APPLICATION NOTE

Liquid Chromatography/ Mass Spectrometry

Authors:

Wilhad M Reuter

Sharanya Reddy

Avinash Dalmia

PerkinElmer, Inc.
Shelton, CT

Analysis of Water-Soluble Vitamins in Infant Formula by UHPLC-MS/MS

Introduction

Water-soluble vitamins (WSV), comprised primarily of the vitamin B complex, are essential ingredients in many foods, particularly in infant formulas. These vitamins play key factors in metabolic pathways and, therefore, impart significant health benefits when included in our daily diet.

As there are human daily nutritional recommendations for these vitamins established by the Food and Drug Administration (FDA) food and supplement manufacturers, as well as independent testing labs, need to be able to quantitatively verify the vitamin content in such products. When analyzing fortified foods, this can be particularly challenging due to the wide range in concentration of vitamins, in keeping with daily allowances (see Table 1). For instance, in infant milk, vitamin B12 (cyanocobalamin) is present at 1-2 ppb while vitamin B2 (riboflavin) is present at 1000-times higher concentration (1-2 ppm). Therefore, any quantitative analytical procedure must be able to accommodate this wide spread in concentration.

PerkinElmer®
For the Better

Table 1. Daily required value (DV) for each of the analyzed vitamins.

Vitamin	DV (daily required value; mg; per FDA guideline[1])
B3 (niacin)	20
B6	2
B3* (niacinamide)	Not available
B1	1.5
B9	0.4
B7	0.3
B12	0.006
B2	1.7

We present an LC-MS/MS method for the quantitative analysis of B-vitamins in a single run. The analyzed vitamins included vitamin B1 (thiamine), B2 (riboflavin), B3 (niacin), B3* (niacinamide), B6 (pyridoxine), B7 (biotin), B9 (folic acid) and vitamin B12 (cyanocobalamin), using three internal standards. A simple liquid-liquid extraction was used for extracting vitamins from infant formula. The sample extraction coupled with the fast analytical method (<6 mins) was found to be robust/reliable and the least time consuming.

Experimental

Hardware/Software

For the chromatographic separations, a PerkinElmer Altus™ UPLC® System was used, including the Altus A-30 Solvent/Sample Module, integrated vacuum degasser and column heater. For detection, a PerkinElmer QSight™ 210 MS/MS detector was used. All instrument control, analysis and data processing was performed using the Simplicity 3Q™ software platform.

Method Parameters

The LC and MS/MS method parameters are shown in Tables 2 and 3, respectively.

Solvents, Standards and Sample Preparation

All solvents, reagents and diluents used were HPLC-grade and filtered via 0.22-µm nylon filters.

For all dilutions, 5-mM ammonium formate, adjusted to pH 4.9 with 10% formic acid, was used.

All B-vitamin standards, including B1 (thiamine), B2 (riboflavin), B3 (niacin), B3* (niacinamide), B6 (pyridoxine), B7 (biotin), B9 (folic acid) and B12 (cyanocobalamin) were obtained from Sigma-Aldrich® Inc., Saint-Louis, MO.

For calibration and quantitation purposes, three internal standards were used: vitamins B1′ (thiamine; $^{13}C_4C_8H_{17}N_4OS^+$), B7′ (biotin; $C_{10}D_2H_{14}N_2O_3S$) and B2′ (riboflavin; $^{13}C_4C_{13}H_{20}{}^{15}N_2N_2O_6$), all obtained from Sigma-Aldrich® Inc. B1′ was used for calibrating B3, B3*, B6 and B1, while B7′ was used for B7, B9 and B12, and B2′ was used for B2.

The analyzed samples included two commercially-obtainable infant formula powders, labeled IF1 and IF2.

Table 2. LC Method Parameters.

Column	PerkinElmer Altus UPLC® BEH C18, 1.7 µm, 2.1 x 50-mm (Part# N2972000)
Mobile Phase	Solvent A: 5 mM ammonium formate, pH to 4.9 with formic acid Solvent B: Acetonitrile (ACN)

	Time (min)	Flow rate (mL/min)	%A	%B	Curve
1	Initial	0.5	100.0	0.0	
2	1.60	0.5	100.0	0.0	6
3	4.50	0.5	70.0	30.0	6
4	5.00	0.5	50.0	50.0	6
5	6.00	0.5	50.0	50.0	6
6	6.05	0.5	100.0	0.0	6

Analysis Time	6 min; re-equilibration time: 4 min
Pressure	6900 psi/460 bar (maximum)
Oven Temp.	40 °C
Injection Volume	3 µL

Table 3. MS/MS Parameters.

Ionization Mode:	ESI - positive				
Drying Gas (Nitrogen)	75	HSID Temp	320 °C;	Electrospray V1	5000 V

Exper. Group 1 (0.20 – 0.85 min)	MRM Transitions (amu)				
	Quantifier Ion	Qualifier Ion	EV	CE(V)	Dwell Time (msec)
B3 (niacin)	124.3/80.2	124.3/53.2	55	-30	100

Exper. Group 2 (0.7 – 2.5 min)	MRM Transitions (amu)				
	Quantifier Ion	Qualifier Ion	EV	CE(V)	Dwell Time (msec)
B1 (thiamine)	265.0/121.7	265.0/144.0	20	-25	50
B1′ (thiamine; $^{13}C_4C_8H_{17}N_4OS^+$)	269.0/122.0		20	-25	50
B3* (niacinamide)	123.3/80.2	123.3/53.2	25	-30	50
B6 (pyridoxine)	170.3/133.7	170.3/105.8	22	-28	50

Exper. Group 3 (3.0 – 4.4 min)	MRM Transitions (amu)				
	Quantifier Ion	Qualifier Ion	EV	CE(V)	Dwell Time (msec)
B7 (biotin)	245.0/96.4	245.0/104.5	24	-45	35
B7′ (biotin; $C_{10}D_2H_{14}N_2O_3S$)	247.0/98.7		24	-45	35
B9 (folic acid)	442.1/295.2	442.1/176.0	21	-22	35
B12 (cyanocobalamin):	678.9/147.2	678.9/399.2	23	-45	35
B2 (riboflavin)	377.0/172.3	377.0/198.2	35	-48	35
B2′ (riboflavin; $^{13}C_4C_{13}H_{20}{}^{15}N_2N_2O_6$)	383.0/175.4		35	-48	35

EV = Entrance voltage; **CE(V)** = Collision energy

To guard against possible standard or sample instability, all stock and working standards were stored under refrigeration until used; all prepared samples were analyzed within four hours and only amber 2-mL LC vials were used.

All calibrants and samples were filtered via 0.22-µm nylon filters.

Experimental

Standard Preparation

A 40-µg/mL stock standard of B2, B9 and B7 was prepared in a 250-mL volumetric flask. As these three vitamins are best dissolved under basic conditions, 50 mL of 0.05% ammonium hydroxide (NH_4OH) was first added to the flask, which was shaken until the standards were thoroughly dissolved. The flask was then filled to mark with diluent.

A 40-µg/mL stock standard of B3, B3*, B6 and B1 and a 5-µg/mL stock standard of B12 were prepared using straight diluent.

For the working standard, 25 mL of each of the three stock solutions plus 25 mL of diluent were added to a 100-mL volumetric flask. After being shaken, the flask was then stored under refrigeration. This working standard also served as the level-6 calibrant, containing 10 µg/mL of each vitamin, except B12, which was at 1.50 µg/mL.

Internal standard stock solutions (ISTDs) were prepared by adding the entire contents of each isotopic vitamin standard, as follows: 2 mg of B1′, 5 mg of B7′ and 1 mg of B2′ were transferred to a 20-mL, 250-mL and 10-mL volumetric flask, respectively. To aid in dissolution, the B1′ and B2′ standards were initially transferred to each flask via 5x rinses of 1 mL 0.05% NH_4OH. All three flasks were then filled to mark with diluent. The resulting stock concentrations were 100 µg/mL for B1′ and B2′ and 20 µg/mL for B7′.

Six calibration levels were prepared by serial dilution of the working standard. The resulting vitamin concentrations are provided in Table 4. Vitamin B12 was calibrated using a much lower concentration range due to its significantly lower DV guideline (0.006 mg). Before running each batch, 50 mL of each calibrant level was transferred to a 50-mL volumetric flask and then spiked to 0.1 µg/mL with internal standards by adding 50 µL of ISTDs B1′ and B2′ and 250 µL of ISTD B7′. Note: If less than 50 mL of any calibrant level is available, the ISTDs should be added proportionate to the available volume. All calibrants were injected in triplicate.

Sample Preparation

The two infant formula powders (labeled IF1and IF2) were prepared by first weighing out the required amount of each in a tared 50-mL centrifuge tube and then adding 20 mL of diluent. The required amount was determined from the recommended proportion of powder-to-water provided in the label claim for each product. Each centrifuge tube was pulse-vortexed for 15 minutes, whereupon the solutions were spiked to 0.2 µg/mL with internal standards by adding 40 µL of ISTDs B1′ and B2′ and 200 µL of ISTD B7′. The samples were spiked to 0.2 µg/mL ISTD to compensate for the 2-fold dilution during the following sample preparation procedure.

Table 4. Vitamin B concentrations at each calibration level.

Calibration Level	Conc. of B1, B3, B3*, B6, B7, B9 and B2 (µg/mL)	Conc. of B12 (µg/mL)
1	0.004	0.0005
2	0.02	0.0025
3	0.10	0.0125
4	0.40	0.050
5	2.00	0.250
6	10.00**	1.250

** Level 6 was not used for B1, B3, B6 and B9.

20 mL of acetonitrile, acidified with 10 µL of 10% formic acid, was then added to each tube. This was followed by pulse-vortexing for five minutes and centrifugation at 7800 rpm for 10 minutes. The addition of the acidified ACN caused a protein crash/precipitation, which allows one to remove the fatty/solid material via centrifugation. 10.0 mL of the supernatant was then carefully transferred from each of the three tubes to separate 50-mL centrifuge tubes and dried down to ~1 mL. Each tube was then filled to the 10-mL mark with diluent, pulse-vortexed for three minutes and centrifuged at 7800 rpm for five minutes. 1 mL of each supernatant was filtered into a 2-mL vial. All samples were injected in triplicate.

Results and Discussion

Using the prescribed method parameters, Figure 1 shows an overlay of 12 replicates of the combined quantifier MRMs of the level-5 calibrant. All eight vitamins are well resolved, eluting in less than four minutes, with the 12-replicate overlays demonstrating excellent chromatographic reproducibility.

Figure 2 shows examples of the calibration results for vitamins B1 and B12, exhibiting exceptional fits (R^2 = ≥ 0.999; n=3). A 6-level calibration was used for vitamins B2, B3*, B7 and B12. For vitamins B1, B3, B6 and B9, a 5-level calibration was used. All eight vitamins had calibration fits > 0.996.

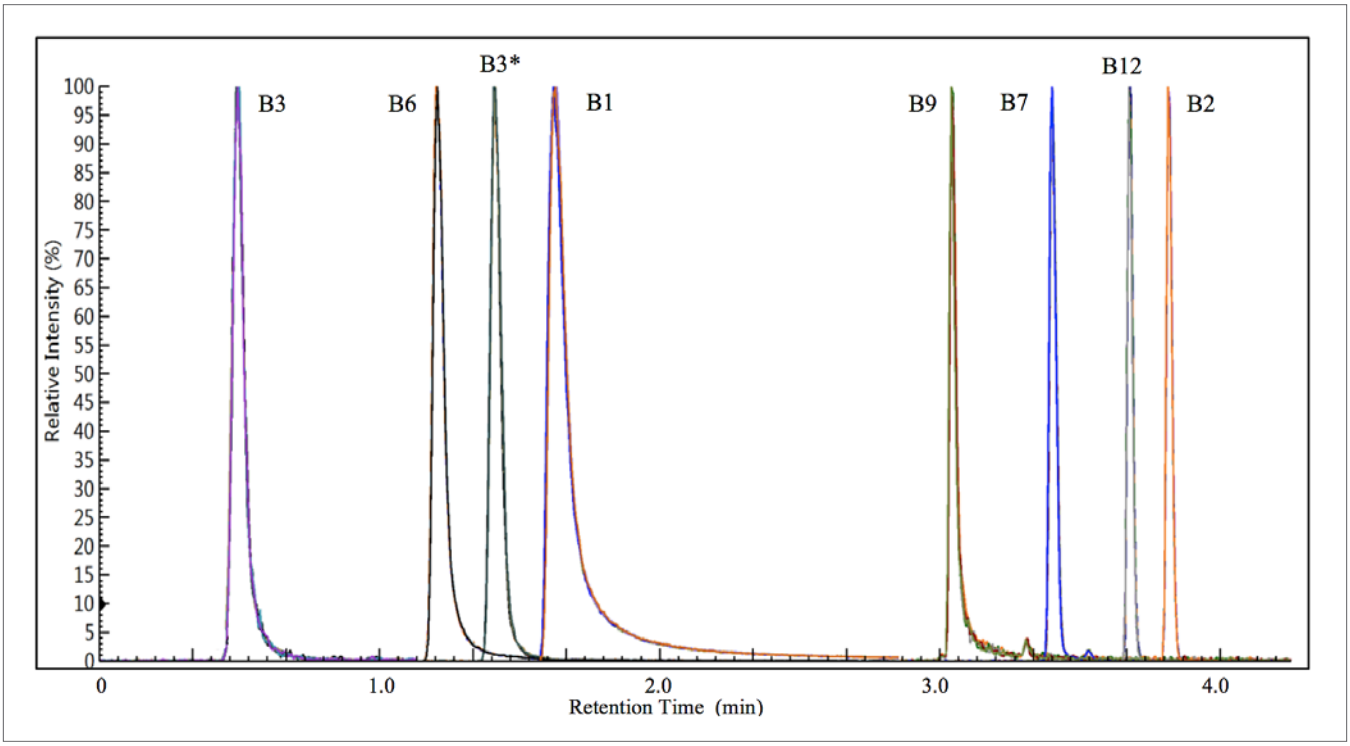

Figure 1. 12 replicate overlays of the combined quantifier MRMs of the level-5 calibrant.

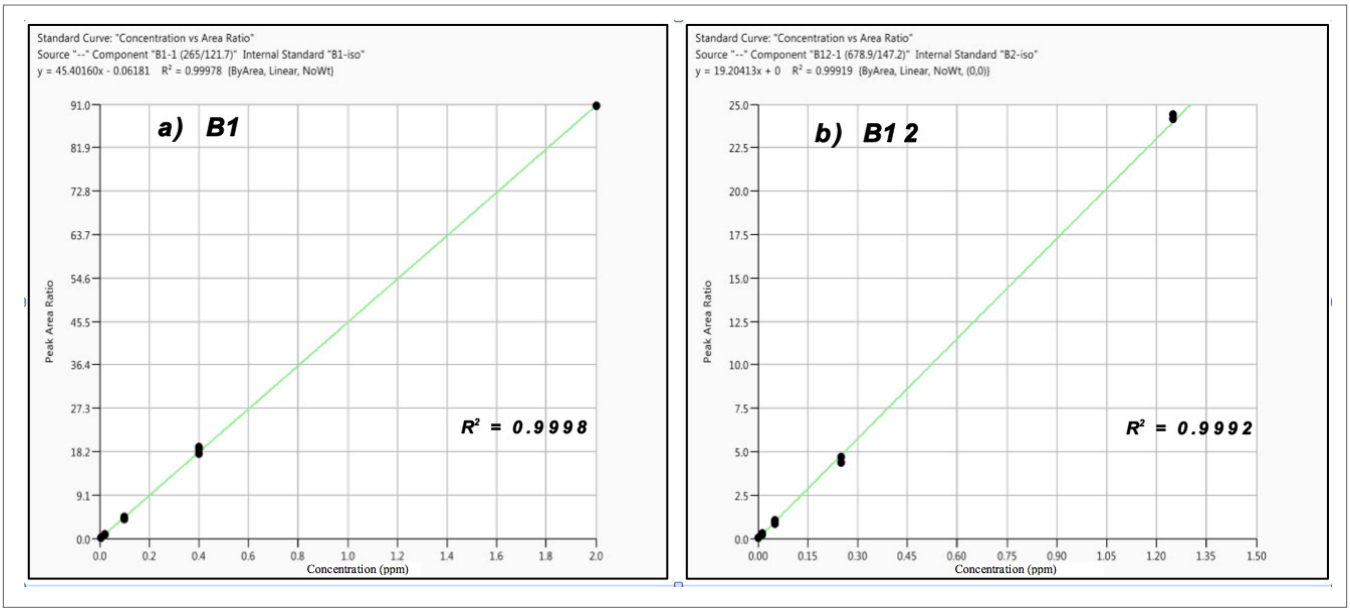

Figure 2. Calibration results for Vitamins B1 (a) and B12 (b); n= 3 at each level.

Upon injection of a diluent blank right after triplicate injections of the level-6 calibrant, no detectable carryover was observed for any of the eight vitamins. An example of carryover test results is shown for vitamins B1and B12 in Figure 3.

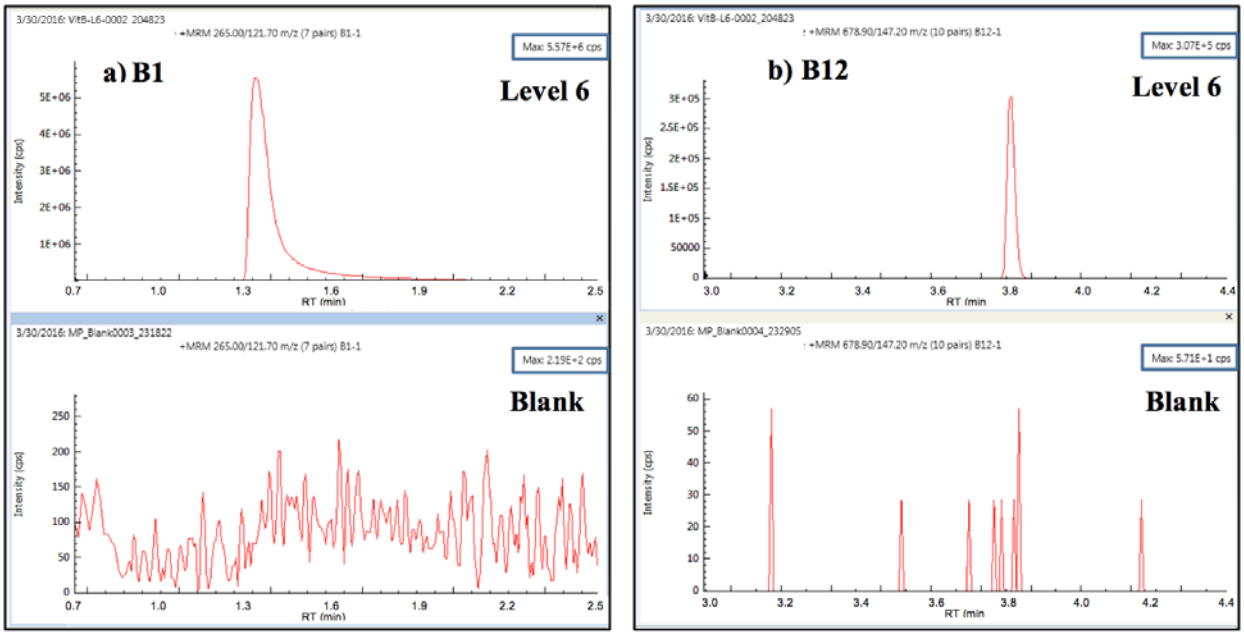

Figure 3. Carryover test MRM for vitamins B1 and B12: 3 replicate injections of level-6 calibrant followed by diluent blank.

Figure 4 shows the quantifier MRMs at the lowest quantitatable level for each analyte. Vitamins B1, B2, B3* and B6 were all quantitatable down to 0.004 µg/ml (4 ppb) and vitamin B12 was quantitatable down to 0.0005 µg/ml (0.5 ppb). Vitamins B3 and B9 were quantitatable down to 0.02 µg/ml (20 ppb). All the analyzed vitamins were easily quantitatable within the expected guidelines set forth by the FDA and the quantitatable limits (LOQ or IDL) for vitamins B1, B6 and B2 are likely to be even lower.

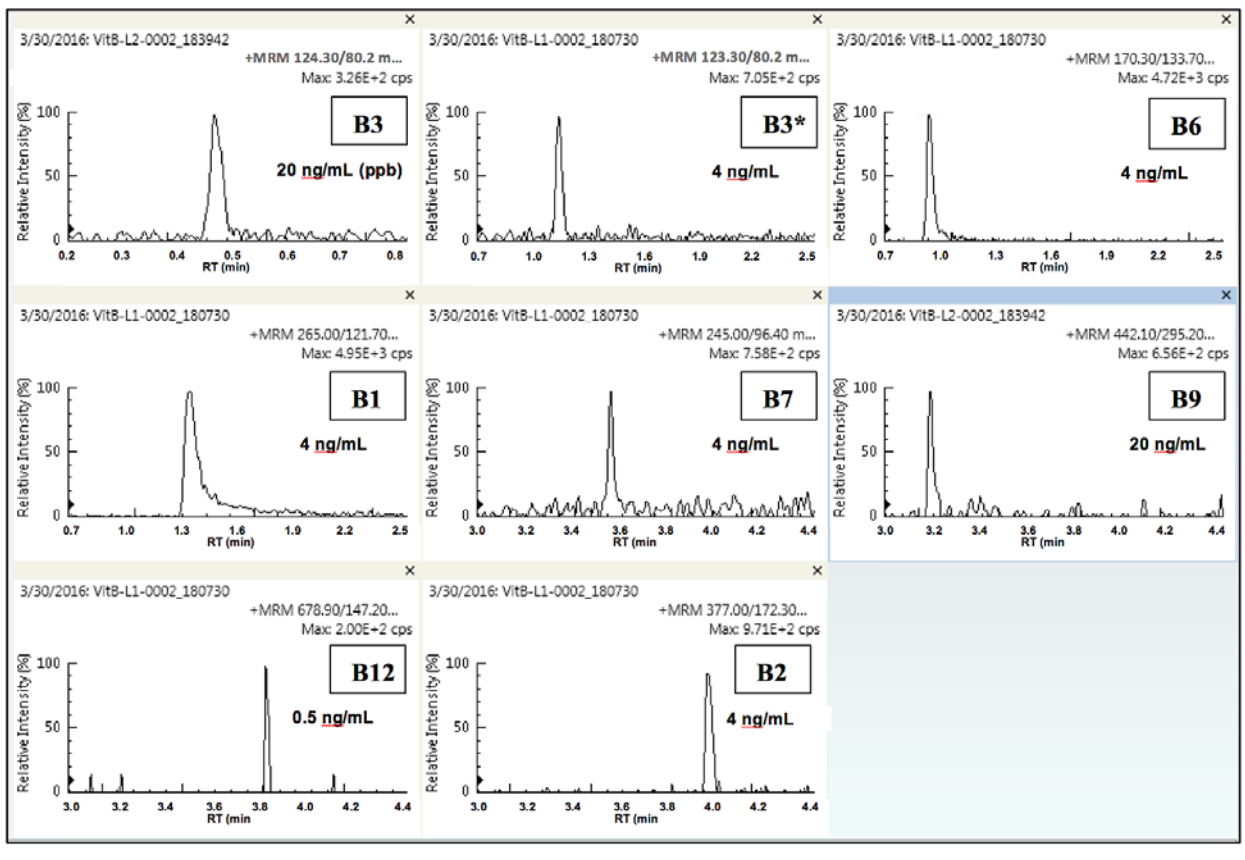

Figure 4. Quantifier MRMs of lowest quantitated level for each of the eight vitamins.

Two store-bought infant formula powders, IF1 and IF2, were analyzed for the eight B-vitamins. Each sample was prepared as described above and injected in triplicate. Figures 5-6 show the individual quantifier MRMs for each vitamin found in the prepared infant formula samples. The two sample profiles look quite similar, which was to be expected considering that the label claims are quite similar for this type of product.

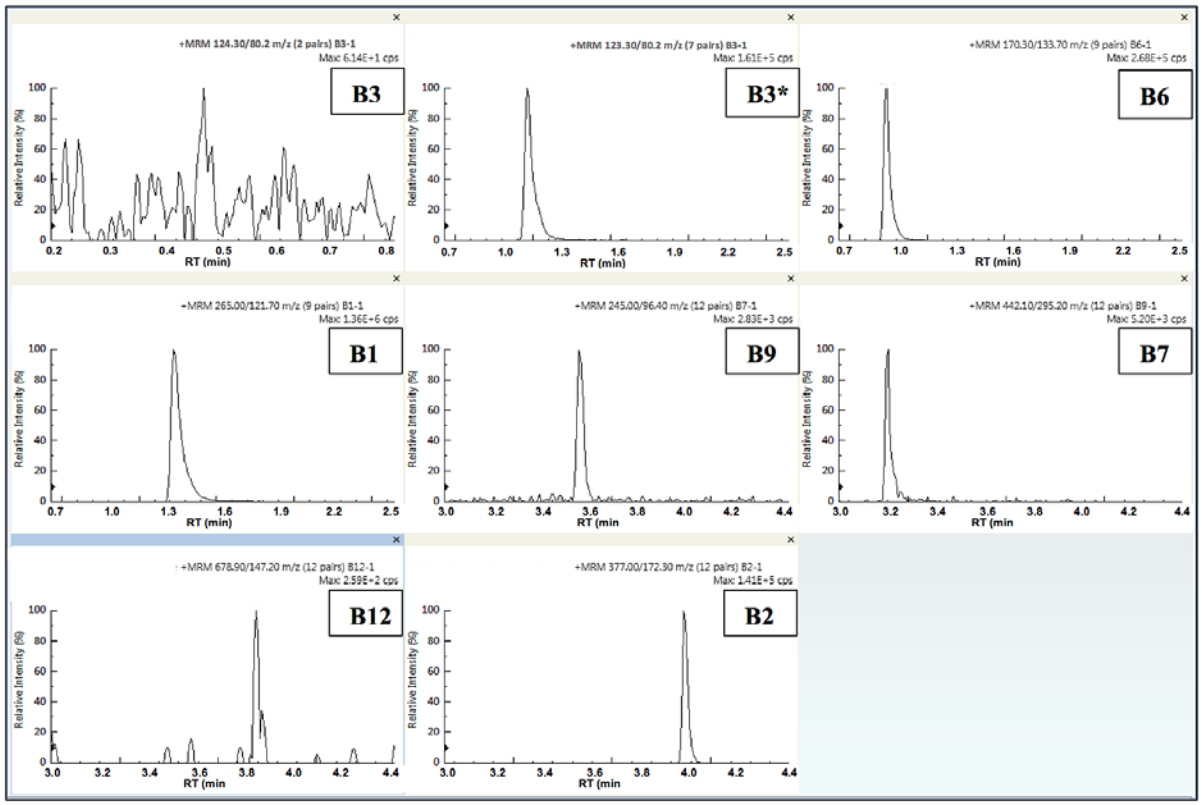

Figure 5. Quantifier MRMs for the 8 B-vitamins in IF1.

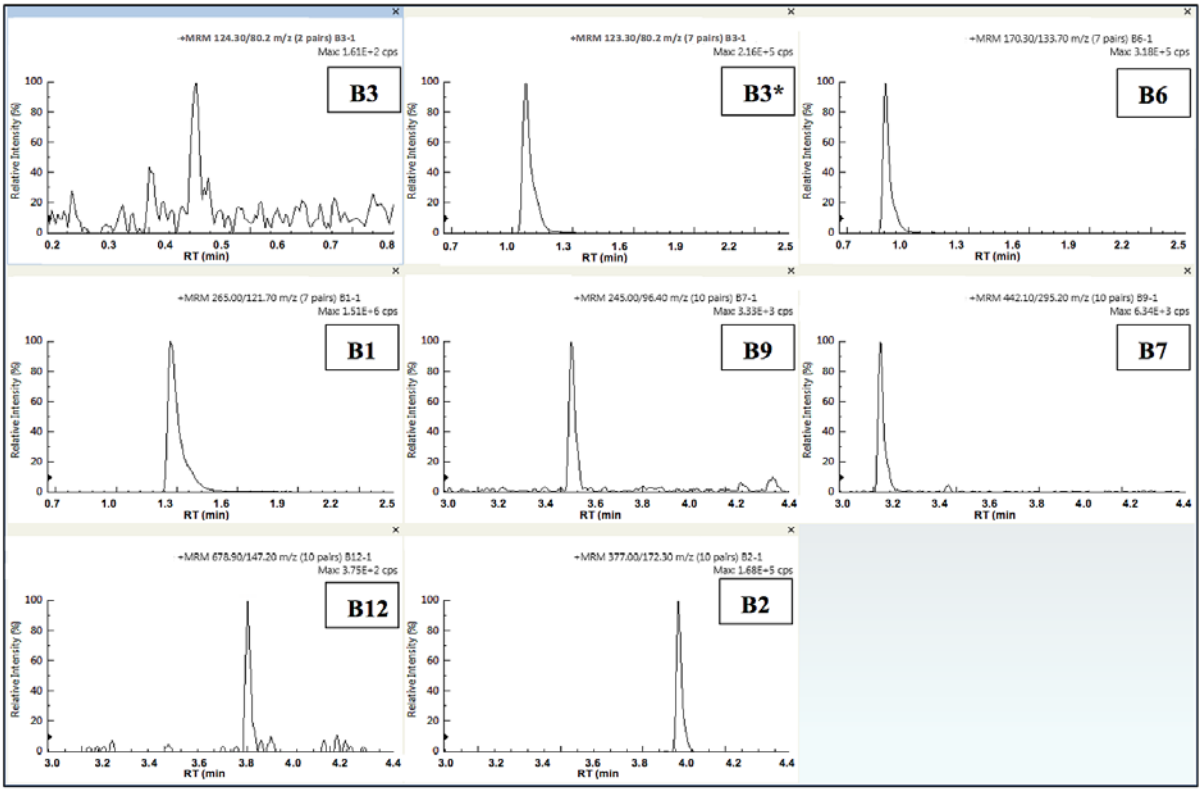

Figure 6. Quantifier MRMs for the 8 B-vitamins in IF2.

Sample reproducibility is demonstrated in Figure 7, showing the replicate MRMs for vitamins B2 and B12 in IF2. The reproducibility at the very low 1-2 ppb level of vitamin B12 was particularly impressive.

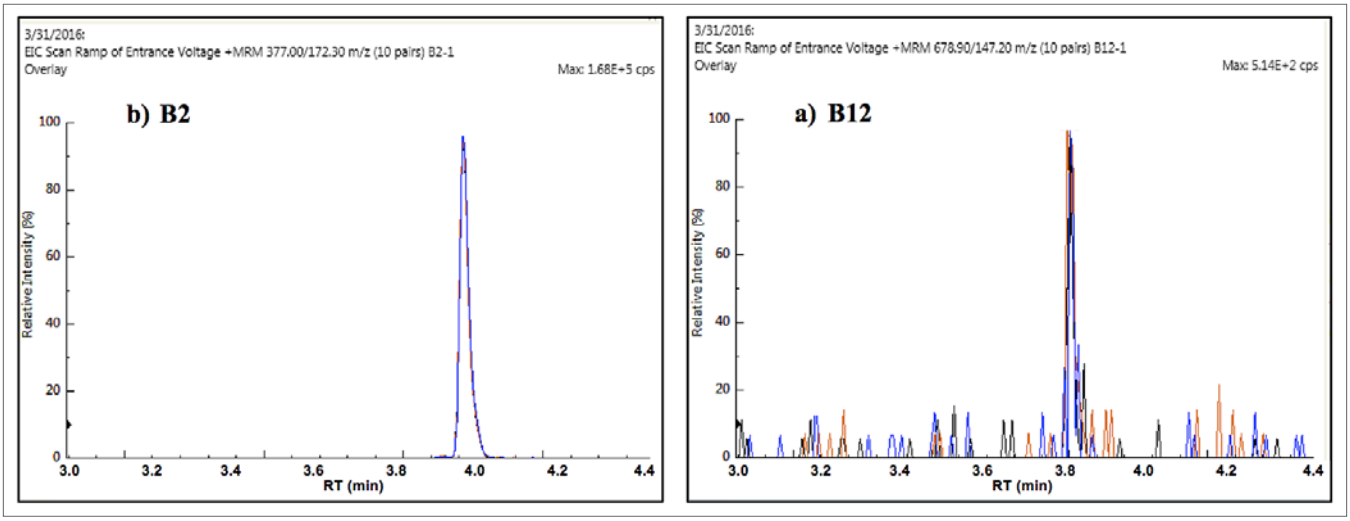

Figure 7. Replicate quantifier MRMs for vitamin B2 (a) and B12 (b) in IF2.

As shown in Table 5, apart from vitamin B1in IF1, the calculated concentrations for the B-vitamins having a clear label claim were all within an acceptable target range for both infant formulas. The specific label claim values for B3 (niacin) and B3* (niacinamide) were not available as these two vitamins are typically reported as a combined group under "B3" or just "niacin". These two vitamins are also known to inter-convert, making quantitative comparisons difficult.[2] The low value for vitamin B1 in IF1 may actually be real, as the value for IF2 was close to label claim, even on the high side, suggesting good vitamin B1 recovery expectations.

Overall, the results support very good recovery performance following the provided sample preparation procedure for infant formula.

To provide additional analyte confirmation, qualifier/quantifier ion ratios were also determined. As shown in Table 6, the ion ratios for the quantitatable vitamins found in both infant formulas were all found to be within, and most well within, the tolerance window (± 20%) of the ion ratios calculated for standards. Niacin (B3) was found at too low a concentration to be ratioed, as any vitamin B3 that was found was predominantly niacinamide (B3*).

Table 5. Quantitative results for B-vitamins found in IF1 and IF2, as compared to label claim; n= 3.

Vitamin	RT (min)	IF1			IF2		
B3	0.457	NA	0.020	--	NA	0.029	--
B6	0.904	0.450	0.42	- 6.7	0.405	0.30	- 25.9
B3*	1.097	NA	2.56	--	NA	2.35	--
B1	1.305	1.50	0.80	- 47	0.643	0.70	+ 8.9
B9	3.180	0.195	0.17	- 12.8	0.103	0.13	+ 20.1
B7	3.540	0.038	0.040	+ 5.3	0.030	0.033	+ 10.0
B12	3.814	0.002	0.002	0.0	0.002	0.002	0.0
B2	3.954	1.50	1.61	+ 7.3	1.029	0.98	- 4.8

** Average of three replicates

Table 6. Analyte ion ratios for IF1 and corresponding standard levels.

Vitamin	MRMs used for Ion Ratio (amu)	Ion Ratio		% Difference (±)
		IF1	Standard	
B3*	123.3/53.2 123.3/80.2	0.165	0.158	+ 4.4
B6	170.3/105.8 170.3/133.7	0.046	0.045	+ 2.2
B1	265.0/144.0 265.0/121.7	0.241	0.241	0.0
B9	442.1/176.0 442.1/295.2	0.473	0.478	- 1.0
B7	245.0/104.5 245.0/96.4	0.692	0.752	- 7.8
B12	678.9/399.2 678.9/147.2	0.773	0.642	+ 20
B2	377.0/198.2 377.0/172.3	0.802	0.773	+ 3.8

Conclusions

The results obtained confirm the applicability of an LC-MS/MS method for the efficient, routine and robust chromatographic analysis of B-vitamins in infant formula. This was accomplished using a single MS method to identify and quantitate the eight water soluble vitamins over a wide concentration range in under four minutes. The results showed excellent retention time repeatability and the method was able to detect vitamin B12 at 0.5 ppb, well below the expected level of ~2 ppb in infant formulas.

References

1. U.S. Food and Drug Administration (FDA), Guidance for Industry: A Food Labeling Guide (14. Appendix F), http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/labelingnutrition/ucm064928.htm
2. Nick Byrd, Campden BRI, United Kingdom

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

PerkinElmer®

012898_01 PKI