EVALUATION OF SEMI-TARGETED AND NON-TARGETED ('DISCOVERY') SCREENING TOOLS

Michelle Wood[1] and Thomas G Rosano[2,3]

[1]Waters Corporation, Wilmslow, UK; [2]Albany Medical Center Hospital and College, Albany, NY, USA; [3]National Toxicology Center, Albany, NY, USA

INTRODUCTION

- The constant emergence of new psychoactive substances poses a significant analytical challenge for toxicology laboratories.
- High resolution mass spectrometry *e.g.*, Time-of-flight (TOF) analysis, is increasingly used for toxicological screening.
- Non-targeted acquisition methods, such as TOF-MS^E, are preferred as these facilitate the collection of a complete, unrestricted dataset thus providing the option to use non-targeted, as well as standard targeted workflows. MS^E also allows retrospective examination of the data.

MS^E

- ✓ Targeted analysis
- ✓ Semi-targeted analysis
- ✓ Non-targeted analysis
- ✓ Retrospective analysis

- A typical targeted workflow involves simple comparison of the acquired data to a characterised library (or list) of targets.
- While comparison with large libraries comprising elemental formulae might be considered attractive, supporting data such as retention time (RT) and high energy fragment ion information, are essential to improve accuracy of the identification.
- Screening for known, well-characterised drug substances is straightforward but remains only part of the analytical challenge where the drug landscape is constantly shifting.
- Discovering potential 'unknown' components within the dataset requires a non-targeted approach.
- Software tools are a key element to fully maximising all available information from the dataset.

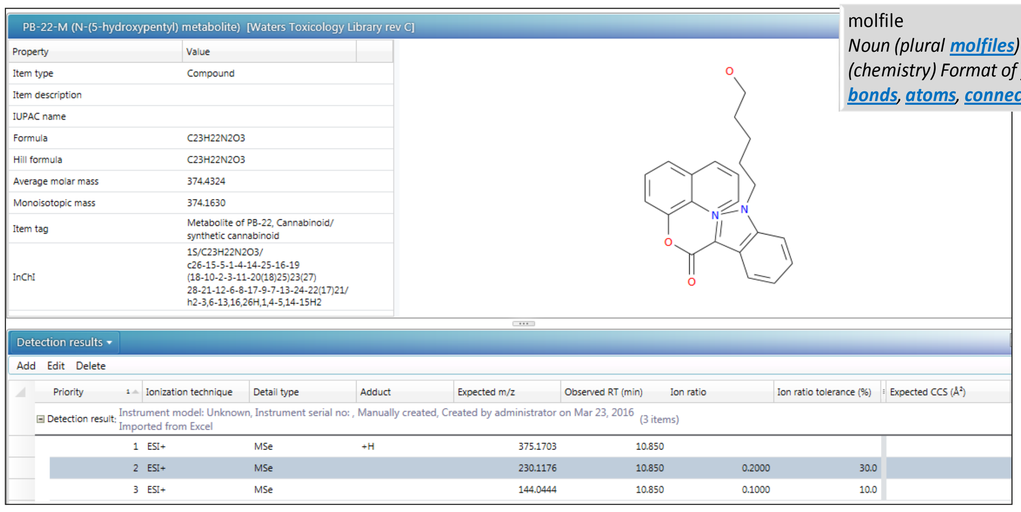


Figure 2. View of the UNIFI® Toxicology Library. *The example shows an entry for the 5-hydroxypentyl metabolite of the synthetic cannabinoid PB-22. The information shown in the 'Detection Results' box details the parameters that are used for high specificity targeted analysis and includes: RT, exact mass for both precursor [H^+] and multiple diagnostic fragment ions. Reference ratios for the fragment ions can also be incorporated into the library and utilised for extra confirmation. Molfiles structures for novel, or existing drug substances can be loaded into UNIFI libraries and used for automated semi-targeted screening.*

molfile
Noun (plural molfiles)
(chemistry) Format of file for holding information about the bonds, atoms, connectivity and coordinates of a molecule

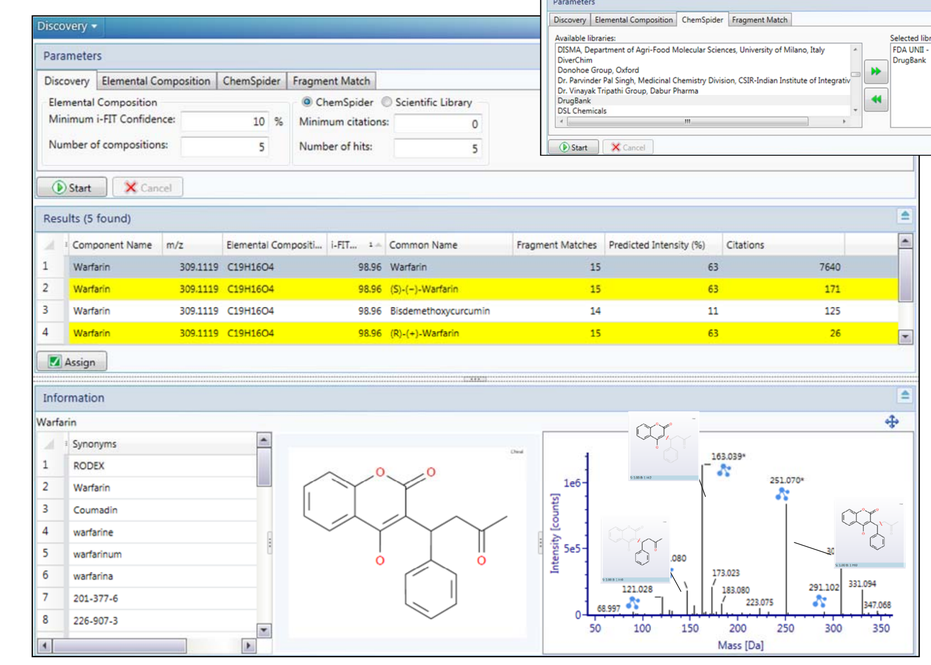


Figure 3. UNIFI® Discovery. *Launching the Discovery tool facilitates Chemspider searching which comprises more than 500 individual libraries, and >80 million chemical structures. The figure shows an example for warfarin; a single elemental composition was proposed based on the measured mass of the precursor i.e., $C_{19}H_{16}O_4$; automatic submission of this formula to the FDA UNII–NLM and DrugBank libraries returned four proposed compounds (middle-table).*

*Molfiles for each proposal are accessed and used for in-silico fragmentation techniques. The table also displays the number of fragment ions (**Fragment Matches**) in the high energy data that match plausible structures for each proposed candidate.*

*The **Predicted Intensity** indicates the percentage, of the total intensity the spectrum, that are covered by these proposed fragment ions e.g., a small number of intense (more-abundant) fragment ions would be better than many non-specific fragment ions of low abundance.*

METHODS

Samples

Seventeen postmortem blood samples.

Screening system:

Waters® Forensic Toxicology Screening Application Solution comprising an ACQUITY UPLC® I-Class and XEVO™ G2-XS QTOF Mass Spectrometer with UNIFI® informatics.

Full accurate mass data was acquired using MS^E mode (**Figure 1**). Data were acquired under two energy conditions: the low energy provides the accurate mass of the precursor ion; the elevated energy leads to the generation of specific accurate mass fragment ions for additional confirmatory purposes.

UNIFI® data processing:

Samples were processed automatically using the standard method which incorporates both targeted (I) and semi-targeted analysis (II). In addition, the Discovery workflow was utilised for selected candidates (III).

I. Targeted analysis

Acquired data is matched against a library containing >1300 drugs and metabolites (**Figure 2**) and uses the following criteria for a *POSITIVE* identification:

- RT ± 0.35 min of reference
- Mass accuracy ± 5ppm
- Minimum of 1 supporting diagnostic fragment ion
- Minimum response 6000 counts

II. Semi-targeted analysis

Acquired data is matched against a Molfile and uses the following criteria for identification:

- A tentative match to the precursor mass triggers automated *in-silico* fragmentation of the molfile to generate theoretical fragment ions (max. of 2 bond breaks)
- Theoretical substructures are compared to any observed fragment ions within the high energy data (± 2mDa)

III. Non-targeted ('Discovery') analysis

Acquired data is submitted to the 'Discovery' tool which automates the following in a single-step (**Figure 3**):

Proposal of elemental composition (max. of 5 formulae taken to next step)

↳ External library (internet) searching for proposed substances (max. of 5 per formulae)

↳ *In-silico* fragmentation of proposed substances and automated comparison with high energy data

RESULTS AND DISCUSSION

Seventeen samples were analysed using the TOF-MS^E mode and processed using UNIFI informatics and the Waters toxicology library. The library comprised data for >1300 drug substances. 1250 substances had associated RT and diagnostic fragments *i.e.,* typically characterised though analysis of reference material. 98% of library entries were also supplemented with a Molfile to allow independent verification of the accuracy of existing targeted data. For 50 emerging drug substances (advisory from early-warning organisations)[‡] where reference material (and therefore RT and diagnostic fragment data) was not available at the time of analysis, a library entry comprising only a Molfile was created.

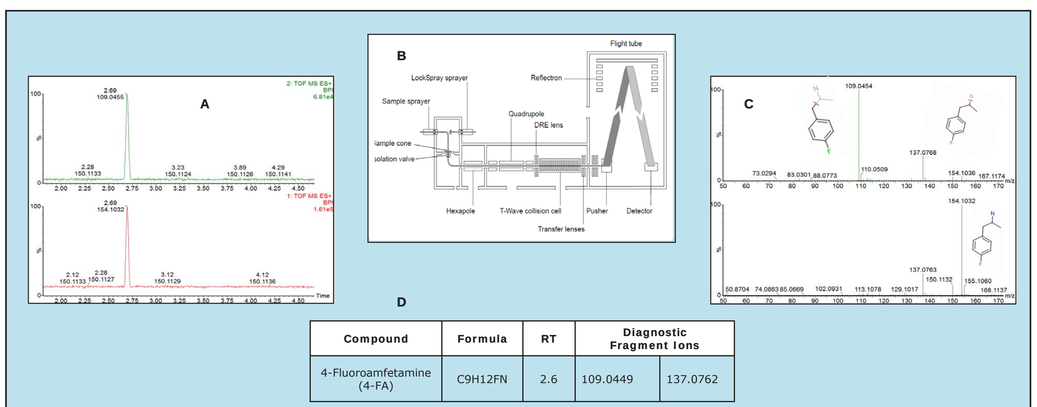
Results are summarised in **Table 1**. Targeted processing (I) led to 138 drug detections involving 74 toxicologically-relevant substances; 128 detections (92.7%) were confirmed by the presence of at least one diagnostic fragment from the library, and satisfied the *POSITIVE* identification criteria. The most commonly detected substances, along with their metabolites, were: cocaine, amiodarone, midazolam, diphenhydramine, mirtazepine, olanzapine and lidocaine.

This study assessed the performance of the automated semi-targeted screening (II) tool. All 138 substances initially detected by targeted analysis were simultaneously confirmed through use of the Molfile; 88% of these were confirmed by theoretical fragment ions as generated by *in-silico* techniques. The same fragmentation tool independently confirmed accuracy of 69% of the associated diagnostic ions, thereby demonstrating good accuracy of the existing library content. Three additional substances were detected by the semi-targeting method; these were subsequently confirmed. The above two screening modes (I and II) are applied automatically to all samples; screening for common fragments and common neutral losses are also available in the routine screening method.

To evaluate the performance of the Discovery tool, each of the initial 138 identifications (from the targeted screen) were de-identified and resubmitted for non-targeted screening (III). Structural elucidation and subsequent external library searching confirmed 85% of the previously detected substances thereby also demonstrating the benefit and accuracy of the Discovery tool.

	Targeted screening (I)	Semi-targeted screening (II)	Discovery (III)
No. of drugs and metabolites detected	138 substances	141 substances (138, as found by targeted analysis + 3 *additional* substances)	117/138
No. of the above detections satisfying the *POSITIVE* criteria	128 (93%) confirmed with ≥ 1 fragment 115 (83%) confirmed with ≥ 2 fragments 75 (54%) confirmed with ≥ 3 fragments	122 (88%) confirmed with ≥ 1 theoretical fragment	Multiple theoretical fragments proposed
Total no. of associated diagnostic ions	365	253 of the 365 (69%) of the diagnostic ions, of the detected substances, were confirmed	N/A

Table 1. *Performance of semi and non-targeted screening — a comparison against the standard targeted screening approach.*



Compound	Formula	RT	Diagnostic Fragment Ions	
4-Fluoroamfetamine (4-FA)	C9H12FN	2.6	109.0449	137.0762

Figure 1. TOF-MS^E analysis. *Data shows analysis of a reference standard for 4-Fluoroamphetamine (4-FA) collected during preparation of the library. With TOF-MS^E, full accurate mass data is acquired simultaneously under low and high energy conditions (**panel A**). Fragmentation of the precursor molecule occurs within the T-Wave collision cell of the instrument (**panel B**). Low (lower-trace) and high energy (upper-trace) spectra are always available for every component (**panel C**). The structure of observed fragment ions are verified prior to their addition into the library along with retention time (RT) and elemental formula for automatic determination of the exact mass of the precursor molecule (**panel D**).*

CONCLUSIONS

- Automated semi-targeted screening confirmed the accuracy of the existing library content. It also improved the overall efficiency of drug screening.
- Semi-targeted screening is a powerful tool allowing the user to accurately screen for substances where reference material is not available; additional specificity and confidence is provided by theoretical fragments.
- The Discovery tool independently confirmed 85% of previously detected substances.
- Semi-targeted and non-targeted workflows significantly enhance the efficiency of toxicology screening.

[‡]Examples of Early-warning organisations
European Monitoring Centre for Drugs and Drug Addiction (EMCDDA); United Nations Office on Drugs and Crime (UNODC); UK Home Office Forensic Early Warning System (FEWS)