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Development and Validation of Two Innovative Quantitative Liquid Chromatography Tandem Mass Spectrometry Methods for Forensic Toxicology Laboratories: Novel Analysis of Designer Drugs and Simultaneous Method for Cocaine and Opioids in Biological Matrices

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Statement of the Problem

With the advent of liquid chromatography tandem mass spectrometry (LC-MS-MS) technology, and an increasing number of toxicology laboratories utilizing these instruments, it is imperative to have methods validated for biological matrices to ensure accurate results are being obtained and reported for quantitative data. The Virginia Department of Forensic Science (VADFS) has made a significant investment in the procurement of LC-MS-MS systems for the Toxicology Sections. Extensive work has been performed within the agency focusing on the best practices for the development, validation, and implementation of new quantitative LC-MS-MS methods. The goal of this research project is to develop and validate two quantitative LC-MS-MS methods for the analysis of whole blood and additional biological matrices in accordance with guidelines promulgated by the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology.[1] The objectives for this project are: 1) develop and validate a technique that addresses the recent proliferation of designer drugs such as cannabimimetic agents and designer stimulants, 2) develop and validate a combined method for cocaine and its metabolites and opioids, and 3) disseminate the methods to the toxicology community.

The inherent increased sensitivity and selectivity of LC-MS-MS makes the technology more advantageous over gas chromatography mass spectrometry (GC-MS) for the analysis of biological specimens which is traditionally viewed as the “gold standard” in forensic toxicology. The superiority of LC-MS-MS is not only due to the increased sensitivity and selectivity, but also in the sample preparation and ionization.[2] GC-MS often requires time-consuming derivatization of compounds prior to analysis, which directly impacts laboratory efficiency. Also, due to the high temperatures utilized in the injection port of GC-MS instruments, thermally labile compounds are difficult to quantitatively evaluate. Also within the injection port, a high degree of fragmentation occurs, making spectral interpretation difficult without database searching capabilities.[3] The two validated methods will directly impact the criminal justice system in a multitude of ways by providing a new quantitative analysis method that currently does not exist for designer drugs as well as increasing laboratory productivity and decreasing turnaround times for cocaine and opioid cases.

An increased submission of designer drugs in VADFS Controlled Substances Section indicates a need for a robust quantitative method for these drugs in the Toxicology Section. Since September 2012, VADFS’s Controlled Substances Section has confirmed 680 cases containing designer drugs. Currently, VADFS does not have a method validated for the quantitative evaluation of these compounds in biological matrices. Designer drugs, including cannabimimetic compounds and designer stimulants are becoming an increased concern in the United States. These structural analogues are manufactured to mimic the effects of scheduled compounds posing a twofold threat to the criminal justice system.[4-8] These drugs pose legal concerns due to their ability to avoid provisions of drug laws and they have a high potential for abuse, which impacts toxicological analysis.[9] In 2012, federal legislation enacted Senate Bill 3187, which includes the addition of twenty-six synthetic drugs to the list of Schedule I substances to the Controlled Substances Act.[10]

Currently, the literature has limited information pertaining to concentrations associated with the effects of these drugs making interpretation for driving under the influence of drugs (DUID) and

medicolegal death investigation nearly impossible.[11, 12] The implementation of a quantitative method for biological matrices validated to exceed the SWGTOX Standard Practices for Method Validation in Forensic Toxicology guidelines will enable the reporting and dissemination of blood and tissue concentrations within the forensic science community to aid in the establishment of human subject concentration information. The target compounds will include those indicated with high prevalence in VADFS Controlled Substances Section, which have been historically tracked since September 2012. The acquisition of concentration data will provide information that will be advantageous for interpreting the meaning of the quantitative results for both DUID cases and death investigation casework, nationwide.

Not only is it crucial for forensic laboratories to validate and implement methods for the quantitative assessment of newly abused/developed compounds such as designer drugs, it is also important to evaluate historical methods to increase laboratory productivity and efficiency. The Toxicology Section of VADFS analyzes approximately 2500 driving under the influence/driving under the influence of drugs (DUI/DUID), 4600 death investigation, and 300 non-implied consent police cases per year. Approximately eight percent of the cases analyzed are reported with quantitative values associated with cocaine, benzoylecgonine, and cocaethylene and approximately thirty one percent are reported with quantitative values associated with opioids. Many cases submitted involve a combination of drugs that require multiple extractions and quantitative analyses. Therefore, combining analytical methods will result in backlog reduction and decreased turnaround times.

The literature contains a variety of methods for the analysis of cocaine and its metabolites using LC-MS-MS. The matrices evaluated include urine, serum, plasma, whole blood, brain tissue, hair, saliva, and meconium.[13-18] The literature also contains similar information for LC-MS-MS analysis of opioids in various matrices.[19-23] Although methods exist for the simultaneous analysis of cocaine and opioids in hair, plasma, placenta, and umbilical cord, limited literature is available for an equivalent method in whole blood and matrices received in medicolegal death investigations such as liver and tissue.[7, 24] In addition to the limited matrices evaluated, the methods are not validated to meet current forensically acceptable method validation guidelines published by SWGTOX. These guidelines are anticipated to become validation requirements with the advent of the Organization of Scientific Area Committees (OSAC), which was constructed by the National Institute of Standards and Technology (NIST). Therefore, there is a significant need for the development and validation of a simultaneous cocaine and opioid quantitative LC-MS-MS method in whole blood and other biologically significant matrices that meets the scope and standards of the forensic community.

Currently, VADFS utilizes GC-MS for the analysis of cocaine and opioids in biological matrices individually. Both extractions utilize a significant volume of blood for analysis, totaling four milliliters for the analysis of the two drug classes. The extractions include a solid phase extraction as well as a time-consuming derivatization prior to GC-MS analysis. Given the nature of limited sample volume in forensic toxicology applications, re-evaluation of historical methods to establish multi-drug class methods, with decreased sample volume, using modern data analysis software will have a significant impact on laboratory efficiency. The target compounds will include the compounds currently evaluated in the VADFS Toxicology Section in multiple methods as well as the addition of other targets that may be useful in circumstances of medicolegal death investigation such as anhydroecgonine methyl ester (AEME) and acetyl

fentanyl. The newly validated method will contain cocaine and its metabolites as well as natural, semi-synthetic, and synthetic opioids enabling the combination of four current VADFS methods into one overarching method. The increased efficiency obtained, from the development and validation of a quantitative LC-MS-MS that simultaneously evaluates cocaine and opioids, will directly impact the criminal justice system. The increased efficiency will reduce turnaround times for case completion enabling Certificates of Analysis to be issued more rapidly. This will also be advantageous for medicolegal death investigations, enabling for a more timely cause of death determination by medical examiners.

This proposed project has the potential to significantly impact the forensic science community. Upon completion of the project, two methods will be fully validated to exceed the generally accepted SWGTOX validation guidelines. The methods will include a comparison and validation of three different sample extraction techniques, including solid phase extraction, liquid-liquid extraction, and protein precipitation as well as complete validations not only in whole blood, but also other common death investigation biological matrices. The comparisons of sample preparation techniques within an individual method will enable for the determination of the most efficient, specific, and cost-effective method that can be immediately implemented into any forensic toxicology laboratory across the United States. This proposed project will facilitate a transition from independent laboratory developed methods to universally standardized methods, enabling a more globalized consistent approach within the toxicology community.

Project Design and Implementation

Within this research project, two methods will be developed and validated to exceed the SWGTOX Standard Practices for Method Validation in Forensic Toxicology guidelines. The method development and validation process will be similar for each method to establish an evaluation of sample preparation techniques as well as optimal instrumental conditions that will not only demonstrate accuracy and precision, but also a level of efficiency and cost analysis for each method. This will be accomplished by completing a validation of three sample preparation techniques including solid phase extraction, liquid-liquid extraction, and protein precipitation for each quantitative method. Finally, the Uncertainty of Measurement will be evaluated for each target contained within the two methods validated enabling further confidence in the methods.

The SWGTOX Standard Practices for Method Validation in Forensic Toxicology guidelines were first published in May 2013 and have become generally accepted as validation guidelines in forensic laboratories. The validation of these two proposed methods will exceed the validation requirements set forth by SWGTOX. The project design is a twofold process consisting of method development prior to method validation. The following experiments will be completed to establish objective evidence that demonstrates the two methods are capable of contributing reproducible and robust methods to the forensic science community.

Method Development

Instrumental Method Optimization

An Agilent Technologies 1290 Infinity liquid chromatography system coupled to a 6430 quadrupole mass spectrometer will be utilized for the development and validation of these two

methods. The Agilent Technologies MassHunter Optimizer software as well as peer-reviewed literature references will be used to optimize ion selection for each target as well as develop optimal mass spectrometer conditions. Appendix B delineates a proposed list of target compounds and internal standards for each method. Upon optimization of individual target compounds, the liquid chromatography separation will be developed for all targets contained within in the method. A standard reverse phase Agilent Technologies Poroshell 120 EC-18 (2.1 x 100 mm, 2.7µm pore size) column will be used as the stationary phase. If acceptable separation is not achieved with standard reverse phase chromatography, a more selective column will be used such as a phenyl-hexyl or diphenyl stationary phase column. Also, mobile phase composition, gradient, and flow rates will be adjusted to achieve optimal separation of the target compounds.

Upon completion of instrumental method development, a neat methanolic calibration curve will be prepared for each method containing all targets as well as associated deuterium labeled internal standards. The calibration range for the designer drugs method will encompass a wide concentration range (at minimum two orders of magnitude) since limited information is known about relative biological concentrations in human subjects. The cocaine and opioids method will contain a concentration range that encompasses therapeutic, toxic, and lethal ranges. The neat methanolic calibration analysis will serve to ensure proper instrument performance without contribution from the matrix.

Sample Preparation Extraction

Sample preparation is a pivotal component to laboratory efficiency. To enable a thorough evaluation of the newly developed methods, at minimum, three extraction techniques will be developed and evaluated. Traditionally, solid phase extractions and liquid-liquid extractions are used extensively in forensic toxicology laboratories. The utilization of protein precipitation is becoming a useful extraction tool in forensic toxicology because of the limited sample volume required and the rapid nature of the extraction. Therefore, at minimum, a solid phase extraction, liquid-liquid extraction, and protein precipitation extraction will be developed for each method. The extraction methods will be developed for whole blood since the majority of evidentiary samples submitted are whole blood. The methods will then be extended to other matrices received in medicolegal death investigations such as liver and tissue where available.

During the development of the sample extraction methods, several parameters will be optimized and evaluated including, but not limited to, sample volume, extraction complexity, and extraction time. The sample volume will be adjusted for each method to determine the smallest sample volume possible to still maintain the integrity of the calibration range. The methods will be developed to have minimal complexity and the three methods will be compared for complexity by evaluating the number of steps for each extraction. The extraction time will be kept to a minimum for each method and a comparison of overall sample extraction time will be compared for each method. This will be significantly impacted by incubation times, solid phase extraction steps, and centrifugation times. The goal is to create three methods with limited sample volume, minimal complexity, and high efficiency. Upon development of three optimal extraction methods for each of the proposed methods, an extensive validation will be completed as delineated in following section of this proposal. Example calculations are provided in Appendix C, which also delineates a validation summary document.

Method Validation

Accuracy/Precision

The accuracy and precision of the methods will be evaluated using pooled matrix samples. To evaluate accuracy, three concentrations distributed equally across the calibration range will be analyzed in triplicate over a minimum of five batches. The percent accuracy shall not exceed ±20 % accuracy at each concentration level. All samples evaluated for accuracy will be included into the accuracy determination. The same data used in the accuracy studies will also be used to determine the within-run and intermediate precision of the methods.

Precision will be expressed as the coefficient of variation (% CV) and two different types of precision studies will be assessed during validation: within-run precision and intermediate precision. To assess within-run precision, the precision of each batch will be evaluated whereas intermediate precision will evaluate the precision of the method over multiple batches. The greatest within-run precision over each batch will be utilized for the within-run precision of the process. Precision, like accuracy, should not exceed ±20 % precision for within-run and intermediate precision.

Sensitivity

The limit of detection and limit of quantitation will be evaluated for each method. To determine the target limit of detection and quantitation, serial dilutions will be prepared and analyzed to determine the lowest concentration that is capable of achieving acceptable predetermined identification criteria. Once estimated, triplicate determinations of fortified samples from at least three different sources will be assessed to verify the concentration meets the identification criteria. The predetermined acceptance criterion for limit of detection is a retention time within ± 5% of the average of the calibrator retention time, a qualifier ion ratio within ±20 % of the average ratio of the calibrators, and a signal to noise ratio greater than three to one. The acceptance criterion for the limit of quantitation is the same as the limit of detection for retention time and ion qualifier ratios, but the signal to noise must be greater than ten to one, in addition the back calculated concentration must be within ±20 % of the target concentration.

Linearity and Calibration Model

The calibration model for each target compound within the two methods will be established by determining the range of analyte concentrations over which the method will be used. The calibration range will be evaluated using at minimum seven calibrators across the calibration range. To establish the calibration model, a minimum of six replicate determinations will be utilized. Although the least squares model for regression is preferred, the best and simplest model (e.g. weighted, non-weighted, liner, quadratic) that fits the data will be chosen. The model will be established by residual plot analysis as well as statistical comparisons. The origin will be ignored in all calibration models, the correlation coefficient must also be ≥ 0.985, and the back calculated calibrator concentrations shall be within ±20 % of the target concentration for acceptance of the calibration model.

The statistical analysis techniques that will be utilized in the determination of the calibration model for each target will be the analysis of variance (ANOVA). The standard deviation of the residuals will be compared between the linear, quadratic, weighted, and non-weighted calibration models. The t-test and f-test will be utilized to determine statistically significant differences between linear and quadratic calibration models. If the two groups are determined not to be statistically different, a linear calibration model will be applied to the target. If the two groups are determined to be statistically different, the quadratic calibration model will be applied. To determine weighting of the calibration model, the sum of the relative residual errors will be evaluated. The weighting that minimizes the sum of relative residual errors for all batches evaluated will be the weighting model applied to the target.

Ion Suppression/Enhancement

Ion suppression and enhancement will be addressed with neat standards and post-extraction fortified samples. Two different sets of samples will be prepared and their peak areas will be compared between sets. Neat standards, at low, medium, and high concentrations, will be prepared in neat extraction solvent and injected a minimum of six times for each concentration level. The responses will be averaged for the three different concentrations. A minimum of ten pairs of post-extraction fortified samples, at three different concentrations, will be prepared to compare to the neat standards. The samples for each pair of post-extraction fortified samples will be from different sources. Post-extraction fortified samples are blank matrix samples that are extracted and then fortified with analytes after extraction. The responses will be averaged for the two concentrations and the ratio between the averages of the sets will then be used to assess ion suppression or enhancement.

Recovery

Recovery will be represented as a percentage of the analyte response after sample preparation compared to that of a solution containing the analyte at a concentration corresponding to 100 %. The solution containing the 100 % (double blank) analyte concentration will be prepared by extracting a minimum of six high, medium, and low concentrations of post extraction fortified samples. A minimum of six extractions of a high, medium, and low concentration of samples fortified prior to extraction will enable a recovery comparison. The post extraction fortified samples nullify the matrix effect enabling an accurate recovery comparison.

Carryover

Carryover will be evaluated with solvent blanks injected immediately following progressively higher concentrations of analyte of interest fortified into blank matrix. A solvent blank shall immediately follow a fortified sample in the injection sequence. The highest analyte concentration at which no analyte carryover is observed in the matrix blank is determined to be the concentration at which the method is free from carryover. This concentration will then be confirmed using triplicate analysis.

Interferences

Interferences from several sources will be evaluated in the validations of these methods. Interferences associated with endogenous compounds will be evaluated by analyzing a minimum of ten negative matrix samples from different sources without the addition of internal standard or analyte. Interferences from the contribution of high concentrations of analyte to internal standard and high concentrations of internal standard to analyte will also be evaluated. This will be accomplished by evaluating three sources of matrix fortified with high concentration of internal standard and no target compounds and high concentration of target compounds with no internal standard. Also, high concentrations of target compounds will be evaluated independently to assess contribution to other compounds within the method. Interferences will also be evaluated by analyzing commonly encountered analytes. A minimum of three matrix samples will be fortified with commonly encountered drugs in the VADFS Toxicology Section as well as metabolites and other structurally similar compounds. The void of peaks is indicative of no interferences associated with endogenous compounds, target compounds, and commonly encountered compounds.

Dilution Integrity

The effect of sample dilution will be assessed using both large and small volume dilutions where applicable. Large volume dilutions are useful for evidentiary samples with sufficient volume, but concentration above the upper limit of detection. The small volume dilution is necessary for evidentiary samples containing limited sample volumes that do not meet the volume requirements of the method. The dilution limit will be determined by preparing fortified pooled samples and analyzing the dilutions in triplicate. The dilutions evaluated will be common dilutions performed on evidentiary samples such as (1:2, 1:5, and 1:10). Accuracy and precision studies will be utilized to determine if the dilution meets the predetermined acceptance criteria of ±20 % of the target concentration for accuracy and precision.

Stability

The stability of extracted samples that are not analyzed immediately will be evaluated. At minimum, two concentrations of samples will be extracted and analyzed at minimum every twenty four hours for a seven day period with triplicate injections at each time point. Samples will be immediately analyzed following extraction for day one instrumental response determination. The samples will then remain in the autosampler of the instrument in normal laboratory conditions and injected over the seven day period. The instrumental response will be evaluated for each target at each time point. Day one will be considered 100 % and subsequent injections will be compared to the initial instrumental response. The samples will be considered stable if the average signal of the triplicate injections for a time point does not increase above 120 % or decrease below 80 % of the original response. This experiment will simulate the stability of the targets if an instrumental run is abruptly interrupted or delayed.

Uncertainty of Measurement

The robustness and reproducibility of the two validated methods will be addressed during the collection of data for the estimation of uncertainty of measurement. During the validation, controls will be analyzed within every validation batch. Control charts will be created to evaluate the historical variance of the methods over the validation period. Also, multiple scientists will

conduct an analysis of each method including the analysis of calibrators, controls, and pooled samples to further assist with an accurate estimation of historical data. Uncertainty of measurement budgets will be developed for every target within the methods including individual budgets for each extraction technique in accordance with the ASCLD/LAB Policy on Measurement Uncertainty and ISO/IEC 17025:2005 General Requirements for the Competence of Testing and Calibration Laboratories.[25, 26]

Dissemination Strategy

Upon completion of the project, the information will be disseminated using various forums including publication in peer-reviewed journals and presentations. Publications will be submitted to a peer-reviewed journal such as the Forensic Science International, Journal of Chromatography B, or the Journal of Analytical Toxicology. Also, the work completed within the scope of this project will be disseminated in the form of presentations at scientific conferences such as the Society of Forensic Toxicologists (SOFT) Conference. There is also the potential for dissemination to a broad audience by presenting the work using an international webinar forum, such as Research Triangle Institute. VADFS has previously presented two international webinars on the validation of quantitative methods. Finally, VADFS publishes all departmental Procedures Manuals on the Department's website providing a continuous means of dissemination of information to its customers, other agencies, or interested parties. Interested parties seeking validation information have the ability to contact the Department to obtain copies of validation information, creating a seamless transition of methods to other laboratories

Potential Impact

This project has the potential to significantly impact the forensic science community in multiple ways. Upon completion of the project, two methods will be fully validated to exceed the generally accepted SWGTOX validation guidelines. The two methods will include a comparison and validation of three different sample extraction techniques as well as complete validations not only in whole blood, but also other common death investigation matrices. The comparisons of sample preparation methods will enable for the determination of the most efficient, accurate, and cost-effective method that can be immediately implemented into any forensic toxicology laboratory.

The validation of a designer drug method including cannabimimetic compounds and cathinone analogues based on the SWGTOX guidelines will enable the forensic community to collect biological concentration information for these increasingly popular compounds. The culmination of data will aid the forensic community in gathering essential information for interpretation of DUID cases as well as medicolegal death investigations.

The validation of a combined cocaine and opioid method has the potential to help decrease laboratory backlogs and turnaround times by decreasing the number of analyses needed to evaluate an individual case. Also, the method has the potential to utilize less sample volume than current methods that require significant volumes, which is critical in forensic applications where case sample volume is limited. The validation will also increase awareness of best practices to the forensic community given the comparative nature of the project as well as inclusion of the estimation of uncertainty of measurement within the validation.

Capabilities/Competencies

[Redacted PI information]

A **Forensic Laboratory Specialist II** will be hired to perform some of the experimentation within this project. The specialist will be under the direct supervision of [PI]. It is anticipated that the specialist will spend no more than twenty nine hours per week working on the project. Please see Appendix D for a biographical sketch of the Forensic Laboratory Specialist II position.

The Virginia Department of Forensic Science Toxicology Section currently has five Agilent Technologies liquid chromatography tandem mass spectrometers. One of these instruments is currently utilized for method development and validation. Therefore, no new analytical instrumentation must be requisitioned for the purpose of this project. The laboratory also currently has all other equipment needed for successful management and validation of the proposed methods, which is demonstrated by the four previously validated and implemented methods within the Department. Project timeline and data archiving plans are delineated in Appendix E and Appendix F respectively. The principal investigator also possesses the statistical knowledge required for method validations of this magnitude. Therefore, statisticians outside of VADFS are not required within the scope of this project. VADFS already possesses a working relationship with the Virginia Office of the Chief Medical Examiner for the acquisition of available death investigation matrices, such as liver and tissue.

APPENDIX A

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APPENDIX B

TARGET COMPOUNDS AND INTERNAL STANDARDS

Proposed compounds for the quantitative analysis of designer drugs method using LC-MS-MS. The method will include, but is not limited to, the compounds delineated in the table.

Compounds for LC-MS-MS Designer Drug Quantitative Method	
Targets	Internal Standards
AB-CHMINACA	JWH-018 4-Hydroxypentyl Metabolite-D_5
FUB-144	JWH-073 3-Hydroxybutyl Metabolite-D_5
AB-PINACA	AM2201 4-Hydroxypentyl Metabolite-D_5
AB-FUBINACA	Butylone-D_3
5-Fluoro-THJ	Ethylone-D_5
FUBIMINA	Methylone-D_3
PB-22	Mephedrone-D_3
5-Fluoro PB-22	
BB-22	
UR-144	
XLR11	
THJ2201	
HU-210	
JWH-018	
JWH-073	
AM1248	
Butylone	
Ethylone	
Methylone	
Mephedrone	
Pentedrone	
Alpha-Pyrrolidinonpentiophenone	
Methcathinone	
Bupropion	
Methylenedioxypyrovalerone	

Proposed compounds for combined quantitative cocaine and opioid method using LC-MS-MS. The method will include, but is not limited to, the compounds delineated in the table.

Compounds for LC-MS-MS Cocaine and Opioid Quantitative Method	
Targets	Internal Standards
Cocaine	Cocaine-D_3
Benzoylecgonine	Benzoylecgonine-D_3
Cocaethylene	Cocaethylene-D_3
Anhydroecgonine methyl ester	Morphine-D_3
Morphine	Codeine-D_3
Codeine	Oxycodone-D_3
Oxycodone	Oxymorphone-D_3
Oxymorphone	Hydromorphone-D_3
Hydromorphone	Hydrocodone-D_3
Hydrocodone	6-Acetylmorphine-D_3
6-Acetylmorphine	Fentanyl-D_5
Fentanyl	Methadone-D_3
Acetyl fentanyl	Meperidine-D_4
Methadone	Buprenorphine-D_4
Meperidine	Norbuprenorphine-D_3
Tramadol	Naloxone-D_5
Buprenorphine	
Norbuprenorphine	
Naloxone	

APPENDIX C

SUPPORTING DATA

Validation Summary

The following is an example of a validation summary for the confirmation and quantitation of target compounds in whole blood using LC-MS-MS.

- I Accuracy and Precision
  - i Accuracy
  - ii Within Run Precision
  - iii Intermediate Precision
- II Sensitivity (LOD, LOQ)
  - i Limit of detection
  - ii Limit of quantitation
- III Linearity and Calibration Model
- IV Ion Suppression/Enhancement
- V Recovery
- VI Carryover
- VII Interferences
  - i Endogenous Compounds (blanks)
  - ii Internal Standard
  - iii Commonly Encountered Analytes
- VIII Dilution Integrity
- IX Stability
- X Robustness
- XI Training
- XII Summary
- XIII References
- XIV Appendix A

Validation experiments were conducted for the following target analytes.

Target	Internal Standard
R(+)Methcathinone	Mephedrone-D_3
R,R(-)Pseudoephedrine	Pseudoephedrine-D_3
Methylone HCl	Methylone-D_3
(±)Amphetamine	Amphetamine-D_{11}
(±)Methamphetamine	Methamphetamine-D_{11}
(±)3,4-Methylenedioxyamphetamine (MDA)	MDA-D_5
Methedrone HCl	Mephedrone-D_3
(±)3,4-Methylenedioxy-N-methylamphetamine (MDMA)	MDMA-D_5
Phentermine	Methamphetamine-D_{11}
Mephedrone	Mephedrone-D_3
α-Pyrrolidinopentiophenone (α-PVP)	Mephedrone-D_3
3,4, Methylenedioxypyrovalerone HCl (MDPV)	Mephedrone-D_3
Bupropion HCl	Mephedrone-D_3

The method includes an alkaline liquid-liquid extraction and subsequent quantitation and confirmation via LC-MS-MS (Agilent 6430) adopted from the literature references provided herein.

I Accuracy and Precision

Accuracy

Accuracy was assessed by analyzing pooled blank blood samples fortified with thirteen target compounds at three different concentrations (low, medium, and high) in triplicate with each batch for a total of five batches. The pooled fortified blood samples were prepared by spiking a large volume of blank blood with the respective concentrations of target analytes (0.03, 0.3, and 1.5 mg/L). Aliquots (1.0 mL) were then taken from the pooled sample and extracted prior to quantitative analysis using LC-MS-MS.

Accuracy was assessed using the following equation:

Equation 1.

$$Accuracy\ (\%) = \left|\left(\left(\frac{Mean\ of\ calculated\ values}{Expected\ value}\right) \times 100\%\right) - 100\right|$$

The acceptance criterion for pooled accuracy was ±20 % for all three concentrations levels. All back calculated concentrations were utilized in determining the overall accuracy for the method. Table 1 represents the accuracy for the pooled blood samples. The percent accuracy also demonstrates bias within the measurements. The *n* was fifteen for all three concentration levels.

Table 1. Percent accuracy for amphetamines, phentermine, and designer stimulants quantitated by LC-MS-MS

	Pooled Accuracy % Accuracy (SD); n=15		
Target	0.03 mg/L	0.3 mg/L	1.5 mg/L
Methcathinone	100(4)	100(6)	102(5)
Pseudoephedrine	100(3)	99(3)	102(5)
Methylone	97(3)	99(3)	97(4)
Amphetamine	102(4)	99(2)	93(5)
Methamphetamine	104(5)	100(2)	95(5)
MDA	100(4)	97(2)	99(5)
Methedrone	101(3)	98(2)	99(6)
MDMA	99(4)	98(2)	97(4)
Phentermine	98(5)	99(3)	99(6)
Mephedrone	99(3)	99(3)	102(5)
α-PVP	105(6)	101(3)	101(6)
MDPV	102(4)	98(3)	97(5)
Bupropion	99(7)	95(5)	97(12)

The accuracy ranged from 93±5 % to 105±6 %. All targets were within the acceptance criteria of ±20 %. The accuracy was within ±5 % for all target compounds.

Precision

The within-run and intermediate precision was assessed using fortified pooled blood samples. The within-run precision was established by analyzing three different concentrations (0.03, 0.3, and 1.5 mg/L) of the target compounds in triplicate over five batches. The highest imprecision was reported for each concentration level. The intermediate precision was calculated by analyzing three different concentrations (0.03, 0.3, and 1.5 mg/L) of the target compounds in triplicate over five batches.

The pooled fortified blood samples were prepared by spiking a large volume of blank blood with the respective concentrations of target analytes (0.03, 0.3, and 1.5 mg/L). Aliquots (1.0 mL) were then taken from the pooled sample and extracted prior to quantitative analysis using LC-MS-MS.

Precision was measured as the coefficient of variance (%CV) for the within-run and intermediate precision analysis. The following equations were utilized to determine the within-run and intermediate precision for the target compounds using LC-MS-MS.

Equation 2.

$$Within-run\ Precision\ (\%CV) = \left(\frac{Standard\ deviation\ of\ batch\ mean}{Calculated\ mean\ of\ batch}\right) \times 100\%$$

Equation 3.

$$Intermediate\ Precision\ (\%CV) = \left(\frac{Standard\ deviation\ of\ combined\ means}{Calculated\ grand\ mean}\right) \times 100\%$$

The predetermined acceptance criterion for within-run and intermediate precision was ±20 % at each concentration level. Table 2 represents the within-run precision data for the pooled samples at three concentrations.

Table 2. Within-run precision of amphetamines, phentermine, and designer stimulants quantitated by LC-MS-MS

	Pooled Within-Run Precision Mean ± SD(%CV); n=3		
Target	0.03 mg/L	0.3 mg/L	1.5 mg/L
Methcathinone	0.029±0.002(7)	0.285±0.010(3)	1.53±0.06(4)
Pseudoephedrine	0.029±0.001(3)	0.301±0.008(3)	1.47±0.04(3)
Methylone	0.028±0.001(5)	0.300±0.005(2)	1.43±0.05(3)
Amphetamine	0.029±0.002(7)	0.300±0.004(1)	1.37±0.05(4)
Methamphetamine	0.030±0.002(8)	0.306±0.006(2)	1.37±0.06(4)
MDA	0.028±0.001(4)	0.284±0.005(2)	1.42±0.05(3)
Methedrone	0.030±0.001(4)	0.297±0.007(2)	1.39±0.05(4)
MDMA	0.028±0.001(3)	0.284±0.005(2)	1.40±0.05(3)
Phentermine	0.029±0.003(11)	0.298±0.008(3)	1.40±0.04(3)
Mephedrone	0.029±0.002(6)	0.298±0.005(2)	1.47±0.05(3)
α-PVP	0.033±0.004(11)	0.308±0.005(1)	1.44±0.10(7)
MDPV	0.031±0.002(8)	0.293±0.014(5)	1.40±0.05(4)
Bupropion	0.029±0.004(14)	0.274±0.025(9)	1.48±0.23(16)

As shown in Table 2, all targets were within the predetermined acceptance criteria of ±20 % for the coefficient of variation. The percent CV ranged from 1 % to 16 % for the target compounds. Bupropion had the largest imprecision (16 %) at the highest concentration. The overall highest percent CV over the five batches for each target was represented in Table 2.

Table 3 represents the intermediate precision for the target compounds at three concentrations levels. The *n* was fifteen for the determination of intermediate precision.

Table 3. Intermediate precision of amphetamines, phentermine and designer stimulants quantitated by LC-MS-MS

	Pooled Intermediate Precision Mean ± SD(%CV); n=15		
Target	0.03 mg/L	0.3 mg/L	1.5 mg/L
Methcathinone	0.030±0.001(5)	0.299±0.017(6)	1.53±0.08(5)
Pseudoephedrine	0.030±0.001(3)	0.296±0.009(3)	1.53±0.07(4)
Methylone	0.029±0.001(3)	0.297±0.009(3)	1.46±0.06(4)
Amphetamine	0.030±0.001(4)	0.297±0.007(2)	1.40±0.07(5)
Methamphetamine	0.031±0.001(5)	0.299±0.006(2)	1.43±0.07(5)
MDA	0.030±0.001(4)	0.291±0.007(2)	1.49±0.07(5)
Methedrone	0.030±0.001(3)	0.295±0.006(2)	1.48±0.09(6)
MDMA	0.030±0.001(4)	0.293±0.007(2)	1.46±0.07(5)
Phentermine	0.029±0.002(5)	0.298±0.009(3)	1.48±0.09(6)
Mephedrone	0.030±0.001(3)	0.298±0.009(3)	1.52±0.07(5)
α-PVP	0.032±0.002(6)	0.302±0.010(3)	1.52±0.09(6)
MDPV	0.031±0.001(4)	0.294±0.009(3)	1.45±0.08(5)
Bupropion	0.030±0.002(7)	0.284±0.016(6)	1.45±0.19(13)

All targets were within the predetermined acceptance criterion of a percent CV within ±20 % for the intermediate precision. The percent CV ranged from 2 % to 13 %. The largest imprecision was bupropion (13 %) at 1.5 mg/L. All other targets were within a percent CV of ±10 %.

The accuracy and precision of the method meets the general acceptance criterion of ±20 % for accuracy, within-run precision, and intermediate precision. All thirteen targets were within the acceptance criterion.

II Sensitivity (LOD, LOQ)

i Limit of Detection

The limit of detection (LOD) was addressed by spiking three different blank blood sources at 0.005, 0.0025, and 0.00125 mg/L along with calibrators (0.01-2.0mg/L). The lowest concentration that was capable of achieving acceptable predetermined identification criteria (e.g. retention time, peak shape, signal-to-noise ratio, etc.) was considered the target LOD.

Predetermine acceptance criteria:

Retention Time - ±5 %
Qualifier Ratio - ±20 %
Signal to Noise – > 3

Table 4 demonstrates the LOD for compounds within the amphetamine and bath salt LC-MS-MS quantitative method.

Table 4. Limit of detection for amphetamines, phentermine, and designer stimulants quantitation by LC-MS-MS

Limit of Detection	
Target	LOD (mg/L)
Methcathinone	0.005
Pseudoephedrine	0.005
Methylone	0.0025
Amphetamine	0.005
Methamphetamine	0.005
MDA	0.00125
Methedrone	0.00125
MDMA	0.00125
Phentermine	0.01
Mephedrone	0.00125
α-PVP	0.0025
MDPV	0.00125
Bupropion	0.00125

The LOD for MDA, methedrone, MDMA, mephedrone, MDPV, and bupropion was determined to be 0.00125 mg/L. The LOD for methylone and α-PVP was determined to be 0.0025 mg/L. Methcathinone, pseudoephedrine, amphetamine, and methamphetamine had an LOD of 0.005 mg/L. Phentermine was the only analyte that did not meet the acceptance criteria at any concentration. Therefore the LOD for phentermine was determined to be 0.01 mg/L.

ii Limit of Quantitation

The limit of quantitation (LOQ) was addressed by spiking three different blank blood sources at 0.005, 0.0025, and 0.00125 mg/L along with calibrators (0.01-2.0 mg/L). The lowest concentration that was capable of achieving acceptable predetermined identification and quantitation criteria was considered the target LOQ.

Predetermine acceptance criteria:

- Retention Time - ±5 %
- Qualifier Ratio - ±20 %
- Signal to Noise - > 10
- Back Calculated Concentration - ±20 %

Table 5 demonstrates the LOQ for compounds within the amphetamine and bath salt LC-MS-MS quantitative method.

Table 5. Limit of quantitation for amphetamines, phentermine, and designer stimulants quantitation by LC-MS-MS

Limit of Quantitation	
Target	LOQ (mg/L)
Methcathinone	0.01
Pseudoephedrine	0.005
Methylone	0.005
Amphetamine	0.01
Methamphetamine	0.01
MDA	0.005
Methedrone	0.005
MDMA	0.005
Phentermine	0.01
Mephedrone	0.0025
α-PVP	0.005
MDPV	0.0025
Bupropion	0.01

The LOQ for mephedrone and MDPV were determined to be 0.0025 mg/L. Pseudoephedrine, methylone, MDA, methedrone, MDMA, and α-PVP had a LOQ of 0.005 mg/L. The LOQ for methcathinone, amphetamine, methamphetamine, phentermine, and bupropion were determined to be 0.01 mg/L.

III Linearity and Calibration Model

The best fit calibration model was determined using multiple statistical analysis techniques as well as the analysis of residual plots. A total of five fortified blank blood sources over nine batches were analyzed to determine the calibration model for each target. The calibration range assessed was from 0.01mg/L to 2.0 mg/L. A total of eight calibrators (0.01, 0.02, 0.05, 0.10, 0.25, 0.50, 1.0, and 2.0 mg/L) were used in the determination of the calibration model. To determine the linear/quadratic nature of the model, ANOVA was used to compare the standard

deviation of residuals from all nine batches. The t-test and f-test were utilized from the ANOVA. The t-test determined if there was a statistically significant difference between the linear and quadratic models.

> If p-value < 0.05 (level of significance), the null hypothesis was rejected
>
> If p-value > 0.05 (level of significance), the null hypothesis was not rejected
>
> The null hypothesis states that there was no statistically significant difference between groups.

The f-test is utilized to determine if there was a statistically significant difference in the variance between two groups.

> If $f > F_{crit}$, the null hypothesis was rejected
>
> If $f < F_{crit}$, the null hypothesis was not rejected
>
> The null hypothesis states that the variances between the two groups were equal.

If the two groups were determined not to be statistically different, a linear calibration model was applied to the target. This was to ensure that a quadratic fitting model was not applied to the data if there was no statistical significance. If the two groups were statistically different, the quadratic calibration model was applied to the target.

To determine the weighting of the calibration model (non-weighted or 1/x weighting), a t-test was used to assess if there was a significant difference between the two groups. The t-test was completed after the linear/quadratic nature of the model was established. The weighted and non-weighted sum of relative error for the residual was compared using the t-test.

> If p-value < 0.05 (level of significance), the null hypothesis was rejected
>
> If p-value > 0.05 (level of significance), the null hypothesis was not rejected
>
> The null hypothesis states that there was no statistically significant difference between groups.

The weighting of the calibration model was determined by applying the weighting that minimizes the sum of relative error for the residuals. The sum of relative error was averaged for an overall relative sum over the nine batches analyzed. The relative residual error was calculated using Equation 4 for each concentration in the calibration curve.

Equation 4.

$$Relative\ Residual\ Error = \frac{|Residual\ Error|}{Theoretical\ Concentration}$$

The relative residual errors for a calibration curve were then summed. The sums of the relative errors for the nine batches were then averaged and the lowest average between the weighted and non-weighted groups was determined to be the best fit weighting model for the curve.

Table 6 summarizes the best fit calibration models for each target in the amphetamines, phentermine, and designer stimulants quantitative LC-MS-MS method.

Table 6. Calibration models for amphetamines, phentermine, and designer stimulants quantitation by LC-MS-MS

Regression Analysis		
Target	Linear/Quadratic	Weighting
Methcathinone	Linear	Weighted (1/x)
Pseudoephedrine	Linear	Weighted (1/x)
Methylone	Linear	Weighted (1/x)
Amphetamine	Quadratic	Weighted (1/x)
Methamphetamine	Quadratic	Weighted (1/x)
MDA	Quadratic	Weighted (1/x)
Methedrone	Quadratic	Weighted (1/x)
MDMA	Quadratic	Weighted (1/x)
Phentermine	Quadratic	Weighted (1/x)
Mephedrone	Linear	Weighted (1/x)
α-PVP	Quadratic	Weighted (1/x)
MDPV	Quadratic	Weighted (1/x)
Bupropion	Quadratic	Weighted (1/x)

The best fit calibration model for methcathinone, pseudoephedrine, methylone, and mephedrone was determined to be a linear weighted (1/x) model. All other targets in the method were determined to have a quadratic weighted (1/x) model. The ANOVA and t-test results are depicted in Appendix A. The linear, linear weighted, quadratic, and quadratic weighted residual plots a reviewable on the validation data CDs.

IV Ion Suppression/Enhancement

Ion suppression or enhancement was addressed by assessing the instrumental response of post-extraction fortified samples and neat standards. Post-extraction fortified samples were prepared from blank matrix that was subject to the liquid-liquid extraction protocol. After the extraction, the blank samples were spiked with both target and internal standard. The neat samples were prepared by spiking a comparable quantity of standard stock solution and internal standard for a total of 200 µL of sample.

Equation 5 was used to calculate the ion suppression/enhancement for all targets and internal standards. The ion suppression/enhancement was assessed at three different concentrations (0.02, 0.25, and 1.0 mg/L) and averaged for an overall suppression/enhancement.

Equation 5.

$$Ion\ Suppression/Enhancement = \left(\frac{Mean\ signal\ of\ double\ blank}{Mean\ signal\ of\ neat\ standard}\right) \times 100\%$$

The post-extraction fortified samples were analyzed in triplicate using blank blood, liver, and urine. Five sources of blank blood, four sources of liver, and two sources of urine were analyzed to determine the suppression/enhancement of each matrix type. Table 7 describes the overall suppression/enhancement for each target compound.

Table 7. Overall suppression/enhancement of target compounds

	%Suppression/Enhancement (SD)		
Analyte	Blood Suppression/ Enhancement (%)	Liver Suppression/ Enhancement (%)	Urine Suppression/ Enhancement (%)
Methcathinone	98(12)	83(33)	92(10)
Pseudoephedrine	75(11)	64(21)	81(7)
Methylone	110(7)	88(33)	97(3)
Amphetamine	94(12)	77(28)	103(3)
Methamphetamine	112(9)	88(37)	109(3)
MDA	112(11)	87(31)	103(5)
Methedrone	120(8)	94(36)	108(4)
MDMA	116(7)	90(36)	109(5)
Phentermine	107(12)	88(35)	107(5)
Mephedrone	111(7)	91(33)	101(3)
α-PVP	113(7)	101(35)	98(7)
MDPV	118(7)	100(36)	102(4)
Bupropion	94(10)	95(28)	97(5)

Values of 100 % were indicative of no ion suppression or enhancement in the samples. Values greater than 100 % indicated ion enhancement and values less than 100 % indicated ion suppression. In blood samples, the ion suppression/enhancement ranged from 75 % to 120 %. The greatest suppression was seen with pseudoephedrine and the greatest enhancement was seen with methedrone. In liver samples, the ion suppression/enhancement ranged from 64 % to 101 %. The greatest suppression was seen with pseudoephedrine and the greatest enhancement was seen with α-PVP. The urine samples had a range of ion suppression/enhancement from 81 % to 109 %. The target with the greatest suppression was pseudoephedrine and the target with the greatest enhancement was MDMA. It was noted that the suppression and enhancement seen in the samples did not affect the quantitative result for the samples.

The overall suppression/enhancement was also assessed in the internal standards as seen in Table 8. The suppression and enhancement of internal standards was evaluated in the same manner as the target compounds.

Table 8. Overall suppression/enhancement of internal standards

	%Suppression/Enhancement (SD)		
Analyte	Blood Suppression/ Enhancement (%)	Liver Suppression/ Enhancement (%)	Urine Suppression/ Enhancement (%)
Amphetamine-D_{11}	99(14)	88(32)	92(2)
MDA-D_5	97(5)	80(26)	93(2)
MDMA-D_5	107(4)	87(35)	96(2)
Mephedrone-D_3	106(7)	93(34)	99(2)
Methamphetamine-D_{11}	105(7)	87(37)	98(2)
Methylone-D_3	101(4)	86(32)	88(2)
Pseudoephedrine-D_3	81(10)	75(25)	83(4)

The range of suppression/enhancement for the blood samples was from 81 % to 106 %. The internal standard with the greatest suppression was pseudoephedrine-D_3 and the internal standard with the greatest enhancement was mephedrone-D_3. In the liver samples, the suppression/enhancement ranged from 75 % to 93 %. There was no enhancement indicated with the liver samples. The internal standard with the most suppression was pseudoephedrine-D_3. In urine samples, the suppression/enhancement ranged from 83 % to 99 %. There was no enhancement indicated in the urine samples. The internal standard with the greatest suppression in the urine samples was pseudoephedrine-D_3.

V Recovery

Recovery was addressed by assessing the instrumental response of pre-extraction fortified samples and post-extraction fortified samples. Pre-extraction fortified blanks were prepared from blank matrix that was spiked with both target and internal standard compounds prior to extraction. Post-extraction fortified blanks were prepared from blank matrix that was subject to the liquid-liquid extraction protocol. After the extraction, the blank samples were spiked with both target and internal standard compounds.

Equation 6 was used to calculate the recovery for all targets and internal standards. The recovery was assessed at three different concentrations (0.02, 0.25, and 1.0 mg/L) and average for an overall recovery for each matrix.

Equation 6.

$$Recovery = \left(\frac{Average\ pre - extraction\ spike}{Average\ post - extraction\ spike}\right) \times 100\%$$

The pre-extraction and post-extraction fortified samples were analyzed in triplicate using blank blood, liver, and urine. Five sources of blank blood, three sources of liver, and two sources of urine were analyzed to determine the average recovery of each matrix type. Table 9 describes the average recovery for the target compound with each of the matrix types.

Table 9. Average recovery of target compounds

	%Recovery (SD)		
Analyte	Blood Recovery (%)	Liver Recovery (%)	Urine Recovery (%)
Methcathinone	77(22)	71(21)	78(2)
Pseudoephedrine	101(25)	89(19)	98(10)
Methylone	82(8)	77(26)	83(1)
Amphetamine	86(17)	75(20)	85(3)
Methamphetamine	100(31)	76(23)	87(3)
MDA	82(9)	73(19)	86(3)
Methedrone	80(8)	70(20)	82(1)
MDMA	85(9)	72(21)	87(2)
Phentermine	86(14)	77(22)	86(2)
Mephedrone	95(36)	75(18)	82(1)
α-PVP	90(12)	85(17)	87(2)
MDPV	88(8)	81(16)	87(3)
Bupropion	98(16)	86(13)	86(2)

The range of target compound recovery in blood was 77 % to 101 % with methcathinone having the lowest recovery. In liver samples, the recovery ranged from 70 % to 89 %. Methedrone had the lowest recovery in the liver samples. The recovery of the compounds in urine ranged from 78 % to 98 % with methcathinone having the lowest recovery and pseudoephedrine having the highest recovery. It was noted that the quantitation was not affected in cases where compounds had a lower recovery.

The average recovery was also assessed in the internal standards as seen in Table 10. The recovery of internal standards was evaluated in the same manner as the target compounds.

Table 10. The average recovery for internal standards

	%Recovery (SD)		
Analyte	Blood Recovery (%)	Liver Recovery (%)	Urine Recovery (%)
Amphetamine-D_{11}	85(16)	71(21)	89(3)
MDA-D_5	83(9)	72(20)	89(3)
MDMA-D_5	87(9)	71(22)	91(2)
Mephedrone-D_3	86(10)	71(17)	86(2)
Methamphetamine-D_{11}	87(12)	71(23)	90(3)
Methylone-D_3	83(9)	70(19)	86(2)
Pseudoephedrine-D_3	96(20)	83(22)	88(3)

In blood samples the range of internal standard recovery was 83 % to 96 %. MDA-D_5 and pseudoephedrine-D_3 had the lowest and highest recoveries respectively. The recovery range for liver samples was 70 % to 83 % with methylone-D_3 having the lowest and pseudoephedrine-D_3 having the highest percent recovery. In urine samples, the recovery ranged from 86 % to 91 %. Methylone-D_3 demonstrated the lowest percent recovery while methamphetamine-D_3 demonstrated the highest percent recovery.

VI Carryover

Carryover was evaluated by injecting solvent blanks immediately following progressively higher concentrations of analyte of interest fortified into blank matrix. The highest concentration of analytes injected was 5.0 mg/L and no carryover was detected at this concentration. The concentration was confirmed by triplicate analysis.

VII Interferences

i Endogenous Compounds (Blanks)

Interferences from endogenous compounds were evaluated by analyzing multiple sources of matrix without the addition of the target compounds or internal standard. A total of six blank blood, three blank liver, and two blank urine matrices were analyzed for interferences. No interferences were detected in the matrices.

ii Internal Standard

To evaluate potential interferences of internal standard by a high concentration of analyte, three negative blood matrix samples were fortified with a high concentration (2.0 mg/L) of the target compounds. The samples were analyzed for the absence of response for the internal standard. No contributions were detected in the internal standard from a high concentration of target compounds.

To evaluate potential interferences of target compounds by a high concentration of internal standard, three negative blood matrix samples were fortified with a high concentration (2.0 mg/L) of internal standard compounds. The samples were analyzed for the absence of response of target compounds. No contributions were detected in the target compounds from high concentrations of internal standards.

iii Commonly Encountered Analytes

Interferences from commonly encountered compounds were evaluated by analyzing three sources of blank matrix fortified with high concentrations of commonly encountered drugs, metabolites, and other structurally similar compounds. Table 11 depicts the compounds that were assessed for interferences.

Table 11. Interferents and concentrations of commonly encountered analytes

Drug Class	Drug	Concentration (mg/L)
Opioids	Codeine, Morphine, Hydrocodone, Hydromorphone, Oxycodone, Oxymorphone	0.8
	6-Monoacetylmorphine	0.2
Cocaine	Cocaine, Cocaethylene, Benzoylecgonine	4.0
Benzodiazepines	Alprazolam, Clonazepam, Lorazepam, Diazepam, Nordiazepam, Oxazepam, Temazepam, Zaleplon, Zolpidem, Zopiclone	2.0
Cannabinoids	THC, Carboxy-THC	0.1/0.5
Barbiturates	Butalbital, Secobarbital, Phenobarbital	30
Carisoprodol and Meprobamate	Carisoprodol, Meprobamate	100
Fentanyl	Fentanyl	0.1
Acetaminophen, Salicyclic Acid	Acetaminophen, Salicylic acid	400/200
Base Drugs	Diphenhydramine, PCP, Tramadol, Methadone, Amitriptyline, Nortriptyline, Cyclobenzaprine, Trazodone, Citalopram, Fluoxetine, Chlorpheniramine, Dextromethorphan, Propoxyphene, Mirtazepine, Sertraline, Diltiazem, Bupropion, Ketamine, Fluvoxamine, Doxylamine, Brompheniramine, Doxepin, Paroxetine	6.0
Acid/Neutral Drugs	Ibuprofen, Butalbital, Acetaminophen, Meprobamate, Caffeine, Gluetheminde, Naproxen, Metaxolone, Carbamazepine, Diazepam	6.0

There were no interferences detected from the three blank matrices fortified with commonly encountered analytes with the exception of bupropion. Three matrix sources were spiked with the

statewide base quantitation controls I, II, and III. Upon analysis, a peak was identified as bupropion in all three matrices. It was determined that bupropion was present in the spiked samples and therefore no interference was detected.

VIII Dilution Integrity

The effect of sample dilution on the accuracy and precision of samples was assessed using both large and small volume dilutions. When assessing large volume dilutions, a pooled blood sample was fortified with the targets at a concentration of 2.0 mg/L. Aliquots of the pooled sample where then taken and analyzed as undiluted, 1:2, 1:5, 1:10, and 1:20 dilution. The 1:2 dilutions were prepared by taking a 2.0 mL aliquot of pooled sample and diluting with 2.0 mL of blank blood. The 1:5 dilutions were prepared by taking 1.0 mL of pooled sample and diluting with 4.0 mL of blank blood. To prepare the 1:10 dilutions, a 1.0 mL aliquot of the pooled sample was diluted with 9.0 mL of blank blood. The 1:20 dilutions were prepared by diluting a 1.0 mL aliquot of pooled sample with 19.0 mL of blank blood. Three 1.0 mL aliquots of each dilution along with an undiluted sample were then extracted and analyzed for accuracy and precision.

The overall accuracy and intermediate precision were evaluated for the large volume dilution as seen in Table 12 and Table 13. The predetermined acceptance criterion for accuracy and precision was ±20 % accuracy and ±20 % percent CV.

Table 12. Large volume dilution integrity percent accuracy for amphetamines, phentermine, and designer stimulants quantitation by LC-MS-MS

Dilution Integrity Accuracy (Large Volume) % Accuracy (SD)				
Target	1: 2 Dilution	1:5 Dilution	1:10 Dilution	1:20 Dilution
Methcathinone	102(3)	103(2)	100(1)	99(2)
Pseudoephedrine	88(2)	88(1)	88(1)	89(2)
Methylone	90(2)	90(1)	90(1)	90(3)
Amphetamine	87(3)	88(1)	91(2)	93(1)
Methamphetamine	88(2)	91(1)	94(2)	97(3)
MDA	89(3)	88(1)	88(1)	88(2)
Methedrone	87(1)	87(2)	88(3)	88(5)
MDMA	89(2)	90(1)	90(1)	91(1)
Phentermine	90(3)	90(1)	93(3)	93(1)
Mephedrone	91(2)	90(1)	90(1)	90(2)
α-PVP	87(2)	83(1)	89(4)	90(5)
MDPV	82(8)	77(4)	80(5)	86(2)
Bupropion	83(11)	81(8)	79(12)	89(8)

The accuracy for all target compounds was within the acceptance criterion of ±20 % accuracy for all large volume dilution ratios with the exception of MDPV and bupropion. MDPV did not meet the predetermined acceptance criteria using a 1:5 large volume dilution ratio. Therefore, casework samples can be diluted by no more than a 1:2 large volume dilution to still maintain the predetermine acceptance criteria for MDPV. Bupropion did not meet the predetermined acceptance criteria using a 1:10 large volume dilution ratio. Therefore, casework samples can be diluted by no more than a 1:5 large volume dilution to still maintain the predetermine acceptance criteria for bupropion.

Table 13. Large volume dilution integrity intermediate precision for amphetamines, phentermine, and designer stimulants quantitation by LC-MS-MS

Dilution Integrity Intermediate Precision (Large Volume) Mean ± SD (%CV)				
Target	1: 2 Dilution	1:5 Dilution	1:10 Dilution	1:20 Dilution
Methcathinone	1.02±0.03(3)	0.41±0.01(2)	0.199±0.002(1)	0.099±0.002(2)
Pseudoephedrine	0.88±0.02(2)	0.35±0.01(1)	0.176±0.001(1)	0.089±0.002(2)
Methylone	0.90±0.02(2)	0.36±0.01(1)	0.179±0.002(1)	0.090±0.003(3)
Amphetamine	0.87±0.03(3)	0.35±0.01(1)	0.181±0.005(3)	0.093±0.001(1)
Methamphetamine	0.88±0.02(2)	0.36±0.01(1)	0.189±0.003(2)	0.097±0.003(3)
MDA	0.89±0.03(3)	0.35±0.01(1)	0.175±0.001(1)	0.088±0.002(2)
Methedrone	0.87±0.01(2)	0.35±0.01(2)	0.177±0.006(4)	0.088±0.004(5)
MDMA	0.89±0.02(3)	0.36±0.01(1)	0.181±0.002(1)	0.091±0.001(1)
Phentermine	0.90±0.03(3)	0.36±0.01(2)	0.186±0.006(4)	0.093±0.001(2)
Mephedrone	0.91±0.02(3)	0.36±0.01(1)	0.180±0.001(1)	0.090±0.002(3)
α-PVP	0.87±0.02(2)	0.33±0.01(1)	0.178±0.009(5)	0.090±0.005(5)
MDPV	0.82±0.08(10)	0.31±0.02(5)	0.160±0.011(7)	0.086±0.002(3)
Bupropion	0.83±0.11(13)	0.32±0.03(10)	0.159±0.023(15)	0.089±0.008(9)

The predetermined acceptance criterion for intermediate precision was a percent CV within ±20 %. All target compounds were within the acceptance criteria at all of the large volume dilution ratios.

To evaluate small volume dilution integrity small volumes of the fortified pooled blood sample were diluted up to a 1.0 mL volume. The 1:2 dilutions were prepared by diluting 0.5 mL of the pooled sample with 0.5 mL of blank blood. The 1:5 dilutions were prepared by diluting 0.2 mL of the pooled sample with 0.8 mL of blank blood. To prepare the 1:10 dilutions, 0.1 mL of pooled sample was dilution with 0.9 mL of blank blood. The 1:20 dilutions were prepared by diluting 0.05 mL of pooled sample with 0.95 mL of blank blood. All dilutions were performed using serological pipettes with the exception of the 1:20 dilutions. The 1:20 dilutions were prepared using a positive displacement pipette.

The overall accuracy and intermediate precision were evaluated for the small volume dilution as seen in Table 14 and Table 15. The predetermined acceptance criterion for accuracy and precision was ±20 % accuracy and ±20 % percent CV.

Table 14. Small volume dilution integrity percent accuracy for amphetamines, phentermine, and designer stimulants quantitation by LC-MS-MS

Dilution Integrity Accuracy (Small Volume) % Accuracy (SD)				
Target	1: 2 Dilution	1:5 Dilution	1:10 Dilution	1:20 Dilution
Methcathinone	109(4)	103(2)	88(10)	94(4)
Pseudoephedrine	95(3)	87(1)	77(9)	84(1)
Methylone	96(3)	89(1)	78(9)	84(1)
Amphetamine	91(2)	88(1)	80(8)	87(2)
Methamphetamine	94(3)	91(1)	83(9)	93(1)
MDA	97(4)	88(1)	77(9)	83(1)
Methedrone	92(5)	86(1)	75(9)	82(3)
MDMA	95(2)	88(1)	80(9)	85(2)
Phentermine	96(3)	92(2)	83(9)	93(2)
Mephedrone	97(3)	90(1)	78(8)	84(1)
α-PVP	85(3)	86(2)	81(3)	97(2)
MDPV	88(4)	76(3)	74(8)	82(6)
Bupropion	92(4)	81(5)	79(9)	87(6)

The accuracy of the 1:5 dilutions was within the predetermined acceptance criterion for all targets with the exception of MDPV. The following target compounds did not meet the predetermine accuracy acceptance criteria for small volume dilutions at a 1:10 dilution ratio: pseudoephedrine, methylone, MDA, methedrone, mephedrone, MDPV, and bupropion. Therefore, those compounds shall be diluted by no more than a 1:5 small volume dilution is casework samples. The remaining compounds passed the acceptance criteria for the 1:20 small volume dilution.

Table 15. Small volume dilution integrity intermediate precision for amphetamines, phentermine, and designer stimulants quantitation by LC-MS-MS

Dilution Integrity Intermediate Precision (Small Volume) Mean ± SD (%CV)				
Target	1: 2 Dilution	1:5 Dilution	1:10 Dilution	1:20 Dilution
Methcathinone	1.09±0.04(3)	0.41±0.01(2)	0.177±0.021(12)	0.094±0.004(4)
Pseudoephedrine	0.95±0.03(3)	0.35±0.01(1)	0.154±0.017(11)	0.084±0.001(1)
Methylone	0.96±0.03(4)	0.36±0.01(1)	0.156±0.017(11)	0.084±0.001(1)
Amphetamine	0.91±0.02(2)	0.35±0.01(1)	0.161±0.015(9)	0.087±0.002(2)
Methamphetamine	0.94±0.03(3)	0.36±0.01(1)	0.166±0.018(11)	0.093±0.001(1)
MDA	0.97±0.04(4)	0.35±0.01(1)	0.154±0.018(11)	0.083±0.001(1)
Methedrone	0.92±0.05(6)	0.34±0.01(1)	0.149±0.017(12)	0.082±0.003(3)
MDMA	0.95±0.02(3)	0.35±0.01(1)	0.159±0.017(11)	0.085±0.002(3)
Phentermine	0.96±0.03(3)	0.37±0.01(2)	0.166±0.018(11)	0.093±0.002(2)
Mephedrone	0.97±0.03(4)	0.36±0.01(2)	0.157±0.016(10)	0.084±0.001(1)
α-PVP	0.85±0.03(3)	0.35±0.01(2)	0.163±0.005(3)	0.097±0.002(2)
MDPV	0.88±0.04(5)	0.30±0.01(4)	0.148±0.016(11)	0.082±0.006(8)
Bupropion	0.92±0.04(5)	0.32±0.02(6)	0.157±0.018(11)	0.087±0.006(7)

The predetermined acceptance criterion for intermediate precision was a percent CV within ±20 %. All target compounds were within the acceptance criteria at all of the small volume dilution ratios. The percent CV significantly increases, but remains within the acceptance criteria, with the 1:10 small volume dilution ratio. Both inaccuracy and imprecision increase with the 1:10

dilution for all target compounds and decrease with the 1:20 dilution ratio. This may be due to the utilization of a more accurate and precise pipette. The 1:10 dilution used a disposable serological pipette where the 1:20 dilution utilized a mechanical positive displacement pipette.

IX Stability

The stability of extracted samples that were not analyzed immediately was evaluated at two concentrations (1.0 mg/L and 0.02 mg/L). The samples were extracted and injected immediately to establish Day 1 instrumental responses. Both concentration levels were injected in triplicate and the instrumental response was compared over a seven day period. If the average instrumental response decreased below 80 % or increased above 120 %, then the target was considered unstable after that time period.

Table 16 and Table 17 show the seven day stability at 1.0 mg/L and 0.02 mg/L for the target compounds. Chart 1 and Chart 2 graphically represent the tabulated accuracy data.

Table 16. Stability of a 1.0 mg/L concentration of analytes over a seven day period

Deviation From Day 1 Response 1.0 mg/L Accuracy(%); n=3							
Target	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Methcathinone	100	112	117	118	113	103	102
Pseudoephedrine	100	90	96	95	97	88	88
Methylone	100	99	102	101	96	90	93
Amphetamine	100	91	99	98	97	90	90
Methamphetamine	100	93	99	99	92	86	85
MDA	100	86	94	93	85	79	79
Methedrone	100	93	96	97	93	86	86
MDMA	100	91	96	96	92	85	85
Phentermine	100	92	98	98	91	87	87
Mephedrone	100	94	98	98	94	88	87
α-PVP	100	97	101	101	93	91	90
MDPV	100	98	101	102	93	92	92
Bupropion	100	95	99	98	93	90	89

Chart 1. Graphic representation of seven day stability of a 1.0 mg/L concentration of analytes

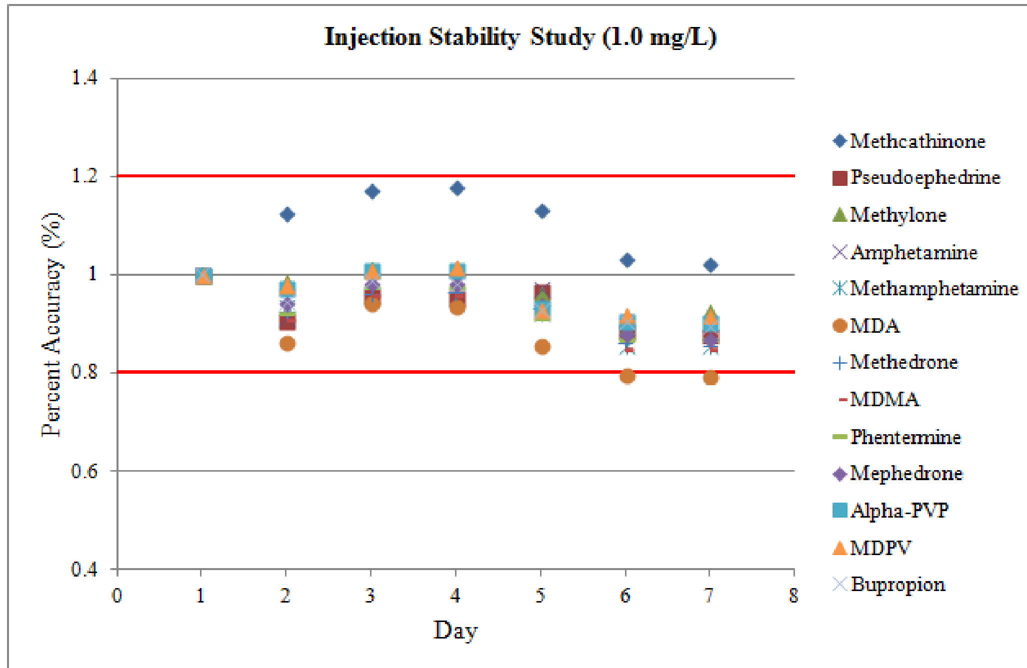
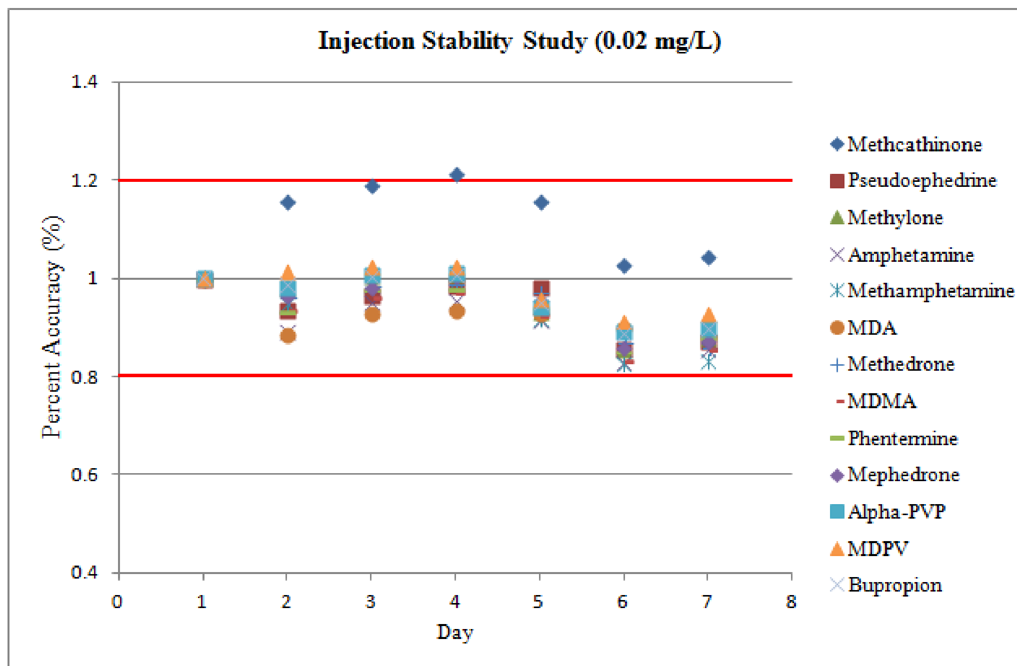


Table 17. Stability of a 0.02 mg/L concentration of analytes over a seven day period

Deviation From Day 1 Response 0.02 mg/L Accuracy(%); n=3							
Target	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Methcathinone	100	116	119	121	116	103	104
Pseudoephedrine	100	94	97	98	98	86	87
Methylone	100	98	100	101	93	86	88
Amphetamine	100	89	95	95	92	83	86
Methamphetamine	100	95	99	99	92	83	83
MDA	100	89	93	93	93	90	88
Methedrone	100	96	99	99	97	87	86
MDMA	100	94	96	97	93	83	86
Phentermine	100	93	98	98	94	85	88
Mephedrone	100	96	98	101	96	86	87
α-PVP	100	98	101	101	94	89	90
MDPV	100	101	102	103	96	91	92
Bupropion	100	99	101	101	95	89	90

Chart 2. Graphic representation of seven day stability of a 0.02 mg/L concentration of analytes



All compounds were stable up to seven days after being extracted with the exception of methcathinone. The day four injection of methcathinone was greater than the upper limit acceptance criteria of 120 %. During analysis, the sample remained in the autosampler of the instrument. After five days it appears that the accuracy is beginning to decrease but still remains within the acceptance criteria for all compounds with the exception of methcathinone.

X Robustness

Robustness was demonstrated by the completion of nine successful batches of samples, completed by three analysts over a six week period. Each batch was comprised of calibrators and controls. Results of the batches were previously described within the validation summary.

XI Training

Scientists trained to perform methods involving liquid-liquid extraction and LC/MS-MS confirmation and quantitation may use this method. Additional training of scientists will follow this validation, as necessary.

XII Summary

All target compounds passed the comprehensive validation. The accuracy and precision for all compounds was within the predetermined acceptance criteria. The LOD and LOQ were also established for each compound. Phentermine had the highest LOD at 0.01 mg/L. The LOQ was

established to be 0.01 mg/L for methcathinone, amphetamine, methamphetamine, phentermine, and bupropion. All other targets have a lower LOQ than 0.01 mg/L.

The best fit calibration model was determined for each target compound using statistical analysis and residual plots to determine linearity as well as weighting. Methcathinone, pseudoephedrine, methylone, and mephedrone were best fit with a linear weighted (1/x) calibration model. The remaining targets were best fit using a quadratic weighted (1/x) model.

Suppression/enhancement was assessed by comparing the instrument response of post-extraction fortified samples with neat samples. The suppression/enhancement was assessed using blank blood, liver, and urine samples. Any suppression or enhancement noted did not have an effect on the overall accuracy and precision of the method. Recovery was also assessed with the same matrices by comparing the instrumental response of pre-extraction and post-extraction fortified samples. The recovery for the three matrices was no less than 70 % and did not have a significant impact on the accuracy and precision of the method.

Carryover as well as interference was evaluated for the method. There was no carryover seen in a blank sample after the injection of a 5.0 mg/L sample. Also, there were no interferences seen from endogenous compounds, internal standard, target analytes, or commonly encountered analytes.

Processed sample stability was evaluated by analyzing two concentrations of target compounds over a seven day period. After seven days there was no significant instability with any of the target compounds. The dilution integrity was also established using large and small volume dilutions. The accuracy and precision of the 1:2, 1:5, 1:10, and 1:20 dilutions were evaluated.

The accuracy for all target compounds was within the acceptance criterion of ±20 % accuracy for all large volume dilution ratios with the exception of MDPV and bupropion. MDPV did not meet the predetermined acceptance criteria using a 1:5 large volume dilution ratio. Therefore, casework samples can be diluted by no more than a 1:2 large volume dilution to still maintain the predetermine acceptance criteria for MDPV. Bupropion did not meet the predetermined acceptance criteria using a 1:10 large volume dilution ratio. Therefore, casework samples can be diluted by no more than a 1:5 large volume dilution to still maintain the predetermine acceptance criteria for bupropion.

The following target compounds did not meet the predetermine accuracy acceptance criteria for small volume dilutions at a 1:10 dilution ratio: pseudoephedrine, methylone, MDA, methedrone, mephedrone, MDPV, and bupropion. Therefore, those compounds shall be diluted by no more than a 1:5 small volume dilution in casework samples. The remaining compounds passed the acceptance criteria for the 1:20 small volume dilution.

This method provides a rapid and sensitive technique for the detection and quantitation of amphetamines, phentermine, and designer stimulants by LC-MS-MS.

XIII References

DFS Quality Manual

Toxicology SOP

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Appendix A

Regression Analysis

Methcathinone

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

> P-value = 0.08758 > 0.05 and therefore the null hypothesis was not rejected and the groups were determined to be insignificantly different.
>
> F = 3.37339 < 4.60011 (F_{crit}) and therefore the null hypothesis was not rejected and the variances were determined to be equal.

From these results, it was determined that there was an insignificant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the linear calibration model will be utilized for methcathinone.

Comparison of Linear Non-weighted and Linear Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

> P-value = 0.025866173 < 0.05 and therefore the null hypothesis was rejected and the groups were determined to be significantly different.

The linear weighted model was determined to be the most appropriate calibration model for methcathinone. The t-test for the comparison of weighted and non-weighted linear models indicated a significant difference between the two groups and therefore the linear weighted model was selected as the best fit. This is due to the average sum of relative error for the residuals being lower than the non-weighted model (weighted 0.3228 and non-weighted 0.9313).

Methcathinone: linear-weighted calibration model

Pseudoephedrine

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

> P-value = 0.61296 > 0.05 and therefore the null hypothesis was not rejected and the groups were determined to be insignificantly different.
>
> F = 0.26769 < 4.60011 (F_{crit}) and therefore the null hypothesis was not rejected and the variances were determined to be equal.

From these results, it was determined that there was an insignificant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the linear calibration model will be utilized for pseudoephedrine.

Comparison of Linear Non-weighted and Linear Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

> P-value = 0.073155106 > 0.05 and therefore the null hypothesis was not rejected and the groups were determined to be insignificantly different.

The linear weighted model was determined to be the most appropriate calibration model for pseudoephedrine. The t-test for the comparison of weighted and non-weighted linear models indicated an insignificant difference between the two groups and therefore the linear weighted model was selected as the best fit. This was due to the average sum of relative error for the residuals being lower than the non-weighted model (weighted 0.1801 and non-weighted 0.3894).

Pseudoephedrine: linear-weighted calibration model

Methylone

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

> P-value = 0.09703 > 0.05 and therefore the null hypothesis was not rejected and the groups were determined to be insignificantly different.
>
> F = 3.16323 < 4.60011 (F_{crit}) and therefore the null hypothesis was not rejected and the variances were determined to be equal.

From these results, it was determined that there was an insignificant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the linear calibration model will be utilized for methylone.

Comparison of Linear Non-weighted and Linear Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

> P-value = 0.052107025 > 0.05 and therefore the null hypothesis was not rejected and the groups were determined to be insignificantly different.

The linear weighted model was determined to be the most appropriate calibration model for methylone. The t-test for the comparison of weighted and non-weighted linear models indicated an insignificant difference between the two groups and therefore the linear weighted model was selected as the best fit. This was due to the average sum of relative error for the residuals being lower than the non-weighted model (weighted 0.2344 and non-weighted 0.5074).

Methylone: linear-weighted calibration model

Amphetamine

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

> P-value = 0.0112 < 0.05 and therefore the null hypothesis was rejected and the groups were determined to be significantly different.
>
> F = 8.52452 > 4.60011 (F_{crit}) and therefore the null hypothesis was rejected and the variances were determined to be different.

From these results, it was determined that there was significant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the quadratic calibration model will be utilized for amphetamine.

Comparison of Quadratic Non-weighted and Quadratic Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

> P-value = 0.000697638 < 0.05 and therefore the null hypothesis was rejected and the groups were determined to be significantly different.

The quadratic weighted model was determined to be the most appropriate calibration model for amphetamine. The t-test for the comparison of weighted and non-weighted quadratic models indicated a significant difference between the two groups and therefore the quadratic weighted model was selected as the best fit. This was due to the average sum of relative error for the residuals being lower than the non-weighted model (weighted 0.2051 and non-weighted 0.7667).

Amphetamine: quadratic weighted calibration model

Methamphetamine

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

P-value = $9.71 \times 10^{-8} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be significantly different.

$F = 99.37609016 > 4.60011$ (F_{crit}) and therefore the null hypothesis was rejected and the variances were determined to be different.

From these results, it was determined that there was significant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the quadratic calibration model will be utilized for methamphetamine.

Comparison of Quadratic Non-weighted and Quadratic Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

P-value = $0.001233953 < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be significantly different.

The quadratic weighted model was determined to be the most appropriate calibration model for methamphetamine. The t-test for the comparison of weighted and non-weighted quadratic models indicated a significant difference between the two groups and therefore the quadratic weighted model was selected as the best fit. This was due to the average sum of relative error for the residuals being lower than the non-weighted model (weighted 0.3500 and non-weighted 1.0318).

Methamphetamine: quadratic weighted calibration model

MDA

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

> P-value = $9.3x10^{-5}$ < 0.05 and therefore the null hypothesis was rejected and the groups were determined to be significantly different.
>
> F = 29.1881 > 4.60011 (F_{crit}) and therefore the null hypothesis was rejected and the variances were determined to be different.

From these results, it was determined that there was significant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the quadratic calibration model will be utilized for MDA.

Comparison of Quadratic Non-weighted and Quadratic Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

> P-value = 0.007963787 < 0.05 and therefore the null hypothesis was rejected and the groups were determined to be significantly different.

The quadratic weighted model was determined to be the most appropriate calibration model for MDA. The t-test for the comparison of weighted and non-weighted quadratic models indicated a significant difference between the two groups and therefore the quadratic weighted model was selected as the best fit. This was due to the average sum of relative error for the residuals being lower than the non-weighted model (weighted 0.2163 and non-weighted 0.5959).

MDA: quadratic weighted calibration model

Methedrone

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

> P-value = $9.5 \times 10^{-5} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be significantly different.
>
> $F = 29.0722 > 4.60011$ (F_{crit}) and therefore the null hypothesis was rejected and the variances were determined to be different.

From these results, it was determined that there was significant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the quadratic calibration model will be utilized for methedrone.

Comparison of Quadratic Non-weighted and Quadratic Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

> P-value = $0.004233459 < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be significantly different.

The quadratic weighted model was determined to be the most appropriate calibration model for methedrone. The t-test for the comparison of weighted and non-weighted quadratic models indicated a significant difference between the two groups and therefore the quadratic weighted model was selected as the best fit. This was due to the average sum of relative error for the residuals being lower than the non-weighted model (weighted 0.1840 and non-weighted 0.4785).

Methedrone: quadratic weighted calibration model

MDMA

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

> P-value = 0.04751 < 0.05 and therefore the null hypothesis was rejected and the groups were determined to be significantly different.
>
> F = 4.71845 > 4.60011 (F_{crit}) and therefore the null hypothesis was rejected and the variances were determined to be different.

From these results, it was determined that there was significant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the quadratic calibration model will be utilized for MDMA.

Comparison of Quadratic Non-weighted and Quadratic Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

> P-value = 0.001078124 < 0.05 and therefore the null hypothesis was rejected and the groups were determined to be significantly different.

The quadratic weighted model was determined to be the most appropriate calibration model for MDMA. The t-test for the comparison of weighted and non-weighted quadratic models indicated a significant difference between the two groups and therefore the quadratic weighted model was selected as the best fit. This was due to the average sum of relative error for the residuals being lower than the non-weighted model (weighted 0.1631 and non-weighted 0.3970).

MDMA: quadratic weighted calibration model

Phentermine

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

> P-value = $3.1 \times 10^{-6} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be significantly different.
>
> $F = 55.4897 > 4.60011$ (F_{crit}) and therefore the null hypothesis was rejected and the variances were determined to be different.

From these results, it was determined that there was significant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the quadratic calibration model will be utilized for phentermine.

Comparison of Quadratic Non-weighted and Quadratic Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

> P-value = $0.0225470281 < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be significantly different.

The quadratic weighted model was determined to be the most appropriate calibration model for phentermine. The t-test for the comparison of weighted and non-weighted quadratic models indicated a significant difference between the two groups and therefore the quadratic weighted model was selected as the best fit. This was due to the average sum of relative error for the residuals being lower than the non-weighted model (weighted 0.4326 and non-weighted 0.7782).

Phentermine: quadratic weighted calibration model

Mephedrone

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

P-value = 0.24563 > 0.05 and therefore the null hypothesis was not rejected and the groups were determined to be insignificantly different.

F = 1.46855 < 4.60011 (F_{crit}) and therefore the null hypothesis was not rejected and the variances were determined to be equal.

From these results, it was determined that there was an insignificant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the linear calibration model will be utilized for mephedrone.

Comparison of Linear Non-weighted and Linear Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

P-value = 0.05867927 > 0.05 and therefore the null hypothesis was not rejected and the groups were determined to be insignificantly different.

The linear weighted model was determined to be the most appropriate calibration model for mephedrone. The t-test for the comparison of weighted and non-weighted linear models indicated an insignificant difference between the two groups and therefore the linear weighted model was selected as the best fit. This was due to the average sum of relative error for the residuals being lower than the non-weighted model (weighted 0.1685 and non-weighted 0.4994).

Mephedrone: linear-weighted calibration model

α-PVP

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

> P-value = 0.00096 < 0.05 and therefore the null hypothesis was rejected and the groups were determined to be significantly different.
>
> F = 17.3103 > 4.60011 (F_{crit}) and therefore the null hypothesis was rejected and the variances were determined to be different.

From these results, it was determined that there was significant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the quadratic calibration model will be utilized for α-PVP.

Comparison of Quadratic Non-weighted and Quadratic Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

> P-value = 0.002566127 < 0.05 and therefore the null hypothesis was rejected and the groups were determined to be significantly different.

The quadratic weighted model was determined to be the most appropriate calibration model for α-PVP. The t-test for the comparison of weighted and non-weighted quadratic models indicated a significant difference between the two groups and therefore the quadratic weighted model was selected as the best fit. This was due to the average sum of relative error for the residuals being lower than the non-weighted model (weighted 0.3145 and non-weighted 0.7673).

α-PVP: quadratic weighted calibration model

MDPV

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

> P-value = 0.00019 < 0.05 and therefore the null hypothesis was rejected and the groups were determined to be significantly different.
>
> F = 25.1836 > 4.60011 (F_{crit}) and therefore the null hypothesis was rejected and the variances were determined to be different.

From these results, it was determined that there was significant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the quadratic calibration model will be utilized for MDPV.

Comparison of Quadratic Non-weighted and Quadratic Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

> P-value = 0.001072084 < 0.05 and therefore the null hypothesis was rejected and the groups were determined to be significantly different.

The quadratic weighted model was determined to be the most appropriate calibration model for MDPV. The t-test for the comparison of weighted and non-weighted quadratic models indicated a significant difference between the two groups and therefore the quadratic weighted model was selected as the best fit. This was due to the average sum of relative error for the residuals being lower than the non-weighted model (weighted 0.2617 and non-weighted 0.7232).

MDPV: quadratic weighted calibration model

Bupropion

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

> P-value = 0.00877 < 0.05 and therefore the null hypothesis was rejected and the groups were determined to be significantly different.
>
> F = 9.26024 > 4.60011 (F_{crit}) and therefore the null hypothesis was rejected and the variances were determined to be different.

From these results, it was determined that there was significant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the quadratic calibration model will be utilized for bupropion.

Comparison of Quadratic Non-weighted and Quadratic Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

> P-value = 0.000664256 < 0.05 and therefore the null hypothesis was rejected and the groups were determined to be significantly different.

The quadratic weighted model was determined to be the most appropriate calibration model for MDPV. The t-test for the comparison of weighted and non-weighted quadratic models indicated a significant difference between the two groups and therefore the quadratic weighted model was selected as the best fit. This was due to the average sum of relative error for the residuals being lower than the non-weighted model (weighted 0.5010 and non-weighted 1.4417).

MDPV: quadratic weighted calibration model

APPENDIX D

LIST OF KEY PERSONNEL
CURRICULUM VITAE FOR [PI]
BIOGRAPHICAL SKETCH FOR FLS II POSITION

NAMES AND AFFILIATIONS OF KEY PERSONNEL

Point of Contact for this Grant:

[Redacted]

BIOGRAPHICAL SKETCH

Forensic Laboratory Specialist II

Virginia Department of Forensic Science
Chemistry Program Division of Technical Services
700 North Fifth Street
Richmond, VA 23219

Required Qualifications

High school diploma or equivalent with college courses in mathematics and science; experience working in a scientific laboratory environment using basic laboratory equipment, techniques, and laboratory safety procedures; the ability to accurately record data and maintain record, follow written protocols, and oral instructions, establish work priorities, and independently perform routine duties; good oral and written communication skills; valid driver's license and/or other means of reliable transportation.

Preferred Qualifications

Bachelor's degree in a natural science are or college level courses in biology, chemistry, and/or biochemistry; experience handling human physiological fluids; experience in the use of mass spectrometry, liquid chromatography, and or liquid chromatography tandem mass spectrometry; experience in a forensic science laboratory; experience with computers in a laboratory environment.

APPENDIX E

PROJECT TIMELINE

Date	Action
January 2015	• Award of grant • Posting and advertising of Toxicology Forensic Laboratory Specialist II (FLSII) position
February 2015	• Interview and award of FLSII position • Ordering of initial reagents and supplies for combined cocaine and opioid method
March 2015	• Start of FLSII position • Training of FLSII • Submission of first quarterly financial report
April 2015	• Instrumental method development for combined cocaine and opioid method • Development of three sample preparation techniques for combined cocaine and opioid method
May 2015	• Completion of sample preparation method development for combined cocaine and opioid method
June 2015	• Begin validation of all three sample preparation methods for whole blood • Submission of second quarterly financial report
July 2015	• Continued validation of three sample preparation method for whole blood • Submission of first semi-annual progress report
August 2015	• Completion of validation studies for three sample preparation methods in whole blood • Begin validation of all three sample preparation methods for available medicolegal matrices
September 2015	• Continued validation of three sample preparation methods for available medicolegal matrices • Submission of third quarterly financial report
November 2015	• Complete validation of three sample preparation techniques for available medicolegal matrices • Completion of data review and begin preparation of cocaine and opioid validation summary
December 2015	• Finalize validation summary for combined cocaine and opioid method • Ordering of initial reagents and supplies for designer drug method • Submission of fourth quarterly financial report • Submission of fist annual progress report
January 2016	• Instrumental method development for designer drug method
February 2016	• Development of three sample preparation techniques for designer drug method
March 2016	• Completion of sample preparation method development for designer drug • Submission of fifth quarterly financial report

April 2016	• Begin validation of all three sample preparation methods for whole blood
May 2016	• Continued validation of three sample preparation method for whole blood
June 2016	• Completion of validation studies for three sample preparation methods in whole blood • Submission of sixth quarterly financial report
July 2016	• Begin validation of all three sample preparation methods for available medicolegal matrices • Submission of second semi-annual progress report
August 2016	• Continued validation of three sample preparation methods for available medicolegal matrices
September 2016	• Complete validation of three sample preparation techniques for available medicolegal matrices • Submission of seventh quarterly financial report
November 2016	• Completion of data review and begin designer drug validation summary • Begin preparation of final report
December 2016	• Finalize validation summary for designer method • Submit documents to peer-reviewed journal articles • Prepare abstract for SOFT conference presentation • Submission of eighth quarterly financial report • Submission of final report

APPENDIX F

DATA ARCHIVING PLAN

The Virginia Department of Forensic Science will comply with the data archiving requested by NIJ. All instrumental data files are maintained on the instrument computer, CD/DVD or other appropriate storage devices, and further stored on a departmental network folder. Instrumental files, such as method and report template files will also be maintained in the same manner. All documentation pertaining to the results of the validation including Microsoft Excel and Microsoft Word files will also be stored on CD/DVD or other appropriate storage devices and on the departmental network folder. All such information and files will also be submitted to NIJ upon completion of the project.

APPENDIX G

VIRGINIA DEPARTMENT OF FORENSIC SCIENCE
PREVIOUS AND CURRENT NIJ AWARDS

1996 **Forensic DNA Laboratory Program, Phase 1, NIJ Grant #96IJ-CX-0059, $375,000, with $125,000 matching state funds**

Funding provided to purchase necessary equipment and supplies and conduct training to accelerate STR DNA analysis capabilities for typing the backlog of convicted offender samples and for typing the backlog of casework samples in three of the Division's four laboratories.

1998 **Forensic DNA Laboratory Improvement Program, Phase 2, NIJ Grant #98-DN-VX-0018, $375,000, with $125,000 matching state funds**

Funding provided to purchase equipment and supplies for DNA STR fluorescence imaging analysis of forensic cases in all four of the Division's laboratories.

1999 **Forensic DNA Laboratory Program, Phase 4, NIJ Grant #98-DN-VX-0018 (S-1) $250,000, with $83,334 state matching funds**

Funding provided to purchase equipment and supplies for validating and implementing the remaining CODIS core STR loci for forensic casework in all four of the Division's laboratories and for the analysis of convicted offender samples in the Central Laboratory. DFS will also purchase CODIS equipment upgrades for the Central and Eastern Laboratories and an additional CODIS workstation for the Central Laboratory to maximize efficiency in this area.

2000 **FY 2000 DNA Backlog Reduction Program, NIJ Grant #2000-RC-CX-0018, $1,800,000**

Funding provided for the analysis of 36,000 convicted offender samples outsourced to vendor laboratory, with 371 no suspect cases analyzed for the state match requirement [360 cases, 1% of the convicted offender samples analyzed, required].

2001 **Vendor services received under the Convicted Offender DNA Backlog Reduction Program (FY2001)**

24,000 convicted offender samples and 1,714 quality assurance samples, totaling 25,714 samples outsourced to vendor laboratory, with more than 257 no suspect cases analyzed as the state match requirement [257, 1% of the 25,714 convicted offender and quality assurance samples analyzed, required].

2002 **Crime Laboratory Improvement Program FY 2002, NIJ Grant #2003-LP-CX-K003, $237,651, with $83,514 state matching funds**

Funding provided to purchase equipment and supplies for the Eastern and Western Laboratories for extracting/isolating DNA from crime scene samples on the Beckman Coulter BioMek® 2000 Laboratory Automation Workstation using Promega's DNA IQ™ System. Funding also provided to purchase an FMBIO® III Plus Fluorescent Imaging Analysis System for the Central Laboratory, newer model CODIS workstations for the Eastern and Western Laboratories, and CODIS file servers for all three of the Division's regional laboratories.

2002 **Vendor services received under the FY2002 Convicted Offender DNA Backlog Reduction Program (Outsourcing)**

Funding provided for the analysis of 18,000 convicted offender samples and 900 quality assurance samples, at the PowerPlex™ 1.1 and 2.1 Systems loci and 50,300 convicted offender samples and 2,515 quality assurance samples at the PowerPlex™ 2.1 System loci.

2003 **No Suspect Casework DNA Backlog Reduction Program, NIJ Grant#2003-DN-BX-K051, $529,964**

Funding provided to contract with the Virginia Institute of Forensic Science and Medicine to train four examiners to conduct DNA analysis on forensic cases (6 months) and to subsequently provide four qualified DNA examiners to screen and conduct DNA analysis on a minimum of 360 no suspect cases (1 year). Laboratory space will be renovated to accommodate four examiners in the Central Laboratory and equipment and supplies purchased for their use.

2004 **DNA Capacity Enhancement Program Formula Grant, FY04, NIJ #2004-DN-BX-K167, $431,770.00**

Funding provided to purchase equipment for the Eastern and Central Laboratory and renovate space in the Central Laboratory to accommodate additional examiners.

2004 **Forensic Casework DNA Backlog Reduction Program Formula Grant, FY04, NIJ #2004-DN-BX-K160, $796,725**

Funding provided for four fully qualified "restricted position" DNA examiners for 18 months and five fully qualified "restriction position" DNA examiners for six months to analyze a minimum of 650 backlogged cases. Funding also provided for five "restricted position" support staff and to purchase supplies to analyze backlogged cases.

2004 **National Institute of Justice Congressionally Directed Awards, FY04, NIJ #2004-LP-CX-0001, $1,490,250 with $496,750 state matching funds**

Funding provides for training examiners for DNA and other forensic disciplines by an outside vendor at the Division's Central Laboratory using the Division's training protocols, equipment, and methods of assessing trainees' performance.

The second component of the project are the following facility modifications and equipment purchases to enhance and/or expand current capabilities: remodeling space and purchasing two comparison microscopes in the Eastern Laboratory's Firearms/Toolmarks Section; replacing the FTIRs in the Central and Western Laboratories' Controlled Substance Sections; purchasing a scanning electron microscope for the Eastern Laboratory's Trace Evidence Section; providing a Crimescope to the Latent Prints Sections in each regional laboratory; and developing an enhanced laboratory management information system.

2005 **DNA Capacity Enhancement Program Formula Grant, FY05, NIJ #2005-DA-BX-K029, $621,250**

Funding provided to purchase equipment for the Eastern and Central Laboratory and to renovate space in the Eastern Laboratory to accommodate additional examiners.

2005 **Forensic Casework DNA Backlog Reduction Program Formula Grant, FY05, NIJ #2005-DN-BX-K070, $594,320.28 (Federal Funds - $539,204; State Funds - $55,116**

Funding provided for six fully qualified "restricted position" DNA examiners for seven months to analyze a minimum of 170 backlogged cases. Funding also provided for one "restricted position" support staff for the Central Laboratory. Additional funding provided to purchase supplies to analyze backlogged cases.

2006 **DNA Capacity Enhancement Program Formula Grant, FY06, NIJ #2005-DN-BX-K153, $768,640**

Funding provided to screen and conduct DNA analysis in-house on a minimum of 768 backlogged cases, and to purchase equipment associated with conducting DNA analysis on these backlogged cases.

2006 **Forensic Casework DNA Backlog Reduction Program Formula Grant, FY06, NIJ #2005-DN-BX-K120, $385,992**

Funding provided to screen and conduct DNA analysis in-house on a minimum of 300 backlogged forcible rape and homicide/non-negligent manslaughter cases and to purchase supplies associated with screening and conducting DNA analysis on these cases.

2007 **Forensic DNA Backlog Reduction Program Formula Grant, FY07, NIJ #2007-DN-BX-K159, $1,019,119**

Funding provided for the salaries and benefits of two "restricted position" full time support personnel (one for the Central Laboratory and one for the Northern Laboratory). The remaining grants funds are to purchase supplies and equipment associated with screening and conducting DNA analysis on backlogged aggravated assault, forcible rape, murder, and robbery cases.

2007 **Convicted Offender and/or Arrestee DNA Backlog Reduction Program Grant, 2007-DN-BX-K004, $360,000**

Funding provided for the overtime salaries and benefits for five DNA Data Bank analysts to analyze offender and arrestee samples and perform technical review of the data, and two support staff to accession the offender and arrestee samples and enter the identifying information into computer databases used to track the samples. The remaining funds will be used to purchase supplies and commercially available STR kits (PowerPlex® 16 BIO System kits) accepted by the National DNA Index System (NDIS) for the DNA Data Bank analysts to conduct DNA analysis on these backlogged offender and arrestee samples.

2008 **Paul Coverdell Forensic Science Improvement Program, 2008-CD-BX-0044, $59,000**

Funding provided to purchase various supplies and equipment items to improve capacity in the Controlled Substances and Toxicology Sections.

2008 **Research and Development in the Area of Controlled Substances Detection and Analysis, 2008-DN-BX-K140, $49,774**

Funding provided for a portion of the salary and benefits for two Forensic Scientists to evaluate the use of CMAs, including β-cyclodextrin (BCD) and hydroxypropyl-β-cyclodextrin (HPBCD), in reverse-phase thin layer chromatography (RPTLC) for the differentiation of enantiomeric drug substances including but not limited to dextromethorphan, levomethorphan, dextrorphanol, levorphanol, dextropropoxyphene, levopropoxyphene, dextro-methamphetamine and levo-methamphetamine in order to find a simple, fast, cost effective alternative for enantiomer determination that any forensic laboratory would be able to use regardless of budgetary or space constraints. Funding also provided to purchase necessary supplies and small equipment items. The results of this research project were presented as follows: "Development of a Thin Layer Chromatography Method for the Separation of Enantiomers Using Chiral Mobile Phase Additives", presented by Robyn Larson at the 2010 NIJ Grantees Meeting (at the annual meeting of the American Academy of Forensic Science in Seattle) and "Investigation of Thin Layer Chromatography Methods for the Separation of Enantiomers Using Chiral Mobile Phase Additives", presented by Kelly Howerter at the 2012 meeting of the Mid-Atlantic Association of Forensic Sciences.

2008 **FY 2008 Forensic DNA Backlog Reduction Program, 2008-DN-BX-K036, $ 942,280**

Funding provided for the salaries and fringe benefits for two "restricted position" part-time support personnel (one for the Central Laboratory and one for the Northern Laboratory). DNA analysis will be conducted in-house on a minimum of 945 backlogged cases giving priority first to rape, murder, aggravated assault, and robbery cases. Funding also provided to purchase supplies and equipment associated with screening and conducting DNA analysis on the backlogged cases.

2008 **Governor-Mandated Post-Conviction DNA Testing of Biological Evidence in Case Files, 2008-DN-BX-K128, $ 4,520,295**

Funding provided to pay the overtime salaries and FICA benefits for fully qualified DFS examiners who will complete reviews of approximately 2,200 post-conviction cases that involved forcible rape, murder, or non-negligent manslaughter in order to identify cases that meet project selection criteria for DNA testing. A minimum of 700 post-conviction cases, with approximately 5,600 samples, will be forwarded to a private laboratory for the DNA analysis. Project funds will be used to pay the contract fees per sample associated with the DNA analysis. As these analyses are completed and data returned to DFS, the eleven DFS examiners will be review and verify the results. Certificates of Analysis will be issued for all case in which DNA analysis is performed. In addition, letters of notification will be sent to the parties as required by Virginia Legislation.

2008 **FY 2008 Using DNA Technology to Identify the Missing, 2008-DN-BX-K154, $443,682**

Funding provided for DFS and the Office of the Chief Medical Examiner (OCME) to initiate a joint effort to conduct DNA analysis and profiling of human remains currently in OCME storage. Depending on the condition of the evidence, short tandem repeat (STR) DNA testing and/or mitochondrial DNA testing will be conducted. DFS will use funds from this grant to pay the salaries and benefits for a mitochondrial DNA examiner and a wage Forensic Laboratory Specialist.

The OCME will use funds from this grant to pay the salaries of a wage Forensic Pathologist and a wage Medicolegal Death Investigator, and will contract with an Anthropologist for case file review and pre-DNA examination of all unidentified remains to determine suitability for testing. DFS will conduct mitochondrial DNA tests of all samples submitted. When DFS completes the DNA testing, the DNA profiles will be entered into the CODIS+mito database.

2009 **FY 2009 Forensic DNA Backlog Reduction Program, 2009-DN-BX-K080, $ 950,167**

Funding provided for the salaries and fringe benefits for five "restricted position" full-time personnel. One full time forensic laboratory specialist to contact Virginia's law enforcement agencies to determine the status of the backlogged cases and to assist the DNA examiners with laboratory support functions. Four Forensic Scientists to conduct scientific exams on items of evidence and reference samples. Additional funds provided for purchasing supplies associated with screening and conducting DNA analysis on the backlogged cases, new robotic systems, and the statewide DNA annual mandatory training in accordance with the FBI Quality Assurance Standards.

2009 **FY 2009 Convicted Offender/Arrestee DNA Backlog Reduction Program, 2009-DN-BX-K020, $171,579**

Funding provided for overtime salaries and FICA benefits for five DNA Data Bank

analysts, who will analyze offender and arrestee samples and perform technical review of the data, as well as two full-time staff to perform Data Bank sample analysis support functions. Funding also provided to purchase the chemistries necessary to analyze the backlogged samples and associated controls.

2009 **Solving Cold Cases with DNA, 2009-DN-BX-K046, $490,960**

Funding provided for two Forensic Scientists and the necessary supplies and equipment to conduct DNA analyses on active cold cases meeting the specified crime category. Eligible DNA profiles will be searched and uploaded into the Combined DNA Index System (CODIS).

2009 **Paul Coverdell Forensic Science Improvement Program, 2009-CD-BX-0043, $164,951**

Funding provided for renovation of the Central Laboratory to accommodate new Forensic Scientists in the Firearm and Toolmarks Section, to expand office space for examiners, to expand/relocate the Digital & Multimedia Evidence Section, and to relocate the Administrative Office and forensic case files. Funding provided covers general construction costs and materials/labor associated with the renovation efforts.

2010 **FY 2010 Paul Coverdell Forensic Science Improvement Program, 2010-CD-BX-0038, $311,246**

Funding provided to DFS to conduct training in the Physical Evidence and Chemical Analysis Services program areas. Funding also provided to purchase balances for the Controlled Substances Section and three Gas Chromatograph-Mass Spectrometers for the Trace Evidence Section.

2010 **FY 2010 Forensic DNA Backlog Reduction Program, 2010-DN-BX-K120, $920,520**

Funding provided to enhance the capacity and to reduce the current backlog in the Forensic Biology Section. Three Forensic Scientists to conduct scientific exams on items of evidence and reference samples and one Forensic Laboratory Specialist to contact Virginia's law enforcement agencies to determine the status of the backlogged cases and to assist the DNA examiners with laboratory support functions. Additional funds provided for purchasing supplies associated with screening and conducting DNA analysis on the backlogged cases and the statewide DNA annual mandatory training in accordance with the FBI Quality Assurance Standards.

2010 **FY 2010 Using DNA Technology to Identify the Missing, 2010-DN-BX-K130, $468,640**

Funding provided to continue joint effort by DFS and the OCME to identify human remains located in Virginia. DFS to conduct DNA analysis and profiling of human remains submitted to the laboratory by OCME and law enforcement agencies and upload resulting DNA profiles into the CODIS+mito database. The OCME to perform an

anthropological pre-DNA examination of all unidentified human remains to determine suitability for DNA testing and to enter each unidentified human remains case into the NamUs database. The OCME Medicolegal Death Investigators to review potential hits and liaison with the local law enforcement agencies. Contract Odontologists to assist in examining unidentified dental remains and make comparisons with dental x-rays of potential matches. Funding also provided for the purchase of necessary supplies and equipment.

2011 **FY 2011 Forensic DNA Backlog Reduction Program, 2011-DN-BX-K421, $1,447,358**

Funding provided to enhance the capacity and to reduce the current backlog in the Forensic Biology Section. Four Forensic Scientists to conduct scientific exams on items of evidence and reference samples and one Forensic Laboratory Specialist to assist the DNA examiners with laboratory support functions. Additional funds provided for purchasing supplies associated with screening and conducting DNA analysis on the backlogged cases and the statewide DNA annual mandatory training in accordance with the FBI Quality Assurance Standards.

2011 **FY 2011 Paul Coverdell Forensic Science Improvement Program, 2011-CD-BX-0051, $230,825**

Funding provided for training related travel and registration fees. Funding also provided to enhance the capacity of the Latent Prints Section with the purchase of software for latent print comparisons.

2012 **FY 2012 DNA Backlog Reduction Program, 2012-DN-BX-0021, $1,165,649**

Funding provided to enhance capacity in the Forensic Biology Section. Four Forensic Scientists to conduct scientific exams on items of evidence and reference samples and one Forensic Laboratory Specialist to assist the DNA examiners with laboratory support functions. Funding also provided to purchase three 3500 genetic analyzers for validation and protocol development for future use in the section. Training for DNA scientists was provided with funds under this award.

2012 **FY 2012 Paul Coverdell Forensic Science Improvement Program, 2012-CD-BX-0022, Total award for VA = $ 185,297. DFS portion = $103,891**

Funding provided to DFS for training related travel and registration fees for scientists in the Chemical Analysis and Physical Evidence program areas. Funding also provided for equipment items for the Firearms and Toxicology sections.

2013 **FY 2013 DNA Backlog Reduction Program, 2013-DN-BX-0079, $990,871**

Funding provided to enhance capacity in the Forensic Biology Section. Four Forensic Scientists to conduct scientific exams on items of evidence and reference samples and one Forensic Laboratory Specialist to assist the DNA examiners with laboratory support

functions. Funding also provided to purchase one 3500 Genetic Analyzer. Training for DNA scientists will be provided with funds under this award.

2013 **FY 2013 Paul Coverdell Forensic Science Improvement Program, 2013-CD-BX-0055, Total award for VA = $170,371. DFS portion = $83,582.** Funding provided for training and continuing education of DFS Forensic Scientists in various scientific disciplines.

2014 **FY 2014 DNA Capacity Enhancement and Backlog Reduction Program, 2014-DN-BX-0044, $906,457.** Funding provided to enhance capacity in the Forensic Biology Section. Five Forensic Scientists to conduct scientific exams on items of evidence and reference samples and one Forensic Laboratory Specialist to assist the DNA examiners with laboratory support functions. Funding also provided to purchase two 3500 Genetic Analyzers and one Qiagility Robot. Training for DNA scientists will also be provided with funds under this award.

2014 **FY 2014 Paul Coverdell Forensic Science Improvement Program, 2014-CD-BX-0055. Total award for VA = $184,994. DFS portion = $92,496.** Funding provided for training and continuing education of DFS Forensic Scientists in various scientific disciplines.