

**DEPARTMENT OF FORENSIC SCIENCE
METHOD VALIDATION SUMMARY FORM**

Section: Toxicology

Method: Utilization of a Circulating Bath in the Amphetamines, Phentermine, and

Designer Stimulants Quantitation and Confirmation by LCMSMS

Results recorded? ☒

Procedure documented? ☒

Method fit for use? ☒

Approved by: James Hutchings Date: 9-16-26

Executive Validation Summary: “Validation of the Utilization of a Circulating Bath in the Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS”

Summary

The validation evaluated the utilization of a circulating bath within the liquid-liquid extraction procedure for the existing Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS method within the Toxicology Procedures Manual (Qualtrax Revision 34). In accordance with the addendum to the validation plan, new internal standards were evaluated for alpha-PVP, MDPV, and bupropion including alpha-PVP-D₈, MDPV-D₈, and bupropion-D₈, respectively.

The bias and precision for all targets was within the acceptance criteria with the expansion of the qualifier ratio acceptance from $\pm 20\%$ to $\pm 30\%$. The estimated limit of detection and limit of quantitation were evaluated at the threshold level and lower limit of quantitation of the current method and the analyses met the predetermined acceptance criteria.

Limitations

It was noted during the frozen sample stability studies that some matrices may not fully freeze within the shorter time frames, therefore the samples should be returned to the circulating bath to fully freeze before transfers. With the implementation of this method, it will be noted that during the transfer step samples should be returned to the circulating bath to ensure that the samples stay frozen prior to transferring.

Studies Performed

1. Bias and Precision
 - a. Bias
 - b. Within-run Precision
 - c. Intermediate Precision
2. Sensitivity
 - a. Estimated Limit of Detection (LOD)
 - b. Lower Limit of Quantitation (LLOQ)
3. Interferences (Endogenous Compounds)
4. Frozen Sample Stability

Validation Results

Validation Summary Results			
Compound	Bias and Precision	LOD (mg/L)	LLOQ (mg/L)
Methcathinone	Pass	0.005	0.010
Pseudoephedrine	Pass	0.005	0.010
Methylone	Pass	0.005	0.010
Amphetamine	Pass	0.005	0.010
Methamphetamine	Pass	0.005	0.010
MDA	Pass	0.005	0.010
Methedrone	Pass	0.005	0.010
MDMA	Pass	0.005	0.010
Phentermine	Pass	0.005	0.010
Mephedrone	Pass	0.005	0.010
Alpha-PVP	Pass	0.005	0.010
MDPV	Pass	0.005	0.010
Bupropion	Pass	0.005	0.010

Memo To: James Hutchings, Ph.D., Toxicology Program Manager
From: Rebecca Wagner, Ph.D., Chemistry Research Section Supervisor
CC: Alka Lohmann, Director of Technical Services
Date: June 25, 2026
RE: Validation Summary
 Validation of the Utilization of a Circulating Bath in the Amphetamines,
 Phentermine, and Designer Stimulants Quantitation and Confirmation by
 LCMSMS

Validation Summary – Utilization of a Circulating Bath in the Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS

The validation evaluated the utilization of a circulating bath within the liquid-liquid extraction procedure for the existing Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS method within the Toxicology Procedures Manual (Qualtrax Revision 34). After centrifugation at approximately 2500 rpm for 15 minutes to achieve separation (Section 26.6.8), the samples were placed into a PolyScience Refrigerated Circulator containing reagent alcohol. The samples incubated in the circulator for 3 minutes at approximately -25°C. After incubation, the supernatant was poured into clean, screw cap tubes, and the procedure continued as established in the Toxicology Procedures Manual (Section 26.6.10-26.6.12). The target compounds and associated internal standards are listed in Table 1.

Table 1 Target compounds and internal standards

Target Compounds	Internal Standard
Methcathinone	Mephedrone-D ₃
Pseudoephedrine	Pseudoephedrine-D ₃
Methylone	Methylone-D ₃
Amphetamine	Amphetamine-D ₁₁
Methamphetamine	Methamphetamine-D ₁₁
MDA	MDA-D ₅
Methedrone	Mephedrone-D ₃
MDMA	MDMA-D ₅
Phentermine	Methamphetamine-D ₁₁
Mephedrone	Mephedrone-D ₃
Alpha-PVP	Alpha-PVP-D ₈
MDPV	MDPV-D ₈
Bupropion	Bupropion-D ₉

In accordance with the addendum to the validation plan, new internal standards were evaluated for alpha-PVP, MDPV, and bupropion including alpha-PVP-D₈, MDPV-D₈, and bupropion-D₉, respectively. The optimized instrumental parameters for these internal standards are listed in Table 2.

Table 2 Internal standard parameters

Compound	Precursor (<i>m/z</i>)	Product (<i>m/z</i>)	Approx Retention Time (min)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator (V)
Alpha-PVP-D ₈	240.2	134.1	6	90	24	7
		<u>91</u>		90	24	7
Bupropion-D ₉	249.2	185	6.5	95	8	7
		166.5		95	16	7
MDPV-D ₈	284.2	175	6.2	95	20	7
		<u>134.5</u>		95	28	7

The validation was conducted pursuant to the validation plan including the addendum to the validation plan.

1. Bias and Precision
 - a. Bias
 - b. Within-run Precision
 - c. Intermediate Precision
2. Sensitivity
 - a. Estimated Limit of Detection (LOD)
 - b. Lower Limit of Quantitation (LLOQ)
3. Interferences (Endogenous Compounds)
4. Frozen Sample Stability
5. Summary
6. References

The validation was performed using an Agilent Technologies 6460 and 6470 liquid chromatograph tandem mass spectrometer. The biological matrices evaluated during the validation included blank blood, antemortem blood, postmortem blood, urine, and liver for quantitative analysis. The utilization of blank blood during the validation refers to the matrix used to prepare calibrators and controls within the Toxicology Procedures Manual (Qualtrax Revision 34). The preparation of blank blood includes the utilization of human packed blood cells (approximately 300 mL) that are diluted with approximately 100 mL of water and approximately 100 mL of 1% sodium fluoride. The lower limit of quantitation was established to be 10 ng/mL with an administratively established threshold of 5 ng/mL (Toxicology Procedures Manual Section 26, Qualtrax Revision 34).

1. Bias and Precision

Bias was assessed by analyzing blank blood, antemortem blood, postmortem blood, urine, and liver with the target compounds at three different concentrations (low, medium, high). The number of matrices evaluated for bias and precision experiments increased to three matrix sources per matrix type. This change is in accordance with the Addendum to the Validation Plan. Each concentration, for each matrix source, was evaluated in triplicate, over a total of five batch analyses. Each matrix type was evaluated with three matrix sources producing an n of 45 for each concentration per matrix type. The calibration range of the method was established to be 10 ng/mL to 2000 ng/mL. The three concentrations evaluated for bias included 30 ng/mL, 800 ng/mL, and 1600 ng/mL.

Given the instability of the target compounds, pooled samples were not prepared for analysis, rather individually fortified samples were prepared by fortifying matrix at the respective concentrations with 1.0 mL aliquots of matrix. Individually fortified matrix samples were then extracted prior to quantitative analysis. Bias was assessed using Equation 1.

Equation 1

$$\text{Bias (\%)} \text{ Concentration}_x = \left(\frac{\text{Mean of Calculated Concentration}_x - \text{Expected Concentration}_x}{\text{Expected Concentration}_x} \right) \times 100$$

The acceptance criterion for bias was $\pm 20\%$ for all three concentration levels. Each matrix source, for each matrix type, was independently evaluated for bias ($n=15$). The bias for each matrix source is represented in Tables 3-7.

Table 3: Blank Blood Bias

Bias (% Bias, n=15)									
	Blank Blood Source 1			Blank Blood Source 2			Blank Blood Source 3		
	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL
Methcathinone	1.24	-1.12	-7.27	0.33	3.88	-1.31	-0.29	1.21	-2.09
Pseudoephedrine	-1.29	1.76	1.42	-0.58	2.40	-0.13	-0.51	5.18	1.41
Methylone	0.93	0.49	-1.15	-1.73	2.70	1.14	-2.91	3.45	0.06
Amphetamine	0.09	-0.10	0.33	2.20	0.78	-1.02	-0.84	2.51	-1.10
MDA	2.09	-0.67	-2.01	1.67	0.92	-0.62	3.07	4.23	0.45
Methamphetamine	1.84	-0.62	0.51	3.13	0.34	-0.45	1.60	0.56	-3.22
Methedrone	0.78	2.36	0.27	0.84	3.80	0.51	0.62	-0.53	-3.58
MDMA	0.87	0.31	0.00	1.29	1.91	-0.44	-1.07	4.26	0.69
Phentermine	2.64	3.90	1.49	2.09	0.68	1.01	0.02	2.33	-2.04
Mephedrone	-0.58	1.04	0.11	1.89	2.43	-0.57	-0.33	3.78	0.57
Alpha-PVP	0.07	0.74	-0.38	0.84	2.73	-0.14	-0.07	4.28	0.63
MDPV	0.16	1.23	-0.01	-0.73	2.23	0.17	0.58	5.00	1.19
Bupropion	1.09	0.18	-0.14	1.07	1.65	-1.22	1.49	2.17	-0.76

Table 4: Antemortem Blood Bias

Bias (% Bias, n=15)									
	Antemortem Blood Source 1			Antemortem Blood Source 2			Antemortem Blood Source 3		
	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL
Methcathinone	-3.80	-2.58	-3.85	-3.09	-1.88	-2.31	-4.31	2.16	-4.14
Pseudoephedrine	2.84	-0.32	0.57	3.09	2.35	1.61	1.96	3.41	1.13
Methylone	2.84	0.32	0.68	-0.78	3.57	1.49	-1.18	3.78	0.05
Amphetamine	-0.82	-2.39	-4.03	3.82	0.23	-0.36	0.53	-0.28	-1.42
MDA	3.22	0.51	0.23	3.96	0.76	-1.15	5.36	4.09	0.57
Methamphetamine	4.31	-1.15	-2.64	5.87	-0.21	-0.04	3.00	1.10	-2.26
Methedrone	-3.67	0.07	2.28	1.67	2.38	2.10	-1.44	4.44	1.56
MDMA	1.58	-0.21	-1.25	2.36	0.92	0.67	0.27	1.74	-0.25
Phentermine	-4.69	1.00	-1.22	-5.00	0.02	1.35	2.60	3.94	1.58
Mephedrone	2.18	-0.50	-0.72	2.87	2.35	0.67	0.51	2.52	0.34
Alpha-PVP	0.78	-3.22	-4.98	1.49	-1.59	-3.30	1.22	3.90	-3.06
MDPV	0.24	-1.99	-3.75	-0.07	-2.42	-3.39	-0.18	3.05	-2.59
Bupropion	2.82	0.27	-3.55	0.76	-1.54	-2.27	-0.53	3.16	-4.13

Table 5 Postmortem Blood Bias

Bias (% Bias, n=15)									
	Postmortem Blood Source 1			Postmortem Blood Source 2			Postmortem Blood Source 3		
	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL
Methcathinone	-6.42	-1.94	-4.53	-5.73	-7.26	-7.01	-3.93	-2.62	-5.10
Pseudoephedrine	-0.51	2.05	1.12	1.33	1.87	0.63	1.60	4.70	3.23
Methylone	1.36	1.52	-0.57	-1.33	1.55	0.97	-1.18	3.02	1.52
Amphetamine	-1.80	1.52	0.52	2.67	1.01	-0.73	1.62	2.64	-0.002
MDA	2.69	-0.04	-2.55	3.56	-1.64	-1.23	6.42	2.53	0.48
Methamphetamine	2.71	0.34	0.16	4.84	-1.54	-0.76	6.04	1.04	-1.40
Methedrone	-1.16	3.24	1.25	3.09	-3.64	-1.32	2.02	0.23	-0.30
MDMA	0.44	1.80	0.66	1.91	0.26	-0.36	2.27	4.19	2.23
Phentermine	1.22	3.86	3.06	2.93	-3.14	-1.29	0.69	1.34	0.40
Mephedrone	0.78	2.87	0.42	1.49	1.81	0.17	0.96	3.36	2.11
Alpha-PVP	2.51	3.00	0.75	1.80	1.40	-0.28	5.64	8.67	4.53
MDPV	2.87	2.27	0.28	1.56	1.21	-1.19	5.09	7.25	2.09
Bupropion	1.09	0.25	-1.56	-2.60	-4.56	-3.18	-0.87	0.57	-2.92

Table 6 Urine Bias

Bias (% Bias, n=15)									
	Urine Source 1			Urine Source 2			Urine Source 3		
	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL
Methcathinone	-3.73	-5.51	-9.51	-5.51	-7.06	-8.69	-12.27	-6.19	-9.72
Pseudoephedrine	1.29	1.81	0.10	2.51	3.91	2.99	1.31	3.59	4.08
Methylone	1.56	0.89	-0.35	-1.00	2.68	0.93	-1.09	2.31	0.10
Amphetamine	-2.27	-1.01	-1.64	4.22	2.51	2.26	1.40	1.89	1.90
MDA	2.71	-1.24	-2.64	3.73	-1.62	-3.09	4.44	2.64	0.29
Methamphetamine	2.18	-1.31	-1.24	6.11	-0.75	0.005	3.69	1.11	-0.05
Methedrone	1.16	1.54	-0.97	4.98	0.01	-2.52	3.04	2.07	-1.30
MDMA	-0.29	0.50	-0.10	0.76	2.76	2.46	1.71	3.62	3.11
Phentermine	-13.16	-2.45	-2.07	-10.80	-3.79	-0.97	0.87	3.86	1.90
Mephedrone	0.64	0.91	0.19	2.67	2.95	1.12	0.38	2.62	1.26
Alpha-PVP	1.56	3.58	2.33	2.89	4.79	4.42	5.38	8.27	6.48
MDPV	2.20	4.49	3.53	2.56	5.06	3.88	5.49	8.93	6.54
Bupropion	1.62	1.41	0.15	-0.51	-0.37	0.96	0.29	1.80	0.43

Table 7 Liver Bias

Bias (% Bias, n=15)									
	Liver Source 1			Liver Source 2			Liver Source 3		
	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL
Methcathinone	-2.67	-1.86	-4.16	-7.76	-4.45	-5.69	-18.89	-15.51	-15.14
Pseudoephedrine	0.98	-0.21	-3.20	1.87	2.57	0.21	2.33	2.91	0.79
Methylone	1.24	3.10	-0.42	-2.24	2.58	0.42	-0.47	4.56	1.95
Amphetamine	-1.18	-0.84	-3.27	0.24	2.03	0.76	2.73	1.91	0.83
MDA	2.07	2.89	-0.55	2.22	0.26	-2.45	6.16	4.26	2.02
Methamphetamine	1.71	1.20	-1.55	4.84	0.97	-0.62	6.13	3.76	2.57
Methedrone	3.69	2.45	-1.47	0.91	-1.09	-2.93	-9.56	-11.02	-11.14
MDMA	0.82	2.27	-0.51	2.96	2.77	1.14	3.33	5.36	3.09
Phentermine	9.38	4.69	1.04	5.78	-1.56	-1.09	16.93	8.42	5.66
Mephedrone	0.69	1.64	-0.98	2.20	3.42	0.45	2.13	4.30	1.82
Alpha-PVP	2.27	6.10	2.72	3.98	5.34	2.63	7.62	10.84	6.77
MDPV	-1.91	2.86	-0.50	3.80	5.13	3.14	8.27	11.93	8.37
Bupropion	0.89	2.11	-1.74	-1.82	-1.89	-1.16	-0.58	1.20	-0.22

All matrix sources for all matrix types had bias values within the predetermined acceptance criterion of $\pm 20\%$ for the target compounds. During the bias evaluation, qualifier ion ratio failures for bupropion-D₉ were noted with one antemortem blood matrix source (source 2) and one urine matrix source (source 2). The qualifier ratio for bupropion-D₉ was on average between 5-7%. This enabled the adjustment of the acceptance criterion for qualifier ratio acceptance from $\pm 20\%$ to $\pm 30\%$ in accordance with ANSI/ASB Standard 098, *Standard for Mass Spectral Analysis in Forensic Toxicology*. With the expansion of the acceptance criterion, the bupropion-D₉ qualifier ion ratios passed for the two matrix sources.

a. Within-run Precision

The within-run precision was assessed utilizing the same individually fortified samples as in the bias evaluation across all matrix types (blank blood, antemortem blood, postmortem blood, urine, and liver) and sources. The three concentrations evaluated included 30 ng/mL, 800 ng/mL and 1600 ng/mL.

Given the instability of the target compounds, pooled samples were not prepared for analysis, rather individually fortified samples were prepared by fortifying matrix at the respective concentrations with 1.0 mL aliquots of matrix. Individually fortified matrix samples were then extracted prior to quantitative analysis. Within-run precision was assessed using Equation 2.

Equation 2

$$\text{Within-run Precision (\%CV)} = \left(\frac{\text{Standard Deviation of Batch Mean}}{\text{Calculated Mean of Batch}} \right) \times 100$$

The acceptance criterion for within-run precision was $\leq 20\%$ for the coefficient of variation (%CV) at each concentration level. The within run-precision for all matrix types and matrix sources is represented in Tables 8-12. The largest %CV observed was utilized for each matrix type at each concentration. All matrix types for all matrix sources had within-run precision (%CV) within the predetermined acceptance criterion of $\leq 20\%$ for the target compounds.

Table 8 Blank Blood Within-Run Precision

Within-Run Precision									
Mean±Standard Deviation (%CV), n=3									
	Blank Blood Source 1			Blank Blood Source 2			Blank Blood Source 3		
	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL
Methcathinone	31±1(2)	800±18(2)	1489±72(5)	31±1(2)	809±19(2)	1666±53(3)	30±1(3)	841±144(17)	1459±48(3)
Pseudoephedrine	30±1(4)	829±15(2)	1643±25(2)	30±1(3)	816±14(2)	1631±46(3)	29±1(2)	839±134(16)	1598±65(4)
Methylone	31±2(6)	769±10(2)	1580±10(1)	29±1(3)	810±8(1)	1659±31(2)	29±1(3)	839±128(15)	1561±57(4)
Amphetamine	31±1(3)	825±23(3)	1646±37(2)	31±1(4)	803±8(1)	1588±52(3)	30±1(2)	843±128(15)	1622±113(7)
MDA	31±2(5)	807±10(1)	1547±28(2)	29±1(5)	806±18(2)	1633±48(3)	31±1(2)	847±134(16)	1587±58(4)
Methamphetamine	31±1(3)	794±18(2)	1577±43(3)	31±1(3)	787±19(2)	1629±48(3)	30±1(4)	831±128(15)	1509±58(4)
Methedrone	30±1(3)	812±13(2)	1612±38(2)	30±1(5)	825±27(3)	1581±52(3)	31±2(7)	825±127(15)	1388±49(4)
MDMA	31±1(5)	806±22(3)	1605±23(2)	30±2(5)	818±13(2)	1573±28(2)	29±1(3)	814±132(16)	1626±60(4)
Phentermine	30±1(3)	923±30(3)	1648±124(8)	34±2(6)	782±25(3)	1648±46(3)	29±1(3)	837±128(15)	1499±67(5)
Mephedrone	31±1(3)	794±21(3)	1580±28(2)	31±1(3)	813±19(2)	1603±13(1)	30±1(3)	840±131(16)	1530±53(4)
Alpha-PVP	30±1(3)	822±11(1)	1589±34(2)	31±1(4)	816±17(2)	1634±37(2)	29±3(11)	832±123(15)	1580±69(4)
MDPV	31±2(6)	816±10(2)	1586±19(1)	30±3(10)	802±16(2)	1601±31(2)	29±1(3)	844±130(15)	1599±73(5)
Bupropion	31±1(2)	811±16(2)	1581±27(2)	31±1(3)	839±15(2)	1585±22(1)	30±1(4)	829±125(15)	1513±69(5)

Table 9 Antemortem Blood Within-Run Precision

Within-Run Precision									
Mean±Standard Deviation (%CV), n=3									
	Antemortem Blood Source 1			Antemortem Blood Source 2			Antemortem Blood Source 3		
	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL
Methcathinone	31±3(11)	757±55(7)	1559±65(4)	30±2(7)	754±33(4)	1565±66(4)	30±1(4)	834±35(4)	1560±50(3)
Pseudoephedrine	31±4(13)	772±55(7)	1645±30(2)	32±3(8)	808±20(3)	1637±58(4)	30±1(2)	811±13(2)	1562±47(3)
Methylone	31±3(10)	777±62(8)	1635±63(4)	31±2(6)	803±36(4)	1646±48(3)	31±2(5)	865±22(3)	1607±37(2)
Amphetamine	31±4(12)	749±47(6)	1465±60(4)	32±3(9)	816±26(3)	1592±85(5)	30±1(3)	784±20(3)	1563±51(3)
MDA	32±4(13)	773±57(7)	1652±64(4)	32±2(6)	803±37(5)	1648±81(5)	30±1(3)	819±35(4)	1685±52(3)
Methamphetamine	32±4(13)	769±58(8)	1537±50(3)	32±2(7)	780±34(4)	1630±70(4)	33±1(4)	820±23(3)	1545±70(5)
Methedrone	32±4(14)	776±57(7)	1669±49(3)	31±2(7)	822±34(4)	1675±87(5)	27±1(4)	823±2(6)	1613±40(2)
MDMA	31±3(11)	790±63(8)	1565±57(4)	31±2(6)	779±36(5)	1629±53(3)	30±1(4)	766±17(2)	1653±65(4)
Phentermine	31±5(17)	758±67(9)	1554±65(4)	29±3(10)	791±24(3)	1588±79(5)	30±3(9)	833±30(4)	1594±75(5)
Mephedrone	31±4(13)	771±58(8)	1590±59(4)	31±2(8)	812±30(4)	1616±51(3)	31±1(4)	808±15(2)	1603±42(3)
Alpha-PVP	31±4(14)	752±56(7)	1442±68(5)	30±2(7)	776±38(5)	1536±74(5)	32±1(4)	817±44(5)	1638±83(5)
MDPV	31±4(14)	760±55(7)	1571±109(7)	31±2(7)	789±47(6)	1577±59(4)	31±2(6)	789±48(6)	1686±137(8)
Bupropion	31±4(13)	768±57(8)	1471±93(6)	30±2(7)	771±38(5)	1604±86(5)	30±1(2)	816±36(4)	1435±50(4)

Table 10 Postmortem Blood Within-Run Precision

Within-Run Precision Mean±Standard Deviation (%CV), n=3									
	Postmortem Blood Source 1			Postmortem Blood Source 2			Postmortem Blood Source 3		
	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL
Methcathinone	27±2(8)	806±32(4)	1529±106(7)	31±1(3)	719±60(8)	1454±57(4)	29±2(6)	771±29(4)	1510±45(3)
Pseudoephedrine	29±1(4)	834±16(2)	1599±33(2)	31±1(2)	782±70(9)	1619±28(2)	32±1(3)	828±12(1)	1660±27(2)
Methylone	31±1(2)	830±20(2)	1579±36(2)	30±1(2)	768±72(9)	1625±14(1)	30±1(4)	827±23(3)	1637±40(3)
Amphetamine	28±1(5)	828±23(3)	1593±42(3)	32±1(3)	780±83(11)	1583±31(2)	30±1(4)	829±22(3)	1640±58(4)
MDA	31±1(2)	798±17(2)	1568±34(2)	32±1(2)	748±66(9)	1574±34(2)	33±2(5)	820±21(3)	1580±34(2)
Methamphetamine	31±1(2)	814±25(3)	1565±67(4)	33±2(5)	769±80(10)	1606±35(2)	33±1(2)	768±15(2)	1634±42(3)
Methedrone	30±2(6)	852±25(3)	1658±23(1)	30±1(2)	753±62(8)	1498±28(2)	31±2(7)	812±43(5)	1642±49(3)
MDMA	30±1(5)	777±13(2)	1584±32(2)	30±2(6)	760±73(10)	1585±27(2)	31±1(5)	859±13(2)	1581±22(1)
Phentermine	31±2(6)	798±31(4)	1570±31(2)	32±1(3)	760±85(11)	1540±44(3)	31±1(2)	821±34(4)	1532±52(3)
Mephedrone	31±1(2)	785±18(2)	1585±43(3)	31±1(3)	782±75(10)	1588±28(2)	31±1(2)	825±13(2)	1675±32(2)
Alpha-PVP	31±1(2)	843±24(3)	1598±32(2)	32±1(3)	777±90(12)	1596±45(3)	33±1(3)	880±15(2)	1620±40(3)
MDPV	31±1(3)	768±16(2)	1649±46(3)	30±1(3)	795±78(10)	1560±48(3)	31±1(4)	878±13(2)	1641±34(2)
Bupropion	30±1(3)	815±36(4)	1556±47(3)	30±1(2)	742±7(10)	1578±46(3)	30±1(2)	808±17(2)	1557±40(3)

Table 11 Urine Within-Run Precision

Within-Run Precision Mean±Standard Deviation (%CV), n=3									
	Urine Source 1			Urine Source 2			Urine Source 3		
	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL
Methcathinone	29±1(3)	705±41(6)	1367±72(5)	29±1(2)	770±18(2)	1501±219(14)	27±1(5)	763±21(3)	1509±28(2)
Pseudoephedrine	31±1(2)	792±61(8)	1561±29(2)	30±1(1)	820±13(2)	1715±188(11)	31±1(2)	851±16(2)	1667±45(3)
Methylone	31±1(2)	776±64(8)	1611±23(1)	30±1(2)	802±10(1)	1608±199(12)	29±1(4)	825±18(2)	1591±22(1)
Amphetamine	28±1(3)	772±68(9)	1509±45(3)	31±1(4)	814±28(3)	1738±206(12)	30±1(2)	822±7(1)	1724±35(2)
MDA	32±1(3)	773±61(8)	1541±45(3)	31±1(2)	754±21(3)	1524±189(12)	31±1(3)	816±21(3)	1651±45(3)
Methamphetamine	31±1(4)	747±74(10)	1540±47(3)	34±1(3)	801±21(3)	1658±194(12)	31±1(5)	804±15(2)	1595±43(3)
Methedrone	30±1(3)	761±59(8)	1592±37(2)	29±1(3)	765±16(2)	1578±223(14)	30±1(3)	819±28(3)	1573±34(2)
MDMA	30±1(2)	774±56(7)	1594±39(3)	31±2(5)	795±19(2)	1696±220(13)	31±1(5)	850±12(1)	1616±35(2)
Phentermine	25±3(12)	787±25(3)	1534±71(5)	25±2(7)	740±40(5)	1648±195(12)	30±3(11)	835±46(6)	1637±72(4)
Mephedrone	30±1(2)	776±60(8)	1559±42(3)	32±1(3)	813±10(1)	1659±214(13)	30±1(3)	825±11(1)	1623±40(3)
Alpha-PVP	30±1(2)	792±66(8)	1616±44(3)	30±1(2)	836±8(1)	1696±237(14)	32±1(2)	866±11(1)	1737±40(2)
MDPV	31±1(2)	791±56(7)	1665±45(3)	30±1(2)	847±11(1)	1707±209(12)	32±1(2)	871±12(1)	1699±28(2)
Bupropion	30±1(2)	796±54(7)	1592±41(3)	29±1(3)	819±11(2)	1704±10(13)	31±1(1)	817±16(2)	1591±23(1)

Table 12 Liver Within-Run Precision

Within-Run Precision Mean±Standard Deviation (%CV), n=3									
	Liver Source 1			Liver Source 2			Liver Source 3		
	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL
Methcathinone	29±5(17)	843±102(12)	1509±74(5)	28±1(5)	789±20(3)	1494±106(7)	24±1(4)	673±18(3)	1437±30(2)
Pseudoephedrine	30±5(16)	850±103(12)	1510±81(5)	30±2(6)	798±19(2)	1548±92(6)	31±1(1)	831±12(1)	1645±36(2)
Methylone	30±6(18)	863±112(13)	1554±97(6)	30±2(5)	842±12(1)	1603±98(6)	31±1(2)	831±8(1)	1639±21(1)
Amphetamine	30±5(18)	819±108(13)	1502±80(5)	29±2(8)	799±12(2)	1559±81(5)	31±1(5)	799±56(7)	1589±46(3)
MDA	30±5(18)	838±102(12)	1485±100(7)	30±1(3)	778±15(2)	1608±208(13)	32±1(3)	848±13(2)	1615±33(2)
Methamphetamine	30±6(19)	835±91(11)	1513±94(6)	30±1(4)	804±22(3)	1574±142(9)	33±1(4)	842±17(2)	1594±47(3)
Methedrone	31±6(18)	856±110(13)	1544±87(6)	30±1(2)	818±18(2)	1600±141(9)	28±1(3)	653±26(4)	1420±30(2)
MDMA	31±4(12)	858±90(10)	1559±97(6)	30±2(7)	785±35(4)	1561±87(6)	30±1(3)	831±16(2)	1650±67(4)
Phentermine	33±1(3)	834±108(13)	1724±226(13)	33±2(5)	757±37(5)	1615±240(15)	35±1(4)	921±20(3)	1723±51(4)
Mephedrone	30±6(19)	825±108(13)	1540±92(6)	30±1(4)	817±13(2)	1573±102(7)	30±1(3)	830±9(1)	1620±18(1)
Alpha-PVP	31±5(17)	873±114(13)	1605±95(6)	31±1(3)	816±14(2)	1618±100(6)	32±1(1)	899±14(2)	1698±39(2)
MDPV	30±6(19)	855±111(13)	1678±175(10)	31±2(5)	837±17(2)	1614±110(7)	33±1(4)	885±16(2)	1774±30(2)
Bupropion	31±5(18)	846±112(13)	1522±89(6)	30±2(7)	803±19(2)	1580±120(8)	30±1(3)	789±13(2)	1608±13(1)

During the within-run precision evaluation, qualifier ion ratio failures for bupropion-D₉ were noted with one antemortem blood matrix source (source 2) and one urine matrix source (source 2). The qualifier ratio for bupropion-D₉ was on average between 5-7%. This enabled the adjustment of the acceptance criterion for qualifier ratio acceptance from ±20% to ±30% in accordance with ANSI/ASB Standard 098, *Standard for Mass Spectral Analysis in Forensic Toxicology*. With the expansion of the acceptance criterion, the bupropion-D₉ qualifier ion ratios passed for the two matrix sources.

b. Intermediate Precision

Intermediate precision was assessed by utilizing the same individually fortified samples as in the bias evaluation across all matrix types (blank blood, antemortem blood, postmortem blood, urine, and liver) and sources. The three concentrations evaluated included 30 ng/mL, 800 ng/mL and 1600 ng/mL. Intermediate precision was assessed using Equation 3.

Equation 3

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

The acceptance criterion for intermediate precision was ≤20% for the %CV at each concentration level. The within intermediate precision data is represented in Tables 13-17.

Table 13 Blank Blood Intermediate Precision

Intermediate Precision									
Mean±Standard Deviation (%CV), n=15									
	Blank Blood Source 1			Blank Blood Source 2			Blank Blood Source 3		
	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL
Methcathinone	30±1(1)	791±19(2)	1483±55(4)	30±1(2)	831±23(3)	1579±57(4)	30±1(3)	810±59(7)	1567±66(4)
Pseudoephedrine	30±1(2)	814±23(3)	1623±25(2)	30±1(3)	819±9(1)	1598±31(2)	30±1(2)	841±55(7)	1623±41(3)
Methylone	30±1(3)	804±20(2)	1582±16(1)	29±1(3)	822±12(1)	1618±35(2)	29±1(2)	828±52(6)	1601±35(2)
Amphetamine	30±1(2)	799±20(3)	1605±46(3)	30±1(3)	806±10(1)	1584±39(2)	30±1(2)	820±53(6)	1582±81(5)
MDA	31±1(3)	795±13(2)	1568±22(1)	31±1(4)	807±15(2)	1590±46(3)	31±1(2)	834±58(7)	1607±43(3)
Methamphetamine	31±1(3)	795±19(3)	1608±39(2)	31±1(3)	803±17(2)	1593±39(2)	30±1(3)	804±55(7)	1548±81(5)
Methedrone	30±1(2)	819±17(2)	1604±34(2)	30±1(4)	830±26(3)	1608±41(3)	30±1(5)	796±59(7)	1543±98(6)
MDMA	30±1(2)	802±18(2)	1600±26(2)	30±1(4)	815±14(2)	1593±33(2)	30±1(2)	834±57(7)	1611±46(3)
Phentermine	31±1(4)	831±52(6)	1624±82(5)	31±2(7)	805±25(3)	1616±36(2)	30±1(3)	819±52(6)	1567±86(5)
Mephedrone	30±1(2)	808±20(2)	1602±31(2)	31±1(2)	819±10(1)	1591±23(1)	30±1(1)	830±52(6)	1609±55(3)
Alpha-PVP	30±1(2)	806±22(3)	1594±33(2)	30±1(4)	822±11(1)	1598±33(2)	30±2(5)	834±51(6)	1610±45(3)
MDPV	30±1(3)	810±18(2)	1600±18(1)	30±1(5)	818±14(2)	1603±23(1)	30±1(3)	840±63(8)	1619±48(3)
Bupropion	30±1(2)	801±16(2)	1598±34(2)	30±1(2)	813±18(2)	1580±22(1)	30±1(2)	817±50(6)	1588±56(4)

Table 14 Antemortem Blood Intermediate Precision

Intermediate Precision									
Mean±Standard Deviation (%CV), n=15									
	Antemortem Blood Source 1			Antemortem Blood Source 2			Antemortem Blood Source 3		
	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL
Methcathinone	29±2(7)	779±44(6)	1538±45(3)	29±2(8)	785±36(5)	1563±71(5)	29±1(3)	817±26(3)	1534±41(3)
Pseudoephedrine	31±2(7)	797±28(3)	1609±36(2)	31±1(4)	819±14(2)	1626±56(3)	31±1(2)	827±32(4)	1618±55(3)
Methylone	31±2(6)	803±33(4)	1611±42(3)	30±1(3)	829±26(3)	1624±37(2)	30±1(4)	830±22(3)	1601±37(2)
Amphetamine	30±2(8)	781±29(4)	1536±101(7)	31±2(6)	802±18(2)	1594±76(5)	30±1(3)	798±27(3)	1577±63(4)
MDA	31±2(7)	804±33(4)	1604±54(3)	31±1(4)	806±20(2)	1582±71(5)	32±1(4)	833±36(4)	1609±66(4)
Methamphetamine	31±2(6)	791±38(5)	1558±55(4)	32±2(6)	798±22(3)	1599±64(4)	31±1(4)	809±25(3)	1564±41(3)
Methedrone	29±3(10)	801±40(5)	1637±51(3)	31±1(5)	819±27(3)	1634±55(3)	30±1(5)	835±40(5)	1625±41(3)
MDMA	30±2(7)	798±29(4)	1580±38(2)	31±1(4)	807±26(3)	1611±42(3)	30±1(3)	814±39(5)	1596±59(4)
Phentermine	29±3(11)	808±49(6)	1581±70(4)	29±3(9)	800±18(2)	1622±53(3)	31±2(6)	832±34(4)	1625±57(4)
Mephedrone	30±2(6)	796±32(4)	1588±43(3)	31±1(4)	819±17(2)	1611±34(2)	30±1(2)	820±22(3)	1605±36(2)
Alpha-PVP	30±2(7)	774±33(4)	1520±70(5)	30±1(5)	787±29(4)	1547±56(4)	30±1(4)	831±43(5)	1551±89(6)
MDPV	30±2(7)	784±33(4)	1540±76(5)	30±1(3)	780±32(4)	1546±62(4)	30±1(5)	824±54(7)	1559±119(8)
Bupropion	31±2(7)	802±34(4)	1543±73(5)	30±1(3)	788±26(3)	1564±86(6)	30±1(2)	825±30(4)	1534±69(4)

Table 15 Postmortem Blood Intermediate Precision

Intermediate Precision									
Mean±Standard Deviation (%CV), n=15									
	Postmortem Blood Source 1			Postmortem Blood Source 2			Postmortem Blood Source 3		
	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL
Methcathinone	28±1(4)	784±42(5)	1527±74(5)	28±2(7)	742±50(7)	1488±78(5)	29±1(4)	779±20(3)	1518±46(3)
Pseudoephedrine	30±1(3)	816±23(3)	1618±26(2)	30±1(2)	815±33(4)	1610±25(2)	30±1(3)	838±10(1)	1652±32(2)
Methylone	30±1(2)	812±21(3)	1591±24(2)	30±1(3)	812±39(5)	1616±25(2)	30±1(2)	824±11(1)	1624±23(1)
Amphetamine	29±1(4)	812±22(3)	1608±33(2)	31±1(2)	808±36(4)	1588±20(1)	30±1(3)	821±15(2)	1600±48(3)
MDA	31±1(2)	800±19(2)	1559±23(1)	31±1(3)	787±41(5)	1580±37(2)	32±1(3)	820±21(3)	1608±37(2)
Methamphetamine	31±1(2)	803±25(3)	1603±48(3)	31±1(4)	788±33(4)	1588±34(2)	32±1(3)	808±24(3)	1578±64(4)
Methedrone	30±1(3)	826±38(5)	1620±28(2)	31±2(5)	771±38(5)	1579±59(4)	31±1(5)	802±34(4)	1595±73(5)
MDMA	30±1(3)	814±22(3)	1610±25(2)	31±1(4)	802±42(5)	1594±25(2)	31±1(2)	834±17(2)	1636±37(2)
Phentermine	30±1(4)	831±58(7)	1649±70(4)	31±1(5)	775±41(5)	1579±37(2)	30±1(3)	811±23(3)	1606±127(8)
Mephedrone	30±1(2)	823±22(3)	1607±31(2)	30±1(2)	814±34(4)	1603±19(1)	30±1(2)	827±8(1)	1634±32(2)
Alpha-PVP	31±1(2)	824±28(3)	1612±29(2)	31±1(4)	811±42(5)	1596±31(2)	32±1(3)	869±14(2)	1673±39(2)
MDPV	31±1(2)	818±30(4)	1605±37(2)	30±1(3)	810±42(5)	1581±35(2)	32±1(3)	858±16(2)	1634±58(4)
Bupropion	30±1(2)	802±28(3)	1575±33(2)	29±1(4)	764±33(4)	1549±33(2)	30±1(2)	805±16(2)	1553±23(1)

Table 16 Urine Intermediate Precision

Intermediate Precision									
Mean±Standard Deviation (%CV), n=15									
	Urine Source 1			Urine Source 2			Urine Source 3		
	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL
Methcathinone	29±1(4)	756±38(5)	1448±72(5)	28±2(6)	744±35(5)	1461±108(7)	26±1(4)	752±15(2)	1445±47(3)
Pseudoephedrine	30±1(2)	814±29(4)	1602±40(3)	31±1(2)	831±12(1)	1648±93(6)	30±1(2)	829±17(2)	1665±38(2)
Methylone	30±1(2)	807±29(4)	1594±17(1)	30±1(3)	821±18(2)	1615±95(6)	30±1(2)	819±11(1)	1602±19(1)
Amphetamine	29±1(4)	792±33(4)	1574±63(4)	31±1(4)	820±22(3)	1636±107(7)	30±1(1)	815±11(1)	1630±74(5)
MDA	31±1(2)	790±26(3)	1558±40(3)	31±1(2)	787±23(3)	1550±105(7)	31±1(3)	821±23(3)	1605±36(2)
Methamphetamine	31±1(4)	790±37(5)	1580±55(3)	32±1(4)	794±16(2)	1600±96(6)	31±1(3)	809±18(2)	1599±66(4)
Methedrone	30±2(5)	812±39(5)	1584±36(2)	31±2(5)	800±29(4)	1560±113(7)	31±2(5)	817±30(4)	1579±96(6)
MDMA	30±1(2)	804±27(3)	1598±31(2)	30±1(3)	822±19(2)	1639±105(6)	31±1(3)	829±16(2)	1650±28(2)
Phentermine	26±3(11)	780±28(4)	1567±62(4)	27±2(7)	770±32(4)	1585±99(6)	30±3(10)	831±26(3)	1630±70(4)
Mephedrone	30±1(2)	807±31(4)	1603±47(3)	31±1(2)	824±8(1)	1618±99(6)	30±1(2)	821±9(1)	1620±41(3)
Alpha-PVP	30±1(2)	829±35(4)	1637±34(2)	31±1(4)	838±13(2)	1671±103(6)	32±1(3)	866±9(1)	1704±28(2)
MDPV	31±1(2)	836±33(4)	1656±33(2)	31±1(2)	841±15(2)	1662±102(6)	32±1(2)	871±14(2)	1705±40(2)
Bupropion	30±1(2)	811±25(3)	1602±33(2)	30±1(3)	797±17(2)	1615±114(7)	30±1(2)	814±13(2)	1609±37(2)

Table 17 Liver Intermediate Precision

Intermediate Precision Mean±Standard Deviation (%CV), n=15									
	Liver Source 1			Liver Source 2			Liver Source 3		
	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL	30 ng/mL	800 ng/mL	1600 ng/mL
Methcathinone	29±2(8)	785±53(7)	1533±82(5)	28±3(9)	764±39(5)	1509±75(5)	24±1(4)	676±22(3)	1358±47(3)
Pseudoephedrine	30±2(7)	798±50(6)	1549±54(4)	31±1(3)	821±17(2)	1603±51(3)	30±1(2)	823±13(2)	1613±29(2)
Methylone	30±2(7)	825±49(6)	1593±53(3)	29±1(3)	821±17(2)	1607±44(3)	30±1(2)	836±8(1)	1631±22(1)
Amphetamine	30±2(8)	793±48(6)	1547±71(5)	30±2(6)	816±16(2)	1612±66(4)	31±1(3)	815±26(3)	1613±42(3)
MDA	31±2(7)	823±42(5)	1591±74(5)	31±1(2)	802±19(2)	1561±90(6)	32±1(3)	834±22(3)	1632±29(2)
Methamphetamine	31±2(7)	810±42(5)	1575±82(5)	31±1(4)	808±16(2)	1590±69(4)	32±1(3)	830±23(3)	1641±41(3)
Methedrone	31±2(8)	820±49(6)	1577±56(4)	30±2(6)	791±34(4)	1553±76(5)	27±1(5)	712±43(6)	1422±73(5)
MDMA	30±2(5)	818±45(6)	1592±56(4)	31±1(4)	822±26(3)	1618±55(3)	31±1(3)	843±15(2)	1649±37(2)
Phentermine	33±2(5)	838±47(6)	1617±117(7)	32±1(4)	788±36(5)	1583±100(6)	35±1(3)	867±40(5)	1691±79(5)
Mephedrone	30±2(7)	813±43(5)	1584±64(4)	31±1(3)	827±14(2)	1607±49(3)	31±1(1)	834±9(1)	1629±30(2)
Alpha-PVP	31±2(7)	849±49(6)	1644±57(3)	31±1(4)	843±20(2)	1642±43(3)	32±1(3)	887±11(1)	1708±32(2)
MDPV	29±2(8)	823±47(6)	1592±99(6)	31±1(3)	841±19(2)	1650±59(4)	32±1(3)	895±25(3)	1734±44(3)
Bupropion	30±2(7)	817±46(6)	1572±60(4)	29±1(3)	785±19(2)	1581±68(4)	30±1(3)	810±13(2)	1597±35(2)

All matrix types for all matrix sources had an intermediate precision (%CV) within the predetermined acceptance criterion of ≤20% for the target compounds.

2. Sensitivity

a. Estimated Limit of Detection

The estimated limit of detection (LOD) for this validation was defined as an administratively defined decision point (threshold concentration). The limit of detection was evaluated on all instrumentation models and is understood to be an estimate based on the condition of the instruments at the time of the evaluation. The lowest calibrator concentration within the method was 10 ng/mL with a threshold concentration of 5 ng/mL.

Nine blank matrix sources per matrix type were analyzed in triplicate across three batch analyses. The number of blank matrix sources was tripled to demonstrate that all identification criteria were achieved even in the presence of ionization suppression. The peak shape, retention time, qualifier ratio, and signal to noise ratio were evaluated for each compound at each concentration. The predetermined identification criteria included a retention time within ±3%, a qualifier ratio within ±20%, and a signal-to-noise ratio ≥3.3. Table 18 lists the matrices and associated number of identification failures.

Table 18 Estimated limit of detection

Estimated Limit of Detection	
Compound	Failures
Methcathinone	1 blank blood signal to noise
Pseudoephedrine	None
Methylone	1 liver qualifier ion
Amphetamine	1 urine qualifier ion
MDA	1 blank blood qualifier ion, 1 liver qualifier ion
Methamphetamine	None
Methedrone	1 blank blood qualifier ion
MDMA	1 liver qualifier ion
Phentermine	None
Mephedrone	None
Alpha-PVP	None
MDPV	None
Bupropion	None

For the determination of estimated limit of detection, at minimum, 95% of threshold samples had to meet the predetermined identification criteria. The estimated limit of detection for all target compounds was determined to be 5 ng/mL, with all compounds having $\geq 95\%$ replicate passing at the threshold concentration.

b. Lower Limit of Quantitation

The lower limit of quantitation for this validation was established by evaluating the lowest non-zero calibrator (10 ng/mL) for the method. For each matrix type, nine blank matrix sources were fortified at 10 ng/mL in triplicate and analyzed over three batch analyses. The replicates were utilized to demonstrate that all detection, identification, and bias criteria were met even in the presence of ionization suppression.

Predetermined acceptance criteria:

Retention Time: $\pm 3\%$
 Qualifier Ratio: $\pm 20\%$
 Signal-to-Noise: ≥ 10
 Back Calculated Concentration: $\pm 30\%$

In addition to the predetermined acceptance criteria, chromatographic peak shape was also monitored. It was noted that some compounds did not meet the originally specified predetermined acceptance criteria for a back calculated concentration within $\pm 20\%$. The Toxicology Procedures Manual (Section 2.4.1.2, Qualtrax Revision 34) expands the acceptance criteria for the lower limit of quantitation to $\pm 30\%$. Therefore, samples were evaluated against this criterion. Table 19 lists the quantitation failures noted for each matrix type.

Table 19 Lower limit of quantitation

Lower Limit of Quantitation	
Compound	Failures
Methcathinone	None
Pseudoephedrine	1 postmortem blood accuracy, 1 urine signal to noise, 1 liver signal to noise, 1 liver accuracy
Methylone	1 antemortem blood signal to noise, 1 liver signal to noise
Amphetamine	1 urine signal to noise, 1 liver signal to noise
MDA	1 postmortem blood signal to noise
Methamphetamine	None
Methedrone	1 liver accuracy
MDMA	1 liver signal to noise
Phentermine	1 blank blood accuracy, 1 postmortem blood accuracy, 1 urine signal to noise and accuracy, 1 liver accuracy
Mephedrone	None
Alpha-PVP	None
MDPV	None
Bupropion	None

Although failures were noted, all matrix types had a 96% or greater passing rate except for pseudoephedrine in liver. Upon evaluation of pseudoephedrine in liver, two failures were noted including one signal to noise failure and one qualifier ion ratio failure. Given these failures, the number of replicates increased from 27 to 45 samples for pseudoephedrine. With these additional replicates, pseudoephedrine in liver had a 95.5% passing rate which meets the predetermined acceptance criteria of $\geq 95\%$ passing rate.

3. Endogenous Interferences

To evaluate samples for endogenous interferences, a total of ten matrix sources per matrix type (blank blood, antemortem blood, postmortem blood, liver and urine) were extracted and evaluated without the addition of internal standard. The samples were evaluated for the presence of instrumental response for the analytes and internal standard. No endogenous interferences were identified.

4. Frozen Sample Stability

The length of time a sample remains frozen was assessed for each matrix type at 10, 20, 40, and 60 minute time intervals after being placed in the -25°C circulating bath for 3 minutes. The validation plan noted an additional time interval of 1 minute which was subsequently discarded given the clear stability at the later time intervals. A total of ten matrix sources were evaluated in duplicate. At the designated time interval, the samples were poured into clean test tubes and the integrity of the frozen sample was evaluated.

At the 10-minute time interval, all matrices had acceptable transfers except for one blank blood and two postmortem blood samples which produced unacceptable transfers. During the transfers it was noted that the samples maintained their frozen state and the cleanliness of the transfer was more dependent on the flaking of the top of the frozen matrix. When assessing a 20-minute time interval, the amount of sample thawing increased. Four blank blood samples, two antemortem blood samples, and two urine samples did not have acceptable sample transfers.

As the time interval increased, the amount of sample thawing increased. When evaluating the 40- and 60-minute time points, a significant number of samples thawed and produced unacceptable transfers. At the 40-minute interval, one postmortem blood, five liver samples, and 16 urine samples produced unacceptable transfers. At the 60-minute interval, six liver samples and all urine samples produced unacceptable transfers. In general, as the time interval increased, partial thawing produced a “plug” between the thawing pellet and the supernatant. This often made transfer acceptable preventing the thawed pellet from transferring.

Although instances of poor sample transfer were noted, throughout the method validation, batches of 50 samples were routinely placed in the circulating bath at one time. The samples were then able to be successfully transferred without a time constraint due to thawing issues. It was noted during the validation that at times, urine matrices did not freeze within the 3-minute incubation period. The samples would be placed back into the circulating bath for an additional 3-minute incubation period with successful freezing. If a sample was not frozen initially and an unsuccessful transfer occurred, the sample was placed back into a clean test tube, centrifuged, and placed back into the circulating bath. Upon analysis, these samples produced acceptable analytical results.

5. Summary

The utilization of a circulating bath to freeze samples prior to supernatant transfer was demonstrated to be appropriate for use within the existing Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS method. The bias and precision, estimated limit of detection, and limit of quantitation experiments assumed significant ionization suppression based on the original method validation results. Therefore, the number of matrix sources were tripled for each experiment. When the circulating bath was employed, bias and precision predetermined acceptance criteria were met. Additionally, the estimated limit of detection and lower limit of quantitation were also confirmed to be 5 ng/mL and 10 ng/mL, respectively.

When evaluating the thawing interval, mixed results were obtained. This is due to the variability of each sample matrix. Overall, the addition of a circulating bath provides a rapid alternative to manual supernatant transfers. It was

noted that if samples are not fully frozen or were inadvertently transferred prematurely, additional time in the circulating bath is appropriate.

6. References

Wagner, R. Validation Plan: Utilization of a Circulating Bath in the Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS Method, June 2025.

Virginia Department of Forensic Science Quality Manual, Qualtrax Revision 36, 2026.

Virginia Department of Forensic Science Toxicology Procedures Manual, Qualtrax Revision 34, 2026.

Memo To: James Hutchings, Ph.D., Toxicology Program Manager
From: Rebecca Wagner, Ph.D., Chemistry Research Section Supervisor
CC: Alka Lohmann, Technical Services Director
Date: June 16, 2025
RE: Validation Plan
 Utilization of a Circulating Bath in the Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS Method

Validation Plan – Utilization of a Circulating Bath in the Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS Method

It is proposed to validate the utilization of a recirculating bath within the liquid-liquid extraction procedure for the existing quantitative analysis method for amphetamines, phentermine, and designer stimulants using LCMSMS. The existing method is delineated in the Toxicology Procedures Manual Section 26, Amphetamines, Phentermine and Designer Stimulants Quantitation and Confirmation by LCMSMS method (Qualtrax Revision 32). The validation will be performed using the liquid-liquid extraction delineated in Section 26.6 of the Toxicology Procedures Manual. After centrifugation at approximately 2500 rpm for 15 minutes to achieve separation (Section 26.6.8), the samples will be placed into a PolyScience Refrigerated Circulator (PN AD15R-40A-11B) containing reagent alcohol (Fisher A962R-4). The samples will be incubated in the circulator for 3 minutes at -25°C. After incubation, the supernatant will be poured into clean, screw-cap tubes. Following the transfer, the remainder of the procedure will be followed as published (Section 26.6.10 – 26.6.12). The target analytes and associated internal standards are listed in Table 1.

Table 1 Target compounds and internal standards

Quantitative Targets	Internal Standard
Methcathinone	Mephedrone-D ₃
Pseudoephedrine	Pseudoephedrine-D ₃
Methylone	Methylone-D ₃
Amphetamine	Amphetamine-D ₁₁
Methamphetamine	Methamphetamine-D ₁₁
MDA	MDA-D ₅
Methedrone	Mephedrone-D ₃
MDMA	MDMA-D ₅
Phentermine	Methamphetamine-D ₁₁
Mephedrone	Mephedrone-D ₃
Alpha-PVP	Mephedrone-D ₃
MDPV	Mephedrone-D ₃
Bupropion	Mephedrone-D ₃

The instrumental parameters delineated in the Toxicology Procedures Manual will remain unchanged for the validation. The impact of the circulating bath will be evaluated in blank blood,

antemortem blood, postmortem blood, urine, and liver. Based on the modification to the sample preparation procedure, bias, precision, estimated limit of detection, lower limit of quantitation, and endogenous interferences will be evaluated for their potential impact. Other validation parameters including regression analysis, ionization suppression/enhancement, carryover, interference from internal standard, interference from commonly encountered analytes, dilution integrity, and stability will not be assessed. In addition to the aforementioned parameters, an evaluation of the length of time the bottom layer remains frozen will be assessed in all matrix types.

A validation plan is outlined herein pursuant to the Quality Manual (Qualtrax Revision 32) and Toxicology Procedures Manual (Qualtrax Revision 32). The validation plan is in accordance with the ANSI/ASB Standard 036, Standard Practices for Method Validation in Forensic Toxicology (First Edition, 2019).

1. Bias and Precision
 - a. Bias
 - b. Within-run Precision
 - c. Intermediate Precision
2. Sensitivity
 - a. Estimated Limit of Detection (LOD)
 - b. Lower Limit of Quantitation (LLOQ)
3. Interferences (Endogenous Compounds)
4. Frozen Sample Stability
5. References

1. Bias and Precision

The method is intended for the quantitative analysis of multiple matrices (i.e., blank blood, antemortem blood, postmortem blood, urine, and liver). Bias and precision experiments shall be conducted for all matrix types.

a. Bias

Bias shall be measured using fortified matrix samples. To evaluate bias, a minimum of triplicate determinations per concentration (low, medium, and high) over a total of five batch analyses shall be evaluated. The working range to be delineated within the method is 0.01 mg/L to 2.0 mg/L. The low concentration shall be no more than approximately three times the lowest end of the working range of the method and high concentration shall be within approximately 80% of the highest end of the working range. The low, medium, and high concentrations to be evaluated for bias will be 0.03 mg/L, 0.8 mg/L, and 1.6 mg/L.

The bias of the fortified pooled samples will be assessed using Equation 1.

Equation 1

$$\text{Bias (\%)} \text{ Concentration}_x = \left(\frac{\text{Mean of Calculated Concentration}_x - \text{Expected Concentration}_x}{\text{Expected Concentration}_x} \right) \times 100$$

Bias should be as low as possible but shall not exceed $\pm 20\%$ at each concentration level when analyzing common biological fluids. The same data used in the evaluation of bias shall also be used for the determination of within-run and between-run precision.

b. Within-run Precision

Precision will be expressed as the percent coefficient of variation (%CV). During method validation, within-run precision is measured using pooled fortified matrix samples. A minimum of triplicate determinations per concentration (low, medium, and high) over a total of five batch analyses shall be evaluated. The low concentration shall be no more than approximately three times the lowest end of the working range of the method and high concentration shall be within approximately 80% of the highest end of the working range. The low, medium, and high concentrations to be evaluated for within-run precision will be 0.03 mg/L, 0.8 mg/L, and 1.6 mg/L. The within-run precision shall be calculated using Equation 2.

Equation 2

$$\text{Within - run Precision (\%CV)} = \left(\frac{\text{Standard Deviation of Batch Mean}}{\text{Calculated Mean of Batch}} \right) \times 100$$

The within-run precision for each analytical run will be calculated. The analytical run with the largest within-run precision shall be utilized for the overall within-run precision of the process. Within-run precision shall not exceed $\pm 20\%$ at each concentration level when analyzing common biological fluids.

c. Intermediate Precision

Intermediate precision will be measured using pooled fortified matrix samples. A minimum of triplicate determinations per concentration (as delineated above) over a total of five batch analyses shall be evaluated. The intermediate precision shall be calculated using Equation 3.

Equation 3

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

The intermediate precision will be calculated using the combined data from the multiple analyses over the minimum of five batches. The standard deviation and mean will be calculated to determine the intermediate precision. Intermediate precision shall not exceed $\pm 20\%$ at each concentration level when analyzing common biological fluids.

2. Sensitivity

a. Estimated Limit of Detection (LOD)

The estimated limit of detection for this validation shall be defined as an administratively-defined decision point (threshold concentration). The administratively-defined decision point was established to be 0.005 mg/L within the Toxicology Procedures Manual. Therefore 0.005 mg/L will be maintained as the decision point for reporting analytes within this method although a lower estimated LOD may be analytically achievable.

The decision point shall be evaluated by fortifying, at minimum, nine different blank matrix sources per matrix type (i.e., blank blood, antemortem blood, postmortem blood, urine, and liver). Nine different blank matrix sources shall be analyzed over a minimum of three analyses to demonstrate that all predetermined detection and identification criteria are met.

Predetermined identification criteria:

Retention Time: $\pm 3\%$

Qualifier Ratio: $\pm 20\%$

Signal-to-Noise: ≥ 3.3

NOTE: Based on previous validation experiments for ionization suppression/enhancement. The laboratory assumed significant ionization suppression. Therefore, the number of matrix sources of the estimated limit of detection was tripled from three to nine.

b. Lower Limit of Quantitation (LLOQ)

The lower limit of quantitation for this validation shall be established by evaluating the lowest non-zero calibrator (0.01 mg/L) for the method. For each matrix type (i.e., blank blood, antemortem blood, postmortem blood, urine, and liver), a minimum of nine different blank matrix sources shall be fortified at the lowest calibrator concentration and analyzed over a minimum of three analyses. The replicate results will be utilized to demonstrate that all detection, identification, bias, and precision criteria are met.

Predetermined acceptance criteria:

Retention Time: $\pm 3\%$

Qualifier Ratio: $\pm 20\%$

Signal-to-Noise: ≥ 10

Back Calculated Concentration: $\pm 20\%$

NOTE: Based on previous validation experiments for ionization suppression/enhancement. The laboratory assumed significant ionization suppression. Therefore, the number of matrix sources of the lower limit of quantitation was tripled from three to nine.

3. Interferences (Endogenous Compounds)

To assess for interference, the qualifier and quantifier ions for each analyte and internal standard within the method shall be monitored. Interferences below the limit of detection for the method may be deemed insignificant. If present, the impact on identification and quantitation shall be evaluated. If the instrumental response is less than 10% of the LLOQ response for the qualifier or quantifier ions, the impact is deemed insignificant.

Where possible, a minimum of ten negative matrix samples from different sources without the addition of an internal standard shall be analyzed for possible endogenous interferences. A minimum of ten matrix samples for each matrix type (i.e., blank blood, antemortem blood, postmortem blood, urine, liver) within the validation should be evaluated, whenever possible.

4. Frozen Sample Stability

The length of time a sample remains frozen will be assessed during the validation. This is to ensure that the incubation time and temperature are appropriate to maintain the frozen conditions while transferring several samples consecutively. Samples will be placed into the -25°C circulating bath for 3 minutes. After incubation, samples will be removed and samples will be poured into clean test tubes at 1, 10, 20, 40, and 60 minute intervals. To assess the conditions in multiple matrices, ten matrix sources of blank blood, antemortem blood, postmortem blood, urine, and liver will be evaluated.

Each matrix source will be evaluated in duplicate at each timepoint. After the designated timepoint, the samples will be transferred by pouring into a clean test tube to ensure the integrity of the frozen sample. An appropriate time interval is one in which all matrix sources for all matrix types remains frozen for the designated interval.

5. References

Virginia Department of Forensic Science Quality Manual, Qualtrax Revision 32, **2025**.

Virginia Department of Forensic Science Toxicology Procedures Manual, Qualtrax Revision 32, **2025**.

Memo To: James Hutchings, Ph.D., Toxicology Program Manager
From: Rebecca Wagner, Ph.D., Chemistry Research Section Supervisor
CC: Alka Lohmann, Technical Services Director
Date: October 10, 2025
RE: Validation Plan - Addendum
 Utilization of a Circulating Bath in the Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS Method

Addendum: Validation Plan – Utilization of a Circulating Bath in the Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS Method

During the evaluation of the validation plan by the Toxicology Subcommittee of the Scientific Advisory Committee, it was suggested to increase the number of matrices evaluated for bias and precision experiments. For bias and precision, a total of three matrix sources per matrix type will be evaluated. The matrices include blank blood, antemortem blood, postmortem blood, urine, and liver.

A minimum of triplicate determinations per concentration (low, medium, and high) over a total of five batch analyses shall be evaluated for bias and precision within each matrix source. The low, medium, and high concentrations to be evaluated for bias and precision will be 0.03 mg/L, 0.8 ng/L, and 1.6 mg/L.

In addition to the aforementioned change, additional internal standards will be evaluated with the validation. The internal standards include alpha-PVP-D₈, bupropion-D₉, and MDPV-D₈. In addition to the experiments delineated in the validation plan, experiments will be performed within the Northern Toxicology Section in accordance with the associated validation plan. The following instrumental parameters (Table 1) will be used for the internal standards.

Compound	Precursor (m/z)	Product (m/z)	Approx Retention Time (min)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator (V)
Alpha-PVP-D ₈	240.2	134.1	6	90	24	7
		91		90	24	7
Bupropion-D ₉	249.2	185	6.5	95	8	7
		166.5		95	16	7
MDPV-D ₈	284.2	175	6.2	95	20	7
		134.5		95	28	7

These internal standards will be utilized with their associated target compounds, alpha-PVP, bupropion, and MDPV respectively.