Two-dimensional HPLC separation of vitamin D in feedstuff

Authors

Zhang Yanhai,[1] Jin Yan,[1] Xu Qun,[1] and Jeffrey Rohrer[2]; [1]Thermo Fisher Scientific, Shanghai, People's Republic of China; [2]Thermo Fisher Scientific, Sunnyvale, CA, USA

Keywords

Fat-Soluble Vitamins, Food Analysis, Food Quality, Acclaim PolarAdvantage II Column, UltiMate 3000 Rapid Separation Dual System

Goal

To develop an efficient high-performance liquid chromatography (HPLC) method for simple and sensitive determination of radiostol (vitamin D) in feedstuff

Figure 1. Structures and UV spectra of ergocalciferol and cholecalciferol.

Introduction

Vitamins are a well-known group of compounds that are essential for poultry health. These compounds can be classified into two main groups: water- and fat-soluble vitamins. The fat-soluble vitamins play specific and vital functions in metabolism and can cause health problems when they are lacking or in excess. One of the fat-soluble vitamins is vitamin D (V_D), and the most important to poultry are ergocalciferol (V_{D2}) and cholecalciferol (V_{D3}), structures of which are shown in Figure 1.

Poultry health depends in part on diet; however, even feedstuff that initially contains the necessary vitamins can have reduced vitamin content after storage and processing. Therefore, multivitamins are sometimes added to maintain poultry health. To ensure that feedstuff contains the necessary amounts of vitamins, a quality control assay is needed to monitor its vitamin content.

Reversed-phase HPLC is a technique well suited for vitamin analysis; however, some samples, such as feedstuff, are too complex to use a routine HPLC method for vitamin quantification.[1] In addition to vitamins, these samples also contain proteins, amino acids, and minerals. These additional compounds interfere with the separation of vitamins, making quantification difficult. Therefore, a two-dimensional HPLC (2D-HPLC) method similar to one designed for a nutritional drink was developed to measure V_{D2} and V_{D3} in feedstuff.[2]
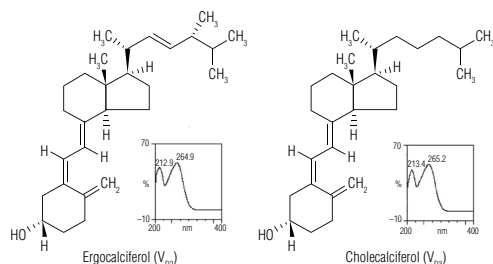
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Equipment

- Thermo Scientific™ UltiMate™ 3000 Rapid Separation Dual system, including:

  – Thermo Scientific™ UltiMate™ 3000 DGP-3600RS Dual Gradient Pump Rapid Separation (P/N 5040.0066) with SRD-3600 Integrated Solvent and Degasser Rack (P/N 5035.9230)

  – Thermo Scientific™ UltiMate™ 3000 WPS-3000TRS Wellplate Sampler, Thermostatted (P/N 5840.0020), with a 25 µL sample loop and a 25 µL syringe

  – Thermo Scientific™ UltiMate™ 3000 TCC-3000RS Thermostatted Column Compartment (P/N 5730.0000) equipped with one 2p–6p and one 2p–10p valve

  – A 500 µL stainless steel sample loop (P/N 6820.2454), for storage of the portion of the separation of the first dimension

  – Thermo Scientific™ UltiMate™ 3000 DAD-3000RS Diode Array Detector (P/N 5082.0020) with a 13 µL flow cell (P/N 6082.0100)

- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2.

Reagents, standards, and consumables

- Deionized (DI) water, 18.2 MΩ-cm resistivity, generated by the Thermo Scientific™ Barnstead™ GenPure™ Pro Ultrapure water system with UV-photo-oxidation and TOC (total organic carbon) monitor (P/N 50131948)

- Acetonitrile (CH_3CN), HPLC grade (Fisher Scientific P/N AC61001-0040)

- Methanol (CH_3OH), 99.8%, HPLC Grade (Fisher Scientific P/N AC61009-0040)

- Ethanol, Anhydrous (Fisher Scientific P/N A405P-4)

- Petroleum Ether (Fisher Scientific P/N E139-1)

- Potassium Hydroxide Solution (KOH), 45%, w/w (Fisher Scientific P/N SP236-500)

- Sodium Sulfate (Na_2SO_4), Anhydrous (Fisher Scientific P/N S415500)

- Ascorbic Acid (V_C), Tocopherol (V_E), Retinol (V_A), Ergocalciferol (V_{D2}), and Cholecalciferol (V_{D3}), ≥97% (National Institute for the Control of Pharmaceutical and Biological Products [NICPBP], China)

- Thermo Scientific™ Target2™ Polypropylene Syringe Filters, 0.45 µm, 30 mm (P/N F2502-9)

Sample preparation

The poultry feedstuff sample was provided by a customer from Zhejiang Province, People's Republic of China.

Add 100 mL of 5 mg/L V_C and 25 mL of 500 g/L KOH to 10 g of a feedstuff sample in a 150 mL conical flask. Mix in a boiling water bath for 30 min. After allowing the conical flask to stand and cool to 40 °C, move the solution to a 250 mL separatory funnel. Extract three times with petroleum ether (100 mL each time). Combine the three petroleum ether extracts and move to a 500 mL separatory funnel. Extract the collected petroleum ether extracts four times with DI water (25 mL each time), then add 1 g of Na_2SO_4 (anhydrous) to remove the residual water. Let the petroleum ether and Na_2SO_4 mixture stand in the 500 mL separatory funnel until fully layered, then release the subnatant to remove Na_2SO_4 from the petroleum ether.

Using rotary evaporation at 55 °C, condense the volume of petroleum ether extract to ~2 mL. Transfer the condensed petroleum ether extract to a 10 mL brown volumetric flask, dry with nitrogen, and dilute to the mark with methanol. Store the sample at 4 °C. Before analysis, pass the prepared sample through a 0.45 µm filter.

Conditions	
First Dimension	
Column	Thermo Scientific™ Acclaim™ 120 C18, 3 µm, 3 × 150 mm (P/N 063691)
Mobile Phase	A) CH_3OH B) H_2O
Gradient	See Table 1
Flow Rate	0.5 mL/min
Injection Volume	5 µL
Temperature	30 °C
Detection	UV, absorbance at 264 nm
Second Dimension	
Column	Thermo Scientific™ Acclaim™ PolarAdvantage II (PA2), 3 µm, 3 × 150 mm (P/N 063705)
Mobile Phase	A) CH_3OH B) CH_3CN C) H_2O
Gradient	See Table 1
Flow Rate	0.5 mL/min
Temperature	30 °C
Detection	UV, absorbance at 264 nm
Valve Switching	See Table 1

Table 1. Gradient program and valve switching.

Time (min)	First Dimension		Valve Switching		First Dimension		
	%A (CH₃OH)	%B (H₂O)	10-Port Valve Position	6-Port Valve Position	%A (CH₃OH)	%B (CH₃CN)	%C (H₂O)
0.0	85	15	1_2	1_6	10	0	90
1.5	85	15	1_2	1_6	10	0	90
15.0	100	0	1_2	1_6	10	0	90
17.0	100	0	1_10	1_6	10	0	90
17.6	100	0	1_2	1_6	10	0	90
18.0	100	0	1_2	1_6	10	0	90
20.0	100	0	1_2	1_6	60	40	0
25.0	100	0	1_2	1_2	60	40	0
30.0	100	0	1_2	1_2	60	40	0
30.2	85	15	1_2	1_2	60	40	0
35.0	85	15	1_2	1_6	60	40	0
36.0	85	15	1_2	1_6	10	0	90

Configuration of the 2D-HPLC system

Figure 2 shows the configuration of the 2D-HPLC system, which has one 6-port valve, one 10-port valve, and two LC columns: Column 1 is the Acclaim 120 C18 column and Column 2 is the Acclaim PA2 column. The treated sample is injected by the autosampler onto the first dimension and separated by Pump 1 using Column 1. Column 2 is simultaneously equilibrated by Pump 2 in the second dimension with the 10-port valve set at 1_2 and the 6-port valve set at 1_6. The portion of the separation of the first dimension that elutes between 17.0 and 17.6 min is stored in a stainless steel 500 µL sample loop with the 10-port valve set at 1_10 and the 6-port valve set at 1_6.[3] The contents of the loop are then eluted by Pump 2 onto the second dimension where the analytes are separated using Column 2 with both the 10-port and 6-port valves set at 1_2.
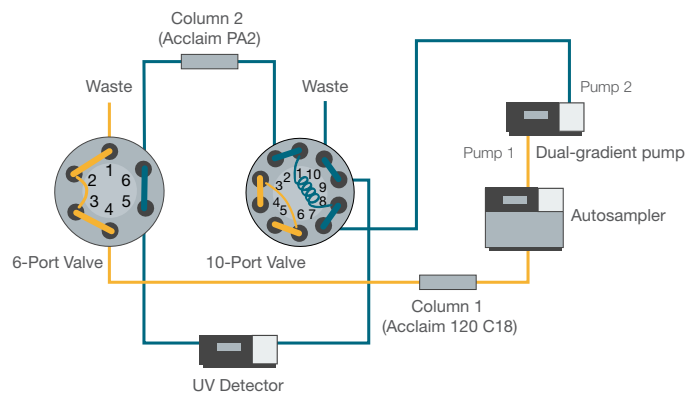

Figure 2. Configuration of the 2D-HPLC system.

Results and discussion

Figure 3 shows a chromatogram of a mixed vitamin standard of V_A, V_{D2}, V_{D3}, and V_E using the specified 2D-HPLC method. In the first dimension (retention time 0–25 min), V_{D2} and V_{D3} were not baseline separated and had a peak resolution of only 1.0. In the second dimension (retention time 25–36 min), they were baseline resolved with a peak resolution of 1.5.

Method precision was estimated using UV detection by making six consecutive injections of V_{D2} and V_{D3} standards with a concentration of 100 µg/L for each. The retention time and peak area reproducibilities RSDs are summarized in Table 2 and show good precision for this 2D-HPLC method.

Figure 4 shows a chromatogram of the feedstuff sample using the 2D-HPLC method. In the first dimension (0–25 min), V_A, V_{D3}, and V_E were detected. As previously described, only the portion of the separation in the first dimension that eluted between 17.0 and 17.6 min was eluted onto the second dimension (25–36 min), where V_{D3} was detected at retention time 30.4 min. V_{D2}, with an expected retention time of 29.7 min, was absent. In the first dimension, the calculated peak purity match factor value was 783 for V_{D3} (the corresponding value for 100% purity is 1000); that, as well as the significant difference between the UV spectra of the V_{D3} standard (Figure 5A) and that measured in the first dimension (Figure 5B), indicate the presence of impurities in the V_{D3} peak. In the second dimension, the peak purity match factor value was 994, and high concordance of the UV spectra between the standard (Figure 5A) and that measured in the second dimension (Figure 5C) demonstrates that using 2D-HPLC can significantly reduce interferences. The peaks for V_A and V_E do not appear to be pure. If their determination is required, then either the 2D method must be modified or another 2D method must be developed.
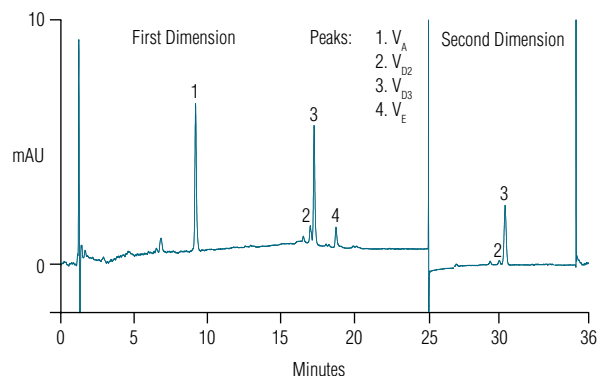

Figure 3. A standard mixture of fat-soluble vitamins resolved using the 2D-HPLC method.

Table 2. Reproducibility of peak retention time and area (n=6).

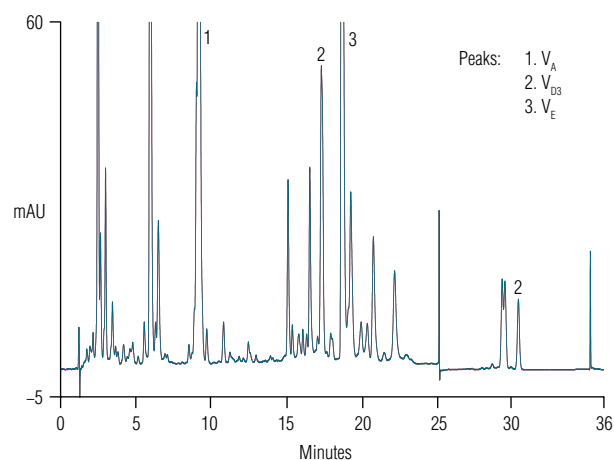| Analyte	Retention Time RSD (%)	Peak Area RSD (%)
V_{D2}	0.04	1.54
V_{D3}	0.05	0.79

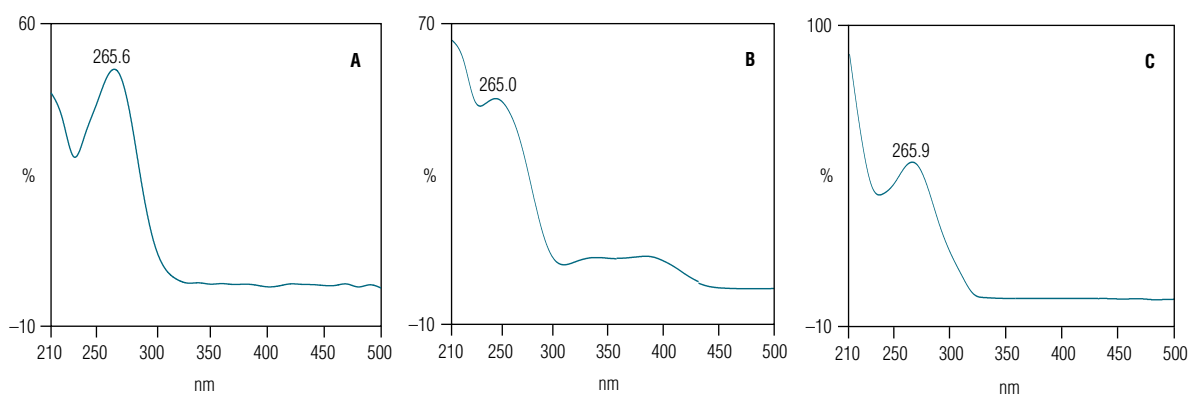Figure 4. A feedstuff sample at 264 nm using the 2D-HPLC method (first dimension, 0–25 min; second dimension, 25–36 min).

Figure 5. UV spectra of the V_{D3} peak of (A) a standard and the feedstuff sample in the (B) first dimension and (C) second dimension.

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Conclusion

2D-HPLC simplifies the determination of V_D content in poultry feedstuff samples. This analysis—performed using an UltiMate 3000 Rapid Separation Dual system and Chromeleon CDS software—is an efficient means to separate V_{D2} and V_{D3}, which are not completely resolved and elute with other peaks in the first dimension during feedstuff sample analysis.

References

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