APPLICATIONS

Separation and Quantitation of Physiologic Cobalamins (Vitamin B_{12}) in Dietary Supplements by LC/MS/MS using Synergi™ Fusion-RP Column

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Analysis of cobalamins (Vitamin B_{12}) on reversed phase columns can be challenging due to the relative chemical instability of the analyte, the complexity of matrix effects and the existence of several vitamer forms of B_{12}. In this tech note, we describe a selective, efficient and reproducible method to separate three B_{12} vitamers (methylcobalamin, cyanocobalamin and adenosylcobalamin) appropriate for the quantitative analysis of dietary supplements via LC/MS/MS.

Introduction

Cobalamin is a micronutrient that animals and humans obtain through diet, symbiotic bacteria or vitamin supplements. Vitamin B_{12} deficiency leads to a degeneration of the sensory in the spinal cord with loss of sensation and paralysis that results in a complex spectrum of pathologies, including pernicious anemia, megaloblastic anemia, peripheral neuropathy, depression, impaired cognition, and autoimmune dysfunction. The four vitamer forms of B_{12} are differentiated based upon the functional group (R) at the β-axial position (**Figure 1**). The two forms found naturally in the body are adenosylcobalamin ($AdoB_{12}$) and methylcobalamin (MeB_{12}), while the two most common vitamin forms are hydroxycobalamin (HOB_{12}) and cyanocobalamin (CNB_{12}). Due to its stability advantages, cyanocobalamin is the vitamer form that is most commonly used in supplements.

Historically, cobalamin analysis has been performed using microbiological methods that are known to have significant shortcomings for accuracy and specificity. More recently, analysis has been done with HPLC separation and various modes of detection including UV, fluorescence and MS. LC/MS/MS analysis can be challenging due to the chemical instability and insource ionization issues for many of the vitamer forms. In this study, we assessed the selectivity, specificity and separation of three forms ($AdoB_{12}$, MeB_{12} and CNB_{12}) that are commonly seen in dietary supplements. We have established a sensitive, selective and reproducible quantitation method for the nutraceutical industry utilizing HPLC separation coupled to a triple quadrupole mass spectrometer.

Experimental Conditions

Reagent and chemicals

All solvents and reagents were HPLC or analytical grade. Reference standards were supplied by Genysis Lab.

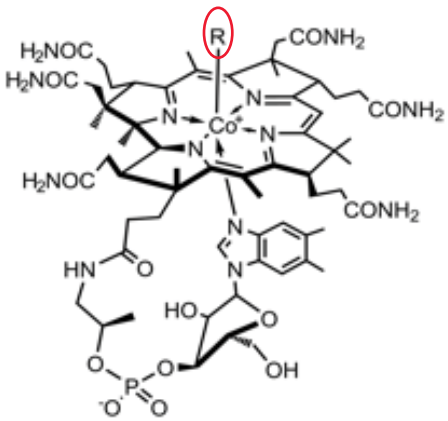
Equipment and materials

Agilent® 1260 pumps and autosampler were used along with a SCIEX QTRAP 4000 and SCIEX 4500, positive polarity, ESI for detection.

LC/MS/MS Method Parameters

Column:	Synergi™ 2.5 µm Fusion-RP
Dimensions:	50 x 2.0 mm
Part No.:	00B-4423-B0
SecurityGuard™ Cartridge:	AJ0-7844
Mobile Phase:	A: 10 mM Ammonium Formate pH 3.5 B: Methanol
Gradient:	**Time (min) B (%)** 0 2 0.2 2 1.8 98 4 98 4.01 2 5.5 2
Flow Rate:	350 µL/min
Injection Volume:	10 µL
Temperature:	21 C°
Instrument:	Agilent 1260 LC
Detection:	MS/MS (ESI+) (SCIEX Triple Quad™ 4500 and SCIEX 4000 QTRAP) @ 600 °C
Sample:	1. Cyanocobalamin (CNB_{12}) 2. Adenosylcobalamin ($AdoB_{12}$) 3. Methylcobalamin (MeB_{12})

Figure 1. Cobalamin Structure



R = CN, Me, OH, Ado

Figure 2. Cobalamin Mobile Phase pH Selection Chart

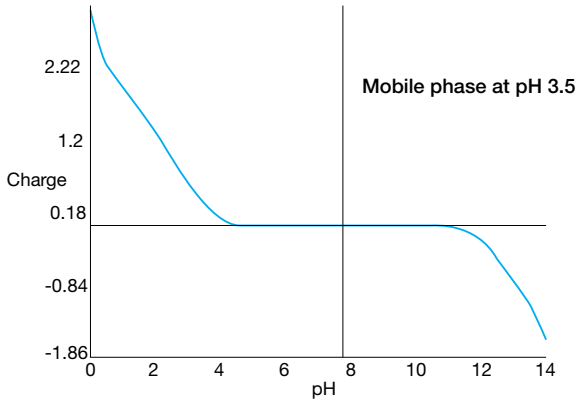
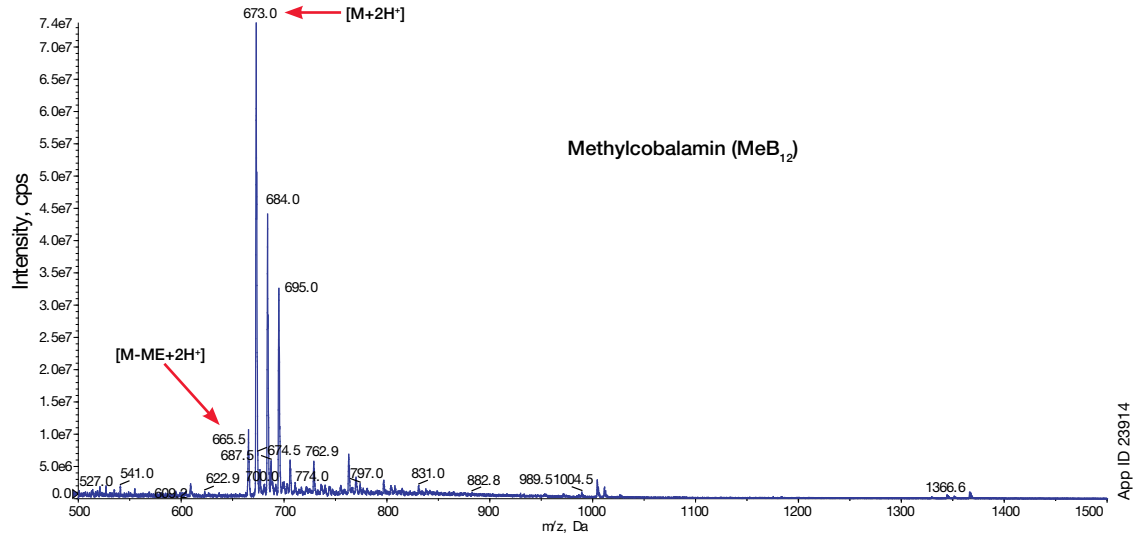
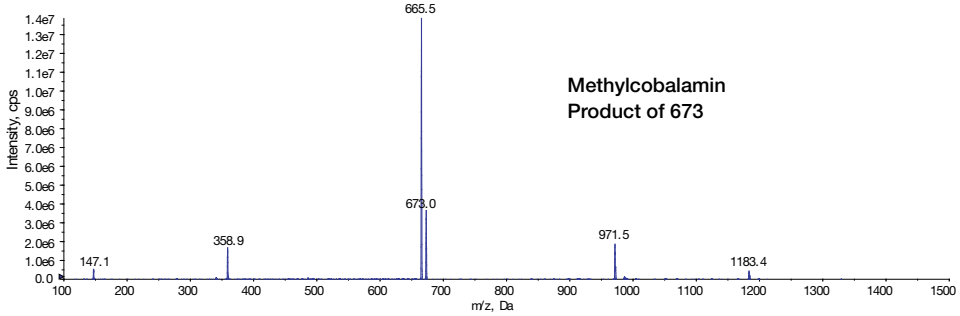


Figure 3. Tuning files _ Methylcobalamin (m.w. 1343.59) by infusion





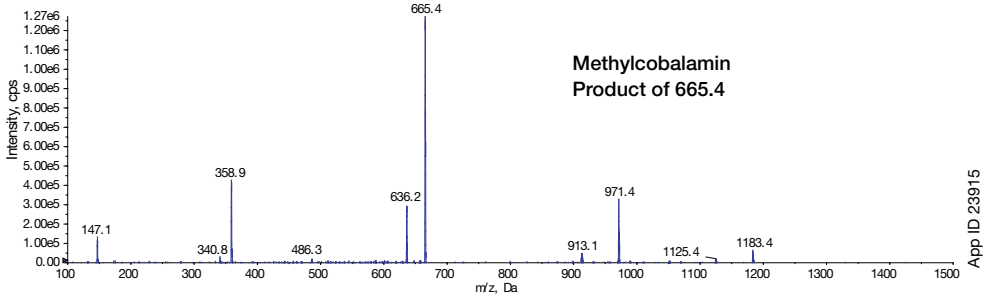
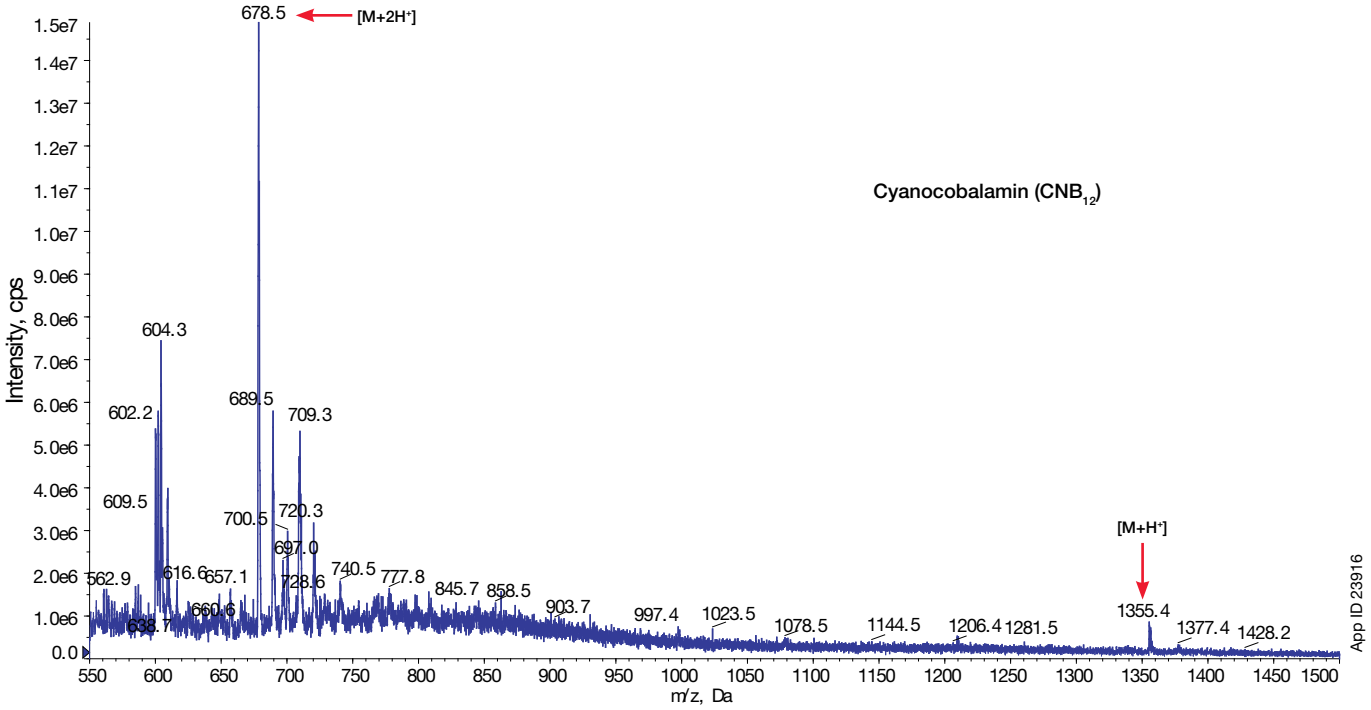


Figure 4. Tuning files _ Cyanocobalamin (m.w. 1354.5) by infusion



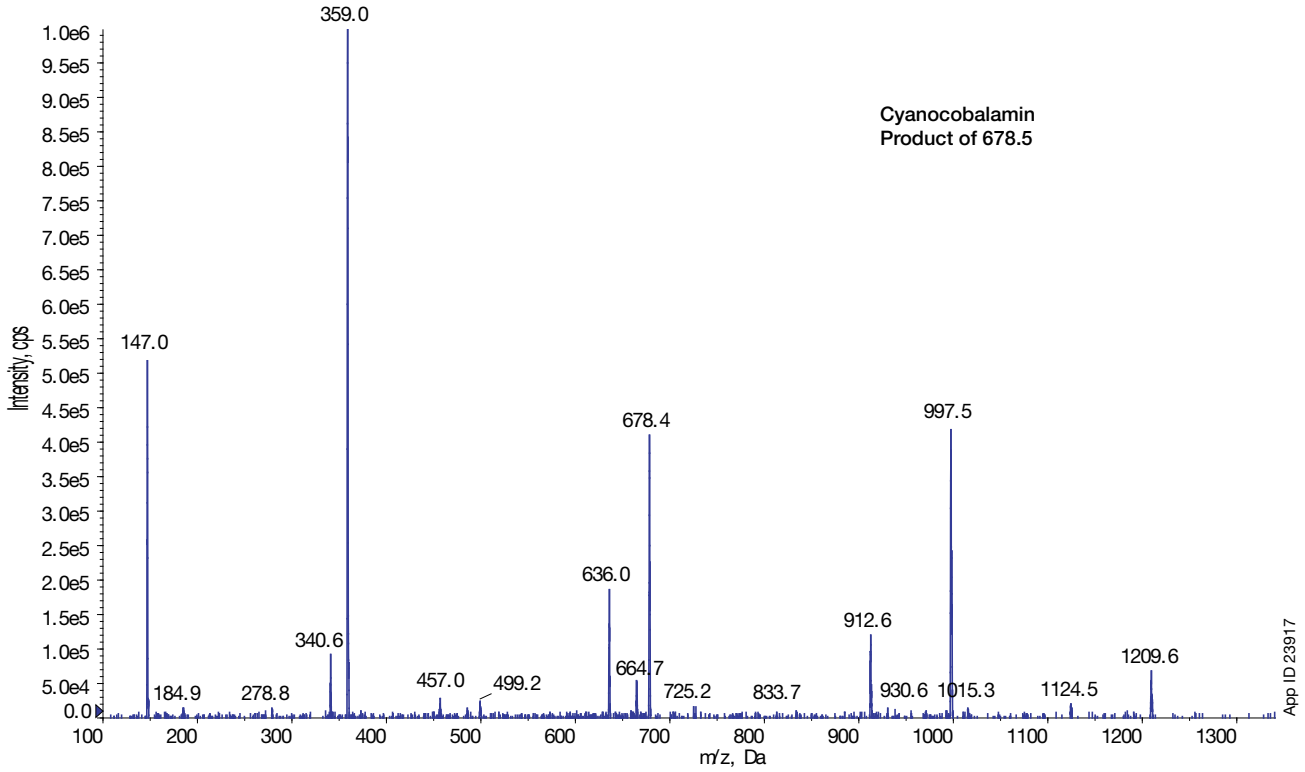
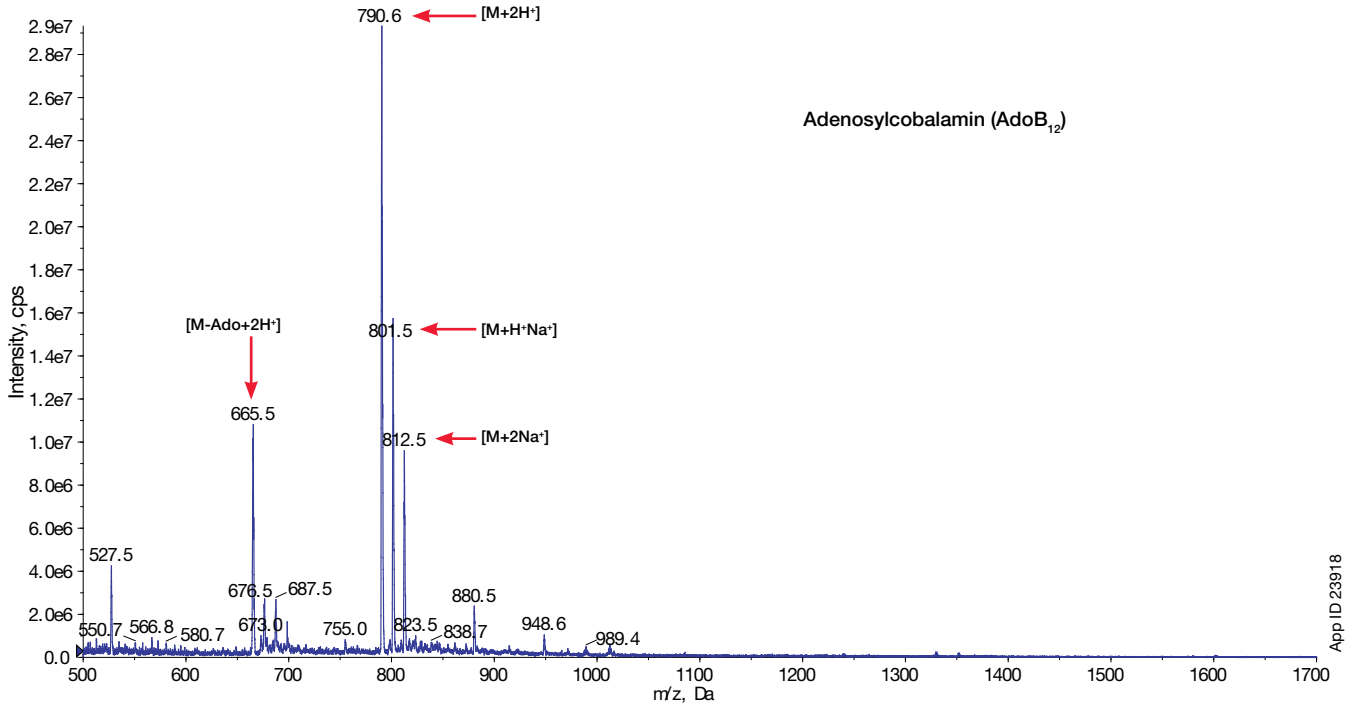
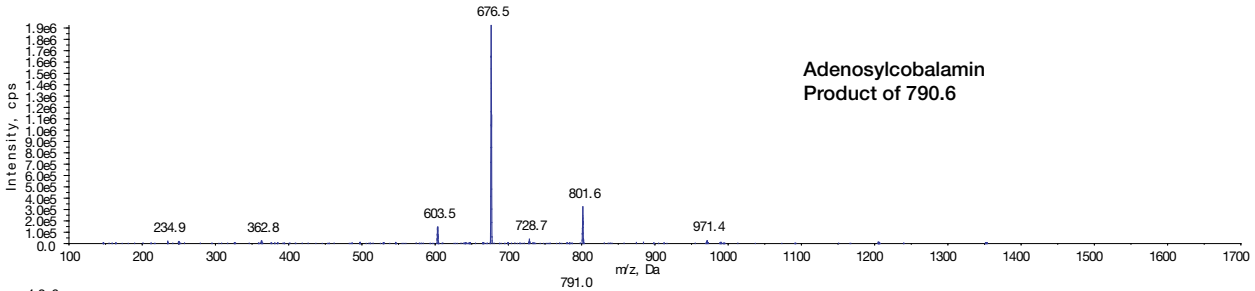
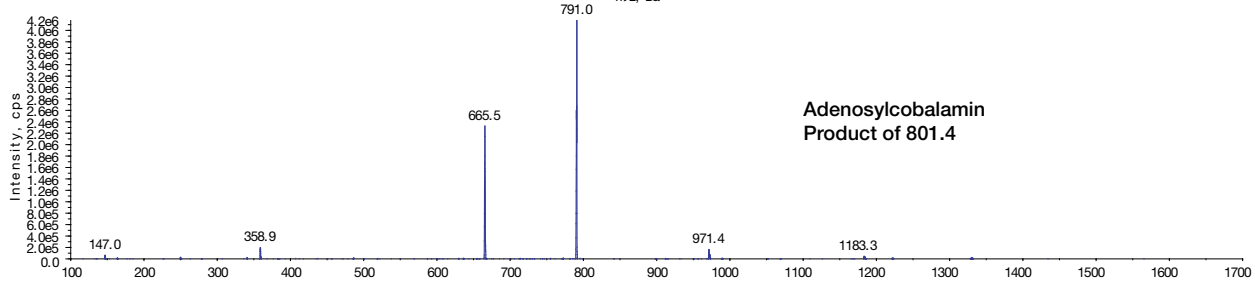


Figure 5. Tuning files _ Adenosylcobalamin (m.w. 1579.58) by infusion







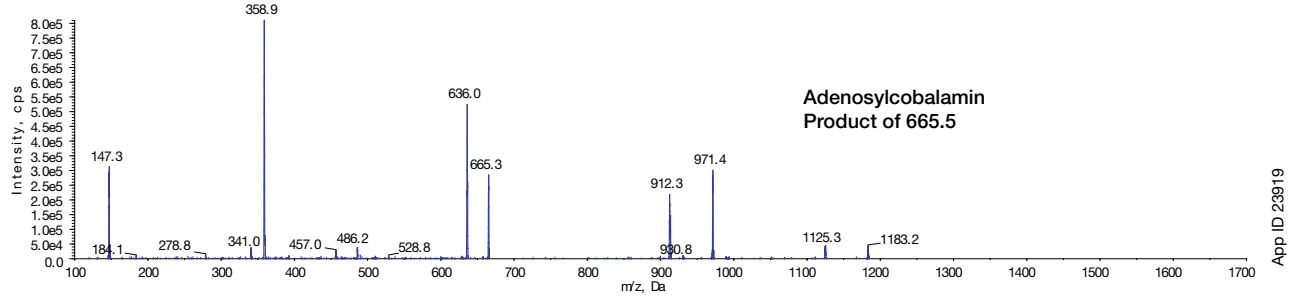


Table 1. Mass Transitions

ID	Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	DP	CE
MeB_{12} 1	673.0	665.5	50	50	25
MeB_{12} 2	673.0	971.5	50	50	38
CNB_{12} 1	678.5	359.0	50	90	32
CNB_{12} 2	678.5	997.5	50	90	28
$AdoB_{12}$ 1*	801.5	676.3	50	70	31
$AdoB_{12}$ 2	790.6	665.6	50	70	31

*An adduct ion $[M+H^+ +Na^+]/2$

Figure 6. Representative Chromatogram of Blank Sample

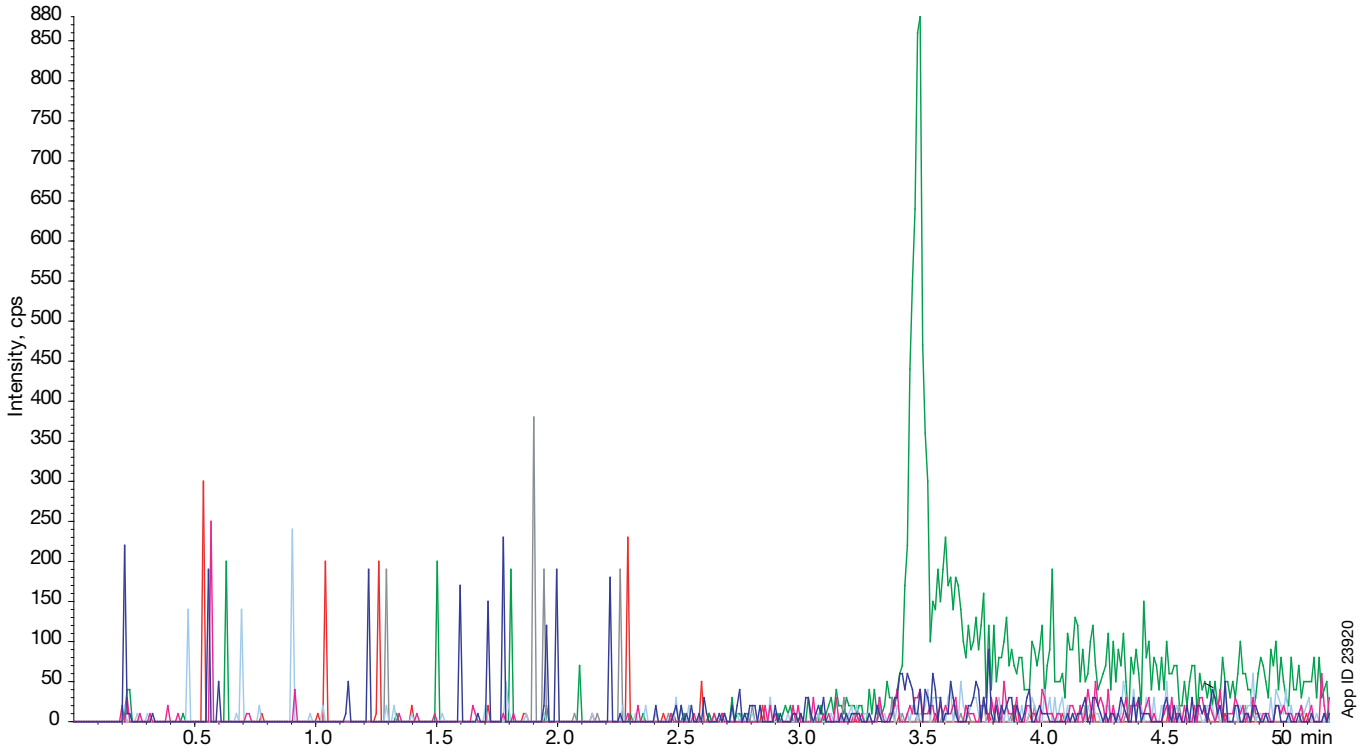


Figure 7. Representative Chromatogram of 15 ng/mL standard

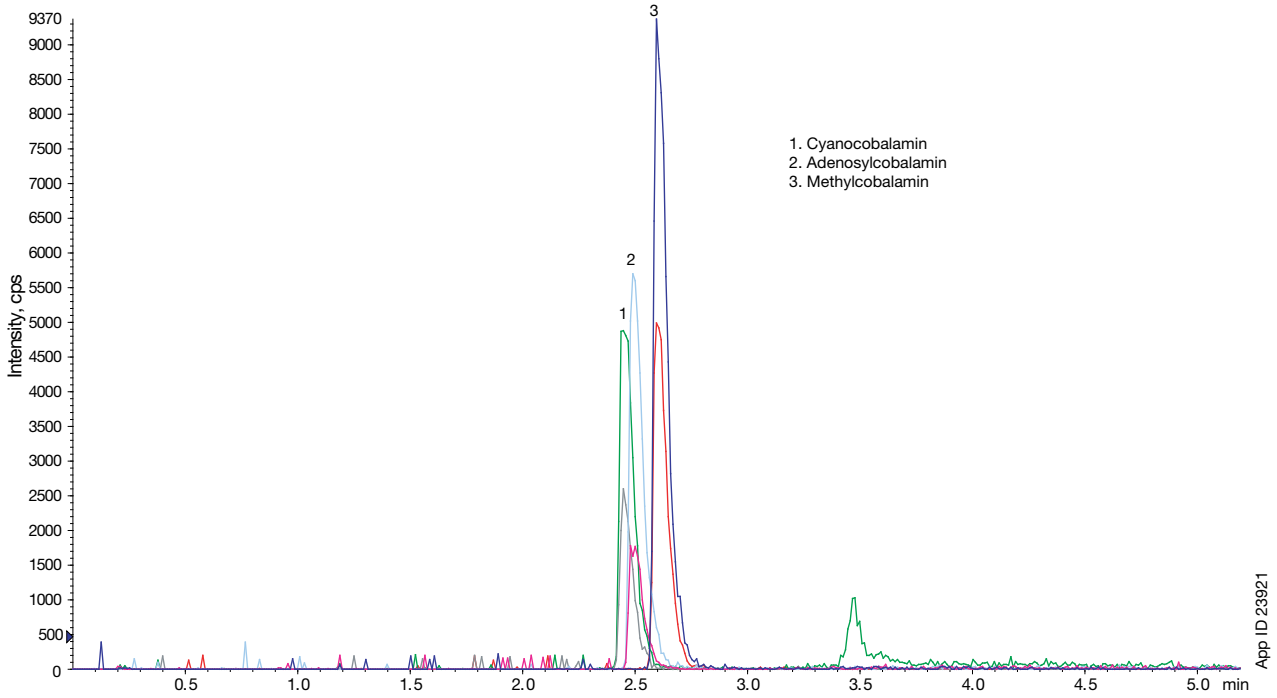


Figure 8. Representative Methylcobalamin Calibration Curve

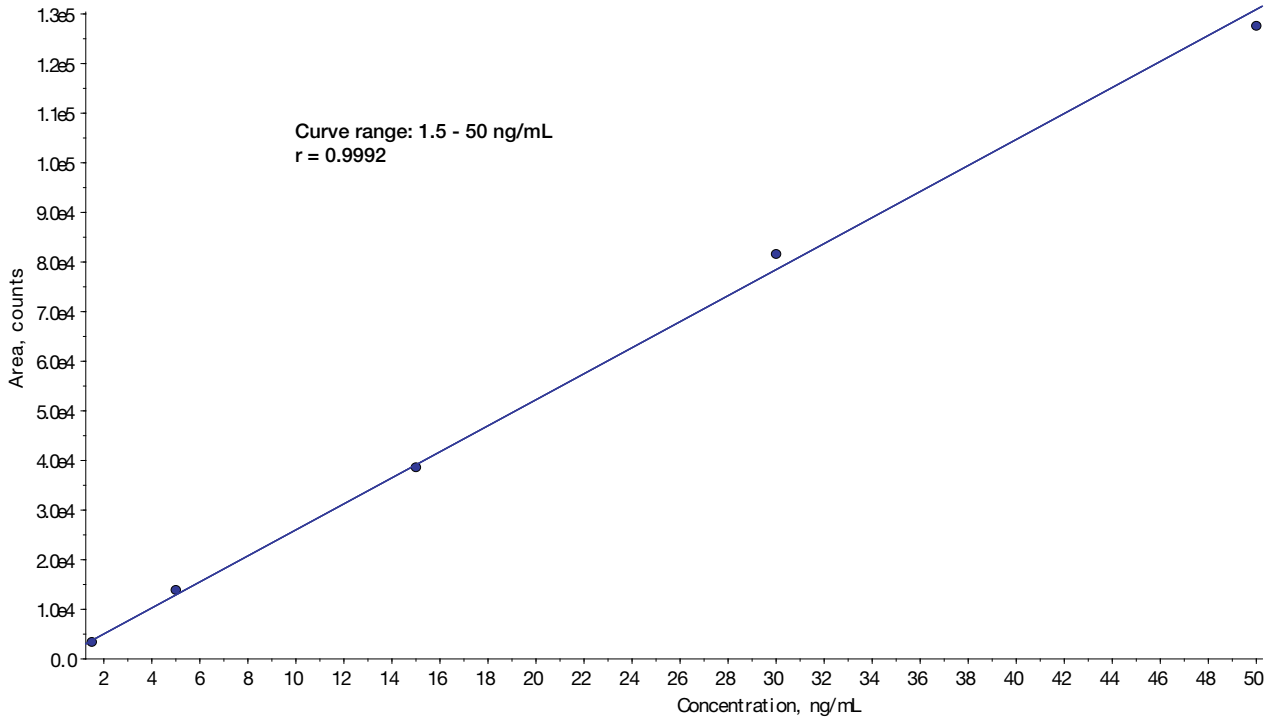


Table 2. Assay Reproducibility at LLOQ (1.5 ng/mL)

Sample 3 B12s at 1.50 ng/mL	Adenosylcobalamin			Cyanocobalamin			Methycobalamin		
	Peak Area	Peak Height	RT (min)	Peak Area	Peak Height	RT (min)	Peak Area	Peak Height	RT (min)
Injection 1	2264	443.8	2.512	2421	491.0	2.469	3104	628.0	2.618
Injection 2	2428	479.8	2.509	2094	401.3	2.471	3450	662.9	2.622
Injection 3	2401	485.6	2.513	2034	419.0	2.468	3314	691.0	2.621
Injection 4	2361	439.0	2.512	2372	450.5	2.474	3341	659.2	2.617
Injection 5	2447	466.1	2.508	2298	428.2	2.466	3391	765.8	2.610
Injection 6	2824	601.2	2.505	2061	392.7	2.466	3382	650.6	2.614
Injection 7	2072	369.4	2.507	2087	403.3	2.462	3486	740.8	2.612
Injection 8	2322	425.0	2.512	2167	463.4	2.473	3268	618.7	2.619
Injection 9	2481	441.2	2.52	2618	548.0	2.473	3462	713.5	2.622
Injection 10	2517	507.8	2.516	2236	448.0	2.473	3359	684.4	2.622
AVG	2412	466	2.51	2239	445	2.47	3356	681	2.62
SD	193	61	0.00	189	48	0.00	112	48	0.00
%RSD	8.00%	13.09%	0.18%	8.44%	10.72%	0.16%	3.33%	6.98%	0.17%

Results and Discussion

All monitored cobalamins showed instability at the mass spectrometer source. TFA added to the mobile phase is reported to increase ionization and peak shape, but TFA is not a suitable for mass spectrometry. A 10 mM ammonium formate at pH 3.5 mobile phase was used to effectively ionize the analytes while providing sufficient separation and sensitivity, (**Figure 2**). The assay was run on a Phenomenex Synergi Fusion-RP 2.5 µm 50 x 2.0 mm column which provided reasonable selectivity.

During method development, all cobalamins showed poor response at $[M+H^+]$. It was found that cobalamins are often multiply charged and/or form strong adduct ions. Furthermore, insource fragmentation can result in the loss of the β-axial ligand leading to adjusted parent masses (**Table 1**). This fragmentation produces identical parent ions for MeB_{12} and $AdoB_{12}$ at m/z of 665.2. To better understand the mass selectivity, please refer to all compound tuning files (**Figures 3-5**).

The assay was further evaluated by Genysis Lab to assess the dynamic range and the assay reproducibility at the LLOQ (lower limit of quantitation) level. The dynamic range of all three cobalamins was 1.5 – 50 ng/mL (**Figure 8**). Representative chromatograms are presented in **Figures 6** and **7**. The reproducibility was assessed by injecting LLOQ standards 10 times and tracking the peak area, peak height and retention times for each analyte.

Conclusion

In this technical note, a quantitative method for the analysis of cyanocobalamin, methylcobalamin and adenosylcobalamin useful for the analysis of dietary supplements was developed. This assay was successfully evaluated and transferred to a customer lab.

References

1. Shawn C. Owen, Manfai Lee and Charles B. Grissom. Ultra-Performance Liquid Chromatographic Separation and Mass Spectrometric Quantitation of Physiologic Cobalamins. J. Chromatographic Science, Vol 49: 228-233 (2011).

APPLICATIONS

Ordering Information

Synergi™ Fusion-RP HPLC Columns

2.5 µm High Speed Technology (HST) Columns (mm)

Phase	30 x 2.0	50 x 2.0	100 x 2.0	50 x 3.0	100 x 3.0	50 x 4.6
Fusion-RP	00A-4423-B0	00B-4423-B0	00D-4423-B0	00B-4423-Y0	00D-4423-Y0	00B-4423-E0

4 µm Capillary Columns (mm)

Phase	50 x 0.30	150 x 0.30	150 x 0.50	Guard Column (mm) 20 x 0.30
Fusion-RP	00B-4424-AC	00F-4424-AC	00F-4424-AF	03M-4424-AC

4 µm Microbore and Minibore Columns (mm)

Phase	50 x 1.0	150 x 1.0	30 x 2.0	50 x 2.0	75 x 2.0	150 x 2.0	250 x 2.0	SecurityGuard™ Cartridges (mm) 4 x 2.0*
Fusion-RP	00B-4424-A0	00F-4424-A0	00A-4424-B0	00B-4424-B0	00C-4424-B0	00F-4424-B0	00G-4424-B0	AJ0-7556 for ID: 2.0-3.0 mm

4 µm MidBore™ Columns (mm)

Phase	50 x 3.0	150 x 3.0	250 x 3.0	SecurityGuard Cartridges (mm) 4 x 2.0*
Fusion-RP	00B-4424-Y0	00F-4424-Y0	00G-4424-Y0	AJ0-7556 for ID: 2.0-3.0 mm

4 µm Analytical Columns (mm)

Phase	50 x 4.6	75 x 4.6	150 x 4.6	250 x 4.6	SecurityGuard Cartridges (mm) 4 x 3.0*
Fusion-RP	00B-4424-E0	00C-4424-E0	00F-4424-E0	00G-4424-E0	AJ0-7557 for ID: 3.2-8.0 mm

* SecurityGuard Analytical cartridges require holder, Part No.: KJ0-4282

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