

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

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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.

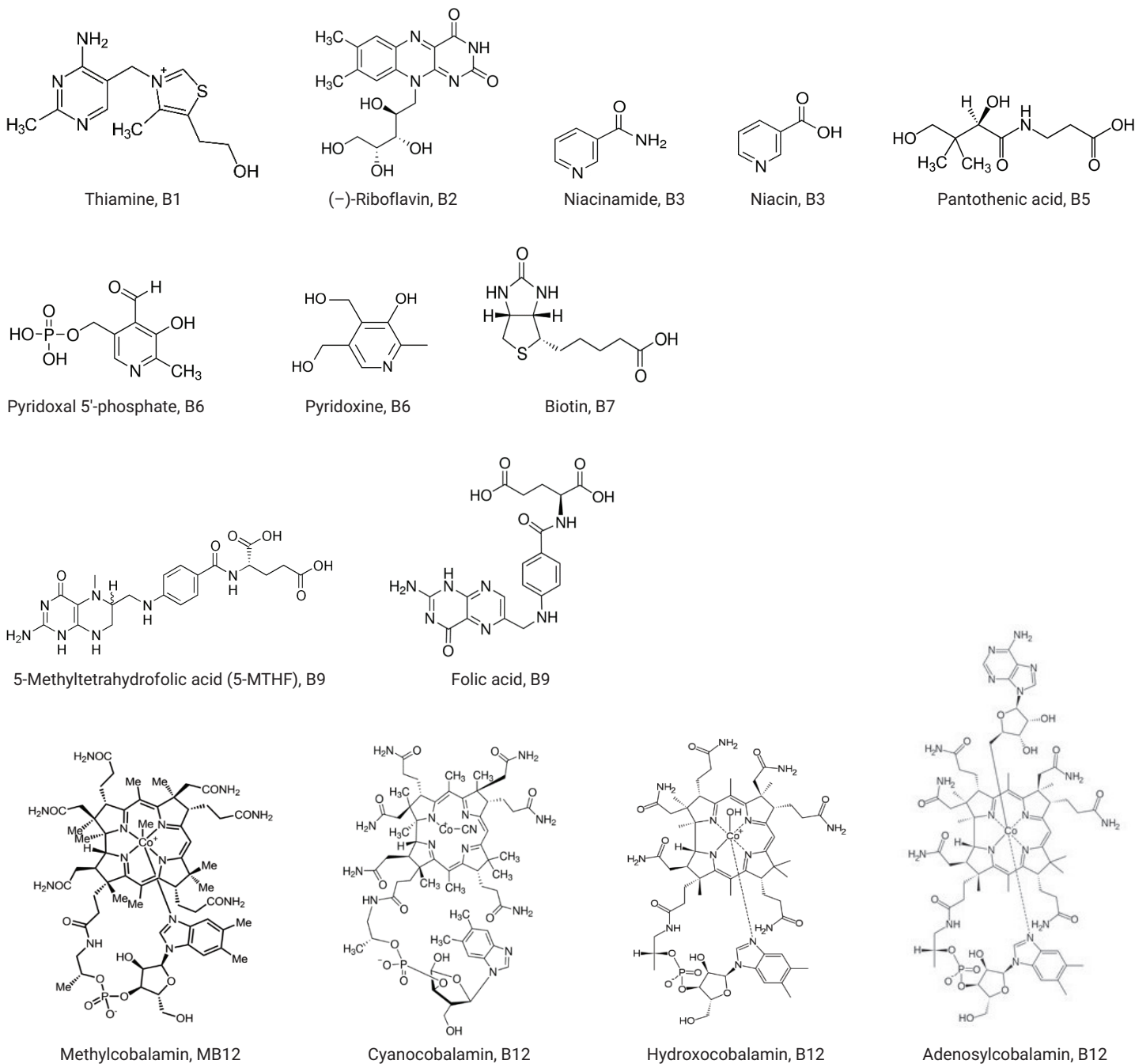


Figure 1. Structure of water-soluble B vitamins.

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-¹³C₄, ¹⁵N solution, and niacinamide-¹³C₆ solution were obtained from Millipore Sigma, Inc. (St. Louis, MO, USA); 5-methyltetrahydrofolate

was obtained from Cayman Chemical (Ann Arbor, MI, USA); pyridoxine-¹³C₄ and biotin-D₂ 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% H₃PO₄ + 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 ₂ O | | | |
| 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 |

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.

- Determine the average weight of the tablet using an analytical balance. Grind the sample into a fine powder to form a homogeneous mixture.
- Weigh out a 5 g sample into a 100 mL amber volumetric flask.
- Add approximately 70 mL extraction solvent (0.1% H₃PO₄ + 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).
- Shake for 20 minutes.
- Fill the flask to the 100 mL volume mark and mix well.
- Draw approximately 1.5 mL of sample extract to a 2 mL microcentrifuge tube.
- Heat the flask to 90 to 95 °C in a water bath for 25 minutes for releasing vitamin B2.
- Cool down to room temperature.
- Draw approximately 1.5 mL of sample extract to a 2 mL microcentrifuge tube.
- Centrifuge the sample extract from step 5 and step 8 for 5 minutes at 13,000 rpm.
- Dilute the supernatants with 0.1% H₃PO₄ + 5% ACN in water as needed.
- Prepare the postspiked samples along with the diluted samples.
- 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 $\pm 30\%$ of the theoretical value
- The calibration standards should bracket the analyte concentration level

Accuracy:

- The tested result for each water-soluble B vitamin meets $\geq 100\%$ 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% H₃PO₄ + 5% ACN in water. Figure 4 shows that a reagent blank is free of analytes in 0.1% H₃PO₄ + 5% ACN in water.

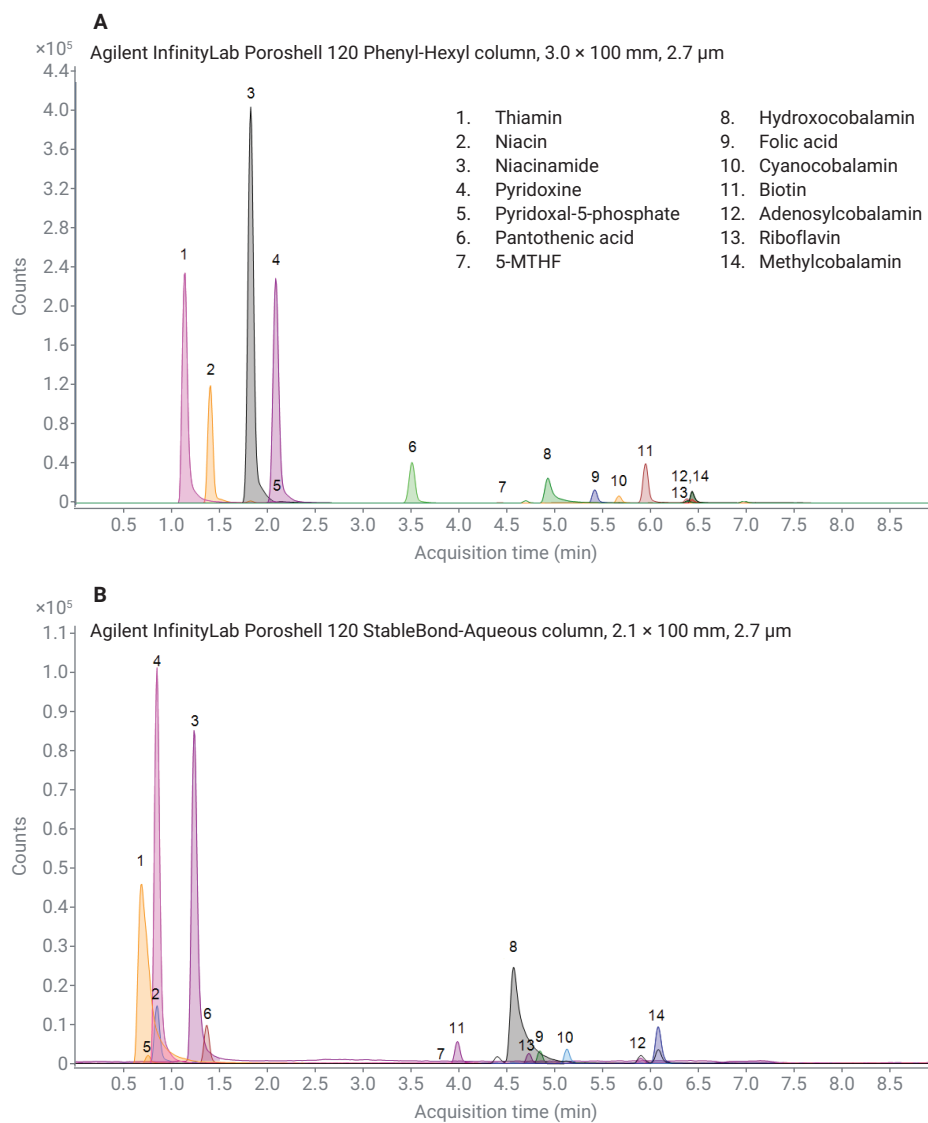


Figure 2. Comparison of the selectivity between Phenyl-Hexyl and StableBond-Aqueous columns.

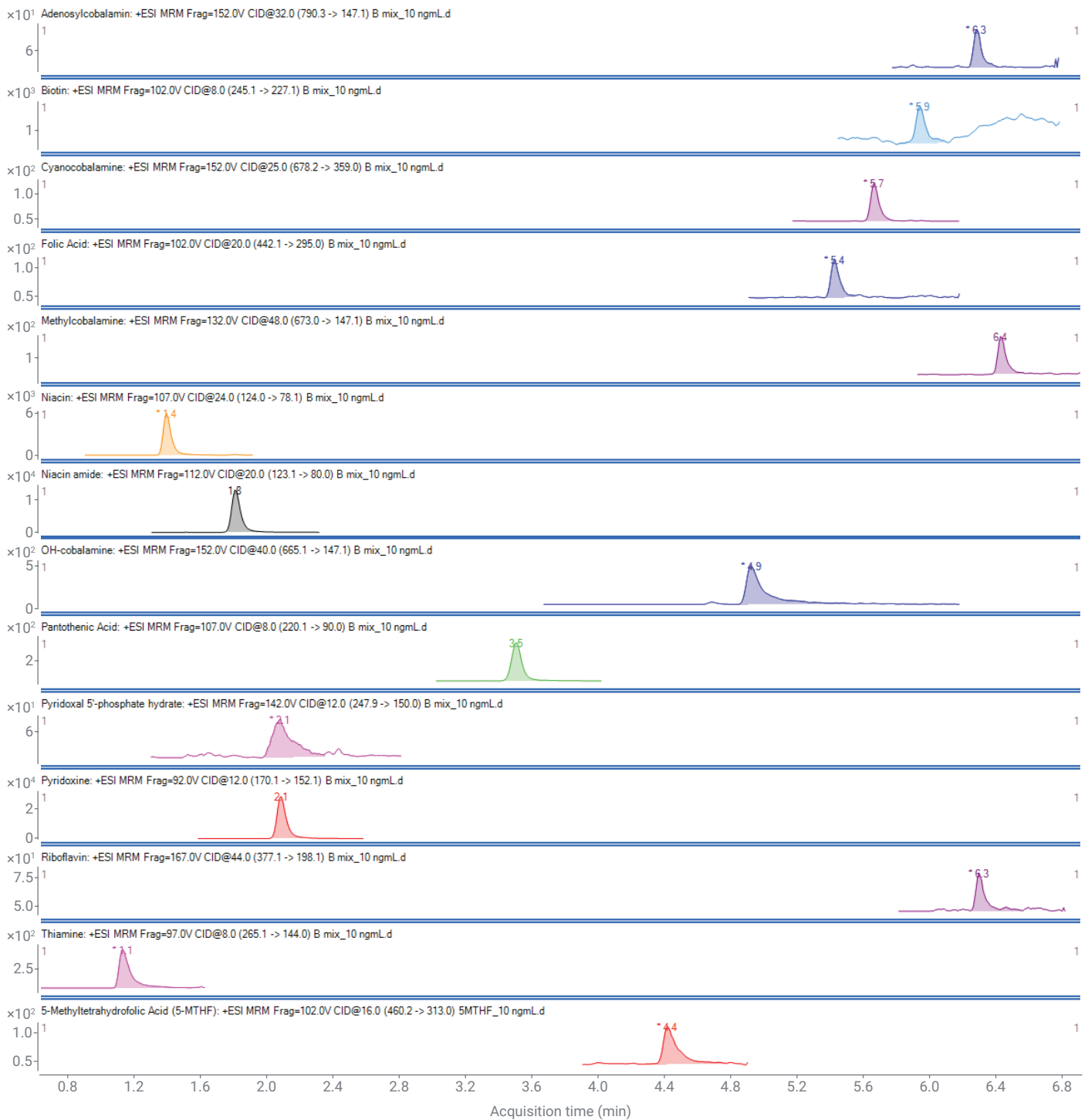


Figure 3. Extracted ion chromatogram of vitamin composite working standards at 10 ng/mL in 0.1% H₃PO₄ + 5% ACN in water, 1 µL injection volume.

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 $\leq 30\%$ 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.

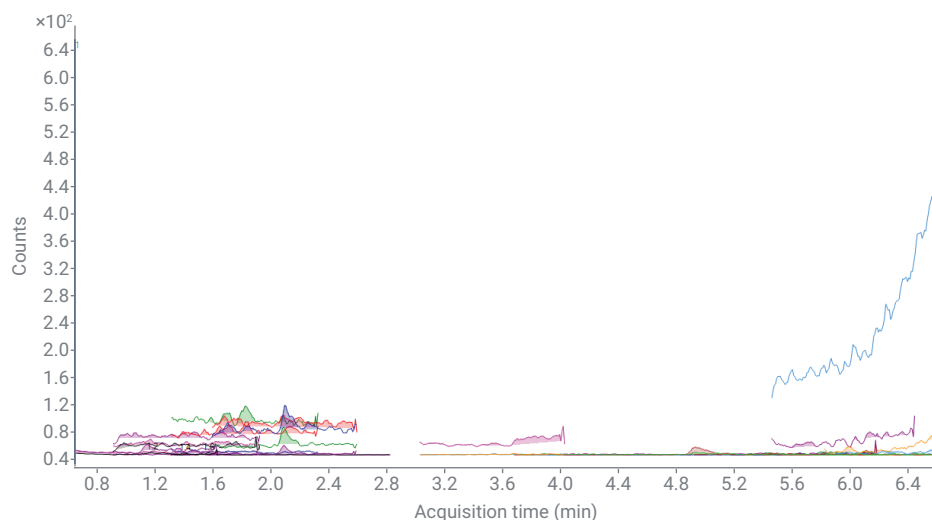


Figure 4. Extracted ion chromatogram of a solvent blank 0.1% H_3PO_4 + 5% ACN in water.

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 $\geq 100\%$ 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).

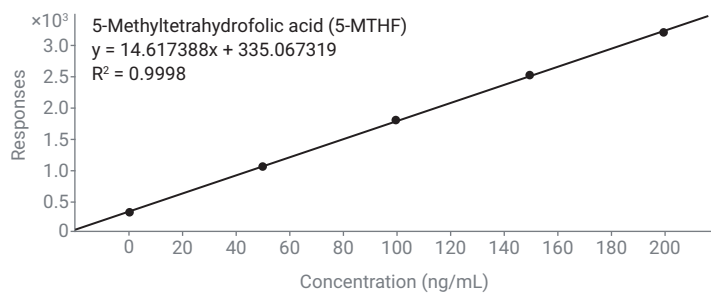


Figure 5. Standard addition curve for 5-MTHF.

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.

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