

Liquid Chromatography

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The Qualitative and Quantitative Analysis of Fat-Soluble Vitamins by UHPLC Using UV Detection

Introduction

Vitamins are essential nutritional elements in the human diet and have diverse biochemical functions. The food industry routinely fortifies many foods with vitamins to

enhance their nutritional value and meet dietary requirements.

In 2009, the United States Pharmacopeial Convention introduced the USP Dietary Supplements Compendium (DSC) – an industry directed resource featuring regulatory guidance, documents, supplemental information, and reference tools.¹ Vitamins are classified not by chemical structure or function, but by solubility. They are divided into two groups; water-soluble (eight B vitamins and vitamin C), and fat-soluble (vitamin A, D, E, and K).²

Over the last decades, there have been increased health concerns raised by many physicians and researchers over the indiscriminant food and supplement intake by humans. Nowadays, many people eating supplements together with fortified foods and whole foods may exceed safe upper limits, potentially leading to a toxic accumulation.³ Water-soluble vitamins are readily excreted from the body, making overdose unlikely. In contrast, fat-soluble vitamins can easily be stored and accumulated in the human body, which may lead to a condition called hyper-vitaminosis.³ Therefore, in many countries, there are strict regulations to limit the maximum intake level of such vitamins.^{4,5,6,7}

Many analytical methods have been developed to analyze fat-soluble vitamins, but they mainly focus on the detection of a few specific vitamins. This application note describes the simultaneous analysis of the most common fat-soluble vitamins by UHPLC using UV detection.

Experimental

Hardware/Software

For all chromatographic separations, a PerkinElmer Altus™ UPLC® System was used, including the Altus A-30 Solvent/ Sample Module, integrated vacuum degasser, A-30 Column Heater, and Altus A-30 UV detector (PerkinElmer, Shelton, CT, USA). 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.

Solvents, Standards and Samples

All solvents and diluents used were HPLC-grade, including reagent alcohol (ethanol with 5% isopropyl alcohol as denaturant).

All vitamin standards were obtained from Sigma-Aldrich® Inc. (Allentown, PA). These included ergocalciferol (D2), cholecalciferol (D3), \pm α -tocopherol (E), DL α -tocopherol acetate (E acetate), phyloquinone (K1), menaquinone (K2), retinyl acetate (A acetate), and retinyl palmitate (A palmitate). The structures of these vitamins are shown in Figure 1.⁸

All secondary standard dilutions were made using a diluent of 80% ACN in water. As the concentration of individual vitamins in dietary supplements and food/beverage products varies considerably, a working standard was prepared to cover the wide range of concentrations.

Table 1. HPLC Method Parameters.

HPLC Conditions							
Column:	PerkinElmer Brownlee™ Analytical DB C18 1.9 μ m, 2.1 x 50-mm (Part# N9303853)						
Mobile Phase:	Mobile Phase B: Acetonitrile (ACN) Mobile Phase C: Water Solvent program:						
		Time (min)	Flow Rate (mL/min)	%A	%B	%C	%D
	1	Initial	0.600	0.0	80.0	20.0	0.00
	2	10.00	0.600	0.0	100.0	0.0	0.00
	3	13.00	0.600	0.0	100.0	0.0	0.00
	4	13.01	0.600	0.0	80.0	20.0	0.00
Analysis Time:	15 min.; 3-minute injection delay time between injections						
Flow Rate:	0.6 mL/min. (max pressure : 6600 psi/~440 bar)						
Oven Temp.:	40 °C						
Detection:	Wavelengths used for calibration/quantitation: 265 nm: for vitamins D2, D3, E, E acetate, K1, and K2 and 325 nm: for vitamins A acetate and A palmitate						
Injection Volume:	5 μ L						
Sampling (Data) Rate:	2 pts./sec						

The working standard (WS) was prepared by weighing out 20 - 2000 μ g/mL of each of the eight vitamins, depending on the vitamin's relative absorptivity and expected concentration in typical dietary supplements. Subsequently, calibration standards were prepared via serial dilution of WS.¹

In preparing the WS, the eight vitamins were first weighed and transferred into individual volumetric flasks. To improve solubility of the K1, K2, D2, and D3 vitamins, reagent alcohol was added to each volumetric flask, adding 30% of flask volume, and then filled to the mark with the 80% ACN/water diluent. Vitamins A (acetate and palmitate), E, and E acetate were also initially dissolved in reagent alcohol, though, for

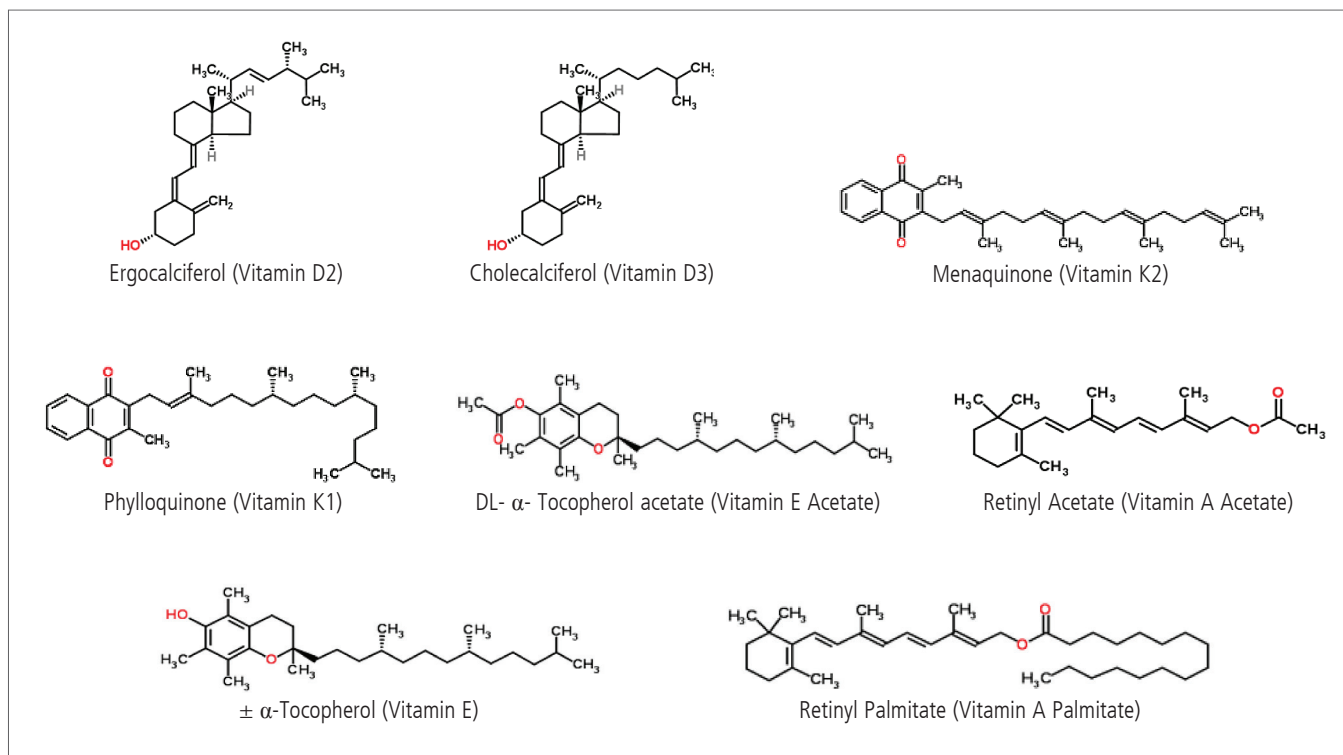


Figure 1. Chemical Structures of Fat-soluble Vitamins.

these, the volumetric flasks were then filled to mark with reagent alcohol.

Each volumetric flask was then sonicated for five minutes. No visual precipitate was observed after sonication. Equal volumes of each of the eight individual stock standards were then added together to produce the WS solution, and the volumetric flask was filled to volume with diluent. Within the WS, the vitamins ranged in concentration from 0.05 to 260 µg/mL, depending on the vitamin. For calibration, all serial dilutions were prepared from the WS.

All standards were filtered through 0.45 µm PTFE filters prior to injection.

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

Results and Discussion

Using the optimized chromatographic conditions described above, Figure 2 shows the UHPLC separation, both at 265 nm and 325 nm, of the eight fat-soluble vitamins contained in the WS.

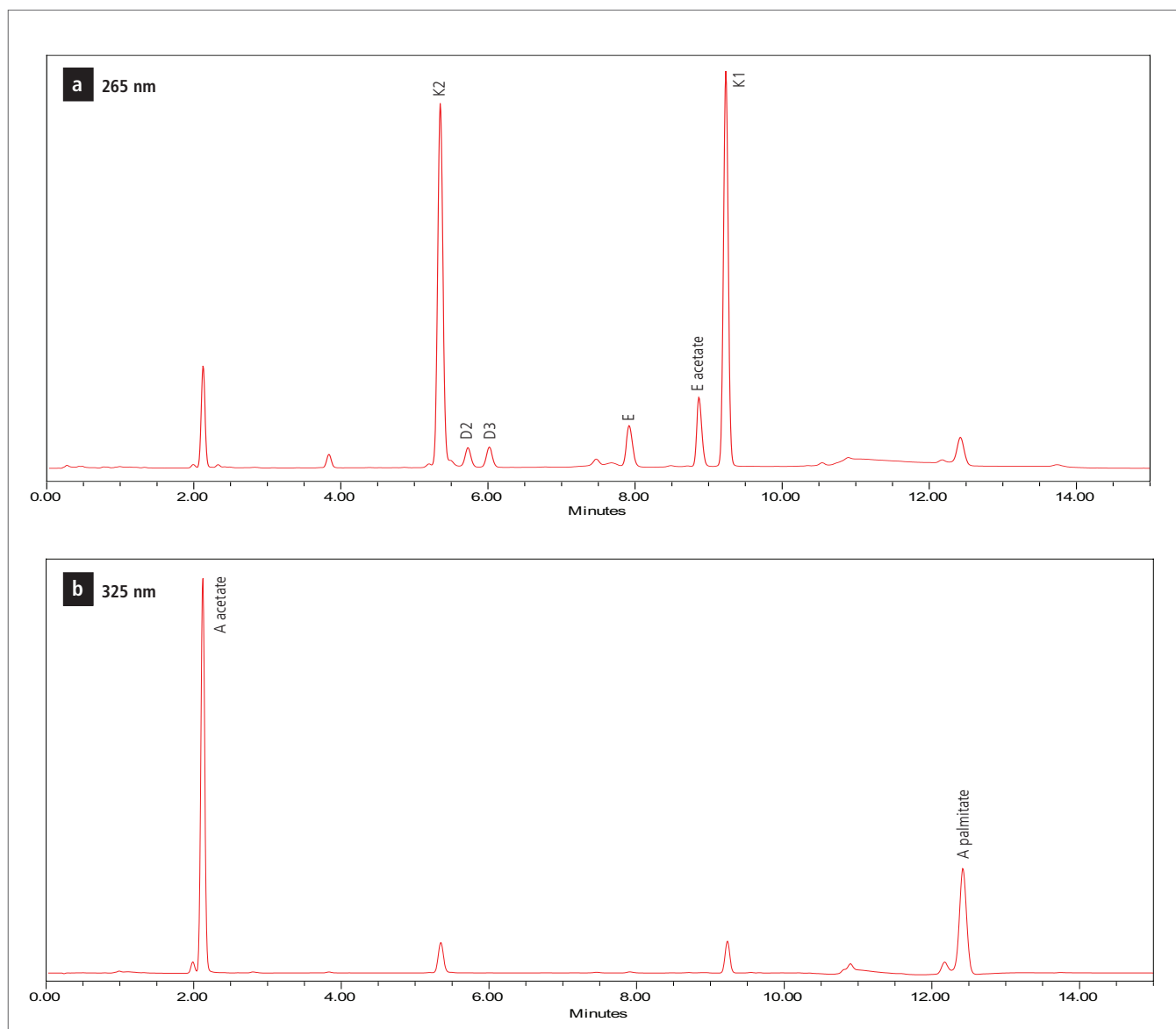


Figure 2. Chromatograms of the fat-soluble vitamin working standard (WS), run at: a) 265 nm and b) 325 nm showing the individual analytes quantitated at each wavelength.

Figure 3 shows the overlay of 12 replicate injections of WS, demonstrating exceptional reproducibility. Retention time % RSDs for all eight vitamins were < 0.3%, with vitamin A palmitate at 0.129%.

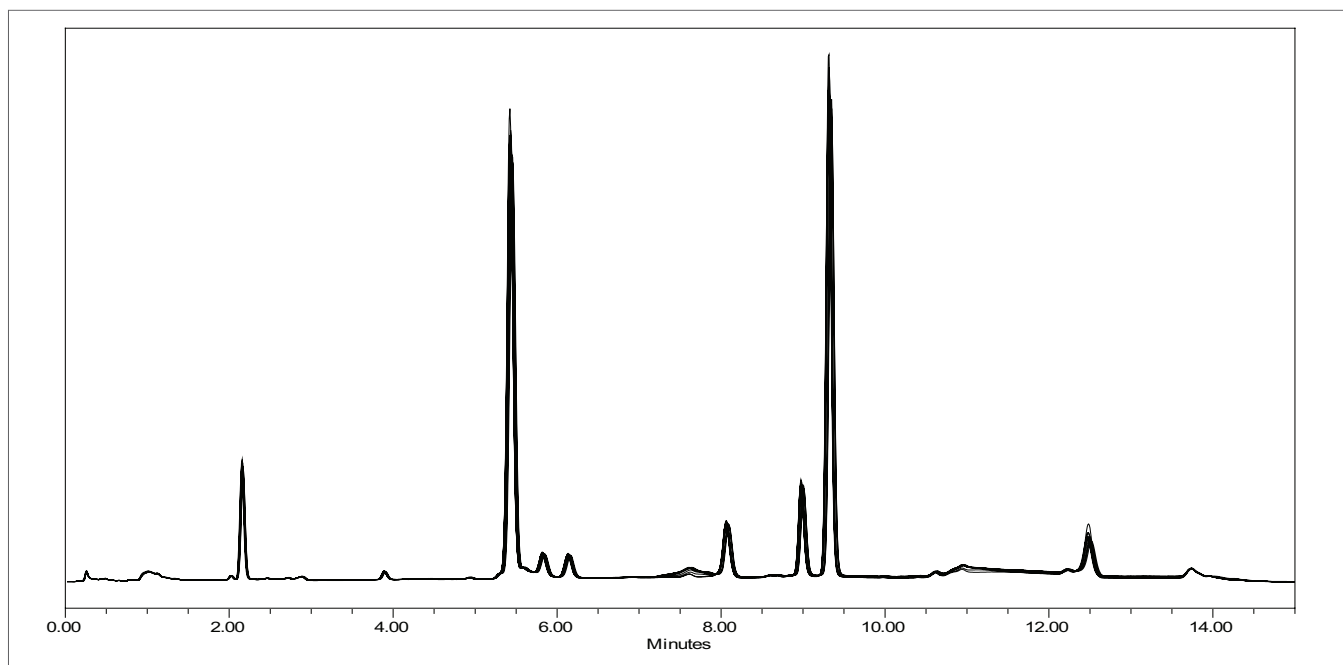


Figure 3. Overlay of 12 replicates of the fat-soluble vitamins WS, wavelength: 265 nm.

Linearity testing was based on a series of WS dilutions. As shown in Figure 4, the linearity results showed exceptional R^2 values of ≥ 0.999 ($n = 3$ at each level).

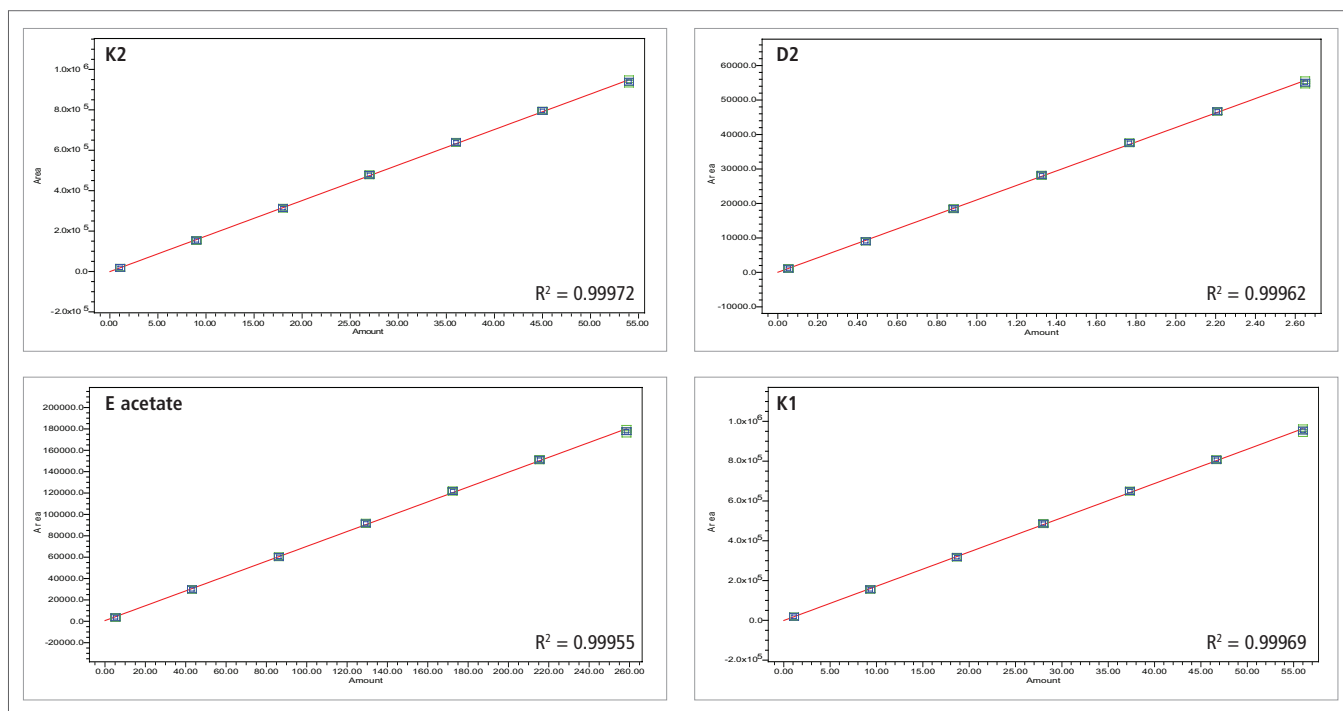


Figure 4. Results of 7-level calibration set for vitamins K2, D2, E acetate and K1; amounts in ppm.

Table 2 presents the limit of quantitation (LOQ) and limit of detection (LOD) levels for all tested fat-soluble vitamins. These limits were obtained using signal-to-noise (s/n) limits, calculated via the respective calibration plots.

Table 2. Calculated LOQ and LOD for all eight fat-soluble vitamins.

Vitamin	LOD ($\mu\text{g/mL}$; ppm) (s/n $\geq 3/1$)	LOQ ($\mu\text{g/mL}$; ppm) (s/n $\geq 10/1$)
A acetate	0.007	0.024
A palmitate	0.127	0.422
D2	0.010	0.034
D3	0.009	0.031
E	0.434	1.448
E acetate	0.270	0.899
K1	0.010	0.035
K2	0.011	0.037

Comparison of LODs and LOQs among the eight fat-soluble vitamins indicated a wide spread in concentrations, with LODs ranging from 0.007 $\mu\text{g/mL}$ for vitamin A acetate to 0.434 $\mu\text{g/mL}$ for vitamin E. This spread is primarily due to the wide range of UV absorbivities of these vitamins at 210 nm.

Conclusion

The results obtained confirm the applicability of this method for the efficient, routine, and robust analysis of eight fat-soluble vitamins. All eight vitamins were completely separated in under 15 minutes. This work has demonstrated the effective chromatographic separation of fat-soluble vitamins using the PerkinElmer Altus A-30 UPLC® system with UV detector.

The obtained results show excellent retention time repeatability as well as exceptional linearity over the tested concentration ranges. Combined with the very low LOQs for most of these vitamins, it is an ideal application for the routine analysis of fat-soluble vitamins in dietary supplements and various food and beverage products.

References

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