Application Note: 529

Quantitative LC-MS Analysis of 14 Benzodiazepines in Urine Using TraceFinder 1.1 Software and High Resolution Accurate Mass

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Key Words

- TraceFinder Software
- Exactive
- Clinical Research

Introduction

Thermo Scientific TraceFinder 1.1 software is developed for quantitative analysis for clinical research laboratories. The software is designed for routine data acquisition, quantitation, qualitative screening and reporting on all Thermo Scientific liquid chromatography mass spectrometry (LC-MS) systems, including high resolution accurate mass (HRAM) instruments, with fully integrated support for the Thermo Scientific Transcend multiplexing system.

TraceFinder™ 1.1 quantitative software simplifies routine analysis for the operator by executing a stepwise workflow from batch creation to reporting. For clinical research laboratories employing multiple types of LC-MS systems, TraceFinder 1.1 software eliminates the need to learn and maintain multiple software programs.

TraceFinder 1.1 software provides many easy approaches to execute workflow routines for operators and lab managers. The work presented here demonstrates the workflow used by lab managers during method development and includes processing method creation using the compound data store (CDS). The operator's workflow includes batch submission, real time monitoring, data review and report generation.

Goal

To demonstrate a new, easy-to-use workflow-driven quantitative method for 14 benzodiazepines in urine using the Thermo Scientific Exactive high performance benchtop mass spectrometer and TraceFinder 1.1 routine quantitative software.

Methods

Sample Preparation

Urine was spiked with internal standards and hydrolyzed with beta-glucuronidase. Acetonitrile was added to the hydrolyzed sample and the resulting mixture was centrifuged. Supernatant was further diluted and subjected to LC-MS analysis.

LC-MS/MS conditions

LC-MS analysis was performed on an Exactive™ mass spectrometer with a heated electrospray ionization (HESI) source coupled with a Transcend™ TLX system used in LX mode. Full scan mass spectrometry analysis was done with resolution of 100,000 (FWHM at *m/z* 200) with a mass isolation window of 3 ppm. Exact mass was used for compound identification. High performance liquid chromatography (HPLC) was carried out on a Thermo Scientific Hypersil GOLD PFP column (100 × 2.1 mm, 5 µm particle size) at room temperature.

The MS conditions were as follows:

Ionization	HESI-II
Polarity	Positive
Vaporizer temp (°C)	350
Capillary temp (°C)	350
Spray voltage (V)	3500
Sheath gas (AU)	40
Auxillary gas (AU)	10
Data acquisition mode	Full scan
AGC target	1.00E+06
Lock mass *(m/z)*	279.2591
Scan range *(m/z)*	135-600
Max injection time (ms)	100
Resolution	100,000

Software

Method development, data acquisition, data processing and report generation were all executed in TraceFinder 1.1 routine quantitation software.

Results and Discussion

Streamlined Workflow:

The entire workflow in TraceFinder 1.1 software is easy to set up and is summarized in Figure 1.

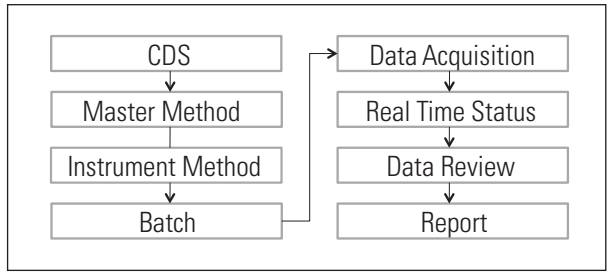

Figure 1. TraceFinder 1.1 workflow for quantitative analysis

Main Tabs in TraceFinder 1.1

Figure 2 shows the four main tabs: Configuration, Method Development, Data Review and Acquisition.

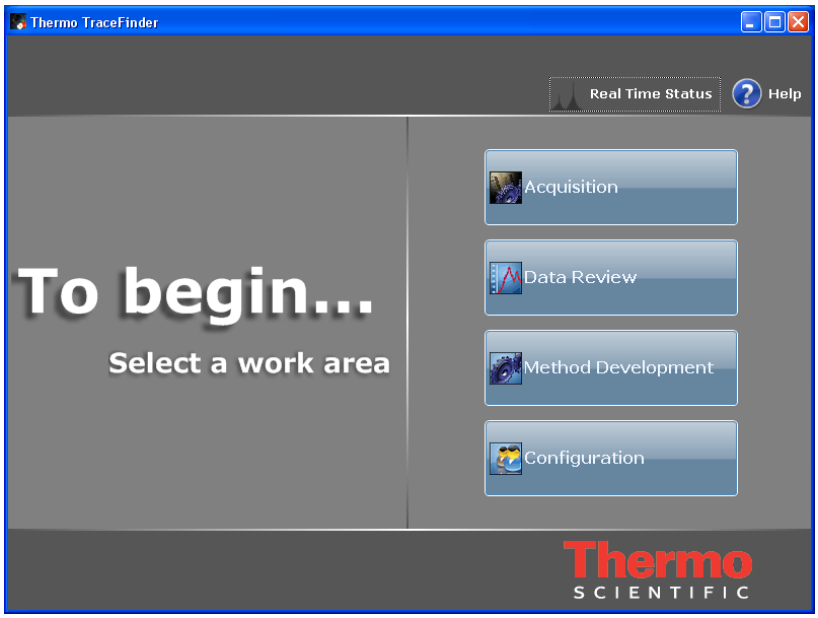

Figure 2. TraceFinder 1.1 welcome screen

Compound Data Store (CDS)

Figure 3 shows the CDS for this benzodiazepines application. Entries in this CDS are built based on the accurate masses. CDS can be later updated with retention times of analytes.

	Compound Name	ExperimentType	Category	Ionization
1	2-Hydroxyethylflurazepam	XIC	Benzo	ESI
2	2-Hydroxyethylflurazepa...	XIC	Benzo	ESI
3	7-Aminoclonazepam	XIC	Benzo	ESI
4	7-Aminoclonazepam-D4	XIC	Benzo	ESI
5	7-Aminoflunitrazepam	XIC	Benzo	ESI
6	7-Aminoflunitrazepam-D7	XIC	Benzo	ESI
7	7-Aminonitrrazepam	XIC	Benzo	ESI
8	a-Hydroxyalprazolam	XIC	Benzo	ESI
9	a-Hydroxyalprazolam-D5	XIC	Benzo	ESI

Compound Name	Mass	RT (min)	Window (sec)
a-Hydroxyalprazolam-D5	330.1160	5.420	240.00

	Compound Name	ExperimentType	Category	Ionization
10	a-Hydroxytriazolam	XIC	Benzo	ESI

Compound Name	Mass	RT (min)	Window (sec)
a-Hydroxytriazolam	359.0460	5.330	240.00

	Compound Name	ExperimentType	Category	Ionization
11	a-Hydroxytriazolam-D4	XIC	Benzo	ESI

Compound Name	Mass	RT (min)	Window (sec)
a-Hydroxytriazolam-D4	363.0710	5.330	240.00

Figure 3. Compound Data Store for benzodiazepines application

Master Method

The "Master Method" contains information on data acquisition (including instrument method), data processing, and analysis. In detail, it contains settings for 5 main categories: General (including method type, injection volume, instrument method, etc), Compound (acquisition list selected from CDS, detection, calibration, etc), Flags, Groups and Reports. Selected tabs in "General" and "Compound" are shown in Figure 4. To complete the master method setup, settings in "Flags", and "Reports" can also be customized. TraceFinder software provides 50 predefined report templates.

Instrument Method

The instrument method is comprised of individual LC, autosampler and MS portions. The software allows for optimization of chromatography and customizable autosampler programming.

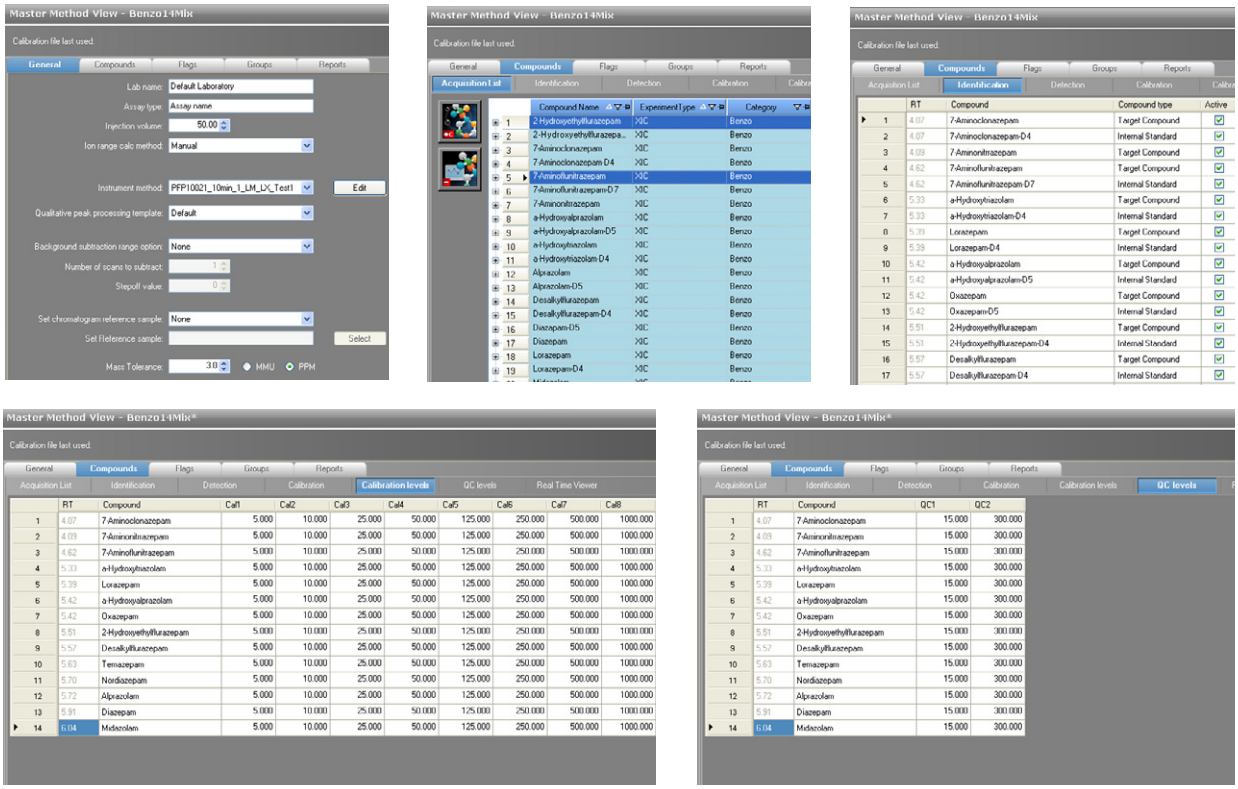

Figure 4. Master Method creation process (selected tabs)

Batch

After creation of the master method, a new sample batch can be created for data acquisition. Creating a batch involves assigning a project, linking to the master method, building a run-sequence and submitting. Figure 5 shows an exemplary batch view containing six calibrators and two levels of "Check Standards" (or QCs, n=5).

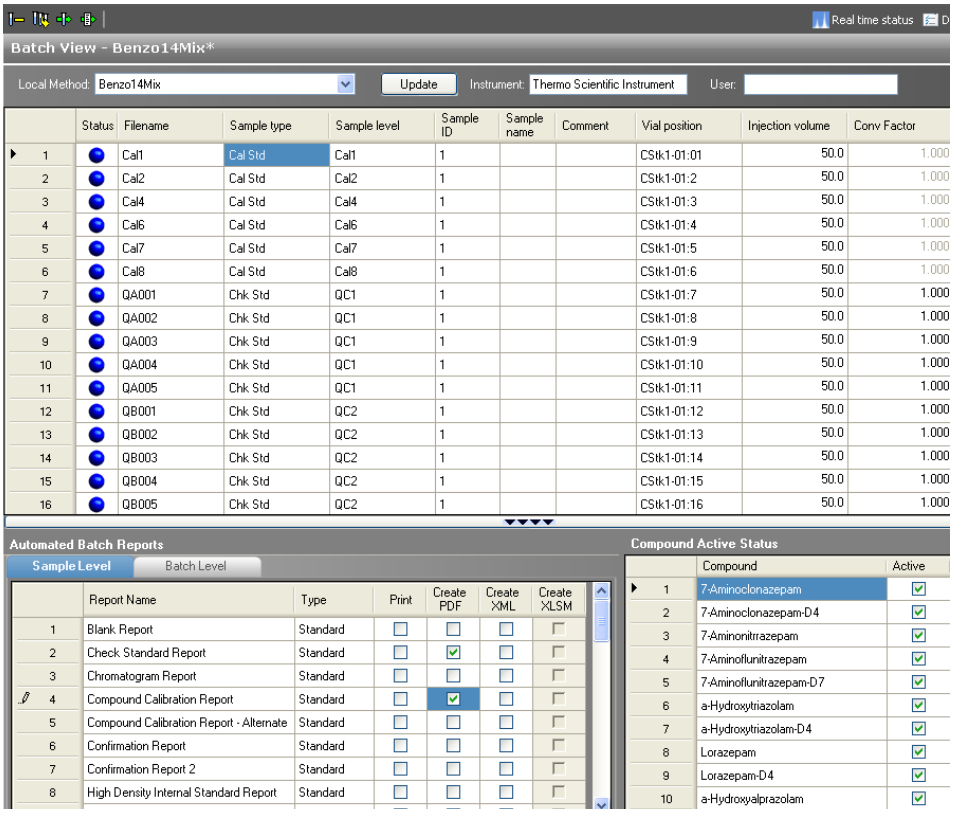

Figure 5. Acquisition Batch view

Data Acquisition and Real Time Status

After batch submission, data will be acquired and real time chromatograms can be shown in customizable ways (Figure 6). Status of acquisition (pressure profile, event log), device status, and sample queue can all be monitored in Real Time Status. TraceFinder software allows for multiple batches submission prioritization.

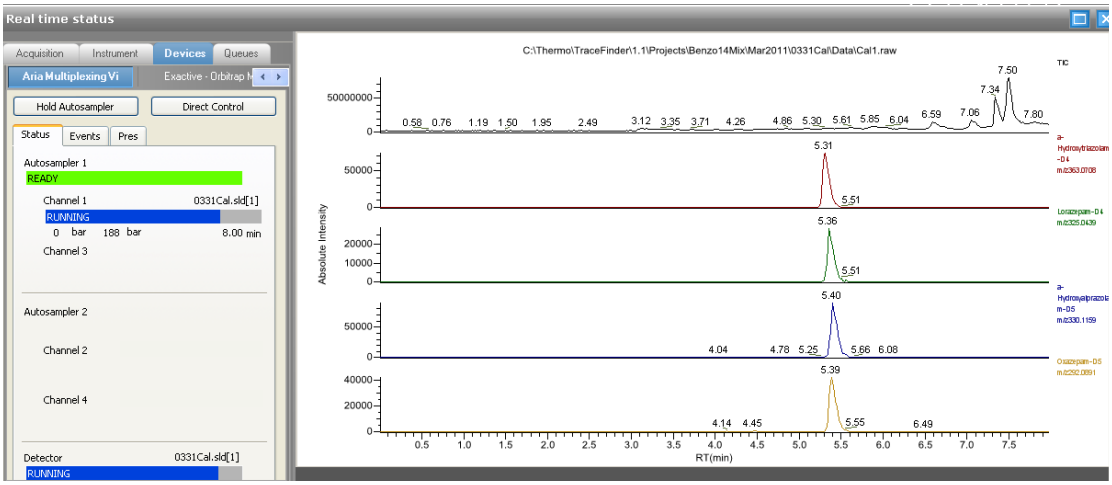

Figure 6. Real Time Status view

Data Review

Data Review (Figure 7) allows for flagging for any items that require attention (retention time drift, limit of quantitation, ion ratio discrepancy, etc.).

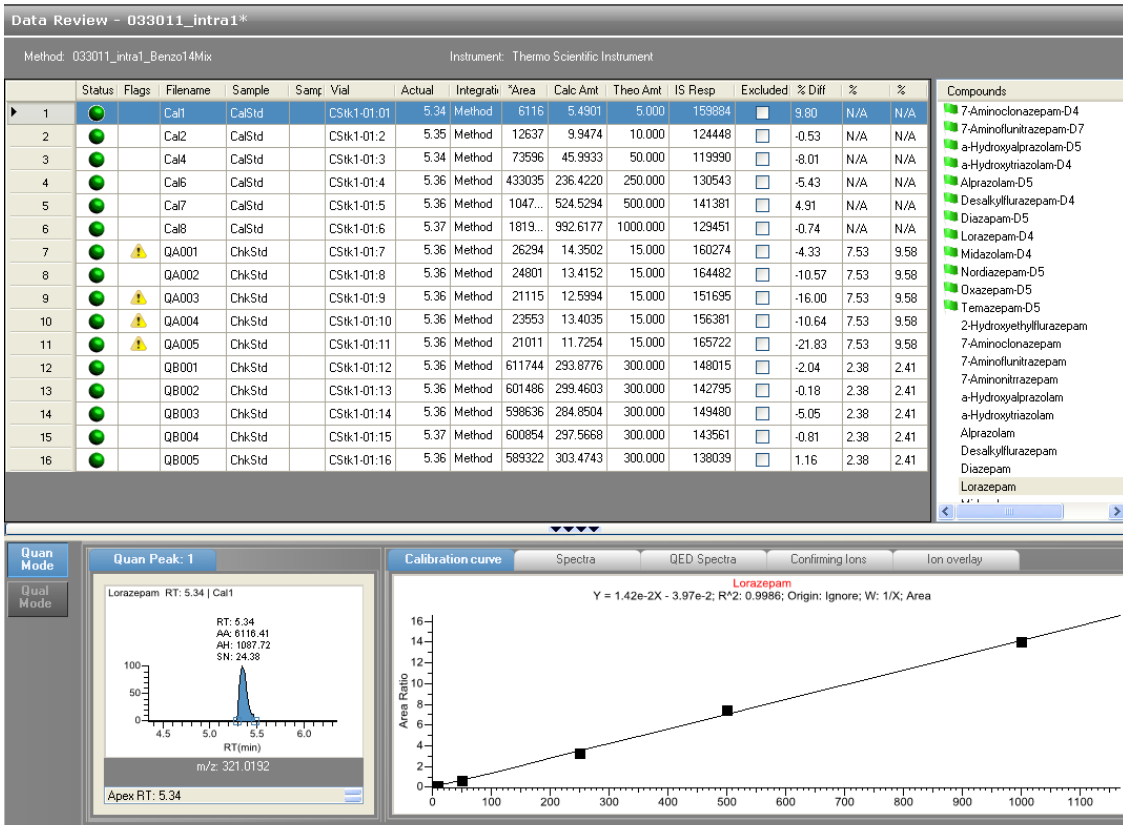

Figure 7. Data Review view for lorazepam, one of the 14 benzodiazepines

Reporting

Figures 8 and 9 are two examples (compound calibration and check standard/quality control) of the Report View.

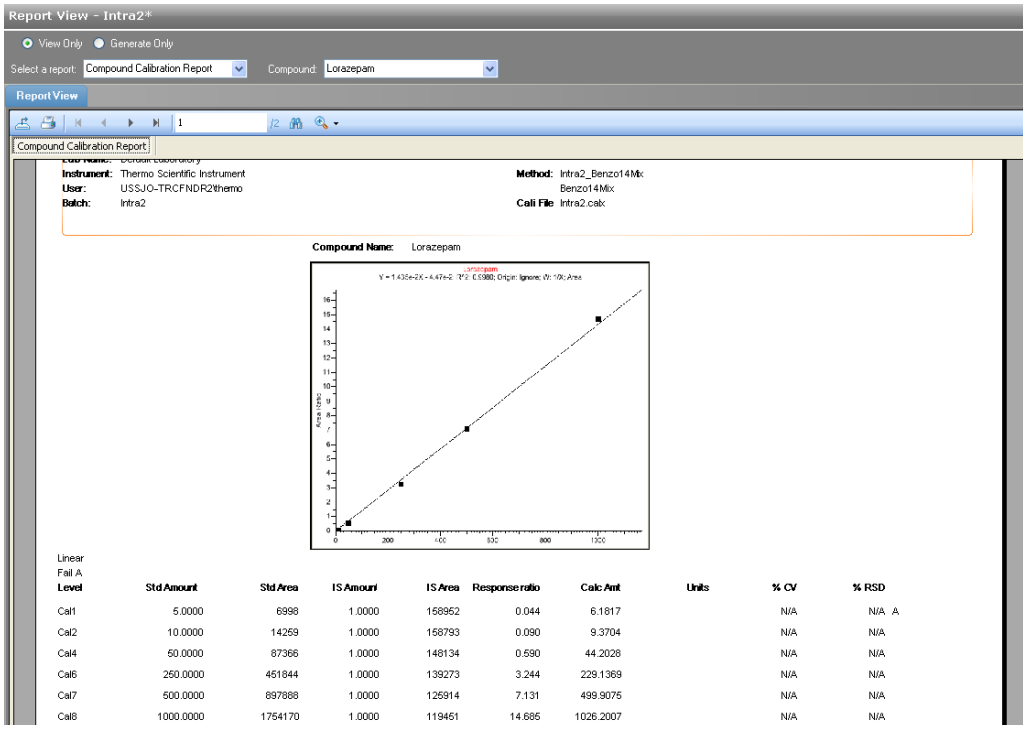

Figure 8. Compound Calibration Report for lorazepam

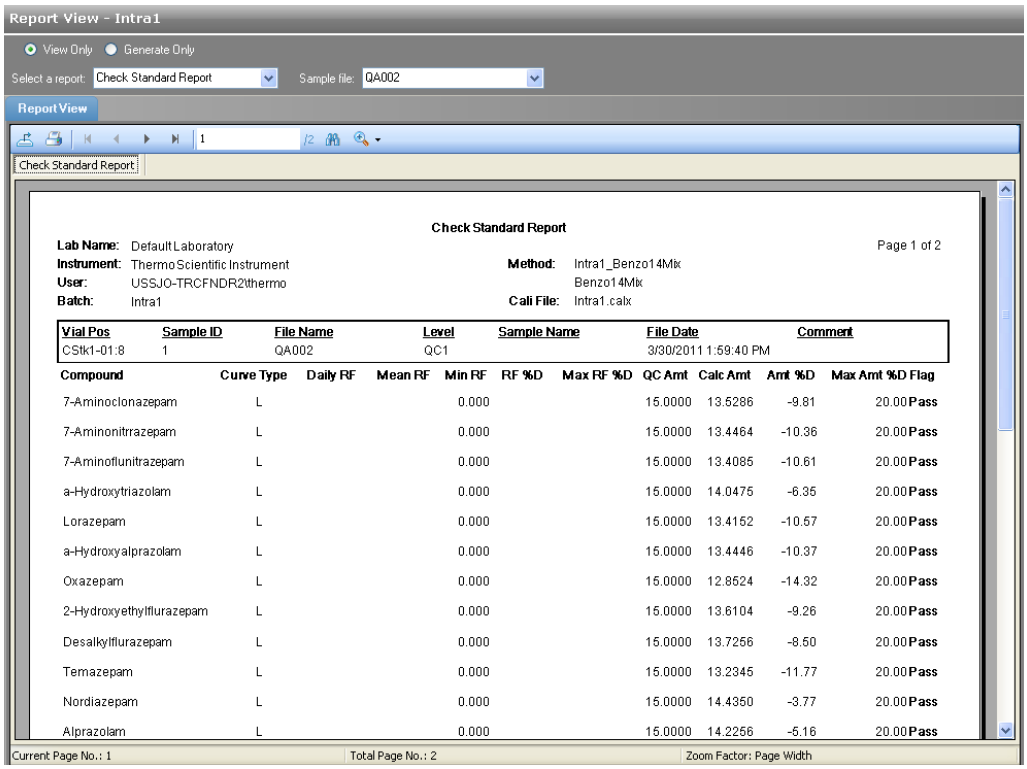

Figure 9. Check Standard (QC) Report for one QC sample

Method Performance

Sample preparation for urine analysis of benzodiazepines was previously done with solid phase extraction (SPE). Here we tested a simple urine dilution strategy. The absolute recovery of deuterated benzodiazepine internal standards was tested with several lots of human urine. It was determined that the absolute recoveries of the internal standards ranged from 83.0% to 100.5% at 100 ng/mL from all lots of urine tested (data not shown).

This method was linear from 5 to 1000 ng/mL for all 14 benzodiazepines with an accuracy of 85.4%-106.0%. Inter- (n=15) and intra-batch (n=5) coefficients of variation (CV) at two different concentration levels ranged from 0.5% to 11.7%. The method has a lower limit of quantitation (LLOQ) of 5 ng/mL for all 14 benzodiazepines tested. The method performance is summarized in Table 1. Figure 10 shows the extracted ion chromatograms (XICs) with 3 ppm mass isolation window of all 14 benzodiazepines at their LLOQ (5 ng/mL).

Table 1. Method performance for 14 benzodiazepines in urine

Name	m/z	QC level 1: 15 ng/mL		QC level 2: 300 ng/mL		Linear Range (ng/mL)	LLOQ (ng/mL)
		% Precision	% Accuracy	% Precision	% Accuracy		
7-Aminonitrazepam	252.1131	2.9	88.7	2.9	106.0	5 - 1000	5
Nordiazepam	271.0633	5.7	89.6	2.9	100.9	5 - 1000	5
7-Aminoflunitrazepam	284.1194	3.4	91.2	4.0	100.9	5 - 1000	5
Diazepam	285.0789	8.8	96.0	2.6	99.7	5 - 1000	5
7-Aminoclonazepam	286.0742	2.0	89.1	2.1	99.4	5 - 1000	5
Oxazepam	287.0582	5.0	85.6	3.5	98.4	5 - 1000	5
Desalkylflurazepam	289.0539	5.5	88.5	2.9	98.6	5 - 1000	5
Temazepam	301.0738	3.4	89.1	2.7	97.6	5 - 1000	5
Alprazolam	309.0902	3.1	90.0	3.2	101.5	5 - 1000	5
Lorazepam	321.0192	7.6	85.4	3.4	95.3	5 - 1000	5
α-Hydroxyalprazolam	325.0851	3.0	87.0	1.8	97.3	5 - 1000	5
Midazolam	326.0855	3.6	91.3	2.6	101.2	5 - 1000	5
2-Hydroxyethylflurazepam	333.0801	3.7	89.0	2.5	99.7	5 - 1000	5
α-Hydroxytriazolam	359.0461	5.9	86.9	2.8	97.5	5 - 1000	5

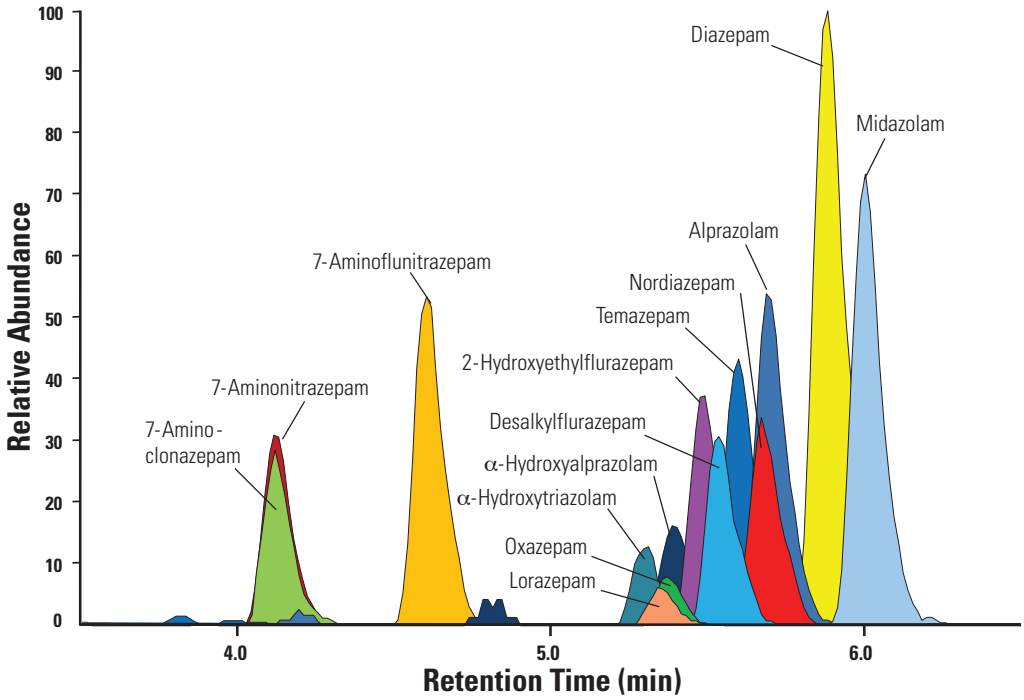

Figure 10. Extracted ion chromatograms of 14 benzodiazepines in urine at their LLOQ (5 ng/mL, mass isolation window=3 ppm)

Conclusion

We have developed a fast and sensitive LC-MS method for 14 benzodiazepines in urine using a benchtop Exactive mass spectrometer with TraceFinder 1.1 software. TraceFinder 1.1 software is easy to use and effective in performing quick routine quantitative analysis of benzodiazepines in urine. The software enables easy method development, batch creation, submission and real time monitoring for clinical research laboratories. The data review functionality was very useful in quick review and verification of the calibration accuracy and linearity. The report templates make selecting and generating reports with all the necessary information easy and quick.

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