

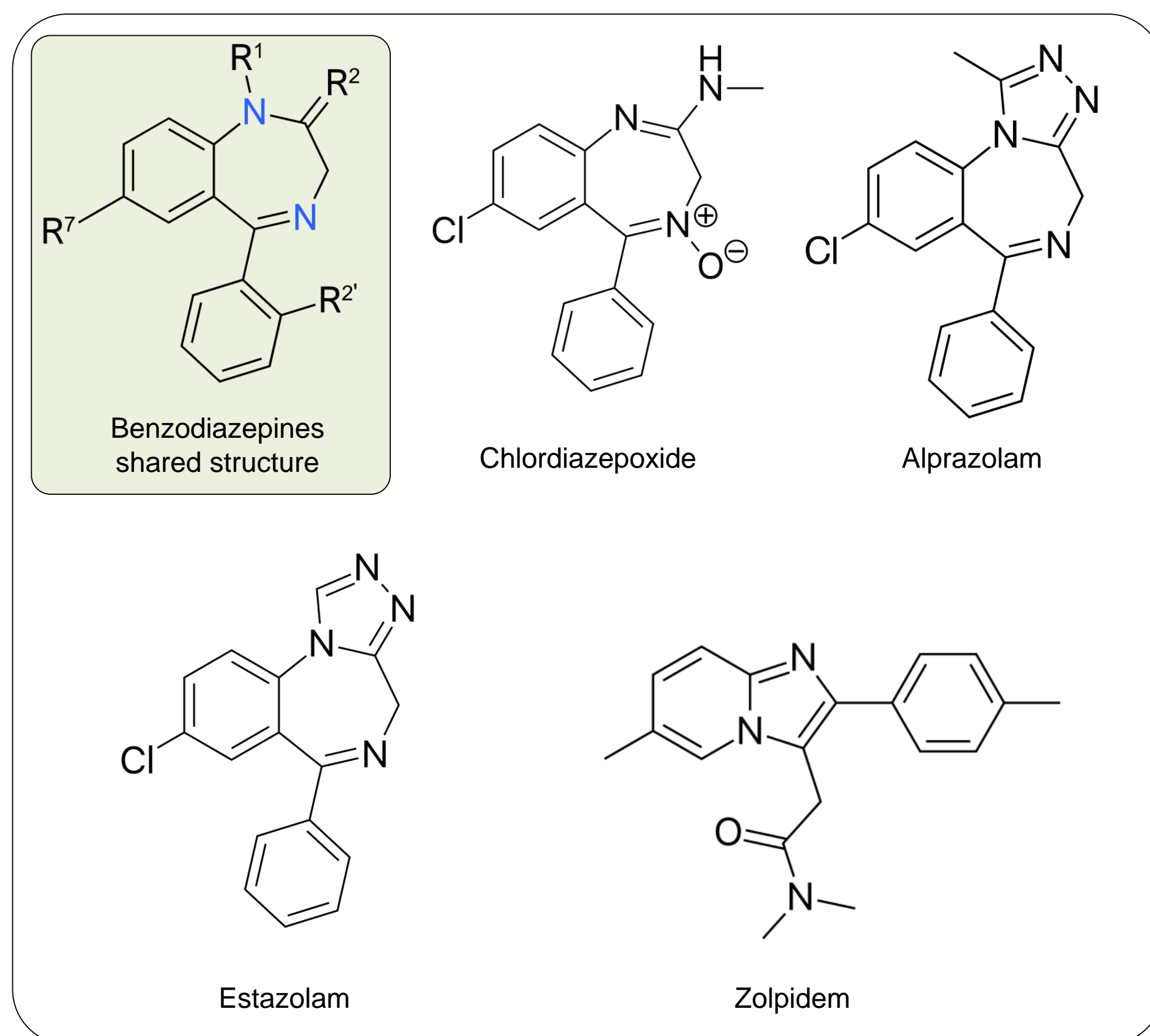
# Fully automated platform for determination of Benzodiazepines in serum

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## 1. Introduction

Benzodiazepines (Figure 1) are a group of psychoactive drugs with a broad range of therapeutic effects. They act as anxiolytics, sedatives and anticonvulsants and belong to the most frequently prescribed drugs all over the world. Most benzodiazepines share a 5-phenyl-1,3-dihydrobenzo[e][1,4]diazepine nucleus with different substituents on the 1, 2, 3, 7 2' positions. Despite this similarity in the chemical structure the drugs differ in pharmacokinetics and metabolic properties. Long-term administration of benzodiazepines require therapeutic drug monitoring (TDM). The quantification is primarily performed from serum in order to optimize the drug dosing, to verify consumption compliance and to identify changes in pharmacokinetics. The therapeutic drug monitoring of these molecules needs to be accomplished by extremely accurate techniques. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) shows high sensitivity and specificity. Nevertheless, LC-MS/MS approaches mostly lack standardization and the necessary throughput for the application in routine analysis. With the aim to reduce the operator involvement and to increase the throughput and data quality, we report a fully automated platform for LC-MS/MS quantifications of Benzodiazepines in serum by the use of a novel automatic preparation unit (CLAM-2000, Shimadzu) (the present work is intended for research use only).



**Figure 1:** Benzodiazepines chemical structure and Zolpidem as example of non-benzo hypnotic agent.

## 2. Methods and Materials

The analysis of benzodiazepines was performed using a fully automatic LCMS preparation Unit (CLAM-2000, Shimadzu, for research use only) online with HPLC-LCMS (NexeraX2-LCMS8060, Shimadzu) starting from serum samples using the "ClinMass® TDM Kit System" (Recipe, MS9500). Samples (Reference material: human serum samples), calibrators and Internal standard mix (Recipe, ClinMass® MS9512) were loaded onto the CLAM-2000 (refrigerated at 8° C). The treated samples were separated by the analytical column (Recipe, MS9030) at 40° C with a binary gradient system (Mobile phase A MS9007, mobile phase B MS9008) at a flow rate of 0.6 ml/min in 7.5 min (Table 1). Quantification was performed using optimized MRM transitions and Internal standard calibration method.



**Figure 2:** CLAM-2000 online with Nexera X2 system and LCMS-8060 triple quadrupole mass spectrometer.

### [LC] NexeraX2 System

Column Temp. : 40°C  
Time Program : gradient A-B 7.5 min  
Injection Volume : 0.5 µL

### [MS] LCMS-8060

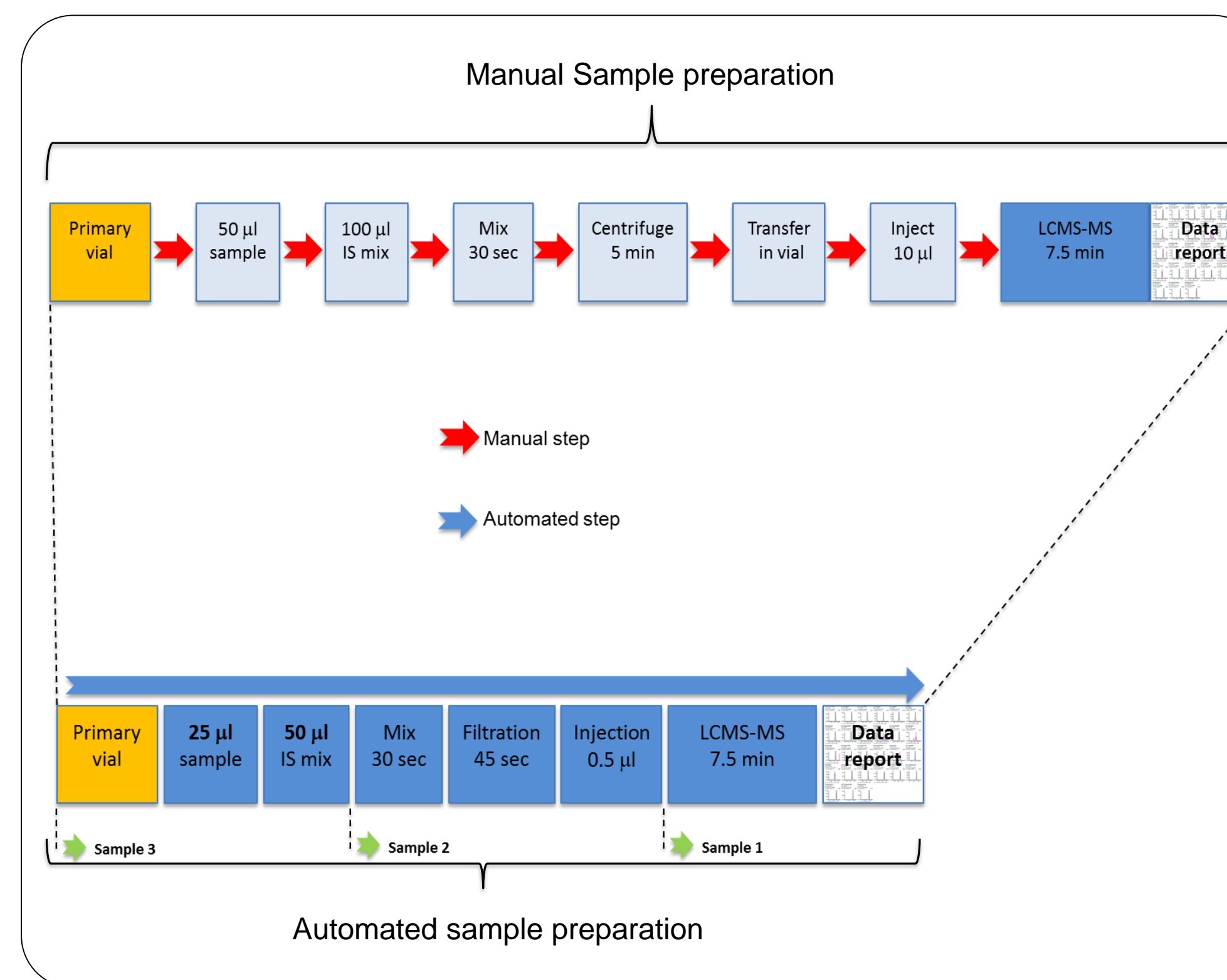
Ionization : ESI Positive  
Nebulizer Gas : 3 L/min  
Interface temperature : 300° C  
Desolvation Line : 250° C  
Heat Block temperature : 400° C  
Drying Gas : 10 L/min  
Scan Type : MRM

**Table 1:** Analytical conditions and source parameters.

## 3. Results

### 3.1 Fully Automated sample preparation

Prior to the LC-MS/MS analysis a sample preparation is carried out in order to remove the sample matrix and to spike the sample with an internal standard. This procedure is time consuming and could be affected by bias caused by the operator due to the liquid transfer steps that are required (Figure 3), moreover it is difficult maintain the traceability of each steps for all the processed samples. Using the CLAM-2000 it was possible to obtain a complete integration of sample preparation steps with the LC-MS/MS quantification. The samples were loaded onto the CLAM-2000 using disposable microvials or primary testing tubes. The fully automatic preparation/analysis procedure was performed as follows: I) 20 µl of methanol were dispensed in a filtration-collection vial; II) 25 µl of serum sample were added; III) 50 µl of IS mix were added (protein precipitation); IV) stirring and incubation (1 min); V) filtration for 0.45 min (deproteinization); the sample was finally transported to the LCMS system without human intervention (Figure 3) for the quantification and the results were directly visualized by CLAM-2000 software control.



**Figure 3:** CLAM2000 fully automated sample preparation and analysis. Due to the overlapped sample preparation the throughput of the instrument was 1 result each 8 minutes for quantification of all Benzodiazepines.

### 3.3 Linearity, Accuracy and Precision

The linearity and accuracy of the method was evaluated using 3 reference serum calibrators levels (Recipe MS6013). For all the analytes linearity and accuracy were within the analytical acceptable range (85%-115%). Furthermore in order to estimate the precision of the method, reference serum samples (Recipe MS6082) spanning from low concentration level to high concentration level were analyzed several times (6 replicates). For all analytes the CV% values were within acceptable analytical ranges. The same experiment was repeated for 4 non-consecutive days in order to estimate the inter-day precision.

Name	R <sup>2</sup>	ACCURACY		PRECISION (%CV)				BIAS %			
		min	max	INTRADAY*		INTERDAY**		INTRADAY*		INTERDAY**	
				Low	High	Low	High	Low	High	Low	High
3-OH-Bromazepam	0.998	98	100	4.7	3.7	4.4	3.5	0.4	1.2	6.7	4.9
7-Aminoclonazepam	0.999	99.2	100	3.5	1.5	4.6	1.9	1.7	3.7	4.7	8.9
7-Aminoflunitrazepam	0.999	99	100	2.8	2.8	2.6	2.6	1.9	0.3	6.3	7.3
7-Aminonitrazepam	0.999	99.5	100	3.3	2.1	3.5	2.0	4.7	2.2	6.5	4.3
alpha-OH-Alprazolam	0.999	100	101	9.7	8.5	9.1	8.4	0.1	5.4	4.6	5.6
alpha-OH-Midazolam	0.999	100	100	2.0	4.0	3.5	2.0	0.4	0.4	5.0	3.0
alpha-OH-Triazolam	0.97	86.2	115	7.1	12.7	11.1	11.0	1.8	10.1	8.5	9.9
Alprazolam	0.997	94.6	107	4.3	5.8	11.4	5.2	4.4	6.9	9.8	9.0
Bromazepam	0.999	96.9	104	4.1	3.8	3.8	2.8	6.8	5.2	7.0	8.3
Chlordiazepoxid	0.999	97.4	102	2.5	4.8	4.7	4.2	8.0	9.4	11.4	12.1
Clobazam	0.999	97.4	103	5.4	3.6	4.1	3.2	1.7	3.2	7.4	7.0
Clonazepam	0.999	96.1	103	3.7	5.5	5.9	5.0	7.8	4.9	6.7	6.0
Demoxepam	0.999	98.1	103	3.3	3.1	2.0	2.2	1.0	0.5	3.9	4.1
Desalkylflurazepam	0.999	96.7	105	6.6	3.2	6.1	2.6	5.8	0.5	6.6	5.8
Desmethylflunitrazepam	0.999	97.4	104	8.2	5.6	8.4	3.4	4.4	5.0	5.2	9.6
Diazepam	0.999	96.7	105	7.8	5.6	6.2	4.2	9.1	6.4	11.6	12.8
Estazolam	0.999	99.9	100	2.0	1.8	2.6	3.2	1.2	2.0	6.2	9.0
Flunitrazepam	0.999	94.6	102	5.8	7.7	6.9	4.9	3.9	3.4	8.1	5.5
Flurazepam	0.999	97.4	102	5.5	8.6	5.3	4.9	0.3	13.3	9.9	15.0
Lorazepam	0.999	99.8	100	4.5	7.9	2.4	4.1	11.8	3.4	10.0	9.2
Lormetazepam	0.996	91	106	6.3	3.9	7.0	2.5	9.8	14.3	9.1	14.1
Midazolam	0.999	96.5	102	4.4	5.2	5.1	6.3	9.8	15.0	9.7	13.6
Nitrazepam	0.998	95.1	107	7.3	5.0	3.4	2.3	1.0	0.6	4.6	6.9
Norclonazepam	0.998	95.6	106	3.7	2.5	2.4	2.0	9.0	12.0	9.2	12.7
Nordiazepam	0.999	98.3	102	1.9	1.5	2.0	2.1	0.8	3.1	5.3	6.5
Oxazepam	0.999	99	101	1.9	1.5	3.2	1.5	3.5	4.8	6.2	8.2
Prazepam	0.999	96.5	102	7.6	1.9	6.9	3.4	1.8	12.7	2.3	10.6
Temazepam	0.999	99	101	6.2	4.1	4.1	3.4	9.8	2.6	10.9	6.2
Tetraazepam	0.999	96.7	105	12.6	11.3	14.4	6.3	6.9	15.0	13.7	14.1
Trazodon	0.997	94.7	107	8.5	4.1	8.7	4.8	4.9	1.9	7.3	9.2
Triazolam	0.998	95.5	106	7.2	2.3	8.8	4.0	0.4	1.7	6.8	4.3
Zaleplon	0.996	93.2	109	7.6	2.5	5.7	4.7	6.3	12.1	5.7	7.3
Zolpidem	0.999	96.2	103	3.0	3.1	3.4	3.2	9.0	5.1	3.7	5.3

**Table 2:** Linearity, Accuracy, and precision evaluated using ClinChek® MS6082 reference serum controls. \*n=6 replicates; \*\*n=4 non-consecutive days.

## 4. Conclusions

- Fully Automated sample preparation procedure resulted suitable for the quantitation of Benzodiazepines by elimination of all manual preparation steps.
- The automation of the method increases the analytical performance, reduces the risk for human operators and due to the reduced reagent consumption, reduces also the cost of the analysis.