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**The Evaluation of Laser Diode Thermal Desorption (LDTD) for High Throughput Analysis of Controlled Substances and Toxicology in Forensic Sciences**

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# ABSTRACT

The abundance of drug-related cases causes backlogs in forensic laboratories, which creates budgetary and policy problems and potentially compromises investigations. The laser diode thermal desorption (LDTD) source, coupled with MS, has demonstrated its applicability in other scientific areas by providing data comparable to traditionally used instrumentation, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS), in less than half of the time without the need for commonly used laboratory consumables such as analytical columns. We evaluated LDTD coupled with triple quadrupole (QQQ) MS for the high-throughput quantitative analysis of controlled substances and drug toxicology in forensic laboratories. One hundred eleven drugs of abuse were optimized in methanolic drug solutions (e.g., designer drugs, controlled substances). Forty-nine compounds across major drug classes were spiked into drug-free human urine and blood for validation of screening or quantitative analysis. Liquid-liquid extraction (LLE) or solid phase extraction (SPE) methods were used for both urine and blood matrices for the extraction of drug analytes of interest. The goal was to utilize minimal sample preparation in order to increase overall sample analysis efficiency. The following parameters were evaluated for screening validation: carryover, interference, limit of detection (LOD), and matrix effect. The same parameters were evaluated for quantitative method analysis, in addition to limit of quantitation (LOQ), linearity, precision and accuracy, calibration model, and stability. Advantages of the LDTD system include (1) quick installation; (2) little instrument training; (3) no additional software requirement; (4) ease of use; (5) minimal maintenance; (6) rapid sample analysis; (7) minimal sample volume; (8) lack of chromatographic solvents and consumables; and (9) the ability to use across multiple MS platforms. Disadvantages include (1) sample destruction; (2) inconsistencies associated with manual spotting; (3) erroneous peaks due to interferences in sample matrix and peak apparitions as a result of the desorption process; (4) inability or difficulty analyzing isomers and isobars due to the lack of chromatography; and (5) the limited amount of ion transitions per method depending on the MS used. Overall, LDTD has the potential for use in screening and quantitation of biological samples in forensic laboratories. Its ability to rapidly analyze a large number of samples in a short amount of time (~12 sec per sample) makes it ideal for high throughput forensic laboratories. However, due to the lack of chromatography, analysts must be diligent in their method development in order to determine the best parameters and extraction techniques.

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# EXECUTIVE SUMMARY

## ES.1  Statement of the Problem

The criminal justice system relies heavily on forensic science to provide scientifically based information on physical evidence through detailed analysis. The U.S. Department of Justice, Bureau of Justice Statistics (BJS) reported that the nation's 411 publically funded crime laboratories received an estimated 4.1 million requests for forensic services. Of these requests, 33% and 15% were controlled substance analysis and toxicology, respectively. Approximately 142,100 controlled substances and 30,400 toxicology cases were backlogged at the end of 2009. Backlogs create budgetary and policy problems that can potentially compromise investigations. One approach to decrease the amount of time for sample analysis is to implement more efficient technologies. The ideal technology reduces analysis time while also preventing errors that may be introduced by extensive sample preparation techniques. It will also improve the quality of the result and increase throughput to address workload demands. Technologies that provide truly broad-spectrum detection are important to improve resolution and sensitivity in forensic analyses. Newer high-throughput technology that allows for rapid sample analysis with limited sample preparation has the potential to significantly decrease both analysis time and per-sample cost of controlled substance and toxicology analyses in forensic laboratories. Laser diode thermal desorption (LDTD) is a new direct-ionization source that can be coupled directly to a mass spectrometer without prior chromatographic separation. Because this is a supplemental instrument to expand the capability of an existing mass spectrometry (MS) technique (e.g., triple quadrupole [QQQ]), it may be more cost efficient for the laboratory to implement than a new mass spectral platform. Although this technique is finding successful application, its further use requires careful consideration of its practicality and demonstration of its validity.

## ES.2  Purpose

The purpose of this study was to evaluate the applicability of the LDTD source in controlled substances and drug toxicology. Specifically, we evaluated the instrument's performance as a high-throughput source coupled with a QQQ MS platform to detect controlled substances, including new emerging designer drugs, drugs used in drug-facilitated crimes, and those relevant to postmortem toxicology. Our goal was to investigate the efficiency and cost effectiveness of this new technology and the feasibility of implementing it in forensic laboratories. The successful implementation of the LDTD source in other scientific areas, such as drug discovery and cytochrome P450 inhibition studies, warrants the investigation of this technology in the area of forensic science. Thus, it is important to verify the potential for the instrument under practical and forensically relevant conditions encountered in a forensic laboratory.

This research was designed to initially optimize MS and LDTD parameters for drugs of abuse prepared in methanol from commercial standards to provide the basis for the evaluation of these drugs in biological matrices, specifically blood and urine. The investigation of urine guided the sample preparatory techniques for blood, each time employing minimal sample preparation required to detect drugs in the specific matrix. In addition to the evaluation of drug-fortified samples, Spice plant material and previously confirmed postmortem case samples were tested (obtained from the North Carolina Office of the Chief Medical Examiner [Raleigh, NC] and The Los Angeles Office of Coroner [Los Angeles, CA]).

## ES.3  Research Design

The project was divided into two stages: Stage I: Instrument Optimization and Stage II: Analysis of Drug Analytes in Specimen Matrices.

### *Stage I: Instrument Optimization and Validation*

#### LDTD Set Up and Training

On-site training and installation of the T-960 LDTD-APCI ionization interface model controlled by LazSoft 4.0 software (Phytronix Technologies, Quebec, Canada) occurred over 5 days. Training involved LDTD operation and maintenance, software operation, compound optimization, and method development. Analytes were subsequently detected using an ABSciex (Framingham, MA) API 4000 QQQ mass spectrometer controlled by Analyst software (Version 1.4.2) (Foster City, CA).The LDTD is operated by a driver though Analyst software.

#### Optimizing of the Mass Spectrometer for the Detection of Drug Standards

Methanolic solutions of 111 compounds consisting of forensically common drug analytes and deuterated internal standards (ISTD) were evaluated to determine optimal MS and LDTD conditions. The analytes included antidepressants, amphetamines, barbiturates, benzodiazepines, designer drugs, opiates, stimulants, hallucinogens, and Z drugs. The precursor ion $[M+H]^+$ of all analytes and ISTDs were optimized for product ions. The two most intense product ions were further optimized for declustering potential (DP) and collision energy (CE) to achieve optimal signal strength of the analyte transition.

### *Stage II: Analysis of Drug Analytes in Specimen Matrices*

#### MS and LDTD Analytical Methods Set-up

Forty-nine compounds across major drug classes were spiked into drug-free human urine and blood for validation of screening or quantitative analysis (**Table ES-1**). Internal standards for these compounds were chosen based off similar structure, class, and/or common functional groups. The drugs were grouped together according to their drug class and analyzed for quantitation or screening analysis. The API 4000 is an older model QQQ and only allows for a minimum dwell time of 10 msec for a total scan time of 100–200 msec given a maximum transition range of 10–20. In order to achieve enough data points across a desorption peak (20–30) and for reliable reproducibility, we used no more than 10 transitions per method. As a result, for both quantitative and screening methods, analytes were pooled into groups of no more than 10 transitions, with the exception of the antidepressants and synthetic cannabinoids.

**Table ES-1. Grouping of drug analytes in quantitative and screening methods**

| Group | Drug Analytes/ISTD | Total Number of Monitored Ion Transitions |
|---|---|---|
| 1 | Amitriptyline/Amitriptyline-d3<br>Citalopram/Citalpram-d6<br>Nortriptyline/Nortriptyline-d3<br>Trazodone/Trazodone-d6 | 12 |
| 2 | AMP/AMP-d6<br>MAMP/MAMP-d9 | 8 |
| 3 | MDA/MDA-d5<br>MDEA/MDEA-d5<br>MDMA/MDMA-d5 | 9 |
| 4 | Alprazolam/Alprazolam-$d_5$<br>Clonazepam/Clonazepam-$d_4$<br>Diazepam/ Diazepam-$d_5$ | 10 |
| 5 | α-hydroxyalprazolam/α-hydroxyalprazolam-$d_5$<br>7-aminoclonazepam/7-aminoclonazepam-$d_4$ | 6 |
| 6 | 4-MEC<br>4-MePPP<br>α-PVP<br>Buphedrone<br>Flephedrone<br>MAMP-$d_9$<br>mCPP<br>MDPV<br>Methylone<br>TFMPP | 10 |
| 7 | 2-AI<br>5-IT<br>5-MeO-AMT<br>5-MEO-DiPT<br>5-MeO-DMT<br>5-MeO-MiPT<br>Amitriptyline-$d_3$<br>MDAI | 8 |
| 8 | AKB48<br>EAM2201<br>JWH-018<br>JWH-073<br>MAM2201<br>MAM2201-$d_5$<br>STS-135<br>UR-144<br>UR-144 5-bromopentyl<br>UR-144 5-chloropentyl<br>XLR11 4-pentenyl<br>XLR11 | 12 |
| 9 | JWH-018 N-(5-hydroxypentyl)<br>JWH-073 N-(4-hydroxybutyl)<br>JWH-122 5-hydroxypentyl<br>JWH-200 4-hydroxyindole<br>MAM2201-$d_5$ | 10 |
| 10 | BZE/ BZE-$d_3$<br>COC/ COC-$d_3$ | 8 |
| 11 | PCP/ PCP-$d_5$ | 4 |

ES-3

## Evaluating LDTD Performance and Sample Preparation of Compounds from Various Drug Classes

Liquid-liquid extraction (LLE) (**Table ES-2**) or solid phase extraction (SPE) (**Table ES-3**) methods were used for both urine and blood matrices for the extraction of drug analytes of interest. The goal was to utilize minimal sample preparation in order to increase overall sample analysis efficiency.

**Table ES-2. Summary of LLE of drug analytes from blood and urine**

| Drug Analyte | Sample Volume (µL) | Acid/Base addition (µL) | Extraction Solvent and Volume (µL) | Post extraction sample preparation (µL) | Amount Spotted onto LazWell (µL) |
|---|---|---|---|---|---|
| Benzodiazepines | 200 | 250 1 N NaOH | 800 ethyl acetate | — | 2 |
| Amphetamines | 100 | 500 1 N NaOH | 500 n-butyl chloride | Remove 300 top layer and add 50 of 0.01 N HCl | 2 |
| Antidepressants | 100 | 400 1 N NaOH | 500 n-butyl chloride | — | 2 |
| Cathinones and Piperazines | 200 | 500 1 N NaOH | 500 n-butyl chloride | Remove 100 top layer and add 10 of 0.01 N HCl | 2 |
| Tryptamines and Indanes | 200 | 500 1 N NaOH | 500 n-butyl chloride | — | 2 |
| Synthetic Cannabinoids and Metabolites | 100 | — | 400 n-butyl chloride | — | 2 |

**Table ES-3. Summary of SPE analytes from blood and urine**

| Drug Analyte | Sample Volume (µL) | Buffer (µL) | Column Wash | Column Wash | Elution | Post extraction sample preparation (µL) | Amount Spotted onto LazWell (µL) |
|---|---|---|---|---|---|---|---|
| COC BZE | 200 | 600 Phosphate buffer (100 mM, pH 6) | 1 mL DI water, 1 mL 0.1 M HCl* | 1 mL MeOH, 1 mL ethyl acetate** | 1 mL CH$_2$Cl$_2$ IPA NH$_4$OH (80:18:2) | — | 4 |
| PCP | 200 | 600 Phosphate buffer (100 mM, pH 6) | 1 mL DI water, 1 mL 0.1 M HCl* | 1 mL MeOH, 1 mL ethyl acetate** | 1 mL CH$_2$Cl$_2$, IPA, NH$_4$OH (80:18:2) | Remove 100 top layer and add 10 of 0.01 N HCl | 4 |

* Blood samples washed with 1 mL sodium carbonate/bicarbonate buffer (100mM pH9) after acid wash
**Blood samples washed with an additional 1 mL of ethyl acetate

### Validation Study Design

Validation was modeled following approaches presented at a workshop during the July 2012 Society of Forensic Toxicologist Annual Meeting in Boston, MA (**Table ES-4**).The following

parameters were evaluated for screening validation: carryover, interference, limit of detection (LOD), and matrix effect. The same parameters were evaluated for quantitative method analysis, in addition to limit of quantitation (LOQ), linearity, precision and accuracy, calibration model, and stability.

**Table ES-4. Validation parameters for quantitative and screening analysis evaluated by LDTD-MS/MS**

| Screening | Quantitative Analysis |
| --- | --- |
| Carryover | Carryover |
| Interference | Interference |
| Limit of Detection (LOD) | LOD |
| Matrix Effect | Matrix Effect |
|  | Limit of Quantitation (LOQ) |
|  | Linearity, Precision and Accuracy (LPA) |
|  | Calibration Curve (including ULOL) |
|  | Stability |

## Calibration Curve

The calibration curve was established by creating a curve spanning the range of biologically relevant concentrations expected in urine and blood. Six non-zero calibrators were used to establish the model, with the exception of alprazolam, clonazepam, and diazepam, whose curves consisted of five non-zero calibrators in urine and blood. Once the appropriate calibration curve was established, it was extracted and analyzed five times (n=5 at each concentration level). For quantitative analysis, each analyte's respective stable isotope labeled compound was used as an internal standard.

## Upper Limit of Linearity

Calibrators in concentrations above the established curve were analyzed to determine at which concentration the curve no longer exhibited linearity.

## Linearity, Precision, and Accuracy

Linearity, precision, and accuracy (LPA) was determined by analyzing three quality control (QC) samples fortified with analyte concentrations at the lower, middle, and upper portion of the calibration curve. Each QC sample was analyzed in triplicate within each linearity run (n=9) over the course of five runs (total n=45).

## Limit of Detection (LOD)/Limit of Quantitation (LOQ)

The LOD was defined as the lowest concentration that produced a reproducible instrument response greater than or equal to three times that of the blank sample. The LOD was determined by fortifying three sources of blank urine or blood matrix in decreasing concentrations. In order to obtain three sources of blank blood, bovine blood was used for the LOD study. The extracted samples were analyzed in duplicate for three runs (n=18). The LOQ was defined as the concentration of the lowest calibrator, which was determined during the establishment of the calibration curve.

**Carryover**

Carryover was determined by analyzing blank sample matrix immediately after a high concentration sample. This was carried out using triplicate analysis. A sample was considered to have carryover if the average of the peak area after the carryover sample was greater than the established LOQ by 20% peak area for quantitation. Validation for screening analysis does not require establishment of a LOQ; therefore, a sample was considered to have carryover if the average of the peak area after the carryover sample was greater than the LOD.

**Interference**

*Evaluation of blank sample matrix*: Five blank matrix samples, fortified with ISTD, were analyzed to demonstrate the absence of interferences originating from the ISTD. In addition five matrix samples were analyzed without the addition of analyte or ISTD to demonstrate absence of interference from matrix. In both cases, a sample was considered to have interference if the average of the peak area of the blank samples was greater than 20% of the established LOQ peak area for quantitative analysis. For screening, a sample was considered to have interference if the average peak area of the blank samples was greater than the LOD by 20%.

*Evaluation of interfering compounds*: Based on known interferences for some drug classes, interference studies were focused on a select group of analytes undergoing quantitative analysis. Potential interferences ephedrine, pseudoephedrine, PPA, and phentermine were fortified in five lots of urine containing AMPs. Potential interferences norcocaine and scopolamine were fortified in five urine lots containing COC/BZE. Finally, potential interferences JWH-073 5'-hydroxyindole and JWH-018 2-hydroxyindole were fortified in five lots of urine containing JWH-018 N-(5-hydroxypentyl), JWH-073 N-(4-hydroxybutyl), JWH-122 5-hydoxypentyl, and JWH 200 4-hydroxyindole. Interference was determined if the calculated concentration was greater than 20% of the target concentration. For screening, interference was determined if the peak area of the sample fortified with potential interfering compounds was greater than the target sample peak area by 20%.

**Matrix Effect**

Matrix effects were evaluated using a modified version of the method described by Matuszewski and colleagues (Matuszewski, 2003). Three sets of samples were created for each target analyte. As described by Matuszewski and colleagues, comparative calculations were used to evaluate the data:

ME (%) = B/A x 100
RE (%) = C/B x 100

where A, B, and C = the mean responses as represented by the area under the peaks for target and internal standard quantitative ions,
ME = matrix effect, and
RE = recovery efficiency.

The mean responses for A, B, and C were determined across these 10 urine or blood lots. The assessment of a relative matrix effect was determined by comparing the MEs between the 10

lots. The variability (%CV) in the MEs between lots is considered to be a measure of the relative matrix effect.

**Stability**

In order to evaluate the stability of dry samples in the LazWell plate, three sets of samples from the LPA study were spotted onto the plates, kept at room temperature, and analyzed after 0, 6, and 15 hours.

**Additional Analysis**

In addition to the validation study, previously confirmed archived postmortem samples were evaluated to determine if the LDTD has the required sensitivity to detect drugs of abuse in samples that have decomposed or contain numerous analytes of potential interferences. Plant Spice material was also analyzed by screening method for the presence of synthetic cannabinoid.

## ES.4 Findings

### Stage I: Instrument Optimization and Validation

#### Optimization of QQQ MS

The precursor ion [M+H]+ for 111 analytes, including their ISTDs, was monitored in product ion scan mode. The two most prevalent product ions were chosen for each analyte, and the DP and CE were adjusted for optimal signal strength. The two product ions were then analyzed in MRM mode while independently varying the DP and CE values. The optimal values were determined by selecting the voltage that produces the greatest intensity.

### Stage II: Analysis of Drug Analytes in Specimen Matrices

#### Laser Pattern Evaluation

In all cases, with the exception of PCP, synthetic cannabinoids, and metabolites, the laser power was decreased from the original methanolic standards optimized at 3-45-2 (**Table ES-5**) for optimal desorption in blood and urine matrices. The benzodiazepines were the only analytes that required a laser power greater than 45%.

**Table ES-5. Summary of LDTD laser patterns of drug analytes that produced optimal desorption in blood and urine**

| Analyte | Laser Power |
|---|---|
| AMP/MAMP | 3-25-0 5 second delay |
| MDA/MDMA/MDEA | 3-25-0 |
| Benzodiazepines | 3-55-0 |
| PCP | 3-45-0 |
| Antidepressants | 3-25-0 |
| COC/BZE | 3-25-0 |
| Synthetic Cathinones | 3-25-0 5 second delay |
| Tryptamines and Indanes | 3-35-0 |
| Synthetic Cannabinoid and Metabolites | 3-45-2 |

An example of the importance of laser pattern optimization was observed early in the method development process. For certain analytes, we observed peak broadening and split peaks in urine and blood extracted samples. Based on consultation with the LDTD manufacturer, it was determined that these undesirable peak characteristics may have occurred as a result of thermal desorption of the matrix, in addition to the analyte of interest. Optimizing the laser pattern to minimize matrix desorption greatly improved the peak shapes. It is important to optimize the laser pattern so that any interference not removed during the sample preparation process will not affect peak desorption of the target analyte, especially at lower analyte concentrations. Subsequent input from other LDTD users has indicated that similar improvements may have been achieved by optimizing the dilution factor at which extracts were loaded onto the plate; however, those approaches were not pursued as part of this project.

The API 4000 is an older model QQQ and only allows for a minimum dwell time of 10 msec for a total scan time of 100–200 msec given a maximum transition range of 10–20. In order to achieve enough data points across a desorption peak (20–30) and for reliable reproducibility, we used no more than 10 transitions per method. As a result, analytes were pooled into groups of no more than 10 transitions, with the exception of the antidepressants and synthetic cannabinoids.

## Issues Encountered during Analysis of Drug Analytes in Blood and Urine

Under usual circumstances, an interferant present in a matrix can be separated from the analyte of interest during gas chromatography (GC) or liquid chromatography (LC) analysis, allowing for successful identification or quantitation of the target analytes. The inability to separate target analytes from interferences using LDTD was a concern and a frequently encountered problem throughout this study.

Early in this study, it was determined that a peak in a blank sample was acceptable if its area was less than 20% of the LOQ. If the criterion was not met, the origin of the peak would be investigated and attempts would be made to either eliminate or decrease the peak by removal of the interferant if contamination persisted or by more extensive sample preparation.

Throughout this project, we encountered the presence of interferant peaks in drug-free urine or blood at the same ion transition of the target analyte. Examples include PCP, 7-aminioclonazepam, amphetamine, and opiates. Transition peaks in the blank for PCP and 7-aminoclonazepam were not eliminated, but quantitative analysis continued by selecting another transition. Amphetamine was resolved by determining that the interferant was coming from deionized water, and use of HPLC-grade water was required. Peaks in the opiates were undetermined.

## Validation Results for Quantitative Method Analysis

### Calibration Model

A six-point calibration curve was evaluated to establish the appropriate calibration model for all analytes in blood and urine. Calibration samples spanned the range of typical concentrations expected and were analyzed in five separate runs (n=5 at each concentration) to establish the model.

The average $r^2$ value of the calibration curves for all drugs extracted in both urine and blood was 0.998. The average slope of the curves for all drugs extracted in urine and blood was 1.00 and

0.993, respectively. For urine and blood analysis, the intercepts ranged from -15.10 to 8.06 and -1.97 to 20.47, respectively. Significance was assigned at the $p < 0.05$ level. All intercept p-values for blood and urine were greater than 0.05, meaning all intercepts were not significantly different from zero. The ULOL ranged from 1,000 ng/mL for trazodone to 15,000 ng/mL for diazepam in urine. The ULOL for blood ranged from 500 ng/mL alprazolam, diazepam, and clonazepam to 10,000 ng/mL for MDEA.

**Evaluation of Precision and Accuracy for All Drugs analyzed by Quantitative Analysis**

For urine, the average overall within-run precision was represented by a %CV < 8.5 for all compounds, with the exception of MDEA, which had greater variability, as shown by a 13.8% CV. PCP had the least variability as shown by a 2.2% CV. The average overall between-run precision was represented by a %CV < 13.0 for all compounds, with the exception of MDEA, which had greater variability, as shown by a 21.0% CV. For blood, the average overall within-run precision was represented by a %CV < 7 for all compounds. The analytes with the highest and lowest variability were MDEA with a %CV of 6.9 and BZE with a 2.0% CV. The average overall between-run precision was represented by a %CV < 12.0, with alprazolam having the highest variability (11.5% CV) and BZE having the lowest (3.4% CV). For the purposes of this study, a %CV < 20.0 was considered acceptable. Overall, all analytes exhibited acceptable precision, with the exception of MDEA, which had a between-run %CV slightly higher than 20.

The % accuracy and its associated %CV for all drugs in urine were 88.9–104.5, with accuracy %CVs less than 16.5, with the exception of MDEA, which had an accuracy %CV of 27.2. The % accuracy and its associated %CV for all drugs in blood were 91.9–107.1 and < 12.0, respectively.

**Evaluation of LOD and LOQ**

In urine, the LOD values ranged from 0.5–15 ng/mL, with MDEA having the highest and COC having the lowest. In blood, the LOD values ranged from 0.25–15 ng/mL, with AMP having the highest while BZE the lowest. In general, the LOD were similar for each analyte across the two matrices, with BZE showing the largest change with an LOD of 2 ng/mL in urine and 0.25 ng/mL in blood.

The LOQ was defined as the value of the lowest non-zero calibrator of calibration range and assessed by analyzing the lowest calibrator to demonstrate reproducibility and accuracy. The number of samples varies as they were pooled from the calibration curves used throughout the validation. In some cases, a sample set was re-extracted if there was an erroneous result for a QC sample. In that case, although the QC sample was unacceptable, the calibration curve was acceptable and therefore was used in the evaluation of the LOQ. All LOQ were acceptable, with %CVs ranging from 3.82–14.2 in urine and 3.13–14.2 in blood. Accuracies ranged from 89.6–107.6% and 90.0–102.1% in urine and blood, respectively.

**Evaluation of Interferences and Carryover**

Based on known interferences for some drug classes, interference studies were focused on a select group of analytes undergoing quantitative analysis. AMPS samples spiked at 200 ng/mL contained the potential interferences ephedrine, pseudoephedrine, PPA, and phentermine at 50,000 ng/mL. The mean results show that the target concentration of with AMP, MDA, MDEA, and MDMA had a %CV < 9, confirming the absence of interference. This was to be expected because MAMP and phentermine are isomers and share a [M+H]+ value of 150.120 m/z. COC

and BZE samples spiked at 40 ng/mL contained the potential interferences norcocaine and scopolamine at 25,000 ng/mL. The results show a mean concentration for COC and BZE > 25% of the target concentration. Again, this was expected because BZE and norcocaine are isomers that share the same [M+H]+ value of 290.139 m/z. Likewise, COC and scopolamine are also isomers with the same [M+H]+ value of 304.154 m/z. The evaluation of blank sample matrix resulted in no interference from the ISTD for all analytes. As previously discussed, the presence of interference in blank samples was not uncommon. As a result, a sample was considered to have interference if the average peak area was greater than 20% of the LOQ.

For carryover, the results show that all analytes in urine and blood, with the exception of, clonazepam and MDA, respectively, did not produce carryover.

**Matrix Effect Evaluation**

A matrix effect value > 100% indicates ion enhancement, while a value < 100% indicates ion suppression. In urine, the antidepressants and benzodiazepines all had ME greater than 140%, indicating ion enhancement. Antidepressant ME ranged from 152.8 to 271.8%, with trazodone being the highest and amitriptyline the lowest. ME for benzodiazepines ranged from 141.1 to 286.8%, with 7-aminoclonazepam being the highest and clonazepam being the lowest. Matrix effects for amphetamines and miscellaneous drugs in urine were within 100 ±20%, with the exception of MDA (149.8%). These large MEs are not unexpected in a process involving thermal desorption, as highly different processes can take place for neat solutions and extracted matrices and do not invalidate the method. The more important characteristic to consider is the presence or absence of a relative ME, and the comparison of ME across different lots of urine or blood, with the absence of relative ME being highly desirable. The relative ME, expressed at %CV, from 10 lots ranged from 9.4 to 84.7%, with amitriptyline being the lowest and α-hydroxyalprazolam being the highest in urine.

For blood, all analytes had a ME greater than 125%, with the exception of the AMPs, BZE, COC, and PCP indicating ion enhancement. ME for antidepressants ranged from 139.6 to 285.4%, with trazodone being the highest and amitriptyline the lowest. ME for AMPs ranged from 46.0 to 119.1%, with AMP being the highest and MDMA the lowest. Benzodiazepines had the highest overall ME, ranging from 123.1 to 238.8%, with α-hydroxyalprazolam being the highest and diazepam the lowest. The relative ME, expressed as %CV, from 10 lots ranged from 22.3 to 77.0%, with MAMP being the lowest and BZE being the highest in blood.

Although the high %CV values in urine and blood are indicative of relative ME, subsequent input from LDTD users suggest that insufficient heating and low laser pattern temperature may have had a negative effect on our reproducibility. Future studies should include determining if further optimization of experimental conditions could mitigate the appearance of this relative ME.

The RE across all analytes in urine ranged from 30.0 to 186.3%, with MDEA being the highest and α-hydroxyalprazolam the lowest. In blood, the RE ranged from 35.7 to 194.6%, with MDMA being the highest and trazodone the lowest.

**Stability**

The results of the stability evaluation, in which QC samples were spotted into sample wells and analyzed at 0, 6, and 15 hours, showed that the accuracy of all analytes was stable up to 15 hours for urine and blood.

## Validation of Results for Screening Method Analysis

### Evaluation of LOD

In urine, the LOD values ranged from 0.1–50 ng/mL, with UR-144 N-(5-bromopently) analog having the highest while JWH-018 N-(5-hydroxypenyl) metabolite and JWH-122 5-hydroxypentyl metabolite have the lowest LOD concentration. Only the synthetic cathinones and piperazines were assessed in blood for screening analysis. In blood, the LOD ranged from 1–10 ng/mL, with mCPP being the highest. In general, the LOD were similar for each analyte across the two matrices. LOD for synthetic cathinones and piperazines in blood were either the same concentration or lower than those in urine, with the exception of buphedrone.

### Evaluation of Interference and Carryover

Since this is a screening method, interference was established if the analyte peak area of the interferant compound analyzed at 25,000 ng/mL had a peak area greater than the peak area by 20% of the analyte of interest at a specific concentration. JWH metabolites were at a target concentration of 20 ng/mL, while all other designer drugs were 100 ng/mL. As expected, JWH-018 2-hydroxyindole, JWH-073 5-hydroxyindole, 2, 3-MDPV, 3-FMC, mephedrone, α-pbp, and pentedrone all interfered with their positional isomers because they have the same nominal mass and transition. It is possible to reduce the interference by selecting another transition not common to the positional isomer, but this is not effective for a quick screening method of unknown compounds. MDAI has the same nominal mass at 6-APDB and mephedrone but not the same exact mass, which can be resolved on a high-resolution instrument. 6-APDB and mephedrone spiked at 25,000 ng/mL and analyzed under the MDAI acquisition method showed interference at MDAI MRM transitions. Their peak area was higher than the MDAI 100 ng/mL sample. This provides an example of compounds not in the same drug class (MDAI and 6-APDB indanes) and mephedrone (cathinone) interfering in the LDTD analysis. Buphedrone did not show interference for MDAI when it was ran as an interferant. However, when MDAI was ran as an interferant against buphedrone, there was interference with the analysis. 5-IT interfered with analysis of 5-MeOAMT. 4-OH-MiPT was an interferant for 5-MeO-Mipt analysis. Methylone, 4-OH-DiPT, and 2-AI showed interference for 5-MeO-DiPT analysis. 5-MeO-AMT interfered with TFMPP acquisition. In urine, with the exception of 2-AI, buphedrone, 4-MEC, XLR-11, UR-144, and XLR-11 4-pentenyl carryover was not observed. In blood, carryover was observed for mCPP.

### Evaluation of Matrix Effect

ME values for all analytes in urine were greater than 125%, with the exception of 2-AI (113.4%) and XLR-11 (124%) indicating ion enhancement. The ME for the indane group ranged from 113.46 to 821.55%, with 5-IT being the highest and 2-AI the lowest. Synthetic cannabinoid had a range of ME from 124.14 to 138.67%, with STS-135 being the highest and AKB-48 the lowest. The ME for synthetic cannabinoids was the most consistent between the analytes compared to all

others groups. The range of ME for synthetic cannabinoid metabolites was from 128.23% for JWH-122 5-hydroxypentyle metabolite to 168.91% for MAM 2201-d$_5$ ISTD. The relative ME, expressed as %CV, from 10 lots ranged from 5.7 to 137.9%, with JWH-073 being the lowest and 5-MEO-DipT being the highest in urine.

Only the synthetic cathinones and piperazines were assessed in blood for ME. The MEs in blood for most of the synthetic cathinones and piperazines were $100 \pm 10\%$, with the exception of α-PVP, buphedrone, flephedrone, and MDPV. The relative ME, expressed as %CV, from 10 lots ranged from 25.6 to 39.0%, with 4-MePP being the lowest and buphedrone being the highest in blood. Future studies should include determining if further optimization of experimental conditions could mitigate the appearance of this relative ME.

The RE for all analyte groups in urine ranged from 30.3 to 157.1%, with XLR11 N-(4-pentenyl) being the highest and 5-MEO-AMT being the lowest. The RE of analytes in blood ranged from 64.5 to 110.5% with values greater than 81%, with the exception of flephedrone.

## ES.5 Conclusion

Advantages of the LDTD system include (1) quick installation; (2) little instrument training; (3) no additional software requirement; (4) ease of use; (5) minimal maintenance; (6) rapid sample analysis; (7) minimal sample volume; (8) lack of chromatographic solvents and consumables; and (9) the ability to use across multiple MS platforms. Disadvantages include (1) sample destruction; (2) inconsistencies associated with manual spotting; (3) erroneous peaks due to interferences in sample matrix and peak apparitions as a result of the desorption process; (4) inability or difficulty analyzing isomers and isobars due to the lack of chromatography; and (5) the limited amount of ion transitions per method depending on the MS used. Overall, LDTD has the potential for use in screening and quantitation of biological samples in forensic laboratories. Its ability to rapidly analyze a large number of samples in a short amount of time (~12 sec per sample) makes it ideal for high-throughput forensic laboratories. However, due to the lack of chromatography, laboratories must invest a significant amount of time in upfront method development to determine the best parameters and extraction techniques.

Future studies should include determining if alternative sample preparation and LDTD parameters will eliminate the challenges of anomalous peaks and peak apparitions encountered during this study.

## ES.6 Implications for Policy and Practice

Forensic laboratories are often backlogged, creating budgetary and policy problems. Backlogs can potentially compromise investigations. Technologies, such as LDTD-MS /MS have the potential to reduce screening time requirements and costs.

# 1.    INTRODUCTION

## 1.1    Statement of the Problem

The National Forensic Laboratory Information System (NFLIS) reported that from January 2012 through June 2012, an estimated 486,452 drug cases were submitted to State and local laboratories in the United States and analyzed by September 30, 2012 (National Forensic Laboratory Information System, 2012). The U.S. Department of Justice, Bureau of Justice Statistics (BJS) reported that the nation's 411 publically funded crime laboratories received an estimated 4.1 million requests for forensic services (Bureau of Justice Statistics [BJS], 2012). Of these requests, 33% and 15% were controlled substance analysis and toxicology, respectively. Approximately 142,100 controlled substances and 30,400 toxicology cases were backlogged at the end of 2009. Backlogs create budgetary and policy problems that can potentially compromise investigations.

The use of minimal sample consumption and preparation, along with higher-throughput technologies for drug analysis, could drastically increase capacity of forensic laboratories, mitigating the current backlog. Mass spectral analyses combined with chromatographic techniques used in controlled substance and toxicology analyses typically require longer sample preparation techniques and sample run times. Previously, RTI International (RTI) successfully evaluated the applicability of direct sample introduction, time-of-flight (AccuTOF™-DART™) technology for screening postmortem toxicology cases (Ropero-Miller et al., 2007; Ropero-Miller et al., 2008 ). It was concluded that the instrument has the potential for extremely rapid analysis and broad detection ability; however, further research is needed to improve sensitivity. Similarly, laser diode thermal desorption (LDTD) is a new direct ionization source that can be coupled directly to a mass spectrometer without prior chromatographic separation. This high-throughput technology has the potential to significantly decrease both analysis time and per-sample cost of controlled substance and toxicology analyses in forensic laboratories. Because this is a supplemental instrument to expand the capability of an existing MS technique (e.g., tandem mass spectrometry), it may be more cost efficient for the laboratory to implement than an entire new mass spectral platform. Although this technique is finding successful application, its further use requires careful consideration of its practicality and demonstration of its validity.

## 1.1    Literature Review

The criminal justice system relies heavily on forensic science to provide scientifically based information on physical evidence through detailed analysis. The timeliness in which a judicial case is processed is often related to the efficiency of the forensic laboratories, which are responsible for analyzing pertinent evidence. One approach to decrease the amount of time for sample analysis is to implement more efficient technologies. The ideal technology reduces analysis time while also preventing errors that may be introduced by extensive sample preparation techniques. It will also improve the quality of the result and increase throughput to address workload demands. Technologies that provide truly broad spectrum detection are important to improve resolution and sensitivity in forensic analyses. Currently, most forensic laboratories rely on MS-based assays coupled to chromatographic separation, such as gas chromatography/mass spectrometry (GC-MS), liquid chromatography/mass spectrometry (LC-MS), and liquid chromatography/ tandem mass spectrometry (LC-MS/MS) for sample analysis

and compound identification. Although these traditional techniques provide high sensitivity and high selectivity, their use of chromatographic separation leads to long per-sample analysis times. During the past several years, the introduction of rapid analytical techniques for the direct analysis of samples has increased. Direct analysis of samples does not include a chromatographic separation step, thus reducing the time involved in sample preparation and analysis and reducing costs by eliminating chromatographic column usage and mobile phase solvent consumption. The most prevalent MS-based direct analysis methods include desorption electro-spray ionization (DESI), atmospheric pressure matrix-assisted laser desorption/ionization (MALDI), and direct analysis in real time (DART) coupled to a mass spectrometer (Van Berkel et al., 2008). Although these techniques are more efficient than traditional chromatographic MS methods, one disadvantage is that quantitative applications can be challenging, making these techniques more suitable for qualitative and semi-quantitative analysis. In a previous NIJ award, RTI evaluated DART-TOF instrumentation screening and established a drug standard reference library that contained controlled substances and drugs commonly analyzed in postmortem toxicology (Ropero-Miller and Stout, 2008; Bynum et al., 2007). It was concluded that the DART-TOF system has a potential application for forensic analyses; however, the issue of sensitivity limits its applicability. RTI also conducted a study comparing the AccuTOF™-DART™ and signature analysis for identifying constituents of refined illicit cocaine (Ropero-Miller et al., 2007). Since our evaluation, DART-TOF has met the Frye Standard, which is a test to determine the admissibility of scientific evidence. As a result, evidence analyzed by DART-TOF is admissible in court.

Introduced by Phytronix Technologies Inc. (Quebec, Canada), the LDTD ion source is a relatively new high-throughput source that allows for ultra-fast sample introduction in MS (Phytronix, 2011). It eliminates the need for chromatographic separation and can be integrated across multiple MS platforms. In LDTD, a small amount of sample, typically 1 to 2 μL, is pipetted into a well in a specially designed 96 or 384 well-plate (LazWell$^{TM}$). After the solvent evaporates from the sample, an infrared laser diode heats the back of the stainless steel sheet of the well, instantly transferring heat to the dried sample. The analyte is vaporized into gas-phase neutral molecules, which are carried, along with water molecules, from the carrier gas through the transfer tube into the corona discharge to undergo atmospheric pressure chemical ionization (APCI). The schematic of the LDTD source is shown in **Figure 1**.
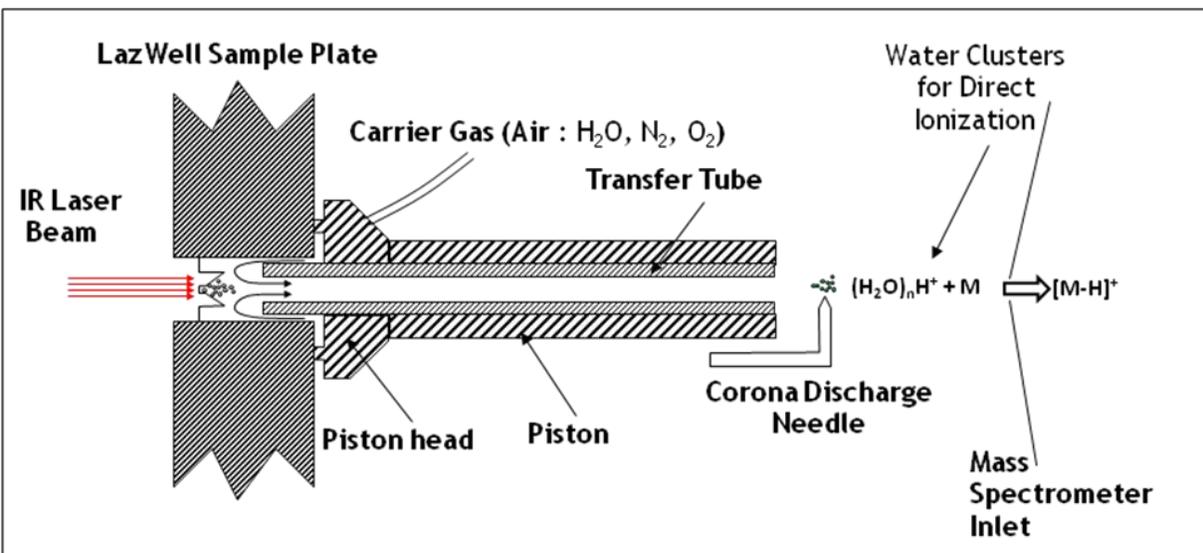
**Figure 1. Schematic of LDTD ionization source (Source: Phytronix)**

Advantages of the LDTD source include (1) minimized occurrence of thermal fragmentation due to the use of the unheated transfer tube and the ultra-fast thermal desorption at an unattended low temperature; (2) elimination of carryover due to the transfer tube; (3) efficient ionization due to solvent-free gas-phase APCI, which allows direct proton transfer from formed water clusters in the corona discharge to the analyte; (4) the use of inexpensive compressed air as the source of carrier gas; (5) a "plug-and-play" interface that can be attached to a variety of mass spectrometers, including QQQs, QTOFs, and ion trap systems; (6) low background signal; (7) low inlet maintenance; and (8) shorter data acquisition time. The LDTD does not require additional software or solvents, making it financially practical.

Many large international pharmaceutical companies, such as Merck, Sanofi Aventis, Astra Zeneca, Novartis, and Abbott, are using LDTD technology in drug discovery and development and production. Other current uses involve environmental applications, endocrine disruptors, and tag profiling; wastewater monitoring; and use in the clinical market to test and implement applications for immunosuppressants. Demonstrating the validity of this cost-effective technology would greatly benefit the field of forensic science because it has the potential for increased use in conjunction with existing MS platforms.

LDTD-MS and LDTD-MS/MS analyses have previously been used to support a variety of research goals, including cytochrome P450 inhibition assays, drug discovery, analysis of residues in dairy milk, analysis of pharmaceuticals and steroid hormones in wastewater, and analysis of drugs in plasma and dried blood spots (DBSs; Boisvert et al., 2012; Fayad et al., 2013; Mohapatra et al., 2012, 2013a, 2013b; Swales et al., 2012; VoDuy et al., 2012). The technique has proven robust for analyzing samples from a variety of matrices with minimal sample preparation and

**Previous Studies**

- LDTD-MS/MS validation results comparable to LC-MS/MS
- Reduction from 7 minutes to 10 seconds per sample for analysis of pharmaceutical compound
- Analysis time of metformin in 84 samples in 50 minutes compared with 3.5 hours using LC-MS/MS

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extraction necessary, including several more unusual matrices such as honey (Blachon et al., 2013), fish muscle (Lohne et al., 2012), and artificial bloom matrix (Lemoine et al., 2013). Heudi and colleagues (2011) developed a sensitive, high-throughput, ultra-fast method for quantitating a pharmaceutical compound (Compound 1) in human plasma using LDTD-MS/MS. They reported recovery, precision, accuracy, and calibration data that were comparable to that of a previously validated LC-MS/MS method but with a run time of 10 seconds per sample compared with the 7-minute LC-MS/MS method. Segura and colleagues (2010) reported results of analyzing sulfonamide residues in dairy milk that were comparable to previously published quantitation methods, but offered the advantage of analysis that was 10 to 150 times faster than similar methods such as LC-MS and LC-MS/MS. The simultaneous analysis of metformin and sitagliptin from mouse and human DBS samples using LDTD-MS/MS was reported (Swales et al., 2011). It was concluded that the use of LDTD, with a total run time of 18 seconds per sample, coupled with a QQQ mass spectrometer increased the efficiency compared with conventional LC-MS methods. The development of a method to analyze metformin in mouse, rat, dog, and human plasma by LDTD-MS/MS resulted in a reduction of analysis time from 3.5 hours (LC-MS/MS) to 50 minutes for 84 samples (Swales et al., 2010). The increased speed of LDTD-MS compared to LC-MS is especially apparent in high-throughput applications, similar to what would be expected in a forensic laboratory. In an in vitro drug discovery assay, where samples are routinely analyzed using 96 well plates, the total analysis time per plate was reduced from 2.5 hours to 45 minutes, with comparable data quality, when researchers switched to an LDTD-based method (Beattie et al., 2012).

Initial studies with LDTD indicated potential shortcomings of the technique, including high quantitative variability and limitations on the range of chemistries that could successfully be ionized in a single assay (Heudi et al., 2011; Swales et al., 2010; Wu et al., 2007). Beattie and colleagues (2012) showed that these could be overcome with careful system optimization. One cause identified for quantitative variability is interaction between the analyte and the metal surface of the LazWell plates. Strategies to minimize variability from this cause include pre-coating the surface of the plate with a solution containing a non-interfering analyte, or adding EDTA (a strong chelating agent) to the well or the sample to reduce interactions without adverse effects on signal for analytes (Lohne et al., 2012).

Without a chromatographic component, LDTD analyses are susceptible to interference problems. Interferences may be components in biological matrices, isomers of the analytes of interest, or other isobaric components in the sample. During the previous AccuTOF™-DART™ evaluation, RTI encountered interferences from isomers, which could not be distinguished by exact mass. The interferences, possibly unresolved compounds and other matrix components interfering with ionization (e.g., creatinine), affect mass determination (Ropero-Miller and Stout, 2008). During the analysis, several potential isomer or interference pairs were identified. These pairs included codeine/hydrocodone, benzoylecgonine/norcocaine, cocaine/scopolamine, and methamphetamine/triethanolamine. For analysis of these compounds, LDTD-MS/MS is preferred over LDTD-MS because the additional fragmentation data can often distinguish between isomers. There are instances, however, where an isobaric interferant also has a shared single reaction monitoring SRM transition. Lemoine and colleagues (2013) encountered such an isobaric interferant while developing a quantitative assay for anatoxin-a. Phenylalanine present in their samples had the same mass as anatoxin-a and dissociated into fragments with the same masses. With careful tuning of instrumental parameters, the researchers were able to favor the ionization and fragmentation of one analyte over the other, enough that a validated quantitative

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method was obtained for anatoxin-a without the need for additional sample preparation steps to remove the phenylalanine interferant.

## 1.2　Research Rationale

Forensic laboratories are often backlogged, creating budgetary and policy problems. Backlogs can potentially compromise investigations. Technologies that reduce screening time requirements, while increasing the range of analytes detected, will provide high-quality criminal investigations at reduced costs. The efficiency with which forensic laboratories produce results depends on the evaluation and implementation of new technologies. The purpose of this study was to evaluate the applicability of LDTD source in controlled substances and drug toxicology. Specifically, we evaluated the instrument's performance as a high-throughput source coupled with QQQ MS platform to detect controlled substances, including new emerging designer drugs, drugs used in drug-facilitated crimes, and those relevant to postmortem toxicology. Our goal was to investigate the efficiency and cost effectiveness of this new technology and the feasibility of implementing it in forensic laboratories. The successful implementation of the LDTD source in other scientific areas, such as drug discovery and cytochrome P450 inhibition studies, warrants the investigation of this technology in the area of forensic science. Thus, it is important to verify the potential for the instrument under practical and forensically relevant conditions encountered in a forensic laboratory

This research was designed to initially optimize MS and LDTD parameters for drugs of abuse prepared in methanol from commercial standards to provide the basis for the evaluation of these drugs in biological matrices, specifically blood and urine. The investigation of urine guided the sample preparatory techniques for blood, each time employing minimal sample preparation required to detect drugs in the specific matrix. In addition to the evaluation of drug-fortified samples, previously confirmed postmortem case samples were tested (obtained from the North Carolina Office of the Chief Medical Examiner [Raleigh, NC] and The Los Angeles Office of Coroner [Los Angeles, CA]).

The final step was to analyze street-prepared drugs in various formulations, such as neat plant material typical of Spice. We considered factors such as the extent of sample preparation and level of extraction necessary for acceptable identification.

## 2. RESEARCH DESIGN AND METHODS

### 2.1 Overview

The original proposed research design was divided into two main phases (A and B): one for evaluating LDTD with a QQQ mass spectrometer and one for evaluating LDTD with a QTOF mass spectrometer. However, at the request of the NIJ, the project was limited to the evaluation of the LDTD source with one MS platform, the AB Sciex API 4000 QQQ (GAN 1 ID#348271). This platform was chosen because it is more frequently encountered in forensic laboratories than a QTOF, which is not feasible for quantitative analysis.

> **Research Design**
>
> - Stage I: Instrument optimization
>   - Equipment set up
>   - Optimization of MS and LDTD
> - Stage II: Analysis of Drugs Analytes in Specimen Matrices
>   - Evaluating LDTD performance and sample preparation
>   - Sample extraction methods
>   - Validation study design

The project was divided into two stages:

- Stage I: Instrument Optimization
- Stage II: Analysis of Drugs Analytes in Specimen Matrices

Throughout the project, we noted how the instrument performed and discussed our observations of its performance, such as ease of use and ability to accurately quantitate or screen for a large amount of drugs in a shorter time period than that typical of LC or GC-MS/MS.

### 2.2 Stage I: Instrument Optimization and Validation

#### 2.2.1 LDTD Set Up and Training

The installation of the T-960 LDTD-APCI ionization interface model controlled by LazSoft 4.0 Software (Phytronix Technologies, Quebec, Canada) was installed. The installation and on-site analyst training occurred over 5 days (December 3–7, 2012). Installation was complete and the instrument was fully operational on December 4. Training occurred for the remainder of the week and involved LDTD operation and maintenance, software operation, compound optimization, and method development. Analytes were subsequently detected using an ABSciex (Framingham, MA) API 4000 QQQ mass spectrometer controlled by Analyst Software (Version 1.4.2) (Foster City, CA).

#### 2.2.2 LDTD and LazWell Sample Plates

**Figure 2** shows the LDTD source installed on the API 4000 at RTI. The housing unit (in blue) can hold up to 10 sample plates and consists of an X-Y moveable stage that aligns the back of the designated well in front of the infra-red laser diode. Samples were spotted into wells on a LazWell (96 sample plate). **Figure 3** shows the front (A) of a LazWell plate. The stainless steel alloy sheet can be seen on the back (B) of the plate in Figure 3. Each well has a hexagonal shape, which allows for concentration of the crystallized analytes within the sample in the heating zone. Once the plate was loaded, the barcode was scanned and loaded into the software.
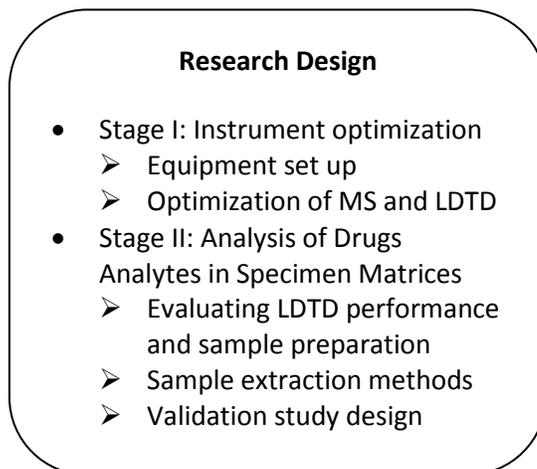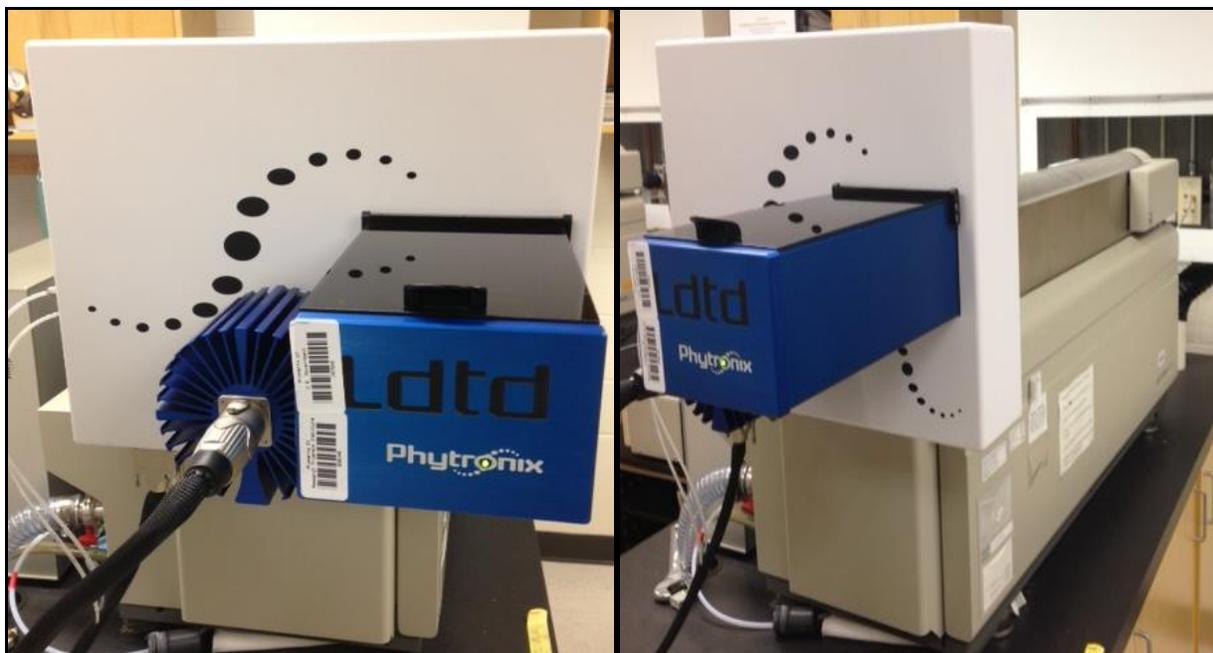
**Figure 2. The LDTD-QQQ system installed at RTI International.**
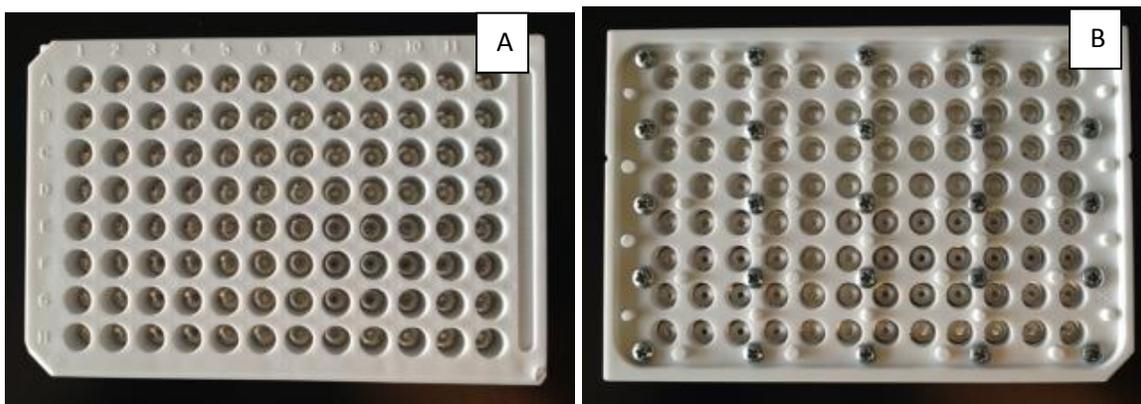


**Figure 3. The front and back of the LazWell 96 sample plate. The stainless steel alloy can be seen in the wells (A) and on the back (B) of the plate.**

Laser power is an essential parameter that controls the power of the laser diode radiation applied to the back of the metal well over a short period of time (< 10 sec). The heating rate of the laser (3000 $^{o}$C/sec) allows for rapid heating of the samples to high temperatures, which provides the energy for vaporization of intact molecules while minimizing fragmentation (Fayad et al., 2010). The amount of heat transferred to the sample can be intensified by increasing the percentage of laser power (i.e., increasing the laser radiation power hitting the back of the well), which ultimately increases the amount of energy transferred to the sample. The laser pattern should be set high enough to favor total desorption of the crystallized compound but not so high that thermal degradation of components in the matrix occurs, generating an increase in background signal.

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### 2.2.3  Optimizing of the MS for the Detection of Drug Standards

Pure drug standards were used for optimization. One hundred eleven compounds consisting of forensically common drug analytes and deuterated internal standards (ISTD) were evaluated in order to determine optimal MS and LDTD conditions. The analytes optimized included antidepressants, amphetamines, barbiturates, benzodiazepines, designer drugs, opiates, stimulants, hallucinogens, and Z drugs (**Table 1**). Each pure methanolic stock solution was diluted for a final concentration of 10,000 ng/mL in methanol, and 4 μL were spotted into the sample wells with a manual pipette. The precursor ion $[M+H]^+$ of all analytes and ISTDs were optimized for product ions. The two most intense product ions were further optimized for declustering potential (DP) and collision energy (CE) to achieve optimal signal strength of the analyte transition.

During optimization, the MS parameters were set to the following: auxiliary gas 0, and sheath gas 0. The LDTD-ACPI parameters (**Figure 4**) for power and timing were as follows: 0% hold for 1 sec, 0% to 45% in 3 sec, hold at 45% for 2 sec, 45% to 0% in 0.1 sec, and hold at 0% for 2 sec. The flow rate was set to 3 L/min.

### 2.2.4  Evaluation of Analyst's Spotting Technique

Prior to data collection, analysts will evaluate their reproducibility of sample deposition (spotting).

## 2.3     Stage II: Analysis of Drug Analytes in Specimen Matrices

### 2.3.1  MS and LDTD Analytical Methods Set Up

After optimization, 49 compounds across major drug classes were spiked into drug-free human urine and blood for validation of screening and/or quantitative analysis (**Table 2).** Many designer drug compounds do not have matched deuterated ISTDs available. Internal standards for these compounds were chosen based off similar structure, class, and/or common functional groups. For example, MAMP-$d_9$ was chosen for synthetic cathinones due to the similarity in structure, and amitryptline-$d_3$ was used for tryptamines and indanes due to amine functional group.

The API 4000 is an older model QQQ and only allows for a minimum dwell time of 10 msec for a total scan time of 100–200 msec given a maximum transition range of 10–20. In order to achieve enough data points across a desorption peak (20–30) and for reliable reproducibility, we used no more than 10 transitions per method. As a result, analytes were pooled into groups of no more than 10 transitions, with the exception of the antidepressants and synthetic cannabinoids.

**Table 3** shows the grouping of all drugs analyzed for quantitation and screening analysis. For quantitation methods, two product ion transitions were monitored for the analyte, and one ISTD product ion was used for quantitative results. However, for screening analysis, only one product ion transition was monitored for the analyte. Current forensic toxicology guidelines recommend that a second product ion is not required for screening analysis.

**Table 1. List of analytes and ISTD optimized for MS conditions.**

| Drug Class | Drug Analytes | Drug Class | Drug Analytes |
|---|---|---|---|
| Antidepressants | Amitriptyline<br>Amitriptyline-$d_3$<br>Citalopram<br>Citalopram-$d_6$<br>Fluoxetine<br>Nortriptyline<br>Nortriptyline-$d_3$<br>Trazodone<br>Trazodone-$d_6$ | Amphetamines | Amphetamine (AMP)<br>AMP-$d_6$<br>Methamphetamine (MAMP)<br>MAMP-$d_9$<br>(±)-3,4-Methylenedioxyamphetamine (MDA)<br>MDA-$d_5$<br>(±)-3,4-Methylenedioxyethylamphetamine (MDEA)<br>MDEA-$d_5$<br>(±)-3,4-Methylenedioxymethamphetamine (MDMA)<br>MDMA-$d_5$<br>Ephedrine<br>Ephedrine-$d_3$<br>Pseudoephedrine<br>Pseudoephedrine-$d_3$<br>Phenylpropanolamine |
| Barbiturates | Amobarbital<br>Butabarbital<br>Butalbital<br>Butalbital-$d_5$<br>Phenobarbital<br>Phenobarbital-$d_5$<br>Pentobarbital<br>Pentobarbital-$d_5$ | Benzodiazepines | 7-aminoclonazepam<br>7-aminoclonazepam-$d_4$<br>α-hydroxyalprazolam<br>α-hydroxyalprazolam-$d_5$<br>Alprazolam<br>Alprazolam-$d_5$<br>Clonazepam<br>Clonazepam-$d_4$<br>Diazepam<br>Diazepam-$d_5$<br>Flurazepam<br>Flunitrazepam-$d_7$<br>Lorazepam<br>Lorazepam-$d_4$<br>Nordiazepam<br>Nordiazepam-$d_5$<br>Oxazepam<br>Oxazepam-$d_5$<br>Temazepam<br>Temazepam-$d_5$ |

(continued)

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**Table 1. List of analytes and ISTD optimized for MS conditions (continued).**

| Drug Class | Drug Analytes | Drug Class | Drug Analytes |
|---|---|---|---|
| Designer Drugs<br><br>Indanes | 2-Aminoindane (2-AI)<br>5-(2-Aminopropyl) indole (5-IT)<br>5,6-Methylenedioxy-2-aminoindane (MDAI) | Designer Drugs<br><br>Synthetic Cannabinoids | AKB-48<br>AM-2233<br>EAM-2201<br>JWH-073<br>JWH-018<br>JWH-018 2-hydroxyindole<br>JWH-018 2-hydroxyindole-$d_9$<br>JWH-018 5-chloropentyl<br>JWH-018 N-(5-hydroxypentyl)<br>JWH-073 5-hydroxyindole<br>JWH-073 N-(4-hydroxybutyl)<br>JWH-122 4-pentenyl<br>JWH-122 5-hydroxypentyl<br>JWH-200 4-hydroxyindole |
| Designer Drugs<br><br>Synthetic Cathinones and Piperazines | 4-Fluoromethcathinone (Flephedrone)<br>4'-methyl-4-pyrrolidinopropiophenone (4-MePPP)<br>4-methylamino-butyrophenone (Buphedrone)<br>3-Trifluoromethylphenylpiperazine (TFMPP)<br>meta-Chlorophenylpiperazine (mCPP)<br>Benzylpiperazine (BZP)<br>α-Pyrrolidinopentiophenone (alpha-PVP)<br>3,4-Methylenedioxypyrovalerone(MDPV)<br>4-Methylethcathinone (4-MEC)<br>3,4-methylenedioxy-N-methylcathinone (Methylone) | Designer Drugs<br><br>Tryptamines | 5-methoxy-N,N-dimethyltryptamine (5-MeO-DMT)<br>5-methoxy-alpha-methyltryptamine (5-MeO-AMT)<br>5-methoxy-N-methyl-N-isopropyltryptamine (5-MeO-MiPT)<br>5-methoxy-diisopropyltryptamine (5-MEO-DIPT)<br>4-hydroxy-diisopropyltryptamine (4-OH-DiPT) |

(continued)

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**Table 1. List of analytes and ISTD optimized for MS conditions (continued).**

| Drug Class | Drug Analytes | Drug Class | Drug Analytes |
|---|---|---|---|
| Miscellaneous | Cocaine (COC)<br>COC-d$_3$<br>Benzoylecgonine (BZE)<br>BZE-d$_3$<br>Scopolamine<br>Norcocaine<br>Phencyclidine (PCP)<br>PCP-d$_5$<br>THC<br>THC-d$_3$<br>THCA<br>THCA-d$_3$<br>11-hydroxy-Δ9THC<br>GHB<br>GHB-d$_6$ | Opiates | 6-acetylmorphine (6-AM)<br>6-AM-d$_3$<br>Morphine (MOR)<br>MOR-d$_3$<br>Codeine (COD)<br>COD-d$_3$<br>Hydrocodone (HCOD)<br>Hydromorphone (HMOR) |
| Z Drugs | Zolpidem<br>Zolpidem-d$_6$<br>Zopiclone<br>Zaleplon | | |
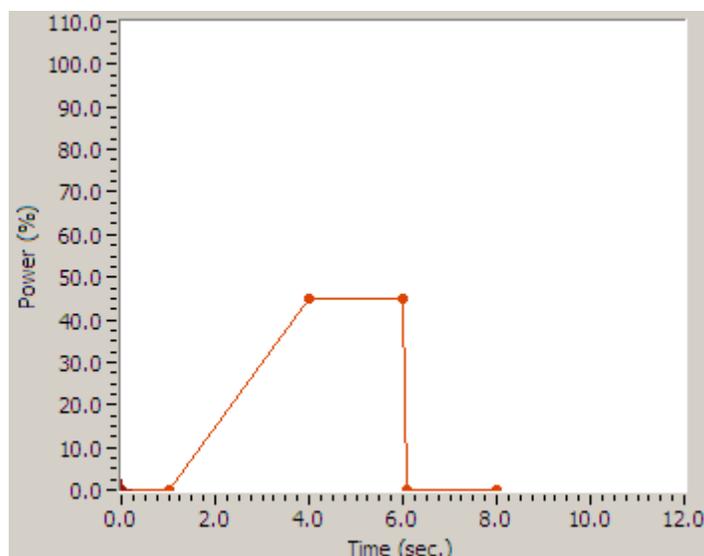
**Figure 4. Example of laser pattern used for analyte optimization**

**Table 2. List of analytes and ISTD analytes for quantitative and/or screening analysis.**

| Drug Class | Drug Analyte | Drug Class | Drug Analyte |
|---|---|---|---|
| Antidepressants | Amitriptyline<br>Citalopram<br>Nortriptyline<br>Trazodone | Amphetamines | AMP<br>MAMP<br>MDA<br>MDEA<br>MDMA |
| Benzodiazepines | Alprazolam<br>Diazepam<br>Clonazepam<br>α-hydroxyalprazolam<br>7-aminoclonazepam | Designer Drugs*<br><br>Indanes | 2-AI<br>5-IT<br>MDAI |
| Designer Drugs*<br><br>Synthetic<br>Cannabinoids | AKB-48<br>EAM-2201<br>JWH-018<br>JWH-073<br>JWH-018 N-(5-hydroxypentyl)<br>JWH-073 N-(4-hydroxybutyl)<br>JWH-122 5-hydroxypentyl<br>JWH-200 4-hydroxyindole<br>MAM-2201<br>STS-135<br>UR-144<br>UR-144 5-bromopentyl<br>UR-144 5-chloropentyl<br>XLR11 4-pentenyl<br>XLR-11 | Designer Drugs*<br><br>Synthetic<br>Cathinones | 4-MEC<br>4-MePPP<br>α-PVP<br>Buphedrone<br>Flephedrone<br>mCPP<br>MDPV<br>Methylone<br>TFMPP |
| Designer Drugs*<br><br>Trytamines | 5-MeO-AMT<br>5-MEO-DIPT<br>5-MeO-DMT<br>5-MeO-MiPT | Miscellaneous | BZE<br>COC<br>PCP |

* Validated for screening analysis

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**Table 3. Grouping of drug analytes in quantitative and screening methods.**

| Group | Drug Analytes/ISTD | Total Number of Monitored Ion Transitions |
|---|---|---|
| 1 | Amitriptyline/Amitriptyline-d3<br>Citalopram/Citalpram-d6<br>Nortriptyline/Nortiptyline-d3<br>Trazodone/Trazodone-d6 | 12 |
| 2 | AMP/AMP-d6<br>MAMP/MAMP-d9 | 8 |
| 3 | MDA/MDA-d5<br>MDEA/MDEA-d5<br>MDMA/MDMA-d5 | 9 |
| 4 | Alprazolam/Alprazolam-$d_5$<br>Clonazepam/Clonazepam-$d_4$<br>Diazepam/ Diazepam-$d_5$ | 10 |
| 5 | α-hydroxyalprazolam/α-hydroxyalprazolam-$d_5$<br>7-aminoclonazepam/7-aminoclonazepam-$d_4$ | 6 |
| 6 | 4-MEC<br>4-MePPP<br>α-PVP<br>Buphedrone<br>Flephedrone<br>MAMP-$d_9$<br>mCPP<br>MDPV<br>Methylone<br>TFMPP | 10 |
| 7 | 2-AI<br>5-IT<br>5-MeO-AMT<br>5-MEO-DiPT<br>5-MeO-DMT<br>5-MeO-MiPT<br>Amitriptyline-$d_3$<br>MDAI | 8 |
| 8 | AKB48<br>EAM2201<br>JWH-018<br>JWH-073<br>MAM2201<br>MAM2201-$d_5$<br>STS-135<br>UR-144<br>UR-144 5-bromopentyl<br>UR-144 5-chloropentyl<br>XLR11 4-pentenyl<br>XLR11 | 12 |

(continued)

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| Group | Drug Analytes/ISTD | Total Number of Monitored Ion Transitions |
|---|---|---|
| 9 | JWH-018 N-(5-hydroxypentyl)<br>JWH-073 N-(4-hydroxybutyl)<br>JWH-122 5-hydroxypentyl<br>JWH-200 4-hydroxyindole<br>MAM2201-$d_5$ | 10 |
| 10 | BZE/ BZE-$d_3$<br>COC/ COC-$d_3$ | 8 |
| 11 | PCP/ PCP-$d_5$ | 4 |

## 2.3.2 Evaluating LDTD Performance and Sample Preparation of Compounds from Various Drug Classes

Originally, we proposed evaluating the ability of the LDTD source to efficiently ionize target compounds in neat urine (i.e., no extraction or other sample preparation treatment). This approach is generally not compatible with APCI-MS due to overloading the source with matrix molecules. LDTD had the potential to overcome that limitation by preferentially desorbing the target analytes; however, during training, we were informed that sample extraction techniques must be performed due to interferences present in the biological matrices that will interfere with the analytes of interest due to the lack of chromatography. Therefore, we omitted analysis of drugs in neat urine and proceeded to sample preparation method development.

### 2.3.2.1 Sample Extraction Methods of Drug Analytes of Interest

#### Materials

All analytical-grade solvents and reagents used in this study were purchased from EMD Chemical (Billerica, MA); Aqua Solutions, Inc. (Deer Park, TX); Fisher Scientific (Fair Lawn, NJ); Strem Chemicals (Newburyport, NJ); and Sigma Aldrich (St. Louis, MO). Stock drug material was purchased from Cerilliant (Round Rock, TX); Cayman Chemical (Ann Arbor, MI); Grace (Albany, OR); LGC (Luckenwalde, Germany); Lipomed (Cambridge, MA); Toronto Research Chemicals (Toronto, Ontario, Canada); and THC Pharm (Frankfurt, Germany). LazWell plates and solid phase extraction (SPE) columns were purchased from Overbrook Scientific (Boston, MA) and SPEware Corporation (Baldwin Park, CA), respectively. Drug-free human urine was collected from volunteers. Drug-free human blood and bovine blood was obtained from the North Carolina Office of the Chief Medical Examiner (NCOCME; Raleigh, NC) and Bioreclamation (Westbury, NY). Postmortem blood samples were collected from the NCOCME and the Los Angeles Country Department of Coroner (Los Angeles, CA).

#### Extraction Methods

**Tables 4 and 5** summarize the LLE and SPE extractions used in this study. The extraction methods were employed for both urine and blood matrices, with any modifications noted in Table 5. After each addition, samples were vortexed and centrifuged at 4000 rpm for 5 minutes prior to spotting onto LazWell plate. Many of the drug analytes were extracted using similar methods with only the amount of sample and solvent volume varying.

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**Table 4. Summary of LLE of drug analytes from blood and urine.**

| Drug Analyte | Sample Volume (µL) | Acid/Base addition (µL) | Extraction Solvent and Volume (µL) | Post extraction sample preparation (µL) | Amount Spotted onto LazWell (µL) |
|---|---|---|---|---|---|
| Benzodiazepines | 200 | 250 1 N NaOH | 800 ethyl acetate | — | 2 |
| Amphetamines | 100 | 500 1 N NaOH | 500 n-butyl chloride | Remove 300 top layer and add 50 of 0.01 N HCl | 2 |
| Antidepressants | 100 | 400 1 N NaOH | 500 n-butyl chloride | — | 2 |
| Cathinones and Piperazines | 200 | 500 1 N NaOH | 500 n-butyl chloride | Remove 100 top layer and add 10 of 0.01 N HCl | 2 |
| Tryptamines and Indanes | 200 | 500 1 N NaOH | 500 n-butyl chloride | — | 2 |
| Synthetic Cannabinoids and Metabolites | 100 | — | 400 n-butyl chloride | — | 2 |

**Table 5. Summary of SPE of analytes from blood and urine.**

| Drug Analyte | Sample Volume (µL) | Buffer (µL) | Column Wash | Column Wash | Elution | Post extraction sample preparation (µL) | Amount Spotted onto LazWell (µL) |
|---|---|---|---|---|---|---|---|
| COC BZE | 200 | 600 Phosphate buffer (100 mM, pH 6) | 1 mL DI water, 1 mL 0.1 M HCl* | 1 mL MeOH, 1 mL ethyl acetate** | 1 mL CH2Cl2, IPA, NH4OH (80:18:2) | — | 4 |
| PCP | 200 | 600 Phosphate buffer (100 mM, pH 6) | 1 mL DI water, 1 mL 0.1 M HCl* | 1 mL MeOH, 1 mL ethyl acetate** | 1 mL CH2Cl2, IPA, NH4OH (80:18:2) | Remove 100 top layer and add 10 of 0.01 N HCl | 4 |

* Blood samples washed with 1 mL sodium carbonate/bicarbonate buffer (100mM pH9) after acid wash
**Blood samples washed with an additional 1 mL of ethyl acetate

## 2.3.2.2 Validation Study Design

Validation was modeled after approaches presented at a workshop during the July 2012 Society of Forensic Toxicologist Annual Meeting (Lebeau and Wagner, 2012). The majority of the validation tests performed in this study was in agreement with the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation (Scientific Working Group for Forensic Toxicology, 2013). Due to the release date of the guidelines, May 2013, some of the validation tests in this study vary slightly from those suggested by SWGTOX.

Forensic toxicology methods are typically categorized as screening methods, qualitative/identification methods, or quantitative methods. SWGTOX suggested validation tests to evaluate for quantitative and screening analyses. These validation study parameters are listed in **Table 6**. The use of LDTD for quantitative analysis would be extremely beneficial from an

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efficiency standpoint due to lack of chromatographic separation, which reduces analysis time compared to typical LC-MS and GC-MS. In some instances, screening method validation parameters were evaluated for certain drug classes such as designer drugs.

**Table 6. Validation parameters for quantitative and screening analysis evaluated by LDTD-MS/MS.**

| Screening | Quantitative Analysis |
|---|---|
| Carryover | Carryover |
| Interference | Interference |
| Limit of Detection (LOD) | LOD |
| Matrix Effect | Matrix Effect |
| | Limit of Quantitation (LOQ) |
| | Linearity, Precision and Accuracy (LPA) |
| | Calibration Curve (including ULOL) |
| | Stability |

### 2.3.2.3 Quantitative Analysis

#### *Calibration Curve*

The calibration curve was established by creating a curve spanning the range of biologically relevant concentrations expected in urine and blood. Six non-zero calibrators were used to establish the model, with the exception of alprazolam, clonazepam, and diazepam, whose curves consisted of five non-zero calibrators in blood. The calibration curve was considered acceptable if all calibrators were within 20% of the target concentration. A simple linear regression model (y=mx+b) was used, and a weighted least squares model (1/x) was applied to the calibration range. The exception was alprazolam, diazepam, and clonazepam analyzed in blood, for which a quadratic equation ($y=ax^2 +bx+c$) was applied. Once the appropriate calibration curve was established, it was extracted and analyzed five times (n=5 at each concentration level). For quantitative analysis, each analyte's respective stable isotope labeled compound was used as an internal standard.

#### *Upper Limit of Linearity*

The upper limit of linearity (ULOL) was established during this step in the validation. Calibrators, in concentrations above the established curve, were analyzed to determine at which concentration the curve no longer exhibited linearity. The ULOL was established by visually determining the concentration level at which the curve appeared linear.

#### *Linearity Precision and Accuracy*

Linear precision and accuracy (LPA) was determined by analyzing three quality control (QC) samples fortified with analyte concentrations at the lower, middle, and upper portion of the calibration curve. Each QC sample was analyzed in triplicate within each linearity run (n=9) over the course of five runs (total n=45).

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## *Limit of Detection (LOD)/Limit of Quantitation (LOQ)*

The limit of detection (LOD) was defined as the lowest concentration that produced a reproducible instrument response greater than or equal to three times that of the blank sample. The LOD was determined by fortifying three sources of blank urine or blood matrix in decreasing concentrations. In order to obtain three sources of blank blood, bovine blood was used for the LOD study. The extracted samples were analyzed in duplicate for 3 runs (n=18). The limit of quantitation (LOQ) was defined as the concentration of the lowest calibrator, which was determined during the establishment of the calibration curve.

## *Carryover*

Carryover was determined by analyzing blank sample matrix immediately after a high concentration sample (**Table 7**). This was carried out using triplicate analysis. All designer drugs (i.e., synthetic cannabinoids and metabolites, indanes, cathinones, piperazines, and tryptamines) were at a carryover concentration of 25,000 ng/mL for screening. A sample was considered to have carryover if the average of the peak area after the carryover sample was greater than the established 20% of the established LOQ peak area for quantitation. Validation for screening analysis does not require establishment of a LOQ; therefore, a sample was considered to have carryover if the average of the peak area after the carryover sample was greater than was greater than the LOD.

**Table 7. Summary of analyte concentrations in urine and blood in the carryover study for quantitative analysis.**

| Drug Class | Drug | Carryover Concentration (ng/ml) |
|---|---|---|
| **Amphetamines** | AMP<br>MAMP | 25,000 |
| | MDA<br>MDEA<br>MDMA | 25,000 |
| | Amitriptyline<br>Nortriptyline<br>Citalopram<br>Trazodone | 100,000 |
| **Antidepressants** | Alprazolam<br>Clonazepam<br>Diazepam | 25,000 |
| **Benzodiazepines** | 7-aminoclonazepam<br>α-hydroxyalprazolam | 25,000 (30,000 in blood)<br>200,000 |
| | COC<br>BZE | 25,000 |
| **Miscellaneous** | PCP | 25,000 |

## Interference

*Evaluation of blank sample matrix*: Five blank matrix samples, fortified with ISTD, were analyzed to demonstrate the absence of interferences originating from the ISTD. In addition, five matrix samples were analyzed without the addition of analyte or ISTD to demonstrate the absence of interference from matrix. In both cases, a sample was considered to have interference if the average peak area of the blank samples was greater than 20% of the established LOQ peak area quantitative analysis. For screening, a sample was considered to have interference if the average peak area of the blank samples was greater than the LOD by 20%.

*Evaluation of interfering compounds*: In order to investigate the potential of interference of isomeric and isobaric compounds with drugs of interest, potential interferences were fortified in five urine lots containing AMPS, COC/BZE, JWH metabolites, and other designer drugs (n=5) and analyzed (**Table 8**). For quantitative analysis of samples containing AMPS, COC, and BZE, samples were fortified with a high concentration of potential interfering compounds. Interference was determined if the calculated concentration was greater than 20% of the target concentration for quantitative analysis. For screening, interference was determined if the peak area of the sample fortified with potential interfering compounds was greater than that of the target sample peak area by 20%.

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**Table 8. Detail of drugs and sample concentrations (ng/mL) included in AMPS, COC/BZE and JWH metabolites interference study.**

| Analyte | Sample 1 | Sample 2 | Analyte | Sample 1 | Sample 2 | Analyte | Sample 1 | Sample 2 |
|---|---|---|---|---|---|---|---|---|
| AMP | 200 | -- | COC | 40 | -- | JWH-018 N-(5-hydroxypentyl) | 20 | -- |
| MAMP | 200 | -- | BZE | 40 | -- | JWH-073 N-(4-hydroxybutyl) | 20 | -- |
| MDA | 200 | -- | | | | JWH-122 5-hydroxypentyl | 20 | -- |
| MDMA | 200 | -- | | | | JWH-200 4-hydroxindole | 20 | -- |
| MDEA | 200 | -- | | | | | | |
| Phentermine | 50,000 | 50,000 | Norcocaine | 25,000 | 25,000 | JWH-073 5-hydroxyindole | 25,000 | 25,000 |
| Ephedrine | 50,000 | 50,000 | Scopolamine | 25,000 | 25,000 | JWH-018 2-hydroxyindole | 25,000 | 25,000 |
| Pseudoephedrine | 50,000 | 50,000 | | | | | | |
| PPA | 50,000 | 50,000 | | | | | | |

## *Matrix Effect*

Matrix effects (MEs) were evaluated using a modified version of the method described by Matuszewski and colleagues (2003). In order to obtain 10 different lots of blood, ME was carried out using bovine blood. Three sets of samples were created for each target analyte. Type A samples (neat) were made by preparing a concentration of target analyte and internal standard in water equivalent to sample types B and C prior to solid-phase or liquid-liquid extraction. Type B samples (post-extraction spike) were made by fortifying the eluent from the solid-phase extraction or organic layer of liquid-liquid extraction of negative urine or blood matrix with target analytes and internal standard. Type C samples (pre-extraction spike) were made by fortifying negative urine or blood matrix with target analytes and internal standard prior to solid-phase or liquid-liquid extraction. **Table 9** shows the target concentrations of each analyte used for the matrix effect study. Ten lots of urine or blood, each from a different donor, were collected, and samples were analyzed once.

As described by Matuszewski and colleagues, comparative calculations were used to evaluate the data:

ME (%) = B/A x 100
RE (%) = C/B x 100

where A, B, and C = the mean responses as represented by the area under the peaks for target and internal standard quantitative ions;
ME = the matrix effect; and
RE = the recovery efficiency.

The mean responses for A, B, and C were determined across these 10 urine or blood lots. The assessment of a relative matrix effect was determined by comparing the MEs between the 10 lots. The variability (%CV) in the MEs between lots is considered to be a measure of the relative matrix effect.

**Table 9. Summary of analyte and internal standard concentrations (ng/ml) used in the matrix effect.**

| Class | Drug | Analyte Concentration (ng/mL) | ISTD Concentration (ng/mL) |
|---|---|---|---|
| **Amphetamines** | AMP MAMP | **500** | **100** |
| | MDA MDEA MDMA | 500 | 100 |
| **Antidepressants** | Amitriptyline Nortriptyline Citalopram Trazodone | 200 | 100 |
| **Benzodiazepines** | Alprazolam Clonazepam Diazepam | 100 | 100 |
| | 7-aminoclonazepam α-hydroxyalprazolam | 100 | 100 |

(continued)

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**Table 9. Summary of analyte and internal standard concentrations (ng/ml) used in the matrix effect (continued).**

| Class | Drug | Analyte Concentration (ng/mL) | ISTD Concentration (ng/mL) |
|---|---|---|---|
| Miscellaneous | COC BZE | 100 | 100 |
| | PCP | 25 | 50 |
| Designer Drugs | Synthetic cathinones Tryptamines Indanes Synthetic cannabinoids | 100 | 100 |
| | Synthetic cannabinoid metabolites | 10 | 10 |

## *Stability*

In order to evaluate the stability of dry samples in the LazWell plate, three sets of samples from the LPA study were spotted onto the plates, kept at room temperature, and analyzed after 0, 6, and 15 hours.

### 2.3.3  Postmortem Sample Analysis

Previously analyzed archived case specimens were evaluated in order to determine if the LDTD has the required sensitivity to detect drugs of abuse in samples that have decomposed or contain numerous analytes of potential interferants. After sample was extracted in duplicate and spotted into the well cavities twice, four samples containing AMPs, three containing benzodiazepines, and three containing COC/BZE were evaluated and quantitated using the established calibration curve. Three specimens containing designer drugs were evaluated under a screening method.

### 2.3.4  Analysis of Spice Samples

The final step was analyzing for Spice plant material. Approximately 15 mg of Spice material was extracted with 1 mL of n-butyl chloride and 2 μL spotted onto the LazWell plate. These samples were previously analyzed to determine their components (Cox et al., 2012). A neat sample in water was extracted with JWH-018 and JWH-073 at 100 ng/mL and used to compare peak area of the Spice material to the standard.

# 3. RESULTS

## 3.1 Stage I: QQQ MS and LDTD Optimization

### 3.1.1 Optimization of QQQ MS

The precursor ion [M+H]+ for 111 analytes, including their ISTDs, was monitored in product ion scan mode. The two most prevalent product ions were chosen for each analyte, and the declustering potential (DP) and CE were adjusted for optimal signal strength. For example, **Figure 5** depicts the product ion scan desorption peak (top) for alprazolam, which has a [M+H]+ value of 309 m/z. The spectrum (bottom) shows 281 m/z and 274 m/z having the greatest intensity. The two product ions were then analyzed in MRM mode while independently varying the DP and CE values. The optimal values were determined by selecting the voltage that produces the greatest intensity. **Figure 6** shows a DP value of 50 V and produces the greatest intensity for product ion transition 309 m/z→281 m/z. The same DP was used for the 309 m/z→274 m/z transition. A CE value of 35 V provided the greatest intensity for both ions (**Figure 7**). A summary of product ions and optimized parameters of all 111 analytes are shown in **Table 10**. Product ion spectra for all analytes validated are presented in **Appendix A**.
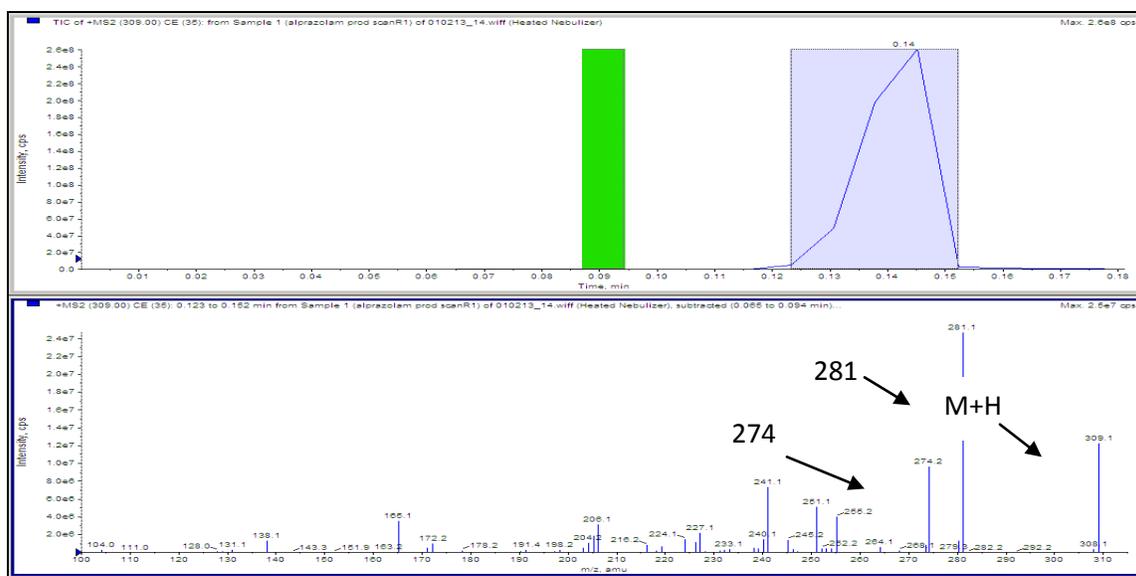


**Figure 5. Alprazolam product desorption peak (top) showing the precursor [M+H]+ ion and the most intense product ions as 281 m/z and 274 m/z (bottom).**
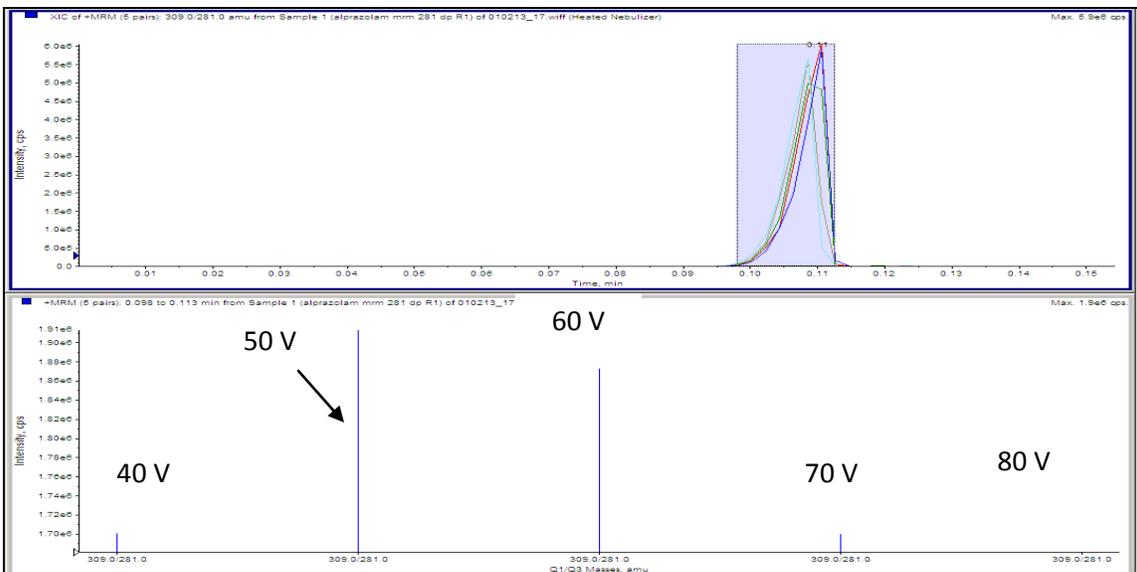
**Figure 6. Ion desorption peak (top) and MRM spectrum (bottom) showing the various intensities of ion transition 309 m/z →281 m/z at different DPs. A DP of 50 V resulted in the greatest intensity.**
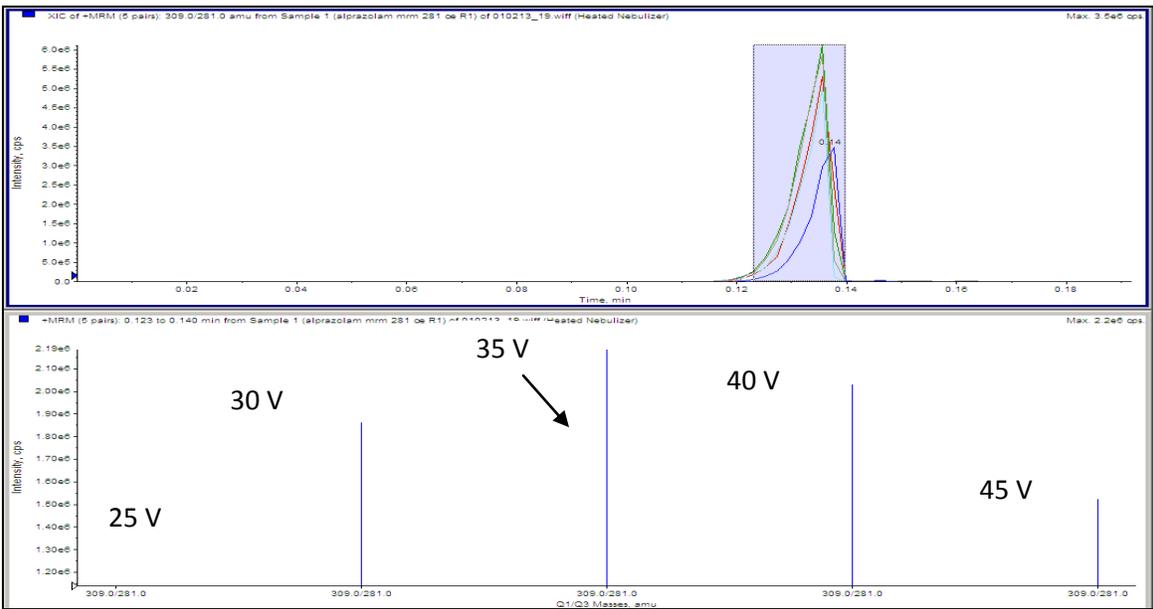


**Figure 7. Ion desorption peak (top) and MRM spectrum (bottom) showing the various intensities of ion transition 309→281 m/z. A CE of 35 V resulted in the greatest intensity.**

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**Table 10. Product ion, CE and DP values for all drug analytes optimized in this study.**

| Drug Class | Drug | Precursor Ion (m/z) | Product Ion 1 (m/z) | CE (V) | Product Ion 2 (m/z) | CE (V) | DP (V) |
|---|---|---|---|---|---|---|---|
| Amphetamines | AMP | 136 | 119 | 15 | 91* | 25 | 30 |
| | AMP-d$_6$ | 142 | 125 | 15 | 93** | 25 | 30 |
| | Ephedrine | 166 | 148 | 15 | 133 | 30 | 40 |
| | Ephedrine-d$_3$ | 169 | 151 | 20 | 136 | 30 | 40 |
| | MAMP | 150 | 119 | 15 | 91* | 25 | 40 |
| | MAMP-d$_9$ | 159 | 125** | 15 | 93 | 25 | 40 |
| | MDA | 180 | 163* | 15 | 133 | 25 | 40 |
| | MDA-d$_5$ | 185 | 168 | 15 | 138** | 25 | 40 |
| | MDEA | 208 | 163* | 20 | 135 | 30 | 40 |
| | MDEA-d$_5$ | 213 | 163** | 20 | 135 | 30 | 40 |
| | MDMA | 194 | 163* | 15 | 105 | 35 | 45 |
| | MDMA-d$_5$ | 199 | 165** | 15 | 107 | 35 | 45 |
| | Phenypropanolamine | 152 | 134 | 20 | 117 | 25 | 30 |
| | Pseudoephedrine | 166 | 148 | 15 | 133 | 30 | 40 |
| | Pseudoephedrine-d$_3$ | 169 | 151 | 10 | 136 | 30 | 40 |
| Antidepressants | Amitriptyline | 278 | 233* | 25 | 91 | 35 | 50 |
| | Amitriptyline-d$_3$ | 281 | 233 | 25 | 91** | 40 | 50 |
| | Citalopram | 325 | 262 | 25 | 109* | 35 | 50 |
| | Citalopram-d$_6$ | 331 | 262 | 30 | 109** | 35 | 50 |
| | Fluoxetine | 310 | 165 | 15 | 148 | 15 | 40 |
| | Nortriptyline | 264 | 233* | 20 | 91 | 30 | 50 |
| | Nortriptyline-d$_3$ | 267 | 233** | 20 | 91 | 30 | 50 |
| | Trazodone | 372 | 176* | 35 | 148 | 45 | 40 |
| | Trazodone-d$_6$ | 378 | 182** | 35 | 150 | 45 | 50 |

**Table 10. Product ion, CE and DP values for all drug analytes optimized in this study (continued).**

| Drug Class | Drug | Precursor Ion (m/z) | Product Ion 1 (m/z) | CE (V) | Product Ion 2 (m/z) | CE (V) | DP (V) |
|---|---|---|---|---|---|---|---|
| Barbiturates | Amobarbital | 227 | 157 | 20 | 97 | 35 | 50 |
| | Butabarbital | 213 | 157 | 20 | 97 | 35 | 50 |
| | Butalbital | 225 | 141 | 25 | 98 | 35 | 50 |
| | Butalbital-d$_5$ | 230 | 141 | 25 | 98 | 35 | 50 |
| | Pentobarbital | 227 | 157 | 20 | 97 | 35 | 40 |
| | Pentobarbital-d$_5$ | 232 | 162 | 25 | 102 | 35 | 50 |
| | Phenobarbital | 233 | 162 | 20 | 117 | 35 | 50 |
| | Phenobarbital-d$_5$ | 238 | 182 | 20 | 167 | 20 | 50 |
| Benzodiazepines | Alprazolam | 309 | 281* | 35 | 274 | 35 | 50 |
| | Alprazolam-d$_5$ | 314 | 286** | 35 | 279 | 35 | 50 |
| | α-hydroxyalprazolam | 325 | 297* | 35 | 279 | 35 | 70 |
| | α-hydroxyalprazolam-d$_5$ | 330 | 302** | 35 | 284 | 35 | 75 |
| | 7-aminoclonazepam | 286 | 222* | 35 | 121 | 43 | 60 |
| | 7-aminoclonazepam-d$_4$ | 290 | 226 | 35 | 121** | 43 | 50 |
| | Clonazepam | 316 | 270* | 35 | 241 | 45 | 50 |
| | Clonazepam-d$_4$ | 320 | 274** | 35 | 245 | 45 | 60 |
| | Diazepam | 285 | 222 | 30 | 154* | 40 | 70 |
| | Diazepam-d$_5$ | 290 | 262 | 30 | 154** | 40 | 60 |
| | Flurazepam | 388 | 315 | 30 | 288 | 35 | 60 |
| | Flunitrazepam-d$_7$ | 320 | 274 | 35 | 247 | 35 | 60 |
| | Lorazepam | 321 | 303 | 20 | 275 | 33 | 55 |
| | Lorazepam-d$_4$ | 325 | 307 | 23 | 279 | 33 | 50 |
| | Nordiazepam | 271 | 243 | 30 | 208 | 38 | 50 |
| | Nordiazepam-d$_5$ | 276 | 248 | 30 | 213 | 40 | 50 |
| | Oxazepam | 287 | 269 | 20 | 241 | 30 | 60 |
| | Oxazepam-d$_5$ | 292 | 274 | 23 | 246 | 33 | 60 |
| | Temazepam | 301 | 273 | 25 | 255 | 30 | 50 |
| | Temazepam-d$_5$ | 306 | 278 | 25 | 260 | 35 | 50 |

(continued)

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**Table 10. Product ion, CE and DP values for all drug analytes optimized in this study (continued).**

| Drug Class | Drug | Precursor Ion (m/z) | Product Ion 1 (m/z) | CE (V) | Product Ion 2 (m/z) | CE (V) | DP (V) |
|---|---|---|---|---|---|---|---|
| **Designer Drugs Indanes** | 2-AI | 134 | 117* | 20 | 115 | 30 | 30 |
| | 5-IT | 175 | 158* | 15 | 130 | 25 | 30 |
| | MDAI | 178 | 131 | 25 | 103* | 35 | 30 |
| **Designer Drugs Synthetic Cannabinoids** | AKB48 | 366 | 215 | 35 | 135* | 30 | 60 |
| | AM-2233 | 459 | 231 | 40 | 98 | 45 | 40 |
| | EAM2201 | 389 | 232 | 35 | 183* | 35 | 60 |
| | JWH-018 | 342 | 214 | 30 | 155* | 35 | 60 |
| | JWH-018 5-chloropentyl | 376 | 248 | 35 | 155 | 35 | 60 |
| | JWH-073 | 328 | 200 | 35 | 155* | 35 | 70 |
| | JWH-122 4-pentenyl | 354 | 212 | 33 | 169 | 35 | 70 |
| | MAM 2201 | 374 | 232 | 35 | 169* | 35 | 70 |
| | MAM 2201-d$_5$ | 379 | 237 | 35 | 169 | 35 | 60 |
| | STS-135 | 383 | 232 | 35 | 135* | 45 | 70 |
| | UR-144 | 312 | 214 | 30 | 125* | 30 | 70 |
| | UR-144 d$_5$ | 317 | 219 | 30 | 125 | 30 | 60 |
| | UR-144 5-bromopentyl | 391 | 294 | 20 | 125* | 35 | 70 |
| | UR-144 5-chloropentyl | 346 | 248 | 35 | 125* | 35 | 50 |
| | URB-602 | 296 | 214 | 15 | 170 | 30 | 30 |
| | XLR11 4-pentenyl | 310 | 212 | 30 | 125* | 30 | 50 |
| | XLR11 | 330 | 232 | 30 | 125 | 30 | 70 |

(continued)

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**Table 10. Product ion, CE and DP values for all drug analytes optimized in this study (continued).**

| Drug Class | Drug | Precursor Ion (m/z) | Product Ion 1 (m/z) | CE (V) | Product Ion 2 (m/z) | CE (V) | DP (V) |
|---|---|---|---|---|---|---|---|
| **Designer Drugs Synthetic Cannabinoid Metabolites** | JWH-018 2-hydroxyindole | 358 | 340 | 18 | 270 | 35 | 60 |
| | JWH-018 2-hydroxyindole-d$_9$ | 367 | 127 | 35 | 155 | 35 | 70 |
| | JWH-018 N-(5-hydroxypentyl) | 358 | 230 | 35 | 155* | 30 | 50 |
| | JWH-073 5-hydroxyindole | 344 | 216 | 33 | 155 | 38 | 70 |
| | JWH-073 N-(4-hydroxybutyl) | 344 | 155* | 35 | 127 | 65 | 80 |
| | JWH-122 5-hydroxypentyl | 372 | 230 | 35 | 169* | 30 | 60 |
| | JWH-200 4-hydroxyindole | 401 | 155* | 35 | 114 | 40 | 50 |
| | RCS-4 N-(5-carboxypentyl) | 352 | 135 | 33 | 71 | 30 | 50 |
| **Designer Drugs Synthetic Cathinones and Piperazines** | 4-MEC | 192 | 174* | 20 | 146 | 20 | 50 |
| | 4-MePPP | 218 | 119 | 30 | 147* | 25 | 60 |
| | α-PVP | 232 | 126 | 35 | 91* | 35 | 70 |
| | Buphedrone | 178 | 160 | 15 | 131* | 30 | 40 |
| | BZP | 177 | 91 | 25 | 85 | 25 | 40 |
| | Flephedrone | 182 | 164* | 20 | 149 | 30 | 40 |
| | mCPP | 197 | 154* | 25 | 119 | 30 | 40 |
| | MDPV | 276 | 175 | 35 | 126* | 35 | 70 |
| | Methylone | 208 | 160* | 25 | 132 | 25 | 50 |
| | TFMPP | 218 | 188* | 30 | 119 | 40 | 40 |

(continued)

**Table 10. Product ion, CE and DP values for all drug analytes optimized in this study (continued).**

| Drug Class | Drug | Precursor Ion (m/z) | Product Ion 1 (m/z) | CE (V) | Product Ion 2 (m/z) | CE (V) | DP (V) |
|---|---|---|---|---|---|---|---|
| **Designer Drugs Trytamines** | 4-OH-DiPT | 261 | 160 | 30 | 114 | 25 | 40 |
| | 5-MeO-AMT | 205 | 188* | 15 | 147 | 30 | 30 |
| | 5-MEO-DiPT | 275 | 174 | 20 | 114* | 25 | 40 |
| | 5-MeO-DMT | 219 | 174 | 20 | 58* | 35 | 30 |
| | 5-MeO-MiPT | 247 | 174 | 25 | 86* | 25 | 40 |
| **Miscellaneous** | 11-OH-delta 9-THC | 331 | 313 | 20 | 271 | 20 | 60 |
| | BZE | 290 | 168* | 27 | 105 | 40 | 40 |
| | BZE-d$_3$ | 293 | 171** | 27 | 105 | 40 | 40 |
| | COC | 304 | 182* | 27 | 82 | 45 | 40 |
| | COC-d$_3$ | 307 | 185** | 27 | 85 | 45 | 40 |
| | GHB | 105 | 87* | 10 | 69 | 10 | 20 |
| | GHB-d$_6$ | 111 | 93* | 10 | 69 | 10 | 20 |
| | Norcocaine | 290 | 168 | 35 | 136 | 35 | 30 |
| | PCP | 244 | 159* | 20 | 91 | 40 | 40 |
| | PCP-d$_5$ | 249 | 164** | 20 | 96 | 40 | 40 |
| | Scopolamine | 304 | 156 | 25 | 138 | 40 | 50 |
| | THC | 315 | 259 | 30 | 193 | 30 | 70 |
| | THC-d$_3$ | 318 | 262 | 30 | 196 | 35 | 70 |
| | THCA | 345 | 299 | 20 | 327 | 25 | 60 |
| | THCA-d$_3$ | 348 | 302 | 20 | 330 | 25 | 50 |
| **Opiates** | 6-AM | 328 | 268 | 30 | 211 | 35 | 80 |
| | 6-AM-d$_3$ | 331 | 271 | 30 | 211 | 35 | 80 |
| | COD | 300 | 215 | 35 | 183 | 40 | 90 |
| | COD-d$_3$ | 303 | 215 | 35 | 183 | 40 | 90 |
| | MOR | 286 | 201 | 35 | 165 | 50 | 70 |
| | MOR-d$_3$ | 289 | 201 | 35 | 165 | 50 | 70 |

**Table 10. Product ion, CE and DP values for all drug analytes optimized in this study (continued).**

| Drug Class | Drug | Precursor Ion (m/z) | Product Ion 1 (m/z) | CE (V) | Product Ion 2 (m/z) | CE (V) | DP (V) |
|---|---|---|---|---|---|---|---|
| Z Drugs | Zaleplon | 306 | 264 | 30 | 237 | 35 | 50 |
| | Zopiclone | 389 | 245 | 20 | 217 | 45 | 40 |
| | Zolpidem | 308 | 263 | 35 | 235 | 48 | 50 |
| | Zolpidem-$d_6$ | 314 | 263 | 35 | 235 | 48 | 40 |

\* Ion used for quantitation and ion used in screening method for detection.
\*\* ISTD product ion for quantitative methods.

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### 3.1.2 Stage II: Analysis of Drug Analytes in Specimen Matrices

### 3.1.2.1 Laser Pattern Evaluation

In all cases, with the exception of PCP, synthetic cannabinoids, and metabolites, the laser power was decreased from the original methanolic standards optimized at 3-45-2 (**Table 11**) for optimal desorption in blood and urine matrices. The benzodiazepines were the only analytes that required a laser power greater than 45%.

**Table 11. Summary of LDTD laser patterns of drug analytes that produced optimal desorption in blood and urine.**

| Analyte | Laser Power |
|---|---|
| AMP/MAMP | 3-25-0 5 second delay |
| MDA/MDMA/MDEA | 3-25-0 |
| Benzodiazepines | 3-55-0 |
| PCP | 3-45-0 |
| Antidepressants | 3-25-0 |
| COC/BZE | 3-25-0 |
| Synthetic Cathinones | 3-25-0 5 second delay |
| Tryptamines and Indanes | 3-35-0 |
| Synthetic Cannabinoid and Metabolites | 3-45-2 |

An example of the importance of laser pattern optimization was observed early in the method development process. **Figure 8, A1−A2,** shows the ion transition desorption peaks of BZE spiked in urine at (A) 10 ng/mL and (B) 500 ng/mL spotted at 4 µL and analyzed with a laser pattern of 3-45-2. The peaks were of poor quality, being broad and containing split peaks, as can be seen in **Figure 8, A1**. Similar instances were observed in other analytes. Based on consultation with the LDTD manufacturer, it was determined that these undesirable peak characteristics may have occurred as a result of thermal desorption of the matrix in addition to the analyte of interest. Optimizing the laser pattern to minimize matrix desorption greatly improved the peak shapes. It is important to optimize the laser pattern so that any interference not removed during the sample preparation process will not affect peak desorption of the target analyte, especially at lower analyte concentration. Plastics and other contaminants may also be a contributing factor. In order to improve peak shape, the laser power was evaluated at 35% and 25% with and without immediately setting the laser power to 0% after the target power was reached (**Figure 9**). A laser pattern of 3-25-0 greatly improved the peak shape at both concentrations (**Figure 8, F1−F2**). This was typical of most drugs analyzed in this study. Subsequent input from other LDTD users indicated that similar improvements may have been achieved by optimizing the dilution factor at which extracts were loaded onto the plate; however, those approaches were not pursued as part of this project. In some cases, such as synthetic cathinones and AMP/MAMP, the laser patterns contained a 5-second delay at the beginning of analysis. This was used to allow the background signal to decrease prior to desorption of each sample.

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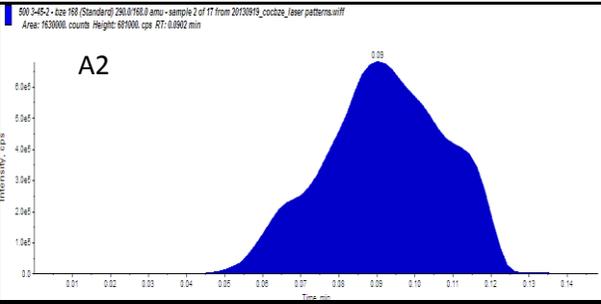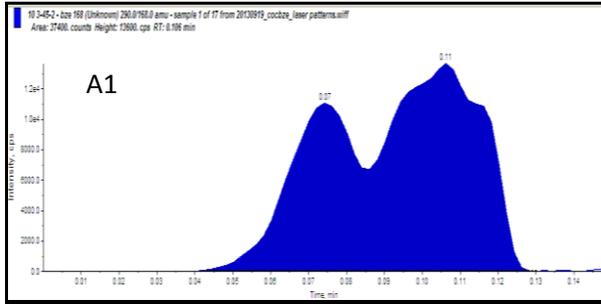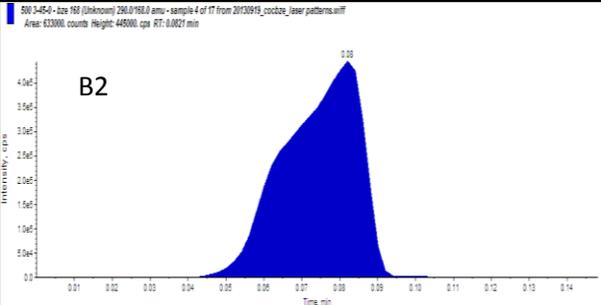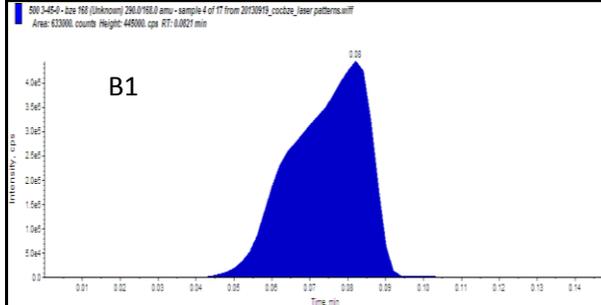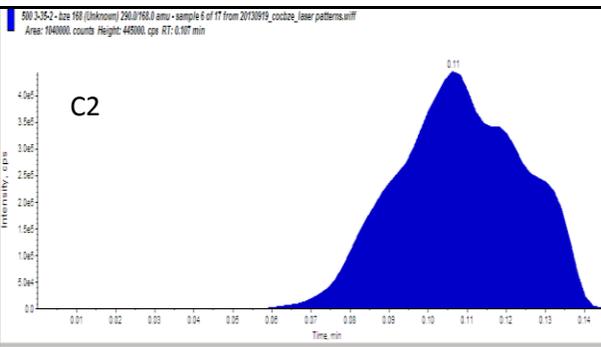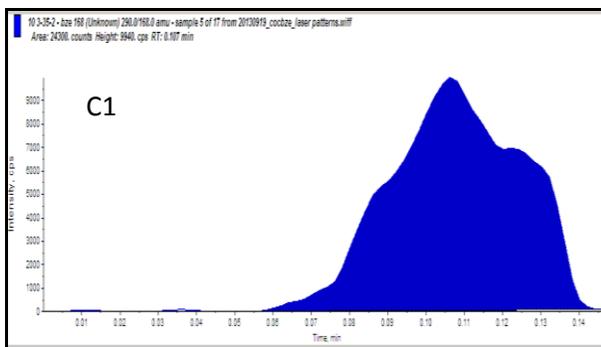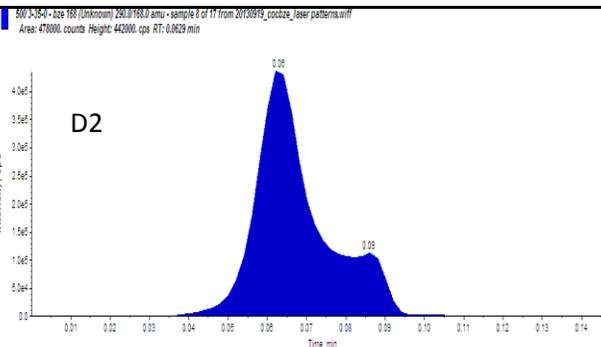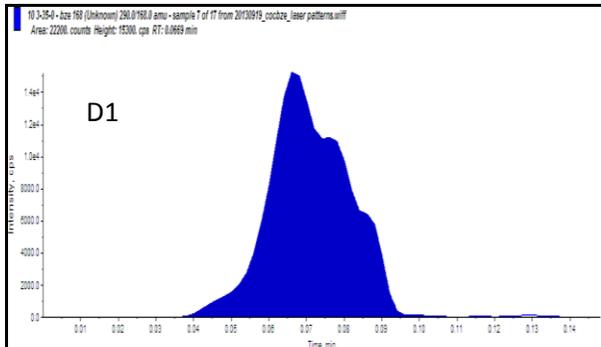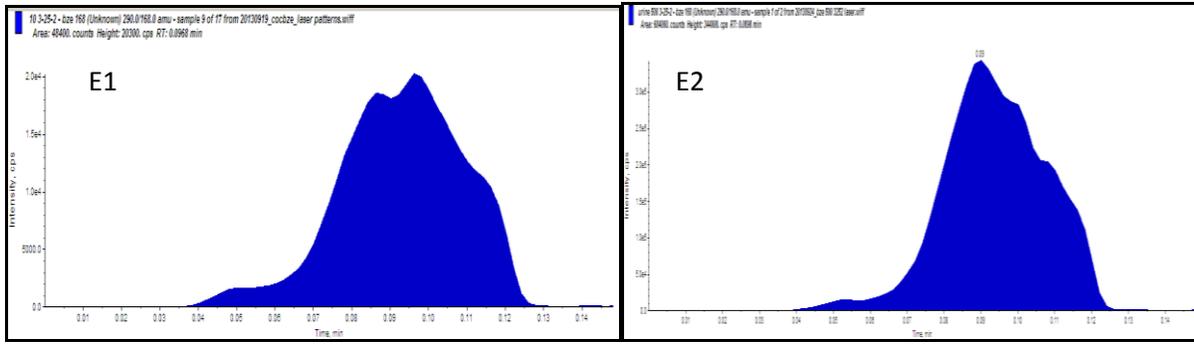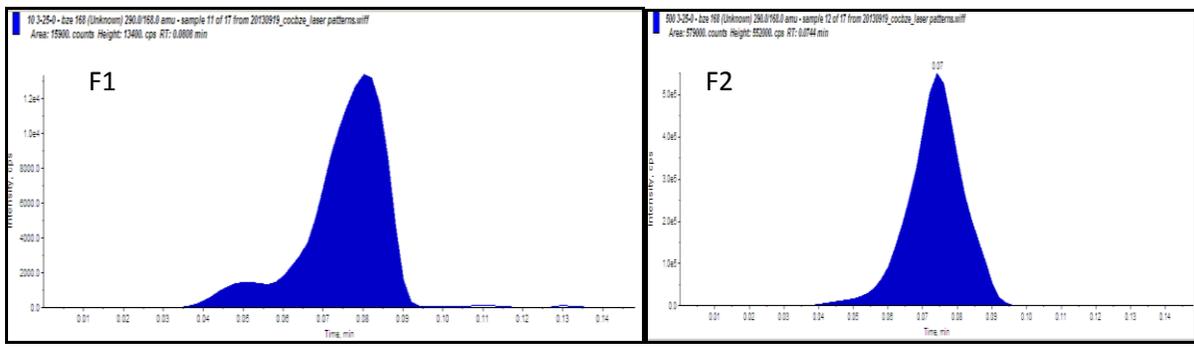
10 ng/mL 500 ng/mL

**Figure 8. Ion transition desorption peaks of BZE spiked in urine at 10 ng/mL (left column) and 500 ng/mL (right column) and spotted at 2 μL analyzed with the following laser patterns: 3-45-2(A1-A2), 3-45-0 (B1-B2), 3-35-2 (C1-C2), 3-35-0 (D1-D2), 3-25-2 (E1-E2), and 3-25-0 (F1-F2).**
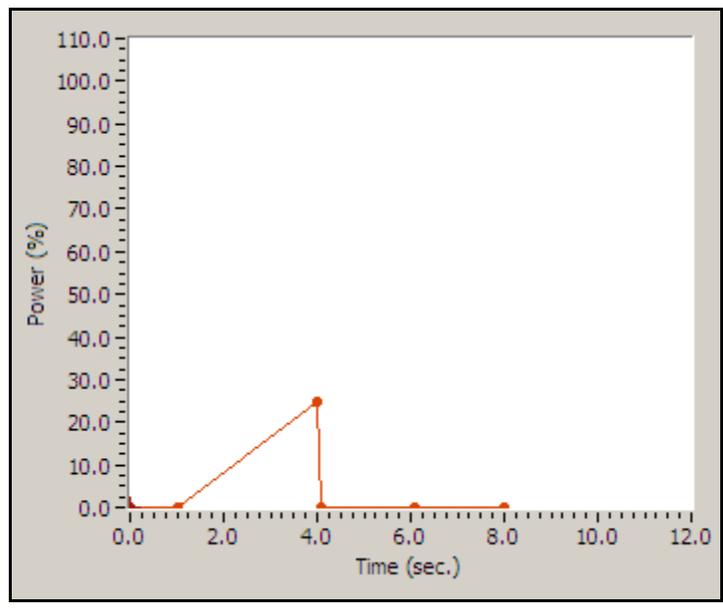


**Figure 9. LDTD laser pattern 3-25-0. After reaching 25% power, the laser immediately decreases to 0%.**

The flow rate was not optimized during this study. A typical flow of 3 L/min was used based on previous analysis of drugs of abuse by LDTD-MS/MS (Auger 2011, 2012 ).

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### 3.1.2.2 Evaluation of Analyst's Spotting Technique

In order to evaluate the analyst's spotting technique, ethyl acetate, MeOH, and n-butyl chloride solutions were fortified with 25 ng/mL of alprazolam. Two analysts spotted samples in replicates of eight at 2 μL each. For all solvents, the %CV of the analyte peak areas were less than 20% for each analyst. **Figure 10, A−C,** shows MS desorption peaks for alprazolam for the ion transition 309 m/z→ 281m/z in different solvents.
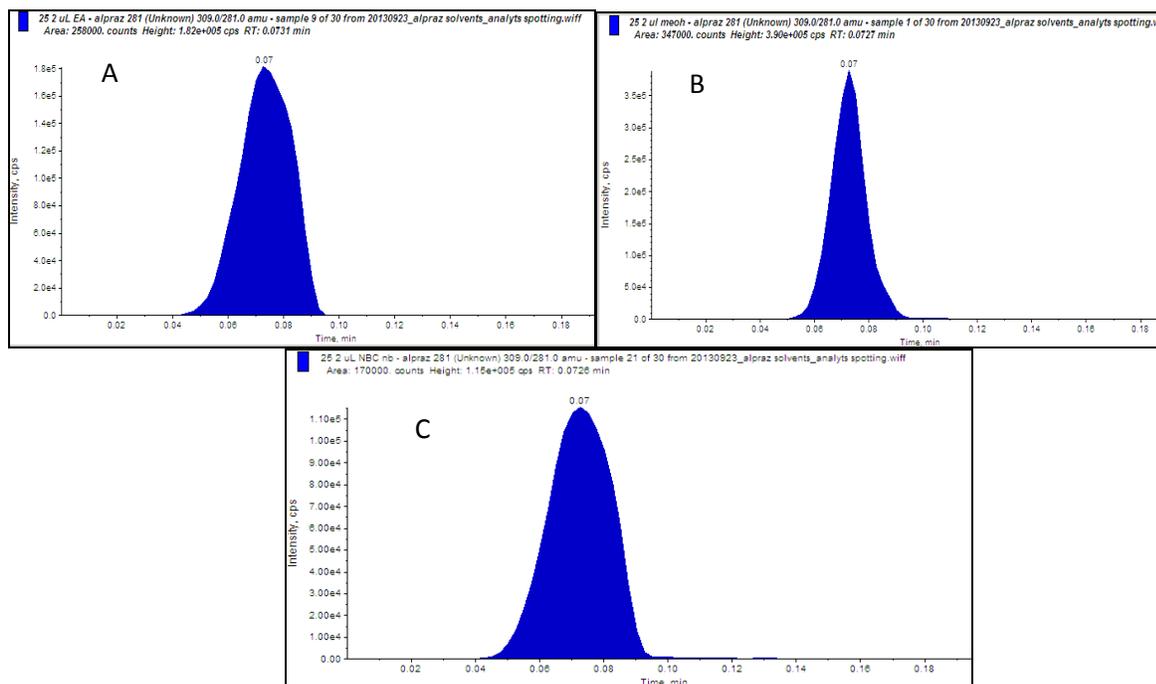


**Figure 10. MS desorption peaks ethyl acetate (A), MeOH (B) and n-butyl chloride (C) solutions fortified with 25 ng/mL solutions of alprazolam.**

## 3.2 Analysis of Drug Analytes in Specimen Matrix

### 3.2.1 Issues Encountered during Analysis of Drug Analytes in Blood and Urine

#### 3.2.1.1 Anomalous Peaks in Drug-free Specimens

Under usual circumstances, an interferant present in a matrix can be separated from the analyte of interest during GC or LC analysis, allowing for successful identification or quantitation of the target analytes. The inability to separate target analytes from interferences using LDTD was a concern and a frequently encountered problem throughout this study. Early in this study, it was determined that a peak in a blank sample was acceptable if its area was less than 20% than that of the LOQ. If the criterion was not met, the origin of the peak would be investigated and attempts would be made to either eliminate or decrease the peak by removal of the interferant if contamination persisted or by more extensive sample preparation.

Throughout this project, we encountered the presence interferant peaks in drug-free urine or blood at the same ion transition of the target analyte. The first encounter occurred during the method development of AMPs in urine. **Figure 11, A−F,** shows the presence of a peak in the MS response for ion transition, 136 m/z→91 m/z, in drug-free (blank) urine without (A) and with

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spiked ISTD (B). Initially, it was thought that an interferant in the urine matrix was responsible for the peak; however, this theory was eliminated when the peak appeared in extracted deionized (DI) water, both with (C) and without ISTD (D). Next, n-butyl chloride was spotted and analyzed to determine if the solvent was contaminated, but there was no peak present and n-butyl chloride was eliminated as the source of the interferant. The remaining sources of contamination were an HCl and NaOH solution, both of which were diluted in DI water obtained from an in-house system. HPLC grade water was obtained from a newly opened bottle and new HCl and NaOH solutions were made. The results showed the absence of a peak for the HPLC water (E) and blank urine (F) samples extracted with the newly made solutions. In light of this discovery, HPLC-grade water was used for all drug analyte extractions for the remainder of the study.
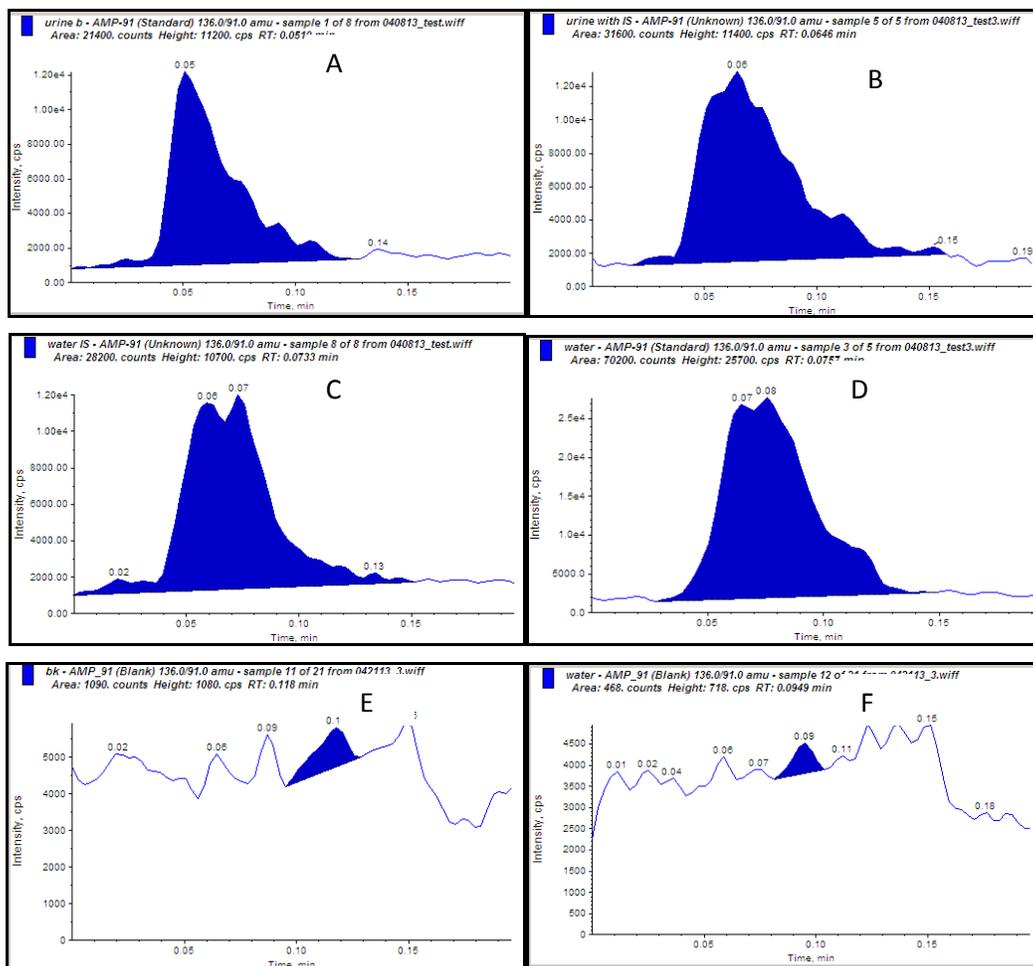


**Figure 11. MS response for ion transition 136 m/z→91 m/z showing the presence of a peak in drug-free urine without (A) and with spiked ISTD (B) and in DI water with (C) without spiked ISTD (D) extracted with solutions made from contaminated DI water. ISTD-spiked drug-free urine (E) and HPLC water (F) extraction with solutions made from HPLC water.**

The problem of erroneous peaks in blank samples occurred again during the method development of PCP. In LDTD-APCI the background signal is typically low except for masses originating from gas impurities. During the thermal desorption process, the increase and decrease of temperature can cause an increase in baseline signal, which results in an integrated peak-shaped apparition. For comparison, turning the flow on and off in LC-ESI would produce a

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similar peak. This baseline increase may also occur as a result of sample preparation or mass ion transition. **Figure 12**, **A–D,** presents MS response for ion transition 244 m/z→91 m/z of a blank sample extracted from urine (A) and blood (B) without the presence of ISTD. As in the case of AMP, all solvents used in the extraction were evaluated and eliminated as a source of contamination. Unlike the previously encountered problem with AMP, the source of interference was not determined. (Subsequent input from other LDTD users suggests that this may have been the result of the baseline increase and not an interfering compound.) After investigating previous PCP analysis by LDTD-MS/MS in the literature, it was determined that although the product ion 91 m/z produced the most intense peak during product ion optimization and has been used in previous publications (Feng et al., 2007; Poklis et al., 2011; Stout et al., 2010), it cannot be used for quantitation in LDTD-MS/MS analysis of PCP. As published in Phytronix Application notes, 159 m/z was used for the quantitative ion in this study (Auger, 2011, 2012). Figure 10 shows the MS response for ion transition 244 m/z→159 m/z in urine (C) and blood (D). Although a small peak is present, its peak area is less than 20% of the LOQ peak area.
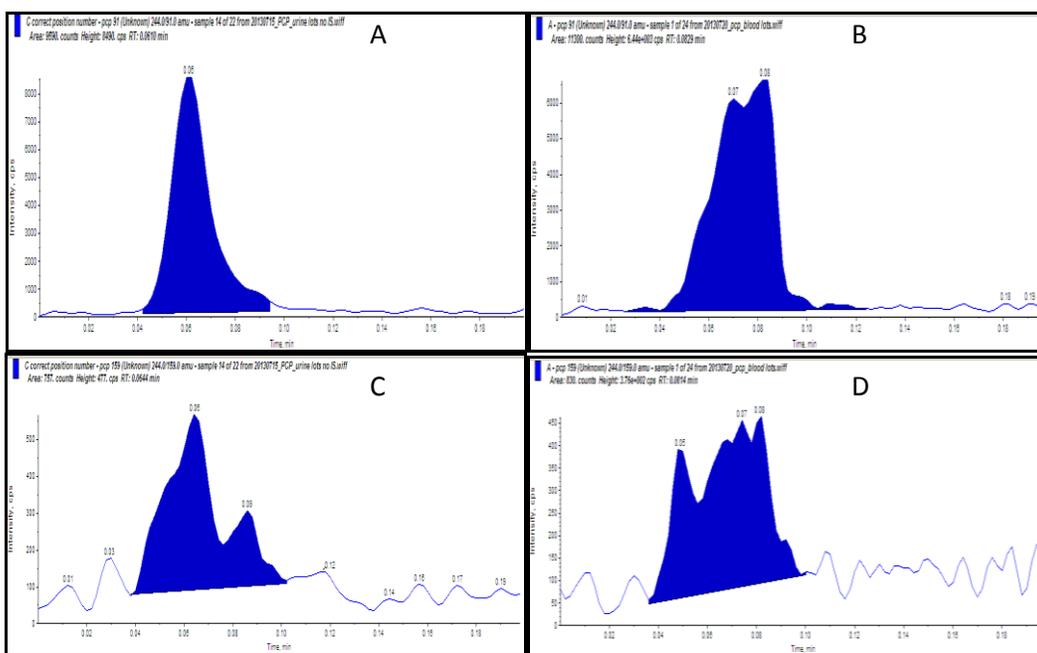


**Figure 12. MS response for PCP ion transition, 244 m/z→91 m/z, shows the presence of an erroneous peak in drug-free urine (A) and blood (B) with no ISTD. MS response for ion transition, 244 m/z→159 m/z, showing the presence of a small peak in drug-free urine (C) and blood (D) with no ISTD; however, this peak is less than 20% of peak area of the LOQ and was considered acceptable.**

A similar problem was encountered during the method development of 7-aminoclonazepam. Product ion 121 m/z was determined to be the most intense during optimization (**Appendix A**) but could not be used as the quantitative ion due to the presence of a peak apparition in both the blank urine and blood matrix without ISTD (**Figure 13, A–B**). The peak was greater than 20% of the LOQ peak area resulting in using 222 m/z as the quantitation ion. **Figure 13, C–D,** shows the typical background signal of product ion 222 m/z in both blank urine (C) and blood (D). The situation was resolved by using a different single reaction monitoring (SRM) transition.

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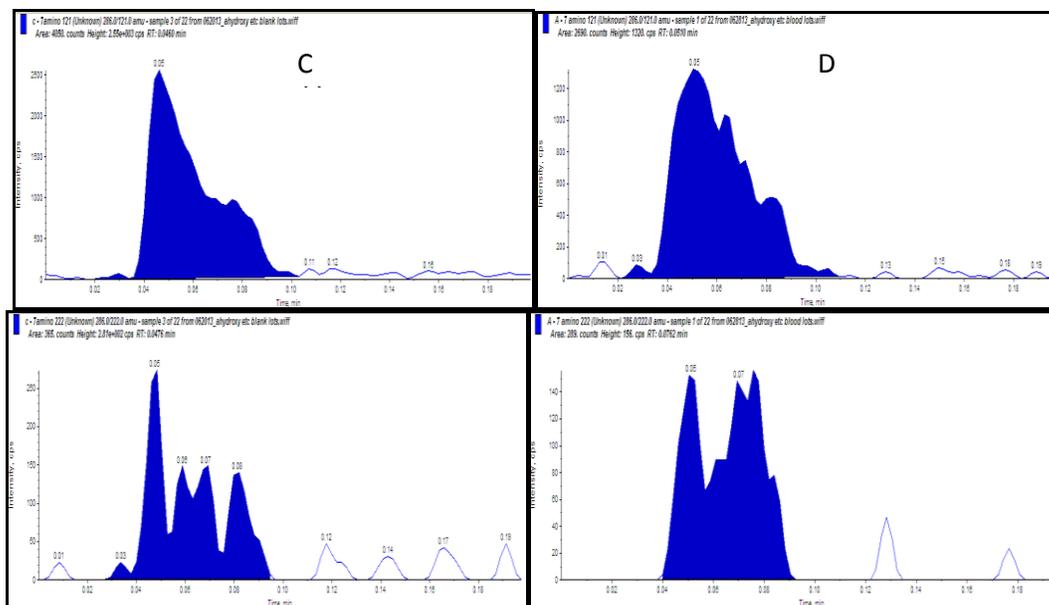
**Figure 13. MS response for the 7-aminoclonazepam ion transition, 286 m/z→121 m/z, showing the presence of an erroneous peak in drug-free urine (A) and blood (B) with no ISTD. MS response for ion transition, 286 m/z →222 m/z, showing a typical background signal in drug-free urine (C) and blood (D) with no ISTD.**

In addition to the issues encountered with AMP, PCP, and 7-aminoalprazolam, erroneous peaks were also present in blank urine matrix during the analysis of COD, MOR, and 6-AM. **Figures 14, A−C,** presents the MS response for the ion transition 286→201 (A) for MOR, 300 m/z→183 m/z (B) for COD and 328 m/z →268 m/z for 6-AM (C) in blank urine. The peak area in the blank for the MOR ion transition was 76% of an extracted urine sample at 100 ng/mL (**Figure 14, D**). After remaking extraction solutions to investigate potential contamination of the solutions and reevaluating extraction techniques without success of eliminating the interferant peak, the samples were analyzed on an Agilent 1290 Infinity LC coupled to an Agilent 6490 QQQ, using the same product ion transitions as on the LDTD-MS/MS, in order to confirm the presence of the opiates in the blank urine matrix. **Figure 15, A,** shows an unextracted standard at 1,000 ng/mL of MOR, COD, and 6-AM in mobile phase (95:5, 5mM ammonium formate:methanol with 0.1% formic acid) with retention times (RT) 0.78, 1.45, and 1.7 min, respectively. **Figure 15, B,** presents the extracted blank urine without the presence of COD, MOR, or 6-AM. Future studies should include alternative sample preparation and LDTD parameters in order to determine ways to decrease the peak apparition.

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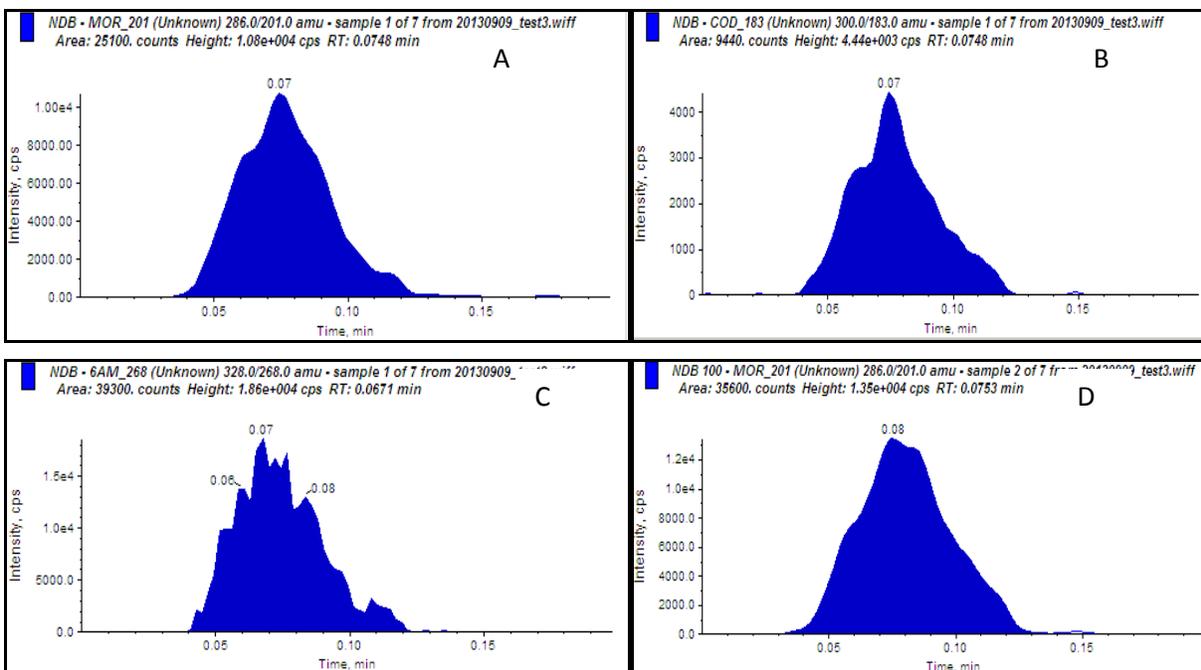
**Figure 14. MS response of ion transition for MOR (286 m/z→201 m/z) (A), COD (300 m/z→183) (B), and 6-AM (328 m/z→268 m/z) (C) showing the presence of an erroneous peak in blank urine with no ISTD. (D) MS response of ion transition for MOR for extracted urine sample spiked at 100 ng/mL**
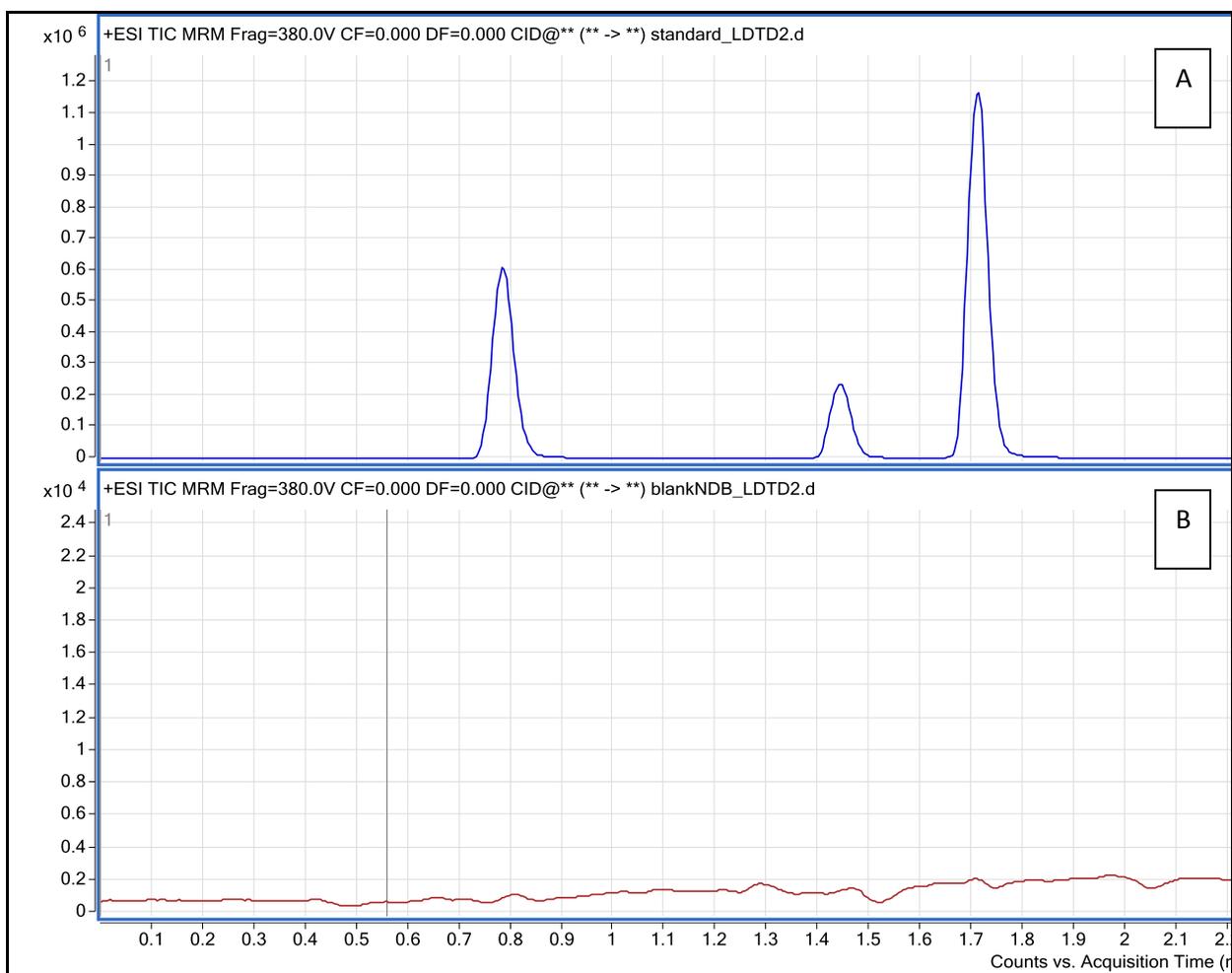
**Figure 15. LC-MS/MS was used to eliminate the presence of MOR, COD, and 6-AM in blank urine. The chromatography for MOR (RT=0.78min), COD (RT=1.45min) and 6-AM (RT=1.7 min) for 1, 000 ng/mL unextracted standard in mobile phase (A) and extracted blank urine sample (B).**

Occasionally, unexplained issues with reproducibility were encountered. Three extracted urine QC samples containing amitriptyline at a target concentration of 45 mg/mL showed inconsistencies in calculated concentrations when the same sample was spotted in triplicate. The calculated concentrations for each QC were 9.6, 7.2, and 11.1 ng/mL (average = 9.3). Initially, improper spotting was suspected, even though it was unlikely that three samples successively were spotted incorrectly. The QC sample contained amitriptyline, citalopram, nortriptyline, and trazodone, which also had a target concentration of 45 ng/mL. If the cause of the low values were due to improper spotting, all analytes would have exhibited a similar decrease in concentration; however, this was not the case. **Table 12** shows the average calculated concentrations of the other analytes in the drug matrix. Although their concentrations are slightly greater than20% than that of the target, they were much closer to the target concentration than amitriptyline. The same decrease was seen for the first QC sample replicate of amitriptyline at a target concentration of 400 ng/mL, which resulted in a calculated concentration of 91 ng/mL. Notriptyline, trazodone, and citalopram had a calculated concentration of 398, 362, and 398 ng/mL, respectively, which meant the cause of the incorrect concentration was not the result of

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inadequate spotting. In both cases, the samples were respotted and reanalyzed, and all results were within acceptable range.

**Table 12. Average calculated concentrations of amitriptyline, citalopram, nortriptyline, and trazodone for the analysis of a QC sample with a target concentration of 45 ng/mL.**

| Drug | Average Calculated Concentration (ng/mL) |
|------|------------------------------------------|
| Amitriptyline | 9.3 |
| Citalopram | 35.6 |
| Nortriptyline | 35.3 |
| Trazodone | 37.1 |

## 3.2.1.2  Importance of Choosing the Correct ISTD

Throughout this study for quantitation analysis, all analytes' respective deuterated ISTDs were used to correct for the loss of analyte during sample preparation or analysis. This proved very important since the presence of biological matrices can affect the sample desorption. In order to keep the number of transitions below 10 during the analysis of the antidepressants, nortriptyline-$d_3$ was used as an ISTD for both nortriptyline and amitriptyline. Even though the drugs are similar in structure, suitable linearity could not be obtained for amitriptyline, although nortriptyline analysis produced acceptable results. Once nortriptyline-$d_3$ was replaced with amitriptyline-$d_3$ (note that amitriptyline-$d_3$ was used in the above example of peak reproducibility), acceptable linearity was achieved.

## 3.3    Validation Results for Quantitative Method Analysis

### 3.3.1  Calibration Model

Calibration and data reduction were performed using Analyst software (version 1.4.2). Details about the calibration ranges used for the quantification of each drug analyte in urine and blood are given in **Tables 13 and 14**, respectively. A six-point calibration curve was evaluated to establish the appropriate calibration model for all analytes in blood and urine. Calibration samples spanned the range of typical concentrations expected and were analyzed in five separate runs (n=5 at each concentration) to establish the model.

A simple linear regression model using the least-squares method was used most often as it is the most often used calibration model (SWGTOX). A theoretically perfect analytical precision would produce an ANOVA analysis, indicating a model fit with a slope significantly different from 0.000 and an intercept not significantly different than 0.000. Hence, a completely accurate calibration curve over the

**Calibration Model Summary**

➢ 6-point calibration curve used to establish model (n=5 at each concentration)
➢ Average $r^2$ value=0.998
➢ Average slope=1.00 and 0.993 in urine and blood, respectively
➢ Intercepts not significantly different from zero (at p<0.05 level)
➢ Quadratic (1/x) calibration model used for alprazolam, clonazepam and diazepam in blood
➢ Linear regression (1/x) calibration model established for all other drug analytes

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entire range would result in an $r^2$=1.000, slope of 1.000, and an intercept of 0.000. The average $r^2$ value of the calibration curves for all drugs extracted in both urine and blood was 0.998. The average slope of the curves for all drugs extracted in urine and blood was 1.00 and 0.993, respectively. For urine and blood analysis, the intercepts ranged from -15.10 to 8.06 and -1.97 to 20.47, respectively. Significance was assigned at the $p < 0.05$ level. All intercept p-values for blood and urine were greater than 0.05, meaning all intercepts were not significantly different from zero. A typical line fit plot, as represented by MAMP fortified in urine over the concentration range 25−2000 ng/mL, is shown in **Figure 16**. Line plots for all other analytes in urine and blood are presented in **Appendix B**.

**Table 13. Summary of calibration, ISTD, QC sample concentration and calibration types used for urine drug analysis. All calibration curves were weighted 1/x.**

| Drug Class | Drug | Calculation type | $r^2$ | Calibration Points (ng/mL) | ISTD Conc (ng/mL) | QC Conc. (ng/mL) |
|---|---|---|---|---|---|---|
| **Amphetamines** | AMP | linear (y=mx+b) | 0.998 | 25, 100, 500, 1000, 1500, 2000 | 100 | 75, 300, 1000 |
| | MAMP | linear (y=mx+b) | 0.998 | 25, 100, 500, 1000, 1500, 2000 | 100 | 75, 300, 1000 |
| | MDA | linear (y=mx+b) | 0.996 | 50, 100, 500, 1000, 1500, 2000 | 100 | 75, 300, 1000 |
| | MEA | linear (y=mx+b) | 0.995 | 50, 100, 500, 1000, 1500, 2000 | 100 | 75, 300, 1000 |
| | MDMA | linear (y=mx+b) | 0.997 | 50, 100, 500, 1000, 1500, 2000 | 100 | 75, 300, 1000 |
| **Antidepressants** | Amitriptyline | linear (y=mx+b) | 0.998 | 15, 50, 100, 500, 800, 1000 | 100 | 45, 400, 800 |
| | Citalopram | linear (y=mx+b) | 0.998 | 15, 50, 100, 500, 800, 1000 | 100 | 45, 400, 800 |
| | Nortriptyline | linear (y=mx+b) | 0.998 | 15, 50, 100, 500, 800, 1000 | 100 | 45, 400, 800 |
| | Trazodone | linear (y=mx+b) | 0.998 | 15, 50, 100, 500, 800, 1000 | 100 | 45, 400, 800 |
| **Benzodiazepines** | Alprazolam | linear (y=mx+b) | 0.998 | 15, 50, 100, 500, 1000 | 100 | 45, 400, 800 |
| | α-hydroxyalprazolam | linear (y=mx+b) | 0.998 | 25, 50, 75, 100, 500, 1000 | 100 | 45, 400, 800 |
| | Clonazepam | linear (y=mx+b) | 0.999 | 15, 50, 100, 500, 1000 | 100 | 45, 400, 800 |
| | 7-aminoclonazpeam | linear (y=mx+b) | 0.998 | 25, 50, 75, 100, 500, 1000 | 100 | 45, 400, 800 |
| | Diazepam | linear (y=mx+b) | 0.998 | 15, 50, 100, 500, 1000 | 100 | 45, 400, 800 |
| **Miscellaneous** | BZE | linear (y=mx+b) | 0.998 | 10, 25, 50, 100, 500, 1000 | 100 | 45, 400, 800 |
| | COC | linear (y=mx+b) | 0.999 | 10, 25, 50, 100, 500, 1000 | 100 | 45, 400, 800 |
| | PCP | linear (y=mx+b) | 0.998 | 10, 20, 50, 100, 250, 500 | 50 | 30, 100, 400 |

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**Table 14. Summary of calibration, ISTD, QC sample concentration and calibration types used for blood drug analysis. All calibration curves were weighted 1/x.**

| Drug Class | Drug | Calculation Type | $r^2$ | Calibration Points (ng/mL) | ISTD Conc (ng/mL) | QC Conc. (ng/mL) |
|---|---|---|---|---|---|---|
| **Amphetamines** | AMP | linear (y=mx+b) | 0.998 | 25, 100, 500, 1000, 1500, 2000 | 100 | 75, 300, 1000 |
| | MAMP | linear (y=mx+b) | 0.998 | 25, 100, 500, 1000, 1500, 2000 | 100 | 75, 300, 1000 |
| | MDA | linear (y=mx+b) | 0.996 | 50, 100, 500, 1000, 1500, 2000 | 100 | 75, 300, 1000 |
| | MEA | linear (y=mx+b) | 0.995 | 50, 100, 500, 1000, 1500, 2000 | 100 | 75, 300, 1000 |
| | MDMA | linear (y=mx+b) | 0.997 | 50, 100, 500, 1000, 1500, 2000 | 100 | 75, 300, 1000 |
| **Antidepressants** | Amitriptyline | linear (y=mx+b) | 0.998 | 15, 50, 100, 500, 800, 1000 | 100 | 45, 400, 800 |
| | Citalopram | linear (y=mx+b) | 0.998 | 15, 50, 100, 500, 800, 1000 | 100 | 45, 400, 800 |
| | Nortriptyline | linear (y=mx+b) | 0.998 | 15, 50, 100, 500, 800, 1000 | 100 | 45, 400, 800 |
| | Trazodone | linear (y=mx+b) | 0.998 | 15, 50, 100, 500, 800, 1000 | 100 | 45, 400, 800 |
| **Benzodiazepines** | Alprazolam | Quadratic (y=ax$^2$+bx+c) | 0.999 | 10, 25, 50, 100, 500, 1000 | 100 | 30, 400, 800 |
| | α-hydroxyalprazolam | linear (y=mx+b) | 0.996 | 25, 50, 75, 100, 500, 1000 | 100 | 60, 400, 800 |
| | Clonazepam | Quadratic (y=ax$^2$+bx+c) | 0.999 | 10, 25, 50, 100, 500, 1000 | 100 | 30, 400, 800 |
| | 7-aminoclonazpeam | linear (y=mx+b) | 0.999 | 25, 50, 75, 100, 500, 1000 | 100 | 60, 400, 800 |
| | Diazepam | Quadratic (y=ax$^2$+bx+c) | 0.999 | 10, 25, 50, 100, 500, 1000 | 100 | 30, 400, 800 |
| **Miscellaneous** | BZE | linear (y=mx+b) | 0.998 | 10, 25, 50, 100, 500, 1000 | 100 | 45, 400, 800 |
| | COC | linear (y=mx+b) | 0.999 | 10, 25, 50, 100, 500, 1000 | 100 | 45, 400, 800 |
| | PCP | linear (y=mx+b) | 0.998 | 10, 20, 50, 100, 250, 500 | 50 | 30, 100, 400 |
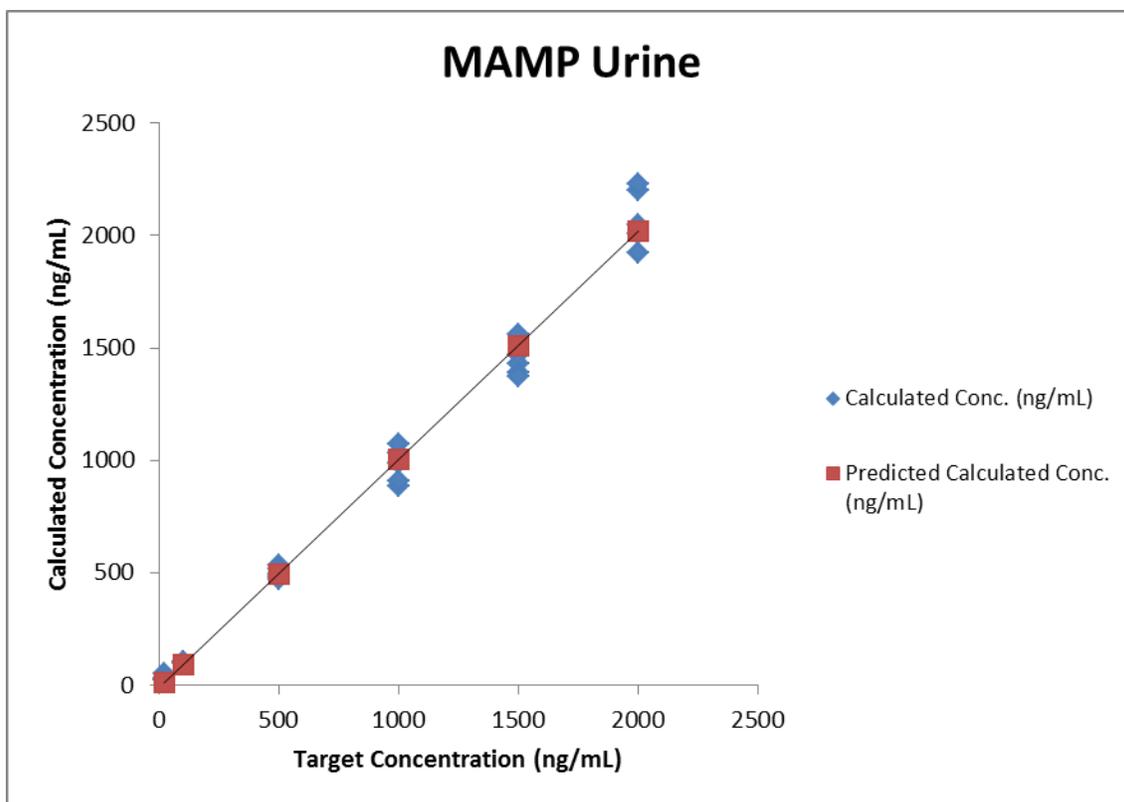
**Figure 16. A typical line fit plot, represented by MAMP, fortified in urine, spanning the concentration range 25-2000 ng/mL.**

After evaluation of all calibration line fit plots (calibration curves), a linear regression calibration model was established for the quantitative analysis of all drugs in blood and urine in this study, with the exception of alprazolam, clonazepam, and diazepam, which followed a non-linear quadratic model for analysis in blood. This was necessary to achieve reproducibility at the upper limit of the calibration curve. Since the concentration range for each curve exceeded two orders or magnitude, a weighted (1/x) least-squares model was applied to all calibration curves. The ULOL was assessed by visually determining the concentration at which linearity was lost.

**Tables 15 and 16** presents ULOL for all analytes in urine and blood, respectively.

**Table 15. ULOL for all drug analytes in urine.**

| Drug Class | Drug | Upper Limit of Linearity (ng/mL) |
|---|---|---|
| **Amphetamines** | AMP | 2000 |
| | MAMP | 2000 |
| | MDA | 2000 |
| | MDEA | 5000 |
| | MDMA | 5000 |
| **Antidepressant** | Amitriptyline | 3000 |
| | Citalopram | 3000 |
| | Nortriptyline | 5000 |
| | Trazodone | 1000 |
| **Benzodiazepines** | Alprazolam | 10000 |
| | Clonazepam | 3000 |
| | Diazepam | 15000 |
| | α-hydroxyalprazolam | 10000 |
| | 7-aminoclonazopam | 5000 |
| **Miscellaneous** | BZE | 10000 |
| | Cocaine | 10000 |
| | PCP | 5000 |

**Table 16. ULOL for all drug analytes in blood.**

| Drug Class | Drug | Upper Limit of Linearity (ng/mL) |
|---|---|---|
| **Amphetamines** | AMP | 2000 |
| | MAMP | 2000 |
| | MDA | 2000 |
| | MDEA | 10000 |
| | MDMA | 2000 |
| **Antidepressant** | Amitriptyline | 2000 |
| | Citalopram | 2000 |
| | Nortriptyline | 2000 |
| | Trazodone | 2000 |
| **Benzodiazepines** | Alprazolam | 500 |
| | Clonazepam | 5000 |
| | Diazepam | 500 |
| | α-hydroxyalprazolam | 5000 |
| | 7-aminoclonazopam | 500 |
| **Miscellaneous** | COC | 1000 |
| | BZE | 1000 |
| | PCP | 5000 |

### 3.3.2 Evaluation of Precision and Accuracy for All Drugs Analyzed by Quantitative Analysis

The precision and accuracy results for QC samples for all drugs analyzed in urine and blood are presented in **Tables 17–20** and **Tables 21–24**, respectively. Each QC level (low, midpoint, and high) was analyzed in triplicate over five runs, for a total of 45 samples. For urine, the average overall within-run precision was represented by a %CV < 8.5 for all compounds, with the exception of MDEA, which had greater variability, as shown by a 13.8% CV. PCP had the least variability as shown by a 2.2% CV. The average overall between-run precision was represented by a %CV < 13.0 for all compounds, with the exception of MDEA, which had greater variability, as shown by a 21.0% CV. For blood, the average overall within-run precision was represented by a %CV < 7 for all compounds. The analytes with the highest and lowest variability were MDEA with a %CV of 6.9 and BZE with a 2.0% CV. The average overall between-run precision was represented by a %CV < 12.0, with alprazolam having the highest variability (11.5% CV) and BZE having the lowest (3.4% CV). For the purposes of this study, a %CV < 20.0 was considered acceptable. Overall, all analytes exhibited acceptable precision with the exception of MDEA, which had a between-run %CV slightly higher than 20.

> **Precision and Accuracy Summary**
>
> ➤ Assessed using QC samples at 3 concentrations (low, midpoint, high)
> ➤ Overall within-run % CV average
>   Urine: < 8.5% (except MDEA)
>   Blood: < 7.0%
> ➤ Overall between-run %CV average
>   Urine: < 13.0%
>   Blood: < 12.0%
> ➤ Overall % accuracy
>   Urine: 88.9-104.5%
>   Blood: 91.9-107.1 %

The % accuracy and its associated %CV for all drugs in urine were 88.9–104.5%, with accuracy %CVs less than 16.5% with the exception of MDEA, which had an accuracy %CV of 27.2. The % accuracy and its associated %CV for all drugs in blood were 91.9–107.1 and < 12.0, respectively.

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**Tables 17–20. Evaluation of precision and accuracy of LDTD-MS/MS results for samples in urine by drug class (n=45).**

| Table 17 | | Precision Evaluation | | Accuracy Evaluation | |
|---|---|---|---|---|---|
| **Drug Class** | **Drug** | **Average Overall Within-Run %CV** | **Average Overall Between-Run %CV** | **Average Overall Accuracy** | **Accuracy %CV** |
| **Amphetamines** | AMP | 2.9 | 8.5 | 101.7 | 10.5 |
| | MAMP | 3.4 | 8.6 | 89.6 | 11.4 |
| | MDA | 6.5 | 12.5 | 95.4 | 16.2 |
| | MDEA | 13.8 | 21.0 | 91.5 | 27.2 |
| | MDMA | 5.4 | 11.7 | 88.9 | 14.6 |

| Table 18 | | Precision Evaluation | | Accuracy Evaluation | |
|---|---|---|---|---|---|
| **Drug Class** | **Drug** | **Average Overall Within-Run %CV** | **Average Overall Between-Run %CV** | **Average Overall Accuracy** | **Accuracy %CV** |
| **Antidepressants** | Amitriptyline | 3.8 | 6.0 | 102.3 | 6.3 |
| | Citalopram | 4.1 | 6.7 | 95.8 | 7.2 |
| | Nortriptyline | 5.0 | 8.4 | 95.2 | 8.1 |
| | Trazodone | 3.3 | 5.2 | 96.4 | 5.3 |

| Table 19 | | Precision Evaluation | | Accuracy Evaluation | |
|---|---|---|---|---|---|
| **Drug Class** | **Drug** | **Average Overall Within-Run %CV** | **Average Overall Between-Run %CV** | **Average Overall Accuracy** | **Accuracy %CV** |
| **Benzodiazepines** | Alprazolam | 6.2 | 8.0 | 90.0 | 9.4 |
| | α-hydroxyalprazolam | 5.2 | 7.5 | 103.4 | 8.2 |
| | Clonazepam | 5.3 | 8.3 | 104.5 | 8.3 |
| | 7-aminoclonazopam | 6.3 | 9.1 | 98.7 | 8.7 |
| | Diazepam | 8.2 | 10.2 | 104.4 | 11.7 |

| Table 20 | | Precision Evaluation | | Accuracy Evaluation | |
|---|---|---|---|---|---|
| **Drug Class** | **Drug** | **Average Overall Within-Run %CV** | **Average Overall Between-Run %CV** | **Average Overall Accuracy** | **Accuracy %CV** |
| **Miscellaneous** | COC | 5.5 | 7.2 | 103.7 | 7.3 |
| | BZE | 8.3 | 12.4 | 96.0 | 12.7 |
| | PCP | 2.2 | 8.3 | 94.0 | 9.6 |

**Tables 21−24. Evaluation of precision and accuracy of LDTD-MS/MS results for samples in blood by drug class (n=45).**

| Table 21 | | Precision Evaluation | | Accuracy Evaluation | |
|---|---|---|---|---|---|
| Drug Class | Drug | Average Overall Within-Run %CV | Average Overall Between-Run %CV | Average Overall Accuracy | Accuracy %CV |
| **Amphetamines** | AMP | 3.1 | 4.3 | 107.1 | 6.1 |
| | MAMP | 3.9 | 7.5 | 91.9 | 8.8 |
| | MDA | 6.6 | 8.1 | 100.4 | 9.6 |
| | MDEA | 6.9 | 7.9 | 93.1 | 9.1 |
| | MDMA | 6.3 | 7.9 | 93.1 | 8.2 |

| Table 22 | | Precision Evaluation | | Accuracy Evaluation | |
|---|---|---|---|---|---|
| Drug Class | Drug | Average Overall Within-Run %CV | Average Overall Between-Run %CV | Average Overall Accuracy | Accuracy %CV |
| **Antidepressants** | Amitriptyline | 3.6 | 5.5 | 104.5 | 5.6 |
| | Citalopram | 2.9 | 5.2 | 103.0 | 7.1 |
| | Nortriptyline | 3.9 | 6.4 | 96.1 | 5.1 |
| | Trazodone | 2.9 | 4.4 | 101.9 | 6.3 |

| Table 23 | | Precision Evaluation | | Accuracy Evaluation | |
|---|---|---|---|---|---|
| Drug Class | Drug | Average Overall Within-Run %CV | Average Overall Between-Run %CV | Average Overall Accuracy | Accuracy %CV |
| **Benzodiazepines** | Alprazolam | 3.7 | 11.5 | 98.1 | 11.5 |
| | α-hydroxyalprazolam | 6.4 | 10.4 | 93.9 | 10.4 |
| | Clonazepam | 3.9 | 7.3 | 97.0 | 7.8 |
| | 7-aminoclonazopam | 4.4 | 10.6 | 97.8 | 10.5 |
| | Diazepam | 5.1 | 10.1 | 93.7 | 10.2 |

| Table 24 | | Precision Evaluation | | Accuracy Evaluation | |
|---|---|---|---|---|---|
| Drug Class | Drug | Average Overall Within-Run %CV | Average Overall Between-Run %CV | Average Overall Accuracy | Accuracy %CV |
| **Miscellaneous** | COC | 3.0 | 6.2 | 106.0 | 6.4 |
| | BZE | 2.0 | 3.4 | 100.6 | 4.0 |
| | PCP | 2.4 | 6.7 | 94.0 | 7.2 |

### 3.3.3  Evaluation of LOD and LOQ

**Tables 25 and 26** present the LOD concentrations for each drug in urine and blood, respectively, at n=18, unless otherwise noted. In urine, the LOD values ranged from 0.5−15 ng/mL, with MDEA having the highest and COC having the lowest. In blood, the LOD values ranged from 0.25−15 ng/mL, with AMP having the highest while BZE had the lowest. In general, the LOD was similar for each analyte across the two matrices, with BZE showing the largest change with an LOD of 2 ng/mL in urine and 0.25 ng/mL in blood. **Figure 17** presents a typical MS ion transition peak of the LOD represented by urine-fortified MAMP at a concentration of 5 ng/mL. Reference **Appendix D** for MS ion transition peaks for the LOD of all analytes in urine and blood.

> **LOD and LOQ Summary**
>
> ➢ LOD (n=18)
>   Urine: 0.5-15 ng/mL
>   Blood: 0.25-15 ng/mL
>
> ➢ LOQ (n values vary)
>   Urine: % CV range 3.8-14.2 %
>   Blood: % CV range 3.1-14.2 %

**Table 25. LOD for drugs analyzed by quantitative analysis in urine.**

| Drug Class | Drug | n | LOD ng/mL |
|---|---|---|---|
| Amphetamines | AMP | 18 | 10 |
| | MAMP | 18 | 5 |
| | MDA | 18 | 10 |
| | MDEA | 18 | 15 |
| | MDMA | 18 | 2 |
| Antidepressants | Amitriptyline | 18 | 5 |
| | Citalopram | 18 | 10 |
| | Nortriptyline | 18 | 2 |
| | Trazodone | 18 | 1 |
| Benzodiazepines | Alprazolam | 18 | 1 |
| | Clonazepam | 18 | 5 |
| | Diazepam | 18 | 5 |
| | α-hydroxyalprazolam | 18 | 10 |
| | 7-Aminoclonazopam | 18 | 10 |
| Miscellaneous | Cocaine | 18 | 0.5 |
| | BZE | 18 | 2 |
| | PCP | 18 | 5 |

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**Table 26. LOD for drugs analyzed by quantitative analysis in blood.**

| Drug Class | Drug | n | LOD ng/mL |
|---|---|---|---|
| **Amphetamines** | AMP | 18 | 15 |
| | MAMP | 18 | 10 |
| | MDA | 18 | 10 |
| | MDEA | 18 | 10 |
| | MDMA | 18 | 2 |
| **Antidepressants** | Amitriptyline | 18 | 5 |
| | Citalopram | 18 | 2 |
| | Nortriptyline | 18 | 10 |
| | Trazodone | 18 | 2 |
| **Benzodiazepines** | Alprazolam | 18 | 2 |
| | α-hydroxyalprazolam | 16 | 10 |
| | Clonazepam | 18 | 10 |
| | 7-aminoclonazopam | 18 | 10 |
| | Diazepam | 18 | 10 |
| **Miscellaneous** | Cocaine | 18 | 0.5 |
| | BZE | 18 | 0.25 |
| | PCP | 18 | 2 |
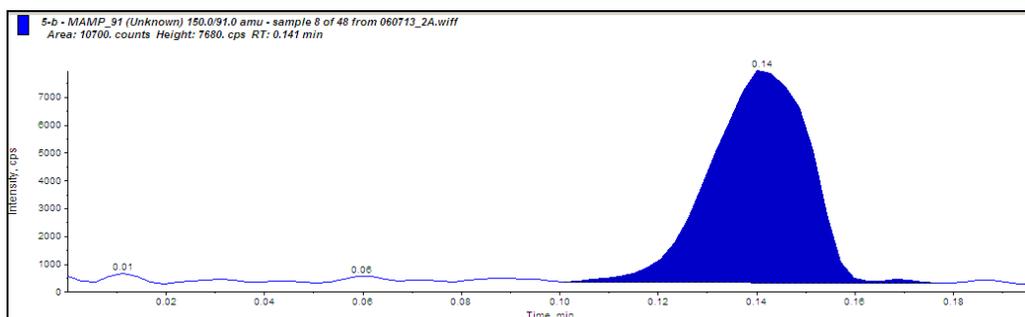


**Figure 17. MS ion transition peak of MAMP with a LOD of 5 ng/mL in urine.**

The LOQ was defined as the value of the lowest non-zero calibrator of calibration range and assessed by analyzing the lowest calibrator to demonstrate reproducibility and accuracy. The number of sample varies as they were pooled from the calibration curves used throughout the validation. In some cases, a sample set was re-extracted if there was an erroneous result for a QC sample. In that case, although the QC sample was unacceptable, the calibration curve was acceptable and therefore was used in the evaluation of the LOQ. **Tables 27 and 28** present the LOQ results for all analytes in urine and blood, respectively. All LOQ were acceptable, with %CVs ranging from 3.8–14.2 in urine and 3.1–14.2 in blood. Accuracies ranged from 89.6–107.6% and 90.0–102.1% in urine and blood, respectively. Reference **Appendix C** for MS ion transition peaks for the LOQ of all analytes in urine and blood.

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**Table 27. LOQ for drugs analyzed by quantitative analysis in urine.**

| Drug Class | Drug | N | Target Conc. (ng/mL) | % CV | %Accuracy |
|---|---|---|---|---|---|
| Amphetamines | AMP | 6 | 25 | 8.8 | 106.3 |
| | MAMP | 6 | 25 | 9.1 | 103.7 |
| | MDA | 6 | 50 | 9.5 | 102.5 |
| | MDEA | 6 | 50 | 9.4 | 106.9 |
| | MDMA | 6 | 50 | 9.5 | 103.2 |
| Antidepressants | Amitriptyline | 8 | 15 | 3.8 | 94.1 |
| | Citalopram | 8 | 15 | 12.9 | 97.8 |
| | Nortriptyline | 8 | 15 | 9.8 | 98.0 |
| | Trazodone | 8 | 15 | 11.8 | 89.6 |
| Benzodiazepines | Alprazolam | 10 | 15 | 14.2 | 105.9 |
| | α-hydroxyalprazolam | 10 | 25 | 7.2 | 107.6 |
| | Clonazepam | 10 | 15 | 9.8 | 95.1 |
| | 7-aminoclonazopam | 10 | 25 | 12.1 | 103.2 |
| | Diazepam | 10 | 15 | 12.5 | 102.5 |
| Miscellaneous | Cocaine | 10 | 10 | 11.5 | 97.0 |
| | BZE | 10 | 10 | 12.1 | 99.1 |
| | PCP | 10 | 10 | 10.4 | 100.0 |

**Table 28. LOQ for drugs analyzed by quantitative analysis in blood.**

| Drug Class | Drug | n | Target Conc. (ng/mL) | % CV | %Accuracy |
|---|---|---|---|---|---|
| Amphetamines | AMP | 6 | 25 | 3.1 | 99.3 |
| | MAMP | 6 | 25 | 5.5 | 97.5 |
| | MDA | 6 | 25 | 8.1 | 96.4 |
| | MDEA | 6 | 25 | 14.2 | 90.0 |
| | MDMA | 6 | 25 | 7.3 | 97.6 |
| Antidepressants | Amitriptyline | 6 | 15 | 6.0 | 98.6 |
| | Citalopram | 6 | 15 | 7.0 | 95.6 |
| | Nortriptyline | 6 | 15 | 8.6 | 98.8 |
| | Trazodone | 6 | 15 | 8.6 | 99.0 |
| Benzodiazepines | Alprazolam | 10 | 10 | 12.0 | 99.2 |
| | α-hydroxyalprazolam | 10 | 25 | 11.3 | 95.5 |
| | Clonazepam | 10 | 10 | 7.7 | 99.8 |
| | 7-aminoclonazopam | 10 | 25 | 6.9 | 102.1 |
| | Diazepam | 10 | 10 | 4.5 | 100.8 |
| Miscellaneous | Cocaine | 10 | 10 | 11.7 | 90.7 |
| | BZE | 10 | 10 | 3.5 | 91.5 |
| | PCP | 10 | 10 | 9.9 | 95.9 |

### 3.3.4  Evaluation of Interference and Carryover

Based on known interferences for some drug classes, interference studies were focused on a select group of analytes undergoing quantitative analysis. **Table 29** presents results for the investigation of AMPs and COC/BZE with potential interferences (see Table 8). AMPS samples spiked at 200 ng/mL contained the potential interferences ephedrine, pseudoephedrine, PPA, and phentermine at 50,000 ng/mL. The mean results show that the target concentration of with AMP, MDA, MDEA, and MDMA had a %CV < 9, confirming the absence of interference. MAMP calculated concentration was greater than the ULOL. This was to be expected because MAMP and phentermine are isomers and share a [M+H]+ value of 150.120 m/z. COC and BZE samples spiked at 40 ng/mL contained the potential interferences norcocaine and scopolamine at 25,000 ng/mL. The results show a mean concentration for COC and BZE > 25% of the target concentration. Again, this was expected because BZE and norcocaine are isomers share the same [M+H]+ value of 290.139 m/z. Likewise, COC and scopolamine are also isomers with the same [M+H]+ value of 304.154 m/z. The evaluation of blank sample matrix resulted in no interference from the ISTD for all analytes. As previously discussed, the presence of interference in blank samples was not uncommon. As a result, it was considered acceptable if the peak was < 20% of the LOQ.

For carryover, the results show that no drugs had carryover except clonazepam in urine and MDA in blood and urine.

**Table 29. Summary of comparison of interference samples containing potential interferences using mean quantitative results of LDTD-MS/MS analysis.**

| Drug Class | Drug | n | Mean (Target 200 ng/mL) | %CV |
|---|---|---|---|---|
| **Amphetamines** | AMP | 5 | 233.2 | 5.2 |
| | MAMP | 5 | >ULOL | NR |
| | MDA | 5 | 241.2 | 8.1 |
| | MDEA | 5 | 212.2 | 4.5 |
| | MDMA | 5 | 215.4 | 8.9 |
| **Miscellaneous** | Cocaine | 5 | >ULOL | NR |
| | BZE | 5 | 130.4 | 2.3 |

### 3.3.5  Matrix Effect Evaluation

ME and relative ME were assessed for quantitative analysis of antidepressants, AMPs, benzodiazepines, and miscellaneous drugs in urine and blood. **Table 30** summarizes the results of ME analysis for analytes and ISTD in urine. A ME value >100% indicates ion enhancement, while a value < 100% indicates ion suppression.

> Urine Matrix Effect Summary
>
> ➤ Relative ME ranged from 9.4 to 84.7%

A ME with a low %CV indicates a small relative ME, while a ME with a high %CV indicates a large relative ME. In urine, the antidepressants and benzodiazepines all had ME greater than 140%, indicating ion enhancement. Antidepressant ME ranged from 152.8 to 271.8%, with

trazodone being the highest and amitriptyline being the lowest. ME for benzodiazepines ranged from 141.10 to 286.8%, with 7-aminoclonazepam being the highest and clonazepam being the lowest. MEs for amphetamines and miscellaneous drugs in urine were within $100 \pm 20\%$, with the exception of MDA (149.8%).

These large MEs are not unexpected in a process involving thermal desorption, as highly different processes can take place for neat solutions and extracted matrices and do not invalidate the method. The more important characteristic to consider is the presence or absence of a relative ME and the comparison of ME across different lots of urine or blood, with the absence of relative ME being highly desirable. The relative ME, expressed at %CV, from 10 lots ranged from 9.4 to 84.7, with amitriptyline being the lowest and α-hydroxyalprazolam being the highest in urine. Although the high %CV values are indicative of relative ME, subsequent input from LDTD users suggest that insufficient heating and low laser pattern temperature may have had a negative effect on our reproducibility. Future studies should include determining if further optimization of experimental conditions could mitigate the appearance of this relative ME.

The RE across all analytes in urine ranged from 30.0 to 186.3%, with MDEA being the highest and α-hydroxyalprazolam the lowest.

In all urine samples, the internal standard behaved similar to the analyte with respect to ME and RE. Despite analytes experiencing significant MEs in urine, the %CV was less than 20 at their targeted concentration in the matrix study. **Table 31** summarizes the accuracy and precision for analytes in urine ME study. Note that BZE does not have accuracy and precision, as it was done at the beginning, prior to including a calibration curve with MEs, but all further analytes included a curve.

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**Table 30. Matrix effect for drugs and ISTD analyzed by quantitative analysis in urine.**

| Drug Class | Drug | Target Ion Response | | ISTD Ion Response | | Relative Matrix Effect from 10 lots |
|---|---|---|---|---|---|---|
| | | ME (%) | RE (%) | ME (%) | RE (%) | %CV |
| **Antidepressants** | Amitriptyline | 152.8 | 77.1 | 155.4 | 73.3 | 9.4 |
| | Nortriptyline | 179.1 | 72.2 | 184.8 | 72.0 | 25.3 |
| | Citalopram | 174.4 | 61.4 | 188.9 | 55.6 | 12.2 |
| | Trazodone | 271.8 | 39.0 | 267.0 | 41.4 | 23.5 |
| **Amphetamines** | AMP | 112.1 | 67.6 | 70.9 | 155.3 | 33.0 |
| | MAMP | 106.4 | 70.1 | 75.6 | 137.1 | 42.3 |
| | MDA | 123.7 | 74.1 | 89.5 | 154.1 | 39.1 |
| | MDEA | 85.8 | 186.3 | 82.4 | 184.6 | 76.3 |
| | MDMA | 98.6 | 178.2 | 99.5 | 186.7 | 79.3 |
| **Benzodiazepines** | Alprazolam | 175.6 | 50.6 | 175.9 | 50.2 | 44.6 |
| | Clonazepam | 141.1 | 49.7 | 151.0 | 50.3 | 45.1 |
| | Diazepam | 157.5 | 68.9 | 167.3 | 68.2 | 19.1 |
| | α-hydroxyalprazolam | 253.2 | 30.0 | 258.3 | 29.3 | 84.7 |
| | 7-aminoclonazopam | 286.8 | 69.7 | 295.7 | 69.5 | 36.4 |
| **Miscellaneous** | BZE | 109.3 | 89.8 | 110.7 | 94.9 | 19.1 |
| | cocaine | 99.0 | 83.9 | 91.1 | 86.2 | 20.6 |
| | PCP | 86.0 | 95.6 | 83.8 | 101.1 | 40.6 |

**Table 31. Accuracy and precision for analytes in urine matrix effect study.**

| Drug Class | Drug | Sample Type | Mean (ng/mL) | %CV | % Accuracy |
|---|---|---|---|---|---|
| Antidepressants | Amitriptyline (200 ng/mL) | A | 222.6 | 4.4 | 111.3 |
| | | B | 219.7 | 5.1 | 109.8 |
| | | C | 230.1 | 4.7 | 115.0 |
| | Nortriptyline (200 ng/mL) | A | 180.2 | 5.7 | 90.1 |
| | | B | 173.1 | 5.5 | 86.5 |
| | | C | 175.7 | 6.7 | 87.8 |
| | Citalopram (200 ng/mL) | A | 216.3 | 3.1 | 108.1 |
| | | B | 200.56 | 4.8 | 100.2 |
| | | C | 222.56 | 4.6 | 111.2 |
| | Trazodone (200 ng/mL) | A | 185.4 | 3.5 | 92.7 |
| | | B | 189.2 | 4.4 | 94.6 |
| | | C | 177.20 | 5.5 | 88.6 |
| Amphetamines | AMP ( 500 ng/mL) | A | 483.10 | 5.7 | 96.6 |
| | | B | 511.60 | 7.4 | 102.3 |
| | | C | 493.10 | 4.6 | 98.6 |
| | MAMP ( 500 ng/mL) | A | 488.00 | 11.1 | 97.6 |
| | | B | 465.60 | 15.7 | 93.1 |
| | | C | 495.90 | 6.8 | 99.1 |
| | MDA (500 ng/mL) | A | 487.20 | 6.1 | 97.4 |
| | | B | 510.10 | 7.0 | 102.0 |
| | | C | 503.30 | 6.0 | 100.6 |
| | MDEA (500 ng/mL) | A | 454.60 | 6.3 | 90.9 |
| | | B | 466.50 | 10.6 | 93.3 |
| | | C | 476.80 | 4.3 | 95.3 |
| | MDMA (500 ng/mL) | A | 457.70 | 5.9 | 91.5 |
| | | B | 498.30 | 8.9 | 99.6 |
| | | C | 477.90 | 6.8 | 95.5 |
| Benzodiazepines | Alprazolam (100 ng/mL) | A | 93.72 | 7.1 | 93.7 |
| | | B | 94.47 | 11.8 | 94.4 |
| | | C | 94.34 | 10.8 | 94.3 |
| | Clonazepam (100 ng/mL) | A | 97.18 | 5.4 | 97.1 |
| | | B | 90.68 | 10.6 | 90.6 |
| | | C | 89.90 | 8.6 | 89.9 |
| | Diazepam (100 ng/mL) | A | 89.25 | 6.7 | 89.2 |
| | | B | 85.10 | 14.9 | 85.1 |
| | | C | 86.81 | 16.1 | 86.8 |
| | α-hydroxyalprazolam (100 ng/mL) | A | 89.66 | 5.7 | 89.6 |
| | | B | 87.56 | 10.5 | 87.5 |
| | | C | 89.07 | 12.8 | 89.0 |

(continued)

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**Table 31. Accuracy and precision for analytes in urine matrix effect study (continued).**

| Drug Class | Drug | Sample Type | Mean (ng/mL) | %CV | % Accuracy |
|---|---|---|---|---|---|
| **Miscellaneous** | 7-Aminoclonazepam (100 ng/mL | A | 88.28 | 8.4 | 88.2 |
| | | B | 86.45 | 12.0 | 86.4 |
| | | C | 81.89 | 15.6 | 81.8 |
| | COC (100 ng/mL) | A | 106.77 | 7.2 | 106.7 |
| | | B | 115.5 | 3.5 | 115.5 |
| | | C | 113.5 | 17.3 | 113.5 |
| | PCP (25 ng/mL) | A | 24.20 | 4.6 | 96.8 |
| | | B | 26.07 | 11.1 | 104.3 |
| | | C | 23.38 | 5.3 | 93.5 |

**Table 32** summarizes the results of ME analysis for analytes and ISTD in blood. All analytes had a ME greater than 125%, with the exception of the AMPs, BZE, and PCP indicating ion enhancement. ME for antidepressants ranged from 139.6 to 285.4%, with trazodone being the highest and amitriptyline being the lowest. ME for AMPs ranged from 46.0 to 119.3%, with AMP being the highest and MDMA the lowest. Benzodiazepines had the highest overall MEs that ranged from 123.1 to 238.8%, with α-hydroxyalprazolam being the highest and diazepam being the lowest. As stated earlier, this was to be expected due to the difference in desorption of a drug spiked into matrix compared to a neat solution. The RE across all analytes in blood ranged from 35.7 to 194.6%, with MDMA being the highest and trazodone the lowest. The relative ME, expressed as %CV, from 10 lots ranged from 22.3 to 77.0%, with MAMP being the lowest and BZE being the highest in blood. Although the high %CV values are indicative of relative ME, subsequent input from LDTD users suggest that insufficient heating and low laser pattern temperature may have had a negative effect on our reproducibility. Future studies should include determining if further optimization of experimental conditions could mitigate the appearance of this relative ME In all blood samples, the internal standard behaved similar to the analyte with respect to ME and RE. Despite analytes experiencing ME in blood, the %CV was less than 20 at their targeted concentration in the matrix study, with the exception of citalopram, COC, and BZE post-extraction and 7-aminoclonazepam neat and pre-extraction sample. In those four cases, the accuracy was within 20%, except COC post-extraction at 120.8%. **Table 33** summarizes the accuracy and precision for analytes in blood matrix effect study.

Blood Matrix Effect Summary

➢ Relative ME ranged from 22.3 to 77.0%

**Table 32. Matrix effect for drugs and ISTD analyzed by quantitative analysis in blood.**

| Drug Class | Drug | Target Ion Response | | ISTD Ion Response | | Relative Matrix Effect across 10 Lots |
|---|---|---|---|---|---|---|
| | | ME (%) | RE (%) | ME (%) | RE (%) | %CV |
| **Antidepressants** | Amitriptyline | 139.6 | 87.7 | 137.7 | 92.6 | 34.9 |
| | Nortriptyline | 195.3 | 54.1 | 203.7 | 54.5 | 41.2 |
| | Citalopram | 176.3 | 71.9 | 191.6 | 66.9 | 33.7 |
| | Trazodone | 285.4 | 35.7 | 266.2 | 36.5 | 39.0 |
| **Amphetamines** | AMP | 119.3 | 67.0 | 119.1 | 67.98 | 25.3 |
| | MAMP | 80.2 | 103.3 | 76.6 | 111.4 | 22.3 |
| | MDA | 113.3 | 105.0 | 65.8 | 109.3 | 36.3 |
| | MDEA | 62.2 | 175.1 | 57.2 | 187.1 | 36.7 |
| | MDMA | 46.0 | 194.6 | 40.8 | 208.9 | 47.8 |
| **Benzodiazepines** | Alprazolam | 173.5 | 42.4 | 181.8 | 43.1 | 36.9 |
| | Clonazepam | 150.3 | 41.2 | 147.5 | 42.7 | 34.0 |
| | Diazepam | 123.1 | 46.5 | 119.6 | 47.8 | 27.1 |
| | α-hydroxyalprazolam | 238.8 | 68.2 | 224.7 | 68.1 | 25.8 |
| | 7-aminoclonazepam | 188.3 | 131.6 | 151.5 | 151.1 | 39.4 |
| **Miscellaneous** | BZE | 102.8 | 178.7 | 106.1 | 183.9 | 77.0 |
| | COC | 75.4 | 106.4 | 74.9 | 106.1 | 22.5 |
| | PCP | 86.0 | 95.6 | 83.8 | 101.1 | 40.6 |

**Table 33. Accuracy and precision for analytes in blood matrix effect study.**

| Drug Class | Drug | Sample Type | Mean (ng/mL) | %CV | % Accuracy |
|---|---|---|---|---|---|
| **Antidepressants** | Amitriptyline (200 ng/mL) | A | 215.8 | 6.7 | 107.9 |
| | | B | 222.6 | 9.5 | 111.3 |
| | | C | 207.6 | 5.4 | 103.8 |
| | Nortriptyline (200 ng/mL) | A | 200.6 | 7.6 | 100.3 |
| | | B | 195.1 | 5.2 | 97.5 |
| | | C | 191.9 | 6.5 | 95.9 |
| | Citalopram (200 ng/mL) | A | 208.5 | 5.6 | 104.2 |
| | | B | 172.00 | 34.4 | 86.0 |
| | | C | 205.70 | 3.0 | 102.8 |
| | Trazodone (200 ng/mL) | A | 194.4 | 6.9 | 97.2 |
| | | B | 208.8 | 5.4 | 104.4 |
| | | C | 203.80 | 9.2 | 101.9 |
| **Amphetamines** | AMP ( 500 ng/mL) | A | 519.30 | 3.3 | 103.8 |
| | | B | 520.80 | 4.3 | 104.1 |
| | | C | 513.10 | 4.1 | 102.6 |
| | MAMP ( 500 ng/mL) | A | 507.70 | 4.1 | 101.5 |
| | | B | 529.00 | 5.4 | 105.8 |
| | | C | 492.50 | 3.6 | 98.8 |
| | MDA (500 ng/mL) | A | 486.30 | 8.4 | 97.2 |
| | | B | 518.80 | 10.6 | 103.7 |
| | | C | 499.00 | 6.1 | 99.8 |
| | MDEA (500 ng/mL) | A | 540.2 | 14.8 | 108.0 |
| | | B | 578.1 | 15.7 | 115.6 |
| | | C | 581.00 | 14.8 | 116.2 |
| | MDMA (500 ng/mL) | A | 514.9 | 8.03 | 102.9 |
| | | B | 558.00 | 7.2 | 111.6 |
| | | C | 521.5 | 8.1 | 104.3 |
| **Benzodiazepines** | Alprazolam (100 ng/mL) | A | 117.10 | 6.5 | 117.1 |
| | | B | 111.26 | 8.3 | 111.2 |
| | | C | 110.60 | 6.9 | 110.6 |
| | Clonazepam (100 ng/mL) | A | 119.10 | 6.3 | 119.1 |
| | | B | 121.40 | 6.3 | 121.4 |
| | | C | 115.1 | 7.8 | 115.1 |
| | Diazepam (100 ng/mL) | A | 115.5 | 5.6 | 115.5 |
| | | B | 119.10 | 6.5 | 119.1 |
| | | C | 115.4 | 7.0 | 115.4 |
| | α-hydroxyalprazolam (100 ng/mL) | A | 92.3 | 6.52 | 92.3 |
| | | B | 98.16 | 11.6 | 98.1 |
| | | C | 98.05 | 10.3 | 98.0 |
| | 7-aminoclonazepam (100 ng/mL | A | 95 | 23.1 | 95.0 |
| | | B | 120.05 | 15.2 | 120.0 |
| | | C | 102.82 | 24.7 | 102.8 |

(continued)

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**Table 33. Accuracy and precision for analytes in blood matrix effect study (continued).**

| Drug Class | Drug | Sample Type | Mean (ng/mL) | %CV | % Accuracy |
|---|---|---|---|---|---|
| **Miscellaneous** | BZE (100 ng/mL) | A | 114.40 | 6.1 | 114.4 |
| | | B | 110.27 | 26.5 | 110.2 |
| | | C | 106.08 | 5.1 | 106.0 |
| | COC (100 ng/mL) | A | 115.10 | 5.06 | 115.1 |
| | | B | 120.86 | 26.3 | 120.8 |
| | | C | 115.90 | 5.4 | 115.9 |
| | PCP (25 ng/mL) | A | 20.79 | 5.2 | 83.1 |
| | | B | 20.95 | 4.1 | 83.7 |
| | | C | 20.90 | 9.4 | 83.6 |

### 3.3.6 Stability Assessment

The results of the stability evaluation in which QC samples were spotted into sample wells and analyzed at 0, 6, and 15 hours showed that the accuracy of all analytes was stable up to 15 hours for urine and blood.

### 3.3.6.1 Postmortem Sample Analysis

Postmortem blood samples were analyzed with LDTD and compared to previously reported Medical Examiner results. **Table 34** summarizes concentrations of MDMA, AMP, MAMP, alprazolam, diazepam, COC, and BZE analyzed by LDTD. BZE samples K, L, and M were also analyzed on a LC-MS/MS at RTI. The % difference ranges from 8.8 to 63.1%.

**Table 34. Postmortem blood concentrations determined with LDTD compared to laboratory report.**

| Sample | Analyte | Average LDTD Concentration (ng/mL) | Laboratory Report (ng/mL) | % difference |
|---|---|---|---|---|
| A | MDMA | 69.6 | 80 | 14.9 |
| B | MDMA | 496 | 590 | 18.9 |
| C | AMP | 544 | 670 | 23.1 |
| D | AMP | 141.5 | 130 | 8.8 |
| E | MAMP | 6560 | 7400 | 12.8 |
| F | MAMP | 843.5 | 860 | 1.9 |
| G | Alprazolam | 42.3 | 66 | 56.0 |
| H | Alprazolam | 35.9 | 48 | 33.7 |
| I | Diazepam | 1120 | 860 | 30.2 |
| J | BZE | 423 | 690 | 63.1 |
| K | BZE | 711 | 970 | 36.4 |
| L | BZE | 292 | 380 | 30.1 |

## 3.4   Validation Results for Screening Method Analysis

### 3.4.1 Evaluation of LOD

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**Tables 35 and 36** presents the LOD concentrations for each drug in urine and blood, respectively, with an n=18, unless otherwise noted. In urine, the LOD values ranged from 0.1 to 50 ng/mL, with UR-144 N-(5-bromopently) analog having the highest while JWH-018 N-(5-hydroxypenyl) metabolite and JWH-122 5-hydroxypentyl metabolite have the lowest LOD concentrations. Only the synthetic cathinones and piperazines were assessed in blood for screening analysis. In blood, the LOD ranged from 1 to 10 ng/mL, with mCPP being the highest. In general, the LOD were similar for each analyte across the two matrices. LOD for synthetic cathinones and piperazines in blood were either the same concentration or lower than those in urine with the exception of buphedrone. **Figure 18** presents a typical MS ion transition desorption peak of the LOD represented by urine fortified with MDPV at a concentration of 2 ng/mL. Reference **Appendix D** for MS ion transition peaks for the LOD of all analytes in urine and blood for screening analysis.

> LOD Summary
>
> ➢ Urine: 0.1-50 ng/mL
>
> ➢ Blood: 1-10 ng/mL
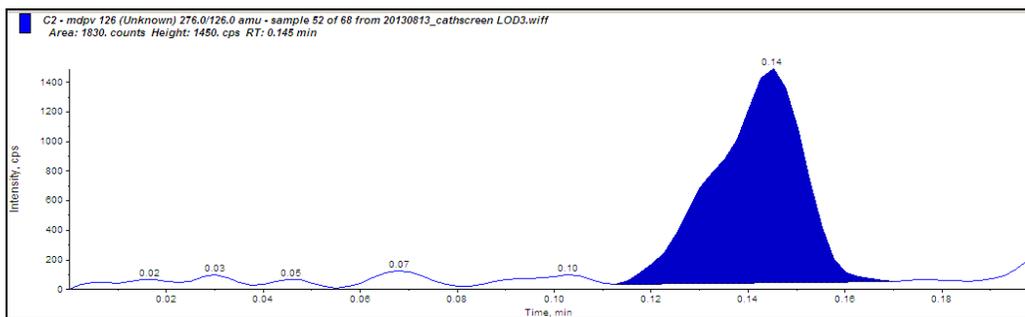


**Figure 18. MS ion transition peak of MDPV at a LOD of 2 ng/mL in urine.**

**Table 35. LOD for drugs analyzed by screening analysis in urine.**

| Drug Class | Drug | n | LOD (ng/mL) |
|---|---|---|---|
| **Designer Drugs Indanes** | 2-AI | 18 | 1 |
| | 5-IT | 18 | 5 |
| | MDAI | 18 | 2 |
| **Designer Drugs Synthetic Cannabinoids** | AKB-48 | 18 | 1 |
| | EAM-2201 | 18 | 5 |
| | JWH-018 | 18 | 0.5 |
| | JWH-073 | 18 | 1 |
| | MAM-2201 | 18 | 0.5 |
| | STS-135 | 18 | 5 |
| | UR-144 | 18 | 5 |
| | UR-144 N-(5-bromopentyl) | 18 | 50 |
| | UR-144 N-(5-chloropentyl) | 18 | 2 |
| | XLR11 N-(4-pentenyl) | 18 | 5 |
| | XLR-11 | 18 | 1 |

(continued)

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**Table 35. LOD for drugs analyzed by screening analysis in urine (continued).**

| Drug Class | Drug | n | LOD (ng/mL) |
|---|---|---|---|
| **Designer Drugs Synthetic Cannabinoid Metabolites** | JWH-018 N-(5-hydroxypentyl) | 18 | 0.1 |
| | JWH-073 N-(4-hydroxybutyl) | 18 | 0.5 |
| | JWH-122 5-hydroxypentyl | 18 | 0.1 |
| | JWH-200 4-hydroxyindole | 18 | 2 |
| **Designer Drugs Synthetic Cathinones and Piperazines** | 4-MEC | 18 | 5 |
| | 4-MePPP | 18 | 5 |
| | α-PVP | 18 | 5 |
| | Buphedrone | 18 | 1 |
| | Flephedrone | 18 | 5 |
| | MCPP | 18 | 20 |
| | MDPV | 18 | 2 |
| | Methylone | 18 | 5 |
| | TFMPP | 18 | 1 |
| **Designer Drugs Tryptamines** | 5-MEO-AMT | 16 | 2 |
| | 5-MEO-DiPT | 16 | 5 |
| | 5-MEO-DMT | 18 | 5 |
| | 5-MEO-MiPT | 18 | 5 |

**Table 36. LOD for drugs analyzed by screening analysis in blood.**

| Drug Class | Drug | n | LOD (ng/mL |
|---|---|---|---|
| **Designer Drugs Synthetic Cathinones and Piperazines** | 4-MEC | 18 | 2 |
| | 4-MePPP | 18 | 5 |
| | α -PVP | 18 | 5 |
| | Buphedrone | 18 | 2 |
| | Flephedrone | 18 | 5 |
| | MCPP | 18 | 10 |
| | MDPV | 18 | 2 |
| | Methylone | 18 | 1 |
| | TFMPP | 18 | 1 |

### 3.4.2 Evaluation of Interference and Carryover

Spice products containing synthetic cannabinoids and bath salt products with synthetic cathinones and other designer drugs are constantly altering their package components due to compounds becoming regulated. These structure changes are only slight modifications, resulting in many isomers and isobars for designer drugs. LDTD lacks the ability to separate such compounds; therefore, it poses a greater risk of compound interferences. However, such

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compounds were evaluated to determine their effect on identification and analysis. **Table 37** summarizes the compounds analyzed for interference and their [M+H]+ values. JWH metabolites were at a target concentration of 20 ng/mL, while all other designer drugs were 100 ng/mL. As expected, JWH-018 2-hydroxyindole, JWH-073 5-hydroxyindole, 2, 3-MDPV, 3-FMC, mephedrone, α-pbp, and pentedrone all interfered with their positional isomers. MDAI has the same nominal mass as 6-APDB and mephedrone, but not the same exact mass, which can be resolved on a high-resolution instrument. 6-APDB and mephedrone spiked at 25,000 ng/mL and analyzed under the MDAI acquisition method showed interference at MDAI MRM transitions. Their peak area was higher than the MDAI 100 ng/mL sample. Buphedrone did not show interference for MDAI when it was ran as an interferant. However, when MDAI was ran as an interferant against buphedrone, there was interference with the analysis. 5-IT interfered with analysis of 5-MeOAMT. 4-OH-MiPT was an interferant for 5-MeO-Mipt analysis. Methylone, 4-OH-DiPT, and 2-AI showed interference for 5-MeO-DiPT analysis. 5-MeO-AMT interfered with TFMPP acquisition. In urine, with the exception of 2-AI, buphedrone, 4-MEC, XLR-11, UR-144, and XLR-11 4-pentenyl carryover was not observed. In blood, carryover was observed for mCPP.

**Table 37. Summary of compounds analyzed for interferences.**

| Target Analyte | Target Analyte [M+H]+ | Interference Compound | Interference Compound [M+H]+ |
|---|---|---|---|
| JWH-018 N-(5-hydroxypentyl) | 358.1801 | JWH-018 2-hydroxyindole | 358.1801 |
| JWH-073 N-(4-hydroxy butyl) | 344.1566 | JWH-073 5-hydroxyindole | 344.1566 |
| 3,4-MDPV | 276.1594 | 2,3-MDPV | 276.1594 |
| Flephedrone (4-FMC) | 182.0975 | 3-FMC<br>2C-H | 182.0975<br>182.1175 |
| Buphedrone<br>MDAI | 178.1226<br>178.0862 | 6-APDB<br>Mephedrone | 178.1226<br>178.1226 |
| 4-MePPP | 218.1539 | α-PBP | 218.1539 |
| 4-MEC | 192.1382 | Pentedrone | 192.1382 |
| TFMPP | 231.1103 | 5-MEO-AMT | 205.1335 |
| 5-MEO-AMT | 205.1335 | 5-IT | 175.1229 |
| 5-MEO-MiPT | 247.1804 | 4-OH-MiPT | 233.1648 |
| 5-MEO-DiPT | 275.2117 | Methylone<br>4-OH-Dipt<br>2-AI | 208.0968<br>261.1961<br>134.0964 |

### 3.4.3 Evaluation of Matrix Effect

ME was assessed for screening analysis in urine and blood. **Table 38** summarizes the results of ME analysis for analytes and ISTD in urine. Only one ISTD was used for each acquisition batch for the designer drugs. ISTDs were chosen based off similar structure and functional groups to the analytes of interest because of the lack of

> Matrix Effect Summary
>
> ➢ Relative ME ranged from 5.7 to 137.9%

deuterated ISTDs. ME values for all analytes and ISTDs in urine were greater than 125%, with the exception of 2-AI (113.4%) and XLR-11 (124%) indicating ion enhancement. The ME for the indane group ranged from 113.4 to 821.5%, with 5-IT being the highest and 2-AI being the lowest. Synthetic cannabinoid had a range of ME from 124.1 to 138.6%, with STS-135 being the highest and AKB-48 being the lowest. The ME for synthetic cannabinoids was the most consistent between the analytes compared to all other groups. The range of ME for synthetic cannabinoid metabolites was from 128.2% for JWH-122 5-hydroxypentyle metabolite to 168.9% for MAM 2201-$d_5$ ISTD. The RE for all analyte groups in urine ranged from 30.3 to 157.1%, with XLR11 N-(4-pentenyl) being the highest and 5-MEO-AMT being the lowest. The relative ME, expressed at %CV, for 10 lots ranged from 5.7 to 137.9 % with JWH-073 being the lowest and 5-MEO-DipT being the highest in urine. Although the high %CV values are indicative of relative ME, subsequent input from LDTD users suggest that insufficient heating and low laser pattern temperature may have had a negative effect on our reproducibility. Future studies should include determining if further optimization of experimental conditions could mitigate the appearance of this relative ME.

**Table 38. Matrix effect for drugs analyzed by screening analysis in urine.**

| Drug Class | Drug | Target Ion Response | | Relative Matrix Effect from 10 Lots |
|---|---|---|---|---|
| | | ME (%) | RE (%) | %CV |
| **Designer Drugs Indanes** | 2-AI | 113.4 | 84.0 | 60.8 |
| | 5-IT | 821.5 | 34.5 | 87.8 |
| | MDAI | 180.1 | 57.7 | 51.1 |
| | Amitryptyline-$d_3$ | 161.5 | 66.5 | NA |
| **Designer Drugs Synthetic Cannabinoids** | AKB-48 | 127.2 | 65.2 | 8.7 |
| | EAM-2201 | 132.4 | 130.8 | 7.8 |
| | JWH-018 | 129.3 | 70.8 | 8.7 |
| | JWH-073 | 129.5 | 75.1 | 5.7 |
| | MAM-2201 | 132.7 | 71.1 | 8.9 |
| | STS-135 | 138.6 | 75.2 | 11.1 |
| | UR-144 | 129.3 | 70.7 | 6.7 |
| | UR-144 N-(5-bromopentyl) | 135.8 | 72.0 | 7.3 |
| | UR-144 N-(5-chloropentyl) | 131.4 | 137.2 | 7.7 |
| | XLR11 N-(4-pentenyl) | 129.3 | 157.1 | 19.3 |
| | XLR-11 | 124.1 | 77.4 | 8.0 |
| | MAM2201-$d_5$ | 135.7 | 70.5 | NA |

(continued)

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**Table 38. Matrix effect for drugs analyzed by screening analysis in urine (continued).**

| Drug Class | Drug | Target Ion Response | | Relative Matrix Effect from 10 Lots |
|---|---|---|---|---|
| | | ME (%) | RE (%) | %CV |
| **Designer Drugs Synthetic Cannabinoid Metabolites** | JWH-018 N-(5-hydroxypentyl) | 129.5 | 57.8 | 33.4 |
| | JWH-073 N-(4-hydroxybutyl) | 134.7 | 58.2 | 32.0 |
| | JWH-122 5-hydroxypentyl | 128.2 | 59.9 | 35.0 |
| | JWH-200 4-hydroxyindole | 153.3 | 70.5 | 10.2 |
| | MAM2201-$d_5$ | 168.9 | 68.2 | NA |
| **Designer Drugs Synthetic Cathinones and Piperazines** | 4-MEC | 160.9 | 74.2 | 36.4 |
| | 4-MePPP | 137.4 | 87.4 | 51.2 |
| | α-PVP | 136.2 | 90.4 | 42.6 |
| | Buphedrone | 169.7 | 68.1 | 65.3 |
| | Flephedrone | 224.8 | 61.5 | 33.7 |
| | MCPP | 278.1 | 61.4 | 45.4 |
| | Methylone | 177.6 | 72.3 | 61.2 |
| | MDPV | 142.0 | 87.9 | 66.0 |
| | TFMPP | 204.1 | 69.9 | 41.1 |
| | MAMP-$d_9$ | 127.0 | 90.6 | NA |
| **Designer Drugs Tryptamines** | 5-MEO-AMT | 643.9 | 30.3 | 60.1 |
| | 5-MEO-DiPT | 459.3 | 47.5 | 137.9 |
| | 5-MEO-DMT | 545.5 | 42.3 | 94.2 |
| | 5-MEO-MiPT | 460.9 | 46.4 | 99.7 |
| | Amitryptyline-$d_3$ | 161.5 | 66.5 | NA |

Only the synthetic cathinones and piperazines were assessed in blood for ME. **Table 39** summarizes the results of ME analysis for analytes and ISTD in blood. The ME effect in blood for most of the synthetic cathinones and piperazines were $100 \pm 10\%$, with the exception of α-PVP, buphedrone, flephedrone, and MDPV. The RE of analytes in blood ranged from 64.5 to 110.5%. All analytes had a RE of 82% or greater, with the exception of flephedrone. The PE in blood ranged from 84.5 to 105.6%, with α-PVP being the highest and flephedrone being the lowest. The %CV for sample B for Relative Matrix effect from 10 lots ranged from 25.6 to 39.0%, with 4-MePP being the lowest and buphedrone being the highest in blood. Overall, ME and RE were more acceptable in blood matrix than urine.

**Table 39. Matrix effect for drugs analyzed by screening analysis in blood.**

| Drug Class | Drug | Target Ion Response | | Relative Matrix Effect from 10 Lots |
|---|---|---|---|---|
| | | ME (%) | RE (%) | %CV |
| Synthetic Cathinones and Piperazines | 4-MEC | 110.0 | 90.1 | 28.9 |
| | 4-MePPP | 101.0 | 95.4 | 25.6 |
| | α-PVP | 113.4 | 93.0 | 35.7 |
| | Buphedrone | 114.5 | 82.9 | 39.0 |
| | Flephedrone | 131.0 | 64.5 | 36.8 |
| | MCPP | 106.3 | 86.3 | 31.4 |
| | MDPV | 113.0 | 91.8 | 34.9 |
| | Methylone | 90.6 | 100.5 | 28.7 |
| | TFMPP | 98.2 | 97.9 | 31.9 |
| | MAMP-d9 | 94.6 | 110.5 | — |

### 3.4.4 Analysis of Spice Samples

A 100 ng/mL standard of JWH-018 and JWH-073 was analyzed with the samples. The peak area of all four Spice samples was greater than the 100 ng/mL peak and was considered to contain JWH-018 (**Table 40**). Pink Tiger and Humboldt Gold had a peak area higher than the JWH-073 standard and was considered to contain JWH-073, but not JWH-018.

**Table 40. List of Spice samples and confirmed synthetic cannabinoid.**

| Type | Description | JWH-018 | JWH-073 |
|---|---|---|---|
| Happy Shaman Herbs | Humboldt Gold | X | X |
| Happy Shaman Herbs | Pink Tiger | X | X |
| Southern Spice | Herbal incense (mango) | X | |
| Hot Hawaiian | Exotic Herbal Blend | X | |

## 4. CONCLUSIONS

## 4.1 Discussions and Findings

### 4.1.1 LDTD System

The LDTD source provided rapid analysis of samples used in this study; however, the issue of manually pipetting the samples into the sample wells was a source of concern for three reasons. First, in order to achieve reproducible results, the analyst must be able to spot a small amount of sample into a small well. This can be achieved with a little practice, but it is imperative that the analyst be able to reproducibly spot samples for analysis in order to achieve optimal results; therefore, precision should be assessed as part of instrument training. Secondly, since the spotting sample size is minimal and typically consists of the analyte (s) in a solvent, it is important to be diligent when spotting samples to ensure that a sample well has not been omitted

or spotted more than once with a different sample because evaporation occurs quickly and it is often difficult to distinguish a well with dried sample from an unused well. Third, analysts must be diligent when moving from one well to another in order to prevent cross contamination. Although we did not propose the evaluation of automated liquid handler workstations, we recommend they be employed by high sample throughput laboratories.

The LDTD instrument was easy to operate with minimal maintenance, which consisted of periodically cleaning of the transfer tube. When maintenance was required, the source was easily removed and reattached. Since it requires no additional software, the time required for instrument operation training mainly consisted of introduction and operation of the MS software for which the analysts were unfamiliar. We experienced no hardware failures to date.

### 4.1.2  MS and LDTD Optimization for Drug Standards and Analytical Methods Set-Up

It was sufficient to optimize analytes and ISTDs using the same laser pattern of 3-45-2 when analyzed in pure solvents. The laser pattern was essential to optimize after extraction from matrix due to split peaks or other peak issues. It is necessary to have a balance between acceptable peak shape and sensitivity because the lower the laser power, the lower the sensitivity. Analysis of drugs of abuse in urine and blood showed that a quick laser pattern was effective in reducing a lot of the peak shape issues. Subsequent input from other LDTD users has indicated that similar improvements may have been achieved by optimizing the dilution factor at which extracts were loaded onto the plate; however, those approaches were not pursued as part of this project. Future studies with LDTD should also optimize with the flow rate to determine its effects on sensitivity. Using the Absciex 4000 MS series only allowed for approximately 10 transitions per acquisition method. Newer model MS systems allow for increased sensitivity and result in screening or quantitation of more compounds, such as 34 compounds in urine (Phytronix).

### 4.1.3  Sample Preparation

A requirement for extensive methods (e.g., solid phase extraction [SPE], back extractions, and/or derivitization) would significantly limit the application of this technology in forensic science laboratory work; therefore, the goal was to achieve qualitative or quantitative results with minimal sample preparation. Although protein crash is commonly used when minimal sample preparation is desired, based on manufacturer's recommendation, we began by employing minimal liquid-liquid extractions (LLE); however, in some instances, SPE was necessary to achieve better sensitivity.

### 4.1.4  Validation

Based on the data collected during quantitative analysis, the LDTD-MS/MS overall results demonstrated good precision and accuracy over a drug analyte range typically used in forensic laboratories. Dried samples were stable up to 15 hours in the well-plate.

When components from a sample matrix co-elute with the analytes of interest, they can interfere with the ionization process, causing suppression or enhancement of the MS signal. APCI has shown to be less susceptible to MEs than ESI (Ismaiel et al., 2008). If neutral species with a higher proton affinity than the analyte of interest are present in the gas phase, it may neutralize

the ionized analytes in ESI mode. Also, if co-eluting non-volatile compounds are present at a high concentration, they may prevent droplets from reaching the required surface charge and radius for ion transfer into the gas phase. One of the reasons that ACPI is less susceptible to ME may be due to the fact that in ACPI, the target analytes are in the gas phase prior to ionization. This reason, coupled with the fact that the samples introduced into the LDTD are solvent-free, should result in very low gas-phase ion suppression (online presentation). Boisvert and colleagues (2012) reported the importance of the washing step when analyzing pesticides and pharmaceuticals in wastewater samples in order to eliminate interfering contaminates that may co-elute. Large MEs are not unexpected in a process involving thermal desorption, as highly different processes can take place for neat solutions and extracted matrices and do not invalidate the method. The more important characteristic to consider is the presence or absence of a relative ME, which is the comparison of ME across different lots of urine or blood. The absence of relative ME is highly desirable. Although the high %CV values are indicative of relative ME, subsequent input from LDTD users suggest that insufficient heating and low laser pattern temperature may have a negative effect on reproducibility. Future studies should include determining if further optimization of experimental conditions could mitigate the appearance of this relative ME. For quantitative analysis, all % accuracies were $100 \pm 20\%$, with the exception of clonazepam (21%), with %CVs < 20, with the exception of BZE (26%). These values highlight the importance of stable isotope-labeled ISTDs when performing quantitative analysis.

The recovery of the many of the analytes were low; however, for the purpose of this research, we employed the extraction technique that required the minimum amount of sample preparation to achieve sensitivity and acceptable peak shape, precision, and accuracy. In order to determine if more extensive sample preparation would improve the recovery values, AMP and MDEA were reevaluated after SPE. The recoveries for both improved, indicating that a more extensive extraction technique would provide better recovery values for the analytes.

### 4.1.5  Interferences

One reason for limited application of LDTD is the presence of isomeric and isobaric compounds within particular drug classes. Since isomers have the same molecular weight, it makes identification and quantitation difficult or even impossible without chromatographic separation. An example of a drug class for which identification is difficult is synthetic cannabinoids. Many analogues will have the same product ions and will be therefore indistinguishable. In some drug classes, this issue can be overcome with derivitization. MOR (Figure 19 C) and HMOR (Figure (19 A) have the same primary mass (286). During analysis, MOR loses water (-18 amu); however, HMOR does not. Using a primary mass of 268 (285-18+1) produces a more specific mass for MOR. The primary mass 286 will generate a good signal, but the result will be the sum of MOR and HMOR because not all of the MOR will result in a loss of water. Derivitization with methoxamine will produce a specific transition for HYM, yielding a primary mass of 315. The same situation occurs with COD (D) and HCOD (B). Although derivitization makes it possible to differentiate between these opiate isomers, it no longer allows for minimal sample preparation, which is ideal in a high-throughput scenario; therefore, screening analysis of the opiates without derivitization was performed.
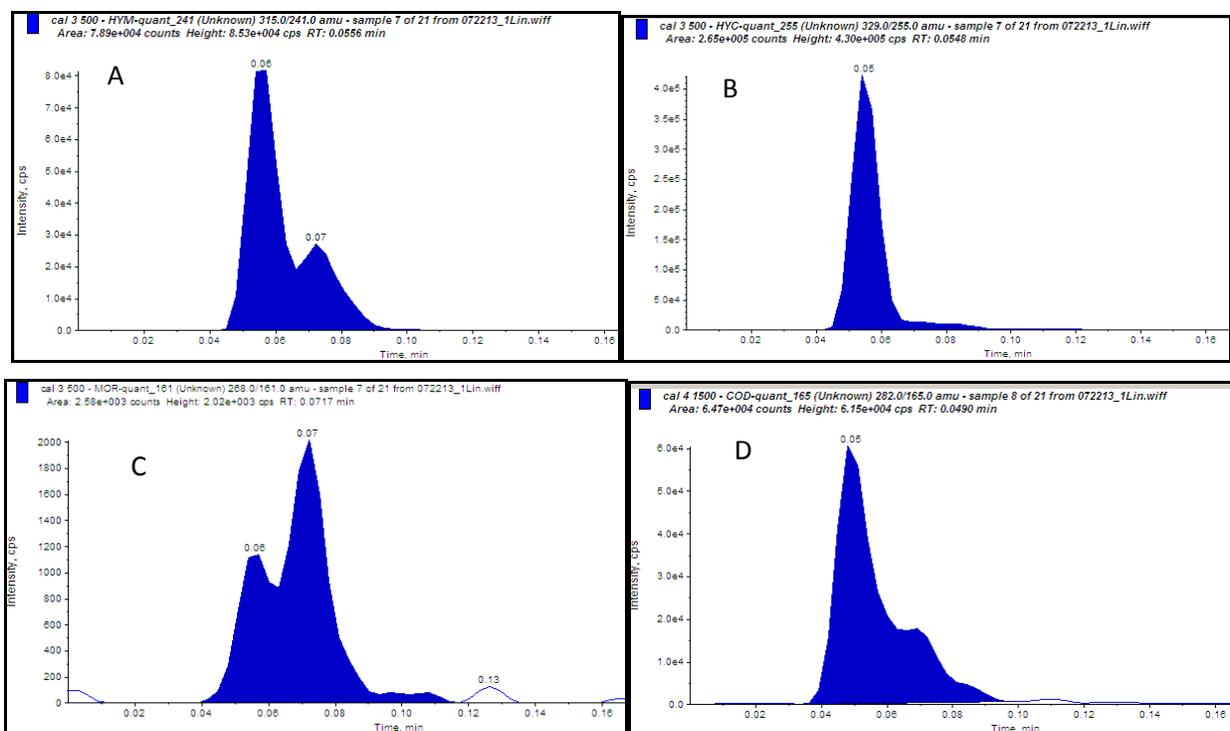
**Figure 19. MS ion ratio of HMOR (A), HCOD (B), MOR (C), and COD (D) after MOX derivitization.**

Other articles reference interferences but remedy the issue by selecting another transition for analysis. However, caution should be taken by selecting another product ion because it may not be as unique to the structure.

### 4.1.6  *Efficiency and Cost Effectiveness*

The LDTD system provided rapid sample analysis throughout this study. The efficiency for sample preparation and analysis was compared between LDTD-MS/MS and LC-MS/MS. The time required for the preparation (including aliquoting and fortifying calibrators, etc.) and quantitative analysis of 27 samples containing AMP, MAMP, MDA, and MDEA in blood were evaluated. The time for LLE of all samples analyzed by LDTD-MS/MS was approximately 40 minutes. For LC-MS/MS analysis, approximately one hour and thirty minutes was required for SPE of the samples. Two analytical methods, one for AMP and MAMP and another for MDA, MDEA, and MDMA, were required for LDTD-MS/MS analysis. This was done in order to have no more than 10 ion transitions per method. As a result, the samples were spotted twice, and each set was acquired under separate methods. The total time for instrument analysis for LDTD-MS/MS and LC-MS/MS were approximately 20 minutes and 3.3 hours, respectively. The run times per sample were 12 sec and 7.5 minutes for LDTD-MS/MS and LC-MS/MS, respectively. Although the need to reanalyze samples did not occur during this instrument comparison study, it is important to note that due to the destruction of samples during the desorption process, a sample must be respotted and analyzed if needed. Unlike LC-MS/MS for which a sample can be reinjected if needed. However, the time required for respotting and analysis is still less than that needed for reinjection and analysis by LC-MS/MS.

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Direct analysis of samples in LDTD does not include a chromatographic separation step; therefore, the need to purchase chromatographic consumables such as columns and mobile phase solvents was eliminated. During this study, the majority of the cost came from the purchase of 96-sample LazWell plates. These plates cost $75 per plate for 10–99 plates, $61 for 100–499 plates and $49 for 500–999 plates. The cost of autosampler vials, inserts, and caps was approximately $87, or $0.87 per sample. A LazWell plate (purchased for $75) had a per-sample cost of $0.78, resulting in a savings of $0.09 per sample. However, it is important to note that because sample wells cannot be reused, the cost per sample may increase slightly depending on the need for sample reanalysis. Although this instrument eliminates the need for chromatographic consumables, the majority of cost savings come from the reduced labor needed as a result of the rapid sample analysis. The high-throughput analysis ability of this system allows laboratory personnel to complete sample analyses and subsequent data reduction in a minimal amount of time. In addition, the rapid sample analysis means that more samples can be run on a single instrument, possibly reducing the number of mass spectrometers an individual laboratory may need to meet their analysis demands.

### 4.1.7  Implications for Policy and Practice in the United States

Forensic laboratories are often backlogged, creating budgetary and policy problems. Backlogs can potentially compromise investigations. Technologies such as LDTD-MS/MS have the potential to reduce screening time requirements and costs.

### 4.1.8  Implications for Further Research

Advantages of the LDTD system include (1) quick installation; (2) little instrument training; (3) no additional software requirement; (4) ease of use; (5) minimal maintenance; (6) rapid sample analysis; (7) minimal sample volume; (8) lack of chromatographic solvents and consumables; and (9) the ability to use across multiple MS platforms. Disadvantages include (1) sample destruction; (2) inconsistencies associated with manual spotting; (3) erroneous peaks due to interferences in sample matrix and peak apparitions as a result of the desorption process; (4) inability or difficulty analyzing isomers and isobars due to the lack of chromatography; and (5) the limited amount of ion transitions per method depending on the MS used. Overall, LDTD has the potential for use in screening and quantitation of biological samples in forensic laboratories. Its ability to rapidly analyze a large number of samples in a short amount of time (~12 sec per sample) makes it ideal for high-throughput forensic laboratories. However, due to the lack of chromatography and the inherent nature of the desorption process, laboratories must invest a significant amount of time in upfront method development to determine the best parameters and extraction techniques. Future studies should include determining if alternative sample preparation and LDTD parameters will eliminate the challenges of anomalous peaks and peak apparitions encountered during this study.

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## 5.1 Dissemination of Research Findings

Society of Forensic Toxicologists Annual Meeting. November 1, 2013 in Orlando, Fl.

American Academy of Forensic Science Annual Meeting. February 16-20 in Seattle Washington.

# APPENDIX A: PROD ION SCANS OF VALIDATED ANALYTES AND ISTD AT 10,000 NG/ML IN METHANOL

**Figure A-1. <u>Antidepressants</u>: Product ion scans of amitriptyline (A), amitriptyline-d$_3$ (B), citalopram (C), citalopram-d$_6$ (D), nortriptyline (E), nortriptyline-d$_3$ (F), trazodone (G), and trazodone-d$_6$ (H).**

74
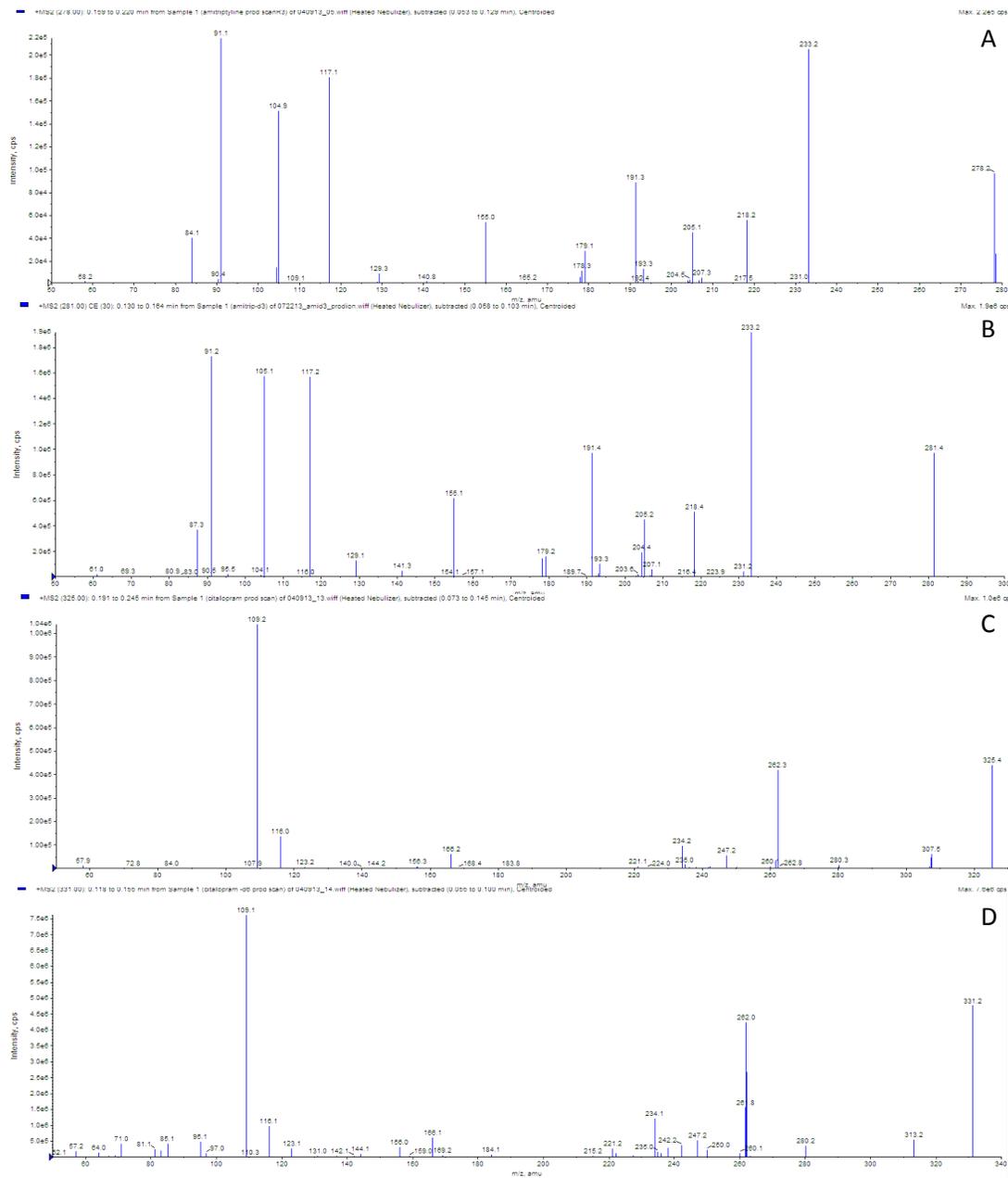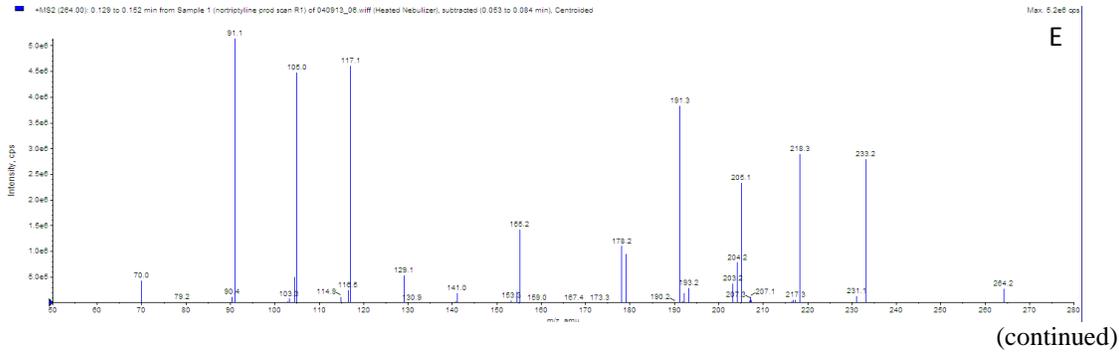
**Figure A-1. <u>Antidepressants</u>: Product ion scans of amitriptyline (A), amitriptyline-d$_3$ (B), citalopram (C), citalopram-d$_6$ (D), nortriptyline (E), nortriptyline-d$_3$ (F), trazodone (G), and trazodone-d$_6$ (H). (continued)**
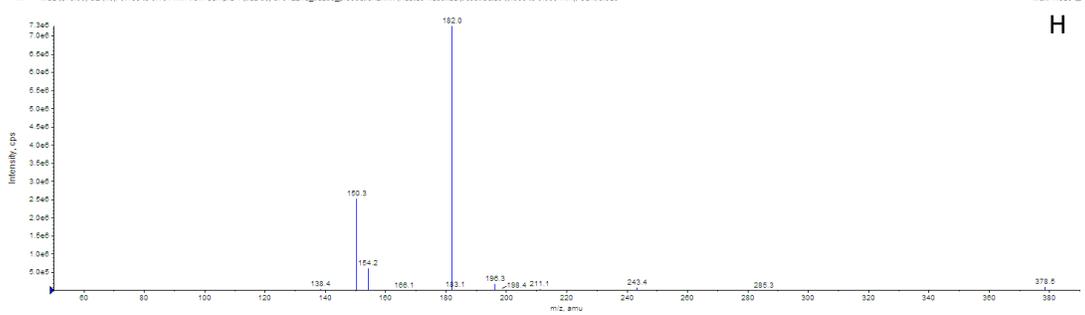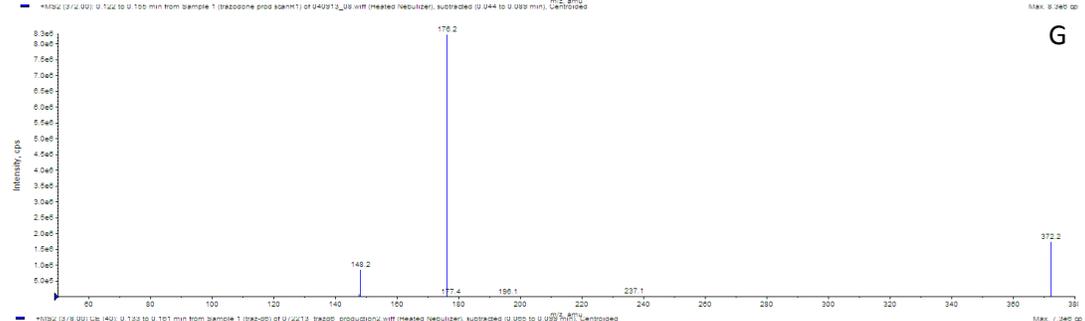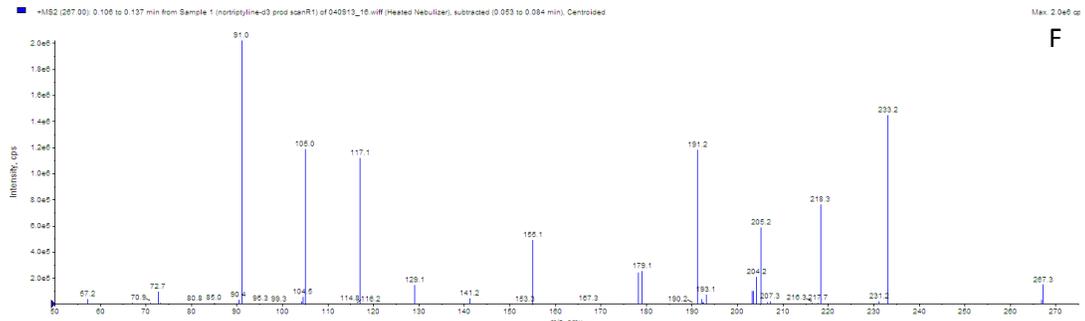


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**Figure A-2. <u>Amphetamines</u>: Product ion scans of AMP (A), AMP-d$_6$ (B), MAMP (C), MAMP-d$_9$ (D), MDA (E), MDA-d$_5$ (F), MDEA (G), MDEA-d$_5$ (H), MDMA (I) and MDMA-d$_5$ (J).**

(continued)

**Figure A-2. <u>Amphetamines</u>: Product ion scans of AMP (A), AMP-d$_6$ (B), MAMP (C), MAMP-d$_9$ (D), MDA (E), MDA-d$_5$ (F), MDEA (G), MDEA-d$_5$ (H), MDMA (I) and MDMA-d$_5$ (J). (continued)**

Figure A-3. <u>Benzodiazepines</u>: Product ion scans of alprazolam (A), alprazolam-$d_5$ (B), clonazepam (C), clonazepam-$d_4$ (D), diazepam (E) and diazepam-$d_5$ (F) α-hydroxyalprazolam (G), α-hydroxyalprazolam -$d_5$ (H), 7-aminoclonazepam (I), 7-aminoclonazepam-$d_4$ (J).
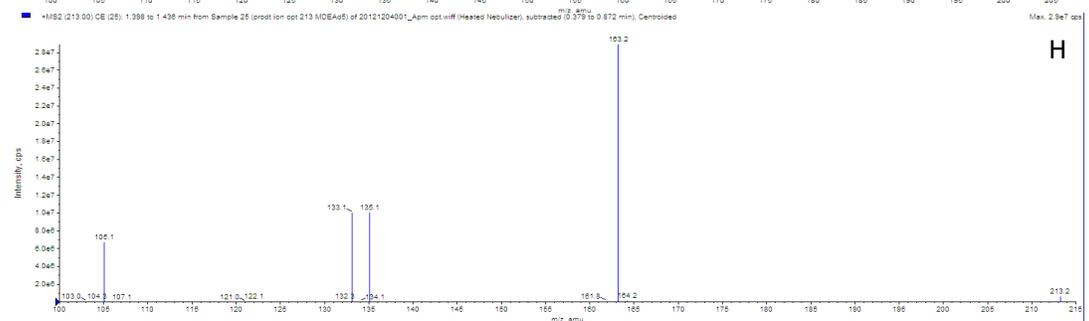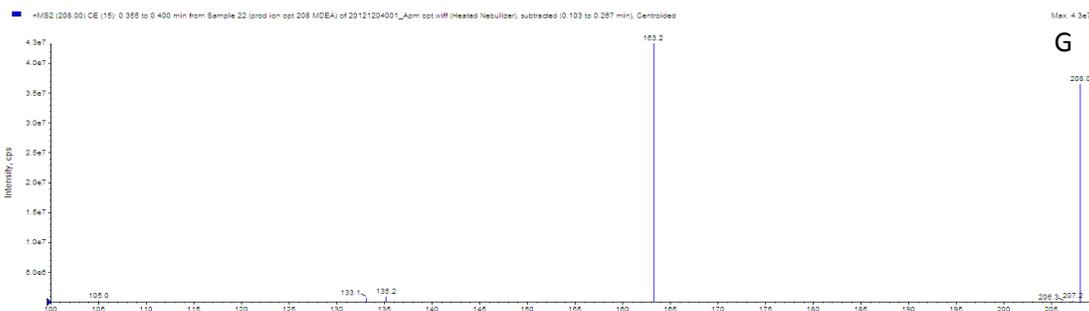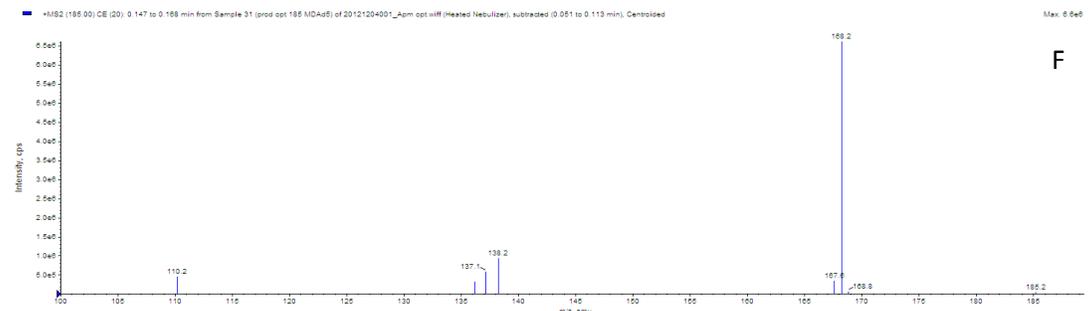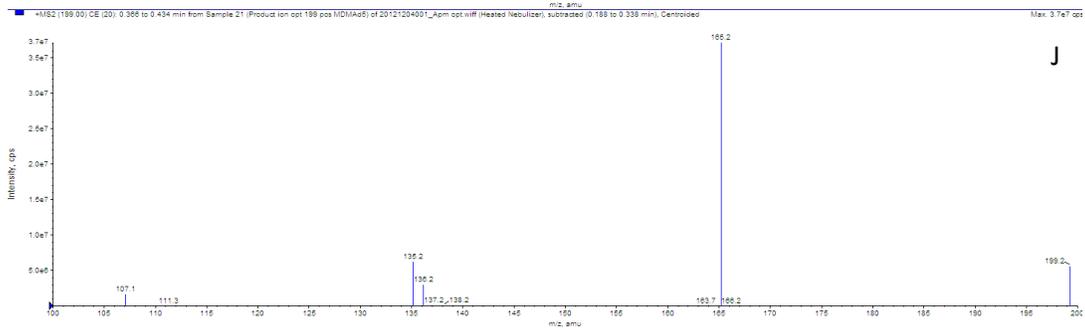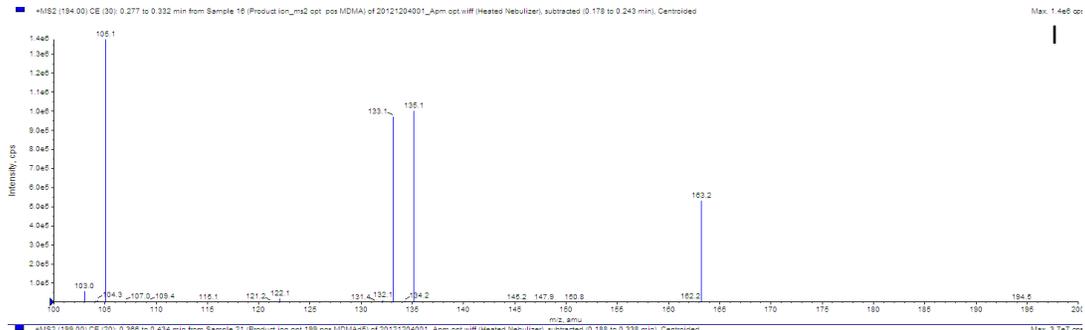
**Figure A-3. <u>Benzodiazepines</u>: Product ion scans of alprazolam (A), alprazolam-d$_5$ (B), clonazepam (C), clonazepam-d$_4$ (D), diazepam (E) and diazepam-d$_5$ (F) α-hydroxyalprazolam (G), α-hydroxyalprazolam -d$_5$ (H), 7-aminoclonazepam (I), 7-aminoclonazepam-d$_4$ (J). (continued)**

(continued)

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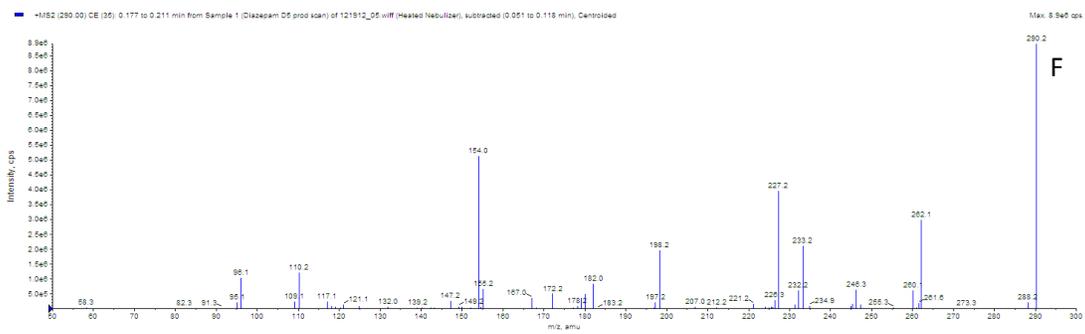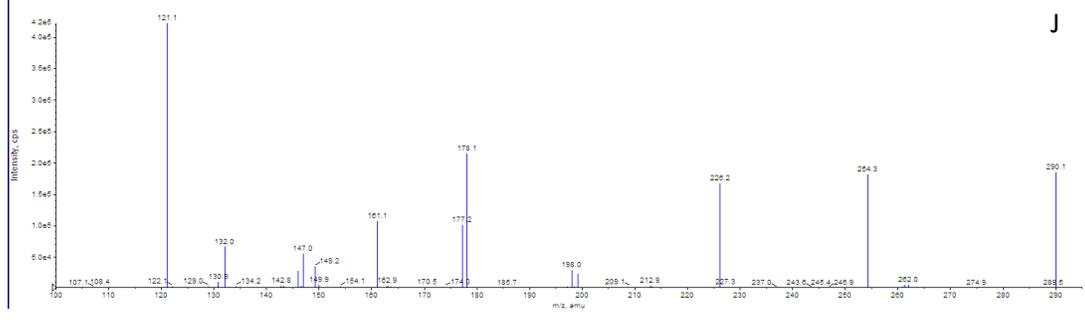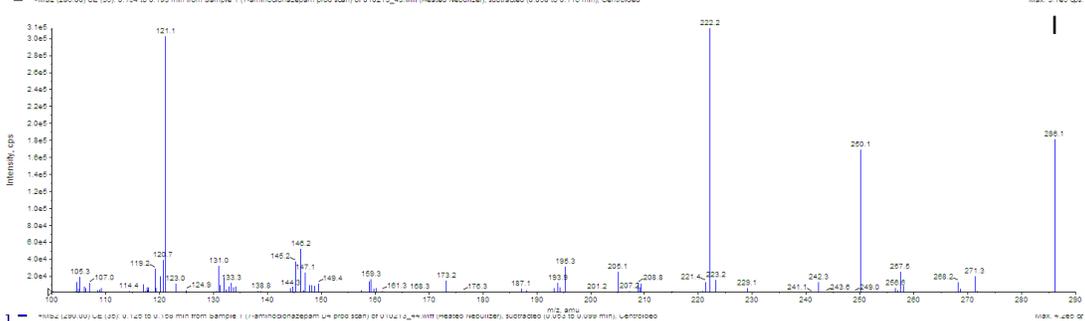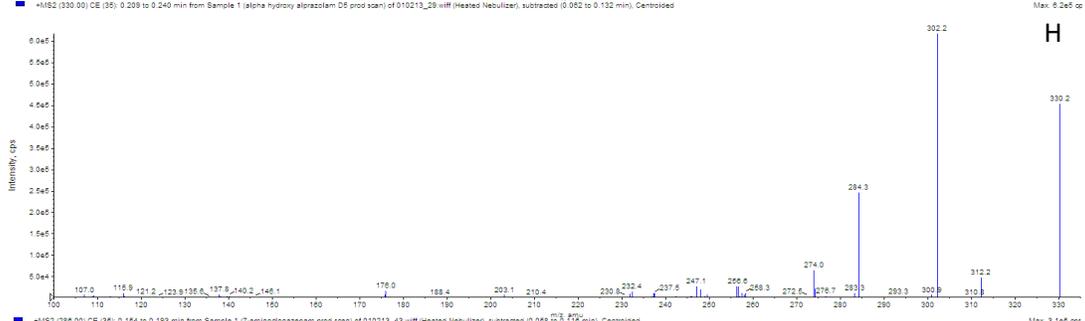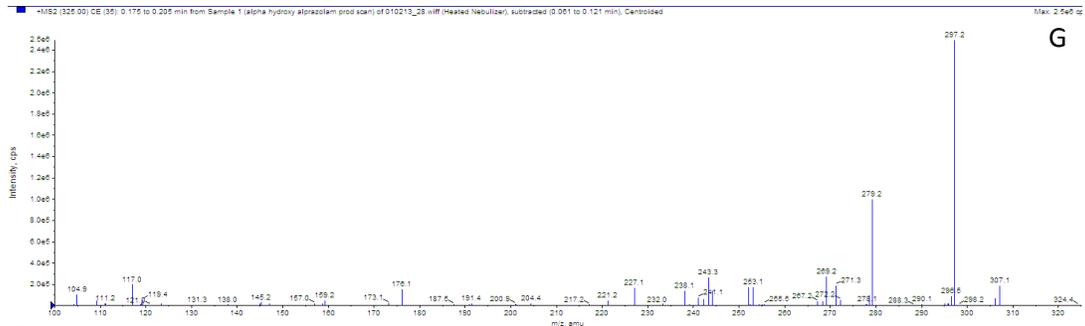
Figure A-4. <u>Designer Indanes</u>: Product ion scans of 2-AI (B), 5-IT (A), and MDAI (C).
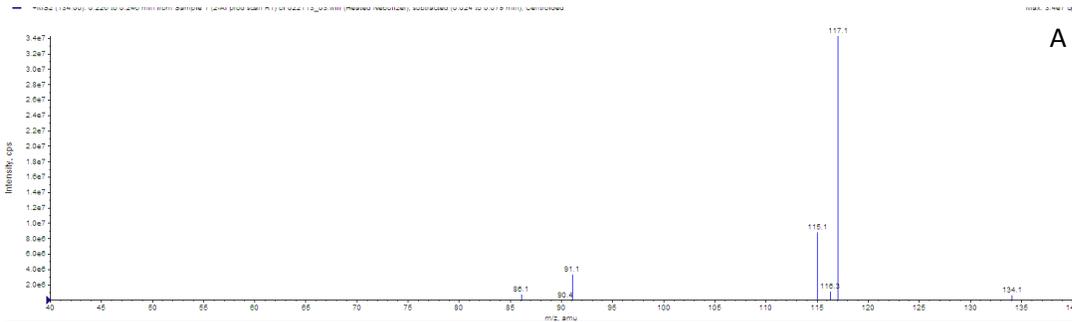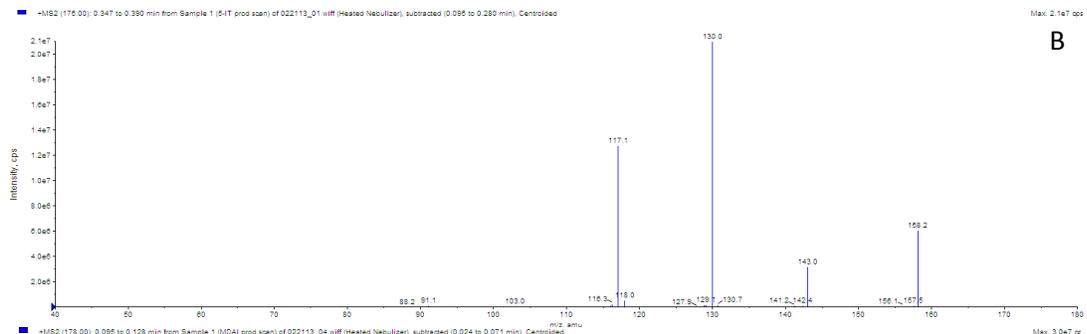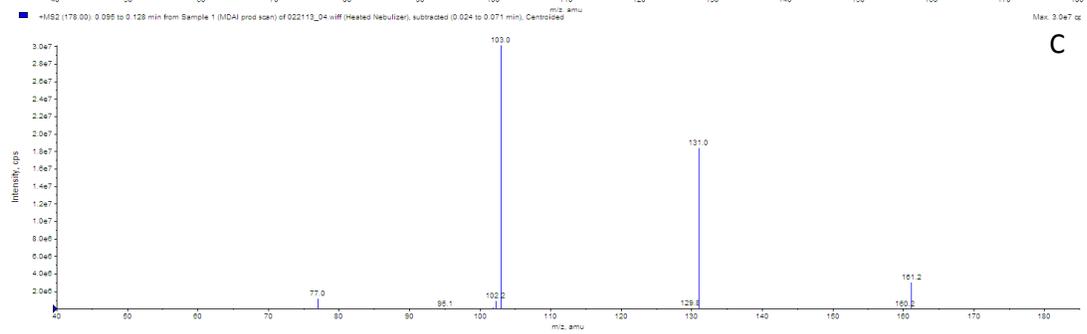


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B

C

81

**Figure A-5. <u>Synthetic Cannabinoids</u>: Product ion scans of AKB48 (A), EAM2201 (B), JWH-018 (C), JWH-073 (D), MAM2201 (E), MAM2201-d$_5$ (F), STS-135 (G), UR-144 (H), UR-144 5-bromopentyl (I), UR-144 5-chloropentyl (J), XLR11 4-pentenyl (K) and XLR11 (L).**
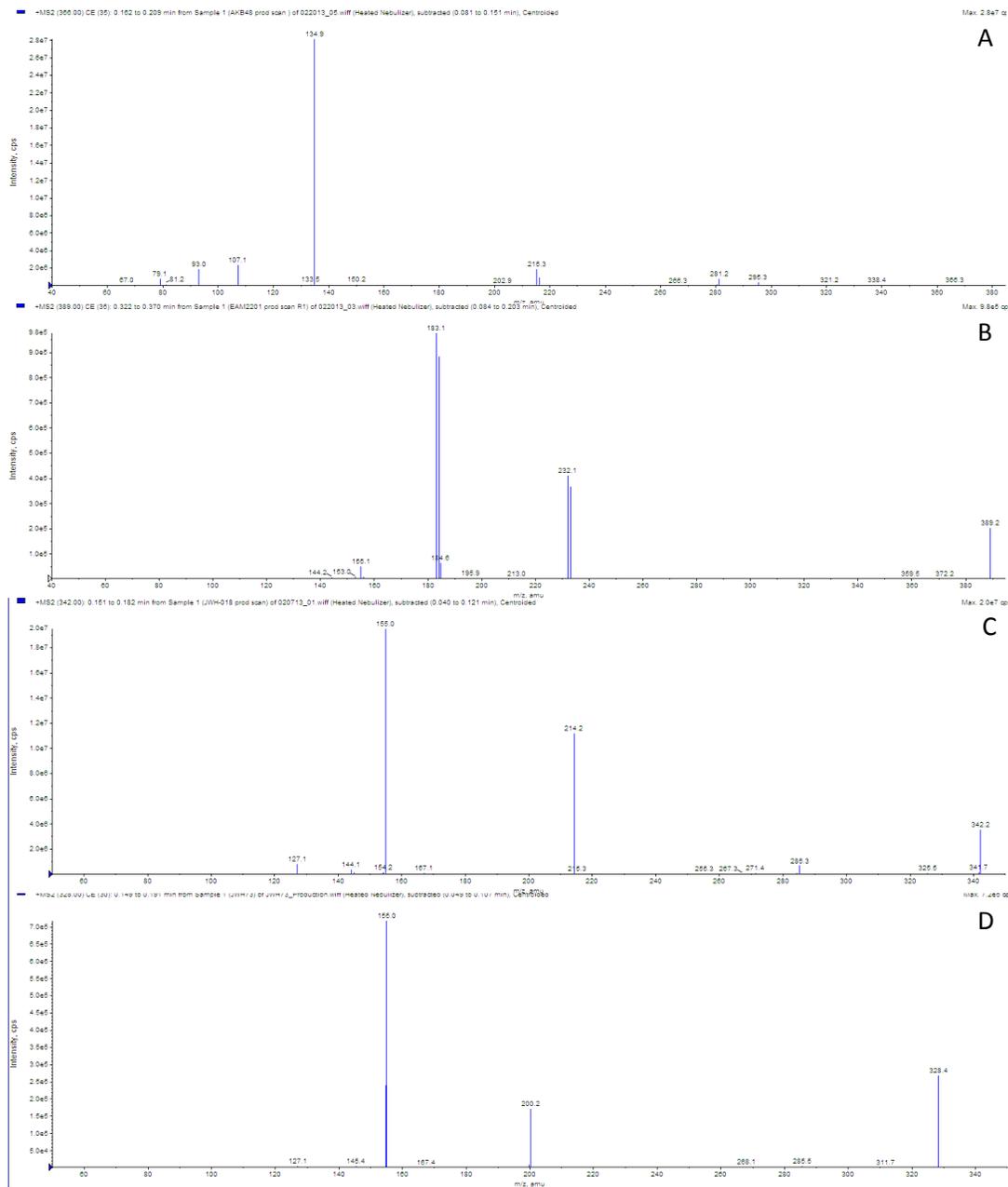


(continued)

**Figure A-5. <u>Synthetic Cannabinoids</u>: Product ion scans of AKB48 (A), EAM2201 (B), JWH-018 (C), JWH-073 (D), MAM2201 (E), MAM2201-d$_5$ (F), STS-135 (G), UR-144 (H), UR-144 5-bromopentyl (I), UR-144 5-chloropentyl (J), XLR11 4-pentenyl (K) and XLR11 (L). (continued)**

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**Figure A-5. <u>Synthetic Cannabinoids</u>: Product ion scans of AKB48 (A), EAM2201 (B), JWH-018 (C), JWH-073 (D), MAM2201 (E), MAM2201-d$_5$ (F), STS-135 (G), UR-144 (H), UR-144 5-bromopentyl (I), UR-144 5-chloropentyl (J), XLR11 4-pentenyl (K) and XLR11 (L). (continued)**
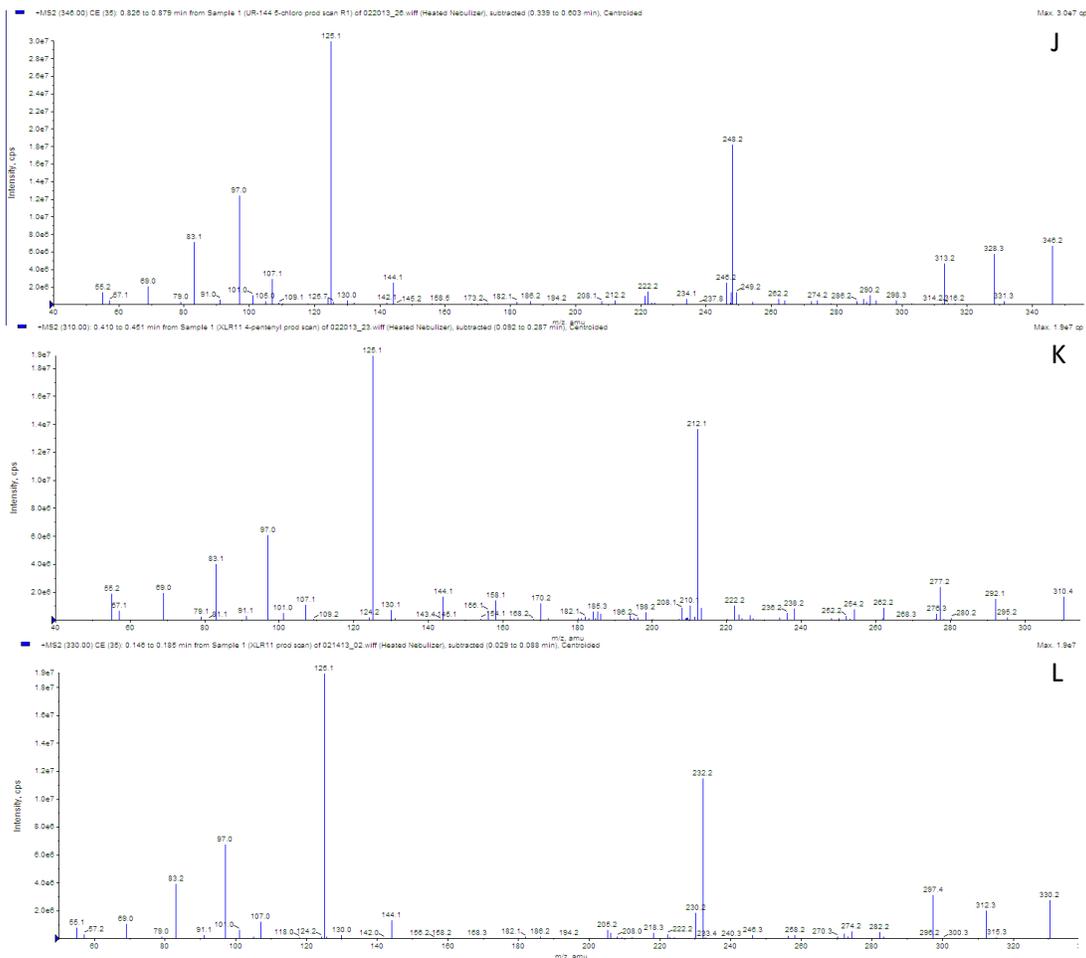
**Figure A-6. <u>Synthetic Cannabinoid Metabolites</u>: Product ion scans of JWH-018 N-(5-hydroxypentyl) (A), JWH-073 N-(4-hydroxybutyl) (B), JWH-122 5-hydroxypentyl (C) and JWH-200 4-hydroxyindole (D).**
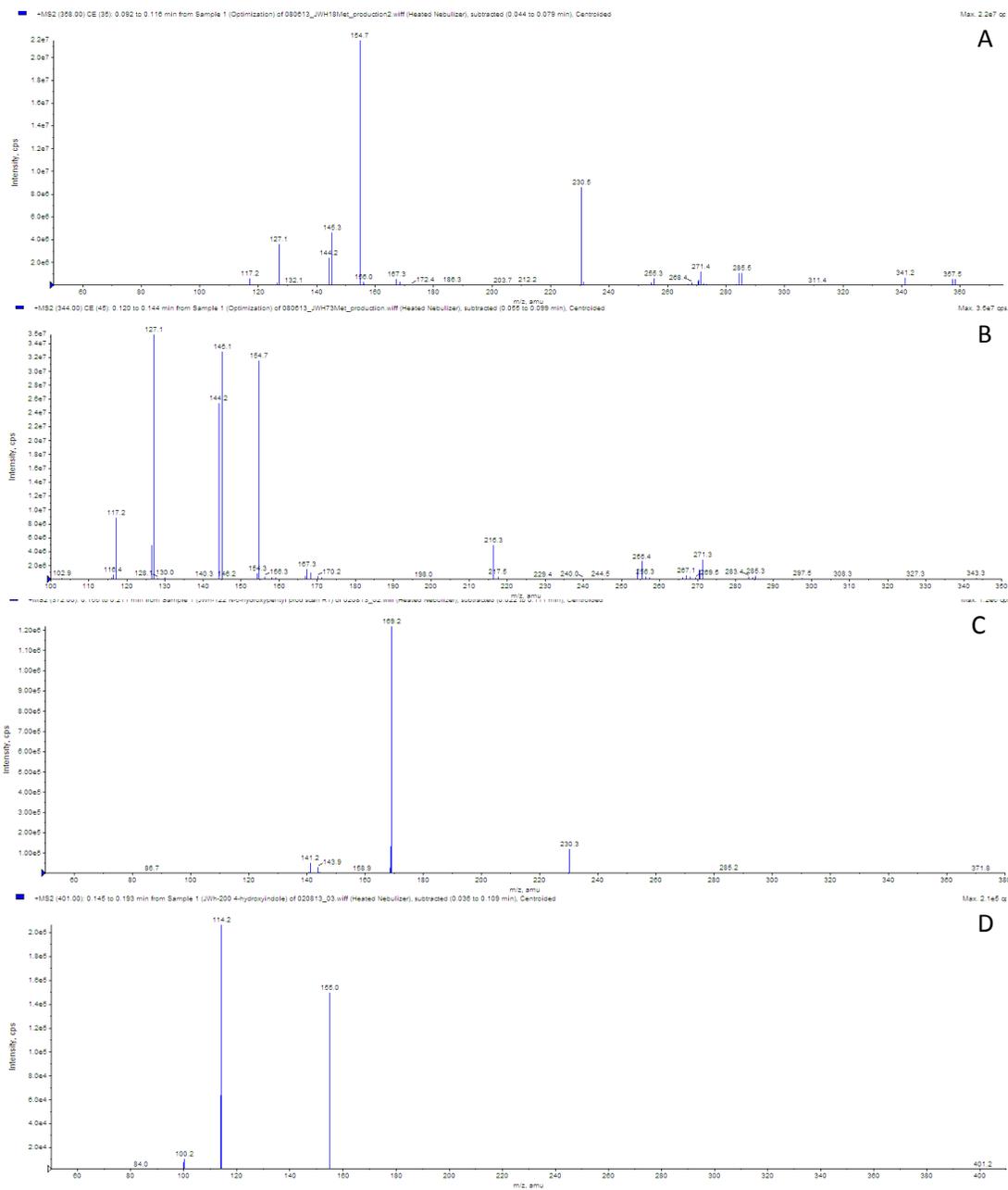
**Figure A-7. <u>Synthetic Cathinones and Piperazines</u>: Product ion scans of 4-MEC (A), 4-MePPP (B), α-PVP (C), buphedrone (D), flephedrone (E), mCPP (F), MDPV (G), methylone (H), and TFMPP (I).**
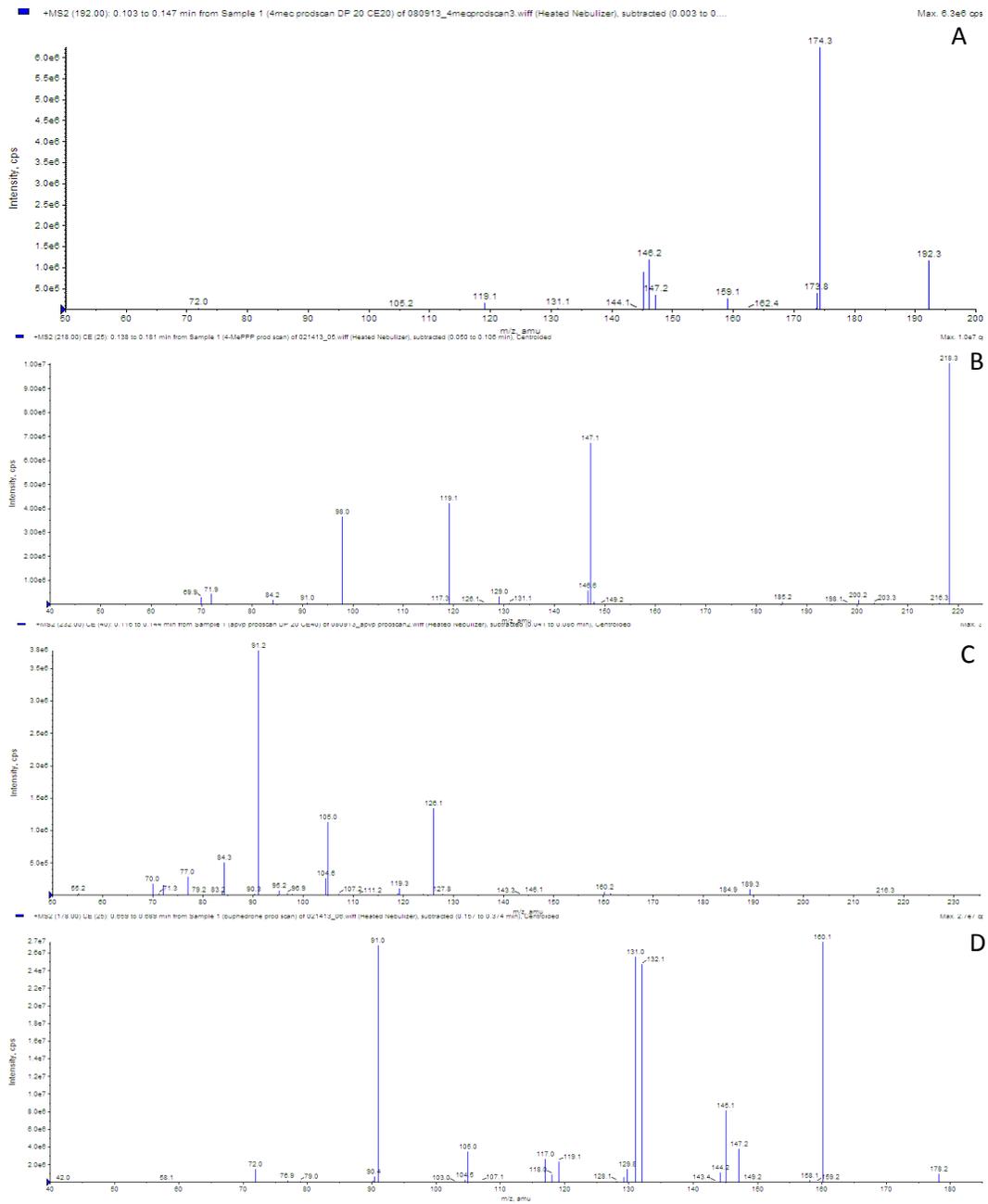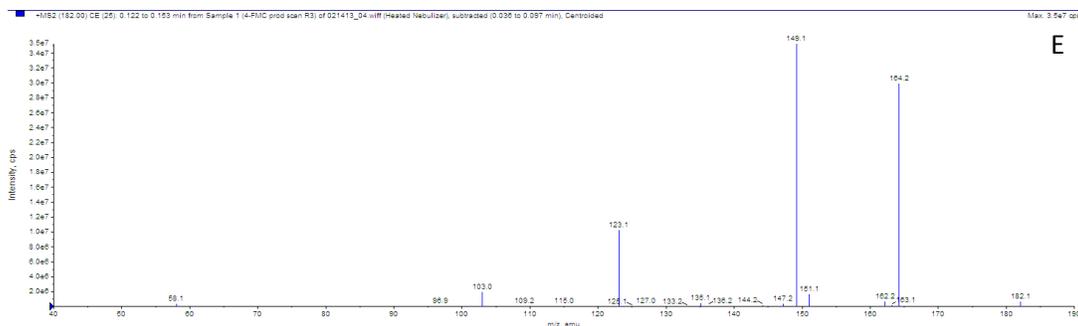
(continued)

**Figure A-7. <u>Synthetic Cathinones and Piperazines</u>: Product ion scans of 4-MEC (A), 4-MePPP (B), α-PVP (C), buphedrone (D), flephedrone (E), mCPP (F), MDPV (G), methylone (H), and TFMPP (I). (continued)**



87
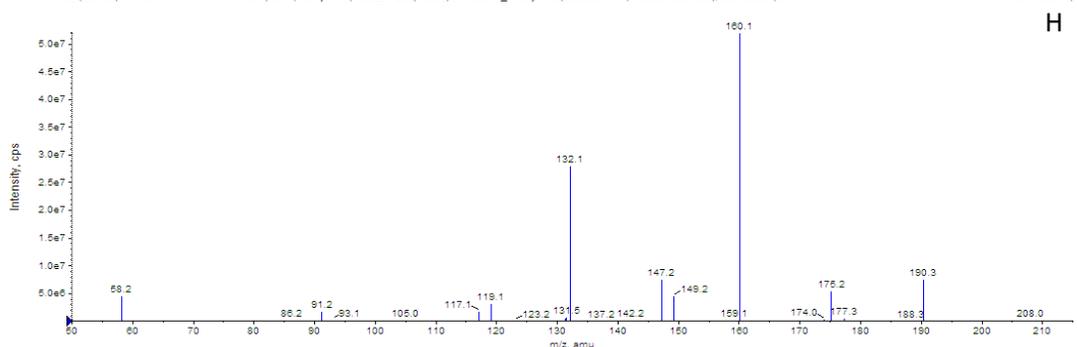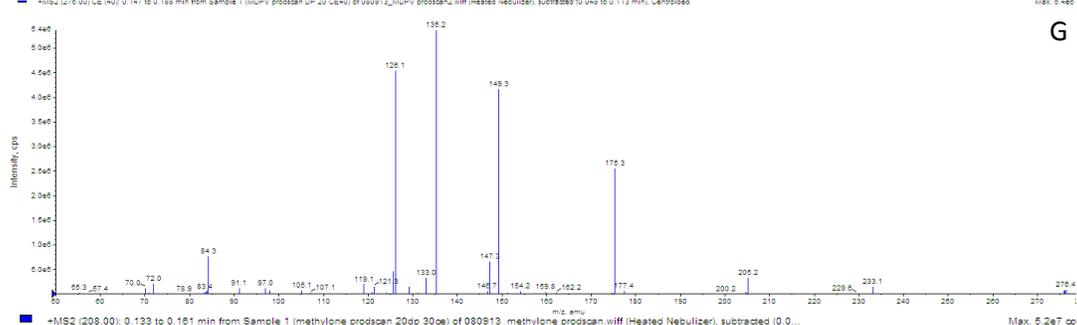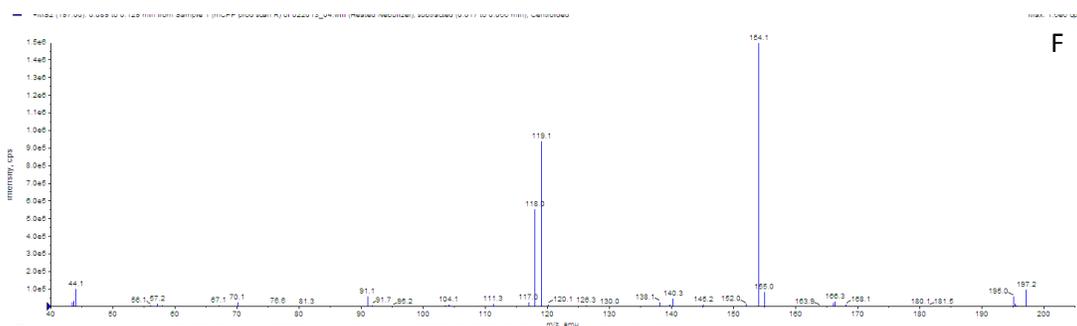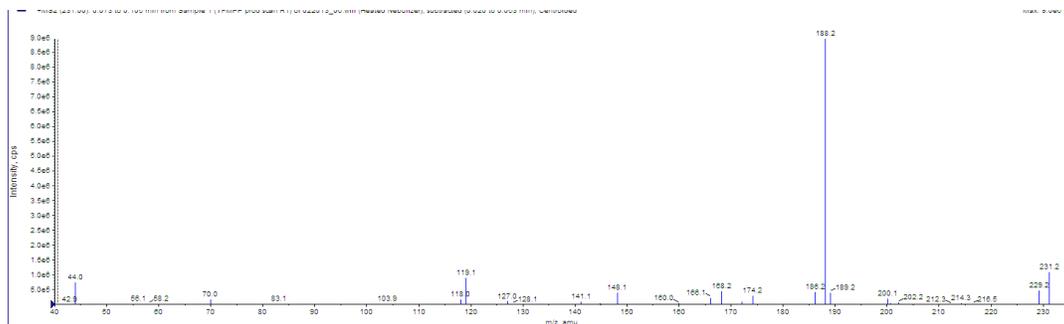
**Figure A-8 <u>Tryptamines</u>: Product ion scans of 5-MeO-AMT (A), 5-MeO-DiPT (B), 5-MeO-DMT (C), and 5-MeO-MiPT (D).**
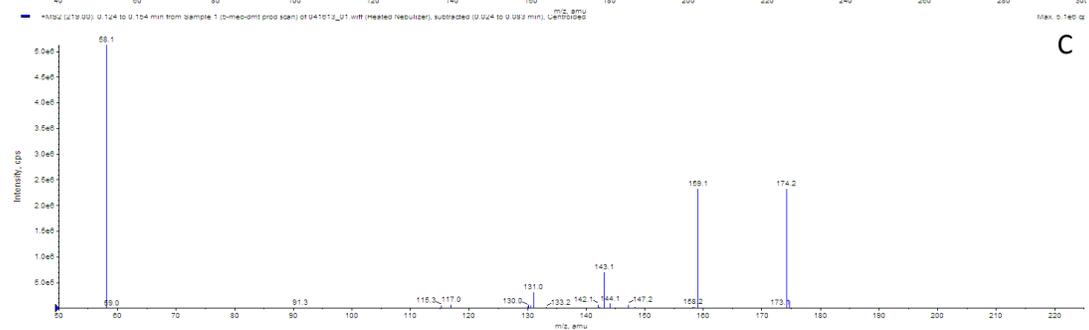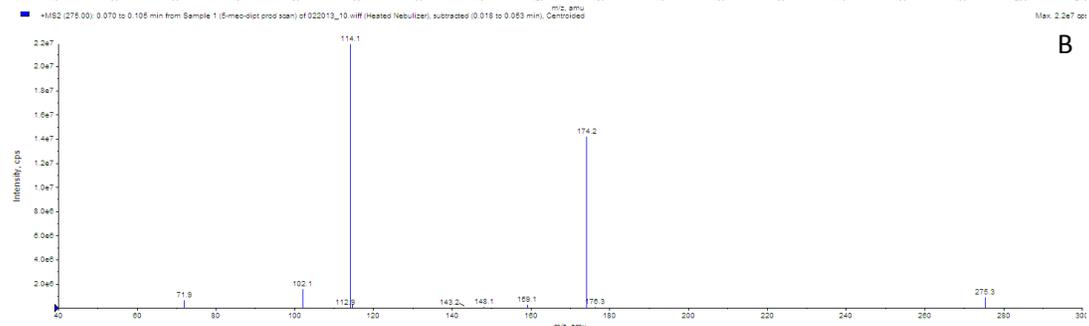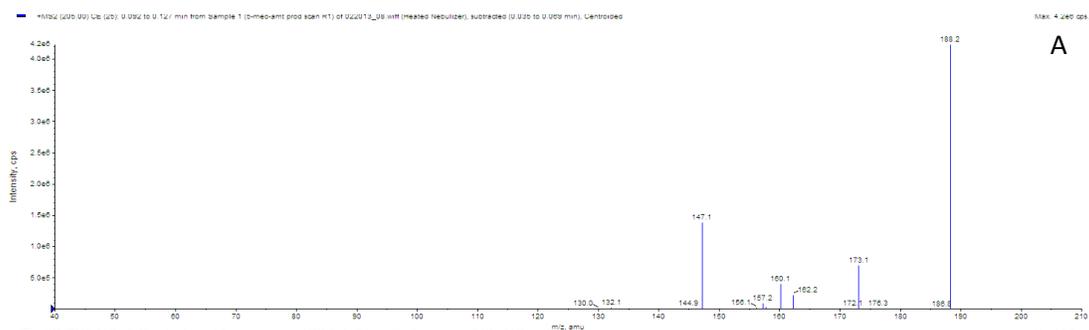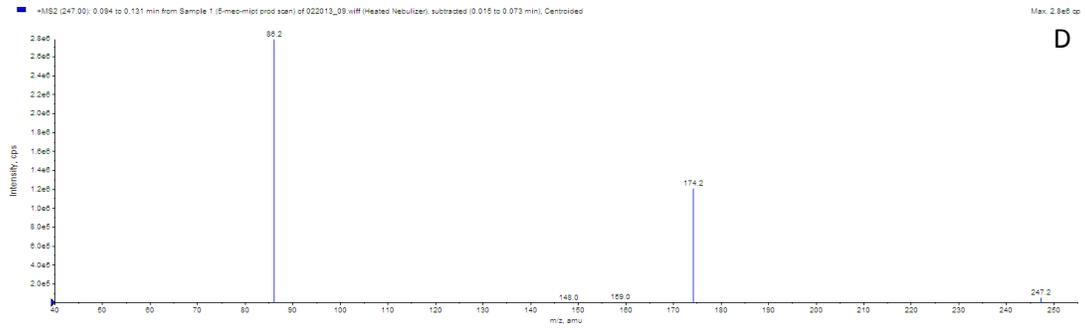
Max. 2.9e6 cp

D



89

**Figure A-9. <u>Miscellaneous</u>: Product ion scans of BZE (A), BZE-d$_3$ (B), COC (C), and COC-d$_3$ (D), PCP (E) and PCP-d$_5$ (F).**

E
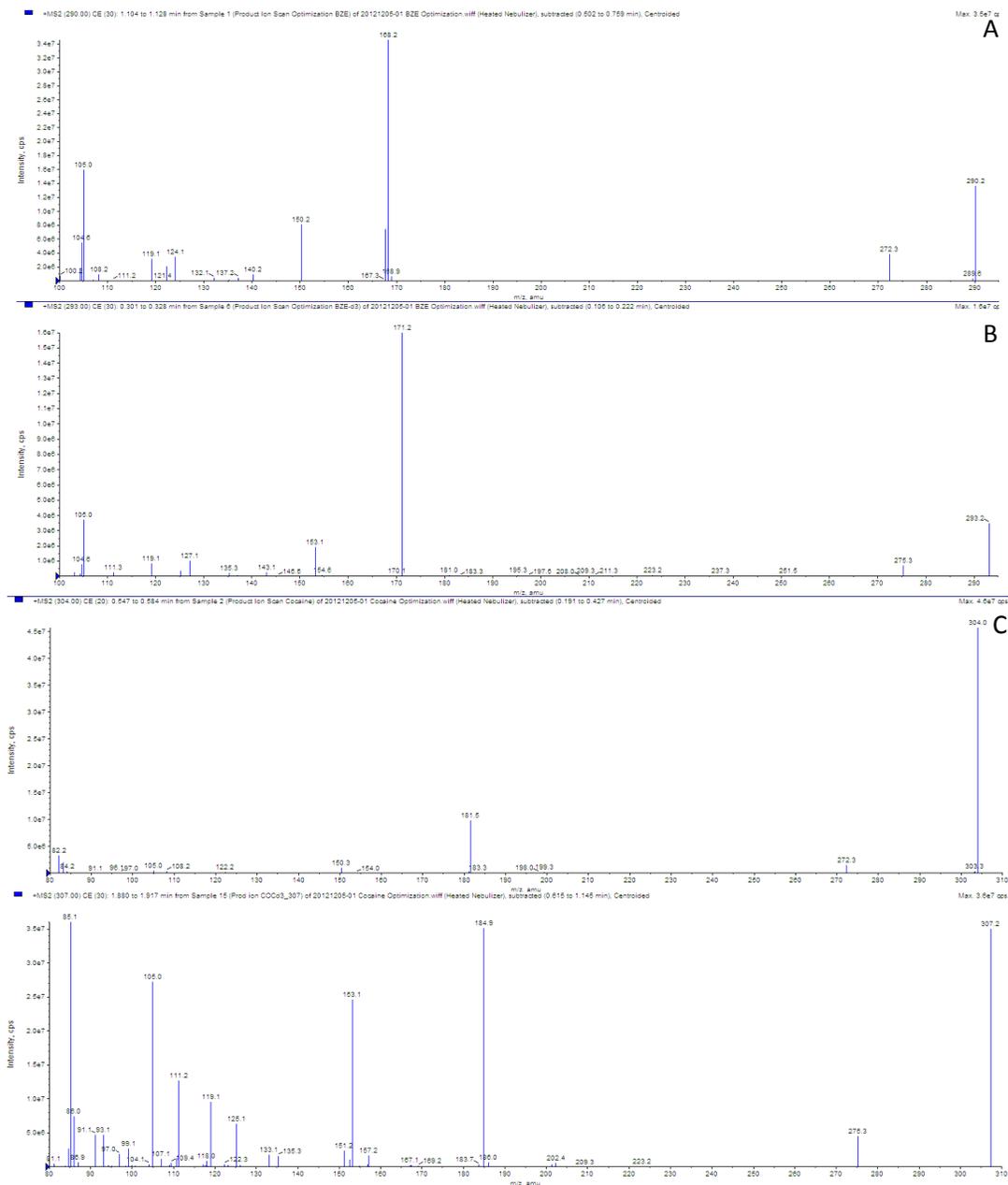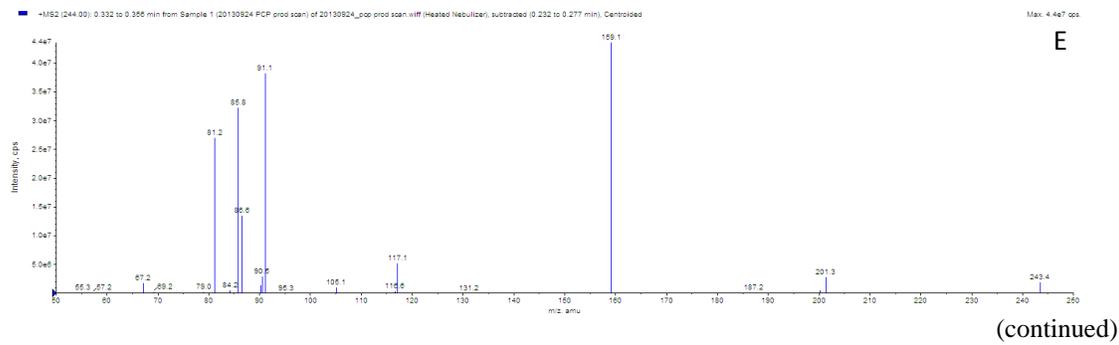
(continued)

**Figure A-9.** <u>**Miscellaneous**</u>**: Product ion scans of BZE (A), BZE-d$_3$ (B), COC (C), and COC-d$_3$ (D), PCP (E) and PCP-d$_5$ (F). (continued)**



F

91
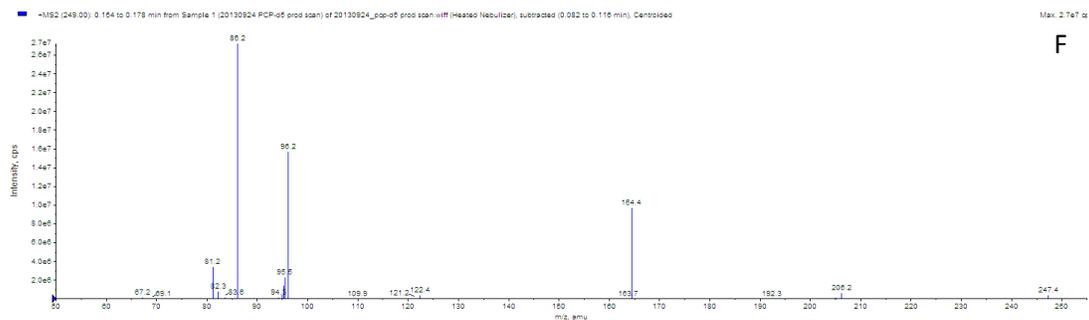
# APPENDIX B: LINE FIT PLOTS FOR QUANTITATION VALIDATION

**Figure B-1. <u>Antidepressants</u>: Urine line fit plot of amitriptyline (A1), citalopram (B1), nortriptyline (C1) and trazodone (D1). Blood lint fit plot of amitriptyline (A2), citalopram (B2), nortriptyline (C2) and trazodone (D2).**



(continued)

**Figure B-1. <u>Antidepressants</u>: Urine line fit plot of amitriptyline (A1), citalopram (B1), nortriptyline (C1) and trazodone (D1). Blood lint fit plot of amitriptyline (A2), citalopram (B2), nortriptyline (C2) and trazodone (D2). (continued)**

**Figure B-1. <u>Antidepressants</u>: Urine line fit plot of amitriptyline (A1), citalopram (B1), nortriptyline (C1) and trazodone (D1). Blood lint fit plot of amitriptyline (A2), citalopram (B2), nortriptyline (C2) and trazodone (D2). (continued)**



(continued)

**Figure B-1. <u>Antidepressants</u>: Urine line fit plot of amitriptyline (A1), citalopram (B1), nortriptyline (C1) and trazodone (D1). Blood lint fit plot of amitriptyline (A2), citalopram (B2), nortriptyline (C2) and trazodone (D2). (continued)**

**Figure B-2. <u>Amphetamines</u>: Urine lint fit pot of AMP (A1) and MAMP (B1) MDA (C1), MDEA (D1) and MDMA (E1). Blood line fit plot AMP (A2), MAMP (B2), MDA (C2), MDEA (D2) and MDMA (E2).**

**Figure B-2.** <u>Amphetamines</u>**: Urine lint fit pot of AMP (A1) and MAMP (B1) MDA (C1), MDEA (D1) and MDMA (E1).  Blood line fit plot AMP (A2), MAMP (B2), MDA (C2), MDEA (D2) and MDMA (E2). (continued)**



(continued)

**Figure B-2. <u>Amphetamines</u>: Urine lint fit pot of AMP (A1) and MAMP (B1) MDA (C1), MDEA (D1) and MDMA (E1).  Blood line fit plot AMP (A2), MAMP (B2), MDA (C2), MDEA (D2) and MDMA (E2). (continued)**



(continued)

**Figure B-2.** <u>Amphetamines</u>**: Urine lint fit pot of AMP (A1) and MAMP (B1) MDA (C1), MDEA (D1) and MDMA (E1). Blood line fit plot AMP (A2), MAMP (B2), MDA (C2), MDEA (D2) and MDMA (E2). (continued)**



(continued)

**Figure B-2.** <u>Amphetamines</u>: Urine lint fit pot of AMP (A1) and MAMP (B1) MDA (C1), MDEA (D1) and MDMA (E1). Blood line fit plot AMP (A2), MAMP (B2), MDA (C2), MDEA (D2) and MDMA (E2). (continued)

**Figure B-3. <u>Benzodiazepines</u>: Urine line fit plot of alprazolam (A1), clonazepam (B1), diazepam (C1), α-hydroxyalprazolam (D1) and 7-aminoclonazepam (E1).  Blood line fit plot of alprazolam (A2), clonazepam (B2), diazepam (C2), α-hydroxyalprazolam (D2) and 7-aminoclonazepam (E2).**



(continued)

**Figure B-3.** <u>Benzodiazepines</u>**: Urine line fit plot of alprazolam (A1), clonazepam (B1), diazepam (C1), α-hydroxyalprazolam (D1) and 7-aminoclonazepam (E1). Blood line fit plot of alprazolam (A2), clonazepam (B2), diazepam (C2), α-hydroxyalprazolam (D2) and 7-aminoclonazepam (E2). (continued)**





(continued)

**Figure B-3. <u>Benzodiazepines</u>: Urine line fit plot of alprazolam (A1), clonazepam (B1), diazepam (C1), α-hydroxyalprazolam (D1) and 7-aminoclonazepam (E1).  Blood line fit plot of alprazolam (A2), clonazepam (B2), diazepam (C2), α-hydroxyalprazolam (D2) and 7-aminoclonazepam (E2). (continued)**



(continued)

**Figure B-3.** <u>Benzodiazepines</u>**: Urine line fit plot of alprazolam (A1), clonazepam (B1), diazepam (C1), α-hydroxyalprazolam (D1) and 7-aminoclonazepam (E1). Blood line fit plot of alprazolam (A2), clonazepam (B2), diazepam (C2), α-hydroxyalprazolam (D2) and 7-aminoclonazepam (E2). (continued)**



(continued)

**Figure B-3. <u>Benzodiazepines</u>: Urine line fit plot of alprazolam (A1), clonazepam (B1), diazepam (C1), α-hydroxyalprazolam (D1) and 7-aminoclonazepam (E1). Blood line fit plot of alprazolam (A2), clonazepam (B2), diazepam (C2), α-hydroxyalprazolam (D2) and 7-aminoclonazepam (E2). (continued)**

**Figure B-4. <u>Miscellaneous</u>: Urine line fit plot s of BZE (A1), COC (B1) and PCP (C1). Blood lint fit plot of BZE (A2), COC (B2) and PCP (C2).**

**Figure B-4. <u>Miscellaneous</u>: Urine line fit plot s of BZE (A1), COC (B1) and PCP (C1). Blood lint fit plot of BZE (A2), COC (B2) and PCP (C2). (continued)**

**Figure B-4. <u>Miscellaneous</u>: Urine line fit plot s of BZE (A1), COC (B1) and PCP (C1). Blood lint fit plot of BZE (A2), COC (B2) and PCP (C2). (continued)**

**APPENDIX C: ANALYTE LOQ CONCENTRATIONS (NG/ML) IN URINE (LEFT COLUMN) AND BLOOD (RIGHT COLUMN)**

**FigureC-1. <u>Antidepressants</u>: Urine LOQ concentrations of amitriptyline (A1), citalopram (B1), nortriptyline (C1) and trazodone (D1) at 15 ng/mL. Blood LOQ concentrations of amitriptyline (A2), citalopram (B2), nortriptyline (C2) and trazodone (D2) at 15 ng/mL.**
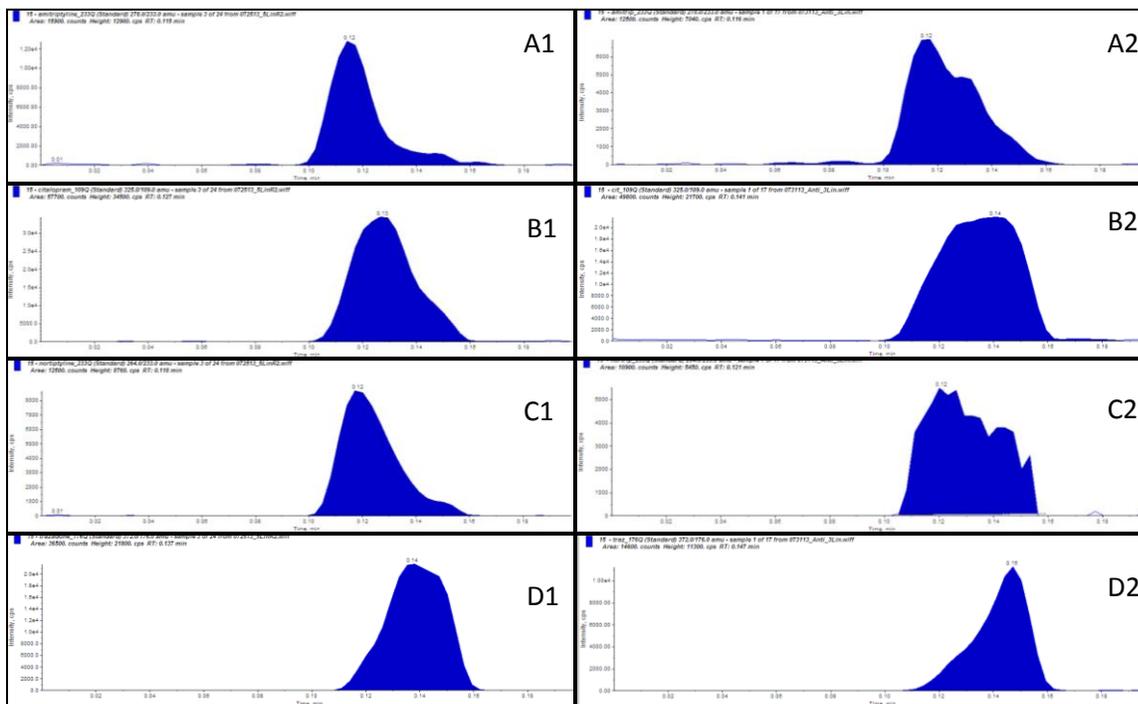
**Figure C-2. <u>Amphetamines</u>: Urine LOQ concentrations of AMP (A1) and MAMP (B1) at 25 ng/mL and MDA (C1), MDEA (D1) and MDMA (E1) at 50 ng/mL.  Blood LOQ concentrations AMP (A2), MAMP (B2), MDA (C2), MDEA (D2) and MDMA (E2) at 25 ng/mL.**
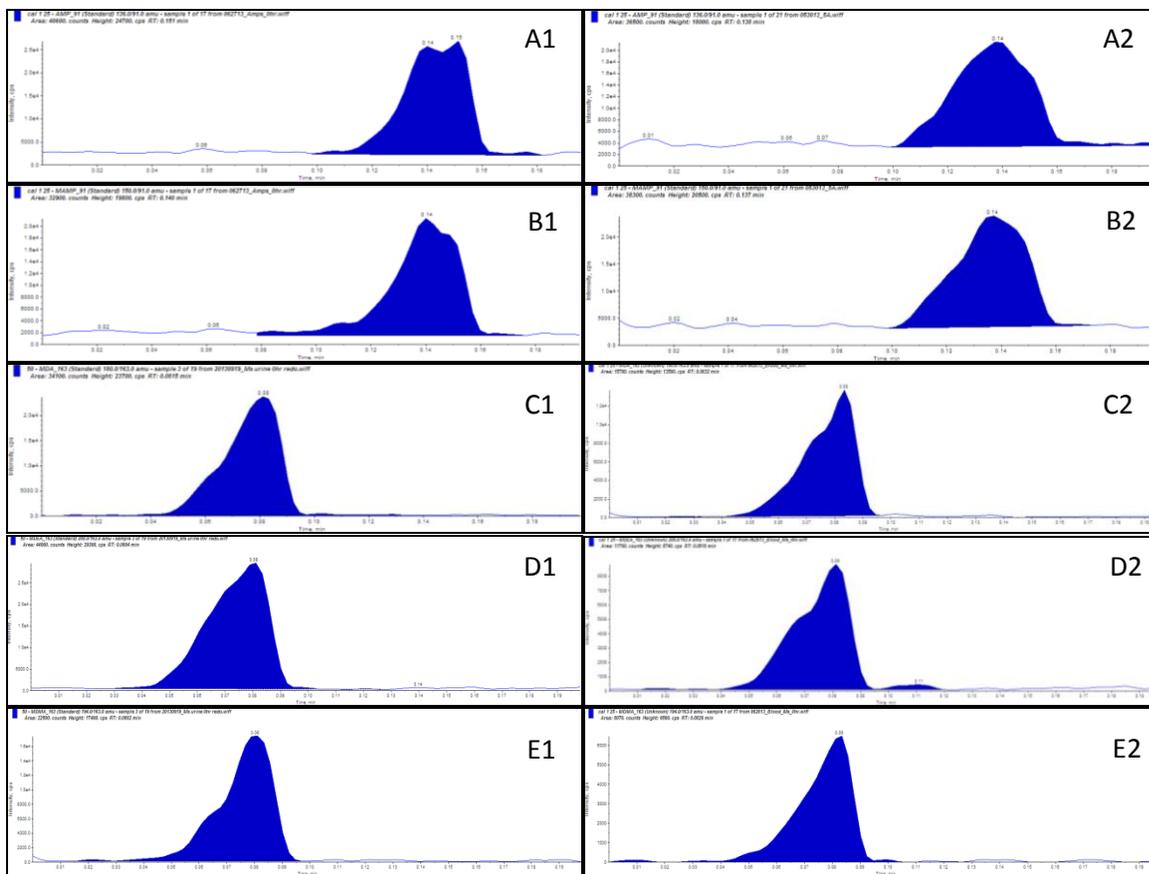
**Figure C-3. <u>Benzodiazepines</u>: Urine LOQ concentrations of alprazolam (A1), clonazepam (B1), diazepam (C1), α-hydroxyalprazolam (D1) and 7-aminoclonazepam (E1) at 15 ng/mL parent compound and 25 ng/mL metabolite.  Blood LOQ concentrations of alprazolam (A2), clonazepam (B2), diazepam (C2), α-hydroxyalprazolam (D2) and 7-aminoclonazepam (E2) at 10 ng/mL parent compound and 25 ng/mL metabolite.**
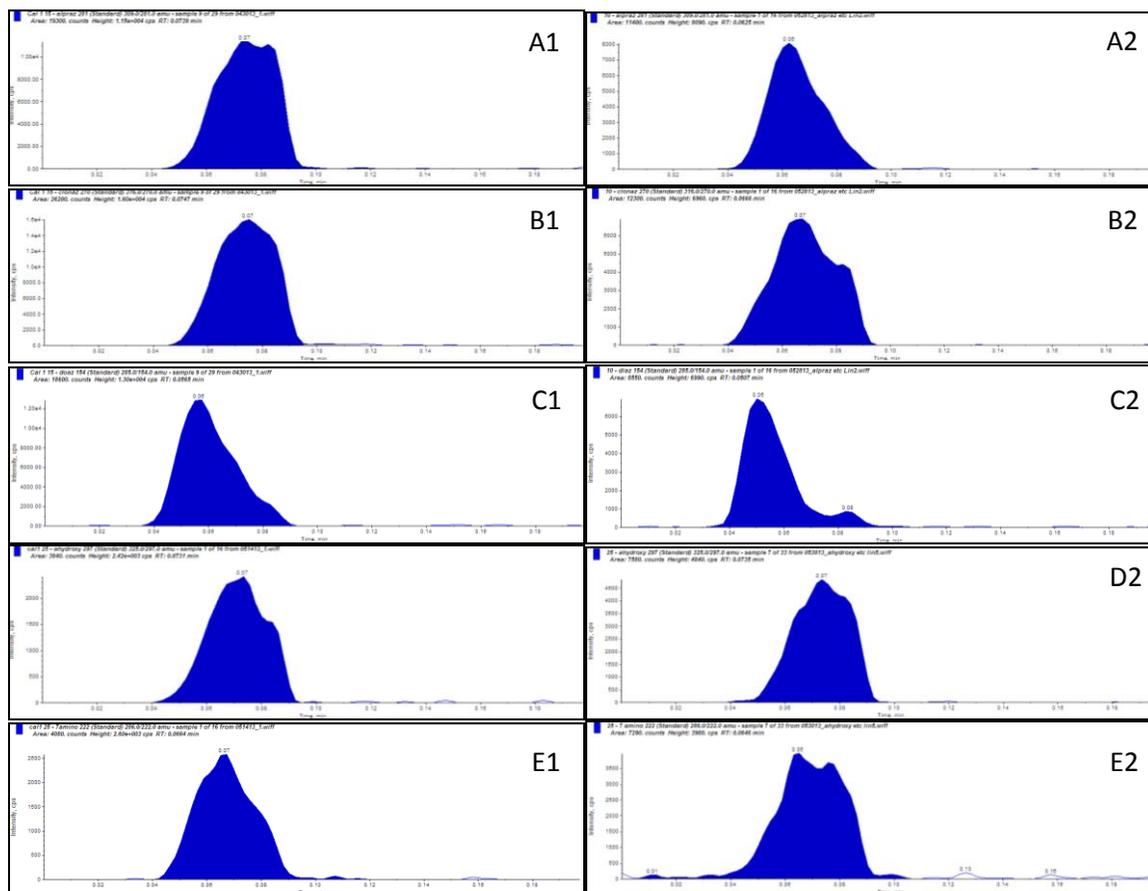
**Figure C-4. <u>Miscellaneous</u>: Urine LOQ concentrations of BZE (A1), COC (B1) and PCP (C1) at 10 ng/mL. Blood LOQ concentrations of BZE (A2), COC (B2) and PCP (C2) at 10 ng/mL.**
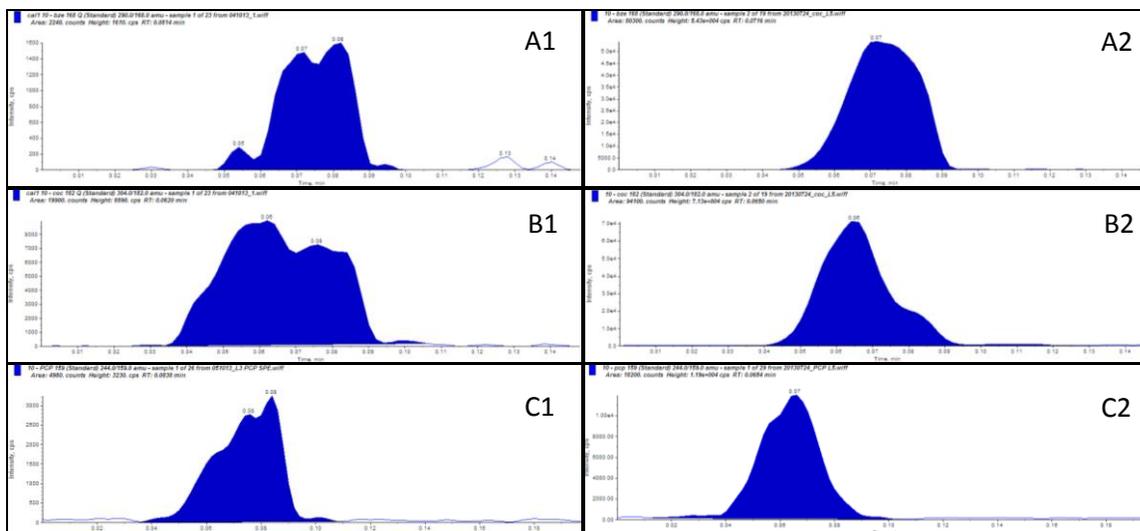
# APPENDIX D: ANALYTE LOD CONCENTRATIONS (NG/ML) IN URINE (LEFT COLUMN) AND BLOOD (RIGHT COLUMN)

**Figure D-1. <u>Antidepressants</u>: Urine LOD concentrations of amitriptyline (A1), citalopram (B1), nortriptyline (C1) and trazodone (D1) at 5, 10, 2, and 1 ng/mL, respectively. Blood LOD concentrations of amitriptyline (A2), citalopram (B2), nortriptyline (C2) and trazodone (D2) at 5, 10, 2, and 2 ng/mL, respectively.**



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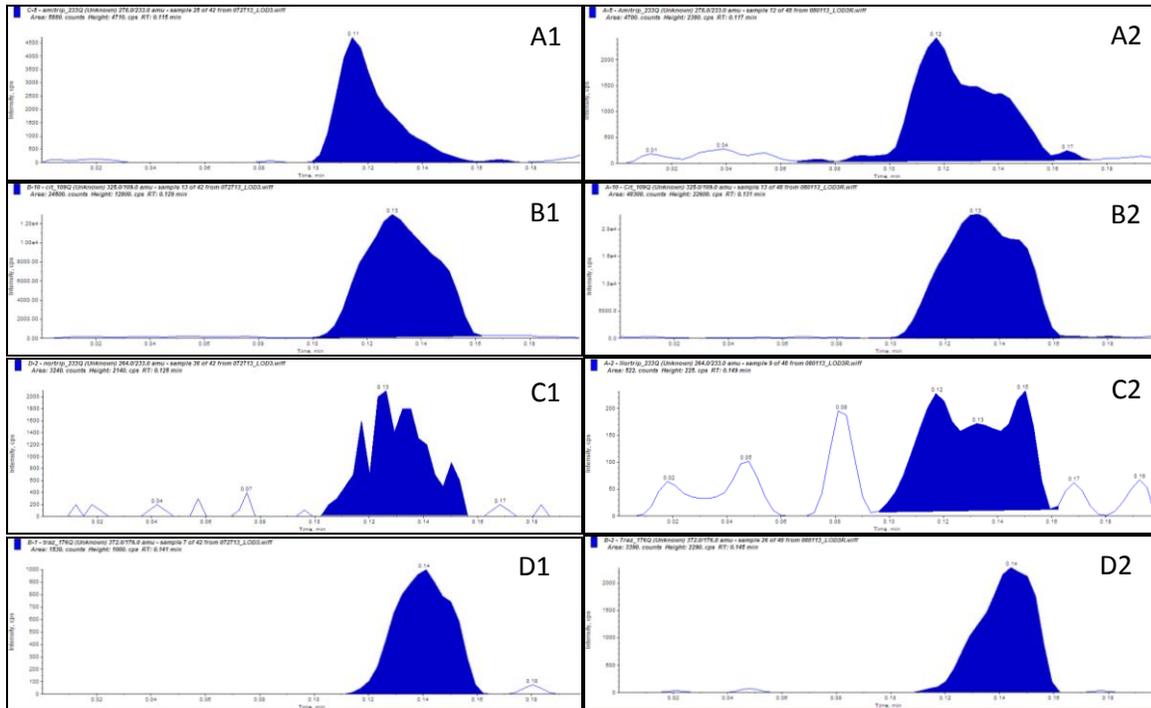
**Figure D-2. <u>Amphetamines</u>: Urine LOD concentrations of AMP (A1) and MAMP (B1), MDA (C1), MDEA (D1) and MDMA (E1) at 10, 5, 10, 15, and 2 ng/mL, respectively.  Blood LOD concentrations AMP (A2), MAMP (B2), MDA (C2), MDEA (D2) and MDMA (E2) at 15, 10, 10, 10, and 2, and ng/mL.**
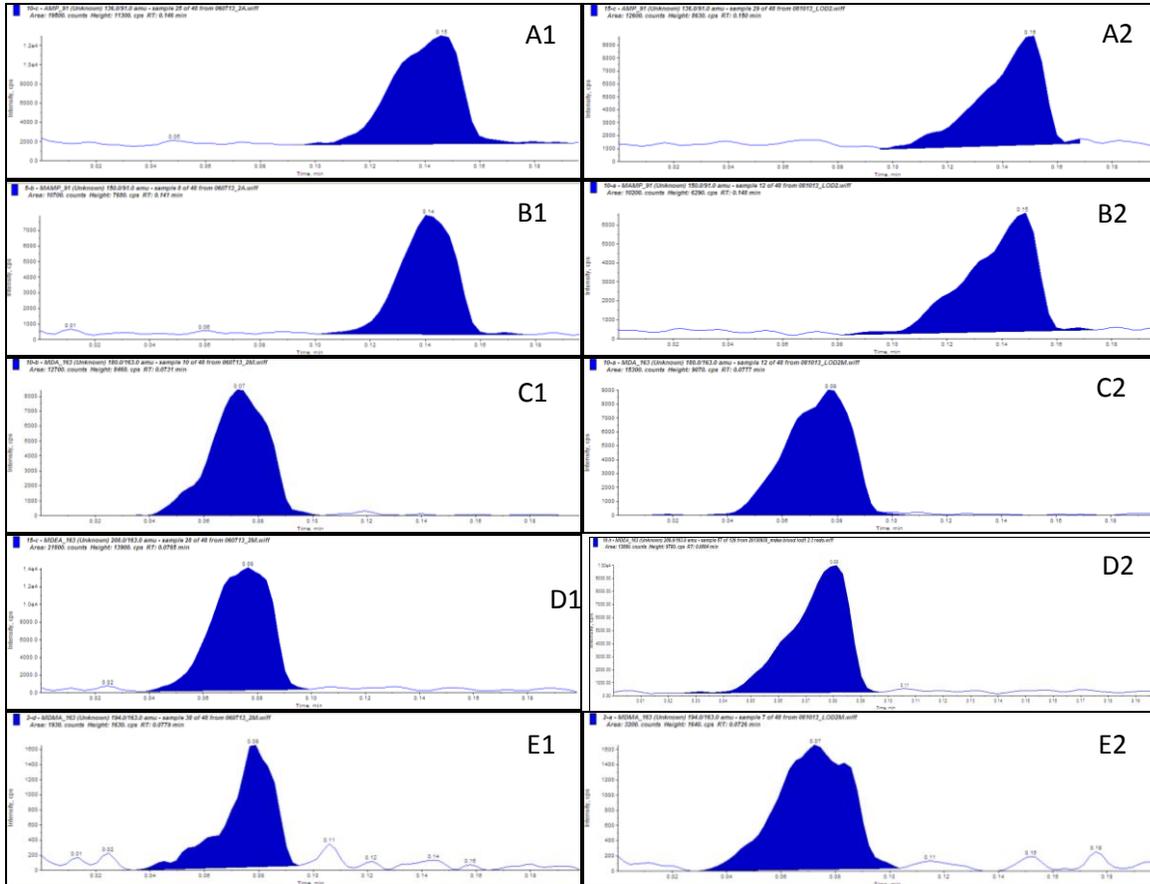
**Figure D-3. <u>Benzodiazepines</u>: Urine LOD concentrations of alprazolam (A1), clonazepam (B1), diazepam (C1), α-hydroxyalprazolam (D1) and 7-aminoclonazepam (E1) at 1, 5, 5, 10, and 10 ng/mL, respectively.  Blood LOD concentrations of alprazolam (A2), clonazepam (B2), diazepam (C2), α-hydroxyalprazolam (D2) and 7-aminoclonazepam (E2) at 2, 10, 10, 10, and 10 ng/mL, respectively.**



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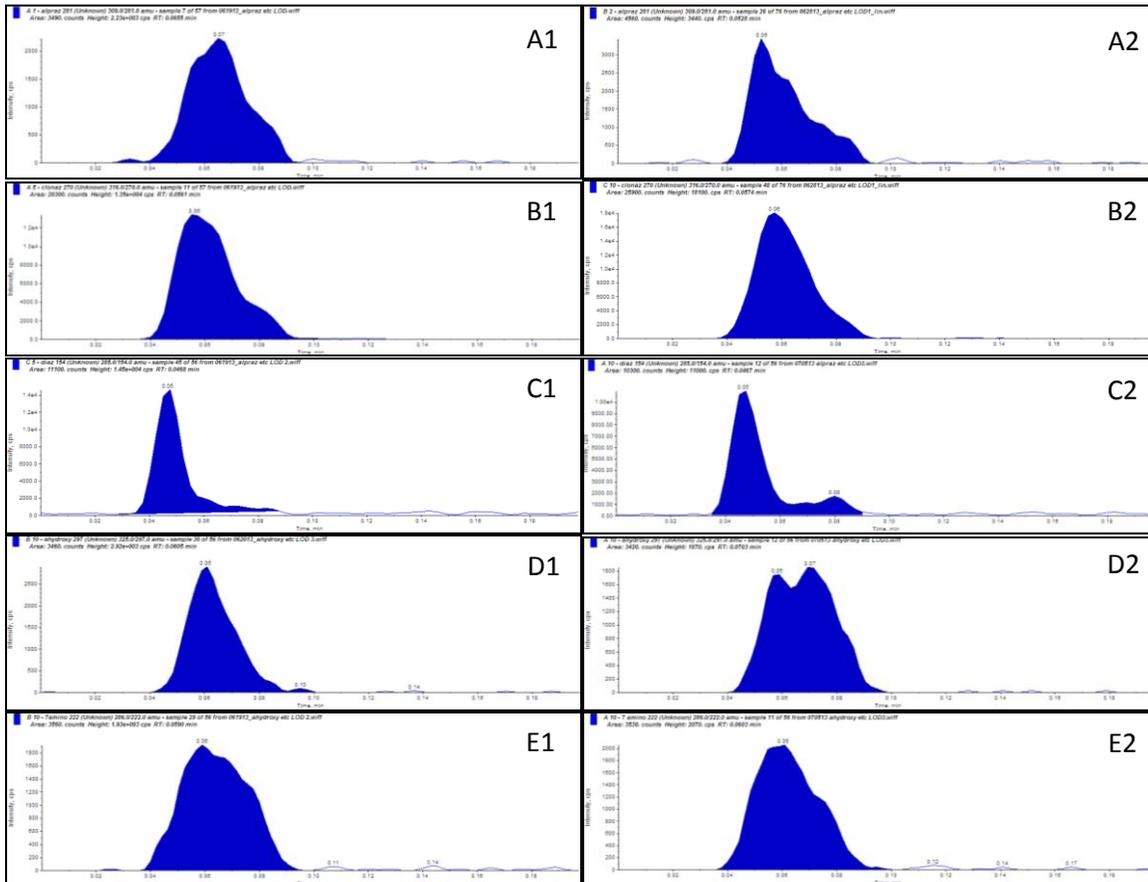
**Figure D-4. <u>Designer Indanes</u>: Urine LOD concentrations of 2-AI (A), 5-IT (B), and MDAI (C) at 1, 5, and 2 ng/mL, respectively.**
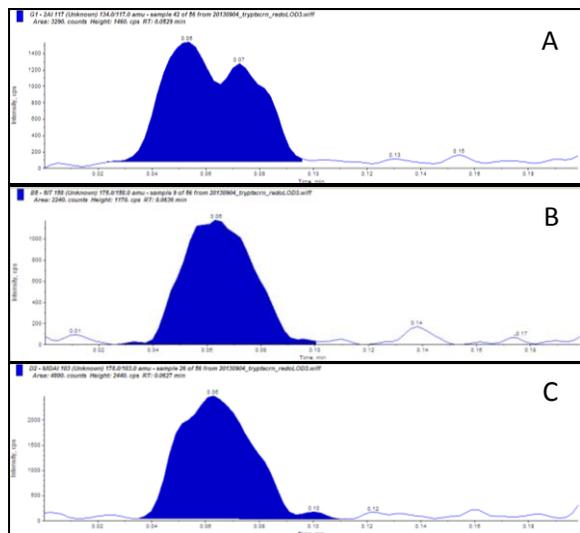
**Figure D-5. <u>Synthetic Cannabinoids</u>: Urine LOD concentrations of AKB48 (A), EAM2201 (B), JWH-018 (C), JWH-073 (D), MAM2201 (E), STS-135 (F), UR-144 (G), UR-144 5-bromopentyl (H), UR-144 5-chloropentyl (I), XLR11 4-pentenyl (J) and XLR11 (K) at 1, 5, 0.5, 1, 0.5, 5, 5, 50, 2, 5, and 1 ng/mL, respectively.**



(continued)

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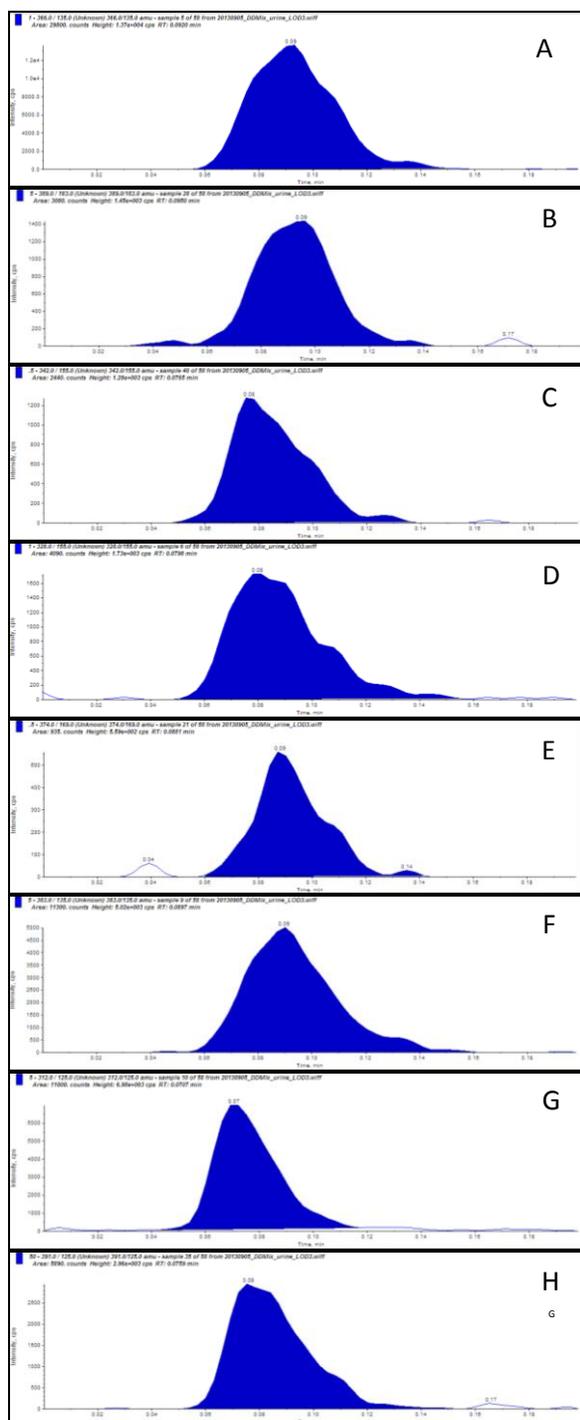
**Figure D-5. <u>Synthetic Cannabinoids</u>: Urine LOD concentrations of AKB48 (A), EAM2201 (B), JWH-018 (C), JWH-073 (D), MAM2201 (E), STS-135 (F), UR-144 (G), UR-144 5-bromopentyl (H), UR-144 5-chloropentyl (I), XLR11 4-pentenyl (J) and XLR11 (K) at 1, 5, 0.5, 1, 0.5, 5, 5, 50, 2, 5, and 1 ng/mL, respectively. (continued)**
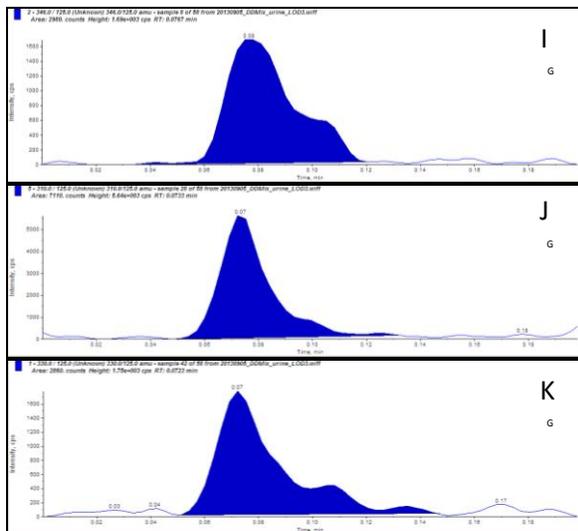
**Figure D-6. <u>Synthetic Cannabinoid Metabolites</u>: Urine LOD concentrations of JWH-018 N-(5-hydroxypentyl) (A), JWH-073 N-(4-hydroxybutyl) (B), JWH-122 5-hydroxypentyl (C) and JWH-200 4-hydroxyindole (D) at 0.1, 0.5, 0.1, and 2 ng/mL, respectively.**

**Figure D-7. <u>Synthetic Cathinones and Piperazines</u>:** Urine LOD concentrations of 4-MEC (A1), 4-MePPP (B1), α-PVP (C1), buphedrone (D1), flephedrone (E1), mCPP (F1), MDPV (G1), methylone (H1), and TFMPP (I1) at 5, 5, 5, 1, 5, 20, 2, 5, and 1 ng/mL, respectively. Blood LOD concentrations of 4-MEC (A2), 4-MePPP (B2), α-PVP (C2), buphedrone (D2), flephedrone (E2), mCPP (F2), MDPV (G2), methylone (H2), and TFMPP (I1) at 2, 5, 5, 2, 5, 10, 2, and 1 ng/mL, respectively.

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**Figure D-7. <u>Synthetic Cathinones and Piperazines</u>:  Urine LOD concentrations of 4-MEC (A1), 4-MePPP (B1), α-PVP (C1), buphedrone (D1), flephedrone (E1), mCPP (F1), MDPV (G1), methylone (H1), and TFMPP (I1) at 5, 5, 5, 1, 5, 20, 2, 5, and 1 ng/mL, respectively.  Blood LOD concentrations of 4-MEC (A2), 4-MePPP (B2), α-PVP (C2), buphedrone (D2), flephedrone (E2), mCPP (F2), MDPV (G2), methylone (H2), and TFMPP (I1) at 2, 5, 5, 2, 5, 10, 2, and 1 ng/mL, respectively. (continued)**

**Figure D-8. <u>Tryptamines</u>: Urine LOD concentrations of 5-MeO-AMT (A), 5-MeO-DiPT (B), 5-MeO-DMT (C), and 5-MeO-MiPT (D) at 2, 5, 5, and 5 ng/mL, respectively.**
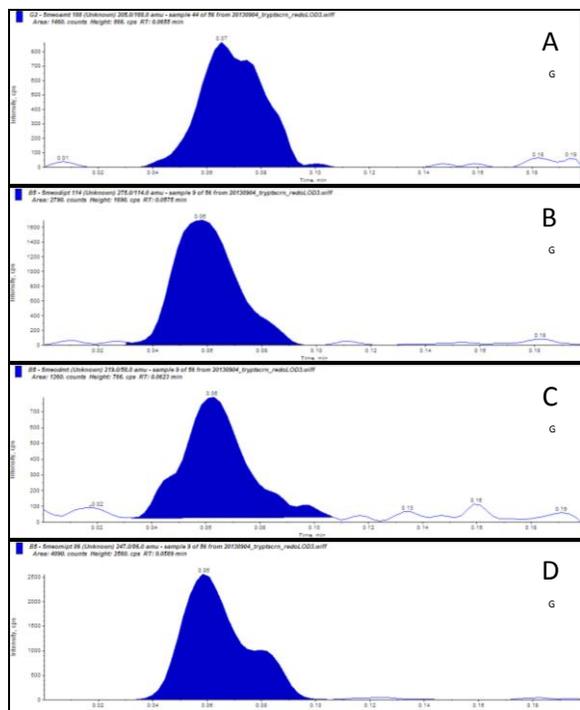
**Figure D-9. <u>Miscellaneous</u>: Urine LOQ concentrations of BZE (A1), COC (B1) and PCP (C1) at 2, 0.5, and 5 ng/mL respectively. Blood LOQ concentrations of BZE (A2), COC (B2) and PCP (C2) at 0.25, 0.5, and 2 ng/mL, respectively.**



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