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TECHNICAL NOTE 21965

Quantitative determination of seven synthetic cathinones (stimulants) from stabilized human urine by UHPLC-MS/MS for forensic toxicology

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Keywords

Syncronis, cathinones, “bath salts”, khat, plant food, legal highs, urine, HILIC, SOLA SCX, bioanalysis, Vanquish Horizon UHPLC system, TSQ Quantiva, triple quad, MS/MS, hydrophilic interaction liquid chromatography

Goal

To develop and assess a quantitative analytical method for the determination of seven synthetic cathinones from stabilized human urine. The Thermo Scientific™ Syncronis™ HILIC 1.7 µm column is used in combination with the Thermo Scientific™ Vanquish™ Horizon UHPLC system and Thermo Scientific™ TSQ Quantiva™ triple-stage quadrupole MS/MS system. Thermo Scientific™ SOLA™ SCX solid phase extraction technology is used for sample clean-up prior to analysis.

Application benefits

- Fast, easy and sensitive detection of seven synthetic cathinones
- No need to derivatize
- Orthogonal approach using mixed mode solid phase extraction (SPE) and hydrophilic interaction liquid chromatography (HILIC) to prevent matrix effects

Introduction

In recent years, the synthesis and availability of new and novel psychoactive substances have been increasingly reported. The availability of analytical methods to detect these new compounds from biological matrices has not kept up with this pace of change.

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Cathinone is a naturally occurring compound found in the leaves of the *Catha edulis* (Khat) plant. The leaves are chewed for the euphoric effect produced by cathinone, which is considered a psychotropic substance. It has amphetamine-like effects and, as such, synthetic cathinones are stimulants. Drug mixes are commonly known as “bath salts” or “plant food”.

Many analytical methods currently in the literature employ limited sample clean-up followed by reversed-phase chromatography using C18-type phases and MS/MS detection. Alternatively, gas chromatography coupled to mass spectrometry (GC-MS) can be used although it is complicated by the need to derivatize the compounds to ensure sufficient volatility.

This technical note describes a novel analytical forensic toxicology method for the determination of seven underivatized synthetic cathinones from acid stabilized human urine using SOLA SCX SPE 10 mg 96-well plates and the Syncronis HILIC 1.7 µm column with a simple isocratic separation. SOLA SPE is based on fritless technology, which removes issues normally associated with conventional SPE. This results in a more robust solution that is far less susceptible to blocking with viscous biological samples.

The combination of mixed mode SPE and HILIC ensures that significant matrix effects are eliminated while maintaining very high analyte recovery. The method was tested intra-day to assess accuracy and precision, specificity, linearity, matrix effects, recovery, and carryover as presented in Table 1. Control urine was obtained and tested for the presence of cathinones. Only cathinone-free urine was used for the matrix effects and specificity experiments.

Table 1. Summary of assay results

Analytes:	Methylenedioxypyrovalerone (MDPV), Buphedrone, Butylone, Ethylone, Mephedrone, Methylone and Methedrone
Species:	Human
Analytical matrix:	Urine/1 M ascorbic acid (90/10, v/v)
Calibration range:	0.100–100 ng/mL
Lower limit of quantification (LLOQ):	0.100 ng/mL
Sample volume:	100 µL
Calibration model:	Linear regression
Weighting factor:	$1/x^2$
Accuracy (bias) and precision:	-9.0–9.3 (CV% 2.1–7.8)
Carryover:	<20% of the LLOQ peak area after 2 double blanks
Specificity:	<20% of the LLOQ peak area in 6 individual sources
Recovery:	85.9–94.1%
Matrix factor:	0.897–1.01 (CV% 1.4–9.5)

Experimental

Chromatography consumables

- Syncronis HILIC 1.7 µm, 100 × 2.1 column (P/N 97502-102130)
- SOLA SCX SPE 10 mg/2 mL 96 well plate (P/N 60309-002)
- Thermo Scientific™ WebSeal™ 96 well non-coated plastic microplates 50 pack (P/N 60180-P202)
- Thermo Scientific™ WebSeal™ nonsterile mats, blue silicone 5 pack (P/N 60180-M122)

Reagents

- Thermo Scientific™ UHPLC-MS grade water (P/N W8-1)
- Thermo Scientific™ UHPLC-MS grade acetonitrile (P/N A956-1)
- Thermo Scientific™ UHPLC-MS grade methanol (P/N A456-1)
- Fisher Chemical™ Optima™ UHPLC-MS grade ammonium formate (P/N A115-50)
- Fisher Chemical™ Analytical grade orthophosphoric acid (P/N O/0500/PB08)
- Fisher Chemical™ Analytical grade ammonia solution 35% (P/N A/3280/PB15)
- Fisher Chemical™ Analytical grade L-ascorbic acid (P/N A/8882/48)

Sample preparation and analysis

Sample preparation protocol

1. Add 100 µL of pre-treated urine to a WebSeal 96 well plate.
2. Add 10 µL of internal standard solution (matrix concentration 5 ng/mL).
3. Add 500 µL of 1% phosphoric acid and vortex mix gently.
4. Condition SOLA SCX well plate with 500 µL of acetonitrile.
5. Condition SOLA SCX well plate with 500 µL of 1% phosphoric acid.
6. Load samples onto SOLA SCX well plate.
7. Wash SOLA SCX well plate with 500 µL of 1% phosphoric acid.
8. Wash SOLA SCX well plate with 500 µL of methanol.
9. Elute SOLA SCX well plate with 2 x 250 µL of 5% ammonia solution (35%) in acetonitrile.
10. Evaporate sample to dryness under nitrogen at 40°C.
11. Reconstitute with 100 µL of acetonitrile.
12. Cap and vortex mix prior to analysis.

Instrumentation

- Vanquish Horizon UHPLC system consisting of the following:
  - System Base Vanquish Horizon (P/N VH-S01-A)
  - Binary Pump H (P/N VH-P10-A)
  - Split Sampler HT (P/N VH-A10-A)
  - Column Compartment H (P/N VH-C10-A)
  - Active Pre-heater (P/N 6732.0110)
- TSQ Quantiva triple-stage quadrupole mass spectrometer

Separation conditions

Table 2. UHPLC parameters

Mobile phase:	Acetonitrile/10 mM ammonium formate (95/5, v/v)
Flow rate:	0.5 mL/min
Run time:	6 minutes
Column temperature:	30°C, with active pre-heating and still air mode
Injection volume:	1 µL

MS/MS conditions

Table 3. MS/MS source parameters

Source:	Thermo Scientific™ Ion Max source with HESI-II probe
Polarity:	Positive ionization
Spray voltage:	4000 V
Vaporizer temperature:	358°C
Sheath gas pressure:	45 Arb
Aux gas pressure:	13 Arb
Ion transfer tube temperature:	342°C
CID gas pressure:	1.5 mTorr

Table 4. Compound transition details

Compound	Polarity	Precursor (*m/z*)	Product (*m/z*)	Collision Energy (V)
MDPV	Positive	276.2	126.2	25
MDPV-d_8	Positive	284.2	135.2	26
Buphedrone	Positive	178.3	131.1	20
Butylone	Positive	222.1	174.1	18
Ethylone	Positive	222.1	174.1	17
Mephedrone	Positive	178.3	145.1	20
Methylone	Positive	208.2	160.1	17
Methedrone	Positive	194.2	161.1	20

Data processing

The Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2.9, was used for data acquisition and analysis.

Results and discussion

Calibration model and range

The calibration model for all compounds was assessed to be a linear regression with $1/x^2$ weighting. Nine calibration standards over the range 0.100–100 ng/mL were freshly prepared in pooled blank human urine pre-treated with 1 M ascorbic acid added 1 part in 10.

A representative calibration curve is shown in Figure 1.

Accuracy and precision

Accuracy and precision (A&P) were determined by analysis of six replicates of quality control (QC) samples at four concentrations over the calibration range tested.

The tested concentrations were 0.100 ng/mL (LLOQ), 0.250 ng/mL (LQC), 5.00 ng/mL (MQC), and 80.0 ng/mL (HQC) for all cathinones. Intra-batch A&P was acceptable for all compounds with bias being <10% and CV <8% at each of the four level QC levels. Representative A&P data and a chromatogram from the LQC level are presented in Table 5 and Figure 2.

Determination of assay specificity

The specificity of the assay was assessed using blank matrix from six individual sources. For each of these, one double blank sample (no cathinones or internal standard added), one single blank sample (internal standard only added), and one upper limit of quantification (ULOQ) sample (no internal standard added) was extracted.

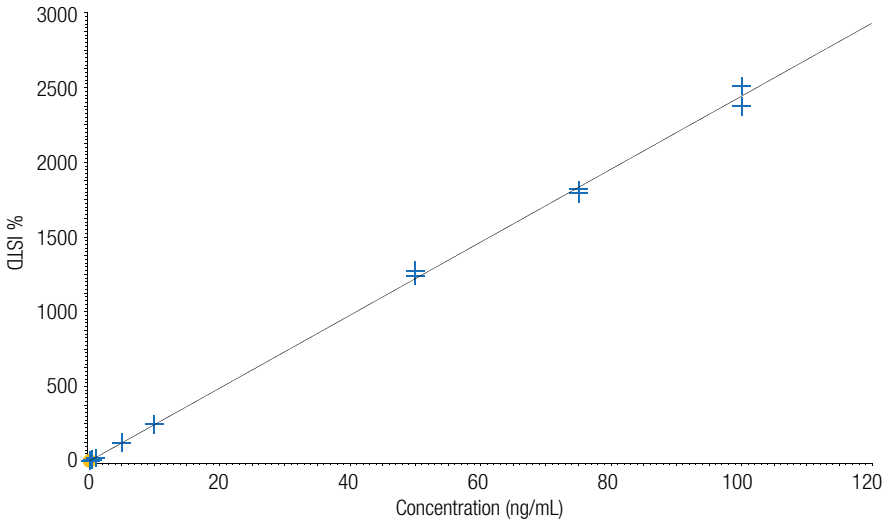

Figure 1. Calibration curve for MDPV

Table 5. Accuracy and Precision at LQC

LQC - 0.250 ng/mL						
MDPV	Buphedrone	Butylone	Ethylone	Mephedrone	Methylone	Methedrone
0.246	0.256	0.247	0.243	0.255	0.242	0.258
0.248	0.236	0.240	0.232	0.243	0.243	0.239
0.240	0.251	0.247	0.245	0.239	0.244	0.233
0.240	0.227	0.234	0.232	0.230	0.230	0.239
0.249	0.254	0.240	0.236	0.249	0.245	0.244
0.263	0.259	0.247	0.247	0.251	0.252	0.262
Mean						
0.248	0.247	0.243	0.239	0.245	0.243	0.246
Bias %						
-0.8	-1.2	-2.8	-4.4	-2.0	-2.8	-1.6
CV %						
3.4	5.2	2.2	2.8	3.7	2.9	4.7

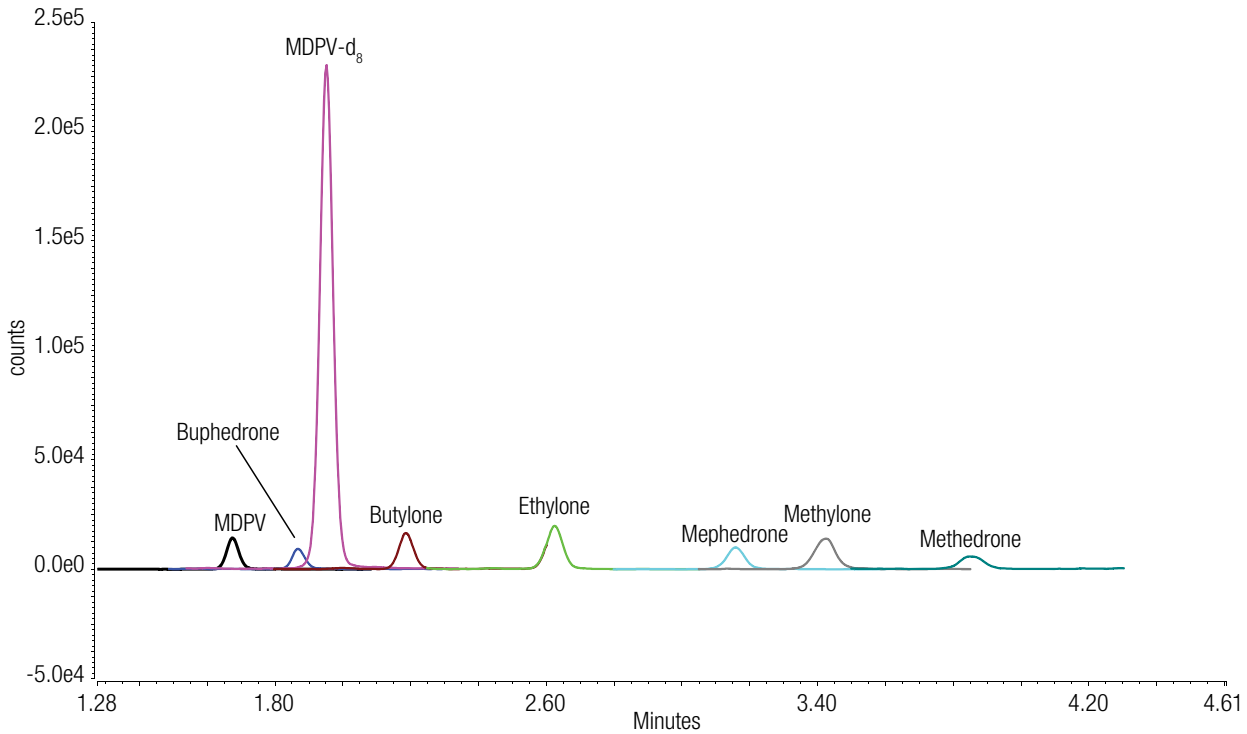

Figure 2. Example chromatogram at LQC level

Acceptable specificity for determination of all cathinones and the internal standard was demonstrated in pre-treated human urine as no interfering peaks were detected in the blank samples. A representative chromatogram of a single blank and a ULOQ with no internal standard are shown in Figures 3 and 4.

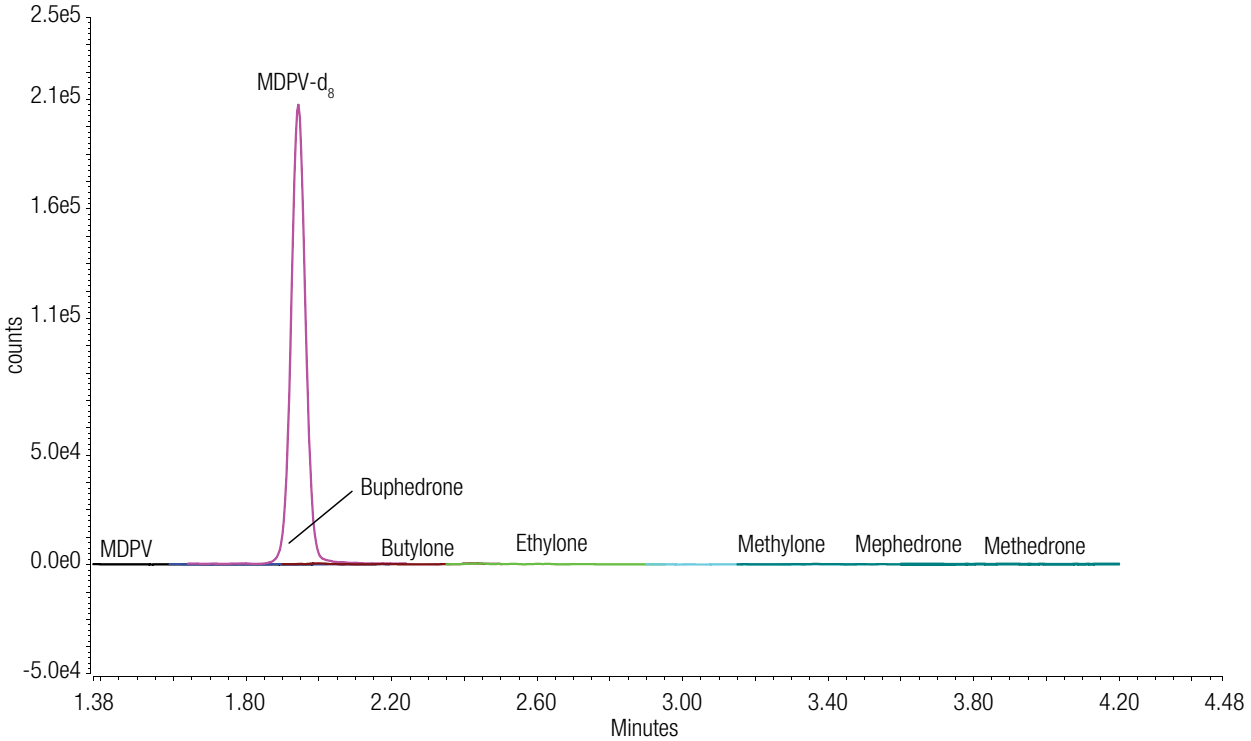

Figure 3. Example chromatogram of a single blank

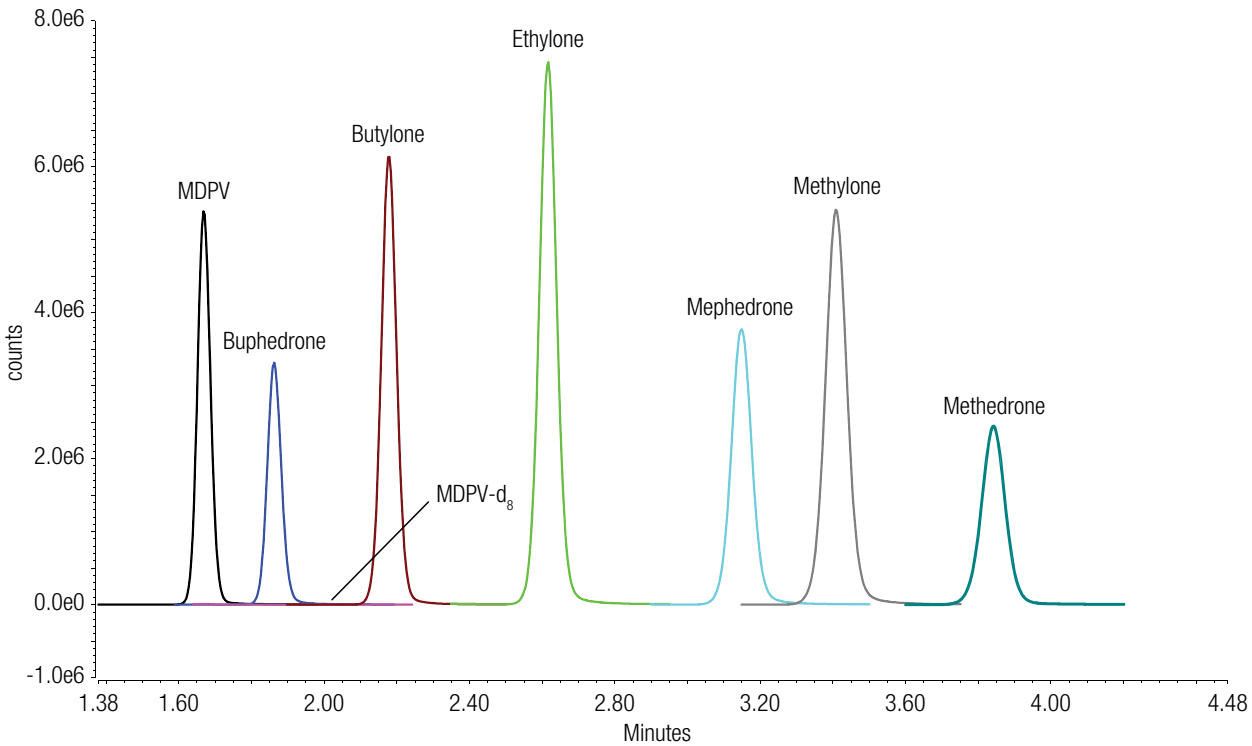

Figure 4. Example chromatogram of a ULOQ no internal standard

Determination of assay carryover

The carryover was assessed by injecting two double blank samples after the injection of the highest calibration sample (ULOQ). The area response of the analyte and internal standard in the blank samples was compared to the area responses of the LLOQ sample after them. No carryover was present using the Vanquish Horizon UHPLC system as shown in Figure 5.

Determination of internal standard normalized matrix factor

The elimination or reduction of matrix effects is arguably the most important aspect of any LC-MS/MS assay. The phenomenon is generally accepted to be the impact of co-eluting compounds on the ionization efficiency and the reproducibility of ionization. As this has a direct impact on the reliability of the data generated, especially as the chemical and biological make-up of matrices vary from individual to individual, the assessment of matrix effect is a key component of assay development and validation.

The use of stable labeled internal standards is one way to correct for matrix effects, although for many multicomponent assays and those for novel compounds, labeled internal standards may not be available. The best practice is to develop assays that employ orthogonal approaches that reduce the chance of ion suppression without relying too heavily on the internal standard. A good example of this is demonstrated within this assay where the outstanding efficiency and selectivity of the Syncronis HILIC 1.7 µm column is so high that we can chromatographically resolve MDPV from MDPV-d_8 based purely on the polarity differences between hydrogen and deuterium. Therefore, where you may expect the labeled internal standard to have the same retention time as the non-labeled version, this is not always the case. Because of this retention difference it is in a region where the co-eluents, which may cause suppression or enhancement, are completely different.

The presence of matrix effects was assessed by using blank matrix from six individual sources. For each of these, three replicate double blank samples were extracted and fortified post extraction with a solution containing the cathinones and internal standard at a concentration equivalent to the LQC level.

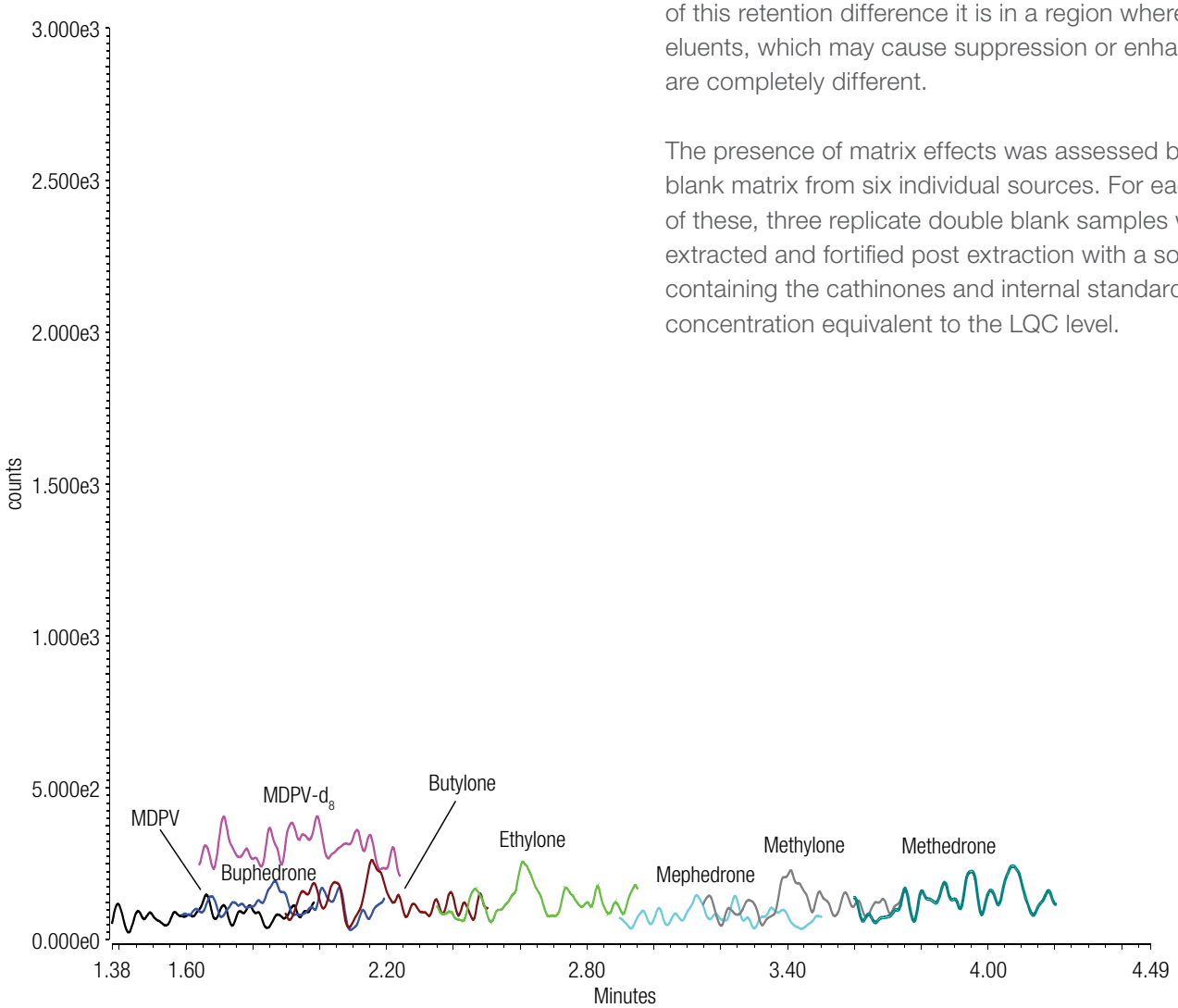

Figure 5. Chromatogram of a double blank sample following a ULOQ sample

An acceptable normalized matrix factor of 1±0.15 with a CV% ≤15 was achieved for all cathinones using MDPV-ds_8 as an internal standard. The matrix factor ranged from 0.897 (CV 9.5%) for methedrone to 1.01 (CV 1.5%) for ethylone. The matrix factor and CV% for the other five cathinones fell between these upper and lower performance levels. An example matrix factor calculation for MDPV is presented in Table 6. LQC 1–6 in the table represent the post extract fortified (or overspiked) samples.

Determination of recovery

The assay recovery was assessed by comparing the mean analyte peak area for each analyte in the LQC accuracy and precision samples to the mean area of the same source of blank urine fortified post extraction.

No specific acceptance criteria are applied for recovery though the range of recovery for the cathinones was high and consistent using the SOLA SCX 10 mg 96 well plate. It was calculated as 85.9–94.1%.

Table 6. Matrix factor assessment for MDPV

			MDPV				
Sample Name	**Analyte Area (counts)**	**IS Area (counts)**	**Mean Analyte Peak Area**	**Analyte Matrix Factor**	**Mean IStd Peak Area**	**IStd Matrix Factor**	**IStd Normalized Matrix Factor**
LQC 1	266787	5066472					
LQC 1	221518	4429615	239230	1.03	4639920	1.07	0.964
LQC 1	229386	4423672					
LQC 2	227830	4375671					
LQC 2	229575	4437028	230800	0.990	4447644	1.02	0.970
LQC 2	234996	4530234					
LQC 3	238407	4592349					
LQC 3	242247	4606309	241201	1.04	4548623	1.04	0.991
LQC 3	242948	4447212					
LQC 4	240548	4514565					
LQC 4	238090	4394061	240556	1.03	4491971	1.03	1.00
LQC 4	243031	4567288					
LQC 5	267744	5211974					
LQC 5	239467	4632336	254656	1.09	4894218	1.12	0.972
LQC 5	256758	4838345					
LQC 6	250193	4728707					
LQC 6	237937	4608329	244643	1.05	4649717	1.07	0.983
LQC 6	245799	4612114					
Low NE	232665	4377282					**Mean** 0.980
Low NE	241209	4478076	233048		4355326		
Low NE	225269	4210620					**CV%** 1.4

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Conclusion

- Fast, easy, and novel separation of seven synthetic cathinones from acid-stabilized human urine for forensic toxicology with a 6-minute cycle time
- Isocratic separation using a Syncronis HILIC column ensures excellent sensitivity by LC-MS/MS
- Simple, robust sample preparation using SOLA SCX 10 mg 96 well plates that is easy to automate
- LLOQ of 0.100 ng/mL from only 100 µL of urine
- Orthogonal technique with high recovery and no matrix effects for reliable quantitation
- Streamlined integration of a robust and powerful analytical solution combining the SOLA SCX SPE 10 mg 96-well plate, Syncronis HILIC 1.7 µm column, Vanquish Horizon UHPLC system, TSQ Quantiva triple-stage quadrupole MS/MS system, and Chromeleon CDS with advanced MS data processing

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