Application Note: 294

Simultaneous Analysis of Water-Soluble Vitamins in Vitamin-Enriched Beverages and Multivitamin Dietary Supplements by UHPLC-MS/MS

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Key Words

- TSQ Quantum Access
- WSV
- B Vitamins
- Vitamin C
- Food Analysis

Introduction

Vitamins are nutrients essential to human health, and are present in almost all types of foods. In addition to food sources, vitamin supplements are often consumed to ensure adequate vitamin intake, as a customary diet does not always provide sufficient vitamins due to bias and/or limitation in the choice of foods, or malfunctions in digestion and ingestion. Vitamin supplements are available in various forms such as single- or multivitamin tablets, formula, and vitamin-enriched beverages (VEB). Certain foods are commercially fortified with vitamins and/or other nutritional essentials such as minerals. Based on their solubility, vitamins are divided into two categories: water-soluble vitamins (WSV) and fat-soluble vitamins (FSV). WSVs include vitamin C (ascorbic acid), B_1 (thiamine), B_2 (riboflavin), B_3 (niacin, niacinamide), B_5 (pentothenic acid), B_6 (pyrodoxine), B_7 (biotin), B_9 (folic acid), and B_{12} (cyanocobalamine). Accurate quantitative measurements for vitamins are required to ensure product quality and regulatory compliance as well as to monitor vitamin intake.

Established methods for vitamin analysis include microbiological methods, which are typically designed for single vitamin analysis and are time consuming,[1,2] and chromatographic methods, including gas chromatography,[3,4] capillary electrophoresis,[5,6] and liquid chromatography (LC) with various methods of detection.[7–16]

LC methods are generally used for simultaneous determination of multiple vitamins of interests and for establishing vitamin profiles in a variety of matrices with various modes of detection.[7–10, 13–16] Here we present a high-throughput method for simultaneous determination for the above mentioned ten WSVs using ultrahigh-performance LC and tandem mass spectrometry (UHPLC-MS/MS). Chromatography was optimized for the total resolution of all target analytes on a Thermo Scientific Acclaim C30 reversed-phase (RP) column. An MS/MS instrument was operated in selected reaction monitoring (SRM) mode for the best selectivity and sensitivity, and an isotope labeled internal standard (IStd) was used for accurate quantitation.

Randomly selected VEBs and multivitamin supplement tablets (MVSTs) were assayed by this method for selected vitamins. Much higher values were observed for most vitamins in VEBs than indicated on product labeling. Results also showed close agreement between observed and label values for MVSTs.

Equipment

Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC (RSLC) system including:

- DGP-3600RS Dual Ternary Rapid Separation Pump System
- SRD-3600RS Integrated Solvent and Degasser
- WPS-3000TRS Analytical Autosampler with 130 µL sample loop
- TCC-3000RS Thermostatted Column Compartment
- VWD-3400RS Four-Channel Variable Wavelength Detector

Thermo Scientific TSQ Quantum Access MAX Triple Stage Quadrupole Mass Spectrometer with heated electrospray ionization (HESI II) probe

Thermo Scientific Dionex DCMS^{Link} 2.11 software

Thermo Scientific Xcalibur 2.1 with the Foundation 1.0.2 Framework and TSQ Series 2.3 control software

Reagents

Deionized (DI) water, 18.2MΩ–cm resistivity

Acetonitrile (CH_3CN), HPLC grade or equivalent, Fisher Scientific (AC610010040)

Ammonium formate, ≥99.995% trace metals basis, Sigma-Aldrich Co. LLC (516961)

Formic acid, Sigma-Aldrich Co. LLC (06440)

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Standards

A set of water-soluble vitamin standards was purchased from AccuStandard, Inc. (VIT-WSK-R1-SET) including:

VIT-001N: Vitamin B1 hydrochloride (B_1)

VIT-002N: Vitamin B_2

VIT-003N: Vitamin B_6

VIT-004N: Vitamin C

VIT-005N: Niacin (B_3)

VIT-006N: Nicotinamide (B_3')

VIT-007N: Vitamin M (B_9)

VIT-008N: D-Pantothenic acid (B_5)

VIT-009N-R1: Vitamin H (B_7)

VIT-010N-R1: Vitamin B_{12}

Isotope labeled IStd: pyrodoxine-d_2, C/D/N Isotopes, Inc. (D-6819)

Prepare individual stock solution by dissolving appropriate amount of pure chemical in 1% formic acid at 1 mg/mL (1000 parts per million, ppm) unless noted. Prepare folic acid and riboflavin in basic solution at 1000 ppm and 100 ppm respectively (basified by ammonia, 4% and 0.7% respectively). Prepare IStd in 1% formic acid at 10 ppm to prepare calibration standards and spike unknown samples.

Prepare calibration standards in 0.1% formic acid from 10 parts per billion (ppb) to 5000 ppb at seven levels: 10 ppb, 50 ppb, 100 ppb, 500 ppb, 1000 ppb, 2000 ppb, and 5000 ppb from stock solution by series dilution.

Divide target analytes into three groups: Group 1 to contain only B_9 (folic acid); Group 2 to contain B_7 (biotin) and B_{12} (cyanocobalamine); and Group 3 to contain B_1, B_3 (niacin and niacinamide), B_5 (pantothenic acid) and B_6 (pyridoxine). Spike IStd in each calibration standard at 500 ppb.

Preparation of Vitamin-Enriched Beverage Samples

Vitamin-enriched beverage (VEB) samples were randomly selected and purchased from a local grocery store and kept at room temperature until analysis. Degas carbonated VEBs using a sonication bath for 30 s. Transfer 1 mL of each sample to a 1.5 mL autosampler vial, spike with IStd at 500 ppb, vortex mix, and analyze for Group 1 and Group 2 vitamins. Pipet 10 µL of each sample to another 1.5 mL autosampler vial, dilute with 990 µL DI H_2O, spike with IStd at 500 ppb, vortex mix, and analyze for Group 3 vitamins.

Preparation of Multivitamin Tablet Samples

Three bottles of multivitamin tablets were purchased from the same grocery store. Weigh 20 tablets from each bottle to calculate the average weight of one tablet. Grind the 20 weighed tablets to fine powder in a coffee grinder (Cuisinart, DCG-12BC) for 1 min (20 s × 3). Weigh three subsamples from each ground sample to 0.1 g in a 15 mL centrifuge tube, recording exact weight. Dissolve each subsample in 10 mL DI H_2O in a sonicator bath for 30 min, spiking the IStd at 500 ppb. Centrifuge the samples for 15 min at 4000 RPM. Pipet 1 mL of the clear supernatant from each sample to a 1.5 mL amber autosampler vial for the analysis of Group 1 and Group 2 vitamins. Pipet 10 µL of supernatant from each sample to another 1.5 mL amber autosampler vial, dilute with 990 µL DI H_2O, spike with IStd to 500 ppb, vortex mix, and analyze for Group 3 vitamins.

UHPLC-MS/MS Conditions

Chromatographic Condition

System:	UltiMate™ 3000 RSLC
Column:	Acclaim™ C30 (P/N 075725)
Dimensions:	2.1 × 150 mm, 3 µm
Mobile Phases:	A) Ammonium formate, pH 4.0 B) Ammonium formate, pH 3.0 C) 90% Acetonitrile/10% NH_4OOCH pH 3.0 Buffer at 10mM in each component Gradient events listed in Table 1
Flow Rate:	0.6 mL/min
Temperature:	15 °C
Injection:	10 µL, 20 ppm of each vitamin
Detection:	UV at 260 nm or TSQ Quantum Access MAX™ Triple Stage Quadrupole MS/MS detection mode

Mass Spectrometric Condition

System:	TSQ Quantum Access MAX Triple Stage Quadrupole Mass Spectrometer
Interface:	Heated electrospray (HESI II)
Spray Voltage:	4000 V
Vaporizer Temp.:	350 °C
Capillary Temp.:	200 °C
Sheath Gas:	40 arbitrary units
Auxiliary Gas:	60 arbitrary units
Detection Mode:	SRM (see Table 2 for details of SRM events)

Table 1: Mobile phase gradient events.

Time (min)	% A	% B	% C
-5.0	100	0	0
0.0	100	0	0
3.5	100	0	0
3.6	0	100	0
12.0	0	70	30
12.1	0	20	80
14.9	0	20	80
15.0	100	0	0

Table 2: SRM MS/MS events and parameters.

Analyte		Retention Time (min)	Scan Time (min)	Precursor (*m/z*)	Product (*m/z*)	Collision Energy (V)
Ascorbic Acid	C	1.2	0–1.4	-175	-87	21
					-115	13
Niacin	B_3	1.7	1.4–2.5	124	80	22
					78	22
Thiamine	B_1	3.0	2.5–4.7	265	122	15
					124	13
Pyridoxine	B_6	5.0	4.7–7.0	170	134	21
					152	13
IStd: Pyrodoxine-d_2	IStd	5.0	4.7–7.0	172	136	21
					154	13
Niacinamide	B_3'	5.8	4.7–7.0	123	80	20
					78	24
Pantothenic Acid	B_5	7.2	7.0–9.0	220	90	14
					184	13
Cyanocobalamine	B_{12}	10.6	9.0–15.0	679	147	37
				1356	1209	53
Folic Acid	B_9	10.9	9.0–15.0	-440	-311	23
					-175	39
Biotin	B_7	11.2	9.0–15.0	245	227	15
					97	33
Riboflavin	B_2	11.7	9.0–15.0	377	295	16
					243	21

Results and Discussion

Chromatography

Although many LC methods have been reported for simultaneous analysis of WSVs, these methods usually suffer from low throughput or incomplete chromatographic resolution, and several highly hydrophilic analytes are poorly retained on the commonly used C18 RP columns. In this study, a C30 column was used to improve the retention of poorly retained analytes, such as vitamin C and thiamine. In addition, ammonium formate was buffered at two pH conditions: pH 3.0 and pH 4.0, with the higher pH buffer used in the early phase of the gradient to further improve the retention for thiamine, and the lower pH buffer used to provide complete resolution for later eluted vitamins.

Under the optimized conditions, all target vitamins were baseline separated within 12 min. The minimum retention factor was observed for vitamin C at 1.3 (retention time 1.2 min), and the retention factor for thiamin was observed at 4.5, which was significantly improved over previously reported methods where thiamin eluted first with a retention factor of less than 1.[14,15] Vitamins B_5 and B_7 lack a UV chromophore and were not visible in the UV chromatogram. However, the peak labels for both analytes are shown in Figure 1A to demonstrate the chromatographic separation. These two analytes were detected by MS/MS with great sensitivity, as seen in Figure 1B and Figure 1C.

Chromatographic Condition

System:	UltiMate 3000 RSLC
Column:	Acclaim C30
Dimensions:	2.1 × 150 mm, 3 µm
Mobile Phases:	A) Ammonium formate, pH 4.0
	B) Ammonium formate, pH 3.0
	C) 90% Acetonitrile/
	10% ammonium formate, pH 3.0
	Buffer at 10 mM in each component
	Gradient events listed in Table 1
Flow Rate:	0.6 mL/min
Temperature:	15 ˚C
Inj. Volume:	10 µL
	20 ppm of each vitamin
	with UV detection
	50 ppb of each vitamin
	with MS/MS detection
	IS pyridoxine-d_2 at 500 ppb
Detection:	A. UV at 260 nm
	B and C. SRM

Mass Spectrometric Condition

System:	TSQ Quantum Access Max
Interface:	Heated Electrospray (HESI)
Spray Voltage:	4000 V
Vaporizer Temp.:	350 ˚C
Capillary Temp.:	200 ˚C
Sheath Gas:	40 arbitrary units
Auxiliary Gas:	60 arbitrary units
Detection Mode:	SRM (see Table 2 for details of SRM events)

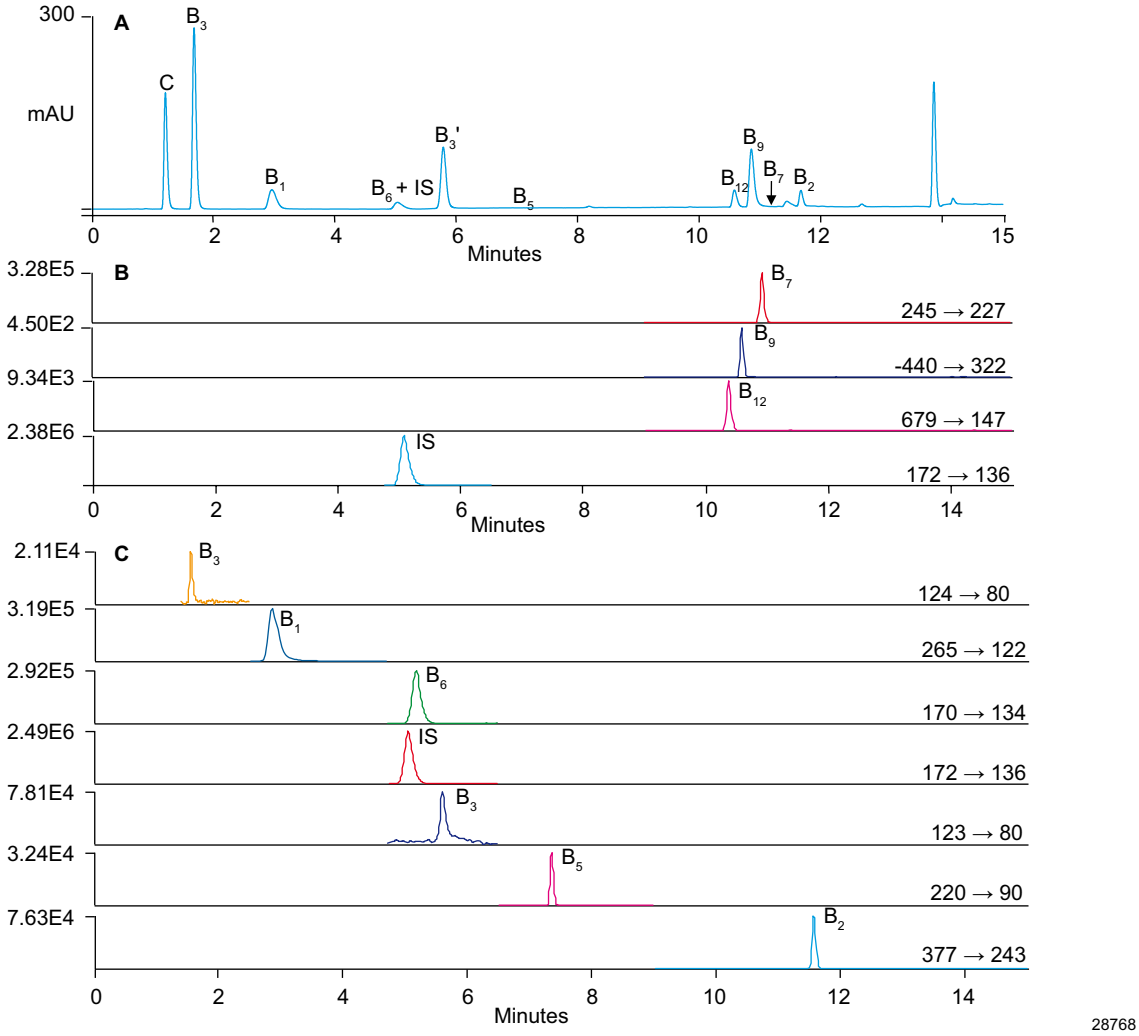

Figure 1: A) UV chromatograms of all target WSVs.
B) Q-SRM chromatograms of Group 1 and 2 vitamins.
C) Q-SRM chromatograms of Group 3 vitamins.

Mass Spectrometry

Electrospray ionization (ESI) was used in this study as the ionization interface due to its suitability and better sensitivity for polar compounds than other atmospheric pressure ionization (API) techniques. Ionization source parameters for the HESI probe used in this study were optimized to provide best sensitivity and were described in the sample preparation section. Under the optimized chromatographic and ionization conditions, most analytes exhibited strong protonated molecular ions (i.e., $[M+H]^+$) except for vitamin C and folic acid where deprotonated molecular ions $[M–H]^-$ were observed as the dominant MS peak. A strong doubly charged MS peak was observed for vitamin B_{12} at 679 *m/z* as well as the $[M+H]^+$ at 1356 *m/z*. For each analyte, the two most intense fragments were selected as the monitored product ions, which are listed in Table 2 along with the optimized collision energies. Thus for each analyte, two SRM transitions were monitored with one being quantitative SRM (Q-SRM), which showed relatively stronger MS response, and the other being confirmative SRM (C-SRM). The Q-SRM chromatograms are shown in Figure 1B and Figure 1C, with each of the vitamins at 50 ppb. The MS/MS detection demonstrated great sensitivity and selectivity for vitamin analysis even at low ppb levels. At 50 ppb, the minimum signal-to-noise ratio (S/N) was observed at 20 for niacin (26 for niacinamide) with the rest of the target analytes showing S/N greater than 100. The great sensitivity provided by MS/MS instrumentation enables the quantitation for low level vitamins such as B_{12} and folic acid in complex matrices, which was not achievable with previously reported methods using only UV detection.

Quantitation

One of the challenges encountered in this study was the large differences in concentration of the vitamins present in beverages or tablets. In the tested samples, the concentrations of Group 3 vitamins (mg levels per serving) were roughly 1000 times the concentrations of Group 1 and 2 vitamins (µg levels per serving). The lowest concentration observed was 0.6 µg per serving (B_{12}) while the highest concentration was at 20 mg per serving (B_3).

A single assay trying to cover the whole concentration range is beyond the linear response range of any mass spectrometer. Two approaches are usually practiced to address this challenge among reported methods covering these wide concentration ranges. Some reported methods use MS for lower concentration analytes and less sensitive detectors such as UV for the quantitation of high concentration vitamins,[14] thus losing the selectivity of MS quantitation and may suffer from interferences and/or lower quantitation accuracy. Another approach performs several assays for each sample with different dilution factors and results are reported with the most appropriate dilution. In this study, the latter approach was used and two assays were performed: the primary one quantitating low concentration vitamins including B_7, B_9, and B_{12}, which were assayed directly after spiking IStd; and the secondary assay quantitating the remaining vitamins at higher concentrations after a 100-fold dilution and respiking the diluted sample with IStd to 500 ppb. This technique took full advantage of the selectivity and specificity provided by MS detection, thus ensuring quantitation accuracy.

Stability of vitamins in solution was another challenge. Vitamin C was extremely unstable in multivitamin solutions, and degradation was observed within 20 min even though the sample was prepared in acidic solution and placed in a thermostatted autosampler at 4 °C. Instability of the analyte itself can cause substantial variance in the quantitative determination of vitamin C, and thus it was not included for quantitation in this study. Instability was also reported for other vitamins, such as riboflavin, pyridoxine, and thiamin, which are light sensitive,[17,18] and thiamin, pentothenic acid (in acid or basic condition), folic acid, and pyridoxine, which are heat labile. To avoid loss of analytes during analysis, samples were prepared in amber autosampler vials and promptly placed in the refrigerated autosampler at 4 °C.

Table 3: Calibration, coefficient of determination, precision, accuracy and detection limits.

Analyte		Calibration Range	r^2	50 ppb*			2000 ppb			LOQ (S/N)
				Mean	% RSD	% Accuracy	Mean	% RSD	% Accuracy	
B_3	Niacin	50–5000	0.9998	43.6	6.14	87.2	1960	3.34	98.0	50 (>10)
B_1	Thiamine	10–5000	0.9999	42.0	3.59	84.0	2029	2.92	101.5	10 (>200)
B_2	Riboflavin	10–5000	0.9996	44.8	3.03	89.6	1864	4.12	93.2	10 (>10000)
B_3	Nicotinamide	50–5000	0.9999	46.0	3.60	91.9	1844	2.93	92.2	50 (>14)
B_5	Pantothenic acid	10–5000	0.9999	44.7	4.57	89.4	1882	3.27	94.1	10 (>100)
B_6	Pyridoxine	10–5000	1.0000	50.4	1.22	101.0	1940	1.66	97.0	10 (>40)
B_7	Biotin	10–5000	0.9985	47.8	4.22	95.6	1910	4.15	95.5	10 (>1000)
B_9	Folic Acid*	100–5000	0.9984	113.0	15.8	113.0	1946	4.73	97.3	100 (>1000)
B_{12}	Cyanocobalamine	10–5000	0.9977	47.7	10.2	95.4	1735	4.61	86.8	10 (>1000)

All precision and accuracy results were summarized from seven replicate assays. *Precision and accuracy results for folic acid obtained from 100 ppb and 2000 ppb standards.

An additional challenge for accurate quantitation was the interactions of vitamins when present together in solution. Interactions between B12, folic acid, and riboflavin have been reported,19–22 thus targeted vitamins were divided into three groups with additional consideration of their concentrations in samples: Group 3 included higher concentration vitamins (B1, B2, B3, B5, and B6) and Group 2 included lower concentration vitamins (B7 and B12). Although folic acid was also present in lower concentration in samples and could be included in Group 2 vitamins, observations revealed that quantitation of low concentration folic acid could be significantly interfered with by the presence of B7 and B12. Thus three calibration standard sets were prepared for the three groups of vitamins to generate individual calibration curves for quantitation.

Method Performance

Method performance was evaluated against calibrations, coefficients of determination, precision, and accuracy. Calibration curves for each analyte were generated from calibration standards with concentrations from 10 ppb to 5000 ppb at seven levels. Quadratic fits were used to fit the experimental data and 1/x was used as the weighting factor. Detailed results are shown in Table 3. Excellent coefficients of correlation, precision, and accuracy were achieved for each target vitamin. Limits of quantitation (LOQs) were determined as the lowest concentration in calibration standards exhibiting signal-to-noise ratios (S/N) greater than 10. LOQ was observed at 10 ppb for most analytes, except for niacin and niacinamide at 50 ppb, and folic acid at 100 ppb. Although S/N for folic acid was achieved with values much greater than 10 at lower concentrations, poor quantitation accuracy was observed, which was believed to be due to the reduced stability of this analyte when present in solution at low concentration. The instruments used in this study are capable of quantification of target vitamins below the LOQs set in this method, proven by the S/N values observed at LOQ. However, this method was designed and the calibration range set to minimize sample preparation procedures, number of dilutions, and assays to be run in order to maintain a high analytical throughput.

Analysis of Vitamin-Enriched Beverages Samples

As described in the sample preparation section, ten beverage samples were selected and analyzed for their vitamin content. Among the selected beverage samples, five were vitamin-fortified water samples with different flavors, and the other five were vitamin-enriched energy drinks. The results are shown in Table 4. Large differences were observed between measured and labeled values, and this observation agreed with previously conducted studies.[23,24] An explanation for these descrepancies could be that the fortification was performed at levels higher than label claims, deviating in the direction of no harm,[24] to compensate for extrapolated degradations during storage and shelf life.

Analysis of Multivitamin

Three types of MVST samples were randomly selected and analyzed for target vitamins. Contrary to results for VEBs, the measured amounts were within good agreement to their label values, as seen in Table 5. The observed label agreement variance between beverage and tablet samples may suggest differences in vitamin stability when in different formulations, i.e., solution or tablet.

Table 5: Water-soluble vitamins in multivitamin tablets.

Analyte	MVST-1	MVST-2	MVST-3
Thiamine (mg/serving)	1.20 (1.5)	1.3 (1.5)	1.7 (1.5)
Riboflavin (mg/serving)	2.40 (1.7)	2.1 (1.7)	2.0 (1.7)
Nicotinamide (mg/serving)	20.00 (20)	19.0 (20)	20.0 (20)
Pantothenic acid (mg/serving)	11.00 (10)	11.0 (10)	11.0 (10)
Pyridoxine (mg/serving)	2.70 (2)	2.5 (2)	3.8 (3)
Biotin µg/serving)	29.00 (30)	25.0 (30)	25.0 (30)
Folic Acid (µg/serving)	590.00 (400)	173.0 (400)	247.0 (400)
Cyanocobalamine (µg/serving)	7.10 (6)	6.0 (6)	27.0 (25)

Label values of vitamins are included in parentheses.
Duplicate assays were performed for each sample.

Table 4: Water-soluble vitamins in vitamin-enriched beverages.

Analyte	VEB-1	VEB-2	VEB-3	VEB-4	VEB-5	VEB-6	VEB-7	VEB-8	VEB-9	VEB-10
Riboflavin (mg/serving)							2.2 (1.7)		5.3 (3.4)	1.1 (0.7)
Nicotinamide (mg/serving)	9.7 (8)	2.2 (2)	3.9 (4)	2.6 (2)	12.0 (8)	20.0 (20)	24.0 (20)	23.0 (20)	26.0 (20)	20.0 (10)
Pantothenic acid (mg/serving)	4.3 (4)	1.6 (1)	3.0 (2)	3.5 (1)	8.3 (4)	5.3 (5)		24.0 (10)	24.0 (10)	3.9 (2.5)
Pyridoxine (mg/serving)	1.1 (0.8)	0.35 (0.2)	0.75 (0.4)	0.40 (0.2)	1.6 (0.8)	6.3 (5)	2.8 (2)	3.8 (2)	4.1 (2)	6.5 (2.5)
Cyanocobalamine (µg/serving)					4.7 (2.4)	3.6 (4.8)	5.4 (6)	7.9 (6)	8.0 (6)	1.1 (2.4)

Label values of vitamins are included in parentheses. Duplicate assays were performed for each sample.

Conclusion

This study describes a UHPLC-MS/MS method for simultaneous quantitation of WSVs in beverages and supplement tablets. This method demonstrated excellent correlation of determination, precision, accuracy, and selective and sensitive detection with low quantitation limits. This method was successfully applied to the determination of WSVs in beverages and supplement tablets with presented results.

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