Application note | 004305

Food & beverage

Enhanced analytical flexibility: simultaneous normal and reversed phase chromatography

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Keywords

Vanquish Flex Duo UHPLC System, normal phase, reversed phase, Vitamin D, Cholecalciferol, Vitamin B, Niacinamide, Pyridoxine, Riboflavin, Thiamine, pregnancy and breastfeeding vitamins, fat-soluble vitamins, water-soluble vitamins

Application benefits

- Utilize a single Thermo Scientific™ Vanquish™ Flex Duo UHPLC System to perform both normal phase for fat-soluble vitamins and reversed phase for water-soluble vitamins complementary analysis
- High accuracy and precision in quantifying both fat-soluble and water-soluble vitamins in capsules

Goal

Running normal phase and reversed phase chromatography simultaneously to achieve comprehensive, efficient, and accurate analysis of vitamins using the unique Vanquish Flex Duo UHPLC System in a hybrid configuration.

Introduction

The Vanquish Flex Duo UHPLC System, with the installation of the normal phase kit, allows you to perform both reversed phase (RP) and normal phase (NP) analysis simultaneously using a single instrument. Running the analysis according to USP monographs, revalidation of methods is not required because these methods have already been validated by the USP. Ensure that laboratory conditions and procedures align with the USP specifications to maintain the integrity and accuracy of the results.

This application note illustrates the versatility of the Vanquish Flex Duo HPLC System's ability to analyze water-soluble vitamins (WSVs) and fat-soluble vitamins (FSVs) in a multivitamin supplement formulated for pregnancy. Under RP conditions, the system was used to analyze WSVs (B1, B2, B3, and B6) according to USP.[1] Simultaneously, NP conditions were applied to analyze FSVs (vitamin D3 assay), also adhering to USP.[2]

The multivitamin sample is designed to be suitable for use during all stages of pregnancy, including trimesters 1, 2, and 3, as well as postpartum during breastfeeding. This comprehensive supplement provides essential vitamins and minerals that work in conjunction with the natural rhythm of the body. It helps support a healthy diet, ensuring that both the mother and baby receive necessary nutrients during these critical periods.

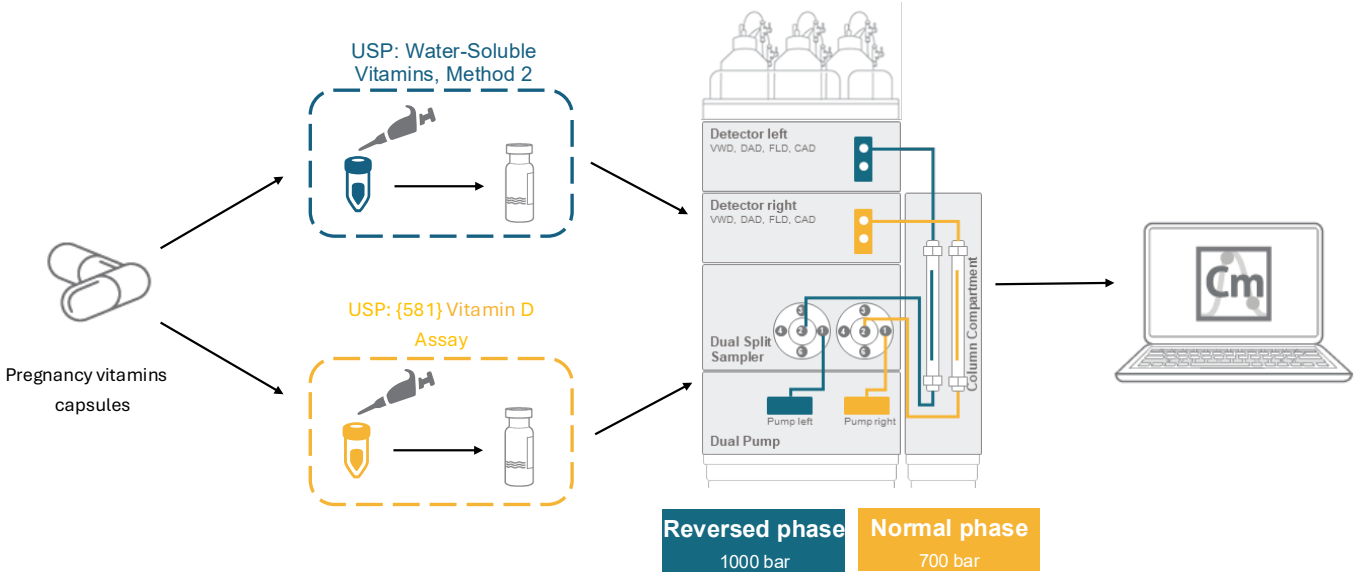

Figure 1. Workflow for FSV (vitamin D3) and WSV (vitamins B) analysis in pregnancy capsules using the Vanquish Flex Duo UHPLC System, running RP and NP (with the NP kit) simultaneously, for accurate identification and quantification according to USP monographs.

Experimental details

Chemicals

- Deionized Water, 18.2 MΩ·cm, MilliporeSigma™ Milli-Q™ Academic Water Purification System (ZMQS50001)
- Fisher Scientific™ Methanol Optima™ LC/MS grade (A456-212)
- Fisher Scientific™ Isopropanol Optima™ LC/MS grade (A461-212)
- Fisher Scientific™ Acetonitrile HPLC grade (A998-4)
- Fisher Scientific™ 2-Propanol (IPA) (HPLC) (A451-4)
- Water with 0.1% Formic Acid (FA) (v/v), Optima™ LC/MS grade (LS118-4)
- Fisher Scientific™ n-Hexane (H303-4)
- Sigma Aldrich™ Dimethyl sulfoxide (472301)
- Fisher Chemical™ Potassium dihydrogen phosphate (10429570)
- Thermo Scientific™ ACROS Organics™ Phosphoric acid, for analysis 85 wt% solution in water (201140025)
- Sigma Aldrich™ Ethylenediaminetetraacetic acid disodium salt dihydrate (E4884)
- Sigma Aldrich™ Sodium hexanesulfonate (H5269)
- USP Cholecalciferol RS (PHR1237)
- USP Niacinamide RS (1462006)
- USP Pyridoxine hydrochloride RS (1587001)
- USP Thiamine hydrochloride RS (1656002)
- USP Riboflavin RS (1603006)

Sample handling

- Thermo Scientific™ Finpipette™ F2 Variable Volume Single-Channel Pipette: 100–1000 µL (4642090)
- Thermo Scientific™ Finpipette™ F2 Variable Volume Single-Channel Pipette: 10–100 µL (4642070)
- Thermo Scientific™ Finpipette™ F2 Variable Volume Single-Channel Pipette: 1–10 µL (4642030)
- Thermo Scientific™ Orion Star™ A111 Benchtop pH Meter (1112106)
- Atlas HD Automated Chemical Synthesis System (2101000)
- Fisherbrand™ Mini Vortex Mixer (14-955-152)
- Corning® 50 mL Centrifuge Tubes (62.547.254)
- Thermo Scientific™ Nalgene™ Syringe Filters, 0.45 µm Nylon Filter (726-2545)

- Fisherbrand™ Plastic PP Two-Part Syringes, Luer Lock (12981021)
- Thermo Scientific™ SureSTART™ 2 mL Glass Screw Top Vials, Level 2 High-Throughput Applications, 100/pack (6ASV9-2P)
- Thermo Scientific™ SureSTART™ 9 mm Screw Caps, Level 2 High-Throughput Applications, White Silicone/Red PTFE, 100/pack (6PSC9ST101)

The Fisher Scientific part numbers can be unique to different countries; the codes given above should be compatible across the EU and US.

Instrumentation

Thermo Scientific Vanquish Flex Duo UHPLC System

- System Base Vanquish Flex (VF-S01-A-03)
- Vanquish Dual Pump F (VF-P32-A-01)
- Vanquish Dual Split Sampler FT[1] (VF-A40-A-02)
- Vanquish Column Compartment H (VH-C10-A-03)
- Vanquish Variable Wavelength Detector F (VF-D40-A)
- Standard Flow Cell, 11 µL, 10 mm, SST (6077.0250)
- Diode Array Detector HL (VH-D10-A)
- LightPipe, 10 mm, Standard Flow Cell (6083.0100B)
- Normal Phase (NP) Kit VQ Flex Systems[2] (6036.3501)

[1] For long-term exposure of normal-phase solvents, please ensure full compatibility of all wetted parts in the autosampler. Please contact Thermo Fisher Scientific for further details.

[2] For optimal analysis results, it is recommended to review the guide on system modifications and identify any required updates. Detailed information and instructions can be found on the System Modifications for Analysis page.

Sample preparation

Vitamin D3 assay preparation (NP)

Standard solution (2 µg/mL of cholecalciferol solution): weigh 0.2 mg of USP Cholecalciferol RS and dilute with n-hexane to 100 mL.

System suitability solution: weigh 0.2 mg of USP Cholecalciferol RS and dilute with n-hexane to 100 mL. Heat this solution under reflux at 60 °C for 1 hour under a nitrogen atmosphere, and cool to room temperature.

Sample solution: transfer the content of 6 capsules to a 50 mL volumetric flask. Add 7 mL of dimethyl sulfoxide and 10.5 mL of n-hexane. Heat this solution under reflux at 60 °C for 1 hour under a nitrogen atmosphere, and cool to room temperature. Centrifuge at 3000 rpm for 10 min. Transfer n-hexane layer to 50 mL volumetric flask and dilute with n-hexane.

WSVs, method 2 preparation (RP)

Solution A: weigh 13.61 g of potassium dihydrogen phosphate. Dilute in 800 mL of water, adjust with 10 N phosphoric acid to a pH of 3.0 and adjust the volume to 1 L with water.

Solution B: weigh 50 g of ethylenediaminetetraacetic acid disodium salt dihydrate. Dissolve in approximately 800 mL of water. Heat the solution while stirring for complete dissolution. Cool to room temperature and adjust the volume to 1 L with water.

Extraction solution: solution A and solution B (4:1) (v/v).

1.5 mg/mL niacinamide standard stock solution: weigh 150 mg of USP Niacinamide RS and dissolve in 100 mL solution A.

350 µg/mL pyridoxine standard stock solution: weigh 35 mg of USP Pyridoxine hydrochloride RS and dissolve in 100 mL solution A.

275 µg/mL thiamine standard stock solution: weigh 27.5 mg of USP Thiamine hydrochloride RS and dissolve in 100 mL solution A.

120 µg/mL riboflavin standard stock solution: weigh 12 mg of USP Riboflavin RS and dissolve in 100 mL solution A. Heat the solution while stirring for complete dissolution.

Standard solution: accurately transfer 16 mL of thiamine standard stock solution, 40 mL of riboflavin standard stock solution, 32 mL of niacinamide standard stock solution, 26 mL of pyridoxine standard stock solution into a 200 mL volumetric flask and fill to volume with solution A and mix well.

Sample solution: add 4 capsules of vitamins into a 100 mL volumetric flask. Add 50 mL of extraction solution, and heat to 90 °C. Boil for 5 min, vortex for 20 s, cool to room temperature and add 15 mL of acetonitrile. Vortex, dilute with water to final volume, and mix well. Pass a portion of the solution through a 0.45 µm nylon filter and discard the first 2 mL of the filtrate.

Mobile phase preparation

RP analysis

Solvent A: Weigh 9.73 g of sodium hexanesulfonate and 10.21 g of potassium dihydrogen phosphate. Dissolve the salts in approximately 800 mL of water. Adjust the pH to 3.5 with 10 N phosphoric acid. Add 25 mL of acetonitrile and mix thoroughly. Adjust the volume to 1 L with water.

Solvent B: Solvent A and acetonitrile (2:1) (v/v).

Chromatographic conditions

Table 1. Chromatographic conditions for NP and RP analysis

	Normal phase	Reversed phase	
Column	Hypersil GOLD Amino, 150 mm x 4.6 mm, 3 µm (PN 25703-154630)	Hypersil GOLD, 150 mm x 4.6 mm, 5 µm (PN 25005-154630)	
Solvent A	n-hexane	45 mM sodium hexanesulfonate, 75 mM potassium dihydrogen phosphate, 2.5% acetonitrile in water, adjusted with 10 N phosphoric acid to a pH of 3.5	
Solvent B	2-propanol	Solvent A:acetonitrile (2:1) (v/v)	
Isocratic/ gradient	Solvent A:solvent B (99:1) (v/v)	Time [min]	B [%]
		0.0	0
		6.0	0
		15.0	28
		19.0	28
		20.0	70
		23.0	80
		23.2	100
		24.8	0
		32.0	0
Flow rate	1 mL/min		
Column temperature	25 °C Thermostatting mode: Still air		
Autosampler temperature	5 °C		
Needle wash solution	100% IPA	MeOH:water (90:10) (v/v)	
Needle wash mode	Wash mode: After draw Wash time: 6.0 s Wash speed: 24.0 µL/s		
Injection volume	100 µL	10 µL	
Detector settings	Wavelength: 265 nm Data collection rate: 10.0 Hz Response time: 0.50 s	Wavelength: 254 nm for vitamins B1, B3, B6, and 280 nm for B2 Data collection rate: 1.0 Hz Response time: 5.00 s	

Chromatography data system

The Thermo Scientific™ Chromeleon™ Software 7.3.2 Chromatography Data System (CDS) was used for data acquisition and analysis.

Results and discussion

The Vanquish Flex Duo UHPLC System features two independent flow paths, each equipped with its own dedicated pump head, injector valve, column, and detector. Each injector valve has its own needle wash solution, which is particularly important to prevent cross-contamination and ensure accurate results. The NP kit includes all parts to modify UHPLC modules in a system for use with NP-compatible solvents and additives. This configuration allows for the simultaneous operation of RP and NP chromatography in a single system (Figure 1).

Before running any analytical sequence, it is crucial to ensure proper equilibration of the column. Allowing the column to equilibrate for an extended period is essential to achieve consistent and reliable results. It is particularly important for normal phase analysis where longer equilibration times are often necessary to stabilize the column and ensure optimal performance.

The standard solution for the vitamin D3 assay evaluation was injected six times (Figure 2A). The average peak area for cholecalciferol was 17.54 mAU*min with a relative standard deviation (RSD) of 0.13%. Additionally, the average asymmetry was 0.98. According to the system suitability requirements, the RSD of peak area for standard solution injections should not exceed 3.0%. The obtained RSD value of 0.13% is well within this limit, indicating excellent system performance and reliability for the vitamin D3 assay.

For the preparation of the system suitability solution, it is necessary to heat the sample under reflux at 60°C for one hour under a nitrogen atmosphere and then allow it to cool to room temperature. This process is essential to obtain the vitamin D3 isomer of cholecalciferol (precholecalciferol). The system suitability requirement specifies that the resolution (Rs) between the vitamin D3 form and precholecalciferol should not be less than 10. The results, based on an average of six injections, showed a resolution of 12.8 (Figure 2 B). This result meets the USP requirement, demonstrating that the system is capable of adequately distinguishing between the vitamin D3 form and precholecalciferol.

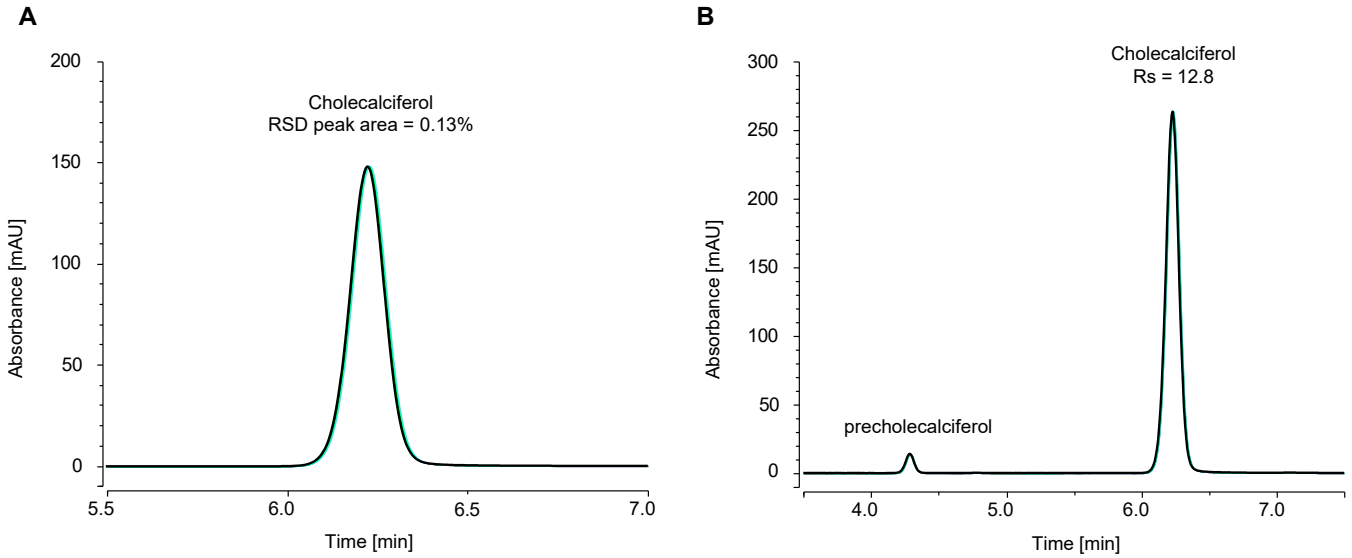

Figure 2. Overlay of the UV chromatograms of six consecutive injections (265 nm) of vitamin D3 standard, run under NP conditions: A. standard solution with peak labels of compound name and RSD peak area and B. system suitability solution with peak labels of compound names and resolution (Rs).

The results for the vitamin D3 assay sample solution are illustrated in Figure 3. According to the pregnancy capsules supplier, the cholecalciferol amount should be 10 mg. After analysis, the measured amount was found to be 9.67 mg +/– 0.01 mg, which is very close to the expected value.

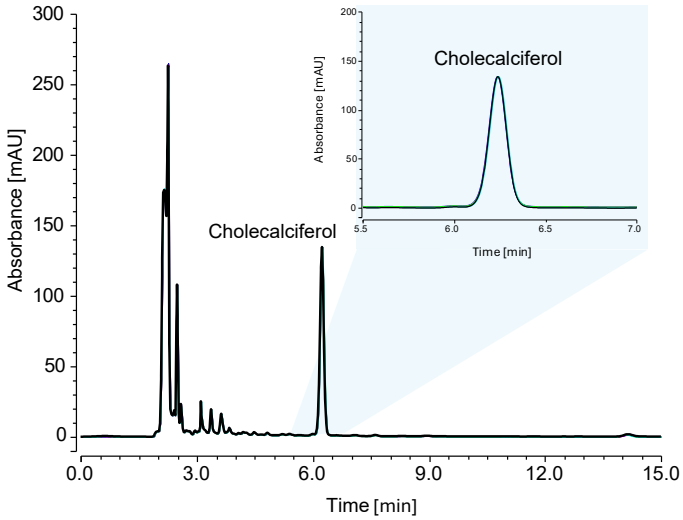

Figure 3. Vitamin D3 assay sample solution UV chromatogram at 265 nm. Overlay of six consecutive injections, run under NP conditions with zoom of cholecalciferol peak.

The quantitative analysis of WSVs B1 (thiamine), B2 (riboflavin), B3 (niacinamide), and B6 (pyridoxine) was conducted following the USP monograph "Water-Soluble Vitamins, Method 2".[1] This method utilizes UV detection at 254 nm for vitamins B1, B3, and B6, and at 280 nm for vitamin B2.

Figure 4 presents an overlay of six consecutive injections of the WSVs standard solution, with Figure 4A showing vitamins B1, B3, and B6 detected at 254 nm, and Figure 4B displaying vitamin B2 detected at 280 nm. According to USP requirements, the RSD of peak area should not exceed 2.0% for each individual compound. The highest value was observed for pyridoxine being 0.16% (Table 2). The results easily met the USP requirements for system suitability testing, showcasing remarkable performance.

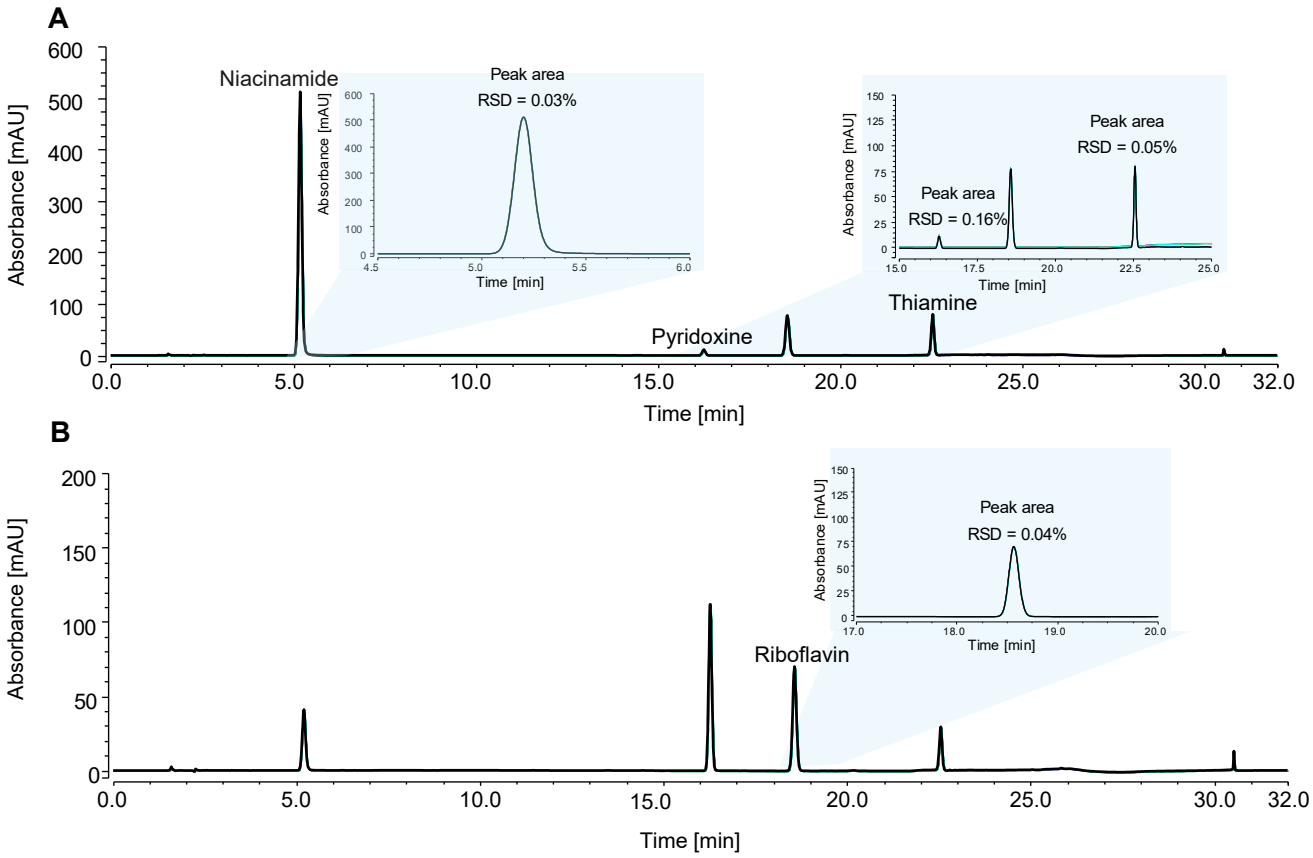

Figure 4: Overlay of six consecutive injections of the WSV standard solution, run under RP conditions. Figure 4A displays vitamins niacinamide (B3), pyridoxine (B6), and thiamine (B1) detected at 254 nm, while Figure 4B shows vitamin riboflavin (B2) detected at 280 nm with their respective peak area RSD

To quantify the WSVs in the capsules, the sample solution was injected six times, and the amount was calculated as described in the USP monograph. The resulting chromatograms for WSVs sample injections can be seen in Figure 5. Table 2 shows the summary of the WSVs quantification.

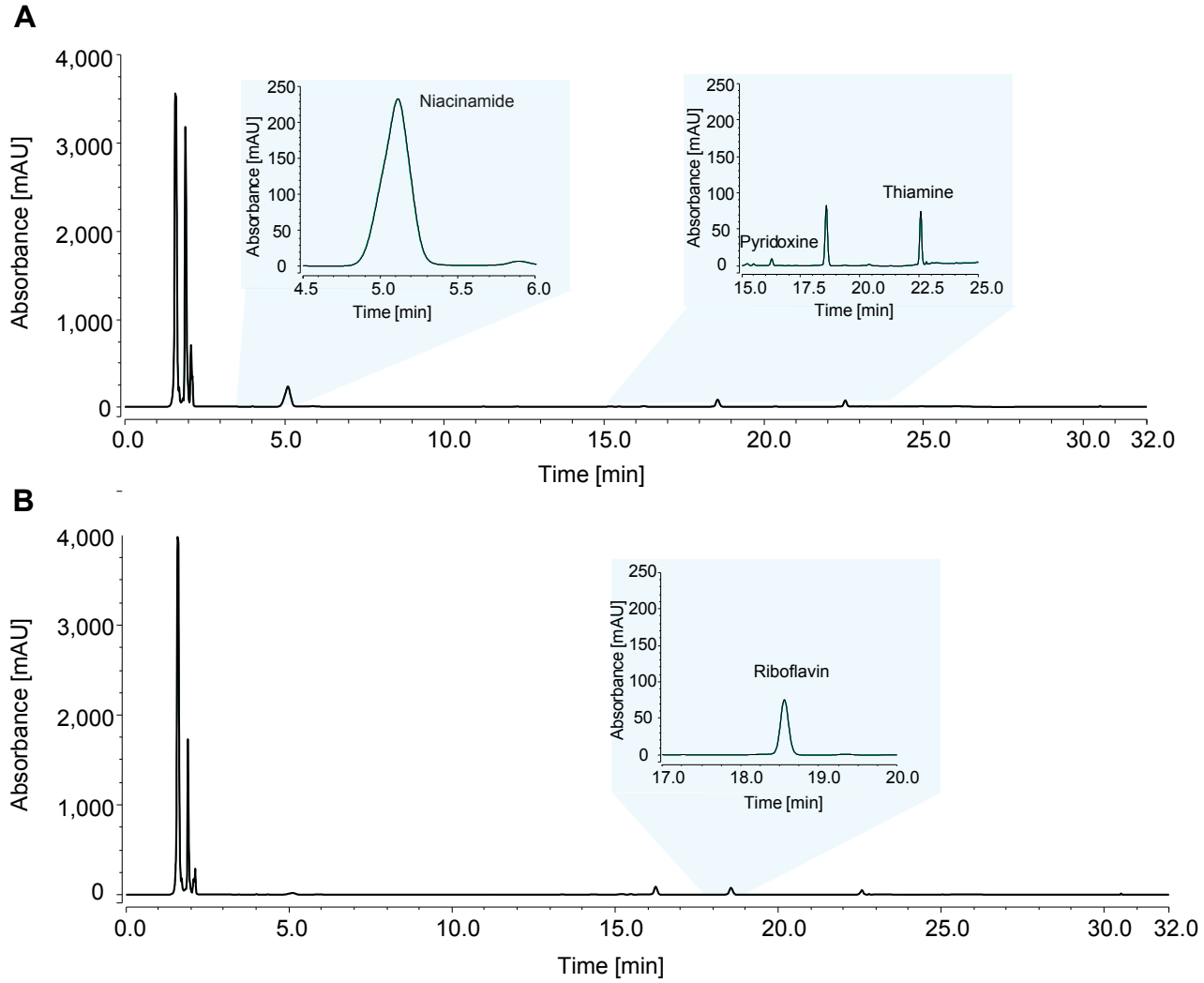

Figure 5. Overlay of six consecutive injections of the WSVs sample solution, run under RP conditions. Figure 5A shows the chromatograms at wavelength 254 nm for the detection of niacinamide (B3), pyridoxine (B6), and thiamine (B1). Figure 5B shows the chromatograms at wavelength 280 nm for riboflavin (B2)

Table 2. Summary of WSV quantification: vitamin content on package label and calculated amount from analysis. Niacinamide, pyridoxine, and thiamine were evaluated from 254 nm and riboflavin from 280 nm.

	Niacinamide	Pyridoxine	Riboflavin	Thiamine
Vitamin content on package label, mg	13.0	2.5	1.6	1.4
Calculated amount, mg (average of six injections)	13.5	2.4	1.8	1.4
Percentage of analyzed content normalized to labelled content	103.8	96.0	112.5	100.0

According to the USP acceptance criteria, which require 90.0%–125.0% of the indicated amount for each individual vitamin, the following results were obtained: niacinamide 103.8%, pyridoxine 96.0%, riboflavin 112.5%, and thiamine 100.0% (Table 2). This evaluation supports the product's nutritional claims, ensuring that it provides the expected levels of tested vitamins. Meeting the USP limits reinforces the quality and reliability of the supplement, making it a trustworthy choice for users.

Conclusion

The Vanquish Flex Duo UHPLC System enabled the simultaneous analysis of fat-soluble and water-soluble vitamins, with one flow path being used for NP (with NP Kit) and the other flow path for RP chromatography. This dual-LC capability significantly increased analytical efficiency, flexibility, and throughput by allowing parallel separations within a single platform while maintaining method robustness and data quality. In conclusion, the study demonstrated:

- Normal phase chromatography effectively analyzed the fat-soluble vitamin D3, while reversed phase chromatography was suitable for water-soluble vitamins B1, B2, B3, and B6.
- For vitamin D3, the standard solution RSD of peak area was 0.13%, meeting the USP requirement of not more than 3.0%. The system suitability solution resolution between vitamin D3 and precholecalciferol was 12.8, exceeding the USP requirement of not less than 10.
- For water-soluble vitamins, standard solution RSDs of peak area were all well below the USP requirement of 2.0%.
- Quantitative determination of the vitamins confirmed that the stated amount on the capsule packaging was adhered to and was found between 96.0% and 112.5%.

References

1. United States Pharmacopeia (**2021**). *Dietary Supplement Monographs, Water-Soluble Vitamins Preparation.* USP-NF. Rockville, MD: United States Pharmacopeia.
2. United States Pharmacopeia (**2023**). General Chapter, *{581} Vitamin D Assay.* USP-NF. Rockville, MD: United States Pharmacopeia.

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