

## Overview

Using protein precipitation procedure, a sensitive and specific liquid chromatographic-tandem mass spectrometric (LC/MS/MS) method capable of quantifying a modified insulin in rat plasma is described.

In this method, the drug was extracted from a 0.05 mL of rat plasma using simple extraction method. Separation was performed on a reverse phase C18 column. Detection was achieved using a SCIEX 6500 Triple Quad system in the positive ion mode along with multiple reaction monitoring (MRM). The lower limit of quantitation was 2 ng/mL.

This method has been successfully applied to preclinical pharmacokinetic studies.

## Introduction

Development of LC-MS/MS assay for intact large biomolecules present many advantages compared to traditional ELISA assay. In recent years, some analytical methods have been developed for determination of intact insulin using LC-MS/MS methods. However, there has been no report on quantitation of any modified insulin that has more than twice of the molecular weight as insulin. In this study, we have developed a rugged, fast method for quantitation of a modified insulin (MW >12,000 to, for example, 18,000 Da) in rat plasma using Sciex Triple Quad 6500 mass spectrometer in high-mass positive ESI mode. The lower limit of quantitation is 2 ng/mL (~0.17 pmol/mL).

## Methods

### Sample Preparation:

Plasma samples were extracted by using the 50- $\mu$ L aliquot of rat plasma. After extraction, the extracts must be centrifuged, and the supernatant were dried and reconstituted. The extract was then transferred to LC vials for LC-MS/MS or stay in the 96-well plate for the analysis.

### Liquid Chromatography:

Pump: Shimadzu UFLC LC-30AD  
Autosampler: Shimadzu UFLC SIL-30AC  
System Controller: Shimadzu CBM-20A

Analytical Column:  
C18 column, 50 x 2.1 mm, 1.9  $\mu$ m

### Gradient:

The analyte was eluted using a gradient of mobile phase A (0.1% acetic acid in water) and mobile phase B (0.1% acetic acid in acetonitrile) from 20% to 60% mobile phase B in 3.0 min.

Injection Volume: 5-10  $\mu$ L

### Mass Spectrometry:

MS System: Sciex 6500 Triple-Quad  
Condition: LC/(+ESI-MS/MS, (High Mass MRM)

### MRM transitions:

Modified Insulin: 1208.4 $\rightarrow$ 1375.3  
Bovine Insulin (IS): 956.7 $\rightarrow$ 315.2

## Results and Discussion

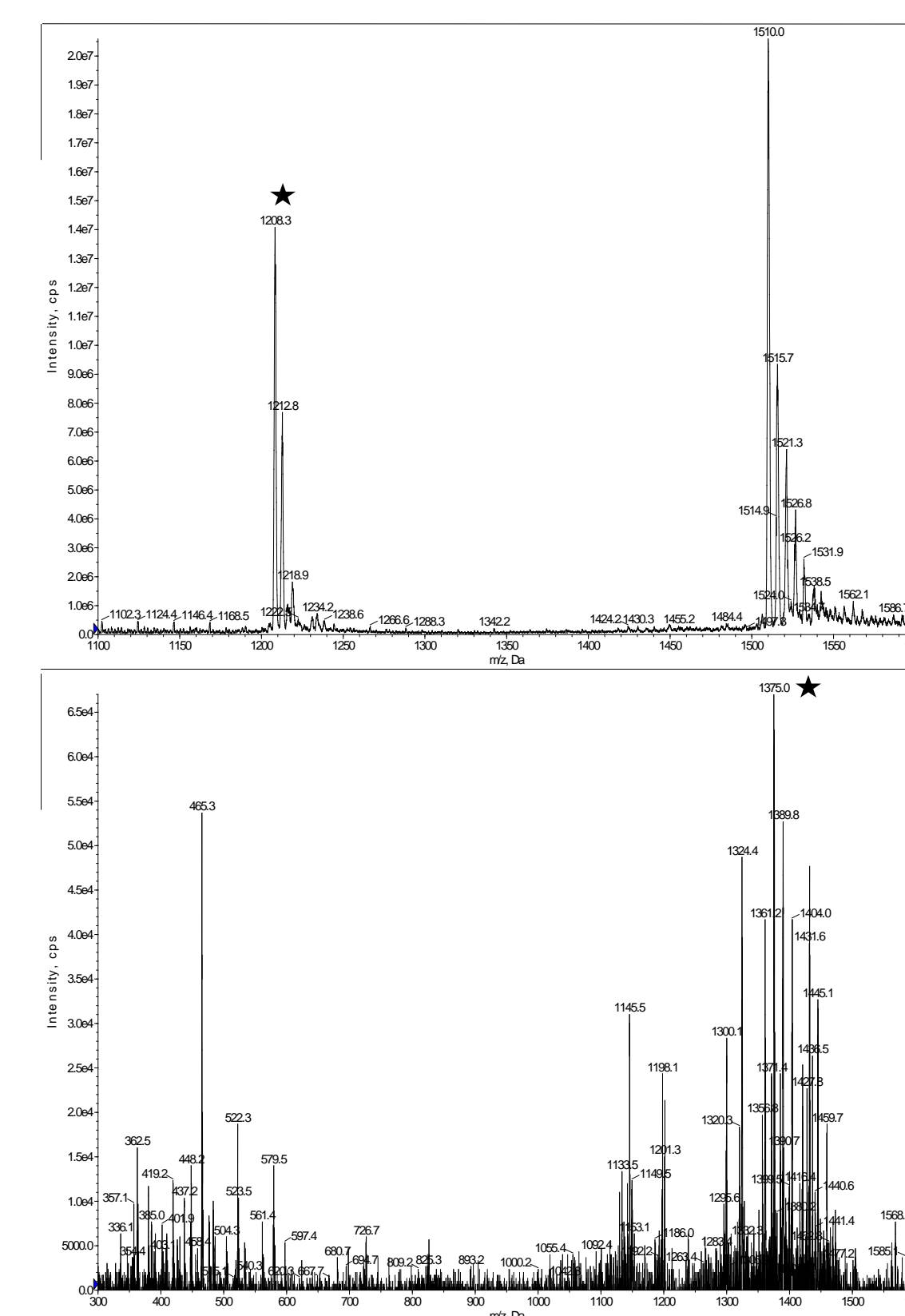


Figure 1. Intact Modified Insulin: Q1scan (Top), Daughter ion scan for m/z: 1208.3 (Bottom).

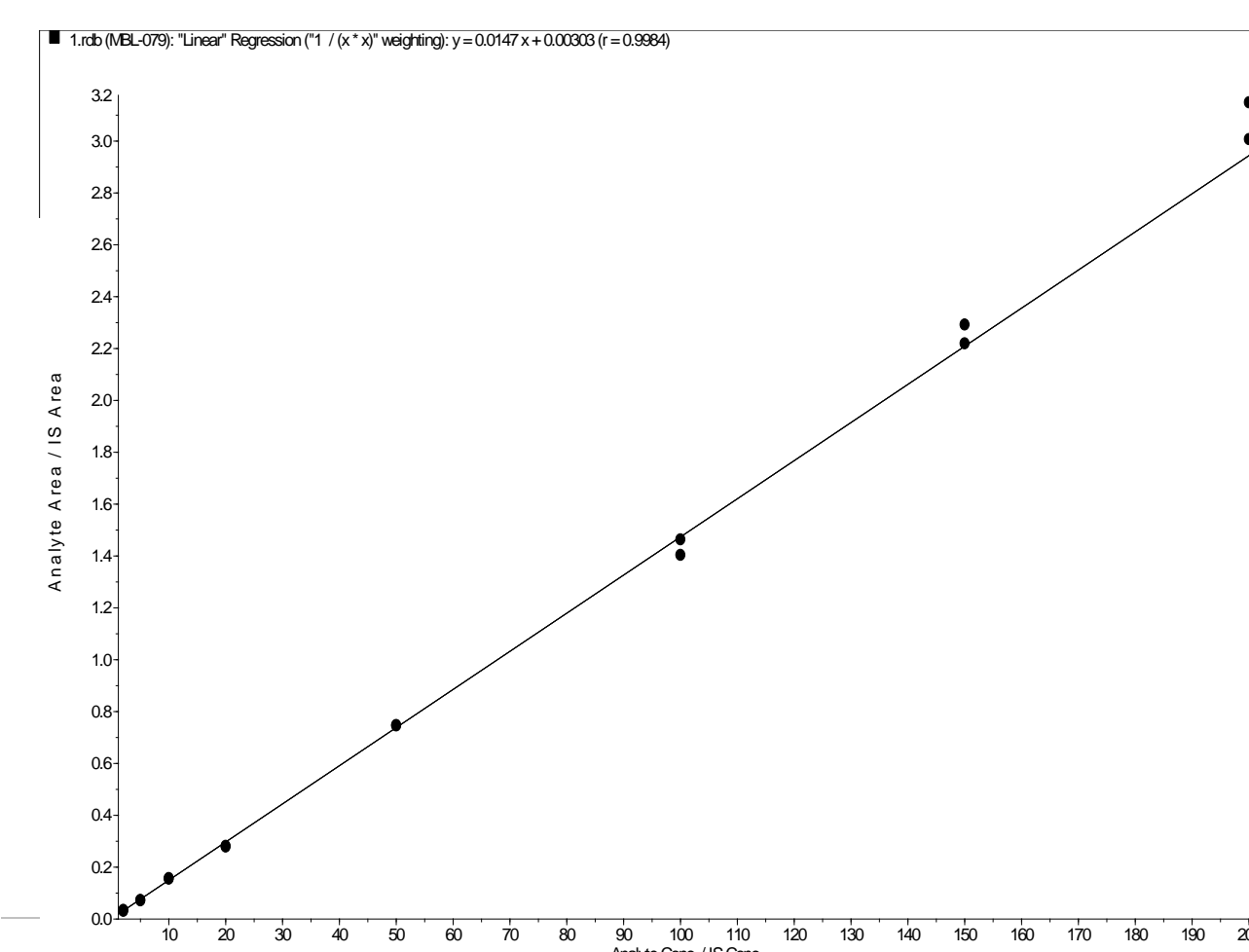


Figure 2. Representative calibration curve of modified insulin from 2 ng/mL to 200 ng/mL.

Table I. Validation Data Summary

Calibration Range		2 to 200 ng/mL		
Correlation coefficient( $r^2$ , mean)		0.9941		
Accuracy & Precision		Accuracy		Precision
	QC	Conc. (ng/mL)	RE%	CV%
Inter-Batch (n=18)	LLOQ	2	2.5	13.6
	Low	6	-0.2	8.9
	Medium	30	3.7	6.2
	High	160	0.6	4.3
Compared with Nominal Value (%)				
Method Recovery		>45		
		Condition		Accuracy
				RE%
Freeze/Thaw	3 Cycles, <-70 °C		<14.7	
Bench-Top	2 hrs, in ice-water bath		<5.0	
Autosampler Extract Stability	25 hrs, 4°C		<2.5	
Long-Term Storage Stability	42 Days, <-70 °C		<12.3	

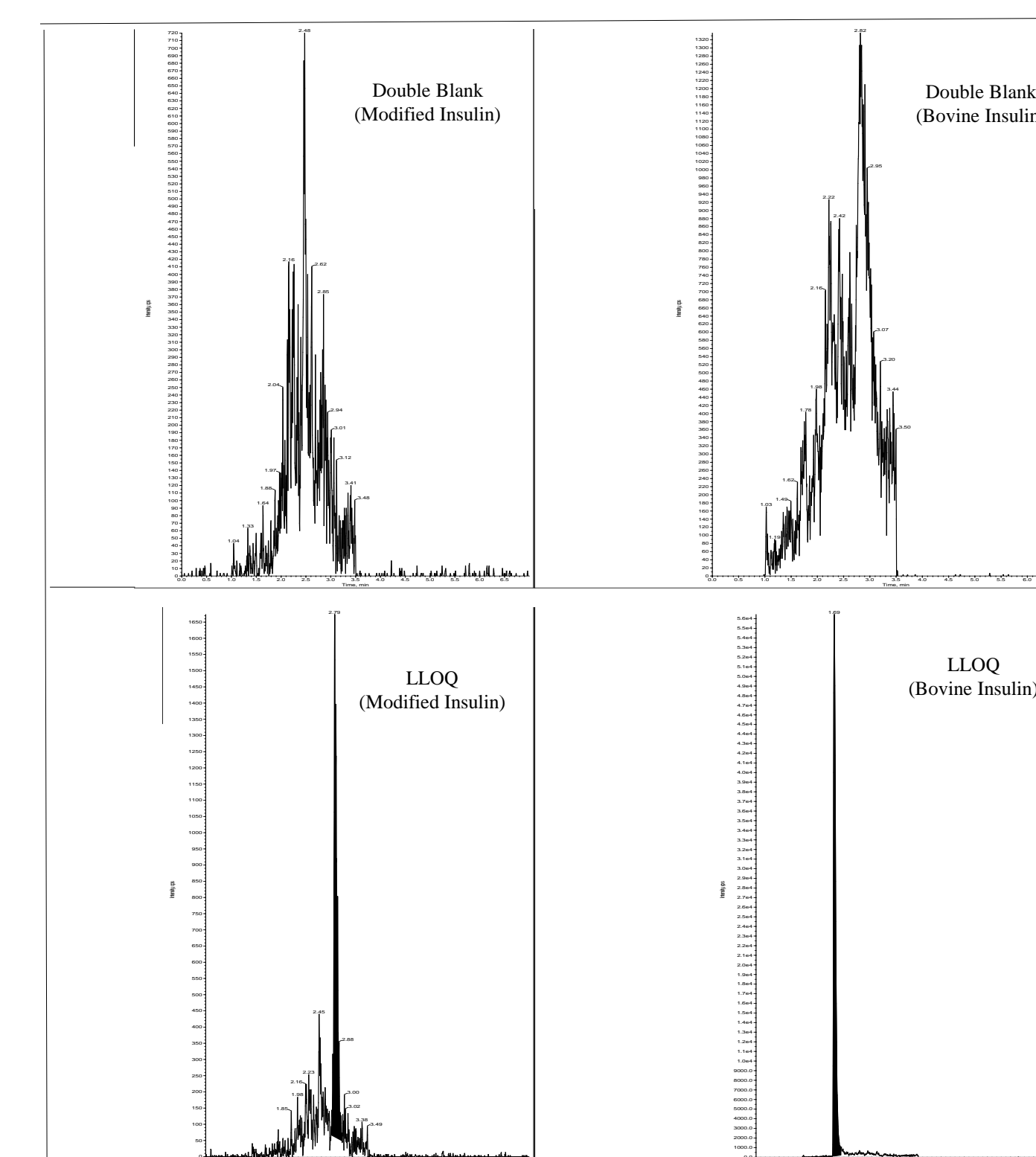
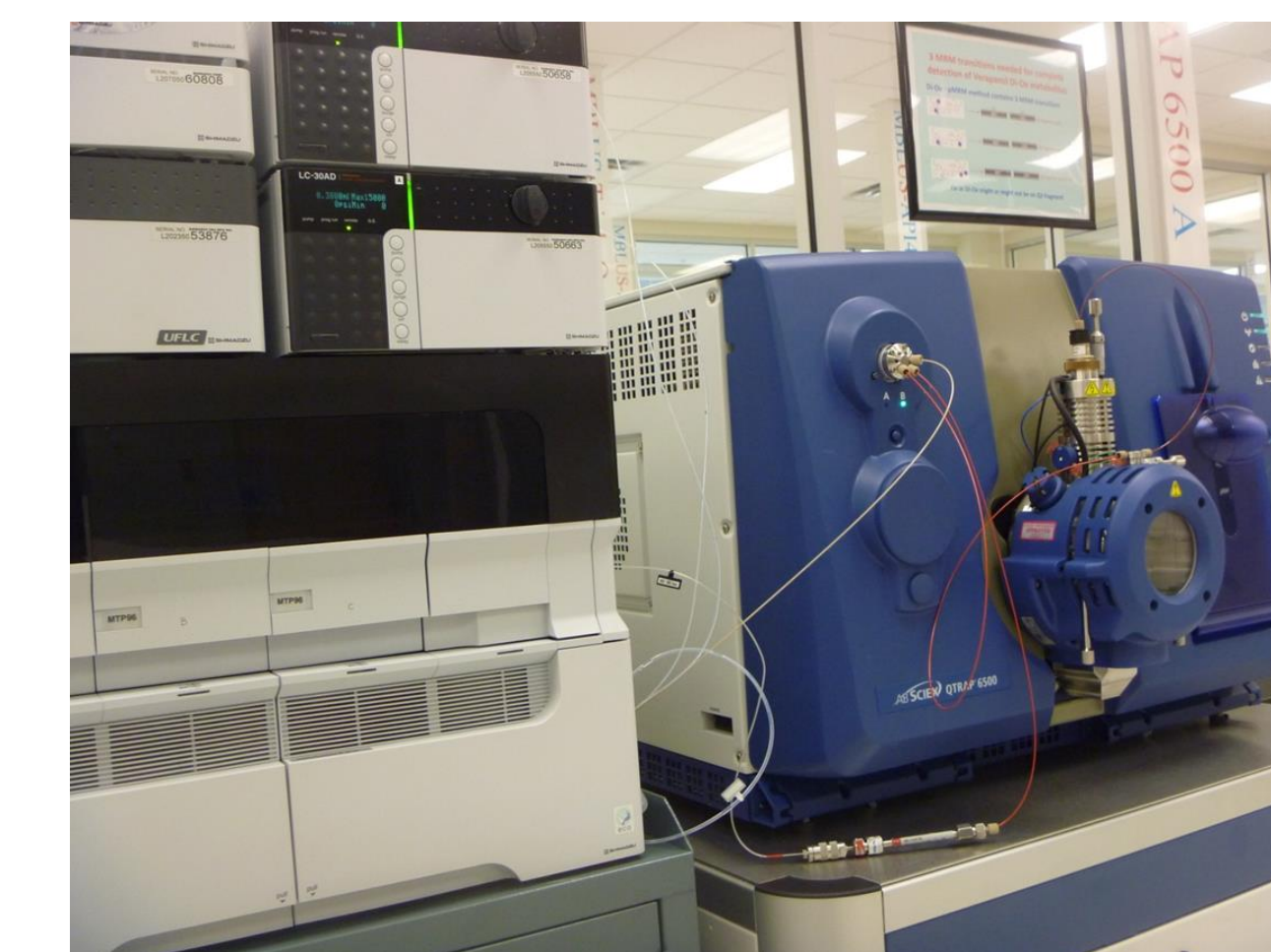


Figure 3. Representative chromatograms for double blank (Top) and LLOQ (Bottom) (Note: modified insulin (Left) and bovine insulin (Right)).

- Distinct charge states (Figure 1) and selective MRM transitions for the intact modified insulin (Figure 3) facilitates development of a simple, selective and sensitive quantitation method for the modified insulin.
- Excellent linearity was obtained with a correlation coefficient  $\geq 0.9941$  for the modified insulin (Figure 2).
- Reproducibility: including LLOQ, the inter-day CV ranged from 4.3% to 13.6% and the biases of the means ranged from -0.2% to 3.7%. (Table I).



## Conclusions

A rapid, simple and specific LC/MS/MS method has been developed and validated for quantifying a modified insulin with a lower limit of quantitation of 2 ng/mL from a 0.05 mL plasma sample. For large intact biomolecule quantitation, high sensitivity and high mass MRM with fast scan rate on 6500 Triple Quad will significantly reduce the time to struggle such bioanalytical methods with high quality data.