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Application News

No. C191

Supercritical Fluid Chromatography

Analysis of Fat-soluble Vitamins Using the Nexera™ UC Supercritical Fluid Chromatograph

Vitamins are essential micronutrients to maintain the normal functioning of organisms, but they must be ingested from food because they are not synthesized sufficiently in the body. Deficiencies of vitamins in the body cause symptoms such as disease and growth disorders. Vitamin A is associated with night blindness and skin abnormalities, vitamin D with rickets and osteomalacia, vitamin E with anemia and blood circulation disorders, and vitamin K with hemorrhaging, osteoporosis, etc. Therefore, it is very important to accurately analyze the fat-soluble vitamins in foods and supplements. The fat-soluble vitamins are broadly classified into vitamin A, vitamin D, vitamin E, and vitamin K according to their chemical structure and physiological effects. Due to the large number of isomers, good separation is required for fat-soluble vitamin analysis.

Here we introduce an example of a simultaneous analysis of fat-soluble vitamins using a supercritical fluid chromatograph (SFC) and triple quadrupole mass spectrometer for detection.

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Target Components and Analysis Conditions

Table 1 lists fat-soluble vitamins and related substances targeted in this study, and the MRM conditions for MS. In addition, the analysis conditions for SFC are summarized in Table 2. Fig. 1 shows a chromatogram obtained from the analysis of standard samples in which the concentration of each fat-soluble vitamin is 1 µmol/L.

Table 1 Target Components and MRM Conditions

No.	Compounds	MRM Transition		Classification
1	Retinol	ESI (+)	269.30>93.00	Vitamin A
2	Retinal	ESI (+)	285.20>161.00	
3	Retinoate	ESI (+)	301.20>122.90	
4	beta-Carotene	ESI (+)	536.60>444.00	Precursor of Vitamin A
5	Ergocalciferol (Vitamin D2)	ESI (+)	397.50>107.10	Vitamin D
6	Cholecalciferol (Vitamin D3)	ESI (+)	384.80>367.10	
7	Calcidiol (25(OH)D3)	ESI (+)	401.40>383.00	
8	Calcitriol (1,25-(OH)2D3)	ESI (+)	399.30>398.80	
9	25-Hydroxyergocalciferol (25(OH) D2)	ESI (+)	412.80>395.00	
10	1-alpha-25-Dihydroxyergocalciferol (1,25-(OH)2D2)	ESI (+)	411.40>134.80	
11	alpha-Tocopherol	ESI (+)	430.80>164.80	Vitamin E
12	beta-Tocopherol	ESI (+)	416.80>151.00	
13	gamma-Tocopherol	ESI (+)	416.80>150.80	
14	delta-Tocopherol	ESI (+)	402.80>136.80	
15	alpha-Tocotrienol	ESI (+)	424.80>164.80	
16	gamma-Tocotrienol	ESI (+)	410.80>150.80	
17	delta-Tocotrienol	ESI (+)	396.80>136.80	
18	alpha-Tocopherolquinone	ESI (+)	429.20>165.15	
19	Phylloquinone (Vitamin K1)	ESI (+)	451.40>186.80	Vitamin K
20	Menaquinone (Vitamin K2)	ESI (+)	445.30>186.80	

Table 2 Analysis Conditions

Column	:	Shim-pack™ UC-RP (150 mm L. × 2.1 mm I.D., 3 µm)
Mobile phase	:	A; CO_2 B; 0.1 % (w/v) Ammonium formate in methanol
Gradient	:	B.conc. 5 % (0 min) - 10 % (6 min) – 30 % (10 min) – 50 % (10.1-15 min) - 5 % (15.1-18 min)
Flow rate	:	0.8 mL/min
Column temp.	:	40 °C
BPR pressure	:	10 MPa
Detector	:	LCMS™-8050 (ESI, MRM mode)
Makeup	:	0.1 % (w/v) Ammonium formate in methanol
Makeup flow rate	:	0.05 mL/min
Injection vol.	:	1 µL

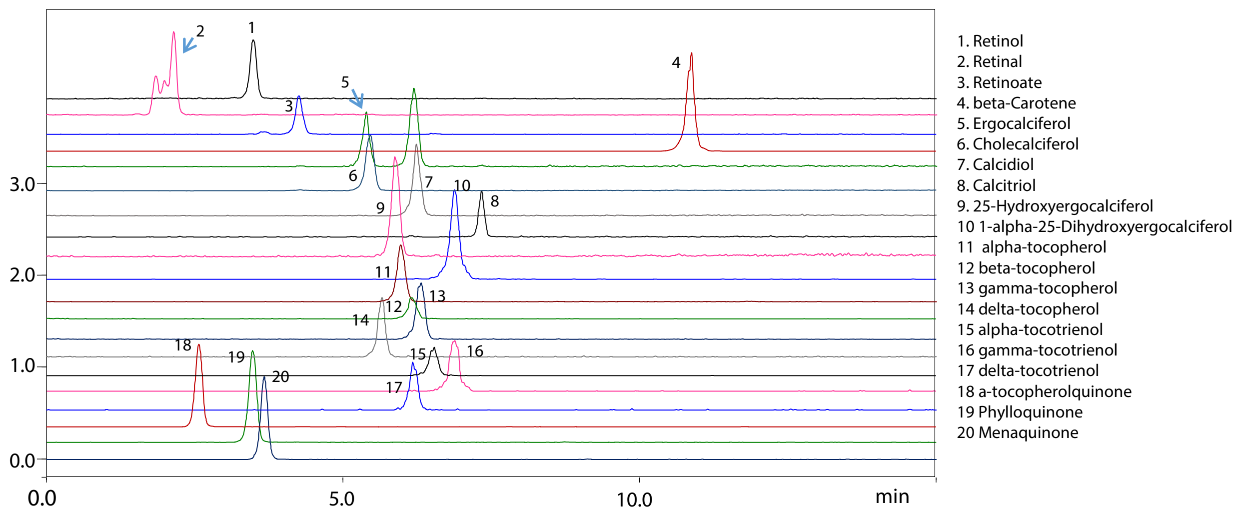

Fig. 1 Chromatogram for Standard Solutions (1 µmol/L)

Calibration Range and Sensitivity

Table 3 gives the information on the calibration curves and limits of detection obtained by analyzing standard samples in which the concentration of each fat-soluble vitamin was 0.01 to 10 µmol/L.

Table 3 Linearity and Limits of Detection

Compounds	Concentration (µmol/L)	R^2	LOD (nmol/L)
Retinol	0.01-10	0.9997	20
Retinal	0.01-10	0.9993	9.1
Retinoate	0.01-10	0.9964	14
beta-Carotene	0.01-10	0.9986	0.40
Ergocalciferol	0.05-10	0.9993	47
Cholecalciferol	0.01-10	0.9953	15
Calcidiol	0.01-10	1.0000	24
Calcitriol	0.05-10	0.9988	25
25-Hydroxyergocalciferol	0.01-10	0.9988	17
1-alpha-25-Dihydroxyergocalciferol	0.01-10	0.9995	0.80
alpha-Tocopherol	0.01-10	0.995	2.2
beta-Tocopherol	0.01-10	0.9973	16
gamma-Tocopherol	0.01-10	0.9992	32
delta-Tocopherol	0.02-10	0.9988	12
alpha-Tocotrienol	0.01-5	0.9988	1.1
gamma-Tocotrienol	0.02-10	0.9999	8.3
delta-Tocotrienol	0.01-10	0.9983	4.3
alpha-Tocopherolquinone	0.01-10	0.9971	0.90
Phylloquinone	0.01-10	0.9991	2.3
Menaquinone	0.01-10	0.9983	4.8

* beta-Carotene only: secondary approximation

Repeatability

Table 4 gives the repeatability of the retention time and peak area of each fat-soluble vitamin, which was obtained by analyzing the 1 µmol/L standard samples six times.

Table 4 Repeatability

Compounds	Retention Time		Peak Area	
	(min)	RSD (%)	Average	RSD (%)
Retinol	3.485	0.112	512700	3.72
Retinal	2.149	0.365	730359	3.99
Retinoate	4.270	0.307	374720	2.71
beta-Carotene	10.882	0.114	15022154	2.74
Ergocalciferol	5.376	0.357	572484	3.24
Cholecalciferol	5.459	0.239	2113522	3.22
Calcidiol	6.237	0.0880	1898273	3.84
Calcitriol	7.324	0.202	378473	3.74
25-Hydroxyergocalciferol	5.902	0.219	1076576	3.36
1-alpha-25-Dihydroxyergocalciferol	6.883	0.197	2088882	4.83
alpha-Tocopherol	5.979	0.240	1137057	3.92
beta-Tocopherol	6.174	0.0820	283144	4.32
gamma-Tocopherol	6.308	0.138	411577	5.94
delta-Tocopherol	5.646	0.247	205092	2.24
alpha-Tocotrienol	6.531	0.394	343701	3.18
gamma-Tocotrienol	6.882	0.264	298561	3.62
delta-Tocotrienol	6.182	0.467	192646	2.61
alpha-Tocopherolquinone	2.583	0.371	7867923	3.94
Phylloquinone	3.485	0.300	2055829	3.76
Menaquinone	3.678	0.336	1637582	2.17

Analysis of Fat-soluble Vitamins in Supplements

Fig. 2 shows the chromatogram obtained from the analysis of the supplement extract containing vitamin D and vitamin K. The supplement was crushed, subjected to extraction with n-hexane, centrifugation and then filtration with a membrane filter. Cholecalciferol and menaquinone were detected in the same sample. Table 5 shows the results of spiking the extract with the equivalent of 1 µmol/L of cholecalciferol and menaquinone and checking the recovery rate. A good recovery rate was obtained with both components.

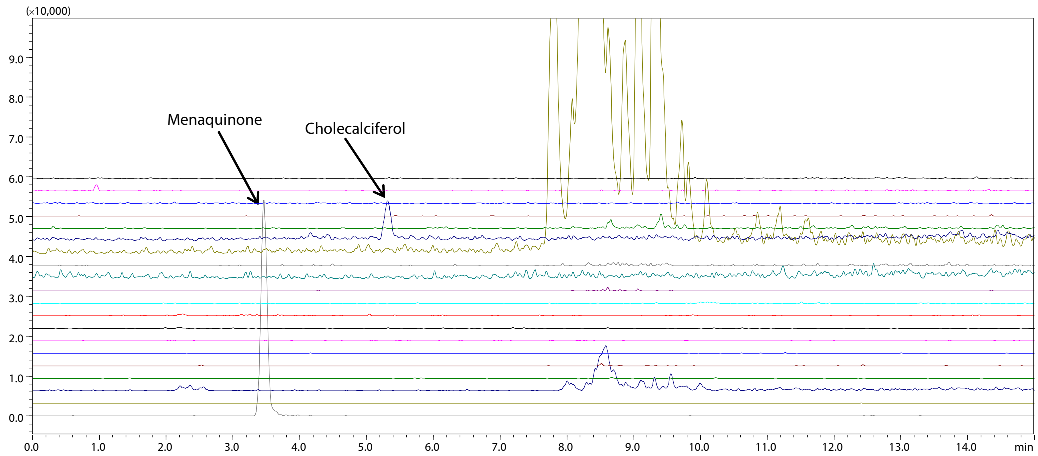

Fig. 2 Chromatogram for Supplement Extracts

Table 5 Spike-and-Recovery Test Results

Component		Cholecalciferol	Menaquinone
Concentration (µmol/L)	No spike	0.05	0.116
	1 µmol/L equivalent added	1.109	1.062
Recovery rate (%)		106	94.6

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