A QuEChERS-Based Approach for Extracting Prescription and Designer Benzodiazepines from Blood and Urine

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UCT Featured Products

ECQUUS15CT
QuEChERS AOAC 15 mL Centrifuge Tube (400 mg $MgSO_4$ + 100 mg Sodium Acetate)

SCS27-DA1021
SelectraCore® DA 100 x 2.1 mm, 2.7 µm

SLGRDHLDR-HPOPT
Selectra® Direct Connect Guard Holder

SCS27-DAGDC21
SelectraCore® DA Guard Column 5 x 2.1 mm, 2.7 µm

Abstract:

This application note presents a fast and efficient method for extracting prescription and designer benzodiazepines from blood and urine samples. Utilizing UCT's AOAC QuEChERS salts for sample preparation and the SelectraCore® DA (biphenyl) HPLC column, the approach streamlines sample preparation by reducing solvent usage and extraction time while maintaining strong analytical performance. Recovery ranged from 69% to 103%, and matrix effect ranged from -19% to 26%, making this method well suited for forensic and clinical applications.

Introduction:

Benzodiazepines are psychoactive compounds characterized by a fused benzene and diazepine ring structure. They act by enhancing the effects of gamma-aminobutyric acid (GABA), producing calming effects on the central nervous system.[1] Based on their duration of action, benzodiazepines are classified as short-acting (e.g., alprazolam, lorazepam) or long acting (e.g., diazepam, clonazepam), each with varying clinical uses and abuse potential.[2]

Designer benzodiazepines (DBZDs) are unregulated analogs developed to mimic traditional benzodiazepines while avoiding legal control. Often sold illicitly, compounds such as etizolam and flubromazepam pose increased risks due to variable potency, impurities, and frequent use alongside other depressants. Their presence complicates drug detection, as many evade routine toxicological screening methods.[3, 4]

QuEChERS (quick, easy, cheap, effective, rugged and safe) is a multiresidue method developed by M. Anastassiades and S. Lehotay in 2002 for the analysis of pesticide residues in fruits and vegetables.[5] Today, QuEChERS is widely adopted across many fields, including forensic toxicology, because of its efficiency and versatility. The salts and sorbents used in this sample preparation technique assist with partitioning the aqueous and organic phases and removing matrix interferences. Most drugs are hydrophobic in nature and will partition into the organic phase, while the matrix will separate into the aqueous phase. This study presents a QuEChERS-based method for the simultaneous extraction and quantification of a panel of both prescription and designer benzodiazepines in blood and urine samples.

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Experimental:

a) Sample Preparation

Sample Pretreatment:

Add 0.5 mL of sample + ISTD + 2 mL of acetonitrile (ACN) to a 15 mL centrifuge tube containing QuEChERS salts (400 mg $MgSO_4$ and 100 mg sodium acetate). Cap and shake vigorously for 1 minute. Vortex and centrifuge samples for 10 min

Extraction:

Transfer 1 mL of the top (ACN) layer to a test tube. Evaporate at 45°C and reconstitute samples with 0.5 mL H_2O:MeOH (60:40 v/v).

b) Analytical Conditions

Table 1. LC-MS-MS parameters

LC-MS-MS Parameters	
LC-MS/MS	Shimadzu Nexera LC-30AD with MS-8050
UHPLC Column	SelectraCore® DA Column 100 x 2.1 mm, 2.7 µm
Guard Column	SelectraCore® DA Guard Column 5 x 2.1 mm, 2.7 µm
Column Temperature	40°C
Flow Rate	0.4 mL/min
Injection Volume	1 µL
Mobile Phase A	5 mM ammonium formate + 0.1% formic acid in water
Mobile Phase B	5 mM ammonium formate + 0.1% formic acid in methanol

Gradient Program		
Time (min)	**Mobile Phase A (%)**	**Mobile Phase B (%)**
0	60	40
9-12	0	100
12.1-15	60	40

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Table 2. MRM Transitions

Compound	Retention Time (min)	Precursor Ion	Product Ion	Collision Energy
7-aminoclonazepam	3.29	285.7	222.1 250.0	-25 -21
7-aminoflunitrazepam	4.17	283.6	135.0 227.0	-27 -26
Adinazolam	4.68	351.7	58.0	-23
Lorazepam	5.46	320.6	275.0 302.9	-23 -17
Oxazepam	5.58	286.6	241.1 269.0	-25 -13
Clonazepam	5.61	315.6	370.0 214.0	-27 -39
α-hydroxyalprazolam	5.97	324.6	297.0 216.0	-38 -39
Clonazolam	5.97	353.7	308.0 280.0	-28 -37
Flubromazepam	6.06	334.6	226.0 186.0	-30 -31
Nordiazepam	6.25	370.7	140.1 208.1	-28 -28
Flunitrazepam	6.41	313.7	268.0 239.0	-27 -36
Flualprazolam	6.49	326.6	299.0 223.0	-29 -43
Temazepam	6.59	300.6	255.0 283.0	-21 -15
Alprazolam	6.80	308.7	281.0 205.0	-27 -43
Bromazolam	7.03	354.8	326.9 338.3	-28 -11
Diazepam	7.26	284.7	193.1 154.0	-32 -28
Etizolam	7.40	342.5	314.0 259.0	-27 -34
Deschloroetizolam	7.58	308.5	280.0 255.1	-25 -26

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Chromatogram:

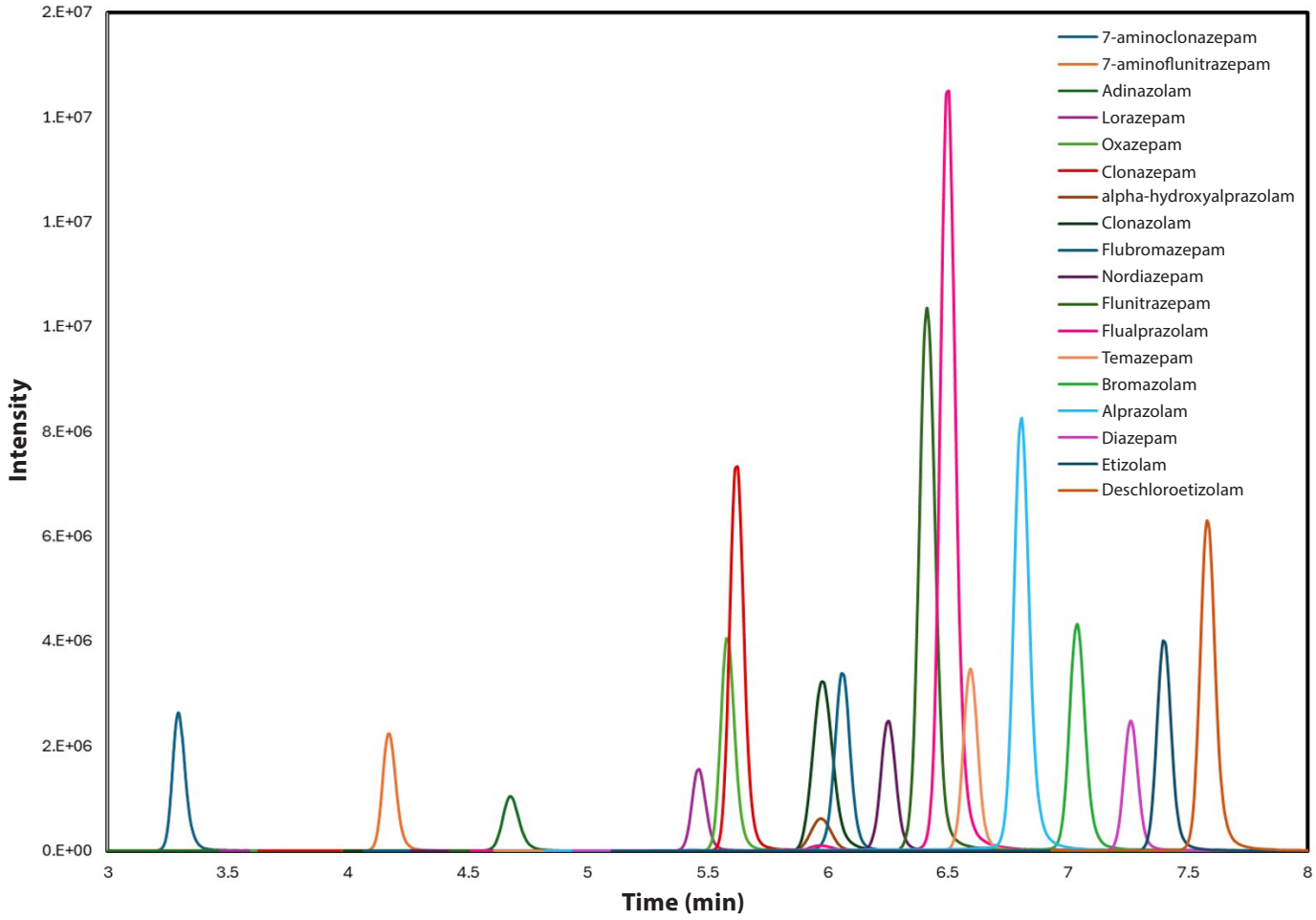

Figure 1. Chromatogram of 300 ng/mL extracted blood sample

Calibration Curves:

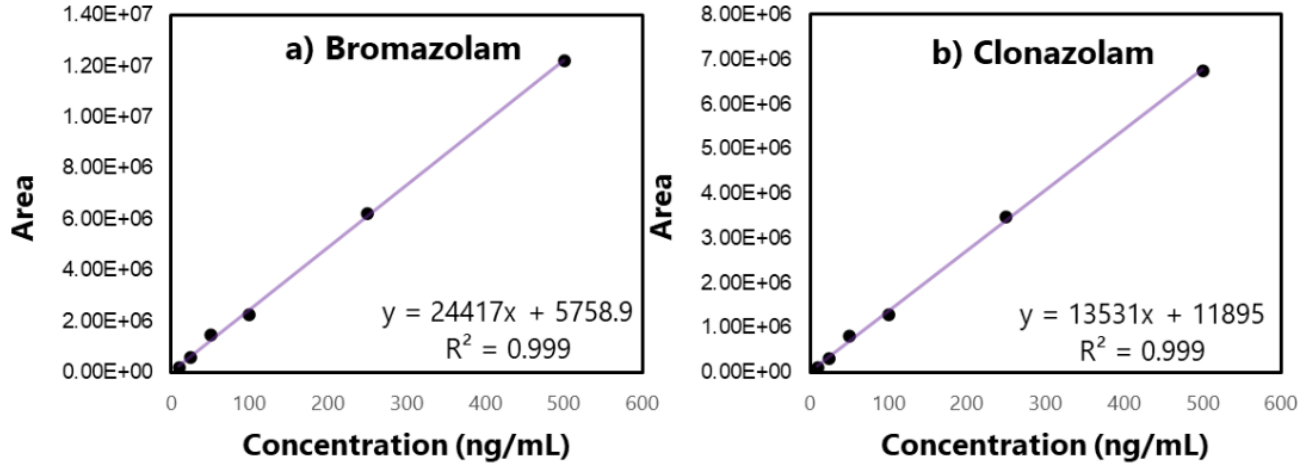

Figure 2. Extracted blood calibration curves for (a) Bromazolam and (b) Clonazolam. Linear range from 10 ng/mL to 500 ng/mL

Results & Discussion:

The method was evaluated by examining parameters such as recovery, matrix effect, and relative standard deviation (RSD) at both low and high analyte concentrations. Across both concentrations, recovery in blood samples (n=3) ranged from 69% to 92%, and matrix effects varied between -3% and 26%, with RSD≤13% (**Table 3**). In urine (n=3), recovery and matrix effect ranged from 92% to 130% and -19% to 28%, respectively, with RSD≤5% (**Table 4**).

When comparing the QuEChERS method to a traditional protein precipitation using 2 mL of acetonitrile as the crash solvent, both approaches yielded comparable recoveries, shown in **Figure 3**. However, the QuEChERS method consistently produced significantly lower matrix effects than protein precipitation, with the exception of 7-aminoflunitrazepam (**Figure 4**). Additionally, the acetonitrile layer from the QuEChERS method evaporated faster than that from the protein precipitation technique. Magnesium sulfate acts as a desiccant and may have removed additional water during the partitioning of the organic and aqueous layers that traditional protein precipitation does not.

Compared to solid phase extraction (SPE) methods, the QuEChERS approach described in this study uses less solvent volume and requires less extraction time. Shakers or mixers can be used to help process large sample batches by saving time and ensuring reproducibility. Therefore, this method can be utilized for screening and confirmation testing.

Table 3. Average recovery, matrix effect, and precision values obtained from spiked blood samples.

Blood						
Analytes	30 ng/mL (n=3)			300 ng/mL (n=3)		
	Recovery (%)	Matrix Effects (%)	RSD (%)	Recovery (%)	Matrix Effects (%)	RSD (%)
7-Aminoclonazepam	85	3	4	82	8	7
7-Aminoflunitrazepam	84	4	5	82	13	6
Adinazolam	91	6	1	87	7	6
Lorazepam	69	2	13	81	13	4
Oxazepam	89	5	2	85	14	6
Clonazepam	92	1	7	86	10	5
α-hydroxyalprazolam	91	1	4	85	12	8
Clonazolam	89	0	1	87	7	5
Flubromazepam	90	4	2	84	17	5
Nordiazepam	90	-3	5	88	6	6
Flunitrazepam	88	3	4	86	15	6
Flualprazolam	86	11	6	82	26	7
Temazepam	89	4	8	85	13	6
Alprazolam	88	5	5	85	19	6
Bromazolam	91	3	2	84	14	5
Diazepam	91	0	4	84	11	7
Etizolam	88	7	5	84	17	6
Deschloroetizolam	88	10	3	82	25	7

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Table 4. Average recovery, matrix effect, and precision values obtained from spiked urine samples.

Urine						
Analytes	30 ng/mL (n=3)			300 ng/mL (n=3)		
	Recovery (%)	Matrix Effects (%)	RSD (%)	Recovery (%)	Matrix Effects (%)	RSD (%)
7-Aminoclonazepam	130	14	4	106	-13	2
7-Aminoflunitrazepam	119	28	3	102	-9	2
Adinazolam	102	-13	3	95	7	3
Lorazepam	103	-19	0	97	3	3
Oxazepam	103	-15	5	96	5	2
Clonazepam	100	-14	1	96	6	2
α-hydroxyalprazolam	104	-18	2	94	10	2
Clonazolam	100	-14	5	96	12	2
Flubromazepam	104	-15	4	93	15	2
Nordiazepam	101	-17	3	93	11	2
Flunitrazepam	98	-14	2	96	9	1
Flualprazolam	100	-12	3	93	16	2
Temazepam	100	-17	2	94	8	1
Alprazolam	102	-14	4	94	9	2
Bromazolam	104	-14	2	95	9	2
Diazepam	105	-19	2	92	9	3
Etizolam	104	-13	5	95	11	3
Deschloroetizolam	102	-13	3	93	11	3

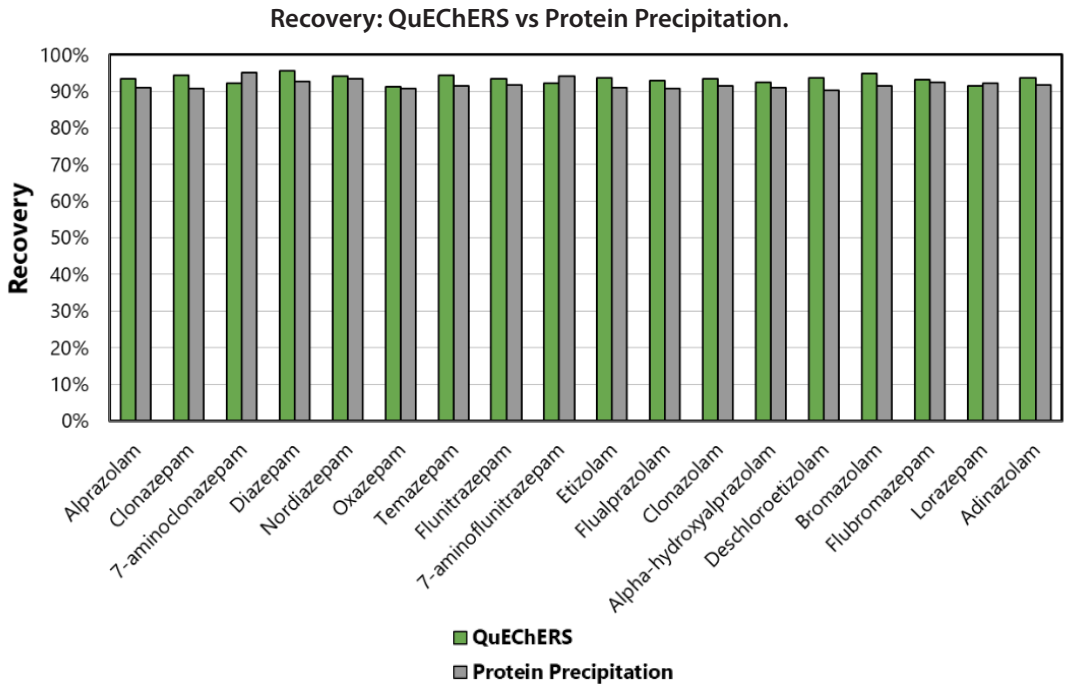

Figure 3. Recovery values from 100 ng/mL spiked blood samples (n=3) using QuEChERS compared to a traditional protein precipitation using 2 mL of acetonitrile

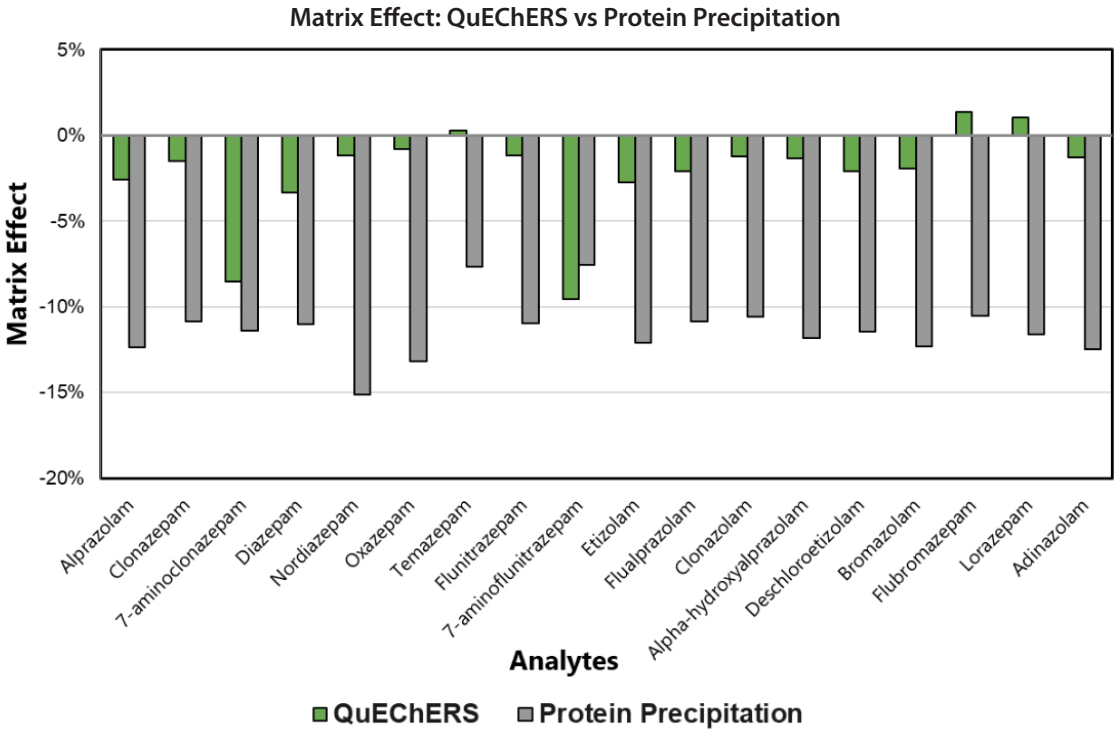

Figure 4. Matrix effect values from 100 ng/mL spiked blood samples (n=3) using QuEChERS versus a traditional protein precipitation using 2 mL of acetonitrile.

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Conclusion:

UCT's QuEChERS was used to successfully develop an alternative sample preparation method for extracting prescription and designer benzodiazepines from blood and urine. This approach can help increase laboratory productivity by reducing sample preparation time and minimizing solvent usage and waste.

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