

Tackling the chromatographic analysis of novel psychoactive substances with High Resolution Mass Spectrometry

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On the 26th of May, 2016 the United Kingdom introduced the 'Psychoactive Substances Act 2016' [1], this law is intended to restrict the production, sale and supply of novel psychoactive substances (NPS) or as they are more commonly known, legal highs or designer drugs.

This law defines a 'psychoactive substance' as anything, which by stimulating or depressing the person's central nervous system affects the persons mental functioning or emotional state.

In November 2016 Germany also introduced 'The New Psychoactive Substances Act' (NpSG) [2]. This law prohibits the acquisition, possession and sale of new psychoactive substances (NPS) as well as sanctioning the passing on of NPS. The NpSG specifically controls over two NPS groups; they are phenethylamines and synthetic cannabinoids.

As governments around the world make new laws to combat the production of the NPS, the effects of these substances are still real. Announcements in November 2016 on the 'UNODC Early Warning Advisory on New Psychoactive Substances' [3] documents increasing numbers of serious adverse events and deaths associated with several emerging synthetic opioids.

Authorities need to be able to rapidly detect the NPS with confidence and accuracy to tackle the problem. However, having the reference standard to confirm the presence of NPS is often difficult if the chromatography and analytical instrumentation such as a mass spectrometer rely on already knowing the details of specific compounds. This includes information such as retention time (RT), their mass to charge ratio (m/z) and the multiple reaction monitoring (MRM) transitions. Advanced systems that are accompanied by libraries which deliver high quality spectral data to confirm a known NPS, and contain these details (RT, m/z, MRM and libraries) are vital if you wish to conduct a screening assay using LC-MS/MS for example.

However, the NPS situation constantly

evolves, as the clandestine laboratories that manufacture them make

modifications to the structure (analogues), stereoisomers and derivatives. In doing

Table 1:
Compounds list/Cutoff concentrations

Compounds	(ng/mL)	Compounds	(ng/mL)	Compounds	(ng/mL)
6-MAM	10	EDDP	100	Mitragynine	10
7-Aminoclonazepam	50	Fentanyl	2	Morphine	50
7-Hydroxymitraglyne	10	Gabapentin	100	Naloxone	50
Acetyl Fentanyl	2	Hydrocodone	50	Naltrexone	50
Alpha-Hydroxyalprazolam	50	Hydromorphone	50	N-desmethyltapentadol	50
Alpha-hydroxymidazolam	50	Imipramine	50	Norbuprenorphine	20
Alpha-hydroxytriazolam	50	JWH-018 4-OH pentyl	10	Norcodeine	50
Alpha-PPP	10	JWH-018 pentanoic acid	10	Nordiazepam	50
Alpha-PVP	10	JWH-019 6-OH hexyl	10	Norfentanyl	2
Alprazolam	50	JWH-073 3-OH butyl	10	Norhydrocodone	50
AM-2201 4-OH pentyl	10	JWH-073 butanoic acid	10	Normeperidine	50
Amitriptyline	50	JWH-122 5-OH pentyl	10	Noroxycodone	50
Amphetamine	100	JWH-210 5-OH pentyl	10	Norpropoxyphene	100
Benzoylcegonine	50	JWH-250 4-OH pentyl	10	Nortriptyline	50
Buphedrone	10	Lorazepam	50	O-Desmethyltramadol	50
Buprenorphine	20	MDA	100	Oxazepam	50
Carisoprodol	100	MDEA	100	Oxycodone	50
Clomipramine	50	MDMA	100	Oxymorphone	50
Codeine	50	MDPV	10	PCP	25
Cotinine	50	Meperidine	50	Pregablin	100
Cyclobenzaprine	50	Mephedrone	10	Propoxyphene	100
Desalkylflurazepam	50	Meprobamate	100	Protriptyline	50
Desipramine	50	Methadone	100	Ritalinic acid	50
Desmethyldoxepin	50	Methamphetamine	100	Sufentanil	2
Dextromethorphan	50	Methedrone	10	Tapentadol	50
Diazepam	50	Methylone	10	Temazepam	50
Dihydrocodeine	50	Methylphenidate	50	Tramadol	50
Doxepin	50	Midazolam	50	Zolpidem	50
				THC-COOH	20

With any drug screen, the time it takes to get a result is critical and in this paper the development of a robust LC configuration and gradient was key to getting accurate and confident data. The HPLC separation was performed with a Phenomenex Synergi Hydro-RP column (20 × 2 mm, 2.5 μm). Mobile phase A was 10 mM ammonium formate in water and mobile phase B was 0.1% (v/v) formic acid in methanol. The image (Figure 1) details the gradient conditions that were employed in this study.

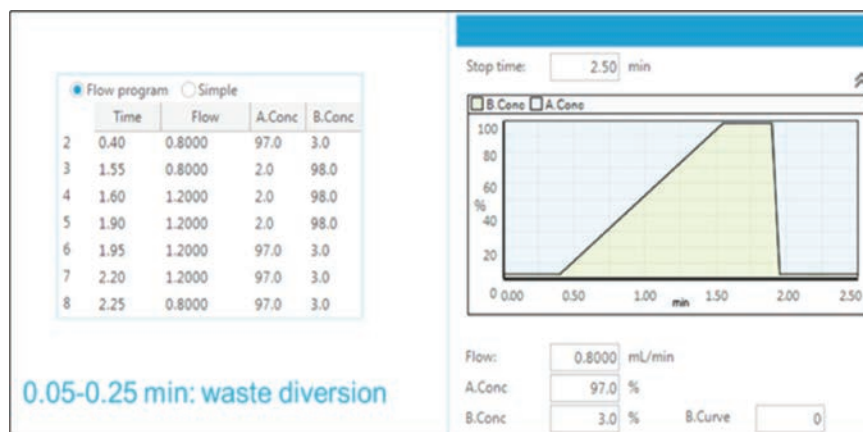


Figure 1 – LC Gradient conditions

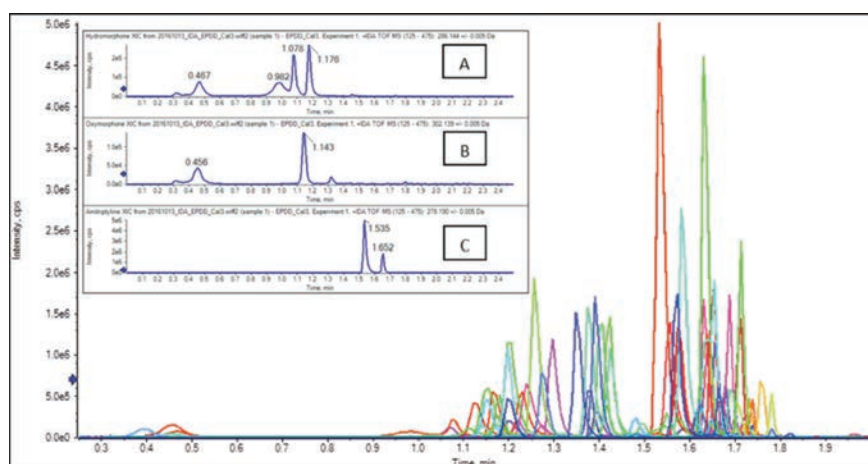


Figure 2 Extracted ion chromatograms of multiple drugs including NPS

this, the NPS may increase potency and alter the side effects of the designer drug. Once the NPS has been modified, it becomes difficult if not impossible to analyse.

In this study 85 analytes were selected which would challenge chromatographic separation and test the instrument to detect and screen the compounds in an ultra-fast time frame. Although in a real-life case it would be highly unlikely that any sample would contain this many forensic drugs.

Experimental

In a recent application note 'Ultra-Fast Forensic Toxicological Screening and Quantitation under 3 Minutes using Sciex X500R QTOF System and Sciex OS 1.0 Software' [4] novel psychoactive substances were addressed in the study of urine samples. Twenty samples were diluted in 10% methanol and centrifuged to reduce the possibility of matrix interference. The clear supernatants were transferred to autosampler vials which were spiked with 85 compounds including opioids and synthetic cannabinoids. The urine samples contained a calibrator set

spiked with compounds described in Table 1. There are 7 levels of calibrators: 40%, 80%, 100%, 200%, 300%, 500% and 1000% which are against the ng/mL value of each compound in table one, for example for the compound of 6-MAM, a metabolite of heroin, the calibration range was 4ng/mL to 100ng/mL. Using such a calibration range enable low level detection, sub cut off level through to a high level of concentration at 100ppb. There were also 20 urine samples with unknown number of compounds. The injection volume was 5 μ L with a dilution factor for this sample set was a x4, again this reduces matrix interferences and the need for instrument maintenance.

Prior to the development of this method a 2-minute LC method [5] had been created using a Phenomenex Kinetex Phenyl-Hexyl column 50 \times 2.1mm, 2.6 μ m 00B-4495-E0, however to improve the retention of the polar species the Hydro RP column was selected and the LC runtime was extended by 0.5 minute. Ensuring sufficient retention of the very polar species was important to allow the diversion of the salt-containing eluates in the beginning of the gradient to waste.

The aim of refining the LC conditions was to evenly distribute all the eluting analytes throughout the duration of the data acquisition window to reduce the number of co-eluting analytes thus minimising ion suppression and matrix effects.

To preserve the column lifetime an organic wash was performed at the end of the gradient run followed by aqueous re-equilibration so retention time reproducibility was maintained. Within the 2.5 min LC runtime several groups of isomers could be resolved, in Figure 2 Morphine at 0.47 mins, hydromorphone at 0.98 mins, norcodeine at 1.08 mins were shown in Inset A, oxycodone at 0.46 min and noroxycodone at 1.14 min were shown in Inset B, and amitriptyline at 1.54 min and EDDP at 1.65 min were shown in Inset C.

In this study the LC was coupled with the Sciex X500R QTOF system, a high-resolution quadrupole time-of-flight (QTOF) mass spectrometer. The X500R is suited for screening applications because the data generated from these systems provides structural information for every possible analyte. Typically, scanning across the full mass range provides the fine spectral details of the precursor ions of the analytes. After coupling to a quadrupole mass filter, such a mass spectrometer (Quadrupole-TOF or QTOF) can provide the full-scan information of not only the precursors ions, but also all the product ions in very high resolving power. Modern QTOF systems provide the capability of switching between MS and MS/MS scans instantly, enabling structural information to be obtained very quickly.

The analytical experiment to acquire the data was MS/MS^{All} with. In this technique's data cycle, the instrument begins by acquiring the TOF-MS information first, and then sequentially follows that by acquiring the MS/MS information of all precursor ions across a specified mass range in pre-divided Q1 mass isolation windows. SWATH[®] acquisition records MS/MS information of everything all the

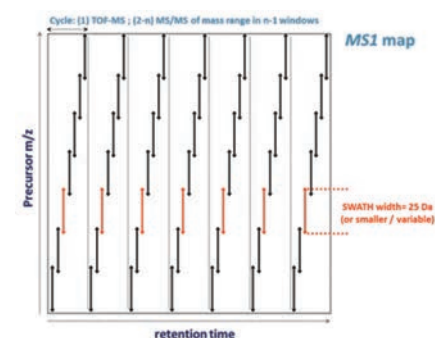


Figure 3 Principle of the analytical technique.

TOF MS						
TOF start mass	100 Da	Declustering potential	50 V	Collision energy	10 V	
TOF stop mass	500 Da	DP spread	0 V	CE spread	0 V	
Accumulation time	0.1 s					
TOF MS/MS						
TOF start mass	25 Da	TOF stop mass	500 Da	Dynamic collision energy	<input type="checkbox"/>	
Accumulation time	0.025 s	Charge state	1			
Mass Table						
	Precursor ion start mass (Da)	Precursor ion stop mass (Da)	Declustering potential (V)	DP spread (V)	Collision energy (V)	CE spread (V)
1	125.0000	160.0000	50	0	35	15
2	159.0000	195.0000	50	0	35	15
3	194.0000	230.0000	50	0	35	15
4	229.0000	265.0000	50	0	35	15
5	264.0000	300.0000	50	0	35	15
6	299.0000	335.0000	50	0	35	15
7	334.0000	370.0000	50	0	35	15
8	369.0000	405.0000	50	0	35	15
9	404.0000	440.0000	50	0	35	15
10	439.0000	475.0000	50	0	35	15

Figure 4: Details of the settings for the acquisition on the X500R QTOF system

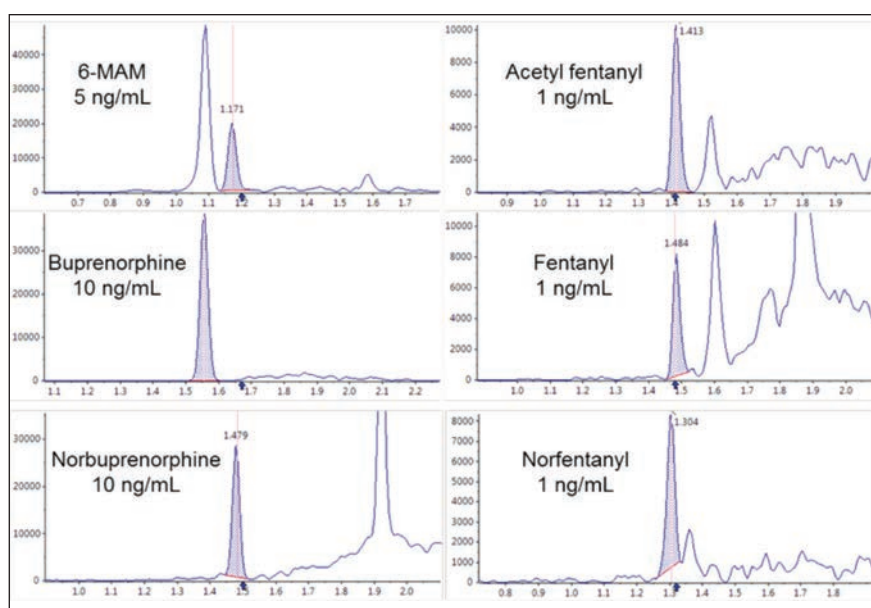


Figure 5: Examples of extracted chromatograms at level 1 spike in urine.

time, and it significantly improves the MS/MS data quality by allowing sequentially programmed Q1 isolations therefore more selective MS/MS data collection compared to other MS/MS^{All} techniques. Figure 3 shows the principal behind this acquisition experiment and Figure 4 details the settings for this acquisition on the X500R QTOF system.

Analysis of the data at spiking level 1 shows clear resolution and separation with confident identification. Figure 5 shows the extracted ion chromatograms of selected

	Cut off value	2ng/mL	10ng/mL	20ng/mL	50ng/mL	100ng/mL	True Positive rate %
Cal Level	% of Cut off value	ng/mL	ng/mL	ng/mL	ng/mL	ng/mL	SWATH acquisition
Level 1	40	0.8	4	8	20	40	96%
Level 2	80	1.6	8	16	40	80	100%
Level 3	100	2	10	20	50	100	99%
Level 4	200	4	20	40	100	200	100%
Level 5	300	6	30	60	150	300	100%
Level 6	500	10	50	100	250	500	99%
Level 7	1000	20	100	200	500	1000	99%

forensic analytes in urine at spiking level 1.

This analytical technique shows that even with a rapid LC gradient of less than 3 minutes, a full comprehensive profile of the sample can be built. Figure 6 shows the true positive rate at the varying concentration levels. Even at the low levels found in sample 1 there is still a 96% positive detection.

Unknown 1 and 2 were quality control urine samples spiked with all the analytes at different levels. Sample 3 through 20 were true unknown samples. There was a total of 82 true positive identifications from Sample 3 to Sample 20. For quantitation, the TOF-MS data from SWATH[®] acquisition can be used. It is worth mentioning that the MS/MS data from this acquisition can also be used for quantitation and compound identification through ion ratios, but we did not test that in this study but has been demonstrated in a previous study [6].

This full scan acquisition technique yielded excellent positive detection due to its complete coverage of MS/MS information. One of the key features of this scan type is the ability to create a digital archive of your sample, as this is an MS^{All} experiment of everything is scanned. The sample only needs to be injected once and the analysts will have a full insight as to what compounds are present in their sample, the analyst can return to this sample and re-interrogate the data at any time. There is no need to re-run a sample, which may have been subjected to degradation and thus forensic analytes may no longer be traceable. This means that if a brand new NPS enters the drug scene the acquisition will view the unique spectra attributed to the new forensic compound and begin to facilitate identifying the type of designer drug that has entered supply.

Conclusion

In this example, it has been demonstrated that tackling the chromatographic analysis of novel psychoactive substances with High Resolution Mass Spectrometry can be highly effective using an ultra-fast chromatographic separation technique coupled with a QTOF mass spectrometer. Non-targeted SWATH acquisition affords retrospective data analysis which is critically important for the rapidly emerging new designer drugs.

References

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