Application Note

Food Testing

Agilent Trusted Answers

Simultaneous Detection and Quantitation of 14 Fat-Soluble Vitamins and Carotenoids by LC/MS/MS Triple-Quadrupole

Author

Hui Zhao
Agilent Technologies, Inc.

Abstract

This application note describes a method for the simultaneous determination of 14 fat-soluble vitamins and carotenoids in a dietary supplement using an Agilent 1290 Infinity II LC coupled to an Agilent 6470 triple quadrupole LC/MS with Agilent MassHunter Workstation software. The method was used to quantify the fat-soluble vitamins and selected carotenoids in a highly complex multivitamin tablet matrix. All tested ingredients met the claimed concentrations. It was concluded that the method can be used for quality control and establishment of the nutrition labels for fat-soluble vitamins and selected carotenoid-containing supplement products.

Introduction

Fat-soluble vitamins and carotenoids have free radical-scavenging properties that allow them to function as antioxidants.[1,2] The importance of the fat-soluble vitamins (A, D, E, and K) and carotenoids in nutrition is well recognized. Supplements that contain fat-soluble vitamins are often consumed to ensure adequate vitamin intake, and are available in various forms such as tablets, capsules, gummies, softgels, and drinks. Accurate quantitative measurements for fat-soluble vitamins and carotenoids are required for a variety of reasons; these include ensuring product quality, confirming regulatory compliance, identifying possible degradation (by processing of the supplement encapsulation), and evaluating composition quantities matching the claims.

Fat-soluble vitamins can be found with free forms, and their ester forms. The esters of vitamin A and vitamin E are often fortified in supplements due to their increased stability. In this study, the fat-soluble vitamins vitamin A (retinol, retinyl acetate, and retinyl palmitate), vitamin D (cholecalciferol (D3) and ergocalciferol (D2)), vitamin E (*alpha*-tocopherol, *alpha*-tocopherol acetate and *alpha*-tocopherol succinate), vitamin K (phytonadione (K1), menaquinone (K2-4), and menaquinone (K2-7)) and selected carotenoids including beta carotene, lutein, and lycopene were analyzed in a single method. The structures of fat-soluble vitamins and carotenoids are given in Figure 1.

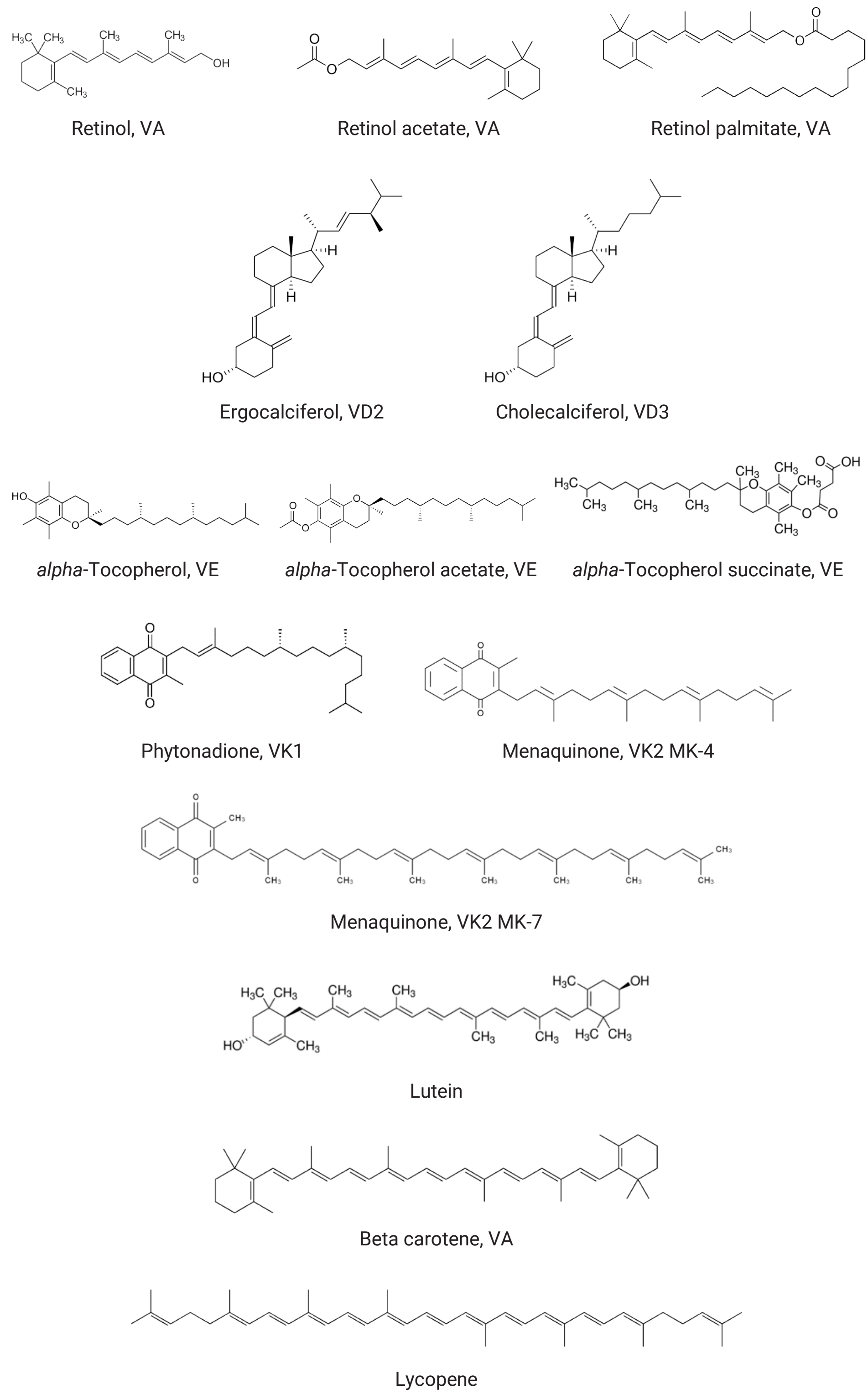

Figure 1. Structure of fat-soluble vitamins.

Traditional methods include microbiological methods, which have significant shortcomings for accuracy, specificity, and throughput; and LC-UV methods, which have poor sensitivity for low-level vitamins (such as vitamin D and K), and poor selectivity within complex matrices. These methods often involve multiple assays to quantify all fat-soluble vitamins and carotenoids. The problem of sensitivity, selectivity, and accuracy can be solved by usage of triple-quadruple MS detection. However, few LC/MS/MS methods are available for the simultaneous analysis of carotenoids and fat-soluble vitamins in supplements.

In this study, a unique LC/TQ method was developed and evaluated to provide fast and sensitive identification and accurate quantification of fat-soluble vitamins and selected carotenoids in a complex multivitamin tablet matrix. All 14 fat-soluble vitamins and carotenoids were analyzed simultaneously in a single run. The postextraction matrix-matched standard was included to compensate for matrix effects. Method criteria for data acceptance were established.

Experimental

Equipment

All experiments in this study were performed using an Agilent 1290 Infinity II LC consisting of an Agilent 1290 Infinity II multisampler (G7167B), an Agilent 1290 Infinity II high speed pump (G7120A), and an Agilent 1290 Infinity II multicolumn thermostat (G7116B) coupled to an Agilent 6470 triple quadrupole LC/MS (G6470B). The system was controlled by Agilent MassHunter acquisition software, version 10.1. Data processing was performed with MassHunter Quantitative Analysis software (version 10.2) and MassHunter Qualitative Analysis software (version 10.0).

Chromatographic conditions

Parameter	Setting
Analytical Column	Agilent InfinityLab Poroshell 120 Stable Bond-Aqueous, 2.1 × 150 mm, 2.7 µm (p/n 683775-914)
Column Oven	45 ±2 °C
Injection Volume	1 µL
Run Time	12 min
Autosampler	5 ±2 °C
Mobile Phase A	0.1% formic acid in water
Mobile Phase B	0.1% formic acid in methanol
Needle Wash	0.1% formic acid in acetonitrile
Gradient	Time (min) / Flow (mL/min) / %A / %B 0 / 0.25 / 20 / 80 7.0 / 0.25 / 0 / 100 9.5 / 0.25 / 0 / 100 10.0 / 0.25 / 20 / 80 12.0 / 0.25 / 20 / 80

MS parameters

Parameter	Setting
MS Acquisition	dMRM
Ion Source Type	Agilent Jet Stream Electrospray ionization (AJS ESI positive)
Drying Gas Temperature	250 °C
Drying Gas Flow	11 L/min
Nebulizer	40 psi
Sheath Gas Heater	350 °C
Sheath Gas Flow	12 L/min
Capillary	4,000 V
Nozzle Voltage	1,000 V
Precursor Ion and Production Ion Resolution	Unit
Compound-Specific Conditions	See Table 1

Sample and standards

The study matrix is a complex Multivitamin tablet with food-based blends. The standards of retinol, retinyl acetate, retinyl palmitate, cholecalciferol, ergocalciferol, alpha-tocopherol, *alpha*-tocopherol acetate, *alpha*-tocopherol succinate, phytonadione, menaquinone (K2-4), menaquinone (K2-7), and beta carotene were obtained from Millipore Sigma, Inc. (St. Louis, MO, USA); lutein and lycopene were obtained from Chromadex, Inc. (Los Angeles, CA, USA); cholecalciferol-D_6 and ergocalciferol-D_3 solutions (100 µg/mL) were obtained from IsoSciences (West Chester, PA, USA).

The individual analyte and internal standard stock solutions were made at concentrations of 100 µg/mL in ethanol and took purity into account. The sample and standards were stored at –20 °C.

Method

Sample preparation

Below is the detailed description of the optimized sample preparation protocol followed in this study. Table 1 gives the analyte-specific LC/MS/MS conditions.

1. Determine the average weight of the tablet using an analytical balance. Grind the sample into a fine powder to form a homogeneous mixture.
2. Weigh out a 1 g sample into a 20 mL glass scintillation vial.
3. Add 5 mL DMSO to the vial and vortex for 1 to 2 minutes.
4. Heat the vial to 50 to 60 °C in a water bath for 5 minutes.
5. Add 15 mL ethanol and shake for 15 minutes.
6. Draw approximately 1.5 mL of sample extract into a 2 mL microcentrifuge tube.
7. Centrifuge the sample extract for 5 minutes at 13,000 rpm.
8. Dilute the supernatants with ethanol, as needed.
9. Prepare the postspiked samples along with the diluted samples.
10. Inject samples into LC/MS/MS using positive ESI mode for analysis.

Method evaluation procedure

The method performance was evaluated by analyzing a complex multivitamin tablet sample. The quantitation was performed using isotopically labeled internal calibration curves with 1/x weighting, or external calibration curves with 1/x weighting and single point postmatrix spike correction.

Table 1. Analyte-specific LC/MS conditions: precursor to product ion transitions, fragmentor, collision energies (CE), cell accelerator voltage (CAV), and retention times (RT).

Compound Group	Compound Name	Precursor Ion (*m/z*)	Product Ion (*m/z*)	RT (min)	Delta RT (min)	Fragmentor (V)	CE (V)	CAV (V)
Carotenoids, VA	Beta carotene	537.5	445.1	8.60	1.5	144	12	5
Carotenoids, VA	Beta carotene	537.5	104.8	8.60	1.5	144	68	5
Carotenoids, VA	Beta carotene	537.5	91.0	8.60	1.5	144	80	5
Carotenoids, VA	Beta carotene	537.5	119.2	8.59	1.5	144	52	5
Carotenoids	Lutein	569.2	171.0	6.80	1	160	40	5
Carotenoids	Lutein	569.2	339.1	6.80	1	160	20	5
Carotenoids	Lutein	569.2	477.2	6.80	1	160	15	5
Carotenoids	Lycopene	537.5	69.1	9.25	1.5	140	45	5
Carotenoids	Lycopene	537.5	144.8	9.25	1.5	140	35	5
Carotenoids	Lycopene	537.5	334.0	9.25	1.5	140	10	5
VA	Retinol	287.2	41.2	3.03	1.5	104	60	5
VA	Retinol	287.2	69.1	3.04	1.5	104	28	5
VA	Retinol	287.2	81.1	3.03	1.5	104	24	5
VA	Retinol	287.2	55.1	3.03	1.5	104	48	5
VA	Retinol Acetate	329.2	269.2	3.83	1.5	104	8	5
VA	Retinol Acetate	329.2	121.1	3.84	1.5	104	28	5
VA	Retinol Acetate	329.2	202.9	3.83	1.5	104	48	5
VA	Retinol Acetate	329.2	191.7	3.83	1.5	104	56	5
VA	Retinyl Palmitate	525.5	269.1	7.74	1	128	12	5
VA	Retinyl Palmitate	525.5	145.0	7.74	1	128	20	5
VA	Retinyl Palmitate	525.5	105.1	7.72	1	128	56	5
VA	Retinyl Palmitate	525.5	144.1	7.75	1	128	20	5
VD	Cholecalciferol	385.1	107.1	5.33	1	104	28	5
VD	Cholecalciferol	385.1	105.1	5.33	1	104	56	5
VD	Cholecalciferol	385.1	81.1	5.33	1	104	44	5
VD	Cholecalciferol	385.1	159.1	5.33	1	104	25	5
VD	Cholecalciferol	385.1	259.1	5.33	1	104	25	5
VD	Ergocalciferol	397.1	69.1	5.43	1	104	36	5
VD	Ergocalciferol	397.1	107.1	5.43	1	104	28	5
VD	Ergocalciferol	397.1	105.1	5.43	1	104	56	5
VD	Ergocalciferol	397.1	41.2	5.43	1	104	60	5
VD	VD2 IS	400.1	69.1	5.43	1	104	40	5
VD	VD3 IS	391.1	105.0	5.32	1	128	48	5
VE	*alpha*-Tocopherol	431.4	165.1	5.60	1	128	24	5
VE	*alpha*-Tocopherol	431.4	55.1	5.60	1	128	60	5
VE	*alpha*-Tocopherol	431.4	69.1	5.60	1	128	44	5
VE	*alpha*-Tocopherol	431.4	57.1	5.60	1	128	48	5
VE	*alpha*-Tocopherol succinate	531.4	265.0	5.87	1.5	176	20	5
VE	*alpha*-Tocopherol succinate	531.4	165.0	5.87	1.5	176	48	5
VE	*alpha*-Tocopherol succinate	531.4	149.0	5.87	1.5	176	36	5
VE	*alpha*-Tocopherol acetate	473.4	207.1	6.47	1	172	16	5

Compound Group	Compound Name	Precursor Ion (*m/z*)	Product Ion (*m/z*)	RT (min)	Delta RT (min)	Fragmentor (V)	CE (V)	CAV (V)
VE	*alpha*-Tocopherol acetate	473.4	165.0	6.47	1	172	40	5
VE	*alpha*-Tocopherol Acetate	473.4	137.0	6.47	1	172	60	5
VE	*alpha*-Tocopherol acetate	473.4	55.2	6.47	1	172	76	5
VK	Menadione, VK3	173.1	77.0	1.89	1	104	36	5
VK	Menadione, VK3	173.1	105.1	1.89	1	104	30	5
VK	Menadione, VK3	173.1	145.0	1.89	1	104	15	5
VK	Menadione, VK3	173.1	50.9	1.89	1	104	60	5
VK	Menaquinone, VK2 MK-4	445.3	69.1	5.36	1	128	44	5
VK	Menaquinone, VK2 MK-4	445.3	81.1	5.36	1	128	56	5
VK	Menaquinone, VK2 MK-4	445.3	187.0	5.36	1	128	24	5
VK	Menaquinone, VK2 MK-4	445.3	95.1	5.36	1	128	40	5
VK	Menaquinone, VK2 MK-7	649.5	187.0	8.02	1	104	32	5
VK	Menaquinone, VK2 MK-7	649.5	95.0	8.02	1	104	32	5
VK	Menaquinone, VK2 MK-7	649.5	81.0	8.02	1	104	32	5
VK	Phytonadione, VK1	451.4	187.0	6.45	1	128	28	5
VK	Phytonadione, VK1	451.4	57.1	6.45	1	128	40	5
VK	Phytonadione, VK1	451.4	43.2	6.45	1	128	60	5
VK	Phytonadione, VK1	451.4	71.1	6.45	1	128	28	5

Method evaluation criteria

Specificity:

- The retention time of each analyte peak to the average of standard peaks is within ±0.2 minutes
- The ion ratio is within the tolerance of 30%

Linearity and range:

- The calibration curve has R^2 >0.99
- Calculated working standard values should be within ± 30% of the theoretical value
- The calibration standards should bracket the analyte concentration level

Accuracy:

- The tested result for each fat-soluble vitamin or carotenoid meets ≥100% claimed on the product label (see Table 3)
- Postspike recovery is within 70 to 130% (due to the variations in method and instrument performance, the criteria should be determined by each individual lab)

Results and discussion

Organic mobile phase selection

In this study, two organic mobile phases were evaluated with the same LC conditions, acetonitrile and methanol. Equivalent sensitivity or better was achieved using methanol for all compounds. Compounded with the fact that MeOH is cheaper than ACN, MeOH was selected as the final solvent. See Figure 2 for a sensitivity comparison between the two solvents. Methanol provided enhanced ionization for improving compound sensitivity.

Specificity

A dynamic multiple reaction monitoring (dMRM) acquisition method was used for vitamin quantitation. Monitoring MS/MS transitions with evaluation of the ratio of their relative intensities and RT of analyte peaks enables the target analyte to be distinguished from potential

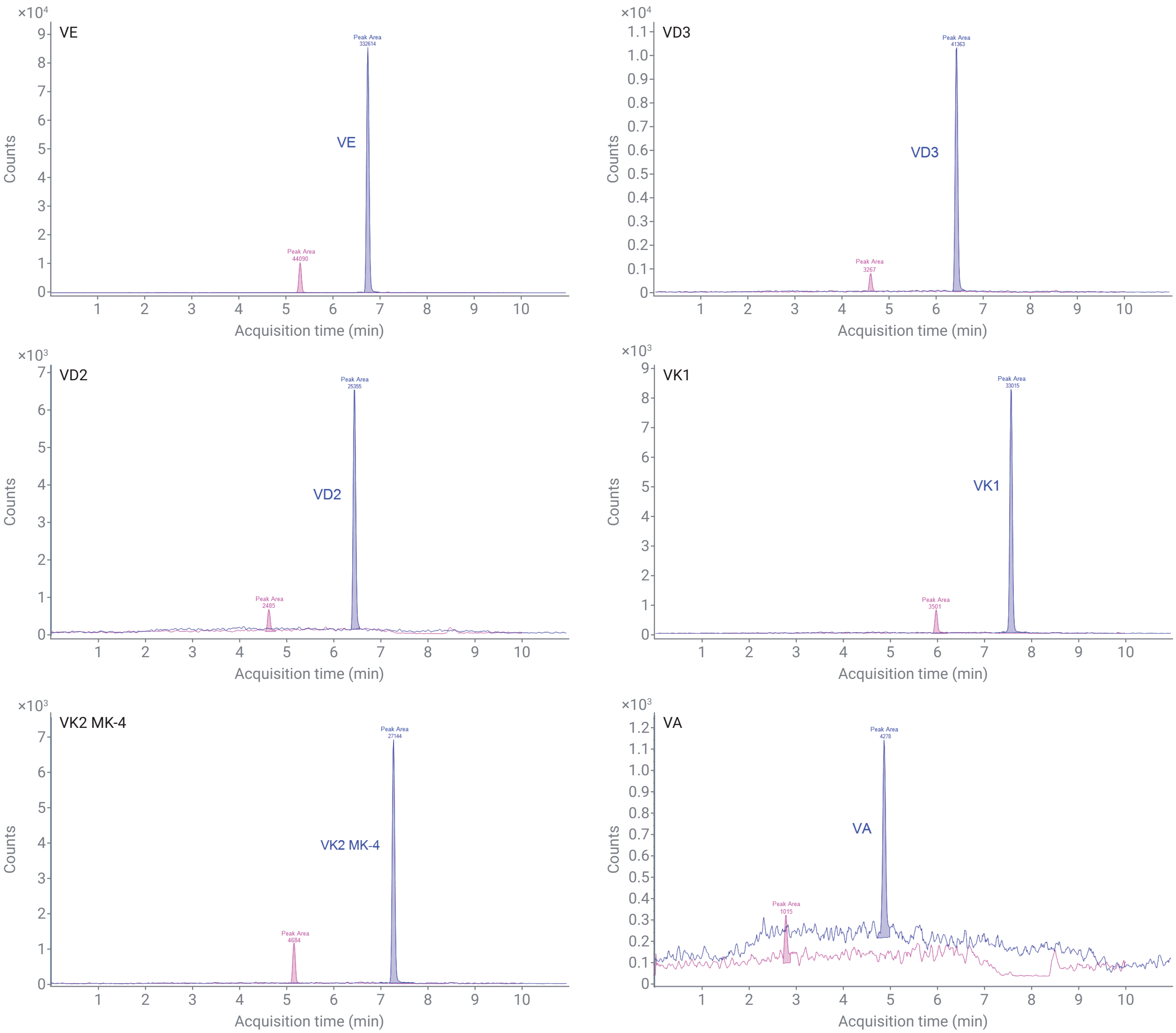

Figure 2. Comparison of the sensitivity between acetonitrile and methanol as organic mobile phase. The peak in blue color is with 0.1% formic acid in methanol as mobile phase B. The peak in pink is with 0.1% formic acid in acetonitrile as mobile phase B. Mobile phase A is 0.1% formic acid in water.

interferences in quantitative analysis. Figure 3A shows the elution profile of all target analytes, and Figure 3B shows an example of an extracted ion chromatogram of a 5 ng/mL working standard in ethanol. Figure 4 shows that a reagent blank is free of analytes in ethanol.

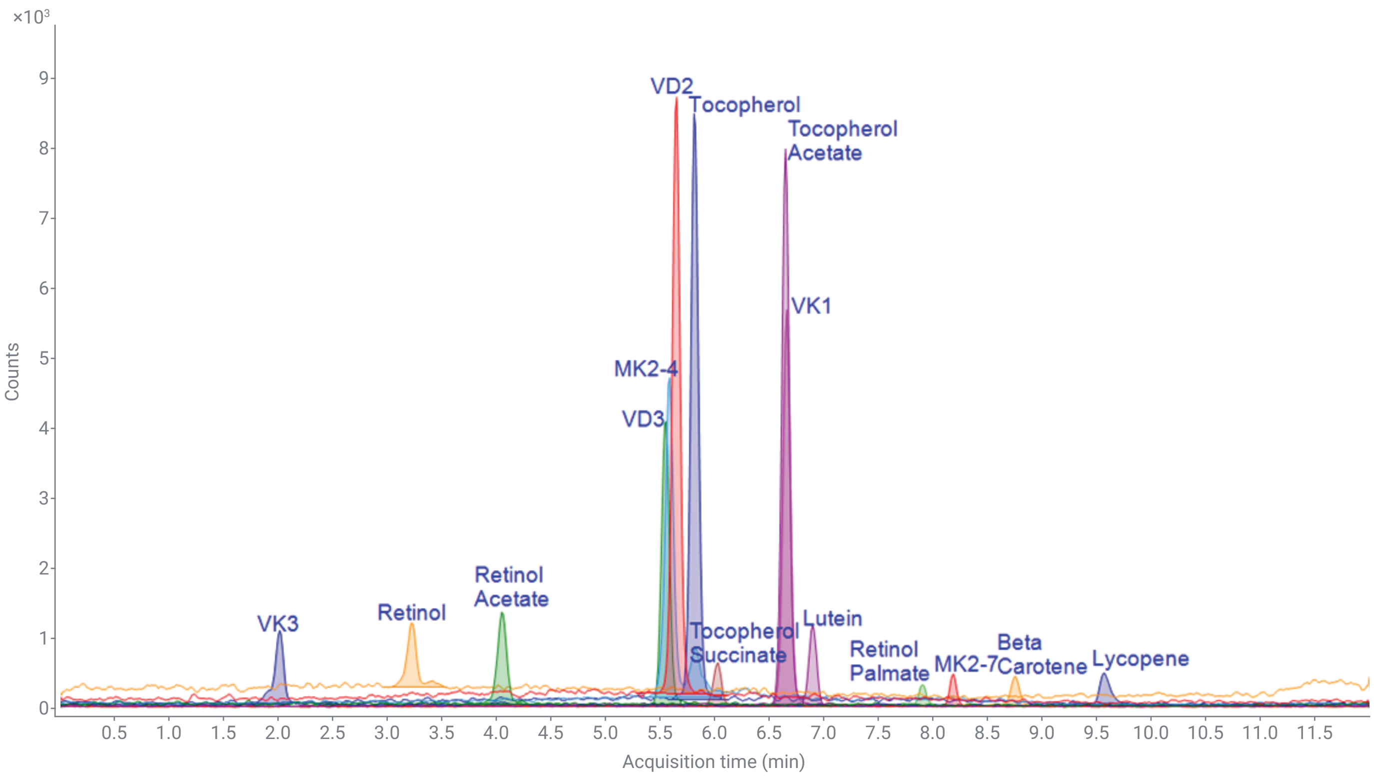

Figure 3A. Elution profile of all fat-soluble vitamins.

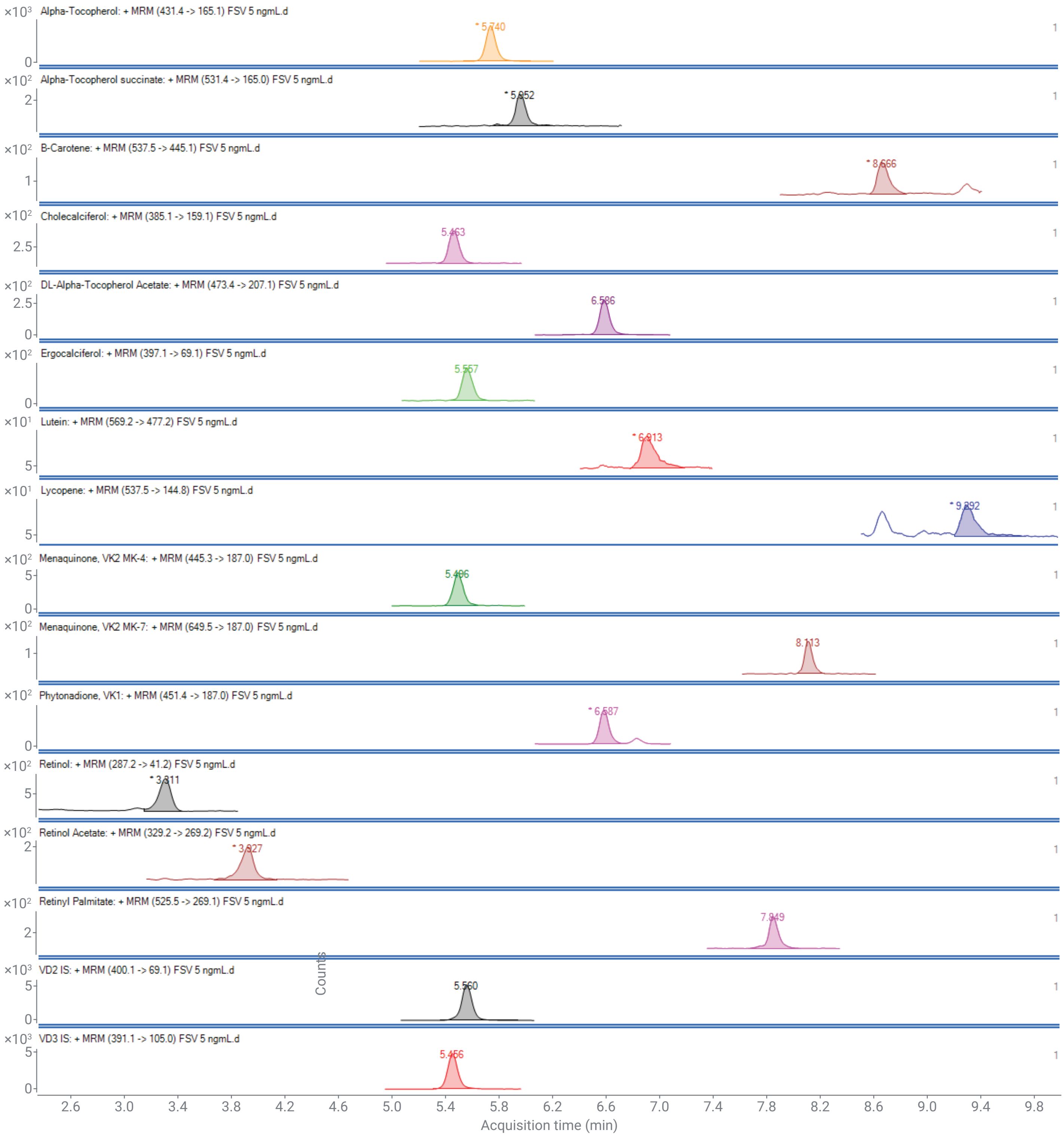

Figure 3B. Extracted ion chromatogram of vitamin composite working standards at 5 ng/mL in ethanol, with 1 µL injection volume.

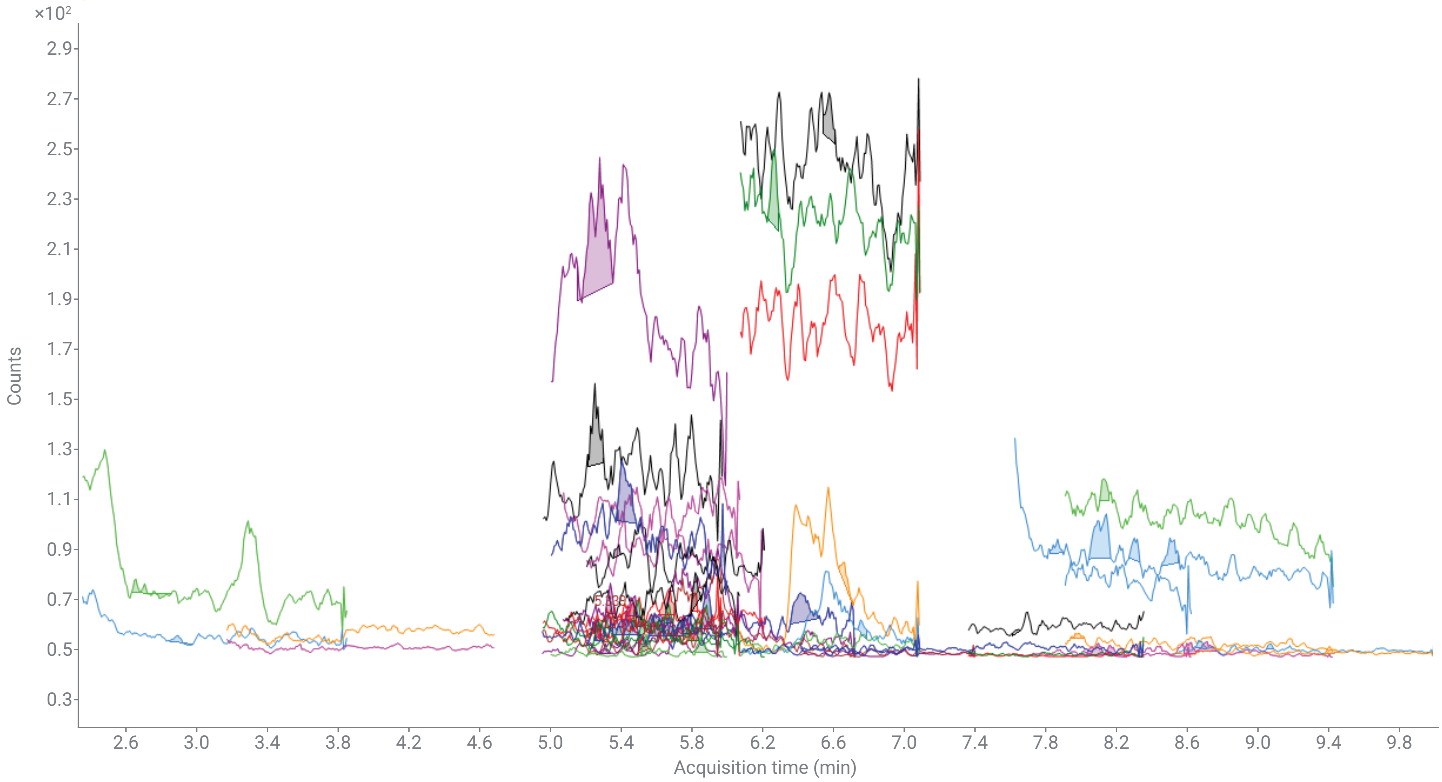

Figure 4. Extracted ion chromatogram of a solvent blank (ethanol).

Range and linearity

The method was evaluated over the range of 0.2 to 500 ng/mL. To evaluate the linearity of the method, nine working standard (WS) solutions were made at 0.2, 0.5, 1, 5, 10, 50, 100, 200, and 500 ng/mL. The calibration curve residuals were ≤30% for WS1 to WS9. The linearity was determined by using a linear calibration with a 1/x weighting factor. The coefficients of determination (R^2) value were >0.99. Table 2 lists the statistical data of the calibration curve residuals, linear range, and coefficients of determination.

Table 2. Calibration curve statistical data.

	Calibration Curve Residual, in Percent										
	WS1 0.2	WS2 0.5	WS3 1	WS4 5	WS5 10	WS6 50	WS7 100	WS8 200	WS9 500		
Compound Name	ng/mL									Range (ng/mL)	R^2
alpha-Tocopherol	16.5	2.6	4.9	−12.5	−12.6	−11.3	−3.6	−1.6	2.9	0.2 to 500	0.9976
alpha-Tocopherol Acetate	12.9	13.7	−5.8	0.1	−7.4	−8.6	−1.3	−4.6	2.6	0.2 to 500	0.9980
alpha-Tocopherol Succinate	–	4.7	3.3	−1.3	1.2	−4.2	−0.2	1.2	0.3	0.5 to 500	0.9996
Cholecalciferol	–	−4.8	11.1	−3.0	6.4	−5.7	0.6	−2.3	2.1	0.5 to 500	0.9992
Ergocalciferol	–	10.4	−1.3	1.8	−5.0	−5.0	−0.6	0.8	0.5	0.5 to 500	0.9998
Beta Carotene	–	–	−24.1	1.5	−2.5	7.9	5.6	6.5	−8.1	1.0 to 500	0.9943
Lutein	–	–	–	−7.7	17.4	1.3	3.6	5.2	−1.4	5 to 500	0.9965
Lycopene	–	–	–	12.2	−10.5	0.0	−6.1	3.8	0.5	5 to 500	0.9978
Menaquinone, VK2 MK-4	–	18.9	−2.1	11.1	−2.3	−4.1	0.1	−1.1	0.5	0.5 to 500	0.9993
Menaquinone, VK2 MK-7	–	24.7	−11.8	12.6	−2.2	−0.7	7.1	2.3	−4.5	0.5 to 500	0.9971
Phytonadione, VK1	–	−20.0	−17.2	5.6	5.8	4.5	7.1	4.7	−6.8	0.5 to 500	0.9948
Retinol	–	–	–	−15.5	−10.9	−0.8	2.1	1.7	−7.1	5 to 500	0.9932
Retinol Acetate	–	–	4.2	−8.4	2.3	−1.6	−1.3	−5.8	–	1 to 200	0.9918
Retinyl Palmitate	–	17.4	−18.3	3.0	−2.1	4.8	−1.6	−1.2	0.1	0.5 to 500	0.9936

Approaches to accurate quantitation

Interfering substances in the matrix may be observed, and can affect the electrospray ionization process, causing ion suppression or enhancement. Currently, there is no guideline for matrix effects in vitamins due to the variations in method and instrument performance. However, matrix effects need to be compensated. A postspiked matrix-matched standard or standard addition can address the matrix effect or any other matrix interactions for quantitation when the internal standard is not available or not easy to obtain.[3]

Sample tests

The multivitamin tablet sample contains food-based blends, including fruits, veggies, digestive enzymes, and more plant-based blends. The complexity of the product formulation served as an excellent matrix to demonstrate unambiguous identification, accurate quantitation, and high sensitivity of a variety of fat-soluble vitamins and carotenoids by LC/MS/MS. The high sensitivity of LC/MS/MS allows for a large dilution after sample extraction. The postspike recoveries for retinyl acetate, beta carotene, cholecalciferol, *alpha*-tocopherol succinate, and phytonadione fell into the accepted range (70 to 130%), and the results were corrected. The corrected results for all fat-soluble vitamins met ≥100% of claims on the product label. The remaining fat-soluble vitamins and carotenoids that are not ingredients of the product formula were also postspiked, and the postspike recoveries were all in the accepted range. Sample results and postspike recoveries for each analyte are shown in Table 3. The result of cholecalciferol was also corrected using internal standards, for comparison with those from post matrix-match correction. Both results were found to be in good agreement (see Table 3).

Table 3. Sample results for a multivitamin tablet.

Compound Name	Vitamins	Serving Size (g)	Claim on Product Label (mg/serving)	Corrected Results by IS (mg/serving)	Corrected Results by Postspike Recovery (mg/serving)	Postspike Recovery (%)
Retinyl Acetate	A	5	0.54		0.60	87.8
Beta Carotene	A; Carotenoids	5	1.26		1.85	103
Cholecalciferol	D3	5	0.05	0.08	0.078	97.1
alpha-Tocopherol Succinate	E	5	60		87	106
Phytonadione	K1	5	0.12		0.18	106
Retinol	A	5				86.6
Retinyl Palmitate	A	5				102
Ergocalciferol	D2	5				99.7
alpha-Tocopherol	E	5				115
alpha-Tocopherol Acetate	E	5				99.1
Menaquinone, VK2 MK-4	K2	5				91.2
Menaquinone, VK2 MK-7	K2	5				104
Lutein	Carotenoids	5				96.4
Lycopene	Carotenoids	5				105

Conclusion

A rapid, sensitive, and accurate LC/TQ method for the identification and quantitation of fat-soluble vitamins and selected carotenoids in a complex supplement matrix was presented. The method used an Agilent 1290 Infinity II LC system coupled to an Agilent 6470 triple quadrupole LC/MS. Fourteen fat-soluble vitamins and carotenoids were simultaneously detected in one run for high efficiency, throughput, and cost reduction, when compared to the traditional involvement of multiple assays. All fat-soluble vitamins and carotenoids in the supplement product met the specifications with the quantitation approach of the matrix-matched standard. The evaluation demonstrated that the method can achieve the necessary specificity, linearity, and accuracy required for fat-soluble vitamin analysis.

References

1. National Research Council. Diet and Health: Implications for Reducing Chronic Disease Risk; The National Academies Press, **1989**.
2. Young, A. J.; Lowe, G. L. Carotenoids-Antioxidant Properties. *Antioxidants* **2018**, 7, 28.
3. Simultaneous Detection and Quantitation of 14 Water-Soluble Vitamins in a Supplement by LC/MS/MS Triple-Quadrupole. *Agilent Technologies application note*, publication number 5994-3016EN, **2021**.

www.agilent.com

DE16169462

Printed in the USA, July 20, 2022
5994-5064EN

Agilent
Trusted Answers