

# Simultaneous Determination of Vitamins A, E, and D<sub>3</sub> in Milk-Based Nutritionals by On-Line Two-Dimensional HPLC

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## Key Words

Cheese, Cholecalciferol, Fat-soluble Vitamins, Food Analysis, Food Quality, Infant Formula, Milk, Milk Powder, Radiostol, Retinol, Tocopherol, UltiMate 3000 x2 Dual HPLC System, Yogurt

## Goal

To develop an efficient high-performance liquid chromatography (HPLC) method for simple and sensitive determination of retinol (vitamin A), cholecalciferol (vitamin D<sub>3</sub>), and tocopherol (vitamin E) in milk-based nutritionals such as infant formula, adult formula, milk, yogurt, and cheese.

## Introduction

Vitamins are a well-known group of compounds that are essential for human health. These compounds can be classified into two main groups, water- and fat-soluble. Retinol (vitamin A), radiostol (vitamin D) and tocopherol (vitamin E) belong to the fat-soluble group, and play specific and vital functions in metabolism. Health problems can be present when these vitamins are either lacking or in excess. Our intake of vitamins depends on our diet. However, even foods that contain the necessary vitamins can have reduced vitamin content after storage, processing, or cooking. Therefore, people take vitamin tablets and/or consume milk-based products such as infant formula, adult formula, milk, yogurt, and cheese to supplement or incorporate these nutrients into their diet. For example, vitamin D is the name given to a series of compounds with antirachitic activity. The most common compounds having vitamin D activity in foods are ergocalciferol (vitamin D<sub>2</sub>) and cholecalciferol (vitamin D<sub>3</sub>).<sup>1</sup> Vitamin D<sub>2</sub> is primarily found in wild mushrooms, while vitamin D<sub>3</sub> is primarily present in foodstuffs of animal origin (e.g., cow's milk and breast milk); therefore, vitamin D<sub>3</sub> is usually found in milk-based formulas such as infant formula and adult nutritionals.<sup>2</sup> However, the amount of vitamin D that is derived from milk in milk-based formulas is usually not adequate for the needs of infants, and thus vitamin D<sub>3</sub> is added to infant formulas.



To ensure that these foodstuffs contain the labeled amounts of vitamins, there needs to be a quality control assay for them. Figure 1 shows the structures of vitamins A, E, and D; in this work these vitamins will be determined simultaneously. Reversed-phase HPLC is a technique well suited for vitamin analysis;<sup>3-6</sup> however, milk-based nutritionals are too complex to use a routine HPLC method for vitamin quantification. For example, the determination of vitamin D in milk-based nutritionals is difficult because of the low content and lack of vitamin D stability in response to heat, light, oxidation, and the presence of other milk components (e.g., fat, emulsifiers, amino acids, proteins, sterols, and minerals) that can interfere with the determination. Emulsifiers cause fat and protein to interact strongly, and successful analytical methods must be capable of disrupting this interaction and release the fat, so that the vitamin D can be separated and detected.<sup>7</sup> Therefore, sample preparation is key to vitamin D determination in these types of samples. The sample preparation steps for milk-based nutritionals in the European Union (EU) and China regulatory methods each include saponification, extraction, concentration, dilution, and sample clean-up.<sup>2,8</sup>

The clean-up procedure is performed using a semi-preparative normal phase HPLC method that is laborious as well as time and organic solvent (*n*-hexane and 2-propanol) consuming. Therefore, an on-line two-dimensional HPLC (2D-HPLC) method, that is similar to the reported design for the separation of vitamin D<sub>2</sub> and D<sub>3</sub> in feedstuff,<sup>9</sup> was developed to measure vitamin D<sub>3</sub> as well as vitamins A and E in milk-based samples.

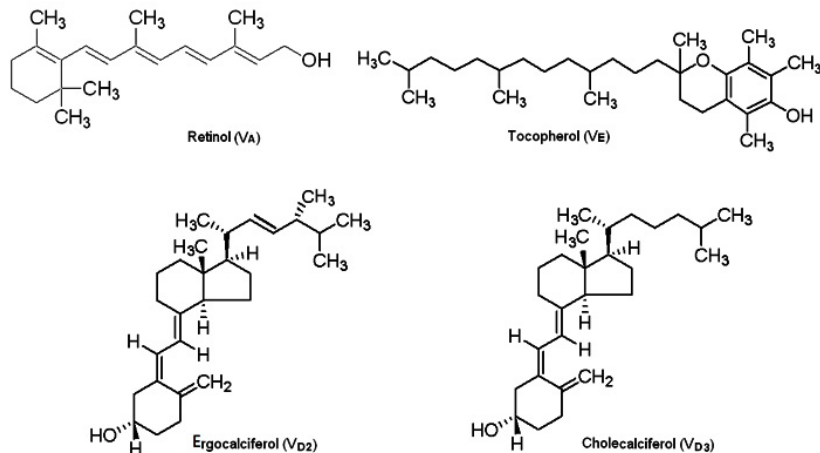


Figure 1. Structures of vitamins A, E, D<sub>2</sub>, and D<sub>3</sub>.

## Equipment

- Thermo Scientific™ Dionex™ UltiMate™ 3000 Rapid Separation Dual HPLC system, including:
  - DGP 3600RS Dual Ternary Pump System (P/N 5040.0066) with SRD-3600 Integrated Solvent and Degasser Rack (P/N 5035.9230)
  - WPS 3000TRS Wellplate Sampler, Thermostatted (P/N 5840.0020) with a 25 μL sample loop (P/N 6820.2415) and a 25 μL syringe (P/N 6822.0001)
  - TCC 3000RS Thermostatted Column Compartment (P/N 5730.0000) equipped with one 2–6 p and one 2–10 p valve
  - A 500 μL stainless steel sample loop (P/N 6820.2454), for storage of the portion of the separation of the first dimension
  - DAD-3000RS Diode Array Detector (P/N 5082.0020) with 13 μL flow cell (P/N 6082.0100)
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2
- Thermo Scientific RT Stirring Hotplate (P/N SP136320-33Q)

## Reagents and Standards

- 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<sub>3</sub>CN), HPLC Grade (Fisher Scientific™ P/N AC610010040)
- Methanol (CH<sub>3</sub>OH), 99.8%, HPLC Grade (Fisher Scientific P/N AC610090040)
- 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<sub>2</sub>SO<sub>4</sub>), anhydrous (Fisher Scientific P/N S415500)
- Sodium chloride (NaCl), ≥99% (Fisher Scientific P/N S271-1)
- Ascorbic acid (vitamin C), Tocopherol (vitamin E), retinol (vitamin A), ergocalciferol (vitamin D<sub>2</sub>), and cholecalciferol (vitamin D<sub>3</sub>), ≥97%, (National Institute for the Control of Pharmaceutical and Biological Products [NICPBP], China)

## Consumables

- Thermo Scientific™ Target2™ Polypropylene Syringe Filters (0.45 μm, 30 mm, P/N F2502-9)
- Thermo Scientific Sun-SRi Luer-Lock Syringe (Fisher Scientific P/N 14-823-261)

## Preparation of Standard Solutions

In the EU regulatory method, to calibrate the loss of vitamin D in sample preparation, an internal standard (I.S.) calibration method is used for the quantitative determination of vitamins A, D, and E. If vitamin D<sub>3</sub> is to be determined, vitamin D<sub>2</sub> is used as an I.S.<sup>2</sup> Vitamin D<sub>2</sub> is thus used as an I.S. in this work.

### Stock Standards 1

Three individual stock standards of vitamin A, E and D<sub>3</sub> are prepared. Dissolve ~0.025 g of vitamins A and D<sub>3</sub>, and ~0.05 g of vitamin E in 25 mL methanol, respectively. The concentration of Stock Standard 1 of vitamin D<sub>3</sub> is 1000 mg/L; that of vitamin A is 1250 mg/L, and that of vitamin E is 2050 mg/L.

### Stock Standards 2

Dilute 200 μL of each Stock Standard 1 to 10 mL with methanol, respectively. The concentration of Stock Standard 2 of vitamin D<sub>3</sub> is 20 mg/L, that of vitamin A is 25 mg/L, and that of vitamin E is 41 mg/L.

### Stock Internal Standard (I.S.) Solution

Vitamin D<sub>2</sub> is used as an internal standard. Dissolve 0.025 g of vitamin D<sub>2</sub> in 25 mL of methanol. The concentration of the Stock I.S. Solution is 1000 mg/L.

### Mixed Standards for Calibration

For calibration, prepare eight mixed working standard solutions with different concentrations by diluting the proper amount of the Stock Standards with methanol. Vitamin D<sub>2</sub> is used as I.S. for the quantitative determination of vitamin D<sub>3</sub>. The volumes of each solution needed to make the mixed standards are shown in Table 1.

Table 1. Preparation of Mixed Standards for Calibration.

Stock Standard	Volume of Stock Standard (mL)	Volume of Methanol (mL)	Final Volume (mL)	Vitamin	Final Concentration of Calibration Standard (mg/L)	Serial No. of Mixture Standards for Calibration
Stock Standard 1 of Vitamin A	0.48	4.46	10.0	A	60.0	1
Stock Standard 1 of Vitamin E	5.00			E	1025	
Stock Standard 1 of Vitamin D <sub>3</sub>	0.05			D <sub>3</sub>	5.00	
Stock I.S. Solution	0.01			D <sub>2</sub>	1.00	
Stock Standard 1 of Vitamin A	0.20	8.77	10.0	A	25.0	2
Stock Standard 1 of Vitamin E	1.00			E	205	
Stock Standard 1 of Vitamin D <sub>3</sub>	0.02			D <sub>3</sub>	2.00	
Stock I.S. Solution	0.01			D <sub>2</sub>	1.00	
Stock Standard 1 of Vitamin A	0.10	9.68	10.0	A	12.5	3
Stock Standard 1 of Vitamin E	0.20			E	41.0	
Stock Standard 1 of Vitamin D <sub>3</sub>	0.01			D <sub>3</sub>	1.00	
Stock I.S. Solution	0.01			D <sub>2</sub>	1.00	
Stock Standard 1 of Vitamin A	0.05	9.59	10.0	A	6.25	4
Stock Standard 1 of Vitamin E	0.10			E	20.5	
Stock Standard 2 of Vitamin D <sub>3</sub>	0.25			D <sub>3</sub>	0.50	
Stock I.S. Solution	0.01			D <sub>2</sub>	1.00	
Stock Standard 1 of Vitamin A	0.01	9.86	10.0	A	1.25	5
Stock Standard 1 of Vitamin E	0.02			E	4.10	
Stock Standard 2 of Vitamin D <sub>3</sub>	0.10			D <sub>3</sub>	0.20	
Stock I.S. Solution	0.01			D <sub>2</sub>	1.00	
Stock Standard 2 of Vitamin A	0.25	9.19	10.0	A	0.625	6
Stock Standard 2 of Vitamin E	0.50			E	2.05	
Stock Standard 2 of Vitamin D <sub>3</sub>	0.05			D <sub>3</sub>	0.10	
Stock I.S. Solution	0.01			D <sub>2</sub>	1.00	
Stock Standard 2 of Vitamin A	0.16	9.555	10.0	A	0.40	7
Stock Standard 2 of Vitamin E	0.25			E	1.025	
Stock Standard 2 of Vitamin D <sub>3</sub>	0.025			D <sub>3</sub>	0.05	
Stock I.S. Solution	0.01			D <sub>2</sub>	1.00	
Stock Standard 2 of Vitamin A	0.10	9.69	10.0	A	0.25	8
Stock Standard 2 of Vitamin E	0.10			E	0.41	
Stock Standard 2 of Vitamin D <sub>3</sub>	0.10			D <sub>3</sub>	0.02	
Stock I.S. Solution	0.01			D <sub>2</sub>	1.00	

Note: Vitamin D<sub>2</sub> is used as an internal standard (I.S.) for the quantitative determination of vitamin D<sub>3</sub>.

## Sample Preparation

Twenty five milk-based samples including milk, infant formulas, adult formulas, cheeses, and yogurt were provided by one of the largest dairy manufacturers in China.

### Milk-Based Samples

Sample preparation is based on the preparation specified in the China regulation (GB 5413.9-2010).<sup>8</sup> For milk and yogurt samples, add 50 g of a sample and 1.0 mL of 10 mg/L vitamin D<sub>2</sub> (dilute 0.1 mL of vitamin D<sub>2</sub> Stock Standard 1 to 10 mL with methanol) to a 250 mL conical flask. For infant/adult formula and cheese samples, add 10 g of a sample and 1.0 mL of 10 mg/L vitamin D<sub>2</sub> to a 250 mL conical flask, and then dissolve it in 30 mL of 70 °C DI water; allow it to cool to room temperature (~25 °C), and then move the sample solution to a 250 mL conical flask. Add 100 mL of 15 g/L vitamin C-ethanol solution (dissolve 3 g of vitamin C in 200 mL of ethanol), and 25 mL of 1.25 g/mL KOH (dissolve 125 g of KOH in 100 mL DI water). After stirring at 1200 rpm and 60 °C for 45 min, and then allowing the conical flask to stand and cool to room temperature (~25 °C), move the solution to a 250 mL separatory funnel and extract three times with petroleum ether (100 mL each time). If there is emulsification (i.e., a foam forms) when extracting add 15 mL of saturated NaCl solution (dissolve 40 g of NaCl in 100 mL of DI water). 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), and then add 1 g of Na<sub>2</sub>SO<sub>4</sub> (anhydrous) to remove the residual water. Let the mixture (petroleum ether and Na<sub>2</sub>SO<sub>4</sub>) stand in the 500 mL separatory funnel until fully layered, and release the supernatant to remove Na<sub>2</sub>SO<sub>4</sub> from the petroleum ether. Use rotary evaporation at temperature not exceeding 40 °C according to BS EN 12821:2009 or GB5413.9 to 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. The sample is stored at 4 °C. Before analysis, pass the prepared sample through a 0.45 µm filter.

### Spiked Milk-Based Formula Samples

For milk and yogurt samples, add 50 g of a sample, 1.0 mL of 10 mg/L vitamin D<sub>2</sub>, and proper amounts of vitamins A, E, and D<sub>3</sub> to a 250 mL conical flask. For infant/adult formula and cheese samples, add 10 g of a sample, 1.0 mL of 10 mg/L vitamin D<sub>2</sub>, and proper amounts of vitamins A, E, and D<sub>3</sub> to a 250 mL conical flask, and then dissolve it in 30 mL of 70 °C DI water. After allowing the sample solution to cool to room temperature (~25 °C), move it to a 250 mL conical flask. Sample preparation is completed using the procedures specified above. The spiked concentrations of vitamins A, E and D<sub>3</sub> in the milk-based samples are shown in the section titled *Analysis of Milk-Based Samples*.

## Chromatographic Conditions

### First Dimension

Column:	Thermo Scientific™ Acclaim™ 120 C8, 3 µm, 2.1 × 100 mm (P/N 059123)
Column Temperature:	30 °C
Mobile Phase:	A: CH <sub>3</sub> CN B: CH <sub>3</sub> OH C: H <sub>2</sub> O In gradient (Table 2)
Flow Rate:	0.4 mL/min
Injection Volume:	5 µL
UV Detection:	Absorbance at 263 nm for vitamin D, 296 nm for vitamin E, and 325 nm for vitamin A

### Second Dimension

Column:	Thermo Scientific™ Accucore™ Polar Premium, 2.6 µm, 3.0 × 150 mm (P/N 28026-153030)
Column Temperature:	30 °C
Mobile Phase:	A: CH <sub>3</sub> CN B: CH <sub>3</sub> OH C: H <sub>2</sub> O In gradient (See Table 2)
Flow Rate:	0.5 mL/min
UV Detection:	Absorbance at 263 nm for vitamin D
Valve Switching:	See Table 2

## Results and Discussion

### Configuration of the On-Line Two-Dimensional HPLC

Figure 2 shows the configuration of the 2-D HPLC system, which has two valves, one 6-port and one 10-port, and two HPLC columns. The treated sample is injected by the autosampler onto the first dimension and separated with pump 1 using column 1, while column 2 is simultaneously equilibrated with pump 2 in the second dimension (1\_6 position on the 6-port valve and 1\_2 position on the 10-port valve). The portion of the first dimension that elutes between 15.75 and 16.20 min is stored in a 500 µL loop (1\_6 position on the 6-port valve, and 1\_10 position on the 10-port valve) (Table 2).<sup>10</sup> This loop connects positions 1 and 8 on the 10-port valve. The contents of the loop will be eluted with pump 2 onto the second dimension, where the analytes are separated using column 2 (1\_2 position on both the 10-port and 6-port valves). Compared to reported 2-D HPLC methods that use a SPE column to trap the analytes,<sup>12</sup> the contents of the 500 µL loop are diluted by the aqueous mobile phase of the second dimension so that the solvent from the first-dimension mobile phase does not induce the vitamin peaks from being bound on the second-dimension column.

Table 2. Gradient program and valve switching.

Time (min)	First Dimension			Valve Switching			Second Dimension				
	%A (CH <sub>3</sub> CN)	%B (CH <sub>3</sub> OH)	%C (H <sub>2</sub> O)	6-Port Valve Position	10-Port Valve Position	Description	%A (CH <sub>3</sub> CN)	%B (CH <sub>3</sub> OH)	%C (H <sub>2</sub> O)		
0.00	50	0	50	1_6	1_2	Connect UV detector and the column on the first dimension, for the determination of vitamins A and E, and clean-up of vitamin D	10	0	90		
15.00	100	0	0			1_2				Store the elution from 15.75 to 16.2 min in the 500 µL sample Loop	
15.75						1_10				Elute the contents of the loop onto the second dimension	
16.20					1_2						
17.00											
19.00				1_2	1_10	Connect UV detector and the column on the second dimension, for the determination of vitamin D	100	0	0		
20.00	0	100	0								
25.00											
27.00							50	50			
28.00	50	0	50							60	40
29.00											
30.00							1_6	1_2	Complete the analysis, the valves return to their original positions	0	100

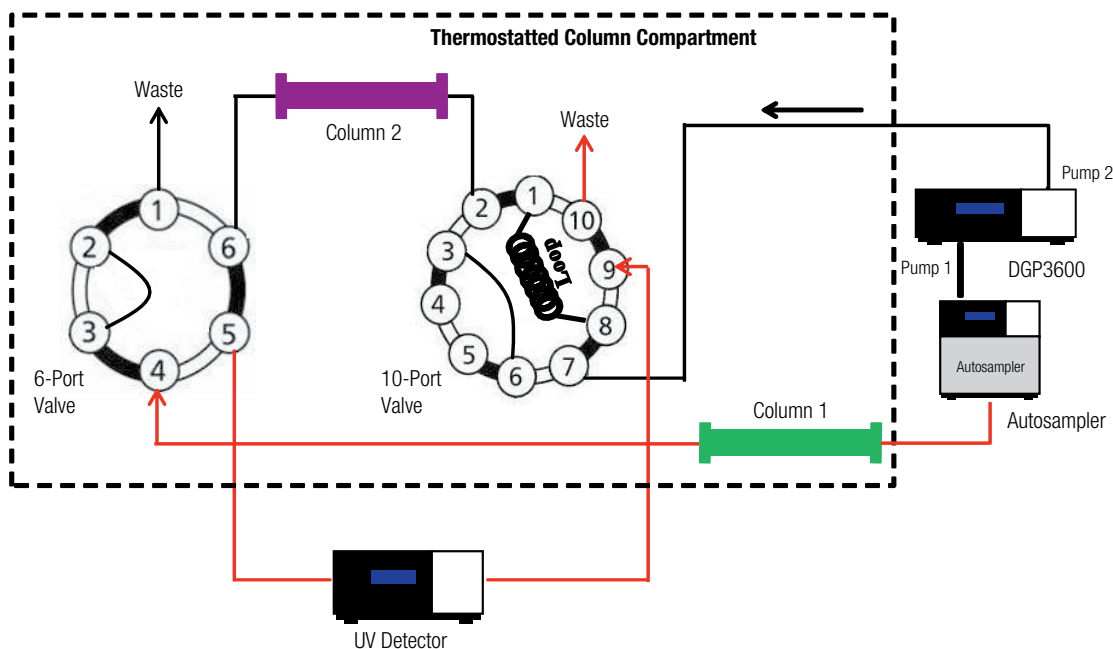


Figure 2. Configuration of the on-line 2-D HPLC system.

### Optimization of the On-Line Two-Dimensional HPLC Method

Having different retention mechanisms in the first and second dimensions is important to the construction of a successful on-line two-dimensional HPLC method. Normal phase (NP) as the first dimension and reversed-phase (RP) as the second dimension is ideal. However, for the on-line two-dimensional HPLC system, solvent incompatibility of the two dimensions can result in the analytes not being retained in the second dimension. Using a micro column (e.g., 1 × 10 mm) in the first dimension and a solvent evaporation interface were reported to eliminate the problem of solvent incompatibility.<sup>11-13</sup> However, the use of the micro column for first dimension and a typical format column for the second dimension decreases detection sensitivity;<sup>11,12</sup> and analytes may be lost due to evaporation in the vacuum assisted dynamic solvent evaporation interface.<sup>13</sup>

Therefore, in this work, reversed-phase is used for both dimensions to decrease the effects of solvent incompatibility. Compared to the C18 stationary phase, the C8 stationary had weak retention for the fat-soluble vitamins

when using acetonitrile and methanol, the commonly used HPLC mobile phase solvents. Moreover, use of a small column in the first dimension is beneficial to reducing elution volume, as well as decreasing any solvent incompatibility. Therefore, the Acclaim 120 C8 (3 μm, 2.1 × 100 mm) and Accucore Polar Premium (C18, 2.6 μm, 3.0 × 150 mm) columns are used for the first and second dimensions, respectively. Figures 3 and 4 show chromatograms of a mixed standard of vitamins A, D<sub>2</sub>, D<sub>3</sub> and E and an infant formula sample (vitamin D<sub>2</sub> already added as an I.S.), respectively, using the specified two-dimensional HPLC method. On the first dimension (retention time 0–20 min), vitamins A and E are separated (Figures 3A and 4A), while vitamin D peak in the infant formula sample cannot be discriminated, although it is observed in the mixed vitamin standard. In the second dimension (retention time 20–30 min), chromatograms of the infant formula sample (Figure 4B) show efficient elimination of interferences, and baseline resolution between vitamins D<sub>2</sub> and D<sub>3</sub> with a peak resolution of 2.2. Therefore, vitamins A and E can be determined in the first dimension, and vitamin D can be determined in the second dimension.

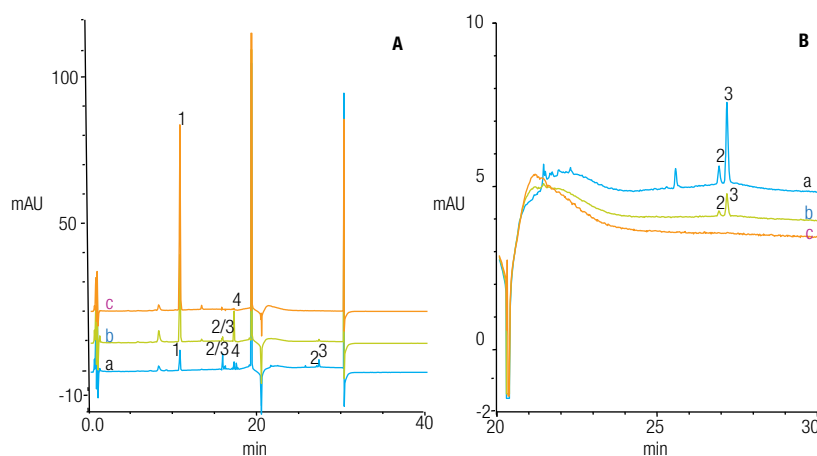


Figure 3. Chromatograms of (A) a mixed standard vitamins A, D<sub>2</sub>, D<sub>3</sub>, and E resolved using the on-line two-dimensional HPLC method, and detected at (a) 263 nm, (b) 296 nm, and (c) 325 nm in the first dimension (0–20 min) and the second dimension (20–30 min), and (B) enlarged chromatograms of the second dimension (20–30 min). Peaks: 1- V<sub>A</sub>, 2- V<sub>D2</sub>, 3- V<sub>D3</sub>, 4- V<sub>E</sub>.

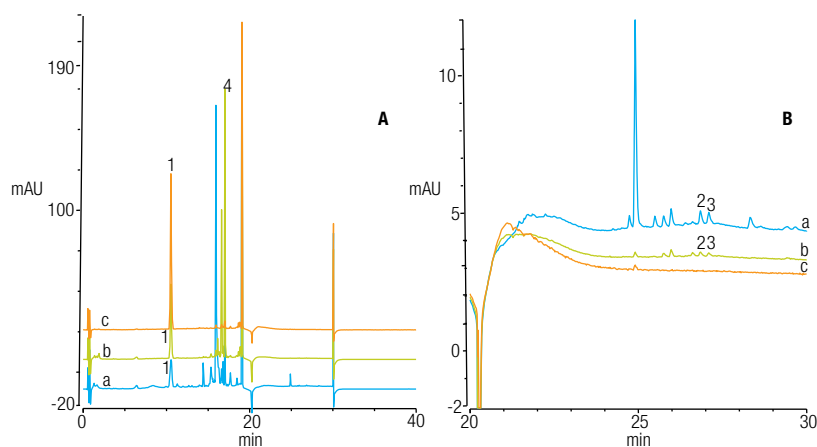


Figure 4. Chromatograms of (A) an infant formula with vitamin D<sub>2</sub> added as an I.S. resolved using the on-line two-dimensional HPLC method, and detected at (a) 263 nm, (b) 296 nm, and (c) 325 nm in the first dimension (0–20 min) and the second dimension (20–30 min), and (B) enlarged chromatograms of the second dimension (20–30 min). Peaks: 1- V<sub>A</sub>, 2- V<sub>D2</sub>, 3- V<sub>D3</sub>, 4- V<sub>E</sub>.

In this on-line two-dimensional HPLC method, vitamins D<sub>2</sub> and D<sub>3</sub> co-elute in the first dimension, and are sent to the 500 µL loop. This may remove interferences (Figure 5).

#### Method Reproducibility, Linearity, and Detection Limits

Method precision was estimated by making six consecutive 5 µL injections of a Mixed Standard for Calibration (No. 6, Table 1) with concentrations of 0.625 mg/L for vitamin A, 2.05 mg/L for vitamin E, 0.10 mg/L for vitamin D<sub>3</sub>, and 0.10 mg/L for vitamin D<sub>2</sub>. Retention time RSDs all are less than 0.1%, and peak area RSDs all are less than 1.0%, demonstrating good short-term precision for this method.

Calibration linearity of vitamins A, E, and D<sub>3</sub> was investigated by making three consecutive 5 µL injections of the Mixed Standards for Calibration prepared at eight different concentrations (i.e., 24 total injections). The internal standard method using vitamin D<sub>2</sub> as the I.S. is used to establish the calibration curves and to quantify vitamins A, E, and D<sub>3</sub> in samples. Different linearity ranges for three compounds were observed when plotting concentration versus peak area. Detailed calibration data calculated by Chromeleon CDS software are shown in Table 3.

Six consecutive 5 µL injections of a mixed standard for calibration (No. 6, Table 1) with concentrations of 0.25 mg/L for vitamin A, 0.41 mg/L for vitamin E, 0.02 mg/L for vitamin D<sub>3</sub>, and 0.10 mg/L for vitamin D<sub>2</sub> were used for estimating method detection limit (MDL) using a signal-to-noise ratio (S/N) = 3. The calculated S/Ns and MDLs are summarized in Table 4.

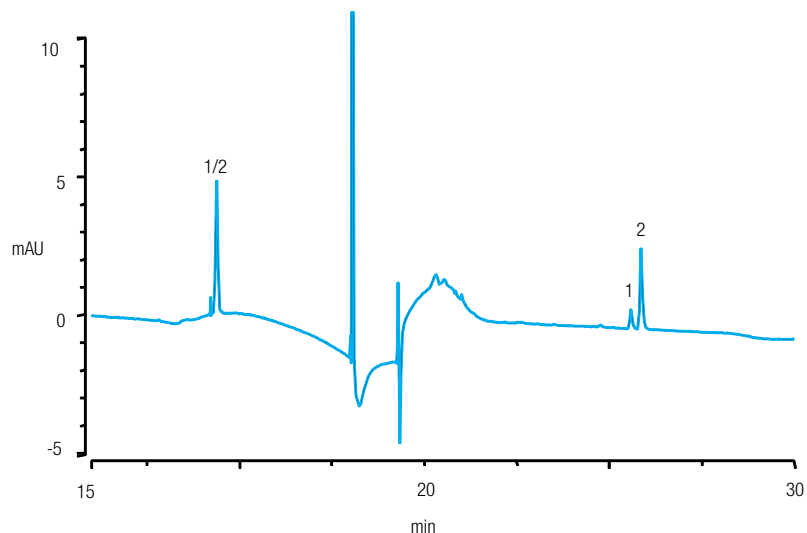


Figure 5. Chromatogram of a mixed standard solution of vitamin D<sub>2</sub> (1.0 mg/L) and vitamin D<sub>3</sub> (0.5 mg/L) in the first dimension (15–20 min) and the second dimension (20–30 min). Peaks: 1- V<sub>D2</sub>, 2- V<sub>D3</sub>.

Table 3. Calibration data.

Vitamin	Regression Equation	r <sup>2</sup>	Range (mg/L)
A	$A = 1.291c + 0.124$	0.9991	0.25–60
E	$A = 0.487c + 1.491$	0.9998	0.41–1025
D <sub>3</sub>	$A = 960.3c - 22.91$	0.9993	0.02–5.0

Table 4. S/Ns of vitamins A, E and D<sub>3</sub> and calculated MDLs for each compound.

Vitamin	Detection Wavelength (nm)	Concentration (mg/L)	S/N	MDL (mg/L)
A	325	0.25	65	0.012
E	296	0.41	12	0.103
D <sub>3</sub>	264	0.02	6.3	0.01

### Analysis of Milk-Based Formulas

Twenty five milk-based samples were determined using this on-line two-dimensional HPLC method. The results are summarized in Table 5. Vitamin D<sub>3</sub> was found in all the samples, while vitamin D<sub>2</sub> was not found in any sample. Vitamin A was found in some formula and cheese samples; and vitamin E was detected only in some formula samples. The results obtained by using the GB method<sup>8</sup> are also listed in Table 5. Comparison of the results obtained by the two methods shows equivalent results. Figure 6 shows chromatograms of infant formula No. 10 and the same sample containing I.S. vitamin D<sub>2</sub>. To judge method accuracy, recoveries of the milk-based samples spiked with vitamin A, E, and D<sub>3</sub> standards (spiked concentrations shown in Table 6) were investigated. The recovery ranges are 91.2–103.7% for vitamin A, 89–95% vitamin E, and 75.5–85.0% (absolute recovery), for vitamin D<sub>3</sub>, demonstrating that this method is suitable for the determination of fat-soluble vitamins in milk-based nutritional.

### Conclusion

Two-dimensional HPLC simplified the determination of vitamins A, E, and D<sub>3</sub> in milk-based nutritional samples. This analysis using a dual UltiMate 3000 system and Chromeleon chromatography data system software is an efficient means to determine Vitamin D<sub>3</sub>, which is not completely resolved in the first dimension of the milk-based nutritional samples analyzed.

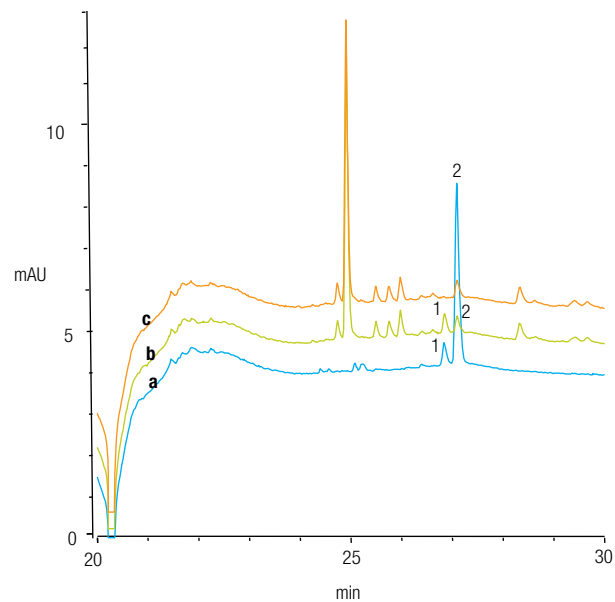


Figure 6. Chromatograms of (a) a mixed vitamin D<sub>2</sub> and D<sub>3</sub> standard, (b) an infant formula sample (No. 10) with vitamin D<sub>2</sub> added as an I.S., and (c) the same infant formula sample using the on-line two-dimensional HPLC method, and detected at 263 nm on the second dimension (20–30 min). Peaks: 1- V<sub>D2</sub>, 2- V<sub>D3</sub>.



Table 5. Detected amounts of vitamins A, E, and D<sub>3</sub> in milk-based nutritional samples.

Sample No.	Sample	Vitamin D <sub>3</sub> (µg/100 g)		Vitamin A (µg/100 g)		Vitamin E (mg/100 g)	
		2-D HPLC Method	GB Method <sup>9</sup>	2-D HPLC Method	GB Method <sup>9</sup>	2-D HPLC Method	GB Method <sup>9</sup>
1	Adult Formula	2.85	2.53	90.0	78.9	2.91	2.99
2		2.70	2.65	86.8	76.9	2.70	2.91
3		2.66	2.58	88.5	79.1	2.80	2.92
4		10.9	11.1	–	–	–	–
5		9.92	9.87	–	–	–	–
6		9.55	9.02	–	–	–	–
7		8.86	7.98	–	–	–	–
8	Infant Formula	7.20	7.41	543	506	9.67	8.07
9		8.24	7.78	556	504	8.80	8.09
10		11.13	10.5	–	–	–	–
11	Cheese	6.46	6.62	411	422	–	–
12		6.90	6.15	358	392	–	–
13		6.75	5.73	401	414	–	–
14		9.88	9.24	401	414	–	–
15		8.26	8.04	411	451	–	–
16		8.53	7.70	–	–	–	–
17		16.4	17.0	–	–	–	–
18	Milk	1.18	1.57	–	–	–	–
19		1.13	1.07	–	–	–	–
20		0.92	1.14	–	–	–	–
21		2.58	2.58	–	–	–	–
22	Yogurt	0.73	0.78	–	–	–	–
23		0.69	0.60	–	–	–	–
24		0.73	0.65	–	–	–	–
25		0.77	0.67	–	–	–	–

Note: "–" represents "not detected".

Table 6. Recoveries of vitamins A, E, and D<sub>3</sub> in the spiked milk-based nutritional samples.

Sample	Vitamin D <sub>3</sub> (µg/100g)		Vitamin A (µg/100g)		Vitamin E (mg/100g)	
	Added	Recovery (%)	Added	Recovery	Added	Recovery
Adult Formula	20.0	82	–	–	–	–
	24.4	76	–	–	–	–
Infant Formula	37.4	77	–	–	–	–
Cheese	11.9	85	–	–	–	–
Milk	9.54	81	62	94	1.03	92
	2.34	82	124	91	2.06	89
	7.56	80	186	104	3.09	96

Note: "–" represents "not detected".

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