



Analysis of Benzodiazepines using Liquid Chromatography Mass Spectrometry-Mass Spectrometry

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Introduction:

Novel benzodiazepines are prevalent within the community and are used to circumvent prosecution under the Controlled Substances Act. However, it has been demonstrated that benzodiazepines can cause impairment and thus affect the ability to safely operate any vehicle on roadways. At DFS, only one novel benzodiazepine is within the current scope (etizolam). Currently, DFS's procedure includes hydrolysis and solid phase extraction on day one which takes up to six hours to complete. These derivatized samples are analyzed by Gas chromatography Mass Spectrometry for five benzodiazepines (diazepam, 7-amino-flunitrazepam, alprazolam, etizolam and midazolam). On day two, samples are derivatized for one hour and analyzed for the remaining eight benzodiazepines (7 amino-clonazepam, alpha-hydroxy alprazolam, alpha-hydroxy triazolam, nordiazepam, oxazepam, temazepam, hydroxy ethyl-flurazepam, and lorazepam). At the minimum, two and half days are required to complete analysis before the data can be reviewed.

Objectives:

1. Shorten analysis time
2. Reduce sample volume
3. Expand the scope of drugs
4. Move from GC/MS to LC/MS/MS

Extraction:

- Preparation: 0.5 mL blood + 3 mL phosphate buffer, vortex
- UCT Clean Screen DAU SPE columns (200 mg)
- Condition: Methanol, water, 0.1 M phosphate buffer (pH 6)
- Load samples
- Wash: Water, 5% ACN+1 M acetic acid (pH 2.4), hexanes
- Dry columns
- Elution: 2% NH₄OH in ethyl acetate (prepare daily)

Urine Hydrolysis:

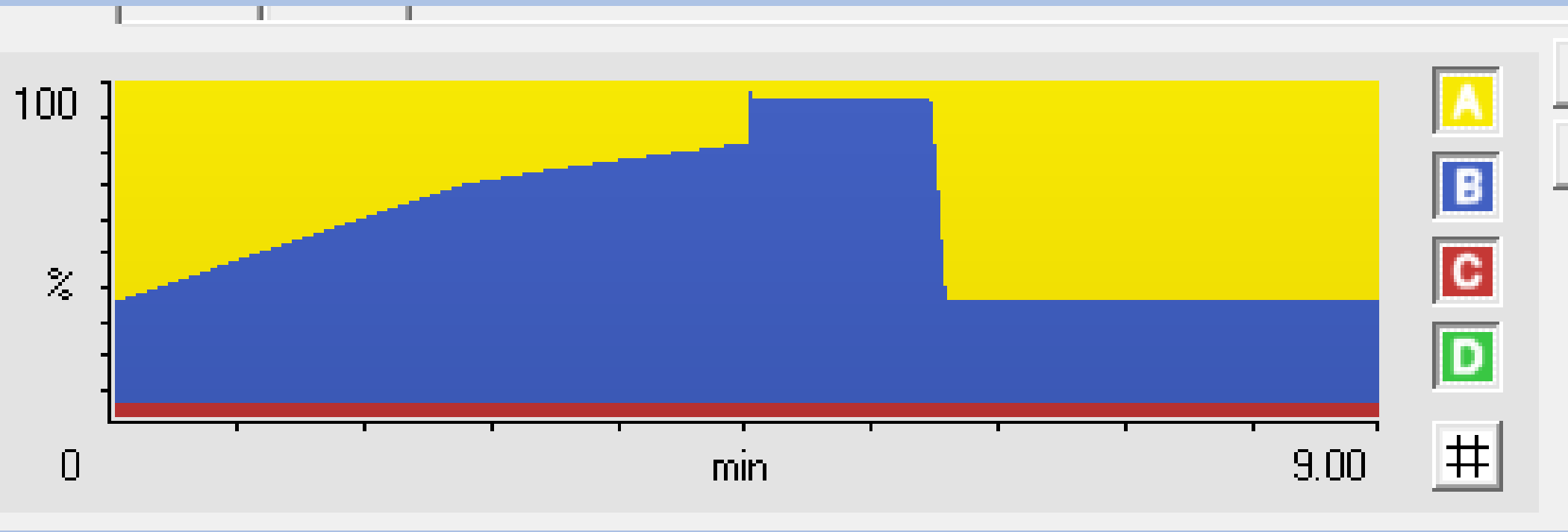
- room temperature/no wait
- 100 µL of Kura Biotech BGTurbo High efficiency β-glucuronidase enzyme
- 200 µL of Instant Buffer I
- 550 µL of DI water
- Add 2 mL of phosphate buffer, and vortex
- Continue with extraction

Instrument:

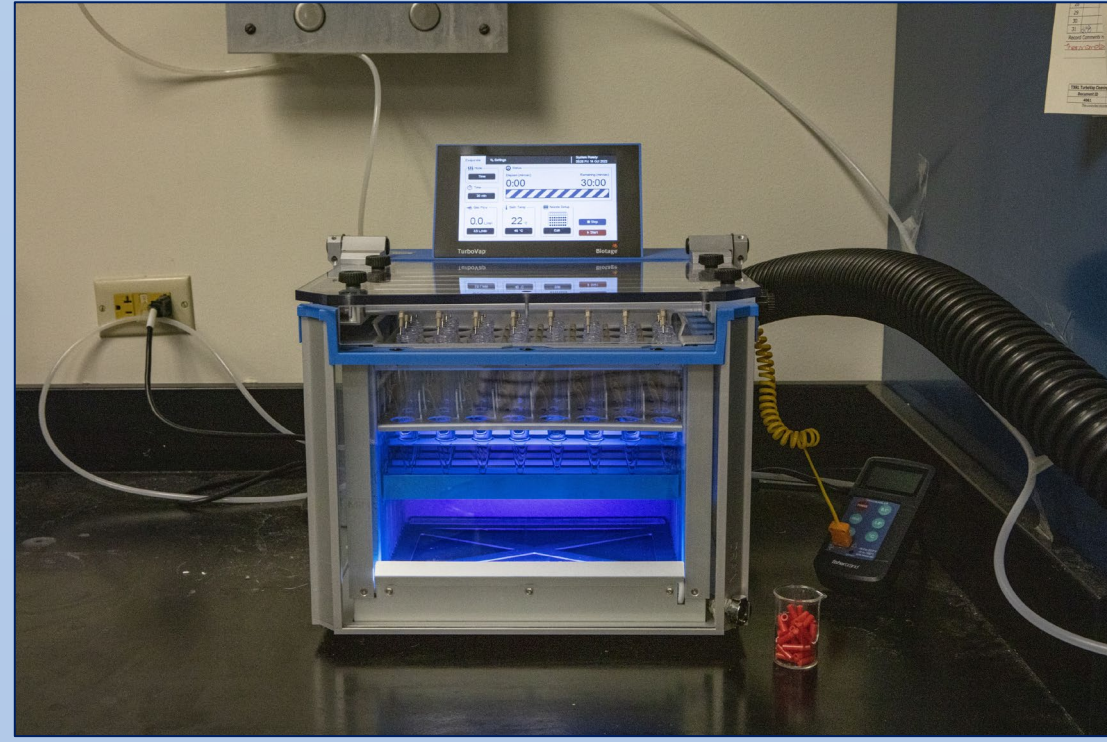
Waters Acquity UPLC H-Class quaternary solvent manager with FTN and Xevo TQD Mass Spectrometer

LC Parameters:

Sample Manager Temperature: 4°C
Column Temperature: 50 °C
Injection Volume: 1 µL



Time (minutes)	% Water	%MeOH	FA (2%)	Curve
Initial	65	30	5	6
2.50	30	65	5	6
3.25	25	70	5	6
4.50	18	77	5	6
4.51	5	90	5	6
4.80	5	90	5	6
5.91	65	30	5	6
9.00	65	30	5	6



MS/MS parameters:

Capillary	0.7 kV
Ionization	ESI/positive
Source	150 °C
Desolvation	500 °C
Cone gas	20 L/hr
Desolvation	800 L/hr

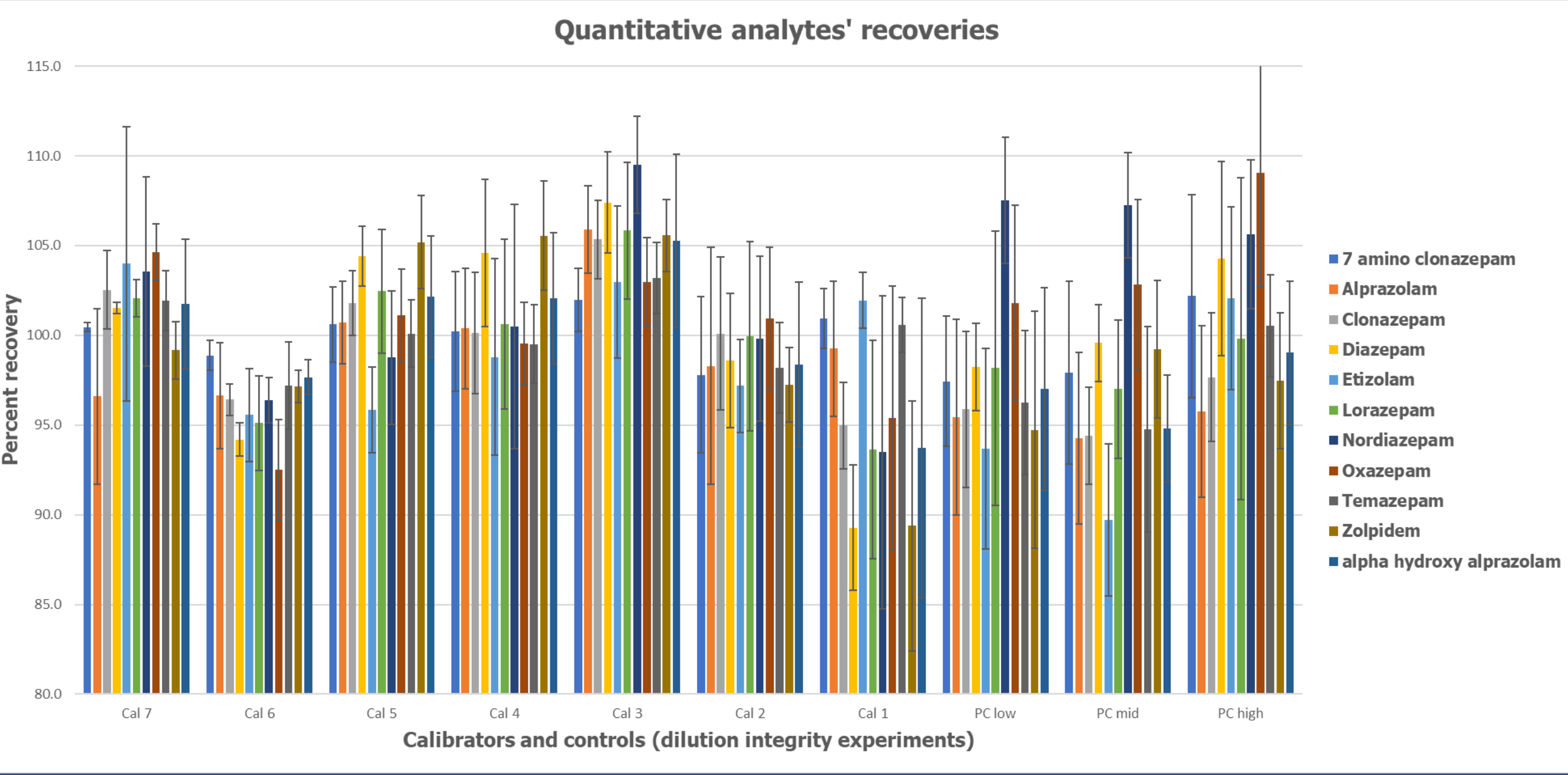
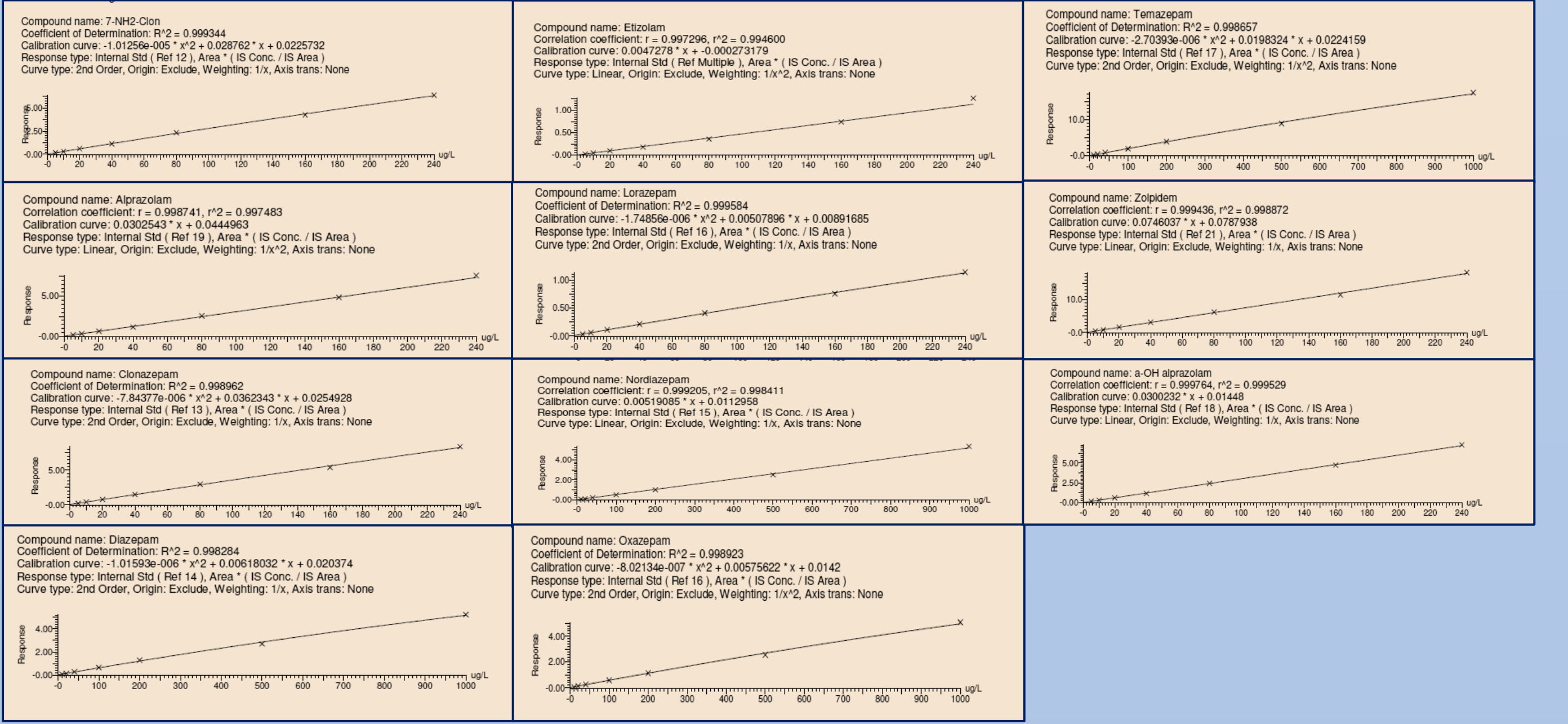
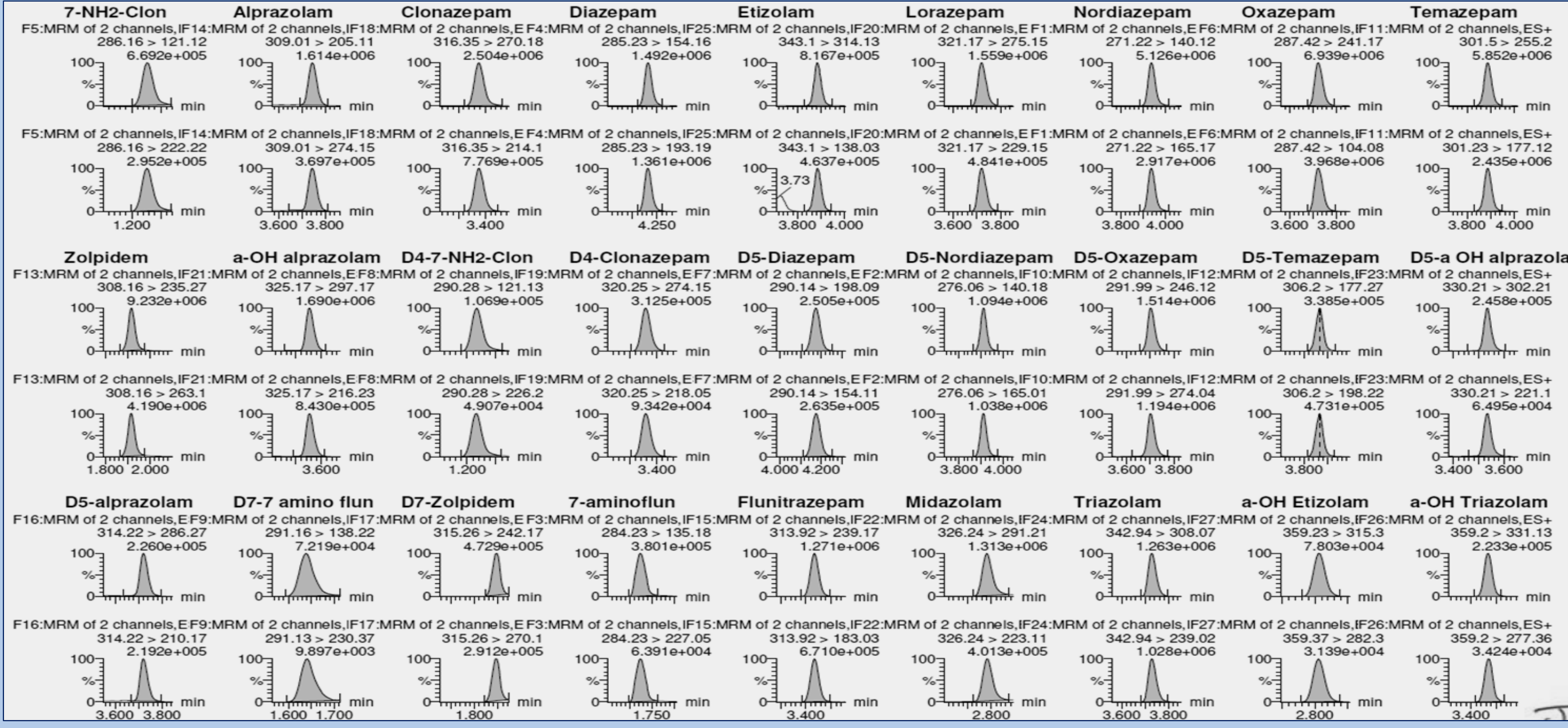
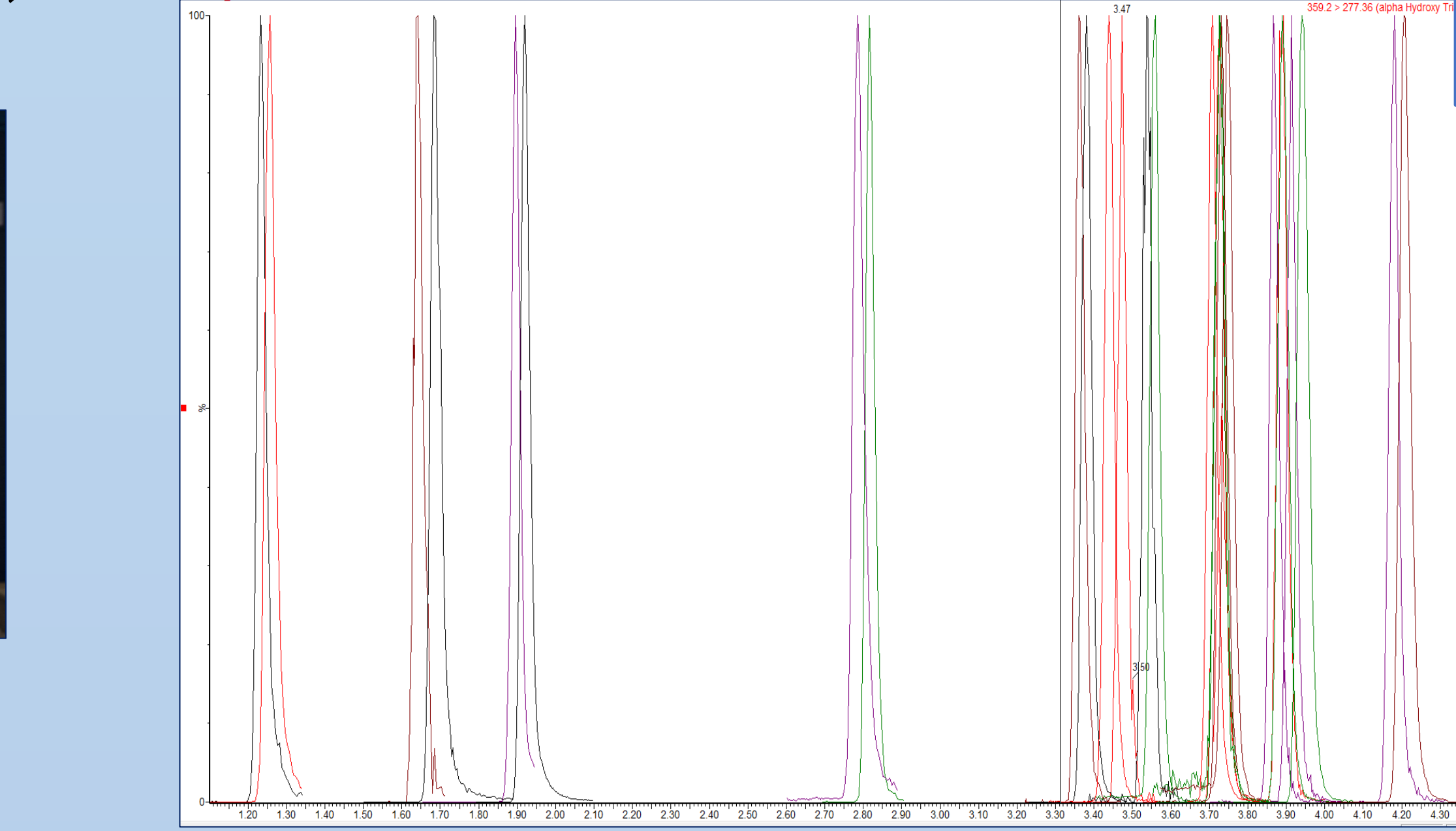
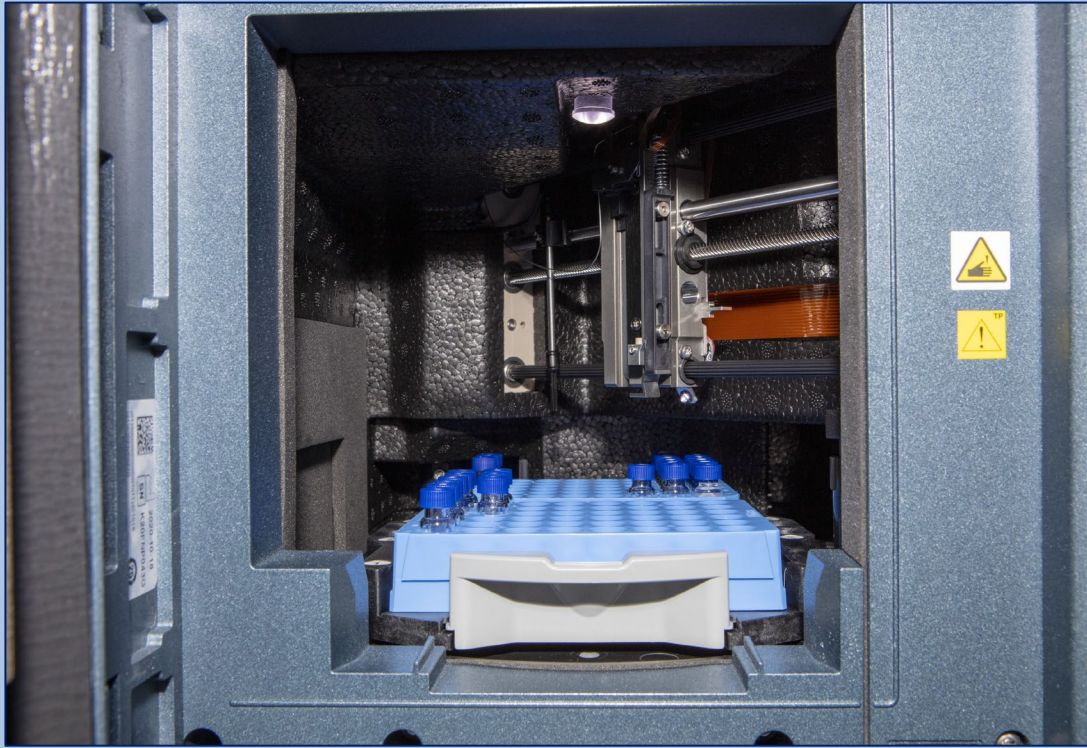
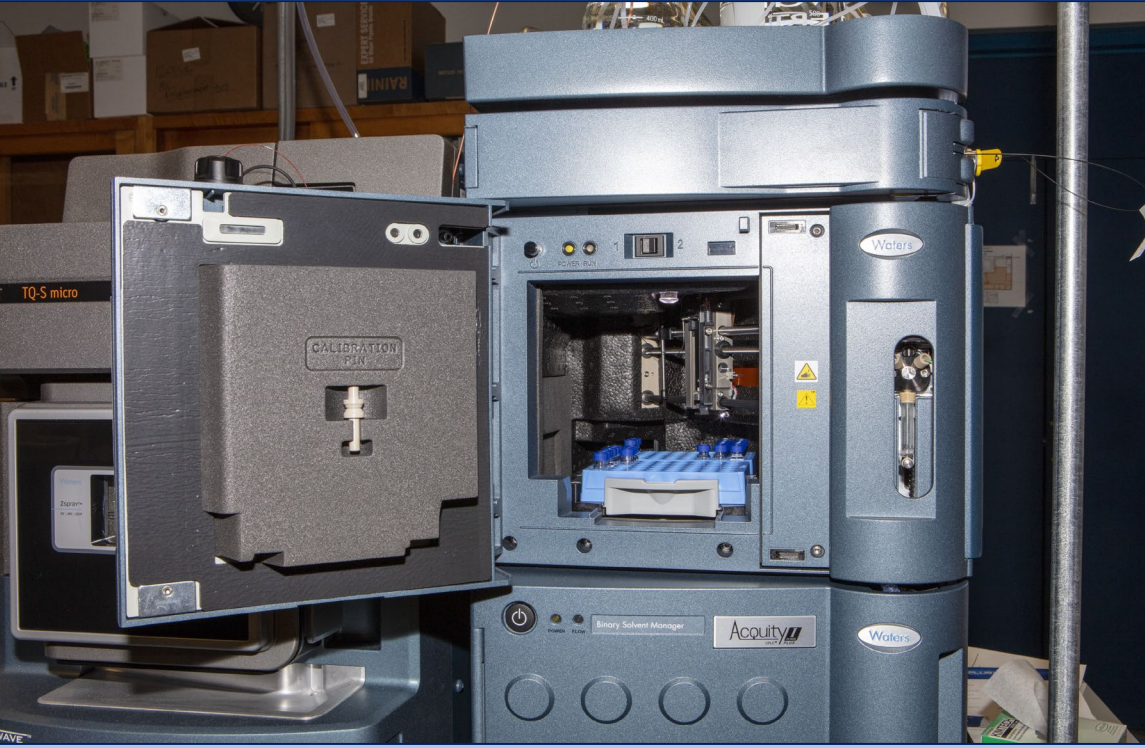
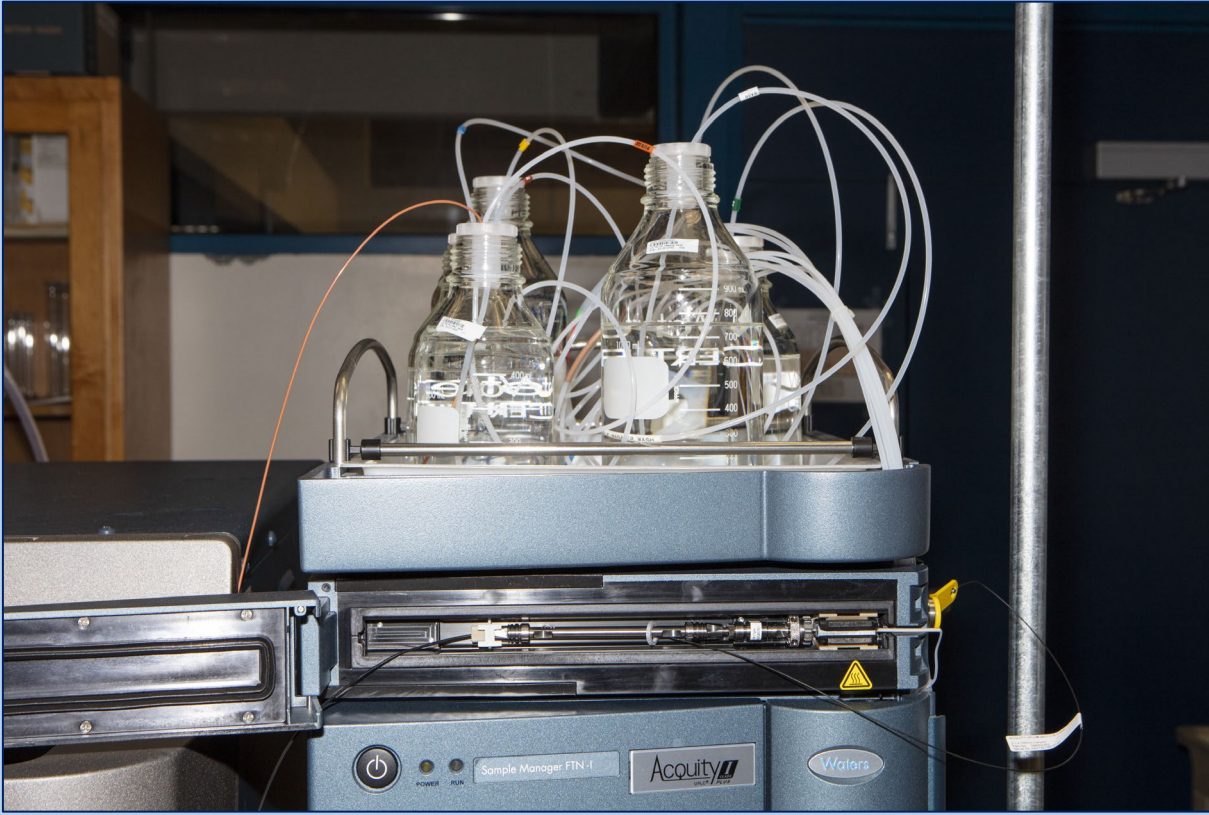
- **Nordiazepam**
- D5-Nordiazepam
- Nitrazepam
- 7-amino flunitrazepam
- **Diazepam**
- **7-amino clonazepam**
- N-chlordiazepoxide
- Demoxepam
- D4-7 amino clonazepam
- D5-Diazepam
- D7-7 amino flunitrazepam
- D5-Oxazepam
- Estazolam
- Nimetazepam
- Chlordiazepoxide
- D5-Estazolam
- **Temazepam**
- Clobazam
- Delorazepam
- D5-Temazepam
- Zaleplon
- **Zolpidem**
- **Alprazolam**
- D5-alprazolam
- Flunitrazepam
- D7-zolpidem
- **Clonazepam**
- Bromazepam
- Diclazepam
- D4-Clonazepam
- **Lorazepam**
- 8-amino-clonazolam
- **α-OH-Alprazolam**
- Midazolam
- D5- α-OH-Alprazolam
- Hydroxy ethyl-flurazepam
- Flumetazepam
- Flubromazepam
- Triazolam
- **Etizolam**
- Phenazepam
- Adinazolam
- Bromazolam
- Pyrazolam
- Clonazolam
- α-OH Etizolam
- α-OH-Triazolam
- α-OH-Clonazolam
- Flubromazolam
- α-OH-Flubromazolam
- Flurazepam
- Zopiclone/eszopiclone

Drug	Calibration	Weighting	Quantitative range (ng/mL)	LOQ/LOD (ng/mL)
7-amino clonazepam	quadratic	1/x	5-240	5
Alprazolam	linear	1/x^2		
Clonazepam	quadratic	1/x		
Etizolam	quadratic	1/x		
Lorazepam	linear	1/x^2		
Zolpidem	quadratic	1/x	10-1000	10
OH-Alprazolam	linear	1/x		
Diazepam	quadratic	1/x^2		
Nordiazepam	quadratic	1/x^2		
Oxazepam	linear	1/x		
Temazepam	linear	1/x		

Interferences:

- None from blank blood or blank urine (synthetic and human)
- None from high drug concentrations and internal standard (IS)
- None from other commonly encountered drugs
 - Fentanyl, nor-fentanyl, codeine, hydrocodone, oxycodone, oxymorphone, morphine, hydromorphone, 6-monoacetyl morphine, amphetamine, methamphetamine, ephedrine, pseudoephedrine, MDA, MDMA, phentermine, tetrahydrocannabinol and metabolites, cocaine and metabolites

	7-amino clonazepam	Alprazolam	Clonazepam	Etizolam	Lorazepam	Zolpidem	OH-Alprazolam	Diazepam	Nordiazepam	Oxazepam	Temazepam
Bias (low)	6.11	2.14	3.34	2.30	-0.93	4.95	2.75	5.41	13.60	9.07	3.94
Bias (mid)	4.90	1.30	3.47	1.52	-1.60	5.95	5.70	7.48	13.37	7.69	3.42
Bias(high)	1.01	0.56	1.60	7.11	-3.62	0.66	1.39	3.89	5.18	4.04	3.27
Within run CV (low)	4.39	5.52	3.38	5.43	6.35	4.38	5.42	4.23	2.48	4.02	4.93
Within run CV (mid)	4.04	3.39	3.07	5.37	3.56	2.70	4.56	3.42	2.67	2.71	4.84
Within run CV (high)	3.82	4.33	2.94	4.16	4.45	2.00	3.74	2.65	2.02	2.02	3.22
Between run CV (low)	9.79	8.15	5.59	15.64	8.06	9.53	14.40	8.60	4.96	8.30	6.87
Between run CV (mid)	6.48	4.06	5.05	10.24	8.62	7.48	9.43	7.30	5.21	7.35	5.93
Between run CV (high)	6.97	4.43	3.79	8.74	6.67	8.17	10.90	3.98	3.98	19.38	9.45



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