Application Note

Food Testing

Agilent

Trusted Answers

Simultaneous Detection and Quantitation of 14 Water-Soluble Vitamins in a Supplement by LC/MS/MS Triple-Quadrupole

Authors

Hui Zhao and
Jonathan Osborn
Agilent Technologies, Inc.

Adam Horkey and
Stacey Murphy
Nature's Way

Abstract

This application note describes a method for the simultaneous determination of 14 water-soluble B vitamins in a dietary supplement using an Agilent 1290 Infinity II LC system coupled to an Agilent 6470 triple quadrupole LC/MS system with Agilent MassHunter Workstation software. The method was used to quantify the water-soluble B vitamins in a highly complex multivitamin tablet matrix. All tested water-soluble vitamins met the manufacturer claimed concentrations. It was concluded that the method can be used for quality control and establishment of nutrition labels for water-soluble vitamin-containing supplement products.

Introduction

Vitamins are essential nutrients for human health. Vitamin supplements are often consumed to ensure adequate vitamin intake and are available in various forms such as tablets, capsules, gummies, softgels, and drinks.

In particular, B vitamins are essential for proper nervous system function and for converting food into cellular energy.[1] The water-soluble B vitamins include B1 (thiamine), B2 (riboflavin), B3 (nicotinic acid and nicotinamide), B5 (pantothenic acid), B6 (pyridoxine and pyridoxal-5-phosphate), B7 (biotin), B9 (folic acid and 5-methyltetrahydrofolate), and B12 (cyanocobalamin, adenosylcobalamin, methylcobalamin and hydroxocobalamin). The structures of these vitamins are given in Figure 1.

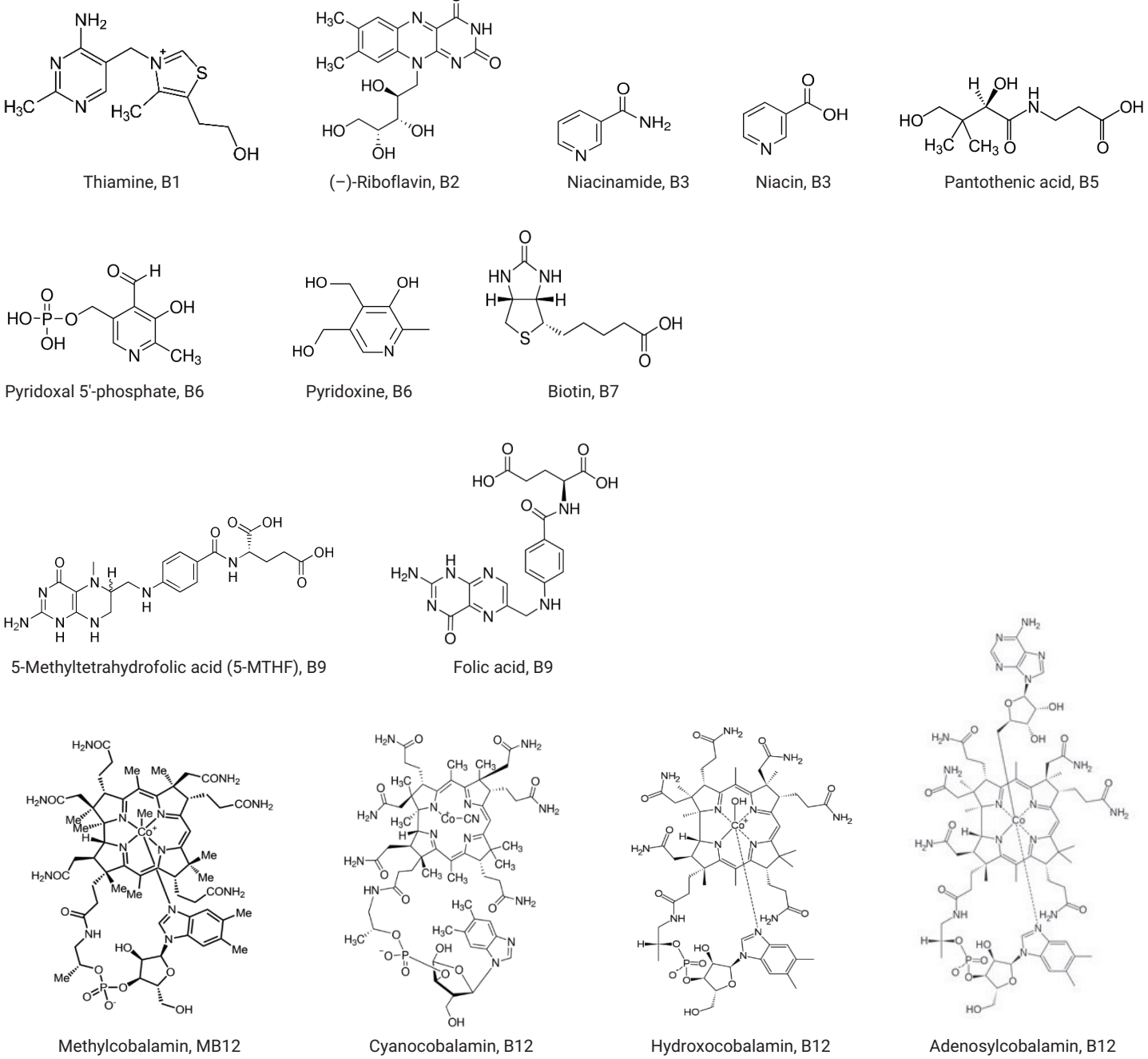

Figure 1. Structure of water-soluble B vitamins.

Accurate quantitative measurements for B vitamins are required to ensure product quality and regulatory compliance. Traditional measurements include microbiological methods, which have significant shortcomings for accuracy, specificity and throughput; and LC-UV methods, which have poor sensitivity for low-level vitamins and poor selectivity within complex matrices. These techniques often involve multiple assays to quantify all B vitamins. Instead, LC/MS/MS has become increasingly popular for vitamin detection due to the high sensitivity, selectivity, and accuracy it provides.

In this study, a fast and sensitive LC/MS/MS method was developed and evaluated to provide identification and accurate quantification of B vitamins in a complex multivitamin tablet matrix. More efficiently, all 14 B vitamins were analyzed simultaneously in one run. The postextraction matrix-matched standard and standard additions were included to compensate for matrix effects. Method criteria for data acceptance were also established.

Experimental

Equipment

All experiments in this study were performed using an Agilent 1290 Infinity II LC consisting of an Agilent 1290 Infinity II multisampler (G7167B), an Agilent 1290 Infinity II high speed pump (G7120A), and an Agilent 1290 Infinity II multicolumn thermostat (G7116B) coupled to an Agilent 6470 triple quadrupole LC/MS system (G6470A). The system was controlled by Agilent MassHunter Acquisition software version 10.1. Data processing was performed with MassHunter quantitative analysis software version 10.1 and MassHunter qualitative analysis software version 10.0.

Sample and standards

The study matrix was Nature's Way Alive! Men's Max Potency Daily Multivitamin (Nature's Way, WI, USA). Standards of thiamine, riboflavin, niacin, niacinamide, pantothenic acid, pyridoxine, pyridoxal-5-phosphate, biotin, folic acid, cyanocobalamin, adenosylcobalamin, methylcobalamin, hydroxocobalamin, riboflavin-$^{13}C_4$, ^{15}N solution, and niacinamide-$^{13}C_6$ solution were obtained from Millipore Sigma, Inc. (St. Louis, MO, USA); 5-methyltetrahydrofolate was obtained from Cayman Chemical (Ann Arbor, MI, USA); pyridoxine-$^{13}C_4$ and biotin-D_2 were obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA).

Individual analyte and internal standard stock solutions were made at concentrations of 100 µg/mL in 0.1% H_3PO_4 + 5% ACN in water and took purity, water content, and counter-ions into account. The sample and standards were stored at 5 °C or –20 °C accordingly.

Chromatographic conditions

Parameter	Setting
Analytical Column	Agilent InfinityLab 120 Poroshell 120 Phenyl-Hexyl, 3.0 × 100 mm, 2.7 µm (p/n 695975-312)
Column Oven	30 ±2 °C
Injection Volume	1 µL
Run Time	9 min
Autosampler	15 ±2 °C
Mobile Phase A	*5 mM ammonium formate + 0.1% formic acid in water
Mobile Phase B	0.1% formic acid in methanol
Needle Wash	50/50 MeOH/H_2O
Gradient	Time (min) Flow (mL/min) %A %B 0 0. 5 97 3 1.0 0. 5 94 6 4.5 0. 5 55 45 5.5 0. 5 10 90 6.5 0. 5 10 90 6.6 0. 5 97 3 9.0 0. 5 97 3

* To achieve the best peak shape, the column needs a relatively long time to equilibrate, or up to 20 mM ammonium formate can be used.

MS parameters

Parameter	Setting
MS Acquisition	Dynamic MRM (dMRM)
Ion Source Type	Agilent Jet Stream electrospray ionization (AJS ESI)
Drying Gas Temperature	270 °C
Drying Gas Flow	13 L/min
Nebulizer	40 psi
Sheath Gas Heater	375 °C
Sheath Gas Flow	11 L/min
Capillary	2,500 V
Nozzle Voltage	0 V
Precursor Ion and Production Ion Resolution	Unit
Compound-Specific Conditions	See Table 1

Method

Below is the detailed description of the optimized sample preparation protocol followed in this study. Table 1 gives the analyte-specific LC/MS/MS conditions.

1. Determine the average weight of the tablet using an analytical balance. Grind the sample into a fine powder to form a homogeneous mixture.
2. Weigh out a 5 g sample into a 100 mL amber volumetric flask.
3. Add approximately 70 mL extraction solvent (0.1% H_3PO_4 + 5% ACN + 0.5% EDTA + 0.5% vitamin C in water) to the flask (Vitamin C was added to the solution to prevent oxidation of the target analytes).
4. Shake for 20 minutes.
5. Fill the flask to the 100 mL volume mark and mix well.
6. Draw approximately 1.5 mL of sample extract to a 2 mL microcentrifuge tube.
7. Heat the flask to 90 to 95 °C in a water bath for 25 minutes for releasing vitamin B2.
8. Cool down to room temperature.
9. Draw approximately 1.5 mL of sample extract to a 2 mL microcentrifuge tube.
10. Centrifuge the sample extract from step 5 and step 8 for 5 minutes at 13,000 rpm.
11. Dilute the supernatants with 0.1% H_3PO_4 + 5% ACN in water as needed.
12. Prepare the postspiked samples along with the diluted samples.
13. Inject samples to LC/MS/MS using positive ESI mode for analysis.

Table 1. Analyte-specific LC/MS conditions: precursor to product ion transitions, fragmentor, collision energies (CE), cell accelerator voltage (CAV), and retention times (RT).

Compound Group	Compound Name	Type	Precursor Ion (*m/z*)	Product Ion (*m/z*)	RT (min)	Delta RT (min)	Fragmentor (V)	CE (V)	CAV (V)
B1	Thiamine	Target	265.1	144.0	1.14	1	97	8	4
B1	Thiamine	Target	265.1	121.9	1.14	1	97	8	4
B1	Thiamine	Target	265.1	80.9	1.14	1	97	28	4
B2	Riboflavin	Target	377.1	243.0	6.31	1	167	16	4
B2	Riboflavin	Target	377.1	198.1	6.31	1	167	44	4
B2	Riboflavin	Target	377.1	172.0	6.31	1	167	16	4
B2	Riboflavin-$^{13}C_4$, $^{15}N_2$	IS	383.1	249.0	6.31	1	167	16	4
B3	Niacin	Target	124.0	80.1	1.41	1	107	20	5
B3	Niacin	Target	124.0	78.1	1.41	1	107	24	5
B3	Niacin	Target	124.0	53.0	1.41	1	107	32	5
B3	Niacinamide	Target	123.1	80.0	1.82	1	112	20	4
B3	Niacinamide	Target	123.1	53.0	1.82	1	112	36	4
B3	Niacinamide-$^{13}C_6$	IS	129.0	86.0	1.81	1	112	20	5
B5	Pantothenic acid	Target	220.1	202.1	3.52	1	107	4	4
B5	Pantothenic acid	Target	220.1	90.0	3.52	1	107	8	4
B5	Pantothenic acid	Target	220.1	71.9	3.52	1	107	16	4
B6	Pyridoxal 5'-phosphate	Target	247.9	150.0	2.06	1.5	142	12	4
B6	Pyridoxal 5'-phosphate	Target	247.9	94.1	2.06	1.5	142	28	4
B6	Pyridoxine	Target	170.1	152.1	2.09	1	92	12	4
B6	Pyridoxine	Target	170.1	134.0	2.09	1	92	24	4
B6	Pyridoxine	Target	170.1	77.0	2.09	1	92	40	4
B6	Pyridoxine-$^{13}C_4$	IS	174.1	138.0	2.09	1	92	24	4
B7	Biotin	Target	245.1	227.1	5.95	1	102	8	4
B7	Biotin	Target	245.1	123.0	5.95	1	102	28	4
B7	Biotin	Target	245.1	97.1	5.95	1	102	32	4
B7	Biotin-D_2	IS	247.1	125.0	5.94	1	102	28	4

Compound Group	Compound Name	Type	Precursor Ion (*m/z*)	Product Ion (*m/z*)	RT (min)	Delta RT (min)	Fragmentor (V)	CE (V)	CAV (V)
B9	Folic acid	Target	442.1	295.0	5.43	1	102	20	4
B9	Folic acid	Target	442.1	176.0	5.43	1	102	44	4
B9	Folic acid	Target	442.1	120.0	5.43	1	102	44	4
B9	5-Methyltetrahydrofolic acid (5-MTHF)	Target	460.1	313.0	4.40	1	102	16	4
B9	5-Methyltetrahydrofolic acid (5-MTHF)	Target	460.1	180.1	4.40	1	102	48	4
B12	Adenosylcobalamin	Target	790.1	359.1	6.28	1	152	28	4
B12	Adenosylcobalamin	Target	790.1	147.1	6.28	1	152	32	4
B12	Cyanocobalamine	Target	678.1	456.9	5.68	1	152	30	4
B12	Cyanocobalamine	Target	678.1	359.0	5.68	1	152	25	4
B12	Cyanocobalamine	Target	678.1	341.0	5.68	1	152	25	4
B12	Methylcobalamine	Target	673.0	665.1	6.42	1	132	14	4
B12	Methylcobalamine	Target	673.0	359.1	6.42	1	132	20	4
B12	Methylcobalamine	Target	673.0	147.1	6.42	1	132	48	4
B12	OH-cobalamine	Target	665.1	359.1	4.93	2	152	20	4
B12	OH-cobalamine	Target	665.1	147.1	4.93	2	152	40	4

Evaluation procedure

Method performance was evaluated by analyzing a complex multivitamin tablet sample. The product label for water-soluble B vitamins is listed in Table 2.

Quantitation was performed using isotopically labeled internal calibration curves with 1/x weighting, or external calibration curves with 1/x weighting and single point postmatrix spike correction, or postmatrix standard addition curve.

Evaluation criteria

Specificity:

- The relative error (RE %) of retention time of each analyte peak to the average of standard peaks is less than 5%
- The ion ratio is within the tolerance of 30%

Linearity and range:

- Calibration curve has R^2 >0.99
- Calculated working standard values should be within ±30% of the theoretical value
- The calibration standards should bracket the analyte concentration level

Accuracy:

- The tested result for each water-soluble B vitamin meets ≥100% claimed on the product label (see Table 2)
- Postspike recovery is within 70% to 130% (due to the variations in method and instrument performance, the criteria should be determined by each individual lab)

Table 2. Supplement facts (serving size: 3 tablets/serving).

Compound Name	Vitamins	Compound Form in Tablet	Claim (mg/serving)
Thiamin	B1	As thiamin mononitrate	40
Riboflavin	B2	As free riboflavin	40
Niacin	B3	As niacinamide	80
Pantothenic Acid	B5	As calcium pantothenate	62.5
Vitamin B6	B6	As total of pyridoxine HCl and pyridoxal 5'-phosphate	42.5
Biotin	B7	As free biotin	0.033
Folate	B9	As 5-methyltetrahydrofolate glucosamine salt	0.42
Vitamin B12	B12	As methylcobalamin	0.2

Results and discussion

Column selection

In this study, two columns were evaluated with the same LC conditions, Agilent InfinityLab Poroshell 120 Phenyl-Hexyl (100 × 3.0 mm, 2.7 µm) and Agilent InfinityLab Poroshell 120 StableBond-Aqueous (100 × 2.1 mm, 2.7 µm). Better selectivity was achieved using the phenyl-hexyl column with a balanced mixture of retention mechanisms, including hydrophobic interactions, the π-π interactions of the phenyl group and the hexyl linkage of the stationary phase. Methanol is a good choice when paired with phenyl columns.[2] See Figure 2 for a selectivity comparison between the two columns. The phenyl-hexyl column provided an enhanced molecular interaction and selectivity to improve the chromatographic separation and peak shape.

Specificity

A dynamic multiple reaction monitoring (dMRM) acquisition method was used for vitamin quantitation. Monitoring MS/MS transitions with evaluation of the ratio of their relative intensities and RT of analyte peaks allows the target analyte to be distinguished from potential interferences in quantitative analysis. Figure 3 shows an example of an extracted ion chromatogram of a 10 ng/mL working standard in 0.1% H_3PO_4 + 5% ACN in water. Figure 4 shows that a reagent blank is free of analytes in 0.1% H_3PO_4 + 5% ACN in water.

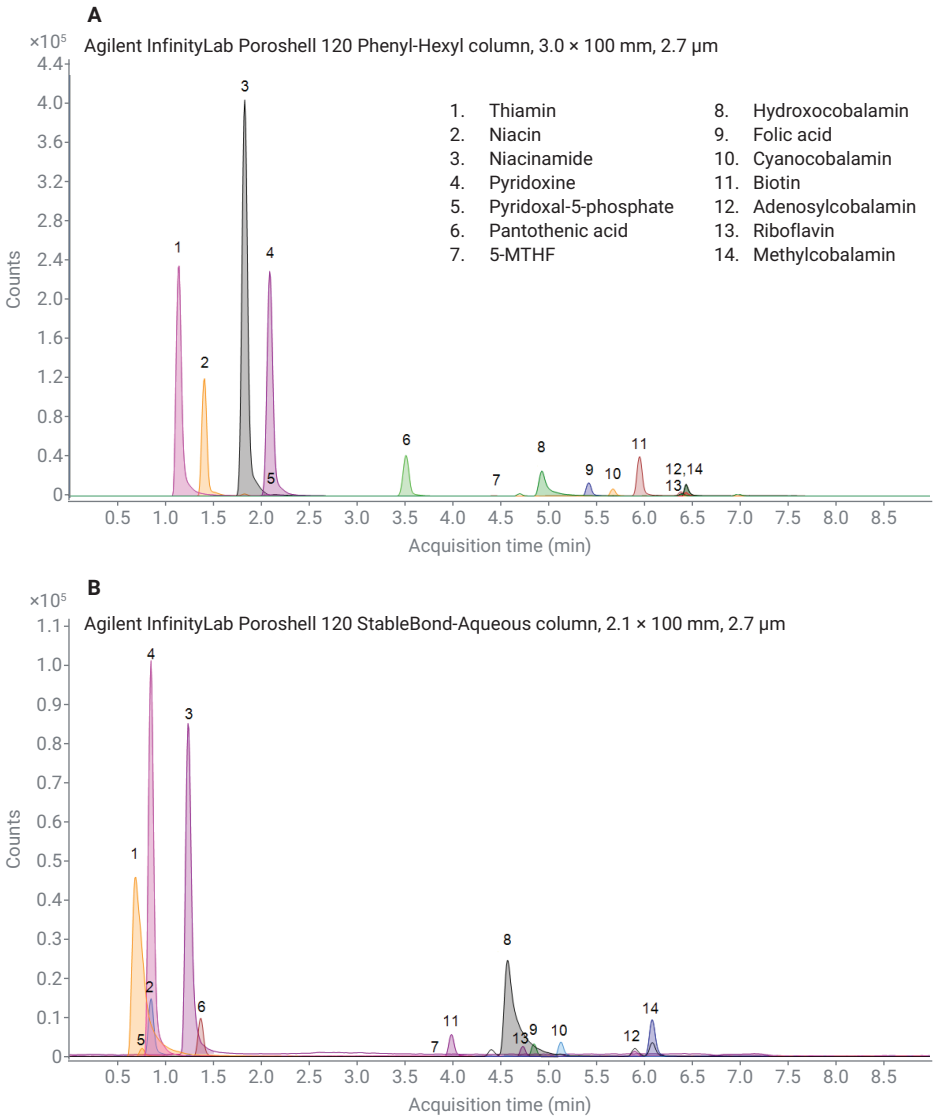

Figure 2. Comparison of the selectivity between Phenyl-Hexyl and StableBond-Aqueous columns.

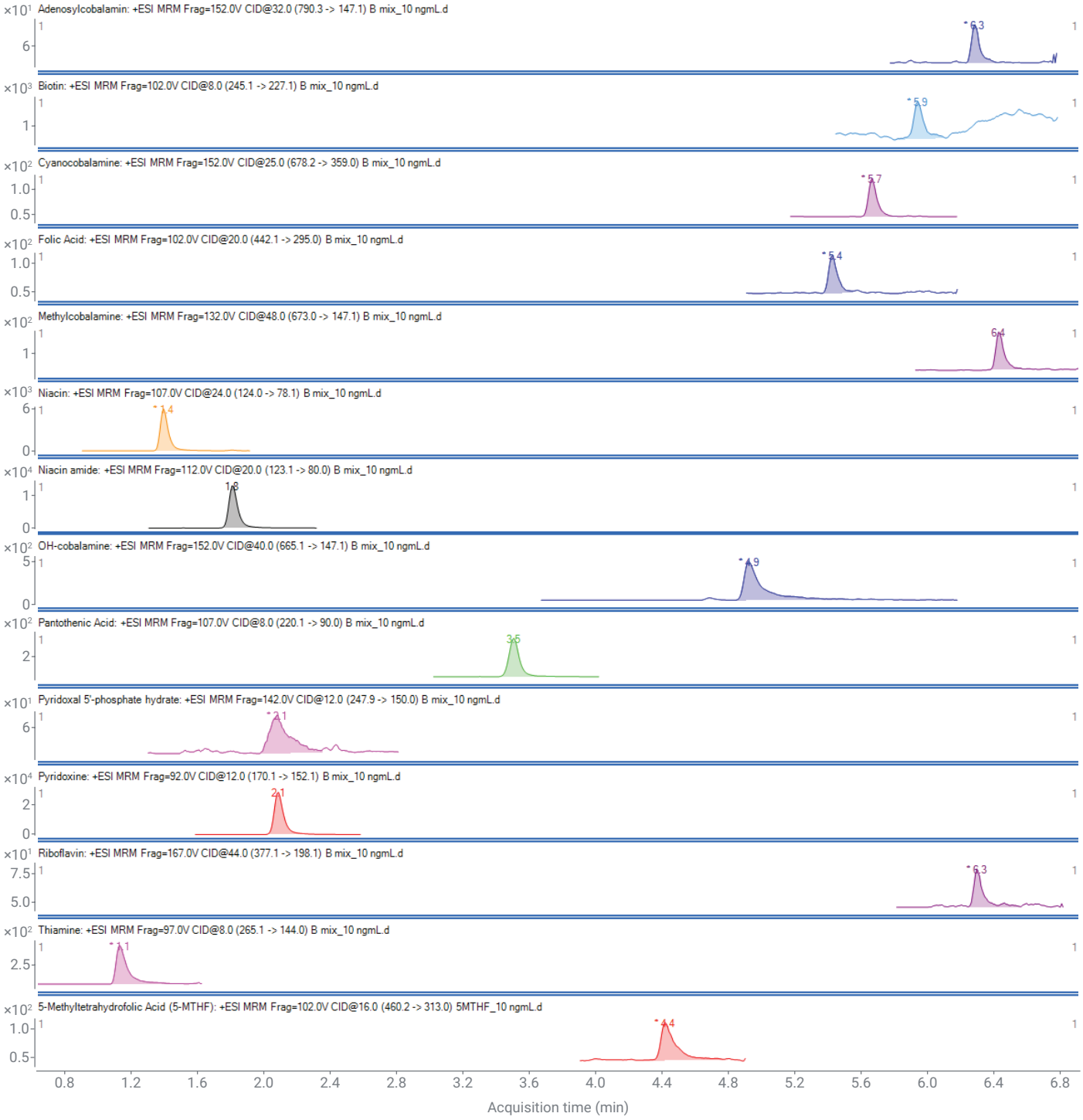

Figure 3. Extracted ion chromatogram of vitamin composite working standards at 10 ng/mL in 0.1% H_3PO_4 + 5% ACN in water, 1 µL injection volume.

Range and linearity

The method was evaluated over the range of 0.5 to 500 ng/mL. To evaluate response linearity of the method, eight working standard (WS) solutions of B vitamins were made at 0.5, 1, 2, 5, 10, 50, 100, and 500 ng/mL. The calibration curve residuals were ≤30% for WS1 to WS8. The linearity was determined by using a linear calibration with a 1/x weighting factor. The coefficient of determination (R^2) values were >0.99. Table 3 lists the statistical data of the calibration curve residuals, linear range and coefficients of determination.

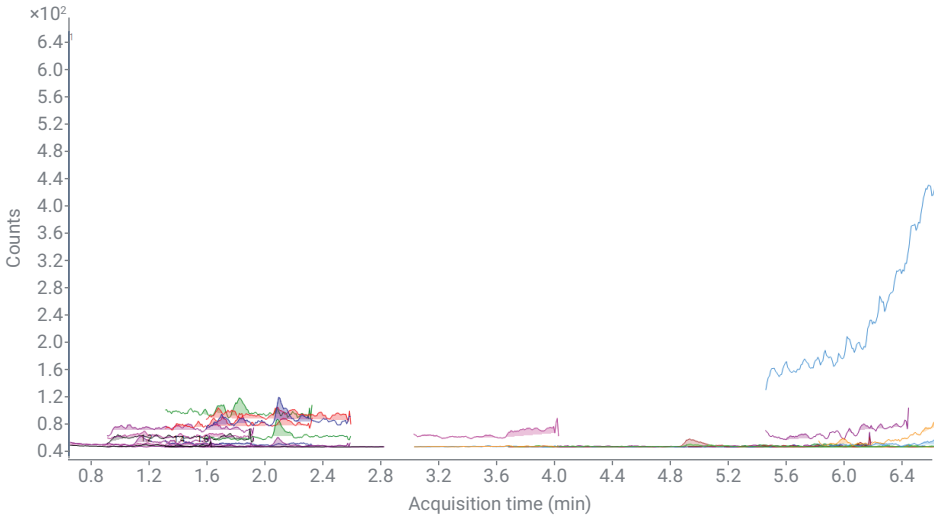

Figure 4. Extracted ion chromatogram of a solvent blank 0.1% H_3PO_4 + 5% ACN in water.

Table 3. Calibration curve statistical data.

Compound Name	Calibration Curve Residual (%)								Range (ng/mL)	R^2
	WS1 0.5	WS2 1	WS3 2	WS4 5	WS5 10	WS6 50	WS7 100	WS8 500		
	ng/mL									
Thiamine	28	17	7	−3	−20	−3	−3	2	0.5 to 500	0.9980
Riboflavin	18	9	12	6	−10	3	−8	3	0.5 to 500	0.9970
Niacin	7	2	−14	−7	−2	−3	3	0	0.5 to 500	0.9995
Niacinamide	23	−10	−4	3	−11	5	7	−2	0.5 to 500	0.9985
Pantothenic Acid	–	17	10	−9	−13	−5	0	2	1 to 500	0.9980
Pyridoxine	27	2	−1	1	−15	0	4	0	0.5 to 500	0.9991
Pyridoxal 5'-phosphate	–	–	–	−13	−17	−3	−2	2	5 to 500	0.9992
Biotin	–	–	–	28	−12	−5	−3	2	5 to 500	0.9978
5-Methyltetrahydrofolic Acid (5-MTHF)	–	–	–	–	25	−12	−8	−10	25 to 500	0.9913
Folic Acid	–	–	–	12	−1	−4	−4	1	5 to 500	0.9993
Methylcobalamine	–	25	3	−13	−4	−14	2	1	1 to 500	0.9981
Cyanocobalamine	−23	8	21	−6	1	0	0	9	0.5 to 500	0.9991
OH-cobalamine	–	30	−7	−2	−13	−6	−4	2	1 to 500	0.9987
Adenosylcobalamin	–	–	–	−23	12	−12	−11	4	5 to 500	0.9936

Approaches for accurate quantitation

Interfering substances in the matrix may be observed and can affect the electrospray ionization process, causing ion suppression or enhancement. Currently, there is no guideline for matrix effects in vitamins due to the variations in method and instrument performance. However, matrix effects need to be addressed for proper quantitation. A postspiked matrix-matched standard or standard addition can address the matrix effect or any other matrix interactions when the internal standard is not available or not easy to obtain.[3]

Postspike recovery was determined by fortifying samples after extraction with an analyte composite standard solution. The results were corrected using postspike recovery if it was within 70 to 130%. If the postspike recovery was outside the range of 70 to 130%, a standard addition curve was generated.

Sample tests

An increasing number of methylcobalamin B12 based supplements have entered the market due to its natural form and higher bio-availability. Naturally occurring folate (5-methyltetrahydrofolate) is a

biologically active form of folic acid and is rapidly gaining popularity as the preferred supplemental form. Nature's Way Alive! Men's Max Potency Daily Multivitamin incorporates high potency B-vitamins, including the active forms of B12 and folate, and food-based blends including Daily Greens, Orchard Fruits and Garden Veggies, Cardio, Digestive Enzyme, and more blends.[4] The complexity of the product formulation served as an excellent matrix to demonstrate unambiguous identification, accurate quantitation, and high sensitivity for a variety of B vitamins by LC/MS/MS.

The high sensitivity of LC/MS/MS allows a large dilution after sample extraction. The postspike recoveries for B1, B2, B3, B5, B6, B7, and B12 fell into the accepted range and the results were corrected. Given that significant ion enhancement for vitamin B9 (folate) was observed, a standard addition curve was generated for quantitation. See Figure 5 for this curve generated from the MassHunter quantitative analysis software. The corrected results for all B vitamins met ≥100% claims on the product label. Sample results and postspike recoveries for each analyte are shown in Table 4. The results of riboflavin, niacinamide, pyridoxine and biotin were also corrected using internal standards to compare with those from post matrix-matched correction. The results from both techniques were found to be in good agreement with each other (see Table 4).

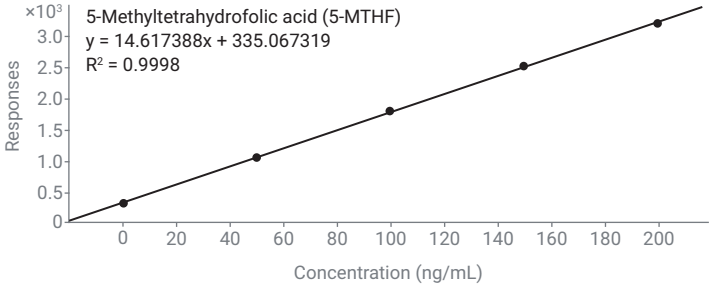

Figure 5. Standard addition curve for 5-MTHF.

Table 4. Sample results for Nature's Way Alive! Men's Max Potency Daily Multivitamin.

Compound Name	Vitamins	Serving Size (g) (3 tablets)	Claim on Product Label (mg/serving)	*Corrected Results by IS mg/serving	*Corrected Results by postspike Recovery (mg/serving)	Postspike Recovery (%)
Thiamin	B1	5.2	40		41	102
Riboflavin	B2	5.2	40	57	57	97.1
Niacinamide	B3	5.2	80	89	87	108
Pantothenic Acid	B5	5.2	62.5		75.9	97.0
Total of Pyridoxine and Pyridoxal 5'-Phosphate	B6	5.2	42.5	47.1	43.0	111
Biotin	B7	5.2	0.033	0.047	0.050	94.7
5-Methyltetrahydrofolate Glucosamine Salt	B9	5.2	0.42		0.44	By standard addition
Methylcobalamin	B12	5.2	0.20		0.21	70.8

Conclusion

A rapid, sensitive, and accurate UHPLC/MS/MS method for the identification and quantitation of water-soluble B vitamins in a complex supplement matrix was presented. The method used an Agilent 1290 Infinity II LC system coupled to an Agilent 6470 triple quadrupole LC/MS system. All 14 B vitamins were detected simultaneously in one run for high efficiency, throughput, and cost reduction when compared to the traditional involvement of multiple assays. This method demonstrated linearity over three orders of magnitude for all analytes tested with an R^2 value of 0.99. All water-soluble B vitamins in the supplement product met the specifications with the quantitation approaches of matrix-matched standard or standard addition. The evaluation demonstrated that the method can achieve the necessary specificity, linearity, and accuracy required for water-soluble vitamin B analysis.

References

1. Kennedy, D. O. B Vitamins and the Brain: Mechanisms, Dose and Efficacy—A Review. *Nutrients* **2016**, *8*, 68-96.
2. Henderson Jr., J. W.; Long, W. J. Exceptional Selectivity of Agilent ZORBAX Eclipse Plus Phenyl-Hexyl Columns to Separate Estrogens. *Agilent Technologies application note*, publication number 5989-9130EN, **2008**.
3. Zhao, H.; Zulkoski, J.; Mastovska, K. Development and Validation of a Multiclass, Multiresidue Method for Veterinary Drug Analysis in Infant Formula and Related Ingredients Using UHPLC-MS/MS. *J. Agric. Food Chem*. **2017**, *65*, 7268–7287.
4. https://www.naturesway.com/Product-Catalog/Alive-Max-Potency-Men-s-Multi-90-Tabs. 2020.

www.agilent.com/chem

DE44209.565474537

Printed in the USA, August 30, 2021
5994-3016EN

Agilent
Trusted Answers