

## Simultaneous Analysis of Vitamin A and D<sub>3</sub> in Vitamin Premixes and Concentrates by Convergence Chromatography/PDA Detection

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### APPLICATION BENEFITS

- Simultaneous determination of vitamin A and D<sub>3</sub> in premixes and concentrates.
- Simplified sample preparation – no purification or dilution in sample preparation.
- Direct injection of sample extract – no reconstitution.
- Much lower solvent waste compared to LC.

### WATERS SOLUTIONS

[ACQUITY UPC<sup>2</sup>® System](#)  
[with PDA detection](#)

[Empower 3 Chromatography](#)  
[Data Software](#)

[ACQUITY UPC<sup>2</sup> BEH Column](#)

### KEY WORDS

UPC<sup>2</sup>, Convergence Chromatography, fat-soluble vitamin, vitamin premix, vitamin A, vitamin D<sub>3</sub>, retinyl acetate, cholecalciferol, ergocalciferol

### INTRODUCTION

Simultaneous analysis of fat-soluble vitamins in foods is challenging due to their different properties and concentrations. A typical method involves extraction and saponification of fat, followed by high performance liquid chromatography (HPLC) with UV/Vis detection. After saponification the extracts can be analyzed for vitamin A directly, but they have to be diluted due to high abundance of the vitamin A in foods and its high molar extinction. Unfortunately, the dilution makes it impossible to detect vitamin D<sub>3</sub> in the same solution. To measure vitamin D<sub>3</sub>, the extracts have to be cleaned on a semi-preparative chromatograph and concentrated. For these reasons vitamins A and D<sub>3</sub> had to be analyzed separately. The HPLC of these compounds suffers from a long runtime, slow equilibration, and poor reproducibility.

As the extraction and saponification of separate vitamin A and D<sub>3</sub> methods are identical, we investigated whether it would be possible to apply Waters® UltraPerformance Convergence Chromatography™ (UPC<sup>2</sup>®) to analyze extract for vitamin A and D<sub>3</sub> in a single chromatographic run.

UPC<sup>2</sup> is a separation technique that uses compressed carbon dioxide as the primary mobile phase. It takes advantage of sub-2 µm particle chromatography columns, the low viscosity of CO<sub>2</sub>, and an advanced chromatography system. This differs from traditional HPLC and improves the sensitivity of this assay. UPC<sup>2</sup> also generates much less solvent waste compared to conventional liquid chromatography.<sup>1</sup> In this application note, we report a method for analysis of vitamin A and D<sub>3</sub> in vitamin premixes and concentrates in one analytical run without purification or dilution. The metrological properties of the UPC<sup>2</sup> and the advantages of the method compared to the HPLC are also discussed.

## EXPERIMENTAL

UPC<sup>2</sup> conditions

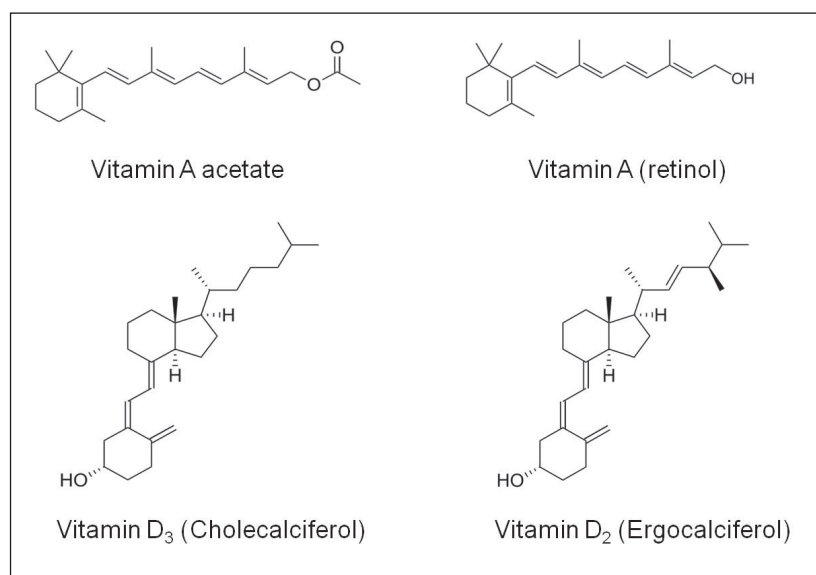
System:	ACQUITY UPC <sup>2</sup>
Detector:	PDA
Software:	Empower 3
Column:	ACQUITY UPC <sup>2</sup> BEH 3.0 mm x 100 mm, 1.7 $\mu$ m
Mobile phase A:	Compressed CO <sub>2</sub>
Mobile phase B:	isopropanol
Wash solvent:	Methanol
Flow rate:	1.7 mL/min
APBR:	2,000 psi
Column temp.:	55 °C
Sample temp.:	10 °C
Injection volume:	7.0 $\mu$ L
Detection:	UV 260 nm
Gradient:	0.5% to 20% B in 9.9 min Hold at 20% for 2 min, re-equilibrate for 3 min

## Sample description

Retinyl acetate (vitamin A acetate) was purchased from Sigma-Aldrich, cholecalciferol (vitamin D<sub>3</sub>) and ergocalciferol (vitamin D<sub>2</sub>) were purchased from the US pharmacopeia. The vitamin premix was from DSM Nutritional Products, Switzerland. Figure 1 shows the structures of relevant compounds used in this study.

## Sample preparation

1.5 g vitamin raw sample was weighed and spiked with 1 mL of vitamin D<sub>2</sub> solution (internal standard) into a 250-mL Erlenmeyer flask. 50 mL ( $\pm$ 10%) ethanol was added, along with 10 mL of 50% KOH, and 2 mL of 33% sodium ascorbate. The samples were saponified for about 1 hour at 80 °C to 85 °C in a water bath. After saponification, the samples were cooled down to room temperature and extracted with a n-hexane diethyl ether mixture. The extract was washed with de-ionized water until neutral, and the extracts were made up to 50-mL with n-hexane. The solution was filtered through a 0.2  $\mu$ m syringe filter into 2-mL autosampler vials and analyzed by UPC<sup>2</sup>/PDA.



## RESULTS AND DISCUSSION

Due to the fact that the molar absorbance of vitamin D<sub>3</sub> is low and its concentration in samples is ten times lower than vitamin A, both vitamins A and D<sub>3</sub> were quantified at the wavelength of the maximum absorbance of D<sub>3</sub>. The typical chromatograms obtained at 260 nm UV wavelengths for vitamin standards in solvent are shown in Figure 2. Chromatograms of the sample extract are shown in Figure 3.

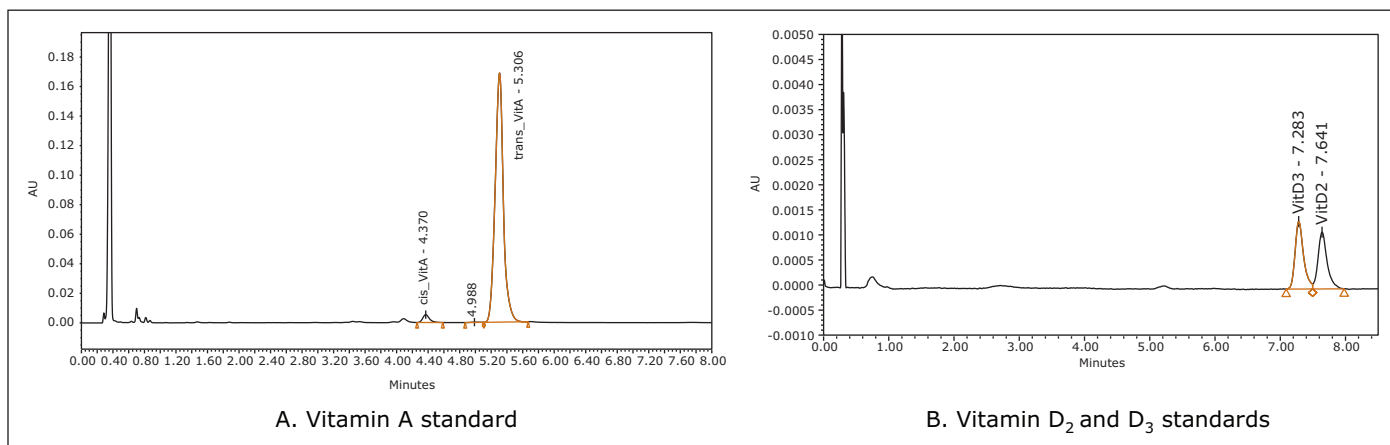


Figure 2. Chromatograms for the vitamin standard solutions at 260 nm.

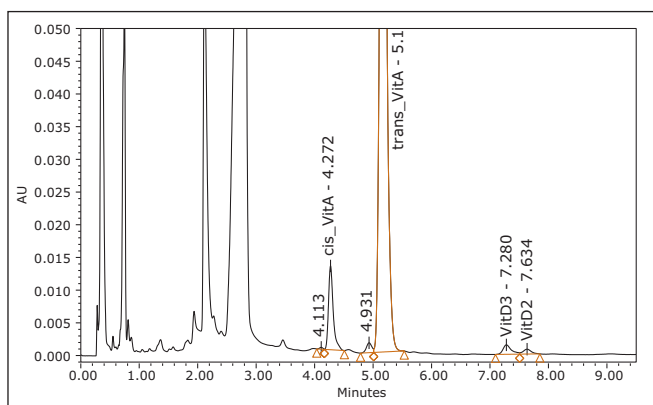


Figure 3. Chromatogram for the sample extract at 260 nm.

The linearity of the assay was investigated using standard solutions. The average peak areas of triplicate injections of seven concentrations were used. Vitamin D<sub>2</sub> was used as the internal standard for the quantification of vitamin D<sub>3</sub>; there was no internal standard used for vitamin A. The coefficient of the determinations  $R^2$  for vitamins A and D<sub>3</sub> were 0.9998 and 1.0000, respectively. The intermediate precision and reproducibility of the assay over a three-month period are shown in Table 1. The intermediate precision of the method for vitamin A was 5.1% and 5.7% for vitamin D<sub>3</sub>, both falling well within the requirement of  $\leq 8\%$ .

Analyte	Intermediate precision, %	Requirement %, <sup>2,3</sup>	Product range, IU/g
Vitamin A	5.1	$\leq 8$	38,000 to 55,800
Vitamin D3	5.7	$\leq 8$	2,280 to 4,230

Table 1. Metrological properties of the method evaluated over three-month period.

To evaluate ruggedness, changes were made to the flow rate, system pressure, and column temperature to observe their effects on the results. There was no statistically significant difference to the  $\pm 2\%$  changes on the results, and the chromatographic resolution of the critical D<sub>2</sub>/D<sub>3</sub> pair was maintained above 1.6 (requirement for R  $\geq 1.2$ ).

The utilization of supercritical fluid chromatography using CO<sub>2</sub> combined with separation on sub 2- $\mu$ m particle size columns provided fast and sufficient separation of the vitamins A and D within the same chromatographic run in a broad range of concentrations. The excellent selectivity of UPC<sup>2</sup> provided baseline separation of analytes from interfering impurity peaks and allowed quantification of the cis- and trans- retinols and the vitamin Ds at 260 nm UV in the same analytical run. Vitamins D<sub>3</sub> and D<sub>2</sub> (internal standard) were separated from each other, and eluted before the matrix peak, in contrast to our traditional method. The extracts in n-hexane can be injected directly into the system, which eliminates the solvent exchange step previously required in our laboratory.

## CONCLUSIONS

A simple and cost-effective assay to analyze vitamin A and D<sub>3</sub> in different vitamin raw materials, premixes, and concentrates using UPC<sup>2</sup> has been developed and validated. The UPC<sup>2</sup>/PDA method demonstrates excellent linearity, resolution, and repeatability. The intermediate precision of the method calculated over a three-month period was less than 6%. The introduction of the ACQUITY UPC<sup>2</sup> System significantly reduced the consumption of HPLC solvents. Beside CO<sub>2</sub>, the only other solvent required was isopropanol. The consumption of isopropanol was 0.12 mL per test. The laboratory has therefore significantly reduced the consumption of HPLC solvents as well as the disposal of waste solvents. By simplifying the procedures and eliminating the repartitioning steps, the reduction of solvent usage has streamlined our workflow and decreased the potential exposure of both laboratory staff and the environment to harmful solvents.

## References

1. Aubin A, Analysis of Fat Soluble Vitamin Capsules using UltraPerformance Convergence Chromatography UPC<sup>2</sup> [Waters Application Note No. 720004394EN](#) (2012).
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3. Standard Method Performance Requirements for Vitamin D3 in Pre-Blends, Pre-Mixes, and Pure Materials – AOAC SMPR 2012.004.

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