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Application of statistical design of experiments to assess pre-treatment parameters in forensic hair analysis for amphetamine

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HIGHLIGHTS

- Application of a 2^4 factorial DoE approach evaluated parameters in hair pre-treatment.
- Optimal hair pre-treatment conditions for amphetamine were identified.
- Combinatorial effect of all parameters was consistently significant.
- Results confirmed importance of evaluating pre-treatment protocols as complete procedures.

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ABSTRACT

Toxicological analysis of human head hair for detecting drugs of abuse requires multiple pre-treatment steps before analysis, such as washing to remove external contamination, pulverization/homogenization of the sample matrix, and subsequent extraction of the drug from matrix components. The predominant approach to developing and assessing the efficacy of hair pre-treatment protocols uses a traditional one factor at a time (OFAT) approach, in which one independent variable at a time is changed to observe the effect on the dependent variable. An alternative approach to assessing pre-treatment protocols is statistical design of experiments (DoE), which involves the systematic variation of all independent variables (*i.e.,* pre-treatment conditions) simultaneously, allowing for the variation in the dependent variable (*i.e.,* extraction efficiency) associated with each independent variable and combinations thereof to be observed. In the present study, a 2^4 factorial block DoE design was used to compare the efficacy of parameters in the decontamination and extraction of amphetamine from human head hair. DoE studies identified that the most effective pre-treatment conditions included 1% SDS washes followed by methanol washes for 30 s each time, milling the hair into a powder, and final extraction of drug with 12.5 µL of 2 mM proteinase K solution/mg of hair. The most notable result of these DoE studies was that the combinatorial effect of all parameters was consistently significant, confirming that it is necessary to consider pre-treatment protocols as complete sets of procedures, as is done in DoE, compared to optimizing individual factors using an OFAT approach.

1. Introduction

Forensic toxicological analysis for the purposes of protecting human health and supporting criminal justice activities can be achieved, in part, by analysis of human head hair for detection of abused substances. Drug analysis in any biological matrix serves to monitor exposure to a variety of licit and illicit substances, recognize pre-natal drug exposure, evaluate abstinence from drug use, and detect exposure to drug facilitated crime substances. Blood and urine are the most commonly tested biological matrices for detecting the use of or exposure to drugs because these matrices are well understood and standard operating procedures exist [1]. Alternatives to blood and urine include hair, nail clippings, and oral fluid [1]. These alternative matrices have been explored because their collection is less invasive than obtaining blood or urine from an individual. Additionally, some alternative matrices, like hair and nail clippings, provide information that blood and urine cannot. This is because hair and nails grow from the body over time, which allows for, via segmental analysis, the detection of substances

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incorporated at different times associated with different growth periods [2].

Prior to toxicological analysis, human head hair requires significant processing. Pre-treatment steps in the analysis of drugs in hair include decontamination and segmentation of the hair strand, extraction of the compounds of interest from the hair matrix, and often, purification of the isolate [3]. Different mechanisms to recover drugs from hair are used with different extraction techniques, *e.g.,* alkaline or enzymatic degradation of the hair proteins or solvent swelling of the hair structures. Although no standardized protocol for hair processing exists, the Society of Hair Testing (SoHT) has made recommendations on a general scheme of analysis [4]. These recommendations list minimally necessary steps for hair analysis but suggest that laboratories investigate and validate methods to fit within these guidelines. Although a great variety of methods for hair analysis have been developed and validated [3,5–10], there is little reported work rigorously comparing the benefits and limitations of one method over another. Additionally, most of the research to date involves the comparison of the individual parameters that contribute to the pre-treatment protocol, without comparing the whole combined protocols.

Statistical design of experiment (DoE) is an alternative approach to analyzing the cause and effect relationships between independent and dependent variables. Traditionally, the approach to determining the effect of each independent variable on the dependent variable is to vary the levels “one factor at a time” (OFAT) to consider their individual effects on the response. Subsequently, another variable can be tested at different levels while holding the first variable and the others at constant values. The OFAT method of testing requires an abundance of time and materials, making it inefficient and often infeasible. In addition, it does not allow for more than one variable to be tested at the same time, nor does it allow for a direct comparison between magnitudes of the effects of each independent variable on the dependent variable. Alternatively, DoE allows for the variation of all parameters simultaneously to observe the effect, the magnitude of that effect, and any effect due to the interaction between factors (*i.e.,* “interaction effects”) on the optimizable dependent variable [11,12].

To date, only limited work using multivariate statistical approaches such as DoE to compare protocols associated with the pre-treatment of hair has been reported. For example, some efforts have been focused on examining selected pre-treatment parameters for analysis of the ethanol consumption biomarker ethyl glucuronide (EtG) in hair [13,14]. In contrast, the present report describes a proof of principle study for the application of DoE in the systematic comparison of the full range of pre-treatment parameters, with amphetamine as a test compound. Separate DoE studies were conducted for decontamination parameters and for all other pre-treatment parameters, including two approaches for final drug extraction from the hair matrix. In the study, a 2^4 factorial block design together with ANOVA and post-hoc testing was employed to demonstrate the potential utility of DoE to facilitate comparison of pre-treatment parameters in forensic hair analysis.

2. Materials and methods

2.1. Chemicals and solvents

Solvents and chemicals used for the preparation of mobile phases and sample preparation for LC–MS/MS analysis (water, methanol, 2-propanol, dichloromethane, formic acid, ammonium formate) were of HPLC-grade and obtained from Fisher Scientific (Hampton, NH, USA). Stock solutions of amphetamine (AMP) and AMP-D11 (1.0 mg/mL in methanol), used in the preparation of the drug incorporated hair reference material (HRM) and for calibration curves, were purchased from Cayman Chemical (Ann Arbor, MI, USA). D-AMP sulfate powdered drug (Sigma-Aldrich, St. Louis, MO, USA) was used for intentional external contamination of hair for the DoE experiments in which decontamination protocols were compared. 1,4-Dithiothreitol (DTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and proteinase-K was obtained from Invitrogen (Carlsbad, CA, USA). Three kDa molecular weight cut off PTFE spin filters were obtained from MilliporeSigma (Burlington, MA, USA). Single donor, unprocessed, natural black color human hair (~300 g) was obtained from a commercial source.

2.2. Production of AMP incorporated and surface contaminated hair

Hair reference material (HRM) with externally incorporated AMP was produced and employed for all DoE studies examining the pre-treatment parameters of extraction solvent volume and sample size ratio, particle size of homogenized hair, aqueous decontamination solvent, organic decontamination solvent, extraction temperature, extraction time, and final extraction method. This approach was deemed appropriate for the present proof-of-principle study, as large amounts of hair with defined drug concentrations were required, a criterion that would be difficult to achieve with hair from drug users.

Using a method modified from that of Ropero-Miller *et al.*[15] samples of drug free human head hair (800 mg) were soaked in 1X PBS, pH 6, spiked with AMP at 1500 pg/mg hair. Samples were incubated at 37 °C while on an orbital shaker in a temperature-controlled oven. Aliquots of hair and spiked PBS solution were taken at 0, 24, 48, 72, 96, and 120 h to monitor drug incorporation. The hair aliquots were washed three times with 1X phosphate buffered saline (PBS), pH 6, and once with 2-propanol for 1 min with agitation. The washed samples were then pulverized using a Mini-Bead Beater 24 ball mill (Biospec; Bartlesville, OK, USA) at 3200 rpm for 30 s. Subsequently, the pulverized hair was subjected to solvent extraction, followed by centrifugation, SPE, and LC–MS/MS analysis as described below. LC–MS/MS analysis was performed on the spiked buffer solutions, extracted samples, and wash solutions. The total mass of hair was washed after incorporation with 3 volumes (150 mL each) of 1X PBS followed by 1 vol of 2-propanol for 1 min each. The hair was dried at 40 °C for 24 h in an oven, and then stored in aluminum foil at room temperature until use in DoE experiments.

For the DoE studies of decontamination parameters, drug free hair was surface treated with powdered drug to model externally contaminated hair. An aliquot of 30 mg of hair was weighed into a stainless-steel milling jar containing 1 mg of AMP sulfate powder. The samples were agitated using the ball mill, without milling beads, at 3000 rpm for 30 s to coat the hair strands. After intentional contamination, the hair was transferred to a clean 20 mL amber scintillation vial and washed by adding 1 mL of solvent and agitating on an orbital shaker. The original vessel used for contamination of the hair was rinsed with 1 mL of methanol and the solution was collected for analysis. The hair was then dried for 24 h at 37 °C in an oven. Once dried, the samples were homogenized using the ball mill with 10 chrome-steel milling beads at 3200 rpm for 30 s. Drug was extracted using the technique (*i.e.,* enzymatic degradation or solvent swelling) identified as most effective for extraction of AMP from the pre-treatment experiments. Extracted samples and wash solutions were purified using a 3 kDa molecular weight cut-off PTFE spin filter and SPE before LC-MS/MS analysis, as described below.

2.3. Isolation of drugs from hair

Two extraction techniques were compared. Enzymatic degradation of the hair matrix was conducted by first incubating 30 mg of hair with 12 mg/mL aqueous DTT for 2 h at 37 °C, then with 2 mg/mL aqueous proteinase K [16]. Solvent swelling of the hair matrix to allow for the extraction of drug held within the cortex was achieved by incubation in a mixture of methanol, acetonitrile, and 2 mM ammonium formate (25:25:50 v/v) at 37 °C [17]. Samples extracted for the decontamination DoE studies were incubated for 24 h, while for the pre-treatment DoE studies the samples were incubated for the amount of time

indicated by the design matrix. Subsequently, the extracted samples were centrifuged at 10,000 × *g* to separate the hair particulates from the solvent mixture containing recovered drug. The supernatants from the enzymatically degraded samples were then centrifuged at 10,000 × *g* using a spin filter. The eluent solution was subjected to SPE cleanup and LC–MS/MS analysis.

2.4. Solid phase extraction (SPE)

For cleanup of hair extracts, Agilent Technologies (Santa Clara, CA, USA) Bond Elut Certify mixed mode cartridges were used following a method characterized for hair samples by Miller *et al.* [18]. The cartridges were installed on an SPE manifold with clean manifold liners. Under 3 mm Hg vacuum, the cartridges were conditioned with two 1 mL volumes of methanol and then two 1 mL volumes of 1X PBS, pH 6. Next, the samples were spiked with a deuterated surrogate of the drug of interest to serve as an internal standard. The samples were loaded to the cartridges at 1 mL/min under 3 mm Hg vacuum. The cartridges were then washed to eliminate matrix components with 1 mL of deionized water followed by 0.5 mL of 0.01 M acetic acid. The cartridges were then dried under vacuum for 10 min. A third wash was then conducted with 50 μL of methanol, and the cartridges were dried again for 2 min. Elution of the compounds of interest was conducted by addition of 0.75 mL of ethyl acetate with 2% ammonium hydroxide at 1 mL/min and subsequently with 0.75 mL of a mixture of dichloromethane, 2-propanol, and 2% aqueous ammonium hydroxide (78:20:2 v/v). The eluted samples were evaporated to dryness in a vacufuge for 1.5 h at 45 °C. Finally, the samples were reconstituted in 400 μL of methanol for LC–MS/MS analysis.

2.5. HPLC-MS/MS analysis

For drug analysis, 2 μL of each isolate was injected onto a reversed-phase LC column and subsequently analyzed by QqQ-MS. The compounds were separated using an Agilent Technologies 1.8 μm Zorbax Eclipse Plus C_{18} rapid resolution HD column (2.1 × 150 mm; 1.8 μm). The aqueous mobile phase used was water with 0.1% formic acid and 5 mM ammonium formate. The organic phase was MeOH with 0.1% formic acid. The separation was conducted using a gradient LC solvent system as follows: at 0 min the mobile phase was a 95% aqueous and 5% organic mixture, from 0 to 5 min the gradient adjusted to 100% organic mobile phase, from 5 to 8 min the gradient was held at 100% organic to flush the column. The mobile phase flow rate was 0.3 mL/min.

An LC-MS/MS multiple reaction monitoring (MRM) method was used for the identification of analytes using an Agilent 6470 triple quadrupole mass spectrometer operated in positive ESI mode. The cycle time was 500 ms. The cell accelerator voltage was 4 V. The drying gas (N_2) was heated at 350 °C, and had a flow rate of 12 L/min. The sheath gas was also heated to 350 °C and had a flow rate of 11 mL/min. The peaks were confirmed by MRM transitions corresponding to the integrated chromatographic peak. MRM peaks were specified in the LC-MS method based on the two most abundant transitions, one as a quantifier and the other as a qualifier. These transitions were identified by Agilent's MassHunter Optimizer program and are listed in Table 1. The data were acquired in Agilent MassHunter Qualitative software. The spectral peak areas were integrated, and the data files were then uploaded into the MassHunter Quantitative software.

A calibration curve was generated using drug standards with a 1 mg/mL concentration and the corresponding deuterated compound. The drug standards were diluted in MeOH to a final volume of 500 μL to generate calibration solutions with 1, 5, 15, 35, 75, 150, 200, and 250 ng/mL concentrations, which represents a wide range of expected concentrations in hair [19–21]. Each calibrator also included 100 ng/mL of deuterated internal standard.

Table 1
MS parameters and MRM transitions selected for LC–MS/MS analytical method for AMP.

Compound	Precursor Ion (*m/z*)	Product Ions (*m/z*)	Fragmentor Voltage	Collision Energy	Accelerator Voltage
AMP	136	119	72	0	4
		91	72	16	4
AMP-D11	147	130	72	4	4
		98	72	16	4

2.6. 2^4 Design matrices for comparison of decontamination and other pretreatment parameters

In general, samples for DoE studies were prepared by first weighing 30 mg of incorporated or surface contaminated hair into a 20 mL amber vial. The aqueous wash was performed by adding 1 mL aqueous decontamination solvent, and agitated on an orbital shaker for 1 min. Next, the organic wash was performed with 1 mL of solvent and agitation on an orbital shaker for 1 min. The hair was then dried for 24 h at 37 °C in an oven. Once dried, the samples were either cut into snippets or powdered. Samples that were to be snippets were cut with surgical scissors into ~1 mm segments. Samples that were powdered were milled with 10 chrome-steel milling beads in the ball mill at 3200 rpm for 30 s. Drug was then extracted using one of the two previously described techniques with a volume of extraction agent.

Table 2 defines each factor and each level of the factors that were studied in the DoE designs. Main factors were chosen by observing which parameters were most variable in literature reports. Blocking factors were selected as the factors that were difficult to change between samples, making them hard to control, but that still may have an impact on the dependent variable. Blocking was used in the design and the statistical analysis because it can be separated from the effects caused by the main factors so that it reduces variability of estimating the main effects. While the effects of these parameters were not of primary interest in the present study, they nonetheless needed to be controlled.

Table 3 outlines the 2^4 factorial block design used to study the decontamination and extraction parameters. Each design point in Table 3 corresponds to a single sample to which different levels of these parameters were applied. A randomized list of design points was used to determine the order in which samples were prepared and analyzed. Three replicate samples for each design point as listed in Table 3 were analyzed. One replicate study of the entire DoE protocol was conducted for each of the two extraction techniques. Extracted samples were purified by spin filter and SPE before LC–MS/MS analysis as previously described.

2.7. Statistical analysis

LC–MS/MS analysis allowed for the quantification of drug from each sample. The quantity of drug detected for each sample was then used to find the statistical parameters necessary to complete an ANOVA table. If a significant difference was observed by means of the ANOVA F-test, Tukey's HSD post-hoc analysis was conducted to determine which parameters or combinations of parameters resulted in this difference. JMP software (13th ed.) was used for all statistical analyses. Complete details on the statistical design parameters are included in the Supplementary Information.

3. Results

3.1. Analytical method performance

Table 4 shows analytical figures of merit for the LC-MS/MS method

Table 2
Definition of factors and factor levels for each DoE design.

Factor	Pre-treatment DoE		Decontamination DoE	
	Definition	Level	Definition	Level
A	Extraction solvent volume ratio to sample size	+; 25 μL/mg −; 12.5 μL/mg	Aqueous decontamination solvent	+; 1% SDS −; HPLC water
B	Particle size of homogenized hair	+; 1 mm snippets −; powder	Organic decontamination solvent	+; dichloromethane −; methanol
C	Aqueous decontamination solvent	+; 1X PBS −; HPLC water	Number of consecutive aqueous washes	+; 3 −; 1
D	Organic decontamination solvent	+; dichloromethane −; 2-propanol	Number of consecutive organic washes	+; 3 −; 1
Block 1	Extraction temperature	+; 40 °C −; 20 °C	Sequence of washes	+; Organic before aqueous −; Aqueous before organic
Block 2	Extraction time	+; 24 h −; 2 h	Wash time	+; 30 min −; 30 s

Table 3
Augmented 2^4 factorial block design.

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

employed for analysis of AMP in hair extracts and washing solutions. Results were determined with 5 replicates of QC samples, prepared in methanol, at concentrations of 5, 75, and 250 ng/mL. The relative standard deviation was below 20% for all QC levels of AMP. Accuracy was ±20% of the nominal concentration for all samples. The figures of merit for the LC–MS/MS method when analyzing for AMP met ANSI/ASB requirements [22].

The SPE protocol used [18] was previously applied to a variety of drugs in the laboratory, including AMP, diazepam, cocaine, morphine, and the major metabolites of these drugs in hair. The average recovery of AMP after SPE was 98%, similar to previous reports using the same method (Fig. 1). Calibration curves for AMP, with AMP-D11 as the internal standard for quantitation, demonstrated linearity ($R^2 > 0.99$) between 1 and 250 ng/mL. The LOD was determined to be 1 ng/mL, as the lowest calibration solution that was detected accurately and precisely.

Table 4
Accuracy and precision measures for the LC-MS/MS method with AMP.

Concentration (ng/mL)	SD (%)	RSD (%)	Accuracy (%)
5	0.3	6.5	98
75	1.2	1.6	101
250	5.5	2.2	100

n = 5 for all samples.

3.2. Incorporation of AMP into hair

The incorporation of AMP into blank human head hair was monitored every 24 h over the course of 120 h. An ANOVA was conducted on the triplicate extracts for each sampling hour. The means were compared by Dunnett's method, using the 0 h time point as a control, and by Tukey's HSD. ANOVA F-test for sampling hour was significant (p-value = 0.002) at an α-level of 0.05. Fig. 1 shows the ANOVA post hoc comparison of the amount of drug extracted from the incorporated hair samples at different times during the process. Comparison between the means for different sampling hours was conducted using Dunnett's method, with the 0 h samples as the control. The p-values for each sampling hour indicated significant differences, at a 0.05 level, ($p \leq 0.003$) for all time points, as compared to the 0 h control.

Tukey's HSD was used to compare the means of all sampling hour AMP concentrations in a pair-wise manner. Incorporation at each time point was significantly different from the 0 h control. However, differences between each non-zero time point were not significant, indicating that maximum incorporation had occurred within 24 h of incubation. After 120 h, 1,105±146 pg/mg of AMP was found to be incorporated into hair; this was ~75% of the 1500 pg/mg concentration of the incorporation solution.

Table 5 shows the concentration of AMP present in three sequential aqueous (1X PBS, pH 6) washes and one organic (2-propanol) wash after 120 h of incorporation. The three aqueous washes removed decreasing amounts of AMP, while the organic wash did not remove any additional incorporated drug from the incorporated hair.

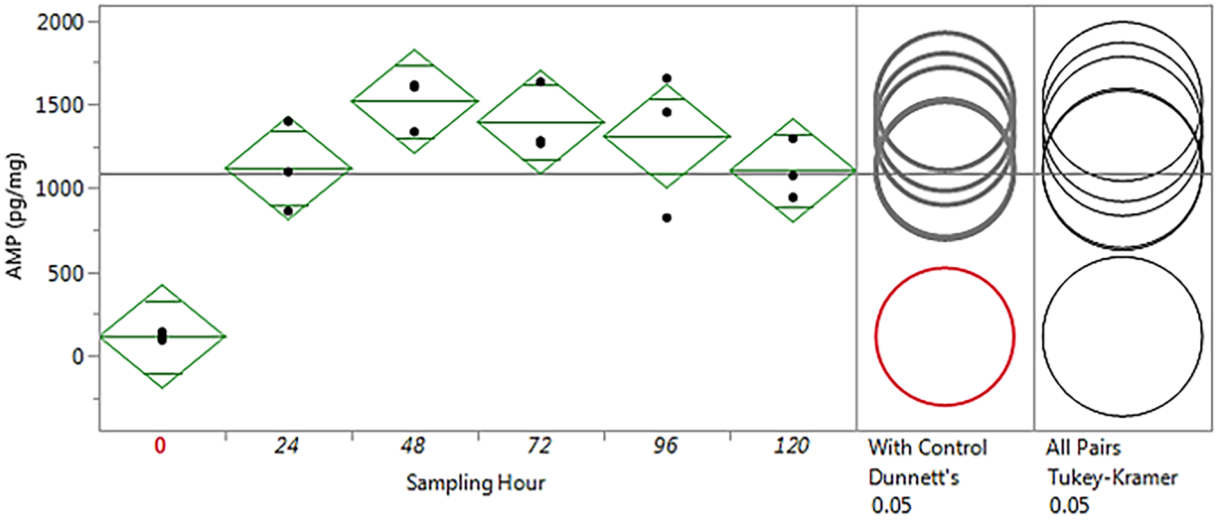

Fig. 1. AMP incorporation into hair over 120 h. The green diamond at each time point depicts the quantiles (indicated with horizontal lines) for the replicate measures, with the center line indicating the mean concentration of AMP extracted. The red circle in the first box to the right of the plot shows the 0 h time point, used as the control for the Dunnett's analysis. The grey rings indicate the subsequent time points. The black rings in the next box represent the variation within the groups at each time point. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 5
Concentration of AMP in sequential washes.

Wash	AMP (ng/mL)
First Aqueous	3.7 ± 1.1
Second Aqueous	2.8 ± 0.7
Third Aqueous	1.8 ± 0.3
Organic	< LOD

3.3. Comparison of extraction techniques

Fig. 2 shows the results of the pooled *t*-test comparing the two hair extraction techniques, which yielded a t*-statistic of 1.985 and a calculated p-value of 0.0048, indicating that the two techniques produced statistically different results. The comparisons were performed with 48 replicates for each extraction technique. The enzymatic hydrolysis technique resulted in the highest mean extraction of 1268 pg/mg of AMP. The solvent extraction technique resulted in a mean extraction of

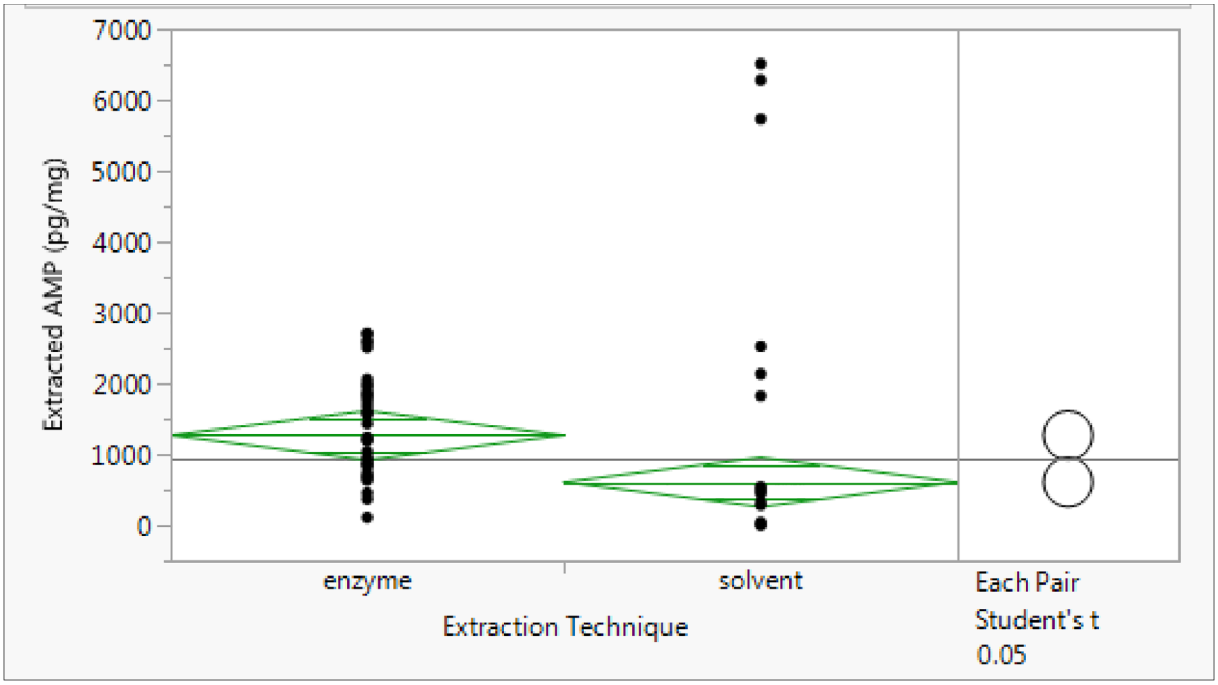

Fig. 2. ANOVA and Tukey's HSD comparison of the extracted AMP with each of the two extraction techniques. Individual points represent each sample extracted using the designated technique (n = 48), with different pre-treatment protocols as outlined in the DoE matrix. The outlines in green represent the quantiles of the data. Solid horizontal line represents the average extracted quantity for both techniques. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

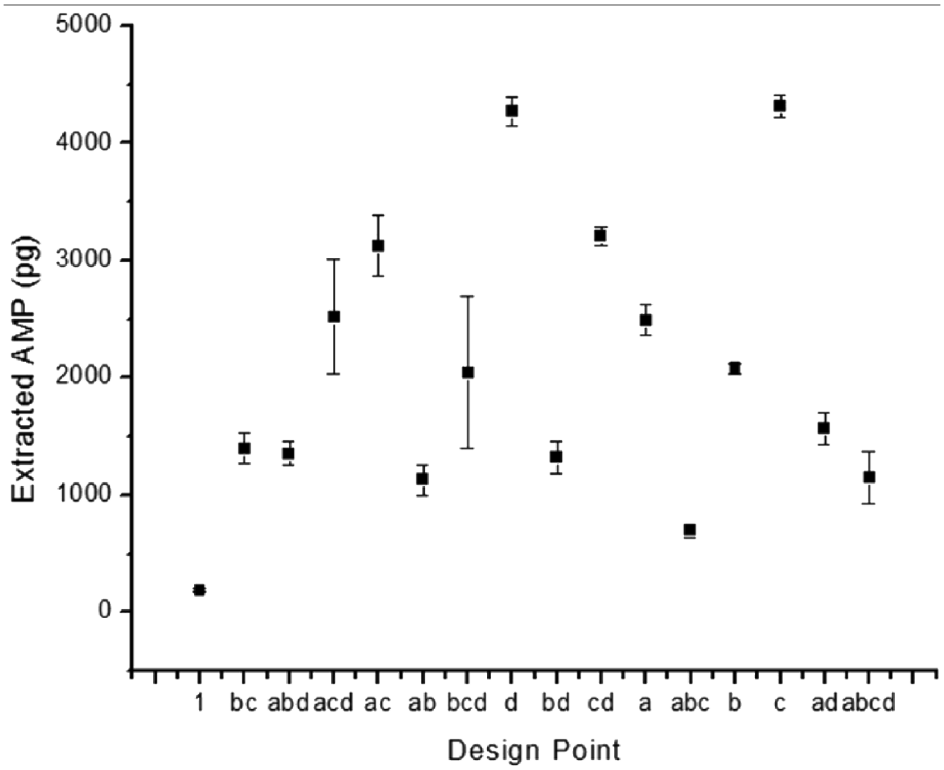

Fig. 3. Plot of enzymatically extracted AMP by design point (n = 3). The y-axis is the recovered amount of AMP (pg); the x-axis shows the design points.

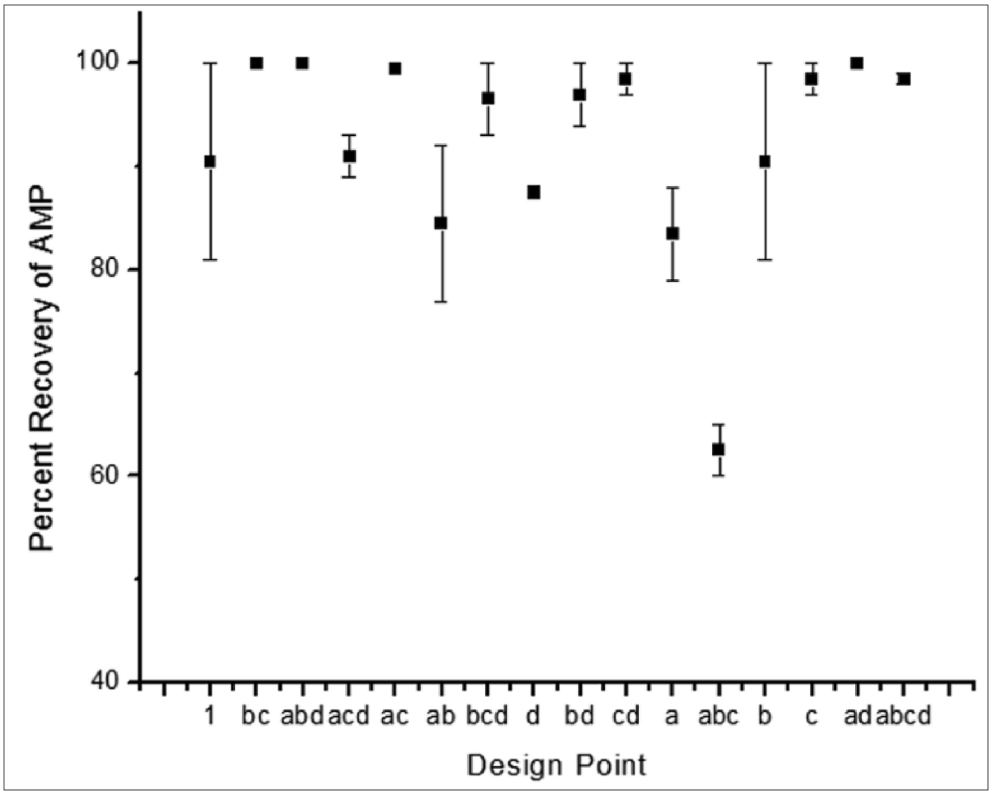

Fig. 4. Plot of the percent of recovered drug from the washes, in duplicate. The y-axis is percent recovery of AMP; x-axis shows the design points.

608 pg/mg of AMP. The solvent extraction technique had higher variance than the solvent method, with six replicates showing noticeably higher amounts of extracted drug. Variance for the enzymatic technique (data range 110 to 2709 pg/mg) was lower than that of solvent extraction (data range 0 to 6,511 pg/mg). Consequently, based on these data, the enzymatic technique was chosen for the subsequent pre-treatment and decontamination DoE studies.

3.4. DoE comparison of pre-treatment parameters

Following the overall statistical comparison of the two extraction techniques, further analysis of the enzymatic hydrolysis protocol was conducted to observe the effect, if any, of the pre-treatment factors and combinations of these factors on the amount of AMP extracted from the incorporated hair samples within each treatment group associated with each design point in the DoE matrix. ANOVA F-Test results indicated that the p-values for all factors and combinations of factors were significant at $\alpha = 0.05$ ($p < 0.00001$).

Fig. 3 shows a plot of the extraction results for each design point ($n = 3$). Large variations in extracted AMP as a function of design point are apparent, with values ranging from 0 to 6,511 pg of AMP, consistent with a substantial effect of different combinations of the treatment factors on extraction efficacy. The treatments associated with design points d and c resulted in the highest extraction of AMP from hair samples, (4273±127 pg and 4317±96 pg, respectively). Design point d was washed with dichloromethane and HPLC water, then pulverized into a powder before extracting with 12.5 μL of extraction solvent/ mg of hair for 24 h at 20 °C. Design point c was washed with 2-propanol and 1X phosphate buffered saline before homogenizing into a powder and extracting with 12.5 μL of extraction solvent/ mg of hair for 24 h at 40 °C. In contrast, the poorest extraction results were noted with design points 1 and abc (191 ± 7 pg and 698 ± 62 pg, respectively). Design point 1 was washed with 2-propanol and HPLC water before milling the hair into a powder and extracting with 12.5 μL of extraction solvent/mg of hair for 2 h at 20 °C. Design point abc was washed with 1X phosphate buffered saline and 2-propanol before cutting the hair into snippets and extracting with 25 μL of extraction solvent/mg of hair for 2 h at 40 °C.

3.5. DoE comparison of decontamination protocols

ANOVA comparison of the impact of factors and combinations of factors on removal of externally contaminated drug from hair was conducted, resulting in p-values less than the α-level (0.05) for all factors except B (particle size of homogenized hair) and C (aqueous decontamination solvent). Fig. 4 shows the recovery of AMP in the combined aqueous and organic washes for each design point. Average recovery was highest for design points d, ac, and abd at 100%. Design point bc was washed with HPLC water three times for 30 s before washing with dichloromethane once for 30 s. Design point abd was washed with one volume of 1% SDS for 30 s before washing with three volumes of dichloromethane for 30 s. Design point ad was washed with three volumes of methanol before washing with one volume of 1% SDS, each for 30 min. In contrast, AMP content of washes for design point abc was lowest (62%), indicating that this combination of decontamination parameters was least effective in removing externally contaminated AMP from hair. Design point abc was washed with one volume of dichloromethane, then three volumes of 1% SDS, each wash for 30 s.

In contrast, AMP content of washes for design point abc was lowest (62%), indicating that this combination of decontamination parameters was least effective in removing externally contaminated AMP from hair. The hair extracts of each sample were also analyzed for AMP content, all showing concentrations below the LOD, indicating that no AMP had been incorporated into the deeper hair matrix during the intentional contamination procedure or as a result of processing.

4. Discussion

Much of the published literature comparing different techniques that have been employed for the pre-treatment and analysis of drugs in hair samples presents tabulated results of different methods without comparing their performance [3,5,8,10,23–25]. Previous work has generally focused on comparing pre-treatment parameters in forensic hair analysis by evaluating individual factors with a OFAT approach. For example, Salomone *et al.* determined that pulverizing hair before determining EtG content was more effective than cutting the hair into snippets [26]. Studies conducted by Romano *et al.* and Stout *et al.* aimed to compare decontamination protocols that were previously established for removal of cocaine from hair, both finding that different protocols resulted in different amounts of drug being removed. They also determined that the tested protocols were insufficient in removing all of the contamination and, in the worst case, actually encouraged incorporation of contaminating drug into the hair [27,28]. Others have examined the effect of heat and ultrasonication on the efficacy of solvent extraction [17], the effect of a variety of aqueous and organic decontamination solvents for the removal of drug from the surface of hair [29], and the efficacy of different extraction solvents for a wide variety of drugs and their metabolites [30]. However, none of these works directly compared all hair pre-treatment parameters in a single experiment.

A few recent studies have applied DoE techniques to hair analysis pre-treatment parameters on a limited basis. For example, Mueller *et al.* conducted DoE studies to evaluate hair processing protocols for EtG. They compared several pre-treatment parameters, including extraction solvent, the use of ultrasonication, incubation time, incubation temperature, solvent amount, and hair particle size using a Plackett-Burman experimental design [14]. This design assumes that the 2-factor effects (*i.e.,* interactive effects coming from 2 factors as opposed to a single factor) are negligible. The factors identified to have significant influence on the detection of EtG included type of extraction solvent, extraction temperature, and particle size of the homogenized hair [14]. A study by Alladio *et al.* investigated optimized methodology for EtG in hair using DoE. First, a 2-factor full factorial design was used to investigate extraction temperature and solvent formulation. Then, an optimization analysis employing a face-centered central composite design compared extraction time, temperature, and pH [13]. While useful as first approximation analyses, such Plackett-Burman experimental designs do not address the possibility of combinatorial effects.

In the present study, the DoE approach employed a 2^4 factorial block design, which allowed for the observation of both the individual and combinatorial effects of the parameters under study. Blank human hair was treated with AMP, either by soaking in buffer solution containing the drug to yield drug “incorporated” HRM, or by coating with drug powder to produce externally contaminated hair. Using incorporated hair for DoE studies, as was done here, has both benefits and limitations. The greatest advantage of incorporated hair over other types of HRM is that it can be prepared to contain either single or multiple analytes with adequate concentrations and in amounts necessary for large DoE studies. The concentration of AMP incorporated into hair in the present study was consistent with that reported by other authors using similar incorporation methods [15]. A limitation of using incorporated hair is that the exact mechanism by which drugs are held within the hair matrix is not fully understood, and while the mechanism of drug incorporation in such material may not be identical to that following systemic drug exposure, incorporated hair is often employed as a reference material in forensic hair analysis [15]. Nevertheless, as the primary goal of the present investigation was to demonstrate proof-of-principle for the useful application of DoE in method development for hair pre-treatment, use of incorporated hair was deemed valid and appropriate.

DoE results with AMP revealed that all hair pre-treatment factors and combinations of factors were found to be significant. These findings indicate that the factors alone did have a significant effect on the level of drug detected in each sample and that the higher-level effects, representing combinations of the main effects, also had significant impact on extracted drug quantities. For example, results demonstrated that the treatments associated with design points c and d resulted in the highest quantity of extracted AMP. The commonalities between the two conditions included the extraction solvent and sample size ratio (12.5 μL/mg), the high time block (extraction for 24 h), and

minimization of particle size through pulverization. The latter effect is likely because drugs that are incorporated into hair are hypothesized to be localized in the cortex of the hair. When the hair strand is pulverized, more of the cortex is exposed to the extraction solution than when the hair is cut into segments. Previous studies have also shown the significance of particle size on drug recovery from hair [26,31].

Further DoE studies were conducted to give additional attention to decontamination protocols specifically designed to remove externally deposited drug prior to extraction of incorporated drug. Decontamination DoE studies indicated that the organic solvent identity and number of consecutive aqueous washes as individual factors did not have a significant effect on the amount of drug removed from the surface of hair intentionally contaminated with AMP. Treatments associated with design points d, ac, and abd resulted in the highest recovery of drug after decontamination. Each of these treatments included four washes, indicating that one aqueous wash and one organic wash was insufficient for the removal of external AMP contamination on hair.

Methanol was used as the organic decontamination solvent for design points d and ac, which suggests that it was more effective than the less polar solvent dichloromethane, in combination with other factors. Design points d and ac also utilized 30 s washes, indicating that this protocol, in combination with the other factors, was more effective at decontamination of AMP. These samples also had three sequential washes with the organic solvent and one wash with the aqueous solvent. In addition, for all of these design points, the aqueous wash was conducted before the organic wash. The aqueous decontamination solution for design points ac and abd was 1% SDS, indicating that this protocol, in combination with the other factors, was more effective at removing AMP from the surface of hair.

Design point abc had the lowest recovery of AMP, at 62.5%. The differences between the effective protocols and samples treated under design point abc were the use of dichloromethane as the organic solvent, washing with the organic solvent before the aqueous, and each wash lasting 30 min. The observed similarities and differences between effective and ineffective decontamination protocols emphasize the importance of considering all of the parameters under study at the same time, as opposed to individually, which is the major strength of the DoE statistical approach.

Results of the present study confirm the potential utility of a DoE approach to optimize decontamination and other pre-treatment parameters for specific drugs in hair analysis. Furthermore, it is important to note that all hair pre-treatment factors and combinations of factors were found to have a significant effect on the efficacy of decontamination and extraction of AMP from the matrix, suggesting that it is necessary to study pre-treatment conditions as whole protocols. Additional work is being conducted with other drugs incorporated into hair and with authentic hair specimens to further explore the applicability of the DoE approach.

5. Conclusions

A 2^4 factorial screening design was successfully applied to the problem of systematically comparing a limited number of the existing techniques for decontaminating and extracting AMP from human head hair. Significant impact on the recovery of AMP from human head hair was observed when considering specific pre-treatment parameters. The most effective pre-treatment parameters were determined as washing with 1% SDS and dichloromethane for 30 s, with a minimum of four consecutive washes, followed by pulverization of the hair into a powder and extracting AMP using the enzymatic extraction technique with a 12.5 μL volume of aqueous proteinase K/mg of hair. It is important to note that the 4-factor interaction effects in both the pre-treatment experiments and the decontamination experiments were significant; this suggests that all factors examined in each of these studies should be considered in combination with one another, not just on their own. Consequently, when attempting to optimize pre-treatment methodology for the analysis of drugs in hair, one must consider the interaction effects, an approach that has not previously been reported. Results obtained in the present study indicate that DoE is a promising approach for this purpose.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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