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Application Note

Simultaneous Analysis of Fat-Soluble Vitamins A, D, and E in Food Using ACQUITY Arc Two-Dimensional Liquid Chromatography

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Abstract

In this application, a fast analytical method is established based on the two-dimensional liquid chromatography to simultaneously assay the contents of vitamin A, α/β/γ/δ-tocopherol, vitamin D_2, and vitamin D_3 in infant milk powder. The method is simple, fast, highly automated, precise and accurate. It is suitable for the assay of vitamin A, α/β/γ/δ-tocopherol, vitamin D_2, and vitamin D_3 in formula milk powder or other milk products.

Benefits

- The two-dimensional column switchingultraviolet detection method can separate vitamins A, D, and α/β/γ/δ-E in a single sample injection, greatly increasing the efficiency of analysis;
- With the heart-cutting technique, the vitamin D is cleaned-up on the first dimension column, and then separated into vitamin D_2 and D_3 peaks. This approach helps to eliminate co-elution, which reduces the interference from complex matrix for the vitamin D_2 and D_3 determination.

Introduction

Vitamins A, D, and E are fat-soluble vitamins essential for the body to maintain normal metabolism and functions. Vitamin A, also known as retinol, plays an important role in promoting body growth, maintaining the integrity of the epidermis, etc. Vitamin D includes two major forms, i.e. vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol), which promote calcium and phosphorus metabolism and bone formation in mammals. Vitamin E includes tocopherols and tocotrienols. There are 8 active forms of vitamin E due to the variation of methyl substitution on the parent tocopherol and tocotrienol ring, including α-, β-, γ-, and δ-tocopherols. Among them, α-tocopherol is usually singled out in food science as it has the highest activity and antioxidative and anti-aging properties.[1] Infant formula and adult nutritional products and animal feeds are two important forms of fat-soluble vitamin fortified products. In actual samples, vitamin A and vitamin E can be quantified directly because their content levels are relative high and matrix interference is negligible; however, vitamin D is generally added in a small amount, has relatively low UV absorption, and suffers severe interference from the matrix, so liquid chromatography-mass spectrometry or semi-preparative normal-phase clean-up is specified for separation and assay of vitamin D in the current national standards.[2] However, the liquid chromatography-mass spectrometry is relatively expensive; while, the normal-phase purification is susceptible to mobile phase conditions and its process is relatively tedious and time-consuming.

This application is based on Waters' existing solutions of two-dimensional ultra-high performance liquid chromatography (UPLC 2D).[3] Using ACQUITY Arc (UHPLC) 2D technology under reversed-phase conditions, the separation and assay of vitamin A, α, β, γ, δ-tocopherol, vitamin D_2, and vitamin D_3 can be completed simultaneously with one sample injection, and the entire assay only takes 15 min. Vitamin A and α, β, γ, δ-tocopherol are separated and quantified on the 1^{st} dimension column, while vitamin D is heart-cut into the trap column after preliminary separation and clean-up on the 1^{st} dimension column, followed by transfer into the 2^{nd} dimension column for further separation and assay to achieve baseline separation of vitamin D_2 and D_3.

Through the matrix spiking tests done on actual samples provided by relevant companies and the analysis of QC samples, the results showed good linearity, excellent correlation coefficient and excellent repeatability. For five replicated injections, the RSD of retention time was <0.5%, the RSD of peak area was <2%. The limit of detection of vitamin D (D_2 and D_3) was as low as 0.5 ug/kg.

Experimental

LC conditions

Instrument system:	Waters ACQUITY Arc 2D system: QSM1 (quaternary pump 1) + QSM2 (quaternary pump 2) + FTN injector + Column Manager (CM-A) equipped with 2-position 6-port valve + 2998 PDA Detector
Mobile phase:	A: water; B: acetonitrile; C: methanol
1st dimension analytical column:	Poroshell 120 PFP (4.6 × 100 mm, 2.7 µm)
2nd dimension analytical column:	XSelect HSS C_{18}SB (3.0 × 150 mm, 3.5 µm)
Trap column:	XBridge BEH C_{18} Direct Connect HP Trap Column (2.1 mm × 30 mm, 10 µm)
Column temp.:	1st dimension column 35 °C, 2nd dimension column 40 °C
Detection wavelength:	1st dimension VA: 325 nm (0–5.5 min); VD:264 nm (5.5–6.8 min); VE:294 nm (6.8–10 min); 2nd dimension VD2 and VD3: 264 nm (10–15 min)
Injection volume:	10 µL

Gradient method: 1st dimension

Time	Flow rate (mL/min)	A (%)	B (%)	C (%)	Curve
0	1	20	0	80	6
9	1	0	0	100	6
10	1	0	0	100	6
11	1	20	0	80	6
15	1	20	0	80	6

2nd dimension

Time	Flow rate (mL/min)	A (%)	B (%)	C (%)	Curve
0	0.5	0	100	0	6
4	0.5	0	100	0	6
10	0.5	0	20	80	6
10.5	0.5	0	100	0	6
15	0.5	0	100	0	6

Valve switching time:

	0 min	5.74 min	6.04 min	8.5 min	15 min
Right valve position	2	1	2	2	2
Left valve position	1	1	1	2	1

Sample Preparation

In this application, the test samples were actual samples of milk powder, and sample supply and preparation were provided by our collaborating laboratory. The sample preparation steps were performed using the "GB 5009.82-2016 Assay of Vitamins A, D, and E in Food"2 as a guide. The specific process is as follows:

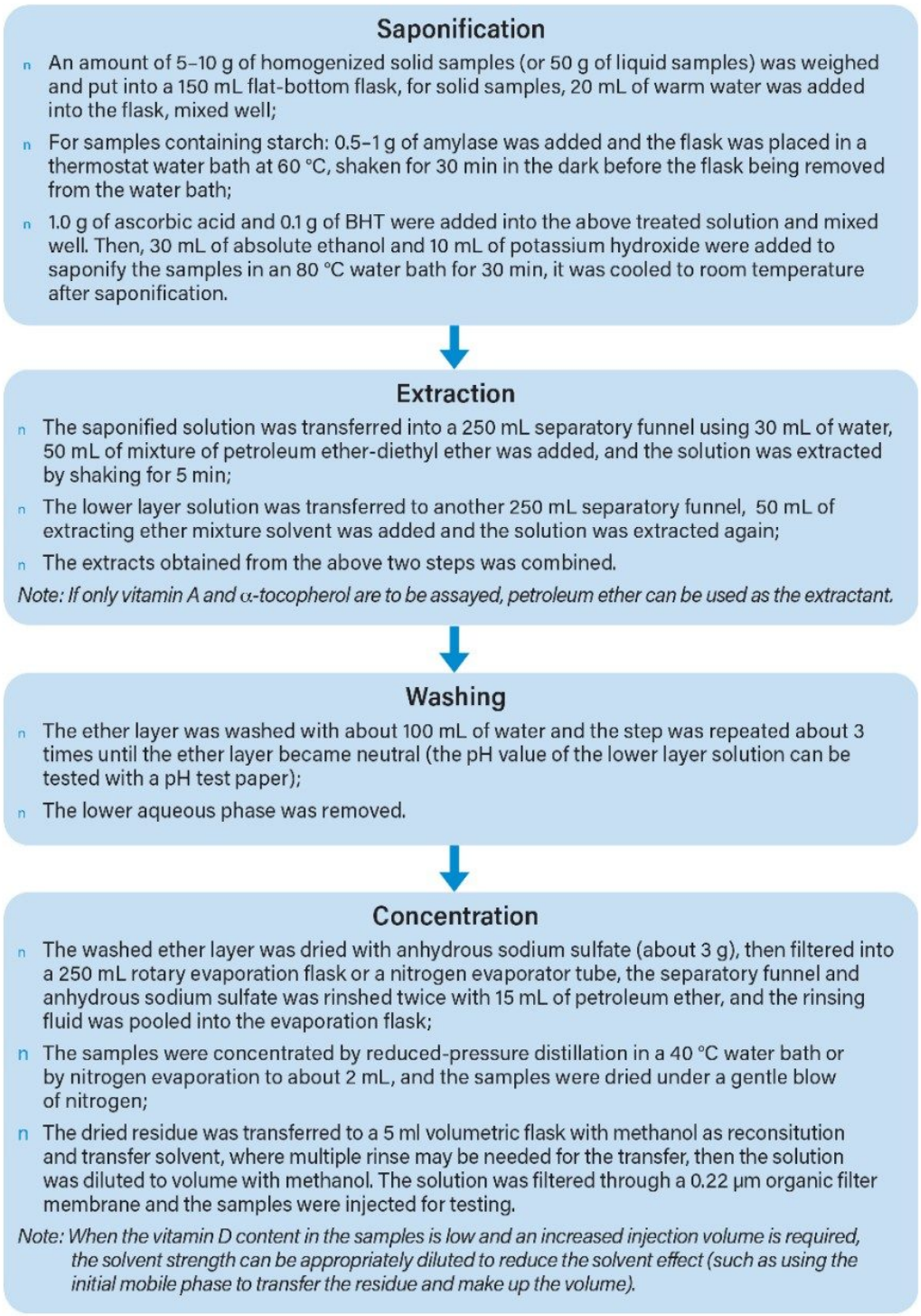

Figure 1. Sample preparation process.

Instrument Connection and Configuration

Vitamin A and vitamin E were measured on the 1st dimension LC, and vitamin D was measured on the 2nd dimension LC after heart-cutting. The key to the method is that the 1st dimension LC should be able to separate the vitamin A and vitamin E from the matrix inferference peaks, and it also should have excellent retention time repeatability to ensure the accuracy of heart-cutting with minimal cutting window. In order to more intuitively determine whether the cutting is accurate, and to allow a timely and convenient adjustment in the retention time when it is needed, two 2-position 6-port valves were used to connect a detector, so that both the 1st dimension and the 2nd dimension peaks were collected on the same chromatogram. The method was as follows: Vitamins A, D and E were passed through the 1st dimension column and the detector, and all peaks showed up. At the same time, vitamin D was subjected to heartcutting so that it was separated again on the 2nd dimension column and the peaks were detected again.

The specific configuration used in this application is shown in Figure 2.

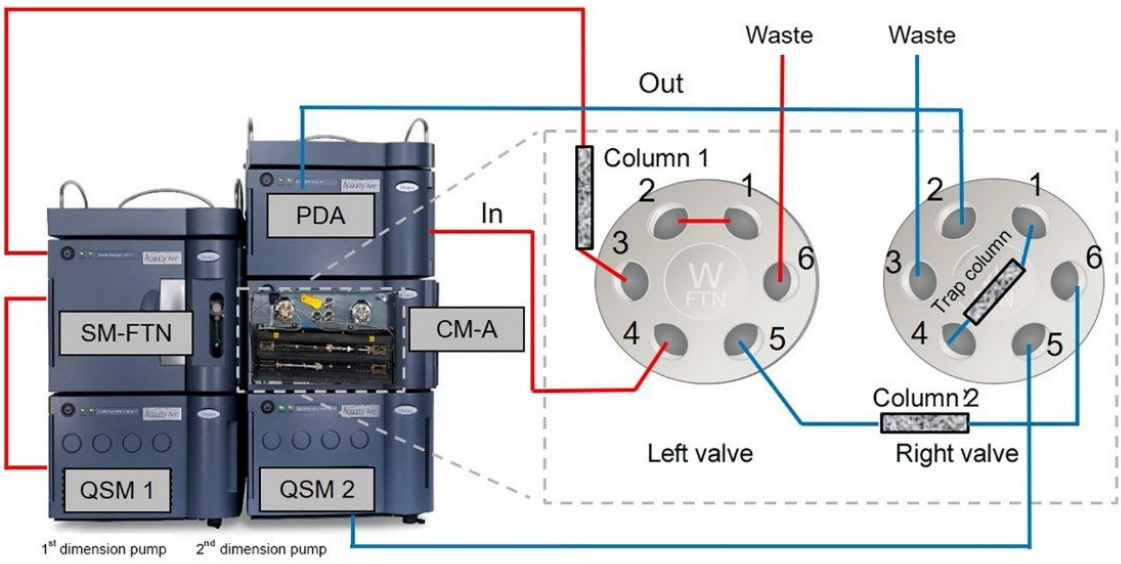

Figure 2. Connection and configuration of the heart-cutting system in the ACQUITY Arc 2D two-dimensional system.

Results and Discussion

Method Development

The goal of the 1st dimension separation is to ensure that the vitamin A and the four isoforms of vitamin E are separated and quantified without any interference from matrix. In addition, the vitamin D peak needs to be completely separated from the vitamin A and vitamin E with peak width as narrow as possible, so that it can be completely heart-cut and trapped without any extra amount of matrix background being also cut and transferred to the 2nd dimension, which could affect the further separation of vitamin D on the 2nd dimension LC. The

advantage of this method's connection and configuration is that by using only one detector, one can flexibly adjust the heart-cutting time window based on the retention time of vitamin D in different samples on the 1st dimension LC, so as to avoid any loss of the target substance during the heart-cut process. The goal of the 2nd dimension LC is to not only separate the vitamin D peak from matrix interferences, but also separate the vitamin D_2 and the vitamin D_3 at baseline resolution in order to achieve qualification and accurate quantitation purposes. Also, the vitamin D_2 and D_3 peaks should be as far away as possible from the pressure disturbing peaks that is caused by valve switching, as well as other major interfering peaks to ensure the accuracy of quantitation.

From the separation chromatogram of the standards in Figure 3 and the chromatogram of the actual spiked sample in Figure 4, it can be seen that good separation of vitamin A, four vitamin E isoforms and impurities can be achieved on the 1st dimension column, and good separation of two vitamin Ds and impurities can be achieved on the 2nd dimension column.

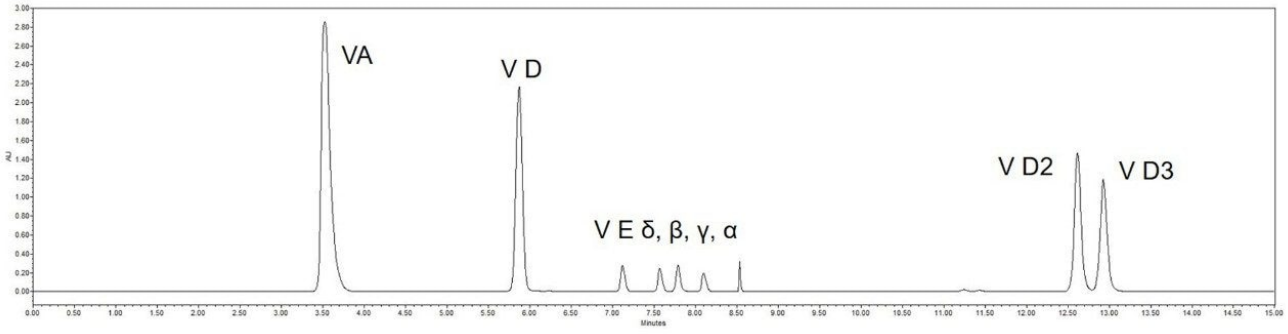

Figure 3. Chromatogram for the standard of vitamin A, four isoforms of vitamin E, vitamin D_2, and vitamin D_3.

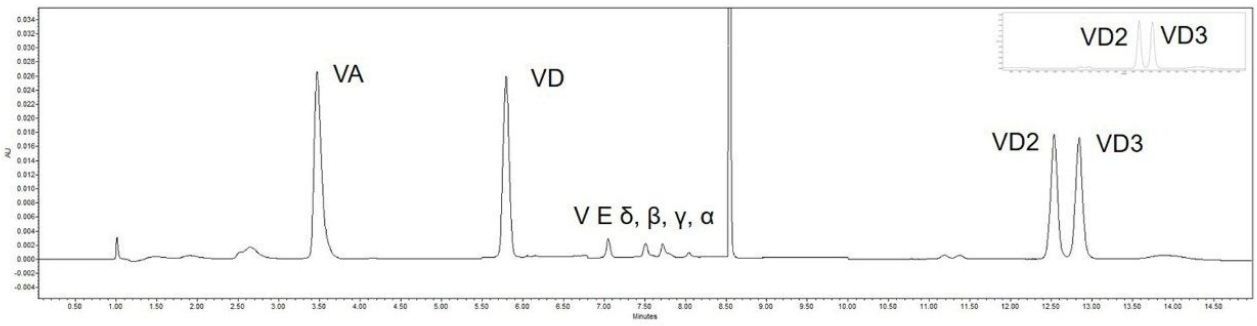

Figure 4. Chromatogram of the actual milk powder spiked sample.

Method Performance

Repeatability

The results of repeated injections (n = 5) of the standards at a 5 µg/L concentration are shown in Figure 5. The RSD for the retention time of vitamin A and four vitamin E isoforms on the 1st dimension LC and the RSD for the retention time of vitamin D_2 and vitamin D_3 on the 2nd dimension LC was all <0.5%, and the RSD for their peak area was <2%, which ensures the accuracy of the heart-cutting method.

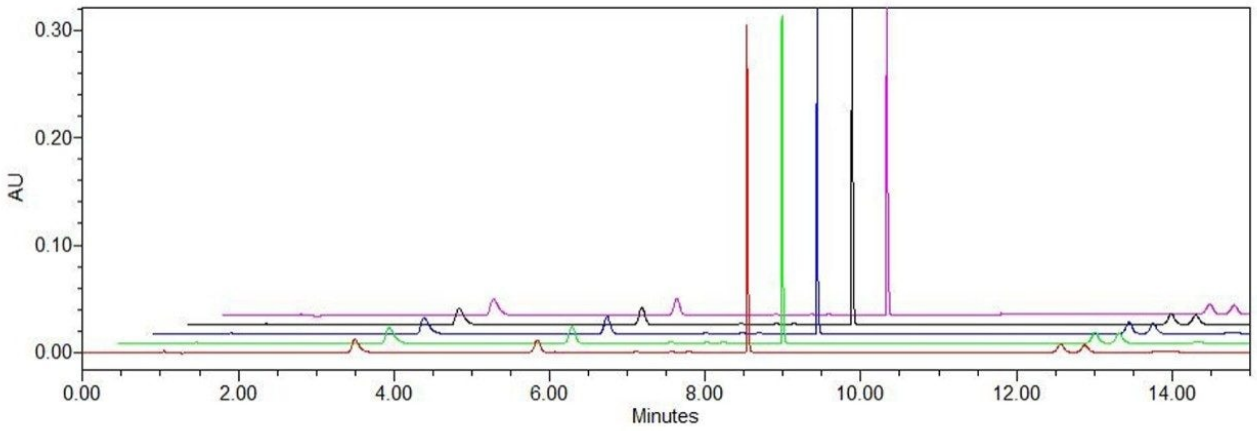

Figure 5. Chromatogram of repeated injections (n = 5) of the standard at a 5 µg/L concentration.

Linearity

In this application, the linearity of vitamin A, four vitamin E isoforms, vitamin D_2, and vitamin D_3 was examined on the basis of the requirements specified in the national standards. The results are shown in Figure 6. Different vitamins all maintained good linear relationships in different linear ranges, and the coefficient of determination R^2 was greater than 0.998.

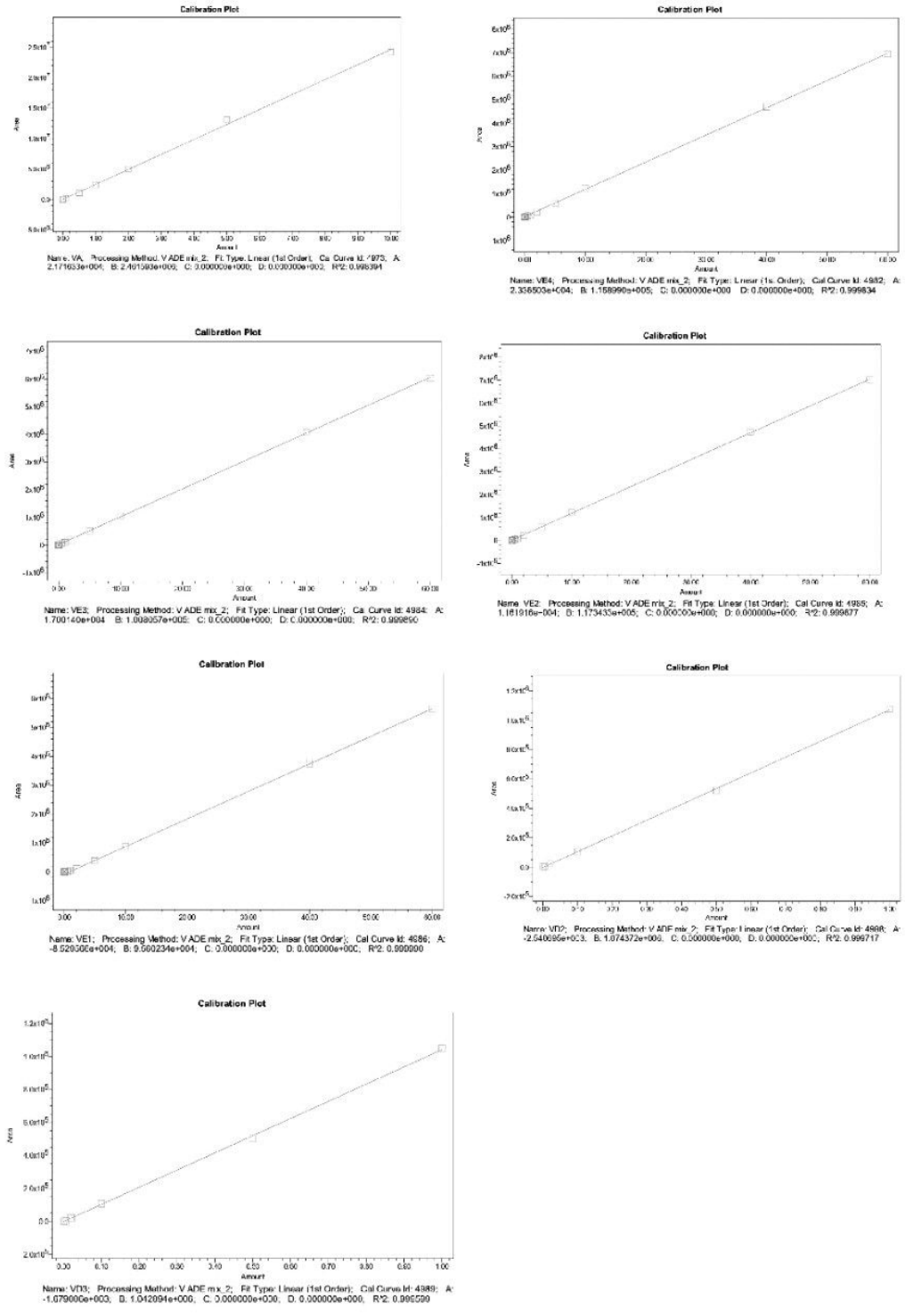

Figure 6. Standard curves in different linear ranges: Vitamin A (0.02–10 mg/L), four isoforms of vitamin E (2–60

mg/L), vitamin D_2 and vitamin D_3 (0.001–1 mg/L); the injection volume was 250 µL.

Limit Of Detection (LOD) and Limit Of Quantitation (LOQ)

In actual samples, the contents of vitamin A and vitamin E are generally high and can be accurately quantified, while the content of vitamin D is generally low and is prone to matrix interference, and hence the quantitation of vitamin D is relatively difficult. Therefore, the LOD of vitamin D is an important parameter in the evaluation of this instrument method. Detection sensitivity was investigated in this work. When the injection volume was 250 µL, the measured S/N ratio of vitamin D_2 and vitamin D_3 was 11 at an injection concentration of 0.5 µg/L, as shown in Figure 7. The refore, 0.5 µg/L was used as the LOQ of vitamin D. The LOD of vitamin D was calculated at 0.17 µg/L using a S/N of 3. This LOQ meets the assay requirement for the lowest concentration point of 50.0 µg/L on the standard curve in liquid chromatography methods specified in national standards. The LOD and LOQ results are shown in Table 1. Therefore, if the content of vitamin D in the sample is relatively high, an injection volume of 10 µL can be used to meet the requirements described in national standards.

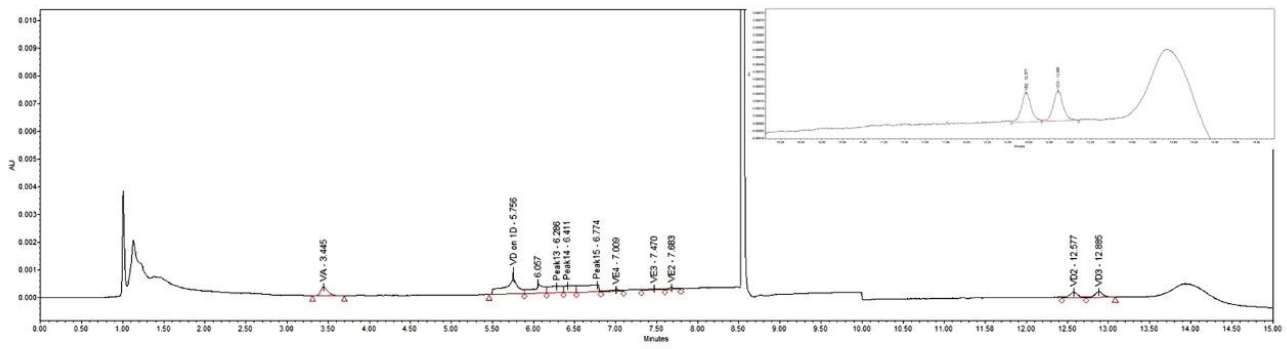

Figure 7. LOQ of vitamin D_2 and D_3 (0.5 µg/L).

Table 1. LOD and LOQ

	LOD (µg/L)	Milk powder sample (µg/100 g)	LOQ (µg/L)	Milk powder sample (µg/100 g)
Vitamin D_2	0.17	0.017	0.50	0.050
Vitamin D_3	0.17	0.017	0.50	0.050

Note: The sample size was 10 g.

Assay Of Actual Samples And Evaluation Of Matrix Effect

Using the method established in this application, milk powder samples provided by our clients were analyzed. Sample preparation was performed by the client according to the preparation method described in this document. The results show that the vitamins added to this batch of samples are mostly vitamin D_3 and α-tocopherol (Table 2).

The magnitude of the matrix effect has a direct impact on the quantitative results. This application also evaluated the matrix effect at the same time. The above samples provided by the client were also spiked and the test results were used as the basis for evaluating the matrix effect. The test results of the actual samples and the evaluation of the matrix effect are shown in Table 2.

Table 2. Test results of actual samples and the matrix effect

	Sample 1			Sample 2			Sample 3			Sample 4		
	Background concentration (mg/kg)	Spiked concentration (mg/kg)	Matrix effect (%)	Background concentration (mg/kg)	Spiked concentration (mg/kg)	Matrix effect (%)	Background concentration (mg/kg)	Spiked concentration (mg/kg)	Matrix effect (%)	Background concentration (mg/kg)	Spiked concentration (mg/kg)	Matrix effect (%)
Vitamin A	7.3	1.0	-18	8.2	1.0	-14	3.3	1.0	-5.0	9.5	1.0	-8.0
δ-tocopherol	ND	1.0	-17	5.6	1.0	-10	ND	1.0	-13	ND	1.0	-7.0
β-tocopherol	ND	1.0	-15	ND	1.0	-12	ND	1.0	-11	ND	1.0	-9.0
γ-tocopherol	ND	1.0	-10	3.2	1.0	-20	2.1	1.0	-18	ND	1.0	-7.0
α-tocopherol	29	1.0	-12	50	1.0	-19	49	1.0	-7.0	54	1.0	-8.0
Vitamin D_2	ND	1.0	-3.0	ND	1.0	-5.0	ND	1.0	-14	ND	1.0	-4.0
Vitamin D_3	0.067	1.0	-10	0.079	1.0	-13	1.2	1.0	-10	2.3	1.0	-9.0

Note: ND means not detected.

Conclusion

In this application, a fast analytical method is established based on the two-dimensional liquid chromatography to simultaneously assay the contents of vitamin A, α/β/γ/δ-tocopherol, vitamin D_2, and vitamin D_3 in infant milk powder. The method is simple, fast, highly automated, precise and accurate. It is suitable for the assay of vitamin A, α/β/γ/δ-tocopherol, vitamin D_2, and vitamin D_3 in formula milk powder or other milk products.

References

1. Mcmahon A, Christiansen S, Shinel, et al. *J AOAC* Int, 2013, 96 (5): 1073.

2. GB 5009.82-2016. Assay of Vitamins A, D, and E in Food.

3. ACQUITY UPLC Systems with 2D Technology Product Solution, 720003899EN

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720006655, March 2020