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APPLICATION NOTE 73351

Simultaneous determination of vitamin A, D and four vitamin E isomers in infant formula by offline solid phase extraction and online two-dimensional liquid chromatography

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Goal

This paper establishes a rapid and quantitative analytical method for the simultaneous detection of seven vitamins (vitamin A, vitamin D_2 and D_3, and four vitamin E isomers) in infant formula using the new Thermo Scientific™ Vanquish™ Core HPLC system, with offline solid phase extraction (SPE) followed by two-dimensional liquid chromatography (2D-LC) analysis. Here, we show that solid phase extraction is a time- and cost-effective alternative to the classical liquid-liquid extraction pretreatment. This method offers a simplified sample pretreatment, effective sample analysis, and improved detection sensitivity. Additionally, this method is a useful guide for product quality control, supplements detection in dairy milk powder, and subsequent improvement of relevant detection standards.

Introduction

Vitamins are essential nutrients for human growth and development. Infant formula is required to be fortified with fat-soluble vitamins A, D, and E.[1] Studies have found that isomers of vitamin E, such as the common β, γ, and δ-vitamin E isomers, also play an important physiological role in the human body.[2] Therefore, lower levels of detection of these vitamins, including the four vitamin E isomers, are necessary.[2] Liquid chromatography is currently the foremost technique used for the analysis of vitamins A, D, and E.[3-8] With respect to the complex nature of the matrix of milk powder samples, the current Chinese

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national standard method[8] uses a relatively cumbersome pretreatment method to achieve the purification and concentration, such as saponification, liquid-liquid extraction, washing, concentration, and reconstitution. The liquid-liquid extraction process uses large amounts of toxic organic extraction solvents, and it is time-consuming and labor-intensive. It also easily causes target analyte degradation and loss, greatly affecting the sample analysis efficiency and the method reproducibility.[4] In this work, a method for the simultaneous determination of vitamin A, vitamins D_2 and D_3, and four vitamin E isomers was developed. The method is based on solid phase extraction pretreatment, instead of the liquid-liquid extraction, and subsequent chromatographic analysis via 2D-LC.[9] The solid phase extraction conditions simplify the sample preparation process, shorten the sample analysis time, and further improve the overall analysis efficiency.

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific™ Acetonitrile, Optima™ LC grade (P/N A955-4)
- Fisher Scientific Methanol, Optima LC grade (P/N A456-4)
- Fisher Scientific Ethanol, HPLC grade (P/N A995-4)
- Fisher Scientific Ethyl acetate, HPLC grade (P/N E195-4)
- Fisher Scientific Diethyl ether, HPLC grade (P/N E199-4)
- Fisher Scientific Acetone, HPLC grade (P/N A949-4)
- Fisher Scientific Petroleum ether, Optima grade (P/N E120-4)
- Fisher Scientific n-Hexane, HPLC grade (P/N H320-4)
- Fisher Scientific Dichloromethane, HPLC grade (P/N M501-4)
- Potassium hydroxide, analytically pure, CNW Technologies (P/N 4.120063.0500)
- Ascorbic acid, analytically pure, CNW Technologies (P/N 4.120019.0100)
- 2,6-di-tert-butyl-p-cresol (BHT), analytically pure, CNW Technologies (P/N 4.440238.0100)
- Anhydrous sodium sulfate, analytically pure, CNW Technologies (P/N 4.120126.1000)
- Vitamin A (all-trans retinol, ≥95%), Sigma-Aldrich (P/N R7632)
- Vitamin D_2 (98.1%), Dr. Ehrenstorfer GmbH (P/N DRE-C17923900)
- Vitamin D_3 (99.6%), Dr. Ehrenstorfer GmbH (P/N DRE-CA17924000)
- α-Vitamin E (α-tocopherol, 98.0%), Sigma-Aldrich (P/N 47783)
- β-Vitamin E (≥ 96%), Toronto Research Chemicals Inc. (P/N T526130)
- γ-Vitamin E (≥ 96%), Sigma-Aldrich (P/N 47785)
- δ-Vitamin E (≥ 96%), Sigma-Aldrich (P/N 47784)

Consumables

- Solid phase extraction column: Thermo Scientific™ HyperSep™ Retain PFP (200 mg, 6 mL) (P/N 60107-212)
- ^{1}D column: FOODKIT 2 ADE 3.0 × 150 mm, 2.6 µm (P/N VITADE-005K2)
- ^{2}D column: FOODKIT 1 & 2 ADE 4.6 × 100 mm, 2.6 µm (P/N VITADE-004K1)

Instrumentation

- Thermo Scientific Vanquish Core HPLC consisting of:
  - System Base Vanquish Core (P/N VC-S01-A-02)
  - Dual Pump CN (P/N VC-P33-A-01)
  - Split Sampler CT (P/N VC-A12-A-02)
  - Column Compartment C (P/N VC-C10-A-03)
  - Diode Array Detector CG (P/N VC-D11-A-01)
  - Flow cell, standard, 13 µL, VC-D11, VF-D11 (P/N 6083.0510)
  - Fluorescence Detector F Dual-PMT (P/N VF-D51-A)
  - Standard flow cell 8 µL, biocompatible, FLD (P/N 6079.4230)
  - 2× 2-position/6-port column switching valves, 70 MPa (P/N 6230.1520)
  - Vacuum manifold

Chromatography software

- Thermo Scientific™ Chromeleon™ 7.3 Chromatography Data System

Standard solutions preparation

Appropriate amounts of vitamin A, D_2, D_3, α-, β-, γ-, and δ-vitamin E standards were accurately weighed, each into respective 10 mL brown volumetric flasks. Methanol was added to dissolve the above listed reagents and diluted for preparation: 0.41 mg/mL vitamin A solution, 0.16 mg/mL vitamin D_2 solution, 0.28 mg/mL vitamin D_3 solution, 4.25 mg/mL α-vitamin E solution, 2.50 mg/mL β, γ-vitamin E solution, and 4.00 mg/mL δ-vitamin E solution. Then, appropriate amounts of each standard solution were precisely measured into 10 mL brown volumetric flasks, diluted to volume with methanol to form a series of standard mixed working solutions. The concentration of each standard solution is shown in Table 1.

Sample solutions preparation

Following the method of GB 5009.82-2016,[8] 5 g (±0.01 g) of solid milk powder sample was accurately weighed into a 150 mL flat-bottomed flask. Then about 20 mL of warm water (45–50 °C) was added to dissolve the sample. Samples of two different brands of infant formula were used.

Saponification

After the milk powder sample solution was prepared, 1.0 g of ascorbic acid, 0.1 g of BHT, 30 mL of absolute ethanol, and 20 mL of 0.5 g/g potassium hydroxide aqueous solution were added. After complete dissolution, the mixture was saponified via magnetic stirring under a constant water bath temperature of 80 °C for 30 minutes. This saponified liquid was then removed from the warm water bath and cooled to room temperature with cold water.

The saponified liquid was treated with two different procedures, liquid-liquid extraction or solid phase extraction, as described below:

Liquid-liquid extraction

The above saponified liquid and 50 mL of a petroleum ether/diethyl ether mixture (1:1, v/v) were mixed into a 250 mL separatory funnel. After a 5 min vigorous shake and intermittent deflation, the aqueous lower layer solution was transferred into another 250 mL separatory funnel. Then, 50 mL of a petroleum ether/diethyl ether mixture was added for extraction, and the organic extracts were combined. The organic solution was washed with 100 mL of water. The liquid-liquid extraction process was repeated three times with water until the pH of organic solution was neutral. The organic solution was dehydrated with anhydrous sodium sulfate and filtered. The filtrate was transferred into a 250 mL round bottom flask and rotary evaporated to 2 mL within a water bath at 40 °C. The sample was immediately dried with nitrogen and reconstituted with methanol into a 10 mL volumetric flask, diluted to the mark, and then analyzed by HPLC.

Solid phase extraction

The solid phase extraction column was conditioned with 6 mL of methanol followed by 3 mL of ultrapure water. Then 6 mL of the saponified liquid was accurately measured for loading. When the liquid level was reduced to the stationary phase bed, the column was rinsed with about 10 mL of methanol/water solution (60/40, v/v) until the pH of effluent was neutral. 6 mL of acetone was pipetted for elution of the solid phase extraction column. The extracted liquid was collected, dried by nitrogen, reconstituted with 1 mL of methanol, and analyzed by HPLC.

Table 1. Concentrations of standard working solutions for each vitamin (µg/mL)

Vitamin (µg/mL)	Retention time (min)	Concentration level							
		1	2	3	4	5	6	7	8
A	5.54	0.01	0.05	0.1	0.5	1	5	10	50
α-VE	14.98	0.01	0.05	0.1	0.5	1	5	10	50
β-VE	13.43	0.01	0.05	0.1	0.5	1	5	10	50
γ-VE	13.92	0.01	0.05	0.1	0.5	1	5	10	50
δ-VE	12.15	0.01	0.05	0.1	0.5	1	5	10	50
VD_2	19.98	0.02	0.05	0.1	0.2	0.5	1	2	5
VD_3	20.33	0.02	0.05	0.1	0.2	0.5	1	2	5

Chromatographic conditions

The chromatographic conditions are shown in Table 2. The ¹D and ²D gradient programs and valve switching times are shown in Table 3. The HPLC fluidic connection diagram is shown in Figure 1.

Table 2. Chromatographic conditions

Parameter	Value
Column temperature	25 °C
Mobile phase	A: acetonitrile; B: methanol; C: water
Flow rate	¹D analysis pump: 0.5 mL/min, ²D analysis pump: 0.8 mL/min
Detectors	DAD: 264 nm (VD_2, VD_3); 296 nm (α, β, γ, δ-VE), 325 nm (VA); FLD: λ_{ex}: 295 nm, λ_{em}: 330 nm
Injection volume	10 µL

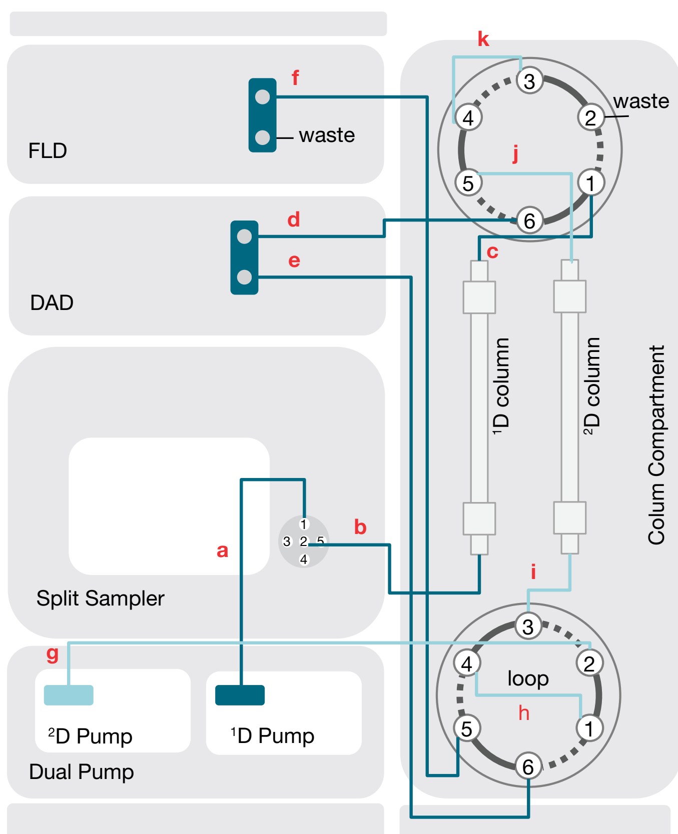

Figure 1. HPLC fluidic connection diagram

#	Connection
a	Thermo Scientific™ Viper™ Capillary, ID × L 0.18 × 350 mm, SST (P/N 6040.2375)
b	Passive pre-heater, ID × L 0.18 × 530 mm, SST (P/N 6040.2375)
c	Viper Capillary, ID × L 0.18 × 250 mm, SST (P/N 6040.2385)
d	Viper Capillary, ID × L 0.18 × 350 mm, SST (P/N 6040.2375)
e	Viper Capillary, ID × L 0.18 × 850 mm, SST (P/N 6040.2380)
f	Viper Capillary, ID × L 0.18 × 950 mm, SST (P/N 6040.2390)
g	Viper Capillary, ID × L 0.18 × 650 mm, SST (P/N 6040.2395)
h	Viperized custom trapping loop, 500 µL, ID × L 0.5 × 3300 mm, SST
i	Passive pre-heater, ID × L 0.18 × 530 mm, SST (P/N 6732.0170)
j	Viper Capillary, ID × L 0.18 × 250 mm, SST (P/N 6040.2385)
k	Viper Capillary, ID × L 0.18 × 150 mm, SST (P/N 6040.2360)

Table 3. ¹D and ²D gradient programs and valve switching times

¹D analysis pump (Target: VA, VE)				²D analysis pump (Target: VD)				Valve switching		
Time (min)	A%	B%	C%	Time (min)	A%	B%	C%	Time (min)	LowerValve	UpperValve
0	0	88	12	0	60	40	0	0	1_2	6_1
2	0	88	12	1	60	40	0	8.93	6_1	6_1
15	0	97	3	2	40	0	60	9.45	1_2	6_1
16	0	100	0	10	40	0	60	16	1_2	1_2
19	0	100	0	12.5	100	0	0			
19.5	0	88	12	20.5	60	40	0			
24	0	88	12	24	60	40	0			

Results and discussion

Optimization of chromatographic conditions

This work employed the new Thermo Scientific Vanquish Core HPLC system, based on our previous method[5]. This method has further optimized the gradient elution procedure to improve analysis efficiency and subsequently reduced the total analysis time from 35 min to 24 min. The different steps of the ^{2}D-LC separation and the valve positions are shown in Figure 2. The sample was injected on ^{1}D column and analyzed by diode array detection (DAD) and fluorescence detection (FLD) detection.

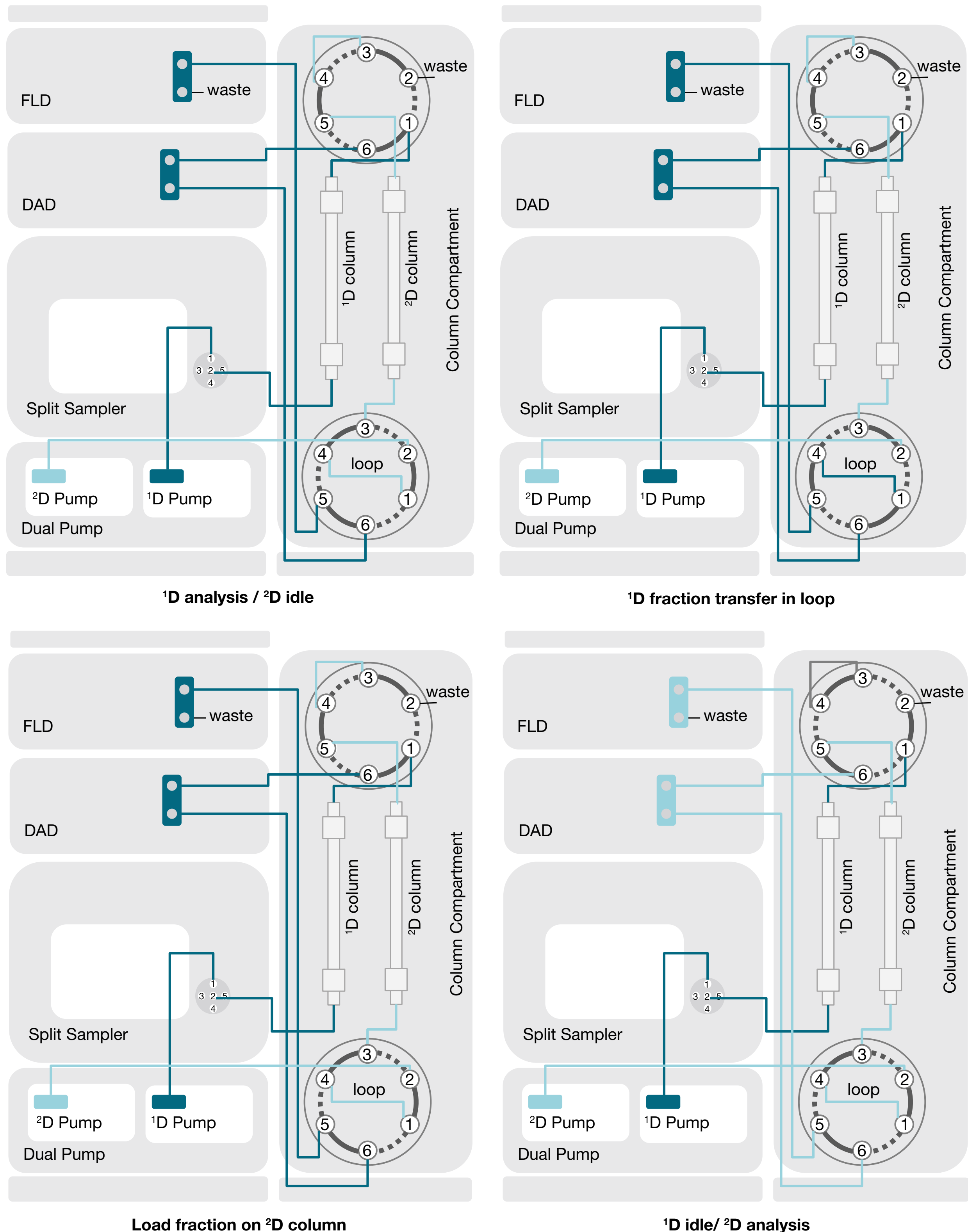

Figure 2. The different steps of the 2D-LC separation and the valve positions

Then the vitamin D fraction was transferred into the loop, loaded on the ²D column, and detected by DAD via valve switching. The method can baseline separate and accurately quantify the four isomers of vitamin E on a ¹D column. The purification of vitamin D and separation of vitamin D_2 and D_3 can be achieved on the ²D column. Under the above premise, our work has further improved the analysis efficiency and detection sensitivity compared to traditional liquid-liquid extraction. Specifically, this method has increased sensitivity for vitamin E and its isomers, reduced the effects of matrix interference on the content determination, and determined the vitamin E isomers on the ¹D with a fluorescence detector. The mixed standard solutions and typical sample analysis spectra are shown in Figures 3 and 4, respectively.

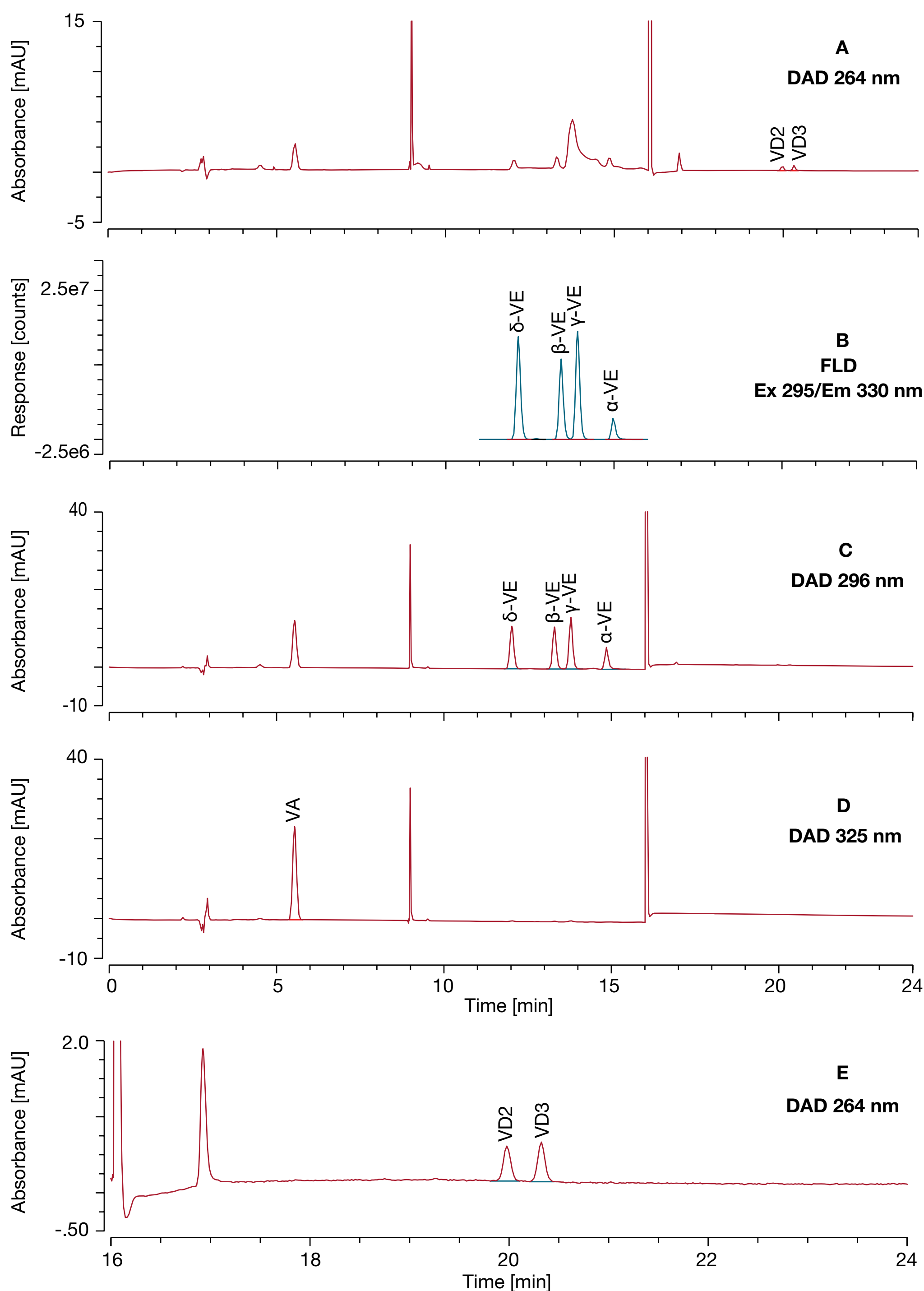

Figure 3. Analytical profile of a mixture of vitamin A, D, and E solutions (A~D, 0~16 min is the first dimension chromatogram, 16~24 min is a second dimension spectrum; E, 2nd-dimensional magnified image)

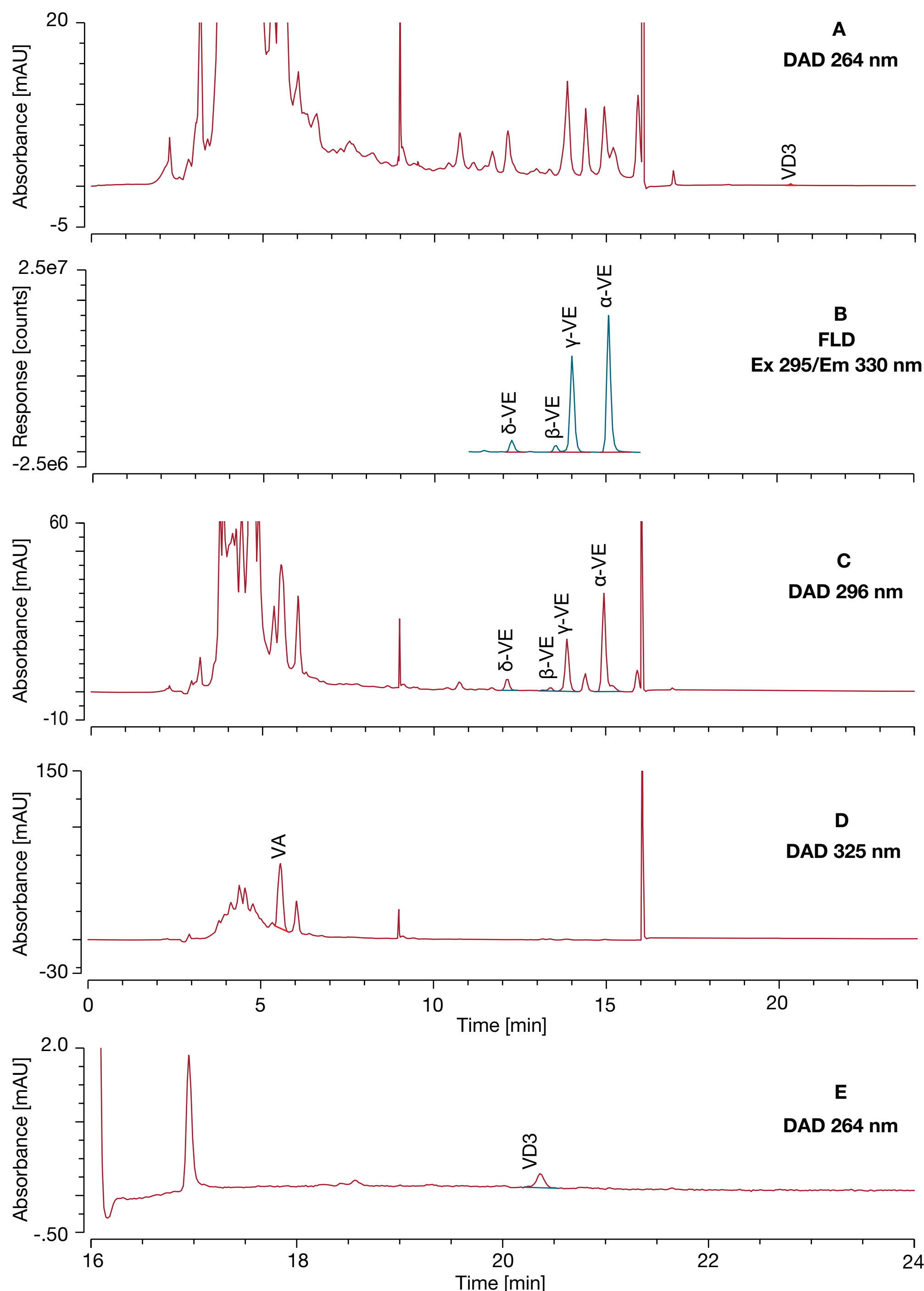

Figure 4. Analytical profile of infant formula samples (A–D, 0–16 min is a first dimension chromatogram, 16–24 min is a second dimension chromatogram; E, second dimension expanded image)

Optimization of pretreatment conditions

Due to the matrix complexity of milk powder samples, the instability of vitamin D to light and heat, and the low concentration of vitamin D, the Chinese national standard method requires a series of tedious pretreatment steps to purify the sample and concentrate the target analytes. In addition, a large amount of organic solvent was required for multi-step liquid-liquid extractions. This not only led to degradation and loss of the target analyte, but also affected the sample analysis efficiency and the method reproducibility. In this paper, the liquid-liquid extraction was replaced by solid phase extraction. As a result, the pretreatment time was reduced from 3 hours to 1 hour. Due to the strong alkalinity of the sample treated by saponification, SPE columns packed with polymeric material were chosen. We compared the elution effects

of five different elution solvents (acetone, ethyl acetate, dichloromethane, methanol, n-hexane) on the target sample. Finally, the Thermo Scientific HyperSep Retain PFP was selected as the optimal solid phase extraction column and acetone was used as the optimal elution solvent.

Method linearity, sensitivity, and precision results

The appropriate amounts of vitamin A, D2, D3, α, β, γ, δ-vitamin E standard solution were accurately weighed into a 10 mL brown volumetric flask and diluted with methanol to prepare a series of standard mixed solutions. Ten microliters of solution were injected, and the linear range and detection limit of the method were examined by the concentration as the abscissa and the peak area as the ordinate. The results showed that each compound provided good linearity in its concentration range and the correlation coefficient was higher than 0.9993. The sensitivity of the method was high. The detection limit of vitamin D_3 can reach 3.5 ng/mL, as shown in Figure 5. The method linearity and its detection limit results are shown in Table 4. The Level 5 mixed standard solution was injected six times sequentially to evaluate the peak area precision. The results showed that the RSD of vitamin A peak area was 0.15%, the peak area RSD of vitamin D_2 and D_3 were 0.40% and 0.99%, respectively, and the peak area RSD of α, β, γ, δ-vitamin E was 0.10% and 0.13%, 0.05% and 0.14%, respectively, indicating the precision of the method.

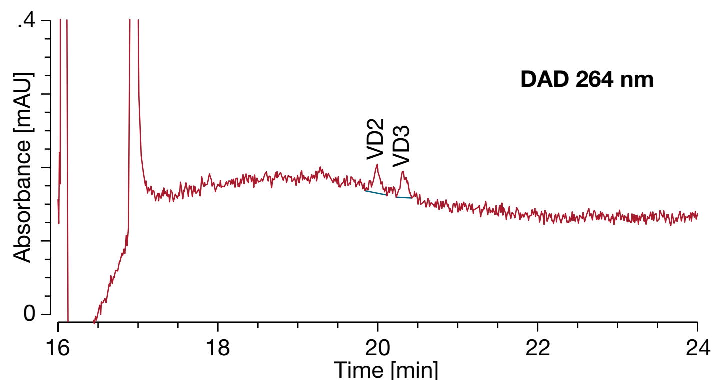

Figure 5. Second dimension chromatogram of vitamin D_3 detection limit (S/N = 4.3), 5 ng/mL

Sample analysis result

The samples of the two brands of infant formula were saponified and the two pretreatment methods—solid phase extraction and the national standard liquid-liquid extraction—were compared to determine the content of each vitamin. The results in Table 5 show that the two pretreatment methods are comparable. However, solid phase extraction is superior to liquid-liquid extraction in terms of time and solvent savings.

Table 4. Method linearity and detection limit results

Vitamin	Detection wavelength (nm)	Range (µg/mL)	Linearity equation	R^2	LOD (ng/mL)
VA	DAD, 325	0.01–50	Y = 3.2362x - 0.0098	0.9999	1.8
α-VE	FLD, λ_{ex} 295, λ_{em} 330	0.05–50	Y = 84078.5x - 3463.5	0.9996	2.7
β-VE		0.05–50	Y = 196839.8x + 5479.7	0.9995	1.9
γ-VE		0.05–50	Y = 257541.1x + 3773.2	0.9996	1.6
δ-VE		0.05–50	Y = 261596.9x + 13364.2	0.9993	1.4
VD_2	DAD, 264	0.02–5	Y = 0.4084x - 0.0013	0.9999	4.0
VD_3	DAD, 264	0.02–5	Y = 0.4585x - 0.0036	0.9999	3.5

Table 5. Comparison of determination results of vitamins in samples

Sample	Pretreatment method	Content (µg/100 g for VA, VD; mg/100 g for VE)					
		VA	VD3	α-VE	β-VE	γ-VE	δ-VE
1	Solid Phase Extraction	538.55	5.76	12.14	0.30	0.45	0.86
	Liquid-liquid extraction	475.38	5.53	10.19	0.31	0.44	0.67
2	Solid Phase Extraction	446.11	6.53	10.82	0.22	2.47	0.30
	Liquid-liquid extraction	533.55	6.84	10.51	0.23	2.35	0.21

Sample 2 was used to compare the recovery of the two pretreatment methods. Vitamin A, α-vitamin E, and vitamin D_3 were spiked at a certain concentration. The recovery ratios of the two pretreatment methods are shown in Table 6. The recovery ratio of typical vitamins in the sample is generally within the acceptable range and the actual vitamin content in the sample is basically consistent with both methods. In addition to the main α-vitamin E in the sample, the content of γ-vitamin E was also higher, while the content of β and δ-vitamin E was relatively low. Different brands of formula milk powder have certain differences in the content of vitamins E isomers. Therefore, analysis and quantification of vitamin E and its isomers in samples have reference significance for evaluating and controlling product quality.

Table 6. Comparison of spiked recovery results

Pretreatment method	Spiked recovery ratio, %		
	VA	VD3	α-VE
Solid Phase Extraction	100.4	101.2	112.1
Liquid-liquid extraction	106.4	118.9	117.3

Conclusion

In this application note, a 2.5× faster 2D-LC method for the simultaneous analysis of vitamin A, vitamin D, and four vitamin E isomers in infant formula was optimized using the Vanquish Core HPLC system in a 2D-LC setup.

This method adopts the solid phase extraction technique instead of the liquid-liquid extraction technique, which simplifies the sample pretreatment process, decreases the sample analysis time, and further improves the detection sensitivity and the sample analysis efficiency.[5,8] This is of great significance for enterprise product quality control and milk powder testing and evaluation.

References

1. Stillwell, W. An introduction to biological membranes (second edition) [M]. Amsterdam: Elsevier Science, 2016.
2. Kamal-Eldin, A.; Görgen, S.; Lampi, A.M. Normal phase high performance liquid chromatography of tecopherols and tocotrienols. Comparison of different chromatographic columns. *Journal of Chromatography A,* **2000,** *881,* 217–227.
3. Chen, J.B. Rapid detection of vitamins A, D and E in food by on-line purification two-dimensional high performance liquid chromatography. *Journal of Food Safety and Quality,* **2019,** *10*(6), 1726:1733.
4. Zhang, Y.H.; Zhu, X.Y.; Yang, X.L.; Jin, Y. Simultaneous qualification of vitamins A, D_3 and E in fortified infant formulae by liquid chromatography coupled with fully automated online two-dimensional column switching method. *Chinese Journal of Analytical Chemistry,* **2013,** *5,* 771–775.
5. Zhang, Y.H.; Jin, Y. Simultaneous determination of vitamins A, D_3, and E in infant formula and adult nutrition by online two-dimensional liquid chromatography. *Chinese Journal of Chromatography,* **2015,** *33*(3), 291–297.
6. Zhang, Y.H.; Zhang, D.W.; Cao, Y. Simultaneous determination of vitamins A, D in vitamin A and D pharmaceutical by online two-dimensional reverse phase liquid chromatography. *Journal of Instrumental Analysis,* **2016,** *35*(1), 28–34.
7. Woollard, D.C.; Bensch, A. Determination of vitamin A and vitamin E esters in infant formulae and fortified milk powders by HPLC: Use of Internal Standardization. *Food Chemistry,* **2016,** *197*(A), 457–465.
8. GB 5009.82-2016, Determination of vitamins A, D, E in foods. National food safety standard.
9. Aurea, A.; Victor, C. Solid-phase extraction of organic compounds: A critical review. Part II. *Trends in Analytical Chemistry,* **2016,** *80,* 655–667.

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