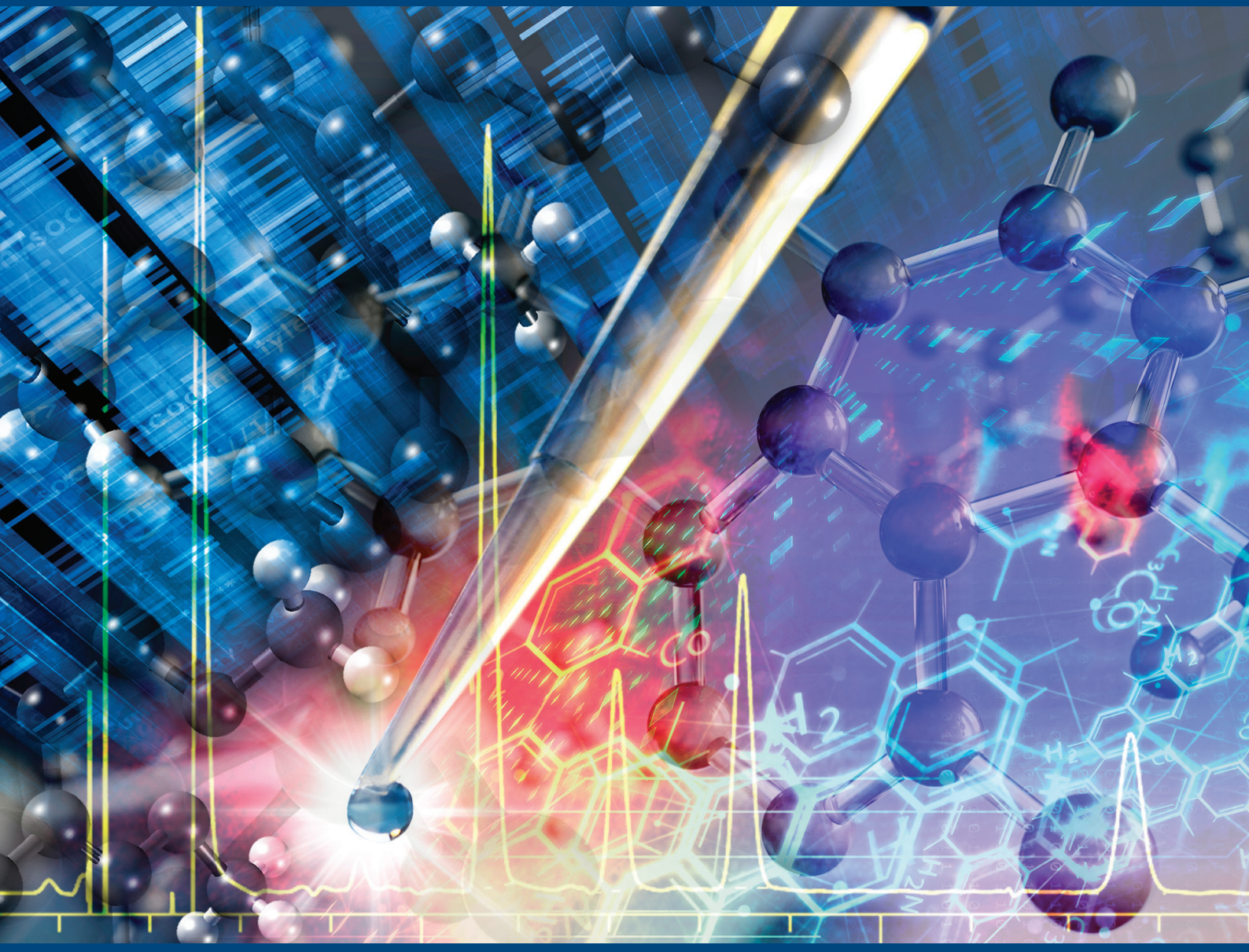
ISSN 1615-9306 · JSSCCJ 48 (5) 2025 · Vol. 48 · No. 5 · May 2025

Journal of SEPARATION SCIENCE

5|2025



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ORIGINAL ARTICLE OPEN ACCESS

Simultaneous Determination of 10 Fat-Soluble Vitamins by Ultra-Performance Liquid Chromatography in Multivitamins With Minerals Capsules

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Received: 3 April 2025 | **Revised:** 18 April 2025 | **Accepted:** 23 April 2025

Funding: The authors are thankful to United States Pharmacopeial Convention for the funding of this project.

Keywords: fat-soluble vitamins | multivitamin capsules | simultaneous determination | ultra-high performance liquid chromatography

ABSTRACT

A novel and accurate ultra-high performance liquid chromatography (UHPLC) method combined with UV detector was developed for the simultaneous determination of 10 fat-soluble vitamins (retinyl acetate, retinyl palmitate, beta carotene, alpha tocopherol, alpha tocopheryl acetate, alpha tocopheryl acid succinate, ergocalciferol, cholecalciferol, phytonadione, and menaquinone-7) in multivitamin with mineral capsules. The chromatographic separation was achieved on a Waters XBridge Sheild C18 (100 × 2.1 mm, 1.7 µm) column. The mobile phase comprised of 0.1% formic acid (v/v) in water and 0.1% formic acid in acetonitrile (v/v) delivered in a gradient mode. An enhanced and simple sample preparation procedure involving enzyme digestion of the gel coating of the capsules and ultra-sonication was developed compared to the complex and time-consuming saponification methods. The method was validated to fulfill International Conference on Harmonization (ICH) requirements and included specificity, linearity, accuracy, precision, and robustness. The linearity of the method was excellent ($R^2 > 0.999$), the RSD for the precision was < 5% and the recovery of the vitamins was in the range of 99.2%–101.9%, demonstrating that the method is suitable for analysis of fate-soluble vitamins in multivitamin capsules. The developed method could be incorporated into the USP-NF Multivitamin with minerals capsules monograph.

1 | Introduction

As one of the essential constituents of food, vitamins play important roles in the processes of metabolism, cell regulation, and are necessary for the maintenance of normal physiological activities [1–3]. Based on their solubility, vitamins have been classified as fat-soluble vitamins (A, D, E, and K) and water-soluble vitamins (B-complex and C vitamins) [4]. Fat-soluble vitamins are essential for the synthesis of nutrients, enhancing immune function, and growth performance. Several biological functions in the body have been associated with the fat-soluble vitamins [5–13]. The phytochemicals present in the fat-soluble vitamins are known to show antioxidant, antibacterial, antifungal, antiviral, and anticarcinogenic properties [14].

As vitamins are not naturally synthesized in the human body, a balanced diet is mandatory to maintain the quantity of vitamins at the required level. For people who are at risk of vitamin deficiencies, vitamin supplementation is regarded as an effective treatment (e.g., intake of multivitamin capsules). However, an overdose of vitamins can be toxic in nature [15–19]. In addition, interactions of vitamins and other drugs are often reported [20]. Therefore, it is essential to develop, accurate, reliable, and efficient methods for the simultaneous separation and quantitation

of multiple vitamins in different matrices for quality control [21, 22].

Many analytical methods have been developed for vitamin analysis such as fluorimetry [23], chemiluminescence [24], colorimetry [25], and high-performance liquid chromatography [26–28]. Among them, determination by reverse phase high-performance liquid chromatography due to its rapid, sensitive, and accurate quantitation has become popular in the determination of vitamins in various matrices [29, 30] mainly with a focus on the detection of a few specific vitamins. Due to the matrices' complexity, and the vitamins' stability, developing and validating fast and simple methods are challenging. Multiple methods have been developed in various matrices including foods, pharmaceuticals, biological samples, and feeds [31]. However, the simultaneous determination of 10 fat-soluble vitamins using ultra performance liquid chromatography (UHPLC) in multivitamins with minerals capsules is rarely reported. In addition, the reported methods mainly consist of the tedious sample pretreatment processes of saponification and liquid–liquid extraction. In them, the regular alkaline saponification procedure readily causes the oxidation of vitamins which leads to low recovery and reproducibility, and the methods are time-consuming and complicated [32–33]. Liquid–liquid extraction uses highly toxic volatile solvents which make the experimental procedure not suitable for routine use. This determined the objective of this study to develop a fast, precise, and sensitive RP-UHPLC/UV method and simple sample preparation procedure for routine qualitative and quantitative simultaneous analysis of fat-soluble vitamins in hard shell and soft gel multivitamin capsules. The developed method provides an effective chromatographic separation, sensitivity, reproducibility, and a new method of enzyme digestion and ultra-sonic extraction instead of the traditional saponification or liquid–liquid extraction.

Altogether, this new method would be a great value to ensure the safety and quality of fat-soluble vitamins in dosage forms.

2 | Experimental

2.1 | Chemicals and Reagents

Retinyl acetate (A-acetate), retinyl palmitate (A-palmitate), beta carotene (A provitamine), alpha tocopherol (E), alpha tocopheryl acetate (E-acetate), alpha tocopheryl acid succinate (E-succinate), ergocalciferol (D_2), cholecalciferol (D_3), phytonadione (K_1), and menaquinone-7 (K_2) were obtained as USP Reference Standards. Twelve samples from different manufacturers of multivitamins with minerals capsules (hard shell and soft gel) were purchased from suppliers (Table 1). Protease from *Aspergillus oryzae* was purchased from Sigma Aldrich. All HPLC-grade solvents were obtained from Fisher Chemicals. Deionized water (R = 18.2 MΩ.cm) was obtained using a Milli-Q system (Millipore, Billerica, MA, USA).

2.2 | Instrumentation and Analytical Parameters

UHPLC analysis was performed on Agilent 1290 Infinity Quaternary UHPLC system using a Diode Array Detector (Santa Clara, CA, USA) and Waters UHPLC H-Class PLUS system using a Photodiode Array Detector (Milford, MA, USA). Data acquisition, analysis, and reporting were performed using Waters Empower 3 chromatography data software. Separation of vitamins was carried out on Waters Acquity BEH Shield C18, 2.1 × 100 mm, 1.7 µm column, using a mobile phase system consisting of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). The gradient program (Time (min)/%B) was set as 0/77, 2/77, 8/86, 17/98, 21/98, 21.1/98, and 25/77. The column temperature was maintained at 30°C with a flow rate of 0.4 mL/min. The detection wavelengths of the method were 265 (Vitamin D and K), 285 (Vitamin E), and 325 nm (Vitamin A). The sample injection volume was 3 µL.

Method validation with respect to parameters such as specificity, linearity, accuracy, precision, robustness, and solution stability was performed.

2.3 | Solution Preparation

A solution of dimethyl sulfoxide (DMSO) and isopropyl alcohol (IPA) (1:1 v/v) was used as Diluent I and a solution of DMSO and IPA (3:7 v/v) as Diluent II for hard shell capsules. A total of 10% protease from *Aspergillus oryzea* in water was used to disintegrate the gel capsules. A resolution solution was prepared at concentration of 0.04 mg/mL of each USP retinyl acetate RS, USP retinyl palmitate, and USP beta carotene RS, 0.5 mg/mL of each USP alpha tocopherol RS, USP alpha tocopheryl acid succinate RS, and USP alpha tocopheryl acetate RS, 4 µg/mL of each USP cholecalciferol RS, USP ergocalciferol RS, USP phytonadione RS, and USP menaquinone-7 RS in Diluent I.

2.4 | Standard Solution for Hard Shell Capsules

The standard solutions for assay procedure were prepared by dissolving 0.1 mg/mL of retinol from USP retinyl acetate RS and USP retinyl palmitate RS, 0.1 mg/mL of USP alpha tocopheryl acid succinate RS and USP alpha tocopheryl acetate RS, and 2 µg/mL of USP Phytonadione RS in Diluent I. The standard solution for Vitamin D was prepared by dissolving 1 µg/mL of USP cholecalciferol RS in Diluent II. Assay linearity solutions at 50%, 100%, 150%, 200%, and 250% levels were prepared by sequentially diluting a linearity stock solution prepared at concentration of 30 µg/mL of retinol from retinyl acetate and retinyl palmitate, 0.5 mg/mL of alpha tocopheryl acid succinate and alpha tocopheryl acetate, and 20 µg/mL of phytonadione in Diluent I, 10 µg/mL of cholecalciferol in Diluent II.

2.5 | Sample Preparation for Hard Shell Multivitamin Capsules

The contents of 20 hard shell capsules from six different manufacturers were emptied and weighed, and the average capsule weight (ACW) was determined. A portion of the composite,

TABLE 1 | Vitamin content of the multivitamin with mineral samples analyzed.

Dietary supplement product	Label claim			
	Vitamin A	**Vitamin E**	**Vitamin D**	**Vitamin K**
Prenatal multivitamins and multi-minerals (hard shell)	750 IU	100 IU	200 IU	30 mcg
Observed amount	1160 IU	123 IU	286 IU	45 mcg
Doctor's best multivitamin (hard shell)	1500 mcg	20 mg	—	—
Observed amount	1725 mcg	26 mg	—	—
True balance, multivitamins, and minerals (hard shell)	—	200 IU	500 IU	—
Observed amount	—	276 IU	525 IU	—
Multi-vita mega-mineral (hard shell)	9500 IU	200 IU	—	—
Observed amount	7600 IU	260 IU	—	—
Women's multi whole food blend (hard shell)	—	15 mg	25 mcg	50 mcg
Observed amount	—	21 mg	33 mcg	75 mcg
Men's multi whole food blend (hard shell)	—	17 mg	25 mcg	60 mcg
Observed amount	—	23 mg	35 mcg	90mcg
Multi complete (soft gel)	—	37 mg	1000 IU	40 mcg
Observed amount	—	49 mg	1300 IU	41 mcg
Prenatal multi + DHA, (soft gel)	—	15 mg	25 mcg	80 mcg
Observed amount	—	17 mg	34 mcg	112 mcg
Liquid multi gels (soft gel)	1600 mcg	268 mg	10 mcg	—
Observed amount	2320 mcg	246 mg	11 mcg	—
Women's multi + omega-3 (soft gel)	450 mcg	15 mg	20 mcg	—
Observed amount	63 mcg	16 mg	20 mcg	—
Multi-vita (soft gel)	2400 mcg	13.4 mg	62.5 mcg	—
Observed amount	3048 mcg	12.3 mg	66 mcg	—
Multi for her (soft gel)	750 mcg	22.2 mg	25 mcg	40 mcg
Observed amount	1310 mcg	29 mg	27 mcg	37 mcg

equivalent to 5 mg of retinol, alpha tocopheryl acid succinate or alpha tocopheryl acetate, and 10 μg of phytonadione was dissolved in 50 mL of Diluent I. The solution was sonicated for 30 min. The suspension was then centrifuged for 5 min, and the supernatant was used for UHPLC analysis. For Vitamin D, a composite equivalent to about 50 μg of cholecalciferol was mixed in 10 mL water. The solution was sonicated for 20 min, and 35 mL of Diluent II was added to the solution. The solution was sonicated for another 30 min and diluted to volume with Diluent II to 50 mL. The suspension was then centrifuged for 5 min, and the supernatant was used for UHPLC analysis.

2.6 | Standard Solution for Soft Gel Capsules

The standard solutions for the assay procedure were prepared at concentrations of 0.1 mg/mL of USP alpha tocopherol RS and USP alpha tocopheryl acetate RS, 2 μg/mL of USP phytonadione RS, and 1 μg/mL of USP cholecalciferol RS in 1-propanol. Assay linearity solutions at 50%, 100%, 150%, 200%, and 250% levels of the vitamin nominal concentration were prepared by sequentially diluting a linearity stock solution prepared at concentration of 0.5 mg/mL of alpha tocopherol and alpha tocopheryl acetate, 20 μg/mL of phytonadione, and 10 μg/mL of cholecalciferol in 1-propanol.

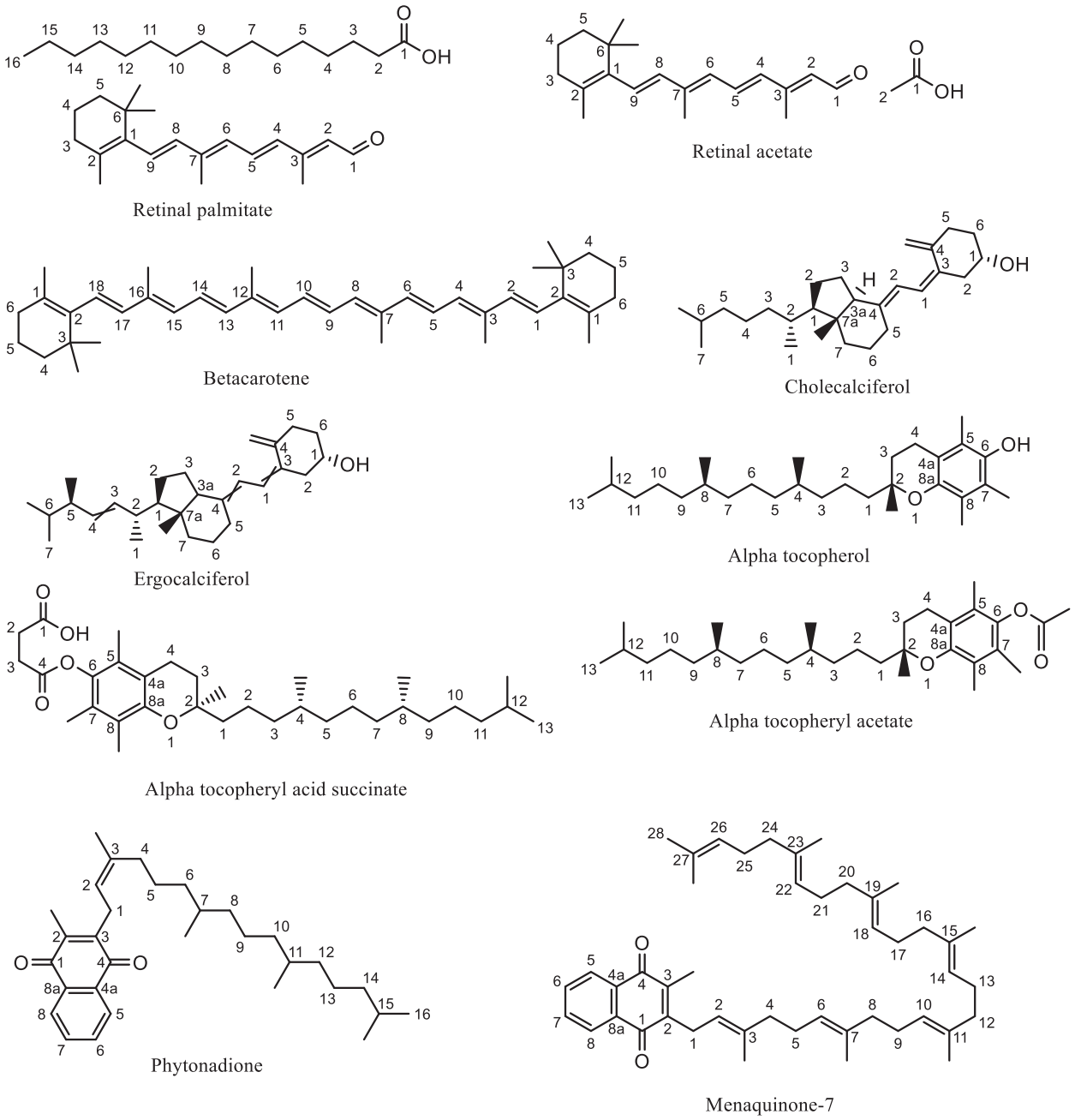

FIGURE 1 | Structures of vitamins.

2.7 | Sample Preparation for Soft Gel Multivitamin Capsules

Transferred an appropriate number of capsules to obtain a solution containing nominal concentrations of 0.5 mg/mL of alpha tocopherol and alpha tocopheryl acetate, 2 µg/mL of phytonadione, and 1 µg/mL of cholecalciferol and transferred into a 100-mL volumetric flask, added 10.0 mL of 10% protease solution, and sonicated for 20 min at 40°. Added about 80 mL of 1-propanol and sonicated for 20 min. Allowed to cool to room temperature and diluted to volume with 1-propanol. The suspension was then centrifuged, and supernatant was analyzed.

3 | Results and Discussion

3.1 | UHPLC Method Development

This study's main objective was to develop an UHPLC-UV method that accurately quantitates and separates 10 fat-soluble vitamins present in multivitamin and mineral capsules. The chemical structures of 10 oil soluble vitamins are shown in Figure 1. The Resolution solution containing retinyl acetate, retinyl palmitate, beta carotene, alpha tocopherol, alpha tocopheryl acid succinate, alpha tocopheryl acetate, cholecalciferol, ergocalciferol, phytonadione, and menaquinone-7 in Diluent I was used for method development. Different compositions of

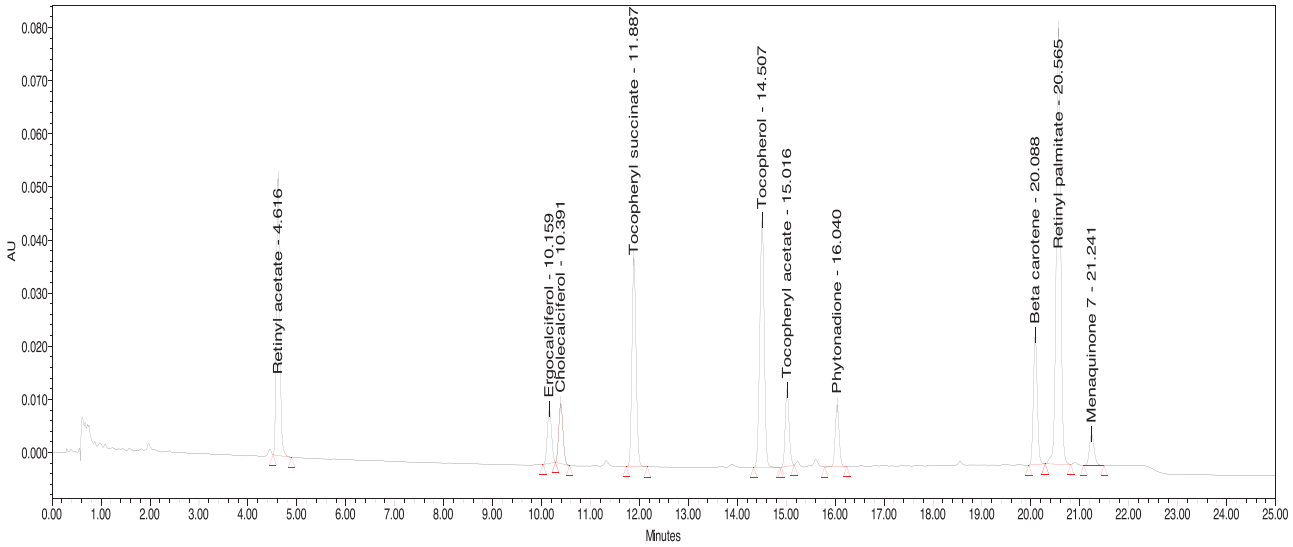

FIGURE 2 | Chromatogram depicting separation of fat-soluble vitamins at 265 nm.

TABLE 2 | Chromatographic performance data.

Compound	RT (min)	Resolution[a]
Retinyl acetate	4.3	—
Ergocalciferol	9.7	38.5
Cholecalciferol	9.9	1.5
Alpha tocopheryl acid succinate	11.4	9.1
Alpha tocopherol	13.9	16.1
Alpha tocopheryl acetate	14.4	3.2
Phytonadione	15.5	7.3
Beta carotene	19.2	29.1
Retinyl palmitate	19.6	3.0
Menaquinone 7	20.1	3.8

[a]Resolutions were calculated between two adjacent vitamin peaks.

water, methanol, and acetonitrile with 0.1% TFA as an added modifier were tested as mobile phases. It was found that the pH of the mobile phase does not have any effect on the elution order of the vitamin peaks. Finally, 0.1% formic acid in water (v/v) and 0.1% formic acid in acetonitrile (v/v) delivered in a gradient mode demonstrated a symmetric peak shape for all 10 fat-soluble vitamins and were chosen as the mobile phase. Different C18 columns, Waters Acquity BEH (100 × 2.1 mm, 1.7 µm), Phenomenex Kinetex (100 × 2.1 mm, 1.7 µm), and Agilent Zorbax Eclipse Plus (100 × 2.1 mm, 1.8 µm) were also tested. All these columns strongly retained retinyl palmitate, beta carotene, and menaquinone-7, and the elution of these vitamins required lengthy run time. The desired separation with a resolution of not less than 1.5 between adjacent peaks of 10 vitamins in 25-min run time was achieved on Waters Acquity BEH Shield RP C18 (100 × 2.1 mm, 1.7 µm) with the column maintained at 30° and flowrate of 0.4 mL/min. The chromatogram of the resolution solution and the chromatographic performance data are presented in Figure 2 and Table 2, respectively.

3.2 | Selection of the Extraction Solvent for Vitamins in Hard Shell Capsules

For maximum recovery of vitamins DMSO and IPA in different ratios were evaluated. The higher recoveries of Vitamins A, E, and K were achieved when DMSO and IPA 1:1, v/v (Diluent I) was used as a diluent and the higher recovery of Vitamin D was achieved when DMSO and IPA 3:7, v/v (Diluent II) was used as a diluent (Figure 3). The simple sample preparation procedure for Vitamins A, E, and K in hard shell capsules was developed which included adding Diluent I to the capsule composites and sonicating for 30 min. The solution was finally diluted to volume and centrifuged or filtered through a nylon filter of 0.45-µm pore size. No difference in the recoveries were seen when the sample solutions were either centrifuged or filtered. The highest recovery of Vitamin D was achieved when water equivalent to 20% of the total volume was added to the capsule composite, the solution was then sonicated for 20 min. Diluent II was added to the solution and the solution was sonicated for additional 30 min and diluted to volume with Diluent II.

3.3 | Selection of Extraction Solvent for Vitamins in Soft Gel Capsules

Previously reported sample preparation procedure for soft gel capsules included cutting of the gelatin shell and washing the capsule with chloroform or hexane [34]. To avoid the use of hazardous solvent, attempts were made to disintegrate the gelatin covering and release the contents of the capsules into the volumetric flask using the capsules as is. A total of 10% protease solution was used to completely disintegrate the soft gel capsule with the aid of ultrasonication for 20 min.

Once the complete disintegration of the capsules was achieved, the recovery of Vitamins D, E, and K, was investigated using different extraction solvents, that are, methanol, acetonitrile,

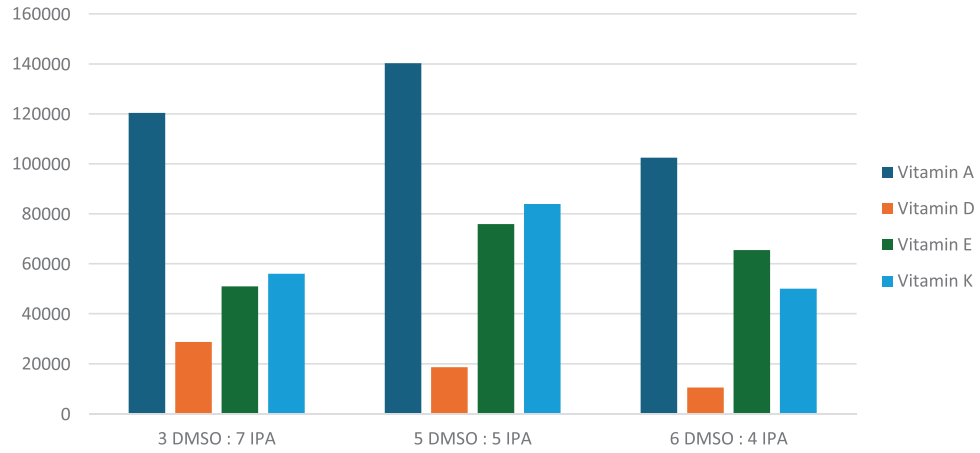

FIGURE 3 | Effect of different solvent ratios of DMSO and IPA on extraction of vitamins in hard shell capsules.

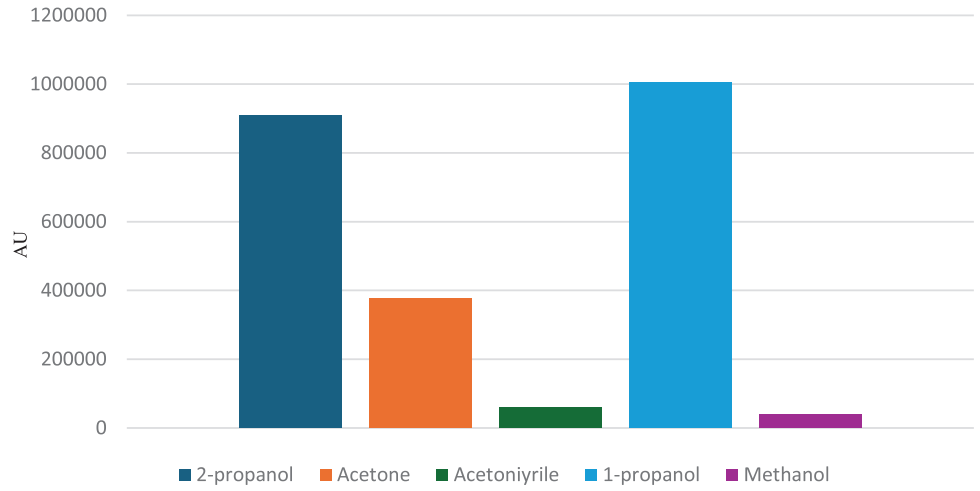

FIGURE 4 | Effect of different solvents on extraction of Vitamin E in soft gel capsules.

acetone, 2-propanol, and 1-propanol. The higher recoveries of vitamins D, E, and K were achieved when 1-propanol was used as an extraction solvent. Figure 4 shows the extraction of Vitamin E with different solvents, similar trends were seen for Vitamin D and K as well.

Different sonication times, that are, 0, 10, 20, and 30 min, for the extraction step were also evaluated. Vitamin's recovery increased with increasing sonication time. However, no significant difference in assay values was seen between 20 and 30 min of sonication time. With 0 sonication time the %Assay for the vitamins was about 20%. When the sample solutions were sonicated for 10 min it was 67.4% and after sonication for 20 and 30 min %Assay was 104.9% and 105.5%, respectively. Thus, 20 min sonication time was set for the sample preparation.

3.4 | Method Validation

The developed method was validated for specificity, precision, linearity, accuracy, and robustness for Vitamins A, D, E, and K as per ICH guidelines [35].

3.4.1 | Specificity

Specificity is the ability to separate and analyze the analyte in the presence of other components in the sample matrix. The specificity of the method was demonstrated by analyzing diluent, standard solution, and sample solutions. No interference to the analyte peak was detected in the sample solutions prepared from hard shell and soft gel multivitamin capsules (Figure 5).

3.4.2 | Linearity

Linearity of the methods was determined in the range of 50%–250% of the nominal concentration of each vitamin (A, D, E, and K) present in the multivitamin capsule samples. The method was shown to be linear with the correlation coefficient of greater than 0.99 (Table 3).

3.4.3 | Accuracy and Precision

The accuracy of the developed method was evaluated by calculating the mean percentage recovery of the added standard

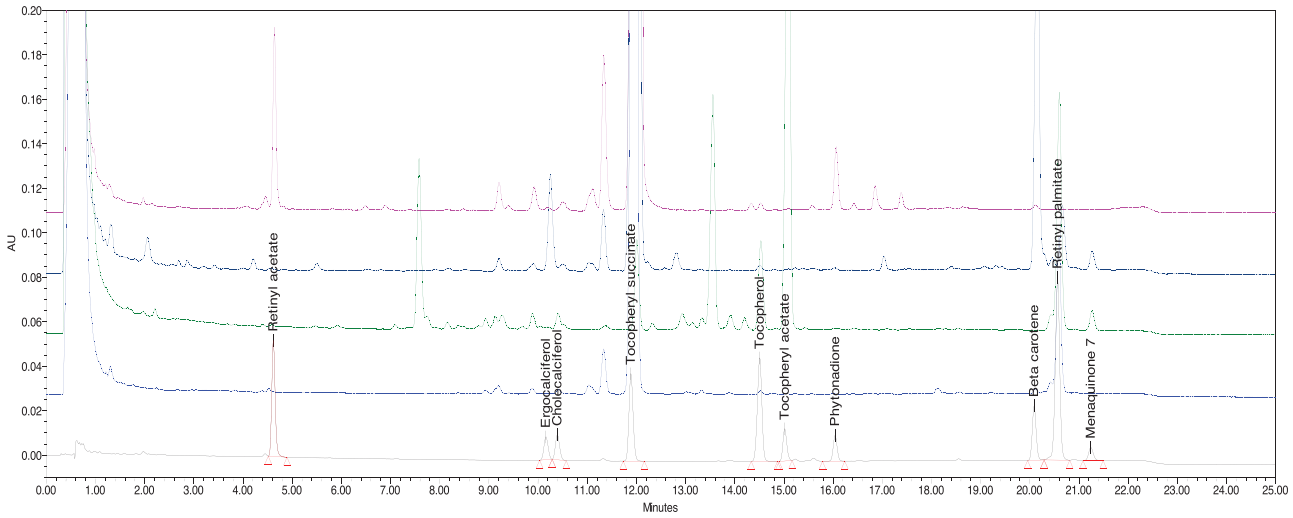

FIGURE 5 | Overlaid chromatograms of the resolution solution and sample solutions from hard shell capsules.

TABLE 3 | Linearity of vitamins.

Compound	Range (μg/mL)	Linearity equation	Bias of intercept (%)	*r*
Retinyl acetate	5.0–25	$y = 72\,900\,000x - 5610$	0.49	1.00
Retinyl palmitate	5.0–25	$y = 76\,700\,000x - 6280$	0.54	1.00
Alpha tocopheryl succinate	50–250	$y = 598\,000x - 192$	0.21	1.00
Alpha tocopheryl acetate	50–250	$y = 652\,000x - 101$	0.09	1.00
Cholecalciferol	0.5–2.5	$y = 19\,100x - 273$	0.9	1.00
Phytonadione	1–5	$y = 15\,400x - 168$	0.3	1.00

TABLE 4 | Accuracy and repeatability.

	%Recovery[a]					
Amount spiked[b]	Retinyl acetate	Retinyl palmitate	Alpha tocopheryl succinate	Alpha tocopheryl acetate	Cholecalciferol	Phytonadione
50%	99.1	99.2	99.9	101.0	99.7	101.6
120%	99.2	99.6	99.5	100.4	100.1	101.9
160%	99.5	100.0	99.6	100.5	100.0	101.7

[a]Mean for six determinations for 120% and three determinations for other levels.

[b]Amount of vitamin spiked into the placebo with respect to the nominal vitamin concentration in dietary supplement formulation.

stock solutions to the analyte-free matrix (placebo) at three different concentration levels (50%, 120%, and 160%), in triplicate. The percentage recovery of each vitamin was in the range 99.2–101.9 which revealed that the method is accurate (Table 4). To access the repeatability, six sample solutions were prepared and relative standard deviations (RSD) of the recoveries of vitamins were calculated. The RSD of the recoveries six sample solutions of each vitamin was less than 3%. The intermediate precision was determined by evaluating the repeatability of the analytical method by second chemist on a different day using a different column lot of the same column and UHPLC instrument from different manufacturer. The precision was calculated based on the RSD, the RSD of the 12 sample solutions of each vitamin analyzed by first and second chemist was less than 5%. The results show that the developed method was precise.

3.4.4 | Robustness

Deliberate but small changes to the UHPLC parameters were made to demonstrate the robustness of the developed method. The UHPLC parameter variations studied included the composition of mobile phase (±10% buffer), column temperature (±3°), and flow rates (±10%), initial isocratic hold time (± 0.5 min). The retention times, resolution, and tailing for the peaks of interest were evaluated. No significant change in resolution and

TABLE 5 | Solution stability over 24 h.

Time in hours	%Deviation[a]					
	Retinyl acetate	Retinyl palmitate	Alpha tocopheryl succinate	Alpha tocopheryl acetate	Cholecalciferol	Phytonadione
4	0.11	−0.16	0.02	0.01	0.06	0.04
8	0.24	−0.75	−0.49	0.09	−0.49	0.06
12	−0.36	−1.22	−0.40	−0.18	0.09	−0.25
16	−0.16	−1.66	−0.21	−0.27	−0.36	−0.26
20	−0.24	−1.87	−0.54	−0.10	−0.17	−0.28
24	−0.11	−1.71	−0.80	−0.22	−0.16	−0.51

[a]%Deviation is the change in peak area from the initial time point over the period of 24 h.

tailing of each vitamin peak was seen under any of the modified chromatographic conditions.

3.4.5 | Solution Stability

The stability of each vitamin in the standard solution and sample solution was determined over 24 h. The solutions were stable for at least 20–24 h at 4°C, and the change in peak area from the initial time was less than 2.0% (see Table 5).

4 | Conclusions

A novel UHPLC-UV method for the simultaneous determination of 10 fat-soluble vitamins in multivitamin and mineral capsules was developed and validated as per the ICH guidelines. Method validation results have proven the method to be specific, linear, precise, accurate, and robust. The simplicity of the sample preparation procedure from the complex matrices should make it a highly desirable method for quality control of multivitamin products in the supplement industry. The UHPLC-UV procedure could be incorporated into the USP-NF monograph.

5 | Disclaimer

The authors do not approve or endorse, any brand or product, nor are the equipment and vendors mentioned in the paper certified by USP. Certain commercial equipment, instruments, or materials may be identified in this presentation to specify adequately the experimental procedure. Such identification does not imply approval, endorsement, or certification by USP of a particular brand or product, nor does it imply that the equipment, instrument, or material is necessarily the best available for the purpose or that any other brand or product was judged to be unsatisfactory or inadequate.

Acknowledgments

The authors are thankful to United States Pharmacopeial Convention for the funding of this project and to Nadejda Soukhova and Qun Xu for providing the comments to the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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