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Toxicology

LC-MS/MS toxicology workflow on the new Orbitrap Exploris 120 mass spectrometer for screening, quantitation, and confirmation of drugs

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Keywords

Tox Explorer Collection, Orbitrap Exploris, Orbitrap, drugs of abuse, ultra-high resolution, accurate mass, quantitation, targeted screening, toxicology, anti-doping, sports anti-doping, forensics, unknown screening, retrospective data analysis

Application benefits

- Comprehensive method from sample acquisition to reporting

- Over 1,800 compounds of interest in spectral library with the addition of 200+ fentanyl analogs

- Separation of isomers of interest in forensic toxicology

- Screen, identify, confirm, and quantitate with one instrument

- Analyze compounds of different drug classes with a wide range of hydrophobicities, positive and negative polarities

Goal

Demonstration of a targeted screening and quantitation method for a large panel of drugs of abuse and other toxins. The Thermo Scientific™ Tox Explorer™ Collection is a workflow-based approach utilizing the high-resolution, accurate-mass Thermo Scientific™ Orbitrap™ Exploris™ 120 mass spectrometer. Identified compounds are screened against an extensive HRAM MS/MS spectral library and a compound database containing molecular formula, exact mass, retention times, and fragment ions.

Introduction

One of the major challenges faced by most toxicology laboratories is analyzing hundreds of drugs of abuse in biological samples. This is further complicated with the constantly increasing emergence in the volume of designer and illicit drugs. Toxicology laboratories also need to rapidly screen for the presence of drugs, quantitatively confirm compounds, and have access to retrospective analytical data. Not to mention, all of these analytical challenges must be addressed while minimizing cost per sample (or per analysis).
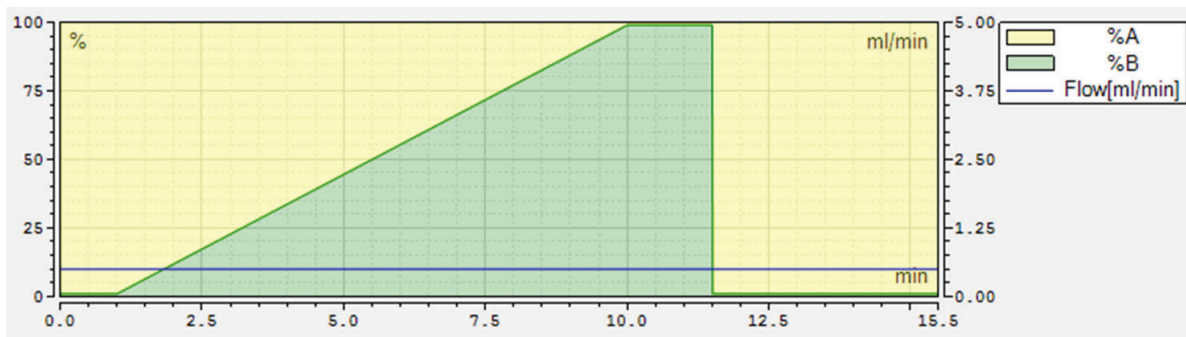
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Figure 1. Graphical representation of gradient used in this note

While each of the above-mentioned challenges can be addressed at a given time, it is difficult to address all of them with a single approach. An all-in-one workflow that combines efficient liquid chromatography with high-resolution, accurate-mass (HRAM) mass spectrometry enables detection and quantitation of a large panel of analytes in a single automated run, providing a fast turn-around time for confident results.

In this technical note, we highlight the benefits of using the Thermo Scientific Tox Explorer Collection for robust, reliable, and reproducible screening and quantitation of over 1,800 drugs of abuse in biological matrices. The Tox Explorer Collection consists of a proven HPLC-MS method with sample preparation guidelines. The method is standardized using a Thermo Scientific™ Vanquish™ Flex ultra-high performance liquid chromatography (UHPLC) system and an Orbitrap Exploris 120 mass spectrometer. A comprehensive HRAM MS/MS spectral library stored in the Thermo Scientific™ mzVault™ mass spectral database and corresponding compound database are fully integrated and searchable using Thermo Scientific™ TraceFinder™ software for efficient identification of compounds.

Experimental
Sample preparation
Over a hundred compounds covering a wide range of drug classes, hydrophobicities, and polarities were prepared in five mixes in urine matrix, with eight internal standards (cotinine-d_3, amphetamine-d_5, naloxone-d_5, 6-MAM-d_3, benzoylecgonine-d_8, 7-aminoflunitrazepam, imipramine-d_3, diazepam-d_5). The samples were diluted twenty-fold in water, and the calibration curves from 0.1 to 1,000 ng/mL were run in triplicate.

Liquid chromatography
Gradient elution was performed using a Vanquish Flex UHPLC system for the separation of analytes. Mobile phases consisted of 2 mM ammonium formate with 0.1% formic acid in (A) water and (B) acetonitrile:methanol (1:1) (v:v) mixture. The column used

for separation was a Thermo Scientific™ Accucore™ Phenyl-Hexyl column (2.6 µm, 100 × 2.1 mm). The method duration for the quantitation and target screening was 15.5 minutes (Figure 1). Thermo Scientific™ Viper™ tubing and fittings were used to decrease the dead volume within the LC.

Mass spectrometry
The Orbitrap Exploris 120 mass spectrometer was used for the targeted screening and quantitation analysis (Figure 2). Full scan and targeted data-dependent MS/MS scanning were used with an inclusion list for the targeted compounds. The inclusion list contains the exact mass of the compound, polarity, and retention time. Resolution setting of 60,000 (FWHM at *m/z* 200) was used for the full scan and 15,000 for the MS^2 scan. The isolation window was *m/z* 1.5 and stepped collision energies (18.75, 37.5, 56.25) were used to generate the MS/MS spectrum. Polarity switching was performed, which allows the acquisition of both positively and negatively ionized analytes during a single analysis.

Figure 2. The Orbitrap Exploris 120 MS coupled with the Vanquish Flex UHPLC

Software

Data acquisition, processing, and reporting were all completed under one software platform, TraceFinder software, version 5.1.

Library generation

The spectral library and compound database were derived by injecting prepared stock solutions to obtain over 1,800 MS/MS spectra. The spectra that were acquired were then imported into an mzVault library (see Figure 3 for workflow). The expansion of the database includes fentanyl analogues from the **CDC TOM Kits™**. Addition of new compounds to a database and method can be done with ease because manual optimization of individual precursors is not required. New compounds can easily be added to the existing method by collecting full scan data with targeted MS^2 spectra. Once the retention time is known, the information is simply added to the compound database and library; there is no need for re-validation because cycle time/dwell time does not change in the method. This library utilizes the mzVault search algorithm for confidence in HRAM spectral library matching. The chromatographic separation of the standard solutions injected provided the retention times that were used to create the compound database.

Results and discussion

Over a hundred compounds were analyzed to demonstrate the capability of the method described here. The compounds were selected based on different drug groups, retention times across the entire chromatographic run, and different polarities.

Targeted screening

TraceFinder software stores the compound database including molecular formula, exact mass, retention time, and fragment ions for all compounds of interest. Five parameters were set to ensure positive confirmation during screening: exact mass of the precursor ion, retention time of UHPLC chromatography, isotopic pattern match, fragment ion match and spectral match with the mzVault library. Additional details on the criteria for each of these parameters can be found in Table 1.

Table 1. Criteria assigned in TraceFinder software for targeted drugs of abuse screening

| Parameter	Criteria
m/z of the parent ion	<5 ppm mass deviation for an intensity threshold set at 5,000 au
Retention time	Within a 30 s window
Isotopic pattern match	<10 ppm mass deviation, <20% intensity deviation, fit >70%
Fragment ion search	At least 2 fragments with <5 ppm deviation and an intensity threshold of 5,000 au
Spectral mzVault library	Reverse search, passing value >70%

Workflow for adding compounds to database
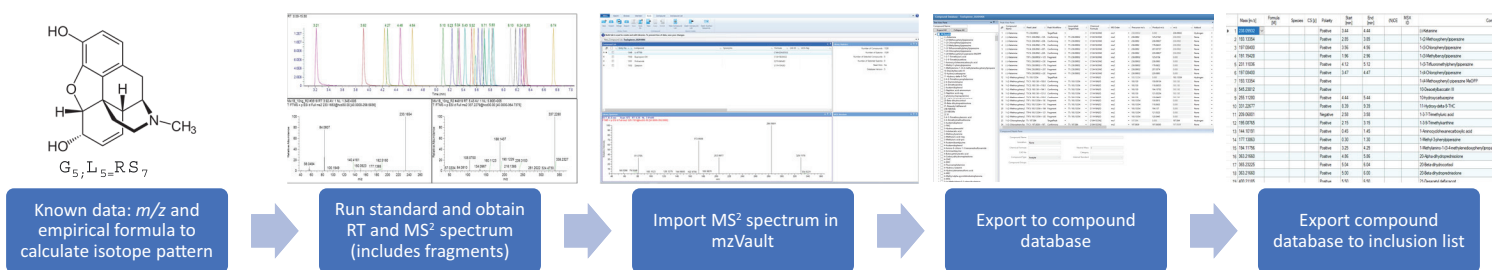
Figure 3. Workflow of library generation using the Thermo Scientific suite of software

The ability to utilize color-based flagging and column filtering allows the user an easy-to-understand summary of analytes detected in each sample. The overlay of expected and experimental data highlights any corresponding match between the isotopes, fragments, and library spectra. As part of the LC-MS/MS screening method, analytes were found within a mass accuracy of 5 ppm and within a 30 s retention time window.

An overlay of extracted ion chromatograms (XIC) of all the compounds is shown in Figure 4. Figure 5 further illustrates an example of mass spectra eluting at the beginning and end of chromatography to demonstrate the low delta ppm information that can be obtained from one MS/MS scan only, unlike other quadrupole time-of-flight mass spectrometry techniques that require averaging of multiple MS/MS scans.
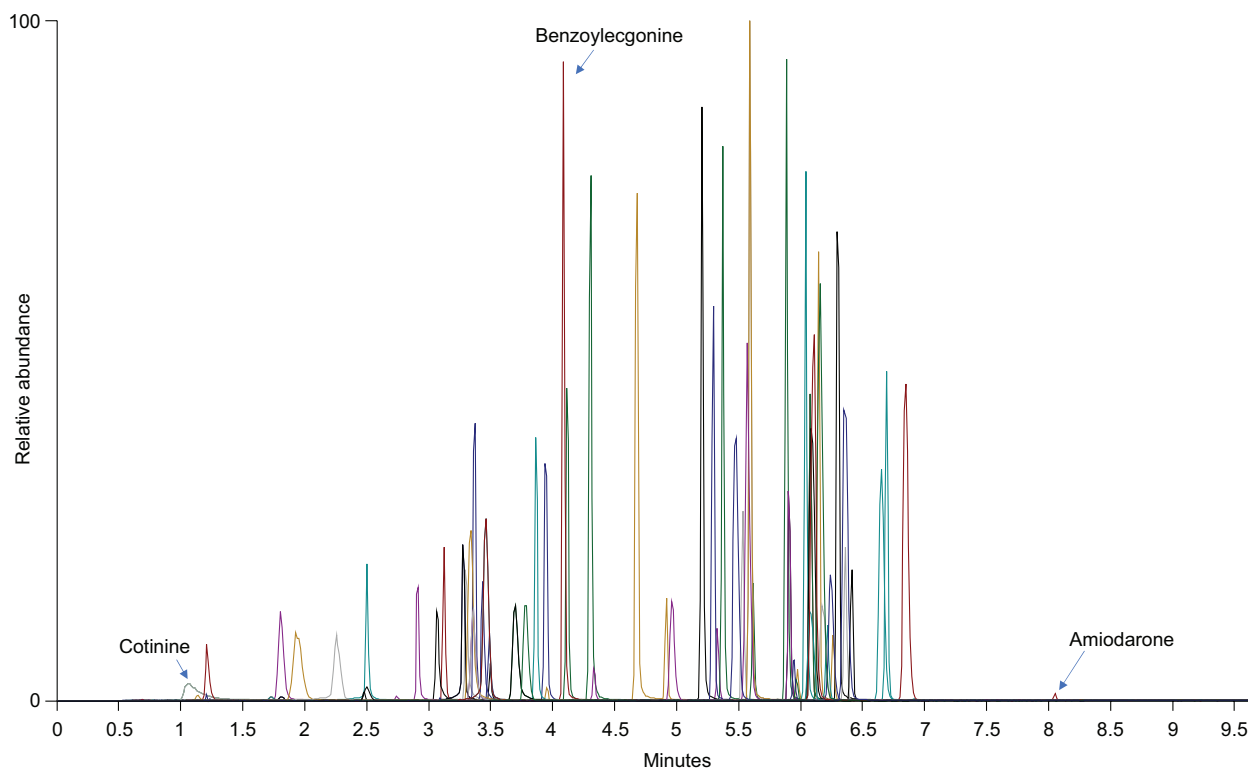

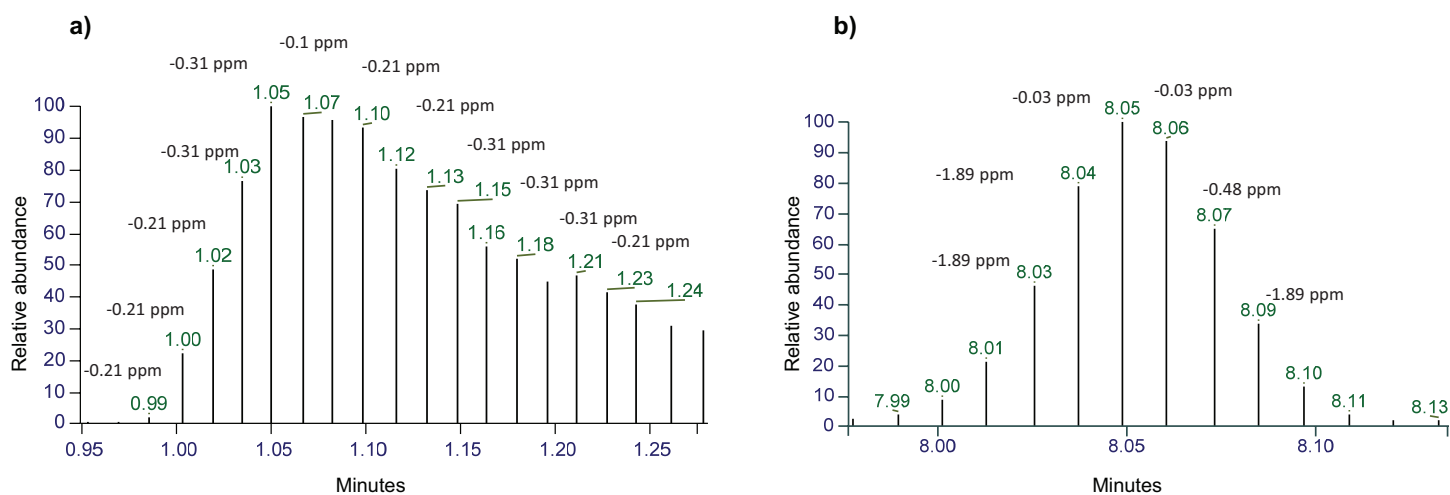**Figure 4. XIC of analytes**

Figure 5. From full scan spectrum, mass accuracy at different points under the chromatography peak of (a) early eluter, nicotine, and (b) later eluter, THC-COOH
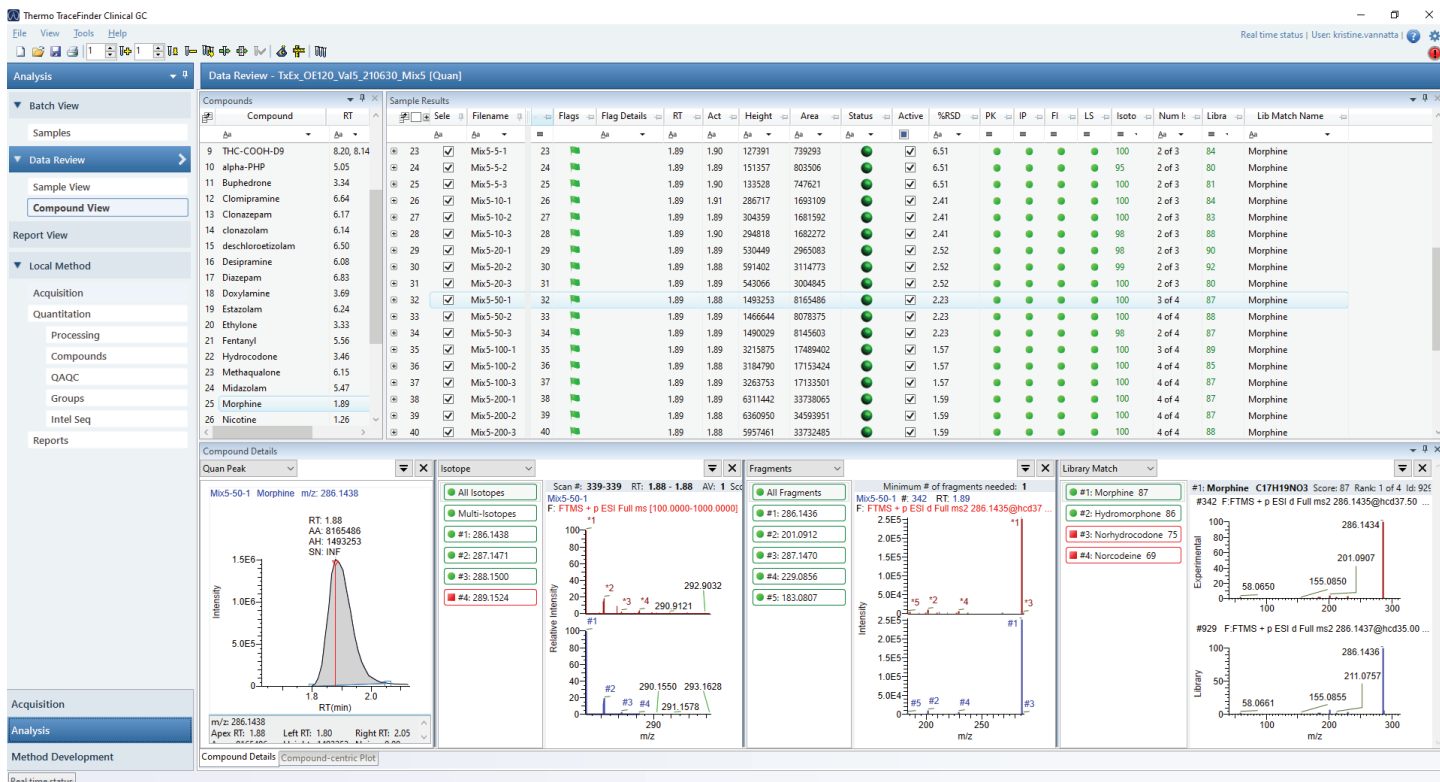
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Figure 6. Data review for screening of the analyte, morphine demonstrating retention time, isotope matching, fragment ions, and library match

An example of the screening data review is found in Figure 6, where the software quickly indicates which compound was positively detected in the sample. Morphine was found in Mix 5 for the entire calibration curve. The screening criteria are depicted in the bottom portion of the figure: extracted chromatogram, isotope matching, fragmentation pattern, and library matching. The exact mass of 286.1436 *m/z* is an isomer for four drugs of abuse compounds. Two out of the four were positive for library matching. However, with the Tox Explorer Collection, chromatography of these isomers is resolved with different retention times.

Quantitation of compounds

For most quantitation methods, analytical verification typically involves evaluation of the limit of quantitation (LOQ) and the intra-day and inter-day accuracy and precision. Over hundred analytes of different classes, retention times, and polarities were evaluated for limit of detection (LOD), LOQs, and limit of identification (LOI). Specific criteria for quantitation that were used can be found in Table 2.

TraceFinder software not only performs a fast screening workstream with confident identification and confirmation of the compounds, but also enables quantitative results based on calibration curves and standards used for quality control measures. The Orbitrap Exploris MS platform enables confirmation and quantitation on one instrument.

Table 2. Criteria assigned for quantitation of drugs of abuse compounds

Calibration parameters	Passing criteria
LOD = limit of detection	Presence of peak at correct retention time
LOQ = limit of quantitation, back-calculated concentration within	20% for the compounds with their own deuterated analog and internal standard (IS) (marked with IS)
	30% for all other compounds (use non-self IS)
LOI = Limit of Identification	IP = passing isotopic pattern score (70) FI = presence of diagnostic fragment ions LS = passing library score (70)

The samples with the selected analytes were prepared in the concentration range of 0.1 ng/mL to 1,000 ng/mL in urine. Eight internal standards were used, and quantitation for all compounds was processed using full scan spectra. An example of the calibration curve for 7-aminoflunitrazepam is shown in Figure 7. Figure 8 is representative XICs of 0.1 ng/mL for different analytes in each of the five mixes tested. The %CV for intra- assay calculated concentration were always less than 20%.
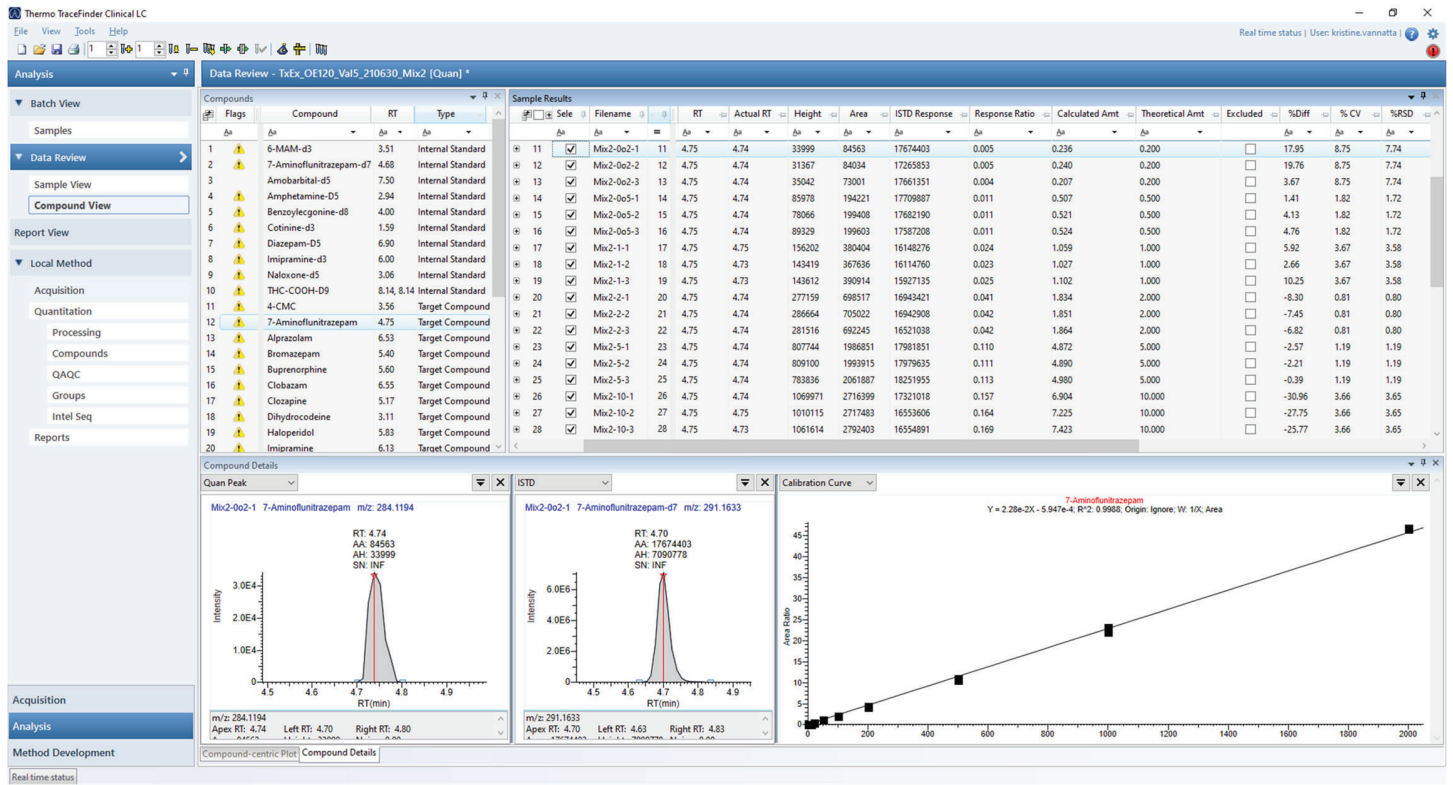

Figure 7. Data review for quantitation of 7-aminoflunitrazepam demonstrating peak area, percent different and percent RSDs for calibration curve of 0.1 to 1,000 ng/mL
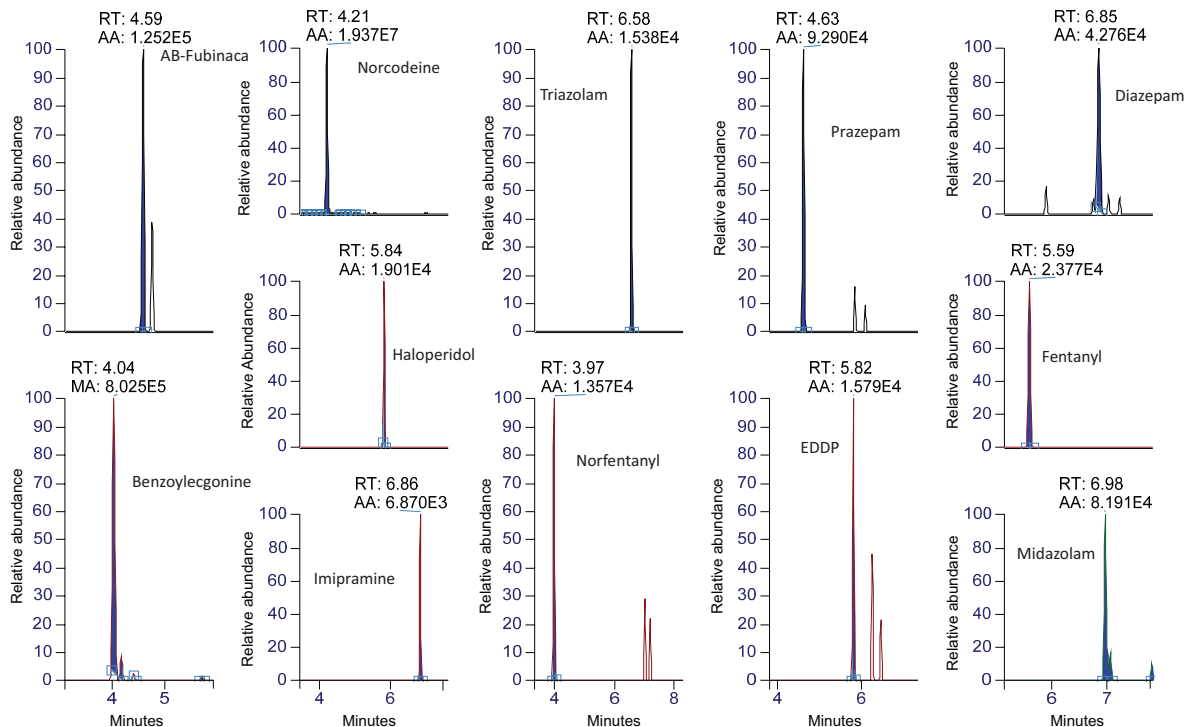

Figure 8. Analyte XIC at the concentration 0.1 ng/mL for various compounds in the five mixes run for quantitation using the Tox Explorer Collection method

The results obtained in this study fulfill standard clinical research requirements,[1] and this method can be optimized to achieve higher sensitivity by utilizing an alternative sample preparation and/or more targeted mass spectrometry methods. These results confirm the suitability of the Orbitrap Exploris 120 mass spectrometer for quantitative analysis, with an excellent dynamic range and a highly accurate mass measurement for a wide range of compounds.

Robustness of method

Robustness of the analytical method instills confidence in the data. This robustness can be demonstrated by showing high mass accuracy and mass stability across multiple injections. To demonstrate mass accuracy, internal standards 6-MAM-d_3, amphetamine-d_5, diazepam-d_5, and imipramine-d_3 were used, and the ensuing results are shown in Figure 8.
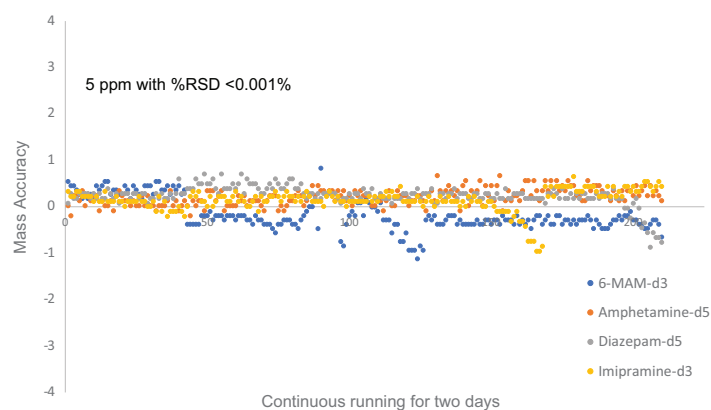

Figure 9. Robustness of method. Mass accuracy of four internal standards for continuous injections spanning over two days.

The masses were within 1.3 ppm with %RSD <0.1% over 2 days (48 hrs) without needing system recalibration procedures. The %CV for peak abundance for the internal standards were below 10% (imipramine-d_3 was 13% in Mix 4), and retention time for the compounds was ±0.01 min, demonstrating the reproducibility and robustness of this method.

Polarity switching

This extensive panel of drugs of abuse contains compounds, such as barbiturates and THC-COOH metabolite, that ionize in negative mode. Having compounds of different polarities generates a need to analyze mass spectra in both positive and negative mode. The Orbitrap Exploris 120 mass spectrometer performs fast polarity switching, resulting in comprehensive data obtained in a single analytical run.

Retrospective analysis

To keep methods up to date on current substances and allow identification of unknown compounds, retrospective analysis provides the ability to reprocess data that was previously collected in a non-targeted manner. With the data acquired for Tox Explorer Collection, a common plasticizer was identified within the sample with excellent mass accuracy (Figure 10).
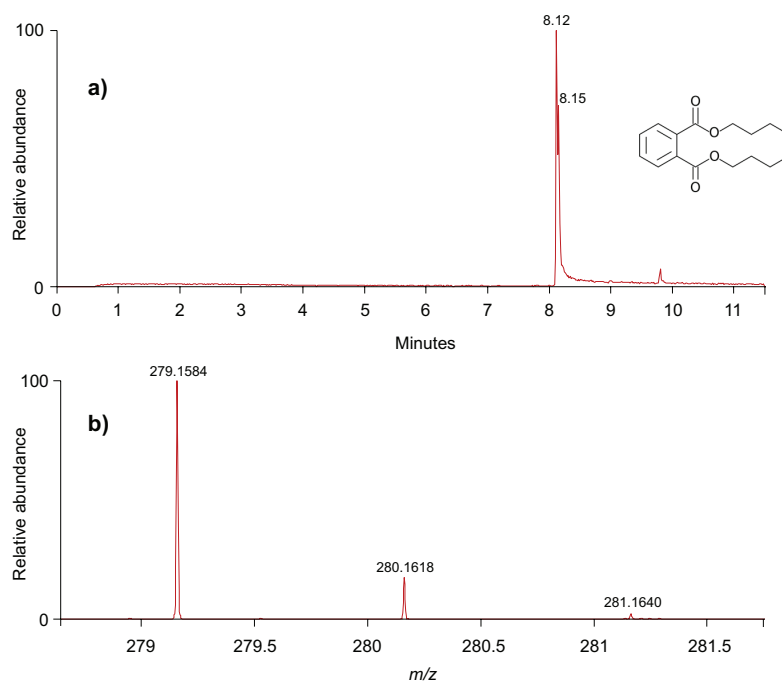

Figure 10. (a) XIC of exact mass of dibutylphthalate (*m/z* 279.1591) and (b) the spectrum at retention time 8.07, demonstrating mass accuracy

Conclusion

Tox Explorer Collection ensures a robust, reliable, sensitive, and easy-to-implement workflow for toxicology laboratories challenged with the task of analyzing hundreds of samples containing drugs of abuse analytes in complex biological matrices. The method implemented here uses a Vanquish Flex UHPLC system connected to an Orbitrap Exploris 120 mass spectrometer and leverages the power of Thermo Scientific™ Orbitrap™ high-resolution, accurate-mass technology to perform accurate targeted screening and quantitation with high efficiency, reliability, and confidence. The design of the Orbitrap Exploris mass spectrometers allows for ultra-high resolution and improved duty cycle. The compact footprint is ideal for space-constrained laboratories. The method reported in this study demonstrates the outstanding data quality for a drugs of abuse panel in urine obtained using an LC-HRAM-based quantitation and screening approach. Unlike other high-resolution platforms, Orbitrap mass spectrometers can quantify a larger panel of analytes with no averaging of MS/MS spectra to obtain informative fragment information.

TraceFinder software stores the compound database and enables data acquisition, monitoring, processing, reviewing, and customized reporting—all on one software platform. In addition, TraceFinder software screens for targeted analytes (using exact mass, retention time, isotope patterns, fragment ions, and matching to a compound library) and quantifies based on full scan data.

The method reported in this study incorporates a quick and simple dilute-and-shoot sample preparation option and meets typical requirements, such as sensitivity, linearity of response, accuracy, and precision, that most toxicology laboratories regularly address.

Reference

1. https://www.samhsa.gov/workplace/drug-testing

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