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.

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**.