

Application Note

## Enhancing the LC-MS/MS Analysis of B-group Vitamins with MaxPeak High Performance Surfaces Technology

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## Abstract

Waters MaxPeak High Performance Surfaces (HPS) provide an effective solution to mitigate interactions between analytes and metal surfaces in liquid chromatography. This application note investigates the effects of the MaxPeak HPS on the LC-MS/MS analysis of B-group vitamins and demonstrates the key benefits of using the MaxPeak HPS in the simultaneous analysis of B-group vitamins in energy drinks and vitamin B complex dietary supplements. The key benefits observed include high response, improved sensitivity, less peak tailing, better calibration linearity, and no carry-over compared to the stainless-steel surfaces in a conventional LC system setup. Greater sensitivity (3–10 times) was observed for riboflavin, thiamine, nicotinamide, flavin mononucleotide, pyridoxal 5'-phosphate, and 5-methyltetrahydrofolate using the Waters ACQUITY Premier Solution. The analytical performance (accuracy and repeatability) of the simultaneous LC-MS/MS analysis of B-group vitamins in energy drinks and vitamin B complex dietary supplements are also presented. The ACQUITY Premier Solution (system and column) showed clear advantages, such as improved sensitivity, less peak tailing, better calibration linearity, and no carry-over issue, over the conventional liquid chromatography solution for the analysis of B vitamins.

### Benefits

- Waters ACQUITY Premier Solution improves LC-MS/MS analysis of B vitamins
- 3 to 10 times better sensitivity were observed for six B vitamins using the MaxPeak HPS than the conventional stainless-steel surfaces
- Higher response, less peak tailing, and less carry-over are observed with Waters ACQUITY Premier Solution

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## Introduction

The B-group vitamins include eight B vitamins, which are thiamine (B<sub>1</sub>), riboflavin (B<sub>2</sub>), niacin (B<sub>3</sub>), pantothenic acid (B<sub>5</sub>), pyridoxine (B<sub>6</sub>), biotin (B<sub>7</sub>), folate (B<sub>9</sub>), and cyanocobalamin (B<sub>12</sub>). Each B vitamin has different forms (or vitamers). Recently, new vitamin B vitamers, such as flavin mononucleotide (FMN) and pyridoxal 5'-phosphate (PLP), have been formulated in dietary supplements. These phosphate containing compounds are often called "native" or coenzymated B vitamins and are believed to be readily absorbed by the human body. The structures of the B vitamins and their vitamers are shown in Figure 1. The interactions

between phosphate containing compounds and stainless-steel surfaces and their detrimental effects on high performance liquid chromatography (HPLC) have been reported.<sup>1-2</sup> These interactions can cause severe peak tailing, reduced peak height, and/or carry-over, which lead to inaccurate and unreliable results in HPLC analysis. There are a few workarounds available to address this analyte metal interactions issue. However, these workarounds have their own limitations,<sup>3</sup> and a better approach is needed.

Waters MaxPeak High Performance Surfaces (HPS) technology, which are implemented in the Waters ACQUITY Premier Solution (including system and columns), provides an effective way to mitigate the analyte metal interactions. Dramatic improvements have been observed using the MaxPeak HPS in HPLC analyses for organic acids, oligonucleotides, peptide, glycans, and phospholipids.<sup>4-7</sup> This application note investigates the effects of the MaxPeak HPS on the analysis of B-group vitamins using liquid chromatography with tandem mass spectrometry (LC-MS/MS). The advantages of applying the MaxPeak HPS to the analysis of B vitamins, including those non-phosphate containing B vitamins, are highlighted in the simultaneous LC-MS/MS analysis of B-group vitamins in energy drink and dietary supplement samples.

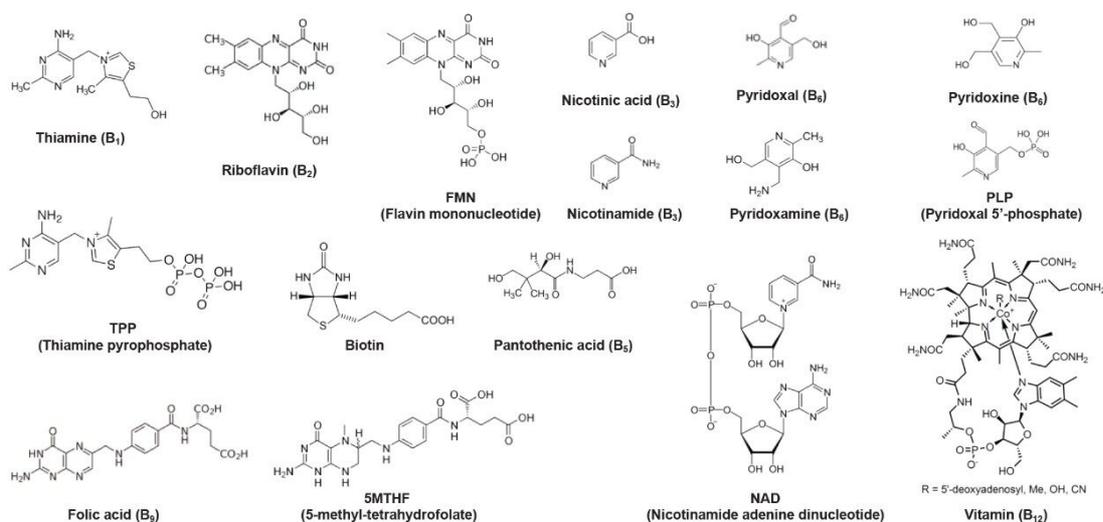


Figure 1. Structures of B Vitamins and their vitamers.

## Experimental

All sample preparation was conducted in dimmed light environment and with amber glassware.

### Standard Preparation

Thiamine, thiamine pyrophosphate (TPP), riboflavin, flavin mononucleotide (FMN), nicotinic acid, nicotinamide, pantothenic acid, pyridoxine, pyridoxamine, pyridoxal, pyridoxal 5'-phosphate (PLP), biotin, folic acid, 5-methyltetrahydrofolate (5MTHF), cyanocobalamin (CN B<sub>12</sub>), methylcobalamin (Me B<sub>12</sub>), adenosylcobalamin (Aden B<sub>12</sub>) were purchased from Sigma-Aldrich (St. Louis, MO). Stable isotope labeled B vitamins: <sup>13</sup>C<sub>5</sub>, <sup>15</sup>N-folic acid, <sup>13</sup>C<sub>4</sub>, <sup>15</sup>N<sub>2</sub>-riboflavin, <sup>13</sup>C<sub>4</sub>-thiamine, <sup>2</sup>H<sub>4</sub>-biotin, <sup>13</sup>C<sub>6</sub>, <sup>15</sup>N-pantothenic acid, <sup>2</sup>H<sub>2</sub>-pyridoxine, and <sup>2</sup>H<sub>4</sub>-nicotinic acid were purchased from Isosciences (isosciences.com, Ambler, PA). Individual B vitamin stock solutions were prepared by dissolving individual vitamins with deionized water at concentrations of 0.5 mg/mL (recorded to 3 significant figures) except riboflavin and biotin, which were prepared as a mixed stock solution at 0.05 mg/mL (recorded to 3 significant figures) for each riboflavin and biotin standard. The folic acid stock solution may need a small amount (one drop or two) of ammonium hydroxide solution (28–30% NH<sub>3</sub>) to assist the dissolution. The PLP stock solution was heated to 50 °C to assist dissolution.

Three intermediate B vitamin mix solutions were prepared by diluting aliquots of the individual stock solutions with deionized water as follows, one intermediate standard mix solution of CN B<sub>12</sub> (0.1 mg/mL) and Me B<sub>12</sub> (0.1 mg/mL), one intermediate standard mix solution of thiamine (0.01 mg/mL), pantothenic acid (0.1 mg/mL), pyridoxine (0.01 mg/mL), nicotinamide (0.1 mg/mL), nicotinic acid (0.1 mg/mL), and pyridoxal (0.01 mg/mL), and one intermediate standard mix solution of folic acid (0.1 mg/mL) and 5MTHF (0.1 mg/mL). The working standard solutions were prepared from these intermediate B vitamin mix solutions and the remaining individual standard stock solutions by mixing and dilution with deionized water to various concentration levels.

The individual stable isotope labeled B vitamin standard stock solutions were prepared in a similar way as previously described for the individual B vitamin stock standards. A mixture of all stable isotope standard stock solution was prepared from these individual stock solutions by mixing appropriate volumes of the individual stock solutions with deionized water to obtain a concentration of 0.02 mg/mL for each standard except <sup>13</sup>C<sub>5</sub>, <sup>15</sup>N - folic acid which was prepared at 0.2 mg/mL. This stable isotope standard mixture stock solution was diluted with deionized water 10 times to make an intermediate stable isotope mixture solution, which was later used to fortify the working standard solutions and the sample solutions as internal reference standards at 0.1 µg/mL, except <sup>13</sup>C<sub>5</sub>, <sup>15</sup>N - folic acid which was added at 1 µg/mL, in the final solutions.

#### Sample Preparation

The energy drinks (ED) from major brands, such as Red Bull, Monster Energy, and Rockstar, were purchased from a local store. They were filtered through a 0.45 µm GMF membrane syringe filter. An aliquot of the filtrate was analyzed without dilution for low concentration B vitamin (CN B<sub>12</sub>). Another aliquot of the filtrate was diluted with deionized water 50 times (1:50 by volume) and analyzed for other B vitamins. A vitamin B

complex dietary supplement (DS, liquid form) was also tested. 50  $\mu$ L of DS was taken and diluted with 200 mL deionized water first, then filtered through a 0.45  $\mu$ m GMF membrane syringe filter and further diluted with deionized water at different ratios (0.95:1, 1:10, 1:100 by volume) prior to the analysis.

## Method Conditions

### LC Conditions

LC system:	ACQUITY Premier System
MS system:	Xevo TQ-S micro MS System
Run time:	9.0 min
Column:	ACQUITY Premier BEH C <sub>18</sub> Column, 1.7 $\mu$ m, 2.1 $\times$ 100 mm (p/n 186009453)
Vial:	LCMS Certified Amber Glass Max Recovery Vial (p/n 600000755CV)
Temp.:	40 °C
Mobile phases:	A: 20 mM ammonium formate in water (pH 5.0) B: Methanol
Flow rate:	0.35 mL/min
Injection volume:	2 $\mu$ L

## Gradient Program

Time (min)	Flow (mL/min)	A%	B%
Initial	0.35	99	1
0.5	0.35	99	1
2.5	0.35	92	8
5	0.35	10	90
6	0.35	10	90
6.1	0.35	99	1
9	0.35	99	1

## MS Conditions

Polarity:	ES+
Capillary voltage:	1.4 kV
Cone voltage:	70V
Source temp.:	150 °C
Desolvation temp.:	350 °C
Cone gas flow:	350 L/Hr
Desolvation gas flow:	650 L/Hr

## MRM Parameters (Quantification Traces are in Bold)

Compound	Molecular ion ( <i>m/z</i> )	Fragment ion ( <i>m/z</i> )	Cone voltage (V)	Collision energy (eV)
CN B <sub>12</sub>	<b>678.3</b>	<b>147.1</b>	85	35
CN B <sub>12</sub>	678.3	359.1	85	35
Me B <sub>12</sub>	<b>673.7</b>	<b>147.1</b>	10	45
Me B <sub>12</sub>	673.7	359.2	10	25
Aden B <sub>12</sub>	<b>791.1</b>	<b>359.2</b>	50	28
Aden B <sub>12</sub>	791.1	486.6	50	33
Riboflavin	<b>377.2</b>	<b>243.2</b>	90	20
Riboflavin	377.2	172	90	20
Thiamine	<b>265.1</b>	<b>122</b>	35	13
Thiamine	265.1	144	35	13
TPP	<b>425.2</b>	<b>122.1</b>	20	23
TPP	425.2	303.8	20	15
Biotin	<b>245.1</b>	<b>227.1</b>	50	13
Biotin	245.1	97	50	13
Pantothenic acid	<b>220.2</b>	<b>90.1</b>	50	18
Pyridoxine	<b>170.1</b>	<b>134</b>	50	21
Pyridoxine	170.1	152.2	50	21
Nicotinic acid	<b>124.1</b>	<b>78</b>	75	18
Nicotinic acid	124.1	80	75	18
Nicotinamide	<b>123.1</b>	<b>80</b>	70	18
Nicotinamide	123.1	78.1	70	18
NAD	<b>664.4</b>	<b>428.2</b>	70	45
NAD	664.4	542.4	70	23
Folic acid	<b>442.2</b>	<b>176.2</b>	70	40
Folic acid	442.2	295.2	70	15
FMN	<b>457.2</b>	<b>359.2</b>	70	25
FMN	457.2	439.3	70	25
PLP	<b>248.1</b>	<b>150</b>	50	25
PLP	248.1	168.1	50	25
Pyridoxal	<b>168</b>	<b>150.1</b>	10	14
Pyridoxal	168	93.9	10	25
Pyridoxamine	<b>169.1</b>	<b>152.1</b>	20	15
Pyridoxamine	169.1	134	20	25
5MTHF	<b>460.2</b>	<b>313.2</b>	70	18
5MTHF	460.2	194.2	70	33
<sup>13</sup> C <sub>4</sub> <sup>15</sup> N <sub>2</sub> -riboflavina	<b>383</b>	<b>249</b>	20	20
<sup>13</sup> C <sub>4</sub> -thiamine	<b>269.1</b>	<b>122</b>	35	13
<sup>13</sup> C <sub>6</sub> <sup>15</sup> N <sub>2</sub> -Pantothenate Ca	<b>224.2</b>	<b>94.1</b>	50	18
<sup>2</sup> H <sub>2</sub> -pyridoxine	<b>172.1</b>	<b>136</b>	50	21
<sup>2</sup> H <sub>4</sub> -nicotinic acid	<b>128.1</b>	<b>84</b>	75	18
<sup>13</sup> C <sub>5</sub> <sup>15</sup> N-Folic acid	<b>448.2</b>	<b>295.2</b>	70	15

## Results and Discussion

The LC-MS/MS conditions used in the AOAC Official Method 2015.14 for simultaneous determination of total

vitamins B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, and B<sub>6</sub> in infant formula and related nutritional<sup>8</sup> were used in this study with two minor modifications. The pH of the mobile phase A was adjusted to pH 5.0 to ensure consistent results. The MRM transitions and MS detection parameters for 18 vitamins, including the additional vitamins B<sub>5</sub>, B<sub>7</sub>, B<sub>9</sub>, and B<sub>12</sub> were optimized for the Xevo TQ-S micro MS System.

## Effects of the MaxPeak HPS on the Analysis of B vitamins

Two LC system setups were used in the comparison study of the effects of the MaxPeak HPS on the analysis of B vitamins. One comprised of an ACQUITY Premier System and an ACQUITY Premier BEH C<sub>18</sub> (1.7 μm, 2.1 x 100 mm) Column (referred to as the HPS setup), and the other one comprised of an ACQUITY UPLC H-Class PLUS System and an ACQUITY UPLC BEH C<sub>18</sub> (1.7 μm, 2.1 x 100 mm) Column (referred to as the SOP setup). These two LC systems were identical to each other except that the HPS setup had MaxPeak HPS while the SOP setup had conventional stainless-steel surfaces. The same Xevo TQ-S micro MS System was used in these two systems to minimize instrumental variables in the comparison study.

## Increased Response with the MaxPeak HPS

Higher peak intensities and larger peak areas were observed for majority of the 18 vitamins with the HPS setup. Figure 2 shows comparison plots of peak areas from 7 replicate injections of a standard mix solution (at a concentration of 1 μg/mL) with both LC setups. The data were obtained when the systems and columns were fresh, i.e., no B vitamins have ever been injected onto the systems. One can see that the same or greater peak areas were obtained with the HPS setup for all 18 vitamins than those with the SOP setup. Figure 3A shows comparison of the chromatograms of the FMN, Thiamine, PLP, and Pantothenic acid from the first injections of the same standard mix solution on both LC setups. The peak intensities of these compounds were significantly larger with the HPS setup. This large difference in LC-MS/MS response was attenuated after repeated injections of B vitamins (as the surfaces, especially the stainless steel surfaces, were conditioned by exposure to the analytes), but was still evident after extended use of the LC-MS System (see Figure 3B). Figure 3B shows a comparison of B vitamin chromatograms for a DS sample obtained on both LC setups. Over a hundred injections were already made on both LC setups when the chromatograms in Figure 3B were collected. Less difference in peak intensity for these B vitamins were observed, but the difference in peak height were still evident between the HPS and the SOP setups. Also, severe peak tailing was still observed for thiamine with the SOP setup (Figure 3B).

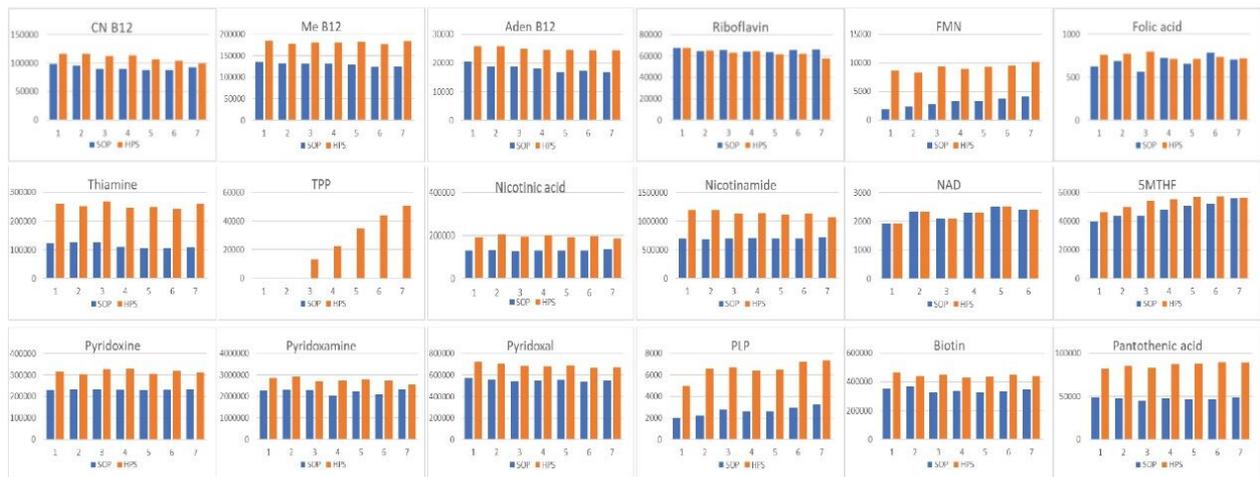


Figure 2. Comparison of LC-MS/MS peak areas of B vitamins and their vitamers with the HPS setup and the SOP setup. Peak areas from stainless steel surfaces (SOP, blue bar) are plotted side by side with those from MaxPeak HPS (HPS, orange bar).

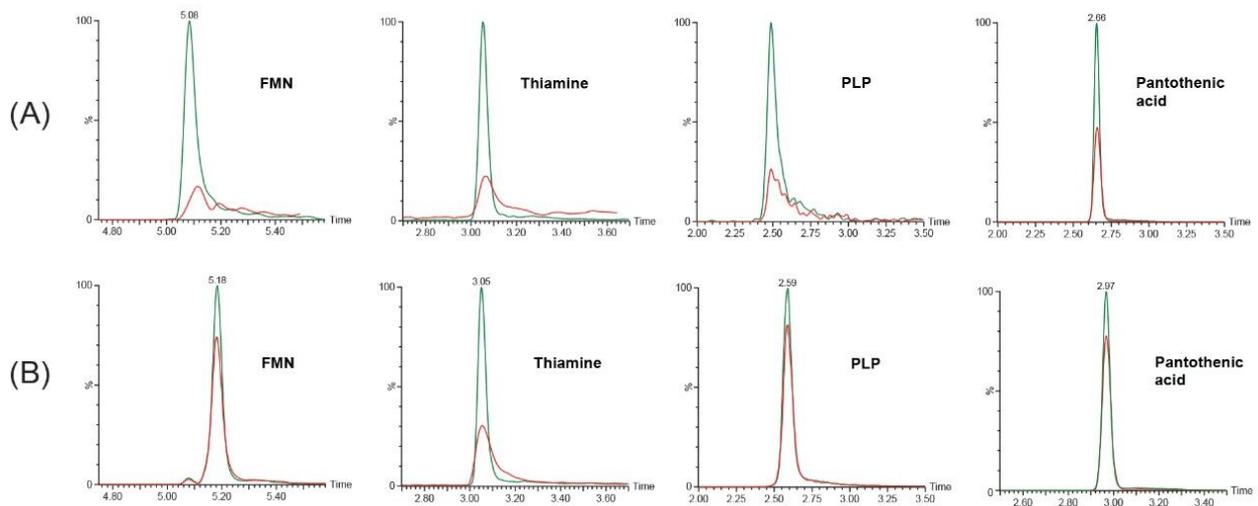


Figure 3. Comparison of LC-MS chromatograms of FMN, Thiamine, PLP, and Pantothenic acid obtained on the HPS setup (green traces) and the SOP setup (red traces). (A) Observed during the initial injections of the same standard mix on fresh LC systems. (B) Observed in the B vitamin analysis of the same DS sample on LC systems that have been extensively used.

## Improved Sensitivity with the MaxPeak HPS

Higher sensitivity was also found with the HPS setup than the SOP setup in the LC-MS/MS analysis of B

vitamins. The limit of quantitation (LOQ) was estimated using the working standard mix solutions at signal to noise (S/N) ratio of 10. Table 1 shows the LOQ values and the calibration results obtained with both LC setups for the simultaneous B vitamin analysis for ED and DS samples. The LOQ with the HPS setup shows the same or better LOQs than those with the SOP setup. Six B vitamins show 3–10 times improvement in LOQ with the HPS setup. Better calibration linearity ( $R^2$ ) was also observed with the HPS setup (Table 1).

Vitamins	LOQ (ng/mL)	Range (ng/mL)	$R^2$	LOQ (ng/mL)	Range (ng/mL)	$R^2$	Order of polynomial fitting	I.S.
	HPS			SOP				
Cyanocobalamin	10	10–3,000	0.99	10	10–3,000	0.97	1st	None
Methylcobalamin	10	10–10,000	0.995	10	10–10,000	0.99	1st	None
Riboflavin	3	3–10,000	0.9993	10	10–10,000	0.998	1st	$^{13}\text{C}_4\text{ }^{15}\text{N}_2$ - Riboflavin
Thiamine	3	3–1,000	0.9991	10	10–1,000	0.99	1st	$^{13}\text{C}_4$ - Thiamine
Biotin	3	3–3,000	0.9991	3	3–3,000	0.99	1st	None
Pantothenic acid	10	10–10,000	0.99	10	10–10,000	0.99	1st	$^{13}\text{C}_3\text{ }^{15}\text{N}$ - Pantothenic acid
Pyridoxine	1	1–1,000	0.998	1	1–1,000	0.998	1st	$^2\text{H}_2$ - Pyridoxine
Nicotinic acid	30	30–10,000	0.993	30	30–10,000	0.99	2nd	$^2\text{H}_4$ - Nicotinic acid
Nicotinamide	10	10–3,000	0.997	30	30–10,000	0.99	2nd	$^2\text{H}_4$ - Nicotinic acid
Folic acid	100	100–10,000	0.99	100	100–10,000	0.93	1st	$^{13}\text{C}_5\text{ }^{15}\text{N}$ - Folic acid
FMN	100	100–100,000	0.995	300	300–30,000	0.95	1st	None
PLP	100	100–30,000	0.991	300	300–3,000	0.96	2nd	$^2\text{H}_2$ - Pyridoxine
Pyridoxal	3	3–300	0.9993	3	3–300	0.99	2nd	$^2\text{H}_2$ - Pyridoxine
5-M-THF	10	10–10,000	0.9991	100	100–3,000	0.98	1st	None

Table 1. LOQ and calibration results for B vitamins with two LC setups.

## No Carry-over with MaxPeak HPS

Figure 4 shows a comparison of the B vitamins LC-MS/MS chromatograms obtained with the HPS and the SOP system setups in a carry-over study. Residual peaks were found in the blank injection on the SOP system setup for riboflavin, pyridoxal, 5MTHF, and MeB<sub>12</sub> while no residual peak was found on the HPS system setup.

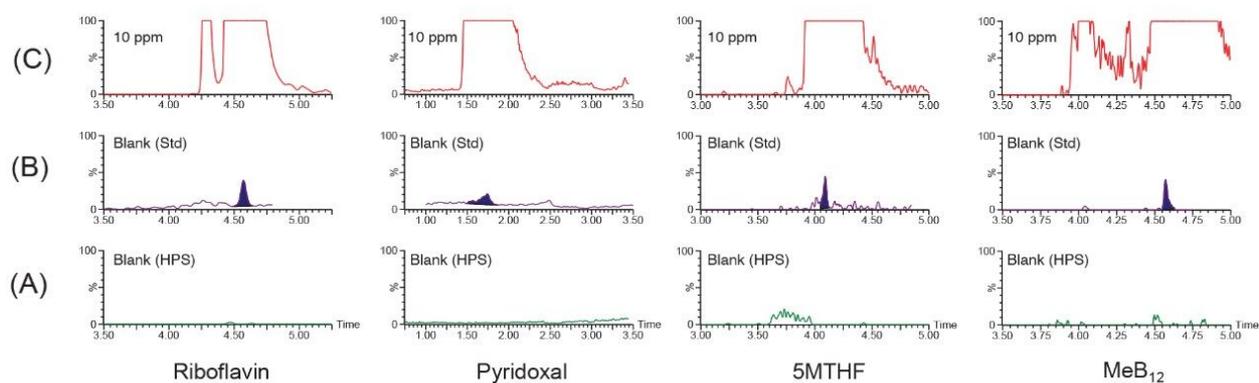


Figure 4. Comparison of LC-MS chromatograms of blank injections obtained with HPS setup (A) and with SOP setup (B) right after the injections of a 10 ppm standard solution (C) for riboflavin, pyridoxine, 5-methyl-THF, and methylcobalamin. Small residual peaks were observed in the SOP system setup (B) for these four vitamins at 0.03–0.1% level of the 10 ppm peaks (C). No residual peak was observed with the HPS system (A).

### Analysis of Energy Drink and Dietary Supplement Samples

The B vitamins in ED and vitamin B complex DS were analyzed using the HPS setup coupled with the Xevo TQ-S micro MS System. Figure 5 and 6 show the B vitamin chromatograms for ED and DS. Not all 18 B vitamins were formulated in these samples, only those B vitamins present in the samples were analyzed.

# Energy drink

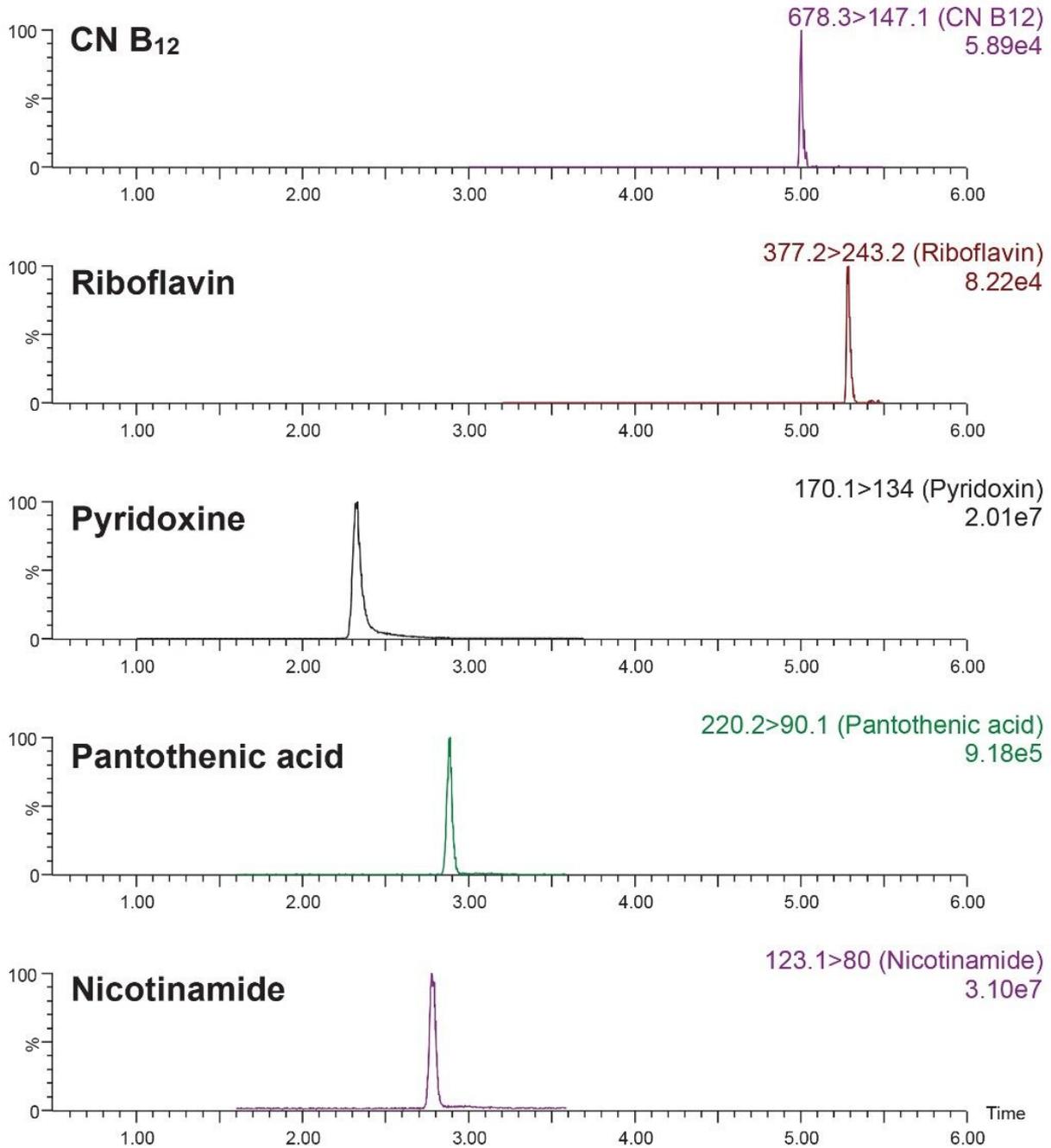


Figure 5. Chromatograms of B vitamins in an Energy Drink sample.

# Dietary supplement

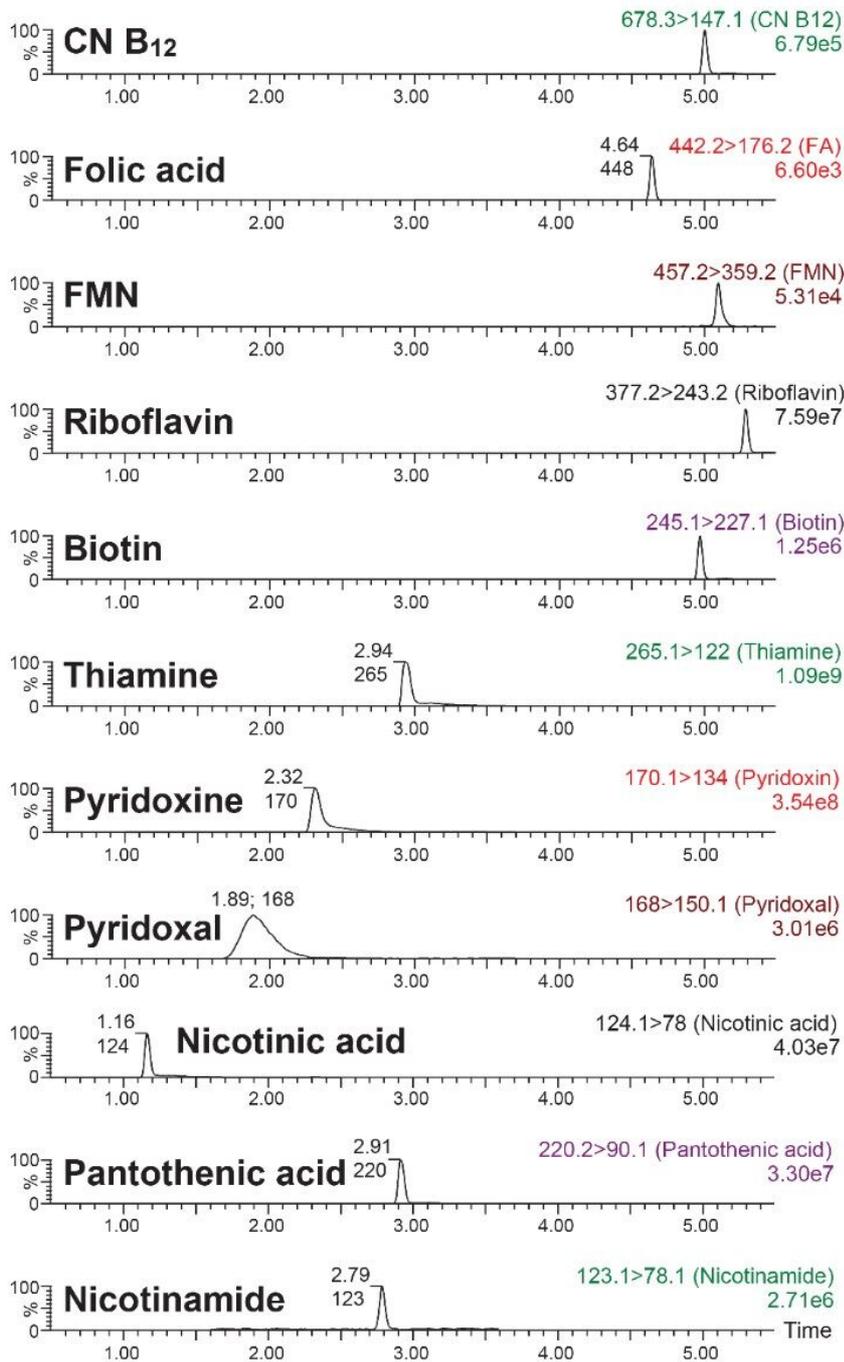


Figure 6. Chromatograms of B vitamins in a vitamin B complex dietary supplement sample.

Table 2 shows the analysis results and the labeled or declared values of B vitamins in ED samples. The accuracy of the LC-MS/MS analysis was assessed by spiking experiment on ED1 sample. Recovery of 91% to

107% were obtained for these B vitamins. Comparing to the labeled values, the determined B vitamin values are in the range of 90% to 124% of the labeled values for ED1 and ED2, and 138% to 180% for ED3. Normally, 25–50% overage in fortified nutrients in food products is commonly used. The relative high B vitamin contents in ED3 indicates excess overage in this product. The analysis repeatability were below 7% RSD for most B vitamins, except that higher RSD (up to 17%) for cyanocobalamin and one result (11%) for nicotinamide in ED2. The high RSD for cyanocobalamin can be explained by the extremely low concentration of cyanocobalamin (near LOQ) in ED1 and ED3. Table 3 shows the analysis and spiking experiment results for a vitamin B complex DS. Excellent recovery for 12 vitamins were obtained (78%–122%). The repeatability of these B vitamins was within 7% RSD in general.

Sample		Cyanocobalamin (B <sub>12</sub> ) (µg/serving)	Riboflavin (B <sub>2</sub> ) (mg/serving)	Pantothenic acid (B <sub>5</sub> ) (mg/serving)	Pyridoxine (B <sub>6</sub> ) (mg/serving)	Nicotinamide (B <sub>3</sub> ) (mg/serving)
ED1	Labeled value	2.88	—	3.5	5.96	22.4
	Assay	3.59	0.23	4.97	7.03	23.54
	RSD (%) (n=4)	10%*	7%	1%	3%	3%
	Assay/Labeled value	124%	—	142%	118%	105%
	Spiking amount	2.14	0.18	15.66	1.49	20.84
	Recovery (%)	96%	103%	95%	107%	91%
ED2	Labeled value	12	3.38	—	4.08	40
	Assay	10.8	3.89	—	4.43	43.3
	RSD (%) (n=3)	1%	3%	—	3%	11%
	Assay/Labeled value	90%	115%	—	109%	108%
ED3	Labeled value	2.4	1.3	5	1.7	16
	Assay	3.78	1.79	8.99	2.74	22.75
	RSD (%) (n=3)	17.4%*	5%	4%	7%	5%
	Assay/Labeled value	158%	138%	180%	161%	142%

Note: \*: The B<sub>12</sub> contents were at low concentrations, about 3xLOQ level.

Table 2. Analysis and spiking recovery results for energy drinks.

Sample	DS						
	Labeled value	Assay (average)	RSD (%) (n=3)	Spiking amount	Assay (after spiking)	RSD (%) (n=3)	Recovery (%)
Cyanocobalamin (B <sub>12</sub> ) (µg/serving)	1000	2124	7.00%	378	2453	6.90%	87%
Methylcobalamin (B <sub>12</sub> ) (µg/serving)	0	0	—	347	270	13%	78%
Folic acid (B <sub>9</sub> ) (µg/serving)	0	0	—	355	323	0.40%	91%
5MTHF (B <sub>9</sub> ) (µg/serving)	0	0	—	400	374	1.40%	94%
FMN (B <sub>2</sub> ) (mg/serving)	1.7	2.86	10%	0.8	3.73	4.30%	107%
PLP (B <sub>6</sub> ) (mg/serving)	0	0	—	44	54	3.10%	122%
Riboflavin (B <sub>2</sub> ) (mg/serving)	0	0.159	9.50%	0.43	0.57	1.30%	95%
Thiamine (B <sub>1</sub> ) (mg/serving)	0	0	—	31	29	5.80%	92%
Biotin (B <sub>7</sub> ) (µg/serving)	0	0	—	441	448	5.20%	102%
Pantothenic acid (B <sub>5</sub> ) (mg/serving)	30*	0	—	36	44	6.80%	120%
Pyridoxine (B <sub>6</sub> ) (mg/serving)	2	2.53	4.20%	4.03	7.09	2.10%	113%
Nicotinamide (B <sub>3</sub> ) (mg/serving)	20	25	1.00%	—	—	—	—
Nicotinic acid (B <sub>3</sub> ) (mg/serving)	0	0	—	40	39	4.40%	98%

Note: \*B<sub>5</sub> as D-Panthenol.

Table 3. Analysis and spiking recovery results for a vitamin B complex dietary supplement.

## Conclusion

This application note investigated the effects of the MaxPeak HPS on the LC-MS/MS analysis of 18 B-group vitamins and showed lasting benefits of using MaxPeak HPS over conventional stainless-steel surfaces for the B vitamins analysis. The key benefits that were found in the LC-MS/MS analysis of B vitamins include higher responses, less peak tailing, improved sensitivity, better calibration linearity, and less carry-over. These benefits were significant at the initial instrument use stage. After extensive use of the LC system and column, the difference between MaxPeak HPS and convention stainless-steel surfaces became less significant but was still evident as observed in the analysis of B vitamins in commercial ED and vitamin B complex DS. Method validation with ED and DS samples showed excellent accuracy and repeatability. The Waters ACQUITY Premier Solution exhibited clear advantages over conventional LC solution in the sensitivity, accuracy, and precision for the analysis of B-group vitamins.

## References

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