

# Identification and Quantitation of Traditional and Designer Benzodiazepines in Urine by UHPLC-MS/MS

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## INTRODUCTION

The increasing abuse of designer benzodiazepines has created the necessity for an expanded analytical method to detect emerging designer benzodiazepine compounds and help identify illicit use. The method developed by our laboratory provides a comprehensive analysis of urine specimens, evaluating the presence of 23 traditional and designer benzodiazepines at concentrations from 20-4000 ng/mL.

## OBJECTIVE

Expand our laboratory's urine benzodiazepine confirmation method to include designer benzodiazepines relevant to workplace drug testing

## EXTRACTION METHOD

Using a Tecan Freedom Evo<sup>®</sup> automated pipetter platform equipped with a BioShake 3000-T elm heated mixer unit, 50 µL of each urine sample, 50 µL internal standard, and 50 µL of 20:80 BG Turbo<sup>®</sup> β-Glucuronidase:0.1M phosphate buffer (pH 6.8) solution were added to a 96-well plate and incubated at 50°C while mixing at 300 rpm for 7.5 minutes. Samples were then diluted with 850 µL of 0.1% Formic Acid in 50:50 DI H<sub>2</sub>O:Methanol.

## INSTRUMENT PARAMETERS

Table 1: UHPLC-MS/MS Parameters

UHPLC System	Shimadzu Nexera	LC-30AD Pumps SIL-30 AC Auto Sampler	Time	Aqueous 1 (%)	Aqueous 2 (%)	Organic (%)
	Shimadzu Prominence	CBM-20A Controller CTO-20A Column Oven DGU-20A5 Degasser	0.00	70.0	25.0	5.0
Injection Volume	8 µL		0.50	51.0	25.0	24.0
Analytical Column	Waters CORTECS Shield RP18 2.7µm, 2.1 x 100 mm (PN 186008664)		1.80	51.0	25.0	24.0
Guard Column	Waters CORTECS Shield RP18 2.7µm, 2.1 x 5 mm (PN 186008712)		3.10	25.0	45.0	30.0
Column Temp.	35°C		3.90	25.0	45.0	30.0
Mobile Phase	Aqueous 1: 10mM Ammonium Formate Aqueous 2: 0.1% Formic Acid in DI Water Organic: 0.01% Formic Acid in Acetonitrile		4.15	0.0	10.0	90.0
Flow Rate	0.800 mL/min		4.16	70.0	25.0	5.0
Run Time	5.00 minutes		5.00	STOP		
Mass Spectrometer	Sciex API6500 Triple Quad					
Ionization	ESI Positive					
Source Temp.	450°C					
Scheduled MRM	Target scan time 100 milliseconds					
Scheduled Ionization	Start 1.35 minutes End 4.60 minutes					

Analyte	Internal Standard	Precursor Ion	Product Ion Quantifier	Product Ion Qualifier	Retention Time (± 0.5 mins)	MRM Detection Window (seconds)	Dwell Weight
<b>7-Aminonitrazepam</b>	7-Aminonitrazepam-D5	252.2	121.0	224.2	1.60	30	6.00
	7-Aminonitrazepam-D5	257.1	121.0	229.0	1.58	30	6.00
	7-Aminonitrazepam-D4	290.0	254.1	226.0	1.88	30	4.00
<b>7-Aminoclonazepam</b>	7-Aminoclonazepam-D4	285.9	250.1	222.2	1.89	30	4.00
	7-Aminoflunitrazepam-D7	291.0	231.1	263.1	2.07	30	3.60
<b>8-Aminoclonazepam</b>	N-Desmethyloclobazam-13C6	324.1	220.0	296.1	2.06	30	4.00
<b>7-Aminoflunitrazepam</b>	7-Aminoflunitrazepam-d7	283.9	227.1	256.1	2.09	30	3.60
<b>α-Hydroxyetizolam</b>	Estazolam-D5	359.1	341.0	315.0	2.28	35	2.60
<b>Bromazepam</b>	Estazolam-D5	316.0	287.9	209.0	2.58	35	0.10
	α-Hydroxymidazolam-D4	346.0	301.0	203.0	2.79	35	3.25
<b>α-Hydroxymidazolam</b>	α-Hydroxymidazolam-D4	342.0	297.2	203.1	2.81	35	3.25
<b>α-Hydroxyfluprazolam</b>	Estazolam-D5	343.1	314.9	234.0	2.91	35	4.00
<b>α-Hydroxyflubromazolam</b>	α-Hydroxyfluprazolam-D5	387.0	358.7	234.0	3.02	35	4.00
<b>α-Hydroxyalprazolam</b>	α-Hydroxyalprazolam-D5	325.2	297.0	205.0	3.05	35	4.20
	α-Hydroxyalprazolam-D5	330.2	302.0	210.0	3.03	35	4.20
<b>α-Hydroxytriazolam</b>	α-Hydroxyalprazolam-D5	359.1	176.1	239.0	3.08	35	4.00
	Estazolam-D5	300.2	272.0	210.0	3.17	35	4.00
<b>Estazolam</b>	Estazolam-D5	295.2	267.1	205.0	3.19	35	4.00
	Oxazepam-D5	292.2	246.1	236.0	3.23	35	4.00
<b>Oxazepam</b>	Oxazepam-D5	287.0	241.0	231.0	3.25	35	4.00
<b>N-Desmethyloclobazam</b>	N-Desmethyloclobazam-13C6	287.1	244.8	181.1	3.38	35	3.20
	N-Desmethyloclobazam-13C6	293.1	250.8	187.1	3.38	35	3.20
<b>2-Hydroxyethylflurazepam</b>	2-Hydroxyethylflurazepam-D4	333.0	305.1	194.2	3.45	35	3.60
	2-Hydroxyethylflurazepam-D4	337.0	309.1	194.1	3.43	35	3.60
<b>Lorazepam</b>	Lorazepam-D4	321.1	229.0	194.0	3.49	35	4.00
	Lorazepam-D4	325.1	232.9	198.0	3.47	35	4.00
<b>Bromazolam</b>	Temazepam-D5	353.0	324.9	204.9	3.63	35	3.20
<b>3-Hydroxyphenazepam</b>	Temazepam-D5	366.9	321.0	274.8	3.70	35	1.75
	Temazepam-D5	306.1	260.2	177.0	3.86	40	1.20
<b>Temazepam</b>	Temazepam-D5	301.0	255.0	177.0	3.89	40	1.20
	Nordiazepam-D5	276.2	140.0	165.0	3.89	40	1.00
<b>Nordiazepam</b>	Nordiazepam-D5	271.2	140.0	165.0	3.93	40	1.00
<b>Lormetazepam</b>	Nordiazepam-D5	335.2	288.8	177.0	4.12	40	3.60
<b>Diazepam</b>	Diazepam-D5	285.2	193.1	154.2	4.25	30	5.00
	Diazepam-D5	290.0	198.1	154.1	4.24	30	5.00
<b>Halazepam</b>	Temazepam-D5	353.1	241.0	325.0	4.47	15	6.00

Figure A: Representative Chromatogram of Method Analyte Components

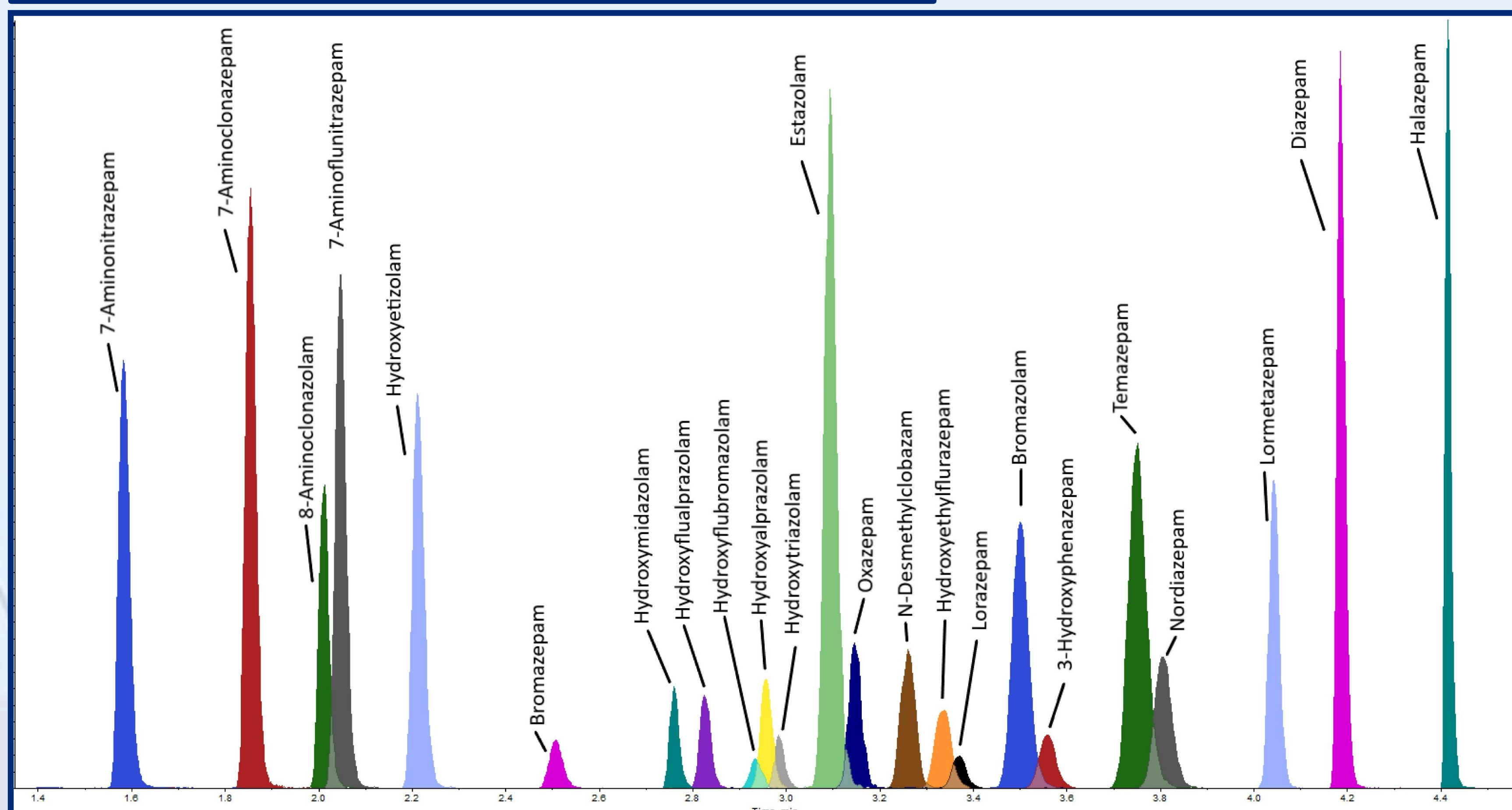


Figure B: Number of Designer Benzodiazepines Above Confirmation Limit Of Detection by Month

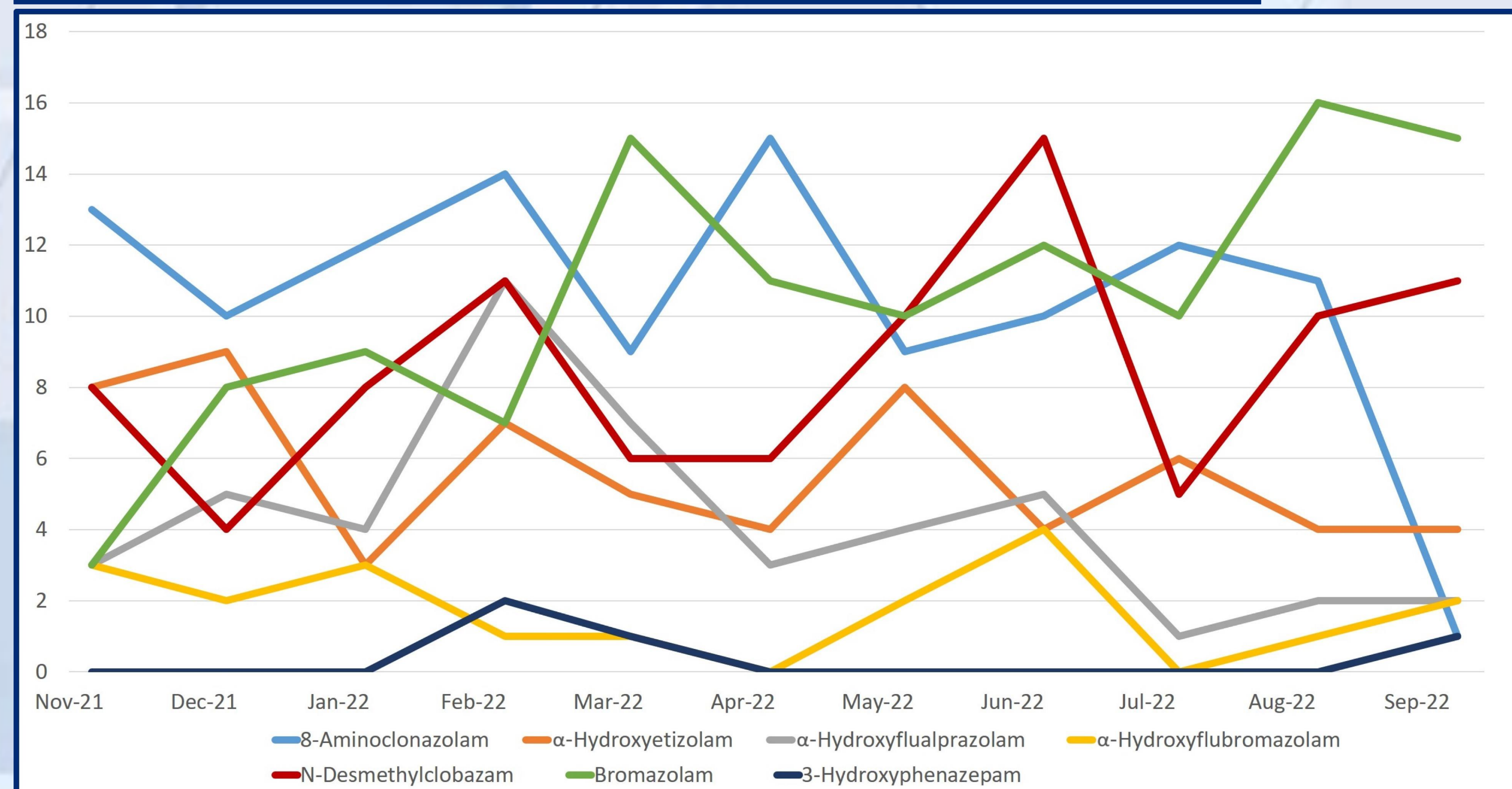
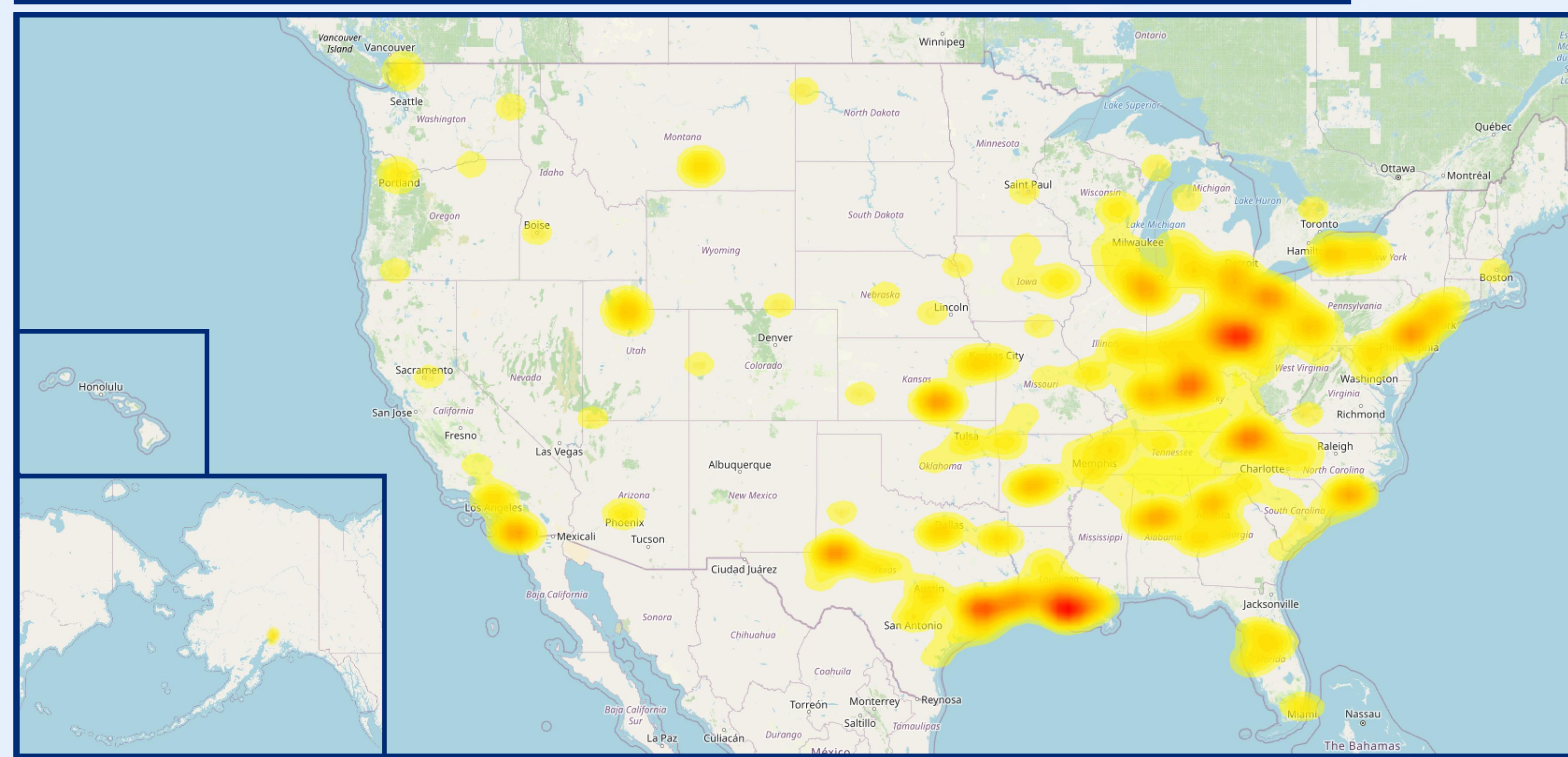


Figure C: Heat Map of Designer Benzodiazepines Positives by Collection Site (Controlled for Duplicate Donors)



## RESULTS / DISCUSSION

Urine specimens screening above the benzodiazepine enzyme immunoassay cutoff that did not confirm for routinely tested benzodiazepines were selected and analyzed using a SCIEX x500R QTOF to identify designer benzodiazepines. Based on detection results, seven new compounds were identified in routine workplace drug test samples to be added to the standard sixteen-analyte benzodiazepine confirmation method: bromazolam, 8-aminoclonazepam, α-hydroxyetizolam, α-hydroxyfluprazolam, α-hydroxyflubromazolam, n-desmethyloclobazam, and 3-hydroxyphenazepam.

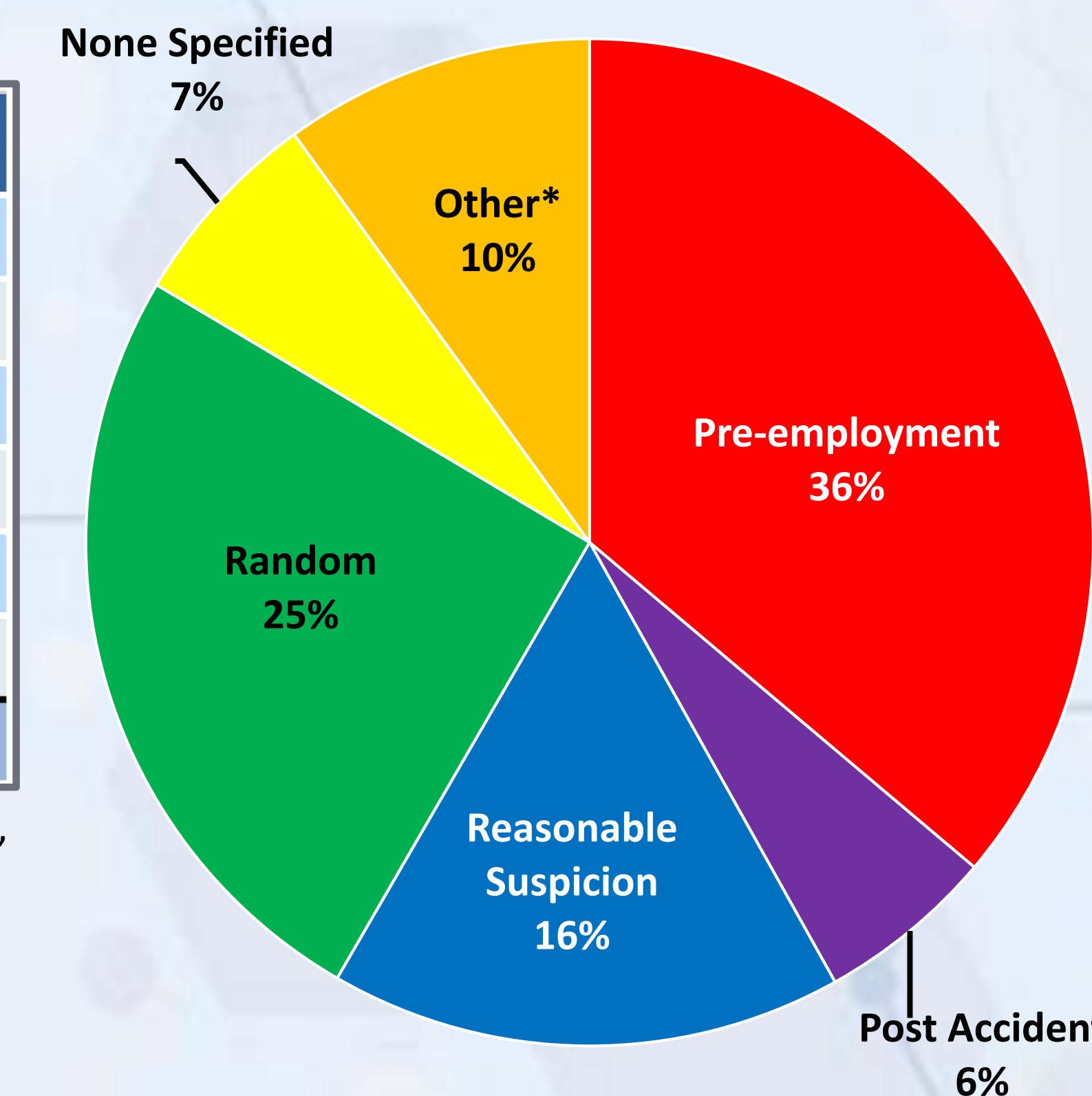
To validate the updated and expanded benzodiazepine confirmation method, urine spiked with known concentrations of 23 traditional and designer benzodiazepines was analyzed to establish linearity and to evaluate matrix and interference effects. Linearity was established through analysis of accuracy and precision data from 5 replicates at 11 concentrations from 20 ng/mL to 4000 ng/mL, where mean accuracy was within ±10% of target concentration and a CV of <10% was observed for each concentration. Carryover was not detected in negative controls injected after the replicates at highest concentrations, which were used to determine analyte upper limits of linearity (1,000, 2,000, or 4,000 ng/mL, analyte dependent). No matrix effects or interferences were observed in the analysis of 10 different donor samples and more than 100 over-the-counter, prescription, and illicit drugs evaluated.

Using the expanded confirmation method, from November 2021 to September 2022, 458 designer benzodiazepines were identified among 401 unique specimens. Bromazolam and 8-aminoclonazepam were the most abundant, having been identified in 116 samples each, while 3-hydroxyphenazepam was the most uncommon, having been found in only 4 samples. Of the 401 samples identified to have at least one designer benzodiazepine, 232 samples also tested positive for the presence of one or more additional illicit substances, such as marijuana metabolite, amphetamines, opiates, methadone, synthetic cannabinoids, and/or traditional benzodiazepines. During this timeframe, the use of the expanded method yielded a total of 172 positive results due to the presence of the newest designer benzodiazepine analytes—positives that would have previously gone undetected using standard benzodiazepine confirmation.

Table 2: Reason for Employer Testing

Reason for Test	Total
Pre-employment	145
Random	101
Reasonable Suspicion	66
None Specified	26
Post Accident	23
Other*	40
<b>Total Samples</b>	<b>401</b>

\*Other includes: Promotion, Probation, Pre-Access, Follow-Up, Baseline, Self, Insurance, Repeat, Scheduled Test, Annual, Personal, Court



## CONCLUSION

Many designer benzodiazepines demonstrate cross-reactivity with immunoassay screening reagents allowing detection, thus creating opportunity for identification and quantitation in confirmation. With the premise of targeting these drugs at concentrations exhibiting adequate cross-reactivity to generate an immunoassay response greater than the cutoff, the lower quantitative limits for confirmation were only explored to 20 ng/mL. Data from expanded benzodiazepine LC-MS/MS confirmation testing indicates that designer benzodiazepines are being used by individuals in the American workforce, and without an expanded method this use would go unreported. Based on these discoveries and the efficacy of immunoassay detection, it is suggested that routine benzodiazepine confirmatory analysis includes designer analogs.

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## DISCLOSURE

No relevant financial or nonfinancial relationships to disclose.

