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## 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,  $\alpha/\beta/\gamma/\delta$ -tocopherol, vitamin D<sub>2</sub>, and vitamin D<sub>3</sub> in infant milk powder. The method is simple, fast, highly automated, precise and accurate. It is suitable for the assay of vitamin A,  $\alpha/\beta/\gamma/\delta$ -tocopherol, vitamin D<sub>2</sub>, and vitamin D<sub>3</sub> in formula milk powder or other milk products.

## Benefits

- The two-dimensional column switching ultraviolet detection method can separate vitamins A, D, and  $\alpha/\beta/\gamma/\delta$ -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<sub>2</sub> and D<sub>3</sub> peaks. This approach helps to eliminate co-elution, which reduces the interference from complex matrix for the vitamin D<sub>2</sub> and D<sub>3</sub> determination.

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## 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<sub>2</sub> (ergocalciferol) and vitamin D<sub>3</sub> (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  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -tocopherols. Among them,  $\alpha$ -tocopherol is usually singled out in food science as it has the highest activity and antioxidative and anti-aging properties.<sup>1</sup> 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 relatively 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.<sup>2</sup> 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).<sup>3</sup> Using ACQUITY Arc (UHPLC) 2D technology under reversed-phase conditions, the separation and assay of vitamin A,  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ -tocopherol, vitamin D<sub>2</sub>, and vitamin D<sub>3</sub> can be completed simultaneously with one sample injection, and the entire assay only takes 15 min. Vitamin A and  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ -tocopherol are separated and quantified on the 1<sup>st</sup> dimension column, while vitamin D is heart-cut into the trap column after preliminary separation and clean-up on the 1<sup>st</sup> dimension column, followed by transfer into the 2<sup>nd</sup> dimension column for further separation and assay to achieve baseline separation of vitamin D<sub>2</sub> and D<sub>3</sub>.

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<sub>2</sub> and D<sub>3</sub>) was as low as 0.5 ug/kg.

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## 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
1 <sup>st</sup> dimension analytical column:	Poroshell 120 PFP (4.6 x 100 mm, 2.7 $\mu$ m)
2 <sup>nd</sup> dimension analytical column:	XSelect HSS C <sub>18</sub> SB (3.0 x 150 mm, 3.5 $\mu$ m)
Trap column:	XBridge BEH C <sub>18</sub> Direct Connect HP Trap

Column (2.1 mm × 30 mm, 10 µm)

Column temp.: 1<sup>st</sup> dimension column 35 °C, 2<sup>nd</sup> dimension column 40 °C

Detection wavelength: 1<sup>st</sup> dimension VA: 325 nm (0–5.5 min); VD:264 nm (5.5–6.8 min); VE:294 nm (6.8–10 min); 2<sup>nd</sup> 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:

## Saponification

- An amount of 5–10 g of homogenized solid samples (or 50 g of liquid samples) was weighed and put into a 150 mL flat-bottom flask, for solid samples, 20 mL of warm water was added into the flask, mixed well;
- For samples containing starch: 0.5–1 g of amylase was added and the flask was placed in a thermostat water bath at 60 °C, shaken for 30 min in the dark before the flask being removed from the water bath;
- 1.0 g of ascorbic acid and 0.1 g of BHT were added into the above treated solution and mixed well. Then, 30 mL of absolute ethanol and 10 mL of potassium hydroxide were added to saponify the samples in an 80 °C water bath for 30 min, it was cooled to room temperature after saponification.



## Extraction

- The saponified solution was transferred into a 250 mL separatory funnel using 30 mL of water, 50 mL of mixture of petroleum ether-diethyl ether was added, and the solution was extracted by shaking for 5 min;
- The lower layer solution was transferred to another 250 mL separatory funnel, 50 mL of extracting ether mixture solvent was added and the solution was extracted again;
- The extracts obtained from the above two steps was combined.

*Note: If only vitamin A and  $\alpha$ -tocopherol are to be assayed, petroleum ether can be used as the extractant.*



## Washing

- The ether layer was washed with about 100 mL of water and the step was repeated about 3 times until the ether layer became neutral (the pH value of the lower layer solution can be tested with a pH test paper);
- The lower aqueous phase was removed.



## Concentration

- The washed ether layer was dried with anhydrous sodium sulfate (about 3 g), then filtered into a 250 mL rotary evaporation flask or a nitrogen evaporator tube, the separatory funnel and anhydrous sodium sulfate was rinsed twice with 15 mL of petroleum ether, and the rinsing fluid was pooled into the evaporation flask;
- The samples were concentrated by reduced-pressure distillation in a 40 °C water bath or by nitrogen evaporation to about 2 mL, and the samples were dried under a gentle blow of nitrogen;
- The dried residue was transferred to a 5 ml volumetric flask with methanol as reconstitution and transfer solvent, where multiple rinse may be needed for the transfer, then the solution was diluted to volume with methanol. The solution was filtered through a 0.22  $\mu$ m organic filter membrane and the samples were injected for testing.

*Note: When the vitamin D content in the samples is low and an increased injection volume is required, the solvent strength can be appropriately diluted to reduce the solvent effect (such as using the initial mobile phase to transfer the residue and make up the volume).*

Figure 1. Sample preparation process.

dimension LC after heart-cutting. The key to the method is that the 1<sup>st</sup> dimension LC should be able to separate the vitamin A and vitamin E from the matrix interference 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 1<sup>st</sup> dimension and the 2<sup>nd</sup> dimension peaks were collected on the same chromatogram. The method was as follows: Vitamins A, D and E were passed through the 1<sup>st</sup> 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 2<sup>nd</sup> dimension column and the peaks were detected again.

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

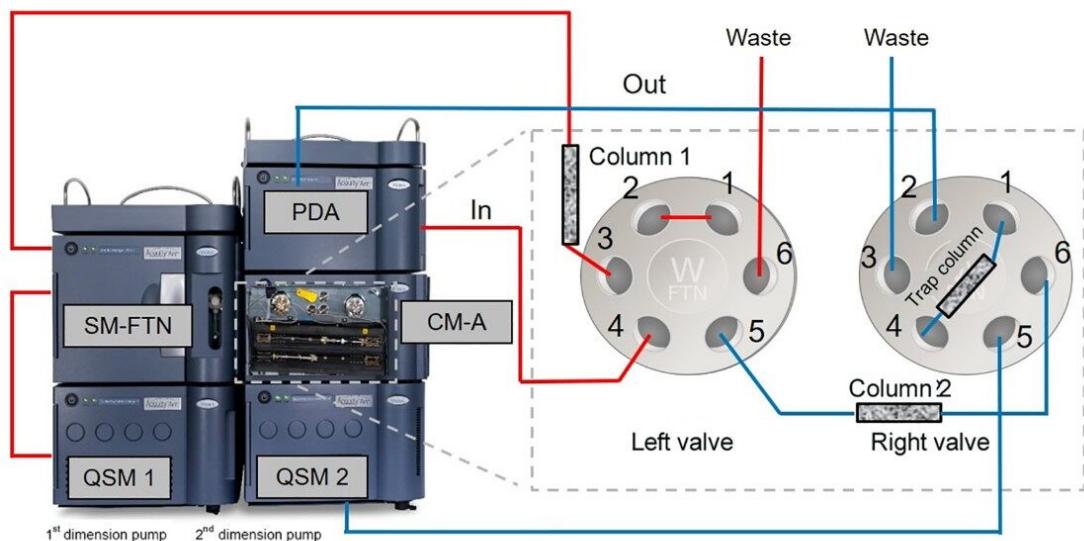


Figure 2. Connection and configuration of the heart-cutting system in the ACQUITY Arc 2D two-dimensional system.

## Results and Discussion

### Method Development

The goal of the 1<sup>st</sup> 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 2<sup>nd</sup> dimension, which could affect the further separation of vitamin D on the 2<sup>nd</sup> 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 1<sup>st</sup> dimension LC, so as to avoid any loss of the target substance during the heart-cut process. The goal of the 2<sup>nd</sup> dimension LC is to not only separate the vitamin D peak from matrix interferences, but also separate the vitamin D<sub>2</sub> and the vitamin D<sub>3</sub> at baseline resolution in order to achieve qualification and accurate quantitation purposes. Also, the vitamin D<sub>2</sub> and D<sub>3</sub> 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 1<sup>st</sup> dimension column, and good separation of two vitamin Ds and impurities can be achieved on the 2<sup>nd</sup> dimension column.

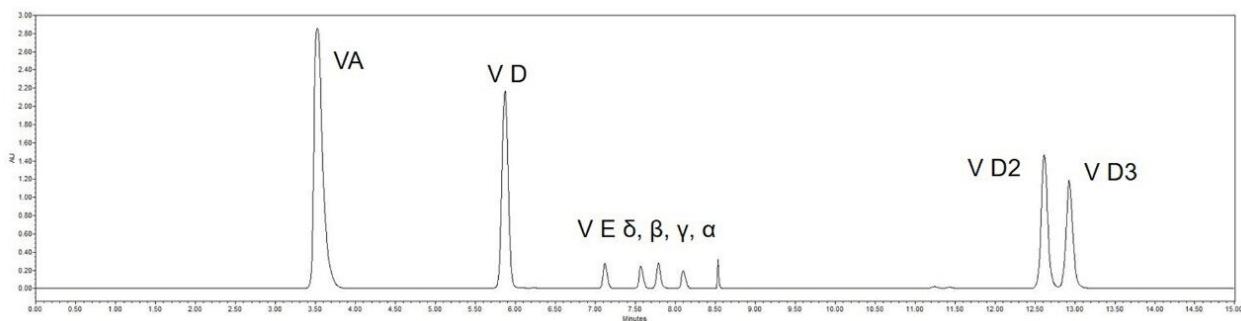


Figure 3. Chromatogram for the standard of vitamin A, four isoforms of vitamin E, vitamin D<sub>2</sub>, and vitamin D<sub>3</sub>.

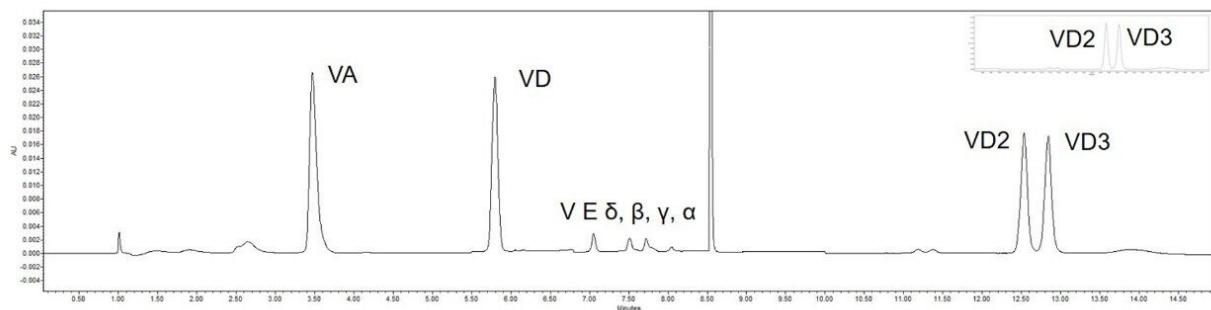


Figure 4. Chromatogram of the actual milk powder spiked sample.

## Method Performance

### Repeatability

The results of repeated injections ( $n = 5$ ) of the standards at a 5  $\mu\text{g/L}$  concentration are shown in Figure 5.

The RSD for the retention time of vitamin A and four vitamin E isoforms on the 1<sup>st</sup> dimension LC and the RSD for the retention time of vitamin D<sub>2</sub> and vitamin D<sub>3</sub> on the 2<sup>nd</sup> dimension LC was all <0.5%, and the RSD for their peak area was <2%, which ensures the accuracy of the heart-cutting method.

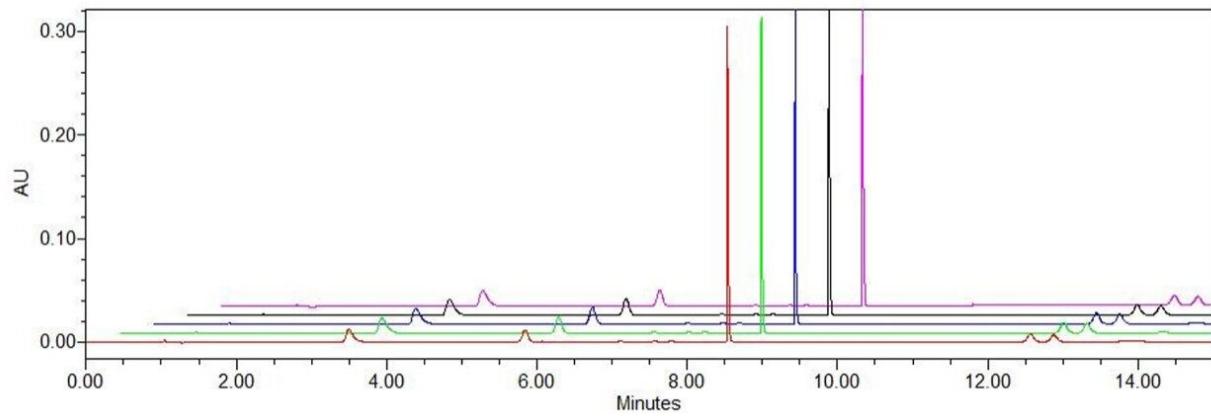


Figure 5. Chromatogram of repeated injections ( $n = 5$ ) of the standard at a 5  $\mu\text{g/L}$  concentration.

### Linearity

In this application, the linearity of vitamin A, four vitamin E isoforms, vitamin D<sub>2</sub>, and vitamin D<sub>3</sub> 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.

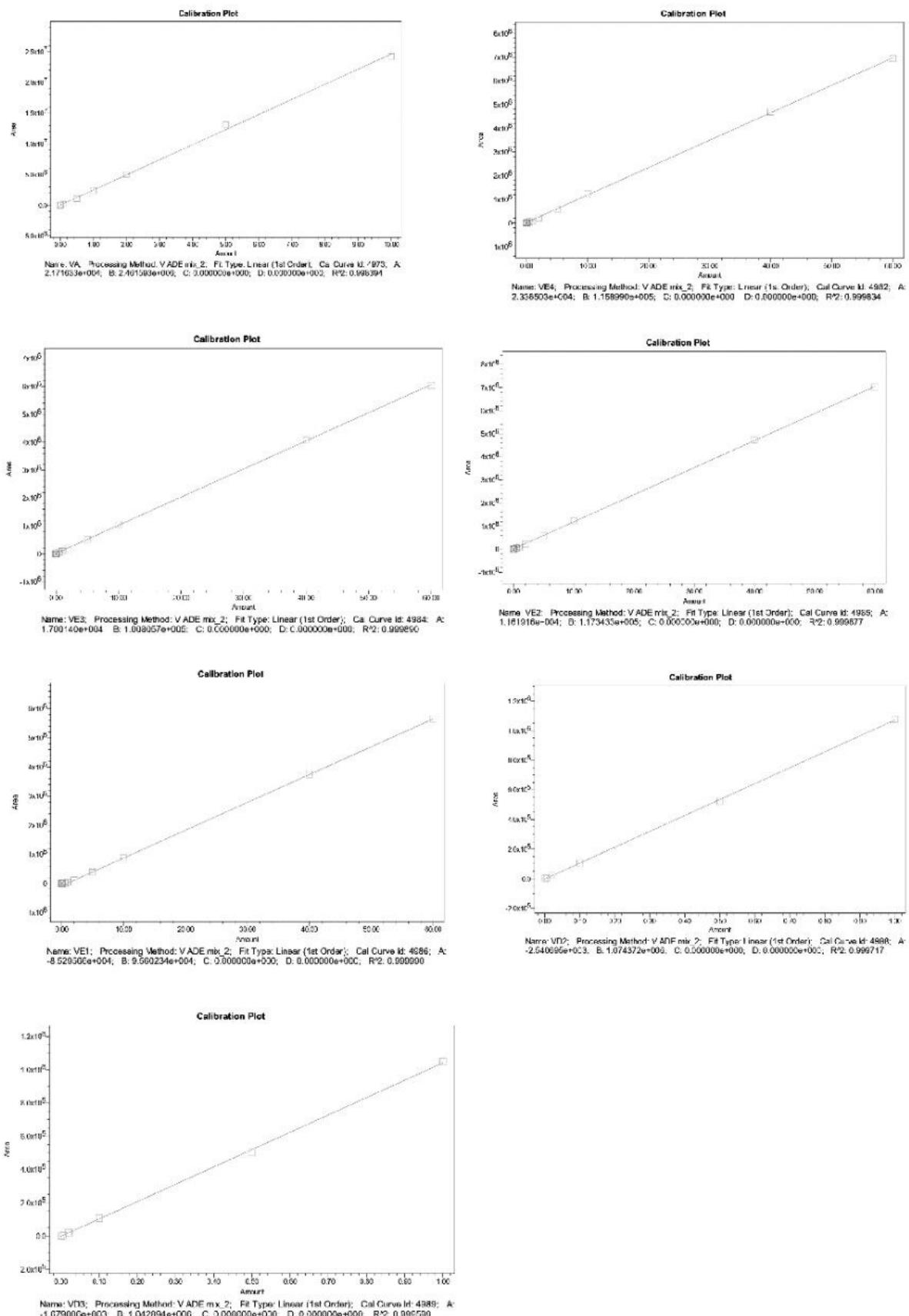


Figure 6. Standard curves in different linear ranges: Vitamin A (0.02–10 mg/L), four isoforms of vitamin E (2–

## 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.

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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## Featured Products

ACQUITY Arc System <<https://www.waters.com/134844390>>

Empower 3 Chromatography Data Software <<https://www.waters.com/10190669>>

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