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Forensic toxicology

Quantitation of an oral fluid drug panel including THC using the new Stellar mass spectrometer

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Keywords

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Goal

Accurately confirm and quantitate 31 drugs of abuse down to the sensitivity required for oral fluid testing in a 4.5-minute method using an offline solid phase extraction (SPE) followed by the Thermo Scientific™ Vanquish™ Horizon ultra-high performance liquid chromatography (UHPLC) system coupled with the Thermo Scientific™ Stellar™ mass spectrometer for forensic toxicology.

Application benefits

- A complete and quantitative workflow for 31 drugs of abuse in oral fluid using the Stellar mass spectrometer
- Ability to use CID and HCD fragmentation on the Stellar MS to obtain optimized fragmentation
- Highly reproducible data and lower limit cutoffs achieved by automated extraction using DPX XTR™ tips with mixed mode SCX/WAX chemistry tips and a Hamilton™ STAR™ liquid handler

Introduction

As forensic communities move towards oral fluid matrix for ease of collection and roadside testing, it is important to be able to test for a wide range of analytes and achieve required sensitivity. With the new SAMHSA guidelines and the National Safety Council's Tier 1 drugs providing LOQ levels, the extraction protocol and instrumentation need to be sensitive enough to accomplish these cut-offs.[1,2] Including tetrahydrocannabinol (THC) into the assay provides challenges in the extraction as most drugs of abuse are basic and THC is neutral. This extraction workflow, which extracts THC alongside other drugs of abuse, coupled with the Stellar mass spectrometer generates data that offers improved sensitivity, selectivity, and accuracy for detection and quantitation of drugs of abuse in oral fluid.

Experimental

Calibration standards and control samples

Thirty-one target analytes were prepared into a mix to be spiked into negative human oral fluid. Eight calibration levels ranging from 0.25 to 1,000 ng/mL (1 to 5,000 ng/mL for higher cutoff drugs) were prepared by serial dilution in negative human oral fluid to a volume of 1 mL. Samples were diluted with Quantisal™ Buffer 1:3 (oral fluid: buffer); 500 µL of the prepared sample was aliquoted for extraction; and samples were spiked with 100 µL of internal standard stock comprising the labeled standards of each of the 31 drugs. Each calibration level was prepared in triplicate.

Dispersive solid phase extraction

Samples were extracted using XTR tips with mixed mode SCX/WAX chemistry for INTip™ dispersive solid phase extraction (dSPE) from DPX Technologies. First, XTR tips were conditioned using 500 µL of 50% MeOH followed by aspirating and dispensing 500 µL of each sample three times to bind the analytes to the resin. The tips were washed by aspirating and dispensing three times 750 µL of 30% MeOH and then samples were eluted by aspirating and dispensing 750 µL of 48% ACN/48% MeOH/4% NH4OH three times. Samples were dried down at 50 ˚C for 12 minutes. Once dried, the samples were reconstituted with 20 µL of MeOH + 0.1% formic acid and 80 µL of H_2O + 0.1% formic acid. Note that it is important to add the MeOH portion of the reconstitution solvent first and vortex before the remaining H_2O portion as this will ensure THC remains in solution. Extraction steps were automated on a Hamilton STAR automated liquid handler, which prepared samples in less than 45 minutes as opposed to several hours by hand. This extraction workflow is shown in Figure 1.

Liquid chromatography

Drug analytes were separated with a Thermo Scientific™ Accucore™ Biphenyl column (2.1 x 50 mm, 2.6 µm, P/N 17826-052130) connected to a Thermo Scientific™ Vanquish™ Horizon UHPLC system. Mobile phases consisted of 0.1% formic acid in water for mobile phase A and 0.1% formic acid in MeOH mobile phase B. 1 µL of each standard was injected and chromatographic separation was accomplished using the gradient conditions in Table 1.

Table 1. LC gradient

Time (min)	Flow rate (mL/min)	% A	% B	Curve
0.000	0.5	85	15	5
0.200	0.5	85	15	5
1.500	0.5	40	60	7
2.000	0.5	15	85	5
3.500	0.5	15	85	5
3.500	0.5	1	99	5
4.000	0.5	1	99	5
4.000	0.5	85	15	5
4.500	0.5	85	15	5

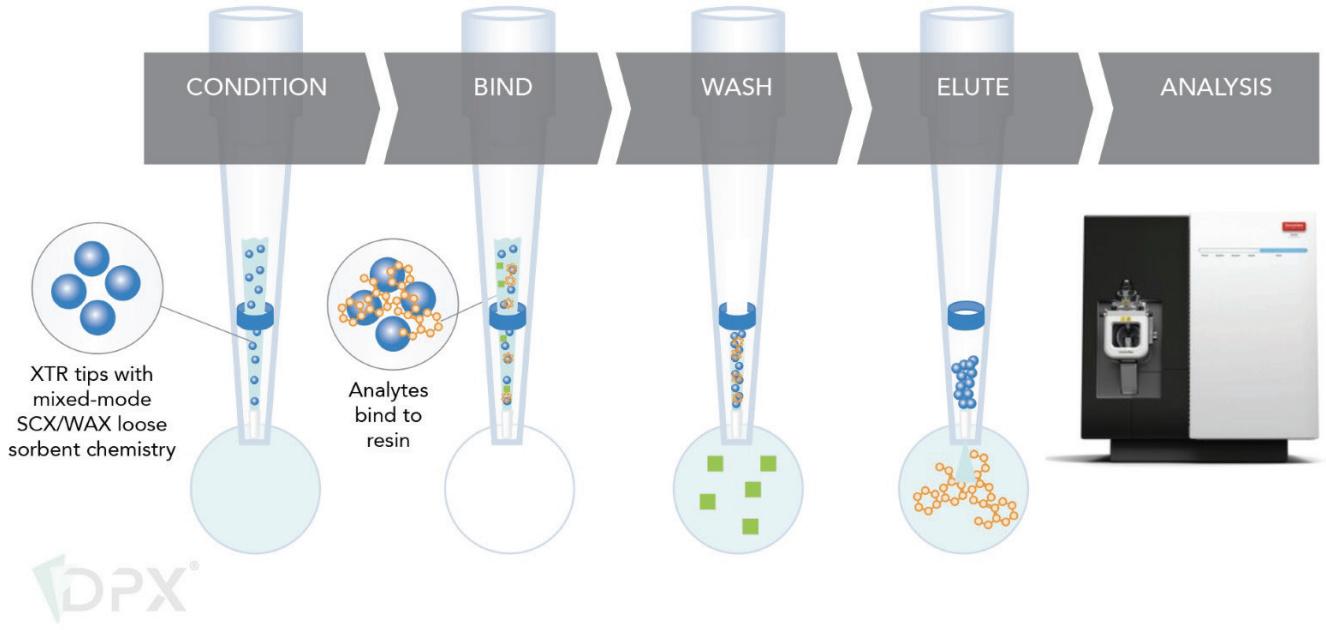

Figure 1. Schematic of dispersive solid phase extraction method. Artwork provided by DPX Technologies.

Mass spectrometry

Data was acquired on the Stellar mass spectrometer using full scan + targeted MS2 method (tMS2) with a mass list for the 31 target drugs and 31 internal standards. Table 2 highlights the source parameters and values. An isolation window of *m/z* 1, scan rate of 125 kDa/s, and RF lens of 30% were used for the tMS2 experiment. The mass list contained the drug precursor *m/z*, optimized collision energies, and retention times (Appendix 1). Both CID (resonance-type, unique to ion trap) and HCD (beam-type) activations were used in the targeted list.

Figure 2 shows the instrument set-up with the Vanquish Horizon UHPLC system and Stellar mass spectrometer.

Figure 2. Stellar mass spectrometer and Vanquish Horizon UHPLC system

Table 2. Source conditions

Parameter	Value
Spray voltage positive ion (V)	3,500
Sheath gas (Arb)	50
Aux gas (Arb)	10
Sweep gas (Arb)	1
Ion transfer tube temp. (°C)	325
Vaporizer temp. (°C)	500

Data analysis

Data was acquired and processed with Thermo Scientific™ TraceFinder™ software, version 5.2, which utilizes the compound database that stores information including molecular formula, mass, retention time, and target/confirming ions for all compounds of interest.

Results and discussion

Chromatography exhibited ample separation for isomers including codeine/hydrocodone and morphine/hydromorphone within a 4.5-minute run. Figure 3 shows the combined extracted ion chromatogram of the 31 drugs in this panel at 100 ng/mL.

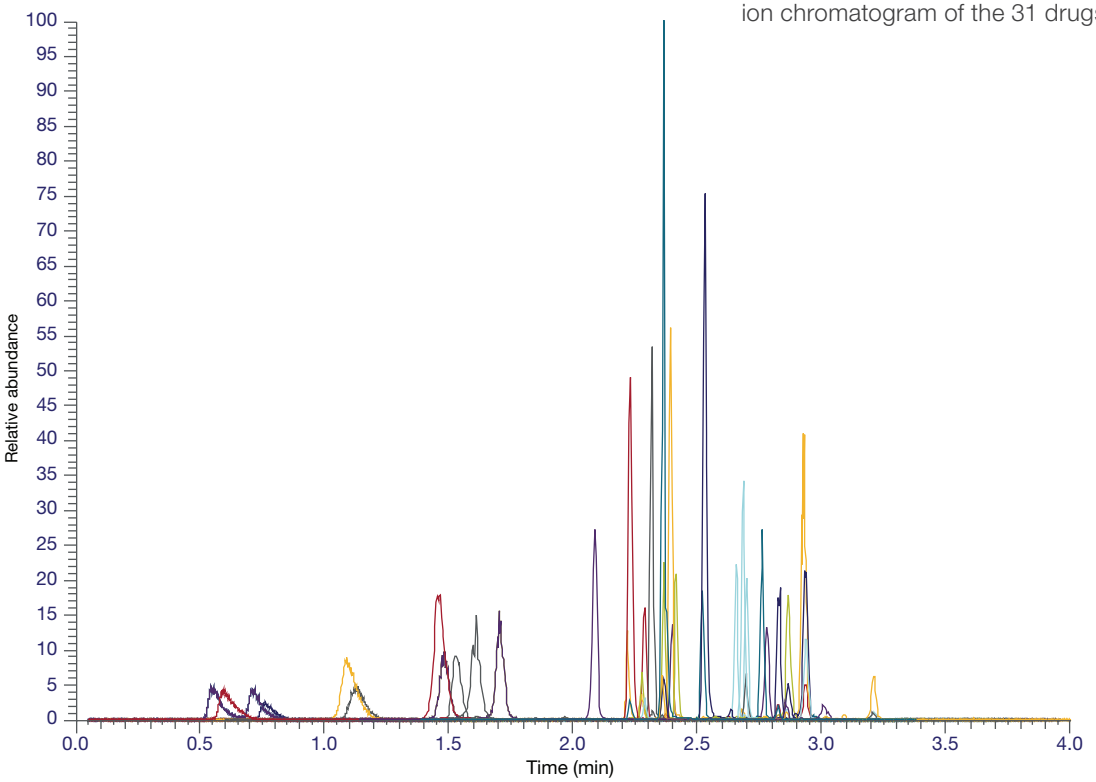

Figure 3. Combined extracted ion chromatogram of 31 drugs in oral fluid

The limit of quantitation (LOQ) and upper limit of linearity (ULOL) were evaluated for each of the 31 analytes. Table 3 shows the criteria for each of these limits. For confirming each drug, ion ratios were used with a tolerance allowance of ±20%.

Table 3. Criteria assigned in TraceFinder software for limits

Parameter	Criteria
Limit of quantitation (LOQ)	Back-calculated concentration on calibration curve within 20% Ion ratios tolerance < ±20% of the average of the calibrators
Upper limit of linearity (ULOL)	Highest calibrator that achieves linearity with an R^2 value ≥ 0.9900

Table 4. Results table depicting LOQ and ULOL achieved for each drug in ng/mL

	LOQ	ULOL
6-MAM	0.5	1,000
7-aminoclonazepam	0.5	1,000
Alprazolam	0.5	1,000
Amphetamine	1	1,000
Benzoylecgonine	0.5	1,000
Buprenorphine	0.5	1,000
Carisoprodol	5	5,000
Clonazepam	0.5	1,000
Cocaethylene	0.5	1,000
Cocaine	0.5	1,000
Codeine	0.5	1,000
Diazepam	0.5	1,000
Fentanyl	0.25	1,000
Hydrocodone	0.5	1,000
Hydromorphone	0.5	1,000
Lorazepam	1	1,000
MDA	5	1,000
MDMA	5	1,000
Meprobamate	5	5,000
Methadone	0.5	1,000
Methamphetamine	0.5	1,000
Morphine	1	1,000
Nordiazepam	0.5	1,000
Oxazepam	1	1,000
Oxycodone	0.5	1,000
Oxymorphone	0.5	1,000
PCP	0.5	1,000
Temazepam	0.5	1,000
THC	1	1,000
Tramadol	0.5	1,000
Zolpidem	5	1,000

Each of the 31 drugs of abuse achieved lower LOQs than the recommended confirmation cutoffs in the new 2023 SAMHSA guidelines. Additionally, each of the compounds had lower LOQs than the National Safety Council's Tier 1 drug cutoffs. The LOQ and ULOL of each analyte are shown in Table 4. The Stellar mass spectrometer uses the new Thermo Scientific™ OptaMax™ Plus HESI source, which allows for increased vaporizer temperatures, thus improving the relative peak areas and overall sensitivity for drugs of abuse. Utilizing a vaporizer temperature of 500 ˚C helped achieve the sensitivity.

The ability to perform both CID and HCD fragmentation assisted in providing optimized collision energies for the highest intensity and unique compound product ions. For most compounds, HCD worked well; however, for buprenorphine, HCD fragmentation produced one high intensity product ion and non-indicative lower mass fragment ions. Additionally, the precursor was still present at a relatively high abundance. Figure 5 shows the impact of using CID fragmentation to fragment buprenorphine and produce two high intensity, high mass product ions that provided consistent ion ratios from 0.5 to 1,000 ng/mL.

Figure 4 depicts the extracted ion chromatograms of two of the analytes, buprenorphine and THC, at their respective LOQ concentrations with their corresponding calibration curves. At their LOQs, they are able to achieve over 8 scans across the peak.

The ion ratio tolerance that was set was ±20% of the average of the triplicate set of calibrators. This was used to determine the LOQs. Every compound had ion ratios of less than ±20%. Five representative compounds are plotted in Figure 6, showing ion ratios of the calibrators and %RSD.

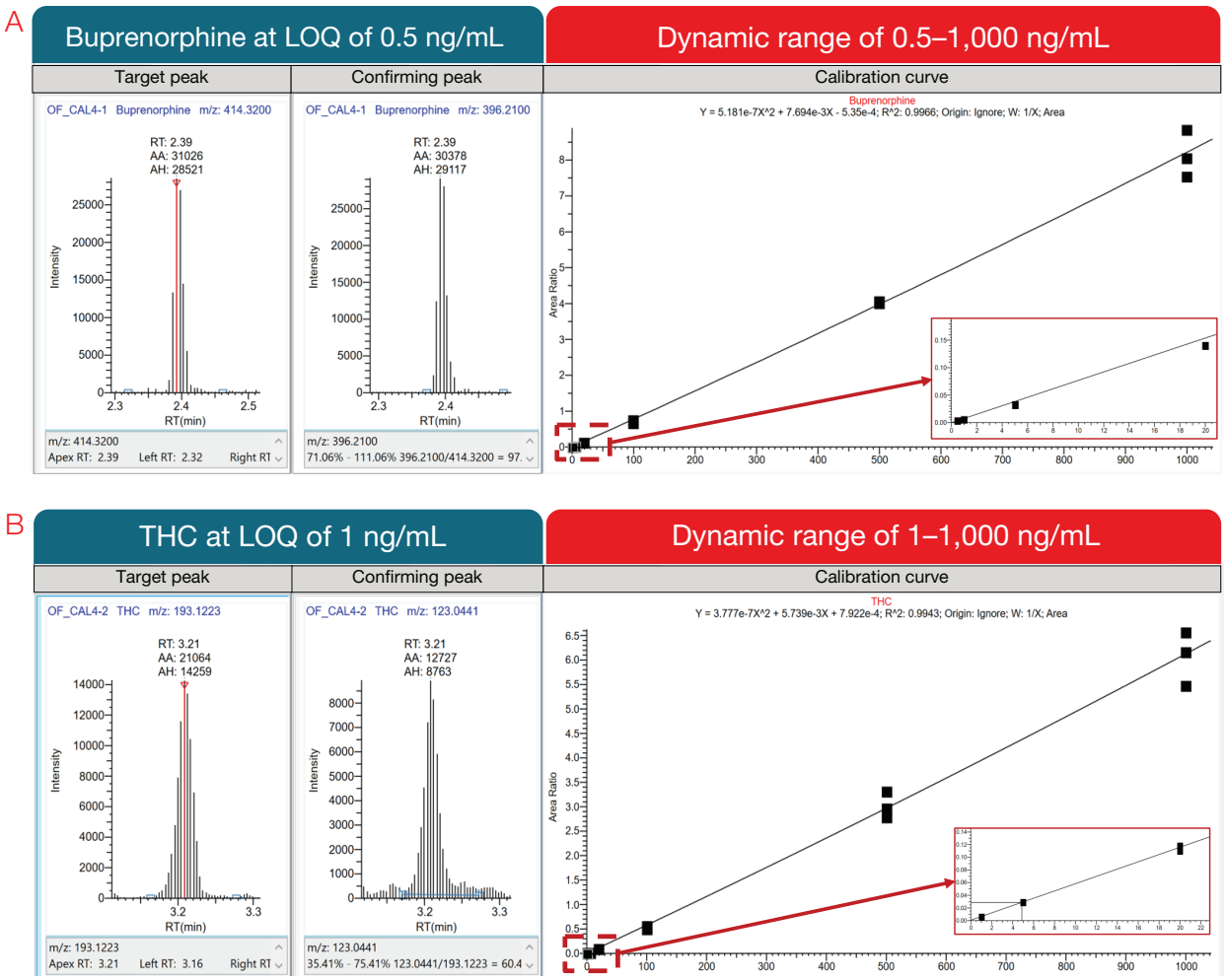

Figure 4. Extracted ion chromatograms of (A) Buprenorphine and (B) THC at their LOQ along with their respective calibration curves

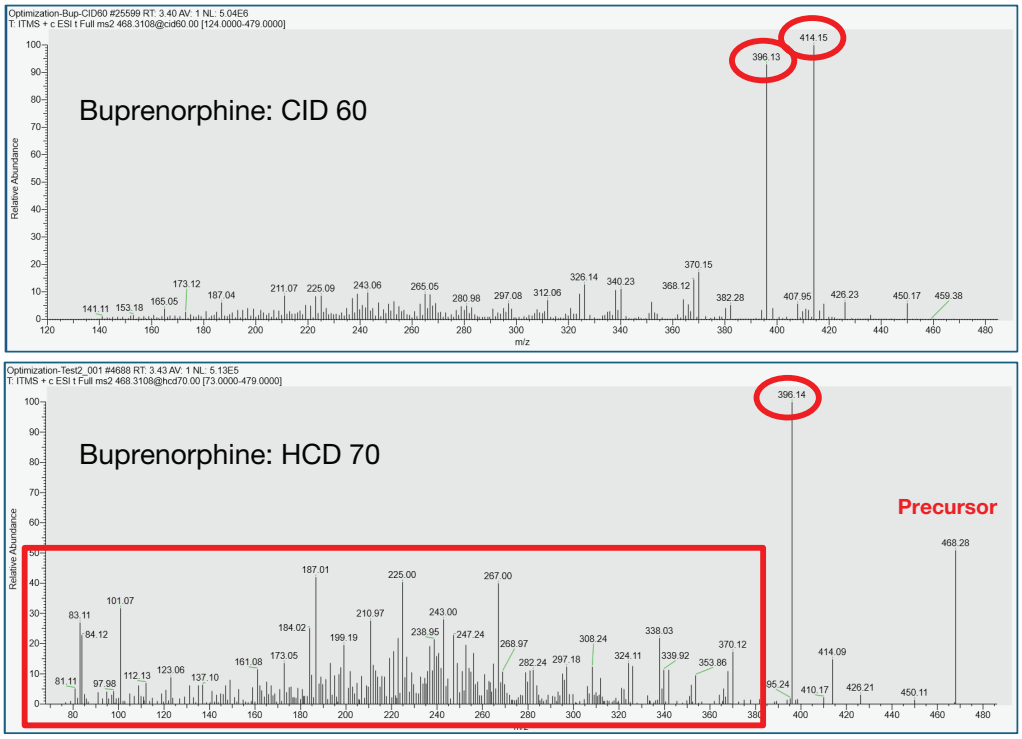

Figure 5. Buprenorphine comparison between CID and HCD fragmentation. The ability to use either fragmentation type gives the user the ability to choose the best option for every compound in an assay.

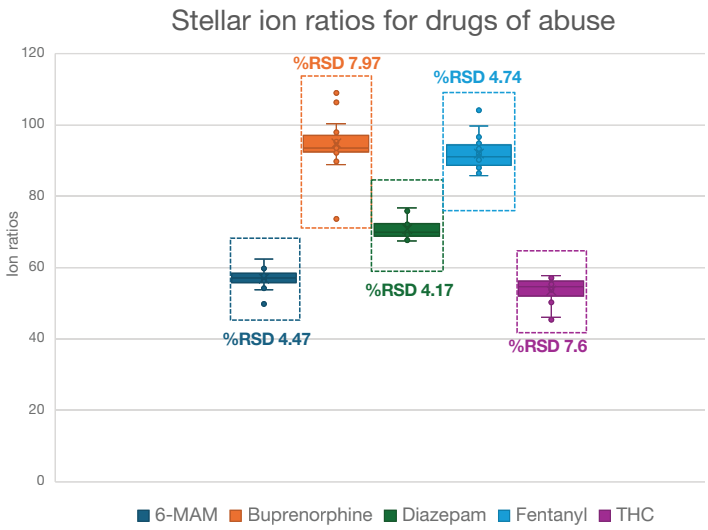

Figure 6. Box and whisker plot of ion ratios for 6-MAM, buprenorphine, diazepam, fentanyl, and THC. Dotted lines indicate ±20% of ion ratio average for calibrators. All analytes had ion ratios of less than ±20%.

Conclusion

This fast and quantitative method was developed around 31 drugs of abuse specified by SAMHSA and the National Safety Council. A complete workflow was presented that involved sample preparation using Hamilton and DPX INTip SPE. Linearity was achieved from LOQs as low as 0.25 ng/mL up to 1,000 ng/mL, which exemplifies the sensitivity of the Stellar mass spectrometer and extraction procedure. The fast scan speeds, instrument sensitivity, and consistent ion ratios allowed us to create a 4.5-minute gradient with LOQs that surpassed SAMHSA and the National Safety Council's Tier 1 drug cutoffs.

References

1. Substance Abuse and Mental Health Services Administration Center for Substance Abuse Prevention. (2023). *Oral Fluid Specimen Collection Handbook for Federal Agency Workplace Drug Testing Programs.*
2. D'Orazio, A. L., Mohr, A. L. A., Chan-Hosokawa, A., Harper, C., Huestis, M. A., Limoges, J. F., Miles, A. K., Scarneo, C. E., Kerrigan, S., Liddicoat, L. J., Scott, K. S., & Logan, B. K. (2021). Recommendations for Toxicological Investigation of Drug-Impaired Driving and Motor Vehicle Fatalities - 2021 Update. In *Journal of Analytical Toxicology* (Vol. 45, Issue 6, pp. 529–536). Society of Forensic Toxicologists. https://doi.org/10.1093/jat/bkab064

Appendix

Table A1. MS parameters for all target analytes and internal standards

Compound	*m/z*	Retention time (min)	Activation type	HCD collision energies (%)	CID collision energies (%)
6-MAM	328.1543	1.53	HCD	55	
6-MAM-d3	331.0732	1.53	HCD	55	
7-aminoclonazepam	286.0742	2.32	HCD	50	
7-aminoclonazepam-d4	290.0993	2.32	HCD	50	
Alprazolam	309.0902	2.81	HCD	60	
Alprazolam-d5	314.1215	2.81	HCD	60	
Amphetamine	136.1121	0.80	HCD	20	
Amphetamine-d5	141.1437	0.80	HCD	20	
Benzoylecgonine	290.1387	2.22	HCD	50	
Benzoylecgonine-d8	298.1889	2.22	HCD	50	
Buprenorphine	468.3108	2.40	CID		60
Buprenorphine-d4	472.3359	2.40	CID		60
Carisprodol	261.1809	2.53	HCD	10	
Carisprodol-d7	268.2248	2.53	HCD	10	
Clonazepam	316.0484	2.71	HCD	55	
Clonazepam-d4	320.0735	2.71	HCD	55	
Cocaethylene	318.1700	2.32	HCD	50	
Cocaethylene-d8	326.2202	2.32	HCD	50	
Cocaine	304.1543	2.26	HCD	50	
Cocaine-d3	307.1732	2.26	HCD	50	
Codeine	300.1594	1.47	HCD	60	
Codeine-d6	306.1971	1.47	HCD	60	
Diazepam	285.0789	2.96	HCD	55	
Diazepam-d5	290.1103	2.96	HCD	55	
Fentanyl	337.2274	2.39	HCD	50	
Fentanyl-d5	342.2588	2.39	HCD	50	
Hydrocodone	300.1594	1.70	HCD	60	

Table A1 (cont.). MS parameters for all target analytes and internal standards (continues)

Compound	*m/z*	Retention time (min)	Activation type	HCD collision energies (%)	CID collision energies (%)
Hydrocodone-d6	306.1971	1.70	HCD	60	
Hydromorphone	286.1438	0.80	HCD	60	
Hydromorphone-d3	289.1626	0.80	HCD	60	
Lorazepam	321.0192	2.67	HCD	60	
Lorazepam-d4	325.0443	2.67	HCD	60	
MDA	180.1019	1.13	HCD	40	
MDA-d5	185.1333	1.13	HCD	40	
MDMA	194.1176	1.45	HCD	40	
MDMA-d5	199.1490	1.45	HCD	40	
Meprobamate	219.1339	2.30	HCD	20	
Meprobamate-d3	222.1528	2.30	HCD	20	
Methadone	310.2165	2.53	HCD	30	
Methadone-d3	313.2354	2.53	HCD	30	
Methamphetamine	150.1277	1.09	HCD	20	
Methamphetamine-d5	155.1591	1.09	HCD	20	
Morphine	286.1438	0.80	HCD	60	
Morphine-d6	292.1814	0.80	HCD	60	
Nordiazepam	271.0633	2.78	HCD	55	
Nordiazepam-d5	276.0947	2.78	HCD	55	
Oxazepam	287.0582	2.69	HCD	30	
Oxazepam-d5	292.0896	2.69	HCD	30	
Oxycodone	316.1543	1.60	HCD	50	
Oxycodone-d6	322.1920	1.60	HCD	50	
Oxymorphone	302.1387	0.80	HCD	40	
Oxymorphone-d3	305.1575	0.80	HCD	40	
PCP	244.2060	2.42	HCD	30	
PCP-d5	249.2374	2.42	HCD	30	
Temazepam	301.0738	2.85	HCD	70	
Temazepam-d5	306.1052	2.85	HCD	70	
THC	315.2319	3.24	HCD	45	
THC-d3	318.2507	3.24	HCD	45	
Tramadol	264.1958	2.14	HCD	20	
Tramadol-13C-d3	268.2185	2.14	HCD	20	
Zolpidem	308.1757	2.38	HCD	50	
Zolpidem-d6	314.2134	2.38	HCD	50	

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