APPLICATION BRIEF

Liquid Chromatography / Mass Spectrometry

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Analysis of Fat-Soluble Vitamins in Methylene Chloride Using Reversed Phase UHPLC-MS/MS

Introduction

Vitamins are micronutrients that are necessary in small amounts for various metabolic functions throughout the human body. Vitamins can be separated into two groups, water-soluble and fat-soluble. Fat-soluble vitamins (FSV), comprised mainly of vitamins A, D, E, and K, are stored in the liver and fatty tissue and are eliminated at a slower rate than the water-soluble vitamins. As such, they do not need to be replenished in the body as frequently. The recommended intake/tolerances of FSV in the body, as proposed by the FDA, are shown in Table 1[1].

Traditionally, due to their limited solubility, FS vitamins in food, food additives, and supplements have been primarily analyzed by normal phase liquid chromatography, often utilizing methylene chloride as a key organic solvent during sample preparation and subsequent chromatographic analysis. This approach can be undesirable due to the inherent hazards associated with the solvents used, as well as their high disposal costs.

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When approached using reversed phase liquid chromatography (RPLC), it can be challenging to solubilize FS vitamins into a suitable solvent. One must also consider that any additional sample preparation steps will increase analysis time. Additionally, when analyzing for FS vitamins by RPLC, as methylene chloride is often used as the sample diluent, it can be a significant challenge to avoid possible peak distortion of the early-eluting analytes due to breakthrough.

Table 1. Daily FS vitamin allowances, per FDA guideline.

Vitamin	DV (daily required value; mg; per FDA guideline[1])
A	5,000 IU
D	400 IU
E	30 IU
K	80 µg

With the above in mind, herein we present a method developed for the analysis of FSV by reversed phase UHPLC-MS/MS, with the analytes being dissolved/diluted in 100% methylene chloride.

Experimental

Solvents and Standards

All solvents, reagents, and diluents used were HPLC-grade or better.

For all dilutions, HPLC-grade methylene chloride was used.

The FSV standards, including vitamin A-acetate (retinol acetate), D3 (cholecalciferol), D2 (ergocalciferol), K2 (menaquinone), E (tocopherol), E-acetate (tocopheryl acetate), K1 (phylloquinone), and A-palmitate (retinol palmitate), were obtained from Sigma-Aldrich® Inc., Saint-Louis, MO. A 100-µg/mL FSV working standard was prepared using methylene chloride. To guard against possible standard instability, all stock and working standards were stored under refrigeration (4 °C) and only amber 2-mL LC vials were used.

Hardware/Software

For the chromatographic separations, a PerkinElmer UHPLC System was used with a PerkinElmer QSight™ 220 MS/MS detector. All instrument control, analysis and data processing was performed using the Simplicity 3Q™ software platform.

Method Parameters

The LC and MS/MS method parameters are shown in Tables 2, 3, and 4, respectively.

Table 2. LC Method Parameters.

Column	PerkinElmer Brownlee 4.6 x 100-mm C8 SPP, 2.7 µm (Part# N9308433)
Mobile Phase	Solvent A: Water with 0.1% formic acid Solvent B: Acetonitrile (ACN) with 0.1% formic acid
Analysis Time	16 min; re-equilibration time: 4 min
Oven Temp.	30 °C
Injection Volume	2 µL

Step	Time (min)	Flow rate (mL/min)	%A	%B	Curve
1	Initial	1.0	15.0	85.0	
2	2.00	1.0	15.0	85.0	6
3	9.00	1.0	0.0	100.0	6
4	15.90	1.0	0.0	100.0	6
5	15.95	1.0	15.0	85.0	6

Table 3. MS/MS Method Parameters.

Experiment Group	Vitamin	Q1 Mass	Q2 Mass	CE (V)	CCL2 (V)	Dwell Time (ms)
2.0 – 5.0 min	A-Acetate	269.5	91.1	-65	-84	100
7.0 – 8.5 min	D3	385.4	259.2	-28	-64	100
	D2	397.4	107.1	-50	-80	100
	K2	445.4	187.0	-34	-84	100
9.5 – 11.5 min	E	431.4	165.0	-33	-92	100
	E-Acetate	473.4	165.1	-54	-60	100
	K1	451.4	187.0	-34	-84	100
14.0 – 16.0 min	A-Palmitate	269.2	91.2	-60	-50	200

Table 4. MS Source Parameters.

Parameter	Setting
Drying Gas	120
HSID Temperature (°C)	320
Nebulizer Gas	160
Electrospray Voltage (V)	5500
Source Temperature (°C)	450

Results and Discussion

As shown in Figure 1, good chromatographic separation of the eight vitamins was achieved in less than 15 minutes and, per Figure 2, the 5-replicate overlays demonstrate excellent chromatographic reproducibility. For three sets of 10-replicate injections of the 100 µg/mL FSV standard prepared in methylene chloride, %RSD's of less than 3% were achieved for all peak areas (Table 5). This demonstrates excellent reproducibility from injection to injection.

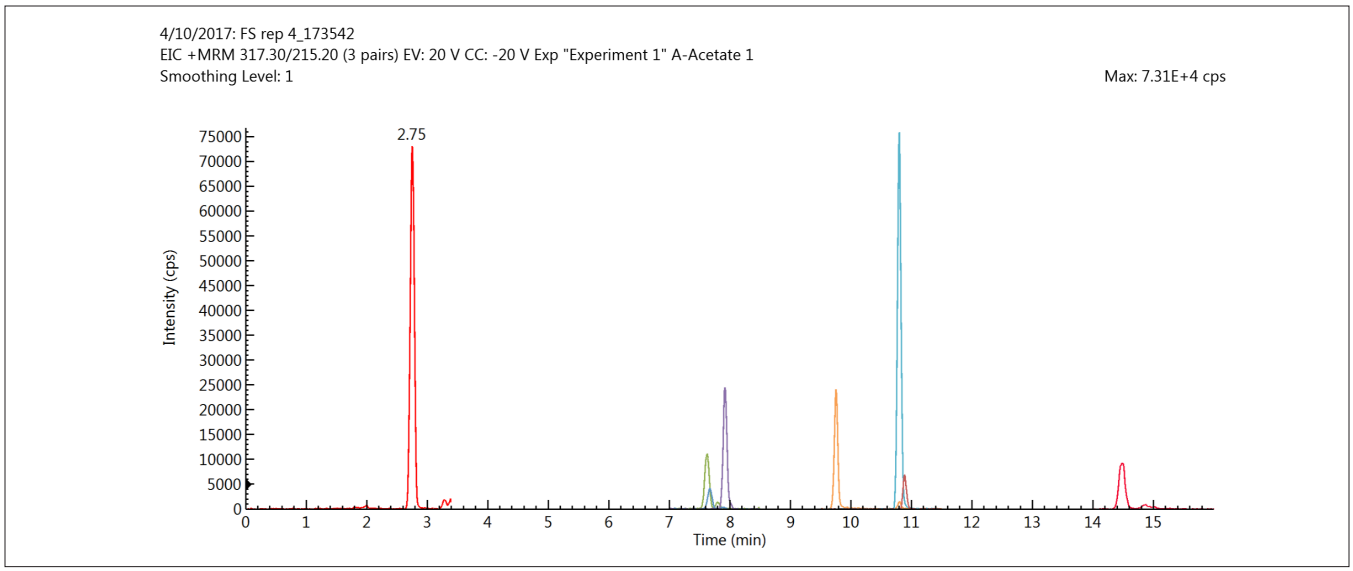

Figure 1. 2-ppm mix of eight FSV standards in methylene chloride.

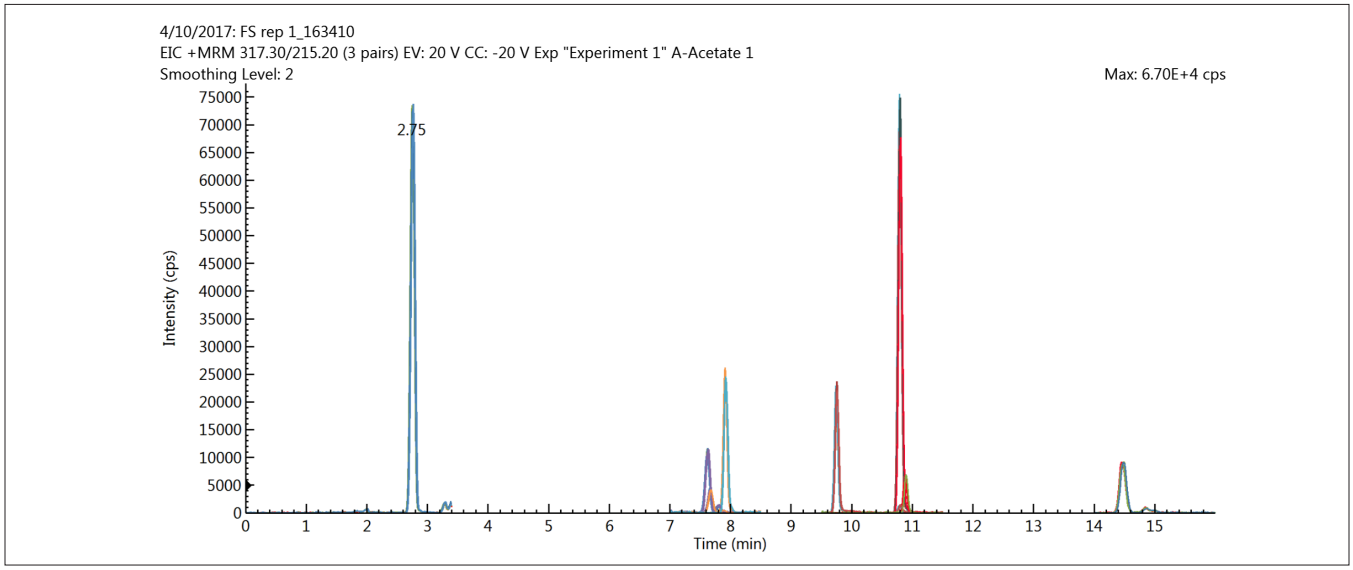

Figure 2. Overlay of five replicate injections of 2-ppm mix of eight FSV standards in methylene chloride.

Table 5. %RSD values for the peak areas of three sets of 10-replicate injections of 100 ppm mix of eight FSV standards in methylene chloride.

	A Acetate	D3	D2	K2	E	E-Acetate	K1	A-Palmitate
Area %RSD (Set 1, n=10)	1.83	1.96	2.04	1.24	2.30	2.17	2.55	2.66
Area %RSD (Set 2, n=10)	2.51	1.84	2.80	2.60	2.94	1.74	2.78	2.23
Area %RSD (Set 3, n=10)	2.78	0.90	1.75	1.81	1.51	1.88	1.34	1.40

Conclusions

By reducing the LC injection volume and increasing the internal diameter of the column used, reverse phase chromatography can be applied directly to the analysis of fat-soluble vitamins prepared in methylene chloride, without the loss of chromatographic performance due to peak distortions of early eluting analytes resulting from breakthrough. Area % RSD's of less than 3% were obtained, demonstrating excellent method reproducibility. The results support the applicability of the prescribed method for the analysis of fat-soluble vitamins by reversed phase UHPLC-MS/MS.

Reference

1. U.S. Food and Drug Administration (FDA), Guidance for Industry: A Food Labeling Guide (14. Appendix F), http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/labelingnutrition/ucm064928.htm

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