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Miami, Florida

ASSESSING PRETREATMENT METHODS AND DRUG-MATRIX BINDING IN FORENSIC HAIR ANALYSIS

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Brianna Spear

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To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This dissertation, written by Brianna Spear, and entitled Assessing Pretreatment Methods and Drug-Matrix Binding in Forensic Hair Analysis, having been approved in respect to style and intellectual content, is referred to you for judgement.

We have read this dissertation and recommend that it be approved.

Watson Lees

Bruce McCord

De Etta Kay Mills

Yukching Tse Dinh

Anthony P. DeCaprio, Major Professor

Date of Defense: October 25, 2022

The dissertation of Brianna Spear is approved.

Dean Michael R. Heithaus
College of Arts, Sciences, and Education

Andrés G. Gil
Vice President for Research and Economic Development
and Dean of the University Graduate School

Florida International University, 2022

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DEDICATION

This dissertation is dedicated to my incredible baby girls. My hope for you is that you learn that you can do ANYTHING if you put in the hard work. I believe in you more than you know. Mommy did this for you, and I hope I made you proud.

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ABSTRACT OF THE DISSERTATION

ASSESSING PRETREATMENT METHODS AND DRUG-MATRIX BINDING IN FORENSIC HAIR ANALYSIS

by

Brianna Spear

Florida International University, 2022

Miami, Florida

Professor Anthony P. DeCaprio, Major Professor

Forensic hair analysis is commonly used in forensic toxicology with regards to drug-facilitated crime, workplace testing, and post-mortem investigation. Hair as a matrix benefits these types of analyses because of its long window of detection, allowing for a determination of drug history of exposure. However, there are currently no standards for forensic hair testing methods or practices, causing bias and inconsistency in forensic hair testing across multiple laboratories. Thus, two of the three aims of this research involved systematically comparing decontamination, pretreatment, and extraction methods to develop optimized forensic hair analysis protocols for multiple drugs and metabolites. Additionally, as hair is a complex matrix, there is limited understanding regarding the physicochemical interactions that occur between drugs of abuse and hair matrix components. Thus, the third aim of this research was to assess relative levels of ionic and non-ionic interactions between drugs and metabolites and the hair matrix.

Major findings of this work included an optimized forensic hair analysis method for multiple drugs and metabolites including decontamination using one 30-min wash with

HPLC water followed by three 30-min washes with dichloromethane, pulverizing the hair into a powder, and a 2-h extraction in a 12.5 μL/mg mixture of methanol acetonitrile, and 2 mM ammonium formate (25:25:50, v/v/v) at 37°C. In addition, binding studies indicated that almost all drugs and metabolites are involved in some degree of both ionic and non-ionic interactions with the hair matrix.

These findings will impact the forensic science community by presenting an optimized forensic hair analysis for drugs and metabolites, as well as providing additional insight regarding the interactions between drugs and metabolites and hair matrix components.

TABLE OF CONTENTS

CHAPTER PAGE

1. INTRODUCTION 1

1.1 Statement of the Problem 1

1.2 Rationale for Research 2

1.2.1 Aim 1 – Assess optimized forensic hair analysis methods for multiple drugs and metabolites 3

1.2.2 Aim 2 – Statistically compare optimized and least effective forensic hair analysis methods for drugs of abuse using authentic user hair 4

1.2.3 Aim 3 – Assess relative levels of ionic and non-ionic binding of drugs and metabolites to authentic HRM 4

1.3 Significance of Study 5

2. BACKGROUND 6

2.1 Forensic Toxicology 6

2.2 Forensic Hair Testing 9

2.3 History of Forensic Hair Testing 11

2.4 Hair Growth and Structure 13

2.5 Hair Reference Materials (HRM) 16

2.6 Drugs of Abuse 18

2.7 Forensic Toxicology Techniques 26

2.8 Liquid Chromatography-Triple Quadrupole-Mass Spectrometry (LC-QqQ-MS) 28

2.9 Introduction to Statistical Design of Experiments (DoE) 30

2.10 Drug-Hair Matrix Interactions 31

3. ASSESSING OPTIMAL FORENSIC HAIR ANALYSIS DECONTAMINATION PARAMETERS FOR EXTERNALLY CONTAMINATED HRM USING A 2^4 FRACTIONAL FACTORIAL BLOCK DESIGN 38

3.1 Introduction 38

3.2 Materials and Methods 39

3.2.1 Chemicals and Solvents 39

3.2.2 Microscopic Examination of Hair 39

3.2.3 Preparation of Externally Contaminated HRM 39

3.2.4 2^4 Fractional Factorial Block Design 40

3.2.5 Decontamination Method....42
3.2.6 Solid Phase Extraction (SPE)....43
3.2.7 HPLC-MS Parameters....45
3.2.8 Metrics for Performance Comparison....45
3.2.9 Analysis of Variance....47
3.3 Results....47
3.3.1 Microscopic Examination of Hair....47
3.3.2 Analytical Method Performance....52
3.3.3 DoE Comparison of Decontamination Parameters....52
3.3.4 Decontamination Studies Summary....55
3.4 Discussion....56
3.5 Conclusion....60
4. ASSESSING OPTIMAL FORENSIC HAIR ANALYSIS PRETREATMENT PARAMETERS FOR AUTHENTIC HRM USING A 2^3 FULL FACTORIAL DESIGN....61
4.1 Introduction....61
4.2 Materials and Methods....62
4.2.1 Chemicals and Solvents....62
4.2.2 Preparation of Incorporated HRM....63
4.2.3 2^3 Full Factorial Design for Authentic HRM....64
4.2.4 HPLC-MS Analysis....65
4.2.5 Metrics for Performance Comparison....66
4.3 Results....67
4.3.1 Analytical Method Performance....67
4.3.2 Incorporated HRM....68
4.3.3 ANOVA F-Tests....68
4.3.4 Recovery by Design Point for Pretreatment Parameters....72
4.3.5 Summary of Pretreatment Parameters....74
4.4 Discussion and Conclusion....74

5. ASSESSING OPTIMAL EXTRACTION TECHNIQUES FOR FORENSIC HAIR ANALYSIS WITH AUTHENTIC HRM 81
5.1 Introduction 81
5.2 Materials and Methods 82
5.2.1 Chemicals and Solvents 82
5.2.2 Extraction Techniques 83
5.2.3 SPE and HPLC-MS Analysis 84
5.3 Results 84
5.4 Discussion....84
5.5 Conclusion....91

6. STATISTICAL COMPARISON OF OPTIMAL AND LEAST EFFECTIVE FORENSIC HAIR ANALYSIS METHODS USING SINGLE DONOR AUTHENTIC USER HAIR....92
6.1 Introduction 92
6.2 Materials and Methods 93
6.2.1 Chemicals and Solvents 93
6.2.2 Microscopic Analysis of Optimized and Least Effective Methods on Hair .93
6.2.3 Extraction Methods....94
6.3 Results and Discussion....96
6.4 Conclusion....102

7. ASSESSING RELATIVE LEVELS OF IONIC AND NON-IONIC BINDING OF DRUGS TO AUTHENTIC HRM....103
7.1 Introduction 103
7.2 HRM, Chemicals, and Solvents....104
7.3 Equilibrium Dialysis (ED) to Assess Drug-Matrix Binding Using a High Salt Buffer 105
7.3.1 ED to Assess Drug-Protein Binding 105
7.3.2 Determining Time to Reach Equilibrium in ED 106
7.4 Assessing Relative Amounts of Drug-Hair Ionic Binding Using High Salt Buffer....108
7.4.1 Developing and Evaluating a ZipTip Procedure....108
7.4.2 Disrupting Ionic Binding Between Drug and Hair Components 109

7.5 Assessing Ionic and Non-Ionic Interactions Between Drugs and Hair Using Changes in pH....110
7.5.1 Method....110
7.5.2 Results....112
7.5.3 Discussion....112
7.5.4 Conclusion....114

8. SUMMARY AND PROSPECTUS....116

REFERENCES....118

VITA....132

LIST OF TABLES

TABLE PAGE

Table 1: Proposed cutoff values for various drugs 13

Table 2: 2^4 fractional factorial block design 42

Table 3: ANOVA F-Test for FEN and MET 53

Table 4: Levels of parameters by design point for FEN and MET 55

Table 5: Decontamination Studies Summary 56

Table 6: 2^3 full factorial design 64

Table 7: List of compounds, internal standards, retention times, and *m/z* transitions 66

Table 8: Bias, precision, matrix effects, and recovery for drugs of interest 68

Table 9: Recovery (%) of MET from incorporated HRM 68

Table 10: ANOVA F-Test results for drugs and metabolites in authentic HRM 70

Table 11: Extraction parameters resulting in optimal recovery for each drug 74

Table 12: Recovery by extraction technique for each drug and metabolite of interest 84

Table 13: Paired T-Test results for authentic specimens 100

Table 14: Summary of recoveries and statistical significance by drug 101

Table 15: Recovery of drugs of interest using ZipTips 109

Table 16: Recovery of drug in TBS 110

Table 17: Relative recoveries (%) of drugs at pH 12 and pH 6 112

LIST OF FIGURES

FIGURE PAGE

Figure 1: Diagram of utilizing segmental hair analysis to provide a drug history of exposure[2] ... 9

Figure 2: Drug detection window ... 10

Figure 3: Hair growth cycle[9] ... 14

Figure 4: Eumelanin and pheomelanin structures ... 15

Figure 5: β-keratin and α-keratin structures ... 15

Figure 6: Example of authentic HRM product data sheet ... 17

Figure 7: Consequences of drug biotransformation ... 18

Figure 8: Structure of MET ... 19

Figure 9: Structure of COC ... 20

Figure 10: Structure of A) COCA, B) NORCOC, and C) HYCOC ... 21

Figure 11: Structure of OXY ... 22

Figure 12: Structure of HER ... 23

Figure 13: Structure of A) 6-MAM and B) MOR ... 23

Figure 14: Structure of DZP ... 24

Figure 15: Structure of NORDZP ... 25

Figure 16: Structure of ALP ... 25

Figure 17: Structure of FEN ... 26

Figure 18: Schematic of QqQ-MS[29] ... 29

Figure 19: Schematic of how drugs incorporate into hair ... 32

Figure 20: Schematic of decontamination DoE procedure ... 43

Figure 21: Schematic of SPE procedure ... 44

Figure 22: Microscopy images at 100X of the top of blank hair of A) without aqueous solution wash, B) wash for 30 s with water, C) wash for 30 min with water, D) wash for 30 s with 1% SDS, and E) wash for 30 min with 1% SDS ... 48

Figure 23: Microscopy images at 100X of the side of blank hair of A) without aqueous solution wash, B) wash for 30 s with water, C) wash for 30 min with water, D) wash for 30 s with 1% SDS, and E) wash for 30 min with 1% SDS ... 49

Figure 24: Microscopy images at 100X of the top of blank hair of A) without organic solvent wash, B) wash for 30 s with MeOH, C) wash for 30 min with MeOH, D) wash for 30 s with DCM, and E) wash for 30 min with DCM ... 50

Figure 25: Microscopy images at 100X of the side of blank hair of A) without organic solvent wash, B) wash for 30 s with MeOH, C) wash for 30 min with MeOH, D) wash for 30 s with DCM, and E) wash for 30 min with DCM 51

Figure 26: Residual plots for FEN and MET 54

Figure 27: Percent recovery by design point for A) FEN and B) MET 54

Figure 28: Preparation of incorporated HRM schematic[68] 64

Figure 29: Schematic of pretreatment DoE procedure 65

Figure 30: Chromatogram of drugs and metabolites of interest 66

Figure 31: Residual plots for pretreatment parameter ANOVA F-Tests 71

Figure 32: Recovery *vs*. design point for A) 6-MAM, B) ALP, C) COCA, D) COC, E) DZP, F) HYCOD, G) MET, H) MOR, I) NORCOC, J) NORDZP, K) OXY, L) HYCOC, and M) FEN...73

Figure 33: Schematic of extraction methods comparison procedure 83

Figure 34: Recovery (%) *vs.* logP for A) solvent swelling extraction, B) base extraction, and C) enzymatic degradation extraction 87

Figure 35: Recovery (%) *vs.* pKa for A) solvent swelling extraction, B) base extraction, and C) enzymatic degradation extraction 87

Figure 36: Recovery (%) *vs.* HBA for A) solvent swelling extraction, B) base extraction, and C) enzymatic degradation extraction 88

Figure 37: Recovery (%) *vs.* HBD for A) solvent swelling extraction, B) base extraction, and C) enzymatic degradation extraction 88

Figure 38: Recovery (%) *vs.* tPSA for A) solvent swelling extraction, B) base extraction, and C) enzymatic degradation extraction 89

Figure 39: Chemical structures for A) COC, B) HYCOC, C) COCA, and D) NORCOC 90

Figure 40: Optimal method for forensic hair analysis 95

Figure 41: Least effective method for forensic hair analysis 95

Figure 42: Microscopic imaging at 100X of A) the top of hair after receiving the optimized treatment, B) the side of hair after receiving the optimized treatment, C) the top of hair after receiving the least effective treatment, and D) the side of hair after receiving the least effective treatment 97

Figure 43: Drug recovery in authentic specimens using optimal and least effective methods 98

Figure 44: Drug (pg) *vs.* time (min) for A) COC, B) COCA, and C) HYCOC...............107

Figure 45: Binding studies design...111

LIST OF ABBREVIATIONS, ACRONYMS, AND SYMBOLS

α	alpha
β	beta
μL	microliter
π	pi
6-MAM	6-Monoacetylmorphine
ALP	Alprazolam
ANOVA	Analysis of Variance
COC	Cocaine
COCA	Cocaethylene
DHI	5,6-dihydroxyindole
DHICA	5,6-dihydroxyindole-2-carboxylic acid
DoE	Design of Experiments
DTT	1,4-Dithiothreitol
DZP	Diazepam
EtG	Ethyl glucuronide
FEN	Fentanyl

h	hours
HBA	hydrogen bond acceptor
HBD	hydrogen bond donor
HER	Heroin
HPLC-QqQ	High performance liquid chromatography coupled with triple quadrupole mass spectrometer
HRM	Hair reference material
HYCOC	p-Hydroxycocaine
LogP	partition coefficient
m/z	Mass-to-charge ratio
MET	Methamphetamine
mg	milligram
min	minute
mL	milliliter
MOR	Morphine
MRM	Multiple reaction monitoring
NORCOC	Norcocaine
NORDZP	Nordiazepam

OXY	Oxycodone
OVAT	One variable at a time
PBS	phosphate-buffered saline
pKa	negative base 10 logarithm of the acid dissociation constant
pg	picograms
ppb	parts per billion
ppm	parts per million
RT	retention time
s	seconds
SAMSHA	Substance Abuse and Mental Health Services Administration
SDS	sodium dodecyl sulfate
SoHT	Society of Hair Testing
SPE	Solid phase extraction
SS	sum of squares
tPSA	total polar surface area
TBS	tris-buffered saline

1. INTRODUCTION

1.1 Statement of the Problem

Forensic hair analysis is commonly used in forensic toxicology with regards to drug-facilitated crime, workplace testing, and postmortem investigation. Hair as a matrix benefits these types of analyses because of its long window of detection. While drug can be detected and quantified in traditional matrices such as blood, urine, and oral fluid for hours to days, drug can be analyzed in hair for weeks to months. This allows for a determination of drug history of exposure. Hair samples are also beneficial due to their minimally invasive collection protocol, as well as their stability at room temperature. However, hair analysis does present some challenges.

The hair analysis process includes decontamination, homogenization, extraction, purification, and instrumental analysis. The Society of Hair Testing (SoHT) gives suggestions regarding the best practice methods for forensic hair analysis, such that at least one aqueous and at least one organic wash should be used during decontamination, and that the hair should be homogenized in some way prior to extraction. The SoHT also suggests that the extraction method should be validated by the laboratory performing the analysis, however, there are currently no guidelines regarding validation standards. While the SoHT does give general guidelines for best practice hair analysis, these cannot be considered standard practices or procedures, as laboratories can choose to disregard the suggestions. This leads to a lack of consistency across multiple laboratories regarding forensic hair analysis methods utilized, and contributes to bias within the hair testing community. Thus, two of the three aims of this research involved systematically

comparing decontamination, pretreatment, and extraction methods to develop optimized forensic hair analysis protocols for multiple drugs and metabolites.

As hair is a particularly complex matrix made up of many proteins, as well as varying amounts of melanin, the physicochemical interactions between drugs and hair are not well understood. Literature reports general trends found to affect drug-matrix binding, as well as some potential binding sites between parent drugs and melanin. However, published work has not assessed the binding of metabolites to hair. Therefore, the last aim of this research was to assess relative levels of ionic and non-ionic interactions between drugs and metabolites with the hair matrix.

1.2 Rationale for Research

Currently, hair is considered an alternative matrix in forensic toxicology, as there are no standardized practices or methods for its analysis, leading to limitations such as bias and inconsistency in testing across multiple laboratories. In order to address these limitations, an optimized method for forensic hair analysis of multiple drugs and metabolites was a major part of the present work. Previous studies in the area of forensic hair analysis method development utilized incorporated HRM, which cannot necessarily represent the interactions between drug and hair *in vitro,* and did not evaluate metabolites in hair or hair samples containing multiple analytes. The present work assessed optimized forensic hair analysis methods using authentic HRM, containing multiple drugs and metabolites. In addition, this work statistically compared the optimized and least effective forensic hair analysis methods to single-donor authentic user specimens containing multiple drugs and metabolites. Finally, to understand the interactions between drugs and

components of the hair matrix, which likely impacts the efficacy of forensic hair analysis procedures, binding studies were completed for multiple drugs.

In summary, Aims 1 and 2 of this research address the problem that there are currently no consistent protocols for forensic hair testing of all drugs. The hypothesis was that there would not be one consistent optimized forensic hair testing method for all drugs. The research goal of Aims 1 and 2 was to investigate optimized protocols for decontamination, pretreatment, and extraction of alprazolam, diazepam and nordiazepam, MET, cocaine and its metabolites, oxycodone, metabolites of heroin, and FEN.

In addition, Aim 3 addresses the problem that the mechanisms of binding for drugs to hair are not well understood. The hypothesis was that both ionic and non-ionic interactions play a role in the binding of drugs to hair. The research goal of Aim 3 was to assess relative amounts of ionic and non-ionic binding of MET, cocaine and its metabolites, oxycodone, metabolites of heroin, and oxycodone to hair.

1.2.1 Aim 1 – Assess optimized forensic hair analysis methods for multiple drugs and metabolites.

Forensic hair analysis procedures require decontamination, homogenization, extraction, and purification prior to analysis.[1] The Society of Hair Testing gives general guidelines regarding each of these steps, however, these guidelines are merely suggestions; each forensic toxicology laboratory may choose the degree to which their methods abide by the guidelines. As each step of the hair analysis method has many variables to consider, the present research assessed the effects of multiple parameters at multiple levels, individually and in combination with each other, on extraction efficiency,

using a statistical technique known as Design of Experiments (DoE). Decontamination studies evaluated the organic and aqueous solvents used for washing the hair, the number of consecutive washes applied to each sample, the sequence of the washes applied, and the wash times, using a 2^4 fractional factorial block design and ANOVA F-tests. A 2^3 full factorial design was then utilized to assess other pretreatment parameters, including extraction solvent volume/sample weight ratio, particle size, and extraction time. Finally, three extraction techniques commonly reported in the literature, solvent swelling, base extraction, and enzymatic degradation, were compared based on relative extraction recovery.

1.2.2 Aim 2 – Statistically compare optimized and least effective forensic hair analysis methods for drugs of abuse using authentic user hair.

When optimizing any method in forensic toxicology, it is important to assess the protocols utilizing authentic specimens. Thus, the optimized and least effective forensic hair analysis methods in Aim 1 were statistically compared for multiple drugs and metabolites using a set of individual authentic user hair specimens and Paired T-Tests.

1.2.3 Aim 3 – Assess relative levels of ionic and non-ionic binding of drugs and metabolites to authentic HRM.

Understanding drug-matrix binding offers explanations regarding the optimization of forensic hair analysis methods. These interactions likely contribute to the efficacy of decontamination, pretreatment, and extraction parameters, in particular how the binding of drug to hair is interrupted using different extraction conditions. Previous research has suggested that a variety of physicochemical factors play a role in the ability of drug to

incorporate in hair and that there are many potential interactions that could be at play. Thus, binding studies evaluating relative levels of ionic and non-ionic binding of drugs and metabolites to authentic HRM were conducted using phosphate buffered saline (PBS) at pH 6 and pH 12. These values were chosen to modify the charge state of the drugs of interest to cationic (at pH 6), for evaluating the disruption of ionic binding, and to neutral (at pH 12), for evaluating the disruption of non-ionic binding.

1.3 Significance of Study

Forensic hair testing has the potential to be routinely used in forensic toxicology with regards to situations that require longer drug detection windows, such as drug-facilitated crime, workplace testing, dependency court cases, and post-mortem investigation. However, the current lack of consensus regarding best practices for forensic hair analysis and lack of comprehension regarding drug-matrix interactions have proven to be limitations that can result in unreliable analysis in the courtroom.

This research reported an optimized forensic hair analysis method for multiple drugs and metabolites developed using authentic HRM, and evaluated using authentic user hair, that can be used in the development of standard practices and methods in forensic hair testing. In addition, this work utilized *in vitro* techniques and authentic HRM to elucidate relative levels of ionic and non-ionic interactions among multiple drugs and metabolites and the hair matrix. By minimizing bias and increasing reproducibility of the analyses, these results will facilitate the further standardization of forensic hair testing methods and practices.

2. BACKGROUND

2.1 Forensic Toxicology

Forensic toxicology focuses on the detection and interpretations of drugs and poisons for purposes in the legal system.[2] Forensic toxicology originated with postmortem testing; detecting poisons in the body that could have caused the death of an individual. However, in the modern era, very few homicides are caused by poisoning, and forensic toxicology has shifted to investigate illicit drugs and legal drugs that are abused. Workplace and correctional drug testing have grown in the field of forensic toxicology as a means of improving workplace safety through eliminating illicit drug use. The most common matrix used in this field is urine testing, however, short windows of detection, many opportunities for adulteration, and invasive collection procedures have sparked interest in alternative matrices such as oral fluid and hair. Drug-facilitated crimes have also become more common in the forensic toxicology field, where determination of whether an individual was exposed to impairing substances such as CNS depressants including benzodiazepines, GHB, antidepressants, and alcohol prior to an alleged offense is critical. In these cases, traditional matrices such as blood and urine typically do not provide a long enough window of detection for determining exposure to impairing substances, while hair testing has potential to address exposure from weeks to months prior.[2]

Forensic toxicology is different than other toxicology disciplines because the specimens analyzed will potentially be used as evidence in a court of law, so the chain-of-custody regarding collection, transport, analysis, and storage of the specimens must be

recorded properly. Such specimens include blood, urine, oral fluid, postmortem specimens, and hair.

Blood is the most commonly used biological matrix in forensic toxicology if it is available and collected within hours of the event in question, as it gives the most direct evidence of drug in the body. Furthermore, drug levels in blood can allow toxicologists to assess the potential pharmacologic effects in an individual.[2] Most forensic toxicology literature reports drugs in whole blood as opposed to plasma or serum, which is most commonly reported in clinical toxicology. When utilizing blood specimens in forensic toxicology, two blood specimens should be collected so that one can be used for screening tests and one can be used for confirmatory and/or repeated testing. In postmortem investigation, blood specimens should be collected from a peripheral site, such as a leg or arm, because collection of blood from a central site such as the heart is likely to be contaminated from drug redistribution from other neighboring organs. It is also preferable to obtain specimens collected prior to death (*i.e.,* antemortem) from the hospital to assess the status of the individual close to the time of the event causing forensic interest.[2]

Urine is an ideal specimen for screening tests for the presence of drugs, as drugs and metabolites are typically present at higher concentrations in urine than in blood, and there typically is not a limited sample.[2] However, urine cannot be used to assess the dose of drug consumed or the effects of the drug on the person at the time of sampling because urine is a waste product stored in the bladder and urine drug levels typically do not correlate well with blood levels. After a urine screening, confirmatory tests utilizing

blood can be used to confirm substances found in the urine. When utilizing the urine matrix, the presence of a drug metabolite can also be useful for assessing a potential exposure. A limitation of urine includes the opportunity for adulteration, such as water loading to dilute urine prior to a drug test. Testing for creatinine and other analytes is utilized to combat this limitation.[2]

Oral fluid testing is based on secretions from salivary glands containing drugs, influenced by pH and the degree of protein binding in the oral fluid.[2] There is generally a good relationship between drug concentrations in blood and in oral fluid; however, smoking drugs or oral administration may deposit drugs into the mucosa of the mouth, which could artifactually increase concentrations found in oral fluid versus the blood. An advantage of oral fluid includes non-invasive collection, so the collector can watch for adulteration and substitution. Current applications of analysis of oral fluid include driving under the influence/driving under the influence of drugs (DUI/DUID) cases and workplace testing.[2]

Common specimens unique to postmortem toxicology include gastric contents, liver, and vitreous humor.[2] Gastric contents assess oral administration of a drug and can supplement blood analysis. Quantification of drugs found in gastric contents is essential to distinguish trace amounts of drug from a prior non-toxic ingestion as opposed to a fatal ingestion. Analysis of the liver is useful for assessing the possible role of a drug in the death when there is a low concentration of the drug in blood or if blood specimen is not available. Vitreous humor should always be collected if possible in postmortem investigation, because concentrations of glucose, urea, and chloride can assess trauma in

the body such as hyperglycemia, kidney dysfunction, and dehydration. Additionally, concentrations of drugs in the vitreous humor can be used to substantiate concentrations of drug present in blood specimens, and vitreous humor is a stable matrix compared to post-mortem blood.[2]

2.2 Forensic Hair Testing

Hair testing has great potential for use in forensic toxicology in regards to drug facilitated crime, workplace testing, and post-mortem investigation.[3] The largest advantage of hair testing is that, since hair grows, on average, one cm/month, a drug history of exposure can be determined by segmental hair analysis. As hair grows out of the follicle, drugs are incorporated into hair and remain in the matrix until the hair is cut off.[2]

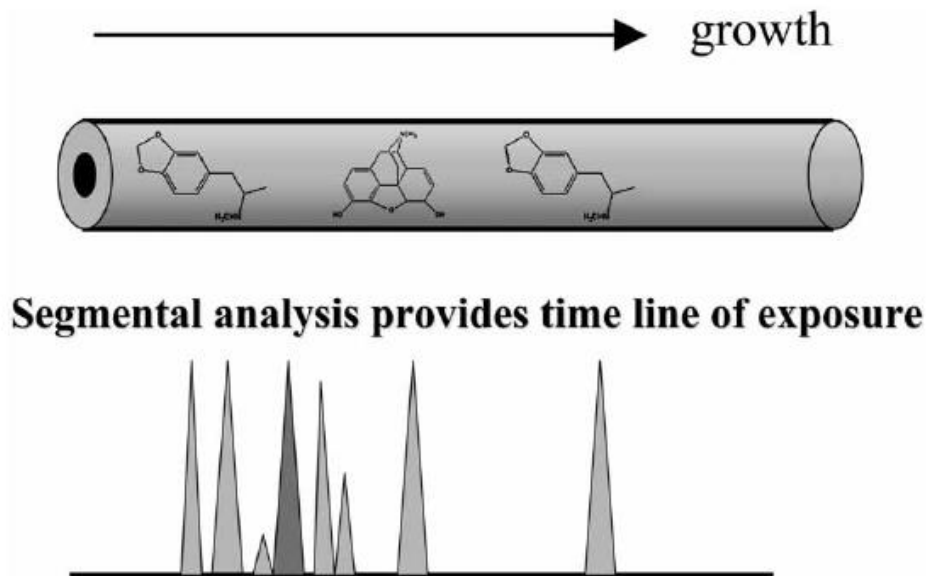


Figure 1. Diagram of utilizing segmental hair analysis to provide a drug history of exposure[2]

When compared to body fluids, hair has a longer window of detection, *i.e.*, from weeks to months instead of hours to days.[3] This is due to the fact that accumulation over time occurs in keratinized matrices.[4] In addition, hair has a simple and minimally invasive collection protocol, while body fluids tend to have a complex and invasive collection protocol.[3] Hair samples are highly stable and can be stored indefinitely at room temperature, while bodily fluids should be frozen and can generally only be stored for a specific amount of time.[3] The ability to determine a drug history of exposure using hair allows for monitoring drug use or abstinence over time, while body fluids can only monitor recent drug history. Additionally, hair is less likely to be adulterated than body fluids.

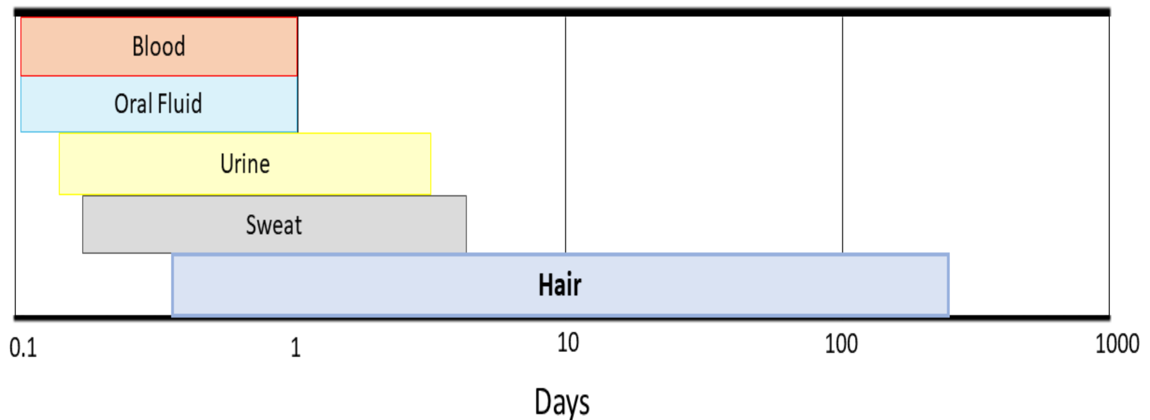


Figure 2. Drug detection window

However, like any biological matrix, hair has limitations.[3] This includes the differentiation between drug ingestion and external contamination, a lack of correlation between administered dose and concentration of drug found in hair, and a bias involving hair color and other individual factors. In addition, hair cannot be used to prove that a person was under the influence at a specific moment in time.[3]

2.3 History of Forensic Hair Testing

Hoppe first reported the use of forensic hair analysis in 1858. He reported an investigation into a suspected arsenic poisoning in Germany, in which the body was exhumed after eleven years.[5] In 1954, almost 100 years later, Goldblum identified amphetamine in guinea-pig hair.[5] Baumgartner, however, reported the most significant progress for forensic hair analysis at the time. In 1978, Baumgartner utilized a 2 h extraction in MeOH to remove opiates from the human hair matrix.[6] Klug reported a chromatographic method in 1980 to quantify morphine and codeine in human head hair.[5] Also in 1980, Valente used radio-immunoassays and compared multiple extraction procedures for detecting cocaine in human hair.[7] Smith and Pomposini extracted phenobarbital in hair using sodium dodecyl sulfate, and Arnold and Püschel used a similar method for methaqualone and barbiturates.[5] However, even up until the early 1990s, hair analysis was not commonly accepted by law enforcement and forensic experts. The use of gas chromatography-mass spectrometry (GC-MS) allowed for the detection of drugs specifically and sensitively, increasing the acceptance of forensic hair analysis as a valid scientific technique for the detection and quantification of drugs in hair.[5]

Currently, hair is considered an alternate matrix because of its many limitations, as well as a lack of consensus and standards regarding the practices and methods used in forensic hair analysis.[8] The international organization known as the Society of Hair (SoHT) testing gives general guidelines regarding the hair analysis process.[1] The overall hair testing process begins with segmentation of the hair when determining a drug history

of exposure. The hair sample is then decontaminated to remove drug and environmental contaminants from the surface of the hair. The SoHT recommends that the decontamination procedure should use at least one aqueous solution and at least one organic solvent wash. The hair is then homogenized in some way, as suggested by the SoHT, either by pulverizing the hair into a powder or cutting the hair into ~1 mm snippets. The drug is isolated from the matrix using a variety of extraction techniques, which the SoHT suggests should be validated by each laboratory. However, the validation guidelines are dependent on the laboratory as well. The extract is purified to remove matrix proteins and other components, commonly using Solid Phase Extraction (SPE). Finally, the purified sample is analyzed using instrumentation, commonly using liquid chromatography-triple quadrupole-mass spectrometry (LC-QqQ-MS).[1]

In forensic hair testing, cutoff values are imperative.[9] A cut off level is the concentration at which a sample is called positive, if the drug concentration is above it, or negative, if the concentration is below it. Cut offs are determined based on instrumental factors, regulatory requirements, policy, and scientific and protective considerations. If the cut off value is low, more drug users may be identified, but people with drug in their hair due to passive exposure may be falsely identified as users. The U.S. Substance Abuse and Mental Health Services Administration (SAMHSA) and the SoHT have both proposed cutoff levels for drugs of interest, as shown in Table 1.[9]

Table 1. Proposed cutoff values for various drugs

Drug	Analyte	SAMHSA Cutoff Level (ng drug/mg hair)	SOHT Cutoff Level (ng drug/mg hair)	Comments
Marijuana	Delta-9-tetrahydro-cannabinol-9-carboxylic acid	0.05	0.0002 (THC-OOH) 0.05 (THC)	It is recommended that the parent drug is measured to assess contamination levels and external removal
Cocaine	Cocaine Benzoylecgonine (BE) Cocaethylene (COCA) Norcocaine (NORCOC)	0.5 > 5% of cocaine level 0.05 0.05	0.5 0.05 0.05 0.05	BE/COC ratio must be greater than 5%, or COC or NORCOC must be present
Opiates	Morphine Codeine 6-acetylmorphine (6-MAM)	0.2 0.2 0.2	0.2 0.2 0.2	Morphine must be present if 6-MAM is detected
PCP	PCP	0.3	No data	N/A
Amphetamines	Amphetamine MET MDMA MDA MDEA	0.3 0.3 0.3 0.3 0.3	0.2 0.2 0.2 0.2 0.2	Amphetamine must also be present in hair from a MET user

2.4 Hair Growth and Structure

2.4.1 Hair Growth Cycle

Hair grows in cycles, beginning with the anagen phase, during which the hair is actively growing. Growth then transitions to the catagen stage, when cell division stops and the follicle begins to degenerate. Finally, the telogen phase takes place and causes the

hair shaft to begin to shut down and then completely stop growing.[3] The relative amount of time spent in each phase varies within and between individuals.[10] Hair contains matrix cells, including melanocytes and keratinocytes, and consists of three layers: the cuticle, cortex, and medulla.[11] The cuticle is the outermost layer of the hair and is made up of overlapping keratinized cells.[12] The body of the hair is made up of the cortex, which contains 12 types of keratins and over 100 types of keratin-associated proteins.[12] The medullary cells are mixed with melanosomes and cortical cells in the hair.[12]

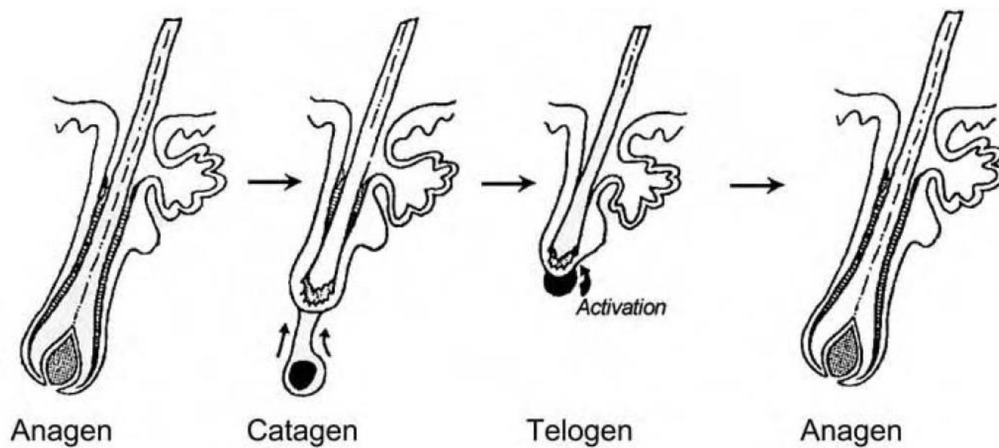
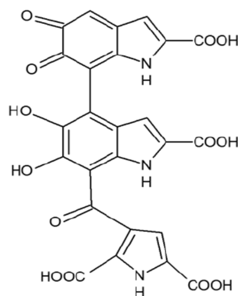
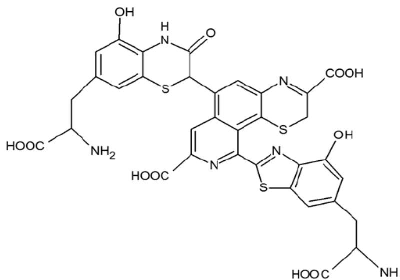


Figure 3. Hair growth cycle[9]

2.4.2 Melanin

The pigmentation in hair comes from melanin, which can be present in all three layers of the hair but is most commonly found within cortical cells.[12] Melanin is derived from tyrosine and dihydroxyphenylalanine (DOPA) through a series of biosynthesis reactions. Eumelanin is most commonly formed from this reaction, but pheomelanin can also be produced in the presence of cysteine.[13] Melanins are polymers with different structures, because they are complex polymers consisting of primarily varying amounts of eumelanin and pheomelanin monomers, as shown in Figure 4.



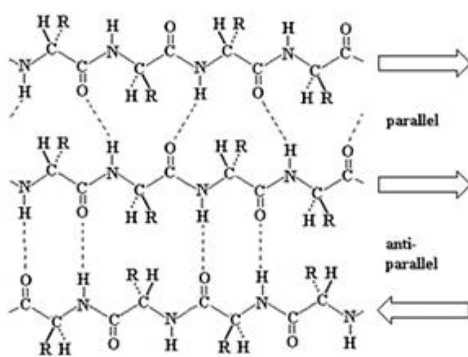


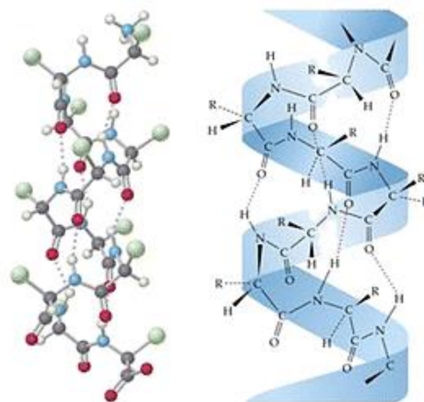
Eumelanin Pheomelanin

Figure 4. Eumelanin and pheomelanin structures

Eumelanin is a dark brown to black pigment containing a high concentration of 5,6-dihydroxyindole-2-carboxylic acid (DHICA), and is made up of indolequinone units. In contrast, pheomelanin is a yellow to reddish-brown pigment, containing a relatively high concentration of 5,6-dihydroxyindole (DHI), and is comprised of benzothiazine, benzothiazole, and isoquinolone units. Hair color is determined by the quantity and type of melanin that is incorporated into the hair shaft.[14]

2.4.3 Keratin





Beta-Keratin Alpha-Keratin

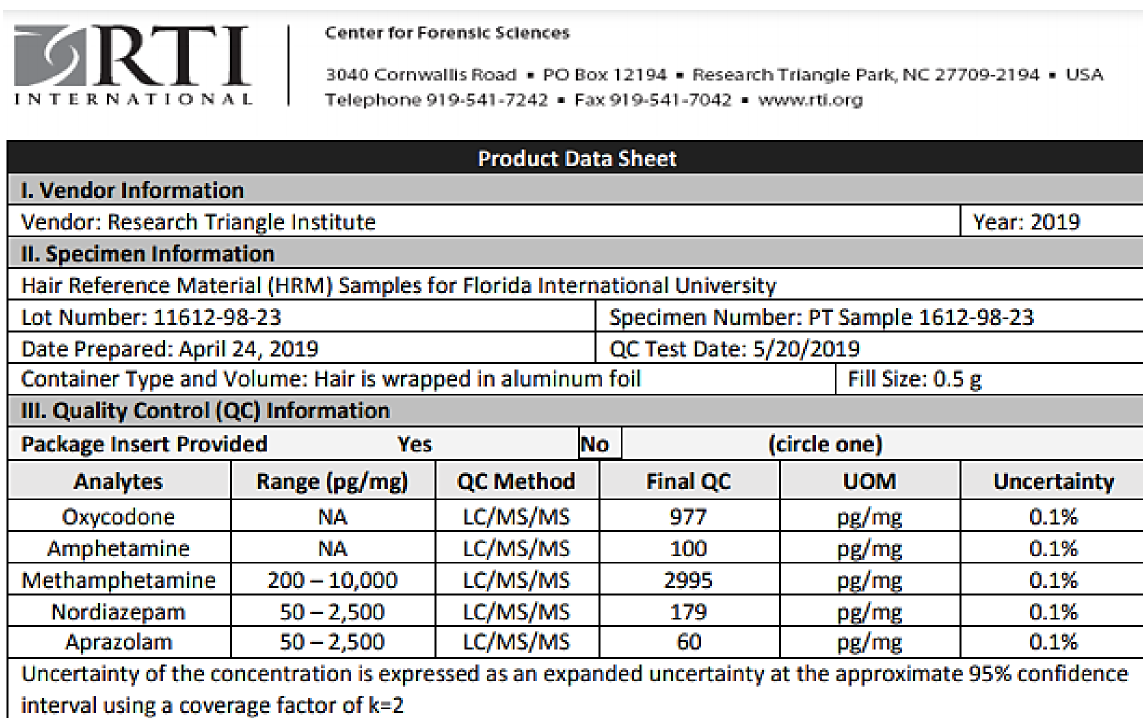
Figure 5. β-keratin and α-keratin structures

Keratin is a protein with mixtures of polypeptide chains that make up an α-helical configuration. These helical configurations are linked and stabilized through disulfide crosslinks.[15] Keratin and its associated proteins make up approximately 65-95% of hair and form filaments that polymerize into higher order structures, creating the hair strand.[8] The structure of keratin and its associated proteins vary based on the individual. However, as shown in Figure 5, the structure is made up of typical peptide bonds, stabilized with multiple disulfide bond linkages between the cysteine amino acids.[16]

2.5 Hair Reference Materials (HRM)

Hair reference material (HRM), prepared as an internal control for analysis as a matrix-matched control sample with a known concentration, is necessary for reliably identifying and quantitating drugs in hair for forensic purposes.[1, 17] Matrix-matched standards are important for use when calibrating an analytical procedure and verifying lab performance. In addition, they compensate for matrix effects, which cause analytical issues such as ion suppression and enhancement.[18] Currently, there are three different types of HRM available: incorporated HRM, authentic HRM, and authentic user hair. Incorporated HRM is generally produced using drug-free hair that has not been chemically treated, which can be obtained from donors or purchased from a commercial source.[1] Incorporated HRM is prepared in-house using a protocol such as that described by Ropero-Miller et al.[17] The advantages of this type of HRM are that it can continuously be produced and that it is relatively inexpensive to make. However, incorporated HRM is not representative of the way that drugs incorporate into hair *in vivo*. Authentic HRM is authentic user hair, generally pooled from multiple users, that has been washed and then

diluted with blank hair to produce a standard with specified concentration range of one or more drugs. The major advantage of this HRM is that the drug is incorporated into the hair through natural processes in the body. The disadvantage is that authentic HRM is expensive, is generally produced in only small quantities, and is currently only available on a limited basis. An example of authentic HRM is shown in Figure 6.



RTI INTERNATIONAL

Center for Forensic Sciences

3040 Cornwallis Road ▪ PO Box 12194 ▪ Research Triangle Park, NC 27709-2194 ▪ USA
Telephone 919-541-7242 ▪ Fax 919-541-7042 ▪ www.rti.org

Product Data Sheet

I. Vendor Information

Vendor: Research Triangle Institute — Year: 2019

II. Specimen Information

Hair Reference Material (HRM) Samples for Florida International University

Lot Number: 11612-98-23 — Specimen Number: PT Sample 1612-98-23

Date Prepared: April 24, 2019 — QC Test Date: 5/20/2019

Container Type and Volume: Hair is wrapped in aluminum foil — Fill Size: 0.5 g

III. Quality Control (QC) Information

Package Insert Provided **Yes** **No** **(circle one)**

Analytes	Range (pg/mg)	QC Method	Final QC	UOM	Uncertainty
Oxycodone	NA	LC/MS/MS	977	pg/mg	0.1%
Amphetamine	NA	LC/MS/MS	100	pg/mg	0.1%
Methamphetamine	200 – 10,000	LC/MS/MS	2995	pg/mg	0.1%
Nordiazepam	50 – 2,500	LC/MS/MS	179	pg/mg	0.1%
Aprazolam	50 – 2,500	LC/MS/MS	60	pg/mg	0.1%

Uncertainty of the concentration is expressed as an expanded uncertainty at the approximate 95% confidence interval using a coverage factor of k=2

Figure 6. Example of authentic HRM product data sheet

Authentic user hair is similar to authentic HRM except that it is from a single donor and is used as a standard without dilution. However, challenges in the use of authentic hair from single donors as a reference standard include limitations in amounts available and variety of drugs represented. Of the various types of standard materials, authentic HRM is the most useful for method development evaluation in forensic hair testing.

2.6 Drugs of Abuse

After dosing of a drug, the drug is absorbed into the blood and transported to the site of action where the drug interacts with its receptor and produces its effect.[19] Once the effect is no longer required, the drug will be eliminated. Some drugs are able to be eliminated from the body without any structural changes, however, most drugs go through chemical modification to increase their water solubility for excretion through urine or bile. This modification is called metabolism or biotransformation. Without metabolism, many drugs would build up in tissues indefinitely.[19] Drug biotransformation due to metabolism results in three different types of metabolites. Most metabolites are inactive and are excreted from the body. However, some metabolites are reactive (electrophilic), forming adducts with DNA and proteins. The metabolites of interest to this research are active metabolites, which are pharmacologically active.

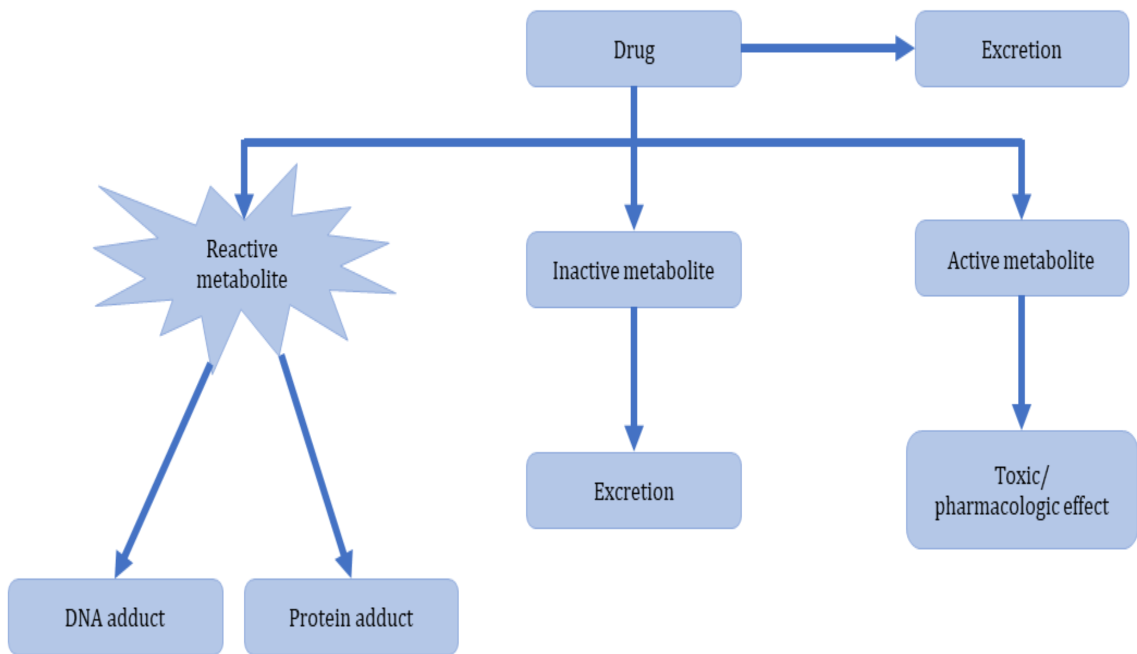


Figure 7. Consequences of drug biotransformation

Metabolism typically occurs in the liver, but other tissues also play a role.[19] Metabolism is broken down into phase I and phase II reactions. During phase I

metabolism, oxidation, reduction, and hydrolysis reactions take place, resulting in the introduction or loss of functional groups. Oxidation reactions are most commonly facilitated with cytochrome P450 (CYP) enzymes, located in the smooth endoplasmic reticulum of the liver. In contrast, phase II metabolism introduces a hydrophilic species to the drug molecule, typically through sulfation, glucuronidation, and glutathione conjugation.[19] Phase I metabolites are of primary interest to the present research.

Different drugs have different physicochemical properties, which should be considered when evaluating forensic hair analysis methods and drug-matrix binding. The drugs and metabolites in this study were chosen based on their range of physicochemical properties, such as logP and pKa. In addition, these drugs and metabolites of interest were evaluated due to their presence in the authentic HRM obtained for this research.

2.6.1 Methamphetamine (MET)

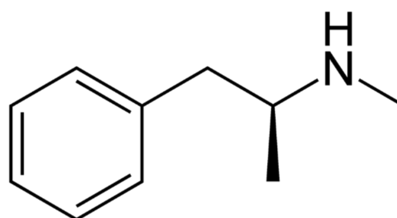


Figure 8. Structure of MET

MET was first synthesized from ephedrine in 1893 and crystallized in 1919.[20] MET is a central nervous system stimulant, resulting in physiological effects including appetite suppression and central nervous system stimulation.[21] It facilitates the release of neurotransmitters such as noradrenaline, dopamine, and serotonin, from nerve terminals in the brain and inhibits their reuptake. This results in increased concentrations of these

neurotransmitters in brain synapses and enhanced stimulation of postsynaptic receptors.[21] MET has a logP of 2.07 in the neutral state, so it is relatively lipophilic.[22] In addition, it has a pKa of 9.87, and is a strong base because of its secondary amine group.[22]

2.6.2 Cocaine (COC)

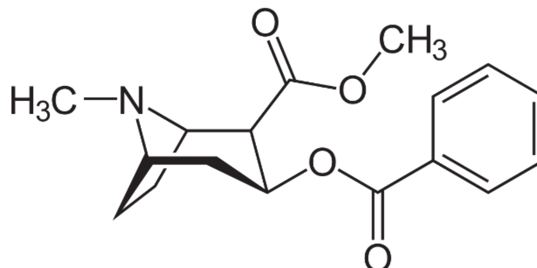


Figure 9. Structure of COC

COC is a naturally occurring compound that is extracted from the coca plant.[23] It is a Schedule II local anesthetic and vasoconstrictor but is rarely used clinically because of its addictive properties. COC binds to dopamine, serotonin, and norepinephrine transporter proteins, and thus inhibits their reuptake into presynaptic neurons. This leads to increased concentrations of these neurotransmitters, causing mood-elevating effects. In addition, COC binds to and blocks sodium channels in the cell membrane of neurons, which inhibits the production of nerve impulses, resulting in loss of sensation.[23] It has a logP of 2.3, indicating that it is relatively lipophilic.[22] Additionally, COC has a pKa of 8.61, and is a weak base due to its tertiary amine group.[22]

COC has many potential metabolites, but cocaethylene (COCA), norcocaine (NORCOC), and p-hydroxycocaine (HYCOC) are of most interest to this research because of their presence in the authentic HRM used.

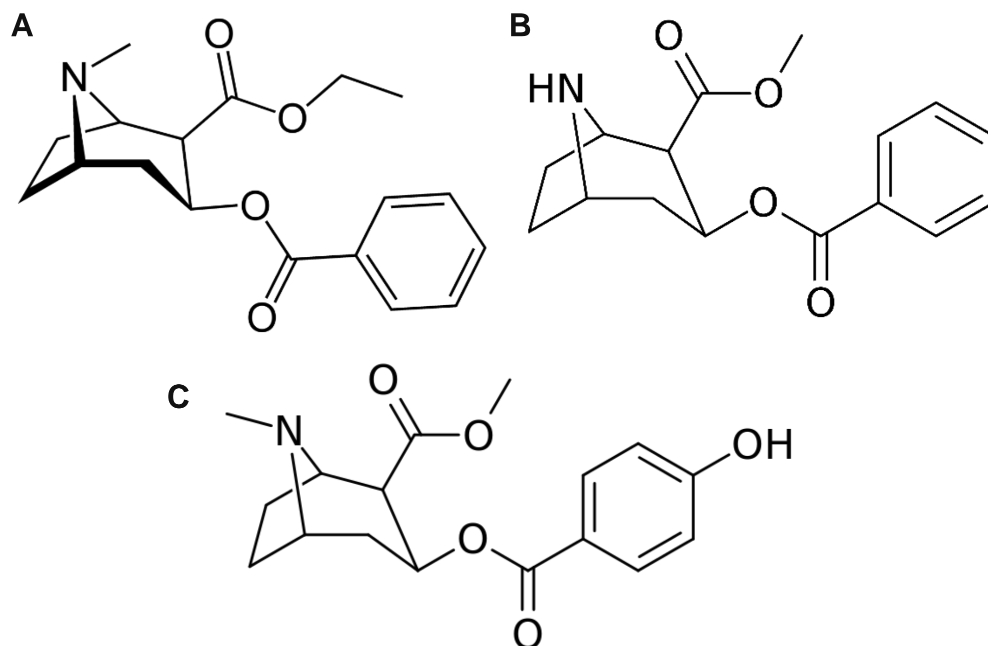


Figure 10. Structure of **A**) COCA, **B**) NORCOC, and **C**) HYCOC

COC is metabolized to COCA in the presence of alcohol through transesterification by hC-1 in the liver, such that the methyl group in COC is replaced with an ethyl group.[24] COCA has the same affinity for the dopamine receptor as COC yet has a half-life many times longer than that of COC. COC is oxidized by CYP3A4 into NORCOC. NORCOC is hepatotoxic to humans and is usually found in the presence of alcohol.[24] HYCOC is metabolized from cocaine by hepatic microsomes. COCA, NORCOC, AND HYCOC have a pKa of 8.77, 9.56, and 9.09, respectively, indicating they are strong bases.[22] COCA and NORCOC have a logP of 2.70 and 1.73, respectively, indicating that they are relatively lipophilic.[22] All metabolites of COC can be detected in hair samples, but COC itself is present at the highest concentration.[24]

2.6.3 Oxycodone (OXY)

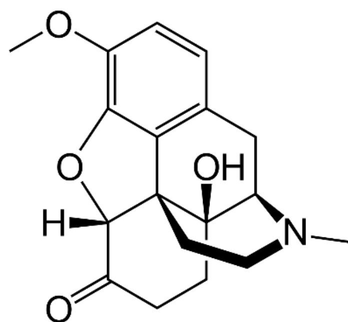


Figure 11. Structure of OXY

OXY is derived from thebaine, a minor component of opium.[24] It was first used as a pain reliever in 1917, and is still used for pain management today. The FDA reported that more people die from fatal overdoses as a result of OXY than HER and COC combined. Detection of OXY is limited in traditional body fluids, with a limit of detection of less than 24 h. However, it can be detected in the hair matrix. It has a pKa of 8.9, and is basic due to its tertiary amine function.[22] OXY has a logP of 0.70, indicating that it is relatively polar.[22]

2.6.4 Heroin (HER)

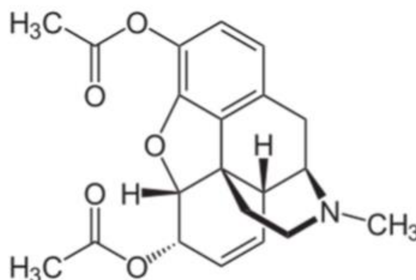


Figure 12. Structure of HER

HER is an opiate, *i.e.,* a natural or semi-synthetic analgesic derived from opium.[25] It binds to nerve cell receptors that normally bind with endogenous opioids in the body. HER was first synthesized from morphine in 1874, and Bayer produced it for the market in 1898.[25] HER has a logP of 1.58, indicating that it is somewhat lipophilic.[22] It has a pKa of 7.95 and is a weak base due to its tertiary amine function. HER is generally considered to be a prodrug of morphine; it readily crosses the blood brain barrier followed by rapid hydrolysis of the acetyl groups.[22]

The primary metabolite of HER is 6-monoacetylmorphine (6-MAM), which is metabolized via reduction of the carboxylic acid to an alcohol.[24] It has a pKa of 9.08, making it basic, and a logP of 1.55, indicating relative lipophilicity.[22] Morphine (MOR) is also a metabolite of HER, metabolized from 6-MAM via hydrolysis.

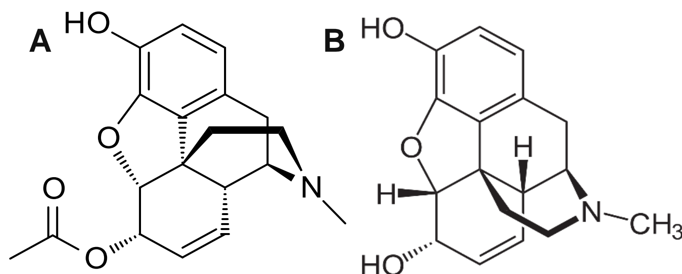


Figure 13. Structure of A) 6-MAM and B) MOR

MOR is also a naturally occurring opiate, which binds to the μ opioid receptor in the presynaptic part of the gray region of the brain. It was first isolated from opium and reported by Friedrich Sertürner in 1805. It has a pKa of 9.12, indicating that it is a strong base and a logP of 0.87, indicating it is relatively polar.[22]

2.6.5 Diazepam (DZP)

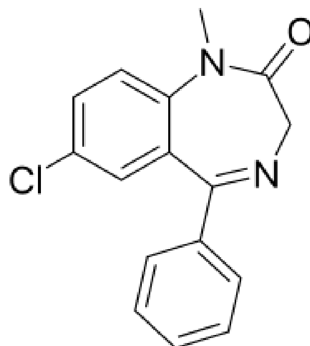


Figure 14. Structure of DZP

The first benzodiazepine was accidentally synthesized in the mid 1950's, while DZP was created for the market as Valium in 1963.[20] Benzodiazepines are used as tranquilizers, anticonvulsants, hypnotics, and sedatives.[26] They can cause drowsiness, memory impairment, amnesia, and transient euphoria when used with alcohol. DZP has a long duration of action as a muscle relaxant.[26] It has a logP of 2.82, indicating that it is relatively lipophilic. Additionally, DZP has a pKa of 3.3, and is weakly basic due to the diazepine ring nitrogen at position 4.[22]

DZP is demethylated in the liver by CYP3A4 and CYP2C19 to nordiazepam (NORDZP).[27] It has a logP of 2.93, indicating it is relatively lipophilic, and a pKa of 2.85, making it a weak base.[22]

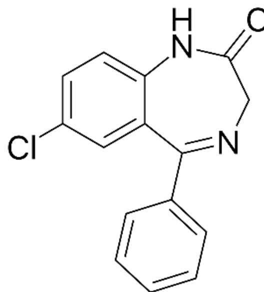


Figure 15. Structure of NORDZP

2.6.6 Alprazolam (ALP)

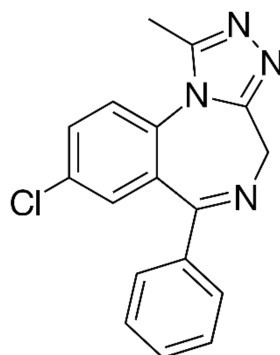


Figure 16. Structure of ALP

ALP is currently one of the most commonly prescribed psychotropic medications in the United States.[20] It is used for treating generalized anxiety and panic disorders. However, ALP has a high rate for misuse and is shown to be more addictive than other benzodiazepines, likely because ALP's antipanic and anxiolytic effects are more rapid than other commonly prescribed antianxiety medications. In addition, its rapid absorption, short half-life, low lipophilicity, and high potency increase the potential for ALP's misuse. ALP is less lipophilic and has a smaller volume of distribution than DZP, giving ALP a faster metabolism and shorter duration of action than DZP.[20] ALP has a pKa of 2.4, and is a weak base due to the diazepine ring nitrogen.[22]

2.6.7 Fentanyl (FEN)

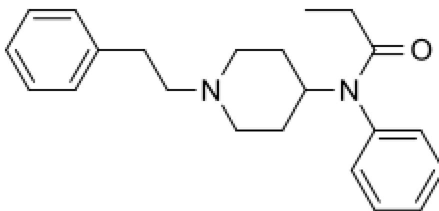


Figure 17. Structure of FEN

FEN was first synthesized by Paul Janssen in 1959.[28] It is a synthetic opioid that is 70 to 100 times more potent than morphine. Its enhanced lipid solubility allows for it to

rapidly cross the blood brain barrier. FEN is an addictive analgesic and anesthetic. It selectively bonds to μ-opiate receptors in the central nervous system and mimics endogenous opiates.[28] This stimulates the exchange of GTP for GDP.[20] The exchange inhibits adenylate cyclase, which decreases cAMP. The decrease of cAMP reduces the release of neurotransmitters including substance P, GABA, dopamine, acetylcholine, and noradrenaline. FEN also blocks the opening of calcium channels, reducing neuron excitability. It has a logP of 4.05, indicating it is a very lipophilic compound. In addition, FEN has a pKa of 7.3, and is a weak base due to its piperidine nitrogen.[20, 22]

2.7 Forensic Toxicology Techniques

The first step of analysis in forensic toxicology is isolation of the drug from the matrix.[2] When analyzing liquid matrices such as urine or blood, a small aliquot of the specimen can be applied directly to an immunoassay, however, for confirmatory tests utilizing chromatographic procedures, clean-up to remove proteins and other matrix components which can affect the accuracy and reliability of the analysis is often necessary. Such techniques commonly include dilute and shoot, crash and shoot, liquid-liquid extraction (LLE), and solid-phase extraction (SPE). Dilute and shoot is commonly used for the analysis of urine samples, diluting the urine with water prior to analysis, to protect instrumentation from the high salt concentration in urine.[29] Crash and shoot is utilized for whole blood, plasma, or serum samples, and denatures and precipitates out proteins present in the blood that could damage the instrument or cause matrix effects.[29] LLE extracts the analytes from the liquid biological material or solubilized solid material using an organic solvent. In contrast, SPE absorbs the analytes of interest onto a solid

support, such as C18 or mixed-phase silica columns, to allow selective absorption. A wash step is utilized to remove analytes that are not of interest from the column, followed by elution steps to collect the analytes of interest.[2]

Forensic toxicology utilizes two main types of detection techniques, immunoassay and chromatography-based methods.[2] Immunoassays utilize antibodies showing selectivity for a specific drug class, and can be automated allowing for high throughput analysis. Chromatographic methods utilized in forensic toxicology include gas chromatography (GC) and liquid chromatography (LC). Traditionally, GC is used for volatile drugs with good thermal stability, while LC is used for separation of polar and semipolar compounds. However, the use of derivatizing agents can be used to increase the stability of polar compounds for use in GC as well.[2]

2.8 Liquid Chromatography-Triple Quadrupole-Mass Spectrometry (LC-QqQ-MS)

LC is used as a separation technique, in which drugs with a higher affinity for the mobile phase elute faster than drugs with a higher affinity for the stationary phase.[30] This research utilized an Agilent 1290 Infinity Ultra High-Pressure LC (UHPLC) with an Agilent 1.8® µm Zorbax Eclipse Plus C_{18} rapid resolution HD column (2.1 x 50 mm; 1.8 µm). The 1.8 µm particle size used in this research, as opposed to the commonly used 3.5-5.0 µm particle sizes, increases resolution and allows for the use of a shorter column, and thus a shorter run time. The advantages of a shorter run time include high throughput, high capacity, and lower cost. The Agilent Rapid Resolution High Definition (RRHD) column used in this research allows for maximized selectivity.[30]

The MS process consists of three parts: ionization, fragmentation, and mass analysis and detection.[31] For this research, an electrospray ionization source (ESI) was used, as it is commonly used for polar and semipolar drugs in aqueous phase. Advantages of ESI include that it is a soft ionization method, has controllable fragmentation, and often results in multiply charged ions, which can be useful in analysis of larger molecules. The sample is introduced to the source via LC.[31] ESI begins with the production of charged droplets from the drug dissolved in mobile phase.[32] The charged droplets shrink due to solvent evaporation and droplet disintegration, producing gas-phase ions which enter the mass analyzer. The gas-phase ions may then be fragmented into smaller ions.[32] The specific ESI source used for this research was an Agilent Jet Stream Technology (AJS) Source.[33] It improves desolvation and ion generation by using superheated nitrogen sheath gas. Additionally, the source is orthogonal to prevent unwanted sample components from being introduced.[33]

The quadrupole analyzer consists of four rods that operate in pairs and apply direct-current (DC) voltage and radio-frequency (RF) potential to separate various ions.[31] The high and low pass rods filter out ions with too low and too high of an *m/z* ratio, respectively. In a QqQ system, every gas-phase ion enters the first quadrupole (Q1), where precursor ions are chosen to move on to the collision cell. Fragmentation of the precursor ions occurs in the collision cell (Q2), resulting in fragment ions that are separated in the third quadrupole (Q3) by *m/z* ratio prior to entering the detector.[31] The QqQ used in this research contains a hexapole in the collision cell which utilizes linear acceleration to optimize MS/MS fragmentation through overall better ion focusing and ion transmission.[33] The detector in this research was an Agilent High Energy Dynode

Detector Assembly. The orientation of the dynodes is perpendicular to the ion beam, reducing the likelihood of neutral molecules impacting the detector, as well as utilizing high voltages to attract the ions. When the ions hit the dynodes, they are converted to electrons prior to impacting the multiplier.[33]

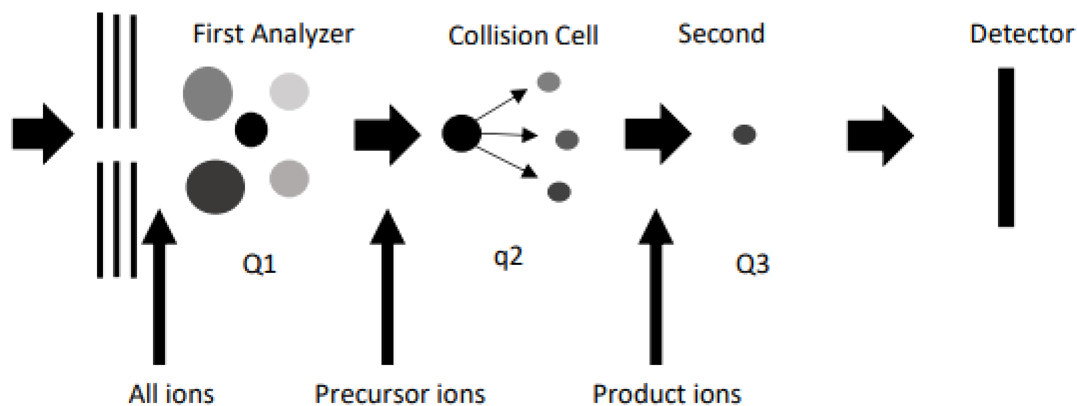


Figure 18. Schematic of QqQ-MS[29]

A dynamic Multiple Reaction Monitoring (dMRM) method was used in this research. dMRM uses a single continuous time segment and up to 4000 transitions.[33] For the purposes of this research, the retention time (RT), ΔRT, precursor ion, and two product ions were utilized for the identification and quantification of drugs and metabolites. dMRM allows for longer dwell times by scanning for transitions within the ΔRT of the RT as opposed to continuously throughout the chromatogram.[33]

2.9 Introduction to Statistical Design of Experiments (DoE)

DoE is commonly utilized in many areas of scientific testing to assess the effect of independent variables, individually and in combination with each other, on the dependent variable.[34] This is in contrast to the traditional approach, One Variable at a Time (OVAT) method, which evaluates the effect of an independent variable on the dependent variable.

In addition, if other variables are held at constant values, another variable can be evaluated. However, this method is laborious and costly due to the amount of materials needed to assess each variable individually. In contrast, DoE allows for assessing all parameters simultaneously by evaluating the associations between the factors and combinations of factors and their impact on the dependent variable.[34]

When analyzing the results of DoE, Analysis of Variance (ANOVA) is initially used to compare more than one parameter to determine which main effects and interactions are important by calculating the p-value.[34] First, the sum of squares (SS) is calculated, which indicates the levels of each factor. The SS is used to calculate mean square and F value, which is converted to p-value. The equation to calculate the F-statistic is:

$$F=\frac{\frac{SSB}{DoFB}}{\frac{SSW}{DoFW}}$$

where SSB is the summation of the squared difference between the mean of each parameter and the overall mean, and SSW is the squared difference between the parameter-mean and each parameter in the group. DoFB is the degrees of freedom between groups, meaning the number of parameters minus 1, while DoFW is the degrees of freedom within groups, meaning the number of data points minus the number of parameters.[34]

When the p-value is ≤ 0.05, the main effects or interactions are considered statistically significant. This statistical significance does not indicate which factors or combinations of factors are optimal, but instead indicates whether DoE was an effective technique for

statistically comparing the parameters of interest. For example, if a single factor was found to be statistically significant, but no combinations of factors were, that would indicate that an OVAT approach would have been sufficient for assessing those particular parameters of interest. In contrast, finding combination(s) of factors to be statistically significant emphasizes the importance of utilizing DoE to assess the effects of those parameters of interest. Finally, a plot of residuals versus sample number is used to determine if ANOVA is the proper means of analysis for the data.[34]

2.10 Drug-Hair Matrix Interactions

It has been proposed that drugs can incorporate into hair in several ways, as shown in Figure 19.[10, 35] External incorporation from environmental exposure has been explored by numerous researchers.[36-38] Additionally, binding of the drug to hair from sweat and sebum, or through excretions and sebum during growth within the follicle, have been explored.[36, 39, 40] There is also passive diffusion directly from the capillaries surrounding the hair follicle.[36, 39, 41-43] Finally, drug may be incorporated into the keratinized hair shaft from the upper dermis.[42, 43]

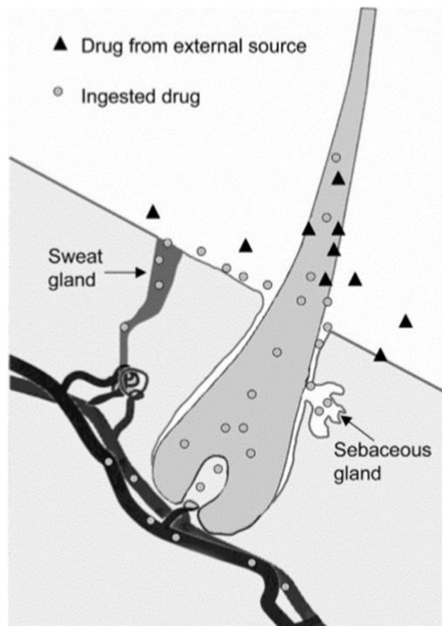


Figure 19. Schematic of how drugs incorporate into hair

Further research regarding the way in which drugs bind to the hair matrix is needed. Some general trends found to affect drug-matrix binding include pKa, structure, size, lipophilicity, protein binding capacity, and melanin affinity of the drug.[1] Correlation between the concentration of a drug in hair and the amount of drug ingested is dependent on the frequency of drug use, hair color, external contamination, use of cosmetic products, and other factors.[1]

There are several known variables that affect the ability of the drug to enter the hair matrix.[2] First, the drug or metabolite needs to be able to cross several membranes and a protein barrier to incorporate into the growing hair shaft. Neutral polar and lipophilic drugs pass through these barriers more easily than charged drugs. Drug metabolites can be present in hair for two reasons.[11] First, the metabolite may incorporate into the hair via the blood-stream post-ingestion. Alternatively, the parent drug may be directly

metabolized in the papilla or hair shaft of the hair, which is known to contain biotransformation enzymes.[11]

Hair is a complex matrix, so there are likely many physicochemical mechanisms by which drugs can bind to hair components.[44] For example, there could be ionic interactions with polar or ionizable functional groups such as amines and carboxylic acids in hair that could be used as binding sites. Neutral polar molecules may bind via hydrogen bonding or diploe-dipole interactions to components in the hair matrix. Lipophilic drugs are likely to be incorporated via hydrophobic or Van der Waals interactions. Factors that affect drug-matrix binding are hypothesized to include diffusional barriers, drug lipophilicities, and hair pigmentation.[44]

For example, some research has demonstrated that electrostatic binding of substances to melanin is strengthened by Van der Waals forces between aromatic indole rings in the melanin polymers and aromatic rings of the organic amines.[14] Charge-transfer may occur when electron-donating complexes interact with melanin. Melanin has a hydrophobic core, which allows for extensive hydrophobic interactions with aliphatic molecules.[14]

When studying binding between drug and matrix, it is important to consider the charge states of melanin and keratin. At low pH, the carboxylate groups in eumelanin and pheomelanin will be neutral, while they will be anionic at high pH.[45] In contrast, the amine groups in pheomelanin will be cationic at low pH 6 but neutral at high pH.[45]

There are two types of keratin present in hair, type I and type II.[46] Type I keratin is a weak acid, with a pKa of 4.5-5.5, while type II keratin is a weak base with a pKa of 6.5-7.5. At physiological pH, the ionizable groups in type I keratin are anionic, while those in

type II are cationic.[45] In contrast, at high pH, type I keratin will still be negatively charged, while those in type II will be neutral. It can also be hypothesized that, for type I keratin, the carbonyl and amide groups can play a role in hydrogen bonding, with the carbonyl group accepting hydrogen bonds and the amide group donating hydrogen bonds.[45]

Many research groups have examined methods of drug binding to the hair matrix.[44] Kidwell and Blank deduced that hair acts as an ion-exchange membrane.[47] Kronstrand et al. found that the concentration of codeine in hair after oral administration was dependent on the melanin content.[14] Nisbet et al. determined that pigmentation plays a role in binding between NBOMes, a group of new psychoactive substances derived from phenethylamines, and the hair matrix, as it was found that drug could only be detected in pooled white hair at the highest dose, while it was detected and quantified in pooled black hair at all doses.[48] Nakahara et al. saw similar results in their experiments, in which phenethylamines, including MET, 3,4-methylenedioxymethamphetamine, benzphetamine, ephedrine, N,N-dimethylamphetamine, and p-nitro-methamphetamine were detected in high concentrations in black hair, while none of these drugs were detected in white hair.[49] Polettini et al. also observed that, the higher the total melanin concentration, the higher amount of drug that was incorporated.[50] Ishiyama et al. proposed that melanin may not be the specific binding site for all drugs, as they saw similar binding with MET to light and dark hair.[51] Stout et al. found that higher MW molecules have higher affinity for melanin.[44] In addition, ionic associations may occur between drug and carboxylic and phenolic hydroxyl groups in melanin-associated proteins. Additionally, they determined that neutral species bind similarly in pigmented

and non-pigmented hair, suggesting that the drug may bind to protein sites other than melanin within the hair. They also found that water-soluble compounds bind less to hair than lipid-soluble compounds.[44]

Stout et al. continued research into drug-matrix interactions using FEN and albino mice to eliminate drug interactions with hair pigments and focus on the drug-protein interactions.[52] A mechanism of drug-matrix interaction in which there is a charge transfer from the N-terminus of the protein to the piperidine nitrogen of the drug. The positively charged drug then associates ionically with the negatively charged C-terminus of the protein. [52]

Polettini et al. and Testorf et al. focused on binding studies between drugs and melanin.[50, 53] Polettini et al. found t that the nature of interaction between MET and amphetamine with melanin was unclear, as ionic, hydrophobic, and possibly covalent binding were detected. In addition, they observed that basic drugs bound more to melanin than acidic or neutral drugs with melanin, and that a higher concentration of melanin resulted in a higher concentration of incorporated MET and amphetamine.[50] Testorf et al. used radiolabeled drugs to directly measure drug binding with melanin.[53] They proposed that the binding may be due to the structure of melanin as an irregular polymer that absorbs light and has multiple electron donors. Therefore, melanin-drug interactions could be primarily electrostatic forces mediated by ionic binding and influenced by Van der Waals forces and hydrophobic interactions.[53]

Shima et al. also studied the effect of melanin on drug incorporation.[43] They collected black and white hair samples from the same individual over time after ingestion of

zolpidem and methoxyphenamine. Their observation was that, in black hair, drugs were incorporated in both Region 1, the hair bulb, and Region 2, the upper dermis zone.[43] Incorporation for Region 1 lasted for one to two weeks after ingestion, while the incorporation for Region 2 lasted up to 24 hours.[43] For white hair, incorporation only occurred through Region 2. This indicated that hair pigments play a role in the incorporation that occurs through Region 1, while incorporation through Region 2 did not depend on the presence of melanin.

Borges et al. studied binding of cocaine, benzoylecgonine, amphetamine, and N-acetylamphetamine to melanin subtypes to understand which chemical functional groups on melanin are responsible for drug binding.[54] They incubated the drugs of interest with melanin overnight and evaporated the samples, followed by reconstitution and analysis using LC-MS/MS. Parameters such as binding capacity and affinity were determined for each drug with each melanin subtype. They found that cocaine and amphetamine bound to the melanin subtypes, while benzoylecgonine did not. It was hypothesized that the positive charge on the tropane nitrogen of cocaine associated with melanin binding; lack of binding of benzoylecgonine was attributed to the carboxylate group that could render the positively charged nitrogen less available for binding with melanin.

Potsch et al. evaluated a biochemical approach to drug-hair matrix binding, based on principles of biological transport across cell membranes, biotransformation, and drug melanin affinity.[55] They determined that drug molecules are conserved during keratinization because they remain in the cell membrane and outside the cells along the intercellular spaces. Alternatively, drug molecules could be captured by keratinocyte

differentiation products and become bound to or associate with keratin intermediate filaments. If melanin polymers are also present in the cell, drug molecules may also adsorb onto their surfaces.[55]

Larsson and Tjalve determined that negatively charged carboxyl groups, phenolic OH groups, and semiquinones on two melanin subtypes (DHI melanin and DHICA melanin) could be responsible for ionic/polar binding, along with Van der Waals and hydrophobic interactions in eumelanin.[56] In addition, they observed that positive radicals and basic drugs have higher binding affinities for melanin.[56] Nakahara et al. determined that binding depends on the melanin affinity and lipophilicity of drug.[49] They also found that basic drugs incorporate better at lower pH.[49] Potsch et al. discovered that the lipid solubility of the drug, molecular mass, ratio of ionized to non-ionized drug, pH gradient, blood flow, and concentration gradient all play a role in drug matrix binding.[57] Kintz et al. discovered that non-acidic, lipophilic drugs with a mass lower than 800 Da, and containing a nitrogen atom for binding to melanin, alkyl chains, and/or phenyl rings are more likely to incorporate into hair.[58]

3. ASSESSING OPTIMAL FORENSIC HAIR ANALYSIS DECONTAMINATION PARAMETERS FOR EXTERNALLY CONTAMINATED HRM USING A 2^4 FRACTIONAL FACTORIAL BLOCK DESIGN

3.1 Introduction

One of the commonly discussed limitations in forensic hair testing is the decontamination process.[59] The SoHT gives general recommendations regarding best practice methods for decontamination, such that at least one aqueous wash and at least one organic wash should be used.[1] However, these criteria may be achieved differently by different laboratories. For example, Stout et al. decontaminated FEN from the surface of hair using water, 1M acetic acid and methanol washes, while Salomone et al. washed using dichloromethane followed by methanol.[52, 60] Additionally, Cairns et al. removed MET from the surface of hair using shampoo treatments and water, while Mantinieks et al. used a 30-min MeOH wash followed by a 60-min 0.1 M PBS (pH 6) wash.[59, 61] These reports demonstrate a lack of consensus regarding the solvents and washing protocols used for decontamination, which can result in a variable amount of external drug contamination being removed, drugs incorporated into the hair being removed, and protic solvents washing contaminated hair into the hair matrix.[62, 63] Previous work in this laboratory utilized a DoE approach to systematically investigate optimized decontamination protocols for several drugs of abuse.[64, 65] The present study extends these findings to include decontamination of MET and FEN.

3.2 Materials and Methods

3.2.1 Chemicals and Solvents

Blank, de-identified, black human hair was obtained from a commercial source (BUW Human Hair, Houston, TX). Authentic HRM was obtained from a collaborating lab (RTI International, Research Triangle Park, NC). Stock solutions of FEN, FEN-D5, MET, and MET-D5 were purchased from Cayman Chemical (Ann Arbor, MI). Solvents and chemicals for decontamination washes (1% SDS, water, methanol, and dichloromethane), LC-MS grade mobile phase solvents and additives (ammonium formate, water, formic acid, acetonitrile, and methanol), and some extraction materials (sodium hydroxide) were HPLC-grade, purchased from Fisher Scientific (Hampton, NH). PTFE spin filters (3 kDa molecular weight cut off) were purchased from Millipore Sigma (Burlington, MA, USA). For extraction, Proteinase-K was ordered from Invitrogen (Carlsbad, CA, USA), and dithiothreitol (DTT) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

3.2.2 Microscopic Examination of Hair

Blank, de-identified, brown color hair was investigated under a Keyence Digital VH-Z100R Microscope to assess microscopic changes that may occur to the hair during decontamination. The hair samples included hair without treatment, as well as after 30 s or 30 min washes with water, 1% SDS, MeOH, and DCM.

3.2.3 Preparation of Externally Contaminated HRM

Externally contaminated hair was prepared by adding 100 µL of 1 mg/mL FEN or MET in methanol to 20 mg of drug-free hair in an Eppendorf tube. The samples were

vortexed to thoroughly coat the hair and then vacufuged for 30 min, allowing the drug to dry onto the surface of the hair. The externally contaminated hair was added to an amber vial, and the Eppendorf tube was washed with 1 mL of MeOH. This wash was subjected to LC-QqQ-MS analysis to assess mass of drug remaining in order to calculate mass of drug coated onto the hair.

3.2.4 2^4 Fractional Factorial Block Design

A 2^k factorial design was chosen for this work because of its ability to reduce the number of experimental runs by including multiple factors of interest in a single experiment.[34] Additionally, DoE allowed for studying both the direct effects of the factors under study and their interactions with each other. The 2^k factorial design consisted of k factors studied at two levels, in this case “high (+)” and “low (–)”. The effect of a factor was designated by a capital letter, such as A or B. Further, AB denoted the interaction between A and B. The treatment combinations of the design, (i.e., “design points”), were designated as lower-case letters, such as “a” and “b”. This notation indicated the levels of factors each sample received. For example, if the treatment combination of a sample was “ab”, this notation would indicate that both A and B were being held at a “high” level.[34]

To determine the most effective method for removing FEN and MET from the surface of the hair, a 2^4 fractional factorial block design (Table 1) was used. Confounding is a technique that allows for the arrangement of a factorial experiment in blocks, causing certain design points to be indistinguishable from the blocks. Blocking plays an important role in DoE, as it reduces the amount of noise.[34] The blocks for this design were

constructed using four combinations, each consisting of two blocking factors. The effects chosen to be confounded with the blocks were ABC and BCD. The defining contrasts for these effects were calculated using the following equations:

$$L1 = x1 + x2 + x3$$

$$L2 = x2 + x3 + x4$$

where, for a 2^k design, xi = 0 (low level), and xi = 1 (high level).[34] Each design point has a specific value for L1 and L2, with four possibilities: (L1, L2) = (0,0), (0,1), (1,0), or (1,1). Treatment groups that have the same value of L1 and L2 are placed in the same block.[34] As a result of the chosen confounding factors, it is found that there is a third natural confounding factor, AD. This is the effect of the generalized interaction between ABC and BCD:

$$(ABC)*(BCD) = AB^2C^2D = AD$$

The factors under study are listed below, with A representing aqueous solvent, B representing organic solvent, C representing number of consecutive aqueous washes, and D representing number of consecutive organic washes. Block 1 studied the sequence of washes and Block 2 studied the wash time. The aqueous solvent was either 1% SDS (+) or HPLC water (-). The organic solvent used was either dichloromethane (+) or methanol (-). There were either 3 (+) or 1 (-) consecutive aqueous and organic washes. The washes were either done organic first (+) or aqueous first (-). The washes were done for either 30 min (+) or 30 s (-). As an example, design point bc would receive the following treatment: three 30-s washes with HPLC water followed by one 30-s wash with DCM.

Table 2. 2^4 Fractional Factorial Block Design

Block (1,2)	Design Points	A	B	C	D	AB	AC	BC	AD	BD	CD	ABD	ACD	BCD	ABC	ABCD	Block
(low, low)	1	-	-	-	-	+	+	+	+	+	+	-	-	-	-	+	+
	bc	-	+	+	-	-	-	+	+	-	-	+	+	-	-	+	+
	abd	+	+	-	+	+	-	-	+	+	-	+	-	-	-	-	+
	acd	+	-	+	+	-	+	-	+	-	+	-	+	-	-	-	+
(low, high)	ac	+	-	+	-	-	+	-	-	+	-	+	-	+	+	+	-
	ab	+	+	-	-	+	-	-	-	-	+	-	+	+	+	+	-
	bcd	-	+	+	+	-	-	+	-	+	+	-	-	+	+	-	-
	d	-	-	-	+	+	+	+	-	-	-	+	+	+	+	-	-
(high, low)	bd	-	+	-	+	-	+	-	-	+	-	-	+	-	-	+	-
	cd	-	-	+	+	+	-	-	-	-	+	+	-	-	-	+	-
	a	+	-	-	-	-	-	+	-	+	+	+	+	-	-	-	-
	abc	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-
(high, high)	b	-	+	-	-	-	+	-	+	-	+	+	-	+	+	-	+
	c	-	-	+	-	+	-	-	+	+	-	-	+	+	+	-	+
	ad	+	-	-	+	-	-	+	+	-	-	-	-	+	+	+	+
	abcd	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

3.2.5 Decontamination Method

The externally contaminated HRM were washed according to the 2^4 Fractional Factorial Block Design matrix. Each wash was collected and analyzed using LC-QqQ-MS. The hair was dried overnight, followed by pulverization into a powder using a Retsch MM200 ball mill with chrome-steel milling beads at 3,200 rpm for 30 s and extracted for 24 h. After extraction, the samples were centrifuged and subjected to solid phase extraction (SPE) prior to LC-QqQ-MS analysis to determine drug remaining in the hair.

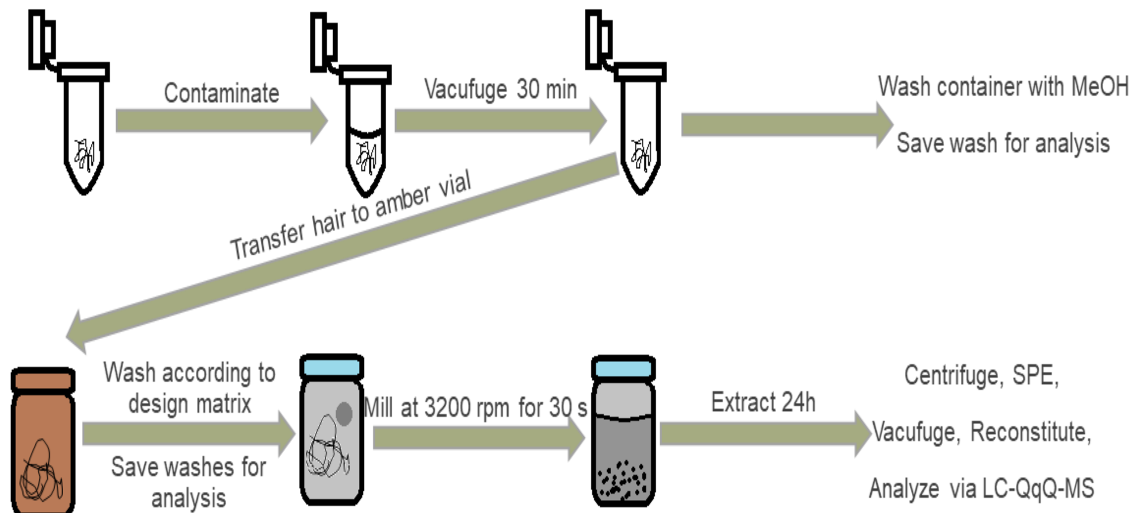


Figure 20. Schematic of decontamination DoE procedure

Recovery was determined using the following equation:

$$\left(\frac{pg\ drug\ in\ washes}{1\frac{mg}{mL} - \ pg\ drug\ in\ Eppendorf\ Tube\ wash}\right) * 100$$

3.2.6 Solid Phase Extraction (SPE)

An SPE protocol developed in previous work for multiple drugs and metabolites of interest was utilized for this study.[66]

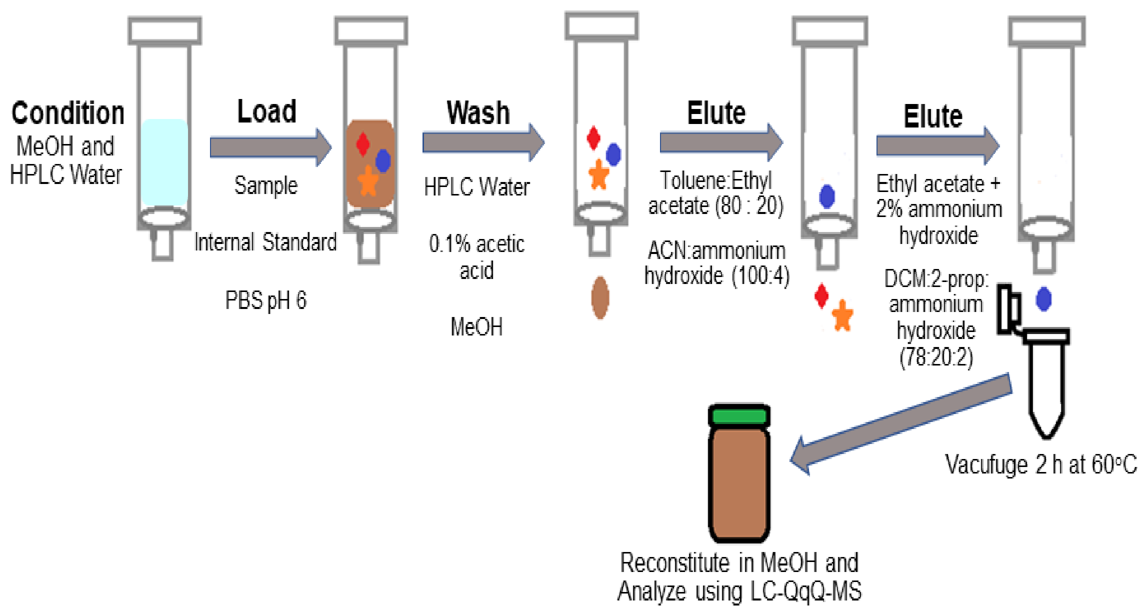


Figure 21. Schematic of SPE procedure

The protocol began with conditioning a Bond Elut Certify mixed mode cartridge (Agilent Technologies; Santa Clara, CA, USA) two times with 1 mL of methanol and two times with 1 mL of HPLC grade water. The sample and internal standard were then loaded onto the cartridge along with 2 mL of 1X phosphate buffered saline (10 mM Na_2HPO_4, 2 mM NaH_2PO_4, 137 mM NaCl, pH 4). The cartridges were washed two times with 1 mL HPLC grade water, followed by 0.5 mL of 0.1% acetic acid and then dried for 10 min. An additional wash step of 0.5 mL MeOH was performed prior to 2 min of drying. The first elution step was 0.75 mL of toluene:ethyl acetate (80:20 v/v), followed by drying the cartridge for 30 s prior to the second elution step using 0.75 mL acetonitrile:ammonium hydroxide (96:4 v/v). The samples were then eluted with 0.75 mL of ethyl acetate/2% ammonium hydroxide, followed by 0.75 mL of a mixture of dichloromethane, 2-propanol, and 2% aqueous ammonium hydroxide (78:20:2). While this method contained multiple elution steps for isolation of multiple drugs and

metabolites of interest, FEN eluted at the ethyl acetate/2% ammonium hydroxide step. Finally, the samples were evaporated to dryness in an Eppendorf Vacufuge Plus, reconstituted in 500 µL of methanol, and run in the LC/MS.

3.2.7 HPLC-MS Parameters

An Agilent 1290 Infinity II LC and 6460 QqQ-MS were used for analysis. The developed LC/MS method utilized a 2 µL injection volume into a Zorbax Eclipse Plus C_{18} rapid resolution HD column (2.1 x 150 mm; 1.8 µm, Agilent Technologies). The gradient elution started at 5% B, went to 75% B over 4.5 minutes, 90% B at 4.75 min, 95% B at 5.5 min, and 100% B by 8 min, at a flow rate of 0.3 mL/min. Solvent A was 5 mM ammonium formate in water with 0.1% formic acid, and solvent B was 0.1% formic acid in methanol. There was a post run time of 2 min.

For QqQ-MS analysis, a multiple reaction monitoring (MRM) method was used in positive ESI mode. A cell accelerator voltage of 4 V and cycle time of 500 ms were used. The drying gas and sheath gas were both at 350°C with flow rates of 12 and 11 L/min, respectively. The retention time for FEN was 4.54 min, with precursor ion 337 *m/z* and product ions 105 and 188 *m/z*. The retention time for FEN-d5 was 4.52 min, with precursor ion 233.2 *m/z* and product ions 84.1 and 55.1 *m/z*. The retention time for MET was 3.47 min, with precursor ion 150.1 *m/z* and product ions 119 and 91 *m/z*. The retention time for MET-d5 was 3.42 min, with precursor ion 155 *m/z* and product ions 96 and 124 *m/z*.

3.2.8 Metrics for Performance Comparison

The goal of this work was not to develop and validate a quantitative method for forensic hair analysis, but rather to compare different pretreatment and extraction parameters and their impact on extraction efficiency using selected performance parameters. Analytical method optimization was completed using calibration model, bias and precision, matrix effects and recovery, and LOD/LOQ as performance metrics. Calibration curves were created using neat drug standards in MeOH with concentrations of 85, 50, 25, 5, 1, and 0.05 ng/mL. Each solution had a total volume of 500 µL and included a deuterated internal standard solution at 100 ng/mL. Bias and precision were measured for drugs and metabolites of interest in MeOH at three different concentration levels (low, medium, and high) over five different runs.

Bias was calculated using the following equation:

$$Bias\ (\%)\ at\ concentration = \left[\frac{(mean\ of\ calculated\ concentration - nominal\ concentration)}{nominal\ concentration}\right] * 100$$

Precision was calculated using the following equation:

$$\%CV = \frac{std\ dev}{mean\ response} * 100$$

Recovery and matrix effects for extracted FEN and MET were evaluated based on three sets of samples. Set 1 included SPE with neat drug standards (15 ng/mL drug in MeOH), Set 2 involved drug-free hair extract spiked with drug after SPE, and Set 3 included drug-free hair extract spiked with drug during the SPE loading step.

Matrix effects were calculated using the following equation:

$$ME = \left[\frac{(set\ 1 - set\ 2)}{set\ 2}\right] * 100$$

Recovery was calculated using the following equation:

$$Recovery = \left[\frac{set\ 3}{set\ 1}\right] * 100$$

The lowest level of the calibration curve (0.05 ng/mL) was reported as the LOD and LOQ.

3.2.9 Analysis of Variance

When analyzing results of a 2^k design, Analysis of Variance (ANOVA) can be used to determine which main effects and interactions are important by calculating the associated p-values.[34] First, the Sum of Squares (SS) is calculated, which indicates the difference between levels of each factor. The SS is used to calculate Mean Square and F value, which is converted to p-value. When the p-value is less than 0.05, the main effects or interactions are considered statistically significant. Finally, a plot of residuals vs. sample number is used to determine if the conclusions made are valid and that ANOVA is the proper means for analysis of the data.[34]

3.3 Results

3.3.1 Microscopic Examination of Hair

Figures 22-25 show the images from the microscopic examination of blank hair from the top and side, before and after washing for 30 s or 30 min with aqueous solution or organic solvent, respectively.

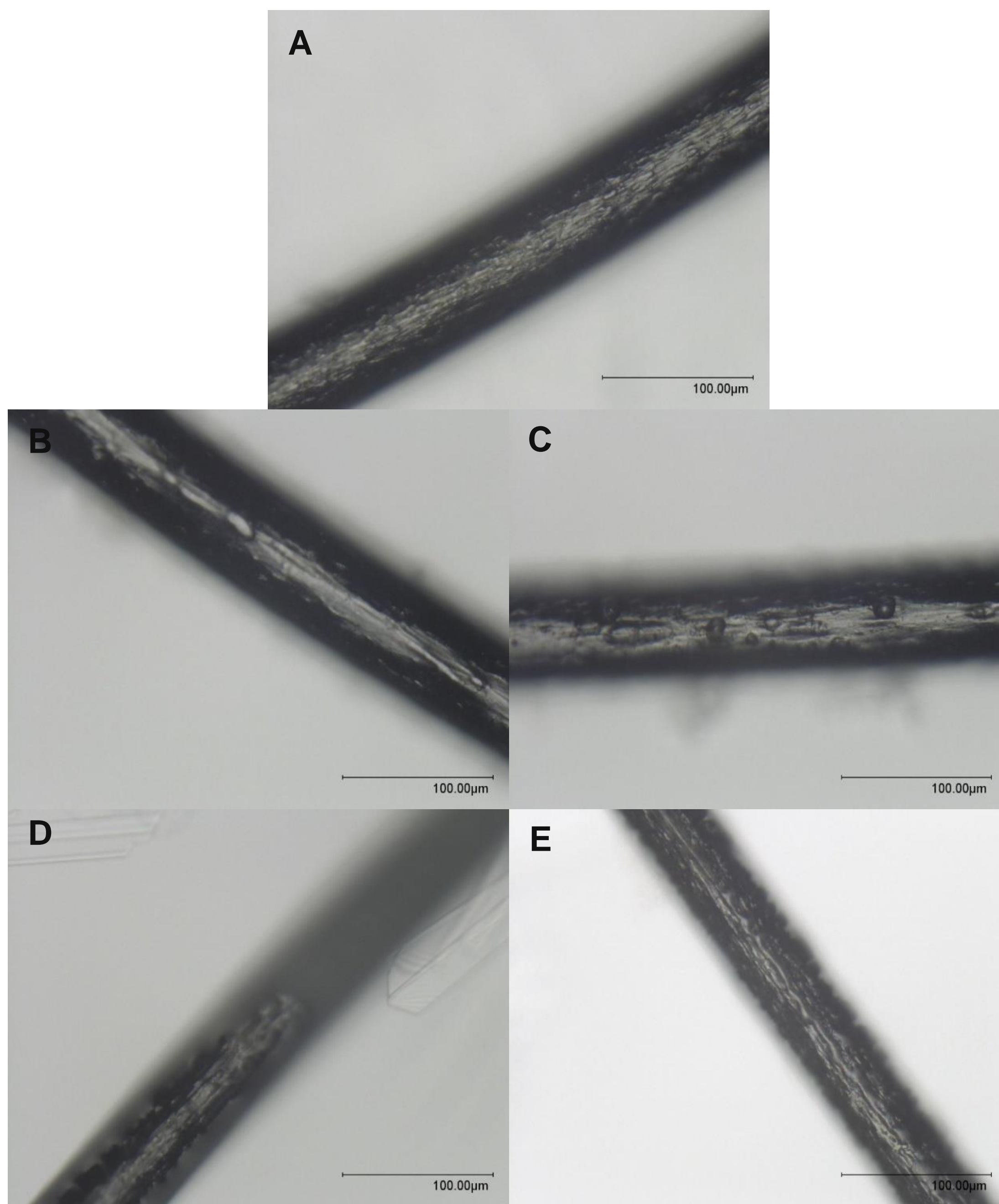


Figure 22. Microscopy images at 100X of the top of blank hair of A) without aqueous solution wash, B) wash for 30 s with water, C) wash for 30 min with water, D) wash for 30 s with 1% SDS, and E) wash for 30 min with 1% SDS

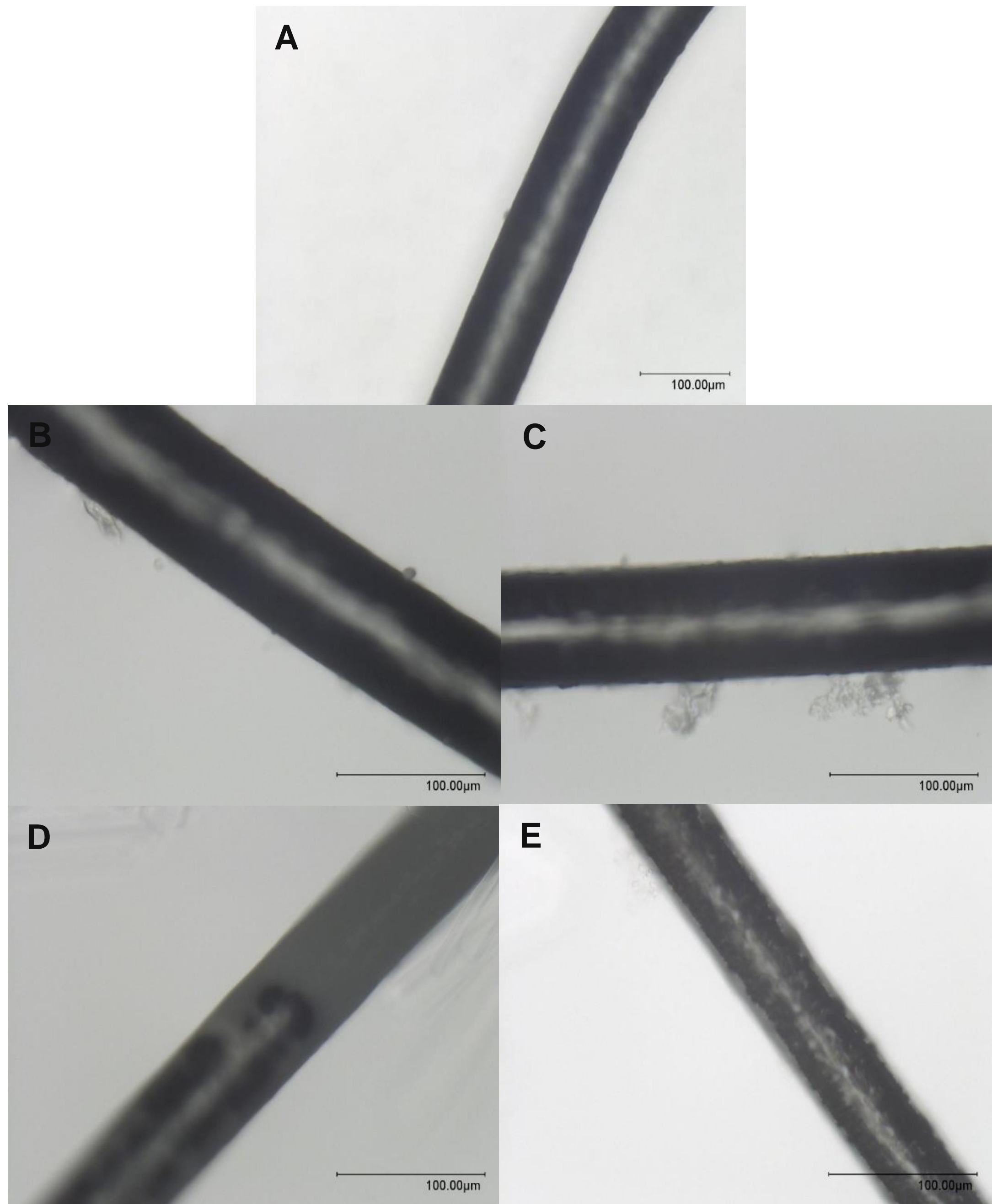


Figure 23. Microscopy images at 100X of the side of blank hair of A) without aqueous solution wash, B) wash for 30 s with water, C) wash for 30 min with water, D) wash for 30 s with 1% SDS, and E) wash for 30 min with 1% SDS

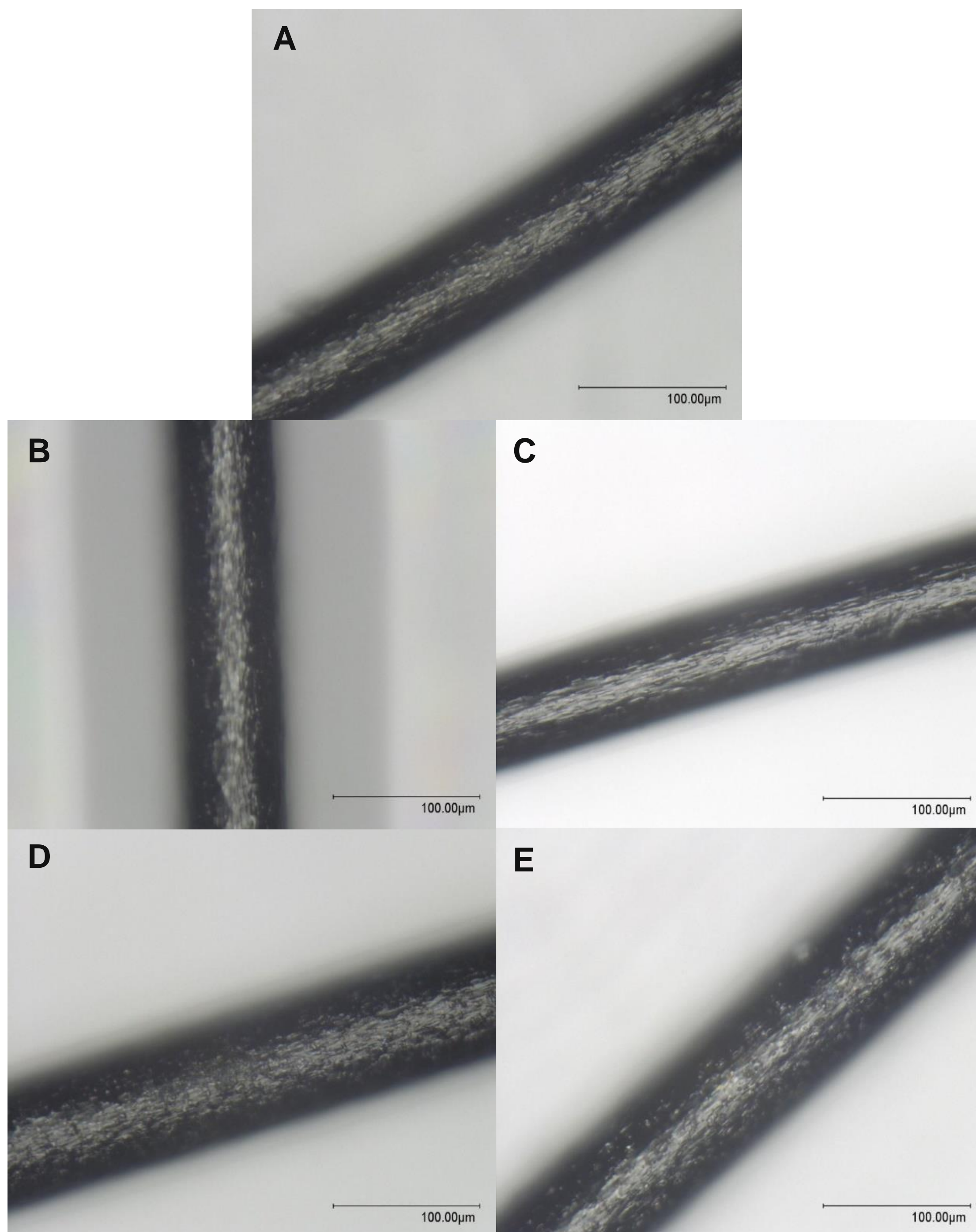


Figure 24. Microscopy images at 100X of the top of blank hair A) without organic solvent wash, B) wash for 30 s with MeOH, C) wash for 30 min with MeOH, D) wash for 30 s with DCM, and E) wash for 30 min with DCM

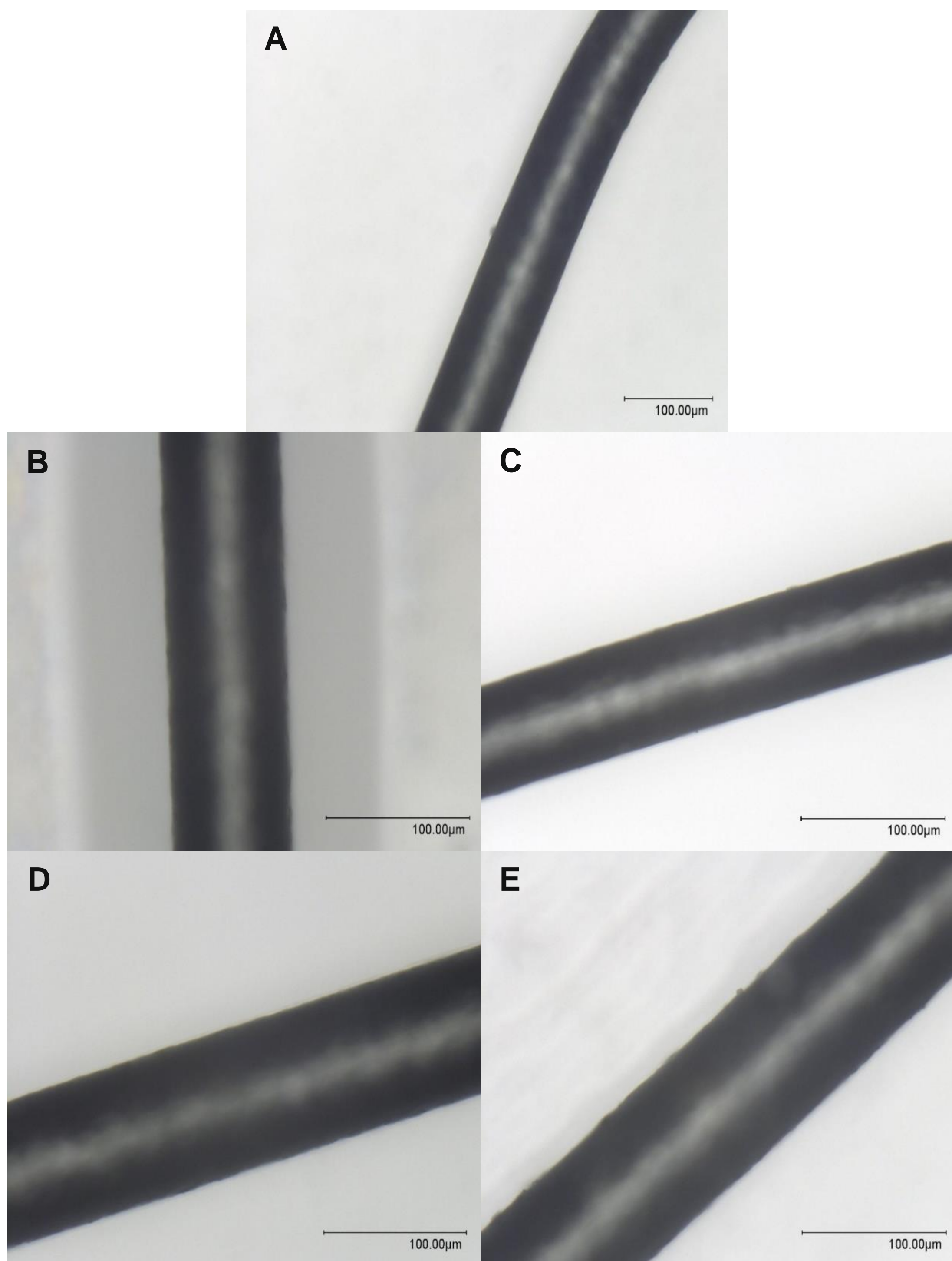


Figure 25. Microscopy images at 100X of the side of blank hair of A) without organic solvent wash, B) wash for 30 s with MeOH, C) wash for 30 min with MeOH, D) wash for 30 s with DCM, and E) wash for 30 min with DCM

3.3.2 Analytical Method Performance

The calibration model was linear, with an R^2 of >0.99. Bias and precision for FEN and MET were within ±20% and acceptable according to current guidelines.[67] Matrix effects and recovery values fell within the acceptable values of <20% and ±20% respectively, indicating that this method was effective in the presence of extracted hair matrix. The LOD and LOQ were determined to be 0.05 ng/mL.

3.3.3 DoE Comparison of Decontamination Parameters

Table 3 shows the results of the ANOVA F-Tests for the FEN and MET DoE. P-values <0.05 shown in Table 3 were considered statistically significant and are denoted in bold font.

Table 3. ANOVA F-Test for FEN and MET

Source	FEN p-value	MET p-value
1	**0.0315**	0.5364
A	**0.0316**	0.2494
B	0.5991	0.9707
C	0.4961	0.1009
D	0.5011	0.1135
AD	**0.0224**	0.1670
AB	**0.0159**	0.5000
BC	**0.0083**	**< 0.0001**
BD	0.3942	0.3492
CD	0.2960	0.1336
AC	**0.0200**	0.1361
ABC	**0.0007**	0.3783
BCD	**0.0070**	0.3153
ACD	**0.0116**	0.0148
ABD	**0.0106**	0.3552
ABCD	**0.0002**	0.3153
BLOCK 1	0.2422	0.8041
BLOCK 2	**0.0001**	**< 0.0001**

As shown in Table 3, FEN factors 1, A, AD, AB, BC, AC, ABC, BCD, ACD, ABD, and ABCD had p-values <0.05, indicating that they were significant. In contrast, factors B, C, D, BD, and CD were determined to be not significant, with p-values ≥0.05. MET factors BC and BLOCK 2 had p-values <0.05, indicating that they were significant. All other MET factors were determined to be not significant, with p-values ≥0.05. Analysis of residuals for both drugs of interest demonstrated equal variance among experimental data points, indicating that ANOVA was the appropriate test for analysis of the data.

Residual plots for FEN and MET were created, as shown in Figure 26. Random distribution of the points above and below the zero line on the residual plots indicate that ANOVA was the proper means for analysis.

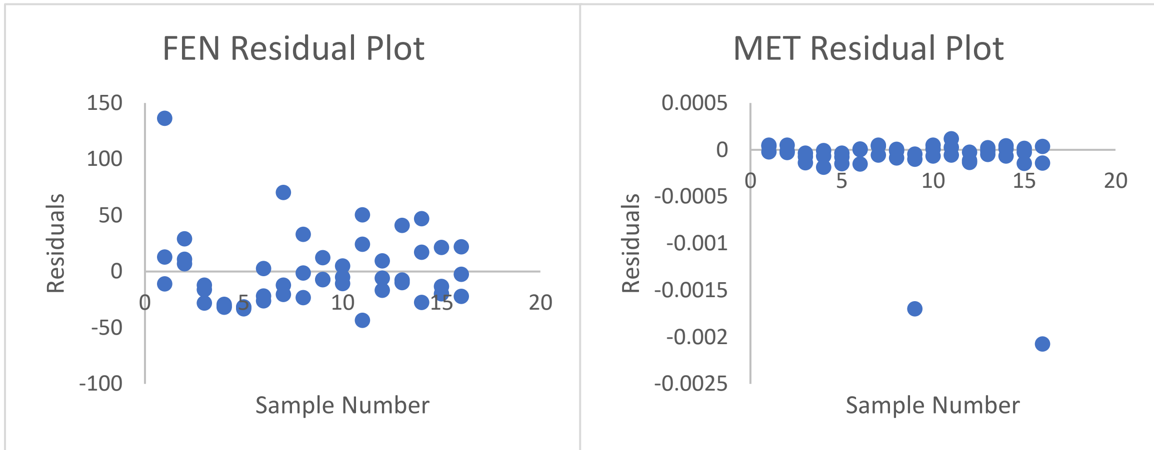


Figure 26. Residual plots for FEN and MET

Plots of the percent recoveries by design point (Figure 27) indicated that the FEN design points with the highest and lowest recovery were b and acd, respectively, with an overall range of 17 to 76% recovery. In contrast, MET design points with the highest and lowest recovery were bcd and 1, respectively, with an overall range of 37-78% recovery.

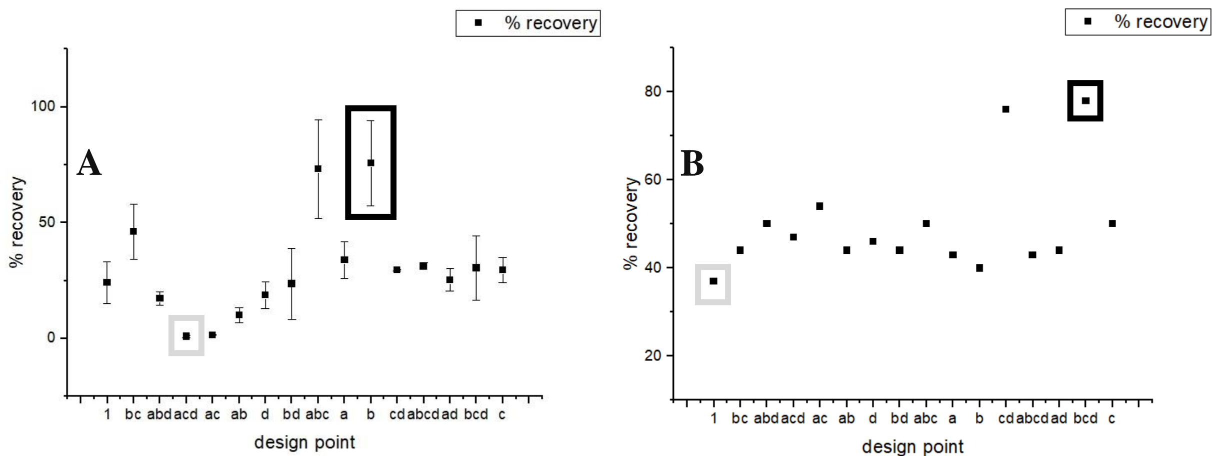


Figure 27. Percent recovery by design point for A) FEN and B) MET

Table 4 shows the levels of parameters by design point.

Table 4. Levels of parameters by design point for FEN and MET

Drug	Design Point	Recovery (±S.D.)	A	B	C	D	BLOCK 1	BLOCK 2
FEN	b	76 (±18)	Water	DCM	1	1	Organic First	30 min
	acd	1 (±1)	1% SDS	MeOH	3	3	Aqueous First	30 s
MET	bcd	78 (±0)	Water	DCM	3	1	Aqueous First	30 min
	1	37 (±0)	Water	MeOH	1	1	Aqueous First	30 s

As shown, the optimal method for removing FEN from the surface of hair was found to be one 30-min wash with dichloromethane followed by one 30-min wash with HPLC water. In contrast, the least effective method for FEN included three 30-s washes with 1% SDS followed by three 30-s washes with MeOH. The optimal method for removing MET from the surface of hair was found to be three 30-min washes with HPLC water followed by one 30-min wash with DCM. In addition, the least effective method for MET included one 30-s wash with HPLC water followed by one 30-s wash with MeOH.

3.3.4 Decontamination Studies Summary

Table 5 shows the levels of parameters by design point for drugs of interest investigated in this study, as well as those previously assessed by Dr. Aijala to evaluate trends regarding best practice decontamination protocols.[64, 65, 68]

Table 5. Decontamination Studies Summary

Drug	A	B	C	D	BLOCK 1	BLOCK 2
FEN	**Water**	**DCM**	**1**	**1**	**Organic First**	**30 min**
MET	**Water**	**DCM**	**3**	**1**	**Aqueous First**	**30 min**
Amphetamine	SDS	DCM	1	3	Aqueous First	30 s
Cocaine	SDS	DCM	1	3	Aqueous First	30 s
Diazepam	SDS	MeOH	3	3	Organic First	30 min
Heroin	Water	DCM	1	3	Organic First	30 s
Δ^9-THC	Water	MeOH	3	1	Aqueous First	30 min

*Bolded values indicate work completed in the present study. Other data from Aijala et al., 2021.

As shown in Table 5, there was not one specific method that is maximally effective for decontamination of all of these compounds. However, a consensus statement can be made that the most effective method for removing multiple drugs of interest from contaminated hair includes one with a 30-min wash with water followed by three 30-min washes with DCM.

3.4 Discussion

Decontamination is an imperative step in forensic hair analysis, to remove any external contamination or drug from the surface of the hair. However, to date, the microscopic impacts of washes on hair has not been reported. Interestingly, the 1% SDS 30-min wash images (Figures 22E and 23E) show differences compared to the unwashed hair sample images, as if the scales of the hair are more pronounced in the wash images. This could be due to adhesion of bubbles from the 1% SDS to the scales of the hair. Other aqueous solution washes did not show a difference compared to the unwashed sample. Of particular interest is Figure 25E, the 30-min wash with DCM. There is

speculation that DCM removes drug from the hair matrix, not just from the surface of the hair,[9] and Figure 25E shows slight swelling open of the scales of the hair from the side angle. However, 100% of the mass of FEN and MET on the externally contaminated HRM was accounted for in the Eppendorf tube wash, aqueous washes, and organic solvent washes. This potential scale swelling was not seen in Figure 25C, the 30-min wash with MeOH. Additionally, microscopy images of hair after organic washes from the top view did not show physical changes.

Decontamination of FEN and MET from hair has been reported by a number of research groups. Washes used for removal of FEN from the surface of the hair ranged from acetone alone to a mixture of dichloromethane, methanol, and water.[60, 69-72] In contrast, washes used for decontamination of MET included water followed by dichloromethane, as well as water or methanol alone.[73, 74] Despite the availability of these data, there are currently no literature reports on best practice methods for decontamination of FEN and MET from hair. The goal of the present study was to identify such methods using a DoE statistical approach and an externally contaminated FEN and MET HRM. Previous work in this laboratory has demonstrated the utility of DoE for this purpose using externally contaminated HRM for a variety of other drugs and metabolites of interest.[64, 65, 68]

Decontamination studies with FEN-contaminated hair indicated that higher level interactions, such as those between 3-4 parameters, were significant in the removal of FEN from the hair surface. The significance of higher-level interactions suggests that studying the combination of factors in decontamination studies is pertinent to understanding the most effective method for FEN, further reinforcing the practicality of

the 2^4 fractional factorial block design. In the decontamination DoE used in the present study, design point b, which included one 30-min wash with dichloromethane followed by one 30-min wash with water, was associated with the highest recovery (*i.e.* removal) of FEN from the hair surface. Design point abc was also associated with high recovery. Interestingly, the latter design point had levels of parameters in common with design point acd, which demonstrated the lowest recovery. For example, abc and acd both used 1% SDS as the aqueous solvent, three consecutive aqueous washes, and a 30-s wash time. However, design point abc employed dichloromethane as the organic wash solvent as compared to methanol for acd, indicating that dichloromethane was likely a key factor in removing FEN from the hair surface. This finding may be explained by relative solvent polarity; dichloromethane is less polar than methanol and FEN is a relatively non-polar molecule.

Decontamination studies with MET-contaminated hair indicated that for BC and BLOCK 2, higher-level interactions were statistically significant. None of the individual factors were found to be statistically significant, indicating that an OVAT approach would not have been effective for studying the parameters associated with the removal of MET from the hair surface. Design point bcd, including three 30-min washes with HPLC water followed by one 30-min wash with DCM, resulted in the highest recovery of MET from the surface of the hair. Design point cd also had a high recovery, yet had many levels of parameters in common with design point 1, which resulted in the lowest recovery of MET. For example, both design points had 30-s washes with HPLC water and MeOH, as well as only one organic wash. However, design point bcd and cd both had three consecutive aqueous washes, while design point 1 only had one aqueous wash.

This suggests that the number of aqueous washes was a key factor in determining the best practice decontamination protocol for MET. An explanation for this result could be that MET participates in hydrogen bonding with water, so more washes with HPLC water results in larger recovery of MET from the hair surface.

As recovery of drug for the final extractions using optimized parameters approached 100%, it can be concluded that digestion or extraction of FEN during the wash steps were not occurring.

One potential limitation of the FEN decontamination study was the large variance in recovery data observed for some design points, particularly those associated with the highest recovery. A potential source of variation may be the process used to externally contaminate the drug-free hair with FEN, which involved immersing hair in FEN solution and then allowing the sample to air dry. When preparing externally contaminated HRM, it can be a challenge to ensure that the drug is equally distributed across the surface of the hair strand. Inhomogeneity of drug applied to the hair surfaces could have contributed to elevated variance in recovery seen with some design points. An additional drawback of DoE is that binary (rather than three or more) comparisons of parameters are generally performed in order to make the size of the experiments manageable. One common approach, as was done here, is to use low and high extremes of the endpoints tested, to maximize the power of the DoE to detect a difference. In the present study, 30-s and 30-min washes were chosen to encompass a range of values used in other method performance experiments for hair reported in the literature.[64, 65, 68, 75, 76]

Previous work in this laboratory examined decontamination efficiency using a DoE approach for amphetamine, cocaine, diazepam, heroin, and Δ^9-THC. When comparing

these data with those of the present study, it is clear that there is not one specific method that is maximally effective for decontamination of all of these compounds.[64, 68, 77] This result is not surprising, considering the varied physicochemical properties of the tested drugs. Nevertheless, some trends are apparent, and a consensus statement can be made that the most effective method for removing multiple drugs of interest from contaminated hair includes one 30-min wash with water followed by three 30-min washes with DCM. This consensus statement may seem counterintuitive, as DCM is hydrophobic and thus may not be able to reach the hair surface without a drying step in between. However, utilizing the shaker during the wash may disrupt water molecules from the hair surface, allowing DCM to access it.

3.5 Conclusion

The present study further established DoE as a useful approach for evaluating individual factors and combinations of variables in method development for forensic hair analysis. In addition, the present study successfully identified an optimized decontamination protocol that can provide potential for consistency in forensic hair analysis methods. Optimal decontamination was accomplished using one 30-min wash with water followed by three 30-min washes with DCM.

4. ASSESSING OPTIMAL FORENSIC HAIR ANALYSIS PRETREATMENT PARAMETERS FOR AUTHENTIC HRM USING A 2^3 FULL FACTORIAL DESIGN

4.1 Introduction

There is currently a lack of consistency in the literature regarding pretreatment methods other than decontamination utilized across multiple laboratories. For example, Tassoni et al. recently analyzed hair for polydrug use by first cutting the hair into snippets, prior to an overnight incubation in 2 mL of 0.1 N HCl solution at 50°C.[78] In contrast, Salomone et al. detected FEN analogues and their metabolites in hair by pulverizing the hair into a powder prior to a 15 h extraction in MeOH at 55°C.[60] Additionally, Kintz et al. recently analyzed for anabolic steroids in finely cut hair followed by an 90 min ultrasonic bath in MeOH at room temperature.[79] These studies are only a small portion of current forensic hair testing procedures reported in the literature. As discussed, a variety of different particle sizes, sample weight/extraction solvent volume ratios, extraction times, and extraction solvents are used across multiple laboratories, increasing bias and decreasing reliability of forensic hair testing. The present study systematically compared such parameters and their effect on extraction recovery of multiple drugs and metabolites.

When assessing methods for forensic hair analysis, matrix-matched standards such as HRM are necessary. Preliminary work in this laboratory utilized incorporated HRM for the purpose of evaluating pretreatment methods for forensic hair analysis because the preparation of such HRM was relatively easy and cost-effective. However, the

incorporated HRM only allowed for the analysis of one parent drug at a time. In contrast, authentic HRM contains multiple drugs of interest, as well as their metabolites. Additionally, while some drugs of interest were not successfully incorporated into the hair, drugs were incorporated into the authentic HRM through natural processes of the body.

4.2 Materials and Methods

4.2.1 Chemicals and Solvents

Authentic HRM was obtained from RTI International (Research Triangle Park, NC). Authentic HRM #1 contained MET, oxycodone (OXY), alprazolam (ALP), and nordiazepam (NORDZP) at levels of 2995, 977, 60, and 179 pg/mg, respectively. Authentic HRM #2 included cocaine (COC), cocaethylene (COCA), norcocaine (NORCOC), p-hydroxycocaine (HYCOC), morphine (MOR), 6-monoacetylmorphine (6-MAM), and hydrocodone (HYCOD) at levels of 906, 31, 38, 18, 527, 696, and 54 pg/mg, respectively. Authentic HRM # 3 contained diazepam (DZP) at 11.4 pg/mg and FEN at 552.7 pg/mg. While this HRM also contained the metabolite norFEN, its concentration was below the LOQ of the analytical method used in this study. Standard solutions of the drugs of interest as well as MET-D5, OXY-D6, ALP-D5, DZP-D5, heroin (HER)-D3, COC-D3, and codeine (COD)-D3 for use as internal standards were purchased from Cayman Chemical (Ann Arbor, MI). LC-MS mobile phases (ammonium formate, water, formic acid, and methanol) and extraction materials (sodium hydroxide and acetonitrile) were HPLC-grade, purchased from Fisher Scientific (Hampton, NH).

4.2.2 Preparation of Incorporated HRM

Eighteen replicate samples of 30 mg of blank, de-identified hair were placed in 20 mL amber scintillation jars. The hair was washed with 1 mL of HPLC water for 5 min on the rotary shaker at 400 rpm 3X, and the solution was discarded after each wash. This was then repeated with 1 mL 2-propanol. Forceps were used to remove the hair samples from the vials and the samples were loosely wrapped in foil and labeled prior to drying overnight in the oven at 37°C. 1X phosphate buffered saline (PBS) solutions were prepared at pH 5, 7, and 9, corresponding to two units below the pKa of FEN, the pKa of FEN, and two units above the pKa of FEN, respectively. The solutions for MET were at pH 7.5, 9.5, and 11.5. Aliquots of 4.995 mL of each buffer was added to six samples. Within each pH group, five of the samples were spiked with 45 µL of 1 ppm FEN or MET. The remaining sample in each pH group was spiked with 45 µL of MeOH to serve as a blank. All samples were shaken at 400 rpm for five days at 37°C.

After the five days, a 1 mL aliquot of the incorporation buffer was collected and analyzed using LC-QqQ-MS. Each hair sample was washed 3X with 1 mL of the appropriate PBS solution, followed by 3X with 1 mL of 2-propanol, and the washes were analyzed using LC-QqQ-MS. The hair samples were dried in the oven overnight and pulverized into a powder prior to extraction using enzymatic degradation, centrifugation,

SPE, vacufugation, reconstitution in MeOH, and analysis using LC-QqQ-MS to determine incorporated drug levels.

Figure 28. Preparation of incorporated HRM schematic[68]

4.2.3 2^3 Full Factorial Design for Authentic HRM

To determine the optimal extraction parameters for authentic HRM within a given set, a 2^3 full factorial design was used. Aliquots of 20 mg of authentic HRM were used for each sample. The factors under study are shown in Table 5, with A as extraction solvent volume/sample weight ratio, B as particle size, and C as extraction time. The solvent/sample weight ratio used was either 12.5 µL/mg hair (-) or 25 µL/mg hair (+). The hair was pulverized into powder (-) using a ball mill or cut into 1 mm snippets (+) with scissors. The extraction time was 2-h (-) or 24-h (+).

Table 6. 2^3 Full Factorial Design

Design Point	A	B	C	AB	BC	AC	ABC
(1)	-	-	-	+	+	+	-
a	+	-	-	-	+	-	+
b	-	+	-	-	-	+	+
c	-	-	+	+	-	-	+
ab	+	+	-	+	-	-	-
ac	+	-	+	-	-	+	-
bc	-	+	+	-	+	-	-
abc	+	+	+	+	+	+	+

Based on previous data, a solvent swelling method was used for extraction of drug, during which the processed hair was incubated in a mixture of methanol, acetonitrile, and 2 mM ammonium formate (25:25:50, v/v/v) at 37ºC.[68] After extraction, the samples were centrifuged and subjected to SPE, as described in Section 3.2.5, prior to LC-QqQ-MS analysis.

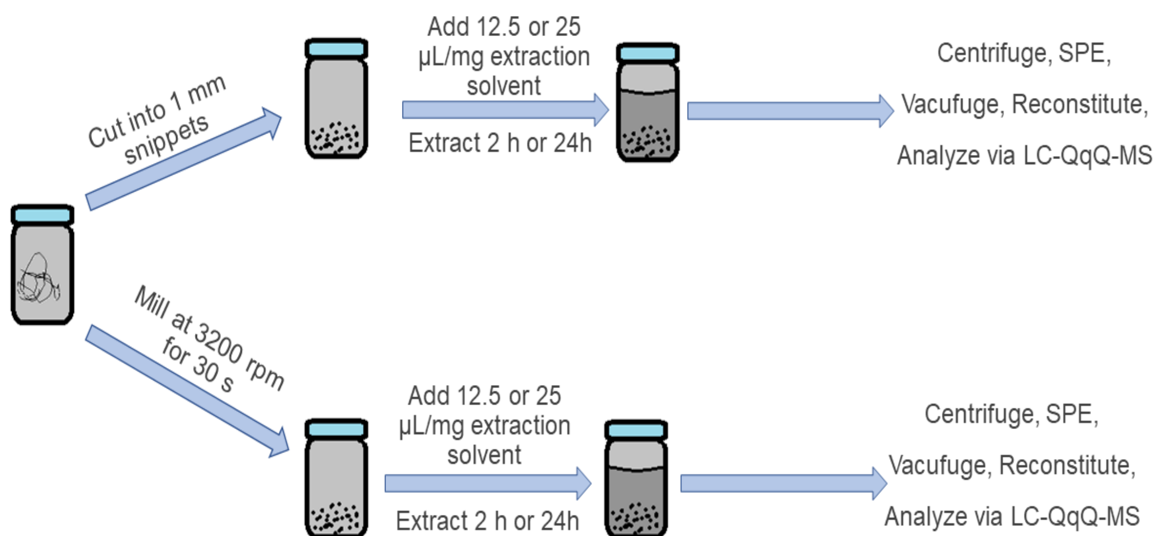


Figure 29. Schematic of pretreatment DoE procedure

Extraction recovery was calculated using the following equation:

$$\left(\frac{pg\ drug\ in\ extract}{\left(\frac{pg\ drug}{mg\ hair}\right) * (20\ mg)}\right) * 100$$

4.2.4 HPLC-MS Analysis

Method protocol for the present study is described in Section 3.2.6. Table 6 shows the retention times and transitions for the drugs of interest relevant to the present study.

Table 7. List of compounds, internal standards, retention times, and *m/z* transitions

Drug	Retention Time (min)	Precursor Ion (*m/z*)	Product Ion 1 (*m/z*)	Product Ion 2 (*m/z*)	Internal Standard
6-MAM	3.30	328	211	165	HER-D3
ALP	5.27	309	281	205	ALP-D5
COCA	4.22	318	196	87	COC-D3
COC	4.03	304	182	82	COC-D3
DZP	5.63	285	222	193	DZP-D5
FEN	4.54	337	105	188	FEN-D5
HYCOD	3.16	300	199	128	COD-D3
MET	3.47	150	119	91	MET-D5
MOR	2.31	286	201	58	HER-D3
NORCOC	4.08	290	136	68	COC-D3
NORDZP	5.57	271	165	140	DZP-D5
OXY	3.08	316	298	241	OXY-D6
HYCOC	3.41	320	182	82	COC-D3

Figure 30 shows a sample chromatogram containing the drugs of interest to the study.

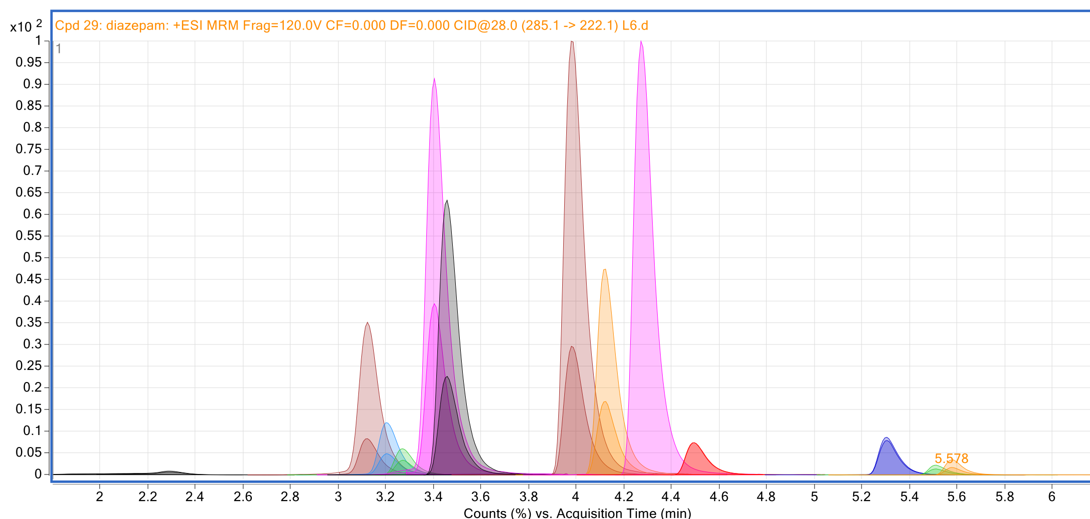


Figure 30. Chromatogram of drugs and metabolites of interest.

All compounds were able to be successfully identified based on RT and major MRM transitions.

4.2.5 Metrics for Performance Comparison

The goal of this work was not to develop and validate a quantitative method for hair analysis, but to compare different pretreatment and extraction parameters and their impact on extraction efficiency using selected performance parameters. Analytical

method optimization was completed using calibration model, bias and precision, matrix effects and recovery, and LOD/LOQ as performance metrics. Calibration curves were created using neat drug standards in MeOH with concentrations of 85, 50, 25, 5, 1, and 0.05 ng/mL. Each solution had a total of 500 µL and included a deuterated internal standard solution at 100 ng/mL. Bias and precision were measured at three different concentration levels (low, medium, and high) over five different runs. Recovery and matrix effects for extracted FEN were evaluated based on the method developed by Peters et al.[80] These studies included extract from drug-free hair as well as neat drug in MeOH. The lowest level of the calibration curve (0.05 ng/mL) was reported as the LOD and LOQ.

4.3 Results

4.3.1 Analytical Method Performance

Bias, precision, matrix effects, and recovery were evaluated for all drugs, metabolites, and internal standards of interest, as shown in Table 8. Bias and precision for all analytes were within ±20% and acceptable according to current guidelines.[67] As shown in Table 8, almost all matrix effects and recovery values fell within the acceptable values, <20% and ±20% respectively, indicating that this method was effective in the presence of extracted hair matrix. The LOD and LOQ were found to be at the lowest calibrator level, 0.05 ng/mL.

Table 8. Bias, precision, matrix effects, and recovery for drugs of interest

Drug	Concentration (ng/mL)						ME (%) (n=2)	CV (%) (n=2)	R (%) (n=3)
	1 (n=3)		8 (n=3)		15 (n=3)				
	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)			
6MAM	1.4	1.3	4.0	1.3	1.1	0.3	7	11	125
ALP	0.6	1.1	0.7	1.5	0.8	0.6	1	5	91
COCA	1.2	0.5	1.9	0.9	0.8	0.6	2	15	101
COC	1.2	1.0	2.3	1.5	1.1	1.0	5	16	126
DZP	0.5	1.6	2.5	1.1	0.3	1.1	0	34	103
FEN	1.5	0.7	3.2	0.8	0.8	0.5	12	2	123
HYCOD	0.4	3.4	0.8	3.3	0.0	2.7	3	24	87
MET	2.7	4.7	3.7	1.5	1.7	2.0	19	4	90
MOR	4.0	13.2	5.5	14.8	6.3	8.4	16	35	110
NORCOC	0.9	1.1	2.5	0.8	0.8	0.7	5	2	125
NORDZP	0.7	1.2	2.4	1.5	0.5	1.1	12	22	110
OXY	0.1	1.0	1.0	1.3	0.4	0.8	5	19	115
HYCOC	1.2	0.5	2.7	1.1	1.0	0.6	27	5	102

4.3.2 Incorporated HRM

Table 9 shows the recovery (%) of MET from the incorporated HRM prepared at each pH level.

Table 9. Recovery (%) of MET from incorporated HRM

pH of buffer	Recovery (%) Extract ± S.D.
7.5	86 ± 17
9.5	58 ± 18
11.5	66 ± 21

As shown in Table 9, MET most effectively incorporated at pH 7.5. In contrast, FEN was not able to be incorporated into the blank hair. Ultimately, this incorporated HRM was not used for further experiments, because authentic HRM containing all drugs of interest became available from a commercial source.

4.3.3 ANOVA F-Tests

Table 10 shows the results of the ANOVA F-tests for all the drugs of interest. P-

values < 0.05 shown in Table 10 were considered statistically significant and are denoted in bold font. Residuals plots were completed (not shown); with the exception of a few points, equal variances were noted, indicating that the conclusions made were valid and that ANOVA was the appropriate test for analysis of the data.

As shown in Table 10, 6-MAM, ALP, COC, HYCOD, MOR, and OXY had individual factors, as well as factors in combination with each other, found to be statistically significant. Additionally, MET and NORCOC had factors in combination with each other found to be statistically significant. These data indicate that DoE was a valuable approach for determining the most effective methods for extraction of these drugs from authentic HRM. In contrast, COCA, DZP, FEN, NORDZP, and HYCOC, had individual factors or no factors found to be statistically significant, indicating that the most effective methods for extraction of these drugs from authentic HRM could have been evaluated using an OVAT approach.

Table 10. ANOVA F-test results for drugs and metabolites in authentic HRM.

6-MAM		ALP		COCA	
Source Factor	p-value	Source Factor	p-value	Source Factor	p-value
1	0.8827	1	**0.0000**	1	0.3965
A	0.7312	A	**0.0006**	A	0.2608
B	**0.0000**	B	**0.0000**	B	0.5980
C	**0.0000**	C	**0.0052**	C	0.4906
AB	**0.0000**	AB	**0.0000**	AB	0.5451
AC	**0.0001**	AC	**0.0004**	AC	0.1359
BC	**0.0000**	BC	**0.0000**	BC	0.8480
ABC	**0.0000**	ABC	**0.0000**	ABC	0.4313
COC		DZP		HYCOD	
Source Factor	p-value	Source Factor	p-value	Source Factor	p-value
1	0.1505	1	0.2232	1	**0.0233**
A	0.7700	A	**0.0303**	A	0.2539
B	**0.0000**	B	0.6047	B	**0.0000**
C	**0.0000**	C	0.5025	C	**0.0001**
AB	**0.0000**	AB	0.1286	AB	**0.0000**
AC	**0.0001**	AC	0.1431	AC	**0.0009**
BC	**0.0000**	BC	0.8045	BC	**0.0000**
ABC	**0.0000**	ABC	0.4282	ABC	**0.0000**
MET		MOR		NORCOC	
Source Factor	p-value	Source Factor	p-value	Source Factor	p-value
1	0.6787	1	0.9965	1	0.6158
A	0.3938	A	0.8399	A	0.4310
B	0.5284	B	**0.0008**	B	0.2808
C	0.4326	C	**0.0108**	C	0.1674
AB	0.6569	AB	**0.0073**	AB	0.4333
AC	**0.0042**	AC	0.0705	AC	**0.0478**
BC	0.4464	BC	**0.0008**	BC	0.2750
ABC	0.6797	ABC	**0.0090**	ABC	0.1395
NORDZP		OXY		HYCOC	
Source Factor	p-value	Source Factor	p-value	Source Factor	p-value
1	0.2895	1	**0.0002**	1	0.5889
A	0.5629	A	**0.0232**	A	0.6629
B	0.6126	B	**0.0000**	B	0.8180
C	0.4879	C	0.0508	C	0.6578
AB	0.6658	AB	**0.0000**	AB	0.2427
AC	0.7755	AC	**0.0355**	AC	0.9567
BC	0.7755	BC	**0.0000**	BC	0.5405
ABC	0.7147	ABC	**0.0001**	ABC	0.3193
FEN					
Source Factor	p-value				
1	0.3402				
A	0.7595				
B	**0.0204**				
C	0.7725				
AB	0.1225				
AC	0.9522				
BC	0.1156				
ABC	0.4502				

Values in **bold** indicate significant factor at p≤0.05.

Residual plots were created for drugs of interest to ensure that ANOVA was the best method for analyzing the data, as shown in Figure 31.

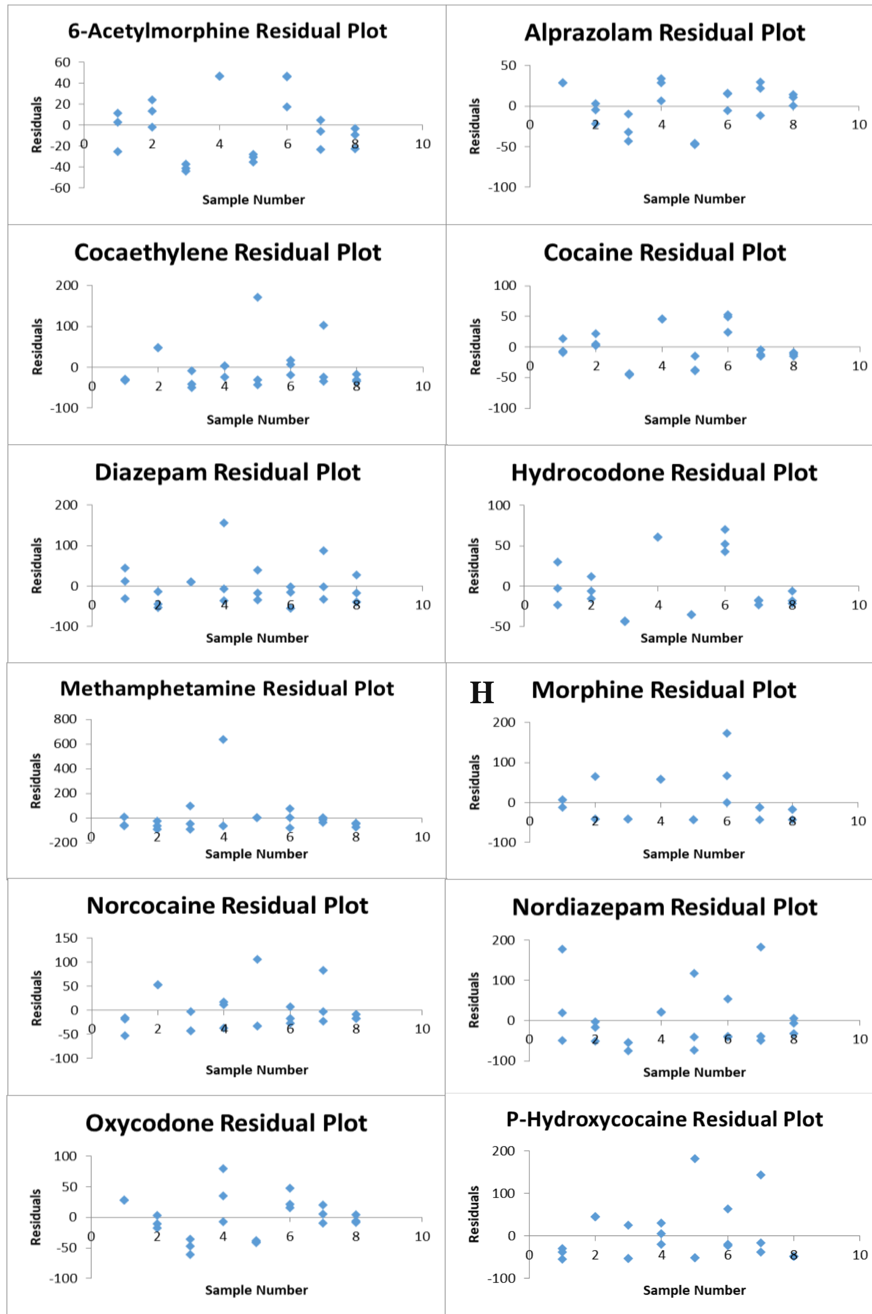


Figure 31. Residual plots for pretreatment parameter ANOVA F-tests

4.3.4 Recovery by Design Point for Pretreatment Parameters

Plots of recovery by design point were created to show the most and least effective conditions with regards to extraction efficiency (Figure 32). For ALP and OXY, highest recovery was observed with design point 1. FEN, COCA, NORCOC, and HYCOC were most effectively extracted from hair using the parameters with design point a. Design point b had the highest recovery for DZP. Additionally, 6-MAM, COC, HYCOD, MOR, and NORDZP were most effectively extracted using the parameters of design point c. For MET, highest recovery was observed with of design point ab.

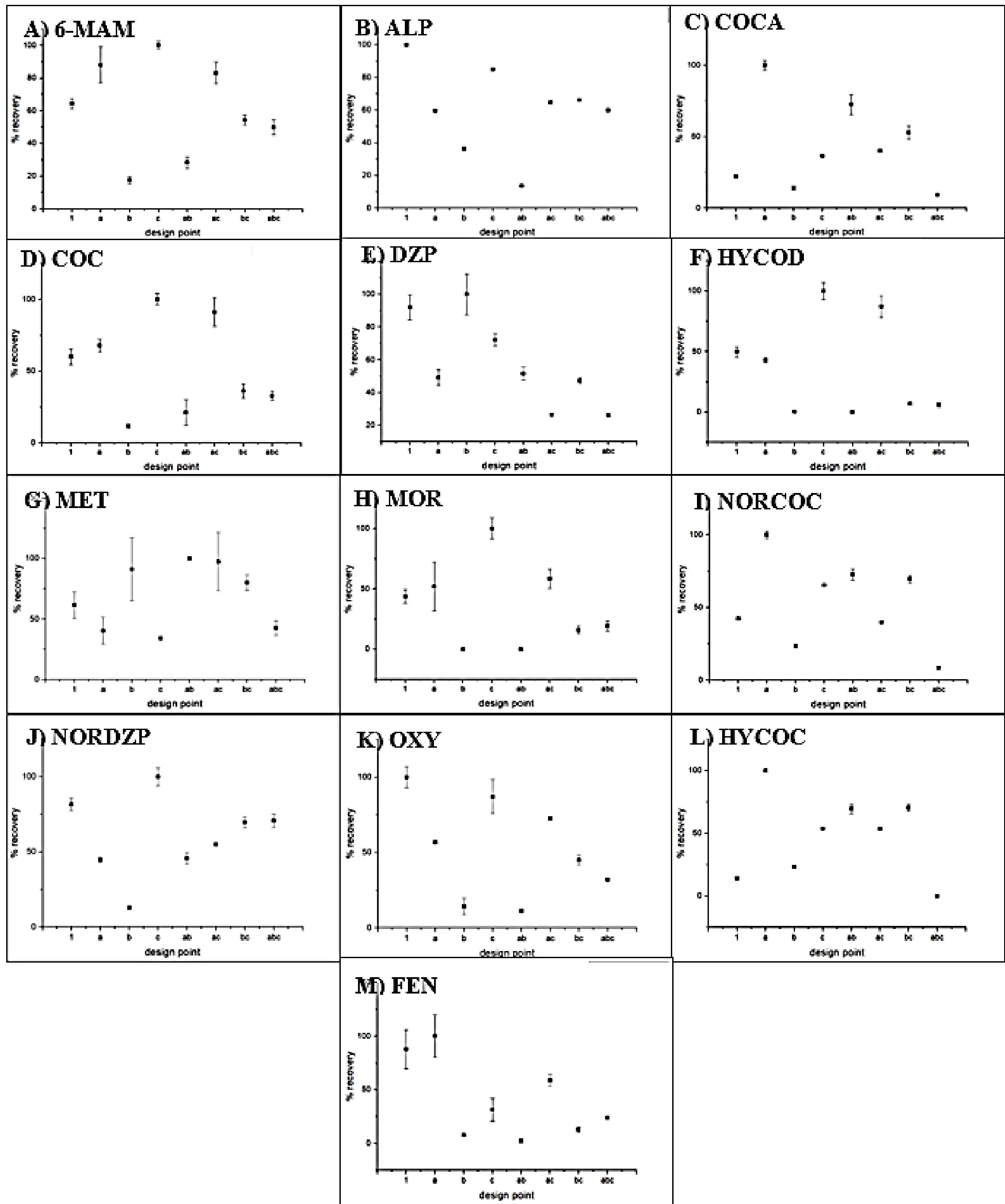


Figure 32. Recovery *vs.* design point for: A) 6-MAM, B) ALP, C) COCA, D) COC, E) DZP, F) HYCOD, G) MET, H) MOR, I) NORCOC, J) NORDZP, K) OXY, L) HYCOC, AND M) FEN

4.3.5 Summary of Pretreatment Parameters

Based on summary DoE data (Table 11), certain trends were noted among the drugs and metabolites of interest extracted from authentic HRM. All of the drugs had high (100%) recovery with at least one combination of extraction parameters (Figure 32). Eight of the drugs had a better recovery with a 12.5 µL/mg solvent volume/sample weight ratio, while five had optimal recovery with a 25.0 µL/mg ratio. Additionally, except for MET and DZP, all drugs were extracted more effectively when the hair was pulverized as compared to snippets. Finally, a 2-h and 24-h extraction time was most effective for eight and five of the drugs, respectively. These data suggest that the best consensus method for extracting multiple drugs from hair would consist of pulverizing the hair prior to a 2-h extraction with 12.5 µL/mg hair solvent volume/sample weight ratio.

Table 11. Extraction parameters resulting in optimal recovery for each drug.

Drug	Source Factor			Recovery (% ± S.D.)
	A	B	C	
6-MAM	12.5 µL/mg	pulverized	24 h	100 ± 2
ALP	12.5 µL/mg	pulverized	2 h	100 ± 0
COCA	25.0 µL/mg	pulverized	2 h	100 ± 3
COC	12.5 µL/mg	pulverized	24 h	100 ± 4
DZP	12.5 µL/mg	snippets	2 h	100 ± 12
FEN	25.0 µL/mg	pulverized	2 h	100 ± 20
HYCOD	12.5 µL/mg	pulverized	24 h	100 ± 7
MET	25.0 µL/mg	snippets	2 h	100 ± 1
MOR	12.5 µL/mg	pulverized	24 h	100 ± 4
NORCOC	25.0 µL/mg	pulverized	2 h	100 ± 3
NORDZP	12.5 µL/mg	pulverized	24 h	100 ± 6
OXY	12.5 µL/mg	pulverized	2 h	100 ± 7
HYCOC	25.0 µL/mg	pulverized	2 h	100 ± 1

4.4 Discussion and Conclusion

Previous literature has been published regarding the creation of incorporated HRM.[17, 68] The advantages of incorporated HRM include cost and availability. The present research intended to compare the DoE results using incorporated HRM to authentic HRM. To accomplish this, incorporated HRM containing MET and FEN needed to be prepared. MET was most effectively incorporated into blank hair at pH 7.5, a pH at which MET is positively charged. This is consistent with literature suggesting that protonated amino groups on basic drugs interact with negatively charged carboxyl groups from melanin.[54] FEN, however, could not be successfully incorporated into blank hair. Since, incorporated HRM most closely represents incorporation of drug through sweat and sebum and not through ingestion of the drug, it was determined that the experiments would move forward utilizing only authentic HRM as the most relevant material.

The analysis of hair for licit and illicit drugs is a complex process, and there are many differing opinions regarding the best methods. Generally, the forensic hair analysis process includes a decontamination step, segmentation/homogenization, isolation of the drug from the matrix, purification of the extracted sample, and instrumental analysis.[3] However, there is no consensus in the literature regarding best practices nor are there comparative studies available that simultaneously compare multiple extraction parameters for common drugs of abuse. The SoHT guidelines for hair analysis only indicate that an organic wash and an aqueous wash should be used during the decontamination and that the sample should be homogenized in some way prior to extraction.[1] However, there are many different ways to meet these criteria. For example, Aleksa et al., Baumgartner et al., Coulter et al., and Dominguez-Romiro et al., all

reported hair analysis methods for amphetamine, cocaine, and opiates. However, each of these groups used different decontamination solvents, including dichloromethane, a water, acetone, and hexane mixture, a methanol and acetone mixture, and shampoo followed by water or acetone.[81-84]

The SoHT also discusses guidelines for hair extraction, specifying that a homogenization step prior to extraction and an extraction technique effective for the drug of interest should be employed.[1] In the same example studies as above, Aleksa et al. used methanol as an extraction solvent, Baumgartner et al. used acidified methanol or KOH, Coulter et al. used PBS at pH 4.2, and Dominguez-Romiro et al. used methanol, HCl, or aqueous NaOH.[81-84] These particular studies are examples a much larger pool of literature reporting a wide range of decontamination and extraction procedures.[3] This lack of consensus in hair analysis protocols can contribute to bias and unreliability in reporting and interpretation of forensic hair testing results.

With regards to the present pretreatment protocols, internal standards were added during the SPE protocol as opposed to before extraction, as more commonly described in the literature when discussing hair testing.[68, 75] This study was not evaluating the extraction method itself, but instead focused on the pretreatment parameters prior to extraction. As there was no viable way to introduce the internal standard prior to homogenization (pulverization or cutting the hair into snippets), and extraction was not of direct interest, the internal standard was added during the SPE protocol. Close to 100% of the drugs of interest were recovered for optimal design points, indicating that it was unlikely that the compounds degraded during incubation and that addition of IS to the hair itself was not warranted.

Some efforts have been reported using OVAT approaches to systematic comparison of hair processing parameters. For example, Eisenbeiss et al. evaluated the best multi-step decontamination, homogenization, and extraction solvent for analytes present in the hair metabolome.[85] However, the OVAT technique only allowed the authors to assess each individual variable and its effect on metabolite recovery; the possible combined effects of these variables on recovery could not be studied. Mantinieks et al. completed four different studies to evaluate the effectiveness of washing solvent, time of wash, and sequence of washes on decontaminating COC and MET from the surface of externally contaminated hair.[86] However, no procedure evaluated in this study was able to comply with the SoHT guidelines while simultaneously removing the contamination.

There are currently only limited reports of DoE being employed in forensic hair testing. Mueller et al. used a Plackett-Burman DoE design, in which representative authentic hair material was used to investigate the impact of ultrasonication, sample solvent, solvent/sample ratio, incubation time, incubation temperature, and hair particle size on the extraction of ethyl glucuronide (EtG), an ethanol metabolite.[75] This study allowed for calculating the effects of individual factors, as well as determining which factors were the most important to the extraction of EtG. However, the Plackett-Burman design does not recognize the significance of interactions between multiple variables and cannot effectively study the combinatorial effects of multiple variables on extraction efficiency. Alladio et al. used a multifactorial experimental DoE design to evaluate the effects of extraction time, temperature, pH, and solvent composition for the extraction of EtG from hair.[76] This work employed hypothesis testing (ANOVA) to determine if the factors were significantly different from each other with regards to extraction efficiency. However, the

study did not evaluate the effects of different types of hair homogenization or extraction solvent volumes on drug recovery.

Previous work in this laboratory by Aijala et al. used an augmented 2^4 factorial block design to systematically evaluate decontamination procedures and extraction parameters for amphetamine, DZP, HER, COC, and Δ^9-THC.[68, 77] It was determined that DoE was particularly useful for this purpose, because the combinatorial effects of the factors were significant.[68, 77] However, Aijala et al. used incorporated HRM, which does not reliably mimic how drugs incorporate into hair *in vivo*. In addition, not all drugs of interest could be assessed, due to issues with preparing incorporated HRM for certain compounds. Work done in the present study expands on these findings by using authentic HRM, which is ideal for evaluating hair extraction procedures for authentic specimens. These HRM can also be used as standards because of the known concentrations of drugs and metabolites present in the hair. Additionally, authentic HRM has the potential to be used in forensic toxicology laboratories for proficiency testing because the identities and concentrations of drugs and metabolites present are known.

In the present investigation, ANOVA F-tests indicated that higher level interactions among two or three individual factors were significant in the extraction of 6-MAM, COC, HYCOD, MOR, OXY, MET, and NORCOC from authentic HRM. These findings, which are consistent with the work done by Alladio et al. and Aijala et al., suggest that studying both individual factors and interactions between factors in hair extraction is pertinent to understanding the most effective parameters for extraction of multiple drugs of interest. The results further reinforce the practicality of the 2^3 factorial design when studying extraction of these multiple drugs and metabolites.[68, 76] In contrast, ANOVA F-tests

suggested that FEN, COCA, DZP, NORDZP, and HYCOC had either single factors or no factors that were statistically significant, indicating that the most effective method for extraction of these drugs could have been evaluated using an OVAT approach or a Plackett-Burman design.

The most effective method for extracting 11 of the 13 drugs examined included pulverizing the hair into a powder prior to extraction. This finding is intuitive and consistent with work done by Salomone et al., who found that extraction of EtG was significantly increased when pulverizing the hair as compared to cutting the hair into snippets.[87] These data align with the concept that when hair is pulverized into a powder, the cuticle, where most drugs bind, is more exposed to the extraction solvent (via increased surface area) than when the hair is cut into snippets.[68, 77] For example, da Rosa Chagas et al. determined that pulverization increased the amount of recovered cocaine and cocaethylene from authentic user hair.[88]

The divergent results for MET and DZP are of note and may involve the impact of physicochemical factors related to drug binding. For example, MET is more strongly basic ($pK_a = 9.9$) compared to the other drugs tested and is likely bound not only to melanin, located in the cortex, but also to other hair proteins.[51] Thus, the swelling of the hair scales accomplished by the solvent extraction technique may have allowed the solvent to reach the cortex and extract the MET without the need for hair pulverization. Additionally, DZP ($pK_a = 3.4$) is essentially neutral at the pH of hair, (~5), and would have weaker interactions with the hair matrix compared to drugs that ionically bind to the matrix, allowing facile extraction without the need for a small particle size. Interestingly, the most effective method for DZP in incorporated HRM as reported by Aijala et al. was

found to be the same as that for authentic HRM in the present study. Evaluation of pretreatment methods using both types of HRM indicated that diazepam is extracted most effectively when the hair is cut into snippets prior to a 2-h extraction in a 12.5 μL/mg extraction solvent/sample size ratio.

Most drugs were effectively extracted using a 2-h, as opposed to a 24-h, extraction time. While this is somewhat counterintuitive, it may be that the longer extraction time allowed for hair matrix components to also be extracted and contribute to interference effects in the LC-MS analysis.[85] Additionally, a 12.5 μL/mg extraction solvent/sample size ratio was most effective for extraction for most drugs of interest. This is consistent with work done by Aijala et al., where amphetamine had the highest extraction efficiency when a 12.5 μL/mg extraction solvent to sample size ratio was used.[68]

A few limitations of this study should be noted. Due to the DoE matrix design chosen, only two levels of each parameter were studied. Additionally, only the solvent swelling extraction method was tested in this work, and the authentic HRM used may not be representative of different hair types and colors present in the general population.

The present study further demonstrated that studying variables both individually and in combination is important in the evaluation of forensic hair analysis methods. As such, DoE was determined to be a valuable approach for determining effective pre-treatment protocols for forensic hair analysis. The most effective method for extracting multiple drugs from authentic HRM was found to include pulverizing the hair into a powder prior to a 2-h extraction with a 12.5 μL/mg extraction solvent/sample size ratio.

5. ASSESSING OPTIMAL EXTRACTION TECHNIQUES FOR FORENSIC HAIR ANALYSIS WITH AUTHENTIC HRM

5.1 Introduction

There are many types of forensic hair extraction techniques discussed in the literature, including enzymatic degradation, acid, base, and buffer extraction, organic solvent extraction, and solvent swelling. Enzymatic degradation works such that the use of the reducing agent dithiothreitol (DTT) in combination with a protease (*e.g.,* proteinase K) can facilitate release of the drug into the hair digest solution without any degradation of the analyte.[89, 90] DTT activates the digesting capability of proteinase K, which causes the hydrolysis of α-keratin, releasing the incorporated drugs from the matrix.[89, 91, 92] Acid and buffer extractions utilize pH to change the charge state of ionizable groups present in the drug molecules, increasing their solubility in aqueous solutions.[93] In contrast, base extraction with NaOH causes damage and removal of hair scales from the cuticle, allowing for the drug to leave the hair matrix.[94] Extraction done with organic solvents, such as MeOH, causes dissolution of neutral and lipophilic compounds, allowing for drugs to leave the hair matrix via diffusion.[95, 96] Finally, solvent swelling allows drugs to solubilize and passively leave the hair cortex along a concentration gradient.[97]

Previous studies in this laboratory demonstrated that enzymatic degradation was more effective than the enzymatic degradation technique for extracting amphetamine.[68] However, that work utilized incorporated HRM, which, as discussed above, may not be representative of how drugs incorporate naturally into hair. In later studies described herein, we reported an optimized pretreatment method using authentic HRM for eleven

drugs and metabolites of interest using the solvent swelling technique. However, no comparisons of other hair analysis extraction techniques were evaluated in this work. Consequently, studies conducted to directly compare the efficacy of solvent swelling, aqueous base, and enzymatic extraction procedures using authentic HRM.

5.2 Materials and Methods

5.2.1 Chemicals and Solvents

Blank, de-identified, black color human hair was obtained from a commercial source (BUW Human Hair, Houston, TX). Authentic HRM was obtained from RTI International (Research Triangle Park, NC). Authentic HRM #1 contained FEN, p-hydroxycocaine (HYCOC), morphine (MOR), 6-acetylmorphine (6-MAM), diazepam (DZP), and nordiazepam (NORDZP) at levels of 552.7 114.7, 2170, 3552, 11.4, and 10.1 pg/mg, respectively. Authentic HRM #2 contained MET and oxycodone (OXY) at levels of 1363 and 1035 pg/mg, respectively. Authentic HRM #3 contained cocaine (COC), cocaethylene (COCA), and norcocaine (NORCOC) at levels of 15968, 225, and 235 pg/mg, respectively. Stock solutions of the drugs of interest as well as FEN-D5, MET-D5, OXY-D6, DZP-D5, heroin (HER)-D3, COC-D3, and codeine (COD)-D2 were purchased from Cayman Chemical (Ann Arbor, MI). Solvents and chemicals used for decontamination washes (1% SDS, water, methanol, and dichloromethane), LC-MS mobile phases (ammonium formate, water, formic acid, and methanol), and some extraction solvents (sodium hydroxide and acetonitrile) were HPLC-grade, purchased from Fisher Scientific (Hampton, NH). 3 kDa molecular weight cut off PTFE spin filters were purchased from MilliporeSigma (Burlington, MA, USA). 1,4-Proteinase-K was

ordered from Invitrogen (Carlsbad, CA, USA), and Dithiothreitol (DTT) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

5.2.2 Extraction Techniques

Approximately 20 mg of authentic HRM were weighed into steel milling jars with steel milling beads. The samples were milled at 3200 rpm for 30 s to pulverize the hair into a powder. 12.5 µL/mg of the appropriate extraction solvent was added to the milling jars, and the extraction was conducted for 2 h at 37°C.

Three different extraction techniques were evaluated, each in triplicate. Enzymatic degradation was completed by incubating the hair in 12 mg/mL dithiothreitol and 2 mg/mL proteinase K (50:50, v/v). To assess solvent swelling, the hair was incubated in a mixture of methanol, acetonitrile, and 2 mM ammonium formate (25:25:50, v/v). Base extraction was completed by incubating the hair in 1 M NaOH.

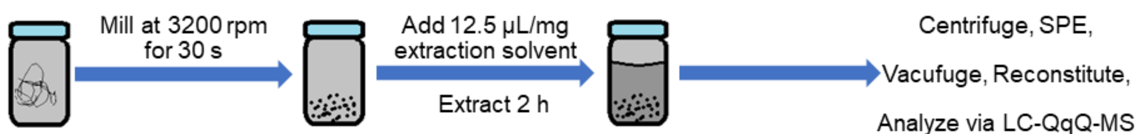


Figure 33. Schematic of extraction methods comparison procedure

After enzymatic degradation and solvent swelling extraction, the samples were centrifuged, subjected to SPE, vacufuged, reconstituted in MeOH, and analyzed using LC-QqQ-MS. After base extraction, the samples were adjusted to pH 7 using HCl prior to centrifugation, SPE, vacufuging, reconstituting in MeOH, and analyzing using LC-QqQ-MS. Extraction recovery was calculated using the equation described in Section 4.2.2.

5.2.3 SPE and HPLC-MS Analysis

SPE was completed according to Section 3.2.5. HPLC-MS method protocols were described in Section 3.2.6, while Section 4.2.3 describes the list of compounds, internal standards, retention times, and *m/z* transitions under study.

5.3 Results

The percent recovery for each drug using each extraction technique is shown in Table 12.

Table 12. Recovery by extraction technique for each drug and metabolite of interest

Drug/Metabolite	HBA[a]	HBD[a]	tPSA[a] (Å^2)	pKa[a]	Log P[a]	% Recovery (± S.D.)		
						Solvent	Base	Enzymatic
6-MAM	5	1	59.0	9.08	1.55	**100 ± 3**[b]	0 ± 0	57 ± 19
MOR	4	2	52.9	9.12	0.87	69 ± 5	65 ± 3	**100 ± 6**
COC	5	0	55.8	8.61	2.30	**100 ± 5**	27 ± 3	78 ± 13
HYCOC	6	2	76.1	9.09	1.90	**100 ± 0**	47 ± 2	10 ± 0
NORCOC	5	1	64.6	9.56	1.73	8 ± 1	0 ± 0	**100 ± 15**
COCA	5	0	55.8	8.77	2.70	40 ± 1	3 ± 1	**100 ± 15**
DZP	2	0	32.7	3.30	2.82	**100 ± 7**	54 ± 3	0 ± 0
NORDZP	2	1	41.5	2.85	2.93	**100 ± 4**	0 ± 0	0 ± 0
OXYCOD	5	1	59.0	8.77	0.70	**100 ± 7**	0 ± 0	41 ± 4
MET	1	1	12.0	9.87	2.07	68 ± 11	45 ± 3	**100 ± 6**

[a] Retrieved from https://pubchem.ncbi.nlm.nih.gov/ or calculated via XLogP3 3.0.
[b] Bolded values indicate highest percent recovery for each technique.

As shown in Table 12, the most effective extraction technique differed from drug to drug. For example, the solvent swelling technique was most effective for 6-MAM, COC, DZP, NORDZP, OXY, and HYCOC, while the enzymatic degradation technique was most effective for COCA, MET, MOR, and NORCOC, and FEN was most effectively extracted using the base technique.

5.4 Discussion

The mode of drug binding in hair can typically include ionic bonding, H-bonding, and hydrophobic/Van der Walls interactions.[14, 44, 56, 98] These in turn will be influenced by pH of the extraction solution and pKa of the drug, the presence or absence of melanin and other keratin-associated proteins, and additional physicochemical factors related to the individual hair specimen.[3] Despite this basic knowledge, the precise mode(s) of binding for the majority of abused drugs is still only poorly understood. One exception may be MET, where previous studies have indicated that bonding with anionic moieties in melanin may be critical.[49] Consequently, it is difficult to predict or account for differences in the relative efficiencies of hair extraction procedures using various techniques for individual drugs. Nevertheless, the present study has clearly revealed optimal extraction conditions for the selected compounds.

The mean recoveries for each drug and extraction technique are shown in Table 12, along with available physicochemical data. As shown in Table 12, the most effective extraction technique differed from drug to drug; there was not one single extraction technique found to be effective for all of the drugs and metabolites of interest. However, the solvent swelling technique was most effective for six of the compounds (6-MAM, COC, DZP, NORDZP, OXY, and HYCOC), while enzymatic degradation was most effective for MOR, NORCOC, COCA, and MET. In contrast, base treatment was only optimal for FEN. In some cases, differences in the efficiency of one extraction technique compared to the others were quite marked, for example with NORCOC (enzymatic) and NORDZP (solvent). In other cases, reasonable recovery was noted with all three methods (*e.g.,* MOR and MET).

The data in Table 12 indicate that there was no clear correlation between optimal extraction method and pKa. Based on pKa values, at pH 4.5, the pH of the solvent swelling extraction solution, eight of the tested compounds would be >99% positively charged. In contrast, DZP and NORDZP would be present in both neutral and positively charged states. Disruption of ionic binding of positively charged compounds to hair components, leading to enhanced recovery, might be expected to occur with base extraction, yet this was not consistently observed in the present study. It can therefore be assumed that extraction efficiency is not a simple function of charge state alone. Scatterplots (Figure 34-38) also show no clear correlations between logP, pKa, HBA, HBD, and tPSA values and extraction recovery. One might predict that the most lipophilic compounds (*i.e.,* highest logP) would prefer solvent extraction. While this was true for DZP and NORDZP (logP values of 2.82 and 2.93, respectively), OXY, the most polar compound tested (log P 0.70) also exhibited optimal extraction with the solvent swelling technique.

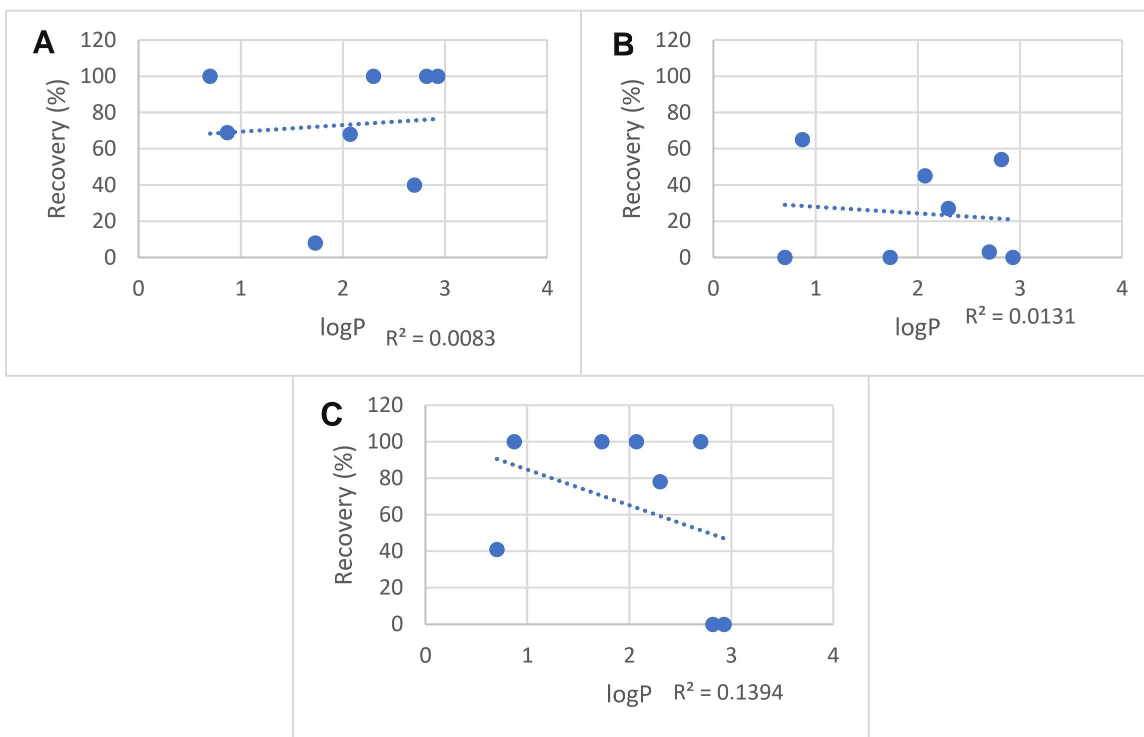


Figure 34. Recovery % vs. logP for A) solvent swelling extraction, B) base extraction, and C) enzymatic degradation extraction

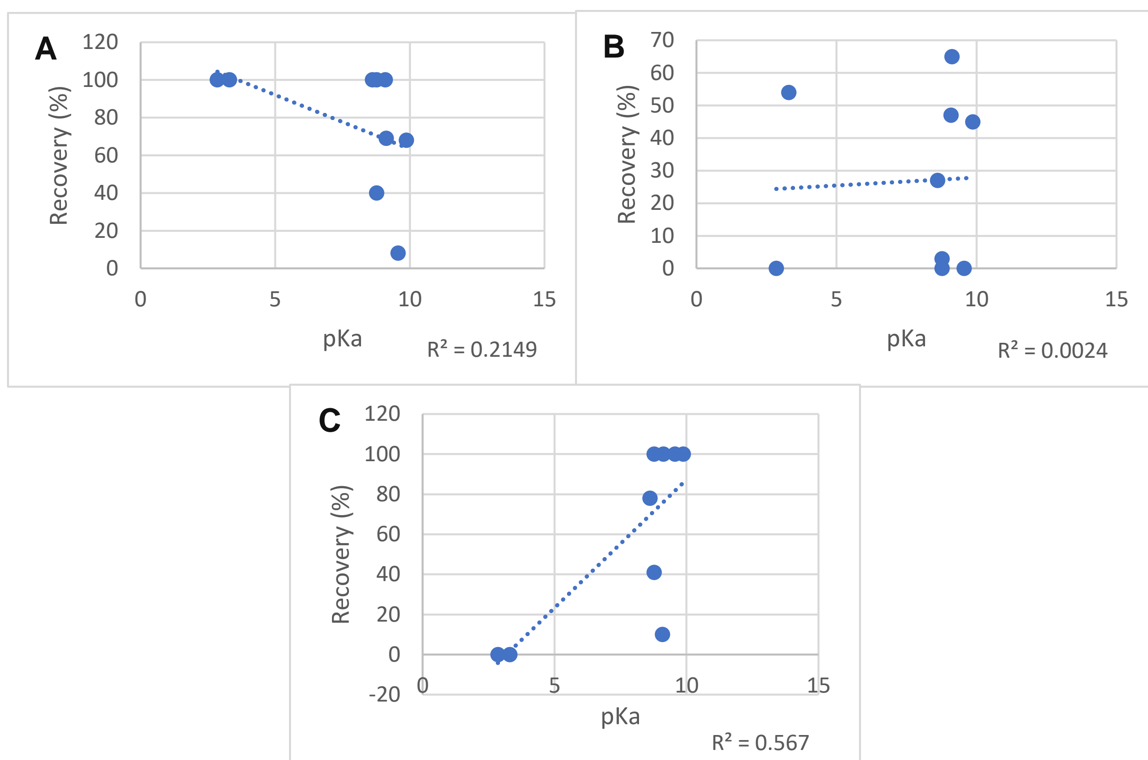


Figure 35. Recovery % vs. pKa for A) solvent swelling extraction, B) base extraction, and C) enzymatic degradation extraction

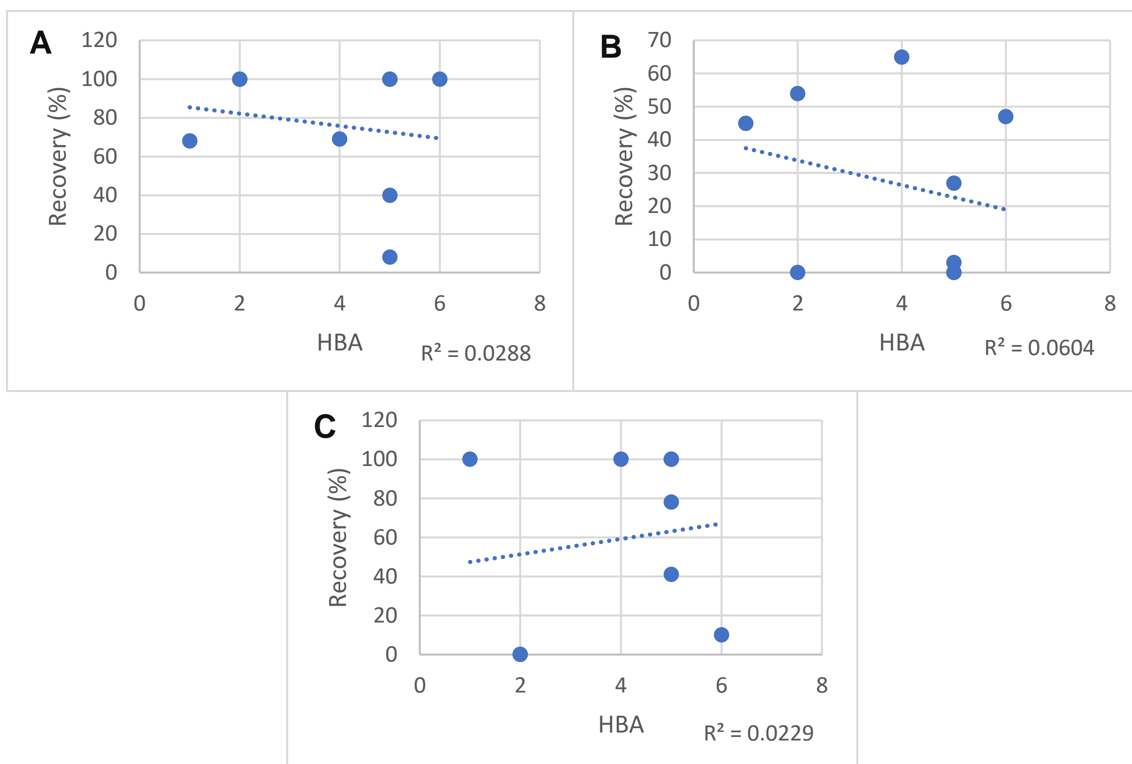


Figure 36. Recovery % vs. HBA for A) solvent swelling extraction, B) base extraction, and C) enzymatic degradation extraction

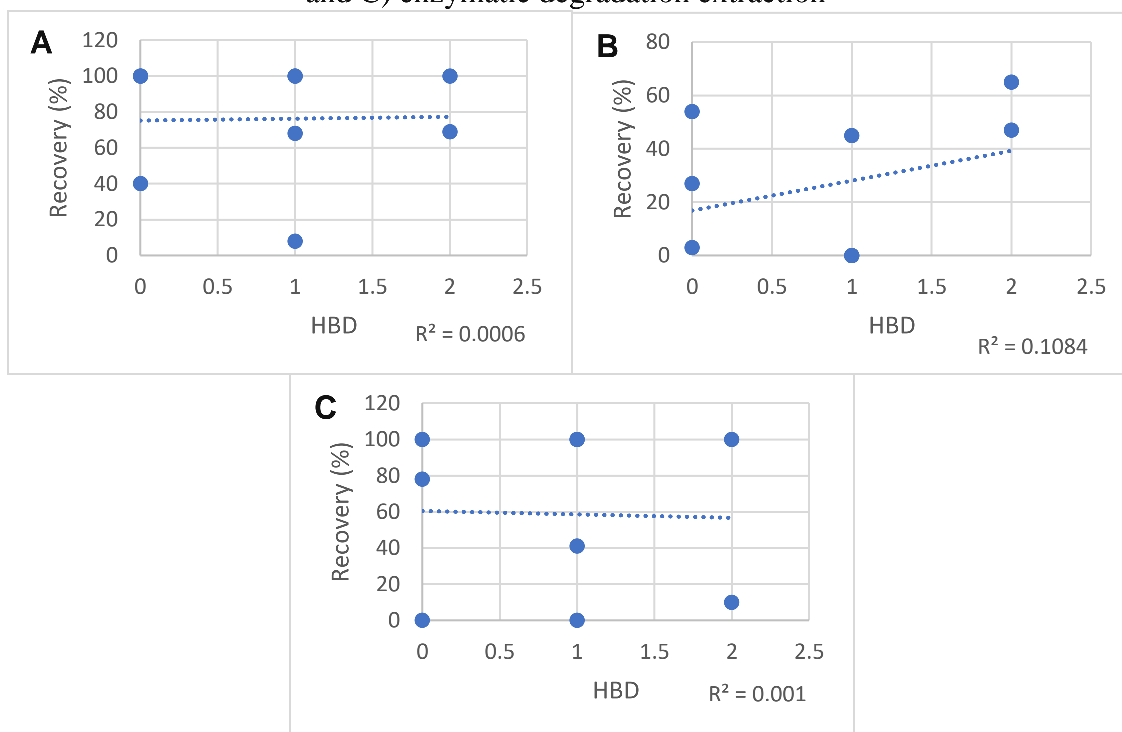


Figure 37. Recovery % vs. HBD for A) solvent swelling extraction, B) base extraction, and C) enzymatic degradation extraction

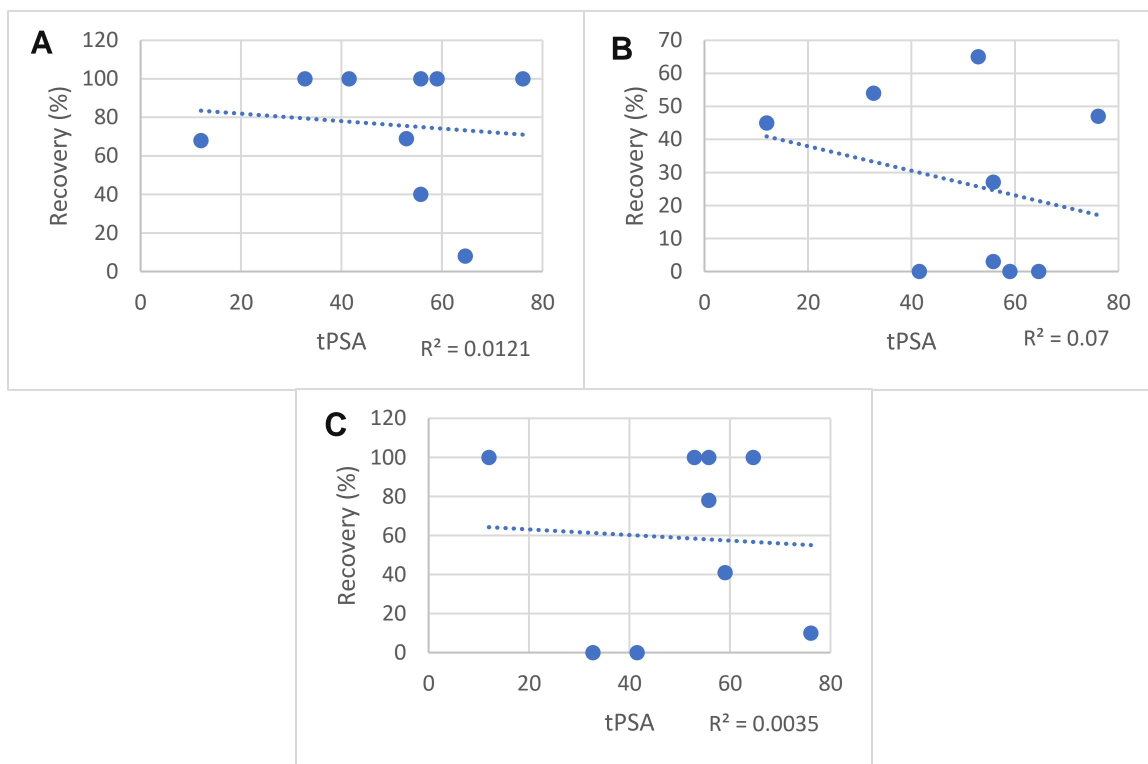


Figure 38. Recovery % vs. tPSA for A) solvent swelling extraction, B) base extraction, and C) enzymatic degradation extraction

Of particular interest are the observed differences in extraction efficiency for COC and three of its metabolites. COC and HYCOC were both extracted optimally using the solvent swelling technique, while NORCOC and COCA were best extracted by enzymatic degradation. There are structural and physicochemical differences among these compounds that could help explain these observations (Figure 39). While COC (logP 2.30) is a methyl ester, COCA (logP 2.70) has an ethyl group, which would facilitate greater hydrophobic interactions with hair components that might be more effectively disrupted by solvent treatment. Furthermore, NORCOC has a secondary amine group on the tropane ring that could better participate in hydrogen bonding with hair components compared to COC. This could help explain the need for harsher enzymatic degradation to disrupt these bonds.

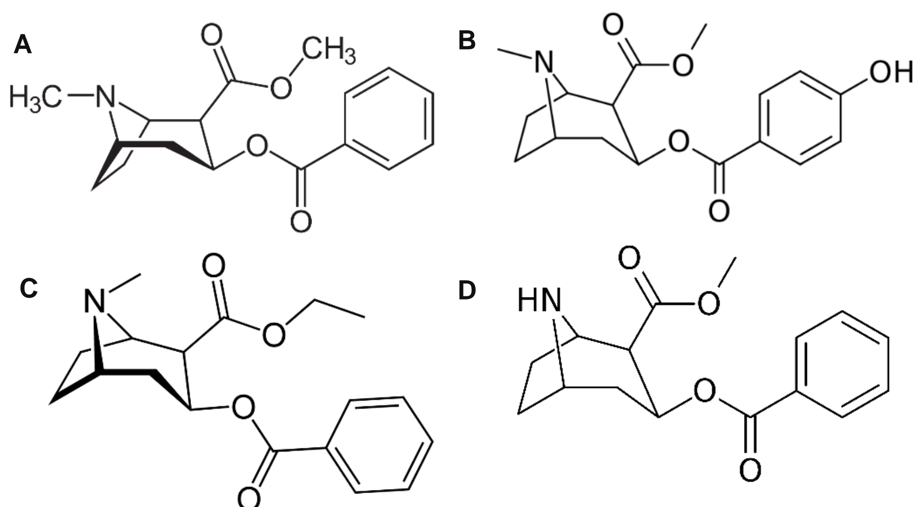


Figure 39. Chemical structures for A) COC, B) HYCOC, C) COCA, and D) NORCOC

In addition, the most effective extraction method for FEN was found to be the base technique. FEN appears to be somewhat of anomaly in this regard, as solvent swelling is generally reported as more effective than base treatment or enzymatic hydrolysis in releasing most drugs from the hair matrix. There are currently no other published data available reporting side-by-side comparison of these three extraction techniques for FEN in hair. The better extraction efficiency in high pH solution is not consistent with previous reports showing lower solubility of FEN as a function of increasing pH.[91] However, it can be hypothesized that aqueous NaOH was most effective for extracting FEN because it may have partially hydrolyzed the keratin in the hair matrix and disrupted hydrogen binding to the protein matrix expected to occur at the 2'-hydroxyl and the piperazine nitrogen on the FEN molecule. In contrast, both the enzymatic and solvent techniques may have been less efficient in the extraction of FEN because they did not as effectively disrupt the molecular interactions between the drug and hair protein.

Based on the results of this study, a consensus statement can be made that solvent swelling may be the best general choice for routine extraction of drugs of abuse from

hair. While some compounds did exhibit better extraction with the enzymatic technique, this is a more labor-intensive procedure that may be more prone to inconsistencies between laboratories. In addition, all of the tested compounds (with the exception of NORCOC) also showed acceptable recoveries with solvent swelling. Finally, our data show that base extraction should probably be avoided as a general extraction procedure for drugs or metabolites in hair.

Some limitations of this work should be discussed. While there are other available methods for isolating drug from hair matrix, such as acid, buffer, and organic solvent extractions, only three extraction techniques were evaluated. Additionally, only ten drugs/metabolites were investigated; additional compounds (such as THC and metabolites) might exhibit more disparate results. These data also emphasize the need for further understanding of the binding interactions between drugs with different physicochemical properties and the hair matrix. However, a consensus statement was made that the majority of drugs and metabolites of interest were most effectively extracted using the solvent swelling technique.

5.5 Conclusions

In conclusion, the most effective extraction technique for these drugs of interest varied based on their physicochemical properties. When optimizing pretreatment parameters for extracting drug from authentic HRM, the solvent swelling technique is most effective overall. This work provides potential for consistent standard procedures for forensic hair testing.

6. STATISTICAL COMPARISON OF OPTIMAL AND LEAST EFFECTIVE FORENSIC HAIR ANALYSIS METHODS USING SINGLE DONOR AUTHENTIC USER HAIR

6.1 Introduction

The SoHT gives general guidelines regarding the best practice methods for forensic hair analysis.[1] They suggest that at least one organic and one aqueous wash should be used for decontamination, and that the hair should be homogenized prior to extraction. However, it is up to the discretion of individual laboratories to determine what methods to utilize based on these guidelines. For example, when analyzing for cocaine, amphetamine, and opiates, Zhuo et al. and Wang et al. decontaminated the hair with water followed by acetone.[99, 100] In contrast, Erne et al. and Rosado et al. washed with water, acetone, and hexane, and deionized water and methanol, respectively.[101, 102] Zhuo et al. and Wang et al. extracted drug from hair using MeOH, while Erne et al. and Rosado et al. used methanol and formic acid-formate buffer, and hydrochloric acid, respectively.[99-102] These papers are a small snapshot of the literature, and emphasize the lack of consistency for forensic hair analysis methods across multiple laboratories, a factor that contributes to bias in forensic hair analysis.

Previous work in this laboratory utilized statistical design of experiments (DoE) to assess best practice decontamination and pretreatment forensic hair analysis protocols.[64, 66] Additionally, we compared multiple types of extraction techniques to determine the most effective method. The present work statistically evaluated the developed optimized (Figure 39) and least effective (Figure 40) forensic hair analysis methods using single

donor authentic hair specimens. These studies provide further support for standardization of methods and practices in forensic hair testing for a variety of drugs and metabolites.

6.2 Materials and Methods

6.2.1 Chemicals and Solvents

Ten single donor authentic hair from drug users, blinded with respect to personal information and drug concentrations, were obtained from RTI International (Research Triangle Park, NC). Standards solutions of MET, oxycodone (OXY), alprazolam (ALP), diazepam (DZP), nordiazepam (NORDZP), cocaine (COC), cocaethylene (COCA), norcocaine (NORCOC), p-hydroxycocaine (HYCOC), morphine (MOR), 6-monoacetylmorphine (6-MAM), hydrocodone (HYCOD), and FEN, as well as MET-D5, OXY-D6, ALP-D5, DZP-D5, heroin (HER)-D3, COC-D3, and FEN-D5 for use as internal standards were purchased from Cayman Chemical (Ann Arbor, MI). LC-MS mobile phases (ammonium formate, water, formic acid, and methanol) and extraction materials (sodium hydroxide and acetonitrile) were HPLC-grade, purchased from Fisher Scientific (Hampton, NH).

6.2.2 Microscopic Analysis of Optimized and Least Effective Methods on Hair

Authentic user specimens were investigated using a Keyence Digital VH-Z100R Microscope to assess microscopic changes that may have occurred during hair treatment procedures. For this analysis, the specimens were washed and extracted using the methods discussed in Section 6.2.3; however, there was no homogenization step prior to extraction and the solvent volume/sample weight ratio was not utilized.

6.2.3 Extraction Methods

Hair samples of 20 mg each were weighed into 1.8 mL steel milling jars. Specimens processed using the previously identified optimized method (Figure 35) were decontaminated with one 30-min wash with HPLC water followed by three 30-min washes with dichloromethane, pulverized into a powder using a Retsch® MM200 ball mill with chrome-steel milling beads at 3,800 rpm for 30 s, and incubated for 2 h in a 12.5 µL/mg solvent volume/sample weight ratio with methanol:acetonitrile:2 mM ammonium formate solution (25:25:50) at 37°C. Specimens processed using the previously identified least effective method (Figure 36) were decontaminated with one 30-s wash with MeOH followed by one 30-s wash with HPLC water, cut into ~1 mm snippets with scissors, and incubated for 2 h in a 25 µL/mg solvent volume/sample weight ratio with 1 M NaOH at 37°C. All samples were centrifuged in 2 mL Eppendorf tubes for 30 min, prior to solid phase extraction using an Agilent Bond Elut LRC mixed mode C8 and strong cation-exchange (SCX) cartridge, vacuum centrifugation, and analysis using an Agilent 1290/6460 LC-QqQ-MS with an Agilent 1.8 µm Zorbax Eclipse Plus C18 rapid resolution HD column (2.1 x 50 mm; 1.8 µm). Paired T-Tests were performed post-analysis to determine if the optimized and least effective forensic hair analysis methods resulted in significantly different results.

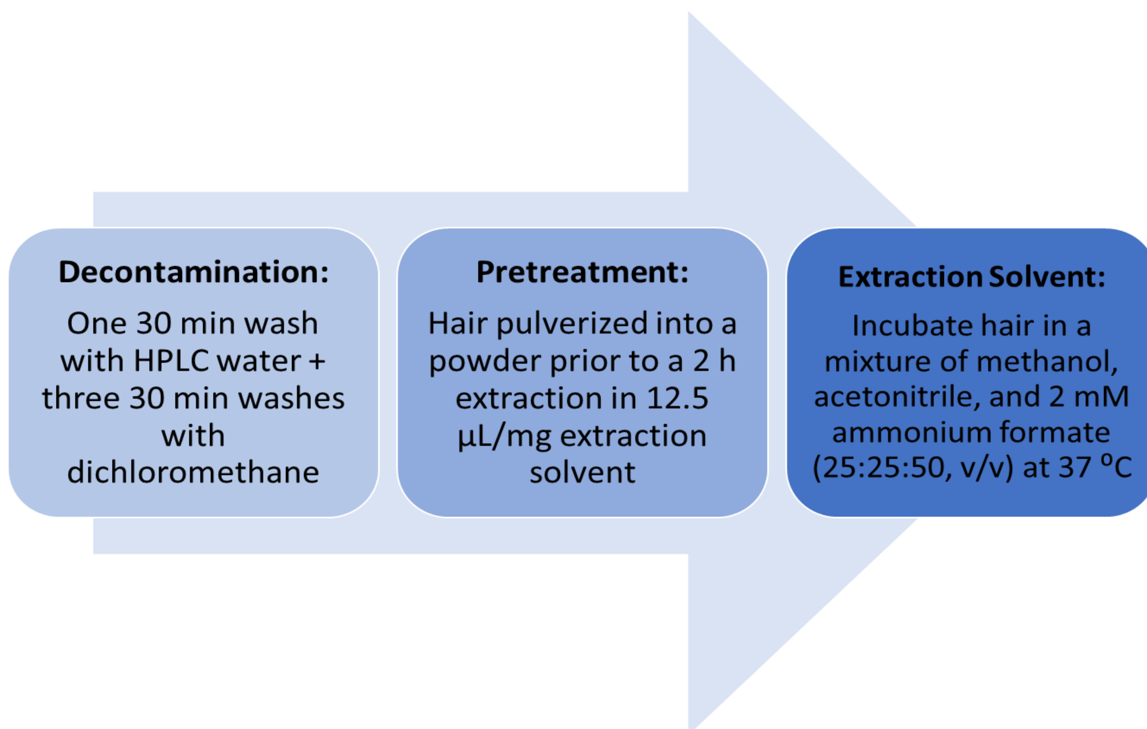


Figure 40. Optimal method for forensic hair analysis

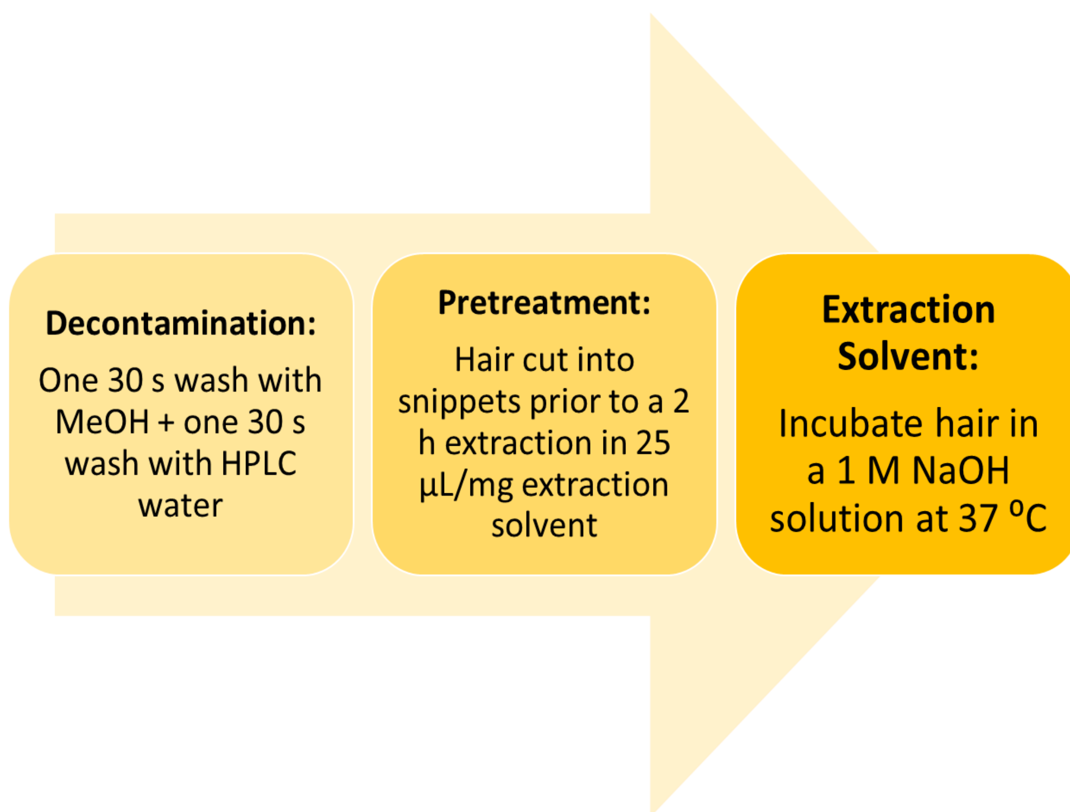


Figure 41. Least effective method for forensic hair analysis

6.3 Results and Discussion

Figure 42 shows the microscopic imaging of hair after receiving the optimized and least effective forensic hair analysis methods. Of particular interest is Figure 42D, where damage done to the hair matrix from 1 M NaOH can be seen, as described in the literature.

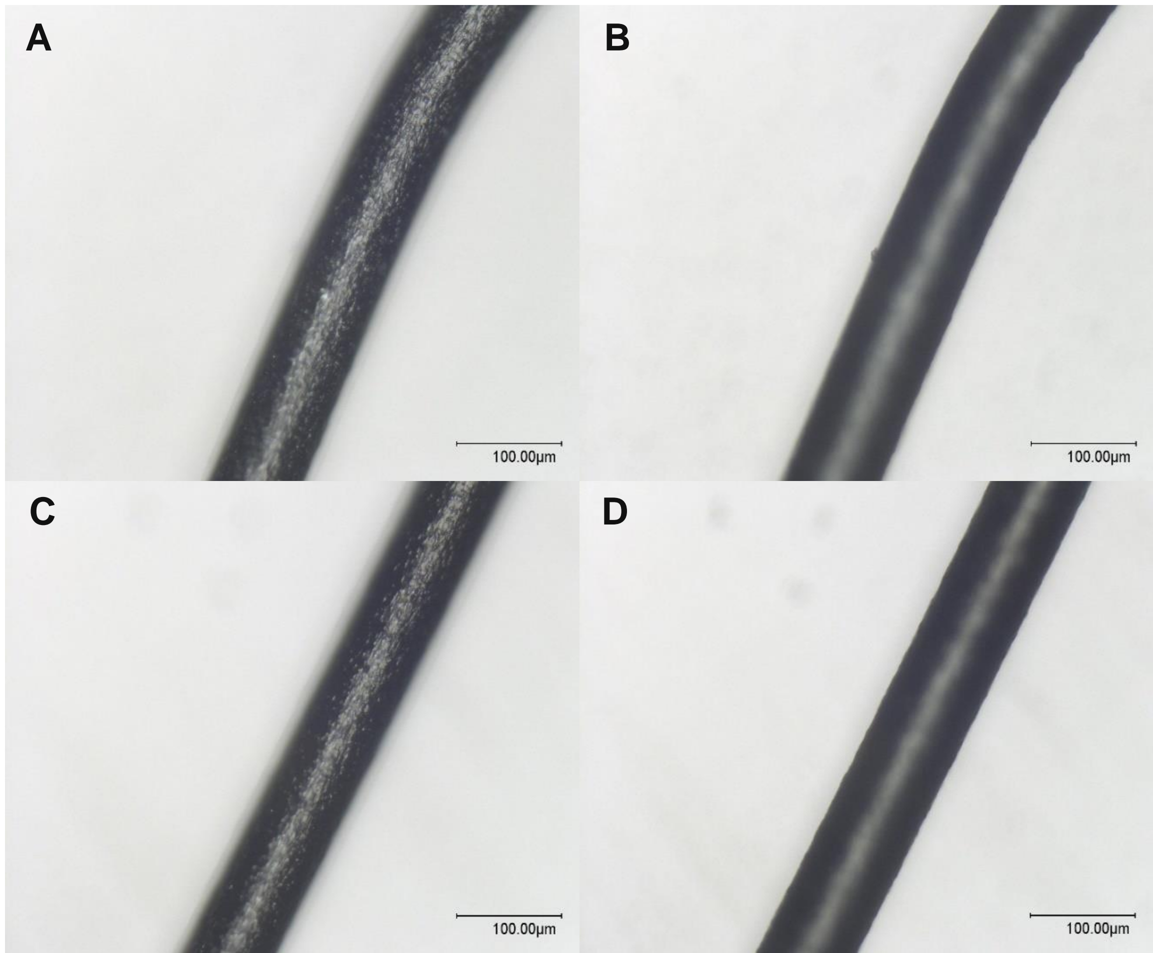


Figure 42. Microscopic imaging at 100X of A) the top of hair after receiving the optimized treatment, B) the side of hair after receiving the optimized treatment, C) top of hair after receiving the least effective treatment, and D) side of hair after receiving the least effective treatment

Figure 43 shows the drug recovery for all ten authentic specimens using the optimized and least effective forensic hair testing methods. Error bars indicate standard deviation.

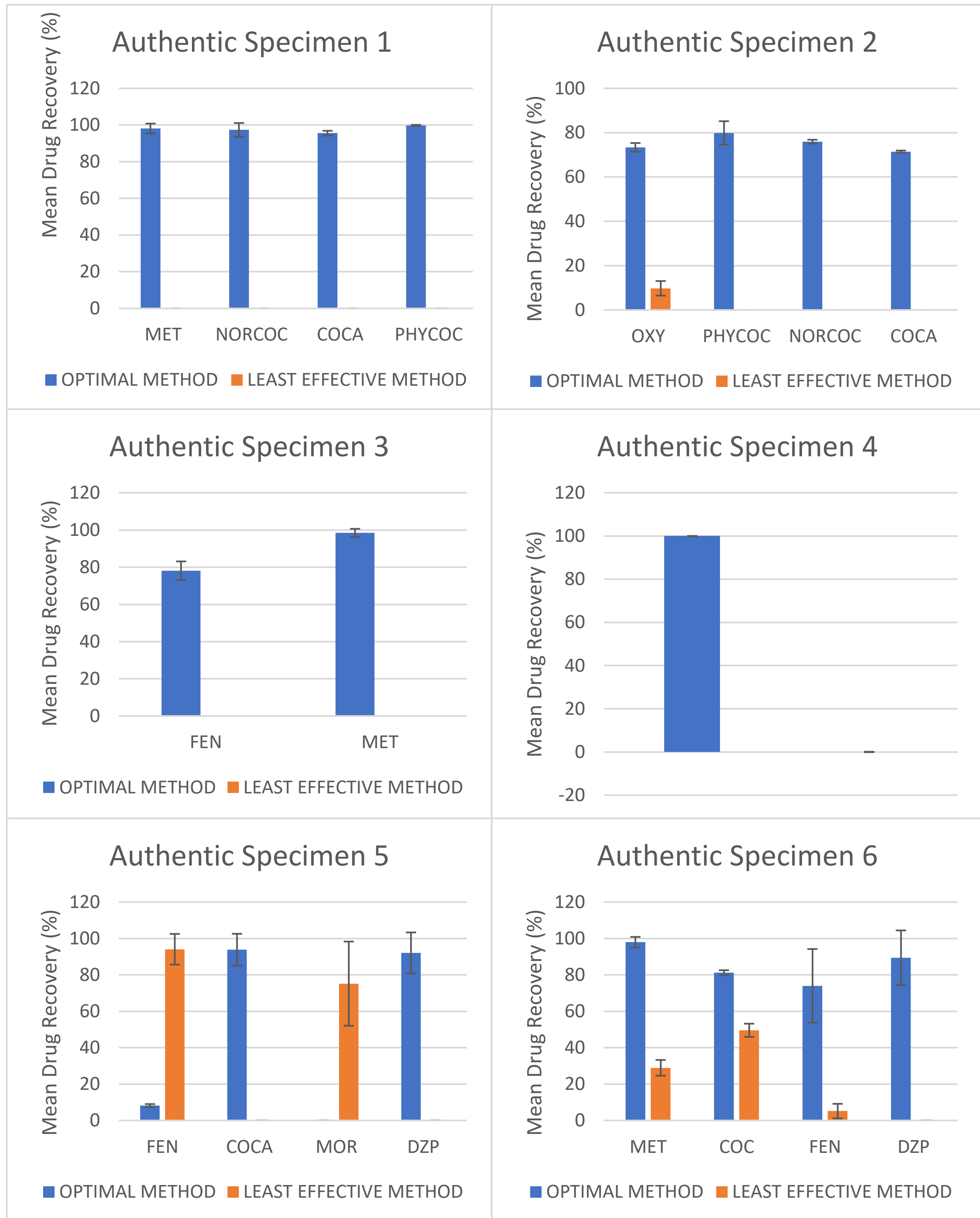


Figure 43a. Drug recovery in authentic specimens using optimal and least effective methods

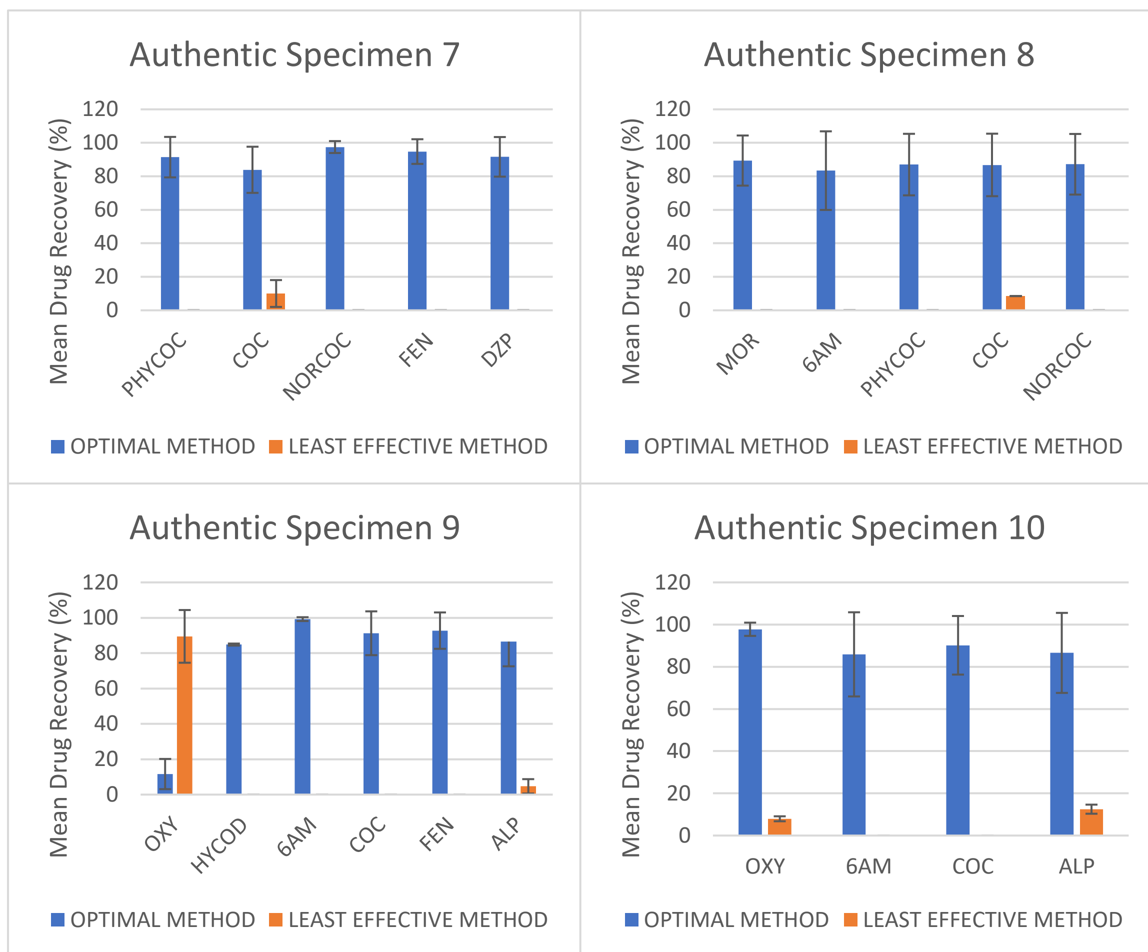


Figure 43b. Drug recovery in authentic specimens using optimal and least effective methods

As shown in Figure 43, the majority of drugs were most effectively extracted using the optimal method. To determine if the differences in recovery using the optimal and least effective methods were statistically significant, Paired T-Tests were completed, as shown in Table 13.

Table 13. Paired T-Test Results for Authentic Specimens

Specimen	Drug	DOF	T
1	MET	1	**63.1**
	NORCOC	2	**2.4**
	COCA	1	**26.4**
	HYCOC	2	1.6
2	OXY	2	**9.9**
	HYCOC	2	**7.8**
	NORCOC	2	**6.7**
	COCA	2	1.6
3	FEN	2	1.8
	MET	1	**78.4**
4	MET	1	**1763**
5	FEN	1	**19.8**
	COCA	2	**4.9**
	MOR	1	**3.7**
	DZP	2	**2.0**
6	MET	1	**85**
	COC	1	0.3
	FEN	2	**2.4**
	DZP	2	**5.0**
7	HYCOC	2	**2.0**
	COC	1	**3.5**
	NORCOC	2	**2.0**
	FEN	1	**22.3**
	DZP	2	0.0
8	MOR	1	**10.3**
	6MAM	1	**6.1**
	HYCOC	2	**2.4**
	COC	1	**3.9**
	NORCOC	2	**2.3**
9	OXY	2	0.2
	HYCOD	2	1.9
	6MAM	2	**2.0**
	COC	2	**2.0**
	FEN	2	**5.5**
	ALP	2	**13.8**
10	OXY	2	**9.5**
	6MAM	1	**7.5**
	COC	1	**11.3**
	ALP	2	**11.7**

The difference between recoveries using the optimal and least effective methods was determined to be statistically significant if T > DOF. These values are indicated in bold.

A summary of recoveries and statistical significance by drug are shown in Table 14.

Table 14. Summary of recoveries and statistical significance by drug

Drug	Highest Recovery With Optimal Method	Statistically Significant
ALP	2/2	1/2
DZP	3/3	2/3
COC	5/5	4/5
COCA	3/3	2/3
NORCOC	4/4	4/4
HYCOC	4/4	3/4
MOR	1/2	2/2
6MAM	3/3	3/3
OXY	2/3	2/3
HYCOD	1/1	0/1
MET	4/4	4/4
FEN	4/5	4/5

The denominator for both columns indicates the number of authentic specimens containing the drug of interest. For example, ALP was present in two authentic specimens. The numerator for the middle column indicates the number of authentic specimens in which the optimal method resulted in higher recovery than the least effective method. For example, ALP was most effectively extracted using the optimal hair analysis method in both authentic specimens it was present in. The numerator in the column on the right indicates the number of authentic specimens in which the difference in recovery between the two methods was statistically significant. For example, there was a statistical difference between the optimal and least effective hair analysis methods for ALP in one of the two specimens it was present in.

As shown in Table 14, the overall recovery of all drugs and metabolites of interest was higher using the optimal method. This indicates a potential for standardization of forensic hair testing for multiple drugs and metabolites. As the optimized forensic hair analysis procedure utilizes the solvent swelling technique, in which drug leaves through the scales

of the hair via passive diffusion, there would be no extraction of matrix components resulting in ion suppression or ion enhancement. These data suggest that there may not be a need for a purification step post-extraction in forensic hair analysis. Additional research should be done evaluating if SPE is a necessary step when utilizing solvent swelling extraction.

An interesting finding of this study is that FEN and MOR in authentic specimen 5, and OXY in authentic specimen 9 did not follow the expected trend. One possibility is that these discrepancies may be related to differences in hair type, color, extent of hair cosmetic treatment, or other individual factors not controlled in the study. Future work should investigate the impact of such variables on optimal forensic hair analysis methods.

6.4 Conclusion

The present study establishes an optimal forensic hair analysis method for multiple drugs and metabolites in authentic user hair specimens. This method consists of decontamination using one 30 min wash with HPLC water followed by three 30 min washes with dichloromethane, pulverizing the hair into a powder, and a 2 h extraction in a 12.5 µL/mg mixture of methanol, acetonitrile, and 2 mM ammonium formate (25:25:50, v/v/v) at 37°C. This work provides potential for consistency in forensic hair analysis methods for multiple drugs and metabolites.

7. ASSESSING RELATIVE LEVELS OF IONIC AND NON-IONIC BINDING OF DRUGS TO AUTHENTIC HRM

7.1 Introduction

It has been proposed that drugs can incorporate into hair in several ways.[10] External incorporation from environmental exposure has been evaluated in multiple studies.[36, 39, 40] Additionally, binding of the drug to hair from sweat and sebum, or through secretions and sebum during growth within the follicle have been assessed.[36, 39, 40] There may also be diffusion of the drug directly from the capillaries surrounding the hair follicle, and potentially the drug incorporating into the hair shaft from the upper dermis.[36, 39, 41-43]

Further research regarding the way in which drugs bind to hair matrix components is needed. Generally, pKa, structure, size, lipophilicity, protein binding capacity, and melanin affinity of the drug affect drug-matrix binding.[10] For the drug to enter the hair matrix, the drug or metabolite needs to be able to cross membranes and a protein barrier to incorporate into the growing hair shaft; neutral drugs more easily pass through these membranes and barriers than charged drugs.[36]

Hair is a complex matrix, so there are likely many ways that drugs bind to hair components.[44] For example, there could be ionic interactions with polar or ionizable functional groups such as amines and carboxylic acids in hair that could be used as binding sites. Neutral polar and lipophilic molecules may bind via hydrogen bonding or other noncovalent interactions to components in the hair matrix. Factors that affect drug-matrix binding are hypothesized to include diffusional barriers, drug lipophilicities, and

hair pigmentation.[44] Finally, to date, published work has not explored mechanisms of binding of drug metabolites in hair.

The original approach planned for this study included separate evaluations of ionic, H-bonding, and hydrophobic modes of drug binding to hair using an equilibrium dialysis-based approach and authentic HRM. However, this approach did not prove successful due to a variety of technical issues. Therefore, the Aim was altered to focus only on determining the relative levels of ionic and non-ionic binding of selected drugs and metabolites to authentic hair reference material (HRM) by assessing recovery of drug from HRM with low salt, high and low buffer pH incubations.

7.2 HRM, Chemicals, and Solvents

A Harvard Apparatus Fast Micro-Equilibrium Dialyzer (250 μL) and regenerated 10 KDa MWCO cellulose membrane were used for equilibrium dialysis studies. Authentic HRM samples were acquired from RTI International (Research Triangle Park, NC, USA) and contained 3,240 pg/mg OXY, 16,526 pg/mg COCA, 92,581 pg/mg COC, 2,338 pg/mg NORCOC, 737 pg/mg HYCOC, 4,675 pg/mg MOR, 12,500 pg/mg 6-MAM, 3,681 pg/mg MET, and 444 pg/mg HYCOD. Standard solutions of oxycodone (OXY), cocaethylene (COCA), cocaine (COC), norcoc (NORCOC), p-hydroxycocaine (HYCOC), morphine (MOR), 6-acetylmorphine (6-MAM), hydrocodone (HYCOD), and MET, as well as MET-D5, OXY-D6, heroin (HER)-D3, MOR-D3, and COC-D3 for use as internal standards were purchased from Cayman Chemical (Ann Arbor, MI). LC-MS mobile phases (ammonium formate, water, formic acid, and methanol) were HPLC-

grade, purchased from Fisher Scientific (Hampton, NH). Pierce C18 ZipTips were purchased from Thermo Scientific (Waltham, MA).

7.3 Equilibrium Dialysis (ED) to Assess Drug-Matrix Binding Using a High Salt Buffer

7.3.1 ED to Assess Drug-Protein Binding

In ED, two solutions are divided by a semipermeable membrane.[103] On one side of the membrane is a binding agent and on the other is the drug solution in a buffer.[103] Typically, phosphate-buffered saline (PBS, pH 7.4) is used.[104] The technique is designed such that only drug molecules can permeate through the membrane until equilibrium is reached.[103]

There are many advantages to using ED, including its ability to evaluate binding of drugs with a range of affinities.[105] Additionally, nonspecific adsorption that may occur during ED is believed to not affect the unbound fraction.[104] A disadvantage of ED is that volume shifts may occur due to the osmotic effect of the buffer molecules, however, this is insignificant when using PBS.[104]

Zamek-Gliszczynski et al. validated a 96-well equilibrium dialysis method with non-radiolabeled drugs (atenolol, diclofenac, diltiazem, imipramine, indomethacin, loperamide, midazolam, nelfinavir, quinidine, sertraline, and warfarin) to assess protein binding in plasma.[106] The % binding was determined for each of the drugs of interest using ED and the following equation:

$$\% \, unbound = \left(\frac{[compound \, in \, buffer]}{[compound \, in \, matrix]}\right) * 100$$

$$\% \, bound = 100\% - \% \, unbound$$

The authors suggest that these methods can be applied to matrices other than plasma.[106]

7.3.2 Determining Time to Reach Equilibrium in ED

7.3.2.1 Method

The cellulose membrane was conditioned using DI water and 1 M NaCl, pH 5. Aliquots of 20 mg of authentic HRM were added with 250 µL of solvent swelling solution to the sample chamber of the dialysis apparatus. An aliquot of 250 µL of 1 M NaCl, pH 5 was added to the dialyzing solution chamber of the dialysis apparatus. The dialysis apparatus was placed on a shaker at 400 rpm. At 30 s, 1 min, 2 min, 3, min, 4 min, 5 min, 30 min, 1 h, and 2 h, 50 µL aliquots of dialysate were removed from the dialyzing solution chamber and put in an Eppendorf tube. An aliquot of 20 µL of 500 ppb internal standard was added to the Eppendorf tube, prior to vacufuging, reconstituting in 50 µL of MeOH, and analysis using LC-QqQ-MS. A time plot of mass of drug recovered versus time was created. The time at which equilibrium was reached was determined as the time at which the concentration of drug reached a plateau.[54]

7.3.2.2 Results and Discussion

Figure 44 shows pg drug vs. time for COC, COCA, and HYCOC.

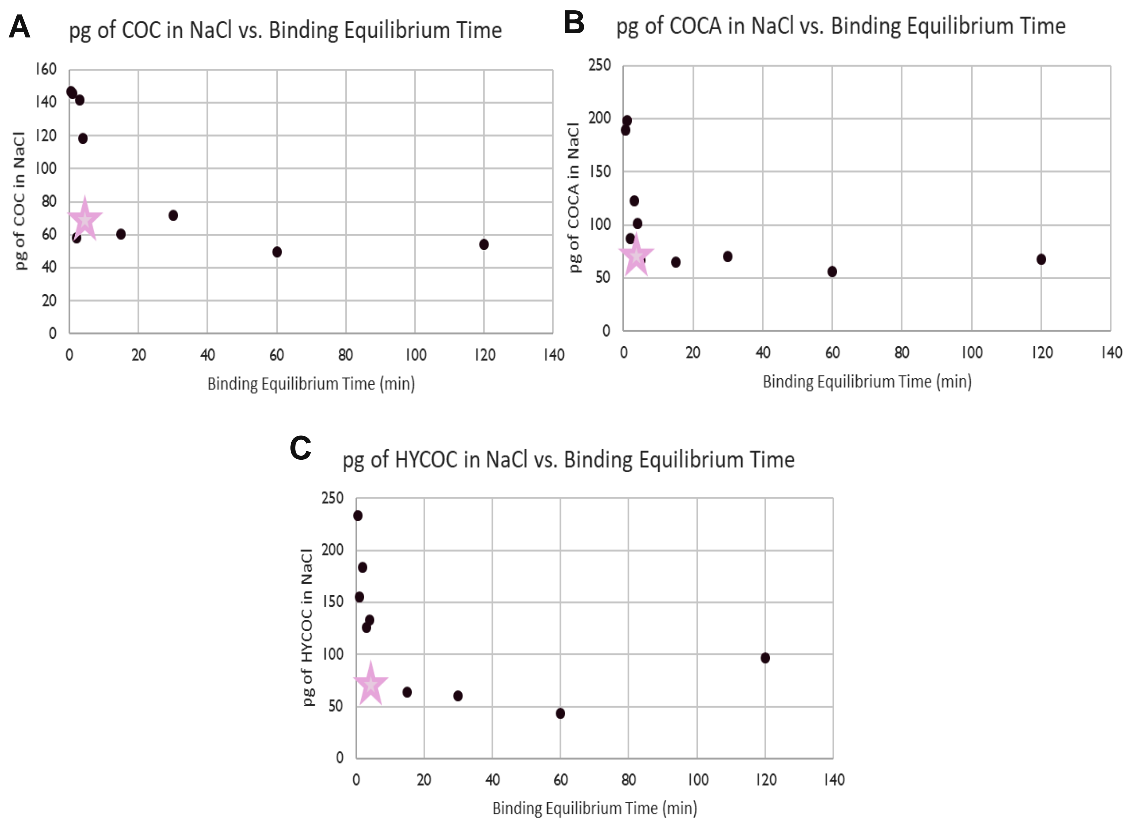


Figure 44. Drug (pg) vs. time (min) for A) COC, B) COCA, and C) HYCOC

As shown in Figure 44, equilibrium was reached for COC and its metabolites at five minutes. However, there was no apparent recovery of MOR, 6-MAM, OXY, or FEN using this technique. It was hypothesized that the overall low recoveries of drugs using this method may be attributed to ion suppression occurring during ESI due to the high salt concentration in the dialysis buffer (1 M NaCl). Therefore, a ZipTip procedure was developed to remove the salt prior to vacufugation, as discussed in Section 7.4.

7.4 Assessing Relative Amounts of Drug-Hair Ionic Binding Using High Salt Buffer

7.4.1 Developing and Evaluating a ZipTip Procedure

7.4.1.1 Method

This method was adapted from a procedure developed by the Advanced Protein Technology Centre, Hospital for Sick Children (Toronto, Canada). The ZipTips were conditioned 7X with 70 µL ACN followed by 7X with 70 µL HPLC water. While the plunger was still down, the ZipTip was filled with 250 µL of a 50 ppb drug + 20 ppb IS mixture in MeOH sample solution and slowly pushed out into the tube 10X. While the plunger was still down, the ZipTip was washed with HPLC water 10X. The wash solution was pipetted out, and with the plunger still down, 100 µL of elution solution 1, (toluene:ethyl acetate; 80:20, v/v), was pipetted in. The solution was held for 20 s prior to being pipetted into an Eppendorf tube. A 10 s drying period was observed before pipetting elution solution 2 (acetonitrile:ammonium hydroxide;100:4, v/v). This same process was repeated with elution solutions 3 (2% ammonium hydroxide in ethyl acetate) and 4 (DCM:2-propanol:2% aqueous ammonium hydroxide; 78:20:2, v/v). Final solutions were evaporated to dryness prior to reconstitution in 250 µL of MeOH and analysis using the LC-QqQ-MS.

7.4.1.2 Results

Table 15 shows the recovery of drugs of interest using ZipTips.

Table 15. Recovery of Drugs of Interest Using ZipTips

Drug	Expected Concentration (ppb)	Average Observed Concentration (ppb ± S.D.)	Accuracy	% Recovery
MOR	50	8 ± 2	-83	17
OXY		102 ± 6	103	203
HYCOD		45 ± 3	-10	90
6-MAM		29 ± 3	-43	57
MET		94 ± 22	88	188
HYCOC		36 ± 6	-28	72
COC		103 ± 4	106	206
NORCOC		143 ± 6	185	285
COCA		145 ± 3	191	291

As shown in Table 15, only HYCOD had an acceptable % recovery, within ± 20%. In contrast, MOR, 6-MAM, and HYCOC showed ion suppression, while OXY, MET, COC, NORCOC, and COCA showed ion enhancement.

7.4.2 Disrupting Ionic Binding Between Drug and Hair Components

7.4.2.1 Method

Twenty mg of authentic HRM were added to a glass test tube with 250 µL of 10X tris-buffered saline (TBS), pH 5. The sample was shaken for 2 h at room temperature and 400 rpm. The samples underwent the ZipTip procedure from Section 7.4.1.1. This was completed in triplicate per HRM. Recovery (%) was determined using the following equation:

$$\left(\frac{\frac{pg\ drug}{mg\ hair}}{concentration\ from\ HRM\ product\ data\ sheet\ \left(\frac{pg}{mg}\right)}\right) * 100$$

7.4.2.2 Results and Discussion

Table 16 shows the recovery of drug present in the dialysate.

Table 16. Recovery of drug in TBS

Drug	Recovery (%)
OXY	0
COCA	0
COC	0.01
NORCOC	0
HYCOC	0
MOR	0
6-MAM	0
MET	0.62
HYCOD	0

As shown in Table 16, only COC and MET had any drug detected in the dialysate, but at <1%. It is hypothesized that the ineffectiveness of the ZipTip salt removal method contributed to these poor recoveries. As an alternative, experiments with low salt buffer at low and high pH were conducted as a basic approach to assess ionic vs. non-ionic interactions between drugs and hair components.

7.5 Assessing Ionic and Non-Ionic Interactions Between Drugs and Hair Using Changes in pH

7.5.1 Method

Aliquots of 20 mg of authentic HRM were added to glass test tubes with 250 µL of 10X PBS, pH 12. At this pH, all drugs and metabolites of interest (COC, HYCOC, OXY, 6-MAM, MET, COCA, NORCOC, MOR) are neutral, since they are basic drugs with pKa values ranging from 8.6-9.9. Samples were rotated at 400 rpm and room temperature for 2 h. The solution was transferred to an Eppendorf tube, followed by the addition of 100 uL of 100 ppb internal standard. The sample was evaporated to dryness, reconstituted

in 250 µL of MeOH, and analyzed using LC-QqQ-MS. This procedure was completed in triplicate per HRM. It is important to note that at pH 12, HER-D3 hydrolyzes; consequently, MOR-D3 was used as an internal standard for MOR and 6-MAM during experiments at this pH. This procedure was replicated using 250 µL of 10X PBS, pH 6. At this pH, all drugs and metabolites of interest (COC, HYCOC, OXY, 6-MAM, MET, COCA, NORCOC, MOR) are cationic, based on their pKa values.

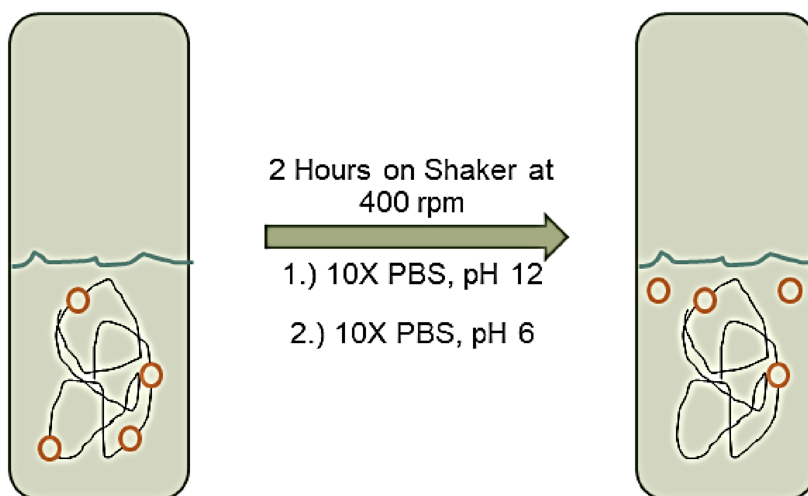


Figure 45. Binding studies design

The absolute recoveries (%) for each drug were calculated according to the following equation:

$$\left(\frac{pg/mg\ (pH\ 6\ or\ pH\ 12)}{(pg/mg\ from\ authentic\ HRM\ product\ data\ sheet)}\right) * 100$$

The relative recoveries (%) for each drug were calculated according to the following equation:

$$\left(\frac{pg/mg\ (pH\ 6\ or\ pH\ 12)}{(pg/mg\ pH\ 6 + pg\ pH\ 12)}\right) * 100$$

7.5.2 Results

Table 17 shows the relative recoveries (%) of drugs at pH 12 and pH 6.

Table 17. Recovery of drugs from HRM at pH 12 and pH 6

Drug	Absolute[a] Recovery (% ± S.D.) pH 12	Absolute Recovery (% ± S.D.) pH 6	Relative[b] Recovery (% ± S.D.) pH 12	Relative Recovery (% ± S.D.) pH 6
COC	**0.115 ± 0.015**	0.006 ± 0.002	**95 ± 2**	5 ± 2
COCA	**0.275 ± 0.004**	0.002 ± 0.000	**99 ± 0**	1 ± 0
HYCOC	**1.194 ± 0.125**	0.000 ± 0.000	**100 ± 0**	0 ± 0
NORCOC	**0.226 ± 0.108**	0.002 ± 0.001	**99 ± 1**	1 ± 1
MET	0.047 ± 0.002	**0.085 ± 0.020**	36 ± 5	**64 ± 5**
MOR	**0.235 ± 0.043**	0.077 ± 0.023	**75 ± 9**	25 ± 9
6-MAM	0.000 ± 0.000	**0.007 ± 0.002**	0 ± 0	**100 ± 0**
OXY	**0.104 ± 0.021**	0.009 ± 0.001	**92 ± 2**	8 ± 2

[a]Calculated based on the pg/mg of drug recovered at each pH and the pg/mg of drug listed in the authentic HRM product data sheet
[b]Calculated based on the pg/mg of drug recovered at each pH and the total pg/mg of drug recovered at both pH values

As shown in Table 17, all drugs, with the exception of MET and 6-MAM, had the highest relative recovery at pH 12. Interestingly, HYCOC and 6-MAM had zero recovery at pH 6 and 12, respectively. In addition, MOR exhibited higher recovery at pH 12, while its metabolite, 6-MAM, had higher recovery at pH 6. It can also be noted that, when calculated based on the concentration of drug provided by RTI, absolute recoveries of all drugs at both pH values were very low. However, the same trends noted with relative recovery were also seen with absolute recovery.

7.5.3 Discussion

Other than MET and 6-MAM, all drugs had recovery at both pH 6 and pH 12. This indicates that with the exception of HYCOC and 6-MAM, all drugs participate in some

degree of ionic and non-ionic binding with hair matrix components. One possibility that should be noted is that HYCOC and 6-MAM had no recovery at pH 6 and 12, respectively, because they were present in very low concentrations in the authentic HRM. Non-ionic interactions with HYCOC could include π-stacking or covalent bonding, while H bonding with the amine group and alcohol, and dipole-dipole interactions with the ester may have occurred on 6-MAM. COC and its metabolites, MOR, and OXY all had higher recovery of drug at pH 12. This indicates that while these drugs exhibit both types of binding, the non-ionic binding between these drugs and hair matrix components is weaker than ionic binding with the matrix, in particular covalent binding or π-stacking between the benzene rings on these drugs and those present in melanin and keratin. In contrast, MET had highest recovery of drug at pH 6, indicating that ionic interactions between MET and the hair matrix are weaker than non-ionic interactions, probably because of H bonding with its amine group.

COC and COCA binding to hair has been previously studied in the literature.[14, 54] In the present study, COCA demonstrated some degree of ionic and non-ionic interactions with hair matrix components. These data could suggest that not only the benzene ring in COCA participates in binding, but also its hydrophobic ethyl group. This is in agreement with findings that demonstrated COCA participates in hydrophobic interactions with melanin's core.[14] However, previous research has only demonstrated ionic interactions between COC and the hair matrix, likely due to the positive charge on the nitrogen.[54] These data are in contrast with the present work, which indicated that some degree of ionic and non-ionic interactions occur between COC and hair matrix components. The

present study is the first in literature to report relative amounts of ionic and non-ionic binding of COC and its metabolites to the hair matrix.

Previous studies have assessed the effect of melanin versus the absence of melanin on binding with MET.[49, 50] Findings suggested that MET binding occurred specifically with melanin, as MET was detected in black hair, not white.[49] Further research suggested that a combination of ionic, covalent, and hydrophobic interactions all played a role in MET binding to melanin.[50] However, the present study was the first in literature to report the relative amounts of ionic and non-ionic binding of MET to the hair matrix.

The effect of melanin presence on binding of MOR to hair has also been evaluated, reflecting the idea that basic drugs are most effectively incorporated into hair in the presence of melanin, probably due to some type of ionic interactions.[107] The present findings provide additional insight that while there are ionic interactions between MOR and the hair matrix, there are also non-ionic interactions. This study is the first to report binding studies data for 6-MAM, HYCOC, NORCOC, and OXY.

One limitation of this study was that only basic drugs were assessed, due to the availability of authentic HRM obtained for this research. Further research should evaluate interactions between acidic and neutral drugs and the hair matrix. Additionally, this work suggests potential sites that these interactions are occurring, however, additional studies should be completed to assess which moieties on the molecules are participating in the specific interactions.

7.5.4 Conclusion

In conclusion, this work reports relative amounts of ionic and non-ionic interactions occurring between COC, COCA, NORCOC, HYCOC, MET, MOR, 6-MAM, and OXY and the hair matrix. These data illuminate potential sites for binding between drugs and metabolites and hair that have not been previously studied in the literature. In addition, this research is the first to report binding studies for 6-MAM, HYCOC, NORCOC, and OXY. These data provide additional understanding regarding drug-matrix interactions, which is imperative to understanding and bettering forensic hair analysis methods.

8. SUMMARY AND PROSPECTUS

The lack of consensus regarding best practice methods for forensic hair analysis in the literature is a cause of bias and lack of consistency in hair. However, with the development of consistent protocols and standardization of practices, forensic hair analysis has the potential to improve forensic toxicology, especially with regards to cases requiring a longer window of detection of drugs and the characterization of a history of drug exposure. Thus, the goals of this work were to develop an optimized procedure for forensic hair analysis and to characterize some basic aspects of the relative levels of ionic and non-ionic binding of drugs to the hair matrix.

It was demonstrated that the most effective method for forensic hair analysis of multiple drugs and metabolites includes decontamination using one 30-min wash with HPLC water followed by three 30-min washes with dichloromethane, pulverizing the hair into a powder, and a 2-h extraction in a 12.5 µL/mg mixture of methanol, acetonitrile, and 2 mM ammonium formate (25:25:50, v/v/v) at 37°C. In addition, binding studies suggested that almost all drugs and metabolites are involved in both ionic and non-ionic interactions with the hair matrix, however, COC and its metabolites, as well as metabolites of HER participate in more non-ionic interactions with the matrix than ionic interactions. In contrast, MET participates in more ionic interactions with the hair matrix than non-ionic interactions. The present work is the first to report relative levels of ionic and non-ionic binding of multiple drugs and metabolites.

Future work should include evaluations of additional extraction techniques, as well as evaluating the effects of other parameters involved in hair analysis, such as

ultrasonication. The effects of hair color and other individual hair characteristics on the optimal forensic hair testing protocols should be evaluated. In addition, binding studies should be evaluated for interactions between hair and acidic and neutral drugs. Future binding studies should also prove the types of ionic and non-ionic interactions occurring between drugs and matrix.

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VITA

BRIANNA SPEAR

2014-2018	B.S., Forensic Science University of New Haven West Haven, CT
2018-2019	Teaching Assistant / Head Teaching Assistant Florida International University Miami, FL
2018-2020	M.S., Chemistry Florida International University Miami, FL
2019-2022	Research Assistant Florida International University Miami, FL
2020-2022	Doctoral Candidate Florida International University Miami, FL

PUBLICATIONS AND PRESENTATIONS

Spear, B. and DeCaprio, A.P. (2022). Evaluation of extraction parameters in authentic reference material using statistical Design of Experiments. *Journal of Forensic Sciences*, https://doi.org/10.1111/1556-4029.15051.

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Spear, B. and DeCaprio, A.P. (2022). Evaluation of three extraction techniques for the analysis of 11 drugs and metabolites in authentic hair reference material; *11th Annual Forensic Science Symposium (Virtual)*; June 6.

Spear, B. and DeCaprio, A.P. (2022). Evaluation of three extraction techniques for the analysis of 11 drugs and metabolites in authentic hair reference material; *American Academy of Forensic Sciences 72nd Annual Meeting (Seattle, WA)*; February 24.

Spear, B. and DeCaprio, A.P. (2021). Evaluation of extraction parameters for the analysis of three authentic hair reference materials (HRM) using statistical Design of Experiments (DoE); *Society of Forensic Toxicologists Meeting (Nashville, TN)*; October 1.

Spear, B. and DeCaprio, A.P. (2021). Assessing pretreatment methods in forensic hair analysis; *Crossing Forensic Borders Global Webinar Series (Virtual)*; April 14.

Spear, B. and DeCaprio, A.P. (2021). Assessing pretreatment methods and drug-matrix binding in forensic hair analysis; *NIJ R&D Symposium (Virtual)*; February 15-19.

Hill, B., Panepinto, M.C., and O'Brien, R.C. (2017). Taphonomic assessment of the dissolution of organic remains in nitric acid; *Northeastern Association of Forensic Scientists Meeting 43rd Annual Meeting; Pocono Manor, PA*; November 8-10.

Spear, B. and DeCaprio, A.P. (2022). Optimization of forensic hair analysis methods using statistical design of experiments (DoE); *NIJ R&D Symposium (Virtual)*; March 1-2.

Spear, B. and DeCaprio, A.P. (2021). Evaluation of extraction parameters for the analysis of authentic hair reference material (HRM) in forensic hair testing using statistical design of experiments (DoE); *American Academy of Forensic Sciences 71st Annual Meeting (Virtual)*; February 15-19.

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