# Single Laboratory Validation of 10 Water Soluble Vitamins in a Multi-Vitamin Mineral Matrix Using HPLC with Photodiode Array Detection

R. Charles Thompson
Albion Advanced Nutrition, Clearfield, Utah USA

## Abstract

P-826 120th AOAC Annual Meeting & Exposition
September 17-21, 2006
Minneapolis USA

Current AOAC methodology for the analysis of water soluble vitamins is both accurate and validated, but only allows for single component analysis of these vitamins. High pressure liquid chromatography (HPLC) provides an excellent tool for the simultaneous analysis of many different water soluble vitamins. The use of UV/Vis Photo Diode Array (PDA) for detection is well suited for the chromaphores present in water soluble vitamins. With the use of HPLC we are able achieve very good analytical ranges, detection and quantitation limits the for simultaneous analysis 10 water soluble vitamins (ascorbic acid, niacin, niacinamide, ca-pantothenate, pyridoxine HCl, thiamine $NO_3$, folic acid, para-aminobenzoic acid, cyanocobalamin, and riboflavin). Here we have evaluated the specificity, accuracy, and precision of this method for use as a single laboratory validated method using HPLC for the simultaneous analysis of water soluble vitamins in a simple multi vitamin mineral matrix.

## Introduction

The essential nature of water soluble vitamins (wsv) has been well established since the turn of the 19th century.[1] Since the early nineteenth century the use of vitamins and minerals for food fortification and supplementation has grown to become commonplace in today's world. With the passage of the Dietary Supplement Health and Education Act in 1994©, the number and complexity of multi-vitamin/mineral (mvm) supplements on the market have become very diverse and complex.[2] There are literally hundreds of different formulas with everything from somewhat simple matrices, to very complex matrices containing at times hundreds of different labeled components. Further complicating the analysis of these products is the fact that some of the components can be herbal extracts containing many different individual chemical compounds. Current AOAC methodology for the analysis of wsv is both accurate and precise, but only allows for individual component analysis which can lead to a very long analysis times for each individual product. Modern chromatographic techniques allow for the simultaneous analysis of multiple wsv. The chromophores present on most wsv provide the opportunity to test over a wide range of analyte concentrations, with good detection limits. This experiment is designed to evaluate the accuracy and precision of a simple extraction procedure for the analysis of 10 water soluble components in a multi-vitamin/mineral dietary supplement containing 100% -200% of RDI of the vitamins and minerals present.

## Purpose

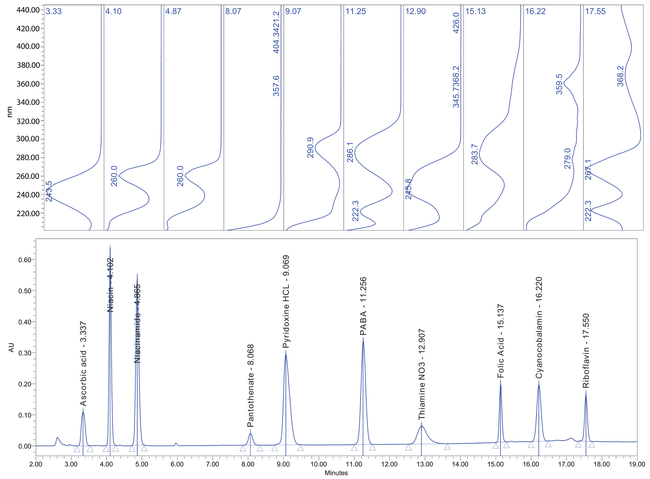
With this experiment we have evaluated a simple extraction procedure from a multi-vitamin/ mineral matrix (mvm) and quantitative HPLC determination for the simultaneous analysis of 10 water soluble vitamins. Ascorbic acid, niacin, niacinamide, ca-pantothenate, pyridoxine HCl, thiamine $NO_3$, folic acid, para-aminobenzoic acid, cyanocobalamin, and riboflavin were evaluated for specificity, range limit of detection, and limit of quantification. With good analytical ranges and quantitation limits established this experiment focuses on the accuracy and precision of the extraction procedure for this analysis procedure.

## Extraction

A simple extraction solvent of 0.05N NaOH was used to extract the water soluble components into solution. This solvent was chosen due to the acidic nature of many of the components being analyzed. Riboflavin is known to be much more soluble in dilute bases, and also has been shown to decompose at high pH.[3] For this reason we chose to extract with a basic solution, sonicating for 10 min, and then immediately buffering the solution at a pH between 6 and 7 with a 0.1M $(NH_4)_2HPO_4$ buffer. The pH range was determined to be optimum for minimizing decomposition based on earlier work presented by Thompson et.al.[4] Samples and standards were weighed into a clean dry 100ml class A volumetric flask at 0.0001g accuracy and then extracted into solution with 30 ml 0.05N NaOH for 10 minutes with sonication. Samples and standards were then diluted to the 100ml total volume with a 100mM $(NH_4)_2HPO_4$ pH 6.0. The pH was then measured to ensure it stayed within the predetermined pH range of 6.0 – 7.0. Each sample or standard was allowed to stand for 10 min, and then mixed thoroughly before being sampled. Three ml aliquots of each sample or standard were taken and centrifuged for 6min to remove undissolved solids prior to filtration with a 13mm 0.5µm nylon syringe filter. Samples were then stored in 2ml amber vials at 20ºC during the HPLC analysis. All samples were injected twice, with results reported as an average of the two injections.

## Testing

All analytes were tested at four concentrations in a multi-mineral matrix. Three separate standard curves were generated testing each matrix a minimum of three times with all analyses taking place over a 5 day time period. At the end of all testing all standard curve points for the three standardizations were plotted on a single curve producing linear correlation coefficients of 0.999 or better. Due to the rapid oxidation of ascorbic acid the curves for this compound did not remain linear.




## HPLC Conditions

HPLC Separation Conditions
HPLC separations were carried out using a Waters 2695 separations module equipped with a 2695 photodiode array detector.

Conditions
Column: YMC ODS-AQ 5µm 120Å 4.6mm x 250mm
Injection: Volume: 10 µl
Run Time: 30min
Detection: Photodiode Array Detector
Wavelength Range 200 – 450 nm
Analytical Wavelengths
205nm Pantothenic Acid
240nm Ascorbic Acid, Thiamine
260nm Niacin, Niacinamide, Riboflavin
280nm para-Aminobenzoic Acid, Pyridoxine, Folic Acid
360nm Cyanocobalamin

Mobile Phase: D - 30mM $H_2NH_2PO_4$, 4mM Sodium Hexanesulfonate pH 2.5
C – HPLC Grade Acetonitrile

**W2690/5 Gradient Table**

| | Time | Flow | %A | %B | %C | %D | Curve |
|---|---|---|---|---|---|---|---|
| 1 | | 1.00 | 0.0 | 0.0 | 7.0 | 93.0 | |
| 2 | 5.00 | 1.00 | 0.0 | 0.0 | 8.0 | 92.0 | 6 |
| 3 | 15.00 | 1.00 | 0.0 | 0.0 | 20.0 | 80.0 | 6 |
| 4 | 17.00 | 1.00 | 0.0 | 0.0 | 20.0 | 80.0 | 6 |
| 5 | 18.00 | 1.00 | 0.0 | 0.0 | 40.0 | 60.0 | 8 |
| 6 | 19.00 | 1.00 | 0.0 | 0.0 | 7.0 | 93.0 | 2 |
| 7 | 20.00 | 1.00 | 0.0 | 0.0 | 7.0 | 93.0 | 1 |
| 8 | 30.00 | 1.00 | 0.0 | 0.0 | 7.0 | 93.0 | 1 |

This HPLC separation was optimized to ensure baseline resolution of all analytes present as well as to minimize matrix interferences that may arise from different mvm matricies.

## Data

**Individual Matrix Data**

| | 2X A | 2X B | 2X C | 2X D | 2X E | 2X F | 2X G | 2X H | 2X I | 2X J | 2X Ave | Std | Rstd(ppt) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ascorbic Acid | 6.7% | 8.8% | 4.6% | 42.7% | 92.7% | 230.1% | 230.1% | 12.9% | 13.6% | 40.9% | **68.3%** | 89.3% | 1307.5 |
| Niacin | 78.1% | 67.3% | 98.3% | 103.2% | 100.9% | 101.6% | 101.6% | 100.1% | 99.5% | 101.0% | **95.2%** | 12.2% | 127.9 |
| Niacinamide | 96.9% | 70.6% | 145.6% | 88.7% | 94.8% | 88.5% | 88.5% | 93.2% | 131.2% | 98.9% | **99.7%** | 22.1% | 221.7 |
| d Calcium Pantothenate | 93.7% | 70.8% | 101.7% | 100.7% | 103.5% | 104.0% | 104.0% | 91.6% | 92.0% | 92.0% | **95.4%** | 10.2% | 106.5 |
| Pyridoxine HCl | 99.1% | 74.8% | 110.4% | 65.8% | 110.7% | 79.6% | 79.6% | 76.5% | 59.5% | 93.5% | **85.0%** | 17.7% | 208.7 |
| p-aminobenzoic Acid | 9.0% | 63.6% | 95.7% | 103.3% | 98.6% | 95.7% | 95.7% | 97.4% | 96.4% | 98.5% | **85.4%** | 29.0% | 339.6 |
| Thiamin Mono-nitrate | 67.9% | 59.2% | 89.2% | 85.2% | 68.9% | 125.7% | 125.7% | 64.7% | 61.1% | 88.2% | **83.6%** | 24.8% | 296.4 |
| Folic Acid | 68.0% | 41.9% | 62.5% | 85.9% | 76.8% | 82.3% | 82.3% | 87.0% | 97.5% | 113.6% | **79.8%** | 19.6% | 245.0 |
| Cyanocobalamin | 61.6% | 45.0% | 64.6% | 90.8% | 54.2% | 95.1% | 95.1% | 53.5% | 55.2% | 119.5% | **73.5%** | 24.7% | 336.1 |
| Riboflavin | 71.5% | 42.3% | 81.5% | 113.6% | 91.3% | 100.2% | 100.2% | 98.7% | 105.3% | 116.5% | **91.9%** | 22.0% | 239.9 |

| | 1X A | 1X B | 1X C | 1X D | 1X E | 1X F | 1X G | 1X H | 1X I | 1X J | 1X Ave | Std | Rstd(ppt) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ascorbic Acid | 19.6% | 7.8% | 7.7% | 9.6% | 24.5% | 54.2% | 5.4% | 23.5% | 21.3% | 20.3% | **19.4%** | 14.2% | 733.6 |
| Niacin | 60.6% | 82.3% | 99.0% | 103.6% | 89.0% | 101.6% | 99.2% | 97.7% | 103.1% | 102.6% | **93.9%** | 13.6% | 144.4 |
| Niacinamide | 78.9% | 50.6% | 84.3% | 88.5% | 78.4% | 87.0% | 89.1% | 85.1% | 91.1% | 85.5% | **83.8%** | 6.2% | 74.2 |
| d Calcium Pantothenate | 94.3% | 82.5% | 99.8% | 103.0% | 89.0% | 103.1% | 100.0% | 87.1% | 93.7% | 93.6% | **94.6%** | 7.0% | 73.6 |
| Pyridoxine HCl | 72.3% | 83.9% | 99.4% | 93.8% | 78.2% | 62.9% | 75.8% | 95.2% | 88.2% | 97.7% | **84.7%** | 12.2% | 144.0 |
| p-aminobenzoic Acid | 90.7% | 77.6% | 92.5% | 105.3% | 85.8% | 100.8% | 92.6% | 96.2% | 98.1% | 99.8% | **93.9%** | 8.0% | 85.2 |
| Thiamin Mono-nitrate | 67.3% | 71.3% | 84.0% | 78.8% | 64.6% | 74.4% | 86.1% | 75.0% | 66.4% | 67.5% | **73.6%** | 7.5% | 102.2 |
| Folic Acid | 62.9% | 51.5% | 56.6% | 76.5% | 113.0% | 70.6% | 70.2% | 124.0% | 116.8% | 90.7% | **83.3%** | 26.3% | 316.1 |
| Cyanocobalamin | 49.3% | 38.7% | 46.0% | 76.1% | 83.2% | 77.8% | 99.5% | 83.6% | 86.2% | 124.5% | **76.5%** | 26.1% | 340.6 |
| Riboflavin | 70.8% | 50.1% | 71.3% | 81.1% | 74.4% | 90.1% | 106.4% | 87.7% | 98.4% | 104.8% | **83.5%** | 17.6% | 210.2 |

| | 1/2X A | 1/2X B | 1/2X C | 1/2X D | 1/2X E | 1/2X F | 1/2X G | 1/2X H | 1/2X i | 1/2X J | 1/2X Ave | Std | Rstd(ppt) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ascorbic Acid | 15.2% | 15.1% | 0.0% | 0.0% | 10.9% | 0.0% | 0.0% | 0.0% | 40.0% | 39.8% | **12.1%** | 16.0% | 1320.3 |
| Niacin | 75.8% | 89.1% | 100.8% | 102.1% | 96.9% | 103.0% | 97.1% | 75.4% | 102.5% | 101.2% | **94.4%** | 10.7% | 113.5 |
| Niacinamide | 91.5% | 97.7% | 116.4% | 96.1% | 89.4% | 98.6% | 90.8% | 67.0% | 91.3% | 85.7% | **92.5%** | 12.3% | 132.8 |
| d Calcium Pantothenate | 88.4% | 89.8% | 98.4% | 101.5% | 93.2% | 101.4% | 97.2% | 67.8% | 91.7% | 92.3% | **92.0%** | 9.8% | 106.8 |
| Pyridoxine HCl | 95.6% | 89.1% | 90.3% | 80.2% | 80.3% | 151.5% | 90.4% | 71.0% | 83.4% | 117.1% | **94.9%** | 23.4% | 246.1 |
| p-aminobenzoic Acid | 85.7% | 83.2% | 93.0% | 99.5% | 91.2% | 97.7% | 102.3% | 72.6% | 98.6% | 98.3% | **92.2%** | 9.3% | 100.6 |
| Thiamin Mono-nitrate | 45.3% | 70.2% | 83.1% | 72.2% | 67.5% | 70.1% | 72.2% | 69.2% | 71.9% | 63.4% | **68.5%** | 9.6% | 139.8 |
| Folic Acid | 41.0% | 36.0% | 42.7% | 64.6% | 110.8% | 62.7% | 54.3% | 157.9% | 110.3% | 74.7% | **75.5%** | 39.2% | 518.7 |
| Cyanocobalamin | 55.1% | 45.1% | 79.5% | 57.7% | 54.6% | 80.6% | 82.8% | 47.3% | 95.3% | 95.7% | **69.4%** | 19.5% | 280.6 |
| Riboflavin | 63.7% | 58.8% | 77.9% | 75.0% | 77.3% | 86.1% | 77.3% | 78.4% | 98.8% | 101.7% | **79.5%** | 13.4% | 168.9 |

| | 1/4X A | 1/4X B | 1/4X C | 1/4X D | 1/4X E | 1/4X F | 1/4X G | 1/4X H | 1/4X i | 1/4X J | 1/4X Ave | Std | Rstd(ppt) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ascorbic Acid | 29.9% | 0.0% | 0.0% | 0.0% | 0.0% | 20.4% | 0.0% | 78.1% | 78.2% | 78.1% | **28.5%** | 35.8% | 1256.6 |
| Niacin | 101.2% | 97.6% | 99.6% | 104.1% | 99.2% | 82.6% | 103.1% | 101.0% | 102.6% | 102.3% | **99.3%** | 6.2% | 62.4 |
| Niacinamide | 84.2% | 102.1% | 89.8% | 87.9% | 81.9% | 85.0% | 85.4% | 113.3% | 81.7% | 83.6% | **89.5%** | 10.3% | 114.8 |
| d Calcium Pantothenate | 104.1% | 96.8% | 97.3% | 96.7% | 93.1% | 94.5% | 94.4% | 91.2% | 94.8% | 89.1% | **95.2%** | 4.0% | 42.5 |
| Pyridoxine HCl | 440.9% | 66.4% | 67.5% | 75.8% | 87.4% | 76.0% | 125.7% | 190.0% | 101.3% | 179.5% | **141.1%** | 114.5% | 811.5 |
| p-aminobenzoic Acid | 92.8% | 87.7% | 92.0% | 99.4% | 92.3% | 94.1% | 92.4% | 95.1% | 95.5% | 96.0% | **93.7%** | 3.1% | 33.0 |
| Thiamin Mono-nitrate | 48.1% | 89.6% | 71.6% | 67.0% | 63.4% | 66.7% | 67.5% | 51.9% | 40.3% | 63.3% | **62.9%** | 13.7% | 217.7 |
| Folic Acid | 54.1% | 92.7% | 40.0% | 48.1% | 51.6% | 62.9% | 51.5% | 83.9% | 74.4% | 98.5% | **65.8%** | 20.3% | 309.0 |
| Cyanocobalamin | 24.4% | 19.4% | 2.9% | 12.2% | 66.8% | 81.3% | 99.3% | 93.0% | 142.9% | 79.8% | **63.2%** | 44.9% | 710.0 |
| Riboflavin | 68.4% | 51.3% | 55.8% | 59.7% | 76.9% | 82.8% | 72.7% | 103.9% | 97.4% | 95.5% | **76.4%** | 18.3% | 239.4 |

**Summary of All Matrices**

| | 2X Ave | 1X Ave | 1/2X Ave | 1/4X Ave | Ave | std | rstd(ppt) |
|---|---|---|---|---|---|---|---|
| Ascorbic Acid | 68.3% | 19.4% | 12.1% | 28.5% | **32.1%** | 25.1% | 782.0 |
| Niacin | 95.2% | 93.9% | 94.4% | 99.3% | **95.7%** | 2.5% | 26.0 |
| Niacinamide | 99.7% | 83.8% | 92.5% | 89.5% | **91.4%** | 6.6% | 72.1 |
| d Calcium Pantothenate | 95.4% | 94.6% | 92.0% | 95.2% | **94.3%** | 1.6% | 16.9 |
| Pyridoxine HCl | 85.0% | 84.7% | 94.9% | 141.1% | **101.4%** | 26.8% | 264.7 |
| p-aminobenzoic Acid | 85.4% | 93.9% | 92.2% | 93.7% | **91.3%** | 4.0% | 44.1 |
| Thiamin Mono-nitrate | 83.6% | 73.6% | 68.5% | 62.9% | **72.2%** | 8.8% | 121.5 |

## Conclusion

The complete extraction of water soluble vitamins from the chose matrix proved to be more challenging than originally anticipated. We did get good recoveries for some wvs at certain levels, but the overall accuracy and precision for this method did not meet our expectations. Our analytical ranges and quantification limits for primary standards are acceptable, and very reproducible but the extraction procedure in clearly inadequate. Ascorbic proved to be problematic in all matrices. Because of it's rapid oxidation at most pH values, we believe that ascorbic acid should be analyzed with a separate analytical procedure better designed to test for the target analyte and it's oxidation products. The cyanocobalamin present in most mvm supplements is at a level that is below the capabilities of this procedure as well. With an improved extraction procedure which may need to be specific to the sample matrix, we believe that an accurate, precise and valid method for the simultaneous analysis of water soluble vitamins can be achieved.

## Primary Standards and Sample Matrix

**Specificity**
Primary standards for all analytes tested were obtained from Chromadex (Santa Ana, Ca). Having previously optimized the HPLC separation, specificity for all analytes present was evaluated by running chromatograms of each individual analyte and comparing capacity factors of each analyete present to those of the primary standard mixture. Along with the capacity factor, the UV/Vis spectrum of each analyte was compared.

**Range**
The analytical range for each analyte present was determined by running individual standard curves as well as a mixture of primary standards. Once linearity and absorption maxima were established the analytical ranges were set based on the limit of detection for each analyte present and the target for each analyte which was based on the dietary reference intakes (DRI)[5] of each analyte. With para-aminobenxoic acid (PABA) there is no DRI, so the target and range for this compound were established by looking at amounts present in common over the counter multi-vitamin/mineral dietary supplements. The following ranges were determined with linear correlation coefficients of 0.999 or better. We did see the most deviation from linearity at the lower end of these curves. The following ranges were chosen for use in the determination of accuracy and precision in the chosen matrix.

**Standard Curve Range Data**

| Analyte | Supplier | CAS# | Lot# | Standard Curve Range in µg/ml |
|---|---|---|---|---|
| Ascorbic Acid | Chromadex | 50-81-7 | 111020-255 | 500 - 7 |
| Niacin | Chromadex | 59-67-6 | 41303-002 | 200 - 3 |
| Niacinamide | Chromadex | 98-92-0 | 108808 | 200 - 3 |
| Ca Pantothenate | Chromadex | 137-08-6 | 99-3276 | 100 - 1.5 |
| 4-Aminobenzoic Acid | Chromadex | 150-13-0 | 22811-001 | 200 - 3 |
| Pyridoxine Hydrochloride | Chromadex | 58-56-0 | 22730-507 | 150 - 2 |
| Folic Acid | Chromadex | 59-30-3 | 02-06170-467 | 50 - 0.8 |
| Thiamin mono-nitrate | Chromadex | 532-43-4 | 22719-224 | 150 - 2 |
| Cyanocobalamin | Chromadex | 68-19-9 | 22742-681 | 100 - 1.5 |
| Riboflavin | Chromadex | 83-88-5 | HS-M693 | 100 - 1.5 |

**Limit of Detection – Limit of Quantification**
Limits of detection were chose to correspond with the bottom of the range for each analyte. This determination was made by observing that with each analyte the lowest point on each standard curve was beginning to deviate from the linear curve. With respect to the signal to noise ratio, there is much more room to drop the limit of detection. The limit of quantification for a 1.5 gram sample in a volume of 100ml would correspond to between 1 and 5 µg/g for the analytes tested.

**Sample Matrix**
The sample matrix chosen for this experiment was based on the formulation in Figure 1, which supplies 100%-200% of all wsv components in a mineral base containing the minerals present in common mvm dietary supplements. Sample matrices were formulated to contain the below labeled amounts of wsv (1X), double the amount present (2X), half the amount present (1/2X), and one quarter the amount present (1/4X). We first formulated and mixed a mineral base mix containing everything but the wsv, and then carefully weighed out the appropriate amounts of wsv to meet the individual 1X, 2X, 1/2X, 1/4X matrices and ground them with a mortar and pestle to make 200g quantities. Each sample was then tested 10 times at 1.5g sample sizes on 5 different days, with three different sets standard curves. PABA was added to this formulation to evaluate along with the other wsv, and because of the very low levels of cyanocobalamin in most mvm formulas, our 1X sample contained 100 times the level found in the mvm formula below.

**References**
1. CH Snider, The Extra Ordinary Chemistry of Ordinary Things, John Wiley and Sons, New York, 2nd Ed., 1995
2. RC Thompson, S Morris, Validation Viewpoint, LC/GC North America, Volume 19, number 11, November 2001
3. Merck Index 13th edition, Merck & Co Inc., Whitehouse Station NJ
4. RC Thompson, Short Term Stability of Water Soluble Vitamins in Solution with Respect to pH, 117th Annual AOAC meeting, Atlanta Ga.
5. Dietary Reference Intakes, National Academy Press, Washington, DC
6. Dietary Supplement Health and Education Act 1994 www.health.gov/dietsupp/ch1.htm

**Aknowledgments**
Thanks to Albion Advanced Nutrition for sponsoring this research, and the research and development department for their help with it. Special thanks to Debra Carey for help in putting the poster together.