Application Note: 522

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Quantitative Analysis of 1,25-dihydroxy-vitamin D_2 and D_3 using Immunoaffinity Extraction with APCI-LC-MS/MS

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Key Words
- Endocrine Testing
- TSQ Vantage
- Accela U-HPLC

Introduction

1,25-dihydroxyvitamin D (1,25D) tests are important in conducting clinical research in chronic renal failure and hypoparathyroidism. Circulating 1,25D levels are a thousand-fold less than 25-hydroxyvitamin D levels, making it a challenging test that benefits from immunoaffinity purification prior to analysis with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). In this work, both $1,25D_2$ and $1,25D_3$ were extracted from human plasma using immunoaffinity extraction and quantified with LC-MS/MS.

Goal

To validate a very sensitive LC-MS/MS method to quantify 1,25-dihydroxyvitamin D by combining immunoaffinity extraction and highly selective atmospheric pressure chemical ionization (APCI).

Materials

ImmunoTube® kits (KM1000) were purchased from Immundiagnostik AG (Bensheim, Germany). Immunoextraction tubes, washing and eluting buffers, and calibrators (CAL1 and CAL2) and controls (CTRL1 and CTRL 2) were provided in the KM1000 kit. The concentrations of the calibrators and controls are specified in Table 1.

Table 1: Calibrators and controls in KM1000 kit

Standards	$1,25D_2$ (pg/mL)	$1,25D_3$ (pg/mL)
CAL1	33	26
CAL2	350	250
CTRL1	63-105	49-81
CTRL2	203-348	146-244

Sample Preparation

Five hundred (500) µL of plasma were spiked with deuterated $1,25D_3$ and processed with the ImmunoTube kit. The immunoaffinity method for processing plasma was provided in the kit.

Instrument Method

A Thermo Scientific Accela UHPLC pump and Accela autosampler were used as the front end system. The detector was a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer run in selected reaction monitoring (SRM) mode and equipped with an APCI probe. The LC gradient consisted of a fast, 5-minute method at a flow rate of 500 µL/min.

Results and Discussion

Figures 1 and 2 display the data collected for $1,25D_2$ and $1,25D_3$ using the calibrators and controls provided in the ImmunoTube kit. Calibration curves were plotted without weighting and set to include the origin of the coordinate (x, y = 0,0).

Conclusion

In this research, ImmunoTube immunoaffinity extraction was used to prepare human plasma prior to LC-MS/MS to quantify $1,25D_2$ and $1,25D_3$. Immunoaffinity extraction allows for the efficient extraction of target compounds from biological samples and almost completely eliminates matrix effects and interferences in LC-MS/MS analysis. The sample preparation is fast, simple, and does not require chemical derivatization. These features make it an ideal method in clinical research for the quantitation of $1,25D_2$ and $1,25D_3$. APCI was used for the method validation with an ImmunoTube kit, and the lowest concentrations tested for $1,25D_2$ and $1,25D_3$ in the kit were 26 and 33 pg/mL, respectively. Based on the S/N ratios at these concentrations, the limit of quantitation (LOQ) of this method was estimated to be around 15 pg/mL.

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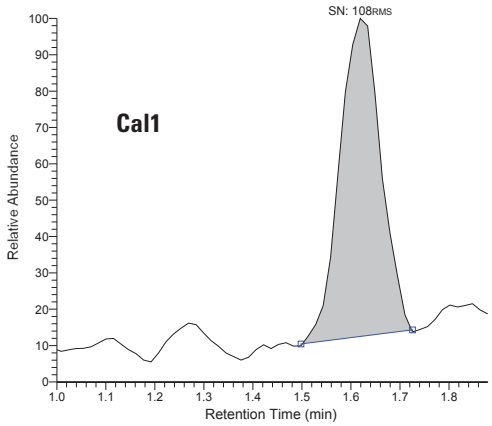
1,25D$_2$	Measured (pg/mL)	Specified (pg/mL)
Cal1	34.8	33
Cal2	349.8	350
Ctrl1	94.3	63-105
Ctrl2	311.6	203-348



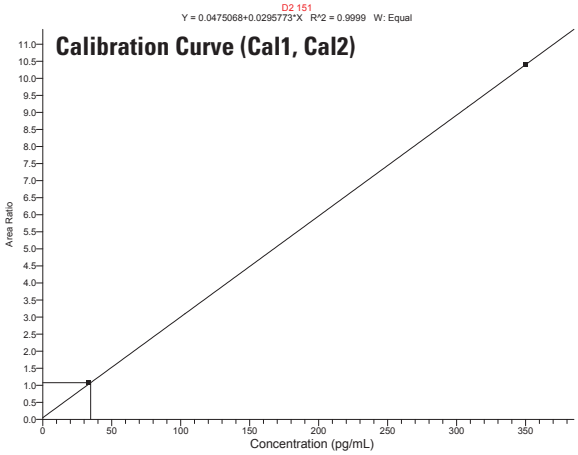
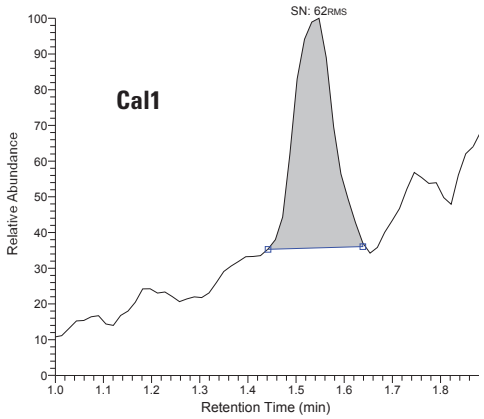


Figure 1: Data for 1,25D$_2$ (Transition 411→151)

1,25D$_3$	Measured (pg/mL)	Specified (pg/mL)
Cal1	21.7	26
Cal2	250.4	250
Ctrl1	59.9	49-81
Ctrl2	200.8	146-244



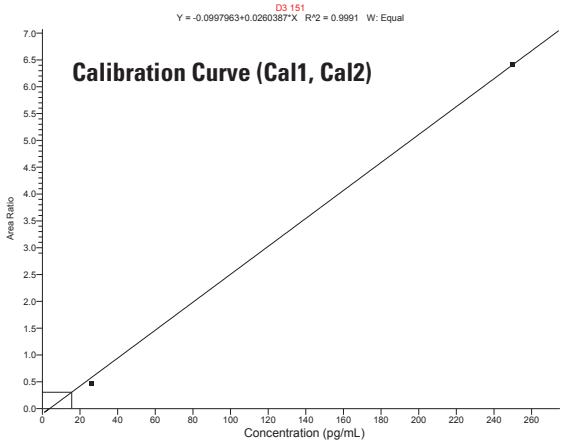


Figure 2: Data for 1,25D$_3$ (Transition 399→151)

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Quantitative LC-MS/MS Analysis of 25-OH Vitamin D_3/D_2 Comparing 1D Chromatography, 2D Chromatography and Automated Online Sample Preparation

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Key Words

- Transcend TLX-1
- TurboFlow Technology
- TSQ Quantum Ultra
- Endocrinology

Introduction

High performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS) is now widely accepted for measurement of vitamin D metabolites. Many clinical research laboratories use 1-dimensional (1D) chromatography (for example, a single HPLC pump and chromatography column) with a triple stage quadrupole mass spectrometer. Various sample cleanup protocols, such as solid phase extraction (SPE), liquid-liquid extraction (LLE), and protein precipitation (PPT), have been applied in these analyses. Frequently, interfering peaks are seen in 25-OH vitamin D3 chromatograms, adversely affecting peak integration and leading to poor accuracy and reproducibility. Here we investigate the use of 2-dimensional chromatography using TurboFlow technology to remove all interfering peaks and significantly improve data quality.

Goal

Compare three methods for the quantitative analysis of 25-OH vitamin D_3/D_2: a validated, online TurboFlow™ method; a commercially available 2D-SPE-LC-MS/MS kit method (Chromsystems MassChrom® 25-OH Vitamin D_3/D_2); and a 1D chromatography method.

Experimental Conditions

Sample Preparation

A 100 µL sample of plasma was mixed with 200 µL internal standard (IS) in acetonitrile, vortexed, and centrifuged. For analysis, 50 µL of supernatant was injected onto the column. Details of the commercial calibrator and QC values (Chromsystems) used in each assay are provided in Tables 1 and 2. (Please note that the control product has since been reformulated to validate borderline D_3 insufficiency and normal levels.) These commercial products were validated against in-house calibration and control material over a wider dynamic range.

HPLC

HPLC analysis was performed using the Thermo Scientific Transcend TLX-1 system powered by TurboFlow™ technology. For analysis, a TurboFlow XL C18 extraction column (50 x 0.5 mm) and a Thermo Scientific Hypersil GOLD analytical column (50 x 2.1 mm, 1.9 µm) were used. For 1D analysis, the analytical column alone was used. For the commercial 2D set up, columns provided within the 2D-SPE-LC-MS/MS kit were used. Eluents for the TurboFlow method were 0.1% formic acid, methanol + 0.1% formic acid, and acetonitrile/IPA/acetone blend (wash solution).

Table 1. Calibrator levels.

Calibrator	Cal 1 (nmol/L)	Cal 2 (nmol/L)	Cal 3 (nmol/L)	Cal 4 (nmol/L)
25-OH Vitamin D_3	9.9	47.8	86.2	174.0
25-OH Vitamin D_2	0.0	37.5	72.3	146.0

Table 2. Quality control levels.

	Mean 25-OH vitamin D_3 (nmol/L)	Mean 25-OH vitamin D_2 (nmol/L)
QC1	77.1	72.7
QC2	167	150

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Mass Spectrometry

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer. Atmospheric pressure chemical ionization (APCI) was used to generate the [M-H_2O]+ ion for 25-OH vitamin D_3, D_2 and the IS.

Results and Discussion

Example calibration lines for the D_3 and D_2 metabolites analyzed by the TurboFlow LC-MS/MS method are presented in Figures 1A and 1B.

Examples of a plasma sample analyzed by the 1D LC-MS/MS method and by the TurboFlow method are provided in Figures 2A and 2B, respectively. There is an interference peak observed in the LC-MS/MS 25-OH-D_3 selected reaction monitoring (SRM) extracted ion chromatogram (XIC). This is commonly observed in analyses where only 1D LC-MS/MS is utilized. When using the TurboFlow method, the interference is removed and larger peak areas with better signal-to-noise ratios are achieved.

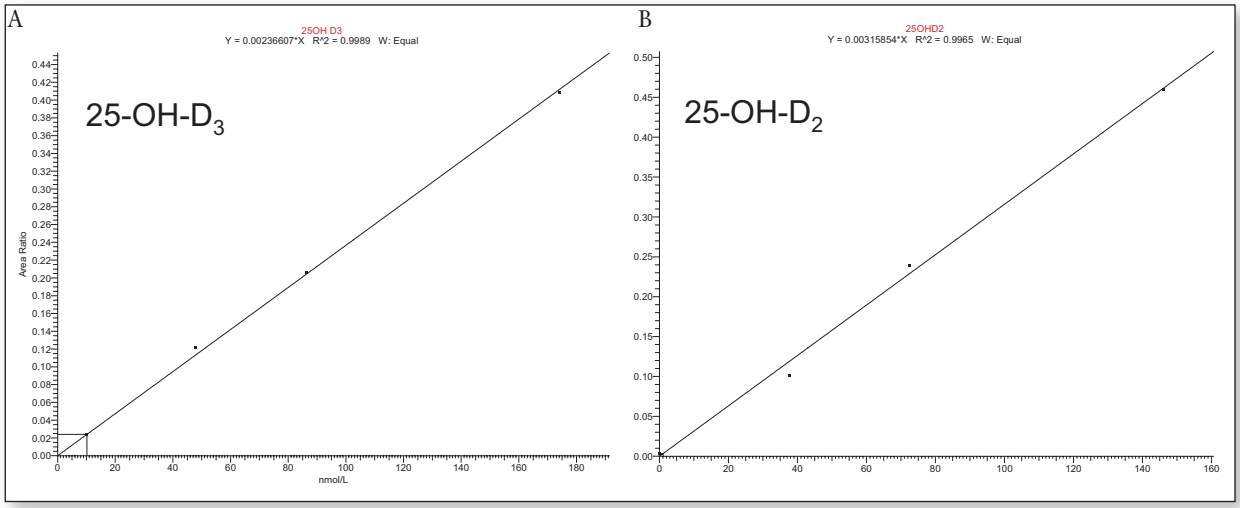


Figure 1. Calibration curves for 25-OH vitamin D_3 (A) and 25-OH vitamin D_2 (B) by TurboFlow LC-MS/MS.

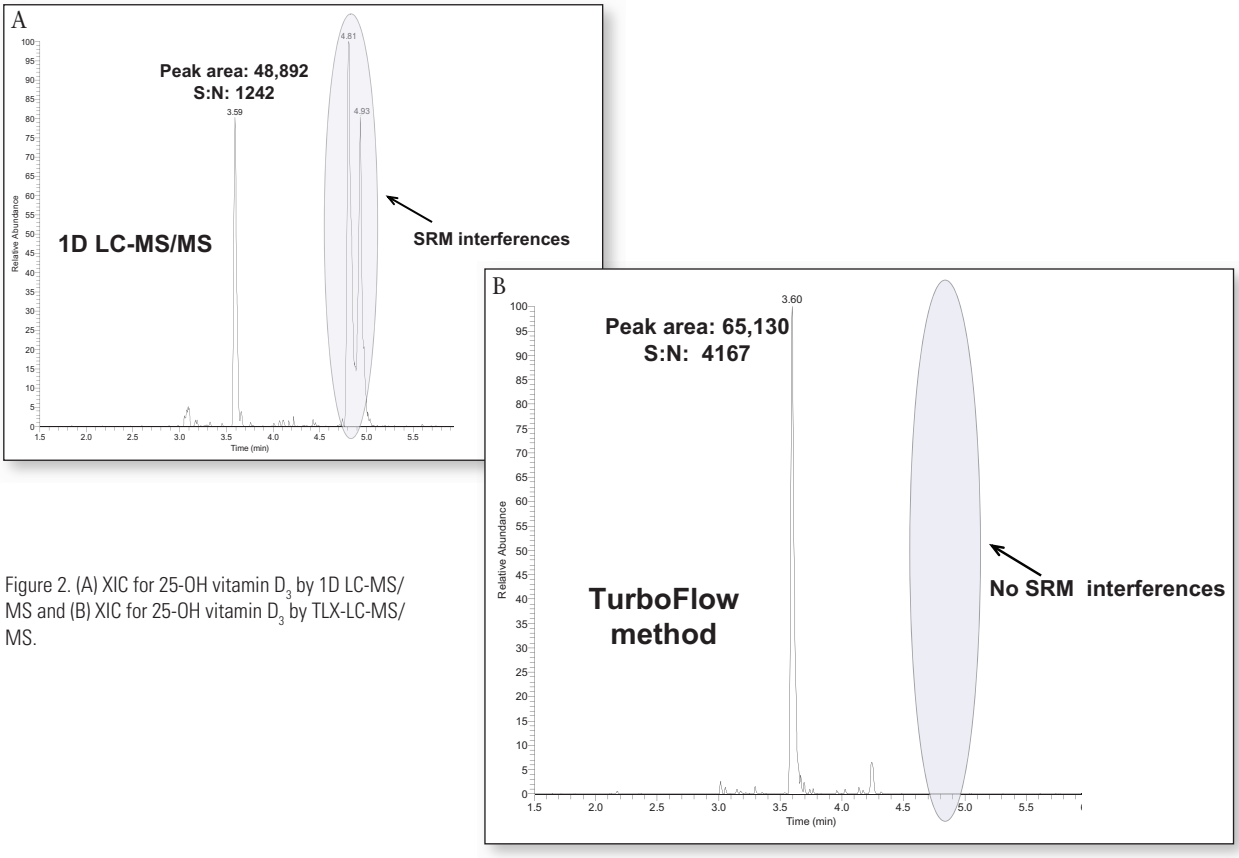


Figure 2. (A) XIC for 25-OH vitamin D_3 by 1D LC-MS/MS and (B) XIC for 25-OH vitamin D_3 by TLX-LC-MS/MS.

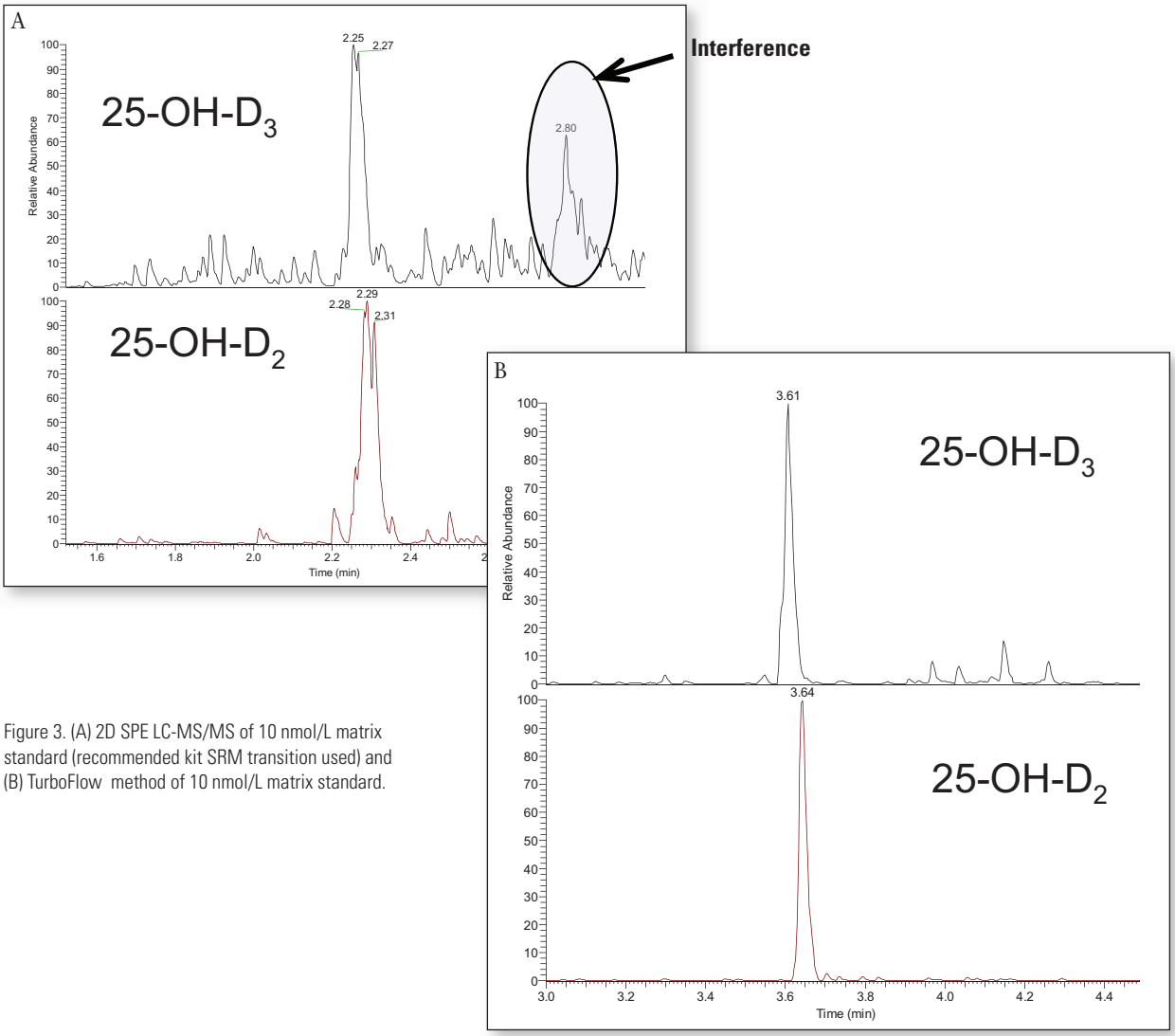


Figure 3. (A) 2D SPE LC-MS/MS of 10 nmol/L matrix standard (recommended kit SRM transition used) and (B) TurboFlow method of 10 nmol/L matrix standard.

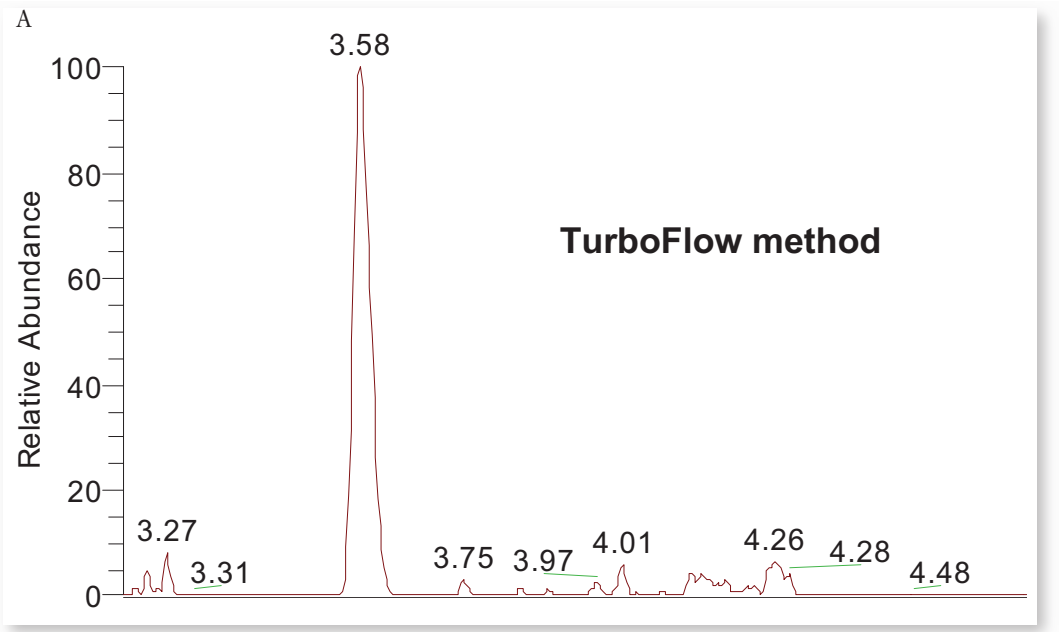
Although cleanup is improved when using other 2D LC-MS/MS methods, interferences are still observed in the 25-OH-D_3 XIC (Figure 3A). Furthermore, at the bottom of the range for 25-OH-D_3 (~10 nmol/L), is detected with greater analytical sensitivity and less noise when analyzed using the TurboFlow method versus a 2D SPE cleanup procedure (Figure 3B).

The 2D-LC-MS/MS approach reduces SRM interferences in the 25-OH-D_3 XICs because the integration of the analyte peak is easier and more accurate. An example of the impact of these interferences on peak integration is shown in Figures 4A and 4B. Here, the result for an individual with normal levels of 25-OH-D_3 would be reported incorrectly due to the high level of interference merging with the analyte peak, and thus, affecting the peak integration.

Conclusion

The TurboFlow method described here has been developed and validated to industry recommended guidelines for clinical laboratories.

Isobaric interferences observed with a 1D LC-MS/MS method at low 25-OH D_3 metabolite concentrations were much reduced by using a 2D-LC-MS/MS approach, and even further improved by using TurboFlow technology. The Transcend™ TLX-1 LC-MS/MS with TurboFlow technology improved the sensitivity and the signal-to-noise ratio.



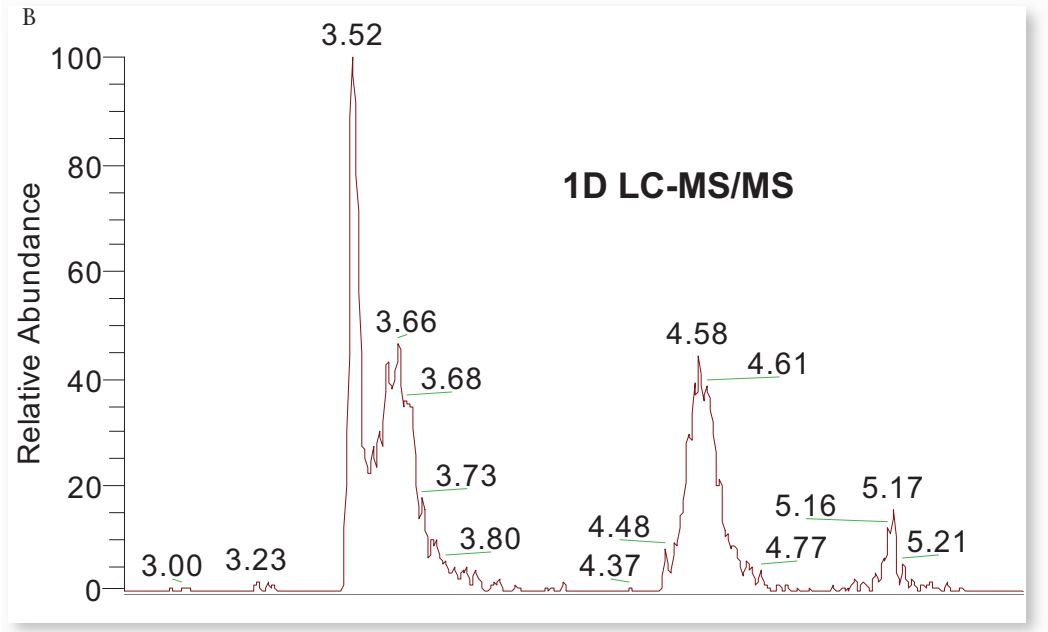


Figure 4. XICs for 25-OH vitamin D_3 by (A) TurboFlow method and (B) 1D LC-MS/MS analysis of a sample at normal levels of analyte (83 nmol/L).

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