VALIDATION AND VERIFICATION OF AN ANALYTICAL METHOD TO IDENTIFY AND QUANTIFY SELECTED AMPHETAMINE-RELATED DRUGS IN WHOLE BLOOD

AHMAD ALAMIR

INTRODUCTION

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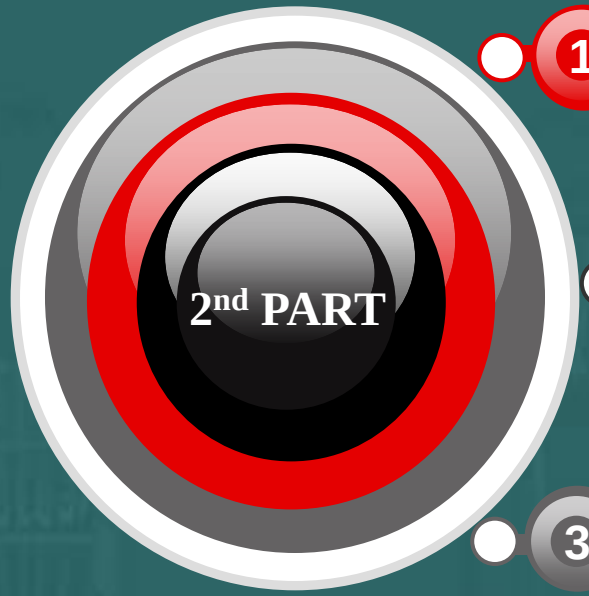

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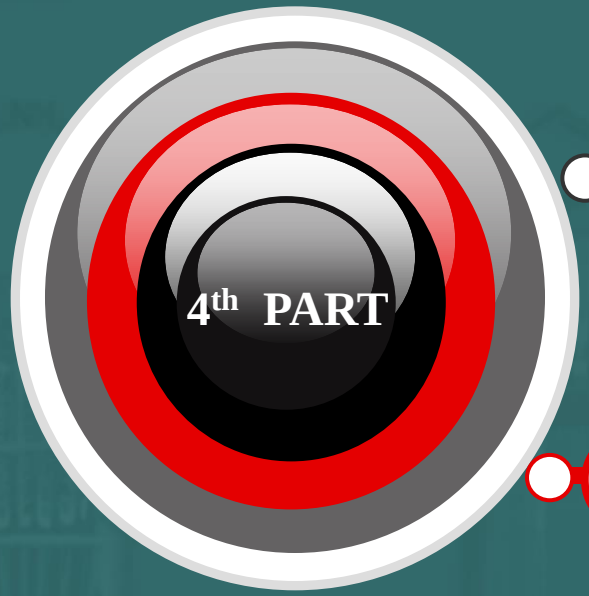

1. Future Work
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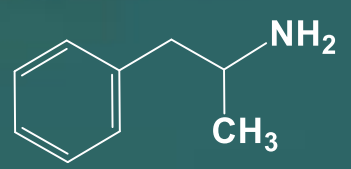
Project's Goals

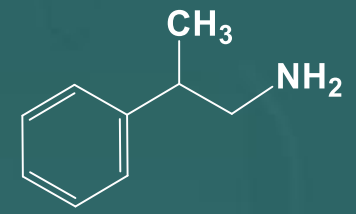
Validation & Verification of an Analytical Method for ARDs

What are ARDs?

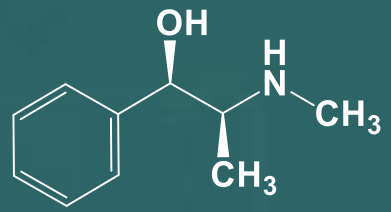
Amphetamine related-drugs (ARDs) is a class of compounds compose of a phenyl ring connected to amine group through a two carbon side chain bearing a methyl group



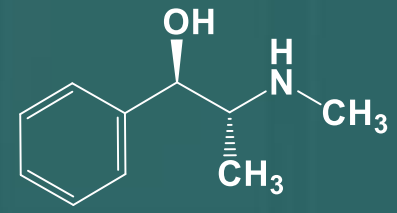
Amphetamine
Nominal Mass: 135 Da



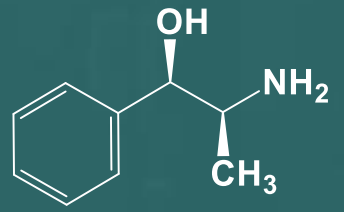
β-methylphenethylamine
Nominal Mass: 135 Da



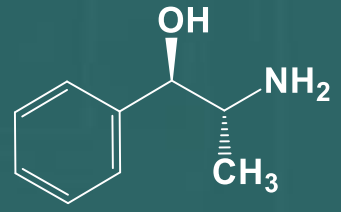
Ephedrine
Nominal Mass: 165 Da



Pseudoephedrine
Nominal Mass: 165 Da



Norephedrine
Nominal Mass: 151Da

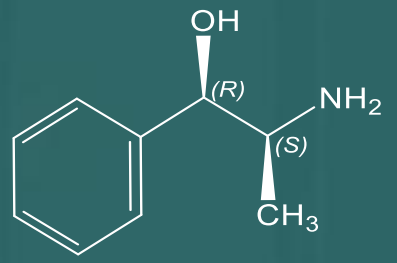
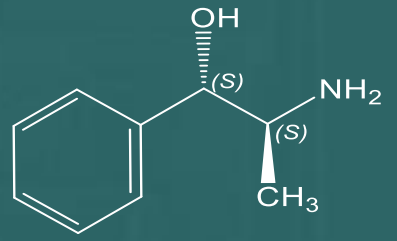
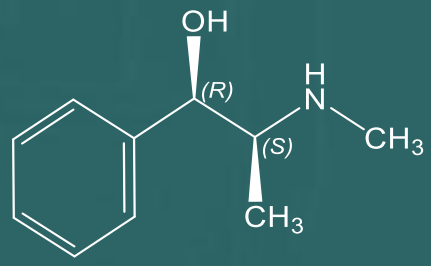


Cathine
Nominal Mass: 151Da

Figure 1: Analytes' Chemical Structure

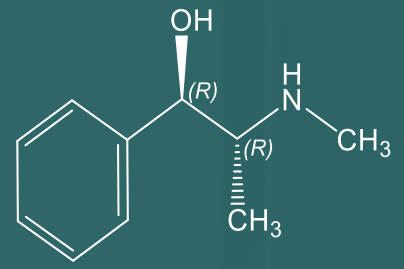
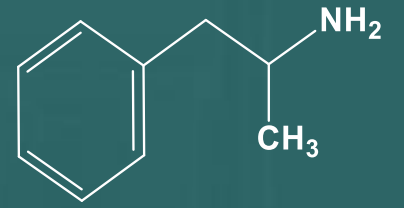
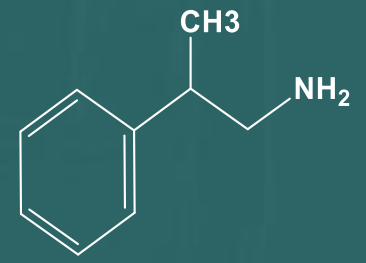
Analytes

Table 1: Amphetamine-related Drugs

No	Analyte	Pharmacological Action	Metabolites	Occurrence	Chemical Structure
1	Norephedrine (NEPH)	Sympathomimetic Amine	4-Hydroxynorephedrine	*Genius Ephedra*	
2	Cathine (CAT)	Sympathomimetic Amine	4-Hydroxycathine	*Genius Ephedra* *Catha Edulis*	
3	Ephedrine (EPH)	Sympathomimetic Amine ά and β-adrenergic receptors agonist	Norephedrine	*Genius Ephedra*	

1-Baselt, R. (2004) Disposition of toxic drugs and chemicals in man. Biomedical publicationsl, Foster City, California.

Analytes cont'd

No	Analyte	Pharmacological Action	Metabolites	Occurrence	Chemical Structure
4	Pseudoephedrine (PEPH)	Sympathomimetic Amine ά and β-adrenergic receptors agonist	Cathine	*Genius Ephedra*	
5	Amphetamine (AMP)	Sympathomimetic Amine Dopamine releasing Agent Dopamine Receptor Agonist inhibit DA reuptake	4-Hydroxynorephedrine 4-Hydroxyamphetamine Norephedrine	*Synthetic*	
6	β–methylphenethylamine (BMP)	Sympathomimetic Amine Dopamine releasing Agent Dopamine Receptor Agonist inhibit DA reuptake	4-Hydroxyβ–methylphenethylamine* 1-amino-2-phenylpropane-2-ol* 4-(1-amino-2-hydroxypropan-2-yl) phenol*	*Synthetic*	

1-Baselt, R. (2004) Disposition of toxic drugs and chemicals in man. Biomedical publicationsl, Foster City, California.

Why These Drugs?

- Amphetamine is one of the most commonly abused drugs in Saudi Arabia in the form of Captagon pills.
- Captagon is a market name for fenethylline which is a conjugate of amphetamine and theophylline
- Roughly one billion Captagon tablets have been sized by Saudi Anti-Drug Enforcement Agency in the last 8 years.
- Khat (Qatt) is a flowering plant that is chewed for its stimulant effect. Khat contains cathinone, cathine and norephedrine.
- Khat is the most commonly abused plant in my town "Jazan" for its euphoria effect.

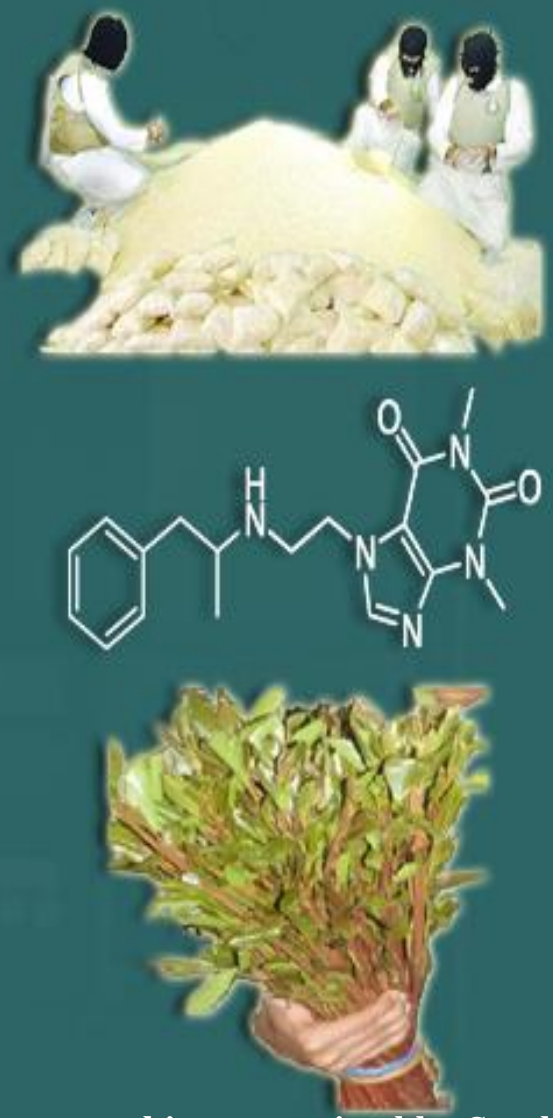


Figure 2: Captagon shipment seized by Saudi anti-drug enforcement (top). Fenethylline chemical structure (middle). Khat plant (bottom)

2. http://www.arabnews.com/node/979806/saudi-arabia

METHODOLOGY

MMSPE

Drug-free Whole Blood Sample

- 250 ul of blood fortified with the Stock working solution
- Adjusted pH with 1 mL of 0.1 M HCl
- Precipitate with 1 mL of ACN

Centrifuge 4000 rpm 15 min

Supernatant extracted by MMSPE

Eluent evaporates to dryness

Evaporated residue reconstituted in MPA

UPLC-qTOF MS

MMSPE Sample Extraction

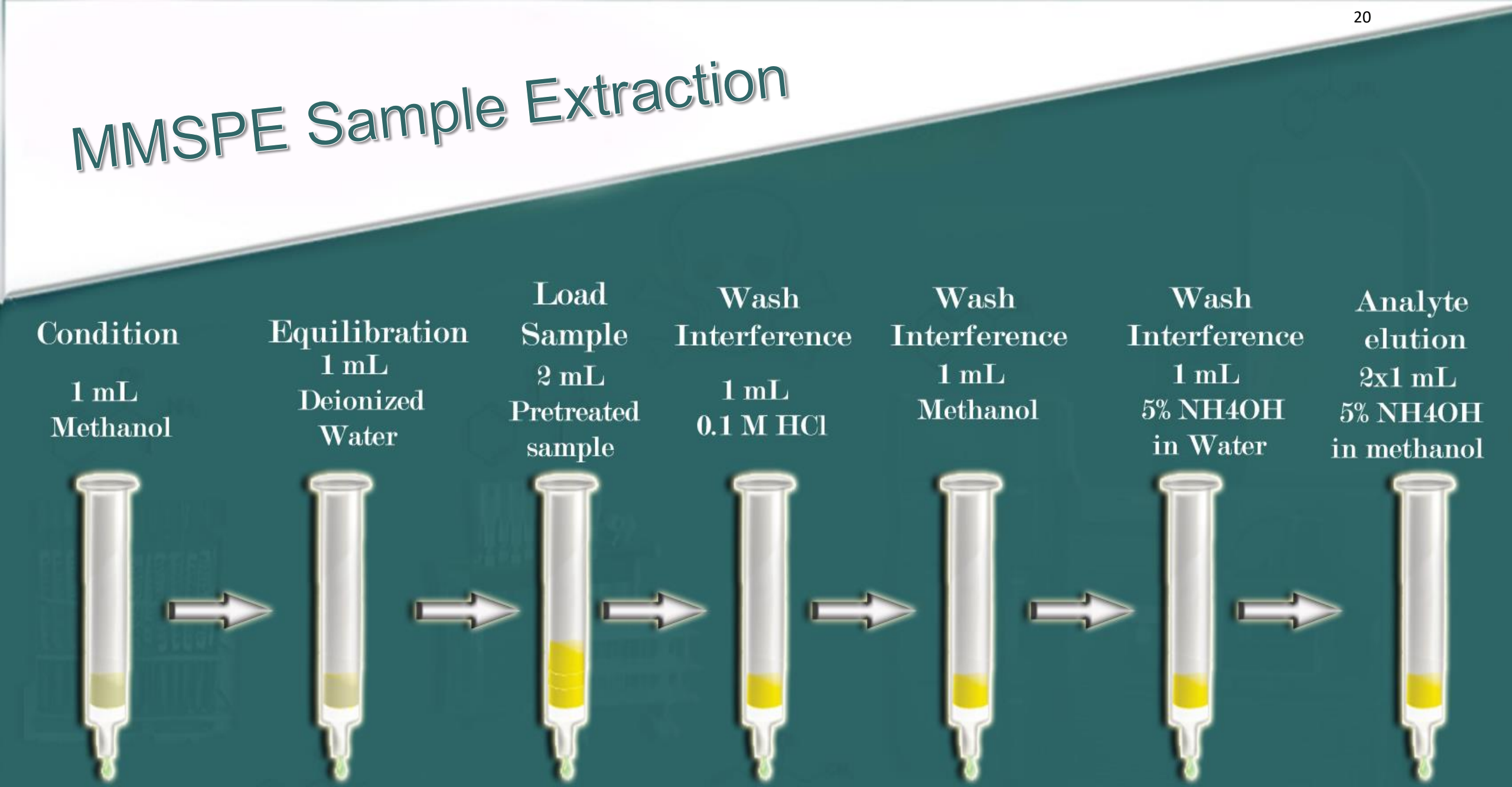


Figure 4: Schematic diagram of the extraction process of the analytes by using MMSPE

LC Condition

- Ultra Performance Liquid Chromatography (UPLC) with a binary mobile phase system equipped with HSS T3 column (2.1 mm x 100 mm, 1.8 um)

- UPLC was run as pseudo-isocratic for 9 minutes (5 mM ammonium formmate, 0.1 formic acid in 100 to 95:5 water:acetonitrile) for baseline resolution of the analytes

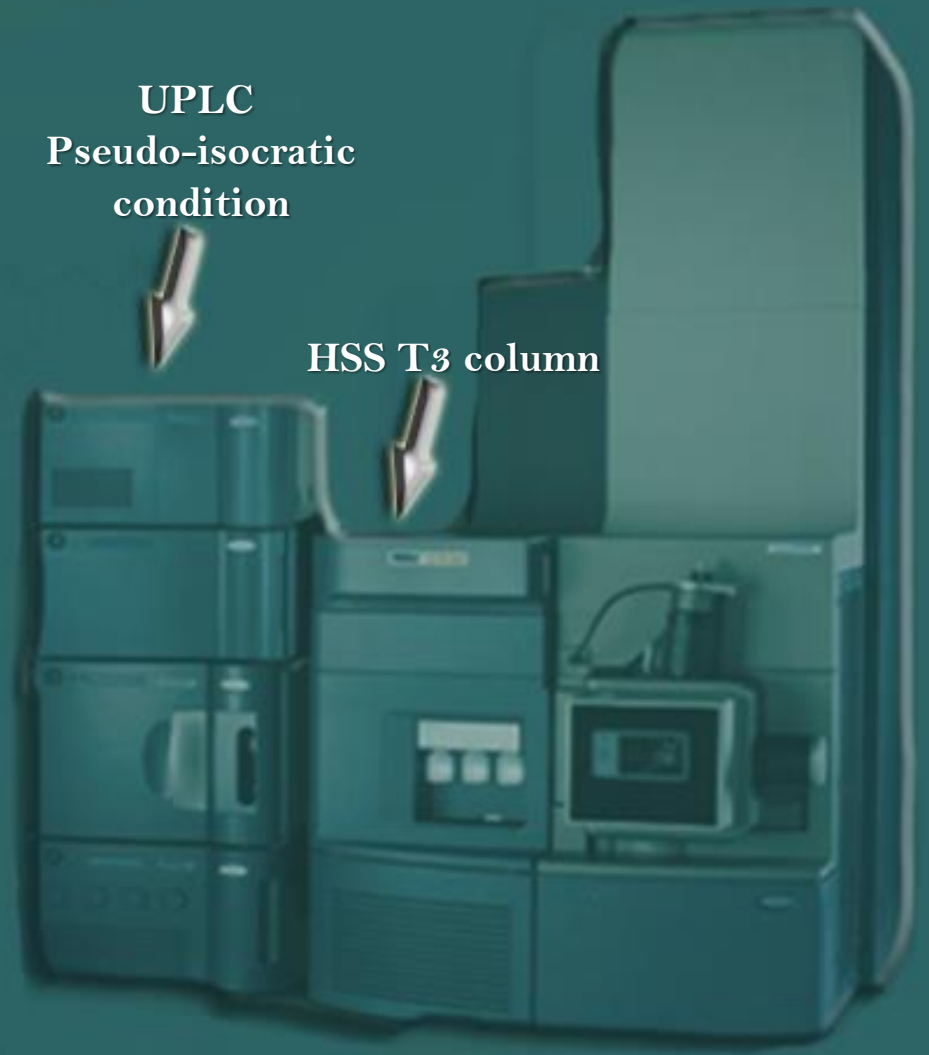


Figure 6: UPLC –qTOF MS
Adopted from http://www.waters.com

Chromatograms

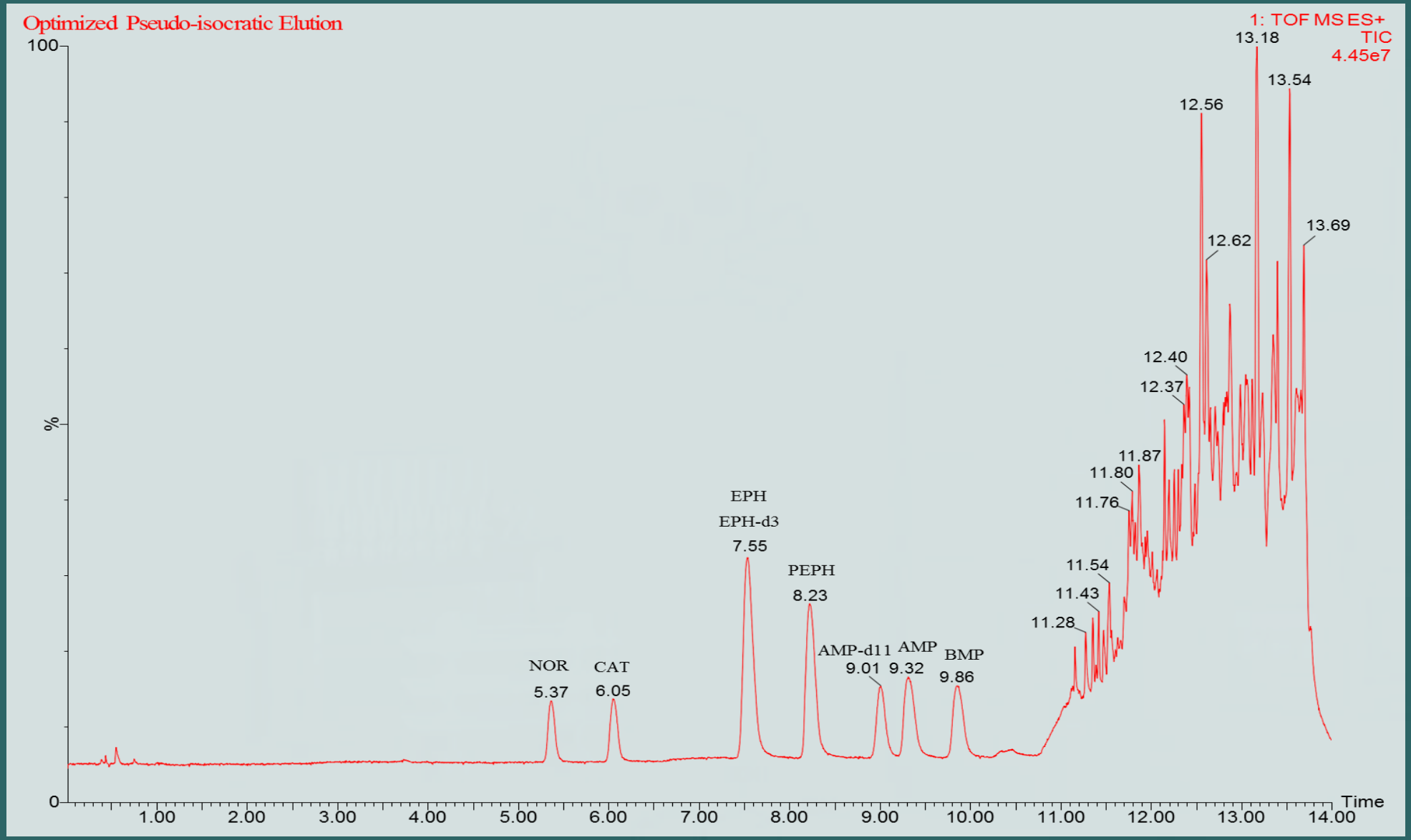


Figure 7: Total ion chromatogram of ARDs by optimized pseudo-isocratic elution

MS Settings

- Mass Spectrometry was performed by Quadrupole Time of Flight Mass (qTOF MS) using positive electrospray ionization and MS^E acquisition mode.
- MS^E Is an acquisition mode in q-TOF MS that allows for acquisition of two accurate full mass spectra sequentially.

Figure 6: UPLC –qTOF MS

9. T.G. Rosano, M. Wood, K. Ihenetu, T.A. Swift, Drug screening in medical examiner casework by high-resolution mass Spectrometry (UPLC-MSE-TOF), Journal of Analytical Toxicology. 37 (2013) 580–593. doi:10.1093/jat/bkt071

MS Settings

- The first mass spectrum is acquired without applying collision energy (LE) in the collision cell.
- This spectrum provides information about the intact molecule (the molecular ion)
- The second spectrum is acquired by applying collision energy (HE) in the collision cell.
- This spectrum provides information about the fragmented ions

Figure 6: UPLC –qTOF MS
Adopted from http://www.waters.com

Mass Spectra

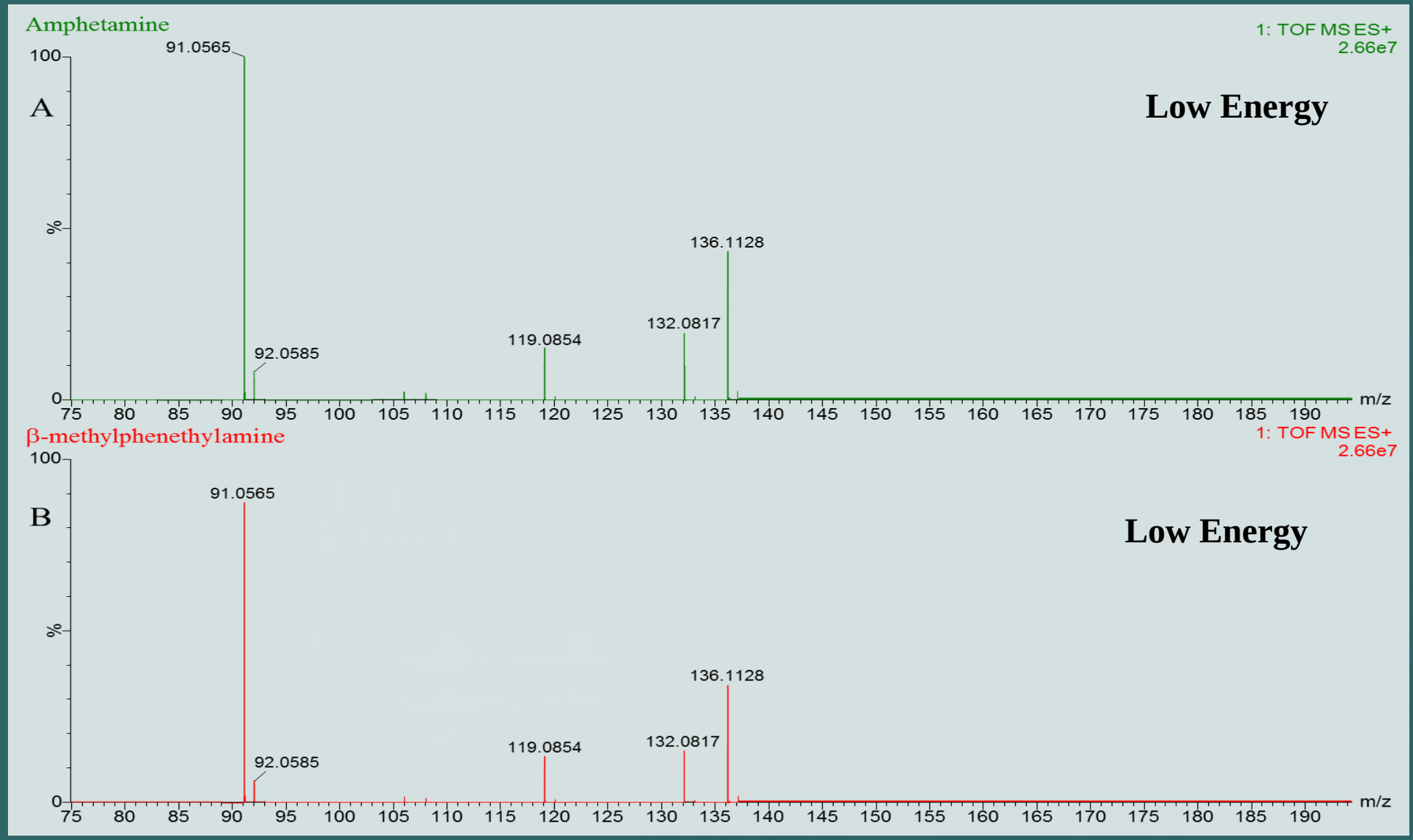


Figure 8: Mass spectra for the 136.11 m/z molecular ions of (A) amphetamine and (B) β-methylphenethylamine at low collision energy

Mass Spectra

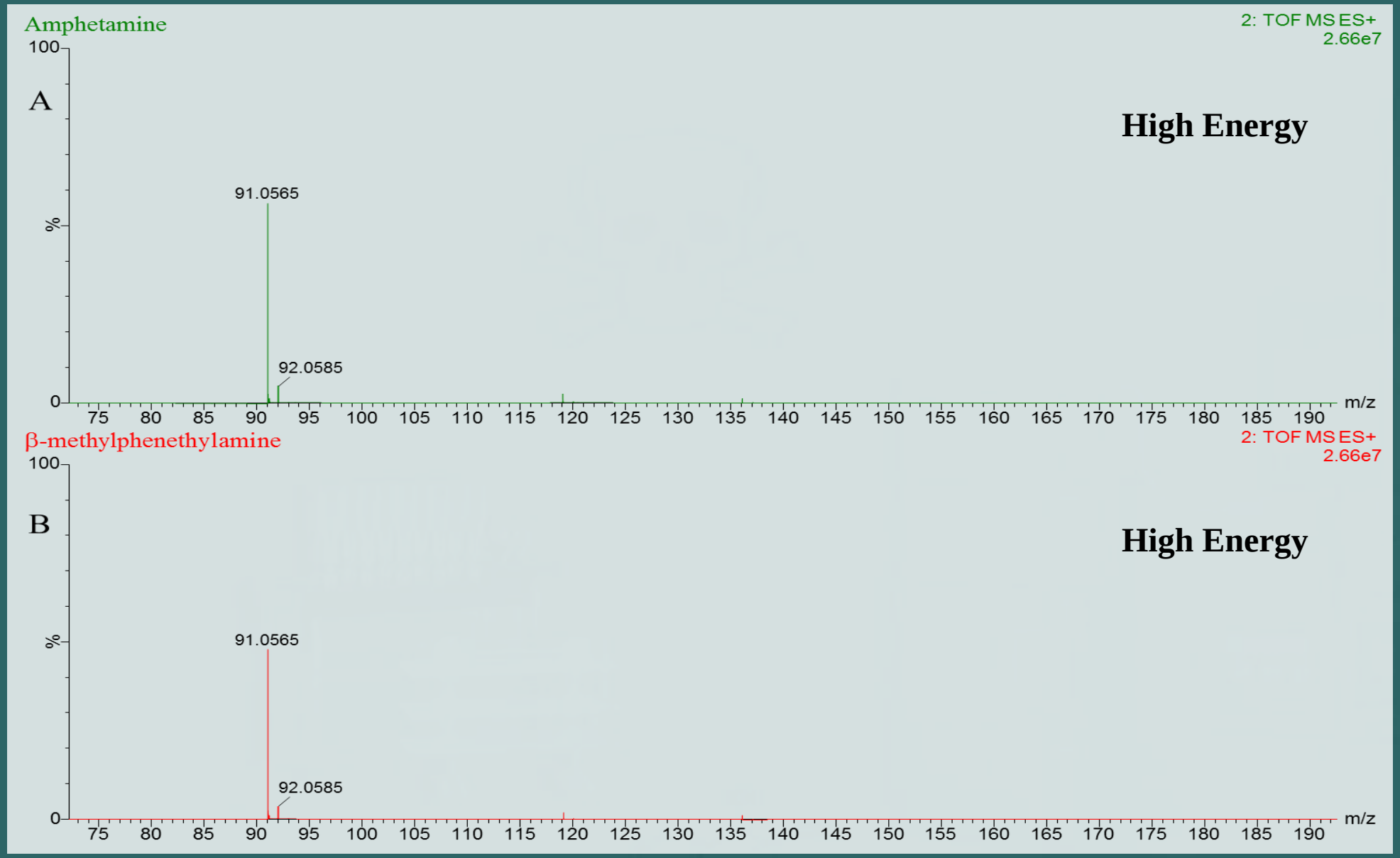


Figure 9: Mass spectra for the 136.11 m/z molecular ions of (A) amphetamine and (B) β-methylphenethylamine at high collision energy

Validated Analytical Method by MMSPE

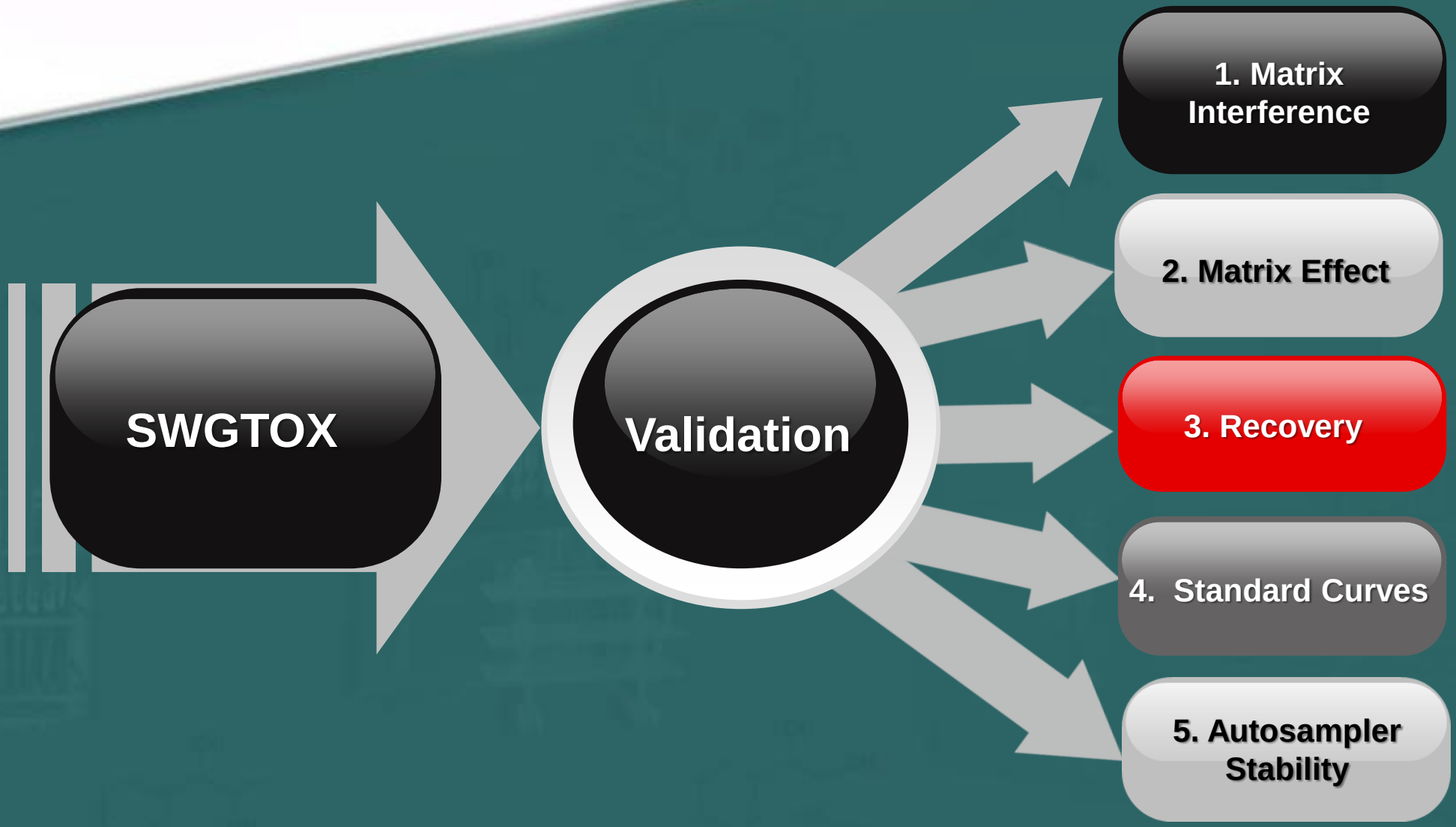
Analytes Parameters

Table 7: Analyte Parameters under Optimized MMSPE and UPLC-qTOF-MS

No	Compound	Ionisation Mode	Chemical Formula	Molecular Ion (m/z)	Fragmented Ions (m/z) (± 0.01 mD)	Retention Time (min) (± 0.05 min)
1	Amphetamine-	Positive		147.1938	98.1078* / 130.1653	8.76
2	Ephedrine-	Positive		169.1568	136.1195 / 151.1433*	7.30
3	Norephedrine	Positive		152.1180	115.0736 / 117.0736 / 134.0975*	5.21
4	Cathine	Positive		152.1180	115.0736 / 117.0736 / 134.0975*	5.90
5	Ephedrine	Positive		166.1378	115.0556 / 117.0713 / 148.1140*	7.31
6	Pseudoephedrine	Positive		166.1378	115.0556 / 117.0713 / 148.1140*	8.00
7	Amphetamine	Positive		136.1219	91.0553 / 119.0868*	9.05
8	β–methylphenethylamine	Positive		136.1219	91.0553 / 119.0868*	9.58

*Quantifier ions

Validation Stage



Matrix Interference Results

Table 8: Evaluation of matrix interferences in five drug-free whole blood matrices after extraction.

Number	Whole Blood Matrix	Result
1	Bovine	No interference
2	Sheep	No interference
3	Human Sample 1	No interference
4	Human Sample 2	No interference
5	Human Sample 3	No interference

Matrix Effect Evaluation

Table 9: Evaluation of matrix effects in five drug-free whole blood matrices after extraction

Number	Whole Blood Matrix	Result
1	Bovine	< ±25%
2	Sheep	< ±25%
3	Human Sample 1	< ±25%
4	Human Sample 2	< ±25%
5	Human Sample 3	< ±25%

Matrix Effect Evaluation

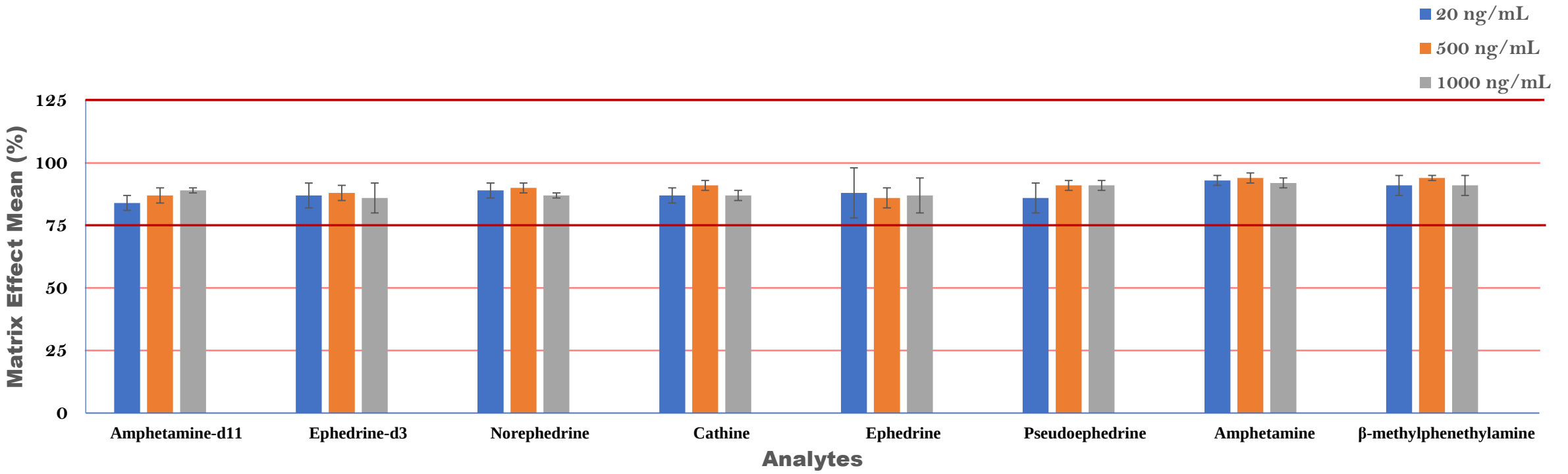


Figure 22: Matrix effects (%) measured in extracts of aged bovine whole blood spiked with amphetamine-related drugs, including two deuterated analogues, at three different concentration levels (20, 500 and 1000 ng/mL). The data shown represent the mean of triplicate analysis, error bars represent the standard error of the mean, and red lines represent acceptable limits of matrix effects.

Recovery Results

Table 10: Evaluation of recovery (%) of amphetamine-related drugs and deuterated analogues from aged bovine whole blood

Analyte	Concentration					
	Low 20 ng/mL	% CV	Medium 500 ng/mL	% CV	High 1000 ng/mL	% CV
Amphetamine—d_{11}	70 ± 3	4.29	70 ± 1	1.43	71 ± 2	2.82
Ephedrine—d_3	77 ± 4	5.19	77 ± 1	1.30	77 ± 4	5.19
Norephedrine	71 ± 3	4.23	72 ± 2	2.78	76 ± 1	1.32
Cathine	68 ± 3	4.41	71 ± 2	2.82	77 ± 1	1.30
Ephedrine	71 ± 4	5.63	75 ± 1	1.33	77 ± 7	9.09
Pseudoephedrine	68 ± 3	4.41	75 ± 4	5.33	90 ± 5	5.56
Amphetamine	70 ± 3	4.29	80 ± 1	1.25	80 ± 2	2.50
β-methylphenethylamine	65 ± 4	6.15	73 ± 1	1.37	75 ± 1	1.33

Recovery Evaluation Result

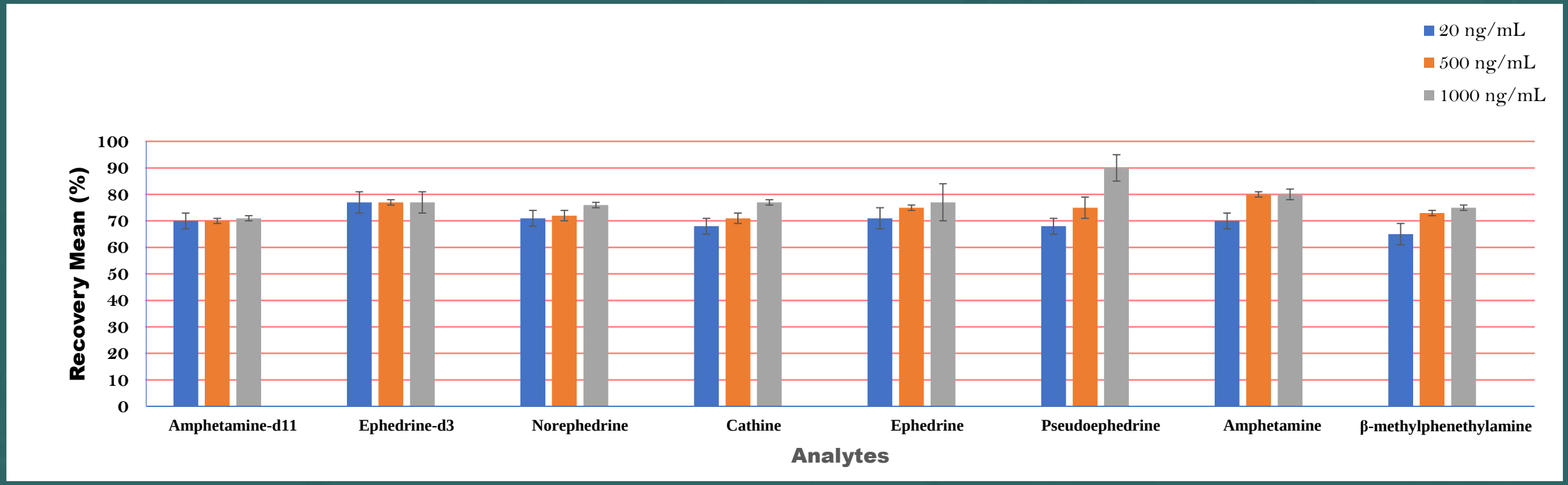


Figure 23: Recovery (%) of amphetamine-related drugs, including two deuterated analogues at three different concentrations from extract of spiked aged bovine whole blood. The data represent the mean of triplicate analysis, and error bars represent the standard error of the mean.

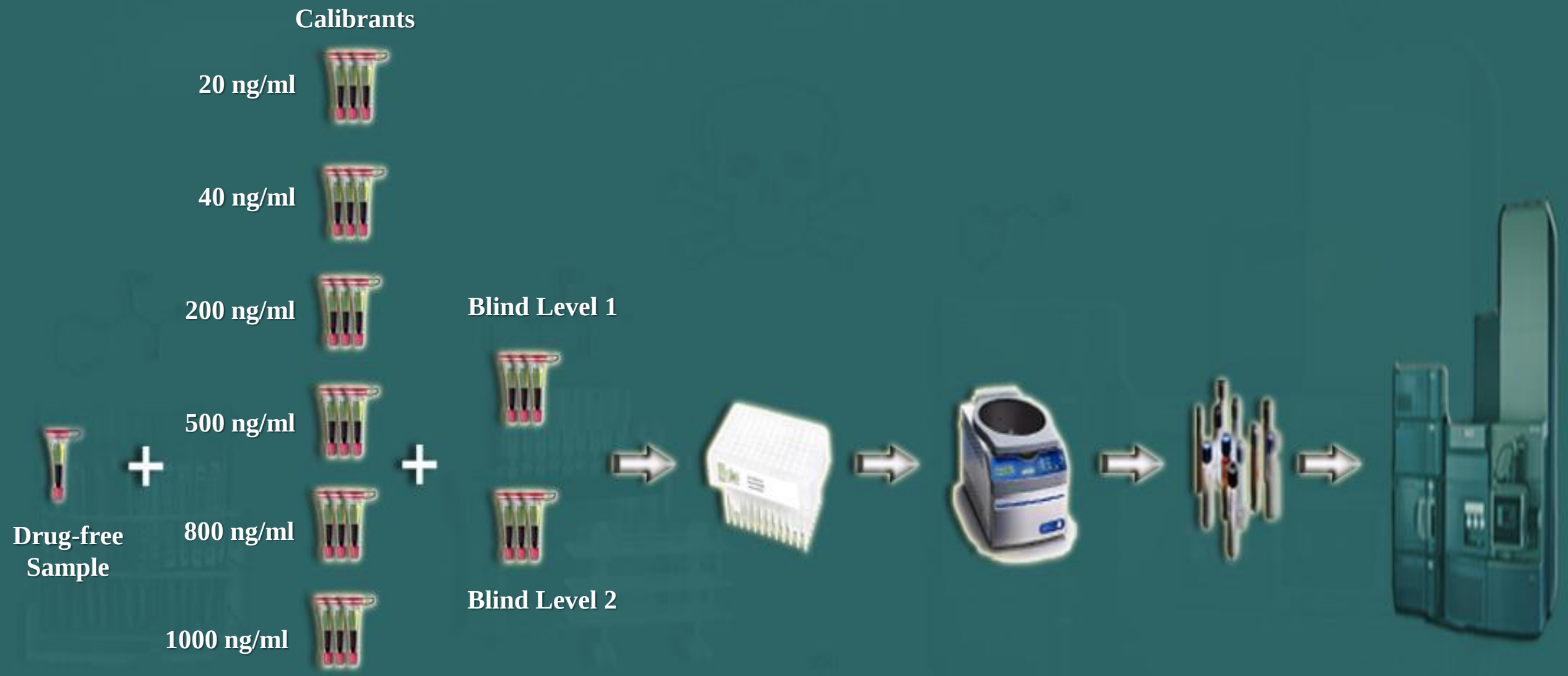
Carryover Evaluation

Carryover Result

- Carryover was evaluated by analysis of three drug-free aged animal WB extracts directly after analyzing the high concentration calibrator (1,000 ng/mL, n = 3) samples

- No carryover was observed upon visual inspection of the chromatograms and after the analysis of EICs.

Calibration Model Experiments



Calibration Model Results

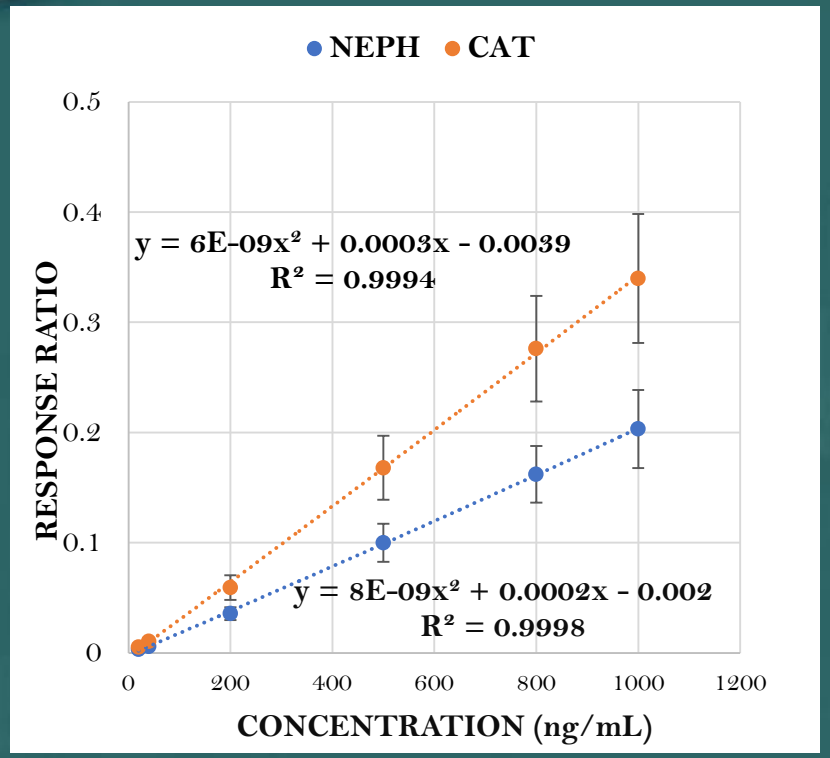


Figure 24: Averaged quadratic calibration curve of NEPH and CAT

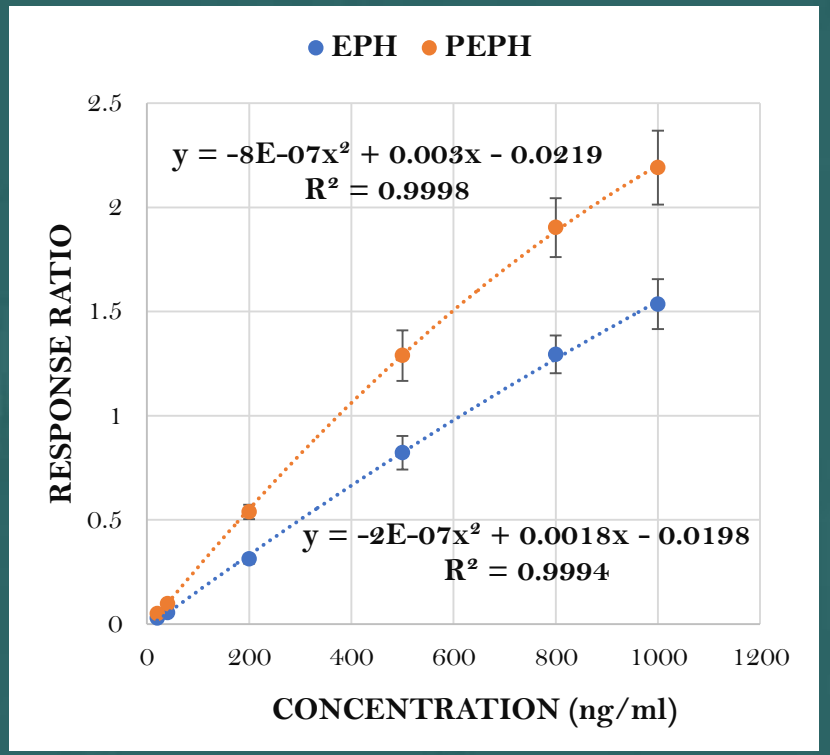


Figure 25: Averaged quadratic calibration curve of EPH and PEPH

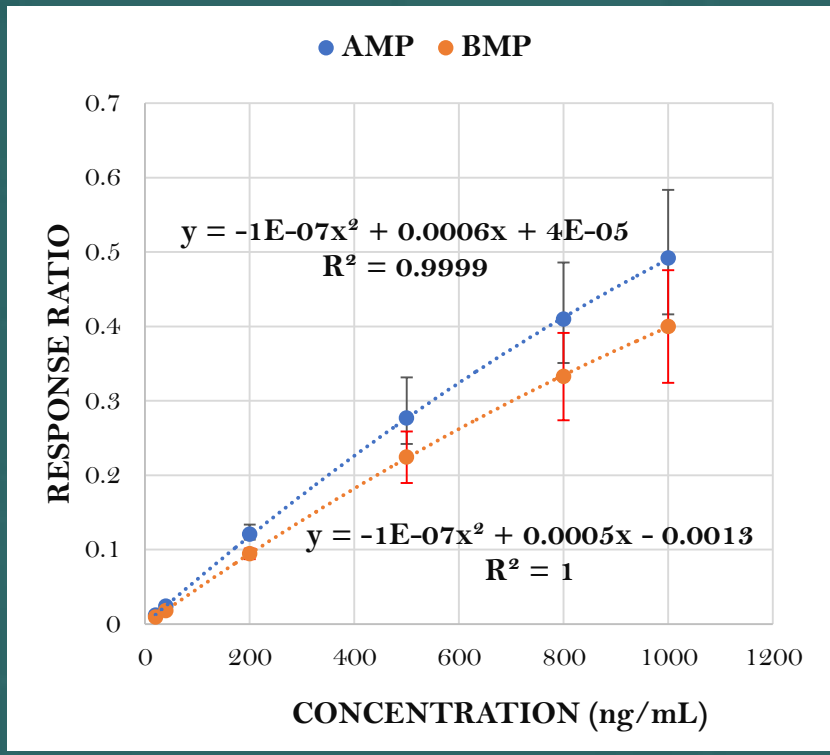


Figure 26: Averaged quadratic calibration curve of AMP and BMP

Calibration Model Results

Table 11: Averaged curve regression equations and correlation coefficients of the analytes in aged bovine whole blood

Analyte	Linearity ng/mL	Regression Equation	R^2
Norephedrine	20 – 1000	$y = 8\,x\,10^{-9}C^2 + 0.0002\,C - 0.002$	0.9998
Cathine	20 – 1000	$y = 6\,x\,10^{-9}C^2 + 0.0003\,C - 0.0039$	0.9994
Ephedrine	20 – 1000	$y = -2\,x\,10^{-7}C^2 + 0.0018\,C - 0.0198$	0.9994
Pseudoephedrine	20 – 1000	$y = -8\,x\,10^{-7}C^2 + 0.003\,C - 0.0219$	0.9998
Amphetamine	20 – 1000	$y = -1\,x\,10^{-7}C^2 + 0.0006\,C + 0.00004$	0.9999
β-methylphenethylamine	20 – 1000	$y = -1\,x\,10^{-7}C^2 + 0.0005\,C - 0.0013$	1

Calibration Model Results

Table 12: Summary of Analytical Performance Parameters

Drug	Limit of detection (LOD, ng/mL)	Limit of quantitation (LOQ, ng/mL)	Within-Run Precision (CV, %) (acceptance criteria: ≤20%) [# failed]	Between-Run Precision (CV, %) (acceptance criteria: ≤20%) [# failed]	Bias (%) (acceptance criteria:≤20%) [# failed]
Norephedrine	20	20	1.17–18.10 [0/90]	15.86–18.99 [0/30]	-4.72-18.25 [0/10]
Cathine	20	20	1.60–13.20 [0/90]	16.2–18.98 [0/30]	-5.00-15.00 [0/10]
Ephedrine	20	20	1.00–11.54 [0/90]	5.31–9.81 [0/30]	-6.90-8.40 [0/10]
Pseudoephedrine	20	20	1.06–12.01 [0/90]	3.50–9.38 [0/30]	-10.82-7.16 [0/10]
Amphetamine	20	20	1.00–18.3 [0/90]	7.95–19.70 [0/30]	-7.98-3.40 [0/10]
β-methylphenethylamine	20	20	1.50–15.78 [0/90]	6.60–18.95 [0/30]	-11.00-9.00 [0/10]

Autosampler Stability Evaluation

40 ng/ml

500 ng/ml

1000 ng/ml

Pretreated Samples

Extraction

Evaporation

Reconstitution

Analyzing at zero hrs

Analyzing at 12 hrs

Analyzing at 24 hrs

Analyzing at 36 hrs

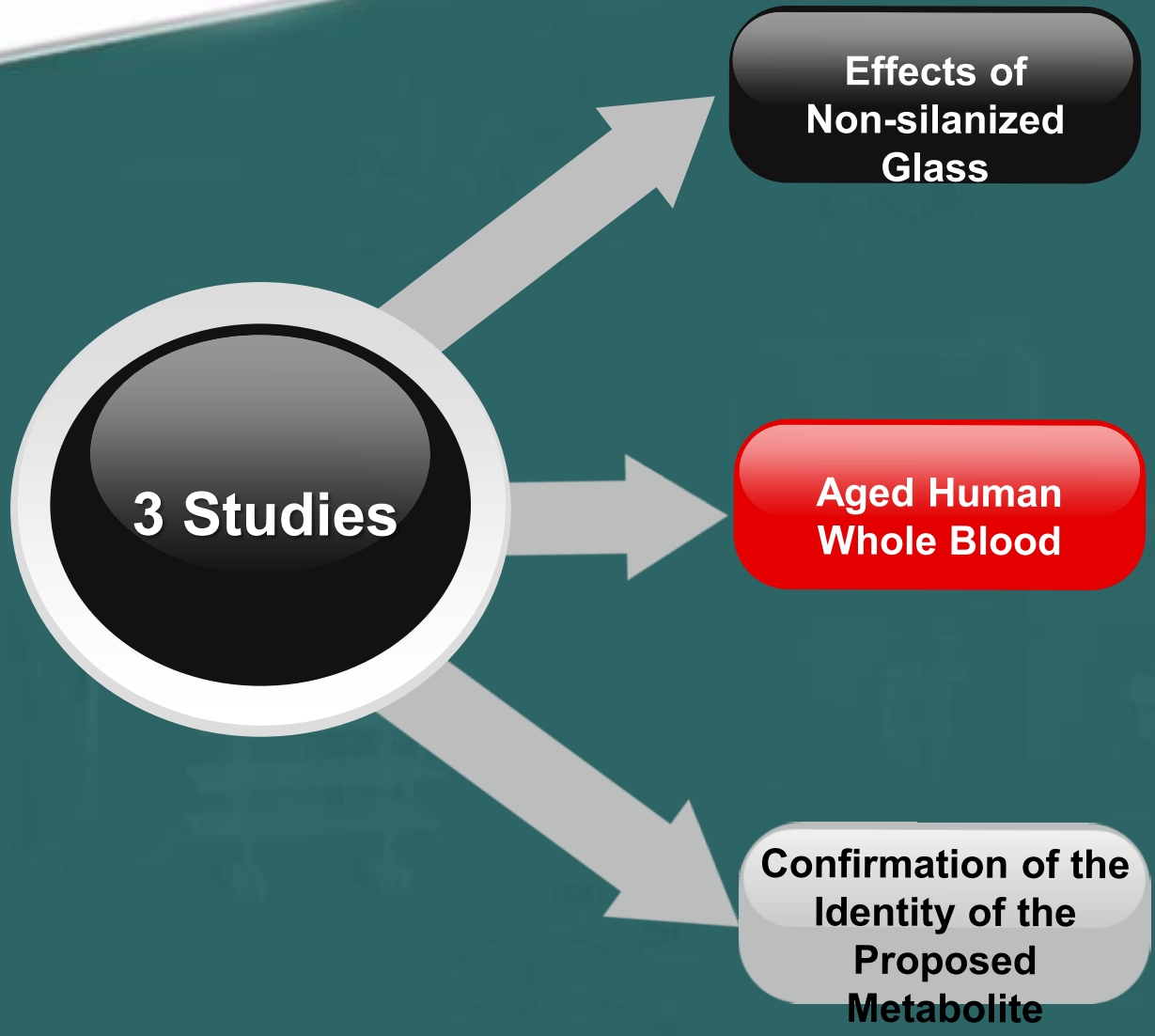
Calibration Model Results

Table 13: Analyte stability data for amphetamine-related drugs at three different concentrations while resident on autosampler (10 °C) over 36 h.

Drug	(CV, %) (acceptance criteria: ≤20%)								
	40 ng/mL			500 ng/mL			1000 ng/mL		
	12 hours	24 hours	36 hours	12 hours	24 hours	36 hours	12 hours	24 hours	36 hours
Norephedrine	5.32	5.32	5.32	1.62	1.35	1.83	2.63	2.63	2.38
Cathine	5.42	1.76	2.34	2.6	4.62	2.22	2.39	1.29	1.16
Ephedrine	2.24	2.37	6.04	1.39	1.07	1.12	1.36	3.35	3.48
Pseudoephedrine	1.56	2.57	2.94	0.57	0.98	1.2	4.22	7.26	7.77
Amphetamine	0.32	0.32	0.32	2.98	2.62	5.45	1.51	3.24	4.17
β-methylphenethylamine	0.30	0.18	0.92	2.81	2.77	5.89	2.46	4.2	4.81

Conclusion

Future Work


3 Studies
Effects of Non-silanized Glass
Aged Human Whole Blood
Confirmation of the Identity of the Proposed Metabolite

Conclusion

EPHEDRINE INTERFERENCE OF THE MIPs

POSTER PRESENTATION IN THE IAFS 2017 CONFERENCE

The abstract of the study was published in Forensic Science International, Volume 277, Supplement 1, p. 230

VALIDATION

An analytical method to identify and quantify selected amphetamine-related drugs in whole blood

VERIFICATION AND METABOLITES STUDY

Determination of BMP and one of its metabolite

وزارة الصحة
Ministry of Health

وزارة التعليم العالي
MINISTRY OF HIGHER EDUCATION

SPECIAL THANK