Technical note | 001736

Clinical research

Comparison of ionization types and precursor ions for quantitative analysis of 25-hydroxyvitamin D on a TSQ Altis MD mass spectrometer

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Keywords

Vitamin D, calciferol, TSQ Altis MD mass spectrometer, Vanquish MD UHPLC system, Hypersil GOLD UHPLC column, TraceFinder software, triple quadrupole, LC-MS/MS, quantitation, clinical research

Application benefits

- Sensitive analysis
- Multiple options depending on user needs
- Registered medical device for compliance

Goal

- Comparison of analytical methods for quantification of 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 on a Thermo Scientific™ TSQ Altis™ MD mass spectrometer
- Evaluate the analytical method performance based on both atmospheric pressure chemical ionization (APCI) and heated electrospray ionization (HESI) sources
- Evaluate the analytical method performance based on different precursor ions

Introduction

Vitamin D (calciferol) is a group of secosteroids essential for several biological functions including the intestinal absorption of calcium, magnesium, and phosphate, promoting healthy growth of bones, as well as other biological effects. Deficiency in vitamin D disrupts proper bone mineralization and can lead to weak or malformed bones, most often in children and the elderly. In children, deficiency usually manifests as rickets with soft and deformed long bones and reduced growth. In adults, deficiency can cause osteomalacia with soft, bending bones and muscle weakness. Vitamin D deficiency can also contribute to osteoporosis characterized by reduced bone mineral density, increased fragility, and increased risk of fractures.

High levels of vitamin D cause hypercalcemia. Symptoms of hypercalcemia include anorexia, nausea, vomiting and increased thirst. The excess calcium is deposited in tissues and organs, especially the kidneys, liver, and heart, leading to organ failure.

The vitamers of interest in humans are vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol). Vitamin D_3 is generated endogenously in human (and most other vertebrates') skin by a photochemical reaction. A similar reaction generates vitamin D_2 in fungi. If supplementation is needed, either vitamer may be taken orally. Vitamin D_3 and D_2 themselves are biologically inactive and are metabolized primarily in the liver to the 25-hydroxyvitamin D forms (calcidiol). Those are then further metabolized by the kidneys to the active 1,25-dihydroxyvitamin D (calcitriol). However, since the biological half-life of calcitriol is on the order of hours while the half-life of calcidiol is weeks, it is this diol form that is most often measured in plasma or serum to determine a person's vitamin D status. While there are many ranges listed in various publications, 20–50 ng/mL of 25-hydroxyvitamin D in serum or plasma is considered optimal. Lower concentrations can lead to deficiency diseases, and higher concentrations can yield toxic effects.

Plasma or serum levels of 25-hydroxyvitamin D can be measured by immunoassay. However, that method does not distinguish between the D_2 versus D_3 forms or several other vitamin D metabolites. Another major challenge is that vitamin D is typically not found free in plasma/serum samples. Liquid chromatography coupled to mass spectrometry gives increased sensitivity and specificity to vitamin D analysis and has become the preferred choice for identification and quantitation of vitamin D in biological matrices.

In this technical note, variations of an analytical method for detection and quantitation of 25-hydroxyvitamin D_2 and D_3 on a TSQ Altis MD mass spectrometer (Figure 1) are compared. Options such as ionization mechanism and precursor ion selection were studied.

Experimental

Sample preparation

Stock solutions of 25-hydroxyvitamin D_2 (25OHD2), 25-hydroxyvitamin D_3 (25OHD3), and d6-25-hydroxyvitamin D_3 (d6-25OHD3), manufactured by Cerilliant, were obtained from Millipore Sigma (Merck KGaA, Darmstadt, Germany). Stock solutions of 25OHD2 and 25OHD3 were combined, diluted in methanol, and spiked into 0.1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) to give a calibration curve in the range of 0.5 to 200 ng/mL. The BSA in PBS was used as a surrogate matrix since it is difficult to obtain vitamin D-free human plasma.

Internal standard was added to a 200 µL aliquot of calibrator at an equivalent concentration of 50 ng/mL. Samples were processed by protein precipitation using 3x volume of acetonitrile. After vortex mixing and centrifugation, the supernatant was transferred to a clean vial and placed on the autosampler, which injected 20 µL of sample on the HPLC column.

Liquid chromatography

Gradient elution was performed using a Thermo Scientific™ Vanquish™ MD ultra-high performance liquid chromatography (UHPLC) system for the separation of analytes. Mobile phases consisted of 0.1% formic acid in water for mobile phase A and 0.1% formic acid in methanol for mobile phase B. The column used for separation was a Thermo Scientific™ Hypersil GOLD™ (1.9 µm, 100 x 2.1 mm, P/N 25002-102130). The gradient ran from 80% to 100% B at a flow rate of 0.5 mL/min, and the total run time including re-equilibration was 3.5 minutes (Figure 2).

Mass spectrometry

Detection of analytes was performed on a Thermo Scientific™ TSQ Altis™ MD mass spectrometer equipped with an Thermo Scientific™ OptaMax™ ion source using either an atmospheric pressure chemical ionization (APCI) or heated electrospray ionization (HESI-II) sprayer. Additionally, both the intact molecular ion as well as a water-loss ion were evaluated as the precursor ion.

Figure 1. Vanquish MD UHPLC system and TSQ Altis MD mass spectrometer

No	Time	Flow [ml/min]	%B	Curve
1	0.000	Run		
2	0.000	0.500	80.0	5
3	0.250	0.500	80.0	5
4	1.000	0.500	100.0	5
5	2.000	0.500	100.0	5
6	2.010	0.500	80.0	5
7	3.500	0.500	80.0	5
8	*New Row*			
9	3.500	Stop Run		

Figure 2. Chromatographic gradient used for separation of 25OHVitD2 and 25OHVitD3. Mobile phases A and B consisted of 0.1% formic acid in water, and methanol, respectively.

Two SRM transitions for each analyte under each condition were included in the acquisition method for quantitation and confirmation. A summary of MS source conditions and SRM properties is presented in Tables 1 and 2.

Software

Data, acquisition, and processing were all completed under one software platform, Thermo Scientific™ TraceFinder™ LDT software, version 1.

Instrument performance evaluation

The instrument performance under each condition was evaluated for limit of quantitation (LOQ), defined as the lowest concentration that showed 1) a linear calibration curve with calibrators back-calculating within 20% of nominal, 2) an $R^2 > 0.99$, and 3) passing ion ratio confirmation within ±20%, relative.

Results and discussion

Using the intact molecule as the precursor, the LOQ for 25OHD2 and D3 were both 5 ng/mL under APCI ionization conditions compared to 1 ng/mL with HESI ionization. If the adduct-loss precursor is used, the LOQs are better, showing 0.5 ng/mL for both compounds using APCI ionization and 0.5 and 1 ng/mL for 25OHD2 and 25OHD3 for HESI ionization, respectively (Table 3). Representative chromatograms at the LOQs and calibration curves are presented in Figure 3.

Table 1. Summary of MS source conditions

Property	TSQ Altis MD	
Ionization	APCI	HESI
Discharge current (µA)	3.5	-
Spray voltage (V)	-	3,500
Sheath gas (Arb)	45	60
Aux gas (Arb)	2	20
Sweep gas (Arb)	0	1
Ion transfer tube temp (°C)	300	325
Vaporizer temp (°C)	400	350

Table 2. Summary of SRM properties

	TSQ Altis MD
Polarity	Positive
Cycle time (s)	0.3
Q1 Resolution (FWHM)	0.7
Q3 Resolution (FWHM)	0.7
CID gas (mTorr)	2
Source fragmentation	0
Chromatographic peak width (s)	3
Use chromatographic filter	Yes

Table 3. Limits of quantitation for 25-hydroxyvitamin D_2 and D_3

	APCI (intact) ng/mL	APCI (adduct-loss) ng/mL	HESI (intact) ng/mL	HESI (adduct-loss) ng/mL
25-hydroxyvitamin D_2	5	0.5	1	0.5
25-hydroxyvitamin D_3	5	0.5	1	1

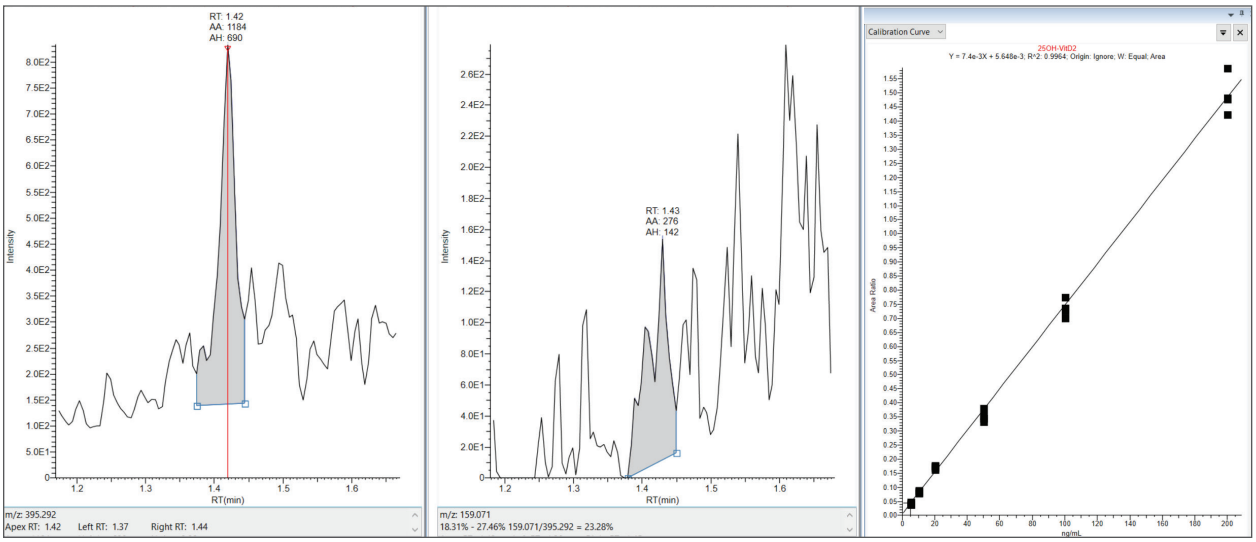

Figure 3a. Calibration curves and chromatograms at LOQ for 25OHD2, APCI, intact

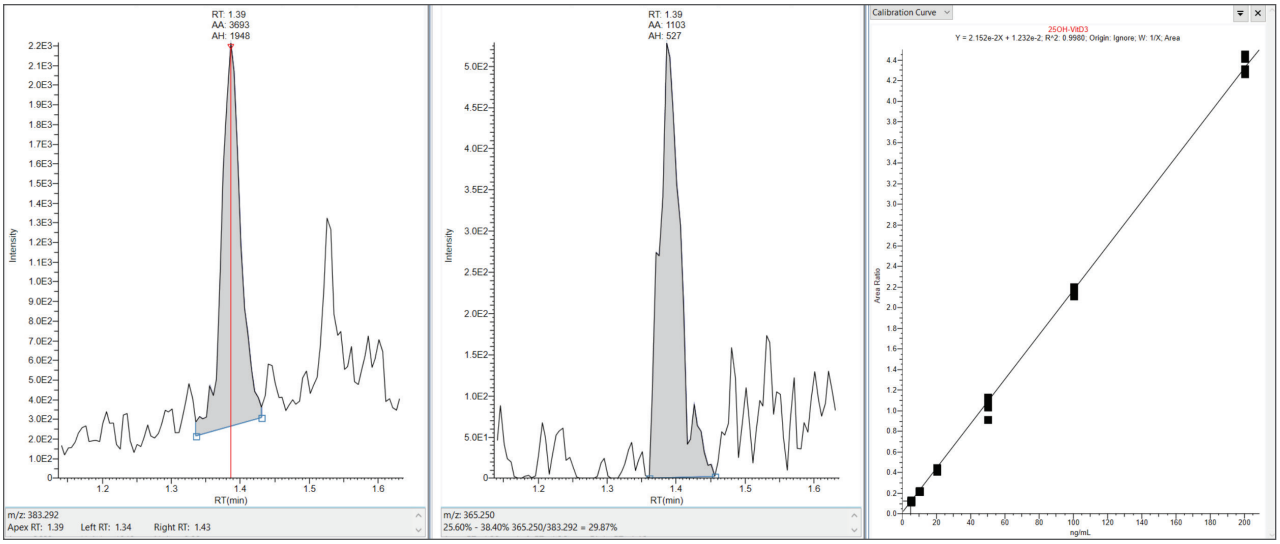

Figure 3b. Calibration curves and chromatograms at LOQ for 25OHD3, APCI, intact

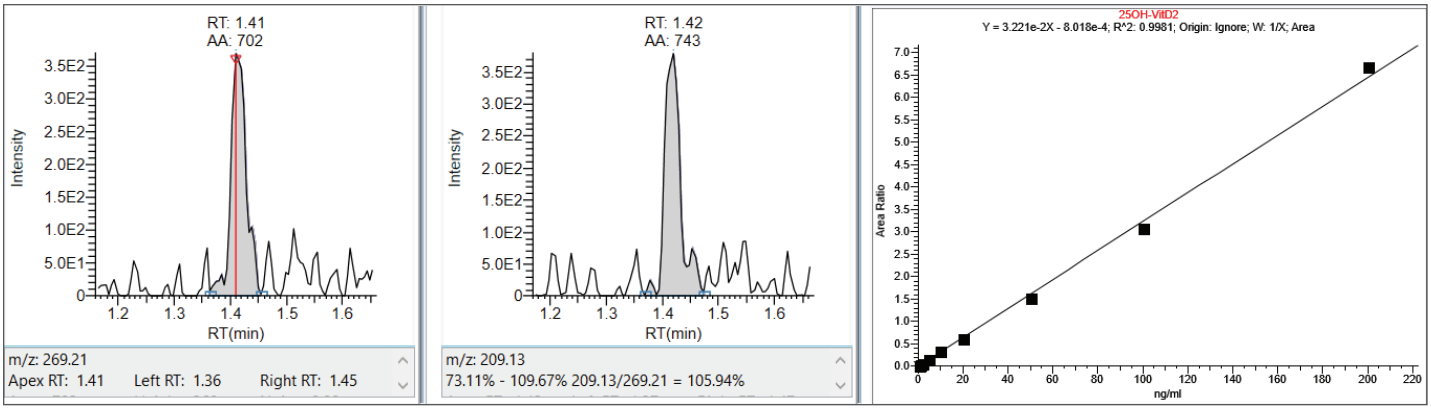

Figure 3c. Calibration curves and chromatograms at LOQ for 25OHD2, APCI, adduct-loss

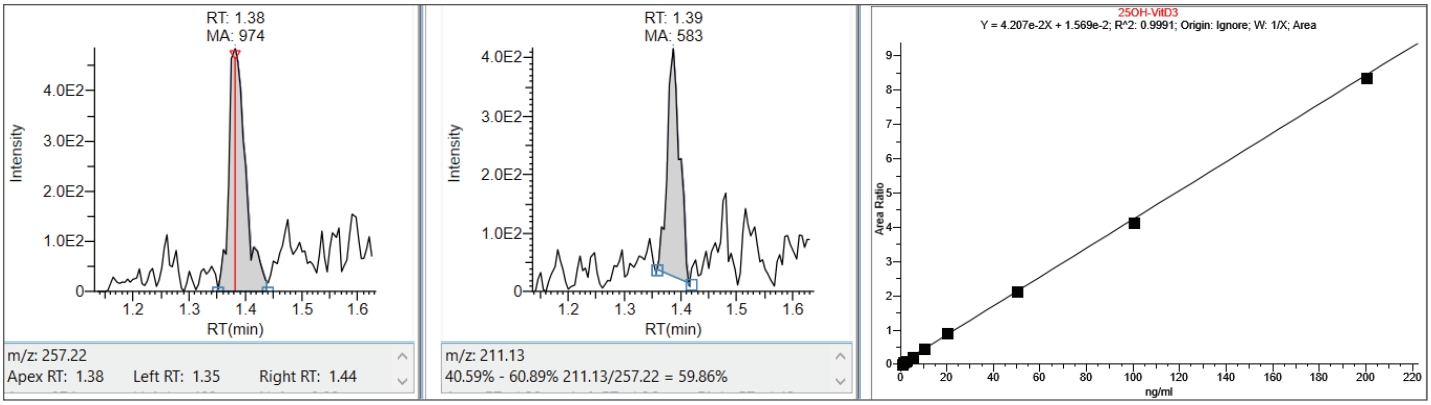

Figure 3d. Calibration curves and chromatograms at LOQ for 25OHD3, APCI, adduct-loss

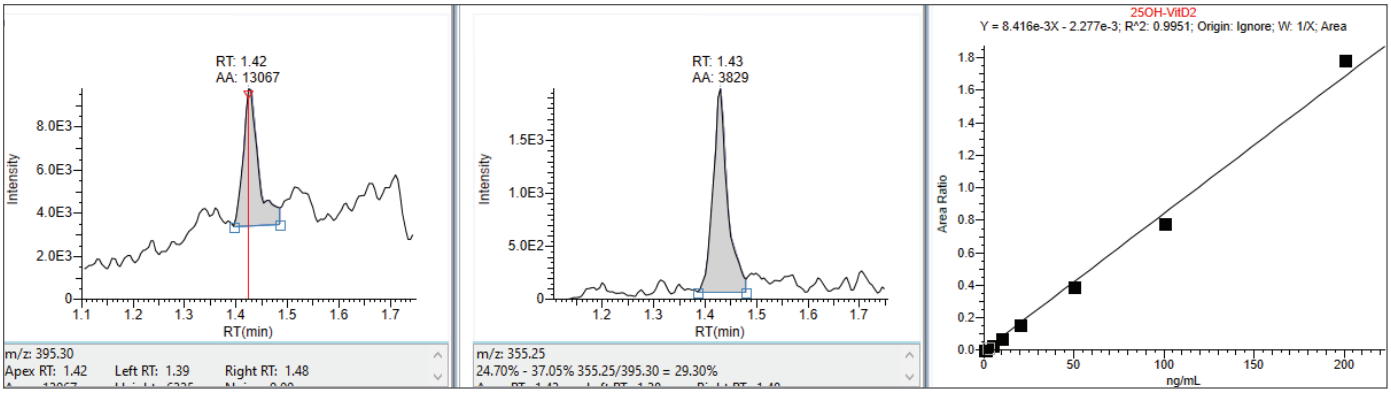

Figure 3e. Calibration curves and chromatograms at LOQ for 25OHD2, HESI, intact

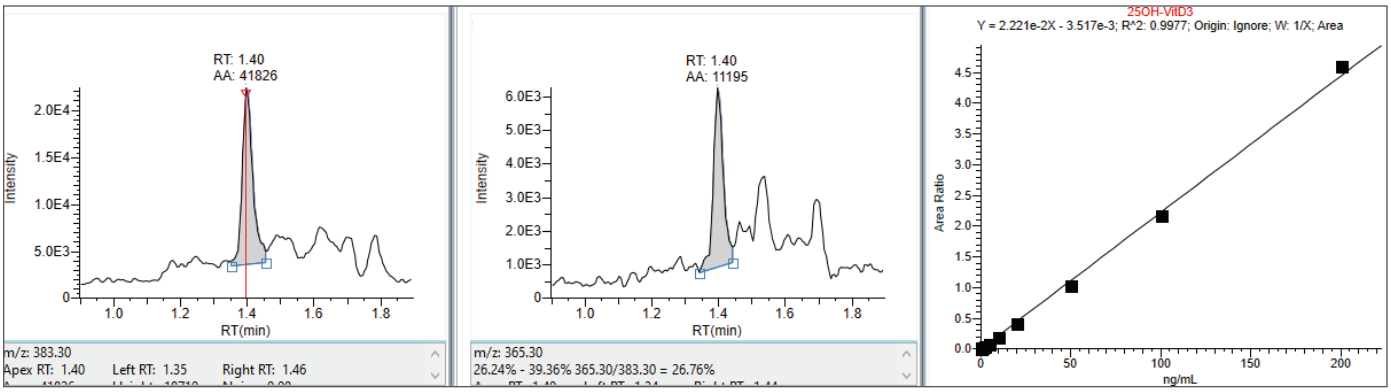

Figure 3f. Calibration curves and chromatograms at LOQ for 25OHD3, HESI, intact

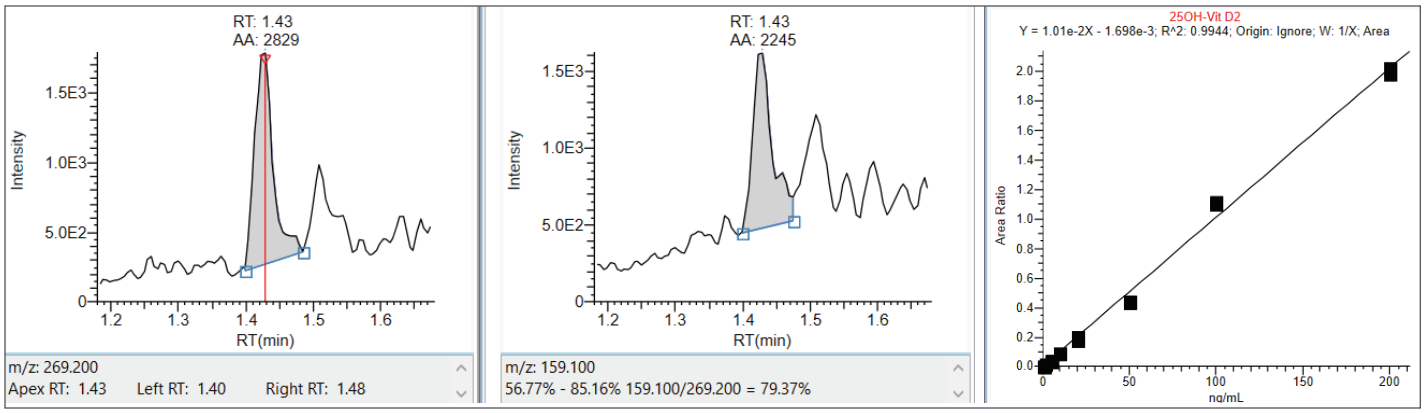

Figure 3g. Calibration curves and chromatograms at LOQ for 25OHD2, HESI, adduct-loss

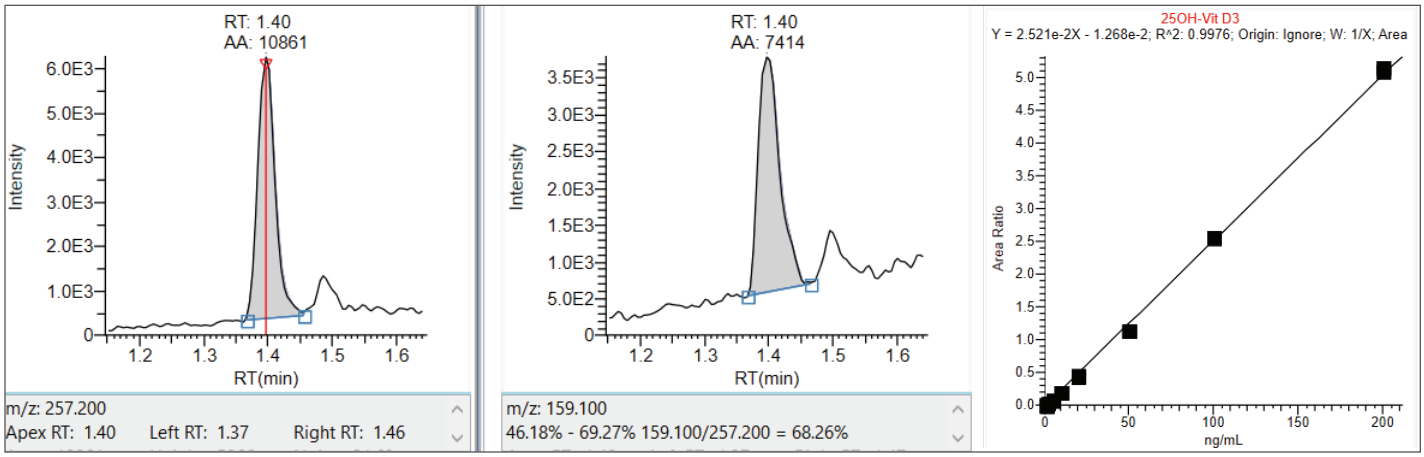

Figure 3h. Calibration curves and chromatograms at LOQ for 25OHD3, HESI, adduct-loss

Conclusion

Multiple analytical methods for the HPLC-MS/MS analysis of 25-hydroxyvitmain D_2 and 25-hydroxyvitamin D_3 were developed on a TSQ Altis MD triple quadrupole mass spectrometer. All the methods showed good sensitivity with limits of quantitation down to 0.5 ng/mL, giving laboratories options to choose from for further development and validation.

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IVD **In Vitro Diagnostic Medical Device***

Liquid chromatography tandem mass spectrometry systems enable in vitro quantification of a variety of compounds in biological matrices. The performance data presented in this paper is for illustrative purposes only and may not represent the performance that laboratories will obtain. Thermo Fisher Scientific does not recommend or suggest analysis of the analytes described herein using its systems. Performance in an individual laboratory may differ from what is presented in this document due to factors, including but not limited to laboratory methods, materials used, operator technique, and system conditions. It is the laboratory's responsibility to validate performance of any assay it intends to utilize in its facility and to comply with all applicable laws and policies.

*Not for Clinical Diagnosis.