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응용 자료

Analysis of Water-Soluble Vitamins and Caffeine in Beverage and Multivitamin Products by Arc HPLC System With PDA Detection

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Abstract

A robust analytical method has been developed to detect and quantify 10 water-soluble vitamins and caffeine in multivitamin tablets and beverages. Arc HPLC-PDA combined with an XSelect HSS T3 XP Analytical Column enables the separation of 10 vitamins and caffeine in 16-minutes. The linearity, accuracy, reproducibility, and robustness of the method were evaluated to assess the system and method performance for the routine analysis of dietary supplements and beverage products.

Benefits

- Efficient test method for multivitamin tablets and vitamin beverages in 16-minutes
- Excellent reproducibility, accuracy, precision of Arc HPLC-PDA with the XSelect HSS T3 XP Column

Introduction

Vitamins are essential to maintain health and promote growth in the human body. Diet and food consumption

may not be sufficient to obtain the required daily vitamin levels. As a result, many manufacturers produce supplements, including multivitamin tablets, and vitamin enhanced beverages, to help consumers achieve the necessary levels. Several multivitamins can be found in many popular products such as fruit juice, vitamin waters, energy drinks, and sports drinks.

The Food and Drug Administration (FDA) has established recommended vitamin intake levels and requires the accurate labeling of products to provide consumers with important information to prevent overdose or overconsumption. Food and beverage manufacturers are required to clearly indicate vitamin compounds, colorings, and other additives that have been included in their products. Manufacturers must comply with strict regulatory requirements, such as European Regulation (EC) No.1925/2006[1] regarding the addition of vitamins and minerals to foods or Title 21 of the U.S. Code of Federal Regulations (CFR) Part 101 – Food Labeling[2] and Part 104 – Nutritional Quality Guidelines for Foods.[3] Once a product has been formulated, food and beverage manufacturers require rapid, reliable, and cost-effective methods to analyze the nutritional content of their products to ensure that their label claims can be substantiated.[4]

This application note will outline a robust analytical method that has been developed to detect and quantify 10 water-soluble vitamins and caffeine in multivitamin tablets and vitamin enhanced beverages. The separation was performed using an Arc HPLC-PDA combined with an XSelect HSS T3 XP Analytical Column.

Experimental

Materials and Reagents

Standards and buffer salts

Vitamins, caffeine standards, and sodium phosphate monobasic were obtained from Sigma-Aldrich.

The phosphoric acid was obtained from Honeywell Research Chemicals.

Multivitamin tablet and vitamin beverages samples were obtained from local sources (Massachusetts).

Sample Preparation

Standards Preparation

Most of the individual vitamin and caffeine standards were prepared at a concentration of 10 mg/mL in water; folic acid, biotin, and riboflavin were prepared in 100 mM sodium hydroxide. The stock standards were stored at 4 °C. A mixed standard of vitamins and caffeine was prepared at 100 µg/mL via serial dilution in water. The calibration curves ranged from 0.4 to 100 µg/mL. Due to the instability of ascorbic acid when mixed with other

vitamins, the mix of standard curve needed to be prepared fresh for analysis.

Adult and Children Multivitamins

Three replicates of individual adult and children's multivitamin tablets were placed into separate 50 mL centrifuge tubes, and an aliquot of water (50 mL) was added. The samples were shaken for 10-minutes, and then centrifuged for 5-minutes. The supernatants were collected, and another 50 mL of water was added to the pellets. The samples were shaken for 10-minutes and centrifuged for 5-minutes. The supernatants were collected and combined with the first supernatant. 20 mL of the 100 mM sodium hydroxide was added to the pellets, shaken for another 10-minutes, and centrifuged for 5-minutes. The supernatants were collected separately and injected. The vitamin concentrations in the samples were different; therefore, 1 µL and 10 µL injection volumes were used to quantify the samples by reducing the sample dilution.

Vitamins Beverages

The vitamin enhanced beverages were filtered with 0.2 µm PVDF syringe filter. The concentrations of the vitamins in the samples were different. Therefore, 1 µL and 10 µL injection volumes were used to quantify the samples without sample dilution.

LC Conditions

LC system:	Arc HPLC System
Detection:	PDA single wavelength at 205 nm, 254 nm 2998 PDA Spectrum 200–400 nm at 1.2 nm resolution
Vials:	Polypropylene 12 x 32 mm Screw Neck Vial (p/n: 186005220)
Filter:	Syringe Filter 0.2 µm PVDF (p/n: WAT200806)
Column(s):	XSelect HSS T3 XP Column, 100 Å, 2.5 µm, 4.6 mm x 150 mm (p/n: 186006741)

Column temp.:	30 °C
Sample temp.:	5 °C
Injection volume:	1 µL and 10 µL
Flow rate:	1.0 mL/min
Mobile phase A:	25 mM Sodium phosphate pH 3.0
Mobile phase B:	Methanol
Weak wash:	95:5, Water:methanol
Strong wash:	50:25:25, Acetonitrile:water:isopropanol
Seal wash:	95:5, Water:methanol

Gradient Table

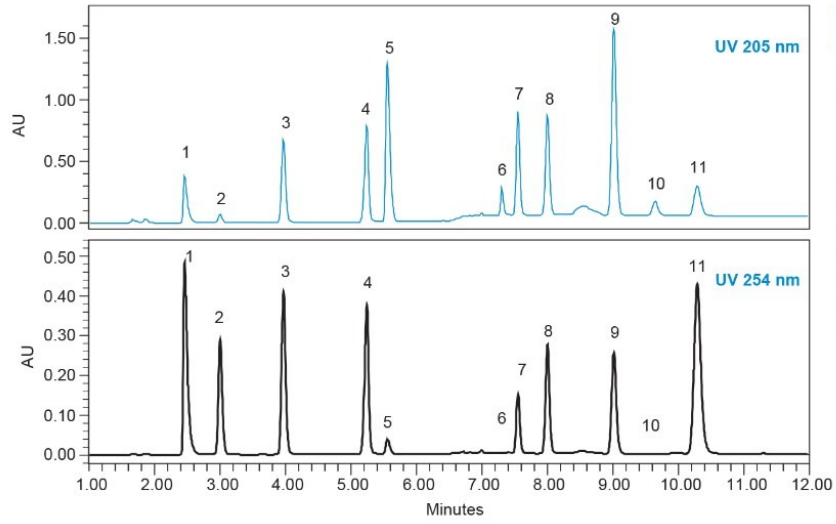
Time (min)	Flow (mL/min)	%A	%B	Curve
0.00	1.00	99.0	1.0	0
3.60	1.00	92.0	8.0	6
4.00	1.00	65.0	35.0	6
10.00	1.00	65.0	35.0	6
12.00	1.00	99.0	1.0	6
14.00	1.00	99.0	1.0	6

Data Management

Chromatography software:	Empower 3 Chromatography Data Software (CDS)

Results and Discussion

The method conditions for the analysis of water-soluble vitamins and caffeine were evaluated by performing several experiments with different mobile phases, pH conditions, and temperature conditions. 25 mM sodium phosphate, at pH 3 with a temperature of 30 °C, was selected for the separation method. The absorbance of the vitamins and caffeine were evaluated for maximum absorption of each individual vitamin. Most of the vitamins can be detected at wavelengths 254 nm and 205 nm except calcium pantothenate (B5), and biotin (B7) can only be seen at 205 nm. To reduce the analysis time, absorbance wavelengths 205 nm and 254 nm were used to analyze the vitamins and caffeine in this study. Retention time and UV absorbance of vitamins and caffeine are shown in Figure 1.



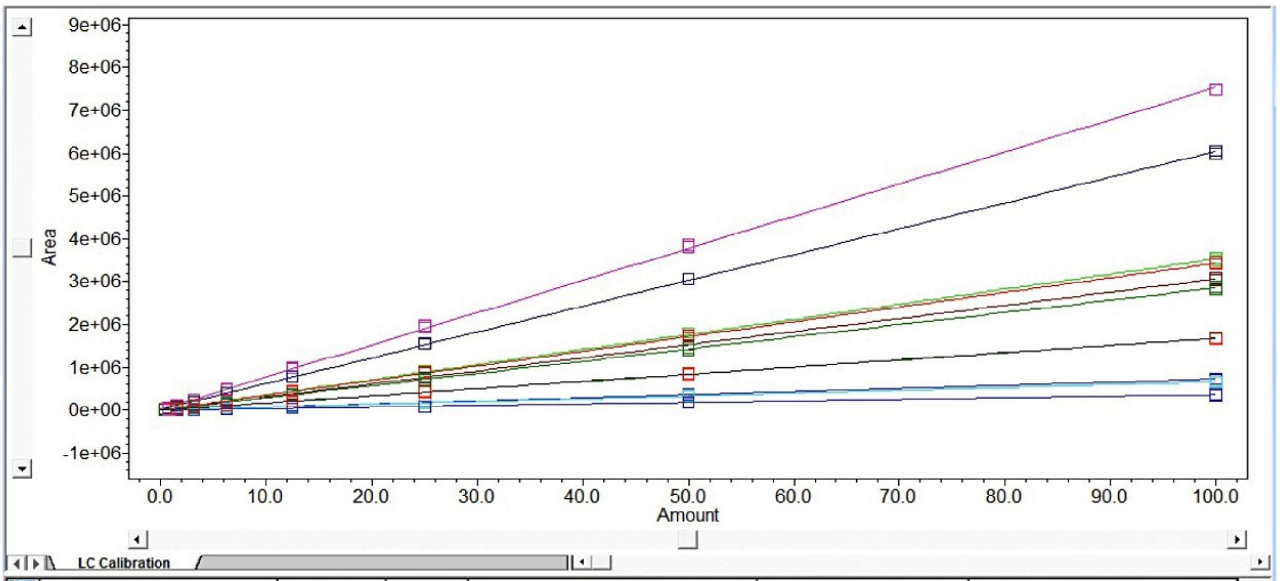
	Name	RT (min)	UV (absorbance)
1	Thiamine (C)	2.45	254
2	Ascorbic acid C	2.99	254
3	Nicotinic acid (B3-OH)	3.95	254
4	Nicotinamide (B3-NH2)	5.21	254
5	Pyridoxine (B6)	5.54	205
6	Calcium pantothenate (B5)	7.30	205
7	Cyanocobalamin (B12)	7.55	254
8	Folic acid (B9)	8.00	254
9	Caffeine	9.02	254
10	Biotin (B7)	9.65	205
11	Riboflavin (B2)	10.29	254

Figure 1. Separation of 10 vitamins and caffeine at 205 nm and 254 nm.

Calibration curve: Linearity, precision of area, and retention time

Multi-point calibration curves for water-soluble vitamins and caffeine were prepared via serial dilution in water. The standards were prepared in duplicate, and 6 replicates were injected for each concentration level.

Calibration curves were generated from 0.4 to 100 µg/mL and showed good linearity (R^2 > 0.999). The degradation of ascorbic acid (Vitamin C) was seen after approximately 4 hours. (R^2 > 0.9983). Standards were run immediately after preparation to be able to obtain the linear curve for Vitamin C due to the compound stability. Calibration curves of vitamins and caffeine are shown in Figure 2.

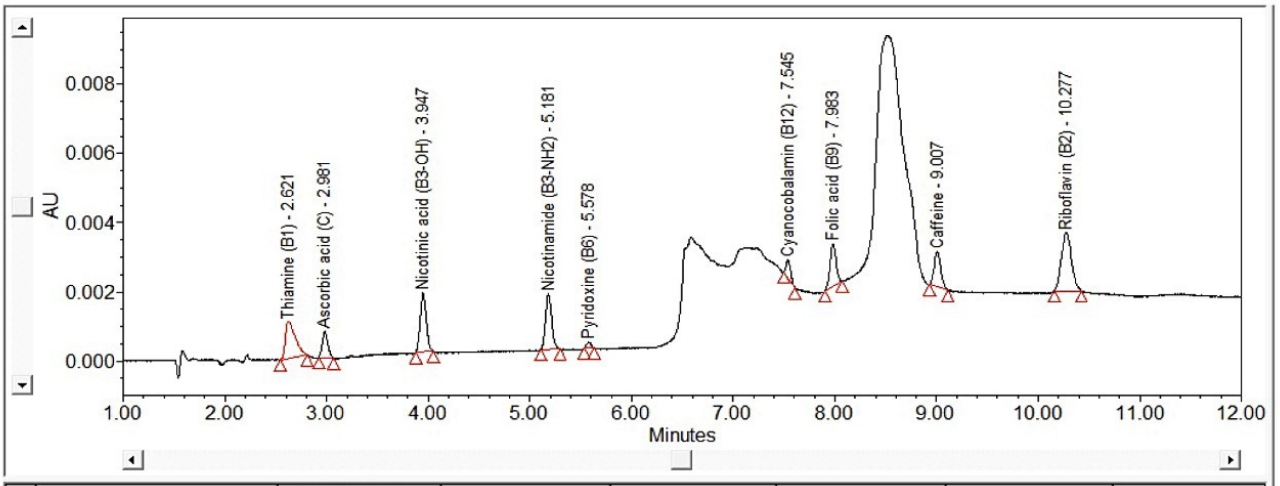


	Name	R	R^2	Equation	Fit Type	Channel
1	Thiamine (B1)	0.999898	0.9998	Y = 1.68e+04 X + 1.34e+03	Linear (1st Order)	2998 Ch2 205 nm@1.2 nm
2	Ascorbic acid (C)	0.999139	0.9983	Y = 3.60e+03 X + 2.44e+03	Linear (1st Order)	2998 Ch2 205 nm@1.2 nm
3	Nicotinic acid (B3-OH)	0.999811	0.9996	Y = 2.85e+04 X + 5.86e+03	Linear (1st Order)	2998 Ch2 205 nm@1.2 nm
4	Nicotinamide (B3-NH2)	0.999928	0.9999	Y = 3.43e+04 X + 7.78e+03	Linear (1st Order)	2998 Ch2 205 nm@1.2 nm
5	Pyridoxine (B6)	0.999926	0.9999	Y = 6.02e+04 X + 2.14e+04	Linear (1st Order)	2998 Ch2 205 nm@1.2 nm
6	Calcium pantothenate (B5)	0.999864	0.9997	Y = 6.63e+03 X + 4.42e+03	Linear (1st Order)	2998 Ch2 205 nm@1.2 nm
7	Cyanocobalamin (B12)	0.999934	0.9999	Y = 3.06e+04 X + 9.25e+02	Linear (1st Order)	2998 Ch2 205 nm@1.2 nm
8	Folic acid (B9)	0.999888	0.9998	Y = 3.52e+04 X + 8.06e+03	Linear (1st Order)	2998 Ch2 205 nm@1.2 nm
9	Caffeine	0.999873	0.9997	Y = 7.50e+04 X + 2.72e+04	Linear (1st Order)	2998 Ch2 205 nm@1.2 nm
10	Biotin (B7)	0.999853	0.9997	Y = 7.30e+03 X - 9.00e+02	Linear (1st Order)	2998 Ch2 205 nm@1.2 nm
11	Riboflavin (B2)	0.999932	0.9999	Y = 1.68e+04 X - 2.48e+01	Linear (1st Order)	2998 Ch2 205 nm@1.2 nm

Curve | Individual Points | Average Points

Figure 2. Calibration curves for 10 vitamins and caffeine at 205 nm from 0.4 to 100 µg/mL at UV 205 nm.

The signal to noise (s/n) of the vitamins and caffeine for calculating the LOD (3:1) and LOQ (10:1) were based on a peak-to-peak measurement in the Empower Software. The s/n values were obtained from the analysis of both UV absorbance 205 nm and 254 nm. A representative chromatogram at the concentration used to calculate the s/n 0.4 µg/mL at 254 nm, shows the LOQ level for most vitamins (Figure 3). The s/n for biotin, calcium pantothenate, and cyanocobalamin was obtained at different concentration levels using the 205 nm wavelength data. Table 1 provides a summary of the LOD, LOQ, and peak tailing for the vitamins and caffeine.



	Name	Retention time (min)	Area (µV*sec)	s/n	Resolution	Amount	USP tailing
1	Thiamine (B1)	2.62	7213	19		0.39	1.9
2	Ascorbic acid (C)	2.98	2940	13	2.6	0.39	1.2
3	Nicotinic acid (B3-OH)	3.95	7028	30	9.1	0.39	1.1
4	Nicotinamide (B3-NH2)	5.18	6770	29	11.3	0.39	1.2
5	Pyridoxine (B6)	5.58	473	3	4.2	0.39	1.1
6	Calcium pantothenate (B5)	7.38					
7	Cyanocobalamin (B12)	7.55	1603	9	23.3		1.2
8	Folic acid (B9)	7.98	5050	22	4.5	0.39	1.0
9	Caffeine	9.01	4637	18	9.0	0.39	1.2
10	Biotin (B7)	9.60					
11	Riboflavin (B2)	10.28	11409	30	8.4	0.39	1.1

3D Channels | 2D Channels | Peaks | Fractions

Figure 3. Representative chromatograms of the vitamins and caffeine calibration standard tested at 254 nm (0.4 µg/mL) with the s/n for LOQ and LOD.

	Compound name	LOD (µg/mL)	s/n	LOQ (µg/mL)	s/n	USP tailing
1	Thiamine (B1)	<0.4	19	<0.4	19	1.9
2	Ascorbic acid C	<0.4	13	<0.4	13	1.2
3	Nicotinic acid (B3-OH)	<0.4	30	<0.4	30	1.1
4	Nicotinamide (B3-NH2)	<0.4	29	<0.4	29	1.2
5	Pyridoxine (B6)	<0.4	31	<0.4	31	1.1
6	Calcium pantothenate (B5)	<0.8	9	<1.6	18	1.2
7	Cyanocobalamin (B12)	<0.4	17	<0.8	17	1.2
8	Folic acid (B9)	<0.4	22	<0.4	22	1.1
9	Caffeine	<0.4	18	<0.4	18	1.1
10	Biotin (B7)	<1.6	9	3.13	19	1.2
11	Riboflavin (B2)	<0.4	30	<0.4	30	1.1

Table 1. LOD and LOQ of vitamins and caffeine at UV 205 and 254 nm.

The repeatability of the retention time and area were calculated by %RSD for each concentration level of the standards curve. The %RSD of the retention time of most vitamins and caffeine was about 0.8% except Vitamin C, which was about 2%. The %RSD area of most vitamins was under 2%. Results of the %RSD for retention time precision and %RSD for area precision are shown in Figures 4A and 4B.

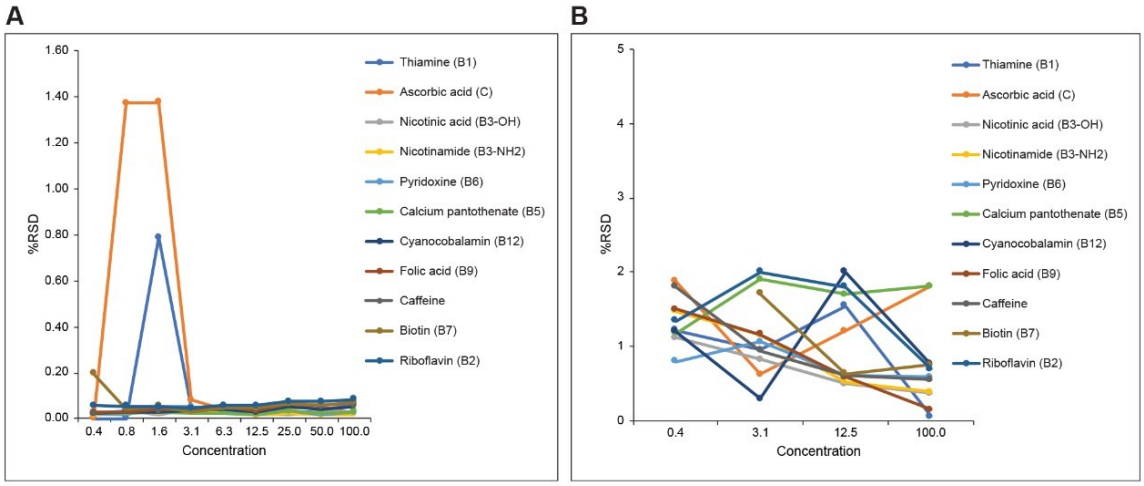


Figure 4A. %RSD of retention time precision of some vitamins at different level of standards (n=6). B . %RSD of Area precision of some vitamins at different level of standards (n=6).

Robustness of the method

Temperature and pH effect:

Different temperatures (28 °C, 30 °C, 32 °C) were evaluated for the method.

When the temperature was increased to 32 °C, most of the vitamins and caffeine still maintained good resolution. However, the resolution between nicotinamide and pyridoxine decreased as the temperature increased. Results are shown in Figure 5A.

Different pH levels (pH 2.8, pH 3.0, pH 3.2) were evaluated for the method.

When the pH was increased to pH 3.2, most of the vitamins and caffeine still maintained good resolution. The resolution between thiamine and ascorbic acid (Vitamin C) decreased as the pH increased. Results are shown in Figure 5B.

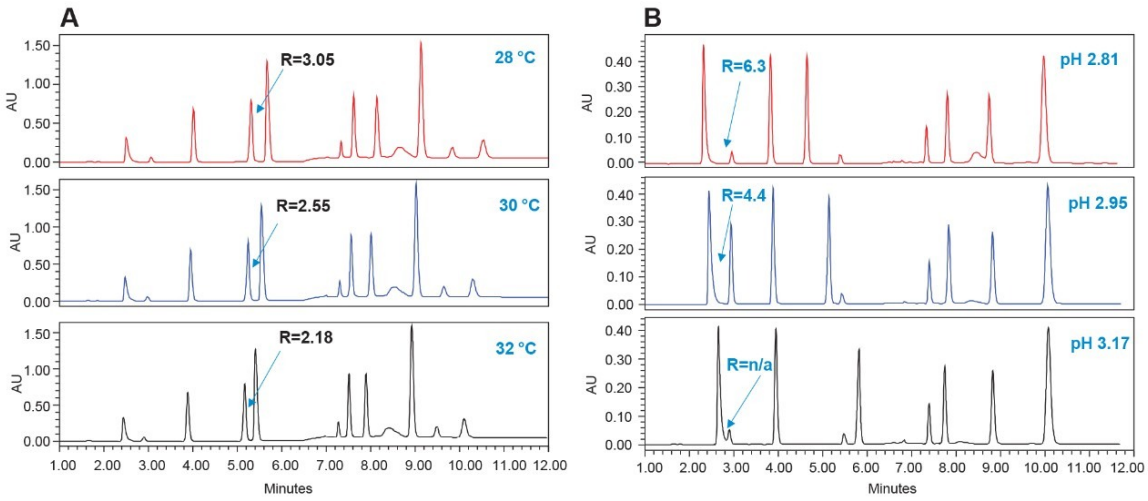


Figure 5A. Effect of temperature on resolution of nicotinamide and pyridoxine at UV 205 nm. B. Effect of pH on resolution of thiamine and ascorbic acid at UV 254 nm.

Column lifetime and pressure trace

The column was used intensively to evaluate the conditions for the separation method and analyzed vitamins products. The reproducibility of the column lifetime was based on the retention time of injected new 100 µg/mL standards from the beginning gradient and after approximately 800 injections; a new standard of 100 µg/mL was injected again. The pressure trace of the column was monitored from injection #120 to injection #1000; approximately 200 psi increase at the #1000 injection. Representative chromatograms are shown in Figure 6A for column lifetime and Figure 6B for pressure trace.

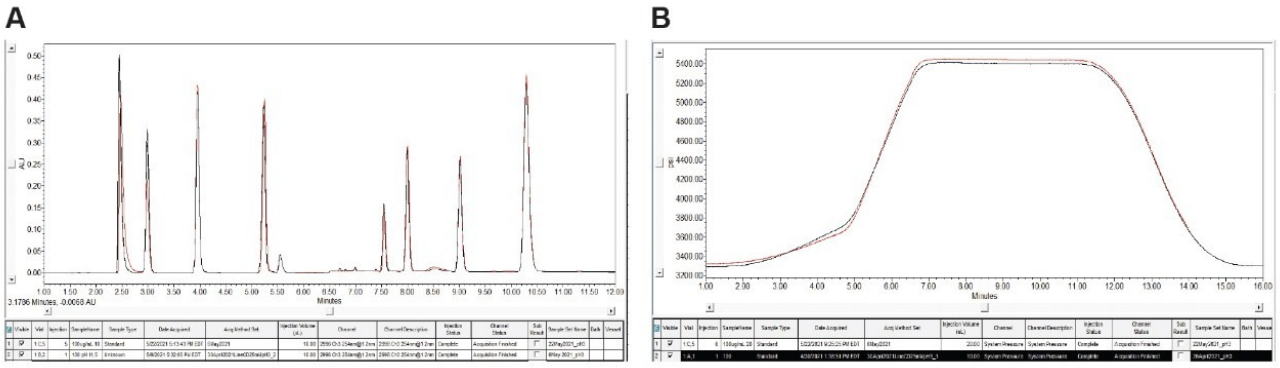


Figure 6A. Column performance after 700 injections of the same gradients. B. Pressure trace of the column after 1000 injections of the same gradients.

Carryover

Figure 7A shows the chromatograms of the blank injections that were injected before and after 3 injections of

children's vitamin tablets. Figure 7B shows the chromatogram of the blank injection that was injected before and after a mix of vitamin and caffeine standards at 100 µg/mL. There was no carryover observed from these samples.

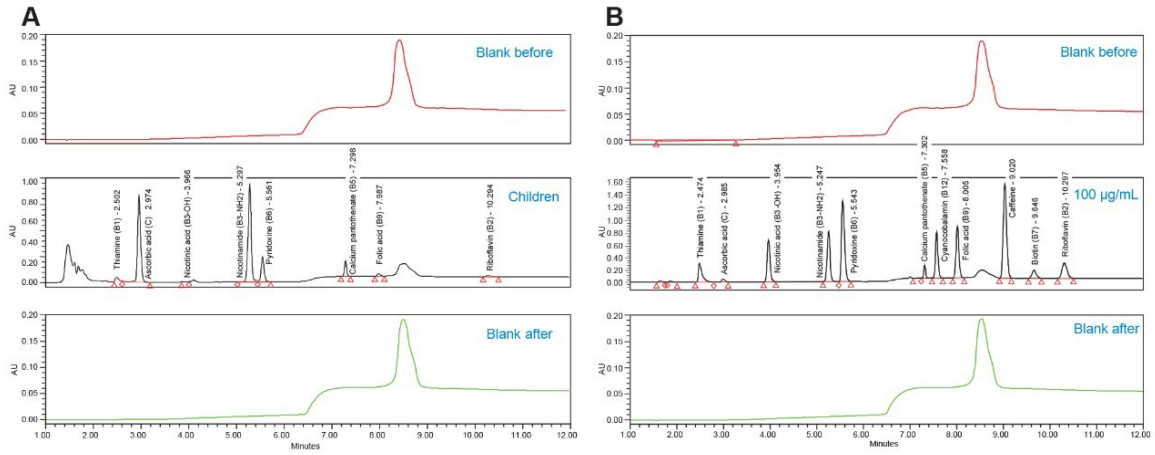


Figure 7A. Carryover observed for children's vitamin. B. Carryover observed for vitamin and caffeine at 100 µg/mL.

Analysis of multivitamin tablets and beverages

The quantitative results generated from the analysis of 10 vitamins in adult and children's multivitamin tablets are shown in Table 2. Samples were from different manufacturers that had several vitamins at varying concentrations. An injection volume of 1 µL was used to quantify the vitamins with high concentrations present in the sample such as Vitamin C. A 10 µL injection volume was used to quantify the biotin and other vitamins with low concentrations in the samples. The smaller injection volume can be applied to concentrated samples to reduce the need for dilution of the samples. However, if the samples contain very high concentrations of the vitamins and caffeine, the samples will need to be diluted to fit the linearity curve. The injection volume on the Arc HPLC injector has been previously study and showed excellent linearity and precision.[5]

	Adult tablet	Adult tablet	Children tablet	Children tablet
Name	Amount label	Amount detected (%RSD)	Amount label	Amount detected (%RSD)
Thiamine (B1) mg	1.5	1.6 (4)	1.2	1.3 (5)
Ascorbic acid (C) mg	90.0	83.6 (2)	90.0	96.5 (1)
Nicotinamide (B3-NH2) mg	20.0	22.5 (2)	12.0	12.6 (1)
Pyridoxine (B6) mg	3.0	3.2 (4)	1.7	1.7 (5)
Calcium pantothenate (B5) mg	10.0	13.3 (4)	5.0	6.5 (4)
Cyanocobalamin (B12) µg	25.0	nd	2.4	nd
Folic acid (B9) µg	500.0	381 (3)	240.0	250 (3)
Biotin (B7) µg	30.0	nd	30.0	nd
Riboflavin (B2) mg	1.7	1.6 (2)	1.3	1.0 (5)

Table 2. Quantitative results from the analysis of vitamin tablet products (n=3).

The tablets were extracted with 50 mL water (2x) to extract water-soluble vitamins and caffeine. Afterward, 20 mL of 100 mM of sodium hydroxide was applied to extract biotin, folic acid, and riboflavin. The supernatant of extraction was injected separately to detect the vitamins that dissolved in sodium hydroxide. The concentration of cyanocobalamin (B12) and biotin (B7) in the tablets were under the limit of detection and quantification of the method. A representative chromatogram of vitamins for the adult tablets was analyzed at 205 and 254 nm (Figure 8).

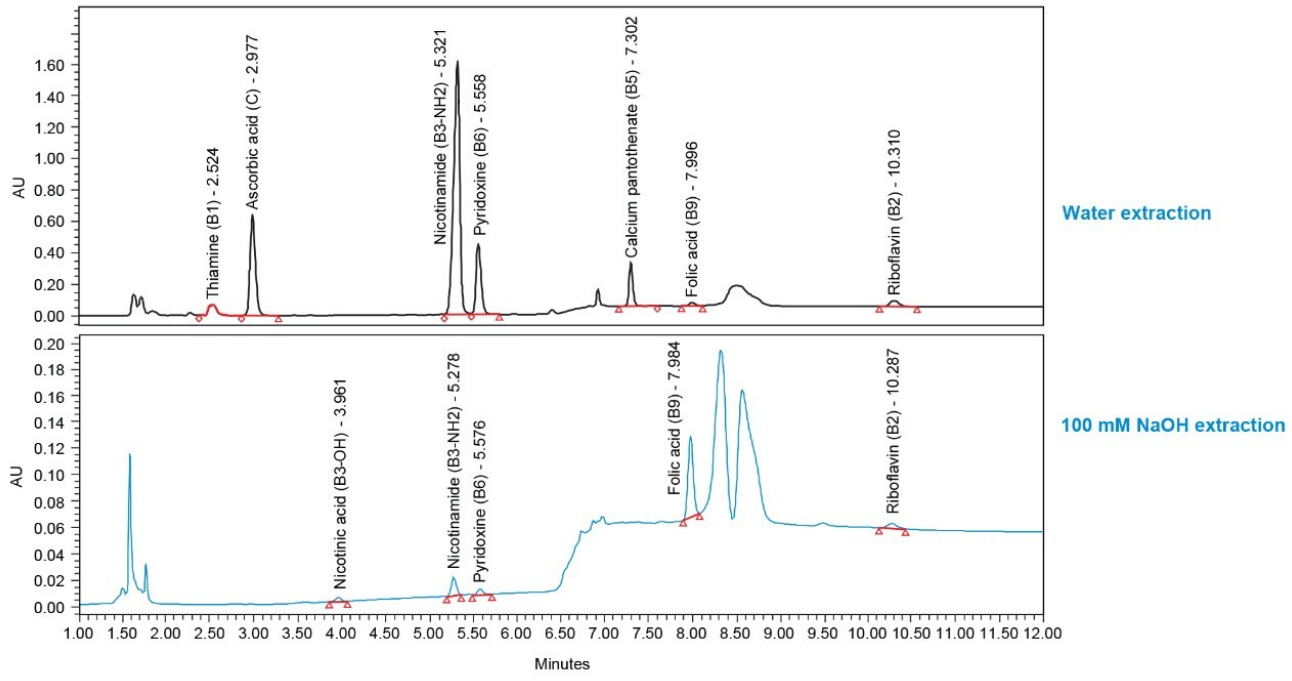


Figure 8. Chromatograms from the analysis of adult multivitamin tablets analyzed at UV 205 nm with water extraction, followed by 100 mM sodium hydroxide.

Some popular beverages such as vitamin enhanced water, sports drinks, and energy drinks were analyzed for

vitamin and caffeine concentration. Injection volumes of 1 µL and 10 µL were used to inject the samples without sample dilution. The concentration of cyanocobalamin (B12) in the beverages were under the limit of detection and quantification for cyanocobalamin of the method. The results are included in Table 3, and the representative chromatogram of energy drinks was analyzed in Figure 9.

	Vitamin waterS4	Vitamin waterS4	Sport drinkS1	Sport drinkS1	Energy drink R8	Energy drink R8
Name	Label value	Detected value (%RSD)	Label value	Detected value (%RSD)	Label value	Detected value (%RSD)
Ascorbic acid (C) mg	180.0	saturated				
Calcium pantothenate (B5) mg	5.0	6.1 (4)	0.8	0.9 (1.0)	3.5	3.91 (5)
Cyanocobalamin (B12) µg	2.4	nd (0)	0.4	nd (0)	2.7	nd
Nicotinamide (B3-NH2) mg	16.0	22.5 (1)	2.4	2.2 (0)	22.4	24 (0)
Pyridoxine (B6) 1.7 mg	1.7	2.1 (3)	0.3	0.1 (0)	6.1	6.4 (0)
Caffeine mg					114.0	118 (0)

Table 3. Quantitative results from the analysis of vitamin beverage products (n=3).

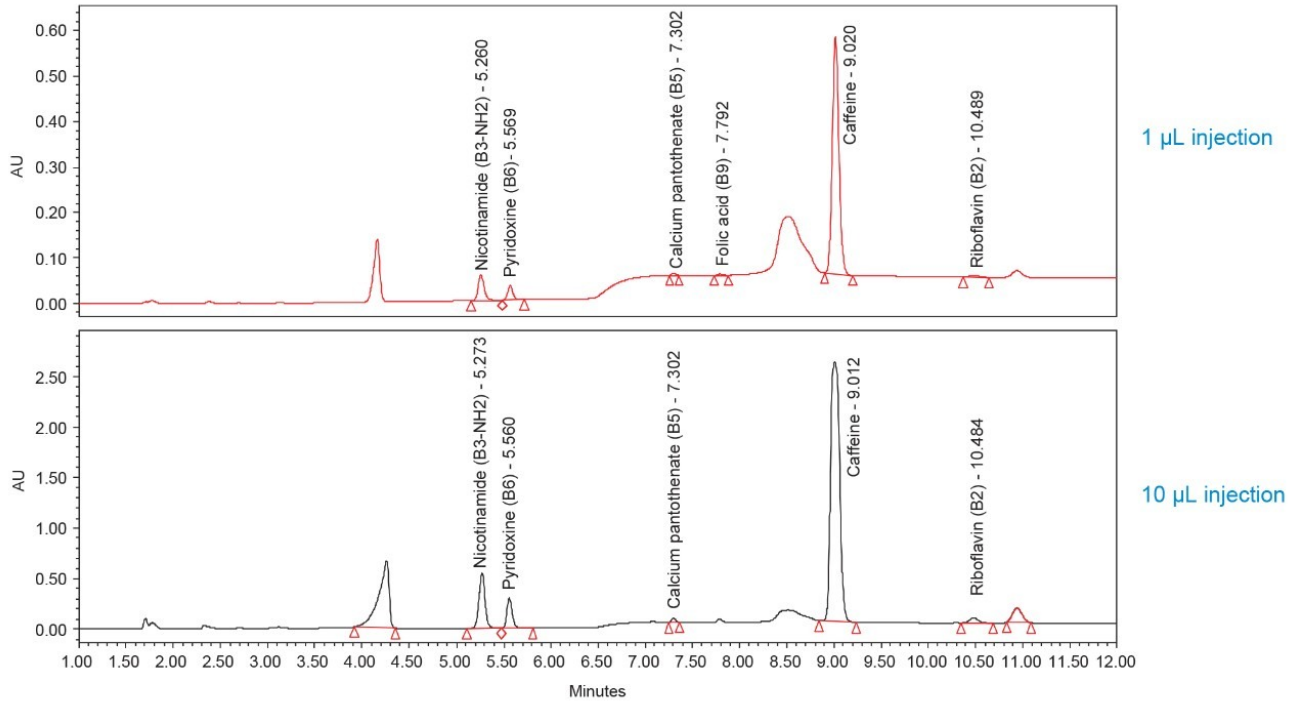


Figure 9. Chromatograms from the analysis of energy drinks analyzed using UV at 205 nm.

Conclusion

The Waters ARC HPLC-PDA System combined with the XSelect HSS T3 2.5 µm column enabled the separation

of 10 vitamins and caffeine under 16-minutes.

The method demonstrated excellent linearity, precision, and accuracy.

The XSelect HSS T3 XP Column showed excellent reproducibility of the retention time and the backpressure.

This analytical workflow is suitable for supporting beverage manufacturers in:

- Standardizing analysis for a range of complex water-soluble vitamins.
- Minimizing overages of vitamins and other raw ingredients.
- Reducing the number of methodologies required.
- Quickly confirming nutritional value, vitamin specifications, and label compliance.

References

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