Validation Report for Quantitation of Select Stimulants in Whole Blood by LC-MSMS

This document describes the validation of select stimulants for quantitation and/or qualitative identification by liquid chromatography with tandem mass spectrometry (LC-MSMS). The target compounds included alpha-PVP, amphetamine, benzoylecgonine, BZP, butylone, cocaethylene, cocaine, ethylone, MDA, MDMA, MDPV, mephedrone, methamphetamine, methylone, methylphenidate, phentermine, and TFMPP. BZP, phentermine and TFMPP were validated for qualitative identification only. Protein precipitation with acetonitrile was used to prepare the whole blood specimens for analysis. A Shimadzu Prominence liquid chromatograph with an AB Sciex 3200 QTrap tandem mass spectrometer, designated as LC-1, was used for quantitation and confirmation using multiple reaction monitoring mode (MRM). This validation included the evaluation of:

Validation Parameter	Acceptance Criteria (1-5)	Results
Selectivity/Specificity	1. No matrix interference from 10 different whole blood sources (if possible) that do not contain the target analyte(s). 2. No interference from at least 10-15 similar compounds that are commonly identified in whole blood case samples. 3. No interference from a high concentration of target compound(s) for the internal standard(s).	1. There was no matrix interference from 10 different whole blood sources with the target compounds or internal standards. 2. There was no interference from 67 drugs and metabolites that are commonly identified in whole blood case samples. 3. There was no interference from a high concentration of target compounds with the internal standards.

Ionization Suppression or Enhancement	1. Isotopically-labeled (deuterium) internal standards that co-elute (within ± 0.05 minutes) with each target analyte will be used for all compounds. Therefore, ionization suppression/enhancement experiments are not required or necessary (5). However, SWGTOX (1) lists this experiment as a requirement and it will therefore be conducted. 2. Average suppression or enhancement must be less than ± 25% and the %CV of the suppression or enhancement must be less than 15%. If any of these values are exceeded then it must be demonstrated that the suppression/enhancement does not impact LOD, LOQ, and bias.	1. BZP and TFMPP demonstrated ion enhancement greater than 25%. These compounds were only validated for qualitative identification and this enhancement did not impact the LOD. 2. Amphetamine and MDA demonstrated average enhancement greater than 25% at 10 ng/mL for the analyte and relative response of the analyte to internal standard, indicating that the use of a deuterated internal standard did not compensate at this concentration. However, the observed ion enhancement did not impact the LOD, LOQ, or bias. 3. None of the other compounds demonstrated average suppression or enhancement greater than ± 25% for the relative response indicating that the use of isotopically-labeled internal standards compensated for any significant ion suppression or enhancement for those compounds. 4. The % CV for the relative response for BZP was 35% at 10 ng/mL. BZP was validated for qualitative identification only. 5. The % CV for the relative response was not greater than 13% for any other compound.

Calibration Model, Linearity	1. At least 6 non-zero concentrations evenly spaced across the calibration range (perhaps more for non-linear models), with five replicates at each level analyzed in 5 separate extractions (one replicate per level per extraction), with the combined data used to establish the calibration model. 2. Coefficient of determination must be ≥ 0.990. 3. Visual inspection of the curve and residual plot should indicate normal random scatter around the calibration curve. 4. Calibrators must be within ± 20% of their prepared concentration.	1. Calibrators at 6 non-zero concentrations for methylphenidate and 7 non-zero concentrations for all other compounds, evenly spaced across the calibration range, with five replicates at each level analyzed in five separate extractions were used to establish the calibration model. 2. The coefficient of determination for all compounds validated for quantitative analysis was greater than 0.990. 3. Visual inspection of the curve and residual plots indicated normal random scatter around the calibration curve. 4. In each separate extraction all calibrators were within ± 20% of their prepared concentration.
Sensitivity – Limit of Detection (LOD)	1. Good chromatographic peak shape. 2. Signal-to-noise ratio of greater than 3:1. 3. MRM ion ratios within 20% and retention time within 0.1 minutes (or 2% relative retention time) compared to a suitable standard (or average of all calibrators). 4. Once determined, the LOD must be verified by at least two replicates of a standard run in three separate extractions prepared in three different sources of blood.	1. All compounds had good chromatographic peak shape at their LODs. 2. All signal-to-noise ratios were greater than 8:1. 3. MRM ion ratios were within 20% and retention times were within 0.1 minutes compared to the average of all calibrators used. 4. The validated LOD was verified by three replicates of a standard run in five separate extractions prepared in five different sources of whole blood.

Sensitivity—Limit of Quantitation (LOQ)	1. Good chromatographic peak shape. 2. Signal to Noise ratio of greater than or equal to 10:1. 3. MRM ion ratios within 20% and retention time within 0.1 minutes (or 2% relative retention time) compared to a suitable standard (or average of all calibrators). 4. Quantitative results must be within ± 20% of their prepared concentration. 5. Once determined, the LOQ must be verified by at least two replicates of a standard run in three separate extractions prepared in three different sources of blood.	1. All compounds had good chromatographic peak shape at their LODs. 2. All signal-to-noise ratios were greater than or equal to 10:1. 3. MRM ion ratios were within 20% and retention times were within 0.1 minutes compared to the average of all calibrators used. 4. Quantitative results were within ± 20% of their prepared concentration. 5. The validated LOQ was verified by three replicates of a standard run in five separate extractions prepared in five different sources of whole blood.
Repeatability—Bias (Accuracy)	1. Evaluated at three concentration levels. A low level less than or equal to three times the LOQ, a high level within 20% of the upper limit of the calibration range, and a medium level near the midpoint of the low and high. Pooled fortified matrix samples will not be used as some target analytes may demonstrate poor stability in matrix. 2. At least 3 replicates from 5 separate extractions should be evaluated for each level. 3. The bias should be within ± 20% of the prepared concentration.	1. Standards prepared at four different concentrations for methylphenidate and five different concentrations for all other compounds—including low, medium, and high levels meeting validation requirements were evaluated for each compound. 2. Three replicates from five separate extractions were evaluated for each level. 3. The bias was within ± 14% of the prepared concentration.

Repeatability— Precision Within-run Between-run	1. Evaluated at least at three concentration levels. These will be the same as those that were used for the bias studies. 2. At least 3 replicates from 5 separate extractions should be evaluated for each level. 3. The coefficient of variation (CV) should be within 20%.	1. Four levels for methylphenidate and five levels for all other compounds were evaluated (the same standards that were used for the bias experiments) for precision. 2. Three replicates from five separate extractions were evaluated for each. 3. The CV was within 15% for those compounds validated for quantitative analysis and within 54% for those compounds validated for qualitative identification.
Reportable Range	1. The reportable range shall be determined after evaluating the calibration model and sensitivity of the assay. 2. It is advantageous but may not be necessary for the reportable range to include the range of desired concentrations noted below.	1. The reportable range was determined after evaluating the calibration model and sensitivity of the assay. 2. The reportable range was determined to at least encompass the concentrations of 50 to 5000 (50,000 with 10x dilution) for benzoylecgonine and 5 to 500 (5,000 with 10x dilution) for all other compounds, but may extend beyond the ranges formally validated to include levels down to the experimental limit of detection and above the highest level evaluated. 3. Compounds meeting acceptable identification criteria with apparent concentrations outside of the range examined in this validation may be reported.

Dilution Integrity	1. Any required dilutions of case samples will be made with whole blood. Dilution integrity will be evaluated at a 1:10 dilution by repeating bias and precision studies at one level using a 1:10 dilution of standards prepared in whole blood. Other dilutions may also be evaluated if it is anticipated that they will be routinely used in casework. 2. All bias and precision criteria stated above must be acceptable when using the dilution.	1. Dilution integrity was evaluated by repeating bias and precision studies at one level using a 1:10 dilution of standards prepared in whole blood. 2. All bias and precision criteria stated above was acceptable for all compounds when using the dilution.
Carryover	1. A blank matrix sample must be analyte free when run after a standard prepared at or above the highest calibrator concentration. 2. Lack of carryover must be determined by triplicate analyses (repeated injection of an extracted standard and blank is acceptable).	1. A blank matrix sample was analyte free when run after a standard prepared at the highest calibrator concentration of 500/5000 ng/mL. 2. The lack of carryover was verified by triplicate analyses.

Extract Stability	1. At least five replicates of controls will be prepared at a low and high concentration. The extracts will be combined and then divided into five different vials. A vial of each level will be injected in triplicate on day 0. The other vials will be stored on the instrument and reinjected on each subsequent day in triplicate. 2. The response of each analyte must be within ± 20% of the response from day 0. If the response falls outside this range then the extract stability of the analyte has been exceeded.	1. Five replicates at a low and high concentration were prepared, combined, and then divided into five different vials. A vial of each level was injected in triplicate on day 0. The other vials were stored on the instrument and reinjected on each subsequent day in triplicate. 2. The response of each analyte validated for quantitative analysis and phentermine was within ± 20% of the response from day 0 on each subsequent day up to day 4. 3. The response for BZP and TFMPP exceeded + 20% on day 1 at the low concentration. However these compounds were only validated for qualitative identification and the MRM ratios were still acceptable and identification was still possible on day 4. 4. All compounds demonstrated stability suitable for quantitative and/or qualitative identification for at least 4 days after the date of extraction.
Ruggedness/ Robustness	1. Validation studies will be performed over multiple days by multiple analysts.	1. Validation studies were performed over multiple days and by 4 analysts and 1 trainee in toxicology.

Case Sample Comparison	1. Any case samples that have been previously determined to contain the target analyte(s) must have identical qualitative results and quantitative results must agree within ± 20% (within the capabilities of the methods being compared). Note: Some analytes may have poor stability in matrix and this should be considered if there are discrepancies in the qualitative and/or quantitative results.	1. Fifteen cases and one proficiency sample were utilized for the case comparison study. Three case samples were negative for the target analytes. 2. All samples negative for the target analytes by the existing methods were negative by the LC-MSMS method. 3. Due to the increased sensitivity with the LC-MSMS method additional related compounds were identified in three case samples and the proficiency sample (e.g. cocaethylene in a case containing cocaine and benzoylecgonine). All other case samples had identical qualitative results. 4. Quantitative results agreed well within the capabilities of the methods being compared. Two contemporaneous benzoylecgonine results were 21% higher by LC-MSMS, but were within the estimated UOM for the GC-MS method of ± 24%. One MDMA result was outside + 20%, but the original result was at the GC-MS LOQ which has an acceptable accuracy of ± 30%. 5. Some cases were in storage for extended periods of time before LC-MSMS analysis was conducted. This led to expected significantly lower results due to the known stability issues with the target compounds in whole blood. One benzoylecgonine result was 52% lower than the GC-MS result for a

		case that had been in storage for greater than two years. One cocaine case was 52% lower by LC-MSMS after storage of greater than 1 year (most of that time in a freezer). Another cocaine case was 25% lower by LC-MSMS after refrigerated storage for 3 weeks.
Estimation of Uncertainty of Measurement	1. The uncertainty of measurement estimation worksheet will be constructed using TX Estimation of Uncertainty of Measurement (UOM) for the replicate data of a suitable control that will be used in routine casework. Note: This requires a minimum of 30 replicates.	1. An uncertainty of measurement estimation worksheet was constructed using TX Estimation of Uncertainty of Measurement (UOM) for the replicate data of the 200/2000 ng/mL control that will be used in routine casework.

Validation Steps

Step 1: Ionization Suppression/Enhancement

Analyst: Nick Tiscione (NBT)
Date: 1/14/16

General Outline of Validation Step

1. Prepare working calibrator(s) and control(s) in acetonitrile or other suitable solvent.
2. Prepare working internal standard in acetonitrile or other suitable solvent.
3. Two different sets of samples will be prepared and the analyte and internal standard peak areas of neat standards will be compared to matrix samples fortified with neat standards after extraction or processing.
4. Set one will consist of neat standards prepared at two concentrations – one low and one high with one replicate at each level. Each of these neat standards will be injected six times to establish a mean peak area for each concentration. If insufficient volume of extract is produced from the sample processing or extraction then multiple replicates will be prepared and combined to yield sufficient volume.
5. Set two will consist of a minimum of ten different matrix sources (if possible). A mixture of sources may count as some of the ten (i.e., if a mixture has six sources, that would count as six of the ten). Each matrix source will be processed in duplicate. After the extraction is complete, each matrix sample will then be fortified with either the low or high concentration neat standard.
6. The average area of each set ($\bar{X}$) is used to estimate the suppression/enhancement effect at each concentration as follows:

$$Ionization\ suppression\ or\ enhancement\ (\%) = \left(\frac{\bar{X}\ Area\ of\ Set\ 2}{\bar{X}\ Area\ of\ Set\ 1} - 1\right) x\ 100$$

Step 2: Sensitivity, Carryover, Linearity, and Selectivity/Specificity

Analyst: NBT, Xiaoqin Shan (XS)
Dates: 1/14/16, 1/27/16, 2/5/16, 2/8/16, 2/9/16

General Outline of Validation Step

1. Extract the following:
    a. A series of at least eight calibrators prepared in whole blood representing anticipated concentrations in whole blood specimens, evenly spaced.

Desired Range (ng/mL)	Suggested Calibrator Levels (ng/mL)
5-500 (All compounds except Benzoylecgonine)	1.25, 2.5, 5, 10, 20, 50, 125, 250, 500
50-5000 (Benzoylecgonine)	12.5, 25, 50, 100, 200, 500, 1250, 2500, 5000

b. A matrix blank with internal standard to be run after the highest calibrator (analyzed in triplicate).
c. A matrix blank fortified with target analytes at the same concentration as the highest calibrator without internal standard.
d. A matrix blank fortified with as many of the following compounds as are available, each at 10 µg/mL or 1 µg/mL (as indicated). Do not add internal standard. Note: Add appropriate amount of each compound to labeled tube (P-1 or P-2, except where indicated), evaporate solvent at room temperature until approximately 100uL remains and add 1mL of whole blood.

1.	6-MAM (1 µg/mL)	CAL 966
2.	7-Aminoflunitrazepam (1 µg/mL)	CAL 967
3.	Acetaminophen*	
4.	Alprazolam	CAL 968
5.	Amitriptyline	CON 1279
6.	Bupropion	CON 1216
7.	Buprenorphine (1 µg/mL)	CON 2030
8.	Butalbital	CAL 957
9.	Caffeine*	
10.	Carbamazepine	CAL 958
11.	Carisoprodol	CON 1981
12.	Chlordiazepoxide	CAL 969
13.	Chlorophenylpiperazine	CON 2076
14.	Chlorpheniramine*	
15.	Clonazepam	CAL 970
16.	Codeine	CAL 971
17.	Cyclobenzaprine	CAL 1015
18.	Desalkylflurazepam	CAL 972
19.	Dextromethorphan	CAL 884
20.	Diazepam	CAL 973
21.	Dihydrocodeine	CAL 974
22.	Diphenhydramine	CAL 914
23.	Doxylamine	CON 1441
24.	Ephedrine	CON 1362

25.	Fentanyl (1 µg/mL)	CAL 975
26.	Flunitrazepam (1 µg/mL)	CAL 862
27.	Fluoxetine	CAL 844
28.	5-MeO-Dipt (Foxy)	CON 1498
29.	Hydrocodone	CAL 976
30.	Hydromorphone (1 µg/mL)	CAL 977
31.	Ibuprofen*	
32.	Lamotrigine	CON 1905
33.	Lidocaine	CON 1653
34.	Lorazepam	CAL 978
35.	Meperidine	CAL 763
36.	Meprobamate	CON 1982
37.	Methadone	CAL 979
38.	Midazolam	CAL 980
39.	Morphine	CAL 981
40.	Naloxone (1 µg/mL)	CAL 826
41.	Naproxen*	
42.	Norbuprenorphine (1 µg/mL)	CON 1960
43.	Nordiazepam	CAL 982
44.	Norquetiapine	CAL 722
45.	Nortriptyline	CON 1930
46.	Oxazepam	CAL 983
47.	Oxycodone	CAL 956
48.	Oxymorphone (1 µg/mL)	CAL 984
49.	Pheniramine	CON 1931
50.	Phenobarbital	CON 1452
51.	Phenytoin	CON 1881
52.	Promethazine	CON 1624
53.	Propoxyphene	CAL 758
54.	Pseudoephedrine*	
55.	Sertraline	CAL 723
56.	Temazepam	CAL 985
57.	THC (1ug/mL)	CAL 1002
58.	OH-THC (1ug/mL)	CAL 998
59.	THCA (1ug/mL)	CAL 916
60.	Topiramate	CON 1985
61.	Tramadol	CAL 885
62.	Trazodone	CAL 937
63.	Venlafaxine	CON 1486
64.	Quetiapine	CAL 1014

65. Zaleplon CAL 986
66. Zolpidem CAL 987
67. Zopiclone CAL 988

*Contained in drug mix purchased from Cerilliant CON 2075 (P-28)

e. A positive control fortified with the above list of compounds (use fortified blood prepared in step d., internal standard, and the target analytes at the concentration that will be used to estimate the uncertainty of measurement.
f. Ten whole blood blanks from different sources that do not contain the target analytes or internal standard. Note: A blood blank that consists of a mixture of different sources may substitute for some of the ten (i.e. a mixture of six sources counts as six of the required ten).

2. Analyze on LC-1.
3. Repeat 1.a. through 1.c. four times in four separate extractions (Steps 3-7) to yield 5 replicates at each calibrator level (Note: the blank need not be analyzed in triplicate after the highest calibrator for these steps).
4. Use the combined data to evaluate the calibration model.
5. Evaluate the calibrators to determine the limit of detection (LOD) and limit of quantitation (LOQ).
6. Determine suitable levels for calibrators to be used in routine analysis (at least 4 for linear models or at least 6 for non-linear models).
7. Determine suitable level(s) for positive control(s) to be used in routine analysis.
8. Evaluate carryover and specificity.

Steps 3-7: Sensitivity, Repeatability, Robustness and Estimation of Uncertainty of Measurement

Analyst: NBT, Russ Miller (RWM)
Dates: 2/5/16, 2/18/16, 4/19/16, 4/20/16, 4/27/16

General Outline of Validation Steps

1. Prepare calibrators, matrix blank, and replicates for each of the positive control(s).
    a. Positive controls
        i. Two replicates at the LOD prepared in at least three different sources or a mixture of at least three different sources of whole blood.
        ii. Two replicates at the LOQ (if different than LOD) prepared in at least three different sources or a mixture of at least three different sources of whole blood.
        iii. Three replicates at a low level within three times the LOQ (may be combined with i and ii).
        iv. Three replicates at a high level within 20% of the upper limit of the calibration range
        v. Three replicates at a medium/mid-level near the midpoint of the low and high.

vi. Three replicates of a 1:10 dilution of a standard prepared at a medium/mid to high level.

2. Analyze on LC-1, running the matrix blank after the highest calibrator.
3. Evaluate the positive controls for precision and accuracy (bias).
4. A suitable control level that will be analyzed for routine analysis will be used to establish an initial estimation of the UOM for the compound(s) being validated for quantitative analysis. See Step 9.

Step 8: Case Sample Comparison/Evaluation

Analyst: NBT, XS, DTY
Dates: 4/22/16, 4/28/16, 5/3/16

General Outline of Validation Step

1. Prepare the calibrators, matrix blank and positive control(s).
2. Prepare at least 5-10 negative and positive cases (as many as available).
3. Perform procedure and run on LC-1.

Step 9: Estimation of Uncertainty of Measurement

Analyst: RWM, Amber Kohl
Dates: 5/10-5/11/16

General Outline of Validation Step

1. Prepare the calibrators and matrix blank.
2. Prepare enough replicates of positive control to be used for UOM estimation (identified in steps 3-7) to yield a total of 30 when combined with replicates from steps 2-8.
3. Perform procedure and run on LC-1.
4. Use TX Estimation of Uncertainty of Measurement (UOM) to establish an initial estimation of the UOM for the compound(s) being validated for quantitative analysis.

Step 10: Extract Stability

Analyst: NBT
Dates: 5/2-5/6/16

General Outline of Validation Step

1. Prepare at least five replicates of controls at a low and high concentration (same as used above in Steps 3-7) with the internal standard.
2. Combine the extracts of the replicates at each level and then divide into five different vials.
3. Inject a vial of each level in triplicate on day 0.
4. Store the other vials on the instrument and re-inject on each subsequent day in triplicate.
5. The response of each analyte must be within ± 20% of the response from day 0. If the response falls outside this range then the extract stability of the analyte has been exceeded.

Results

Ionization Suppression/Enhancement

Working standards for the calibrators, controls, and internal standard were prepared in acetonitrile. Two different sets of samples were prepared and the analyte and internal standard peak areas of neat standards were compared to matrix samples fortified with neat standards after extraction or processing. Set one consisted of neat standards prepared at three concentrations 10/100 ng/mL (100 ng/mL for benzoylecgonine and 10 ng/mL for all other compounds), 200/2000 ng/mL and 400/4000 ng/mL, each with internal standard. Each of the neat standards was injected six times to establish a mean peak area for each concentration. Set two consisted of ten different matrix sources that were extracted in triplicate. After the extraction was complete each matrix sample was fortified with the low, mid, or high concentration neat standard and internal standard. The average area of each set was used to estimate the suppression/enhancement effect at each concentration as follows for the analyte ions, internal standard, and relative response for each drug (quant ion/internal standard ion):

$$Ionization\ suppression\ or\ enhancement\ (\%) = \left(\frac{\bar{X}\ Area\ of\ Set\ 2}{\bar{X}\ Area\ of\ Set\ 1} - 1\right) x\ 100$$

The % CV was also calculated for the analyte ions, internal standard, and relative response for each drug at each concentration.

Analyte1	Analyte2	ISTD	Ionization Suppression or Enhancement (%)			
			10/100 ng/mL			
			Analyte1 Response	Analyte2 Response	ISTD Response	Relative Response
alpha-PVP 1	alpha-PVP 2	IS alpha-PVP d8	**-35%**	**-38%**	**-32%**	-4%
amphetamine 1	amphetamine 2	IS Amphetamine-d6	**31%**	**28%**	-14%	**52%**
benzoylecgonine 1	benzoylecgonine 2	IS benzoylecgonine d3	-4%	-5%	-1%	-4%
Butylone 1	Butylone 2	IS Butylone-d3	-10%	-5%	-7%	-4%
BZP 1	BZP 2	IS BZP-d7	**414%**	**440%**	-21%	**556%**
Cocaethylene 1	Cocaethylene 2	IS Cocaethylene-d3	-7%	-6%	-6%	-1%
Cocaine 1	Cocaine 2	IS Cocaine-d3	-10%	-9%	-6%	-4%
Ethylone 1	Ethylone 2	IS Ethylone-d5	-13%	-9%	-7%	-6%

MDA 1	MDA 2	IS MDA-d5	31%	29%	-4%	36%
MDMA 1	MDMA 2	IS MDMA-d5	-11%	-13%	-10%	-1%
MDPV 1	MDPV 2	IS MDPV-d8	-13%	-15%	-10%	-3%
Mephedrone 1	Mephedrone 2	IS Mephedrone-d3	-15%	-15%	-13%	-3%
Methamphetamine 1	Methamphetamine 2	IS Methamphetamine-d5	-14%	-13%	-12%	-2%
Methylone 1	Methylone 2	IS Methylone-d3	-12%	-11%	-9%	-3%
Methylphenidate 1	Methylphenidate 2	IS Methylphenidate-d9	-14%	-16%	-12%	-2%
Phentermine 1	Phentermine 2	IS Phentermine-d5	-17%	-17%	-14%	-3%
TFMPP 1	TFMPP 2	IS TFMPP-d4	45%	45%	-6%	56%

Analyte1	Analyte2	ISTD	Ionization Suppression or Enhancement (%)			
			200/2000 ng/mL			
			Analyte1 Response	Analyte2 Response	ISTD Response	Relative Response
alpha-PVP 1	alpha-PVP 2	IS alpha-PVP d8	-29%	-29%	-21%	-9%
amphetamine 1	amphetamine 2	IS Amphetamine-d6	-15%	-15%	-5%	-11%
benzoylecgonine 1	benzoylecgonine 2	IS benzoylecgonine d3	1%	1%	19%	-15%
Butylone 1	Butylone 2	IS Butylone-d3	-7%	-6%	9%	-15%
BZP 1	BZP 2	IS BZP-d7	23%	24%	-13%	42%
Cocaethylene 1	Cocaethylene 2	IS Cocaethylene-d3	-6%	-6%	13%	-17%
Cocaine 1	Cocaine 2	IS Cocaine-d3	-3%	-5%	9%	-11%
Ethylone 1	Ethylone 2	IS Ethylone-d5	-9%	-6%	5%	-14%
MDA 1	MDA 2	IS MDA-d5	-4%	-5%	11%	-13%
MDMA 1	MDMA 2	IS MDMA-d5	-8%	-8%	8%	-15%
MDPV 1	MDPV 2	IS MDPV-d8	-9%	-8%	5%	-13%
Mephedrone 1	Mephedrone 2	IS Mephedrone-d3	-15%	-16%	-2%	-13%
Methamphetamine 1	Methamphetamine 2	IS Methamphetamine-d5	-15%	-17%	-2%	-13%
Methylone 1	Methylone 2	IS Methylone-d3	-8%	-9%	6%	-13%
Methylphenidate 1	Methylphenidate 2	IS Methylphenidate-d9	-8%	-10%	1%	-9%
Phentermine 1	Phentermine 2	IS Phentermine-d5	-18%	-18%	-3%	-15%
TFMPP 1	TFMPP 2	IS TFMPP-d4	-6%	-6%	6%	-11%

Analyte1	Analyte2	ISTD	Ionization Suppression or Enhancement (%)			
			400/4000 ng/mL			
			Analyte1 Response	Analyte2 Response	ISTD Response	Relative Response
alpha-PVP 1	alpha-PVP 2	IS alpha-PVP d8	**-28%**	**-28%**	**-34%**	9%
amphetamine 1	amphetamine 2	IS Amphetamine-d6	-15%	-16%	-22%	9%
benzoylecgonine 1	benzoylecgonine 2	IS benzoylecgonine d3	0%	0%	-7%	8%
Butylone 1	Butylone 2	IS Butylone-d3	-7%	-9%	-12%	6%
BZP 1	BZP 2	IS BZP-d7	15%	17%	**-30%**	**67%**
Cocaethylene 1	Cocaethylene 2	IS Cocaethylene-d3	-3%	-4%	-9%	7%
Cocaine 1	Cocaine 2	IS Cocaine-d3	0%	-2%	-9%	10%
Ethylone 1	Ethylone 2	IS Ethylone-d5	-6%	-7%	-12%	6%
MDA 1	MDA 2	IS MDA-d5	-5%	-4%	-8%	4%
MDMA 1	MDMA 2	IS MDMA-d5	-6%	-5%	-8%	3%
MDPV 1	MDPV 2	IS MDPV-d8	-8%	-8%	-11%	4%
Mephedrone 1	Mephedrone 2	IS Mephedrone-d3	-14%	-16%	-20%	8%
Methamphetamine 1	Methamphetamine 2	IS Methamphetamine-d5	-13%	-15%	-20%	8%
Methylone 1	Methylone 2	IS Methylone-d3	-7%	-8%	-12%	6%
Methylphenidate 1	Methylphenidate 2	IS Methylphenidate-d9	-8%	-7%	-15%	9%
Phentermine 1	Phentermine 2	IS Phentermine-d5	-17%	-17%	-20%	5%
TFMPP 1	TFMPP 2	IS TFMPP-d4	-5%	-5%	-15%	12%

Analyte1	Analyte2	ISTD	CV of Ionization Suppression or Enhancement (%)			
			10/100 ng/mL			
			Analyte1 Response	Analyte2 Response	ISTD Response	Relative Response
alpha-PVP 1	alpha-PVP 2	IS alpha-PVP d8	**20%**	**17%**	**17%**	6%
amphetamine 1	amphetamine 2	IS Amphetamine-d6	12%	12%	12%	4%
benzoylecgonine 1	benzoylecgonine 2	IS benzoylecgonine d3	6%	5%	5%	7%
Butylone 1	Butylone 2	IS Butylone-d3	7%	7%	7%	6%
BZP 1	BZP 2	IS BZP-d7	**32%**	**29%**	**29%**	**34%**
Cocaethylene 1	Cocaethylene 2	IS Cocaethylene-d3	4%	4%	4%	3%
Cocaine 1	Cocaine 2	IS Cocaine-d3	4%	4%	4%	3%

Ethylone 1	Ethylone 2	IS Ethylone-d5	8%	8%	8%	5%
MDA 1	MDA 2	IS MDA-d5	8%	5%	5%	7%
MDMA 1	MDMA 2	IS MDMA-d5	7%	7%	7%	5%
MDPV 1	MDPV 2	IS MDPV-d8	5%	6%	6%	5%
Mephedrone 1	Mephedrone 2	IS Mephedrone-d3	15%	13%	13%	5%
Methamphetamine 1	Methamphetamine 2	IS Methamphetamine-d5	13%	13%	13%	3%
Methylone 1	Methylone 2	IS Methylone-d3	8%	8%	8%	3%
Methylphenidate 1	Methylphenidate 2	IS Methylphenidate-d9	9%	7%	7%	6%
Phentermine 1	Phentermine 2	IS Phentermine-d5	15%	16%	16%	3%
TFMPP 1	TFMPP 2	IS TFMPP-d4	10%	12%	12%	13%

Analyte1	Analyte2	ISTD	CV of Ionization Suppression or Enhancement (%)			
			200/2000 ng/mL			
			Analyte1 Response	Analyte2 Response	ISTD Response	Relative Response
alpha-PVP 1	alpha-PVP 2	IS alpha-PVP d8	11%	10%	9%	5%
amphetamine 1	amphetamine 2	IS Amphetamine-d6	8%	8%	8%	3%
benzoylecgonine 1	benzoylecgonine 2	IS benzoylecgonine d3	3%	1%	3%	3%
Butylone 1	Butylone 2	IS Butylone-d3	5%	5%	4%	4%
BZP 1	BZP 2	IS BZP-d7	4%	3%	9%	8%
Cocaethylene 1	Cocaethylene 2	IS Cocaethylene-d3	3%	4%	5%	5%
Cocaine 1	Cocaine 2	IS Cocaine-d3	3%	3%	4%	2%
Ethylone 1	Ethylone 2	IS Ethylone-d5	5%	5%	5%	2%
MDA 1	MDA 2	IS MDA-d5	4%	4%	4%	3%
MDMA 1	MDMA 2	IS MDMA-d5	5%	4%	5%	5%
MDPV 1	MDPV 2	IS MDPV-d8	4%	4%	6%	3%
Mephedrone 1	Mephedrone 2	IS Mephedrone-d3	8%	10%	10%	3%
Methamphetamine 1	Methamphetamine 2	IS Methamphetamine-d5	7%	9%	7%	2%
Methylone 1	Methylone 2	IS Methylone-d3	7%	6%	7%	3%
Methylphenidate 1	Methylphenidate 2	IS Methylphenidate-d9	5%	5%	6%	3%
Phentermine 1	Phentermine 2	IS Phentermine-d5	11%	12%	9%	5%
TFMPP 1	TFMPP 2	IS TFMPP-d4	9%	8%	8%	3%

Analyte1	Analyte2	ISTD	CV of Ionization Suppression or Enhancement (%)			
			400/4000 ng/mL			
			Analyte1 Response	Analyte2 Response	ISTD Response	Relative Response
alpha-PVP 1	alpha-PVP 2	IS alpha-PVP d8	10%	11%	12%	5%
amphetamine 1	amphetamine 2	IS Amphetamine-d6	6%	6%	8%	6%
benzoylecgonine 1	benzoylecgonine 2	IS benzoylecgonine d3	3%	2%	6%	7%
Butylone 1	Butylone 2	IS Butylone-d3	4%	5%	5%	5%
BZP 1	BZP 2	IS BZP-d7	4%	5%	11%	12%
Cocaethylene 1	Cocaethylene 2	IS Cocaethylene-d3	4%	4%	5%	7%
Cocaine 1	Cocaine 2	IS Cocaine-d3	4%	4%	6%	9%
Ethylone 1	Ethylone 2	IS Ethylone-d5	5%	5%	6%	9%
MDA 1	MDA 2	IS MDA-d5	3%	4%	6%	7%
MDMA 1	MDMA 2	IS MDMA-d5	4%	5%	6%	6%
MDPV 1	MDPV 2	IS MDPV-d8	3%	4%	6%	6%
Mephedrone 1	Mephedrone 2	IS Mephedrone-d3	6%	7%	9%	8%
Methamphetamine 1	Methamphetamine 2	IS Methamphetamine-d5	6%	6%	8%	6%
Methylone 1	Methylone 2	IS Methylone-d3	7%	7%	8%	8%
Methylphenidate 1	Methylphenidate 2	IS Methylphenidate-d9	5%	5%	9%	7%
Phentermine 1	Phentermine 2	IS Phentermine-d5	7%	10%	9%	7%
TFMPP 1	TFMPP 2	IS TFMPP-d4	7%	7%	8%	6%

Conclusion

At the low, mid and high concentrations alpha-PVP demonstrated average suppression greater than – 25% for the target analyte ions and at the low and high concentrations greater than – 25% for the internal standard. The CV of the ionization suppression or enhancement was also greater than 15% for the analyte ions and internal standard at the low concentration for alpha-PVP. The relative response and CV of the relative response for alpha-PVP did not exceed ± 9% indicating that the use of a deuterated internal standard compensated for the observed ionization suppression. Amphetamine and MDA demonstrated ionization enhancement greater than 25% at the low concentration that was not compensated for by the use of a deuterated internal standard as the relative response also showed enhancement greater than 25%. This enhancement did not impact the LOD, LOQ, or bias for amphetamine or MDA at 5 or 10 ng/mL. This was confirmed by extracting three replicates at 5 and 10 ng/mL in five different extractions with a different source of whole blood used for the replicates in each extraction. BZP and TFMPP demonstrated very significant enhancement or suppression that was not

compensated for by the use of a deuterated internal standard. BZP also demonstrated a CV of ionization suppression or enhancement greater than 15% at the low concentration that was not compensated for by the use of a deuterated internal standard. Phentermine demonstrated a CV of ionization suppression or enhancement that was at or above 15% for the analyte and internal standard ions that was compensated for by the use of a deuterated internal standard as the CV of the relative response at the low level was 3%. BZP, phentermine, and TFMPP were only validated for qualitative identification and the observed enhancement or suppression did not impact the LOD of these compounds. This was confirmed by extracting three replicates at 5 and 10 ng/mL in five different extractions with a different source of whole blood used for the replicates in each extraction. The use of isotopically-labeled internal standards compensated for any significant ion suppression or enhancement for all other compounds studied.

Selectivity / Specificity

Several different blood samples were prepared and extracted to evaluate the selectivity of the method through an interference study. The specific samples are outlined below.

- A matrix blank fortified with the target analytes at the same concentration as the highest calibrator (5000 ng/mL for benzoylecgonine, 500 ng/mL for all other analytes) without internal standard.
- A matrix blank fortified with 67 related compounds that have been identified in blood drug analysis casework without internal standard. Each compound was fortified at a concentration of 10 µg/mL or 1 ug/mL as applicable.
- A matrix blank fortified with benzoylecgonine at 2000 ng/mL and all other target analytes at 200 ng/mL with internal standard as well as the same 67 related compounds mentioned above.
- Ten whole blood samples from different sources that did not contain the target analytes or internal standard.

There was no matrix interference from 10 different whole blood sources that did not contain the target analytes or internal standards. There was no interference from 67 related compounds that are commonly identified in whole blood case samples. There was no interference from a high concentration (500/5000 ng/mL) of target compounds with the internal standards. The 200/2000 ng/mL control fortified with 67 compounds commonly present in forensic toxicology samples had quantitative results within ± 20% of the prepared concentration for all compounds except BZP and phentermine. Phentermine had quantitative results less than – 20% and therefore will only be determined qualitatively. BZP and TFMPP did not meet multiple other requirements for quantitative analysis and therefore will only be determined qualitatively.

Conclusion

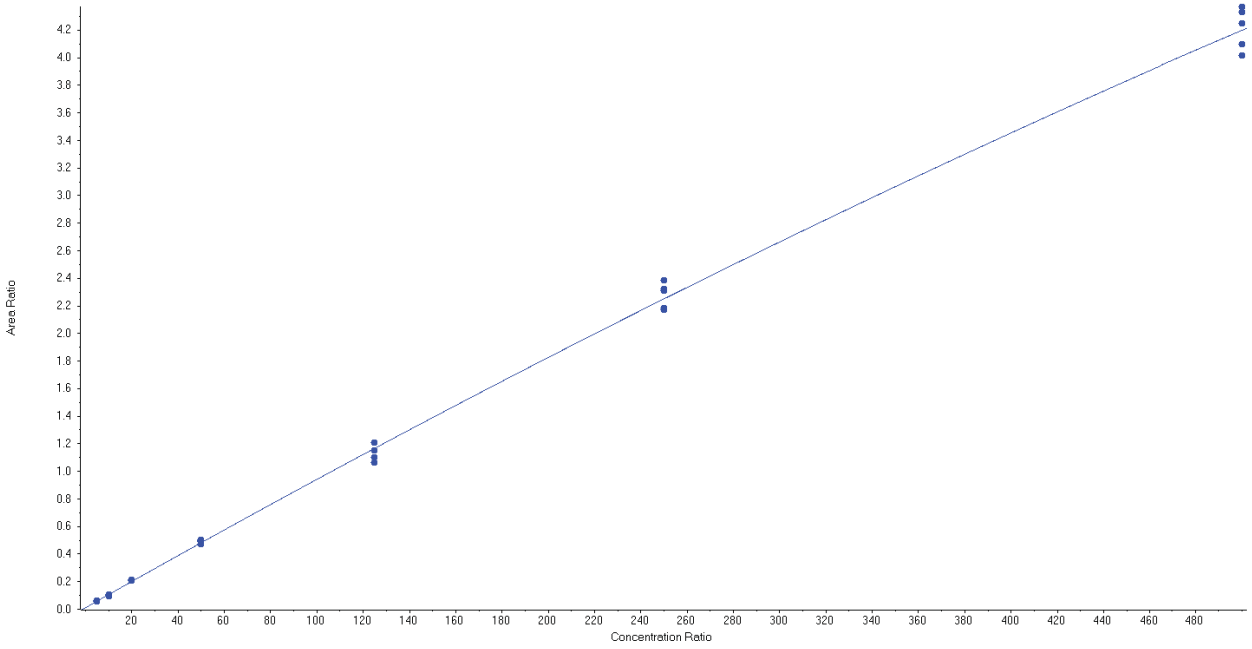
The method is specific for the target analytes and internal standards studied.

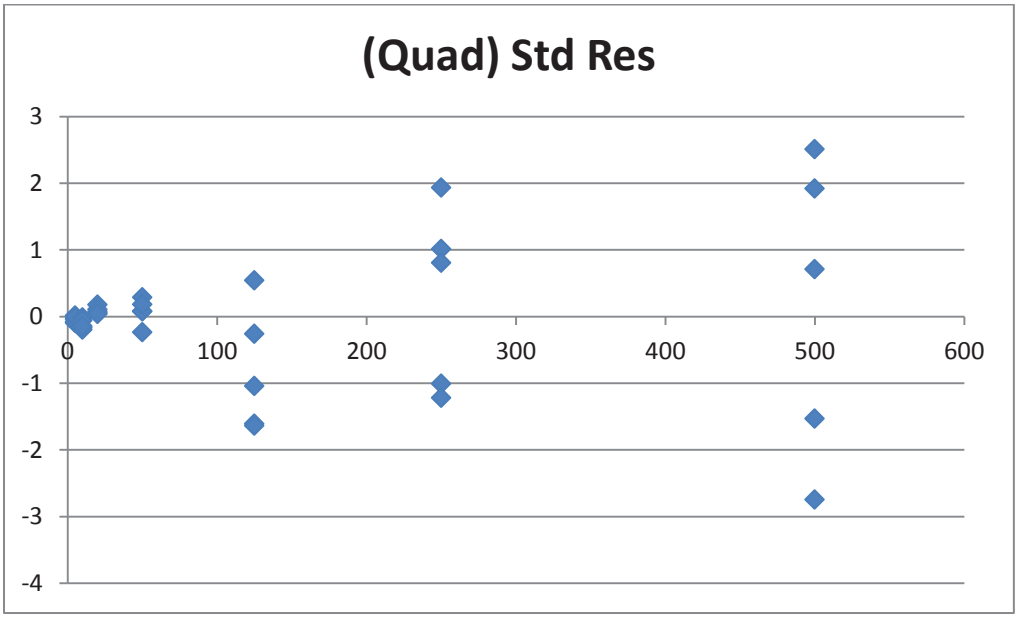
Calibration model/linearity

Calibrators at 9 concentrations from 12.5 to 5000 ng/mL for benzoylecgonine and from 1.25 to 500 ng/mL for all other compounds were prepared and extracted. The combined data of 6 non-zero concentrations from 5 to 250 ng/mL for methylphenidate, 7 non-zero concentrations from 50 to 5000 ng/mL for benzoylecgonine and 7 non-zero concentrations from 5 to 500 ng/mL for all other compounds evenly spaced across the calibration range with five replicates at each level analyzed in 5 separate extractions (one replicate per level per extraction) were used to establish the calibration model. The coefficient of determination (r^2) was ≥ 0.990 for all compounds. Visual inspection of the curves and residual plots indicated normal random scatter around the calibration curve. All calibrators were within ± 20% of their prepared concentration in each extraction. Standardized residual plots for each compound validated for quantitative analysis were constructed. Any outliers (outside ± 3 standard deviations) were eliminated prior to final analysis of the standardized residual plots (Std Res).

Alpha-PVP

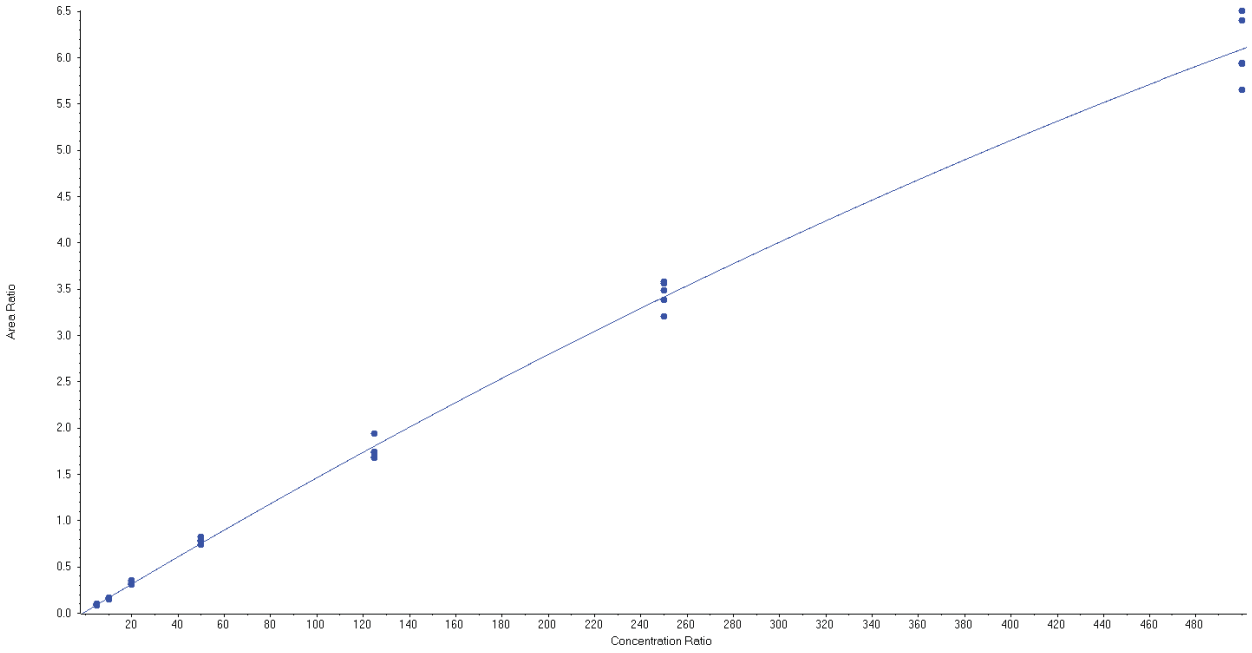
y = -2.33332e-6 x^2 + 0.00954 x + 0.01128 (r = 0.99767) (weighting: 1 / x^2) $r^2 = 0.9953$

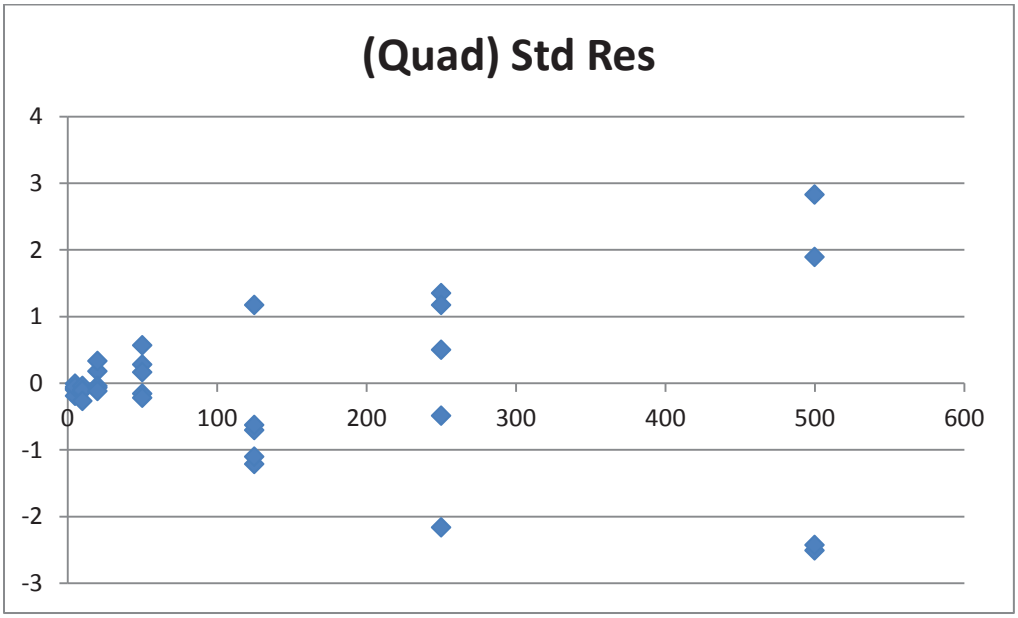




Amphetamine

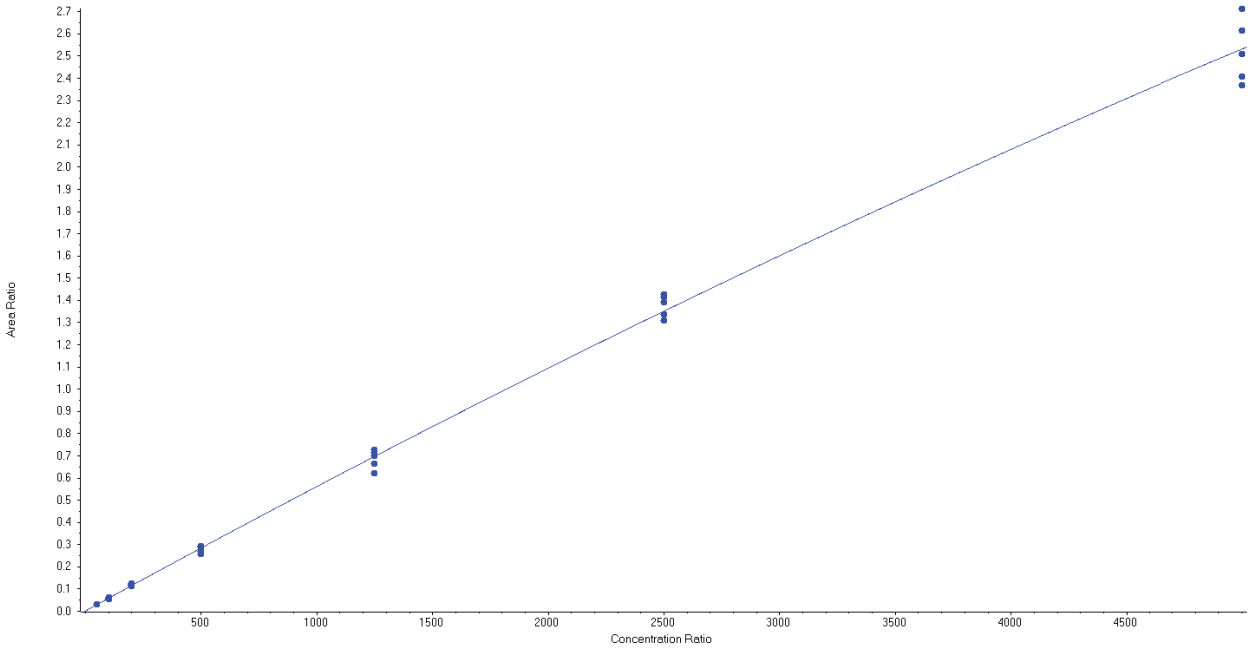
y = -5.79327e-6 x^2 + 0.01505 x + 0.01693 (r = 0.99841) (weighting: 1 / x) $r^2 = 0.9968$

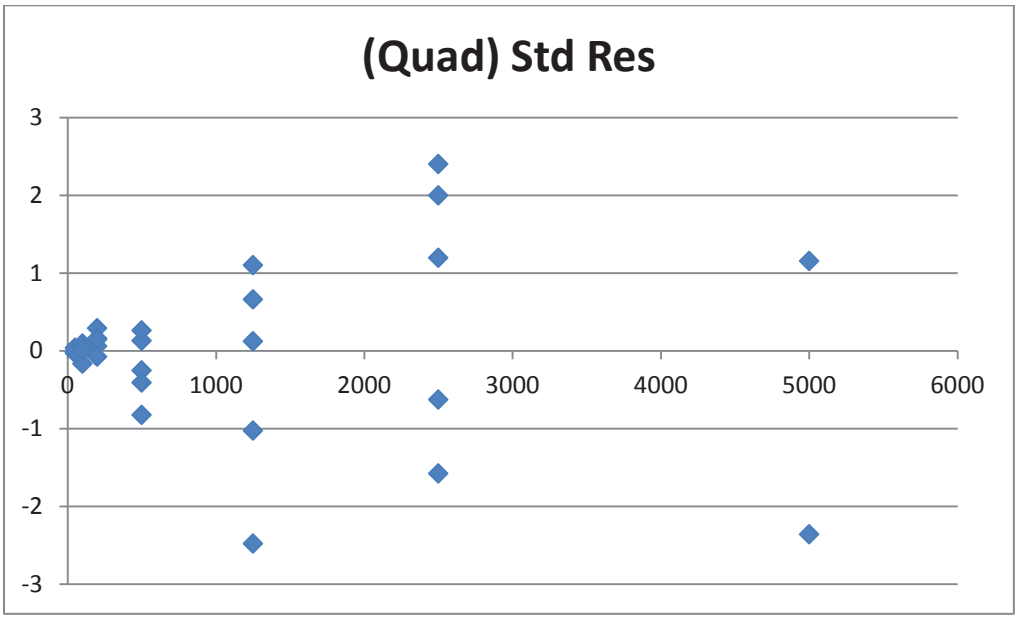




Benzoylecgonine

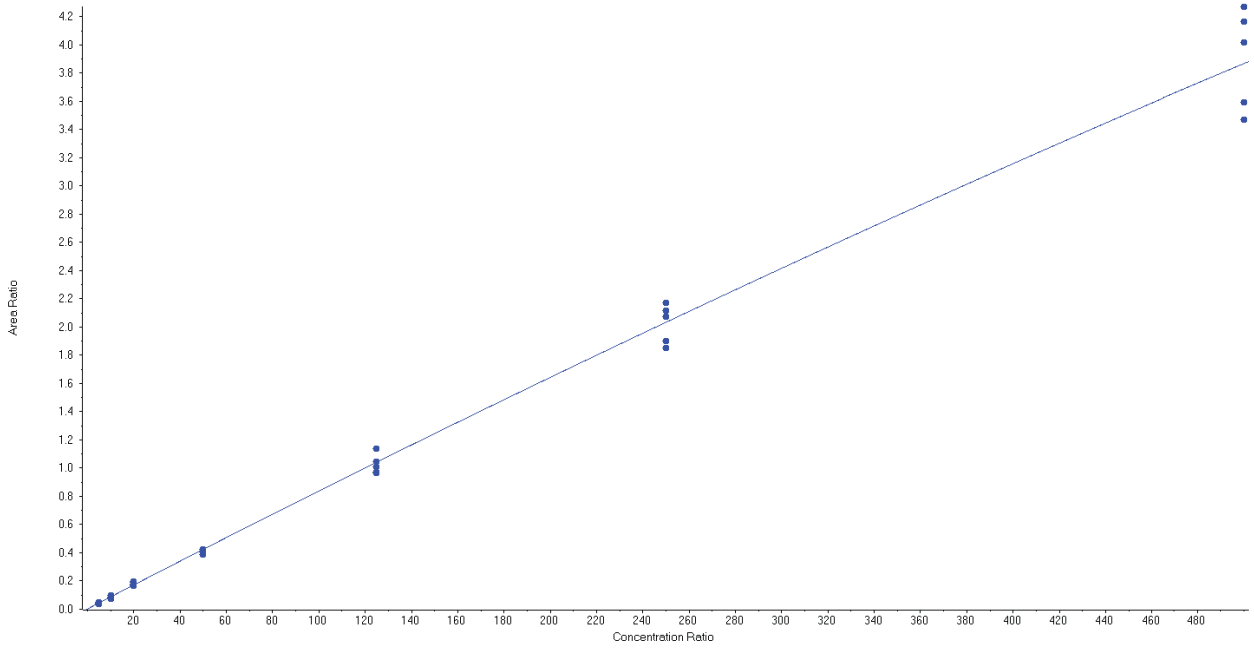
y = -1.35504e-8 x^2 + 5.73797e-4 x + 0.00166 (r = 0.99863) (weighting: 1 / x) r^2 = 0.9973

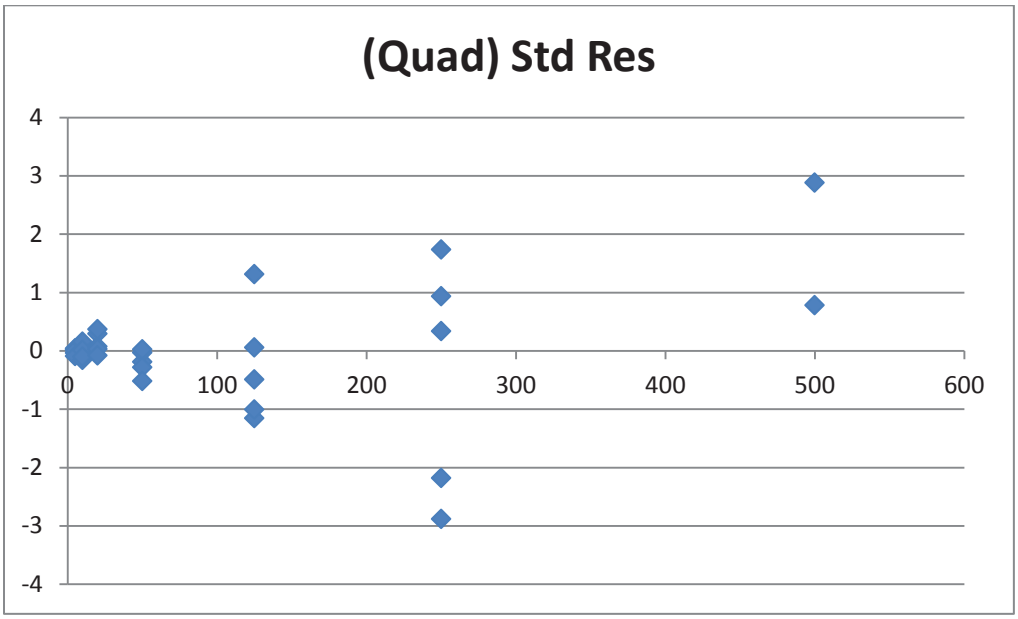




Butylone

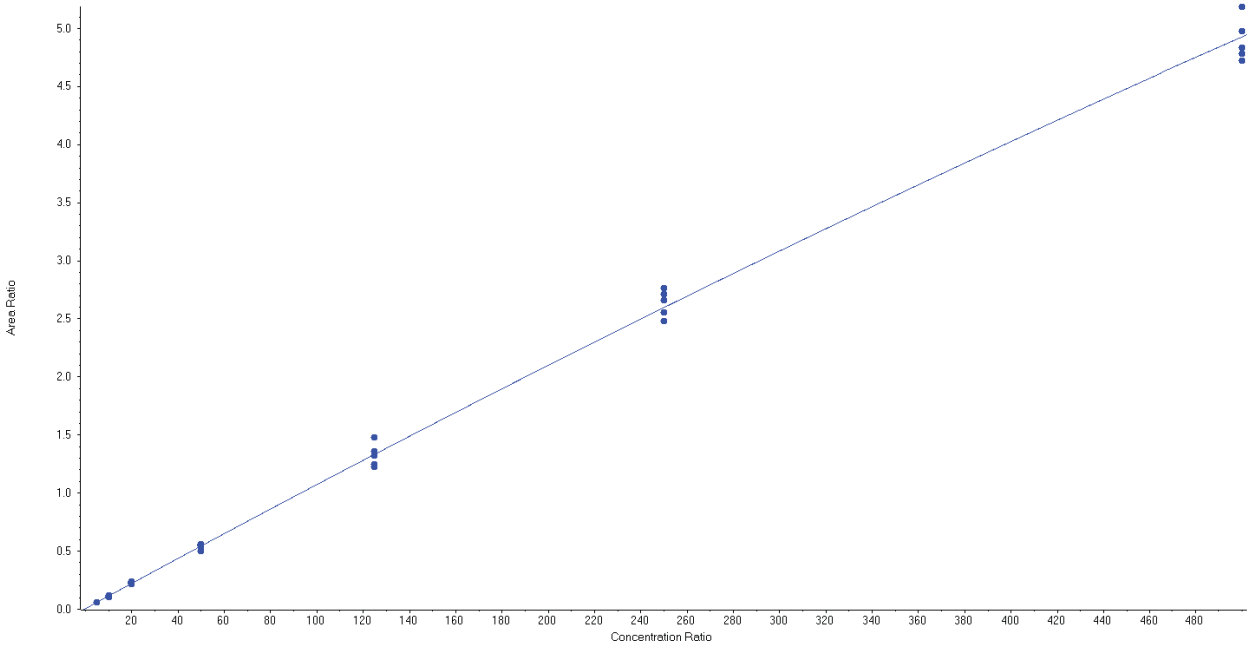
y = -1.57987e-6 x^2 + 0.00852 x + 0.00261 (r = 0.99508) (weighting: 1 / x^2) r^2 = 0.9902

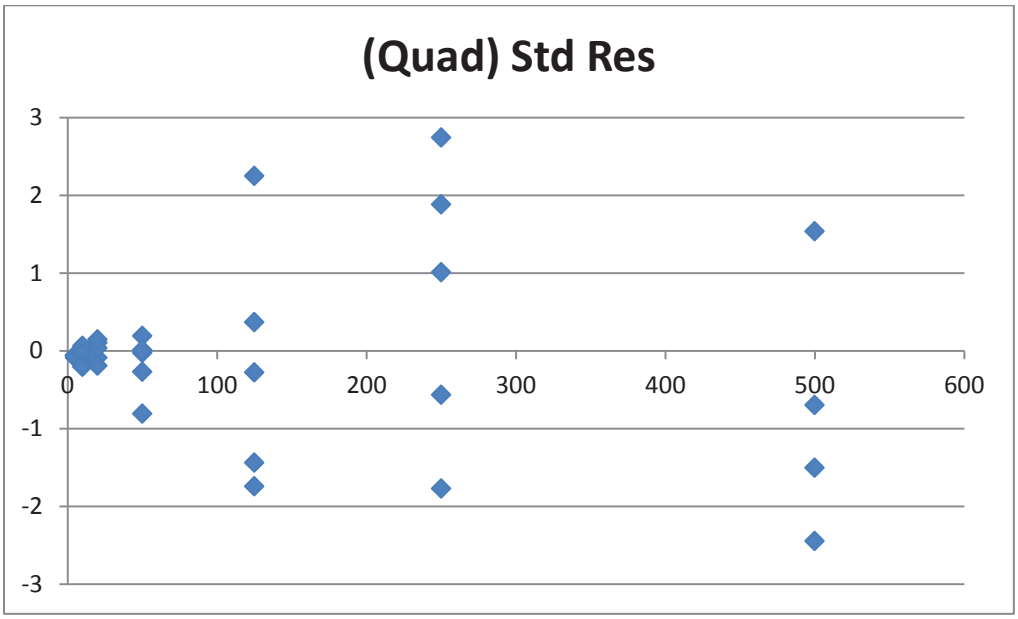




Cocaethylene

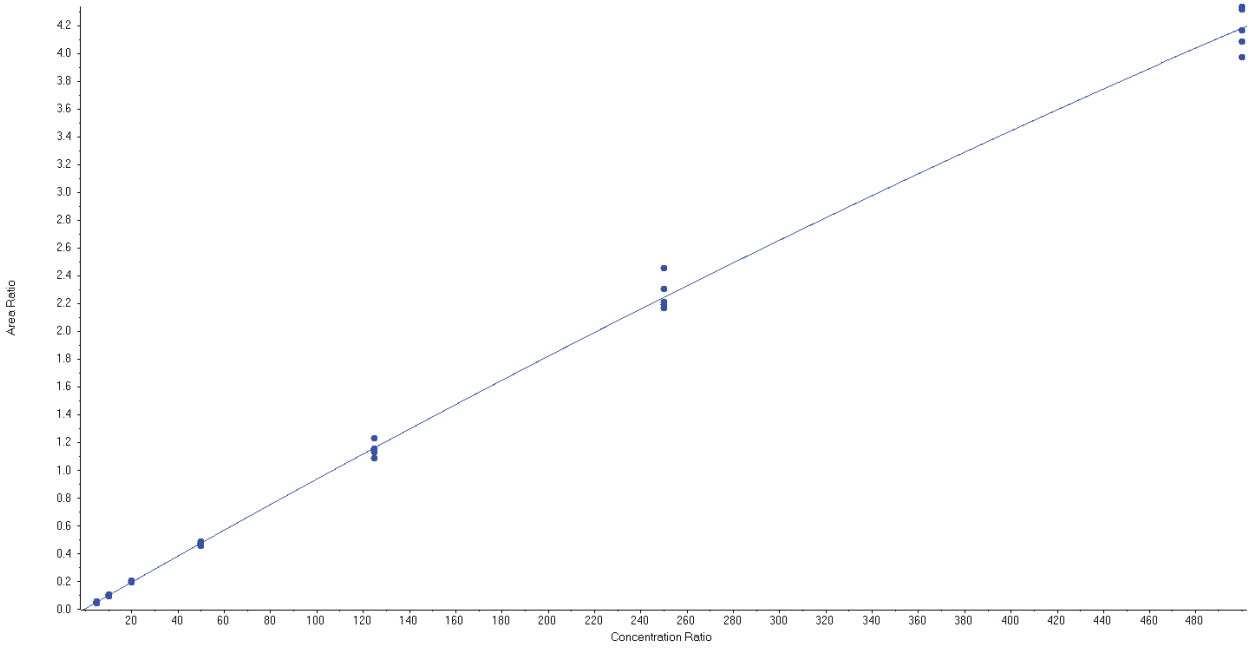
y = -2.09776e-6 x^2 + 0.01089 x + 0.00558 (r = 0.99804) (weighting: 1 / x^2) r^2 = 0.9961

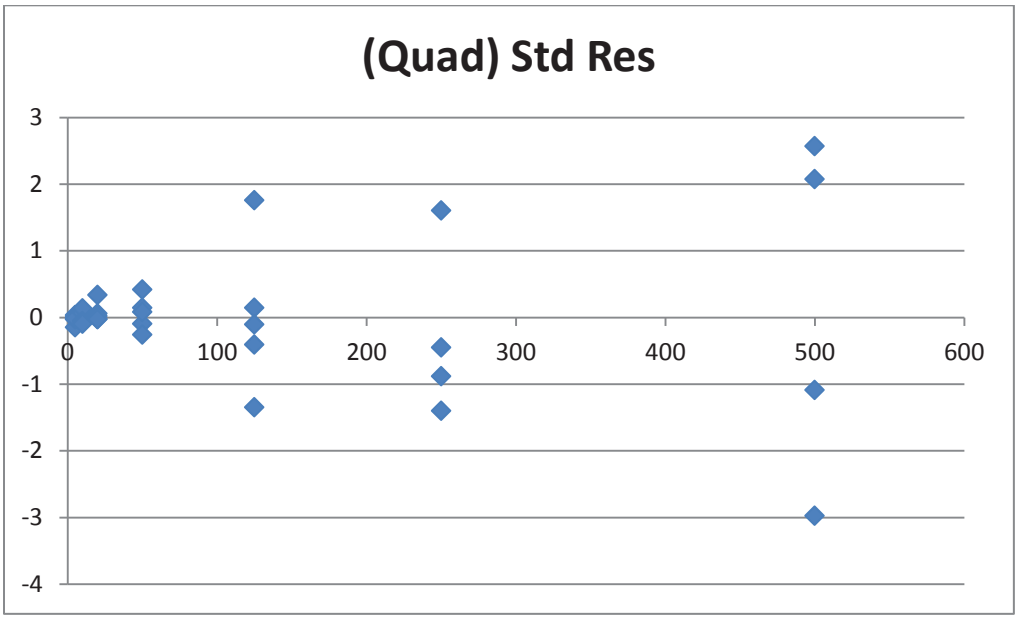




Cocaine

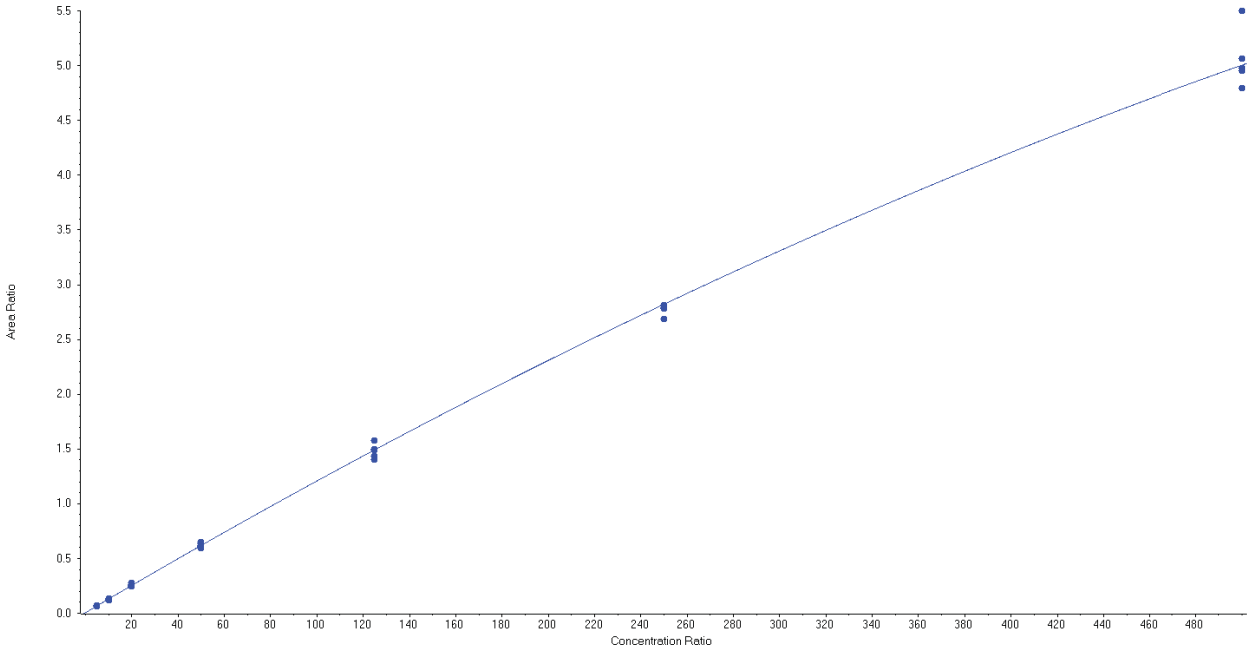
y = -2.43129e-6 x^2 + 0.00957 x + 0.00438 (r = 0.99912) (weighting: 1 / x) r^2 = 0.9982

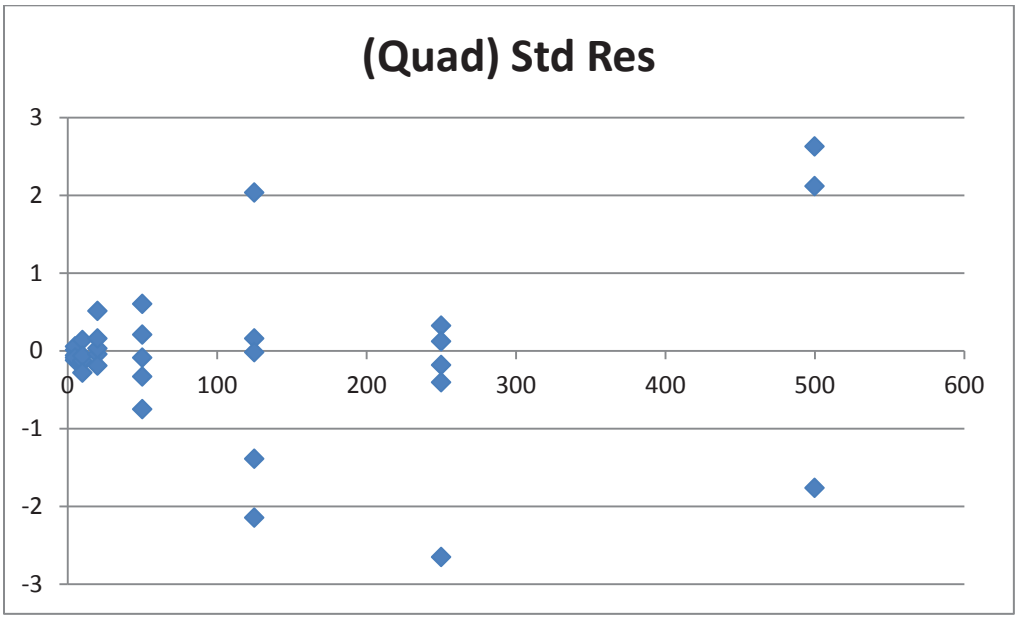




Ethylone

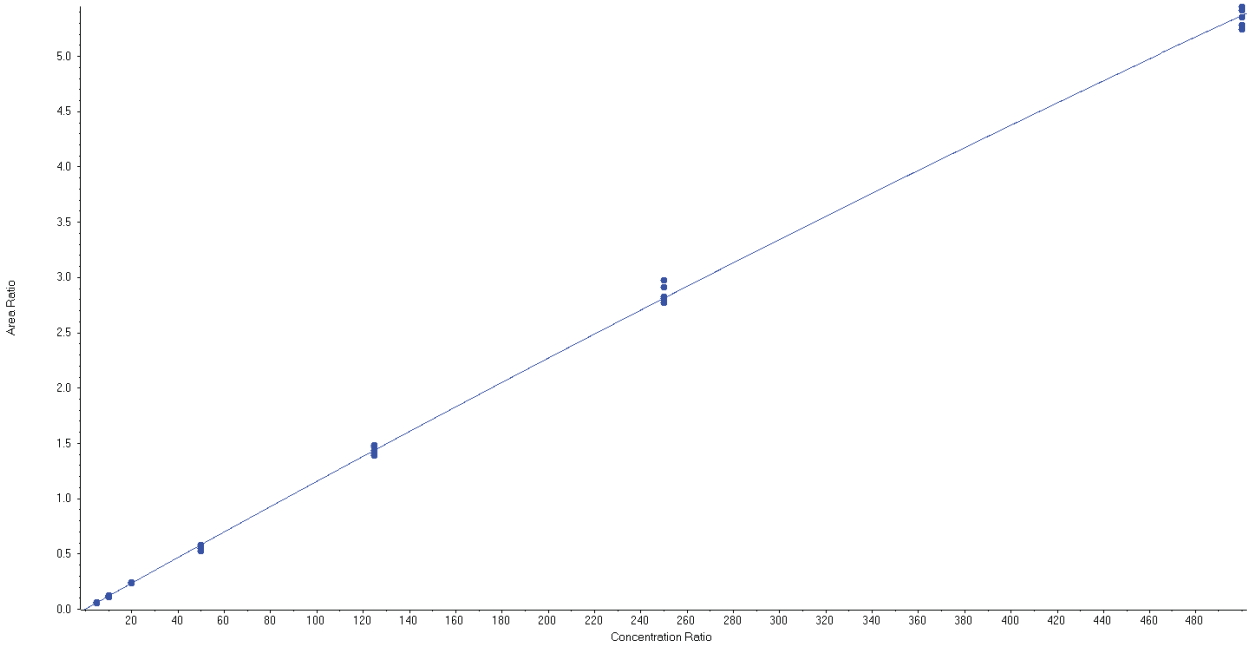
y = -5.06210e-6 x^2 + 0.01253 x + 0.00555 (r = 0.99848) (weighting: 1 / x^2) r^2 = 0.9970

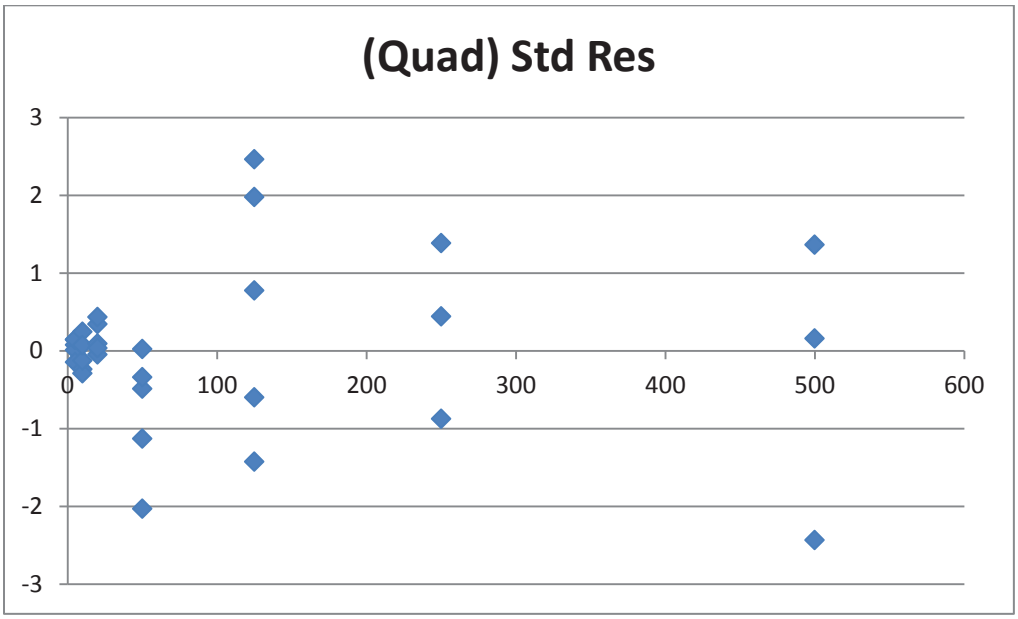




MDA

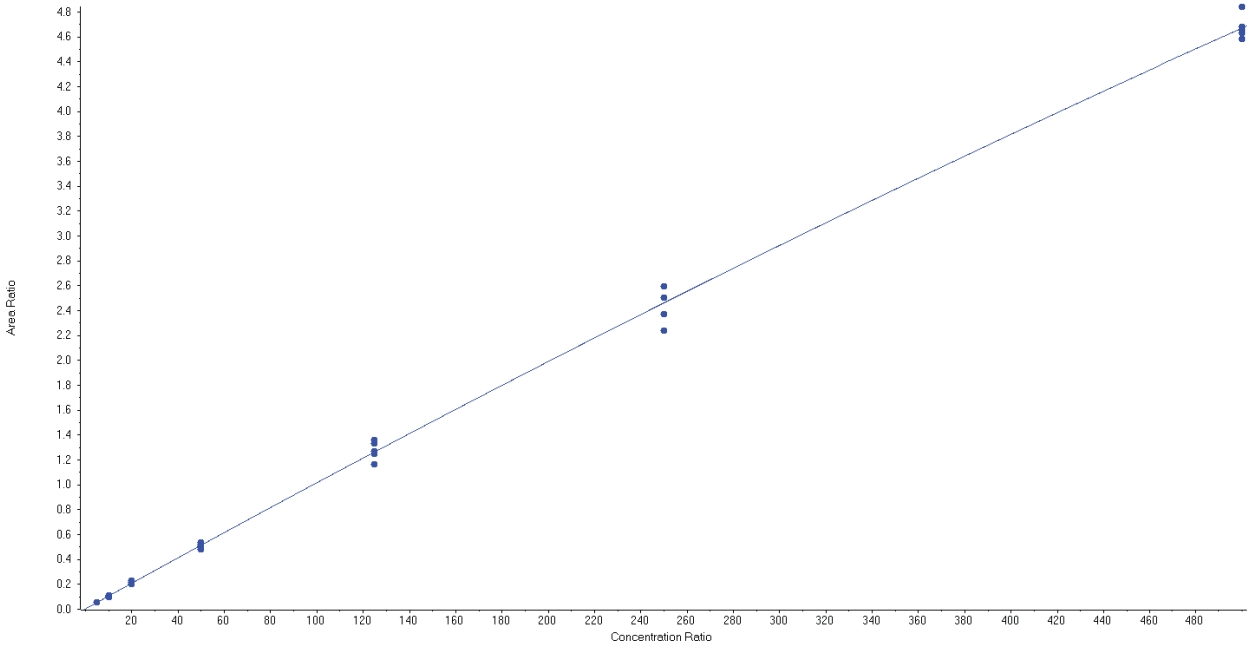
y = -2.03391e-6 x^2 + 0.01175 x + 0.00203 (r = 0.99959) (weighting: 1 / x) $r^2 = 0.9992$

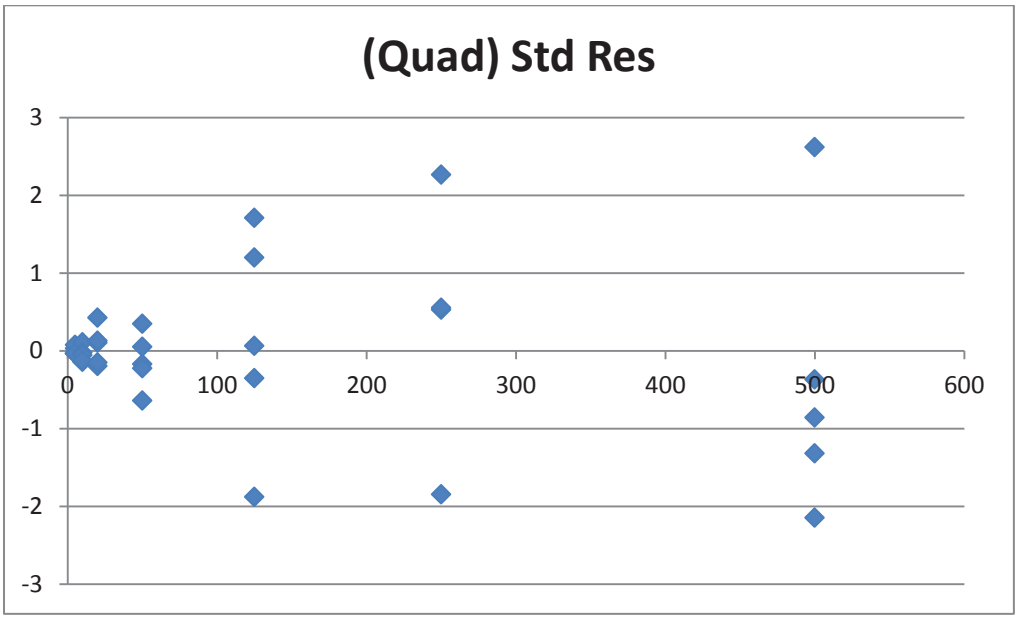




MDMA

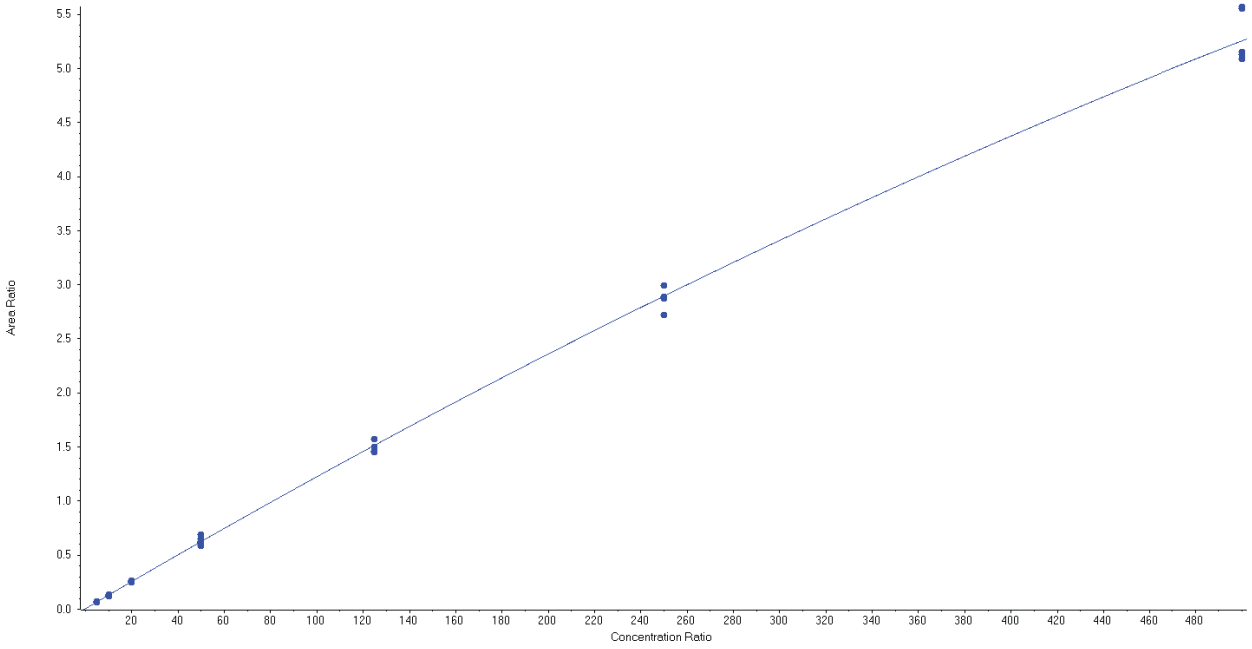
y = -2.02004e-6 x^2 + 0.01035 x + 0.00319 (r = 0.99800) (weighting: 1 / x^2) r^2 = 0.9960

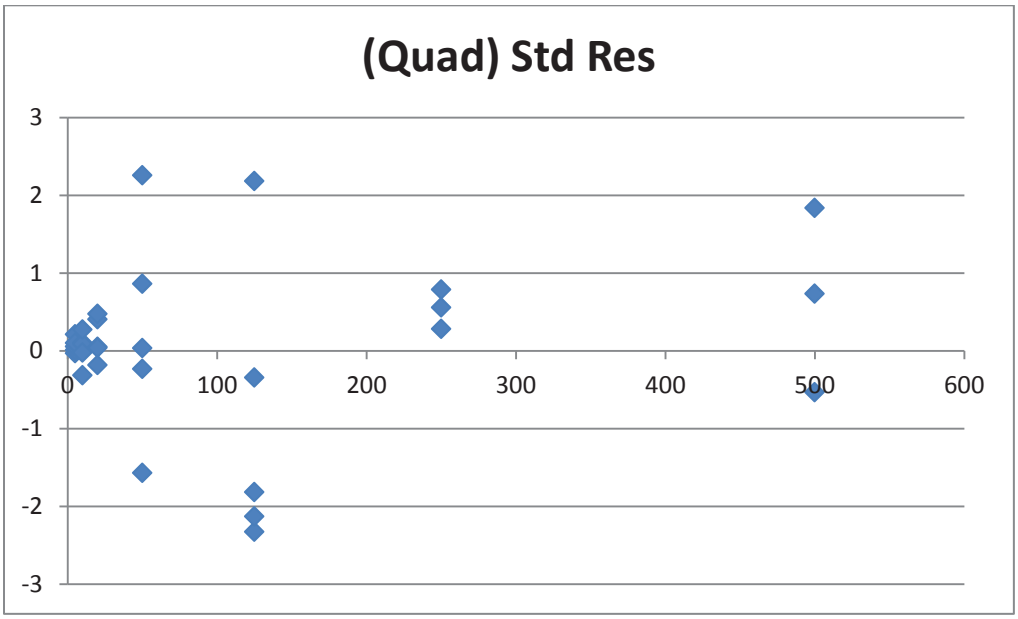




MDPV

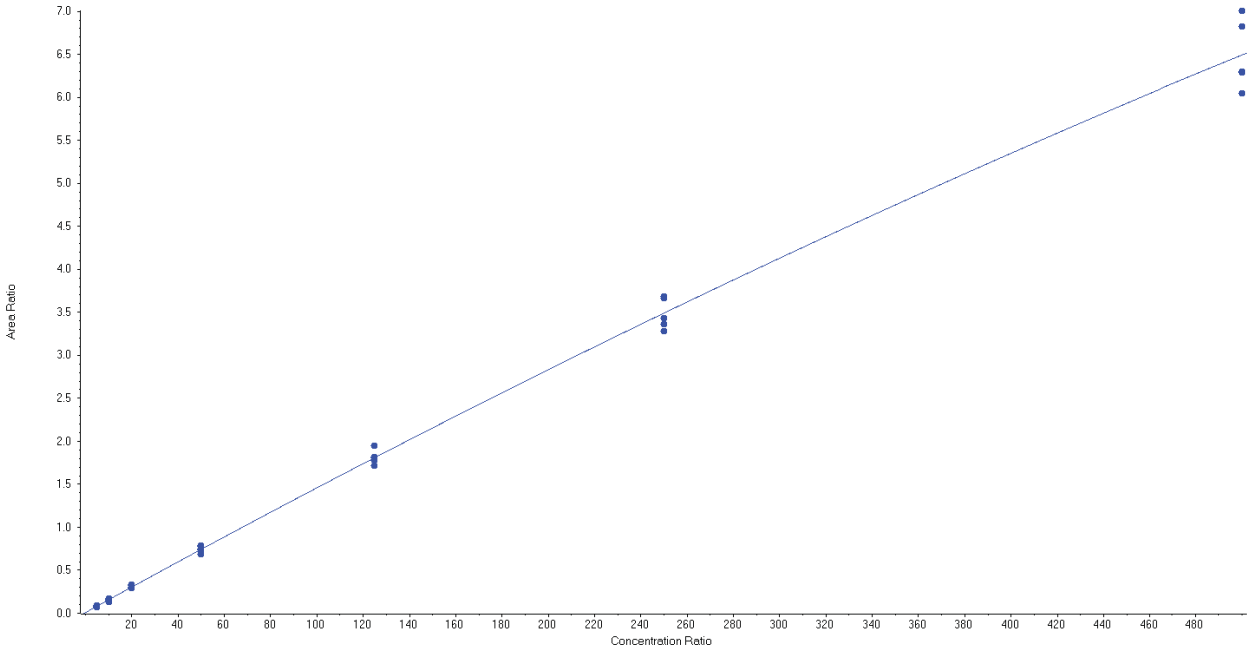
y = -4.24403e-6 x^2 + 0.01262 x + 0.00583 (r = 0.99858) (weighting: 1 / x^2) $r^2 = 0.9972$

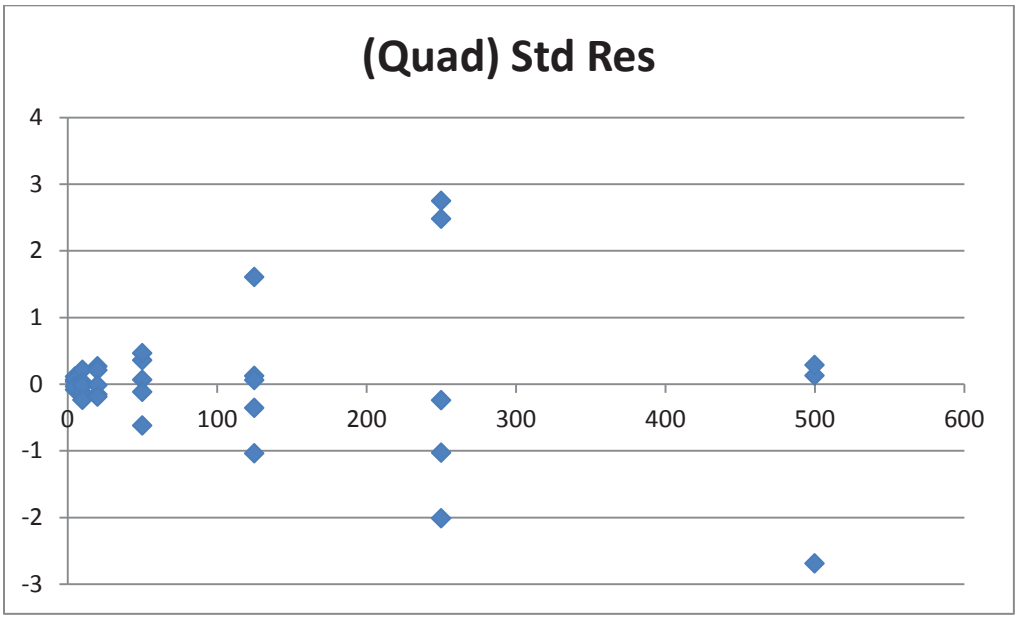




Mephedrone

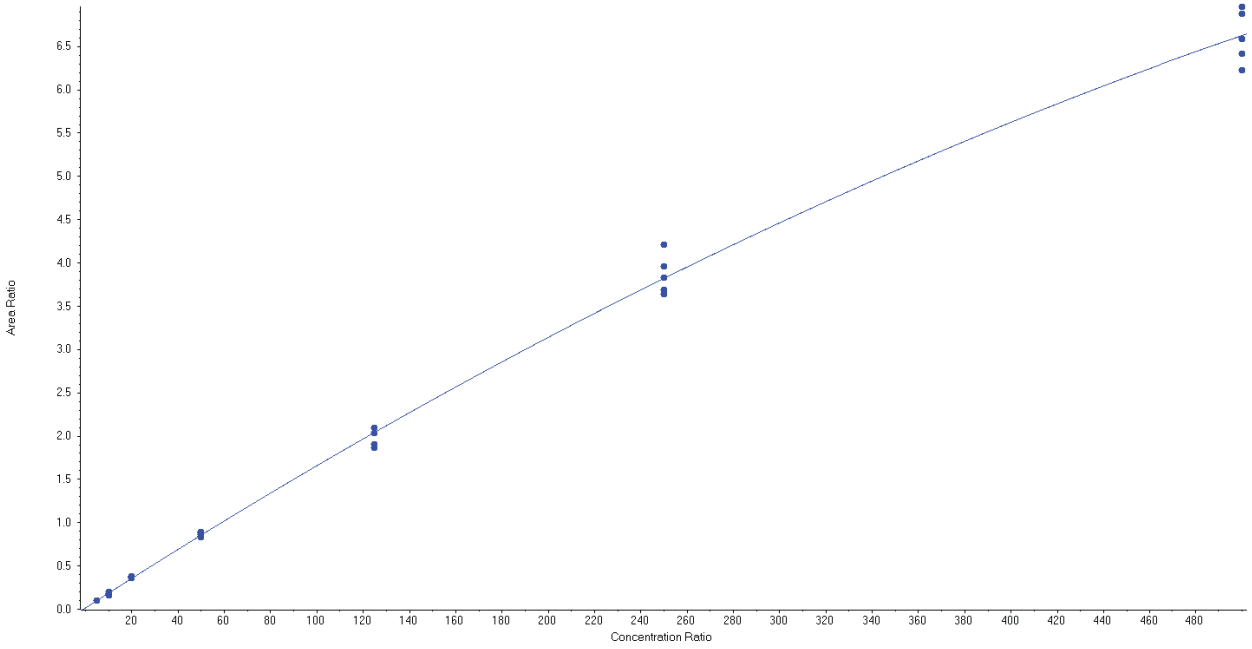
y = -3.84480e-6 x^2 + 0.01489 x + 0.00841 (r = 0.99655) (weighting: 1 / x^2) $r^2 = 0.9931$

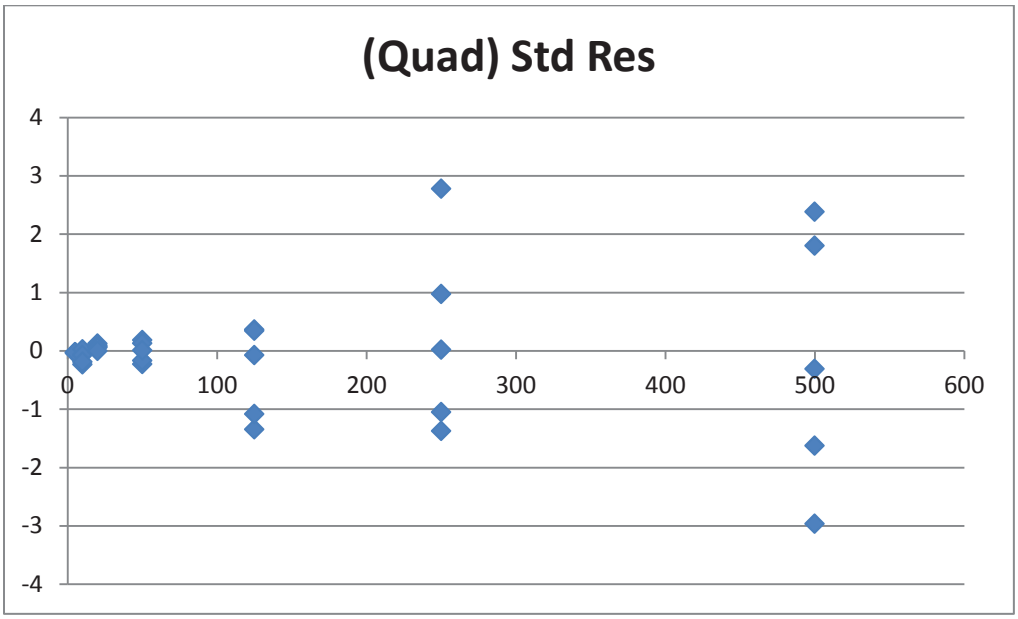




Methamphetamine

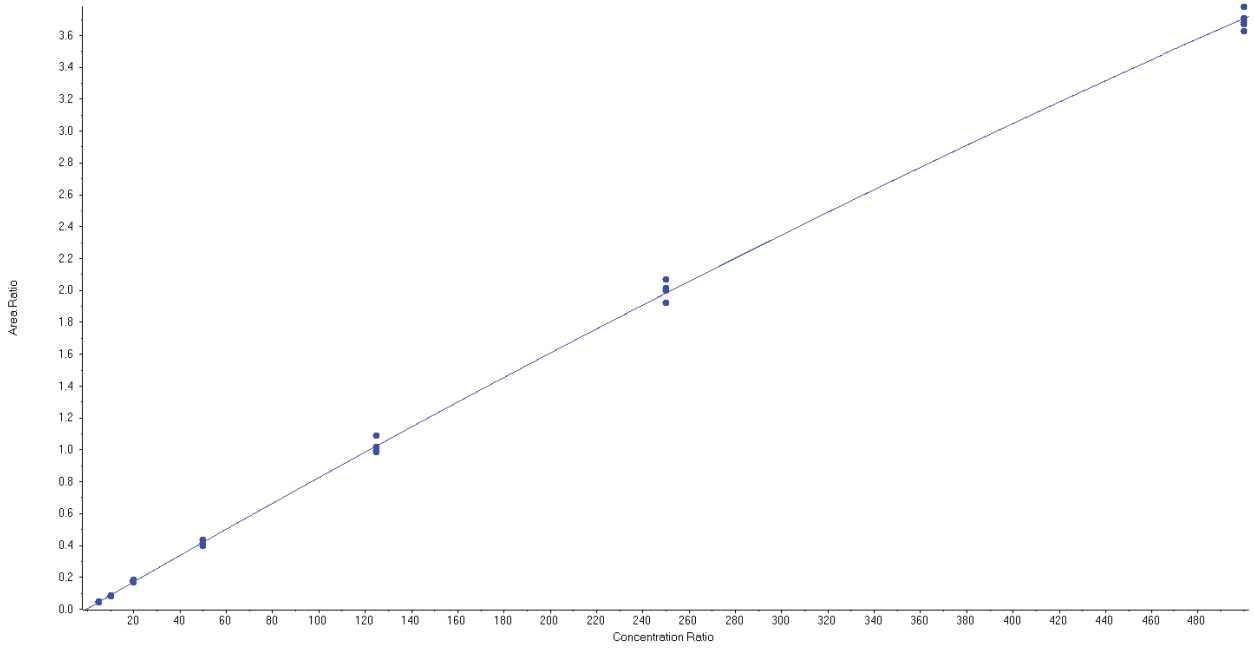
y = -8.02305e-6 x^2 + 0.01724 x + 0.01507 (r = 0.99863) (weighting: 1 / x) $r^2 = 0.9973$

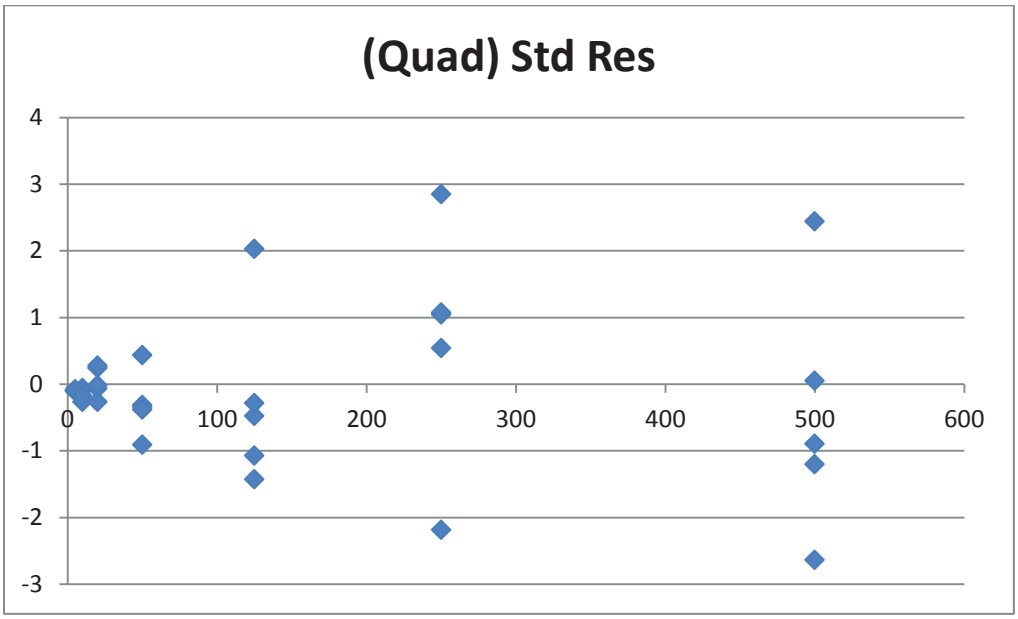




Methylone

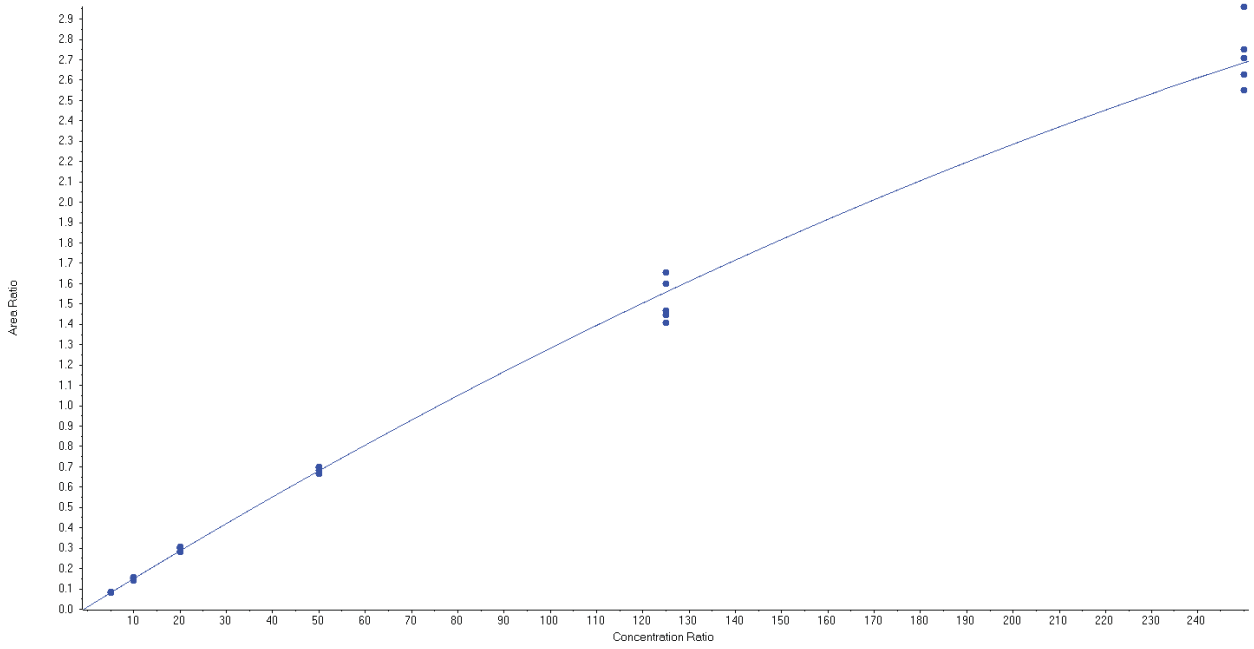
y = -2.03939e-6 x^2 + 0.00842 x + 0.00419 (r = 0.99922) (weighting: 1 / x^2) r^2 = 0.9984

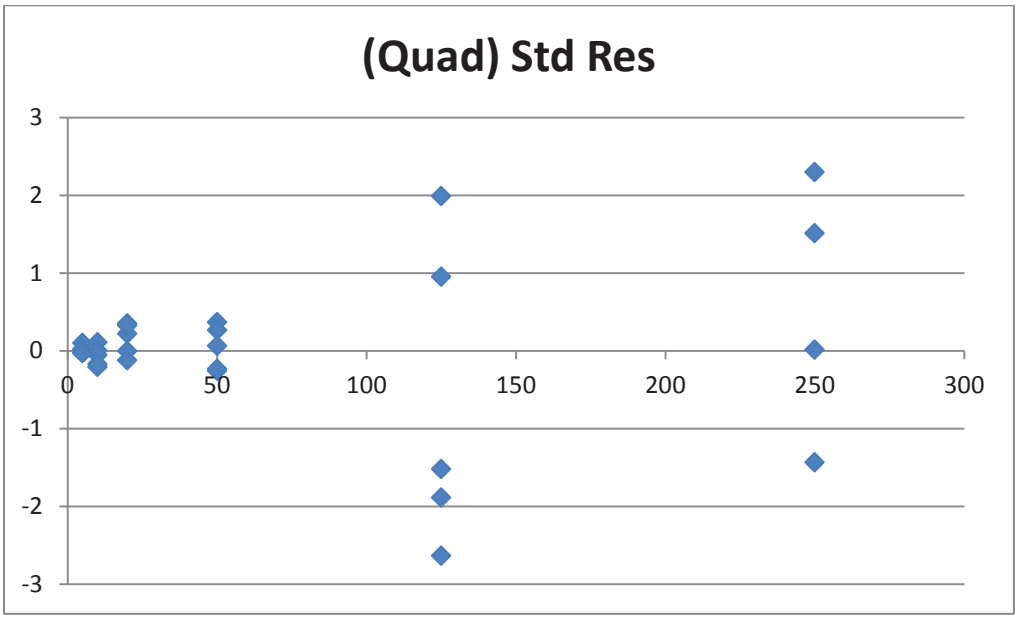




Methylphenidate

y = -1.34451e-5 x^2 + 0.01406 x + 0.01123 (r = 0.99771) (weighting: 1 / x^2) $r^2 = 0.9954$





Conclusion

A suitable calibration model was determined for each compound that was validated for quantitative analysis and is summarized below.

Analyte	Experimental LOD (ng/mL)	Validated LOD (ng/mL)	Validated LOQ (ng/mL)	Quantitative Range (ng/mL)	Curve Fit	Weighting
Alpha-PVP	1.25	5	5	5-500	Quadratic	$1/x^2$
Amphetamine	2.5	5	5	5-500	Quadratic	1/x
Benzoylecgonine	12.5	50	50	50-5000	Quadratic	1/x
Butylone	1.25	5	5	5-500	Quadratic	$1/x^2$
BZP	5	5	Qualitative Only			
Cocaethylene	1.25	5	5	5-500	Quadratic	$1/x^2$
Cocaine	1.25	5	5	5-500	Quadratic	1/x
Ethylone	2.5	5	5	5-500	Quadratic	$1/x^2$
MDA	2.5	5	5	5-500	Quadratic	1/x
MDMA	1.25	5	5	5-500	Quadratic	$1/x^2$
MDPV	1.25	5	5	5-500	Quadratic	$1/x^2$
Mephedrone	2.5	5	5	5-500	Quadratic	$1/x^2$
Methamphetamine	1.25	5	5	5-500	Quadratic	1/x
Methylone	1.25	5	5	5-500	Quadratic	$1/x^2$
Methylphenidate	2.5	5	5	5-250	Quadratic	$1/x^2$
Phentermine	5	5	Qualitative Only			
TFMPP	2.5	5	Qualitative Only			

Sensitivity – Limit of Detection (LOD)

The experimental LOD was evaluated through the analysis of one replicate of a standard run in five separate extractions. Further studies may be conducted to validate the experimentally observed LOD. The validated LOD was verified by three replicates of a standard run in five separate extractions prepared in whole blood from five different sources. All compounds had good chromatographic peak shape at the LODs. All signal-to-noise ratios were greater than or equal to 8:1. MRM ion ratios were within ± 20% and retention times were within 0.1 minutes compared to the average of all calibrators used. Consistent identification of many of the target analytes were observed at the lowest concentration evaluated (12.5 ng/mL for benzoylecgonine and 1.25 ng/mL for all other compounds). These compounds may be identified in case samples below the validated LOD.

Analyte	Experimental LOD (ng/mL)	Lowest Signal to Noise at Experimental LOD	Validated LOD (ng/mL)	Lowest Signal to Noise at Validated LOD
Alpha-PVP	1.25	81	5	188
Amphetamine	2.5	95	5	177
Benzoylecgonine	12.5	241	50	352
Butylone	1.25	8	5	10
BZP	5	58	5	58
Cocaethylene	1.25	214	5	380
Cocaine	1.25	176	5	235
Ethylone	2.5	58	5	65
MDA	2.5	20	5	22
MDMA	1.25	93	5	167
MDPV	1.25	93	5	167
Mephedrone	2.5	25	5	41
Methamphetamine	1.25	68	5	145
Methylone	1.25	14	5	19
Methylphenidate	2.5	18	5	21
Phentermine	5	35	5	35
TFMPP	2.5	33	5	21

Conclusion

The method demonstrated suitable LODs for the compounds validated.

Sensitivity—Limit of Quantitation (LOQ)

The LOQ was verified by three replicates of a standard run in five separate extractions prepared in whole blood from five different sources. All compounds had good chromatographic peak shape at the LOQ. All signal-to-noise ratios were greater than or equal to 10:1. MRM ion ratios were within 20% and retention times were within 0.1 minutes compared to the average of all calibrators. All quantitative results were within ± 20% of their prepared concentration. The relatively low signal to noise ratio observed for butylone was due to the ethylone peak that was within the window where the noise signal was calculated. Even with this confounding factor the calculated signal to noise was 10:1.

Analyte	Validated LOQ (ng/mL)	Lowest Signal to Noise at Validated LOQ
Alpha-PVP	5	188
Amphetamine	5	177
Benzoylecgonine	50	352
Butylone	5	10
BZP	Qualitative Only	
Cocaethylene	5	380
Cocaine	5	235
Ethylone	5	65
MDA	5	22
MDMA	5	167
MDPV	5	167
Mephedrone	5	41
Methamphetamine	5	145
Methylone	5	19
Methylphenidate	5	21
Phentermine	Qualitative Only	
TFMPP	Qualitative Only	

Conclusion

The method demonstrated suitable LOQs for the compounds validated.

Repeatability—Bias (Accuracy)

Standards prepared at five different concentrations for methylphenidate and six different concentrations for all other compounds—including low, medium, and high levels meeting validation requirements and a 1:10 dilution control were evaluated. Three replicates from five separate extractions were evaluated for each level. The replicates fortified at the two lowest concentrations were prepared in five different sources of whole blood. The bias was within ± 14% of the prepared concentration. The control levels and results evaluated for each compound are outlined below.

Analyte	Control Levels	1:10 Dilution Control	Bias					
			5/50 ng/mL (n = 15)	10/100 ng/mL (n = 15)	100/1000 ng/mL (n = 15)	200/2000 ng/mL (n = 15)	400/4000 ng/mL (n = 15)	400/4000 ng/mL 10X (n = 15)
Alpha-PVP	5,10,100,200,400	400	-11%	-7%	-2%	-5%	-4%	-9%
Amphetamine	5,10,100,200,400	400	-4%	-4%	4%	4%	1%	0%
Benzoylecgonine	50,100,1000,2000,4000	4000	-5%	-8%	-4%	-4%	-6%	-10%
Butylone	5,10,100,200,400	400	-2%	-6%	-5%	-1%	-4%	-8%
BZP	5,10,100,200,400	400	Qualitative Only					
Cocaethylene	5,10,100,200,400	400	-2%	-2%	3%	1%	-1%	-2%
Cocaine	5,10,100,200,400	400	3%	-2%	1%	3%	2%	-3%
Ethylone	5,10,100,200,400	400	-4%	-4%	1%	-2%	-3%	-3%
MDA	5,10,100,200,400	400	-6%	-5%	2%	4%	0%	-2%
MDMA	5,10,100,200,400	400	-3%	-2%	2%	3%	-1%	-3%
MDPV	5,10,100,200,400	400	-2%	-3%	0%	0%	0%	-4%
Mephedrone	5,10,100,200,400	400	-11%	-10%	2%	1%	-2%	-4%
Methamphetamine	5,10,100,200,400	400	0%	-2%	2%	0%	-2%	0%
Methylone	5,10,100,200,400	400	-14%	-13%	-7%	-6%	-11%	-12%
Methylphenidate	5,10,100,200	400	-4%	-3%	0%	2%		-4%
Phentermine	5,10,100,200,400	400	Qualitative Only					
TFMPP	5,10,100,200,400	400	Qualitative Only					

Conclusion

The method demonstrated an acceptable bias of within ± 14% evaluated with 15 replicates at each of six levels spanning the calibration range (50, 100, 1000, 2000, and 4000 ng/mL for benzoylecgonine and 5, 10, 100, 200, and 400 ng/mL for all other compounds) and included a 1:10 dilution control (4000 ng/mL for benzoylecgonine and 400 ng/mL for all other compounds).

Repeatability—Within-run and between-run precision

Five levels for methylphenidate and six levels for all other compounds were evaluated (the same standards that were used for the bias experiments) for precision. Three replicates from five separate extractions were evaluated for each level.

Within-run Precision

Analyte	Within-Run Precision (%CV)									
	5/50 ng/mL Control					10/100 ng/mL Control				
	Step 3	Step 4	Step 5	Step 6	Step 7	Step 3	Step 4	Step 5	Step 6	Step 7
Alpha-PVP	1%	7%	11%	10%	5%	2%	5%	2%	2%	7%
Amphetamine	2%	2%	1%	4%	6%	3%	2%	0%	1%	5%
Benzoylecgonine	6%	5%	2%	5%	3%	5%	4%	5%	2%	4%
Butylone	8%	4%	6%	5%	1%	2%	3%	3%	5%	5%
BZP*	2%	7%	24%	5%	8%	1%	6%	17%	17%	11%
Cocaethylene	8%	6%	1%	6%	1%	2%	5%	4%	4%	1%
Cocaine	2%	7%	5%	7%	5%	2%	6%	5%	3%	2%
Ethylone	5%	4%	2%	2%	5%	2%	1%	1%	3%	5%
MDA	14%	8%	3%	7%	2%	4%	6%	6%	4%	1%
MDMA	5%	5%	6%	4%	10%	0%	2%	3%	5%	3%
MDPV	0%	2%	4%	5%	1%	2%	0%	1%	6%	2%
Mephedrone	4%	7%	6%	5%	13%	4%	6%	5%	3%	5%
Methamphetamine	2%	7%	6%	6%	8%	4%	2%	6%	4%	5%
Methylone	4%	5%	5%	2%	1%	1%	5%	2%	3%	5%
Methylphenidate	6%	4%	5%	4%	4%	6%	0%	4%	2%	1%
Phentermine*	5%	11%	6%	7%	10%	1%	7%	8%	3%	8%
TFMPP*	4%	5%	9%	13%	9%	5%	5%	6%	4%	3%

*Qualitative identification only

Analyte	Within-Run Precision (%CV)									
	100/1000 ng/mL Control					200/2000 ng/mL Control				
	Step 3	Step 4	Step 5	Step 6	Step 7	Step 3	Step 4	Step 5	Step 6	Step 7
Alpha-PVP	5%	15%	1%	4%	8%	2%	3%	3%	3%	8%
Amphetamine	1%	11%	3%	2%	7%	2%	2%	3%	2%	5%
Benzoylecgonine	2%	9%	3%	2%	3%	5%	3%	2%	3%	1%
Butylone	2%	8%	3%	3%	5%	2%	3%	2%	5%	2%
BZP*	5%	13%	10%	5%	4%	4%	5%	42%	54%	2%
Cocaethylene	2%	8%	2%	2%	7%	2%	1%	3%	1%	1%
Cocaine	1%	10%	6%	4%	4%	3%	1%	3%	2%	6%
Ethylone	6%	12%	2%	5%	3%	3%	2%	3%	3%	5%
MDA	6%	9%	1%	5%	2%	2%	3%	2%	4%	1%
MDMA	1%	11%	2%	5%	1%	2%	3%	5%	6%	1%
MDPV	2%	9%	3%	4%	1%	1%	5%	2%	4%	5%
Mephedrone	3%	11%	1%	7%	7%	1%	6%	3%	3%	4%
Methamphetamine	3%	13%	4%	2%	6%	4%	6%	2%	2%	6%
Methylone	3%	8%	2%	4%	3%	6%	3%	4%	2%	6%
Methylphenidate	4%	10%	6%	5%	1%	6%	4%	6%	3%	3%
Phentermine*	4%	14%	3%	4%	8%	3%	4%	1%	2%	10%
TFMPP*	2%	14%	3%	6%	5%	5%	5%	7%	7%	4%

*Qualitative identification only

Analyte	Within-Run Precision (%CV)									
	400/4000 ng/mL Control					400/4000 ng/mL 1:10 Dilution Control				
	Step 3	Step 4	Step 5	Step 6	Step 7	Step 3	Step 4	Step 5	Step 6	Step 7
Alpha-PVP	1%	14%	4%	8%	8%	3%	7%	2%	5%	3%
Amphetamine	4%	11%	1%	4%	6%	2%	2%	3%	8%	4%
Benzoylecgonine	0%	3%	2%	9%	4%	1%	6%	6%	5%	5%
Butylone	3%	12%	1%	6%	2%	3%	5%	2%	11%	8%
BZP*	2%	22%	1%	4%	4%	6%	5%	3%	30%	6%
Cocaethylene	3%	3%	2%	4%	4%	3%	6%	3%	3%	1%
Cocaine	2%	11%	4%	5%	2%	2%	6%	3%	9%	3%
Ethylone	1%	11%	3%	3%	5%	3%	5%	6%	12%	4%
MDA	5%	1%	2%	6%	2%	3%	7%	3%	11%	3%
MDMA	3%	1%	2%	8%	3%	3%	6%	2%	9%	2%
MDPV	1%	10%	2%	3%	1%	2%	3%	6%	2%	4%
Mephedrone	2%	11%	3%	6%	6%	4%	6%	8%	6%	6%
Methamphetamine	5%	4%	0%	5%	5%	6%	2%	3%	15%	9%
Methylone	0%	7%	2%	1%	5%	2%	6%	3%	10%	6%
Methylphenidate						3%	3%	3%	4%	4%
Phentermine*	2%	12%	5%	1%	9%	3%	4%	5%	6%	3%
TFMPP*	3%	6%	2%	4%	16%	5%	5%	0%	3%	1%

*Qualitative identification only

Between-run Precision

Analyte	Between-Run Precision (%CV)					
	5/50 ng/mL (n = 15)	10/100 ng/mL (n = 15)	100/1000 ng/mL (n = 15)	200/2000 ng/mL (n = 15)	400/4000 ng/mL (n = 15)	400/4000 ng/mL 10X (n = 15)
Alpha-PVP	9%	5%	7%	5%	9%	6%
Amphetamine	7%	5%	7%	5%	6%	7%
Benzoylecgonine	7%	5%	5%	3%	5%	5%
Butylone	9%	5%	5%	6%	6%	9%
BZP*	14%	12%	10%	33%	21%	15%
Cocaethylene	7%	6%	5%	4%	4%	7%
Cocaine	7%	4%	6%	3%	7%	6%
Ethylone	7%	4%	8%	6%	5%	8%
MDA	8%	4%	5%	3%	5%	6%
MDMA	7%	4%	6%	5%	5%	8%
MDPV	5%	3%	5%	4%	5%	5%
Mephedrone	9%	5%	8%	5%	7%	8%
Methamphetamine	6%	5%	9%	5%	7%	11%
Methylone	5%	4%	5%	5%	5%	8%
Methylphenidate	7%	4%	7%	6%		5%
Phentermine*	9%	6%	9%	6%	6%	6%
TFMPP*	9%	5%	14%	10%	9%	9%

*Qualitative identification only

Conclusion

The method demonstrated acceptable within-run and between-run precision with all CVs within 15% for those compounds validated for qualitative and quantitative analysis and within 54% for those compounds validated for qualitative identification only. Precision was evaluated with 15 replicates at each of five levels for methylphenidate and six levels for all other compounds spanning the calibration range (50, 100, 1000, 2000, and 4000 ng/mL for benzoylecgonine and 5, 10, 100, 200, and 400 ng/mL for all other compounds except methylphenidate which did not include 400 ng/mL) and included a 1:10 dilution control (4000 ng/mL for benzoylecgonine and 400 ng/mL for all other compounds).

Reportable range

The reportable range was determined after evaluating the calibration model and sensitivity of the assay. The reportable range was determined to at least encompass the concentrations outlined below but may extend beyond the ranges evaluated in this validation. Compounds meeting acceptable identification criteria with apparent concentrations outside of the range identified below may be reported. Since a 1:10 dilution was validated the upper end of the reportable range will extend to at least ten times the range indicated below if a dilution is used.

Analyte	Reportable Range (ng/mL)
Alpha-PVP	5-500
Amphetamine	5-500
Benzoylecgonine	50-5000
Butylone	5-500
BZP	Qualitative
Cocaethylene	5-500
Cocaine	5-500
Ethylone	5-500
MDA	5-500
MDMA	5-500
MDPV	5-500
Mephedrone	5-500
Methamphetamine	5-500
Methylone	5-500
Methylphenidate	5-500
Phentermine	Qualitative
TFMPP	Qualitative

Conclusion

The reportable range determined in validation is appropriate for the compounds included in this method.

Dilution Integrity

Dilution integrity was evaluated for a 1:10 dilution. Fifteen replicates of a standard were evaluated in five different extractions. For each extraction a stock standard was prepared by fortifying whole blood at 4000 ng/mL for benzoylecgonine and 400 ng/mL for all other compounds. Three 20 uL replicates of the stock standard were then sampled and combined with 180 uL of whole blood prior to processing to yield a concentration of 400 ng/mL for benzoylecgonine and 40 ng/mL for all other compounds. The bias and precision results for the dilution control are presented in the tables above. The bias for the 1:10 dilution control was within ± 12% and the precision was within 15% for all compounds validated for quantitative analysis.

Conclusion

The 1:10 dilution was verified to meet all bias and precision requirements. If a target compound is identified in a specimen above the concentration of the highest calibrator it may be diluted 1:10 and re-extracted or may be reported as greater than the highest calibrator (i.e. greater than 250, 500 or 5000 ng/mL as applicable).

Carryover

The lack of carryover was determined by triplicate analyses. A blank matrix sample was analyte free when run after a standard prepared at the concentration of the highest calibrator of 5000 ng/mL for benzoylecgonine and 500 ng/mL for all other compounds.

Conclusion

The method demonstrated a lack of carryover up to a concentration of 5000 ng/mL for benzoylecgonine and 500 ng/mL for all other compounds. Matrix or solvent blanks will be run prior to each case sample during routine casework to demonstrate that carryover did not occur.

Extract Stability

Five replicates of controls were prepared at a low and high concentration (200 and 4000 ng/mL for benzoylecgonine and 20 and 400 ng/mL for all other compounds). The extracts were combined and then divided into five different vials. A vial of each level was injected in triplicate on day 0. The other vials were stored on the instrument and re-injected on days 1, 2, 3, and 4. The response of each analyte, internal standard or relative response must remain within ± 20% of the response from day 0. If the response falls outside this range then the extract stability of the analyte was exceeded. Extract stability was confirmed to be at least 4 days after the date of extraction for all compounds validated for quantitative analysis and phentermine. BZP and TFMPP demonstrated an increase in response of greater than 20% on day 2. This did not affect the ability to identify these compounds on days 2-4. Since BZP and TFMPP were only validated for qualitative identification extracts may be analyzed for up to 4 days after the date of extraction without impacting the analysis.

Conclusion

Extracts were confirmed to be stable for at least 4 days after the day of extraction for all compounds validated for quantitative analysis and phentermine which was validated for qualitative identification only. The change in stability observed for BZP and TFMPP did not impact their identification. Therefore extracts may be analyzed for at least 4 days after the date of extraction for all compounds included in this validation.

Ruggedness/Robustness

Validation studies were performed by 4 different analysts and one trainee in toxicology over multiple days and demonstrated repeatable results. Some extracts had to be re-injected and are summarized below.

Step	Issue	Analyte	Resolution
4	5ng/mL CON-1 MRM Ratio outside ± 20%	Phentermine	The control was re-injected and the MRM ratio was within ± 20%.
4	5ng/mL CON-3 quantitative result outside ± 20%	Alpha-PVP, MDA	The control was re-injected and the quantitative result was within ± 20%.
4	400ng/mL CON-1 quantitative result outside ± 20%	Methamphetamine	The control was re-injected and the quantitative result was within ± 20%.
4	400ng/mL CON-3 quantitative result outside ± 20%	Alpha-PVP	The control was re-injected and the quantitative result was within ± 20%.
6	400ng/mL 10X CON-3 quantitative result outside ± 20%	Butylone	The control was re-injected and the quantitative result was within ± 20%.
7	25ng/mL CAL MRM Ratio outside ± 20%	Benzoylecgonine	The calibrator was re-injected and the MRM ratio was within ± 20%.

Conclusion

Overall the method demonstrated acceptable robustness and yielded repeatable results.

Case Sample Comparison

Fifteen case samples and one proficiency that had been previously analyzed were reanalyzed by the LC-MSMS method for a case comparison/crossover study. Three of the case samples were negative for the target analytes by the original methods and the Blood Stimulants LC-MSMS Quant method. Overall there was good agreement of the qualitative results between the original methods and the LC-MSMS method, within the current capabilities of each method.

Due to the increased sensitivity with the LC-MSMS method additional compounds were identified in three case samples and the proficiency sample. In two case samples and the proficiency that contained cocaine and/or benzoylecgonine ([redacted], and 16-FTC01) cocaethylene (CE) was identified at less than 5 ng/mL by LC-MSMS that was not detected by GC-MS. The LOD of the GC-MS procedure was previously determined to be 10 ng/mL. The presence of CE in the proficiency sample was likely due to an impurity that can be observed in cocaine standards that is present as a byproduct of purification of the standard with ethanol

and therefore would not be reported. In two cases ([redacted]) additional amphetamine related compounds were detected by LC-MSMS at concentrations below the GC-MS LOD. All other case samples had identical qualitative results.

The very limited stability of cocaine in blood is well documented (6). In a study on the stability of cocaine in blood preserved with sodium fluoride the cocaine concentration decreased by 89% over three months (6). Two cases that were in storage for greater than a year ([redacted]), with most of that time in a freezer, demonstrated significant decreases in cocaine and benzoylecgonine (BE) concentrations when reanalyzed by LC-MSMS. Another case ([redacted]) containing cocaine was 25% lower by LC-MSMS after refrigerated storage for three weeks. Sufficient volume was not available to repeat the GC-MS analysis contemporaneous to the LC-MSMS testing for these cases. Two of the remaining eight cases containing BE demonstrated a 21% higher quantitative result when retested by LC-MSMS. Although this was higher than the required ± 20% agreement, it was within the 24% estimated uncertainty of measurement for BE by the GC-MS method.

One case ([redacted]) containing a low concentration of MDMA demonstrated greater than 20% higher quantitative results when retested by LC-MSMS. The sample was retested by LC-MSMS and yielded results within 5 ng/mL of the GC-MS result, but still 24% higher due to the low concentration near 20 ng/mL present in the sample. The acceptable accuracy for the GC-MS procedure at or near the LOQ of 20 ng/mL is ± 30%. The retest by the LC-MSMS procedure was within the acceptable accuracy for the GC-MS procedure.

All of the results of the case comparison/crossover study are presented in the table below.

Case #	Original Analysis			LC-MSMS Analysis			% Difference	Comments
	Drug	Concentration (ng/mL)	Date of Analysis	Drug	Concentration (ng/mL)	Date of Analysis		
[redacted]	Cocaine	< 50	11/21/2013	See results below compared to GCMS reanalysis				
	Cocaethylene	< 50	11/21/2013					
	Benzoylecgonine	> 1000	11/21/2013					
[redacted]*	Cocaine	< 100	4/29/2016	Cocaine	9.9	4/28/2016		Original: 2x dilution
	Cocaethylene	< 100	4/29/2016	Cocaethylene	26	4/28/2016		Original: 2x dilution
	Benzoylecgonine	714	4/29/2016	Benzoylecgonine	864	4/28/2016	21%	Original: 2x dilution, UOM for GCMS = 24%
[redacted]	Benzoylecgonine	70	8/28/2013	Benzoylecgonine	32	4/28/2016	-54%	In storage > 2 years
[redacted]	Methylone	156	9/23/2013	See results below compared to GCMS reanalysis				
[redacted]*	Methylone	< 20	5/2/2016	Methylone	6.5	5/3/2016		
[redacted]	Amphetamine	240	3/20/2015	Amphetamine	217	4/28/2016	-10%	
	Cocaine	174	3/13/2015	Cocaine	84	4/28/2016	-52%	In storage > 1 year
				Cocaethylene	< 5	4/28/2016		GCMS LOD = 10ng/mL
	Benzoylecgonine	> 1000	3/13/2015	Benzoylecgonine	916	4/28/2016		In storage > 1 year
[redacted]	Negative		5/11/2015	Negative		4/28/2016		Original: ELISA and Blood Base
[redacted]				Cocaethylene	< 5	5/3/2016		GCMS LOD = 10ng/mL
	Benzoylecgonine	< 50	2/16/2016	Benzoylecgonine	< 50	5/3/2016		
	MDMA	< 20	2/19/2016	MDMA	13	5/3/2016		
				MDA	< 5	5/3/2016		GCMS LOD = 10ng/mL
				Amphetamine	6.9	5/3/2016		GCMS LOD = 10ng/mL
[redacted]	Benzoylecgonine	201	3/21/2016	Benzoylecgonine	197	4/22/2016	-2%	
[redacted]	Negative		3/21/2016	Negative		4/22/2016		Original: ELISA and Blood Base

Case #	Original Analysis			LC-MSMS Analysis			% Difference	Comments
	Drug	Concentration (ng/mL)	Date of Analysis	Drug	Concentration (ng/mL)	Date of Analysis		
[redacted]	Cocaine	84	4/14/2016	Cocaine	63	5/3/2016	-25%	In storage > 3 weeks
	Cocaethylene	< 50	4/14/2016	Cocaethylene	31	5/3/2016		
	Benzoylecgonine	553	4/14/2016	Benzoylecgonine	667	5/3/2016	21%	UOM for GCMS = 24%
[redacted]	Cocaine	< 50	4/26/2016	Cocaine	14	4/28/2016		
	Benzoylecgonine	422	4/26/2016	Benzoylecgonine	461	4/28/2016	9%	
[redacted]	Cocaine	< 50	4/29/2016	Cocaine	5.5	5/3/2016		
	Cocaethylene	< 50	4/29/2016	Cocaethylene	7.5	5/3/2016		
	Benzoylecgonine	152	4/29/2016	Benzoylecgonine	181	5/3/2016	19%	
	Methamphetamine	< 20	5/2/2016	Methamphetamine	19	5/3/2016		
				Amphetamine	< 5	5/3/2016		GCMS LOD = 10ng/mL
[redacted]	Cocaine	< 50	3/21/2016	Cocaine	12	4/22/2016		
	Cocaethylene	< 50	3/21/2016	Cocaethylene	18	4/22/2016		
	Benzoylecgonine	845	3/21/2016	Benzoylecgonine	923	4/22/2016	9%	
[redacted]	Negative		3/21/2016	Negative		5/3/2016		Original: ELISA and Blood Base
[redacted]	Butylone	< 20	4/26/2016	Butylone	22	4/22/2016		GCMS Acceptable accuracy at LOQ = 30%
	MDMA	21	4/26/2016	MDMA	28	4/22/2016	33%	GCMS Acceptable accuracy at LOQ = 30%
	MDA	< 20	4/26/2016	MDA	< 5	4/22/2016		
[redacted]**	Butylone	< 20	4/26/2016	Butylone	20	5/6/2016		GCMS Acceptable accuracy at LOQ = 30%
	MDMA	21	4/26/2016	MDMA	26	5/6/2016	24%	GCMS Acceptable accuracy at LOQ = 30%
	MDA	< 20	4/26/2016	MDA	< 5	5/6/2016		

Case #	Original Analysis			LC-MSMS Analysis			% Difference	Comments
	Drug	Concentration (ng/mL)	Date of Analysis	Drug	Concentration (ng/mL)	Date of Analysis		
[redacted]	Amphetamine	< 20	4/26/2016	Amphetamine	21	4/28/2016		
16-FTCA01	Cocaine	574	3/11/2016	See results below compared to GCMS reanalysis				
	Benzoylecgonine	1370	3/15/2016					Original: 5x dilution
16-FTCA01*	Cocaine	430	4/14/2016	Cocaine	413	4/22/2016	-4%	
				Cocaethylene	< 5	4/22/2016		GCMS LOD = 10ng/mL
	Benzoylecgonine	1470	4/14/2016	Benzoylecgonine	1716	4/22/2016	17%	Original: 10x dilution

*GCMS reanalysis done more contemporaneous to LC-MSMS Analysis

**LC-MSMS reanalysis

Conclusion

Overall the method demonstrated good agreement for qualitative and quantitative results when compared to methods currently in use for casework when taking into account the performance of the original GC-MS methods and known stability issues of cocaine in blood (6). Due to the increased sensitivity of the LC-MSMS method, it is expected that additional compounds will be identified in specimens that cannot be detected by the original GC-MS methods.

Uncertainty of Measurement

An estimation of the uncertainty of measurement was determined for each compound according to the currently approved procedure within the toxicology unit. At least thirty replicates of the 200/2000 ng/mL control performed by 3 different analysts and a trainee in toxicology were used in the estimation. Estimated measurement uncertainty (k = 3) for each compound validated for quantitative analysis is presented in the table below. See the uncertainty worksheets maintained on the network or PBSO portal for detailed results.

Analyte	LC-MSMS UOM (k = 3)	GC-MS UOM (k = 3)
Alpha-PVP	19%	NA
Amphetamine	21%	26%
Benzoylecgonine	15%	24%
Butylone	20%	20%
BZP	NA	NA
Cocaethylene	17%	19%
Cocaine	16%	18%
Ethylone	20%	23%
MDA	15%	17%
MDMA	19%	19%
MDPV	16%	NA
Mephedrone	18%	23%
Methamphetamine	18%	17%
Methylone	17%	32%
Methylphenidate	21%	NA
Phentermine	NA	NA
TFMPP	NA	NA

Conclusion

The estimated uncertainty of measurement (UOM) with a k of 3 was less than or equal to 21%. In most cases the UOM was less than the UOM for the original GC-MS analyses.

Extraction Worksheet

TX BLOOD STIMULANTS LC QUANT EXTRACTION FORM

1. ____ 10uL of the ISTD to labeled conical micro centrifuge tubes (ISTD-_____) (P-_____)
2. ____ Add the following volumes of the 0.5/5ug/mL LOW working calibrator (CAL-_____):
   A)5/50ng/mL → 2uL B)10/100ng/mL → 4uL C)20/200ng/mL → 8uL D)50/500ng/mL → 20uL
   (_____) (_____) (_____) (_____)
3. ____ Add the following volumes of the 5/50ug/mL HIGH working calibrator (CAL-_____):
   E)125/1250ng/mL → 5uL F)250/2500ng/mL → 10uL G)500/5000ng/mL → 20uL
   (_____) (_____) (_____)
4. ____ Add the following volume of the 5/50ug/mL working control (CON-_____):
   A)200/2000ng/mL → 8uL
   (_____)
5. ____ 9 x 200uL blood for each standard and blank (lot/manuf: _____) (P-_____)
6. ____ 200uL Blood for each sample (P-_____)

   or for 1:10 dilution: 20uL blood for each sample (P-_____) with 180uL blank blood (P-_____)

7. ____ Vortex mix all tubes
8. ____ Add 600uL of HPLC or LCMS acetonitrile and vortex mix (lot/manuf:_____/JT Baker)
9. ____ Centrifuge for approximately 5 minutes
10. ____ Transfer supernatant to labeled culture tube
11. ____ Add 10uL of acidified methanol (R44-_____)
12. ____ Evaporate to just dry at ≤ 50°C in Turbovap (approximate time = 7-9 minutes)
13. ____ Add 150uL of LC Sample Diluent (R54, 95:5 of A and B, Make fresh daily) and vortex ≥10 seconds
    Mobile Phase A: LCMS Water with 0.1% Formic Acid (lot/manuf: _____ / JT Baker)
    Mobile Phase B: LCMS Methanol with 0.1% Formic Acid (R52-_____)

14. ____ Transfer to ALS vial with microinsert, and cap for analysis
15. ____ Check and refill rinse and mobile phase solutions as needed (HPLC or LCMS grade)
    Rinse solution: Isopropanol/Methanol/Acetonitrile (60/20/20) (R48-_____)
    Mobile Phase A: LCMS Water with 0.1% Formic Acid (lot/manuf: _____ / JT Baker)
    ☐Mobile Phase A same as above
    Mobile Phase B: Mobile Phase B: LCMS Methanol with 0.1% Formic Acid (R52-_____)
    ☐Mobile Phase B same as above

16. ____ Load method, verify instrument parameters, and run sequence
    LC Column: Phenomenex Kinetex Phenyl-Hexyl 2.6um (50 x 4.6 mm), Cat# 00B-4495-E0,
    Serial # _____

Extraction Performed by: _____ Date: _____ Instrument #: LC-1

Instrument Parameters

Acquisition Information:

Acquisition Method: Stimulants.dam
Created: Tuesday January 12 2016 09:30:45 AM
Last Modified: Tuesday February 09 2016 10:14:44 AM
Comment: Phenomenex Kinetex 2.6u Phenyl-Hexyl
Synchronization Mode: LC Sync
Auto-Equilibration: Off Acquisition Duration: 6min30sec
Number Of Scans: 650
Periods In File: 1
Acquisition Module: Acquisition Method
Software version Analyst 1.6.1

Period 1:

Scans in Period: 650
Relative Start Time: 0.00 msec Experiments in Period: 1

Period 1 Experiment 1:

Scan Type: MRM (MRM)
Scheduled MRM: Yes
Polarity: Positive
Scan Mode: N/A
Ion Source: Turbo Spray MRM detection window: 60 sec Target Scan Time: 0.6000 sec
Resolution Q1: Unit
Resolution Q3: Unit
Intensity Thres.: 0.00 cps
Settling Time: 0.0000 msec
MR Pause: 5.0000 msec
MCA: No
Step Size: 0.00 Da

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
232.000	91.000	3.67	DP	51.00	51.00	alpha-PVP 1
			EP	10.00	10.00	
			CEP	14.00	14.00	
			CE	31.00	31.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
232.000	105.000	3.67	DP	51.00	51.00	alpha-PVP 2
			EP	10.00	10.00	
			CEP	14.00	14.00	
			CE	33.00	33.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
136.000	91.000	2.58	DP	26.00	26.00	amphetamine 1
			EP	6.00	6.00	
			CEP	10.00	10.00	
			CE	23.00	23.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
136.000	119.000	2.58	DP	26.00	26.00	amphetamine 2
			EP	6.00	6.00	
			CEP	10.00	10.00	
			CE	11.00	11.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
290.000	105.000	3.70	DP	46.00	46.00	benzoylecgonine 2
			EP	5.00	5.00	
			CEP	18.00	18.00	
			CE	39.00	39.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
290.000	82.000	3.70	DP	46.00	46.00	benzoylecgonine 1
			EP	5.00	5.00	
			CEP	18.00	18.00	
			CE	41.00	41.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
222.000	174.500	3.16	DP	36.00	36.00	Butylone 1
			EP	5.50	5.50	
			CEP	14.00	14.00	
			CE	21.00	21.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
222.000	131.000	3.16	DP	36.00	36.00	Butylone 2
			EP	5.50	5.50	
			CEP	14.00	14.00	
			CE	41.00	41.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
177.000	91.000	1.31	DP	41.00	41.00	BZP 1
			EP	10.50	10.50	
			CEP	14.00	14.00	
			CE	29.00	29.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
177.000	65.000	1.31	DP	41.00	41.00	BZP 2
			EP	10.50	10.50	
			CEP	14.00	14.00	
			CE	55.00	55.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
318.000	82.000	4.04	DP	46.00	46.00	Cocaethylene 1
			EP	7.50	7.50	
			CEP	22.00	22.00	
			CE	45.00	45.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
318.000	105.000	4.04	DP	46.00	46.00	Cocaethylene 2
			EP	7.50	7.50	
			CEP	22.00	22.00	
			CE	49.00	49.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
304.000	105.000	3.80	DP	41.00	41.00	Cocaine 1
			EP	6.00	6.00	
			CEP	22.00	22.00	
			CE	37.00	37.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
304.000	77.000	3.80	DP	41.00	41.00	Cocaine 2
			EP	6.00	6.00	
			CEP	22.00	22.00	
			CE	83.00	83.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
222.000	174.100	2.96	DP	36.00	36.00	Ethylone 1
			EP	6.50	6.50	
			CEP	14.00	14.00	
			CE	25.00	25.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
222.000	91.000	2.96	DP	36.00	36.00	Ethylone 2
			EP	6.50	6.50	
			CEP	14.00	14.00	
			CE	49.00	49.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
240.000	91.000	3.67	DP	51.00	51.00	IS alpha-PVP d8
			EP	7.50	7.50	
			CEP	16.00	16.00	
			CE	33.00	33.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
142.000	93.000	2.58	DP	26.00	26.00	IS Amphetamine-d6
			EP	6.50	6.50	
			CEP	14.00	14.00	
			CE	23.00	23.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
293.000	105.000	3.74	DP	41.00	41.00	IS benzoylecgonine d3
			EP	6.50	6.50	
			CEP	16.00	16.00	
			CE	39.00	39.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
225.000	177.000	3.16	DP	46.00	46.00	IS Butylone-d3
			EP	5.00	5.00	
			CEP	14.00	14.00	
			CE	23.00	23.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
184.000	98.000	1.31	DP	46.00	46.00	IS BZP-d7
			EP	6.50	6.50	
			CEP	12.00	12.00	
			CE	31.00	31.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
321.000	85.000	4.04	DP	46.00	46.00	IS Cocaethylene-d3
			EP	7.50	7.50	
			CEP	18.00	18.00	
			CE	41.00	41.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
307.000	105.000	3.80	DP	46.00	46.00	IS Cocaine-d3
			EP	6.00	6.00	
			CEP	18.00	18.00	
			CE	41.00	41.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
227.000	179.000	2.96				
			DP	41.00	41.00	IS Ethylone-d5
			EP	6.50	6.50	
			CEP	14.00	14.00	
			CE	25.00	25.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
185.000	168.000	2.83	DP	31.00	31.00	IS MDA-d5
			EP	6.00	6.00	
			CEP	14.00	14.00	
			CE	17.00	17.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
199.000	165.000	3.00	DP	36.00	36.00	IS MDMA-d5
			EP	5.50	5.50	
			CEP	12.00	12.00	
			CE	19.00	19.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
284.000	135.000	3.84	DP	51.00	51.00	IS MDPV-d8
			EP	6.50	6.50	
			CEP	16.00	16.00	
			CE	33.00	33.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
181.000	148.000	3.12	DP	31.00	31.00	IS Mephedrone-d3
			EP	5.50	5.50	
			CEP	12.00	12.00	
			CE	27.00	27.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
155.000	92.000	2.80	DP	31.00	31.00	IS Methamphetamine-d5
			EP	6.00	6.00	
			CEP	12.00	12.00	
			CE	27.00	27.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
211.000	163.000	2.70	DP	36.00	36.00	IS Methylone-d3
			EP	7.00	7.00	
			CEP	14.00	14.00	
			CE	25.00	25.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
243.000	93.000	3.68	DP	41.00	41.00	IS Methylphenidate-d9
			EP	9.00	9.00	
			CEP	14.00	14.00	
			CE	29.00	29.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
155.000	96.000	3.01	DP	26.00	26.00	IS Phentermine-d5
			EP	4.00	4.00	
			CEP	10.00	10.00	
			CE	33.00	33.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
235.000	190.000	3.95	DP	61.00	61.00	IS TFMPP-d4
			EP	6.00	6.00	
			CEP	14.00	14.00	
			CE	29.00	29.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
180.000	163.000	2.83	DP	26.00	26.00	MDA 1
			EP	5.50	5.50	
			CEP	12.00	12.00	
			CE	13.00	13.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
180.000	135.000	2.83	DP	26.00	26.00	MDA 2
			EP	5.50	5.50	
			CEP	12.00	12.00	
			CE	25.00	25.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
194.000	163.000	3.00	DP	31.00	31.00	MDMA 1
			EP	7.00	7.00	
			CEP	12.00	12.00	
			CE	17.00	17.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
194.000	105.000	3.00	DP	31.00	31.00	MDMA 2
			EP	7.00	7.00	
			CEP	12.00	12.00	
			CE	31.00	31.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
276.000	126.000	3.84	DP	51.00	51.00	MDPV 1
			EP	6.50	6.50	
			CEP	20.00	20.00	
			CE	35.00	35.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
276.000	135.000	3.84	DP	51.00	51.00	MDPV 2
			EP	6.50	6.50	
			CEP	20.00	20.00	
			CE	35.00	35.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
178.000	145.000	3.12	DP	26.00	26.00	Mephedrone 1
			EP	6.00	6.00	
			CEP	12.00	12.00	
			CE	23.00	23.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
178.000	91.000	3.12	DP	26.00	26.00	Mephedrone 2
			EP	6.00	6.00	
			CEP	12.00	12.00	
			CE	43.00	43.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
150.000	91.100	2.80	DP	31.00	31.00	Methamphetamine 1
			EP	7.00	7.00	
			CEP	10.00	10.00	
			CE	27.00	27.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
150.000	119.000	2.80	DP	31.00	31.00	Methamphetamine 2
			EP	7.00	7.00	
			CEP	10.00	10.00	
			CE	17.00	17.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
208.000	160.000	2.70	DP	26.00	26.00	Methylone 1
			EP	11.00	11.00	
			CEP	12.00	12.00	
			CE	21.00	21.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
208.000	132.000	2.70	DP	26.00	26.00	Methylone 2
			EP	11.00	11.00	
			CEP	12.00	12.00	
			CE	33.00	33.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
234.000	84.000	3.68	DP	41.00	41.00	Methylphenidate 1
			EP	9.50	9.50	
			CEP	10.00	10.00	
			CE	31.00	31.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
234.000	56.000	3.68	DP	41.00	41.00	Methylphenidate 2
			EP	9.50	9.50	
			CEP	10.00	10.00	
			CE	65.00	65.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
150.000	91.000	3.01	DP	21.00	21.00	Phentermine 1
			EP	5.00	5.00	
			CEP	10.00	10.00	
			CE	27.00	27.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
150.000	65.000	3.01	DP	21.00	21.00	Phentermine 2
			EP	5.00	5.00	
			CEP	10.00	10.00	
			CE	53.00	53.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID
231.000	188.000	3.95	DP	61.00	61.00	TFMPP 1
			EP	5.50	5.50	
			CEP	14.00	14.00	
			CE	31.00	31.00	

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Param	Start	Stop	ID 231.000
	118.000	3.95	DP	61.00	61.00	TFMPP 2
			EP	5.50	5.50	
			CEP	14.00	14.00	
			CE	49.00	49.00	

Parameter Table(Period 1 Experiment 1):

CUR:	30.00
IS:	2000.00
TEM:	650.00
GS1:	60.00
GS2:	40.00
ihe:	ON
CAD:	Medium
CXP	4.00

Valco Valve Diverter

	Total Time (min)	Position
1	0.8	ms
2	4.5	waste

Shimadzu LC Method Properties
Shimadzu LC system
Equlibration time = 0.00 min
Shimadzu LC system Injection
Volume = 5.00 ul Shimadzu LC
Method Parameters

Pumps
═══
Pump A Model: LC-20ADXR
Pump B Model: LC-20ADXR
Pumping Mode: Binary Flow
Total Flow: 0.7000 mL/min
Pump B Conc: 5.0 %
B Curve: 0
Pressure Range (Pump A/B): 0 - 9000 psi

Autosampler
==========
Model: SIL-20ACXR
Use Autosampler: Yes
Rinsing Volume: 1000 uL
Needle Stroke: 50 mm.
Rinsing Speed: 35 uL/sec
Sampling Speed: 5.0 uL/sec
Purge Time: 25.0 min
Rinse Dip Time: 4 sec
Rinse Mode: Before and after aspiration
Cooler Enabled: Yes
Cooler Temperature: 15 deg. C
Control Vial Needle Stroke: 52 mm

Oven
====
Model: CTO-20A
Temperature Control: Enabled
Temperature: 35 deg. C
Max. Temperature: 85 deg. C
Right Valve Position (FCV-12AH): 1

System Controller
=================
Model: CBM-20A Power: On Event 1: Off
Event 2: Off
Event 3: Off
Event 4: Off

Solenoid Valve
==============
Pump A (FCV-11AL)

Port 1 Valve Position: A - LCMS Water 0.1% Formic
Port 2 Valve Position: A
Port 3 Valve Position: A

Pump B (FCV-11AL)

Port 1 Valve Position: A - LCMS MeOH 0.1% Formic
Port 2 Valve Position: A
Port 3 Valve Position: A

Time Program
============

Time	Module	Events	Parameter
0.01	Pumps	Pump B Conc.	5
2.20	Pumps	Pump B Conc.	40
4.50	Pumps	Pump B Conc.	95
5.50	Pumps	Pump B Conc.	95
5.60	Pumps	Pump B Conc.	5
6.50	System Controller	Stop	

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Approved by: Yeatman, Dustin T. Digitally signed by Yeatman, Dustin T. DN: dc=org, dc=pbso, ou=People, ou=Forensic Sciences, cn=Yeatman, Dustin T. Date: 2016.05.13 12:33:57 -04'00'

Chemistry/Toxicology Manager