Application Note: 529

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

Xiang He, Marta Kozak; Thermo Fisher Scientific, San Jose, CA

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 ExactiveTM mass spectrometer with a heated electrospray ionization (HESI) source coupled with a TranscendTM 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
<u>ا</u> ا	5	7-Aminoflunitrazepam	XIC	Benzo	ESI
<u>ا</u> ب	6	7-Aminoflunitrazepam-D7	XIC	Benzo	ESI
<u>ا</u>	7	7-Aminonitrrazepam	XIC	Benzo	ESI
÷.	8	a-Hydroxyalprazolam	XIC	Benzo	ESI
8	9	a-Hydroxyalprazolam-D5	XIC	Benzo	ESI
		Compound Name 🛛 🗢	Mass +⊐	BT (min) 🕂 🕫	Window (sec) 🕒
	••••	a-Hydroxyalprazolam-D5	330.1160	5.420	240.00
	•	a-Hydroxyalprazolam-D5 Compound Name 🛯 🗠 🔽 中			
9.	10				
•	10	Compound Name 🔺 🗸 中	ExperimentType 🍐 🖓 🖶	Category 🖓 🗗	Ionization ⊽+⊧
	10	Compound Name △マ中 a-Hydroxytriazolam	ExperimentType ▲文中 XIC	Category ⊽+¤ Benzo	lonization ⊽+= ESI
	10	Compound Name AV+ a-Hydroxytriazolam Compound Name +	ExperimentType A V + XIC Mass + 359.0460	Category 文+⊅ Benzo RT (min) +⊅ 5.330	lonization ⊽+= ESI Window (sec) += 240.00
	10	Compound Name △文+ a-Hydroxytriazolam Compound Name + a-Hydroxytriazolam	ExperimentType A V + XIC Mass + 359.0460	Category 文+⊅ Benzo RT (min) +⊅ 5.330	Ionization ⊽+= ESI Window (sec) += 240.00
		Compound Name △マ+ a-Hydroxytriazolam Compound Name + a-Hydroxytriazolam Compound Name △マ+	ExperimentType A V + XIC Mass + 359.0460 ExperimentType A V +	Category 文+ Benzo RT (min) + 5.330 Category 文+	Ionization ⊽+ ESI Window (sec) + 240.00 Ionization ⊽+

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.

		Lab name: Default La	boratory				Acquisition List					Calibra	Acquistio		Identification Detection		
		Assay type: Assay nar	10				222			ExperimentType	Catego	, ⊽÷₽		RT	Compound	Compound type	A
		Injection volume: 50	.00 😂				2 2 1 1		frosyethylflurazepam	XIC	Benzo		F 1	4.07	7-Aminoclonazepam	Target Compound	_
		n range calc method: Manual		~				-	droxyethylflurazepa inocionazepam	XIC XIC	Benzo Benzo		2	4.07	7-Aminoclonazepam-D4	Internal Standard	_
								3	nocionazepam D4	XIC	Benzo		3	4.09	7-Aminonitrrazepam	Target Compound	_
									inoflunitrazepam	XIC	Benzo		4	4.62	7-Aminoflunitrazepam	Target Compound	_
		Instrument method: PFP1002	L_10min_1_LM_LX_T	stî 💌	Edit			6 7:Ami	noflunitrazepam-D 7	XIC	Benzo		5	4.62	7 Aminoflunitrazepam D7	Internal Standard	-
		processing template: Default		~					nonitrrazepam	XIC	Benzo		6	5.33	a-Hydroxytriazolam	Target Compound	-
Jan a	ranne hear	processing template. Deraut							droxyalprazolam	XIC	Benzo		7	5.33	a-Hydroxytriazolam-D4	Internal Standard	-
								-	droxyalprazolam-D5 droxytriazolam	XIC	Benzo		8	5.39	Lorazepam	Target Compound	-
*9	ground sub	straction range option: None		*					troxymazoiam troxytriazolam D4	XIC	Benzo		9	5.39	Lorazepam-D4	Internal Standard	-
		r of scans to subtract:	1 🗇					12 Alpra		XIC	Benzo		10	5.42	a-Hydroxyalprazolam	Target Compound	_
		Stepoff value:	0 🔅					13 Alpra	zolam-05	XIC	Benzo		11	5.42	a-Hydroxyalprazolam-D5	Internal Standard	_
			1000						lkylflurazepam	XIC	Benzo		12	5.42	Oxazepam	Target Compound	_
		ram reference sample: None		~				10	kylfurazepam-D4	XIC	Benzo		13	5.42	Oxazepam-D5	Internal Standard	_
				-				16 Diaza	ipam-D5	MC	Benzo		14	5.51		Target Compound	
										140					2-Hydroxyethylflurazepam		-
		iet Reference sample:			Select		۰	17 Diaze		XIC	Benzo		15	5.51	2-Hydroxyethylflurazepam-D4	Internal Standard	
k	lethod ' e last used	Mass Tolerance: View - Benzo14Mik*	30 C MMU				*	17 Diaze 18 Loraz		Mas Cuto	Benzo Benzo Docas Stor Motho bration file last c		15 16 17 2014Mix*	5.51 5.57 5.57	24tjudcovethytkuszerpan 04 Desalkytkuszerpan Desalkytkuszerpan 04		
file ral	lethod ' e last used	Mass Tolerance: View - Benzo14Mik* Compounds Flags	30 S MMU	PPM Report Collocation	ts	stion levels	*	17 Diaze 18 Loraz 19 Loraz	epam	Mas Cab	Benzo Benzo Denne Stor Motho		15 16 17 2014Mik* Flags	5.51 5.57 5.57	24tydrosyntryffurserpom 04 Desafyrffurserpom 0 Desafyrffurserpom 04	Internal Standard Target Compound	cia
ı file cal	lethod ' e last used	Mass Tolerance: View - Benzo14Mik* Compounds Flags	Groups	Repor	ts Calibra		GC levels	17 Diazo 18 Loraz 19 Loraz Rea	repam vepam-D4	Mas Cab	Benzo Benzo Stor Motho bration Re last u General	red. Compounds	15 16 17 2014Mik* Flags	5.51 5.57 5.57	24tydrosyntryffurserpom 04 Desafyrffurserpom 0 Desafyrffurserpom 04	Internal Standard Target Compound Internal Standard	cla
ı file cal	lethod 1 e last used n List	Mass Tolerance: View - Benzo14Mik* Compounds Flags Identification	Groups	Repor	ts Calibra		GC levels	17 Diazo 18 Loraz 19 Loraz Rea	reparm reparm D4	Mas Cab	Benzo Benzo Ster Metho bration file last u General Acquisition List	red. Compounds Identificat	15 16 17 2014Mik* Flogs	5.51 5.57 5.57	24tydroyettythusenpoer:04 Desskythusenpoer:04 Desskythusenpoer:04	Internal Standard Target Compound Internal Standard	cis
file ral	lethod 1 e last used n List RT	Mass Tolerance Vlaw - Benzo 14Mix* Compounds Identification	Groups Detection	Report Colloration	ts Colibra	Cal4 I	QC levels Cat5 C	17 Diazo 18 Loraz 19 Loraz Ros	epam epamD4	XIC XIC Mas Cate	Benzo Benzo Stor Motho bration Rie last u General Acquisition List RT	red. Compounds Identificat Compound	15 16 17 2014Mix* Flegs on 0 20pam	5.51 5.57 5.57	24tychosystlytkuszepam:D4 Desallytkuszepam Desallytkuszepam D4 incuss Pepots Cotocom Coto	Internal Standard Target Compound Internal Standard	cla
file ral	e last used e last used n List RT 4.07	Mass Tolerance View - Benzol-14Mik* Compounds Flags Identification Compound 7 Aninoclonacepan	Groups Detection Call 5.000	Report Colibration Cal2 C 10.000	ts Colibra 25.000	Cal4 50.000	0C levels Ca5 C	17 Diazo 18 Loraz 19 Loraz 19 Loraz 20 Loraz 20 Loraz 19 Loraz 10	Ppam Ppam-D4 I Time Vidwes Ca7 Ca8 500.000 1 500.000 1	NGC NGC Call 000.000	Benzo Benzo Stor Motho bration Rie last u General Acquisition List RT 1 4.07	red. Compounds Identificat Compound 7.Arrinoclory	15 16 17 2014Mik* Flogs on 0 20pam	5.51 5.57 5.57	24bydiouyethyllixanopoinD4 Denallythixanopoin DenallythixanopoinD4 Einxyns Preports Coldroson Coldr 15.000 300.000	Internal Standard Target Compound Internal Standard	cis
ı file cal	e last used n List RT 4.07 4.09	Mass Tolerance View - Benzo 14Mik* Compound: Flags Identification Compound 7 Animonitacepan 7 Animonitacepan	Groups Detection Call 5.000 5.000	Eclibration	ta Calibra 25.000 25.000	Cal4 50.000	Cal5 Cal5.000 125.000	17 Diaze 18 Loraz 19 Loraz 19 Loraz Rec 250.000 250.000	Ppam Ppam-D4 ITime Viewer Ca7 Ca8 500.000 11 500.000 1 500.000 1	NG NG C-82 000.000	Benzo Benzo Stor Motho bration file last o General Acquisition List RT 1 4.07 2 4.03	ed. Compounds Identificat Compound 7.Aminoclon 7.Aminonima 7.Aminonima	15 16 17 2014Mbx* Flogs on 20pam 20pam 20pam 20pam	5.51 5.57 5.57	24getogethythisenpen04 Desallythisenpen04 Desallythisenpen04 calebrain Calebrain Calebrain Calebrain 15000 20000	Internal Standard Target Compound Internal Standard	cia
ı file cal	e last used n List RT 4.07 4.03 4.62	Mass Tolerance View – Benzo 14Mik* Compounds Plays dowlitation Compound 7 Animochasepan 7 Animochasepan	Call Call 5.000 5.000 5.000	Ecoloration Coloration Cat2 C 10.000 10.000 10.000	ts Calibra 25.000 25.000 25.000	Cal4 50.000 50.000 50.000 50.000	Caf5 C 125.000 125.000	17 Diace 18 Losz 19 Losz 19 Losz 19 Losz Res 260.000 250.000 250.000	Ppam Ppam-D4 IT me Viewer Ce/7 Ce/8 500.000 1 500.000 1 500.000 1	XAC XAC XAC C.400 000000 000000	Berzo Berzo Stor Motho Instien file last u General Acquisiton List RT 1 4.07 2 4.03 3 4.62	ed Compounds Identificat Compound 7 Aminoclon 7 Aminoclon 7 Aminofunit 8 Ayekoxptist 8 Ayekoxptist	15 16 17 2014Mbx* Flogs on 20pam 20pam 20pam 20pam	5.51 5.57 5.57	24gdiougettythaansporri D4 Desidlythaansporri D4 Desidlythaansporri D4 Calibration Calibration CC2 15.000 300.000 15.000 300.000	Internal Standard Target Compound Internal Standard	cis
file ral	e last used n List RT 4.07 4.09 4.62 5.33	Mass Tolesurce.	Cell Cell Cell 5.000 5.000 5.000 5.000	Echlorotion Collorotion 10.000 10.000 10.000 10.000	ts Calibra (25.000 25.000 25.000 25.000	Cal4 50.000 50.000 50.000 50.000	C lovels C 2500 125000 125000 125000	17 Diace 18 Loss 19 Loss 19 Loss 19 Loss 10	Ppam Ppam-D4 ITano Viewer Cal? Cal8 500.000 1 500.000 1 500.000 1 500.000 1	Mas c.42	Benzo Benzo Ster Metho bration file last u General RT 1 4.07 2 4.09 3 4.62 4 5.33	ed Compounds Identificat Compound 7 Aminorlina 7 Aminorlina 7 Aminofunit 6 Hydroxytha	15 16 17 20 1 4 Milet* Flogs on 0 20pan repart azepan szepan szepan szepan	5.51 5.57 5.57	24getogethyllivacopenD4 Desallyllivacopen Desallyllivacopen Caloron Caloron 1500 30000 1500 30000	Internal Standard Target Compound Internal Standard	cia
ı file cal	e last used e last used N List RT 4.07 4.09 4.62 5.33 5.39	Mass Tolerance: Vilave – Benzol 1 Milk* Compound Flags dorafilotion Compound 7 Anino characepan 7 Anino characepan 4 Hydroghiacolon Loraepan	Cell Cell 5:000 5:000 5:000 5:000 5:000 5:000	Calbrotion Calbrotion 10.000 10.000 10.000 10.000 10.000 10.000 10.000	calibra 25.000 25.000 25.000 25.000 25.000 25.000 25.000	Cal4 50.000 50.000 50.000 50.000 50.000 50.000 50.000	C loves C 125.000 125.000 125.000 125.000 125.000 125.000 125.000 125.000 125.000	17 Diazo 18 Loraz 19 Loraz 19 Loraz 19 Loraz 20 000 250.0000 250.0000 250.0000 250.0000 250.0000 250.0000 25	Ppam Ppam D4 11 Enc Videwel Car7 Cal8 500.000 1 500.000 1 500.000 1 500.000 1 500.000 1 500.000 1		Berzo Berzo Berzo Ster Metho broton file fast u General Acquisiton List RT 1 4.07 2 4.03 3 4.62 3 4.62 3 5.539	Compounds Identificat Compound 7.Aminoclon 7.Aminoclon 7.Aminofuni eHipdroxytia Lorazepan	15 16 17 20 1 4 Milet* Flogs on 0 20pan repart azepan szepan szepan szepan	5.51 5.57 5.57	24bpdospetflyBlacespeenD4 DesallyBlacespeenD4 DesallyBlacespeenD4 clabeloon clabeloon clabeloon 15000 300.000 15000 300.000 15000 300.000 15000 300.000 15000 300.000	Internal Standard Target Compound Internal Standard	cia
file al	e last uned e last uned h List 4.07 4.03 5.33 5.39 5.42	Mass Tolesnos Vilaw - Benzo 14NH/+* Compound Compound 7 Animochniscopum 7 Animochniscopum 4 Tolesnos 4 Tydroghisectom 6 Hydroghisectom	Call 5000 5000 5000 5000 5000 5000 5000 5	Cathroiden Cathroiden 10.000 10.000 10.000 10.000 10.000 10.000	ts Calibra 25.000 25.000 25.000 25.000 25.000 25.000	Cal4 50.0000 50.0000 50.000 50.000 50.000 50.0000 50.0000 50.0000 50.0000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.00	0C levels Col 25:000 125:000 125:000 125:000 125:000 125:000 125:000	17 Diase 18 Lore 19 Lore 19 Lore 20 Lore 20 Lore 250.0000 250.000 250.000 250.000 250.000 250.000 250.000 2	Ppam Ppam D4 11 Enc Videwel Car7 Cal8 500.000 1 500.000 1 500.000 1 500.000 1 500.000 1 500.000 1		Berzo Berzo Berzo Stor Motho Kration Re last u General Requisiton List R R 1 4.07 2 4.09 3 4.62 4.09 3 4.62 4.09 3 5.53 8 5.42	Ecompounds Identificat Compound 7.Aminoclon 7.Aminoclon 7.Aminofuni 6.Hiydxxytia Lorazepan 6.Hiydxxytia	15 16 17 20 L Milk* 20 pan epan epan epan epan ezelam	5.51 5.57 5.57	24bpdroydflythactopenD4 Deraillythactopen Deraillythactopen Calibration Calibration 1500 300.00 1500 300.00 1500 300.00 1500 300.00 1500 300.00	Internal Standard Target Compound Internal Standard	cls
file ral	e last used e last used 1 List 4.07 4.02 5.33 5.42 5.42 5.51 5.57	Mass Tolesmon	Call Call 5.000 5.000 5.000 5.000 5.000 5.000 5.000 5.000 5.000 5.000	T Report Colbroion Nat2 C 10.000 10.000 10.000 10.000 10.000 10.000 10.000 10.000	ts Calibra 25.000 2	Cal4 50.0000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.0000 50.000 50	Classical Classi	17 Disce 18 Loraz 19 Lor	Roum spame 0.4 I Trace Veneror Cal? Call Source 1 Source 1 S		Berzo Berzo Berzo St Gr Motho Listion Relation Listion Relation Listion Relation Listion Relation Listion Relation Listion Listion	ed Compounds Identificat Compound 7 Antinochan 7 Antinochan 7 Antinochan 0 Atlyckosyth 0 Coaseparn a Hydrosysh 0 Xazeparn 2 Hydrosysh Desakythze	To L4Mibs*	5.51 5.57 5.57	24bpdoxysHyllivacopenD4 DesallyHaceppenD4 DesallyHaceppenD4 Calorizer Calorizer 15000 20000 15000 300.000 15000 300.000 15000 300.000 15000 300.000 15000 300.000 15000 300.000	Internal Standard Target Compound Internal Standard	cis
file al	e last uned e last uned E last 4.07 4.62 5.33 5.39 5.42 5.42 5.51	Viano Tolesson Viano - Benzo 14MHX* Compound Compound 7 Animochniscopon 7 Animochniscopon 7 Animochniscopon 7 Animochniscopon 8 Atydosysteración 6 Atydosysteración 0 Coasepon 8 Atydosysteración 0 Sacepon	¹ Groups 24tection Call 5 000 5 000 5 000 5 000 5 000 5 000 5 000 5 000 5 000 5 000	Editorio Coltoroloco 10.000	ts 25.000 25	Cal4 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000	Cars Cars Cars Cars Cars Cars Cars Cars	17 Diace 18 Loraz 19 Loraz 19 Loraz 19 Loraz 19 Loraz 19 Loraz 10 Lor	repuin spain 0.4 11 mo Venuer CaP CaP 500.000 11 500.000 11 500.000 11 500.000 11 500.000 11 500.000 11 500.000 11		Berso Berso Stor Mother Stor Mother Control Intellector RT 1 4.02 3 4.02 3 4.02 3 4.02 4 5.03 5 5.42 7 5.42 7 5.42 7 5.42 9 5.57 10 5.57	Econopounds Identificat Compound 7.Aminoclon 7.Aminoclon 7.Aminoclon 7.Aminoclon 8.Tychosyal Loracepan a Hychosyal Oxacepan 2.Hychosyel Desakyfike Temacepan	To L4Mibs*	5.51 5.57 5.57	24gebourtlyBluenperi DesallyBluenperi DesallyBluenperi Cellenton C	Internal Standard Target Compound Internal Standard	cia
file al	■ List uned ■ List ■ List	Mass Tolesson Viow - Bonzo L4Ntik* Compound 7 Animochniscopan 7 Animochniscopan 4 Hydroghiazdon Loranpan 6 Hydroghiazdon Loranpan 6 Hydroghiazdon Dasapan Dasapan 7 Temaapan Nordacepan	Groups Carl 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000	T Report Calibration 10.000 10.000 10.000 10.000 10.000 10.000 10.000 10.000 10.000 10.000	te 2500 25000 25000 25000 25000 25000 25000 25000 25000 25000 25000	Cal4 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000	C lovels C love	17. Diace 18. Loraz 18. Loraz 19. Loraz	span span04 ITmeVenue Ca7 Ca8 Socoo 1 Socoo 1		Berso Berso Stor Mother training Intervention RT 1 4.07 2 400 3 4.52 4 5 5.93 6 7 5.42 9 5.57 10 5.57 10 5.57 11 5.70	ed Compound 1 Identificat Compound 7 Animothan 7 Animothan 4 Hydrosylat Oxazepam 2 Hydrosylat Oxazepam 2 Hydrosylat Desalythzos Temazepam Nordiazepam	To L4Mibs*	5.51 5.57 5.57	24getogethyllisengen()4 Desallyllisengen()4 Desallyllisengen()4 Calibrian Ca	Internal Standard Target Compound Internal Standard	cia
file ral	ethod n List RT 4.07 4.08 5.33 5.33 5.42 5.42 5.51 5.53 5.53 5.53 5.70 5.72	Mass Toleracoa Visw - Benzol 14Mik* Ecompoundi Compound 7 Aninofonacepon 7 Aninofonacepon 7 Aninofonacepon 8 Hydrospiteacebon 6 Hydrospiteacebon 9 Hydrospiteacebon 0 Asasypan 8 Hydrospiteacebon 0 Asasypan 7 Aninofonacepon 9 Hydrospiteacebon 0 Asasypan 7 Aninofonacepon 9 Hydrospiteacebon 0 Asasypan 7 Aninofonacepon 9 Hydrospiteacebon 0 Asasypan 7 Aninofonacebon 9 Asasypan	Circups 2010/00 Call 5,000	Calaration Calaration 10.000 10.000 10.000 10.000 10.000 10.000 10.000 10.000 10.000 10.000 10.000 10.000	ta Calibra 25.000 2	Cal4 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000	Corester Corester Carson 125,000 125,000 125,000 125,000 125,000 125,000 125,000 125,000 125,000 125,000 125,000 125,000 125,000 125,000	17. Diace 18. Loraz 19. Loraz 19. Loraz 19. Loraz 20. Conservation 250.0000 250.000 250.000 250.0000 250.0000 250.0000 250.000	sepan sepan-04 lime/Venet CaF CaS		Berao Berao Berao Stor Mother brokon file last or General Acquisition List RT 1 407 2 403 3 4.52 4 5.33 5 5.33 6 5.52 9 5.57 9 5.57 9 5.57 9 5.57 10 5.62 11 5.72 12 5.72	ect. Longound 1 Lonoted Compound Compound Tominordin Tominordin Tominordin Tominordin Conserpain Hightopolit Decallyblace Decallyblace Temacpain Alpracolem Alpracolem	To L4Mibs*	5.51 5.57 5.57	24bpdoxpethyBuscoper/04 DecallyBuscoper/04 DecallyBuscoper/04 Calleron Calleron Calleron Calleron Calleron Calleron 15000 300.000 15000 300.000 15000 300.000 15000 300.000 15000 300.000 15000 300.000 15000 300.000 15000 300.000	Internal Standard Target Compound Internal Standard	cia
file al	■ List uned ■ List ■ List	Mass Tolesson Viow - Bonzo L4Ntik* Compound 7 Animochniscopan 7 Animochniscopan 4 Hydroghiazdon Loranpan 6 Hydroghiazdon Loranpan 6 Hydroghiazdon Dasapan Dasapan 7 Temaapan Nordacepan	Groups Carl 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000 5,000	T Report Calibration 10.000 10.000 10.000 10.000 10.000 10.000 10.000 10.000 10.000 10.000	te 2500 25000 25000 25000 25000 25000 25000 25000 25000 25000 25000	Cal4 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000 50.000	C lovels C love	17. Diace 18. Loraz 18. Loraz 19. Loraz	spom spom 1 Trace Vesser Ca7 Ca8 S0.000 1 S0.000 1		Berso Berso Stor Mother training Intervention RT 1 4.07 2 400 3 4.52 4 5 5.93 6 7 5.42 9 5.57 10 5.57 10 5.57 11 5.70	ed Compound 1 Identificat Compound 7 Animothan 7 Animothan 4 Hydrosylat Oxazepam 2 Hydrosylat Oxazepam 2 Hydrosylat Desalythzos Temazepam Nordiazepam	To L4Mibs*	5.51 5.57 5.57	24getogethyllisengen()4 Desallyllisengen()4 Desallyllisengen()4 Calibrian Ca	Internal Standard Target Compound Internal Standard	cia

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).

- 🔣 📲		Benzo14Mix*										👖 Re	al time status 🛛 🗮 I
Local Met				~	Upd	ate Ir	nstrument:	Thermo	Scientific	Instrument	User:		
	Status	Filename	Sample type	Sample	level	Sample ID	e Sam nam		omment	Vial posi	ion	Injection volume	Conv Factor
▶ 1	•	Cal1	Cal Std	Cal1		1				CStk1-01	:01	50.0	1.00
2	•	Cal2	Cal Std	Cal2		1				CStk1-01	:2	50.0	1.00
3	•	Cal4	Cal Std	Cal4		1				CStk1-01	:3	50.0	1.00
4	•	Cal6	Cal Std	Cal6		1				CStk1-01	:4	50.0	1.00
5	•	Cal7	Cal Std	Cal7		1				CStk1-01	:5	50.0	1.00
6	•	Cal8	Cal Std	Cal8		1				CStk1-01	:6	50.0	1.00
7	•	QA001	Chk Std	QC1		1				CStk1-01	:7	50.0	1.00
8	•	QA002	Chk Std	QC1		1				CStk1-01	:8	50.0	1.00
9	•	QA003	Chk Std	QC1		1				CStk1-01	:9	50.0	1.00
10	•	QA004	Chk Std	QC1		1				CStk1-01	:10	50.0	1.00
11	•	QA005	Chk Std	QC1		1				CStk1-01	:11	50.0	1.00
12	•	QB001	Chk Std	QC2		1				CStk1-01	:12	50.0	1.00
13	•	QB002	Chk Std	QC2		1				CStk1-01	:13	50.0	1.00
14	•	QB003	Chk Std	QC2		1				CStk1-01	:14	50.0	1.00
15	•	QB004	Chk Std	QC2		1				CStk1-01	:15	50.0	1.00
16	•	QB005	Chk Std	QC2		1				CStk1-01	:16	50.0	1.00
	-			-			**	**					1
utomated	l Batch	Reports								Compoun	d Active	Status	
Sampl	e Level	Batch Levi	el								Compo	und	Active
	Benc	nt Name		Туре	Print	Create	Create	Create		▶ 1	7-Amine	oclonazepam	
	· ·					PDF	XML	XLSM	-	2	7-Amino	oclonazepam-D4	V
1	_	Report		Standard						3	7-Amino	onitrrazepam	
2	_	k Standard Report		Standard				Г	-11	4	7-Amino	oflunitrazepam	~
3	_	natogram Report		Standard				Г	-11	5	7-Amino	oflunitrazepam-D7	 Image: A start of the start of
<i>J</i> 4		ound Calibration Rep		Standard				Г		6	a-Hydro	xytriazolam	 Image: A start of the start of
5		ound Calibration Rep	oort - Alternate	Standard				Г	- 1	7	a-Hydro	xytriazolam-D4	 Image: A start of the start of
6	_	mation Report		Standard				Г		8	Lorazep	oam	 Image: A start of the start of
7	_	mation Report 2		Standard				Г		9	Lorazep	oam-D4	V
8	High	Density Internal Stan	dard Report	Standard						10	a-Hydro	xyalprazolam	

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.).

ethod:		intra1_8						Instrument									
_	Status	Flags	Filename	Sample	Samp	Vial	Actual	Integrati	*Area	Calc Amt	Theo Amt	IS Resp	Excluded	% Diff	%	%	Compounds
1			Cal1	CalStd		CStk1-01:01	5.34	Method	6116	5.4901	5.000	159884		9.80	N/A	N/A	🎴 7-Aminoclonazepam-D4
2	•		Cal2	CalStd		CStk1-01:2	5.35	Method	12637	9.9474	10.000	124448		-0.53	N/A	N/A	7-Aminoflunitrazepam-D7 a-Hvdroxvalprazolam-D5
3			Cal4	CalStd		CStk1-01:3	5.34	Method	73596	45.9933	50.000	119990		-8.01	N/A	N/A	a-Hydroxyalprazolam-D 5
4			Cal6	CalStd		CStk1-01:4	5.36	Method	433035	236.4220	250.000	130543		-5.43	N/A	N/A	🍋 Alprazolam-D5
5	•		Cal7	CalStd		CStk1-01:5	5.36	Method	1047	524.5294	500.000	141381		4.91	N/A	N/A	Desalkylflurazepam-D4
6	•		Cal8	CalStd		CStk1-01:6	5.37	Method	1819	992.6177	1000.000	129451		-0.74	N/A	N/A	Diazapam-D5 Lorazepam-D4
7	i i		QA001	ChkStd		CStk1-01:7	5.36	Method	26294	14.3502	15.000	160274		-4.33	7.53	9.58	Midazolam-D4
8	ŏ		QA002	ChkStd		CStk1-01:8	5.36	Method	24801	13.4152	15.000	164482		-10.57	7.53	9.58	🍋 Nordiazepam-D5
9	ŏ		QA003	ChkStd		CStk1-01:9	5.36	Method	21115	12.5994	15.000	151695		-16.00	7.53	9.58	📜 Oxazepam-D5
10	ŏ		0A004	ChkStd		CStk1-01:10	5.36	Method	23553	13.4035	15.000	156381		-10.64	7.53	9.58	Temazepam-D5 2-Hydroxyethylflurazepam
11	ŏ		0A005	ChkStd		CStk1-01:11	5.36	Method	21011	11.7254	15.000	165722		-21.83	7.53	9.58	7-Aminoclonazepam
12	i i		08001	ChkStd		CStk1-01:12	5.36	Method	611744	293.8776	300.000	148015		-2.04	2.38	2.41	7 Aminoflunitrazepam
13	ŏ		08002	ChkStd		CStk1-01:13	5.36	Method	601486	299.4603	300.000	142795		-0.18	2.38	2.41	7-Aminonitrrazepam
14	ŏ		QB003	ChkStd		CStk1-01:14	5.36	Method	598636	284.8504	300.000	149480		-5.05	2.38	2.41	a-Hydroxyalprazolam a-Hydroxytriazolam
15	ŏ		QB004	ChkStd		CStk1-01:15	5.37	Method	600854	297.5668	300.000	143561		-0.81	2.38	2.41	Alprazolam
16	-		QB005	ChkStd		CStk1-01:16	5.36	Method	589322	303.4743	300.000	138039		1.16	2.38	2.41	Desalkylflurazepam
			4														Diazepam Lorazepam
																	Luiazepain Lui L

an		_			_		_		-		· ·		· ·			T	
ode		⊋uan P	'eak: 1				Calibrat	ion curve		Spectra		QED Spectra		Confirmin	g lons		lon overlay
al	L	orazepan	n RT: 5.34 0	Cal1						Y	1.42e-2X -	3.97e-2; R^	Lorazeparr 2: 0.9986; (Oriain: la	nore; W:	1/X: Area	3
de				RT: 5.34			16-										
				AA: 6116.41 AH: 1087.72			14-										
		100-		SN: 24.38			12-									_	
		=		Δ			율 10-										
		50-					Area Ratio					_					
		0-	4.5 5.0	5.5	6.0		-9 Are						_				
			4.5 5.0	RT(min)	6.0		4-			-							
			m/2	: 321.0192			2-										

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

rt View - Intra1	_			_		_
/iew Only 🔵 Generate Only						
a report: Check Standard Report	Sample file	QA002	~			
rtView						
🖼 H 🔺 🕨 1	/2 M	€, -				
Standard Report						
Lab Name: DefaultLaborat Instrument: ThermoScientii User: USSJO-TRCFN Batch: Intra1	fic Instrument	Check Sta	ndard Report Method: Intra1_Ben Benzo14M Cali File: Intra1.calx			Page 1 of 2
Vial Pos Sample II CStk1-01:8 1	D File Name QA002	Level QC1	Sample Name	File Date 3/30/2011 1:59:40 I		nment
Compound	Curve Type Daily R	F Mean RF Min RF	RF%D Max RF%D	QC Amt Calc Amt	Amt%D	Max Amt %D Flag
7-Aminoclonazepam	L	0.000		15.0000 13.5286	-9.81	20.00 Pass
7-Aminonitrrazepam	L	0.000		15.0000 13.4464	-10.36	20.00 Pass
7-Aminoflunitrazepam	L	0.000		15.0000 13.4085	-10.61	20.00 Pass
a-Hydroxytriazolam	L	0.000		15.0000 14.0475	-6.35	20.00 Pass
Lorazepam	L	0.000		15.0000 13.4152	-10.57	20.00 Pass
a-Hydroxyalprazolam	L	0.000		15.0000 13.4446	-10.37	20.00 Pass
Oxazepam	L	0.000		15.0000 12.8524	-14.32	20.00 Pass
2-Hydroxyethylflurazepam	L	0.000		15.0000 13.6104	-9.26	20.00 Pass
Desalkylflurazepam	L	0.000		15.0000 13.7256	-8.50	20.00 Pass
Temazepam	L	0.000		15.0000 13.2345	-11.77	20.00 Pass
Nordiazepam	L	0.000		15.0000 14.4350	-3.77	20.00 Pass
Alprazolam	L	0.000		15.0000 14.2256	-5.16	20.00 Pass
Page No.: 1		Total Page No.: 2		Zoom Factor:	Page Width	



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

		QC level 1	l: 15 ng/mL	QC level 2	: 300 ng/mL			
Name	m/z	% Precision	% Accuracy	% Precision	% Accuracy	Linear Range (ng/mL)	LLOQ (ng/mL)	
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	
lpha-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	
lpha-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.

For Research Use Only. Not for use in diagnostic procedures.

In addition to these offices, Thermo Fisher Scientific maintains a network of representative organizations throughout the world.

Africa-Other

Australia +61 3 9757 4300 Austria +43 1 333 50 34 0 **Belgium** +32 53 73 42 41 Canada +1 800 530 8447 **China** +86 10 8419 3588 Denmark +45 70 23 62 60 Europe-Other +43 1 333 50 34 0 Finland/Norway/ Sweden +46 8 556 468 00 France +33 1 60 92 48 00 **Germany** +49 6103 408 1014 India +91 22 6742 9434 **Italy** +39 02 950 591 **Japan** +81 45 453 9100 Latin America <u>+1 561 688</u> 8700 **Middle East** +43 1 333 50 34 0 Netherlands **New Zealand** +64 9 980 6700 Russia/CIS 43 1 333 50 34 0 **South Africa Spain** +34 914 845 965 Switzerland UK +44 1442 233555 USA +1 800 532 4752

www.thermofisher.com

Legal Notices: ©2016 Thermo Fisher Scientific Inc. All rights reserved. All trademarks are the property of Thermo Fisher Scientific Inc. and its subsidiaries. This information is presented as an example of the capabilities of Thermo Fisher Scientific Inc. products. It is not intended to encourage use of these products in any manners that might infringe the intellectual property rights of others. Specifications, terms and pricing are subject to change. Not all products are available in all countries. Please consult your local sales representative for details.

AN63418_E 06/16S

