TECHNICAL NOTE 73771

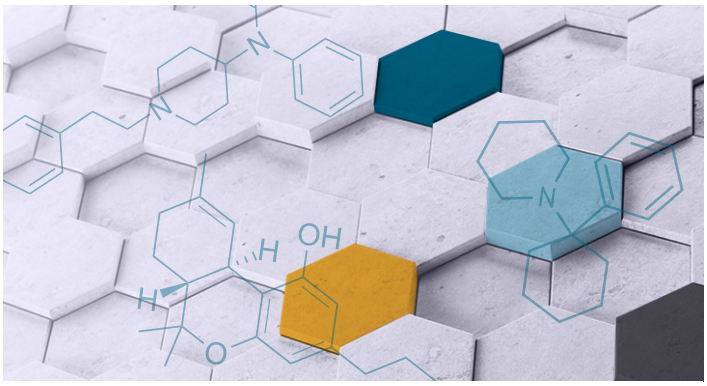
LC-MS/MS toxicology platform and method for high-resolution, accurate mass (HRAM) detection, screening, and quantitation of drugs

Authors: Xiaolei Xie, Kristine L. Van Natta, Kerry Hassell; Thermo Fisher Scientific, San Jose, CA, USA

Keywords: Tox Explorer Collection, Q Exactive Plus, Orbitrap, Drugs Of Abuse, High Resolution, Accurate Mass, Quantitation, Targeted Screening, Toxicology, Anti-Doping, Sports Anti-Doping, Forensics, Unknown Screening, Retrospective Data Analysis, Therapeutic Drug Monitoring

Application benefits

- Comprehensive method from sample acquisition to reporting
- Over 1,500 compounds of interest in spectral library
- Separation of isomers of interest in forensic toxicology
- Screen, identify, confirm, and quantitate with one instrument
- Analyze compounds with a wide range of hydrophobicities, polarities, and different drug classes



Goal

Demonstration of a targeted screening and quantitation method for a large panel of drugs of abuse. The Thermo Scientific™ Tox Explorer™ Collection is a workstream based approach utilizes the high-resolution, accurate-mass Thermo Scientific™ Q Exactive Plus™ Orbitrap™ mass spectrometer. Identified compounds are screened against an extensive HRAM MS/MS spectral library and a 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 samples in biological matrices. This is further complicated with the constantly increasing bulk of designer drugs. Toxicology laboratories also need to rapidly screen for the presence of drugs, quantify and have access to retrospective analytical data. Needless to mention, all of these challenges must be addressed while minimizing

cost per sample (or per analysis). While each of the above-mentioned challenges can be addressed at a given time, it is difficult to address all of them with one approach. An efficient workstream which 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 Thermo Scientific™ Tox Explorer™ Collection for robust, reliable and reproducible screening and quantitation of over 1,500 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 Thermo Scientific™ Q Exactive™ Plus mass spectrometer. A comprehensive HRAM MS/MS spectral library stored in 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

Fifty-four compounds covering a range of drug classes, hydrophobicities and polarities were prepared in four mixes in Surine matrix, with six internal standards (amitriptyline-D5, amphetamine-D5, diazepam-D5, haloperidol-D4, morphine-D3, and THC-COOH-D3). The samples were diluted twenty times in water and the calibration curves were run in triplicate over two days.

Liquid chromatography

Gradient elution was performed using a Vanquish Flex UHPLC system for the separation of analytes. Mobile phases were comprised of ammonium formate and formic acid in water and acetonitrile:methanol 50:50 v/v mixture. The column used for separation was a Thermo Scientific™ Accucore™ Phenyl-Hexyl column (2.6 µm, 100 × 2.1 mm, PN 17926-102130). The method duration for the quantitation and target screening method was 15.5 minutes. Specific Thermo Scientific™ Viper™ tubing and fittings were used to decrease the dead volume within the LC.

Mass spectrometry

The Thermo Scientific™ Q Exactive Plus™ Orbitrap mass spectrometer was used for the targeted screening and quantitation analysis (Figure 1). 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 of 35,000 was used for the full scan and 17,500 for the MS^2 scan. The isolation window was *m/z* 2.0 and stepped collision energies were used to generate MS/MS spectrum. Polarity switching was performed which allowed during one run allows the acquisition of both positively and negatively ionized analytes during a single analysis.

Figure 1. Q Exactive Plus MS coupled with Vanquish Flex UHPLC.

Software

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

Library generation

The spectral library and compound database were derived by injecting prepared stock solutions with this UHPLC method to obtain 1160 retention times and 1519 MS/MS spectra. The spectra that were acquired were then imported into a mzVault library. This library utilizes a new library search algorithm that improves identification confidence in library matching. The injections of the standard solutions provided the retention time that were used to create the compound database.

Results and discussion

Fifty-four compounds were analyzed to demonstrate the capability of the method described herein. 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 for compounds across different drug classes. A representative screenshot of the compound database using TraceFinder software is in Figure 2. Five parameters were set to ensure positive confirmation during screening: exact mass of the parent ion, retention time of UHPLC chromatography, isotopic pattern match, fragment ion match and match with mzVault library. Additional details on the criteria for each of these parameters that were used can be found in Table 1.

Table 1. Criteria assigned in the 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 5000 au
Retention time	Within a 30 s window
Isotopic pattern match	<10 ppm mass deviation, <20% intensity deviation, fit >70%
Fragment ion match	At least 2 fragments with <5ppm deviation and an intensity threshold of 5000 au
Spectral mzVault library matching	Reverse search, passing value >70%

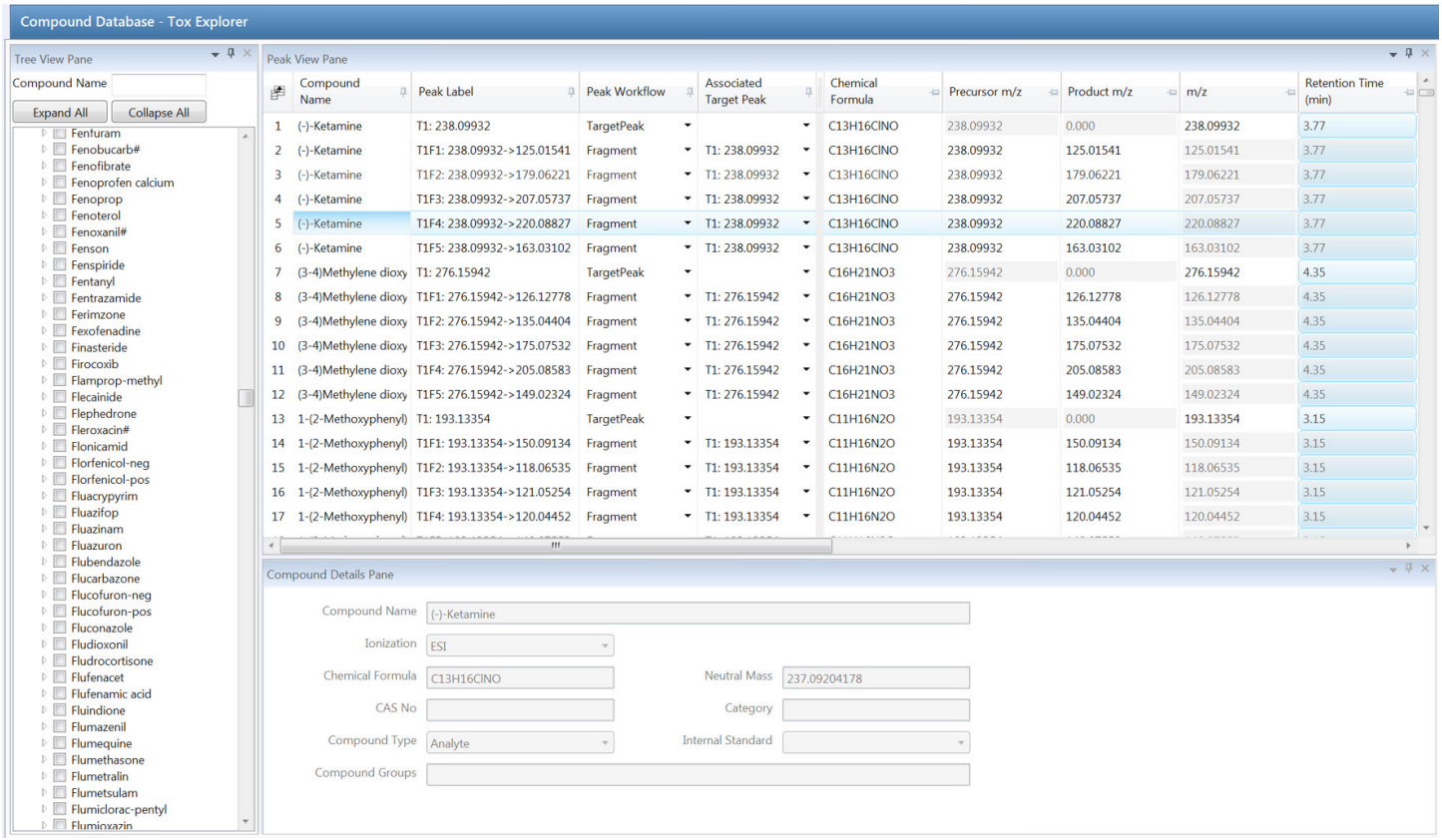


Figure 2. Compound database in TraceFinder software containing all analyte information imported from mzVault library. Retention time, MS/MS spectra, molecular formula, and exact mass information can all be recorded as information associated with a compound within TraceFinder software.

Figure 3 represents data obtained from the screening method using the TraceFinder software. The ability to utilize color-based flagging and column filtering allows the user a unique, 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 screening method, 54 analytes were found within a mass accuracy of 5 ppm and within a 30 s window (retention time). An overlay of Extracted Ion Chromatograms (XIC) of all the compounds is shown in Figure 4. The highlighted inset demonstrates mass accuracy over chromatographic peak. The inset also includes an example mass spectrum to demonstrate the information that can be obtained from one MS/MS scan only, unlike other mass spectrometry techniques, that require averaging of MS/MS scans.

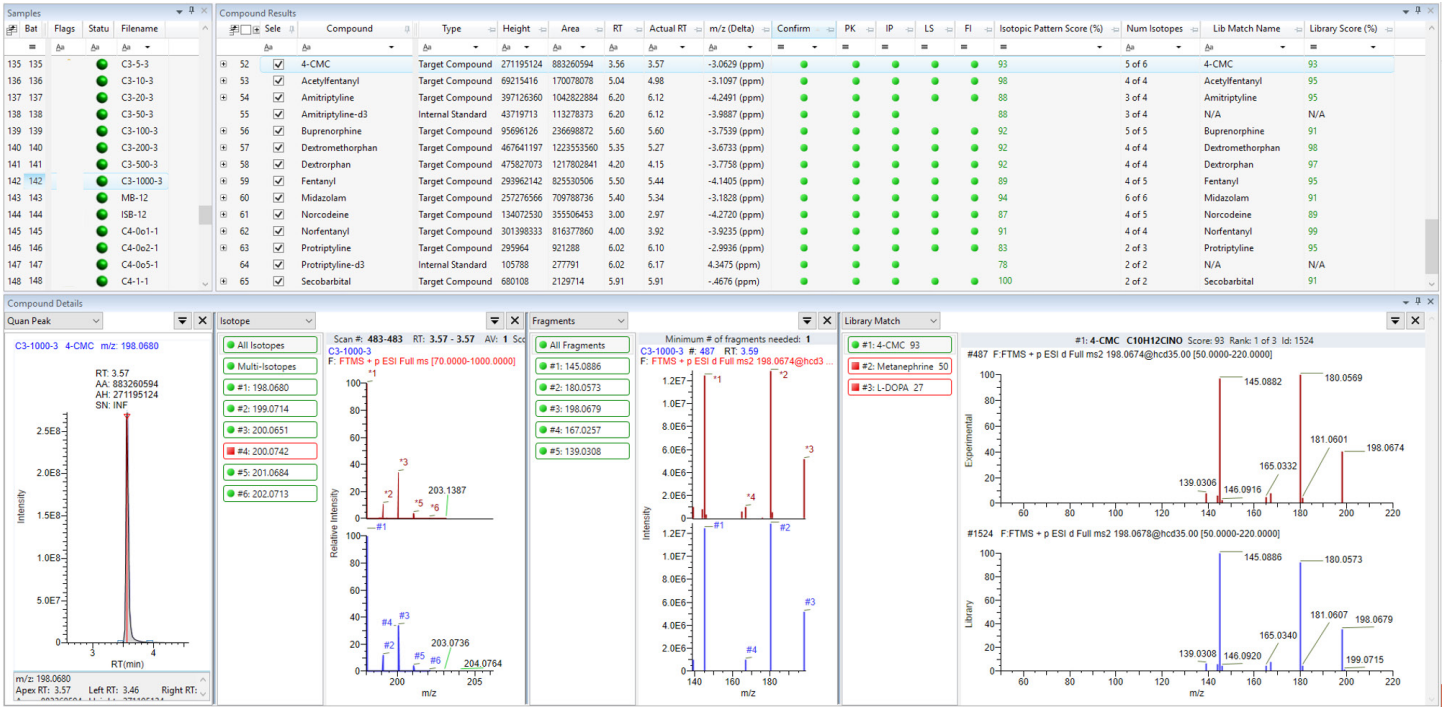


Figure 3. Data review from TraceFinder 5.1 software of targeted screening.

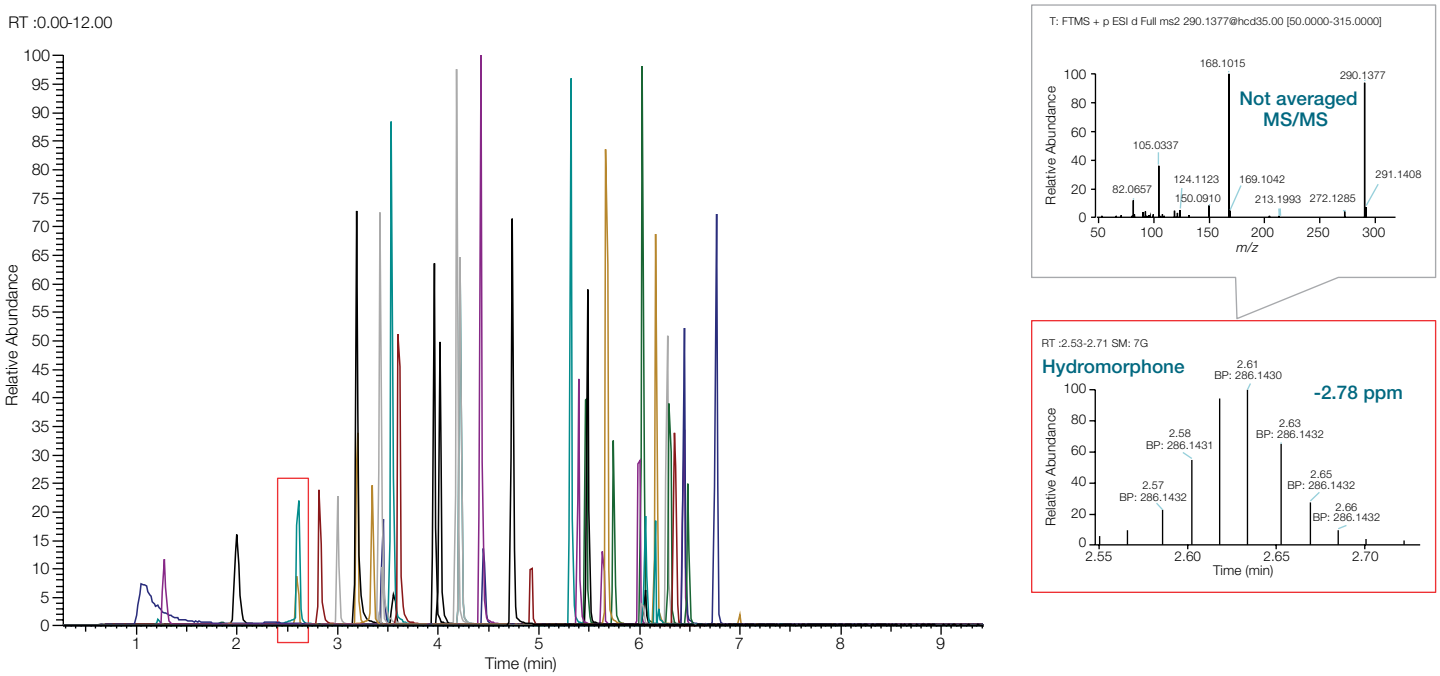


Figure 4. XIC chromatograms of four mixes of 54 drugs of abuse compounds (mass accuracy 5 ppm).

Quantitation of 54 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. Fifty four analytes of different classes, retention times and polarities, were evaluated for limit of detection (LOD), LOQs, and limit of identification (LOI)s. Specific criteria for quantitation that were used can be found in Table 2. The TraceFinder software not only ensures a 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.

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 1000 ng/mL in Surine. Six internal standards were used, and quantitation for all compounds were processed using full scan spectra. An example of the calibration curve for fentanyl can be seen in Figure 5. The LOD, LOQ, and LOI values for each of the analytes can be found in Table 3. The %CV for intra-assay calculated concentration were always less than 20%.

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

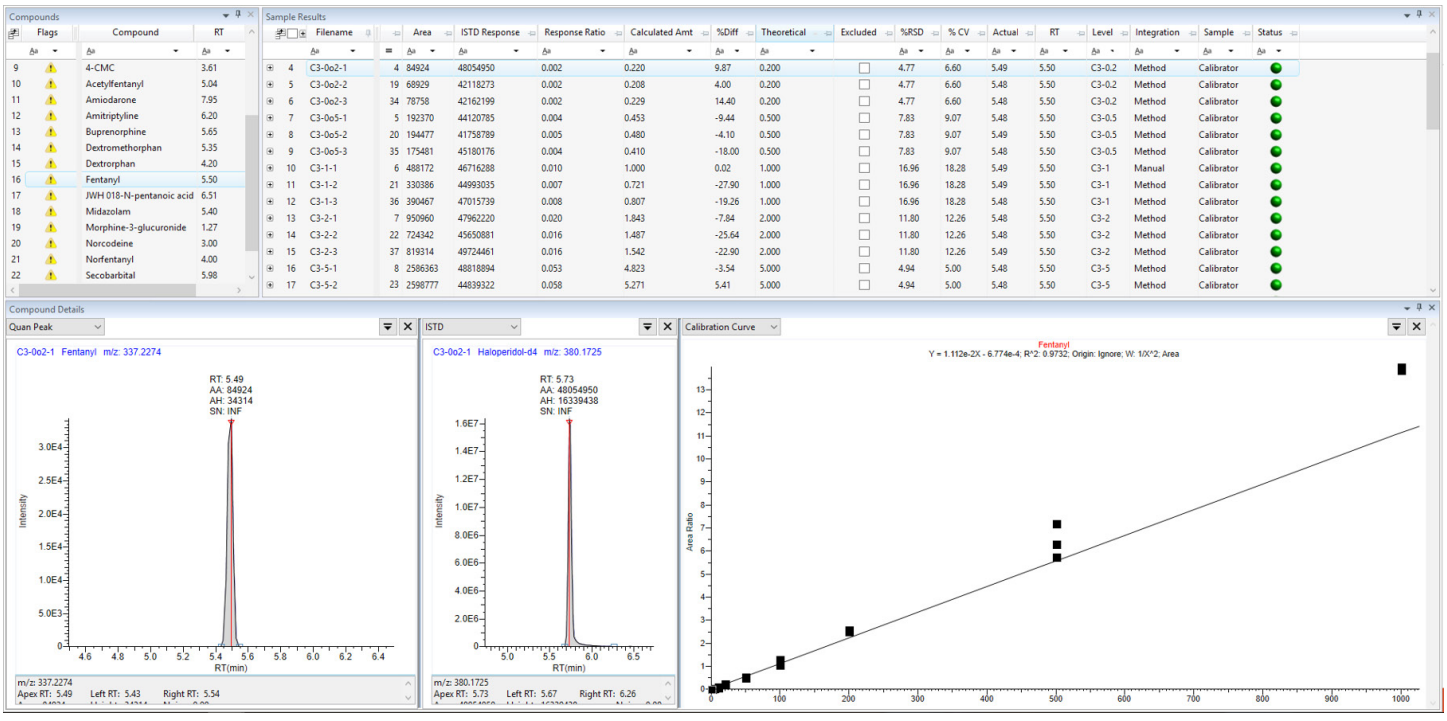


Figure 5. Data review from TraceFinder 5.1 software of quantitation of fentanyl.

Table 3. LOQ, LOD, and LOI for drugs of abuse compounds analyzed for quantitation.

Compound	RT	*m/z*	LOD	LOQ	LOI
25B-NBOMe	6.17	380.0856	0.5	0.5	2
25I-NBOMe	6.44	428.0717	0.1	2	2
2C-B	4.45	260.0281	0.2	1	5
4-CMC	3.61	198.068	0.1	0.2	1
6-AM	3.43	328.1543	0.1	1	1
7-aminonitrazepam	3.19	252.1131	0.1	2	2
AB-FUBINACA	6.99	369.1721	2	5	20
Acetyl fentanyl	5.02	353.2224	1	1	5
Alpha-hydroxyalprazolam	6.06	325.0851	0.5	5	5
ALPHA-PVP	4.41	232.1696	0.5	2	5
Alprazolam	6.44	309.0902	0.1	0.5	2
Amiodarone*	7.94	646.031	200	1000	1000
Amitriptyline IS	6.16	278.1903	0.1	0.2	1
Amphetamine IS	2.83	136.1121	0.5	1	1
Benzoylecgonine	4.01	290.1387	0.1	2	1
Buprenorphine	5.65	468.3108	1	1	5
Caffeine	3.55	195.0877	2	5	5
Desalkylflurazepam	6.34	289.0539	0.2	2	1
Dextromethorphan	5.31	272.2009	0.2	0.2	0.5
Dextrorphan	4.18	258.1852	0.1	0.5	0.5
Diazepam IS	6.76	285.0789	0.1	1	1
Diphenhydramine	5.46	256.1696	0.5	2	1
EDDP	5.68	278.1903	0.1	1	1
EtG	0.7	221.0667(-)	5	5	100
Fentanyl	5.47	337.2274	0.1	0.2	1
Fluvoxamine	6	319.1628	0.2	1	2
Haloperidol IS	5.75	376.1474	0.5	0.5	10
Hydromorphone	2.6	286.1438	0.2	2	2
MDMA	3.4	194.1176	1	2	5
Mephedrone	3.53	178.1226	0.5	0.5	0.5
Methadone	6.27	310.2165	0.2	0.5	0.5
Methamphetamine	3.19	150.1277	1	2	1
Midazolam	5.39	326.0855	0.1	0.2	1
Morphine IS	2	286.1438	0.5	0.5	5
Morphine-3B-glucuronide	1.22	462.1759	10	10	50
Morphine-6B-glucuronide	1.85	462.1759	10	10	100
N-desmethylflurazepam	ND	289.0539	NA	NA	NA
Nicotine	1.2	163.123	5	5	10
Norbuprenorphine	4.92	414.2639	0.5	2	5
Norcodeine	3	286.1438	0.1	0.2	2
Nordiazepam	6.3	271.0633	0.1	0.2	1
Norfentanyl	3.95	233.1648	0.2	0.2	1
Norhydrocodone	3.35	286.1438	0.1	1	2
Normorphine	1.28	272.1281	0.5	1	5
Oxazepam	6.06	287.0582	1	1	2
Phentermine	3.45	150.1277	1	1	1
Pregabalin	2.6	160.1332	1	2	10
Protriptyline	6.03	264.1747	0.1	2	0.5
Secobarbital	5.9	237.1245(-)	50	50	500
Sufentanil	6.03	387.2101	1	1	10
THC	8.89	315.2319	200	500	500
THC-COOH IS	8.14	343.1915	20	20	200
Tramadol	4.22	264.1958	0.1	0.1	1
Triazolam	6.48	343.0512	0.2	0.2	2
Zolpidem	4.73	308.1757	0.1	1	1
Zopiclone	4.06	389.1123	0.2	2	5

Separation of isomers

When analyzing drugs of abuse in biological matrices, it is extremely important to ensure sufficient chromatographic resolution due to isomeric interference found within such panels. Within the 54 analytes reported herein there are four different sets of isomers. Separations of these isomers is achieved using the 15.5-minute chromatographic gradient shown in Figure 6.

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 diazepam-D5, amitriptyline-D3, and morphine-D3 were used and the ensuing results are shown in Figure 7. The same internal standards were plotted for retention time for more than four hundred injections (Figure 8). The masses were within 5 ppm with %RSD <0.001% over 2 days (48 hrs) without recalibration. The %CV for peak abundance for the internal standards were below 7% and the retention time for the compounds were ±0.01 min, demonstrating the reproducibility and robustness of this method.

Polarity switching

This extensive panel of drugs of abuse compounds, contains compounds such as barbiturates and THC-COOH metabolite which ionize in negative mode, due to their specific functional groups losing a hydrogen. Having compounds of different polarities generates a need to analyze mass spectra in both positive and negative mode. The Q Exactive Plus mass spectrometer ensures fast polarity switching (Figure 9) resulting from comprehensive data in a single analytical run.

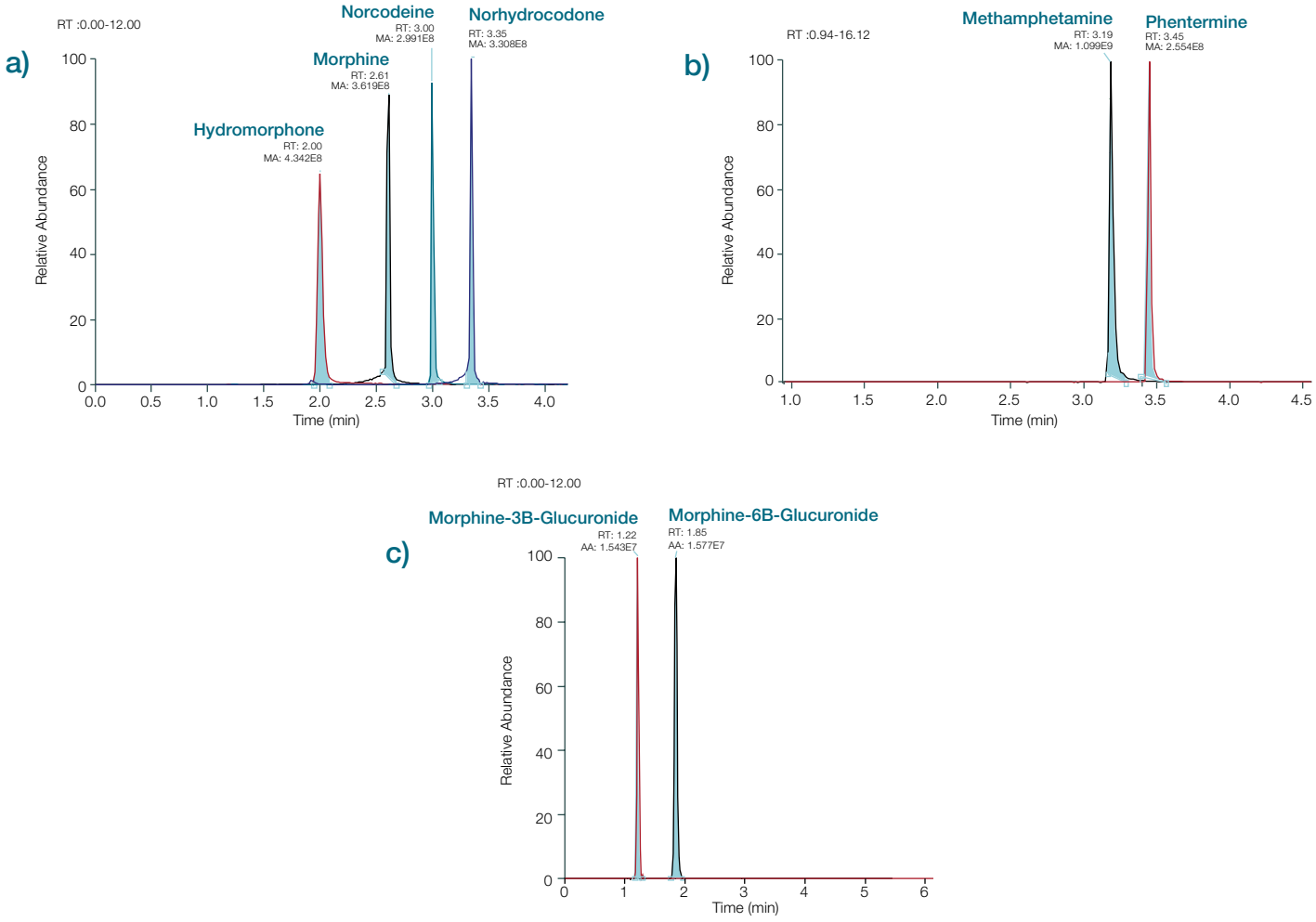


Figure 6. XIC chromatograms of analytes that are isomers, this method provides chromatographic resolution for a) hydromorphone, morphine, norcodeine, and norhydrocodone b) methamphetamine and phentermine and c) morphine-3B-glucuronide and morphine-6B-glucuronide.

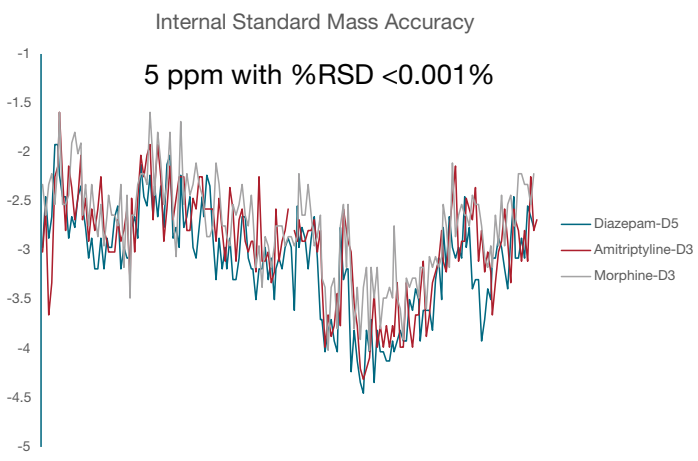


Figure 7. Robustness of mass accuracy over 400 injections for the internal standard, amphetamine-D5, diazepam-D5 and morphine-D3.

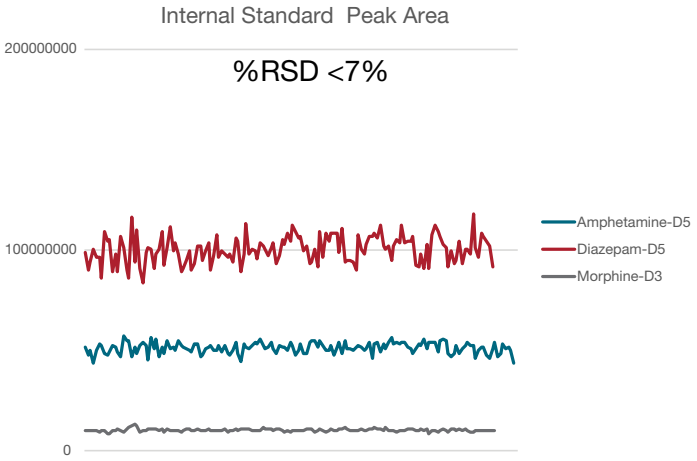


Figure 8. Robustness of peak area for over 400 injections for the internal standard, amphetamine-D5, diazepam-D5 and morphine-D3.

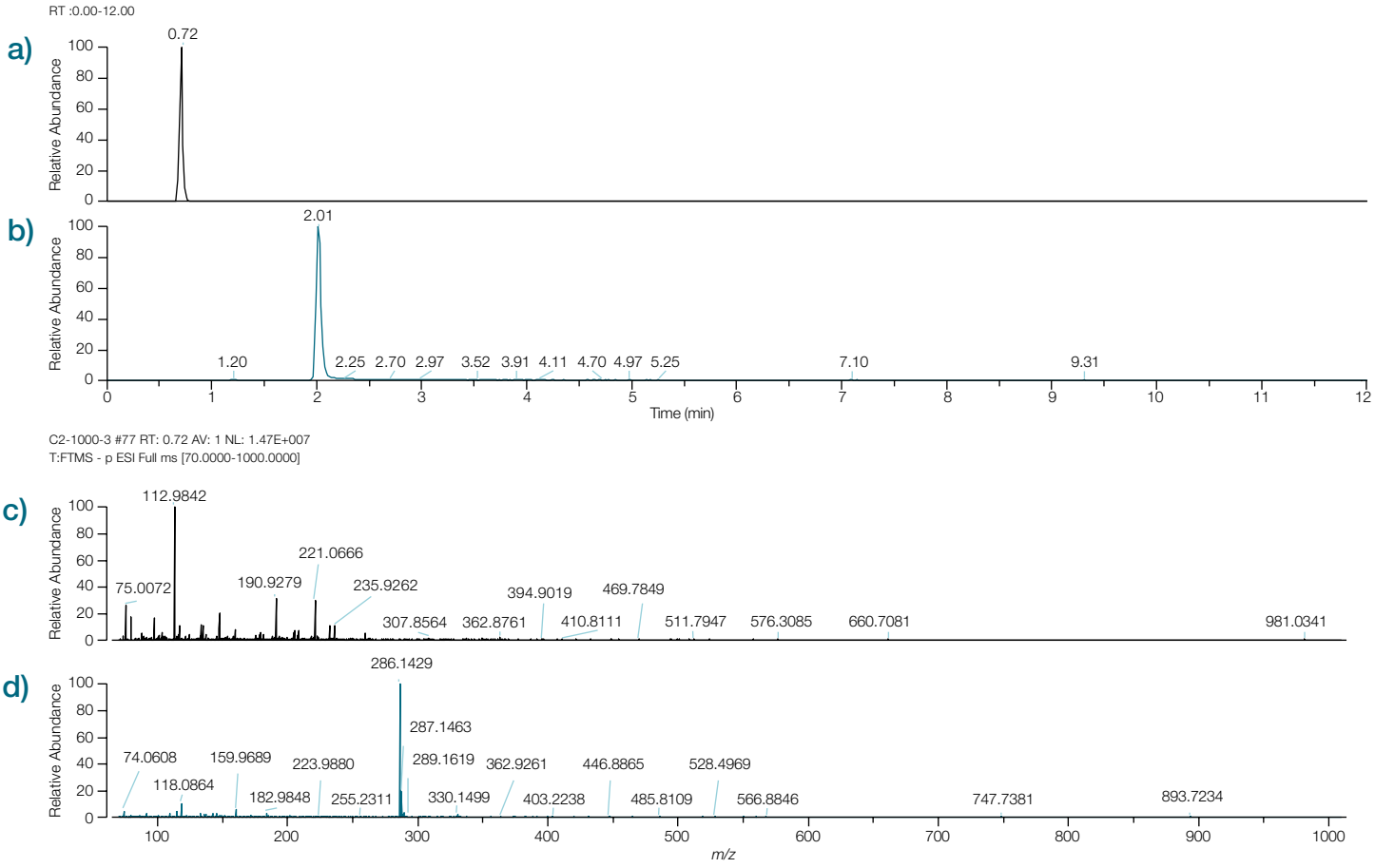


Figure 9. Demonstration of positive and negative switching a) represents XIC of EtG b) XIC of morphine c) full negative spectrum at retention time 0.72 and d) full positive spectrum at retention time 2.01.

thermo scientific

Conclusion

Tox Explorer Collection ensures a robust, reliable, sensitive, easy-to-implement workstream for every toxicology laboratory challenged with the task of analyzing hundreds of samples comprised of drugs of abuse analytes in complex biological matrices. The method implemented herein uses a Vanquish Flex UHPLC system connected to a Q Exactive Plus mass spectrometer and capitalizes the power of Thermo Scientific™ Orbitrap™ technology to perform accurate targeted screening and quantitation with high efficiency, reliability and confidence. The method reported in this study demonstrates outstanding quality of data for a panel of 54 drugs of abuse in Surine obtained using an LC-HRAM based quantitation and screening approach. The TraceFinder software stores the compound database, enables data acquisition, monitoring, processing, reviewing and customized reporting—all on one software platform. In addition, TraceFinder software provides the ability to screen for targeted analytes (using exact mass, retention time, isotope patterns, fragment ions, and matching to a compound library) and quantify 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 need to address.

References

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

Find out more at **thermofisher.com/toxexplorer**

ThermoFisher SCIENTIFIC