Validation of a Quantitative Method for Amphetamines, Phentermine, and Designer Stimulants Using an Agilent 6430 LC/MS/MS

Application Note

Forensics

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Abstract

A method was developed and validated for the quantitation of amphetamines, phentermine, and designer stimulants in biological samples using an Agilent 6430 Triple Quadrupole LC/MS system. Validation studies demonstrated that the LC/MS/MS method provides reliable results that meet acceptance criteria for method validation in forensic toxicology set by the Scientific Working Group for Forensic Toxicology (SWGTOX). The concentration range of target compounds used in this validation was chosen to fit the commonly encountered range of analyte concentrations seen in Driving Under the Influence of Drugs (DUID) and medical examiner casework. The method displays acceptable accuracy and precision for the detection of amphetamines, phentermine, and designer stimulants. Other aspects evaluated during validation include sensitivity, interferences, robustness, carryover, dilution integrity, stability, suppression/enhancement, and recovery for the target compounds in whole blood.

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Introduction

Amphetamines, phentermine, and designer stimulants are analyzed in biological matrixes in forensic toxicology laboratories. Quantitative analysis of amphetamines is important in the investigation of Driving Under the Influence of Drugs (DUID) cases due to the *Per Se* limits set forth by many state governments. Standard GC/MS and GC/MS/MS analysis requires time-consuming sample preparation involving derivatization prior to analysis. Liquid chromatography triple quadrupole mass spectrometry (LC/MS/MS) is becoming an increasingly common technique in forensic and clinical toxicology due to instrumental sensitivity and specificity.

This application note addresses the validation of a LC/MS/MS method on an Agilent 6430 Triple Quadrupole LC/MS System for the quantitation of amphetamines, phentermine, and designer stimulants.

Validation studies included calibration model fits, precision and accuracy, sensitivity measured by the limit of detection (LOD) and the limit of quantitation (LOQ), stability, robustness, dilution integrity, carryover, and ion suppression/enhancement. Validation studies were conducted using the SWGTOX method validation guidelines in conjunction with the Virginia Department of Forensic Science validation guidelines [1,2]. As a result, the method met all criteria for data integrity, and was found to be a reliable method for routine amphetamines, phentermine, and designer stimulants analysis in whole blood.

Experimental

The method includes a liquid-liquid extraction with quantitation and confirmation by an Agilent 6430 Triple Quadrupole LC/MS System, using Agilent MassHunter Acquisition and Quantitative Analysis for data acquisition and analysis. Amphetamines, phentermine, and designer stimulants were extracted from biological samples using saturated trisodium phosphate and 1-chlorobutane in accordance with the Virginia Department of Forensic Science's Procedures Manual. The method was validated for the target compounds shown in Table 1. A comprehensive explanation of the method, including sample preparation and instrumental parameters is detailed in "Amphetamines, Phentermine, and Designer Stimulant Quantitation Using an Agilent 6430 LC/MS/MS" [3].

Table 1. Target Compounds and Corresponding Internal Standards

Target	Internal standard
Amphetamine	Amphetamine-D_{11}
Methamphetamine	Methamphetamine-D_{11}
Phentermine	
3,4-Methylenedioxyamphetamine (MDA)	MDA-D_5
3,4-Methylenedioxymethamphetamine (MDMA)	MDMA-D_5
Mephedrone	Mephedrone-D_3
Methedrone HCl	
α-Pyrrolidinopentiophenone (α-PVP)	
3,4-Methylenedioxypyrovalerone HCl (MDPV)	
Bupropion HCl	
Methcathinone	
Pseudoephedrine	Pseudoephedrine-D_3
Methylone HCl	Methylone-D_3

Sample preparation

Validation studies were performed using pooled and spiked standards. Samples were extracted using the procedure outlined in "Amphetamines, Phentermine, and Designer Stimulant Quantitation using an Agilent 6430 LC/MS/MS" [3]. Pooled standards were prepared by spiking a large volume of blank blood with respective concentrations of target compounds. One-milliliter aliquots were taken from the pooled samples and extracted prior to quantitative analysis by LC/MS/MS. Spiked standards were prepared by pipetting appropriate volumes of working standard solutions into clean test tubes with 1.0 mL of blank blood.

Working standard solution (10 µg/mL): Pipette 100 µL of 1.0 mg/mL standard into a 10-mL volumetric flask and bring to final volume with methanol.

Working standard solution (1 µg/mL): Pipette 1.0 mL of 10 µg/mL working standard solution into a 10-mL volumetric flask and bring to final volume with methanol.

Working internal standard solution (1 µg/mL): Pipette 10 µL of 1.0 mg/mL (or 100 µL of 0.1 mg/mL) internal standard into a 10-mL volumetric flask and bring to final volume with methanol.

Results and Discussion

Linearity and calibration model

The best fit calibration model was determined using multiple statistical analysis techniques as well as the analysis of residual plots. Eight calibrators were analyzed with every batch and were used to assess the instrument response for each target compound. The dynamic range evaluated was 0.01 mg/L to 2.0 mg/L. To establish the calibration model, the origin was ignored and the correlation coefficient (R^2) should be $\geq$ 0.985. The back-calculated concentration should be within ± 20 % of the target concentration.

To determine the linear/quadratic nature of the model, ANOVA was used to compare the standard deviation of residuals from each batch. The weighting of the calibration model was determined by applying the weighting that minimizes the sum of relative error for the residuals. Non-weighted and weighted (1/x) models were evaluated for the lowest average sum of relative error.

A weighted (1/x) linear calibration model best predicted the methcathinone, pseudoephedrine, methylone, and mephedrone response. A weighted (1/x) quadratic calibration model best predicted the response for all other compounds assessed in this method. The dynamic range and calibration models are described in Table 2.

Table 2. Dynamic Range and Calibration Models for Target Compounds

	Regression analysis		
Target	**Dynamic range (mg/L)**	**Linear/ Quadratic**	**Weighting**
Methcathinone	0.010–2.0	Linear	Weighted (1/x)
Pseudoephedrine	0.010–2.0	Linear	Weighted (1/x)
Methylone	0.010–2.0	Linear	Weighted (1/x)
Amphetamine	0.010–2.0	Quadratic	Weighted (1/x)
Methamphetamine	0.010–2.0	Quadratic	Weighted (1/x)
MDA	0.010–2.0	Quadratic	Weighted (1/x)
Methedrone	0.010–2.0	Quadratic	Weighted (1/x)
MDMA	0.010–2.0	Quadratic	Weighted (1/x)
Phentermine	0.010–2.0	Quadratic	Weighted (1/x)
Mephedrone	0.010–2.0	Linear	Weighted (1/x)
α-PVP	0.010–2.0	Quadratic	Weighted (1/x)
MDPV	0.010–2.0	Quadratic	Weighted (1/x)
Bupropion	0.010–2.0	Quadratic	Weighted (1/x)

Accuracy

Accuracy studies were conducted with pooled blood samples fortified with the target compounds. The samples were fortified into 50.0 mL of blank blood and a 1.0 mL aliquot was taken and extracted. Three concentrations (0.03, 0.3, and 1.5 mg/L) were assessed with triplicate analysis for each batch over a total of five batches.

The acceptance criterion for the pooled accuracy was ± 20 % for all concentration levels. Table 3 represents the accuracy of the pooled blood. The percent accuracy also demonstrates any bias within the measurements. The *n* was 15 for all three concentration levels.

Table 3. Percent Accuracy and Bias for Pooled Amphetamines and Designer Stimulants Quantitated by LC/MS/MS

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

The spiked accuracy ranged from 93 ± 5 % to 105 ± 6 %. All targets were within the acceptance criteria of ± 20 % accuracy.

Precision

The within-run and intermediate precision was assessed using the same sample set as the accuracy evaluation. The precision of the samples was measured as the coefficient of variance (% CV). The predetermined acceptance criterion for within-run and intermediate precision was a % CV within ± 20 %. Table 4 represents the intermediate precision at three concentration levels. All targets had an intermediate precision within ± 13 %. Table 5 represents the within-run precision for the target compounds at three concentrations. Bupropion had the largest within-run and intermediate precision. The within-run precision was 13 %, and the intermediate precision was 16 %. All other target compounds had a within-run precision within ± 11 %.

Table 4. Intermediate Precision for Pooled Amphetamines and Designer Stimulants Quantitated by LC/MS/MS

Pooled intermediate precision, mean ± SD (% CV), n = 15

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

Table 5. Within-Run Precision for Pooled Amphetamines and Designer Stimulants Quantitated by LC/MS/MS

Pooled within-run precision, mean ± SD (% CV), n = 3

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

Sensitivity (LOD, LOQ)

The sensitivity of the method was determined by assessing the LOD and LOQ. The LOD and LOQ were established by analyzing triplicate determinations of multiple concentrations (0.005, 0.0025, 0.00125 mg/L) in three different blank blood sources along with calibrators (0.010–2.0 mg/L).

The predetermined acceptance criterion for LOD was a retention time within ± 5 % and a qualifier ratio within ± 20 % of the average qualifier ion ratios of the calibrators within the batch. Also, the signal to noise (S/N) ratio acceptance criteria was a S/N greater than 3:1. These criteria were assessed using the MassHunter Quantitative Analysis Software. The LOQ criteria was a retention time within ± 5 %, and a qualifier ratio within ± 20 % of the average calibrator qualifier ion ratios within the batch. Also, the back-calculated concentration should be within ± 20 % of the spiked concentration, and the S/N ratio should be greater than 10:1.

All targets satisfied the predetermined acceptance criteria for the LOD at 0.01 mg/L or lower, as shown in Table 6. Phentermine was the only target with an LOD of 0.01 mg/L. The LOQ was 0.01 mg/L for methcathinone, amphetamine, methamphetamine, phentermine, and bupropion. All other targets had an LOQ less than 0.01 mg/L.

Table 6. LODs and LOQs

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

Recovery

Recovery was assessed with three different concentrations analyzed in triplicate. The recovery of the extraction was analyzed in blood, liver, and urine. The recovery of each matrix was averaged for an overall recovery for the process over the concentration range. The extracted control response was compared to double blank samples that were spiked with both internal standard and control after extraction. The raw instrumental response was used to calculate the average recovery for each matrix type.

Table 7 represents the average percent recovery for all targets at 0.02, 0.25, and 1.0 mg/L in blood, liver, and urine.

Table 7. % Recovery (SD) for Analytes

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

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

The recovery of the internal standards was also evaluated and is depicted in Table 8.

Table 8. % Recovery (SD) for Internal Standards

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

In blood samples, the range of internal standard recovery was 83 % to 96 %. MDA-D_5 and pseudoephedrine-D_3 had the lowest and highest recoveries respectively. The recovery range for liver samples was 70 % to 83 %, with methylone-D_3 having the lowest and pseudoephedrine-D_3 having the highest percent recovery. In urine samples, the recovery ranged from 86 % to 91 %. Methylone-D_3 demonstrated the lowest percent recovery while methamphetamine-D_{11} demonstrated the highest percent recovery.

Interferences

Interferences from endogenous compounds, internal standards, target analytes, and commonly encountered analytes were evaluated. There should be no source of interference for the method to be accepted. Six blank blood, three blank liver, and two blank urine matrices were analyzed for endogenous compound interference. To test for interferences from internal standard to target, or target to internal standard, two samples were analyzed. One was fortified with only internal standard (2.0 mg/L) and one with only the targets of interest (2.0 mg/L). Three negative matrix samples were fortified for this evaluation. Finally, three sources of blank matrix were fortified at high concentration of commonly encountered drugs, metabolites, and other structurally similar compounds. The results of these studies show that no interferences were detected for all target compounds.

Ion suppression/enhancement

Ion suppression and enhancement was evaluated by analyzing the instrumental reponse of three concentrations of neat standards and post-extraction fortified samples. Five blank blood sources, four liver sources, and two urine sources were used to determine the ion suppression and enhancement of the matrixes. The responses of each sample were used to determine the extent of ion suppression or enhancement.

Suppression and enhancement were evaluated at 0.02, 0.25, and 1.0 mg/L. The suppression was averaged and tabulated as depicted in Table 9. Values greater than 100 % indicate ion enhancement, while values less than 100 % indicate ion suppression. The ion suppression/enhancement ranges from 64 % to 116 % for the targets in this method.

Liver had more significant suppression than both blood and urine. Pseudoephedrine had the most suppression over all matrix types. The suppression in blood was 75 %, while suppression in liver and urine was 64 % and 81 % respectively. The suppression or enhancement seen with the targets had no effect on the pooled accuracy and precision, LOQ, or LOD, and did not impact the quantitative analysis of the targets.

The suppression and enhancement of the internal standard was also assessed (Table 10). The most significant suppression was found in pseudoephedrine-D_3. This was similar to the suppression demonstrated with the target. All other internal standards demonstrated the same level of suppression as the targets and, therefore, there was no effect on accuracy or precision.

Table 9. Overall Analyte Suppression /Enhancement (SD)

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

Table 10. Overall Internal Standard Suppression/Enhancement (SD)

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

Carryover

Carryover was addressed by injecting progressively higher concentrations of target analytes followed by solvent blanks. The highest concentration that did not have a contribution to the quantitative transitions in the subsequent solvent injection was free of carryover. After the highest concentration free of carryover was established, the concentration was confirmed with triplicate analysis. The highest concentration of amphetamines and designer stimulants that was assessed and determined to be free of carryover was 5.0 mg/L. The highest calibrator concentration for the method will be set to 2.0 mg/L.

Stability

Two target concentrations (1.0 mg/L and 0.02 mg/L) were assessed for stability. Samples were extracted and injected immediately to establish an initial (Day 1) instrumental response. Both concentration levels were injected in triplicate every 24 hours and the instrumental response was compared over a seven day period. If the average instrumental response decreased below 80 % or increased above 120 % compared to the Day 1 response, the target was considered unstable after that time period.

Figure 1 represents the average response for each target compound at a concentration of 1.0 mg/L over a seven day period. All 13 compounds were stable up to seven days following extraction with the exception of methcathinone and MDA. Methcathinone is stable at 1.0 mg/L for seven days, but at a concentration of 0.02 mg/L, methcathinone increased above acceptance criteria on Day 4. A similar trend was observed with methcathinone at 1.0 mg/L, although the response did not exceed the 120 % acceptance criteria.

Dilution integrity

Dilution integrity was evaluated in two different ways. A small volume dilution was used in cases where minimal sample volume was available. To evaluate a small volume dilution, a large volume of blood was fortified and diluted 1:2, 1:5, 1:10, and 1:20 with a total volume of 1.0 mL. For example, a 1:2 dilution was prepared by adding 0.5 mL of fortified sample to 0.5 mL of blank blood for a total volume of 1.0 mL.

The large volume dilution was assessed for situations when sample volume was not a factor, but the case sample concentration was greater than the dynamic range. Dilutions of 1:2, 1:5, 1:10, and 1:20 were made in a large volume, and 1.0 mL aliquots were analyzed. For example, a 1:2 dilution, 1.0 mL fortified sample was diluted with 1.0 mL of blank blood, and a 1.0 mL aliquot was used for extraction. The predetermined acceptance criteria for dilution integrity was that the precision and accuracy shall not exceed ± 20 % of the back-calculated concentration.

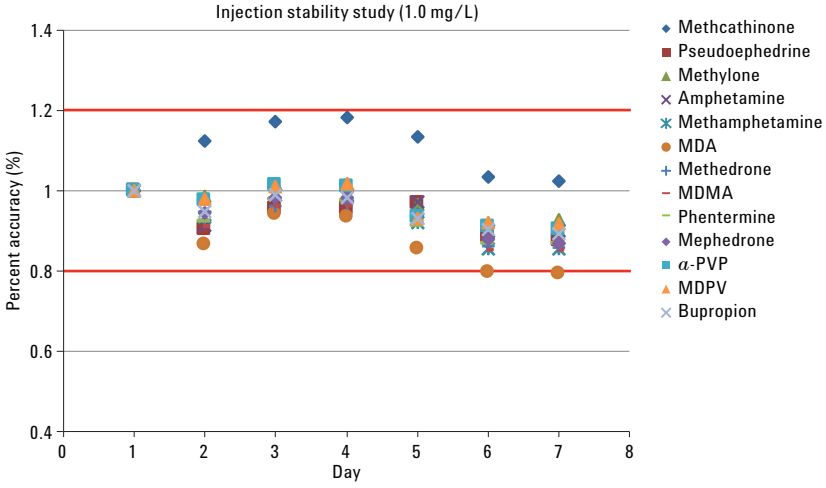

Figure 1. Stability graph. The red lines indicate the 80 % and 120 % acceptance thresholds.

Small volume dilution integrity

In cases of small volume dilution integrity, the accuracy of a 1:5 dilution was within the predetermined acceptance criterion for all targets with the exception of MDPV (Tables 11 and 12). Pseudoephedrine, methylone, MDA, methedrone, mephedrone, MDPV, and bupropion did not meet the predetermined accuracy acceptance criteria for small volume dilutions at a 1:10 dilution ratio. The remaining compounds passed the acceptance criteria for the 1:20 small volume dilution. All targets passed the precision acceptance criteria at all dilution ratios.

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

Table 11. Dilution Integrity Accuracy (Small Volume) % Accuracy (SD)

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

Table 12. Dilution Integrity Intermediate Precision (Small Volume) Mean ± SD (%CV)

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

Large volume dilution integrity

The accuracy for all target compounds was within the acceptance criterion of ± 20 % accuracy for all large volume dilution ratios with the exception of MDPV and bupropion. MDPV did not meet the predetermined acceptance criteria using a 1:5 large volume dilution ratio. Bupropion did not meet the predetermined acceptance criteria using a 1:10 large volume dilution ratio.

The precision for all target compounds was within the predetermined acceptance criterion of ± 20 % for all large volume dilution ratios. The precision for each target compound is shown in Table 14. The largest percent CV was 15 % with a 1:10 dilution for bupropion.

Table 13. Dilution Integrity Accuracy (Large Volume) % Accuracy (SD)

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

Table 14. Dilution Integrity Intermediate Precision (Large Volume) Mean ± SD (%CV)

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

Conclusion

This method development and validation provides a rapid and sensitive technique for the quantitation and confirmation of amphetamines, phentermine, and designer stimulants by LC/MS/MS. The validation addressed the method's linearity and calibration model fit, precision and accuracy, sensitivity (LOD/LOQ), interferences, carryover, dilution integrity, post extraction stability, ion suppression/enhancement, and recovery.

The dynamic range of target compounds used in this validation was chosen to fit the commonly encountered concentrations of the target analytes. All targets passed the comprehensive validation, demonstrating that the method provides reliable quantitative results. This method is a valid means of analyzing amphetamines, phentermine, and designer stimulants for routine drug analysis, providing quick, accurate, and reproducible results.

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