Application Note

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Determination of Fat-Soluble Vitamins in Foods Using Agilent Chem Elut S Extraction with LC/DAD and LC/MS/MS Triple-Quadrupole

Authors

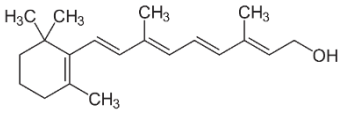
Hui Zhao and Limian Zhao
Agilent Technologies, Inc.

Abstract

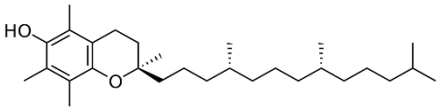
This application note describes a method for the determination of fat-soluble vitamins, including vitamin A (retinol), vitamin D3 (cholecalciferol), vitamin D2 (ergocalciferol), and vitamin E (α-tocopherol) in complex food matrices, including infant formula, egg, canned tuna, and mushroom. Samples were saponified as sample pretreatment, extracted using Agilent Chem Elut S (Supported Liquid Extraction (SLE)) 12 mL cartridges, and fat-soluble vitamins were then simultaneously identified and quantified by an Agilent 1290 Infinity II LC coupled to an Agilent diode array detector (DAD) and Agilent 6470 triple quadrupole LC/MS in series. Data were analyzed using Agilent MassHunter workstation software.

Introduction

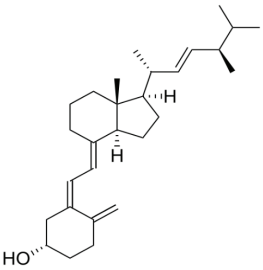
Fat-soluble vitamins, including vitamin A (VA), vitamin D (VD), and vitamin E (VE) play an important role in the stimulation of synthesis and degradation of nutrients, enhancing immune function and growth performance.[1] Accurate measurement of fat-soluble vitamins is often required in clinical studies, nutrition chemistry, and food industry. The structure of fat-soluble vitamins A, D2/D3, and E are given in Figure 1.



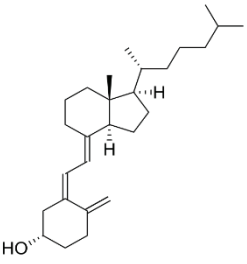
Retinol, VA, log P = 5.7



α-Tocopherol, VE, log P = 10.4



Ergocalciferol, VD2, log P = 7.4



Cholecalciferol, VD3, log P = 7.5

Figure 1. Structure of fat-soluble vitamins.

LC coupled with DAD has been widely used for the determination of VA and VE, due to the high abundance of these vitamins in food samples.[2] In contrast, the trace level of VD in food samples requires LC/MS/MS detection.[3] In this study, a DAD was connected in front of the mass spectrometer, and VA, VE, and VD2/VD3 were simultaneously analyzed with the UHPLC-DAD-MS/MS system.

Fat-soluble vitamins coexist with other lipid contents (triglycerides, sterols, phospholipids, etc.) in the lipid fraction of foods, which makes their isolation and measurement quite challenging. The traditional food sample preparation for fat-soluble vitamin analysis mainly consists of saponification and liquid-liquid extraction (LLE). A saponification procedure will release the vitamins to their free forms, convert the ester forms to free forms, and act as a cleanup step to break down lipids such as triacylglycerols (which interfere with the analysis) into fatty acids. After saponification, the sample mixture is extracted by LLE before sample analysis on the instrument. However, the traditional LLE procedure is time-consuming and labor-intensive, and introduces more deviations and human errors. Supported liquid extraction (SLE) has been demonstrated to be an excellent alternative, providing more sample preparation consistency and simplicity, saving time and effort, and increasing the sample analysis productibility. Instead of traditional diatomaceous earth sorbent, Agilent Chem Elut S products use synthetic sorbent, which improves sorbent batch-to-batch reproducibility and product performance consistency. Considering the sample mixture volume after saponification, Chem Elut S 12 mL cartridges were used in this application. The food matrices selected in this study represent the complex samples that are reported with positive fat-soluble vitamins, and show significant matrix effect.

In this study, an easy, reliable, and sensitive method based on UHPLC-DAD-MS/MS was developed and evaluated to provide identification and quantification of fat-soluble vitamins A, D, and E in a variety of food matrices. The isotopically-labeled internal standards VD2-D_3 and VD3-D_6 were added to compensate for matrix effects that affect MS ionization. Method criteria for data acceptance were established.

Experimental

Instrument method

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 1290 Infinity II DAD (G7117B) and Agilent 6470 triple quadrupole LC/MS system (G6470), in series. Instrument control, data acquisition, qualitative and quantitative data analysis, and reporting was performed using Agilent MassHunter workstation software.

Samples, standards, and reagents

The study matrices, including infant formula, raw egg, canned tuna, and raw baby Bella mushroom were purchased from local grocery stores. The standards of retinol, cholecalciferol, ergocalciferol, and α-tocopherol were obtained from Millipore Sigma, Inc. (St. Louis, MO, USA). Cholecalciferol-D_6 and ergocalciferol-D_3 solutions (100 µg/mL) were obtained from IsoSciences (West Chester, PA, USA). The reagents of pyrogallic acid, potassium hydroxide, and butylated hydroxytoluene (BHT) were obtained from Millipore Sigma, Inc. (St. Louis, MO, USA).

Chromatographic conditions

Parameter	Setting
Analytical Column	Agilent Poroshell 120 SB-AQ, 2.1 × 150 mm, 2.7 µm (p/n 683775-914)
Column Oven	45 ±2 °C
Injection Volume	2 µL
Run Time	10.5 min
Autosampler	5 ±2 °C
Mobile Phase A	0.1% formic acid in water
Mobile Phase B	0.1% formic acid in methanol
DAD Wavelength	Retinol (VA): 326 nm; α-tocopherol (VE): 295 nm
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 8.0 0.25 0 100 8.5 0.25 20 80 10.5 0.25 20 80

MS parameters

Parameter	Setting
MS Acquisition	dMRM
Stop Time	7 min
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

The individual analyte stock solutions were prepared at concentrations of 500 µg/mL in ethanol, and purity was taken into account. The standards were stored at –20 °C.

Sample preparation equipment and consumables

- Agilent Chem Elut S 12 mL cartridges, 3 mL sample, 12 mL tube (part number 5610-2008)
- Agilent Vac Elut SPS 24 manifold (part number 12234004)
- Vortexer, VWR
- Evaporator, CentriVap Complete, Labconco
- Centrifuge
- Incubation oven, VWR

Method

Sample preparation procedure

Below is the detailed description of sample preparation. Before sample weighing, eggs were blended thoroughly, mushrooms were cut, frozen, and then blended while frozen, and canned tuna was blended thoroughly. Table 1 gives the analyte-specific LC/MS conditions.

1. Weigh appropriate amount of thoroughly homogenized sample into a 50 mL centrifuge tube. For infant formula and egg, 1 g of sample was used; for canned tuna, 2 g of sample was used; for mushroom, 8 g of sample was used.
2. Spike with target analyte solution and vortex for 1 minute.
3. Add 5 mL of water and vortex for 15 minutes.
4. Add 10 mL of 2% (w:v) pyrogallol in ethanol and vortex for 3 minutes (pyrogallol was added to prevent oxidation of the target analytes during saponification).
5. Add 5 mL of 50% (w:w) potassium hydroxide in water and vortex for 30 seconds.
6. Place all samples in incubation oven and saponify at 75 to 80 °C for 45 minutes.
7. Remove samples from oven and cool to ambient temperature.
8. Centrifuge at 4,000 rpm for 3 minutes.
9. Place Chem Elut S 12 mL cartridges onto the Vac Elut SPS manifold, with properly labeled 16 × 100 collection tubes beneath.
10. Transfer an aliquot of 2.5 mL sample mixture into Chem Elut S 12 mL cartridges.
11. Apply vacuum to drive sample to sorbent bed, and then equilibrate for 10 minutes.
12. Transfer an aliquot of 5 mL elution solvent, 100 mg/L BHT in hexane and use gravity elution (BHT was added to prevent oxidation of the target analytes during extraction and subsequent steps).
13. Repeat step 12 one more time. At the end of elution, when sample is no longer dripping, apply full vacuum to completely drain the sorbent bed.
14. Evaporate the eluent with N_2 flow at 40 °C to dryness.
15. Reconstitute dried sample with an aliquot of 0.5 mL methanol.
16. Vortex for 2 minutes and sonicate for 10 minutes, then centrifuge for 3 minutes.
17. Transfer the final sample extract into an autosampler vial with a 200 µL glass insert for LC/DAD/MS analysis.

Method evaluation procedure

All sample matrices were first screened for positive detection of targeted vitamins. All matrices except mushroom (usually those mushrooms that have been exposed to sunlight or ultraviolet light are rich in VD2) were confirmed to have positive detection of at least one target. Method sensitivity was evaluated using the mushroom matrix blank to spike in duplicates at each low QC level of 2.5, 5, and 10 ng/g for VD2 and VD3, 25 and 50 ng/g for VA, and 0.5 and 1 µg/g for VE, respectively.

Method accuracy and reproducibility were verified in another three food matrices, including infant formula, raw egg, and canned tuna. Unspiked QCs were prepared at replicates of three or four and QCs were also prespiked at one or three times the presence level of each analyte.

Method quantitation was established using the isotopically labeled internal calibration curve with 1/x weight for VD2 and VD3 on LC/MS/MS, and external calibration curves for VA and VE on LC/DAD.

Method evaluation criteria

Specificity:

- The retention time of each analyte peak to the average of standard peaks is less than ±0.2 minutes.
- The ion ratio for VD2 and VD3 is within the tolerance of 30%.

Linearity and range:

- The calibration curve has a coefficient determination R^2 >0.99.
- The residual of each working standard is within ±15%.
- The calibration standards should bracket the analyte concentration level.

Precision:

- RSDs from at least three replicates are ≤ 20%.

Accuracy:

- The recovery for the spike is within 70 to 130%.

Table 1. Analyte-specific LC/MS conditions: precursor to product ion transitions, fragmentor, collision energies (CE), cell accelerator voltage (CAV), and retention times (RT). Conditions for vitamin A and vitamin E are provided for reference.

Compound Group	Compound Name	Type	Precursor Ion (*m/z*)	Product Ion (*m/z*)	RT (min)	Delta RT (min)	Fragmentor (V)	CE (V)	CAV (V)
VE	α-Tocopherol	Target	431.4	165.1	5.7	1.5	128	24	5
VE	α -Tocopherol	Target	431.4	69.1	5.7	1.5	128	44	5
VE	α -Tocopherol	Target	431.4	57.1	5.7	1.5	128	48	5
VE	α -Tocopherol	Target	431.4	55.1	5.7	1.5	128	60	5
VD3	Cholecalciferol	Target	385.1	259.1	5.4	1.5	104	25	5
VD3	Cholecalciferol	Target	385.1	159.1	5.4	1.5	104	25	5
VD3	Cholecalciferol	Target	385.1	107.1	5.4	1.5	104	28	5
VD3	Cholecalciferol	Target	385.1	105.1	5.4	1.5	104	56	5
VD3	VD3-D_6 IS	IS	391.1	105	5.4	1.5	128	48	5
VD2	Ergocalciferol	Target	397.1	107.1	5.5	1.5	104	28	5
VD2	Ergocalciferol	Target	397.1	105.1	5.5	1.5	104	56	5
VD2	Ergocalciferol	Target	397.1	69.1	5.5	1.5	104	36	5
VD2	Ergocalciferol	Target	397.1	41.2	5.5	1.5	104	60	5
VD2	VD2-D_3 IS	IS	400.1	69.1	5.5	1.5	104	40	5
VA	Retinol	Target	287.2	81.1	3.1	2	104	24	5
VA	Retinol	Target	287.2	69.1	3.1	2	104	28	5
VA	Retinol	Target	287.2	55.1	3.1	2	104	48	5
VA	Retinol	Target	287.2	41.2	3.1	2	104	60	5

Results and discussion

Sample extraction approach

The traditional sample preparation for VA, VD2/VD3, and VE in food samples is based on sample saponification followed by LLE, which usually involves multiple steps of extraction, phase separation, and organic phase transferring. LLE can be time-consuming and labor-intensive, and thus potentially introduces more errors and deviations. In this study, Agilent Chem Elut S-based SLE was used to replace the LLE procedure. Considering the reasonable sample loading size and Chem Elut S cartridge capacity, Chem Elut S 12 mL cartridges were used for extracting 2.5 mL of sample mixture after saponification. Hexane was used as the solvent for 5 mL of extraction, twice.

Compared to traditional LLE, the SLE approach significantly simplified the workflow, providing 30 to 50% time saving and analyst effort on sample preparation. Due to the interaction surface between aqueous and organic phases using SLE being significantly increased, the method performance of high analyte recoveries and great reproducibility was achieved. In addition, SLE provided improved sample matrix cleanup efficiency by complete removal of matrix interferences, such as proteins, phospholipids, saponifiable lipids, and salts. Figure 2 shows the sample final extract dried residue comparison between Chem Elut S extraction and traditional LLE, for egg and infant formula samples. As indicated in Figures 2A and 2B, a lower amount of dried residue shown in the sample prepared by Chem Elut S extraction demonstrated a significantly lower amount of matrix coextractives in the final sample extract.

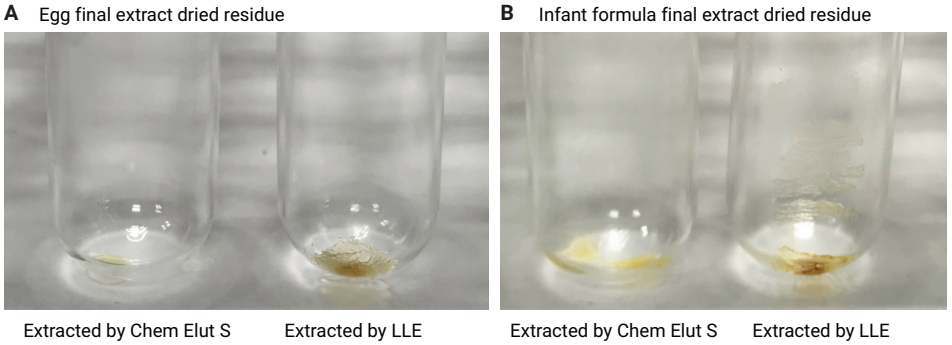


Figure 2. Comparison of the effect of extraction by Agilent Chem Elut S 12 mL cartridges, and by traditional LLE. For both Chem Elut S and LLE, an identical sample mixture after saponification, sample size, and extraction solvent were used.

Specificity

The combination of LC/DAD and LC/MS/MS detection was used for simultaneous fat-soluble vitamin analysis. Monitoring multiple reaction monitoring (MRM) transitions, with evaluation of the ion ratio of their relative intensities and retention time (RT) of analyte peaks enables the target analyte to be distinguished from potential interferences in quantitative analysis. Figure 3 shows an example of the extracted ion chromatograms of a 10 ng/mL composite working standard in ethanol, and an LC/UV chromatogram of a 5 µg/mL A and E working standard in ethanol. Figure 4 shows that a reagent blank is free of analytes in ethanol.

Calibration range and linearity

The method was evaluated over the range of 0.2 to 1,000 ng/mL for LC/MS/MS detection, and 0.05 to 100 µg/mL for LC/DAD detection.

To evaluate the calibration curve linearity of the method, nine working standard (WS) solutions of VA, VD2/VD3, and VE containing internal standards, VD2-D_3 and VD3-D_6, were made at 0.2, 0.5, 1, 5, 10, 50, 100, 500, and 1,000 ng/mL, and three more working standard solutions of VA and VE were made at 5, 20, and 100 µg/mL. The calibration curve residuals were ≤15% for WS1 to WS12. The linearity was determined by using an isotopically labeled internal calibration with a 1/x weighting factor or external calibration. The coefficients of determination (R^2) values were >0.99. Table 2 lists the statistical data of the calibration curve residuals, linear range, and coefficients of determination.

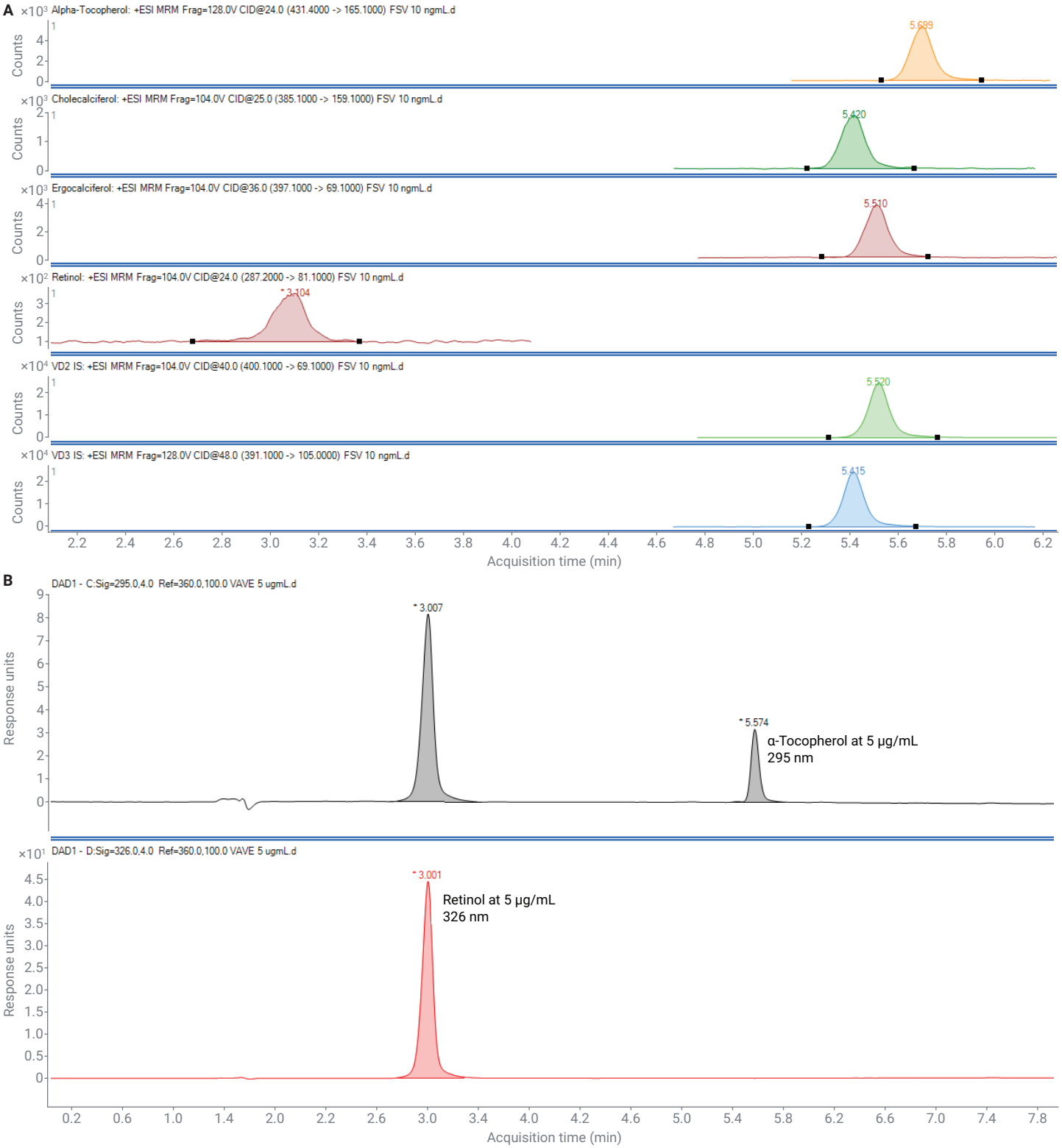


Figure 3. (A) Extracted ion chromatograms of target vitamins composite working standard at 10 ng/mL in ethanol with vitamin D2/vitamin D3 IS at 100 ng/mL, with 2 µL injection volume. (B) LC/UV chromatograms of retinol and α-tocopherol at 5 µg/mL in ethanol, with 2 µL injection volume.

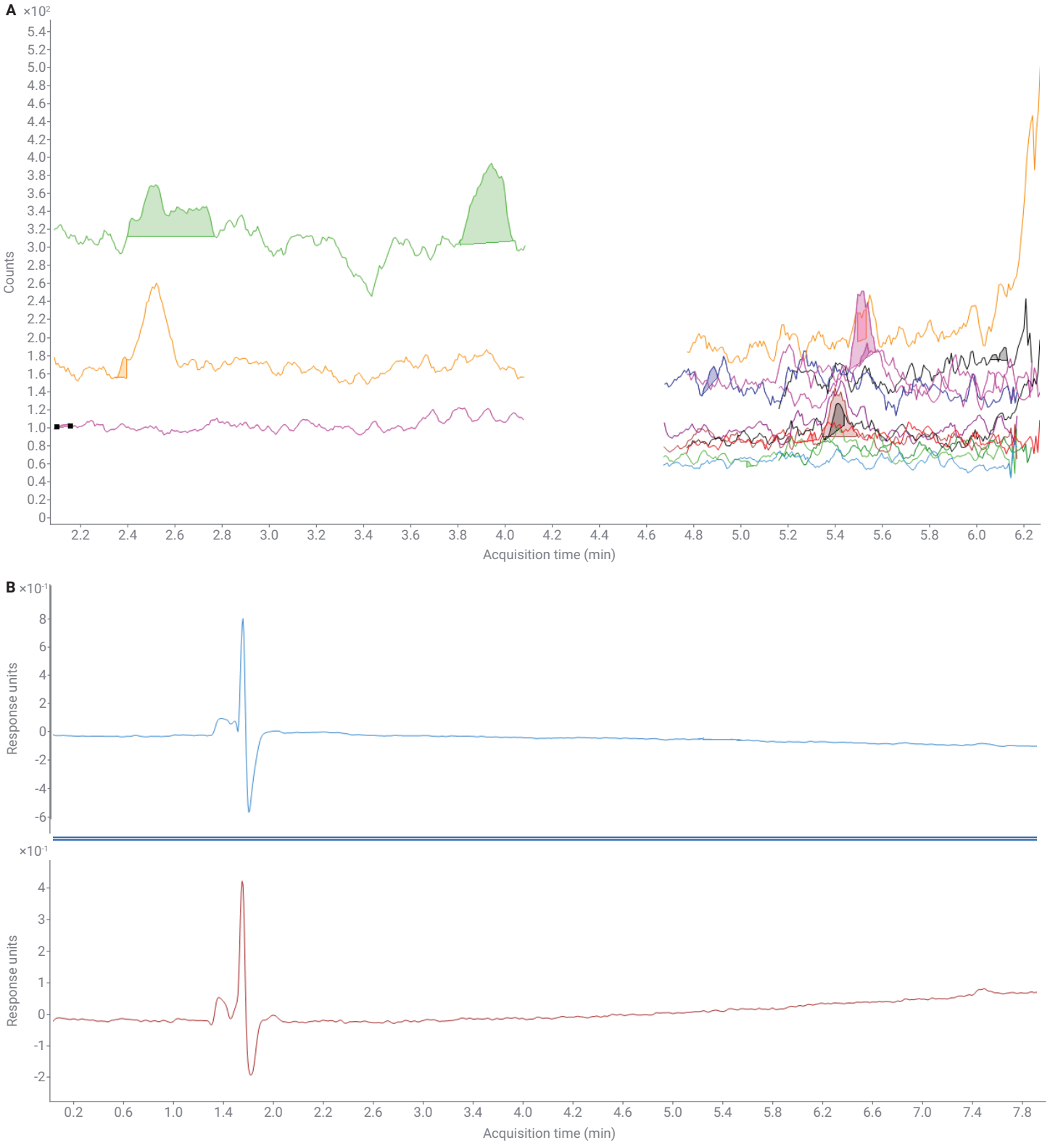


Figure 4. (A) Extracted ion chromatogram of a reagent blank for ethanol. (B) LC/UV chromatogram of a reagent blank for ethanol.

Table 2. Calibration curve statistical data.

Calibration Curve Residual, in Percent														
Compound Name	WS1 0.2	WS2 0.5	WS3 1	WS4 5	WS5 10	WS6 50	WS7 100	WS8 500	WS9 1000	WS10 5	WS11 20	WS12 100	Range	R^2
	ng/mL									µg/mL				
Ergocalciferol (MRM)	–6	15	–4	–9	4	–2	7	5	–3	–	–	–	0.2 to 1,000 ng/mL	0.9981
Cholecalciferol (MRM)	–6	13	3	–8	1	–7	7	5	–3	–	–	–	0.2 to 1,000 ng/mL	0.9985
Retinol (MRM)	–	–	–	9	5	7	5	–4	2	–	–	–	5 to 1,000 ng/mL	0.9986
Retinol (UV at 326 nm)	–	–	–	–	–	–12	–13	–9	–2	–2	–6	1	0.05 to 100 µg/mL	0.9996
α-Tocopherol (MRM)	–9	12	–12	–6	–1	1	13	11	–7	–	–	–	0.2 to 1,000 ng/mL	0.9930
α-Tocopherol (UV at 295 nm)	–	–	–	–	–	–	–	–15	1.4	0	0.1	11	0.5 to 100 µg/mL	0.9991

Accuracy and precision

Method sensitivity was assessed by fortifying a mushroom sample that was confirmed to be free of analytes at levels of 2.5, 5, and 10 ng/g for VD2 and VD3, 25 and 50 ng/mL for VA, and 0.5 and 1 µg/mL for VE. The mushroom sample was then prepared using the developed method. Accuracy was further verified by fortifying three native samples of infant formula, egg, and tuna at 1 or 3 times the presence level of each analyte. VD2/VD3 results were corrected using internal standards, which were fortified at 100 ng/mL for all samples. Accuracy for samples that contained fat-soluble vitamins (see Table 3) was based on spiked/unspiked values, as determined by internal standard calibration or external calibration.

Precision was evaluated using quantitation results from unspiked native samples of infant formula in four replicates, and egg and tuna in three replicates.

Acceptable analyte recoveries (within 70 to 130%) and %RSD (≤ 20%) were obtained for all replicates, except one recovery in the mushroom sample at 67.7% for a spike level at 2.5 ng/g. Table 3 shows the accuracy results, and Table 4 shows the precision results.

Instrument limit of quantification

Because most of the matrices included in this study contained fat-soluble vitamins, the method determined ILOQs. The ILOQ for VD2/VD3, VA, and VE was set at 0.5 ng/mL, 50 ng/mL, and 0.5 µg/mL respectively.

Figure 5 shows each individual extracted ion chromatogram of VD2 and VD3, and their internal standards at an LOQ-corresponding level of 0.5 ng/mL with a signal-to-noise ratio (S/N) >10. Figure 6 shows VA and VE at LOQ corresponding levels of 50 ng/mL and 0.5 µg/mL, respectively, with S/N >10.

Table 3. Accuracy (spike recovery, in percent) of infant formula, egg, canned tuna, and mushroom.

Sample Matrix	Replicates	VD2 in Sample (µg/g)	VD2 Spiked in Sample (µg/g)	VD2 Recovery %	VD3 in Sample (µg/g)	VD3 Spiked in Sample (µg/g)	VD3 Recovery %	VA (326 nm) in Sample (µg/g)	VA Spiked in Sample (µg/g)	VA Recovery %	VE (295 nm) in Sample (µg/g)	VE Spiked in Sample (µg/g)	VE Recovery %
Infant Formula	3	0.156	0.140	90.8	0.133	0.135	112	4.13	6.51	86.0	188	133	81.8
Egg	2	0.201	0.140	95.3	0.148	0.135	107	2.15	6.51	92.6	161	133	112
Canned Tuna	2	ND*	0.0559	94.7	0.0217	0.0540	101	ND	0.542	85.2	1.54	5.57	82.9
Mushroom, Spike Level 1	2	ND	0.00279	67.7	ND	0.00270	74.6	ND			ND		
Mushroom, Spike Level 2	2	ND	0.00559	76.0	ND	0.00540	80.4	ND	0.0271	84.5	ND	0.557	101
Mushroom, Spike Level 3	2	ND	0.0112	76.2	ND	0.0108	97.2	ND	0.0542	78.6	ND	1.11	82.7

* ND: None detected

Table 4. Precision (%RSD) of the detected vitamins in infant formula, egg, and canned tuna.

Sample Identification	Sample Weight (g)	Dilution Factor	VD2 in Sample (µg/g)	VD3 in Sample (µg/g)	VA in Sample (µg/g)	VE in Sample (µg/g)
Infant Formula 1	1	4	0.16470	0.13406	4.0702	192.91
Infant Formula 2	1	4	0.13097	0.12450	4.0998	192.69
Infant Formula 3	1	4	0.17127	0.14350	4.2560	184.42
Infant Formula 4	1	4	0.15604	0.12865	4.1034	182.16
		Average	0.15575	0.13268	4.1323	188.05
		Std Dev	0.018	0.0082	0.084	5.6
		RSD, %	11	6.2	2.0	3.0
Egg 1	1	4	0.18558	0.13836	2.2006	160.10
Egg 2	1	4	0.20791	0.14543	2.2328	170.43
Egg 3	1	4	0.21091	0.16026	2.0205	151.51
		Average	0.20147	0.14802	2.1513	160.68
		Std Dev	0.014	0.011	0.11	9.5
		RSD, %	6.9	7.5	5.3	5.9
Canned Tuna 1	2	4	ND*	0.021978	ND	1.4642
Canned Tuna 2	2	4	ND	0.022440	ND	1.4882
Canned Tuna 3	2	4	ND	0.020674	ND	1.6634
		Average	NA**	0.021697	NA	1.5386
		Std Dev	NA	0.00092	NA	0.11
		RSD, %	NA	4.2	NA	7.1

* ND: none detection

** NA: none applicable

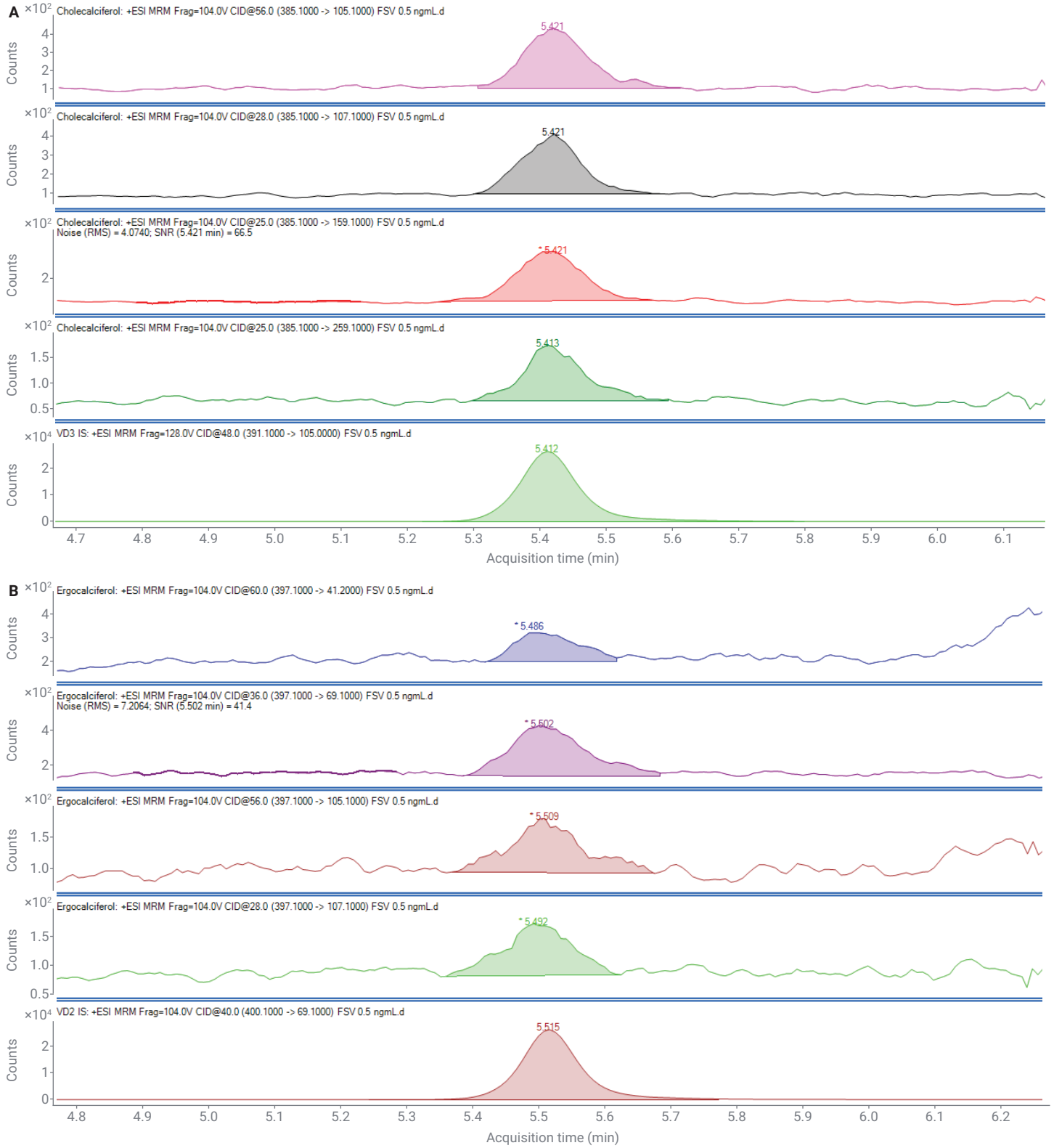


Figure 5. Extracted ion chromatogram of vitamin D3 (A) and vitamin D2 (B) working standard at 0.5 ng/mL (calibration level corresponding to LOQ) with IS at 100 ng/mL in ethanol.

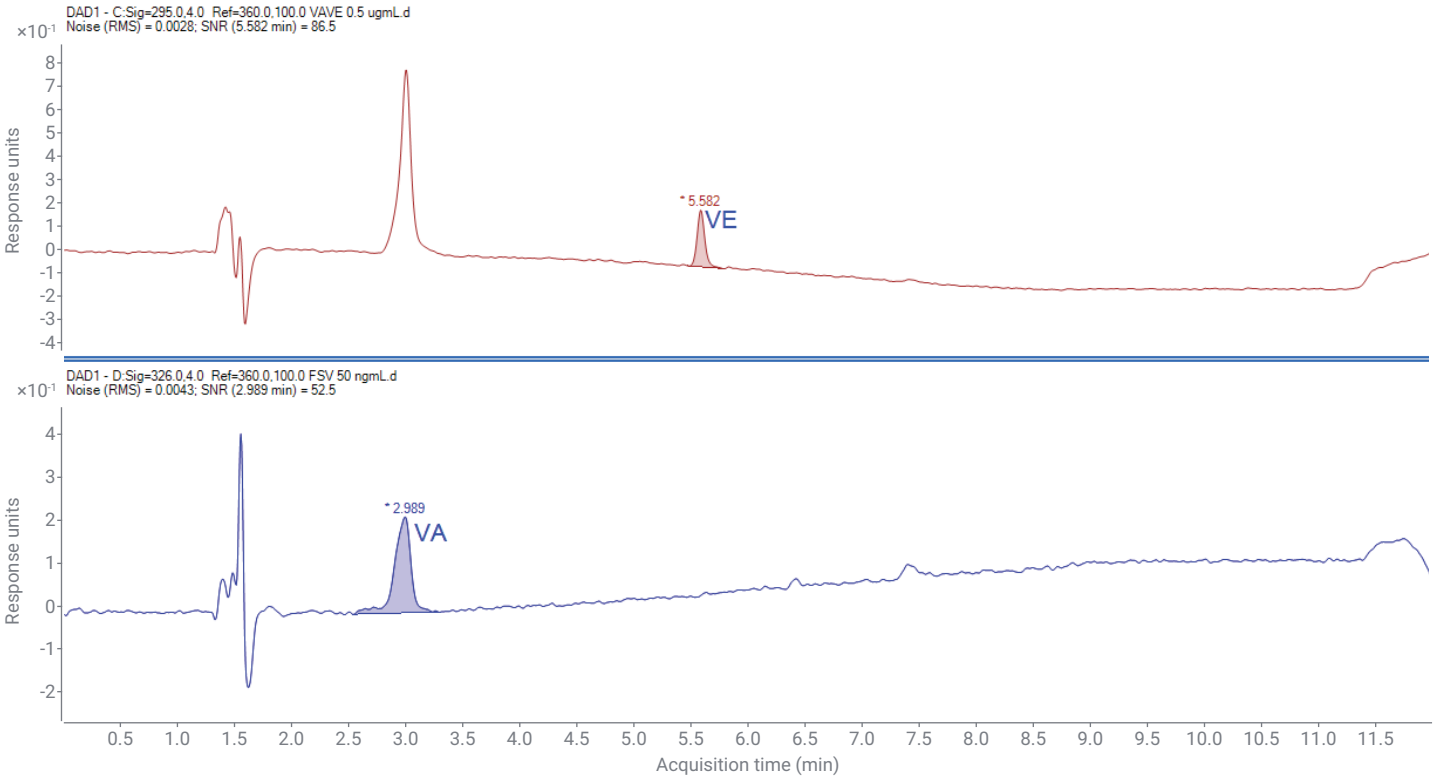


Figure 6. Chromatogram of vitamin A working standard at 50 ng/mL and vitamin E working standard at 0.5 µg/mL (calibration level corresponding to LOQ) in ethanol.

Food samples analysis

The sample results are listed in Table 4. Figure 7 shows an example of the extracted ion chromatogram of VD2 and VD3 in solvent blank (peaks at the left side), native (peaks in the middle), and spiked (peaks at the right side) infant formula and mushroom samples. Figure 8 shows an example of the chromatogram of VA and VE in solvent blank, and native and spiked infant formula and egg samples.

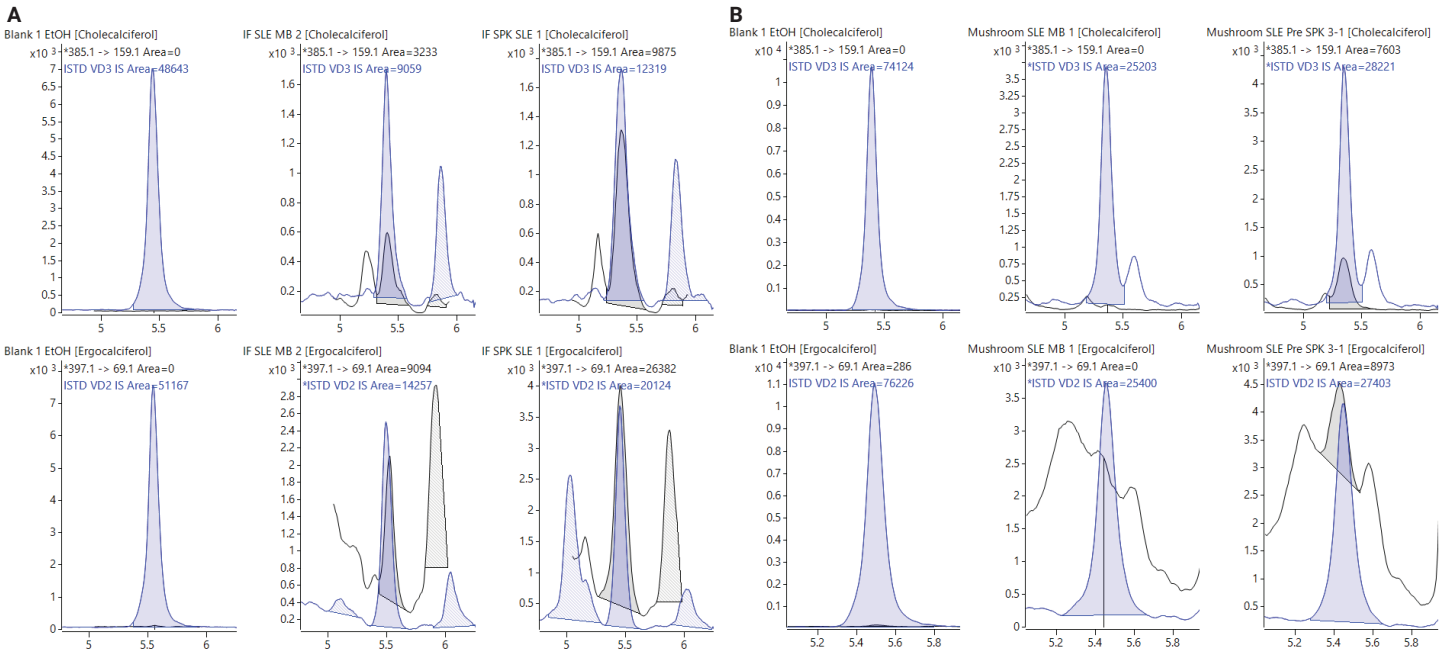


Figure 7. Extracted ion chromatogram of vitamin D2 and vitamin D3 in: (A) native and spiked infant formula and (B) native and spiked mushroom with IS at 100 ng/mL in ethanol. Peaks in blue are vitamin D2 IS or vitamin D3 IS, and peaks overlapped with IS in gray are native vitamin D2 or vitamin D3.

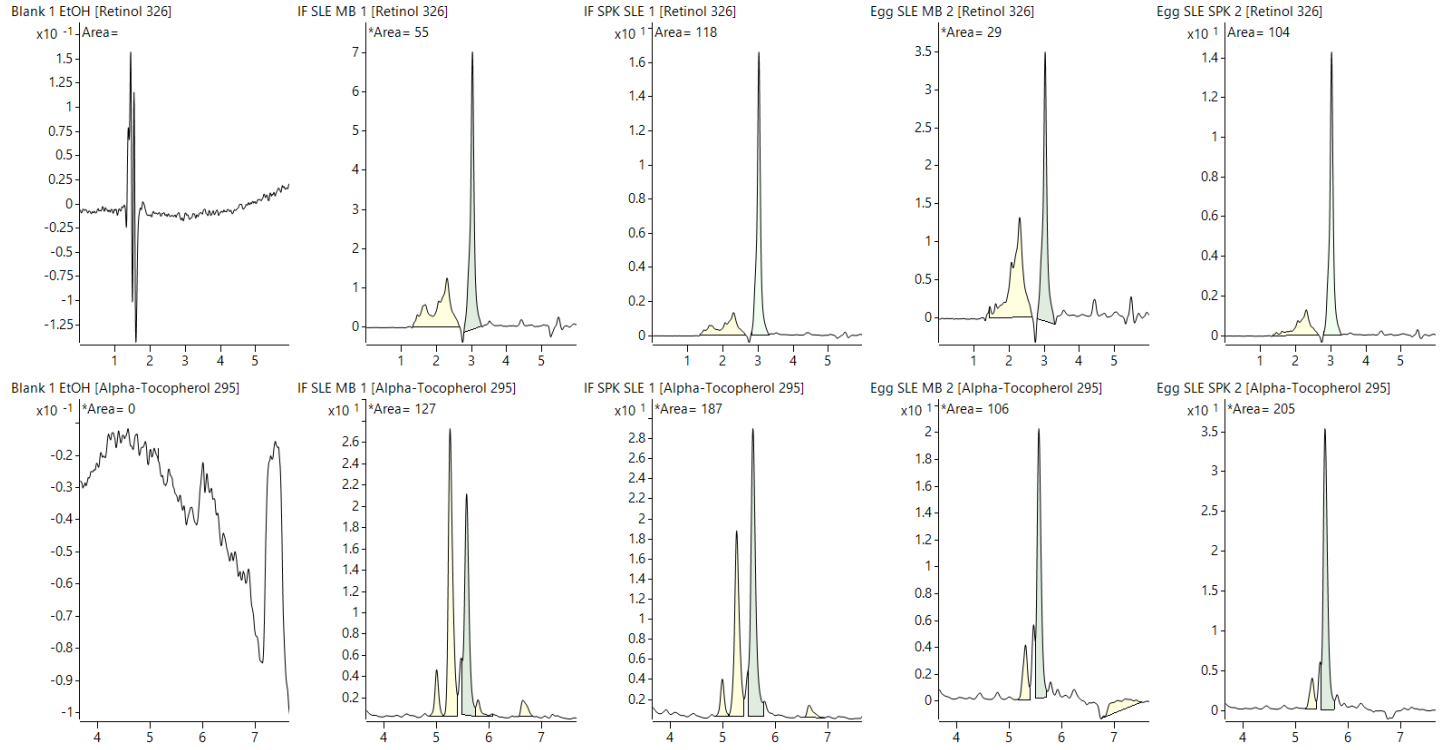


Figure 8. Chromatogram of vitamin A and vitamin E in native and spiked infant formula, and native and spiked egg in ethanol. Peaks in green are vitamin A or vitamin E.

Conclusion

A simple, reliable, and sensitive UHPLC-DAD-MS/MS method for the simultaneous identification and quantification of fat-soluble vitamins, retinol, cholecalciferol, ergocalciferol, and α-tocopherol in food sample matrices was presented. Food samples were first treated for saponification, followed by extraction with the Agilent Chem Elut S. The extracted samples were then analyzed using an Agilent 1290 Infinity II LC coupled to an Agilent DAD and an Agilent 6470A triple quadrupole LC/MS system with Agilent MassHunter workstation software. The method demonstrated adequate specificity, calibration curve linearity, target accuracy, and precision.

References

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