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Project Title: Evaluating Analytical Parameters and Understanding Drug-Matrix Interactions in Forensic Hair Analysis

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Summary of the Project

Major goals and objectives: 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 does not necessarily represent the interactions between drug and hair *in vivo* and is not useful for evaluating metabolites in hair or hair samples containing multiple analytes. The present work assessed optimized forensic hair analysis methods using authentic HRM, which is obtained from drug users and generally contains multiple drugs and metabolites. In addition, this work statistically compared the optimized and least effective forensic hair analysis methods applied 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.

Research questions: This research addresses the problem that there are currently no consistent protocols for forensic hair testing of common abused drugs. The hypothesis was that there would not be one consistent optimized forensic hair testing method for all drugs. The research goal was to investigate optimized protocols for decontamination, pretreatment, and extraction of alprazolam, diazepam and nordiazepam,

methamphetamine, cocaine and its metabolites, oxycodone, metabolites of heroin, and fentanyl.

In addition, this research 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 was to assess relative amounts of ionic and non-ionic binding of methamphetamine, cocaine and its metabolites, oxycodone, metabolites of heroin, and oxycodone in hair.

Research Design, Methods, Analytical and Data Analysis Techniques:

Preparation of externally contaminated HRM - Externally contaminated hair was prepared by adding 100 µL of 1 mg/mL fentanyl (FEN) or methamphetamine (MET) in methanol to 20 mg of drug-free hair in an Eppendorf tube (Figure 1). 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.

Evaluating optimal forensic hair analysis decontamination techniques using 2^4 fractional factorial block DoE protocol - A 2^k factorial design of experiment (DoE) protocol 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.

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.

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. 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, $x_i = 0$ (low level), and $x_i = 1$ (high level). 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. 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 1. 2^4 Fractional factorial block DoE protocol.

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	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

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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.

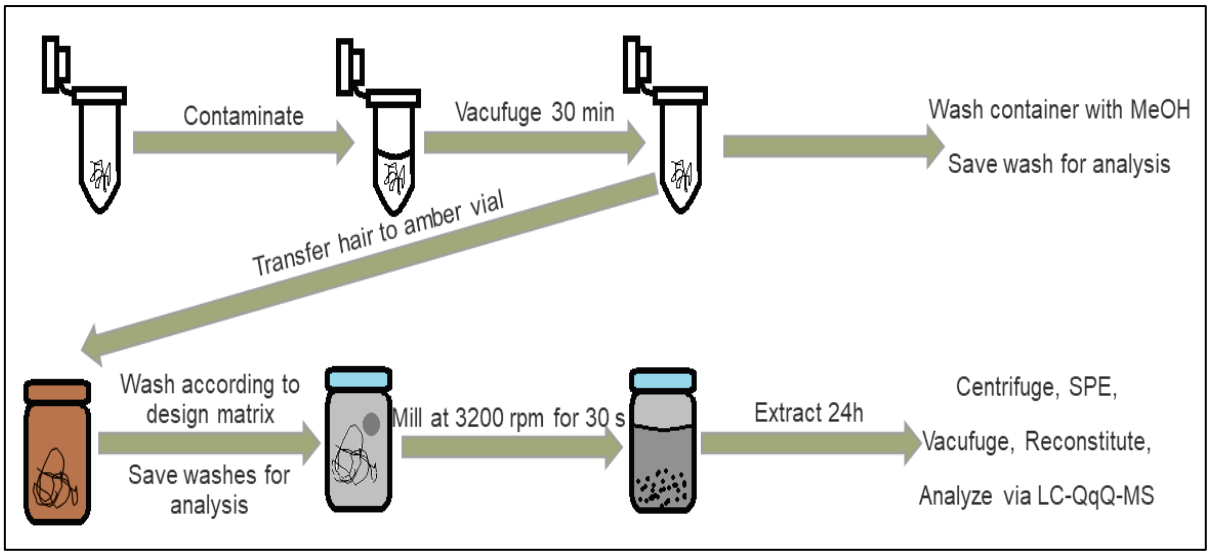

Figure 1. Schematic of decontamination DoE procedure.

Solid Phase Extraction (SPE) - 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

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(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.

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*.

Analysis of Variance (ANOVA) - 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. 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.

Evaluating optimal forensic hair analysis pretreatment techniques using 2^3 full factorial DoE protocol – To determine the optimal extraction parameters for authentic HRM within a given set, a 2^3 full factorial DoE was used. Aliquots of 20 mg of authentic HRM were used for each sample. The factors under study are shown in Table 2, 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 (+).

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. After extraction, the samples

were centrifuged and subjected to SPE, prior to LC-QqQ-MS analysis. Table 3 shows the retention times and transitions for the drugs of interest relevant to the present study.

Table 2. 2^3 Full factorial study DoE protocol.

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

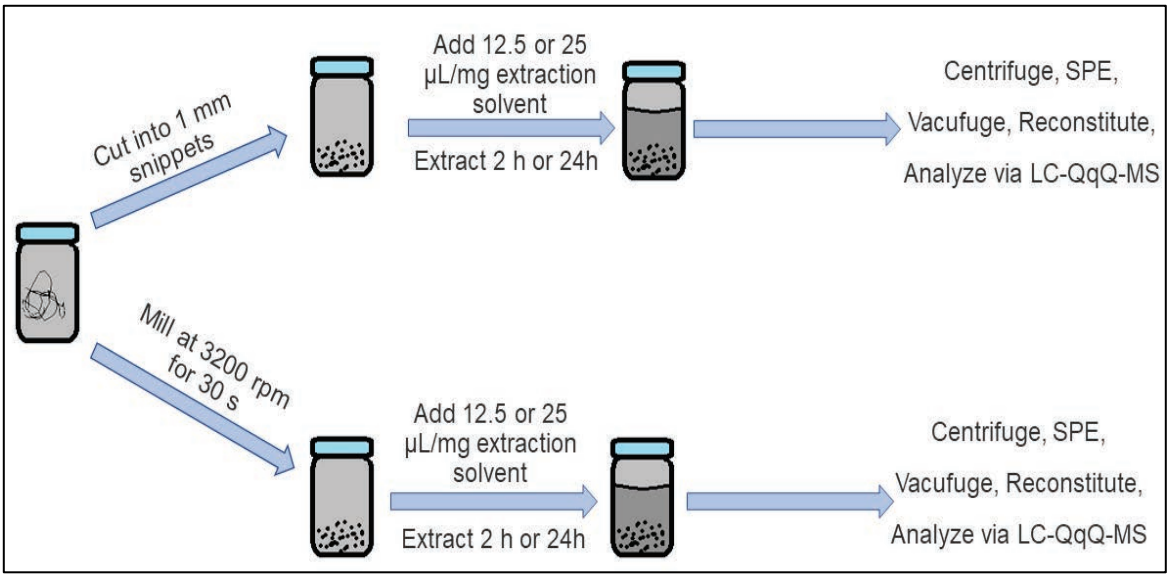

Figure 2. Schematic of pretreatment DoE procedure.

Evaluating optimal forensic hair analysis 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 37ºC.

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Table 3. List of compounds, internal standards, retention times, and *m/z* transitions

Analyte	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

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 in water). 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.

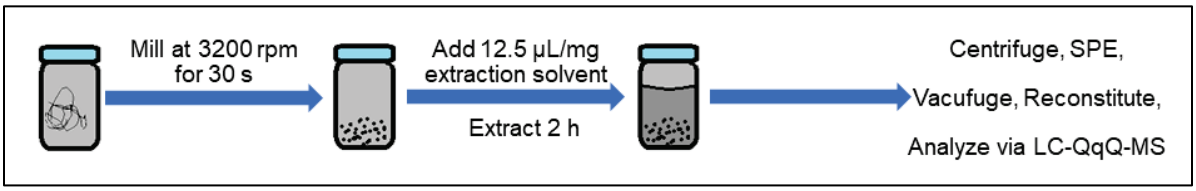

Figure 3. Schematic of extraction methods comparison procedure

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.

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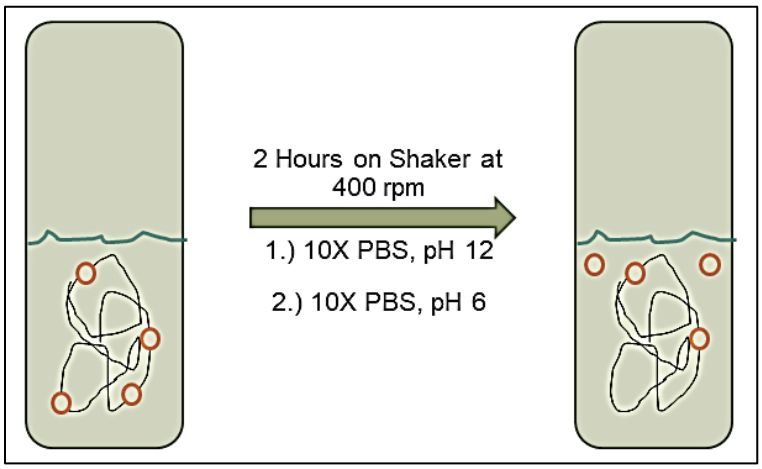
Statistical comparison of optimal and least effective forensic hair analysis methods – Hair samples of 20 mg each were weighed into 1.8 mL steel milling jars. Specimens processed using the previously identified optimized method were decontaminated with one 30-min wash with HPLC water followed by three 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 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 C_8 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 C_{18} 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.

Assessing relative levels of ionic and non-ionic binding of drugs to authentic HRM – 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 the tested drugs and metabolites, including cocaine (COC), p-hydroxycocaine (HYCOC), oxycodone (OXY,) 6-monoacetylmorphine (6-

MAM), MET, cocaethylene (COCA,) norcocaine (NORCOC), and morphine (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, heroin D3 (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 are cationic, based on their pKa values.

Figure 4. 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:

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

Expected applicability of the research: 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 37ºC. 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.

Participants and Other Collaborating Organizations

Anthony P. DeCaprio, Ph.D.

Project Role: Principal Investigator

Brianna Spear, Ph.D.

Project Role: Graduate student (Ph.D.)

RTI International, Inc.

Project Role: Collaborating organization

Changes in approach from original design and reason for change

Substantial delays were encountered during the project period due to Covid-19 pandemic issues which necessitated extended lab shutdowns and which also impacted scheduling of instrument repairs for the LC-QqQ-MS instrument. Two one-year no cost extensions were requested and granted to help ameliorate these delays.

Outcomes

Activities/accomplishments:

Activities and accomplishments under this project included the following achievements to support the ultimate goal of developing an optimized forensic hair analysis method and to obtain a better understanding of drug-matrix binding mechanisms:

- Successful optimization of forensic hair analysis parameters, including decontamination, pretreatment, and extraction methods.
- Statistical comparison between optimized and least effective forensic hair analysis methods using authentic hair specimens.
- Demonstration of the presence of both ionic and non-ionic binding between drugs and hair.
- Presentation of project results at national and international forensic science conferences and publication of peer-reviewed articles.

Results and Findings:

DoE comparison of decontamination parameters:

Table 4 shows the results of the ANOVA F-Tests for the FEN and MET DoE studies. P-values <0.05 shown in Table 4 were considered statistically significant and are denoted in bold font. As shown in Table 4, factors 1, A, AD, AB, BC, AC, ABC, BCD, ACD, ABD, and ABCD for FEN 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. For MET, actors BC and BLOCK 2 had p-values <0.05, indicating that

Table 4. 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**

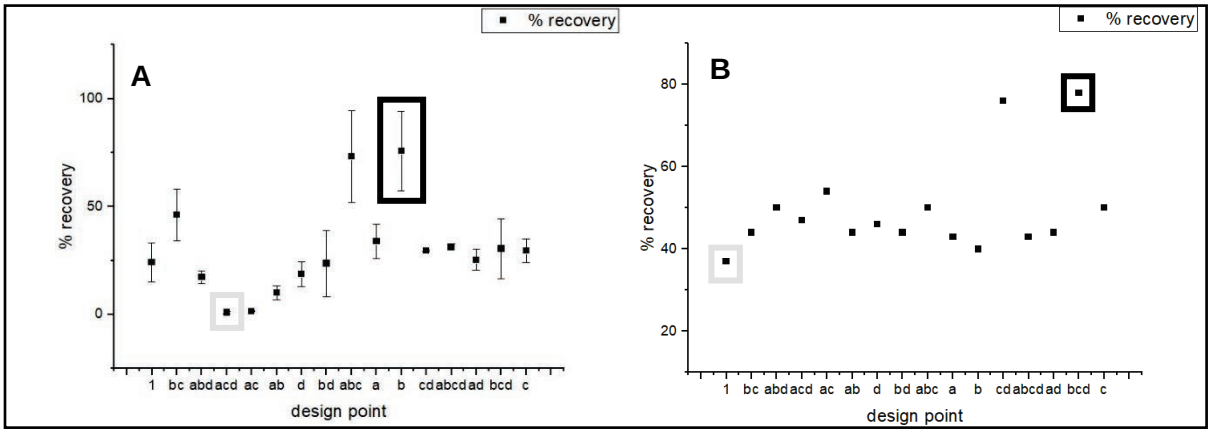

Figure 5. Recovery by design point for A) FEN and B) MET.

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.

Plots of the percent recoveries by design point (Figure 5) 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.

Table 5. Levels of parameters by design point for FEN and MET.

Analyte	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

Table 5 shows the levels of parameters by design point. 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.

Table 6 shows the levels of parameters by design point for drugs of interest investigated in this study, as well as those previously assessed in the PI's lab to evaluate trends regarding best practice decontamination protocols. As shown in Table 6, there was not one single specific method that was 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.

Table 6. Decontamination studies summary.

Analyte	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 and DeCaprio, 2021.

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.[1-5] In contrast, washes used for decontamination of MET included water followed by dichloromethane, as well as water or methanol alone.[6,7] 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.[8-10]

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

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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.

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.[8-12]

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 a “one value at a time” (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.

Previous work in this laboratory examined decontamination efficiency using a DoE approach for amphetamine, COC, diazepam (DZP), HER, 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.[8,10,13] 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.

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.

***DoE Evaluation of Pretreatment Parameters*:**

Table 7 shows the results of the ANOVA F-tests for all the drugs of interest. P-values < 0.05 shown in Table 7 were considered statistically significant and are denoted in bold font. Residuals plots were completed (not shown); with the exception of a few points,

Table 7. 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.

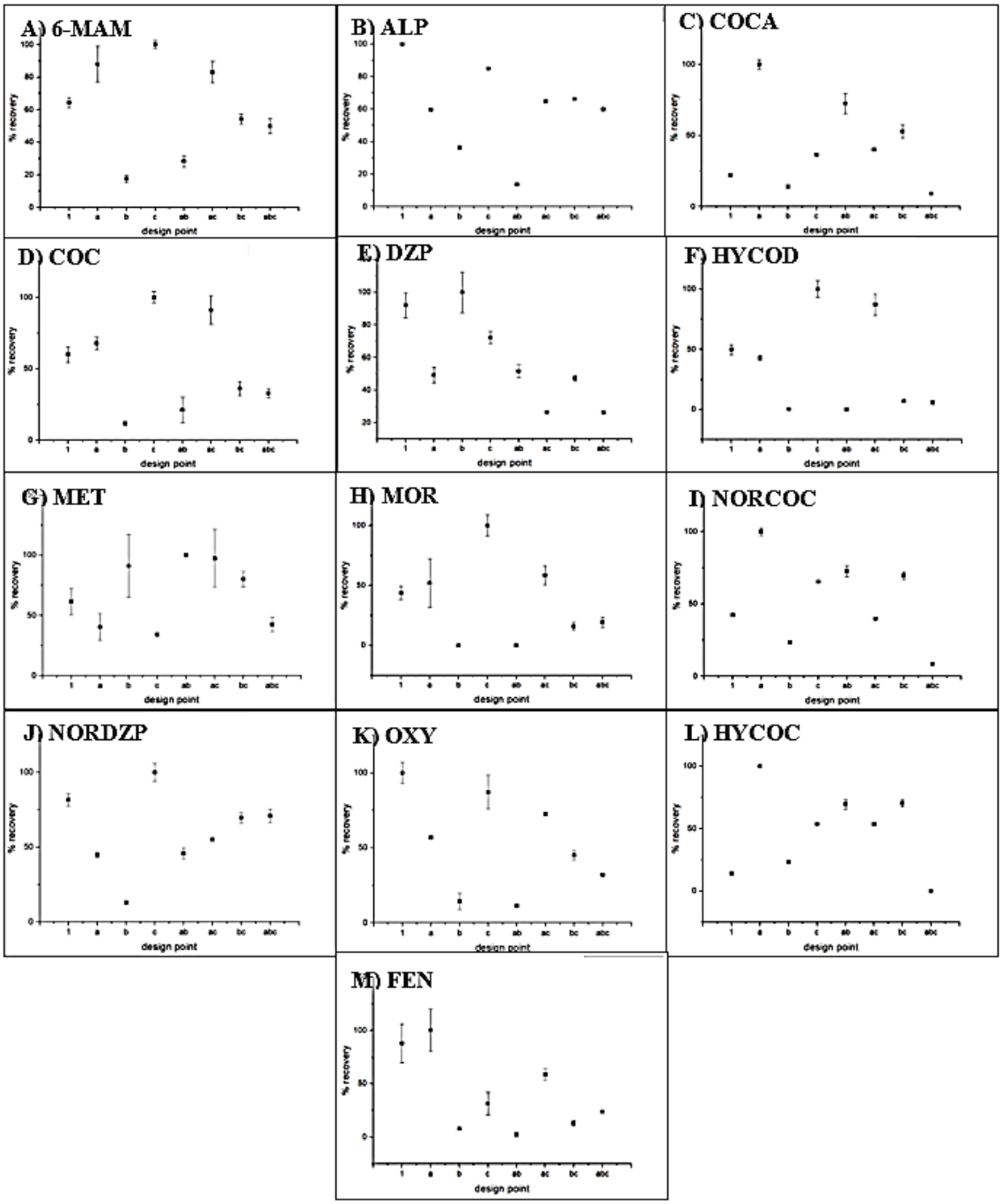

Figure 6. Recovery *vs.* design point for: A) 6-MAM, B) ALP, C) COCA, D) COC, E) DZP, F) HYCOD, G) MET, H) MOR, I) NORCOC, J) NORDZP, K) OXY, L) HYCOC, AND M) FEN

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 7, 6-MAM, alprazolam (ALP), COC, hydrocodone (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, nordiazepam (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 a OVAT approach.

Plots of recovery by design point were created to show the most and least effective conditions with regards to extraction efficiency (Figure 6). 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.

Based on summary DoE data (Table 8), 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 6). 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 8. Extraction parameters resulting in optimal recovery for each drug.

	Source Factor			
Drug	**A**	**B**	**C**	**Recovery (% ± S.D.)**
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

Previous literature has been published regarding the creation of incorporated HRM.[10,14] 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.[15] 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.[16] 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.[17] 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.[18-21]

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.[17] 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.[18-21] These particular studies are examples of a much larger pool of literature reporting a wide range of decontamination and extraction

procedures.[16] 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.[10,11] 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.[22] 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.[23] 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.[11] 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.[12] 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.[10,13] It was determined that DoE was particularly useful for this purpose, because the combinatorial effects of the factors were significant.[10,13] 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.[10,12] 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.[24] 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.[10,13] For example, da

Rosa Chagas et al. determined that pulverization increased the amount of recovered cocaine and cocaethylene from authentic user hair.[25]

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.[26] 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.[22] 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.[12]

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.

***Extraction techniques comparison*:**

The mean recoveries for each drug and extraction technique are shown in Table 9, along with available physicochemical data. As shown in Table 9, 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).

Table 9. Physicochemical parameters and recovery by extraction technique for each drug/metabolite.

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.

The mode of drug binding in hair can typically include ionic bonding, H-bonding, and hydrophobic/Van der Walls interactions.[27-30] 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.[16] 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.[31] 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 data in Table 9 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 7) also show no clear correlations between log P, pKa, HBA, HBD, and tPSA values and extraction recovery. One might predict that the most lipophilic compounds (*i.e.,* highest log P) would prefer solvent extraction. While this was true for DZP and NORDZP (log P 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.

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 8). While COC (log P 2.30) is a methyl ester, COCA (log P 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.

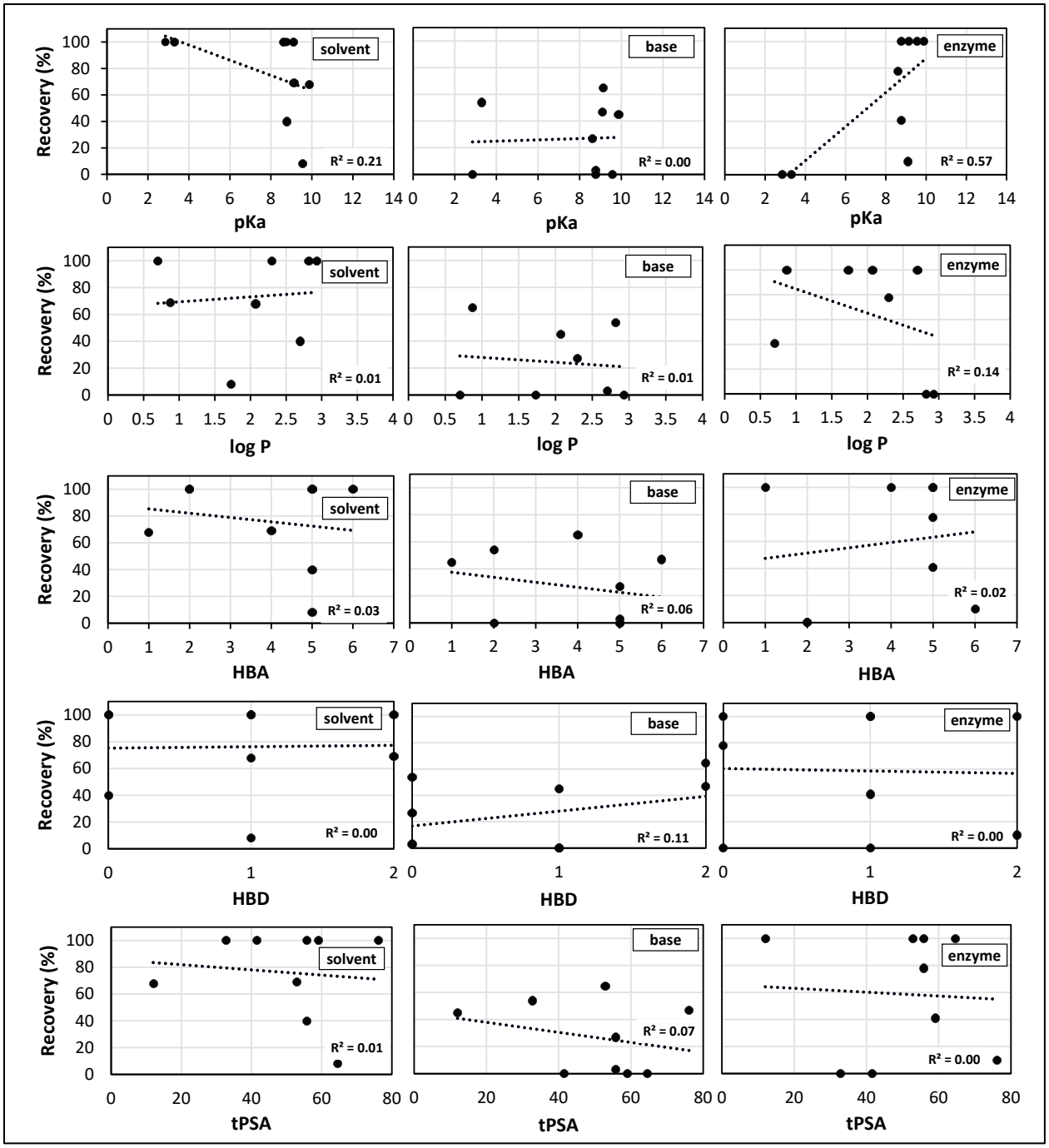

Figure 7. Correlations between recovery for solvent swelling (left panel), base (middle panel), and enzymatic degradation (right panel) extraction methods and a) pKa, b) log P, c) HBA, d) HBD, and e) tPSA.

In addition, the most effective extraction method for FEN was found to be the base technique. FEN appears to be somewhat of an anomaly in this regard, as solvent

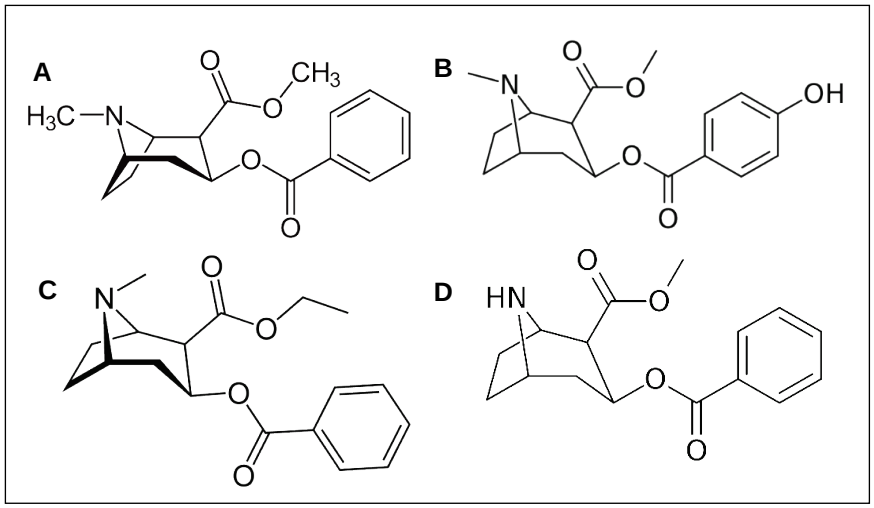

Figure 8. Chemical structures for A) COC, B) HYCOC, C) COCA, and D) NORCOC.

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.[32] 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.

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.

Comparison of optimal and least effective forensic hair analysis methods:

Figures 9 and 10 show the consensus best and least effective methods for hair processing and extraction identified in the present study. These were applied to a series of ten authentic hair specimens obtained from drug users. Each specimen contained

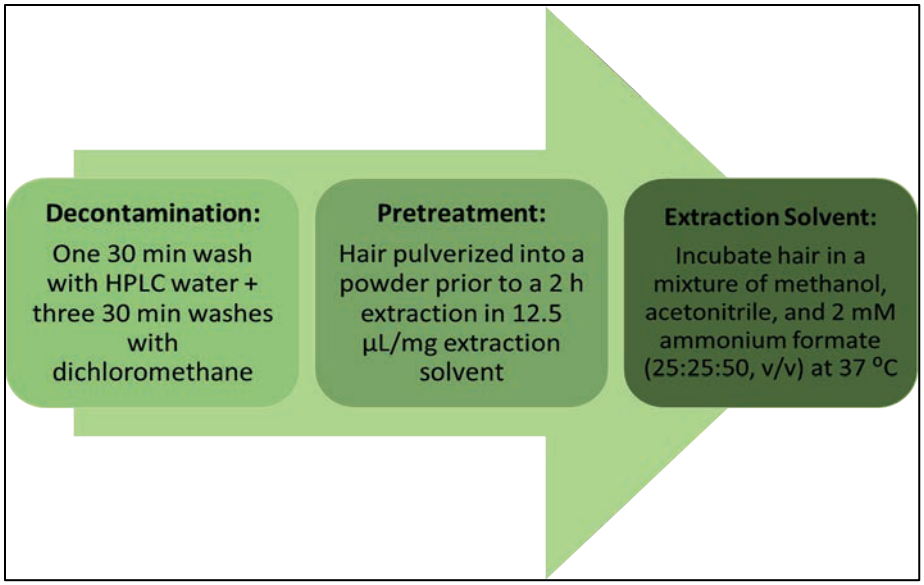

Figure 9. Consensus optimal method for forensic hair analysis.

two or more drugs/metabolites. Figure 11 shows the analyte recovery for each of ten authentic specimens using the optimized and least effective forensic hair testing methods. Error bars indicate standard deviation for triplicate determinations. As shown, the majority of drugs were most effectively extracted using the optimal method. To

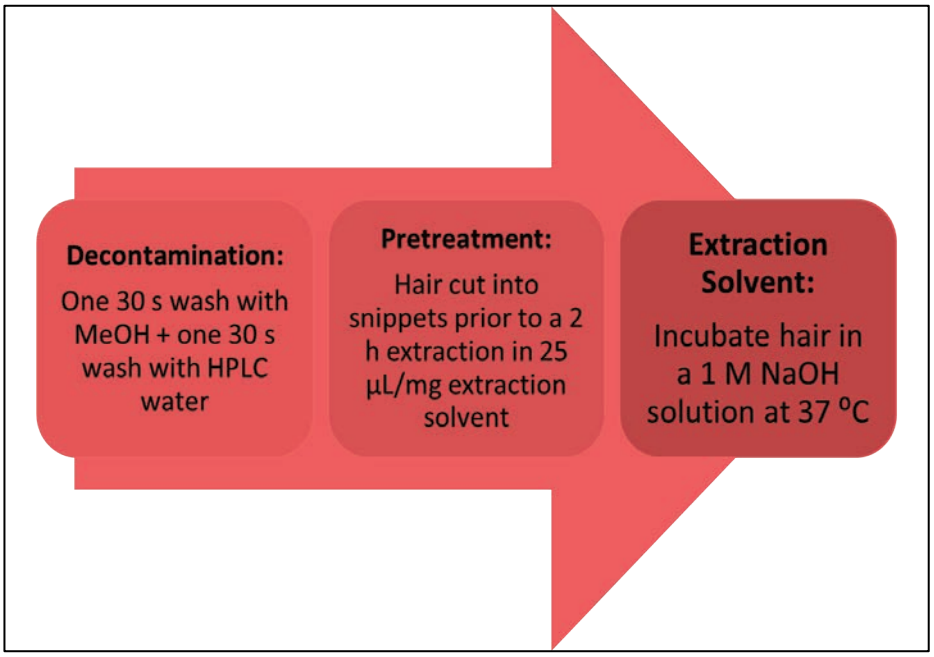

Figure 10. Consensus least effective method for forensic hair analysis

determine if the differences in recovery for the optimal and least effective methods were statistically significant, Paired T-Tests were completed, with results shown in Table 10.

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 in Table 10. A summary of recoveries and statistical significance by drug is shown in Table 11. 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 11, 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.

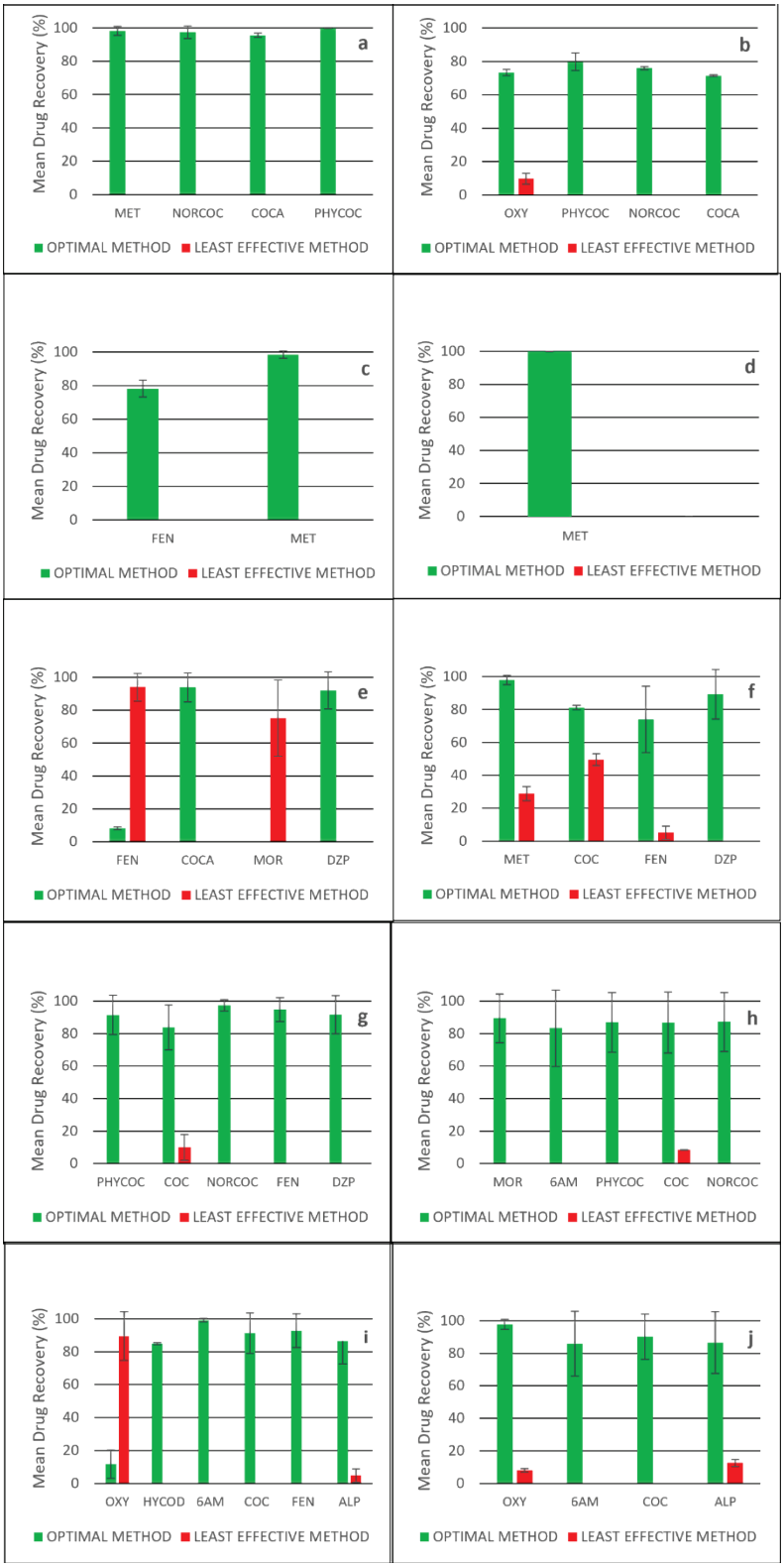

Figure 11. Recovery in authentic specimens with optimal and least effective methods.

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Table 10. 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**

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.

Table 11. 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 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 37ºC. This work provides potential for consistency in forensic hair analysis methods for multiple drugs and metabolites.

Relative levels of ionic and non-ionic binding of drugs to hair:

Table 12 shows the relative recoveries (%) of drugs at pH 12 and pH 6. As shown in Table 12, 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.

Table 12. 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 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 pg/mg of drug recovered at each pH and the total pg/mg of drug recovered at both pH values.

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.[15,27] 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.[27] However, previous research has only demonstrated ionic interactions between COC and the hair matrix, likely due to the positive charge on the nitrogen.[15] 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.[31,33] Findings suggested that MET binding occurred specifically with melanin, as MET was detected in black hair, not white.[31] Further research suggested that a combination of ionic, covalent, and hydrophobic interactions all played a role in MET binding to melanin.[33] 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 reported,

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.[34] 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.

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.

Limitations:

Results from this project provide an optimized forensic hair analysis method. These results are limited in that data were generated without considerations for individualizing characteristics such as race and cosmetic treatment of the hair. Additionally, binding

studies assessed relative amounts of non-ionic and ionic binding; however, further studies are ongoing to further probe the types of interactions occurring between drug and hair.

Artifacts

List of products:

Peer-reviewed publications:

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.

Spear, B. and DeCaprio, A.P. (2022). Evaluation of pretreatment and extraction parameters for the analysis of fentanyl using statistical design of experiments (DoE). *Journal of Analytical Toxicology*, https://doi.org/10.1093/jat/bkac045.

Three additional manuscripts are in preparation.

Invited platform presentations:

Spear, B. and DeCaprio, A.P. (2023). Assessing relative levels of ionic and non-ionic binding of drugs and metabolites in authentic hair reference material (HRM); *American Academy of Forensic Sciences 73rd Annual Meeting* (Orlando, FL); February 17.

Spear, B. and DeCaprio, A.P. (2022). Comparison of forensic hair analysis methods for multiple drugs in authentic user hair; *Society of Forensic Toxicologists Annual Meeting* (Cleveland, OH)*;* November 2.

Spear, B. and DeCaprio, A.P. (2022). Comparison of forensic hair analysis methods for multiple drugs in authentic user hair; *Society of Hair Testing Meeting* (Verona, Italy); June 9.

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 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 Annual 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.

Research poster presentations:

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 Annual Meeting (Virtual)*; February 15-19.

Data sets generated:

Nothing to report.

Dissemination activities:

Two research posters and eight invited talks were presented at international toxicology and forensic science conferences. Two peer-reviewed articles were published and four articles are in preparation.

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