experiments were run. When measuring the impacts of the plastics there was at least a 24-hour incubation time compared to a paper spray MS run which would have had an exposure time of less than 2 minutes. This suggests that exposure time is a relevant factor when choosing a plastic. For the short-term solvent exposure in paper spray MS, any plastic that didn't melt, deform, or discolor when submerged in a solvent didn't have a noticeable impact on detection limits.

6.5.4 Other Factors When Selecting a Plastic

There are a number of other factors to be considered when selecting a 3D printing plastic aside from solvent compatibility. Ease of printing should be considered when first starting out with 3D printing. PP, for example, is useful for its solvent resistance but is difficult to print any

overhanging structures (structures that are printed over gaps in the model). For other plastics, a support material can be printed that serves as a temporary scaffold to hold up the material during printing and is remove after printing. With PP, however, there is a tendency for the heated filament to cool too slowly between points on the scaffold, causing the structure to sag and deform during printing. In addition, PP supports tend to fuse with the structure more solidly and must be cut away instead of snapped off.

Many plastics also have special requirements to be printed successfully. A major consideration is that most 3D printers have a PTFE tube in the heated extruder to feed the filament to the nozzle. This tubing will start to degrade at high temperatures (~250-260°C) and will leave blackened specs in the plastic as it prints. This residue could interfere with the print quality, potentially cause problems with experiments, or damage the 3D printer. To avoid this, all-metal print head can be installed. Additionally, most filaments will deform if cooled too fast. Many 3D printers have an enclosed printing area that is heated either passively (chamber is well insulated) or actively (has an added heating element) to prevent parts from warping or detaching from the build plate. PC in particular requires an elevated build chamber temperature to print correctly, and a 3D printer typically has to be specifically designed to print PC. Finally, filaments like nylon absorb water from the air and swell which can cause problems during printing. It is useful to store these filaments with desiccant between printing jobs or to bake them at 70-100°C for a few hours to dry them out prior to printing. Most manufacturers include a list of special printing instructions with the filament. Suggested printing temperatures can vary from manufacturer to manufacturer.

When selecting a material for a device, it is also important to consider its physical properties. PP, nylon and PET can be slightly flexible at room temperature if the part is thin, whereas PLA, ABS and PC are more rigid. Each plastic becomes soft and bendable at different

temperatures (indicated in table 1), an important consideration if the part will be exposed to elevated temperatures. Organic solvents can discolor, deform, or otherwise melt specific plastics which can lead to poor durability in addition to potentially hurting detection limits as discussed earlier. If solvent compatibility isn't an issue, PLA is generally the easiest plastic to print successfully. PP is more difficult to print, but resistant to many organic solvents. If a more complicated device with overhangs is desired, PET or nylon are more resistant than PLA to different solvents, but easier to print than PP. ABS and PC are more rigid with higher temperature resistance but can be difficult to print unless the 3D printer being used is specifically designed to print those plastics.

The work and discussion presented here focused on fused deposition modeling 3D printing as it is the most cost-effective method to begin 3D printing. Stereolithography (SLA) 3D printers, which use photocurable resins, tend to be more expensive and more difficult to use, but can often print at higher resolution and provide smoother surface finishes. This type of printing was investigated briefly for this work as well, but the cured methacrylate resin crumbled after soaking 24 hours in methanol or acetonitrile and was therefore deemed unsuitable for solvent exposure during MS analysis.

6.6 Conclusion

The work presented here outlined a series of experiments to determine whether long term exposure of 3D printable plastic to organic solvents could hurt the detection limits of analytical methods and whether those results translated into higher detection limits for paper spray mass spectrometry. A selection of small molecule pharmaceuticals in acetonitrile or methanol and the protein cytochrome C in a methanol-water solution were investigated. It was determined that several plastics were deformed or discolored by acetonitrile. With both solvents, and in specific

combinations of plastic, solvent, and analyte, there were measurable decreases in recovery and ionization efficiency of the analytes after 24 hours of exposure. These results were used to select the best and worst materials for paper spray mass spectrometry devices, and the limits of detection were determined using cartridges printed in these materials. The interaction between organic solvent and plastic was much shorter in these experiments; only acetonitrile with PET showing a measurable decrease in detection limits relative to analysis using just an alligator clip. This suggested that as long as the solvent being used doesn't discolor or deform the plastic, brief interactions will not affect the detection limits during the analysis of pharmaceuticals or the protein cytochrome C during paper spray mass spectrometry. This gives the user greater freedom to consider other factors important to 3D printing analytical devices

6.7 Acknowledgments

Funding for this research was provided by a grant from the National Institute of Justice, Office of Justice Programs under award number **2016-DN-BX-007**.

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Education

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Analytical Chemistry

Bachelor of Science, Eastern Illinois University

January 2010 - May 2012

Major: Chemistry

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Associate Degree in Science, Kankakee Community College

Aug 2007 - May 2009

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Professional Experience

Thermo Fisher Scientific, Lead Intern, Summer of 2017

TestAmerica, Analyst I, June 2012-December 2013

Honors and Awards

IUPUI Chancellor's Scholar

NIJ Graduate Fellowship in Science, Technology and Mathematics

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Conference Presentations

Lower detection limits for paper spray mass spectrometry using on paper extraction. Poster presentation at American Society for Mass Spectrometry 67th annual conference in Atlanta. 2019.

Concentrating and preserving THC and designer drugs through paper modifications for paper spray mass spectrometry. Poster presentation at Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy annual meeting in Philadelphia Pennsylvania. 2019.

Detection of Drugs and Threat Chemistries by Paper Spray MS (and Related Methods). Oral presentation at Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy annual meeting in Philadelphia Pennsylvania. 2019.

Using sesame seed oil to concentrate and preserve THC and designer drugs for paper spray mass spectrometry. Poster presentation at Turkey Run Analytical Chemistry Conference (TRACC) at Turkey Run State Park. 2018.

Studying paper properties to improve detection limits of synthetic cannabinoids and fentanyl using paper spray mass spectrometry. Poster presentation at American Society for Mass Spectrometry 66th annual conference in San Diego. 2018.

Studying paper properties to improve detection limits of synthetic cannabinoids and fentanyl using paper spray mass spectrometry. Poster presentation at 2018 Midwest Association for Toxicology and Therapeutic Drug Monitoring annual meeting in Indianapolis. 2018.

Studying paper properties to improve detection limits of synthetic cannabinoids and fentanyl using paper spray mass spectrometry. Poster presentation at Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy annual meeting in Orlando Florida. 2018.

Studying paper properties to improve detection limits of synthetic cannabinoids and fentanyl using paper spray mass spectrometry. Poster presentation at Midwestern Universities Analytical Chemistry Conference annual meeting in Athens. 2017.

Investigating Paper Properties for Ion Suppression and Recovery in Paper Spray Mass Spectrometry. Poster presentation at American Society for Mass Spectrometry 65th annual conference in Indianapolis. 2017.

Evaluation of Blood Fractionation Membranes for the Analysis of Dried Plasma Spots from Whole Blood using Paper Spray Mass Spectrometry. Poster presentation at American Society for Mass Spectrometry 64th annual conference in San Antonio. 2016.

On-cartridge Blood Fraction for Dried Plasma Analysis by Paper Spray Mass Spectrometry. Poster presentation at American Society for Mass Spectrometry 63rd annual conference in St. Louis. 2015.

PUBLICATIONS

1) The Impacts of Paper Properties on Matrix Effects During Paper Spray Mass Spectrometry Analysis of Prescription Drugs, Fentanyl and Synthetic Canabinoids, B. J. Bills, J. Kinkade, G. Ren, N. E. Manicke, Forensic Chemistry, 11 (2018) 15-22.

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3) Rapid Prototyping using 3D Printing in Bioanalytical Research, C. Zhang, B. J. Bills, N. E. Manicke, Bioanalysis, 9 (2017) 329-331.

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