APPLICATION NOTE

Liquid Chromatography

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The Qualitative and Quantitative Analysis of Water-Soluble B Vitamins by HPLC-PDA in Various Multivitamin Tablets

Introduction

Vitamins are essential nutritional elements in a human diet. Though we are able to synthesize certain vitamins, such as Vitamin D when exposed to sunlight, we mainly depend on our diet to supply our vitamins.

Since Ancient Egypt, humans have recognized that eating certain foods guarded us from certain diseases. But only in 1912 did Polish biochemist Kazimierz Funk establish the term *"Vitamine"*, derived from the compound words *Vital* and *Amine,* together meaning *amine of life.* Today, the term *"Vitamin"* is described as an organic compound serving as a vital nutrient that an organism requires in limited amounts.[1]

The food industry routinely fortifies many foods with vitamins to enhance their nutritional value and to help with deficiencies in dietary requirements. However, to meet legal requirements, the manufacturers must label their products in accordance to the specific regulations pertaining to the country in which the products are sold and consumed.

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In 2009, the United States Pharmacopeial Convention introduced the USP Dietary Supplements Compendium (DSC) – an industry-directed resource, including regulatory guidance and reference tools. In the U.S., the Food and Drug Administration (FDA) regulates all dietary supplement products and manufacturing practices under 21 CFR (Code of Federal Regulation), Part 111.[2,3] In Europe, the main EU legislation for vitamins and minerals in food supplements is Directive 2002/46/EC.[4]

Considering the above, this application focused on providing a robust chromatographic method for the separation of eight water-soluble B vitamins in vitamin supplement tablets using HPLC with photodiode array (PDA) detection. Due to the diverse absorptivity range amongst these vitamins, multiple analytical wavelengths were used to provide the best overall response. Method conditions and performance data, including linearity and repeatability, are presented.

Experimental

Hardware/Software

For chromatographic separations, a PerkinElmer Altus™ HPLC System was used, including the Altus A-10 solvent/sample module, integrated vacuum degasser, A-10 column module and Altus A-10 PDA detector. All instrument control, analysis and data processing was performed using the Waters® Empower® 3 chromatography data software (CDS) platform.

Method Parameters

The HPLC method parameters are shown in Table 1.

Table 1. HPLC Method Parameters.

HPLC Conditions	
Column:	PerkinElmer Brownlee™ SPP C18 2.7 µm, 3.0 x 100 mm (Part# N9308410)
Mobile Phase:	Mobile Phase A: 5 mM ammonium formate (NH_4 formate) buffer; pH 4.8 – 4.9 Mobile Phase B: Acetonitrile (ACN) Solvent program:

	Time	Flow	%A	%B	%C	%D	Curve
1		0.60	100.00	0.0	0.0	0.0	
2	2.00	0.60	100.00	0.0	0.0	0.0	6
3	8.00	0.60	70.00	30.0	0.0	0.0	6
4	10.00	0.60	70.00	30.0	0.0	0.0	6
5	10.01	0.60	100.00	0.0	0.0	0.0	6

Analysis Time:	10 min.; 6-minute injection delay time between injections
Flow Rate:	0.6 mL/min. (max. pressure: ~2300 psi/~153 bar)
Oven Temp.:	40 °C
Detection:	Altus A-10 PDA; Wavelengths used for calibration/quantitation: 214 nm: for B3, B3', B6, B7, B9, and B12 267 nm: for vitamins B1 and B2
Injection Volume:	3 µL
Sampling (Data) Rate:	10 pts./sec

Solvents, Standards and Samples

All solvents and diluents used were HPLC-grade and filtered via 0.45-µm filters.

Vitamin B standards were obtained from Sigma-Aldrich Inc® (St Louis, MO). These included: thiamine HCl (B1), riboflavin (B2), niacin (nicotinic acid; B3), niacinamide (B3; labeled "B3'" to distinguish it from niacin), pyridoxine (B6), biotin (B7), folic acid (B9) and cyanocobalamin (B12). The structures of these vitamins are shown in Figure 1.

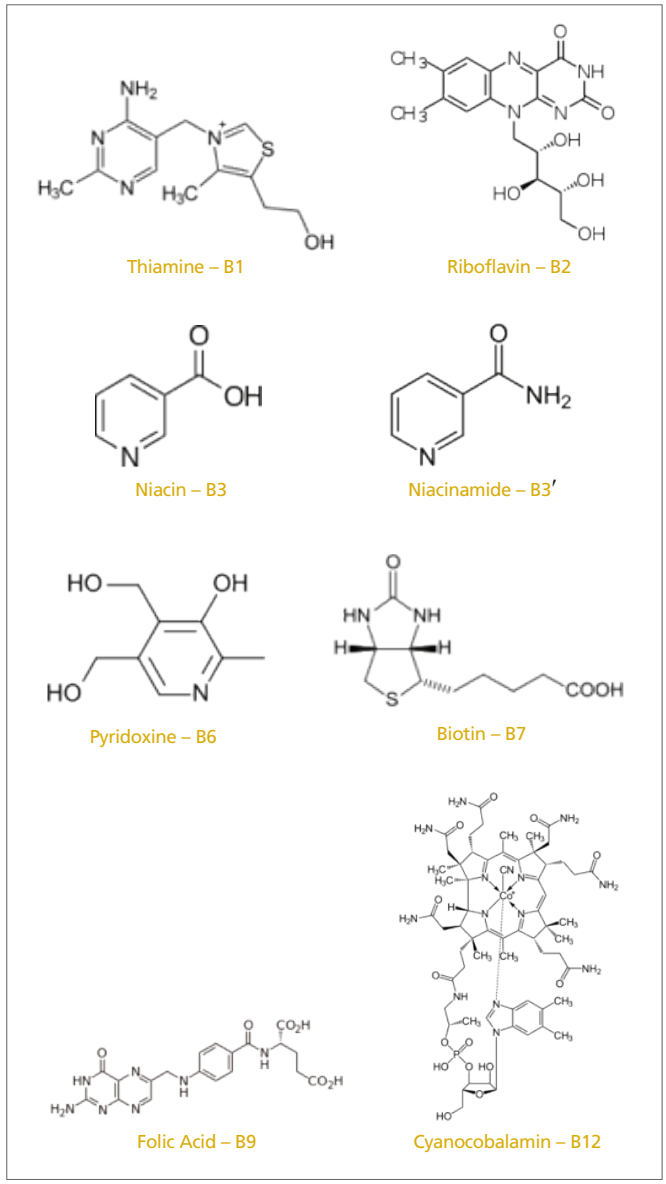

Figure 1. Chemical structures of B vitamins.

As the concentration of individual B vitamins in dietary supplements varies widely from one vitamin to another, two sets of standards were prepared. The first, working standard 1 (WS1), was used for demonstrating chromatographic separation and retention time (RT) reproducibility. It was prepared by weighing out 80 - 320 µg/ml of each of the eight vitamins, depending on the vitamin's relative absorptivity. The second, working standard 2 (WS2), was used for calibration/linearity. It was prepared by weighing individually selected amounts for each vitamin, depending on its expected concentration range in typical dietary supplements.

For both WS1 and WS2, the eight vitamins were weighed out and transferred into individual 50-mL volumetric flasks, to which 30 mL of water was added. Additionally, for vitamins B9, B2 and B7, to improve solubility, 0.5 mL of 0.1N NaOH was added to the corresponding flasks. All flasks were sonicated for five minutes and then filled to the mark with water. Equal volumes of the eight respective stock standards were then added together to produce each of the two WS solutions. For WS1, the resulting concentration for the eight vitamins was between 10 - 40 µg/ml. For WS2, the vitamins ranged in concentration from 0.9 to 525 µg/mL, depending on the vitamin. For calibration, all subsequent concentration levels were prepared from WS2.

All individual stock solutions and working standards were stored at 4.0 °C when not in use.

The multivitamin tablet samples, Multivitamin X and Multivitamin Y, were purchased from a local store. For each sample, a multivitamin tablet was transferred into an individual 50-mL volumetric flask and 30 mL of water was then added. The flask was then stoppered and placed on a heater/stirrer and heated at 40 °C while stirring for 15 min. Once the tablet was completely dissolved, each solution was sonicated for 10 min and then filled to the mark with HPLC-grade water. To remove any small particles, all standards and samples were filtered through 0.45 µm filters prior to injection.

Results and Discussion

Using the optimized chromatographic conditions described above, Figure 2 shows the HPLC separation of the eight B vitamins in WS1, at both 214 and 267 nm. All eight B vitamins are well resolved. The unknown peak at about 1.5 minutes did not interfere with the separation of the B vitamins and was not further characterized.

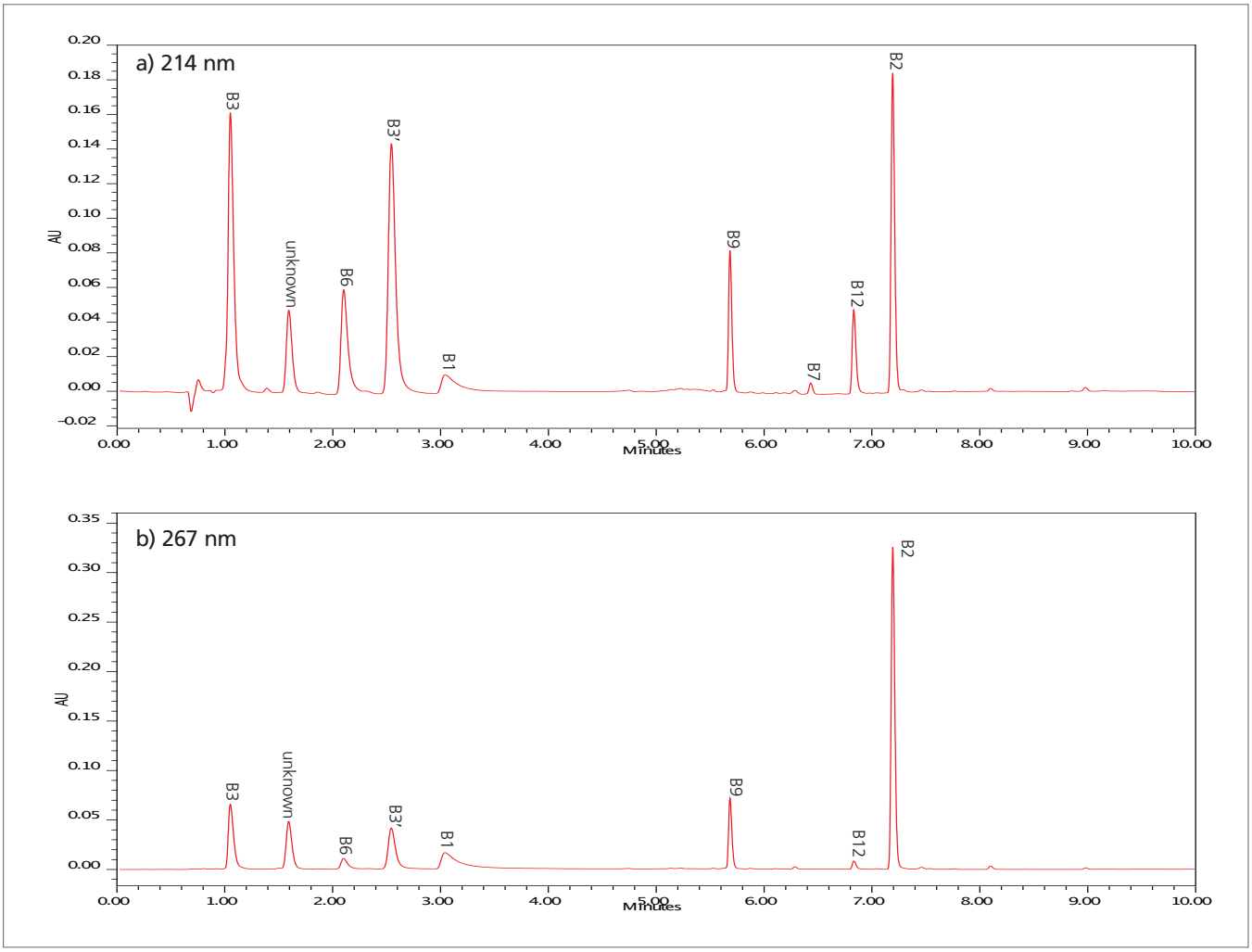

Figure 2. Chromatogram of the vitamin B working standard 1 (WS1); wavelength: a) 214 nm and b) 267 nm.

Figure 3 shows the overlay of 20 replicate WS1 injections, demonstrating exceptional reproducibility. The retention time % RSDs for all eight vitamins were < 0.3% (0.142% for riboflavin).

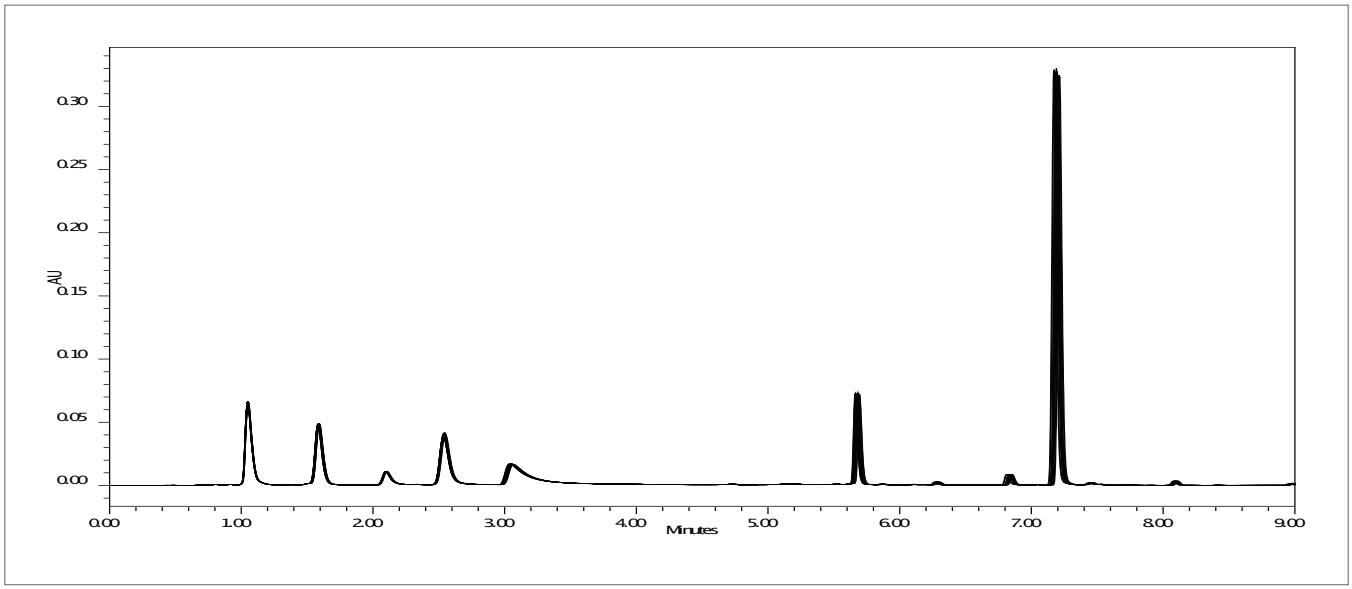

Figure 3. Overlay of 20 replicates of the WS1 vitamin B standard; wavelength: 267 nm.

Table 2 shows the concentration at each calibration level for each of the eight vitamins. Figure 4 shows the calibration results for four example vitamins. All B vitamins displayed linear fits, with most resulting in R^2 values > 0.999 (n = 3 at each level).

Table 2. Concentration (µg/mL) of B vitamins in WS2, for calibration/linearity.

Vitamins	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	Level 7
B1	2.64	8.79	17.58	26.38	35.17	43.96	52.75
B2	2.66	8.88	17.75	26.63	35.50	44.38	53.25
B3	2.65	8.83	17.67	26.50	35.33	44.17	53.00
B3′	26.23	87.42	174.83	262.25	349.67	437.08	524.50
B6	3.13	10.42	20.83	31.25	41.67	52.08	62.50
B7	0.54	1.79	3.58	5.38	7.17	8.96	10.75
B9	0.91	3.04	6.08	9.13	12.17	15.21	18.25
B12	0.04	0.15	0.29	0.44	0.58	0.73	0.88

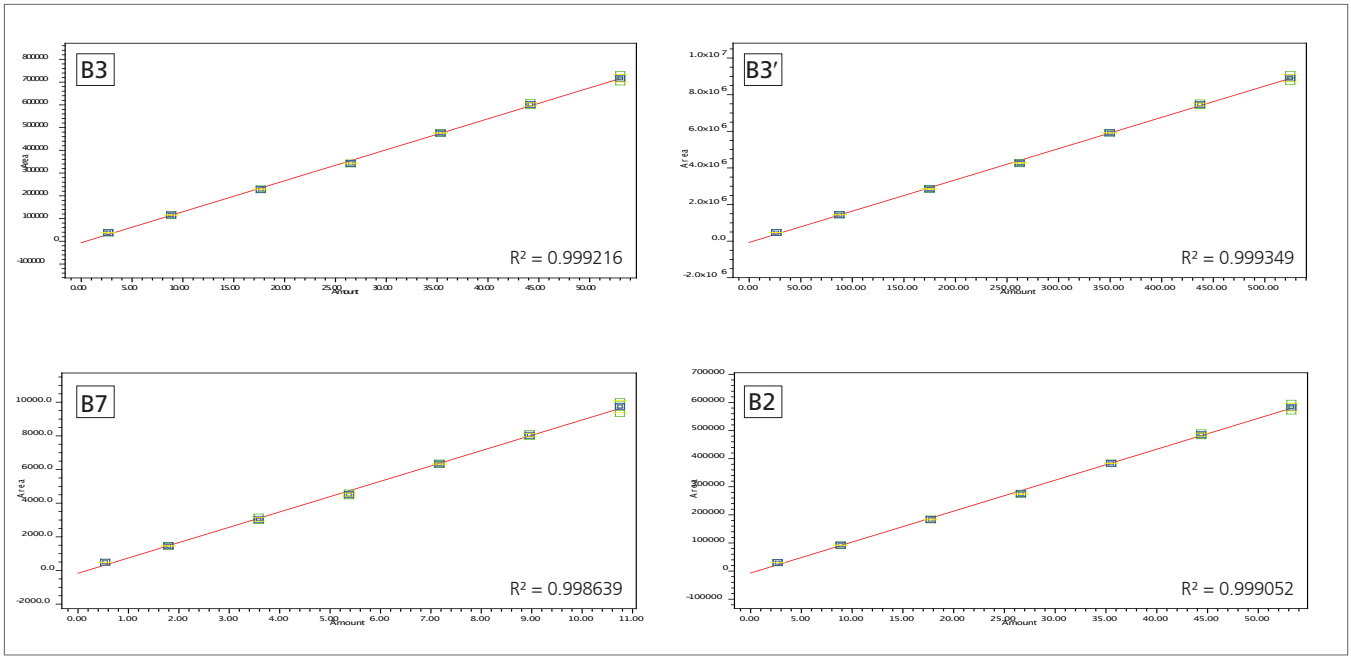

Figure 4. Calibration plots of 7-level calibration set for the vitamins B3, B3′, B7 and B2.

Using the same chromatographic conditions, the two multivitamin tablets, Multivitamin X and Multivitamin Y, were then analyzed. The chromatographic results are shown in Figures 5 and 7-9, displayed at 267 and 214 nm for each of the two samples. Comparing the chromatograms of both of these samples with the WS2 standard mix, it appears that both samples have a similar vitamin B profile. Their presence was further confimed by UV spectral matching, except for vitamins B7 and B9, due to their very low concentration in the samples.

For both samples, the large initial peak eluting very near the void volume was expected to be primarily vitamin C (ascorbic acid). As vitamin C was not the focus of this application, this peak was not further characterized.

Per Figure 5, for Multivitamin X, although a peak eluted at the expected retention time for vitamin B9 (folic acid), the UV spectra for that peak did not match the expected spectra for vitamin B9 (Figure 6). As there was no positive spectral confirmation of vitamin B9 in either sample, the amount of vitamin B9 could not be calculated.

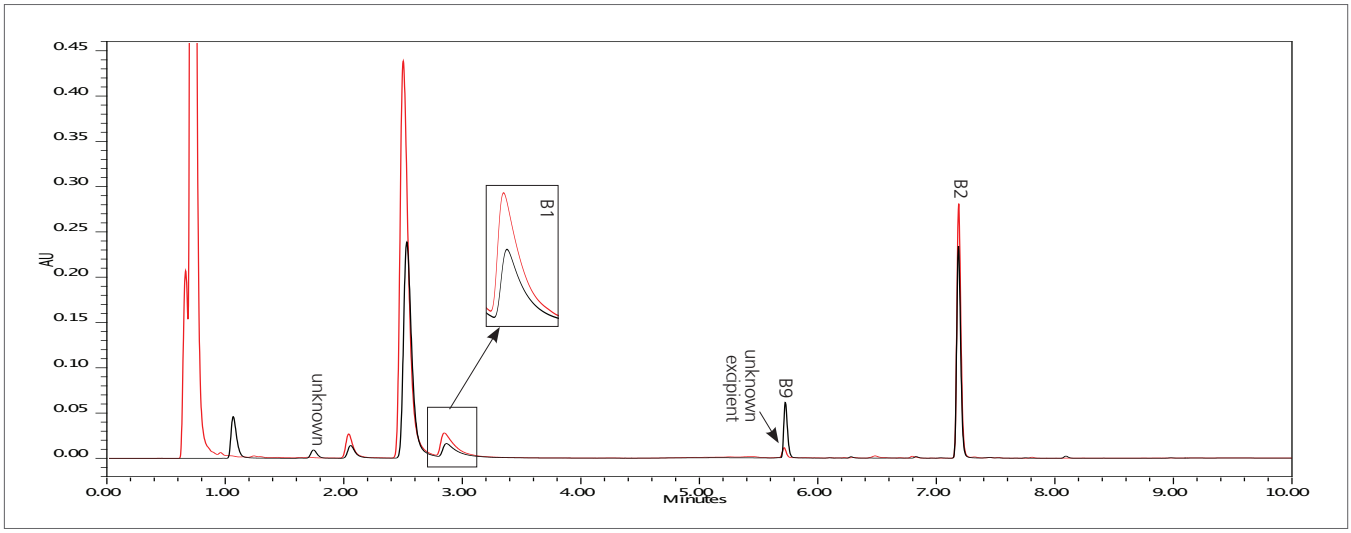

Figure 5. Overlaid chromatograms of Multivitamin X (red) with WS2 (black); wavelength: 267 nm. For optimal sensitivity, only vitamins B1 and B2 were quantitated at 267 nm.

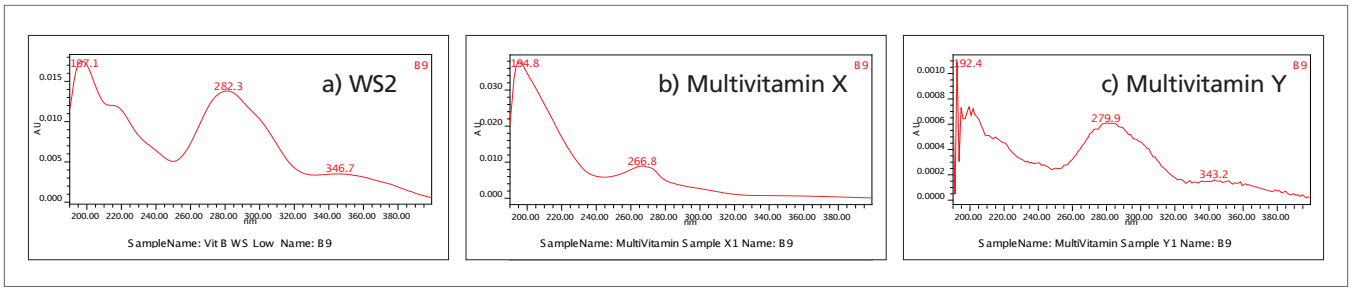

Figure 6. UV spectra captured at the expected elution time for vitamin B9: a) WS2, b) Multivitamin X and c) Multivitamin Y.

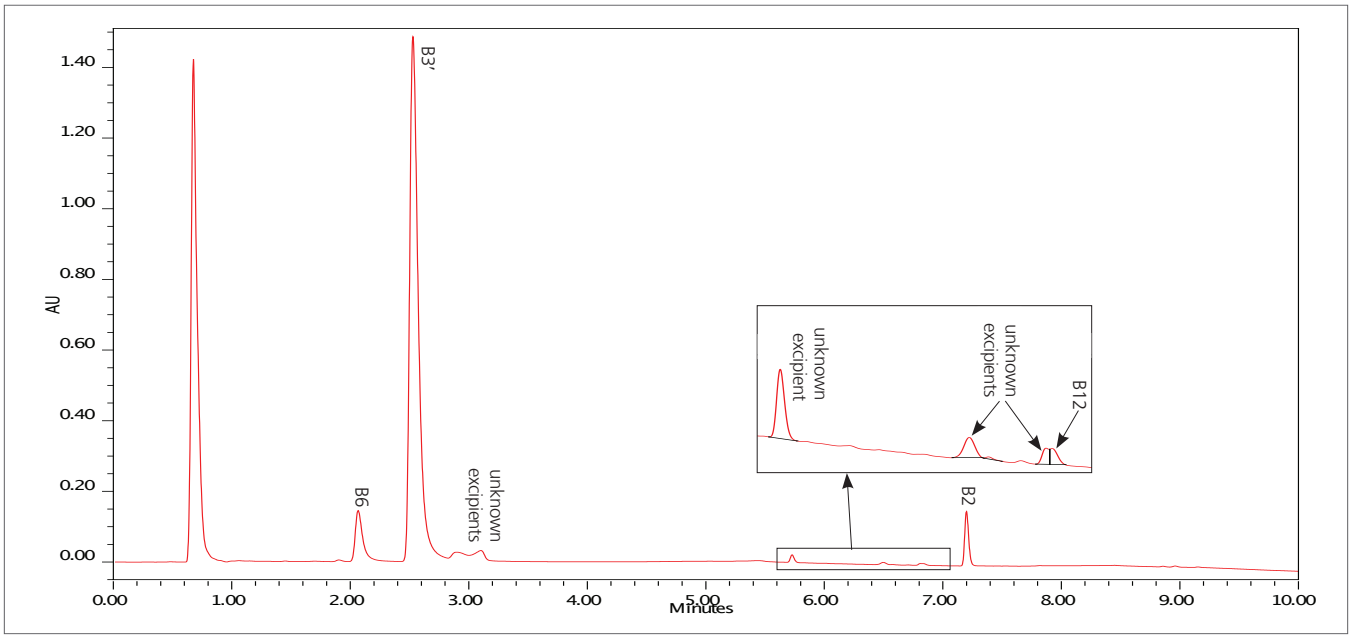

Figure 7. Single chromatogram of Multivitamin X; wavelength: 214 nm. For optimal sensitivity, only vitamins B6, B3' and B12 were quantitated at 214 nm. Vitamins B3 and B7 were not detected..

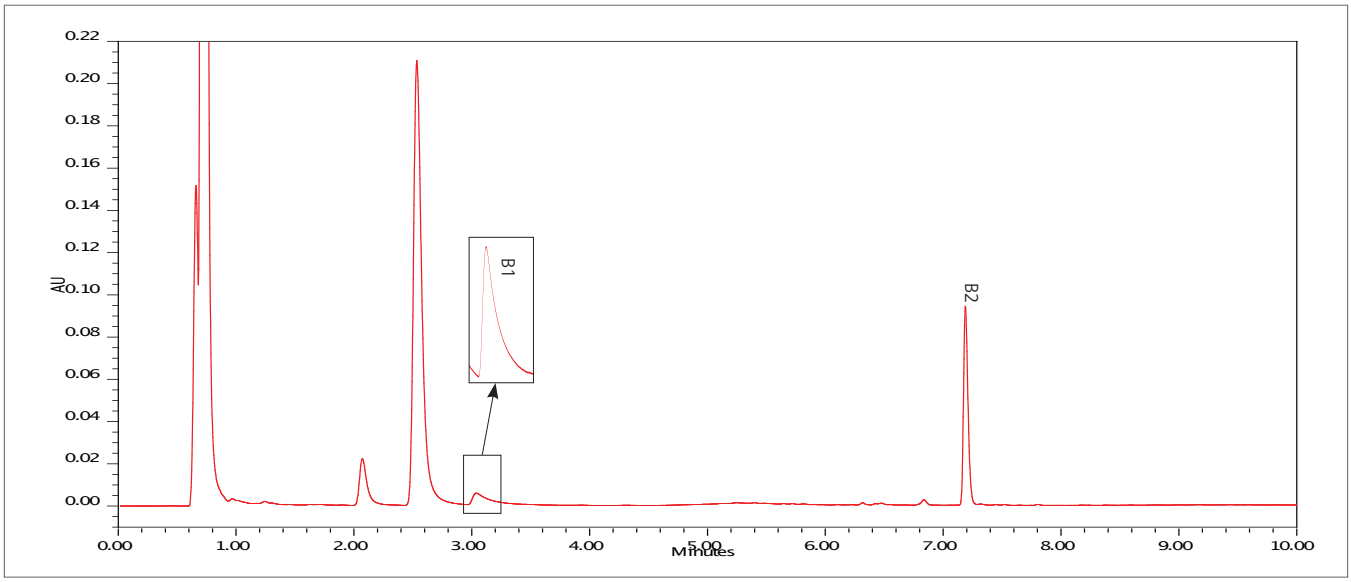

Figure 8. Single chromatogram of Multivitamin Y; wavelength: 267 nm. For optimal sensitivity, only vitamins B1 and B2 were quantitated at 267 nm.

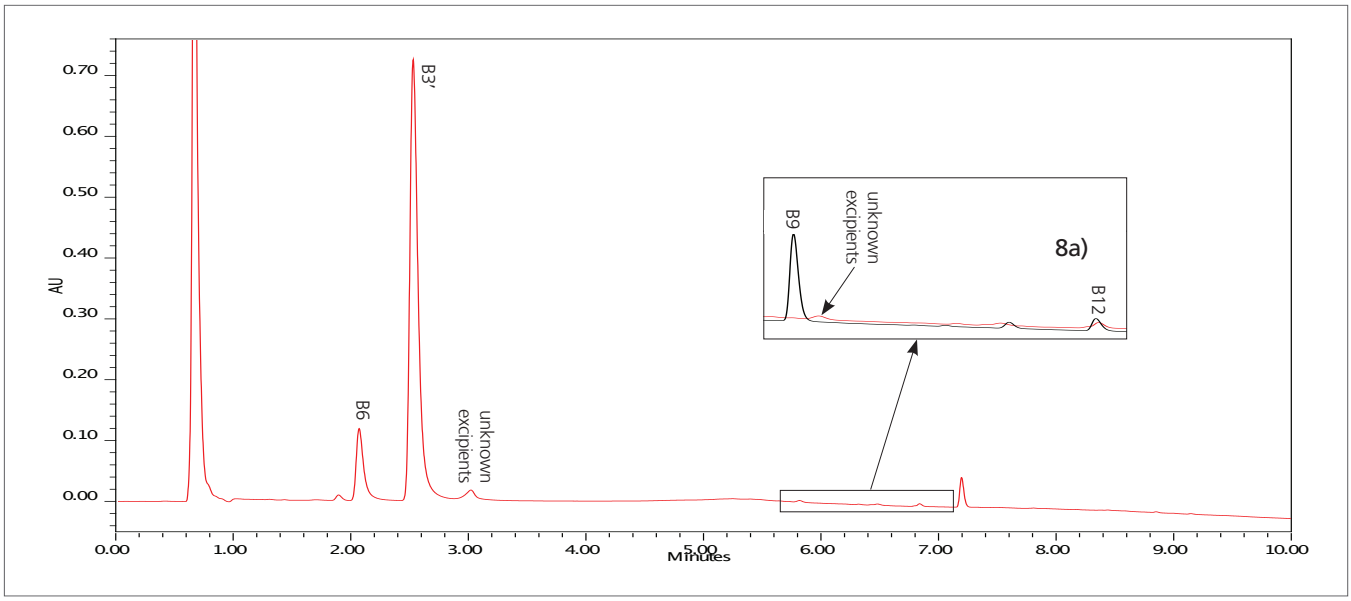

Figure 9. Chromatogram of Multivitamin Y; wavelength: 214 nm. For optimal sensitivity, only vitamins B6, B3' and B12 were quantitated at 214 nm Vitamins B3 and B7 were not detected. The insert (8a) highlights the region in which vitamins B9 and B12 were expected. Multivitamin Y (red), and WS2 (black) were overlaid to show the expected elution times of vitamins B9 and B12. In this region, only vitamin B12 was positively identified and quantitated.

Table 3. Quantitative results for Mutivitamin X and Y.

Multivitamin X:		
Vitamins	**Label claim per serving (µg) (serving size: 1 tablet)**	**Actual results (µg) (based on avg. of 2 indivdual tablets) (3 replicate injections per tablet)**
B1 – Thiamine HCl	1,500	2,237
B2 - Ribolfavin	1,700	1,680
B3 – Niacin (Nicotinic Acid)	0	0
B3' – Niacinamide	20,000	23,625
B6 - Pyridoxine	2,000	2,738
B7 - Biotin	30	ND*
B9 – Folic Acid	400	ND*
B12 - Cyanocobalamin	6	7
Multivitamin Y:		
Vitamins	**Amount per serving (µg) (serving size: 1 tablet)**	**Actual results (µg) (based on avg. of 2 indivdual tablets) (3 replicate injections per tablet)**
B1 – Thiamine HCl	1,500	1,218
B2 - Ribolfavin	1,700	518
B3 – Niacin (Nicotinic Acid)	0	0
B3' – Niacinamide	10,000	10,711
B6 - Pyridoxine	2,000	2,341
B7 - Biotin	30	ND*
B9 – Folic Acid	400	ND*
B12 - Cyanocobalamin	6	11

** Not detected: as vitamins B7 and B9 were not spectrally confirmed, identification was not established and, therefore, amounts could not be calculated.*

Table 3 shows the quantitative results for each multivitamin sample as compared to the label claim. This was based on the average of two analyzed tablets per sample and three replicates per injection. The daily recommended dose for each of these products is one tablet.

For both tablets, the calculated amounts for vitamins B3'and B6 were all close to label claim. Vitamin B3 was shown to be absent in both tablets, also as per label claim. For vitamins B1, though the amount found in Multivitamin Y was closer to label claim, the amount found in Multivitamin X was about 1.5-fold higher than label claim. For vitamin B2, the amount found in Multivitamin X was close to label claim; however, the amount found in Multivitamin Y was about 3-fold lower than label claim. For vitamins B12, though the amount found in Multivitamin X was close to label claim, the amount found in Multivitamin Y was about 2-fold higher than label claim.

The descrepencies from the label claim noted above were certainly of interest but could not be explained. As the sample preparation of these two tablet samples involved merely solubilizing in water followed by filtering through a 0.45 µm filter, there is of course the distinct possibility that some of the vitamin B content in these tablets was indeed other than claimed.

Regarding vitamin B7 (biotin), due to the combination of the low concentration and low-level excipient interference, the amounts could not be calculated.

Conclusion

The results obtained confirm the applicability of this method for the effective and robust analysis of B vitamins in dietary supplement tablets. All eight vitamins are well separated in under eight minutes.

Except for vitamins B7 and B9, the amount of each vitamin B found in two analyzed multivitamin tablets was easily quantitated and any excipients present did not interfere with the peaks of interest. Other than the noted exceptions, the determined amounts were close to the label claim.

References

1. "Vitamins", Wikipedia , The Free Encyclopedia, www.wikipedia.org
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3. U.S. Food and Drug Administration (FDA), U.S. Department of Health and Human Services, www.fda.gov
4. European Food Safety Authority (EFSA) – European Food Safety Authority, www.efsa.europa.eu

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