COMPARISON OF DATA-INDEPENDENT AND DATA-DEPENDENT TECHNIQUES FOR FORENSIC TOXICOLOGY SCREENING ANALYSIS

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INTRODUCTION

- The increase in number, diversity and potential toxicity of drugs is a major concern and presents significant challenges for forensic toxicology laboratories, therefore, analytical methods that can provide reliable results are of interest.
- High-resolution mass spectrometry has gained popularity for broad toxicological screening.
- In addition to monitoring the precursor accurate mass, common analytical practice also includes retention time (RT) and additional mass spectrometry data that is generated through data-independent (DIA) or data-dependent (DDA) techniques.
- The aim of this study was to compare DIA using MS^E and DDA methods with respect to ease of set-up, data generated *e.g.,* richness of information but also screening efficiency *i.e.,* detection accuracy using UPLC-Time of Flight-Mass Spectrometry (UPLC-Tof-MS).

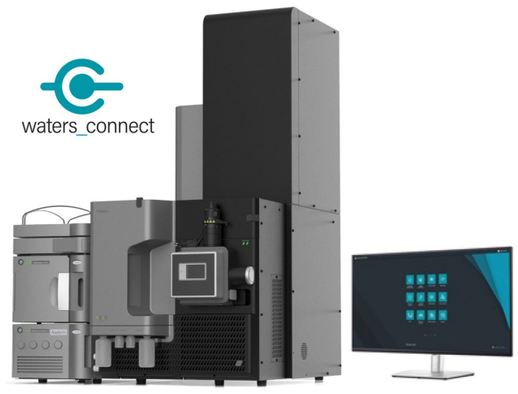

Figure 1. Waters™ ACQUITY™ UPLC I-Class with Xevo™ G3 QTof™

SAMPLES AND PREPARATION

- A system suitability mixture (SSM, Waters p/n: 186007361, see Table 1), was spiked into blank urine to yield a drug concentration of 50 ng/mL. The sample was diluted 5-fold using mobile phase A, prior to analysis.
- Twenty authentic urine samples that had been previously screened for drugs using a variety of techniques (GC-MS, immunoassay and LC-MS based techniques) were anonymized and used for the study. In total, 146 drugs substances were detected by the combined techniques. Samples were diluted 5-fold using mobile phase A prior to analysis.

EXPERIMENTAL

The system comprised an ACQUITY™ UPLC™ I-Class with the Xevo™ G3 QTof™ (Figure 1) and waters_connect™ (UNIFI™) informatics for data acquisition and processing.

ACQUITY UPLC conditions

- Chromatographic separation was achieved using a 15 min gradient elution (Table 2). The same conditions were applied for both the DIA and DDA approaches.

Screening with DIA using MS^E Analysis

- Accurate mass data was acquired in electrospray positive ionization mode using the MS^E technique.[1] MS^E acquisition mode facilitates the simultaneous collection of full MS spectra under two energy levels (collision-cell voltages); the low energy (6 eV) provides accurate mass of the precursor ion while the high energy (10-40 eV ramp) leads to the generation of accurate mass fragment ions for additional confirmatory purposes.

6MAM	EDDP	Morphine	*Milnacipran
Amphetamine	Fentanyl	Nandralone	*Nicotine
Benzoylecgonine	Ketamine	Oxycodone	*Perphenazine
Cetirizine	Ketamine, Nor	Oxymorphone	*Scopolamine
Chlorpheniramine	Lidocaine	Temazepam	*Tianeptine
Cocaine	MDA	Testosterone	*Tiapride
Codeine	MDMA	Tramadol	*Trazodone
Diazepam	Methadone	*Buflomedil	*Triprolidine
Diazepam, Nor	Methamphetamine	*Clozapine	

*Table 1: List of 25 key analytes targeted during the DDA-2 analysis and the 10 system suitability analytes **

Column (Temp.)	ACQUITY UPLC HSS C18, 2.1×150 mm (50°C)
Mobile Phase A	5 mM ammonium formate pH 3.0
Mobile Phase B	Acetonitrile with 0.1% formic acid
Analysis Time	15 min gradient elution
Injection Volume	5 µL
Ionization Mode	ESI positive
Acquisition Range	*m/z* 50 - 1000

Table 2. Summary of LC and MS conditions used with Tof-MS

Screening with DDA

- DDA data was acquired in full scan MS mode. During MS acquisition, a minimum precursor response must be achieved which subsequently trigger data acquisition in MS/MS mode (collision energy ramp from 10-40 eV), before returning back to MS acquisition.
- Several trigger thresholds were evaluated prior to the final analysis; 50,000, 100,000, 200,000, 500,000 and 1 million counts were assessed for both DDA methods (DDA-1 and DDA-2).
- DDA-1 acquisition used a minimum threshold response during a MS survey scan; exceeding this threshold, triggered MS/MS analysis.
- DDA-2 acquisition used the same minimum threshold response, as DDA-1, during the MS survey scan, but the method also included a list for 25 precursor masses (Table 2) to preferentially target key analytes for MS/MS analysis.

MS^E acquisition enabled:
Easier implementation, provides a complete and unrestricted dataset which was processed faster than DDA

Data processing

- Data from all techniques were compared with an established library of >2,000 toxicologically-relevant analytes, identification was based on reference RT (±0.35 min), accurate mass for the precursor (±5 ppm) which attained a minimum response of 10,000 counts and the presence of at least one diagnostic fragment ion (Waters Forensic Toxicology library).[2]

RESULTS AND DISCUSSION

- Figures 2 and 3 show example spectra for a representative compound (4-hydroxy-3-methoxymethamphetamine; HMMA) when analyzed by DIA and DDA respectively. In each case the low energy spectrum shows the accurate mass of the precursor (highlighted in green) which provides high specificity for identification.
- Further confidence in identification is also achieved by detection of analyte-specific product ions which are generated by fragmenting precursor molecules under high energy conditions. Identification involved comparison of component data with a toxicology library comprising exact masses of precursor molecules and analyte-specific fragments; RT was also used in the identification.
- In MS^E, there is no quadrupole selection, therefore all observed precursor ions are fragmented by simple, constant alternation between low and high energy conditions. A unique 3-dimensional algorithm assimilates MS^E data into components based on measured precursor and fragment ions.
- With DDA analysis, only those precursors exceeding the threshold (DDA-1) and corresponding to pre-selected analytes, shown in Table 1 (DDA-2), are selected using the quadrupole for MS/MS fragmentation, thus high energy spectra appear less complex compared to MS^E.
- During DDA-2 acquisition, any substance listed as a key analyte which exceeded the response threshold would take priority in acquiring MS/MS data, before other non-targeted analytes.

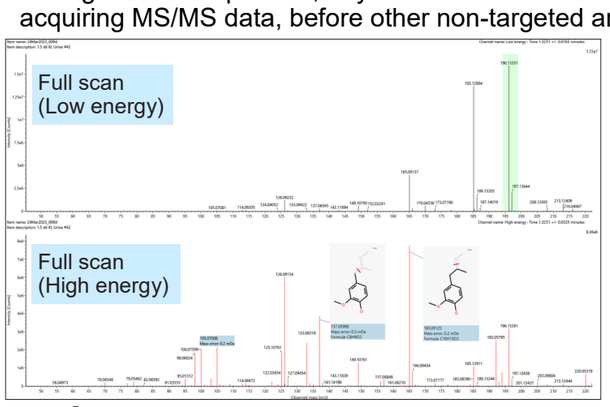

Figure 2: MS^E; low and high energy spectra for HMMA. Low energy spectrum shows the precursor ion. The high energy spectrum shows the diagnostic fragment ions related to the analyte (in blue). Additional ions visible in the high energy spectrum are ions from co-eluting analytes and/or matrix.

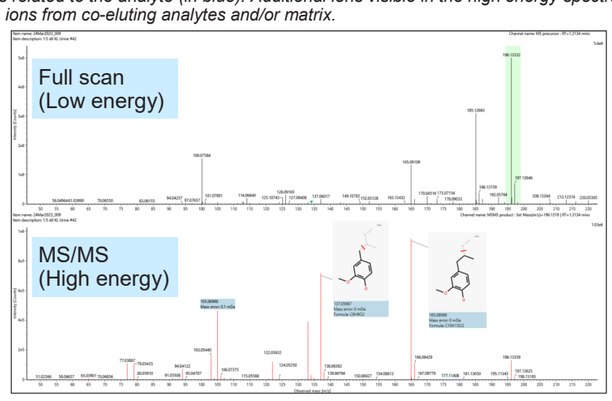

Figure 3: DDA-1; low and high energy spectra for HMMA. Spectral data for DDA-1 and DDA-2 appeared identical.

- Blank urine samples spiked with the SSM analytes (Table 1) were used to investigate the trigger threshold for DDA; a threshold of 100,000 was determined to be optimal for 5-fold diluted urine samples. Lower thresholds led to unnecessary switching to MS/MS while higher thresholds led to some false negatives.
- Twenty authentic urine samples were screened using DIA and DDA techniques (100,000 trigger threshold applied to DDA). Twenty-seven different substances and metabolites were detected by one or more techniques. Table 3 displays the drug detection rate by the three analytical approaches.

Acquisition mode	MS^E	DDA-1	DDA-2
Analytes detected (%)	**94**	**86**	**82**
Processing time per sample (min)	**3**	**12**	**12**

Table 3. Summary of results for DIA (MS^E) and the DDA techniques. Results presented are compared to a total of 146 detections from the reference techniques.

- With respect to screening efficiency, MS^E mode performed better than both DDA techniques with regards to the number of identifications of true positive analytes.
- Despite using a list of precursor ions to preferentially target key analytes of interest for MS/MS acquisition, the DDA-2 approach detected fewer dugs than DDA-1 and MS^E. In 9 instances, the actual targeted drugs were missed with this approach.

CONCLUSIONS

- **The MS^E screening method was easier to implement and use, as optimization of trigger thresholds was unnecessary, unlike with DDA.**
- **Despite the more complex spectra, MS^E mode provided efficient analyte detection and identification of expected drugs in the authentic urine samples (94%). MS^E data was processed 4 times faster than DDA data.**
- **Fewer false negative results were encountered when using MS^E mode for analytes at very low concentration, as no minimum trigger threshold was required.**
- **False negative detections by both DDA-1 and DDA-2 appeared to be due to triggering conflicts, particularly where co-elution was evident.**
- **A disadvantage of DDA is whilst the instrument is collecting MS/MS data, it is not collecting full scan MS data, thus the data is incomplete. A complete and unrestricted dataset is advantageous, as it provides the ability to retrospectively examine data, which MS^E mode of acquisition offers.**

References

1. M Wood. The Utility of MS^E for Toxicological Screening, Waters Application Note, 720005198, Revised Mar 2022
2. M. Wood, N. S. Mistry. Quality Over Quantity—It's Not Always a Numbers Game, Waters White Paper, 720007520, Mar 2022