Application Note

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Determination of Fat-Soluble Vitamins in Infant Formula Using Agilent Bond Elut Plexa Polymeric SPE with HPLC and LC/MS/MS

Authors

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Abstract

An efficient and robust method was developed and validated for analysis of vitamin A, vitamins D2/D3, and *alpha*-, *beta*-, *gamma*- and *delta*-vitamin E in infant formula. An infant formula sample was first saponified, then extracted and cleaned by solid phase extraction (SPE) using the Agilent Bond Elut Plexa polymeric SPE. The cleaned sample extract was analyzed for vitamins A and E by HPLC-DAD, and for vitamins D2 and D3 by LC/MS/MS. The developed method provided good reproducibility (RSD <10%) and excellent recoveries (81.2% to 97.3%), and was a simple, low-cost, efficient, and easy handling technique for determination of fat-soluble vitamins.

Introduction

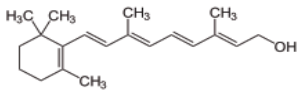
Fat-soluble vitamins such as vitamin A, D, and E (Figure 1) are critical nutrients for human health and children's growth. As a result, the fortification of infant formula with vitamins A, D and E has an important role in the food industry.[1-3] The non-eliminated fat-soluble vitamins accumulate over time and are difficult to excrete from the body.[4] Therefore, it is critical to accurately quantitate the vitamins in infant formula for safety considerations.

There are many publications on the quantitative analysis of fat-soluble vitamins in food using liquid-liquid extraction (LLE) or solid phase extraction (SPE) coupled with HPLC or LC/MS analysis.[5-8] However, these methods are not easily applied to infant formula as the challenges of this formula involve a complex matrix with high fat and a significant difference in the regulated detection levels of vitamins A, D, and E.[9,10] The most frequent forms of vitamins A and E used for infant formula fortification are retinol acetate, retinol palmitate, and α-tocopherol acetate, due to improved stability.[11] Therefore, it is necessary to use saponification or alkaline digestion to transform all esters to the alcohol form, prior to sample extraction and cleanup, to quantitate their total amount.[12] HPLC with DAD is widely used for the analysis of vitamins A and E without internal standards applied, but it is difficult to fulfill trace-level (ppb) detection of vitamins D2 and D3, as baseline chromatography separation for these vitamins is always a challenge.

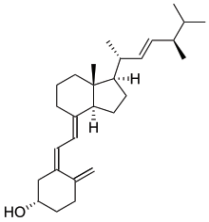
LC/MS/MS can effectively provide the detection sensitivity of vitamins D2 and D3 without the need for complete baseline chromatographic separation. However, the high level of vitamins A and E could cause MS detector saturation, resulting in inaccurate quantification. Additionally, lipid co-extractives from the infant formula matrix could cause significant ion suppression, making it critical to use expensive internal standards for vitamins D2 and D3. Therefore, this method was developed to prepare infant formula samples using saponification, followed by extraction and cleanup on the Bond Elut Plexa. Quantitative determination of vitamins A and E by HPLC-DAD, and vitamins D2/D3 by LC/MS/MS detection could then occur. The complete solution for infant formula analysis demonstrated excellent performance with >80% recoveries and RSD <10% for all vitamin targets.

Equipment and materials

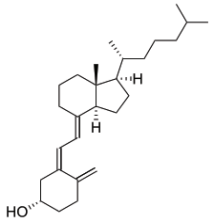
- Agilent 1290 Infinity II LC with flexible pump, multisampler, variable wavelength detector
- Agilent 1290 Infinity II LC and 6470A triple quadrupole LC/MS with an Agilent Jet Stream electrospray ion source
- Agilent Bond Elut Plexa cartridge, 200 mg, 6 mL (part number 12109206)
- Agilent Vac Elut 20 manifold (part number 12234101)
- Nitrogen evaporator with heating system from Organomation Associates, Inc. (MA, USA)



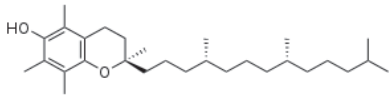
Retinol (Vit A)
Log P: 5.7



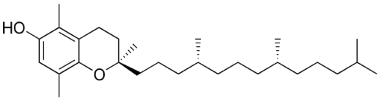
Ergocalciferol (Vit D2)
Log P: 7.4



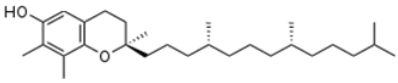
Cholecalciferol (Vit D3)
Log P: 7.5



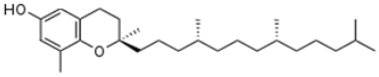
alpha-Tocopherol (α-Vit E)
Log P: 10.7



beta-Tocopherol (β-Vit E)
Log P: 10.3



gamma-Tocopherol (γ-Vit E)
Log P: 10.3



delta-Tocopherol (δ-Vit E)
Log P: 10.0

Figure 1. The structures and Log P values of the fat-soluble vitamins.

Reagents and standards

All reagents and solvents were HPLC or analytical grade. Methanol (MeOH) was from Honeywell (Muskegon, MI, USA); Ethanol (EtOH) was from Acros Organics (Geel, Belgium); Acetone and potassium hydroxide (KOH) were from Sinopharm (Beijing, China). Both ascorbic acid and 2,6-Di-*tert*-butyl-*p*-cresol (BHT) was from CNW (Shanghai, China). The vitamin standards and internal standards were purchased from Alta (Tianjin, China), and prepared in ethanol and stored at –20 °C.

Sample preparation

Stock solutions of vitamins A and E were prepared in ethanol (EtOH) at 5 mg/mL. Vitamins D2, D3, and their corresponding internal standards were prepared in EtOH at 10 µg/mL. The infant formula powder was bought from a local supermarket. Figure 2 shows the step-by-step procedure, featuring two major parts: sample saponification and SPE cleanup.

An aliquot of 5 mL of water was added into 1 g of powdered formula. The sample mixture was thoroughly mixed to generate a homogenous slurry. As ascorbic acid and BHT were reported to be antioxidants that prevent vitamin degradation during sample preparation and detection[13], these components were added at the beginning of sample processing. The addition of EtOH is to increase vitamin solubility and improve fatty sample homogeneity with KOH solution for saponification. During saponification in a water bath at 80 °C, a gentle nitrogen flow was applied to the bottom of the sample to protect the vitamins from oxidation. The sample color changed from yellow-green to dark brown following saponification.

LC/MS/MS Conditions	
Column	Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 100 mm, 2.7 µm (p/n 695775-902)
Column Temperature	40 °C
Autosampler Temperature	15 °C
Injection Volume	5 µL
Mobile Phase	A) H_2O with 0.1% FA, 4.5 mM ammonium formate and 0.5 mM ammonium fluoride B) MeOH with 0.1% FA, 4.5 mM ammonium formate and 0.5 mM ammonium fluoride
Flow Rate	0.4 mL/min
Gradient	Time (min) B% 0 88 1 88 4 90 5 93 5.1 94 5.8 94 6 100
Stop Time	17 min
Ionization Mode	Positive
Cell Accelerator Voltage	4
Gas Temperature	300 °C
Gas Flow	5 L/min
Nebulizer	45 psi
Sheath Gas Temperature	250 °C
Sheath Gas Flow	11 L/min
Capillary	4,000 V

HPLC Conditions	
Column	Agilent InfinityLab Poroshell 120 PFP, 2.1 × 100 mm, 2.7 µm (p/n 695775-408)
Column Temperature	40 °C
Autosampler Temperature	15 °C
Injection Volume	5 µL
Mobile Phase	A) Water B) MeOH
Gradient	Time (min) %B Flow rate (mL/min) 0 80 0.5 2 80 0.5 3 90 0.5 6 90 0.5 7 100 0.5
Stop Time	9 min
DAD Detection	294 nm for Vitamin E and 325 nm for Vitamin A

Name	Retention Time	Ion Transition (*m/z*)	Fragmentor (V)	Collision Energy (eV)
VD2	7.14	397.3 → 379.7 397.3 → 91.0	100	10 70
VD2-d3	7.13	400.3 → 125.4	120	10
VD3	7.19	385.3 →259.5 385.3 → 367.6	100	10 10
VD3-d3	7.18	388.3 → 370.4	100	10

Sample cleanup was performed with the Bond Elut Plexa cartridge, which contains a unique polymer-based SPE. The advanced polymer architecture allows excellent extraction performance of the vitamins, without binding the large endogenous proteins into the pore. This performance remains stable under the extremely alkaline conditions resulting from saponification. During the sample wash, approximately 10 mL of water was applied to wash the cartridge (which can be confirmed by pH test paper) and a mixture of 80/20 MeOH/water was applied to wash off hydrophobic interferences. Following sample cleanup, half of the sample was tested on HPLC-DAD for vitamins A and E analysis, and the other half was tested on LC/MS/MS for vitamins D2 and D3 analysis.

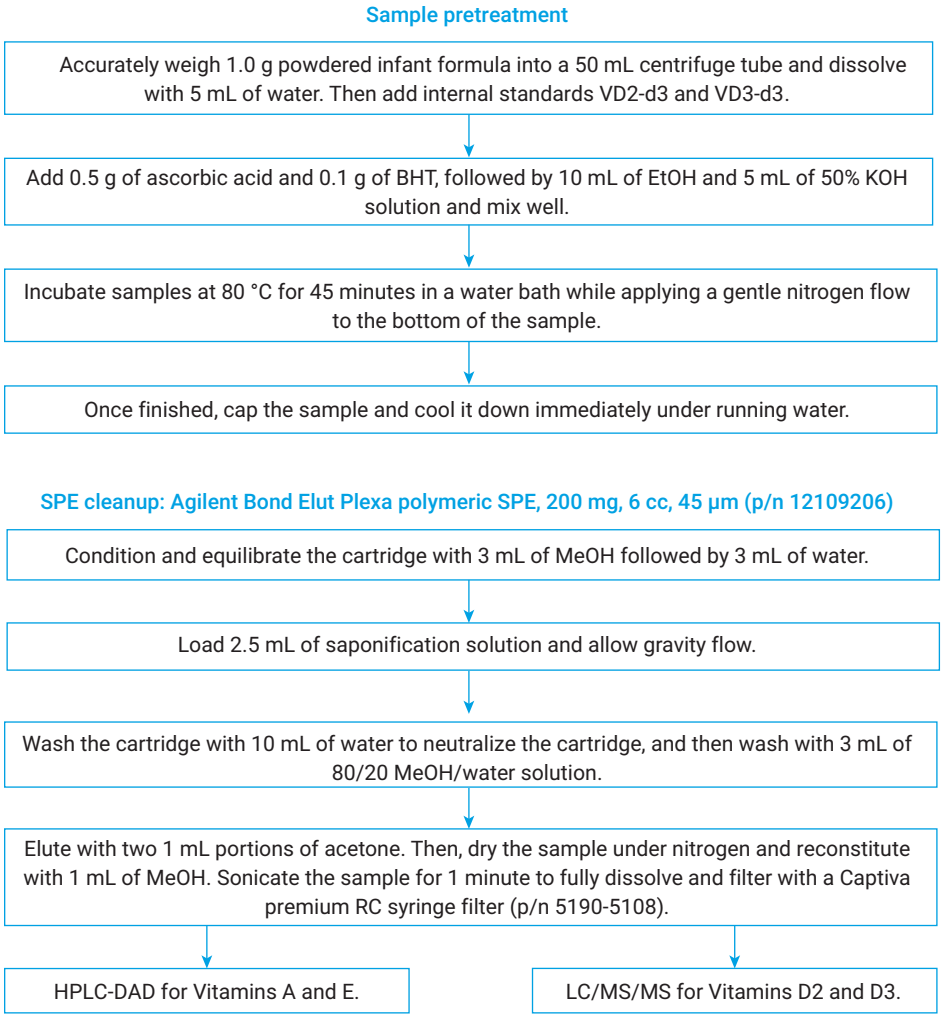


Figure 2. Sample preparation procedure for fat-soluble vitamin analysis in powdered infant formula with the Agilent Bond Elut Plexa polymeric SPE.

Method validation and discussion

Reagent calibration curves were prepared by spiking vitamins A and E into a standard solution at 0.05, 0.1, 0.5, 1, 5, 10, 20, 100, and 200 µg/mL in MeOH for HPLC analysis, and vitamins D2 and D3 standard solution at 1, 5, 10, 20, 50, 100, 200, 500, and 1,000 ng/mL with 80 ng/mL of internal standards in MeOH for LC/MS/MS analysis. An Agilent InfinityLab Poroshell 120 PFP column provided excellent peak shape and baseline separation of vitamin A and four forms of vitamin E on HPLC-DAD. The InfinityLab Poroshell 120 EC-C18 column was used for vitamins D2 and D3, providing substantial chromatographic separations with matrix interferences. Both chromatograms are shown in Figure 3.

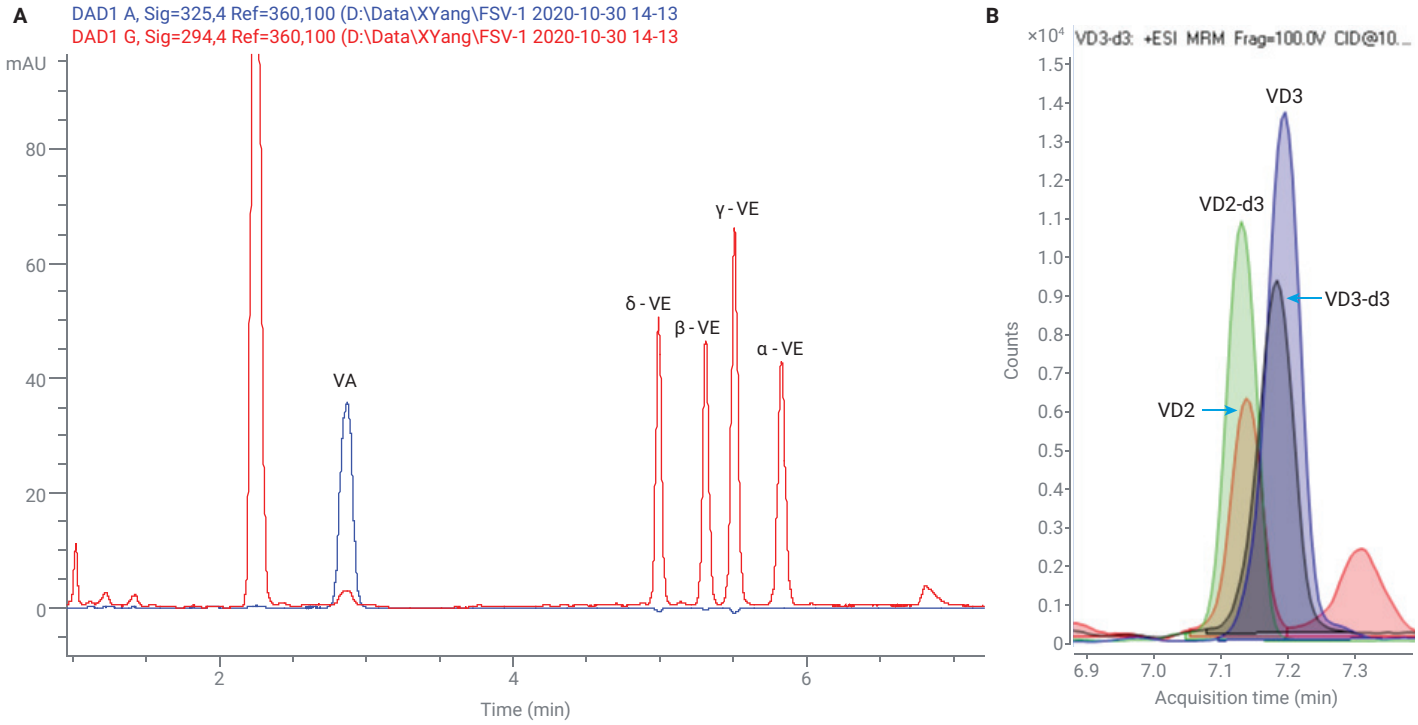
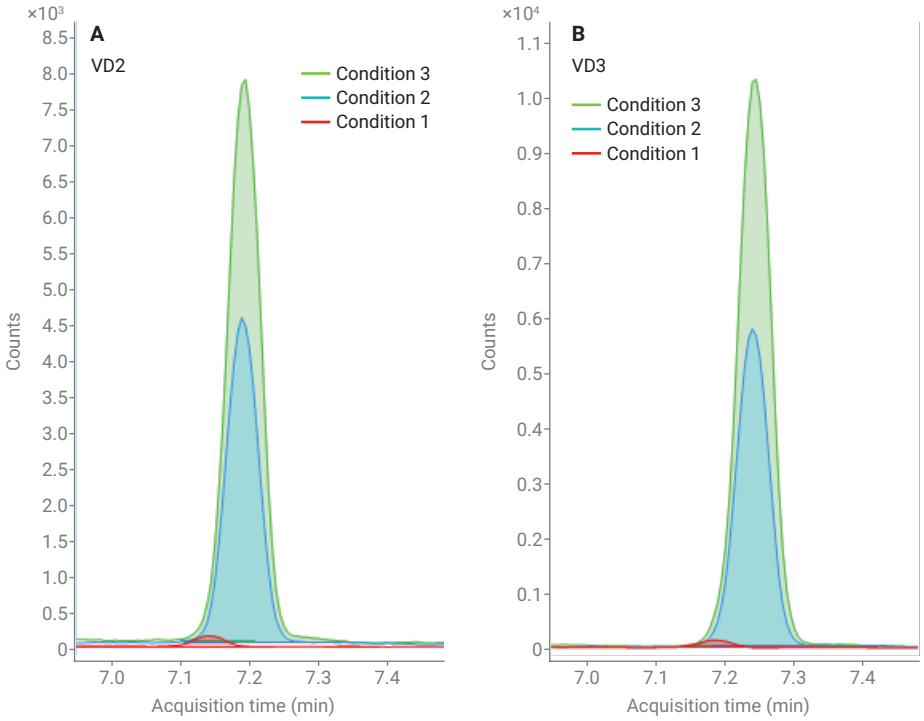


Figure 3. Left: The HPLC chromatogram of vitamin A (325 nm) with 25 µg/g spiking, and *alpha*-, *beta*-, *gamma*-, and *delta*-vitamin E (294 nm) with 250 µg/g spiking in infant formula. Right: The LC/MS/MS chromatogram of vitamins D2 and D3 with 0.8 µg/g spiking and their corresponding internal standards with 0.4 µg/g spiking in infant formula.

Furthermore, the LC/MS/MS sensitivity of vitamins D2 and D3 were significantly improved by using appropriate mobile phase additives, as shown in Figure 4. The addition of 4.5 mM ammonium formate (condition 2) improved the peak intensity dramatically, over formic acid only in the mobile phase (condition 1). Based on condition 2, 0.5 mM ammonium fluoride was further added to improve the analyte ionization efficiency of vitamins D2 and D3 on LC/MS/MS, resulting in doubled responses.



LC/MS Mobile Phase Conditions	Ratio of Peak Height	
	Vitamin D2	Vitamin D3
Condition 1	1	1
Condition 2	30	41
Condition 3	54	73

Figure 4. Peak intensity of vitamins D2 and D3 under different mobile phase conditions. Condition 1: A) MeOH with 0.1% FA; B) H_2O with 0.1% FA; Condition 2: A) MeOH with 0.1% FA, 4.5 mM ammonium formate; B) H_2O with 0.1% FA, 4.5 mM ammonium formate; Condition 3: A) MeOH with 0.1% FA, 4.5 mM ammonium formate and 0.5 mM ammonium fluoride; B) H_2O with 0.1% FA, 4.5 mM ammonium formate and 0.5 mM ammonium fluoride.

The data from LC/MS/MS and HPLC were processed with Agilent MassHunter quantitative analysis software and Agilent ChemStation analytical workstation software, respectively. The neat standard calibration curves are shown in Figure 5. For vitamins A and E, calibration curves were established within the dynamic range of 0.05 to 200 µg/mL with R^2 of 0.999, using unweighted linear regression fit. For vitamins D2 and D3, calibration curves were established within the dynamic range of 1 to 1,000 ng/mL with R^2 of 0.998 and 0.999, respectively, using linear regression fit and 1/x weight.

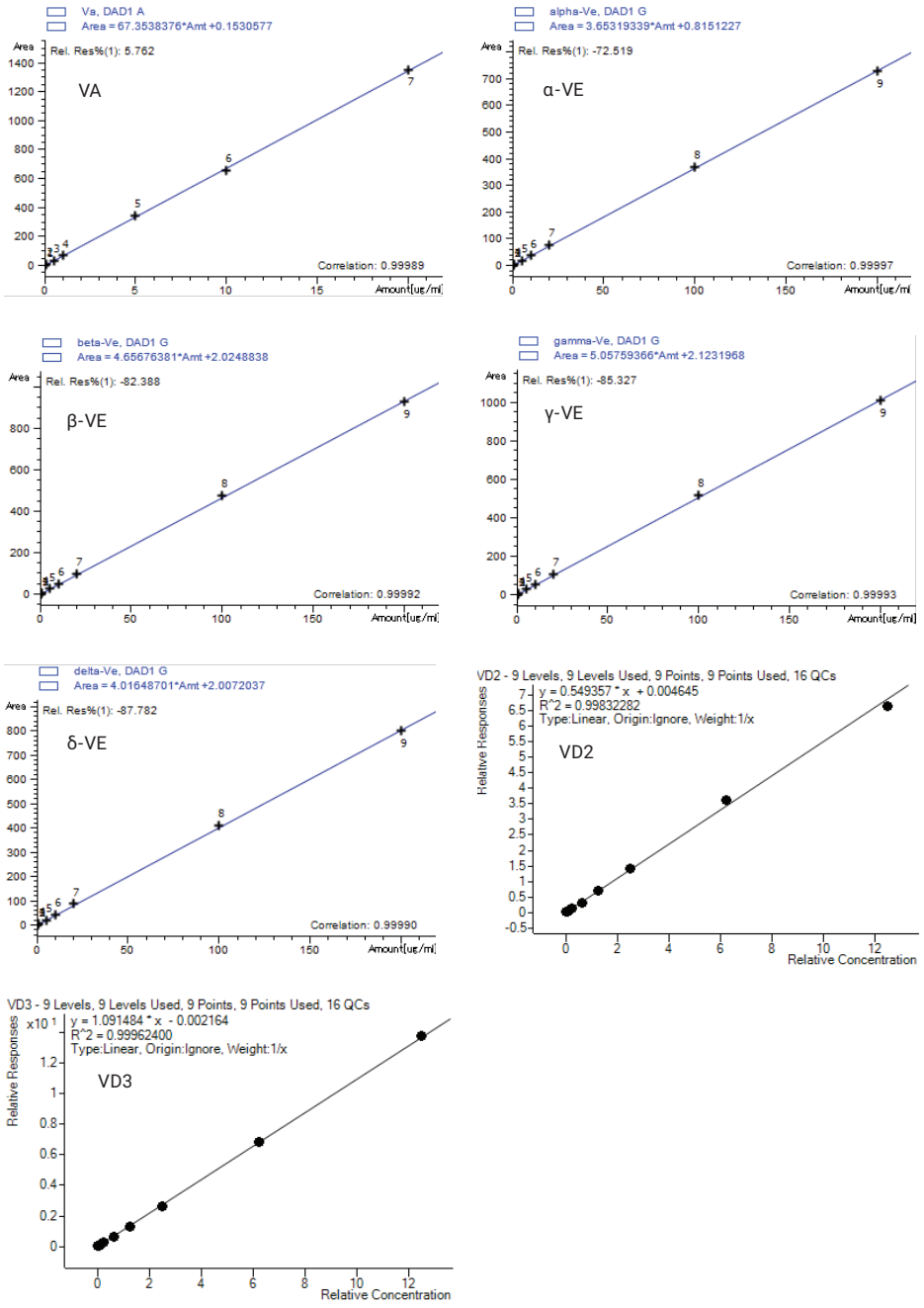


Figure 5. The neat calibration curves of vitamins A and E on HPLC-DAD analysis, and vitamins D2 and D3 on LC/MS/MS analysis.

QC samples with four replicates at low and high spiking levels were tested to evaluate accuracy and precision as shown in Table 1. Due to the widely applied nutrient addition to infant formula, it is not feasible to find a real matrix blank. As a result, analyte quantitation was established based on the neat calibration curves. The matrix effect on LC/MS/MS was corrected with the use of stable labeled-IS for each analyte. The matrix effect on LC-DAD was not an issue as long as good chromatographic separation was observed. The matrix control was preliminarily quantitated, giving the rough concentration of vitamin E at very high levels (a total of 9.8 mg/100 g as α-tocopherol equivalents), while vitamin D3 was shown at a low level (13 µg/100 g). The results confirmed the significant challenges for simultaneous quantitation by either HPLC-DAD or LC/MS/MS. Such results demonstrate the necessity of using HPLC-DAD for high-level quantitation of vitamins A and E, and LC/MS/MS for low level quantitation of vitamins D2 and D3. The two levels of spiking QC samples were then prepared and quantitated for four replicates with matrix control contribution correction. The low-QC samples were spiked at a concentration of 2 to 3 times the matrix control, while the high- QC samples were spiked at a concentration of 4 to 5 times the matrix control. The calculated concentrations were corrected with the matrix control contribution, and the corrected calculated concentration then corresponded to the actual spiking level. Recovery was calculated based on the ratio of the corrected calculated concentration to the actual spiking concentration. All recovery data were above 80%, and RSDs were below 10%.

Table 1. Method quantitation results for vitamins in powdered infant formula at low and high spiking levels (n = 4).

	Matrix		QC-Low					QC-High				
Analytes	Background (µg/g)	RSD% (n = 4)	Spiking Level (µg/g)	Calculated Concentration (µg/g)	Corrected Concentration (µg/g)	Rec%	RSD% (n = 4)	Spiking Level (µg/g)	Calculated Concentration (µg/g)	Corrected Concentration (µg/g)	Rec%	RSD% (n = 4)
VA	5.4	9.1	15.0	19.5	14.1	95.6	8.5	25.0	27.5	22.1	89.9	8.9
VD2	0	0	0.4	0.36	0.36	89.5	2.0	0.8	0.65	0.65	81.2	4.5
VD3	0.13	6.2	0.4	0.46	0.33	87.9	3.4	0.8	0.88	0.75	94.6	2.2
α-VE	87.2	2.4	150.0	229.3	142.1	96.7	8.8	250.0	301.3	214.1	89.4	1.5
β-VE	1.3	6.5	150.0	144.0	142.7	95.2	5.6	250.0	213.3	212.0	84.9	2.2
γ-VE	99.5	1.2	150.0	242.7	143.2	97.3	6.9	250.0	309.3	209.8	88.5	1.5
δ-VE	44.0	6.6	150.0	186.7	142.7	96.2	8.6	250.0	248	204.0	84.4	3.2

Conclusion

This study demonstrates an efficient and robust workflow for fat-soluble vitamin analysis in infant formula using sample saponification followed by Agilent Bond Elut Plexa polymeric SPE for extraction and cleanup. Two separate platforms of HPLC-DAD and LC/MS/MS provided appropriate quantitation levels for the different vitamins in infant formula. The method validation demonstrated excellent recoveries (81.2% to 97.3%) and precision (RSD <8.9%).

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