DEPARTMENT OF FORENSIC SCIENCE
METHOD VALIDATION SUMMARY FORM

Section: Toxicology

Method: Addition of Novel Psychoactive Substances to the Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS Method

Results recorded? ☑

Procedure documented? ☑

Method fit for use? ☑

Approved by: James Hutchings Date: 2-10-26

Executive Validation Summary: "Addition of Novel Psychoactive Substances to the Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS Method"

Summary:
The validation of the addition of novel psychoactive substances to the "Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS" utilizing the liquid-liquid extraction sample preparation with a specific acquisition method on the LCMSMS. The biological matrices evaluated include blank blood, antemortem blood, postmortem blood, liver, and urine.

Limitations:
Due to an interferent with pentylone that was observed and identified to be N,N-dimethylpentylone, the method does not pass the predetermined validation acceptance for pentylone and it was removed from the method. Pseudoephedrine and ephedrine produce an instrumental response for methcathinone therefore methcathinone was removed from the method.

Studies Performed:

1. Sensitivity - Estimated Limit of Detection (LOD)
    a. >95% of samples meet RT (±3%), qualifier ratio (±20%), and S/N (≥3.3)
2. Ionization Suppression/Enhancement
    a. >±25% indicates significant suppression/enhancement – must evaluate impact to sensitivity
3. Carryover
    a. No instrumental response >10% of the administratively defined decision point
4. Interferences - No instrumental response >10% of the administratively defined decision point
    a. Endogenous Compounds
    b. Internal Standard
    c. Commonly Encountered Analytes
5. Stability - >±20% from Day 1 response indicates instability

Compound	LOD (mg/L)	Suppression/ Enhancement	Carryover (passed at conc., mg/L)	Interferences	Stability (days)
4-APDB	0.0025	Suppression (low)	0.5	Passed	6
4-chloro-alpha-PVP	0.0025	Passed	0.5	Passed	6
4/**5**/6-MAPB	0.0025	Passed	0.5	Passed	6
5-APDB	0.0025	Suppression (low)	0.5	Passed	6
5-DBFPV	0.0025	Passed	0.5	Passed	6
6-APDB	0.0025	Suppression (low)	0.5	Passed	6
Alpha-PVP	0.0025	Passed	2.0	Passed	6
Dibutylone	0.0025	Passed	0.5	Passed	6
Ethylone	0.0025	Passed	2.0	Passed	6
MDPV	0.0025/ 0.005 (postmortem blood)	Passed	2.0	Passed	6
Mephedrone	0.0025	Passed	2.0	Passed	6
Methedrone	0.0025	Passed	2.0	Passed	6
Methylone	0.0025	Passed	2.0	Passed	6
N-Ethylpentylone/ N,N-Diethylpentylone	0.0025	Passed	0.5	Passed	6
N,N-Dimethylpentylone	0.0025	Passed	0.5	Passed	6
PV8	0.0025/ 0.005 (liver)	Passed	0.5	Passed	6

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 January 4, 2026
RE: Validation Summary
Addition of Novel Psychoactive Substances to the Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS Method

Validation Summary – Addition of Novel Psychoactive Substances to the Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS Method

The validation of adding novel psychoactive substances to the existing Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS method (Section 26, Qualtrax Revision 33) within the Toxicology Procedures Manual is presented here. The validation included the utilization of the existing liquid-liquid extraction sample preparation procedure delineated in Section 26 of the Toxicology Procedures Manual. The acquisition method delineated within the validation plan was utilized to collect the validation data. The target compounds and internal standards for the method are listed in Table 1.

Table 1 Target Compounds and Internal Standards

Target Compounds	Internal Standards
4-APDB	N-Ethylpentylone-D_5
4-chloro-alpha-PVP	N-Ethylpentylone-D_5
4/**5**/6-MAPB	N-Ethylpentylone-D_5
5-APDB	N-Ethylpentylone-D_5
5-DBFPV	N-Ethylpentylone-D_5
6-APDB	N-Ethylpentylone-D_5
Alpha-PVP	Mephedrone-D_3
Dibutylone	N-Ethylpentylone-D_5
Ethylone	Methylone-D_3
MDPV	Mephedrone-D_3
Mephedrone	Mephedrone-D_3
Methcathinone	Mephedrone-D_3
Methedrone	Mephedrone-D_3
Methylone	Methylone-D_3
Pentylone	N-Ethylpentylone-D_5
N-Ethylpentylone/N,N-Diethylpentylone	N-Ethylpentylone-D_5
N,N-Dimethylpentylone	N-Ethylpentylone-D_5
PV8	N-Ethylpentylone-D_5

Note: The target compound for 4/5/6-MAPB will be 5-MAPB. The target compound for N-ethylpentylone/N,N-diethylpentylone will be N-ethylpentylone.

During the validation, an interferent with pentylone was observed and identified to be N,N-dimethylpentylone. As noted in Table 1, both pentylone and N,N-dimethylpentylone were

compounds of interest for the validation. These compounds were chromatographically resolved but the conversion of N,N-dimethylpentylone to pentylone could not be eliminated or minimized to be insignificant. Therefore, the method does not pass the predetermined validation acceptance for pentylone and it was removed from the method including the presentation of data within the validation summary.

The validation included the following:

1. Sensitivity - Estimated Limit of Detection (LOD)
2. Ionization Suppression/Enhancement
3. Carryover
4. Interferences
    a. Endogenous Compounds
    b. Internal Standard
    c. Commonly Encountered Analytes
5. Stability
6. Robustness
7. Summary
8. References

The validation was performed using an Agilent Technologies 6460 and 6470 liquid chromatograph tandem mass spectrometer equipped with an Agilent Technologies Infinity Poroshell EC-C18, 2.1x150 mm, 2.7 µm column. The matrices evaluated during the validation included blank blood, antemortem blood, postmortem blood, liver, and urine. An administratively defined decision point (threshold control) of 0.005 mg/L was employed.

1. Sensitivity - Estimated Limit of Detection (LOD)

The estimated limit of detection for this validation was established as an administratively defined decision point (threshold concentration). The limit of detection is understood to be an estimate based on the condition of the instrument at the time of the evaluation. The administratively established threshold was determined to be 0.005 mg/L for reporting analytes within the method although a lower estimated LOD may be analytically achievable. In addition to the 0.005 mg/L concentration, a lower concentration of 0.0025 mg/L was also assessed.

To evaluate the estimated limit of detection, nine blank matrix sources, per matrix type were utilized. The number of matrix sources was tripled based on the anticipated ionization suppression with the method. The matrix types evaluated included blank blood, antemortem blood, postmortem blood, liver, and urine. Each matrix source was fortified with the compounds of interest over three batch analyses. The retention time, qualifier ratio, signal-to-noise ratio, and peak shape were evaluated for each compound. The predetermined acceptance criteria are defined in Table 2. The predetermined acceptance criteria had to be met for 95% of the samples per matrix type to be considered acceptable.

Table 2 Predetermined Identification Criteria

Estimated Limit of Detection	
Component	Criteria
Retention Time	±3%
Signal-to-Noise Ratio	≥3.3
Qualifier ratio	±20%
Peak Shape	Good

During the evaluation of the data for the estimated limit of detection the number of failures were noted for each matrix type. A total of 27 replicates per matrix type for a total of 135 replicates across all matrix types was assessed. All compounds and all matrix types passed the predetermined acceptance criteria at a concentration of 0.0025 mg/L with the exception of MDPV in postmortem blood and PV8 in liver. All replicates, for all compounds, met the predetermined acceptance criteria for identification at a concentration of 0.005 mg/L.

When assessing MDPV in postmortem blood, two qualifier ratios failed for the 27 replicate samples. Given that only 92.5% of the replicates met the predetermined acceptance criteria, the 0.0025 mg/L concentration did not meet the estimated limit of detection criteria. The 0.005 mg/L concentration met the predetermined acceptance criteria for all replicate analyses.

When assessing PV8 in liver at a concentration of 0.0025 mg/L, three replicates failed the predetermined acceptance criteria. When assessing the 0.005 mg/L concentration, all replicates passed the predetermined acceptance criteria.

All compounds, in all matrix types, passed the predetermined acceptance criteria at a concentration of 0.005 mg/L which was the administratively defined decision point (threshold concentration).

2. Ionization Suppression/Enhancement

Ionization suppression and enhancement was evaluated by assessing the instrumental response of post-extraction fortified samples to neat standards. Post-extraction fortified samples were prepared from blank matrix that was subject to the liquid-liquid extraction protocol. After extraction, the blank samples were fortified with both target and internal standard. The neat samples were prepared by spiking an appropriate volume of target analyte and internal standard and reconstituting in starting mobile phase.

Equation 1 was used to calculate the ionization suppression/enhancement for the target compounds and internal standards. The ionization suppression was assessed at two different concentrations (0.015 mg/L and 0.4/1.6 mg/L) the concentrations are delineated in Table 3.

Equation 1

$$Ion\ Suppression/Enhancement = \left(\frac{Average\ Post-Extraction\ Fortified\ Sample}{Average\ Neat\ Sample}\right) \times 100$$

Table 3 Ionization suppression/enhancement concentrations

Ionization Suppression/Enhancement Concentrations		
Target Compounds	Low Concentration (mg/L)	High Concentrations (mg/L)
4-APDB	0.015	0.4
4-chloro-alpha-PVP	0.015	0.4
4/5/6-MAPB	0.015	0.4
5-APDB	0.015	0.4
5-DBFPV	0.015	0.4
6-APDB	0.015	0.4
Alpha-PVP	0.015	1.6
Dibutylone	0.015	0.4
Ethylone	0.015	1.6
MDPV	0.015	1.6
Mephedrone	0.015	1.6
Methcathinone	0.015	1.6
Methedrone	0.015	1.6
Methylone	0.015	1.6
N-Ethylpentylone/N,N-Diethylpentylone	0.015	0.4
N,N-Dimethylpentylone	0.015	0.4
PV8	0.015	0.4

To fully evaluate the impact of ionization suppression/enhancement, duplicate determinations of each concentration for each matrix source were evaluated. A total of ten different sources per matrix type (blank blood, antemortem blood, postmortem blood, liver, and urine) were used in the evaluation. The post-extraction fortified samples were compared to six replicate injections of neat standards. The overall ionization suppression or enhancement was calculated for each matrix type at low and high concentrations. Table 4 shows the ionization suppression/enhancement for each matrix type.

Table 4 Ionization Suppression/Enhancement

Ionization Suppression/Enhancement % Suppression/Enhancement±Standard Deviation (%CV)										
	Blank Blood		Antemortem Blood		Postmortem Blood		Liver		Urine	
Compound	Low	High	Low	High	Low	High	Low	High	Low	High
4-APDB	70.4±6.6(9)	89.2±2.8(3)	71.7±2.3(3)	90.8±1.6(2)	80.4±9.0(11)	90.4±2.5(3)	89.1±15.1(17)	85.8±15.7(18)	84.8±3.5(4)	94.0±2.0(2)
4-chloro-alpha-PVP	99.7±2.9(3)	94.7±1.9(2)	98.4±2.5(3)	98.1±1.1(1)	100±2.7(3)	97.2±1.2(1)	95.5±15.7(16)	88.5±9.9(11)	99.5±1.5(2)	101±1.1(1)
4/5/6-MAPB	83.3±4.7(6)	96.9±0.9(1)	83.4±1.2(1)	98.9±1.1(1)	94.0±6.9(7)	97.3±0.6(1)	99.5±14.3(14)	89.4±16.5(19)	94.9±3.8(4)	100±2.0(2)
5-APDB	71.7±6.2(9)	90.8±2.5(3)	71.5±2.1(3)	92.5±1.9(2)	83.3±9.4(11)	92.4±1.9(2)	88.5±15.7(18)	85.5±16.6(19)	86.2±3.9(4)	95.6±2.1(2)
5-DBFPV	100±3.4(3)	97.5±0.8(1)	96.5±2.3(2)	99.8±1.0(1)	103±2.9(3)	98.5±1.0(1)	101±9.6(9)	91.5±11.9(13)	100±1.9(2)	102±0.9(1)
6-APDB	72.1±6.5(9)	90.5±2.4(3)	71.6±2.1(3)	92.5±1.7(2)	82.1±8.1(10)	92.3±1.6(2)	87.3±14.6(17)	85.7±16.0(19)	86.3±3.6(4)	95.5±1.9(2)
Alpha-PVP	97.8±2.1(2)	90.3±4.9(5)	95.6±2.1(2)	94.4±1.7(2)	102±2.6(3)	97.0±1.8(2)	102±9.6(9)	92.4±13.4(15)	99.6±1.9(2)	100±2.5(2)
Dibutylone	90.0±4.1(5)	93.7±2.7(3)	88.6±2.5(3)	96.0±0.9(1)	98.3±3.8(4)	97.2±1.2(1)	102±9.9(10)	92.8±12.7(14)	96.8±4.6(5)	99.3±3.4(3)
Ethylone	88.8±3.9(4)	98.0±0.8(1)	87.0±3.1(4)	98.9±1.1(1)	95.8±4.5(5)	97.5±1.3(1)	91.1±14.5(16)	90.8±14.0(15)	96.3±2.9(3)	101±1.3(1)
MDPV	101±2.5(2)	98.1±0.8(1)	96.3±2.1(2)	100±1.1(1)	104±3.3(3)	99.2±0.5(1)	99.4±10.3(10)	93.7±11.9(13)	98.6±2.1(2)	102±0.8(1)
Mephedrone	82.7±4.2(5)	95.0±1.1(1)	83.4±1.8(2)	96.6±0.8(1)	93.8±6.0(6)	96.8±1.0(1)	92.9±12.8(14)	89.9±14.0(16)	93.7±3.0(3)	99.5±1.3(1)
Methcathinone	81.5±4.7(6)	91.2±2.2(2)	81.3±3.7(5)	92.7±1.4(2)	92.5±6.0(6)	94.1±2.2(2)	88.2±16.1(18)	88.9±14.7(17)	92.2±3.8(4)	97.9±2.1(2)
Methedrone	79.0±5.1(6)	95.4±1.1(1)	78.6±2.9(4)	96.2±1.2(1)	90.8±6.8(8)	95.7±0.9(1)	89.2±15.8(18)	88.5±15.6(18)	92.5±4.2(5)	99.3±1.6(2)
Methylone	81.2±4.6(6)	95.6±1.3(1)	80.1±3.0(4)	96.6±1.4(1)	90.8±5.7(6)	95.5±1.4(1)	89.4±15.5(17)	87.9±15.1(17)	91.9±3.7(4)	98.6±1.7(2)
N-Ethylpentylone/N,N-Diethylpentylone	95.9±2.9(3)	99.0±0.8(1)	95.1±2.0(2)	99.6±1.3(1)	104±3.9(4)	99.4±0.7(1)	96.5±9.9(10)	92.3±12.9(14)	99.8±1.8(2)	101±0.6(1)
N,N-Dimethylpentylone	95.1±3.5(4)	96.4±1.4(1)	92.9±1.7(2)	98.5±0.7(1)	101±3.0(3)	98.7±0.9(1)	105±9.6(9)	96.0±9.4(10)	101±2.8(3)	101±0.9(1)
PV8	103±2.3(2)	95.5±1.5(2)	104±2.0(2)	98.4±0.8(1)	104±3.7(4)	97.1±2.3(2)	102±16.7(16)	88.8±10.9(12)	102±1.3(1)	101±0.9(1)
Methylone-D_3	84.2±4.7(6)	97.6±1.2(1)	82.4±3.0(4)	95.7±1.4(1)	92.9±5.7(6)	96.0±1.3(1)	89.3±15.3(17)	88.3±14.4(16)	93.6±3.7(4)	99.1±1.7(2)
Mephedrone-D_3	85.7±4.0(5)	97.4±1.1(1)	85.9±1.8(2)	96.0±0.7(1)	96.1±5.8(6)	97.2±0.6(1)	92.2±13.3(14)	89.5±14.4(16)	94.3±3.3(4)	100±1.3(1)
N-Ethylpentylone-D_5	95.6±2.5(3)	96.9±1.1(1)	92.5±2.3(2)	98.0±1.3(1)	102±3.4(3)	100.3±1.0(1)	96.3±10.0(10)	92.1±13.6(15)	99.6±2.1(2)	102±1.3(1)

NOTE: Blue highlighted cells indicate significant ionization suppression/enhancement (>±25%).

When evaluating blank blood for ionization suppression or enhancement, it was noted that 4-APDB, 5-APDB, and 6-APDB all had significant ionization suppression (≥25%) at the low concentration (0.015 mg/L). These three compounds also demonstrated significant ionization suppression in antemortem blood at the low concentration. The high concentration (0.4 mg/L) for these compounds did not demonstrate ionization suppression or enhancement in blank blood or antemortem blood. All other compounds, including internal standards, did not have significant ionization suppression or enhancement (≥25%) and also had a %CV less than 20% in all matrix types.

3. Carryover

Carryover was evaluated by analyzing blank matrix samples immediately following a high concentration (0.5/2.0 mg/L) of fortified matrix within the injection sequence. Table 5 establishes the concentrations evaluated for each compound within the method.

Table 5 Carryover concentrations evaluated

Target Compounds	Carryover Concentration Evaluated (mg/L)
4-APDB	0.5
4-chloro-alpha-PVP	0.5
4/5/6-MAPB	0.5
5-APDB	0.5
5-DBFPV	0.5
6-APDB	0.5
Alpha-PVP	2.0
Dibutylone	0.5
Ethylone	2.0
MDPV	2.0
Mephedrone	2.0
Methcathinone	2.0
Methedrone	2.0
Methylone	2.0
N-Ethylpentylone/N,N-Diethylpentylone	0.5
N,N-Dimethylpentylone	0.5
PV8	0.5

Three matrix sources per matrix type (blank blood, antemortem blood, postmortem blood, liver, and urine) were evaluated over three analytical runs. The blank sample immediately following the fortified matrix sample was evaluated for an instrumental response greater than 10% of the administratively defined decision point (0.005 mg/L) instrumental response. No blank matrix samples immediately following any fortified matrix sample had indications of carryover.

4. Interferences

To assess for interference, the qualifier and quantifier ions for each analyte and internal standard within the method were monitored. If an instrumental response was noted and was less than 10% of the administratively established threshold response, the impact of the instrumental response was deemed insignificant.

a. Endogenous Compounds

To evaluate samples for endogenous interferents, 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 each compound within the analytical method. No endogenous interferences were identified.

b. Internal Standard

To evaluate potential interferences of the internal standard by a high concentration of analyte, samples were fortified with the high control concentration (0.5/2.0 mg/L) without internal standard and analyzed for the absence of response for the internal standard. A single matrix sample, per matrix type was evaluated. No interference from a high concentration of analyte was detected.

To evaluate potential interferences from the method's internal standard concentration to a low concentration of analyte, a single matrix sample, per matrix type was fortified at a concentration of 0.02/0.1 mg/L without the analyte of interest and analyzed for the absence of response for the analyte. No interferences from the internal standard were detected.

c. Commonly Encountered Analytes

Interferences from commonly encountered compounds were evaluated by analyzing blank matrix fortified with high concentrations of commonly encountered drugs, metabolites, and structurally similar compounds. In addition to the evaluation of commonly encountered compounds, a blank matrix was fortified individually with the compounds within the analytical method to monitor potential interference with compounds within the method. Table 6 lists the drug class, drug, and concentration evaluated for commonly encountered compounds.

Table 6 Commonly encountered analytes

Class	Drug	Concentration
Opioids and Cocaine	Oxymorphone, Hydromorphone, 6-Methylacetylmorphine, Acetylfentanyl, Fentanyl, Benzoylecgonine, Meperidine, Tramadol, Methadone, Morphine, Codeine, Oxycodone, Hydrocodone, Cocaethylene, Cocaine	0.2/2.0/1.0 mg/L
Cannabinoids	Δ9-THC, Δ8-THC, Carboxy-THC, 11-Hydroxy-THC	0.2/0.4/1.0 mg/L
Anti-Epileptic Drugs	Gabapentin, Levetiracetam, Lamotrigine, Zonisamide, 10,11-dihydro-10-hydroxycarbamazepine, Oxcarbazepine, Topiramate, Carbamazepine, Phenytoin, Pregabalin, Lacosamide	40 mg/L
Benzodiazepines	Alprazolam, Clonazepam, Lorazepam, Diazepam, Nordiazepam, Oxazepam, Temazepam, Zolpidem	2 mg/L
NPS	Dibutylone, N-ethylpentylone, Tenocyclidine, Clonazolam, 4-Chloro-alpha-PVP, PV8, 5-MAPB, 4-APDB, 5-APDB, 6-APDB, Pentylone, N,N-Dimethylpentylone, 3-Methoxy-PCP, Methoxphenidine, Mitragynine, 5-DBFPV, N-cyclohexyl Pentylone, N-ethylpentylone-D_5	0.02/0.5/1.0 mg/L
Carisoprodol and Meprobamate	Carisoprodol, Meprobamate	100 mg/L
Fentanyls	3-Fluorofentanyl, 4-Methoxybutyrylfentanyl, Acetylfentanyl, Acrylfentanyl, alpha-Methylacetylfentanyl, alpha-Methylfentanyl, Benzodioxolefentanyl, beta-Hydroxythiofentanyl, Butyrylfentanyl, Carfentanil, cis-3-Methylfentanyl, Cyclopropylfentanyl, Despropionylfentanyl, Fentanyl, Furanylfentanyl, Methoxyacetylfentanyl, Ocfentanil, ortho-Fluoroacrylfentanyl, ortho-Fluorobutyrylfentanyl, ortho-Fluorofentanyl, ortho-Fluoroisobutyrylfentanyl, para-Fluoroacrylfentanyl, para-Fluorobutyrylfentanyl, para-Fluorofentanyl, para-Fluoroisobutyrylfentanyl, Phenylfentanyl, Tetrahydrofuranfentanyl, trans-3-Methylfentanyl, U-47700, U-49900, Valerylfentanyl	0.05/0.1 mg/L
Acid/Neutral Drugs	Acetaminophen, Carbamazepine, 10,11-dihydro-10-hydroxycarbamazepine, Ibuprofen, Levetiracetam, Oxcarbazepine, Phenytoin, Salicylic Acid, Naproxen	6 mg/L
Basic Drugs	Amitriptyline, Citalopram, Cyclobenzaprine, Dextromethorphan, Diphenhydramine, Nortriptyline, Phencyclidine, Tramadol, Trazodone	6 mg/L
Amphetamines and Designer Stimulants	Amphetamine, Methamphetamine, MDA, MDMA, Bupropion, Phentermine, Methcathinone, Methylone, Ethylone, Methedrone, Mephedrone, Alpha-PVP, MDPV, Pseudoephedrine, Ephedrine, Mephedrone-D_3, Methylone-D_3, Pseudoephedrine-D_3, Amphetamine-D_{11}, Methamphetamine-D_{11}, MDA-D_5, MDMA-D_5, alpha-PVP-D_8, Bupropion-D_9, MDPV-D_8	0.1/2.0 mg/L
Barbiturates	Butabarbital, Butalbital, Pentobarbital, Phenobarbital, Secobarbital	40 mg/L

When evaluating commonly encountered compounds for interference with the target compounds within the qualitative method it was noted that N,N-dimethylpentylone produced an interfering peak within the acceptable retention time window of pentylone. Upon further investigation, the presence of the chromatographic peak suggests the conversion of N,N-dimethylpentylone to pentylone during sample preparation. The chromatographic peak identified when evaluating a high concentration of N,N-dimethylpentylone was nearly equal to the threshold concentration instrumental response. Therefore, this interferent was deemed significant and pentylone does not meet the predetermined acceptance criteria for interference within the analytical method.

During the evaluation of each compound within the qualitative method, in addition to the compounds within the associated Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS method, it was noted that pseudoephedrine and

ephedrine produced an instrumental response for methcathinone. Therefore, methcathinone does not meet the predetermined acceptance criteria for interference and is not fit for purpose within this validated analytical method.

5. Stability

The stability of extracted samples that were not analyzed immediately was evaluated at two concentrations for each matrix type (blank blood, antemortem blood, postmortem blood, liver, and urine). The low and high concentrations evaluated coincide with the administratively defined decision point concentration (low) and the carryover concentration evaluated (Table 6). The samples were extracted and injected immediately, in triplicate, to establish the Day 1 instrumental response. Both concentration levels were subsequently injected in triplicate every twenty-four hours over a six-day period.

The instrumental response of the target compound and internal standard were evaluated at each timepoint. If the average ratioed instrumental response decreased below 80% or increased above 120% of the average Day 1 response, then the target compound was considered unstable at that timepoint. In addition to the assessment of the deviation of instrumental response, predetermined identification criteria were also evaluated. Tables 7-24 show the stability study results for the target compounds and internal standards within the analytical method.

Table 7 4-APDB stability

4-APDB Stability Study 0.005 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	103	102	100	100	98
Antemortem Blood	100	102	99	98	98	96
Postmortem Blood	100	98	97	95	96	94
Liver	100	99	97	97	98	96
Urine	100	98	97	97	97	95
0.5 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	102	100	99	99	98
Antemortem Blood	100	101	99	98	99	97
Postmortem Blood	100	101	99	98	99	96
Liver	100	99	97	97	98	95
Urine	100	99	98	98	98	96

Table 8 4-Chloro-alpha-PVP stability

4-Chloro-alpha-PVP Stability Study						
0.005 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	98	98	98	96	96
Antemortem Blood	100	98	95	95	95	93
Postmortem Blood	100	100	96	96	97	94
Liver	100	98	99	100	98	93
Urine	100	100	98	99	100	96
0.5 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	97	99	100	99	95
Antemortem Blood	100	99	100	100	100	95
Postmortem Blood	100	100	100	100	101	95
Liver	100	98	100	100	100	94
Urine	100	100	101	101	101	95

Table 9 4/**5**/6-MAPB stability

4/**5**/6-MAPB Stability Study						
0.005 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	97	95	93	94	94
Antemortem Blood	100	98	94	93	93	92
Postmortem Blood	100	97	93	92	93	91
Liver	100	98	96	95	95	93
Urine	100	97	93	93	94	92
0.5 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	98	96	95	95	94
Antemortem Blood	100	98	96	95	95	93
Postmortem Blood	100	99	95	95	95	94
Liver	100	99	97	96	97	95
Urine	100	98	95	96	96	94

Table 10 5-APDB stability

5-APDB Stability Study 0.005 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	101	100	99	99	99
Antemortem Blood	100	100	97	97	97	95
Postmortem Blood	100	98	96	95	96	93
Liver	100	99	98	98	98	96
Urine	100	100	98	97	98	95
0.5 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	101	99	98	98	97
Antemortem Blood	100	101	98	98	98	96
Postmortem Blood	100	100	97	97	98	95
Liver	100	99	98	98	98	96
Urine	100	99	97	97	98	95

Table 11 5-DBFPV stability

5-DBFPV Stability Study 0.005 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	101	98	97	97	97
Antemortem Blood	100	99	96	96	96	93
Postmortem Blood	100	98	95	95	96	92
Liver	100	99	99	98	99	96
Urine	100	98	97	96	97	93
0.5 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	98	98	100	100	94
Antemortem Blood	100	99	99	101	101	94
Postmortem Blood	100	101	101	102	102	95
Liver	100	100	103	102	101	95
Urine	100	100	101	102	102	95

Table 12 6-APDB stability

6-APDB Stability Study 0.005 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	103	100	98	99	97
Antemortem Blood	100	99	97	96	96	95
Postmortem Blood	100	99	95	95	96	93
Liver	100	99	99	98	98	96
Urine	100	100	97	96	97	94
0.5 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	101	100	99	98	97
Antemortem Blood	100	101	99	98	98	96
Postmortem Blood	100	101	100	97	98	96
Liver	100	99	98	98	98	95
Urine	100	100	97	97	97	95

Table 13 Alpha-PVP stability

Alpha-PVP Stability Study 0.005 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	97	95	94	93	93
Antemortem Blood	100	96	94	92	91	90
Postmortem Blood	100	97	93	92	93	89
Liver	100	98	95	95	96	93
Urine	100	97	94	94	95	91
2.0 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	96	95	94	93	91
Antemortem Blood	100	97	95	95	95	90
Postmortem Blood	100	97	95	95	95	91
Liver	100	97	96	96	96	92
Urine	100	97	96	96	96	91

Table 14 Dibutylone stability

Dibutylone Stability Study 0.005 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	97	94	92	90	90
Antemortem Blood	100	96	93	92	91	88
Postmortem Blood	100	97	93	92	91	88
Liver	100	99	96	96	96	92
Urine	100	97	94	93	95	90
0.5 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	96	94	93	94	91
Antemortem Blood	100	98	95	95	95	91
Postmortem Blood	100	98	95	94	95	91
Liver	100	98	97	97	97	92
Urine	100	98	96	96	97	92

Table 15 Ethylone stability

Ethylone Stability Study 0.005 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	99	97	94	94	94
Antemortem Blood	100	99	95	94	95	93
Postmortem Blood	100	98	95	94	96	92
Liver	100	100	97	98	100	95
Urine	100	97	94	94	96	92
2.0 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	98	96	96	96	95
Antemortem Blood	100	100	98	98	97	95
Postmortem Blood	100	99	99	99	97	96
Liver	100	100	99	99	100	95
Urine	100	100	98	99	99	95

Table 16 MDPV stability

MDPV Stability Study 0.005 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	99	98	96	97	97
Antemortem Blood	100	98	96	97	97	95
Postmortem Blood	100	98	95	96	96	95
Liver	100	99	97	99	100	99
Urine	100	100	98	97	99	98
2.0 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	98	99	99	99	97
Antemortem Blood	100	99	99	100	100	96
Postmortem Blood	100	99	99	100	100	97
Liver	100	99	100	100	100	97
Urine	100	100	100	101	101	97

Table 17 Mephedrone stability

Mephedrone Stability Study 0.005 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	98	96	94	94	95
Antemortem Blood	100	97	94	93	94	93
Postmortem Blood	100	97	94	93	94	93
Liver	100	99	97	96	98	96
Urine	100	98	95	95	97	95
2.0 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	97	95	94	94	95
Antemortem Blood	100	98	95	95	94	95
Postmortem Blood	100	98	96	95	95	95
Liver	100	99	96	96	96	97
Urine	100	98	96	96	95	95

Table 18 Methcathinone stability

Methcathinone Stability Study 0.005 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	98	95	94	93	95
Antemortem Blood	100	97	94	93	92	92
Postmortem Blood	100	97	94	93	93	92
Liver	100	99	96	96	98	96
Urine	100	97	95	94	95	93
2.0 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	97	95	94	93	94
Antemortem Blood	100	98	95	94	94	94
Postmortem Blood	100	98	96	95	95	96
Liver	100	99	96	96	95	95
Urine	100	98	96	96	95	96

Table 19 Methedrone stability

Methedrone Stability Study 0.005 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	99	98	96	95	97
Antemortem Blood	100	98	95	94	95	94
Postmortem Blood	100	98	95	94	95	94
Liver	100	99	97	98	100	96
Urine	100	98	96	95	97	94
2.0 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	97	95	96	95	94
Antemortem Blood	100	97	94	96	95	94
Postmortem Blood	100	98	96	97	97	96
Liver	100	100	97	98	97	97
Urine	100	98	96	98	98	95

Table 20 Methylone stability

Methylone Stability Study 0.005 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	102	100	100	100	101
Antemortem Blood	100	98	97	97	96	97
Postmortem Blood	100	100	97	97	97	96
Liver	100	103	101	101	103	100
Urine	100	100	99	98	100	98
2.0 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	101	100	99	99	99
Antemortem Blood	100	100	98	98	98	97
Postmortem Blood	100	100	99	99	99	98
Liver	100	102	101	101	101	98
Urine	100	100	98	99	98	97

Table 21 **N-Ethylpentylone**/N,N-Diethylpentylone stability

N-Ethylpentylone/N,N-Diethylpentylone Stability Study 0.005 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	96	96	95	94	92
Antemortem Blood	100	98	95	93	94	91
Postmortem Blood	100	97	94	94	95	89
Liver	100	97	97	96	98	91
Urine	100	98	95	95	96	91
0.5 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	97	95	95	95	90
Antemortem Blood	100	98	96	95	95	90
Postmortem Blood	100	99	97	96	97	91
Liver	100	97	97	96	96	91
Urine	100	98	97	96	97	91

Table 22 N,N-Dimethylpentylone stability

N,N-Dimethylpentylone Stability Study						
0.005 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	98	95	94	93	92
Antemortem Blood	100	97	93	93	93	90
Postmortem Blood	100	98	95	93	95	90
Liver	100	98	96	96	95	92
Urine	100	98	94	94	93	91
0.5 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	97	96	96	95	91
Antemortem Blood	100	98	96	96	95	91
Postmortem Blood	100	98	96	96	96	91
Liver	100	98	98	98	97	92
Urine	100	98	97	96	97	92

Table 23 PV8 stability

PV8 Stability Study						
0.005 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	98	98	97	96	95
Antemortem Blood	100	97	96	96	97	93
Postmortem Blood	100	98	96	96	96	92
Liver	100	100	99	99	100	94
Urine	100	100	98	99	98	94
0.5 mg/L						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	98	98	99	98	94
Antemortem Blood	100	99	97	99	99	94
Postmortem Blood	100	101	99	99	100	95
Liver	100	99	100	100	100	94
Urine	100	100	99	99	100	95

Table 24 Internal standard stability

Mephedrone-D_3 Stability Study						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	97	96	95	94	95
Antemortem Blood	100	98	95	94	94	94
Postmortem Blood	100	98	95	94	94	94
Liver	100	99	96	96	96	95
Urine	100	97	95	95	95	94
Methylone-D_3 Stability Study						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	101	99	99	98	99
Antemortem Blood	100	99	98	97	97	96
Postmortem Blood	100	100	98	98	98	97
Liver	100	102	101	101	101	99
Urine	100	100	98	98	98	96
N-Ethylpentylone-D_5 Stability Study						
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	98	97	96	96	92
Antemortem Blood	100	98	96	95	95	91
Postmortem Blood	100	99	96	95	96	92
Liver	100	98	96	97	97	92
Urine	100	97	95	95	96	91

When evaluating the stability data, the instrumental responses for all compounds were within ±20% of the Day 1 instrumental response for all six days of the study. This includes all matrices evaluated (blank blood, antemortem blood, postmortem blood, liver, and urine) and concentrations assessed. Therefore, all compounds were determined to be stable for six days after extraction in all matrix types.

6. Robustness

The validation was performed using blank blood, antemortem blood, postmortem blood, liver and urine. The experiments for the validation were performed on an Agilent Technologies 6460 and 6470 liquid chromatograph tandem mass spectrometer.

7. Summary

The validation demonstrated that the qualitative addition of certain novel psychoactive compounds to the Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS method is fit for purpose for the analysis of blank blood, antemortem blood, postmortem blood, liver, and urine. During the validation, N,N-dimethylpentylone was discovered to interfere with pentylone. This was due to the conversion of N,N-dimethylpentylone to pentylone during the extraction process. Given the significance of this conversion and the inability to reduce or remove the interferent, this method is not fit for purpose for pentylone.

In addition to the interference of N,N-dimethylpentylone with pentylone, it was noted that pseudoephedrine and ephedrine interfere with methcathinone. Although these two compounds

are not part of this qualitative method, this interferent should be noted when pseudoephedrine and ephedrine are present in a biological matrix.

Significant ionization suppression was noted for 4-APDB, 5-APDB, and 6-APDB in both blank blood and antemortem blood. No other compounds or matrices indicated significant ionization suppression or enhancement. Due to the significant ionization suppression noted, nine blank matrix sources per matrix type were utilized to evaluate the estimated limit of detection for the method. An administratively defined decision point was evaluated as the estimated limit of detection. This concentration was established to be 0.005 mg/L. In addition to this concentration, a concentration of 0.0025 mg/L was also evaluated for all matrix types (blank blood, antemortem blood, postmortem blood, liver, and urine). All identification criteria were met for both concentrations (0.0025 mg/L and 0.005 mg/L) in all matrix types with the exception of MDPV in postmortem blood and PV8 in liver. Both MDPV in postmortem blood and PV8 and liver failed to meet the identification criteria at a concentration of 0.0025 mg/L. These compounds in their respective matrices did meet the predetermined identification criteria at 0.005 mg/L.

Carryover was evaluated for each matrix type by assessing a blank matrix immediately following the injection of a high concentration of fortified matrix. No carryover was detected for any compound in any of the matrices. In addition to carryover, the stability of extracted samples that were not immediately analyzed was evaluated for each matrix type. For this study, it was determined that each compound was stable in blank blood, antemortem blood, postmortem blood, liver, and urine for at least six days after extraction.

Based on the validation data, the qualitative addition of novel psychoactive compounds to the Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS method is fit for purpose for all included compounds with the exception of pentylone.

All data from the validation has been stored on the TX Validation SharePoint.

8. References

Virginia Department of Forensic Science Quality Manual, Qualtrax Revision 33, 2026.

Virginia Department of Forensic Science Toxicology Procedures Manual, Qualtrax Revision 33, 2026.

Memo To: James Hutchings, Ph.D., Toxicology Program Manager
From: Rebecca Wagner, Ph.D., Chemistry Research Section Supervisor 6/4/25
CC: Alka Lohmann, Technical Services Director
Date June, 04, 2025
RE: Validation Plan
Addition of Novel Psychoactive Substances to the Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS Method

Validation Plan – Addition of Novel Psychoactive Substances to the Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS Method

It is proposed to add compounds from the existing Novel Psychoactive Substances (NPSs) Qualitive Screen and Confirmation using LCMSMS method (Section 30, Qualtrax Revision 32) to the existing Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS method (Section 26, Qualtrax Revision 32). The method validation will include the previously validated liquid-liquid extraction sample preparation procedure delineated in Section 26 of the Toxicology Procedures Manual and acquisition method delineated in Section 30 of the Toxicology Procedures Manual (Qualtrax Revision 32). The target analytes and internal standards for the method are listed in Table 1.

Table 1 Target compounds and internal standards

Target Compounds	Internal Standards
4-APDB	N-Ethylpentylone-D_5
4-chloro-alpha-PVP	N-Ethylpentylone-D_5
4/5/6-MAPB	N-Ethylpentylone-D_5
5-APDB	N-Ethylpentylone-D_5
5-DBFPV	N-Ethylpentylone-D_5
6-APDB	N-Ethylpentylone-D_5
Alpha-PVP	Mephedrone-D_3
Ethylone	Methylone-D_3
Ethylpentylone/N,N-Diethylpentylone	N-Ethylpentylone-D_5
Dibutylone	N-Ethylpentylone-D_5
Methcathinone	Mephedrone-D_3
Methedrone	Mephedrone-D_3
Methylone	Methylone-D_3
MDPV	Mephedrone-D_3
Mephedrone	Mephedrone-D_3
Pentylone	N-Ethylpentylone-D_5
N,N-Dimethylpentylone	N-Ethylpentylone-D_5
PV8	N-Ethylpentylone-D_5

Note: The target compound for 4/5/6-MAPB will be 4-MAPB. The target compound for ethylpentylone/N,N-Diethylpentylone will be ethylpentylone.

MEMORANDUM FOR RECORD

From: James Hutchings Date: 01/07/2026

E-Signature: James Hutchings

Subject: Update to NPS Validation Plan Dated June 4, 2025

DISTRIBUTION:

☐ Director	☐ FS Lab #:		
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☐ DTS	☐ Program Manager:		
☐ HR	☐ Supervisor, Section:	Laboratory:	
☐ Counsel	☑ Other:	Validation Plan - NPS Addition to AMPS Method	

The note below Table 1 is updated to the following:

Note: The target compound for 4/5/6-MAPB will be 5-MAPB. The target compound for ethylpentylone/N,N-diethylpentylone will be N-ethylpentylone.

MEMORANDUM FOR RECORD

From: James Hutchings Date: 01/07/2026

E-Signature: James Hutchings

Subject: Update to NPS Validation Plan Dated June 4, 2025

DISTRIBUTION:

☐ Director	☐ FS Lab #:		
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☐ DTS	☐ Program Manager:		
☐ HR	☐ Supervisor, Section:	Laboratory:	
☐ Counsel	☑ Other:	Validation Plan - NPS Addition to AMPS Method	

The note below Table 1 is updated to the following:

Note: The target compound for 4/5/6-MAPB will be 5-MAPB. The target compound for ethylpentylone/N,N-diethylpentylone will be N-ethylpentylone.

The matrices to be evaluated during validation include blank blood, antemortem blood, postmortem blood, liver and urine. The method will employ an administratively defined decision point (threshold control) of 0.005 mg/L. In addition to the decision point, a high control will be evaluated at 0.5/2.0 mg/L (NPS method compounds/bath salts). Agilent Technologies 6460 and 6470 liquid chromatograph tandem mass spectrometers will be used for analysis. The acquisition method is listed in Table 2.

Table 2 Instrumental acquisition parameters

Liquid Chromatography Parameters

Parameter	Setting		
Column	Agilent Technologies Infinity Poroshell EC-C18, 2.1x150 mm 2.7 µm (PN 693575-902)		
Injection Volume	2.0 µL		
Wash Time	20 seconds		
Column Thermostat	60°C		
Mobile Phase A	Water with 0.1% formic acid		
Mobile Phase B	Methanol with 0.1% formic acid		
Flow Rate	0.4 mL/min		
Injection Volume	10 µL with 5 second needle wash		
Stop Time	15.0 minutes		
Post Time	2.0 minutes		
Gradient	Time (minutes)	Mobile Phase A (%)	Mobile Phase B (%)
	0.0	98	2
	0.5	98	2
	10.0	80	20
	12.0	70	30
	12.5	5	95
	13.5	5	95
	14.0	98	2
	15.0	98	2

Mass Spectrometer Parameters

Parameter	Settings
Scan Type	Dynamic MRM
Ion Mode	ESI Positive
Start Time	0.25 minutes
Delta EMV (+)	400 V
Gas Temperature	325°C
Gas Flow	12 L/min
Nebulizer	45 psi
Capillary	3500 V

Compound	Precursor (*m/z*)	Product (*m/z*)	Approximate RT (minutes)	RT Window	Fragmentor (V)	Collision Energy (V)	Cell Accelerator (V)
4/5/6-MAPB	190.1	159.1	10.7	3	90	8	2
		131.0			90	20	
4-APDB	178.1	161.1	8.2	3	75	4	2
		133.0			75	16	
4-chloro-alpha-PVP	266.1	126.1	13.8	3	100	28	2
		125.0			100	24	
5-APDB	178.1	161.1	8.5	3	65	4	2
		133.0			65	20	
5-FPV	274.2	126.1	13.6	3	135	28	2
		161.1			135	24	
6-APDB	178.1	161.1	9	3	75	4	2
		133.0			75	20	
Alpha-PVP	232.2	126.1	12.5	3	115	28	7
		91.0			115	24	
Dibutylone	236.1	161.1	9.2	3	105	20	2
		86.1			105	24	
Ethylone	222.1	174.0	7.5	3	110	18	7
		146.0			110	28	
MDPV	276.3	135.0	13	3	130	25	7
		126.0			130	25	
Mephedrone	178.3	160.0	9.2	3	85	10	7
		144.0			85	30	
Mephedrone-D_3	181.3	163.0	9.2	3	90	9	7
		148.0			90	21	
Methcathinone	164.2	146.0	5.4	3	85	10	7
		130.0			85	34	
Methedrone	194.2	176.0	7.7	3	90	8	7
		161.0			90	20	
Methylone	208.2	190.0	6.2	3	80	14	7
		132.0			80	26	
Methylone-D_3	211.2	163.0	6.2	3	85	13	7
		135.0			85	29	
N-ethylpentylone-D_5	255.2	207.1	12.8	3	95	20	2
		194.1			95	28	
Ethylpenylone/N,N-Diethylpentylone	250.1	232.1	12.8	3	115	8	2
		202.1			115	16	
Pentylone	236.1	218.1	12.2	3	85	8	2
		188.1			85	16	
N,N-Dimethylpentylone	250.1	175.0	12.5	3	105	20	2
		100.1			105	24	
PV8	260.2	154.1	13.8	3	135	28	2
		91.1			135	24	

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. Sensitivity - Estimated Limit of Detection (LOD)
2. Ionization Suppression/Enhancement
3. Carryover
4. Interferences
    a. Endogenous Compounds
    b. Internal Standard
    c. Commonly Encountered Analytes
5. Stability
6. Robustness
7. References

1. Sensitivity - 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 shall be estimated using two concentrations. The concentrations to be evaluated are 0.005 mg/L and 0.0025 mg/L. The defined concentration (0.005 mg/L) will be established 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, liver, and urine). The 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. The number of matrix sources will be increased from three to nine given the anticipated ionization suppression of the analytes.

Predetermined identification criteria:

Retention Time: ±3%
Qualifier Ratio: ±20%
Signal-to-Noise: ≥3.3

To establish the estimated limit of detection, at minimum, 95% of the fortified LOD samples shall meet all identification criteria for each matrix type. During the validation, if the limit of detection concentration (0.005 mg/L) does not meet the anticipated acceptance criteria (95%), a higher limit of detection concentration may be evaluated upon approved by the Toxicology Program Manager.

2. Ionization Suppression/Enhancement

Ionization suppression and enhancement will be addressed with neat standards and post-extraction fortified samples. Two different sets of samples shall be prepared, and their peak areas compared between sets. Neat standards, at low and high concentrations, will be prepared in neat reconstitution solvent and injected a minimum of six times each. Low and high concentrations will be utilized in the determination of ionization suppression or enhancement. The responses will be averaged for the two different concentrations (0.015 mg/L and 0.4/1.6 mg/L). A minimum of ten duplicates of post-extraction fortified samples (matrix that is extracted and then fortified), per matrix type (i.e., blank blood, antemortem blood, postmortem blood, liver, and urine), will be prepared to compare to the neat standards. The responses will be averaged for the two concentrations. The ratio between the averages of the sets will then be used to assess ionization suppression or enhancement as shown in Equation 1.

Equation 1

$$Ion\ Suppression/Enhancement = \left(\frac{Average\ Post - Extraction\ Fortified\ Sample}{Average\ Neat\ Sample}\right) \times 100$$

The ionization suppression or enhancement will be evaluated for the qualifier and quantifier transitions for the analytes and internal standards within the method. If suppression or enhancement exceeds ±25% or the %CV exceeds 20%, an evaluation of the effect on limit of detection shall be evaluated. The influence on the parameters shall be assessed by at least tripling the number of different sources of blank matrices used in the evaluation.

3. Carryover

Carryover will be evaluated by analyzing blank matrix samples immediately following the high control concentration (0.5/2.0 mg/L) of fortified matrix within the injection sequence. The highest analyte concentration at which no analyte carryover is observed, in the blank matrix, is determined to be the concentration at which the method is free from carryover. Analyte carryover is indicated by a response greater than 10% of the limit of detection. This concentration shall be confirmed using triplicate analysis with a minimum of three sources per matrix type. If carryover is detected at the 0.5/2.0 mg/L concentration, mitigation strategies will be evaluated. If mitigation is not an option, the high control concentration will be decreased until no carryover is indicated.

4. Interferences

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

a. Endogenous Compounds

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, liver, and urine) within the validation should be evaluated, whenever possible.

b. Internal Standard

To evaluate potential interferences of the internal standard by a high concentration of analyte, samples shall be fortified with the high control concentration without internal standard and analyzed for the absence of response for the internal standard. A single blank matrix (i.e., blank blood, antemortem blood, postmortem blood, liver, and urine) sample, per matrix type, shall be evaluated.

To evaluate potential interferences from the method's internal standard concentration to a low concentration of analyte, matrix shall be fortified with an appropriate concentration of internal standard (concentration delivered within method) without the analyte of interest and analyzed for the absence of response for the analyte. A single blank matrix (i.e., blank blood, antemortem blood, postmortem blood, liver, and urine) sample, per matrix type, shall be evaluated.

c. Commonly Encountered Analytes

Analytes which may be expected to be present in case samples shall be evaluated for their potential to interfere with the method's analytes. Matrix samples shall be fortified with commonly encountered drugs, metabolites, and other structurally similar compounds at high concentrations (i.e., highest calibrator concentration from current method).

Potential interferents to be evaluated:

Barbiturates (30 mg/L)
Amphetamines (2.0 mg/L)
Benzodiazepines (2.0 mg/L)
Carisoprodol and meprobamate (100 mg/L)
Anti-epileptic drugs (40 mg/L)
Basic drugs from previously made mixes (6.0 mg/L)
Acid/neutral drugs from previously made mixes (6.0 mg/L)
Opioids and cocaine (0.2/2.0/1.0 mg/L)
Fentanyl derivatives (0.05/0.1 mg/L)
Novel psychoactive substance (1.0 mg/L)
Cannabinoids (0.1/0.2/0.5 mg/L)

In addition to commonly encountered analytes, each drug within the method will be evaluated individually including other compounds within the Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS method.

5. Stability

During the validation period, the stability of extracted samples that are not analyzed immediately shall be addressed. Extracted samples shall be stored in autosampler vials on

the instrument throughout the stability evaluation process. This enables the simulation of an abrupt abortion, delay, or interruption during instrumental analysis.

At minimum, a single blank matrix source, per matrix type (i.e., blank blood, antemortem blood, postmortem blood, liver, and urine), will be extracted at two concentrations (high [0.4/1.6 mg/L] and low [0.015 mg/L]) and analyzed at minimum every twenty-four hours for a seven-day period with triplicate injections at each time point. For day one instrumental response, samples will be extracted and immediately analyzed. The responses will be averaged and all other responses from subsequent time points will be evaluated against the average response. The average instrumental responses for each time point will be compared to the day one instrumental response and plotted. Compounds are considered stable if the average signal response of the triplicate injections for a time point falls within the method's predefined acceptable bias (i.e., ±20%). For example, if the peak area increases above 120% or decreases below 80% of the original response the compound is no longer deemed stable. Alternatively, the ratio of peak area of analyte to internal standard may be utilized in the stability evaluation as opposed to peak area.

Stability should be carried out by injecting samples from the same autosampler vial throughout the stability experiments.

6. Robustness

Robustness will be assessed by performing the validation on multiple instruments. Validation experiments should include the current models of instruments within the laboratory.

7. References

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

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