APPLICATION NOTE

Liquid Chromatography/ Mass Spectrometry

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Analysis of Water-Soluble Vitamin Formulations with Significant Phosphate Levels 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),[1] 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 widely ranging concentrations of vitamins, in keeping with the daily required values (DV), as shown in Table 1. For instance, while the DV for B3 (niacin) is 20 mg, it is far lower (0.006 mg) for vitamin B12 (cyanocobalamin). Therefore, any quantitative analytical procedure must be able to accommodate this wide spread in concentrations.

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Table 1. Daily WS vitamin allowances, per FDA guideline.

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

For vitamins present in phosphate-containing matrices, it makes the analysis even more demanding, considering that such non-volatile matrix components are quite undesirable when using MS detectors. What makes this is particularly challenging is the inherent difficulty in performing a liquid extraction or SPE to get rid of the phosphates, without losing some of the WSV analytes as well.

Considering the above, this work presents an LC/MS-MS method for the quantitative analysis of B-vitamins in a single run, using automated diverter valve switching for removal of matrix phosphates. The analyzed vitamins included vitamin B1 (thiamine), B2 (riboflavin), B3 (niacin), B3* (niacinamide), B6 (pyridoxine), B7 (biotin), B9 (folic acid) and vitamin B12 (cyanocobalamin).

Experimental

Hardware/Software

For the chromatographic separations, a PerkinElmer UHPLC System was used with a PerkinElmer QSight™ 210 MS/MS detector. For automated diverter valve switching, a modified six-port two-position valve module was used (N8122251). 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 Samples

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

For all dilutions, 5-mM ammonium formate was used, adjusted to pH 5.3 with formic acid.

The 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.

WS vitamin formulations were provided as samples by a supplier of value-added nutritional food ingredients and were received in stabilized water diluent. They were labeled WSVF-1 and WSVF-2.

To guard against possible standard/sample instability, 1) all stock, working standards and samples were stored under refrigeration until used, 2) all prepared samples were analyzed within four hours, and 3) only amber 2-mL LC vials were used.

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

Table 2. LC Method Parameters.

Column	PerkinElmer Altus UPLC BEH 2.1 x 50-mm C18, 1.7 µm (Part# N2972000)
Mobile Phase	Solvent A: 5 mM ammonium formate, pH to 5.3 with formic acid Solvent B: Acetonitrile (ACN)

Time (min)	%A	%B
Initial	100.0	0.0
1.60	100.0	0.0
4.50	70.0	30.0
5.00	50.0	50.0
7.00	50.0	50.0
7.05	100.0	0.0

Diverter Valve Switches	Start run in diverted-to-waste position; switch to MS in 20 seconds; switch back to diverted-to-waste in nine minutes (two minutes into re-equilibration)
Analysis Time	Seven minutes; re-equilibration time: four minutes
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): 120 Lpm; **HSID Temp:** 275 °C; **Electrospray V1:** 5000; **Detector Voltage:** 2700	

Exper. Group 1 (0.2 – 0.7 min)	MRM Transitions (amu)					
	Quantifier Ion	Qualifier Ion	EV	CCL2	CE(V)	Dwell Time (msec)
B3 (niacin)	124.3/52.6	124.3/79.3	30	-50	-45	150
Exper. Group 2 (0.6 – 2.5 min)	**MRM Transitions (amu)**					
	Quantifier Ion	Qualifier Ion	EV	CCL2	CE(V)	Dwell Time (msec)
B1 (thiamine)	265.0/121.7	265.0/144.0	20	-80	-25	75
B3* (niacinamide)	123.3/80.2	123.3/53.2	30	-45	-35	75
B6 (pyridoxine)	170.3/133.7	170.3/105.8	22	-80	-32	75
Exper. Group 3 (2.8 – 5.0 min)	**MRM Transitions (amu)**					
	Quantifier Ion	Qualifier Ion	EV	CCL2	CE(V)	Dwell Time (msec)
B7 (biotin):	245.0/96.4	245.0/104.5	25	-80	-45	50
B9 (folic acid)	442.1/295.2	442.1/176.0	20	-120	-30	50
B12 (cyanocobalamin)	678.9/147.2	678.9/399.2	23	-145	-40	50
B9 (folic acid)	377.0/172.3	377.0/198.2	33	-100	-52	50

EV = Entrance voltage; **CCL2** = Collision cell lens 2 voltage; **CE(V)** = Collision energy

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 4-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 upper level calibrant for B12 (1.00 µg/mL).

Five calibration levels were prepared by serially diluting the working standard. The resulting vitamin concentrations are provided in Table 4. Vitamin B12 was calibrated using a lower concentration range due to its lower DV guideline (0.006 mg). All calibrants were injected in triplicate.

Table 4. Vitamin B concentrations per calibration level.

Calibration Level	Conc. of B1, B3, B3*, B6, B7, B9 and B2 (µg/mL)	Conc. of B12 (µg/mL)
L1	0.004	0.002
L2	0.02	0.01
L3	0.10	0.04
L4	0.40	0.2
L5	2.00	1.00
B7	0.3	0.3
B12	0.006	0.006
B2	1.7	1.7

Sample Preparation

To avoid column overload of some of the analytes, samples WSVF-1 and WSVF-2 were further diluted 100-fold with initial mobile phase solvent before injection. This also served to bring the expected analyte concentrations to fall within the calibration range.

Results and Discussion

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

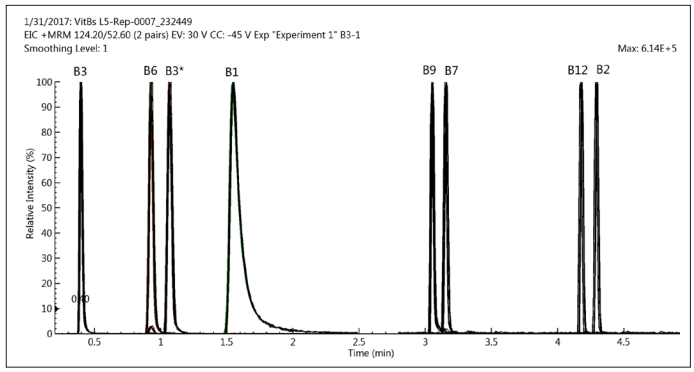

Figure 1. Normalized five replicate overlays of the combined quantifier MRMs of the level-5 calibrant.

Figure 2 shows examples of the 5-level calibration results for vitamins B1 and B9. All eight B-vitamins had calibration fits > 0.998.

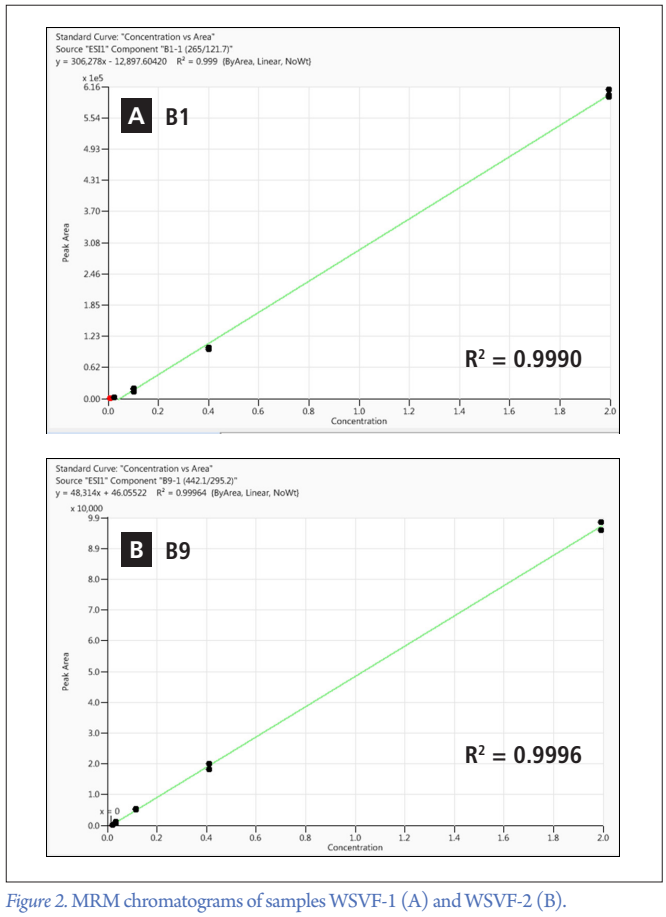

Figure 2. MRM chromatograms of samples WSVF-1 (A) and WSVF-2 (B).

The MRM chromatograms of both samples are shown in Figure 3. It can be noted that the two chromatograms look quite alike, containing a similar distribution of water-soluble vitamins.

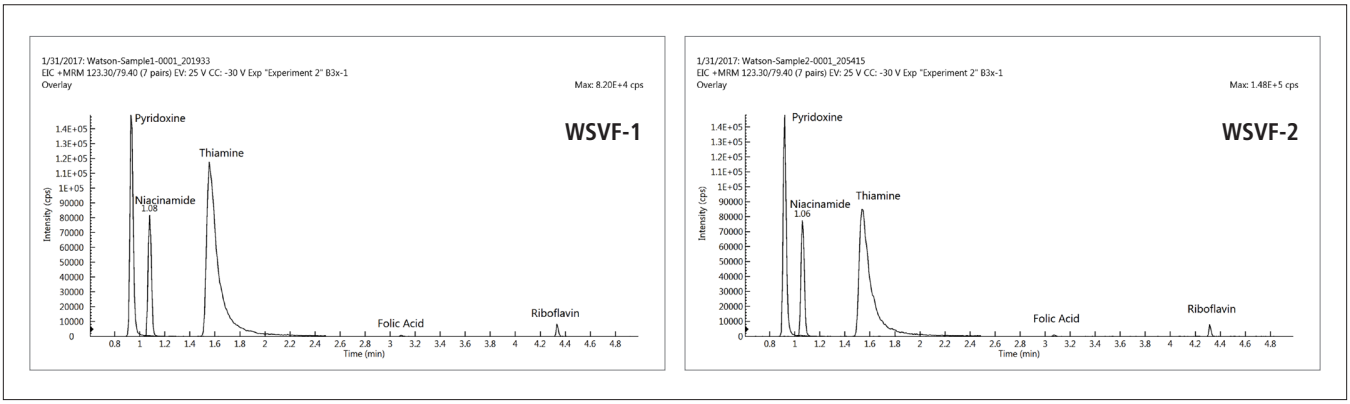

Figure 3. MRM chromatograms of samples WSVF-1 (A) and WSVF-2 (B).

Table 5 shows the calculated vitamin content results for both samples. Comparing the two samples, the quantitative results were quite similar. Overall, the averaged concentrations for B6, B3* (niacinamide), B1and B2 were all better than 80% of the reported values. There was no B12 or B7 detected in any of the samples, as expected. Interestingly, small amounts of both B3 (niacin) and B9 were also detected, though these were not expected. The presence of B3 is not surprising, as niacinamide and niacin are known to interconvert.[2]

Table 5. Resulting vitamin concentrations for samples WSVF-1 and WSVF-2.

	B3 (Niacin)	B6 (Pyridoxine)	B3* (Niacinamide)	B1 (Thiamine)	B9 (Folic Acid)	B7 (Riboflavin)
WSVF-1	0.4 0.6 0.6 **Avg: 0.5**	30.8 30.4 29.9 **Avg: 30.4**	260.4 264.2 264.2 **Avg: 262.9**	24.2 25.6 26.0 **Avg: 25.3**	4.2 5.1 5.6 **Avg: 4.7**	35.1 34.4 34.1 **Avg: 34.5**
WSVF-2	0.4 0.5 0.3 **Avg: 0.4**	29.4 29.2 27.3 **Avg: 28.6**	264.1 260.4 260.7 **Avg: 261.7**	24.3 24.4 25.1 **Avg: 24.6**	6.7 8.0 8.0 **Avg: 7.6**	31.9 34.9 33.2 **Avg: 33.3**

All concentrations in mg/mL (ppm); B12 and B2 weren't detected, nor expected

For added identity confirmation of the analytes, qualifier/quantifier ion ratios were used. The ion ratios of sample WSVF-2 are shown in Table 6, the green background indicating positive confirmation of all the analytes found in the sample. This was based upon the corresponding ion ratios from the Level 3 calibration standard, with all sample ion ratios varying no more than 20% from those of the calibrant.

Table 6. Ion ratios of sample WSVF-2.

Analyte	Mass Transition	Type	Ion Ratio (Area)
B3-1	124.2/52.6	Quantifier	–
B3-2	124.2/79.3	Qualifier	0.32
B3*-1	123.3/52.7	Quantifier	–
B3*-2	123.3/79.4	Qualifier	1.42
B6-1	170.3/133.7	Quantifier	–
B6-2	170.3/105.8	Qualifier	0.10
B1-1	265.0/121.7	Quantifier	–
B1-2	265.0/144.0	Qualifier	0.37
B9-1	442.1/295.2	Quantifier	–
B9-2	442.1/176.0	Qualifier	0.29
B2-1	377.0/172.3	Quantifier	–
B2-2	377.0/198.2	Qualifier	0.86

To verify the effectiveness of the automated diverter valve in diverting the phosphate in the matrix (helping to retain analyte sensitivity), 100 injections of WSVF-1 were made over a 17 hour period. As a representative result, Figure 4 shows riboflavin's MRM chromatographs and height values for injections 2 and 100. Though there was a marginal decrease in retention time due to a shift in mobile phase pH over the 17 hours, the analyte response had decreased only very slightly. This confirmed stability in analyte sensitivity, assuring the robustness of both the procedure and the MS system.

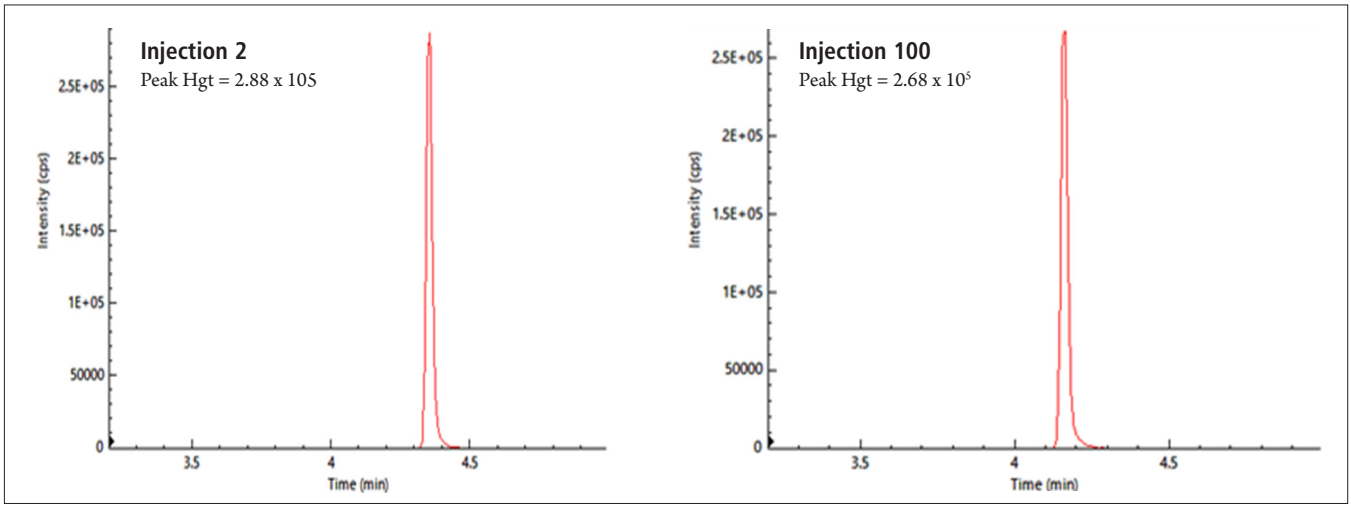

Figure 4. Quantifier MRM chromatograhs of riboflavin for injections 2 and 100 of sample WSVF-1.

Conclusion

The results obtained confirm the applicability of an LC/MS/MS method for the efficient, routine and robust chromatographic and quantitative analysis of B-vitamin formulations containing significant levels of non-volatile phosphate. This was accomplished using automated diverter valve switching and a single MS method to quantitate eight water soluble vitamins over a wide concentration range in under five minutes. The results showed excellent chromatographic repeatability and sample analyte identities were positively confirmed via their qualifier/quantifier ion ratios.

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.

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