Direct Solvent Extraction of Sympathomimetic Amines and Synthetic Cathinones from Biological Samples

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1 INTRODUCTION

Sympathomimetic amines (SMAs) are generally a class of synthetic phenethylamine-derived drugs often generically referred to as "amphetamines". Almost all of these compounds show some degree of stimulant effects, but a wide variety of additional structure-dependent pharmacological effects can be seen in various compounds. Synthetic cathinones (SC) are compounds that may have structural similarities to SMAs and related effects.

2 SCOPE

Analyses	☒ Screening ☒ Confirmation ☒ Quantitation
Matrices	blood, serum, plasma, urine, gastric contents, vitreous humor, or a prepared tissue homogenate.
Analytes	Amphetamine, methamphetamine, ephedrine / pseudoephedrine, methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA), methylenedioxyethylamphetamine (MDEA), methylone, mephedrone and 3,4-methylenedioxypyrovalerone (MDPV). See validation for complete list.
Personnel	This document applies to authorized personnel who perform the described tasks, singly or in combination.

3 PRINCIPLE

Biological specimens are qualitatively analyzed and/or quantitated for SMAs or SCs. Specimens are mixed with an internal standard, adjusted to a basic pH, and extracted with hexane. The hexane is removed, acidified to prevent evaporation of volatile analytes, and taken to dryness. The resulting residue is reconstituted in 10/90 methanol/water and analyzed by LC-ESI-MS with data dependent MS^2 and MS^3. MS^3 detection is included because some analytes yield MS^2 spectra with limited information content.

4 SPECIMEN CRITERIA

This procedure uses a biological sample such as: blood, serum, plasma, urine, gastric contents, vitreous humor, or a prepared tissue homogenate. When available, 0.5 mL of biological fluid or 1.0 g of tissue homogenate (1:1) is used in the analysis.

5 EQUIPMENT

5.1 Equipment

A. Vortex mixer
B. Rotator
C. Centrifuge
D. Evaporator with nitrogen
E. Routine laboratory supplies, including disposable pipettes, wooden sticks, test tube racks, graduated cylinders, etc.

5.1.1 Column

A. HPLC Column: Xterra C18, 2.1 x 150 mm, 5 µm dp; or equivalent
B. Guard Column: 2.1 x 7.5 mm

5.2 Consumables

A. 16x100 mm screw-top tubes with Teflon-lined caps
B. 12x75 mm culture tubes with polypropylene snap-tops

5.3 Instruments

A. Thermo LTQ Orbitrap XL Hybrid Ion Trap/Fourier Transform Mass Spectrometer
B. Shimadzu HPLC

5.4 Software

Component	Software	Version
Operating System	Microsoft Windows	7 Pro SP 1 / XP Professional
Mass Spectrometer	Foundation	1.0.2 or higher
	Xcalibur	2.1.0 SP1 / 2.0.7
	LTQ Tune Plus	2.5.5
	Shimadzu LC Controller	5.4 / 6.5

5.5 Chemicals/Reagents

Storage/stability determined by manufacturer unless otherwise noted.

5.5.1 Purchased

A. Acetonitrile	Optima grade or better
B. Formic Acid	Puriss grade or better
C. Hexane	UV grade or better
D. Hydrochloric acid	ACS grade or better
E. Methanol	Optima grade or better
F. Sodium hydroxide	ACS grade or better
G. Water	Deionized and Optima or better

5.5.2 Prepared

A. Mobile Phase 1 (Aqueous) 0.1% Formic Acid in Water	
	Add 0.5 mL formic acid to 500 mL water (Optima grade or better). Store in glass at room temperature. Stable for 2 weeks.
B. Mobile Phase 2 (Organic) 0.1% Formic Acid in Acetonitrile	

Add 0.5 mL formic acid to 500 mL acetonitrile (Optima grade or better). Store in glass at room temperature. Stable for 1 month.

C. 4% Sodium Hydroxide

Dissolve 2 g sodium hydroxide in 50 mL deionized water. Store in plastic at room temperature. Stable for at least 6 months.

D. Methanol:Hydrochloric Acid (4:1 v:v)

Mix 20 mL methanol with 5 mL hydrochloric acid. Store in glass at room temperature. Stable for at least 1 month.

E. Methanol:Water (10:90 v:v)

Mix 5 mL methanol with 45 mL water (both Optima grade or better). Store in glass at room temperature. Stable for at least 1 year.

5.6 Standards/Controls

Storage and stability determined by manufacturer unless otherwise noted.

5.6.1 Purchased

A. Negative Control:

Purchased from Diagnostics Products Corporation, UTAK Laboratories, Inc., Cliniqa, or prepared in-house from an appropriate blank specimen. Blood and urine will be stored refrigerated, frozen or obtained fresh. Stability determined by manufacturer.

B. Internal Standard Stock Solutions (0.1 mg/mL)

1. Amphetamine-d5
2. Ephedrine-d3
3. MDA-d5
4. MDEA-d5
5. MDMA-d5
6. MDPV-d8
7. Mephedrone-d3
8. Methamphetamine-d5
9. Methylone-d3

Purchased from Cerilliant Corporation or equivalent.

C. Standard Stock Solutions (1 mg/mL)

1. Amphetamine
2. Ephedrine
3. MBDB (N-methylbenzodioxazolylbutanamine, N-methyl-1-3,4-methylenedioxy-phenyl)-2-butanamine)
4. MDA
5. MDEA
6. MDMA
7. MDPV
8. Mephedrone
9. Methamphetamine
10. Methylone

Purchased from Cerilliant (typically used for calibrators), from Lipomed (typically used for controls) or another approved supplier.

5.6.2 Prepared

5.6.2.1 Internal Standards

A. SMA Internal Standard Working Solution (2 µg/mL)

Analyte (0.1 mg/mL stock)	Aliquot (mL)	
1. Amphetamine-d5	0.50	
2. Ephedrine-d3	0.50	
3. MDA-d5	0.50	
4. MDEA-d5	0.50	
5. MDMA-d5	0.50	
6. Methamphetamine-d5	0.50	
i. Add components to		25 mL volumetric flask
ii. Add	2	Methanol (Optima)
iii. QS	25	Water (Optima)
Store in glass at <0ºC. Stable for at least 2 years.		

B. SC Internal Standard Working Solution (2 µg/mL)

Analyte (0.1 mg/mL stock)	Aliquot (mL)	
1. Mephedrone-d3	0.50	
2. Methylone-d3	0.50	
3. MDPV-d8	0.50	
i. Add components to		25 mL volumetric flask
ii. Add	2	Methanol (Optima)

iii. QS	25	Water (Optima)
Store in glass at <0ºC. Stable for at least 2 years.		

A and B are suggested preparation schemes which can be modified depending on case analysis needs. If targeted analysis is desired fewer analytes may be used.

5.6.2.2 Control

A. SMA Control Working Solution (1 µg/mL)

Analyte (1.0 mg/mL stock)	Aliquot (mL)	
1. Ephedrine	0.050	
2. Amphetamine	0.050	
3. Methamphetamine	0.050	
4. MDA	0.050	
5. MDMA	0.050	
6. MDEA	0.050	
i. Add components to		50 mL volumetric flask
ii. Add	9.9	Methanol (Optima)
iii. QS	50	Water (Optima)
Store in glass at <0ºC. Stable for at least 2 years.		

B. SC Control Working Solution (1 µg/mL)

Analyte (1.0 mg/mL stock)	Aliquot (mL)	
1. Mephedrone	0.050	
2. Methylone	0.050	
3. MDPV	0.050	
i. Add components to		50 mL volumetric flask
ii. Add	9.9	Methanol (Optima)
iii. QS	50	Water (Optima)
Store in glass at <0ºC. Stable for at least 1 year.		

5.6.2.2.1 Control Scheme

Control Level (ng/mL)	Blood Volume (µL)	Control Working Solution Spike Volume (1 µg/mL) (µL)	
		SMA	SC
		A	B
0	500	0	0
60	500	30	30
600	500	300	300

SMA and SC Control preparations should be done separately.

5.6.2.3 Calibration

A. SMA Calibration Working Solution (5 µg/mL)

Analyte (1.0 mg/mL stock)	Aliquot (mL)	
1. Ephedrine	0.250	
2. Amphetamine	0.250	
3. Methamphetamine	0.250	
4. MDA	0.250	
5. MDMA	0.250	
6. MDEA	0.250	
i. Add components to		50 mL volumetric flask
ii. Add	8.5	Methanol (Optima)
iii. QS	50	Water (Optima)
Store in glass at <0ºC. Stable for at least 1 year.		

B. SMA Calibration Working Solution (0.5 µg/mL)

Analyte (1.0 mg/mL stock)	Aliquot (mL)	
1. Ephedrine	0.025	
2. Amphetamine	0.025	
3. Methamphetamine	0.025	
4. MDA	0.025	
5. MDMA	0.025	
6. MDEA	0.025	
i. Add components to		50 mL volumetric flask

ii. Add	9.9	Methanol (Optima)
iii. QS	50	Water (Optima)
Store in glass at <0ºC. Stable for at least 1 year.		

C. SC Calibration Working Solution (5 µg/mL)

Analyte (1.0 mg/mL stock)	Aliquot (mL)	
1. Mephedrone	0.25	
2. Methylone	0.25	
3. MDPV	0.25	
i. Add components to		50 mL volumetric flask
ii. Add	9.25	Methanol (Optima)
iii. QS	50	Water (Optima)
Store in glass at <0ºC. Stable for at least 1 year.		

D. SC Calibration Working Solution (0.5 µg/mL)

Analyte (1.0 mg/mL stock)	Aliquot (mL)	
1. Mephedrone	0.025	
2. Methylone	0.025	
3. MDPV	0.025	
i. Add components to		50 mL volumetric flask
ii. Add	9.9	Methanol (Optima)
iii. QS	50	Water (Optima)
Store in glass at <0ºC. Stable for at least 1 year.		

5.6.2.3.1 Calibration Scheme

Cal Level (ng/mL)	Blood Volume (µL)	Calibration Solution Spike Volume (µL)			
		SMA		SC	
		A	B	C	D
25	500	0	25	0	25
50	500	0	50	0	50
75	500	0	75	0	75

100	500	0	100	0	100
250	500	25	0	25	0
500	500	50	0	50	0
750	500	75	0	75	0

SMA and SC Calibrators should be prepared separately.

5.6.2.4 Performance Check

A. Column Performance Evaluation Mix (1 µg/mL)

The current working control solution is used as the performance check.

6 PROCEDURE

This procedure may be used for SMA or SC analysis depending upon the case scenario.

	Step	Note	Reference/Lot
	A. Samples (duplicate for quantitative exams)		
	1. To labeled 16 x 100 mm screw-top tubes add:		
☐	i. 0.5 mL of biological fluid		
☐	a. Add 0.2 mL of deionized water		
☐	ii. 1 g of a prepared tissue homogenate		
	B. Controls (Section 5.6.2.2.1)		
☐	1. Prepare Negative Control(s)		
☐	2. Prepare Positive Control(s) (duplicate for quantitative exams)		
	i. SMA Control Working Solution		
	ii. SC Control Working Solution		
	C. Calibrators (Section 5.6.2.3.1)		
☐	1. SMA Calibrators		
	i. Calibration Solution A		
	ii. Calibration Solution B		
☐	2. SC Calibrators		
	i. Calibration Solution C		
	ii. Calibration Solution D		
	D. Internal Standard(s)		
	1. Add 50 µL of Internal Standard Working Solution		
☐	i. SMA IS Working Solution		
☐	ii. SC IS Working Solution		
	Results in 200 ng/mL internal standard as prepared.		
	E. Adjust pH		
☐	1. Add 0.2 mL of 4% sodium hydroxide		
☐	2. Vortex		
	F. Extract		
☐	1. Add 2mL hexane to each tube		

☐	2. Rotate for 20 minutes		
☐	3. Centrifuge 10 minutes at 3000 rpm		
	i. If emulsions develop, break up with wooden stick and recentrifuge		
☐	4. Transfer organic (top) layer to a 12 x 75 mm tube		
☐	5. Add 0.1 mL of 4:1 Methanol:Hydrochloric acid		
☐	6. Vortex		
	G. Concentrate		
☐	1. Evaporate to dryness under nitrogen at 40°C		
	H. Reconstitute		
☐	1. Add 100 µL of Methanol:Water (10:90) 2. Vortex		
☐	**I. Instrumental Analysis** 1. LC/MS: analyze 10 µL i. Analyze LC/MS Performance Standard prior to batch analysis		
	ii. Mobile Phase 1 (aqueous)		
	iii. Mobile Phase 2 (organic)		
	iv. LC Column		

7 ANALYTICAL PARAMETERS

7.1 Shimadzu HPLC

7.1.1 Gradient

Time (min)	Mobile Phase %		Flow Rate
	1-Aqueous	2-Organic	(mL/min)
0	92.5	7.5	0.3
5	92.5	7.5	0.3
20	40	60	0.3
23	40	60	0.3
28	92.5	7.5	0.3
32	92.5	7.5	0.3

7.1.2 Conditions

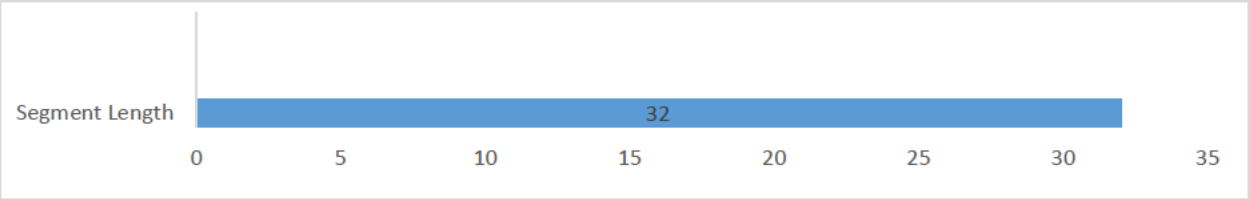
Column Heater (°C)	40
Autosampler (°C)	15
Run Time (min)	32

7.2 Thermo LTQ Orbitrap XL

7.2.1 Source

Mode	ESI
Polarity	(+)
Spray Voltage (kV)	5
Capillary Temperature (°C)	250
Sheath Gas	25
Aux Gas	10
Sweep Gas	0

7.2.2 Segment(s)



7.2.3 *Scan Events*

Event	Mode	Range (m/z)	Details	Isolation Width (m/z)	Collision Energy (rel)	Analyzer	Resolution
1	Full Scan	125-350				ITMS	unit
2	MS^2 DDS	Software control	Most intense ion from Event 1* Exclusion (m/z): 141, 155, 169, 181, 185, 199, 211, 213, 284; threshold 1000 counts	2.0	70	ITMS	unit
3	MS^3 DDS	Software control	Most intense neutral loss from Event 2* Inclusion (m/z): neutral loss of 17, 18, 31, or 45; threshold = 1000 counts	2.0	70	ITMS	unit

*Events may include fewer masses for targeted analysis; events 2 and 3 are optional if MSn has already been performed.

7.2.4 *Dynamic Exclusion*

Repeat Count	10	**Repeat Duration (s)**	30
Exclusion List Size	25	**Exclusion Duration (s)**	30
Expiration Count	5	**Expiration Threshold (S/N)**	<5
Exclusion Width (m/z)	-1 to 2		

8 DATA ANALYSIS

8.1 Decision Criteria

8.1.1 LC/MS Performance Standard

In addition to the performance checks specified in the LC/MS standard operating procedure, a performance standard mix is analyzed through the analytical column to monitor the performance of the column.

8.1.1.1 Chromatography

The analyte's molecular ion traces shall:

A. Have reasonable peak shape (varies by analyte)
B. Compare favorably to the previous analysis of the standard using the same Equipment
    1. Retention times ±0.6 min
    2. Responses 50-200%

8.1.1.2 Mass Spectrometry

The analyte mass assignments shall be present:

Analyte	Unit Mass
MBDB	208
Ephedrine	166
Amphetamine	136
Methamphetamine	150
Phentermine	150
MDA	180
MDMA	194
MDEA	208

8.1.2 Batch Acceptance

A. Negative Control
No target analytes are detected.
B. Positive Control
Qualitative: Target analytes are detected.
Quantitative: Within ±20% of the target value
C. Internal Standards for Controls
The controls meet the recovery criteria from 8.1.3.1

8.1.3 Unknown Sample Acceptance

8.1.3.1 Internal Standard Recovery

The internal standards are detected.

8.1.4 Unknown Sample Compound Identification

In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard or extracted Positive Control.

8.1.4.1 Chromatography

The peak of interest will show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample will compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs.

Additionally, the following two criteria should be met.

8.1.4.1.1 LC Retention Time

The retention time of the peak will be within 5% of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard, Calibrator, or extracted Positive Control.

8.1.4.1.2 Signal-to-Noise

To justify the existence of a peak, its signal to noise ratio will exceed 3. Further, the baseline signal for the peak of interest will be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or solvent blank injected just prior to the sample.

8.1.4.2 Mass Spectrometry

The mass spectrum of the analyte of interest will compare favorably to a reference standard, extracted calibrator, or an extracted Positive Control. See the Guidelines for Comparison of Mass Spectra (TOX104) for further guidance.

8.1.4.2.1 Data Dependent Analysis

Mass spectral fragments of all SMAs tested in validation and found to extract via this procedure are listed in below table. In most circumstances the MS^2 and MS^3 (when present) spectra in an unknown sample should have all the same significant ions as the spectra of the known analyte in a contemporaneously analyzed standard, control, or calibrator, and should not have any significant ions not present in the known spectrum. Additionally, for any compound in the table with two primary ions listed for a given spectral level, the intensity ratio for those ions should meet the requirements given in TOX104.

Compound Name	Precursor from Full Scan MS	Primary MS^2 Product Ion(s)	Primary MS^3 Product Ions(s)
Amphetamine	136	119	91
Cathinone	150	132	117
Methamphetamine	150	119	91

Phentermine	150	133	91
Phenylpropanol amine	152	134	117
Ethylamphetamine	164	119	91
Methcathinone	164	146	131
(pseudo)Ephedrine	166	148	133, 117
PMA	166	149	121
Benzylpiperazine	177	91, 85	not triggered
Propylamphetamine	178	119, 91	not triggered
Mephedrone	178	160	not triggered
MDA	180	163	135, 133
PMMA	180	149	121
2C-H	182	165	150
Dimethoxyphenethyl amine	182	165	150
4-MTA	182	165	137, 117
BDB	194	177	147, 133
MDMA	194	163	135, 133
Dimethoxy-amphetamine	196	179	151
Chlorophenyl piperazine	**197**, 199	154	not triggered
Methylone	208	190, 160	not triggered
MBDB	208	177	135
MDMA	208	163	135, 133
MDEA	208	163	135, 133
DOM	210	193	178,156
Mescaline	212	195	180
DOET	224	207	192, 179
Trimethoxy-amphetamine	226	209	194, 181
Trifluoromethyl phenylpiperazine	231	188	not triggered
Fenfluramine	232	**187**,159	159
Methylphenidate	234	84	not triggered
2-CT-2	242	225	210,164
2-CT-4	256	239	197
2-CT-7	256	239	224, 197, 164
2C-B	**260**, 262	243	228, 164
DOB	**274**, 276	257	229, 178
MDPV	276	205, 175, 126	not triggered
2C-I	308	291	276, 164

8.2 Calculations

8.2.1 Calibration

Model	Linear
Weighting	$1/x^2$

Refer to TOX-101 for further guidance.

8.2.2 Software

Quantitative and qualitative calculations may be performed by one or more of the following software packages:

A. Thermo Xcalibur
    1. QualBrowser
    2. QuanBrowser
    3. Tracefinder
B. Microsoft
    1. Excel

9 Reporting

9.1 Measurement Uncertainty

Refer to CHEM-100 and TOX-101.

10 Corrective Measures

Refer to Quality Control for Toxicology Examinations (TOX-101) for guidance on action steps in the event of a quality control failure.

At concentrations below approximately 25 ng/mL, some analytes may show a strong signal in full MS extracted ion chromatograms, but show no tandem MS signal due to the interaction of data dependent scan conditions and dynamic exclusion parameters. If there is good reason to suspect that this has happened, the questioned sample should be reinjected with scan event #2 changed to target only the ion(s) of interest and dynamic exclusion disabled.

11 Performance Characteristics

Compound	LOD in Blood (ng/mL)	LOD in Urine (ng/mL)	LLOQ (ng/mL)	Linear Range (ng/mL)	Accuracy (average % bias)	Precision (average % intermediate)
Amphetamine	5	5	25	25-750	+5.7	4.8
Methamphetamine	5	5	25	25-750	+0.6	4.2
(pseudo)Ephedrine	10	10	25	25-750	+1.9	3.5
MDA	5	5	25	25-750	+5.1	4.5
MDMA	5	5	25	25-750	+3.9	3.9
MDEA	5	5	25	25-750	-0.7	5.2
Methylone	25	10	25	25-750	-10.9%	3.7

Mephedrone	25	10	25	25-750	-11.5%	3.8
MDPV	25	2	25	25-750	-3.4%	2.0

11.1 Carryover

High analyte concentrations in samples may carryover into subsequent samples. Analysts should investigate evidence for carryover if high sample analytes loads are encountered.

12 Limitations

A. This procedure is not able to distinguish between:
    1. Different optical isomers of SMAs
    2. Ephedrine and pseudoephedrine
    3. 4-chlorophenylpiperazine and 3-chlorophenylpiperazine
B. The following phenethylamine-group compounds were tested and found to not be extractable via this procedure:
        1. HMA (hydroxymethoxyamphetamine)
        2. HHMA (hydroxymethamphetamine)
        3. HMMA (hydroxymethoxymethamphetamine)
        4. Salbutamol
C. Grossly decomposed or putrefied samples may affect both detection and quantitation limits
D. High levels of PMMA may interfere with accurate quantitation of MDA
E. High levels of BDB may interfere with accurate quantitation of MDMA

13 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the FBI Laboratory Safety Manual for guidance.

14 Revision History

Revision	Issued	Changes
04	02/11/2022	Complete document reformat. Changed "bath salts" to "synthetic cathinones/SC" throughout 1, 3, 4 – simplified and clarified phrases 4 – removed specimen volume variability 5 – reformat Equipment for more categorization 6 – Procedure changed to checklist format 7 – Reformatted Instrument parameters 8.2 – provided more specifics on data analysis/calculations 11.1 – added carryover phrase