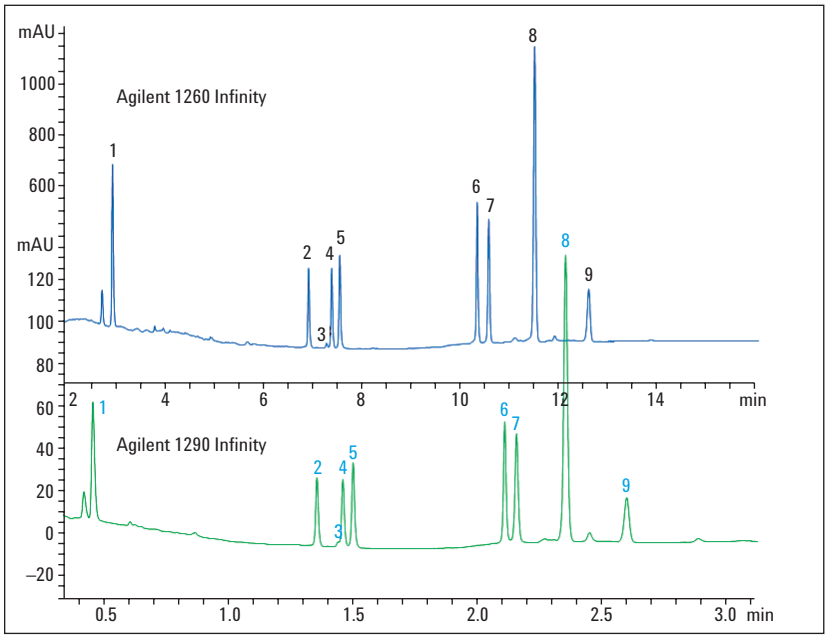
Agilent Application Solution

Analysis of fat-soluble vitamins from food matrix for nutrition labeling

Application Note

Food



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Abstract

This Application Note shows how to carry out qualitative and quantitative analysis of fat soluble vitamins from two different food matrixes. A robust reverse phase high performance liquid chromatographic (RP-HPLC) method for simultaneous determination of nine fat soluble vitamins was developed. Separation and quantification was achieved by an Agilent 1260 Infinity LC System using an Agilent Poroshell EC-C18 column. The advanced feature of the Agilent 1260 Infinity Diode Array Detector (DAD) to select multiple wavelengths was utilized effectively to detect various vitamins at their maximum absorbance. Robustness of the method for routine nutrition labelling analysis was established by partial validation and the method was verified by analyzing vitamin D content from two different food matrices. Finally, using an Agilent 1290 Infinity LC System, this HPLC method was effectively transferred to a short Ultra High Pressure Liquid Chromatographic (UHPLC) method.

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Introduction

Vitamins are nutrients required in trace amounts by an organism for its healthy growth and must be obtained from the diet. In general, vitamins can be classified into two groups, water- or fat-soluble. As the level of vitamins present in a food may vary from nanograms to milligrams, labelling these vitamins in a matrix like infant formula is a mandatory requirement by the US Food and Drug Administration (FDA). The diversity in the chemical nature of vitamins made concurrent extraction and analysis of all vitamins challenging. Due to this, analysis of water soluble and fat soluble vitamins will be usually performed separately. Simultaneous analyses of ten water soluble vitamins are described in another Agilent Application Solution[1]. In this study, simultaneous analysis on nine fat soluble vitamins are discussed. The list of vitamins used in this study covers vitamins A, D, E, and K, which are the common fat soluble vitamins present in food medium. Even though extraction procedures for various fat soluble vitamins are different, developing a single analytical method simplifies the analytical challenge by quantifying the analytes in a single LC run.

In this Application Note, we described an approximately 15 minute long, sensitive, and robust method for simultaneous determination of nine fat soluble vitamins with UV detection. Also, this HPLC method transferred to a 3.5 minute UHPLC method for customers where short analytical times are critical requirement.

Experimental

Instruments and Software

An Agilent 1260 Infinity Quaternary LC System consisting of the following modules was used:

- Agilent 1260 Infinity Quaternary Pump with vacuum degasser (G1311B)
- Agilent 1260 Infinity High-Performance Autosampler (G1367E)
- Agilent 1260 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Diode Array Detector (G4212B) with 60-mm Max-Light flow cell

The UHPLC analysis was developed and performed using an Agilent 1290 Infinity LC System consisting of the following modules:

- Agilent 1290 Infinity Binary Pump with integrated vacuum degasser (G4220 A) and 100-µL Jet Weaver mixer
- Agilent 1290 Infinity High Performance Autosampler (G4226A)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C):
- Agilent 1290 Infinity Diode Array Detector (G4212A) with 10-mm Max-Light flow cell

Columns:

- Agilent Poroshell 120 EC-C18 columns with internal diameters of 2.1 mm and lengths of 75 mm, packed with 2.7-µm particles (697775-902)
- Agilent Poroshell 120 EC-C18 column 3.0 × 150 mm, 2.7-µm (693975-302)

Software:

- Agilent ChemStation B.04.02

Reagents and materials

All the chemicals and solvents used were HPLC grade. Highly purified water was used from a Milli Q water purification system (Millipore Elix 10, Millipore, USA). Acetonitrile and methanol were of super gradient grade and were purchased from Lab-Scan (Bangkok, Thailand) and biotech grade tetra hydro furan (THF) was purchased from Sigma (Germany). Eluent additive grade acetic acid was purchased from Fluka (Germany) and all other chemicals used in this study were from Aldrich (India). Standards of menadione (K3), Linolenic acid (6-omega fatty acid), Retinol (alcohol form of vitamin A), retinoic acid (acid form of vitamin A), 9-cis retinal (aldehyde form of vitamin A), vitamin K2, Cholecalciferol (D3), tocopherol (a form of vitamin E), and vitamin K1 were purchased from Aldrich (India). The infant formula and multivitamin tablets used in this study for recovery and nutrition labelling analysis were purchased locally.

Chromatographic parameters

Chromatographic parameters used for reverse phase liquid chromatography and UHPLC are shown in Table 1.

Parameter	Agilent 1260 Infinity Quaternary LC system	Agilent 1290 Infinity LC system
Column oven:	45 °C	45 °C
Acquisition rate:	20 Hz	80 Hz
Data acquisition:	216, 246, 266, 326, 356, 376 nm	216, 246, 266, 326, 356, 376 nm
Flow cell:	60 mm path	10 mm path
Injection volume:	5 µL (needle with wash, flush port active for 5 seconds)	1 µL (needle with wash, flush port active for 3 seconds)
Sample thermostat:	5 °C	5 °C
Mobile phase A:	95:5; water:THF with 0.05% acetic acid	95:5; water:THF with 0.05% acetic acid
Mobile phase B:	75:25:5; Acetonitrile:Methanol:THF with 0.035% acetic acid	75:25:5;Acetonitrile:Methanol: THF with 0.035% acetic acid
Gradient:	At 0 min → 30%B At 3 min → 75%B At 8 min → 100%B At 15 min → 100%B At 15.1 min → 30%B	At 0 min → 50%B At 0.6 min → 75%B At 1.7 min → 100%B At 3.4 min → 100%B At 3.5 min → 50%B
Post run time:	5 minutes	1 minute
Flow rate:	0.9 mL/min	0.9 mL/min

Table 1
Chromatographic parameters used for the Agilent 1260 Infinity LC system and the Agilent 1290 Infinity LC system.

Fat soluble vitamin standard solution

Vitamin standards of menadione, linolenic acid, retinol, retinoic acid, retinal, vitamin K1, K2, D3, and tocopherol were prepared individually by weighing approx. 50 mg of the vitamin powder and transferring it to a 25-mL volumetric standards flask. Mobile phase B was added to form a stock solution of 2.0 mg/mL (2,000 ppm). Sonication was used when required. Fat-soluble vitamin stock solutions were wrapped with aluminum foil and stored at + 4.0 °C in the dark when not in use.

Mixed standard solution and linearity levels

About 100 µL of each standard were precisely mixed to get a 1,000-µL standard mix of fat soluble vitamins at concentration of 200 ppm each. Linearity levels were prepared by subsequent dilution of this 200-ppm standard spike mix, using mobile phase B as diluent. The linearity standard solutions were covering a range of 5 pg/µL to 100 ng/µL (10 levels and 6 replicates)

Sample preparation for nutrition labelling and recovery studies

Two different types of samples, infant formula and vitamin tablets were used for nutrition labelling and recovery studies. Vitamin D was the analyte used in this study to evaluate recovery efficiency and further perform nutrition labelling analysis. Vitamin D from 5-g infant formula was extracted by alkaline hydrolysis, using ethanolic potassium hydroxide and the refluxing time was about 45 minutes[2]. Pyrogallol was used as an antioxidant during the refluxion. A 10-mL mixture of hexane and diethyl ether in the ratio 1:1 was used to extract vitamin D from the saponified digest. A 1.0 mL amount of this organic layer was evaporated to dryness after successive washing with water and further reconstituted with 200 µL mobile phase B and injected in HPLC for nutrition labelling analysis. Recovery studies were performed using spiked and unspiked samples of infant formula. An on-column concentration of 25 ng vitamin D standard was used for sample spiking. Extraction procedure was same as before.

In the case of multi vitamin tablets, recovery and nutrition labelling studies were carried out from one tablet.

Precautions

Vitamins are known to be highly sensitive to light and heat. To extend the stability in solution form, all the prepared solutions were wrapped in aluminum foils and stored in a refrigerator at 4 °C in the dark, when not in use. The thermosttated autosampler tray was maintained at 4 °C during the analysis. Extractions of vitamin D from samples were performed under dark conditions.

Procedure

5 µL of mobile phase B was injected as blank and followed by each linearity level in six replicates. Area and retention time (RT) information of each level were utilized to calculate standard deviation (SD) and relative standard deviation (RSD) values. LOD and LOQ were established from the lower linearity level injections. Average area of vitamin peaks in each linearity levels was plotted against the concentration to construct linearity curves.

Six critical method parameters were changed to evaluate the robustness of the method. A standard spike mix was injected in six replicates and data was used for robustness study.

For nutrition labelling analysis, amount of vitamin-D present in infant formula and multivitamin tablet were quantified and compared with the label value.

Recovery studies using infant formula and multivitamin tablets were performed by injecting with or without spiking 25 ng vitamin D standard to 5 g or per tablet respectively.

The method was effectively transferred to UHPLC. LOD, LOQ, and linearity of each vitamin were evaluated and precision of the method was established by area and RT RSD. Linearity curves for all vitamins using UHPLC method were also plotted.

Results and Discussion

Separation and detection

Excellent separation of nine fat soluble vitamins in 13 minutes was achieved using an Agilent Poroshell 120 EC-C18 (3.0 mm × 150 mm, 2.7 µm) column. The absorbance maximum was found to be different for different vitamins due to the diversity in molecular structures. The chromatographic elution patterns of nine vitamins are shown in Figure 1 and the list of vitamins with individual absorbance maxima are tabulated in Table 2. The observed base line drift at 216 nm can be explained by the change in amount of modifier in the mobile phase during the gradient run. We used the peak purity feature in the ChemStation software to check the purity of each peak, and thus specificity of method was evaluated. Precision, linear range, accuracy, specificity, recovery, and robustness studies were used to validate the method.

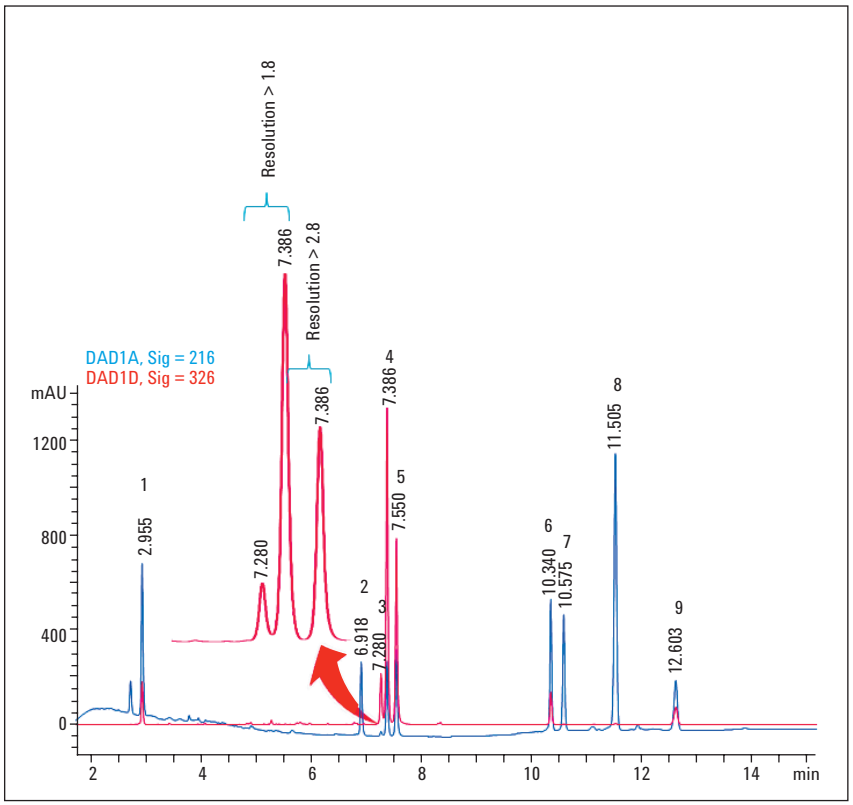

Figure 1
Separation of nine fat soluble vitamins using a 15-cm Agilent Poroshell 120 EC-C18 column. The excellent separation of three different forms of vitamin A is shown in the insert.

Sl no.	Name	Synonym	Absorbance max.	RT
1	Menadione	vitamin k3	250	2.965
2	Linolenic acid	6-omega fatty acid	< 216	6.918
3	Retinol	vitamin A alcohol	326	7.280
4	Retinoic acid	vitamin A acid	356	7.386
5	9-cis Retinal	vitamin A aldehyde	376	7.550
6	Vitamin K2	vitamin K2	246	10.340
7	Cholecalciferol	vitamin D3	266	10.575
8	Tocopherol	a form of vitamin E	< 220	11.505
9	Vitamin K1	vitamin k1	246	12.603

Table 2
List of vitamins used in this study and observed absorbance maxima for each vitamin.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The analyte concentration that provides a signal to noise ratio (S/N) of > 3 was considered as LOD and analyte concentration with S/N > 10 was considered as LOQ. Observed LOD and LOQ value of each vitamin are tabulated in Table 3. As an example, overlay of LOD, LOQ chromatograms of cholecalciferol (Vitamin D) with blank is shown in Figure 2.

Linearity

All the prepared linearity levels were injected in six replicates and linearity curves for each vitamin were constructed from the LOQ level to a highest concentration level in the study using area response and concentration values. This linearity range covers the usual vitamin content values in common food matrices. The observed regression coefficients for all vitamins were also tabulated in Table 3. The linearity curve for cholecalciferol is shown as an example in Figure 3.

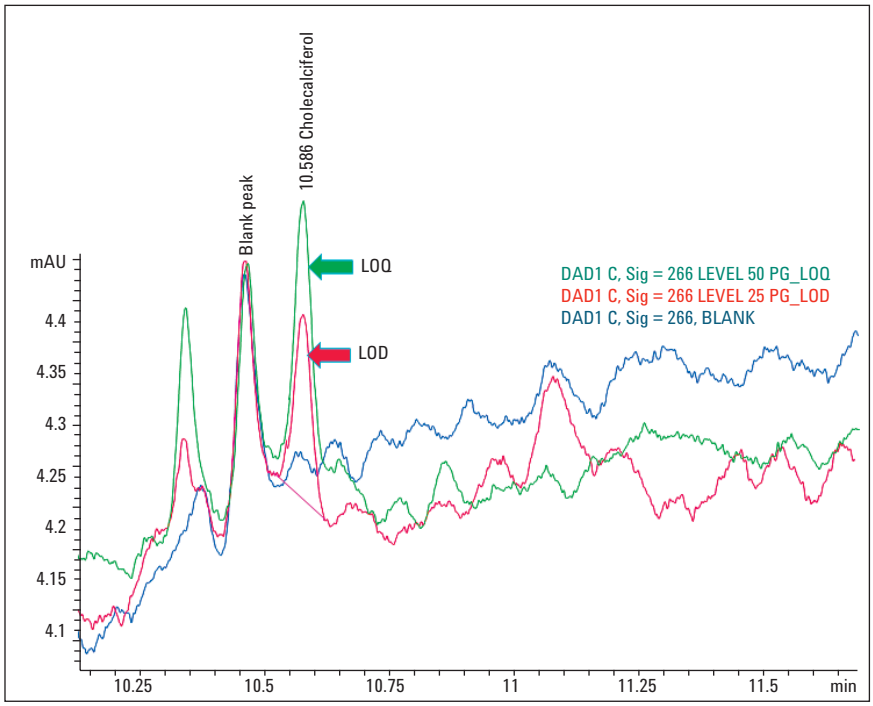

Figure 2
LOD (25 pg) and LOQ (50 pg) chromatograms of cholecalciferol (vitamin D) overlaid with blank.

Sl no.	Name	LOD (ng)	LOQ (ng)	Linearity range (ng) on-column	R^2 value	Total levels, replicates = 6	Linearity equation
1	Menadione	0.025	0.05	0.05–250	0.99997	10	y = 33.0129976 x + 6.4314273
2	Linolenic acid	1.5	2.5	2.5–500	0.99989	6	y =1.86128287 x + 0.1410729
3	Retinol	0.5	1.5	1.5–500	0.99990	7	y = 0.60131392 x + 1.2872412
4	Retinoic acid	0.025	0.05	0.05–250	0.99987	10	y = 40.3653646 x + 70.38627
5	Retinal	0.025	0.05	0.05–250	0.99956	10	y = 42.7776135 x + 50.084652
6	Vitamin K2	0.025	0.05	0.05–500	0.99996	11	y = 15.2586556 x – 16.251493
7	Cholecalciferol	0.025	0.05	0.05–500	1.00000	11	y = 17.9806249 x – 5.667246
8	Tocopherol	5	25	25–500	0.99896	4	y = 9.74071376 x – 229.06229
9	Vitamin K1	0.05	0.15	0.15–500	0.99989	10	y = 14.6792864 x – 19.932064

Table 3
LOD, LOQ, and linearity results of all nine vitamins.

Precision of retention time (RT) and area

To establish the method precision, relative standard deviation (RSD) values for retention time (RT) and area of all nine vitamins at 50 ng on-column concentration were calculated. The highest observed area RSD value was 0.035% and RT RSD was 0.04%. Graphical representation of area and RT RSD values of nine vitamins are shown in Figure 4.

Robustness

Robustness of the method was evaluated by deliberately varying six critical method parameters and the resulting deviation in area and retention time was calculated and compared to the original method. As a sample, a standard spike mix solution of vitamins was injected in six replicates. Allowed deviation for retention time and area was set to ± 3% and ± 5% respectively. Results from robustness study are summarized in Table 4.

The deviation of retention time is within the allowed limit for robustness studies with increasing flow rate, mobile phase composition and column temperature. However, the impact of gradient steepness variation was higher and observed RT deviations were above the limit. Area deviations for eight vitamins were found to be within the limit for majority of tests, except for vitamin K2. The area of vitamin K2 was found to be very sensitive to flow rate, gradient steepness, column temperature, mobile phase composition, and detection wavelength. Another parameter which needs to be carefully controlled is the detection wavelength. Robustness results indicate that, the method is reliable to use for normal usage and, to a great extent, the performance remains unaffected by deliberate change in parameters.

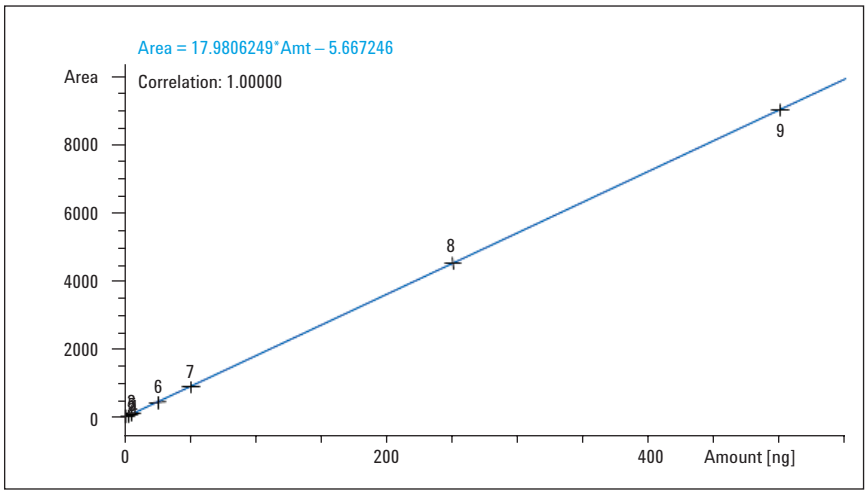

Figure 3
Linearity curve of cholecalciferol from 0.05 ng to 500 ng (on-column concentration) showing excellent coefficient value.

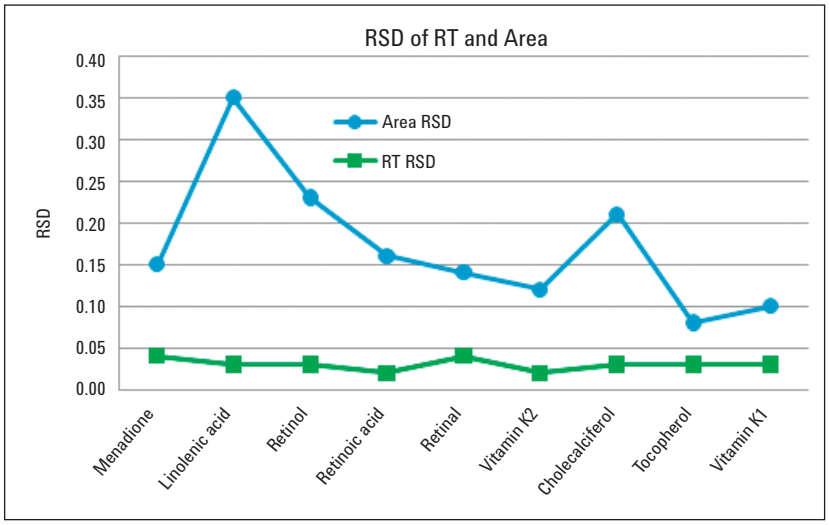

Figure 4
Excellent RT and area RSD values for all vitamins at 50 ng on-column concentration.

Sl no.	Parameter (actual value)	Measured deviation	Modified value	RT deviation (Allowed limit is ± 3.0%)	Area deviation (Allowed limit is ± 5.0%)
1	Flow rate (0.9)	2%	0.92 mL/min	Passed	Passed for 8 compounds
			0.88 mL/min	Passed for 8 compounds	Passed for 6 compounds
2	Injection volume (5 µL)	2%	5.1 µL	Passed	Passed for 8 compounds
			4.9 µL	Passed	Passed for 8 compounds
3	Gradient steepness (5, 75 to 100 in 5 minutes)	10%	4.5 (77.5 to 100 in 5 minutes)	Passed for 5 compounds (Failed for 4 compounds which are eluting in the initial gradient time frame)	Passed for 8 compounds
			5.5 (72.5 to 100 in 5 minutes)	Passed for 5 compounds (Failed for 4 compounds which are eluting in the initial gradient time frame)	Passed for 8 compounds
4	Mobile phase composition (ACN 75%)	5%	Acetonitrile 80% (80:15:5)	Passed	Passed for 7 compounds
			Acetonitrile 70% (70:25:5)	Passed	Passed for 8 compounds
5	Column temperature (45 °C)	5%	47.2 °C	Passed	Passed for 8 compounds
			42.8 °C	Passed	Passed for 8 compounds
6	Wavelength (216, 246, 266, 326, 356, 376 nm)	3 nm	(219, 249, 269, 329, 359, 379 nm)	Passed	Passed for 6 compounds
			(213, 243, 263, 323, 353, 373 nm)	Passed	Passed for 5 compounds

Table 4
Robustness test result summary.

Recovery of vitamin D from sample matrix

Recovery analyses for vitamin D from multi vitamin tablets or infant formula were carried out by a standard addition method[3]. A standard solution of vitamin D at 25 ng/µL was used for this analysis. In this study, the area of the vitamin D peak in the spiked sample, unspiked sample and standard chromatogram were measured separately. The difference in detector response between spiked and unspiked sample was compared against response observed in standard chromatogram and expressed in percentage as recovery. The recovery results from multivitamin tablets and infant formula were 94% and 62% respectively. The low recovery value of vitamin D from infant formula can be justified by the challenge involved in vitamin extraction from a matrix containing a high amount of proteins and fat. The chromatograms observed for spiked, unspiked, and standard vitamin solutions are shown in Figure 5.

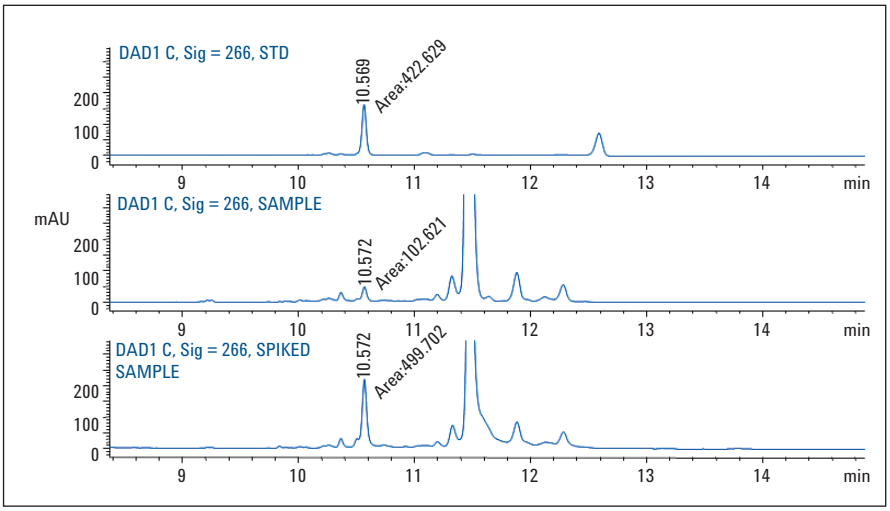

Figure 5
Overlay of spiked, unspiked, and standard chromatograms used for multivitamin tablets.

Nutrition labelling

In this study, the amount of vitamin D present in infant formula and multivitamin tablets was estimated using the area response and compared with the concentration claimed on the label. Linearity equations originated from linearity curves were used for the calculation. Extraction was carried out using ethanolic KOH refluxion. The label claim for vitamin D in infant formula and multivitamin tablets were 8.3 µg/100 g and 10 µg/tablet respectively. The calculated values with recovery corrections were 6.0 µg/100 g and 12.0 µg/tablet respectively. The results show excellent suitability of the method to quantify vitamins in food sample.

UHPLC Method

A UHPLC method with diode array detection was developed for the separation of nine fat soluble vitamins. The resulting UHPLC method shows excellent resolution and saves about 66% percent analysis time and solvent compared to the 15 minutes long HPLC gradient (Figure 6). The Retinol peak is slightly co-eluting with the retinoic acid peak. This will not be a problem for analyzing vitamins, because in a given sample matrix, the possibility of vitamin A being present in the alcohol (retinol) and acid (retinoic acid) form simultaneously is very rare. The LOD and LOQ levels and linearity of each vitamin, except for retinol, was established using the UHPLC method. The observed LOD, LOQ and linearity results are tabulated in Table 5. Excellent linearity observed for vitamin D is shown in Figure 7. RSD values for RT and area for an on-column concentration of 100 ppm with an injection volume of 1 µL was calculated. The highest observed RSD for area was 1.25% and that for RT was 0.12%. The results can be graphically represented as in Figure 8. Low RSD values for area and RT confirmed precision of the method.

These results prove the reliability and sensitivity of the developed UHPLC method. Quick nutrition labeling analysis of food samples is possible using this method.

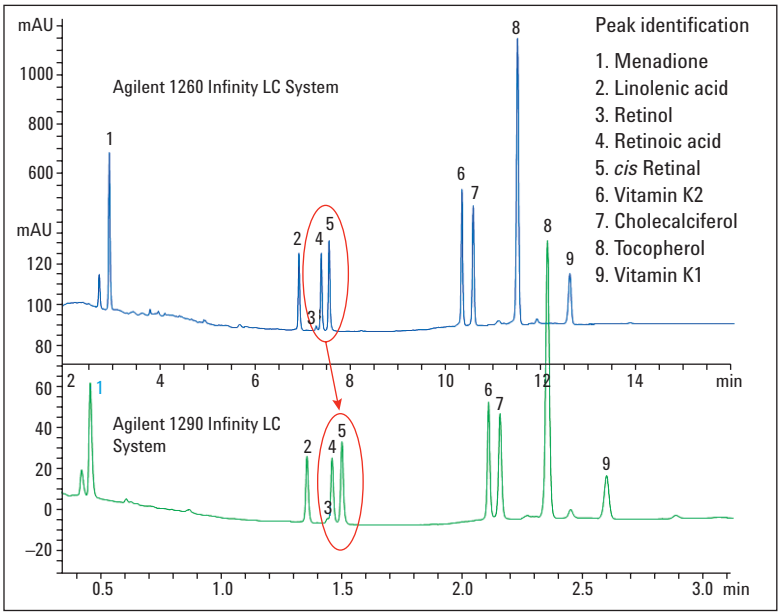

Figure 6
Overlay of separation of nine fat soluble vitamins using HPLC method on Agilent 1260 infinity and UHPLC method on 1290 Infinity LC system.

Sl no.	Name	LOD (ng)	LOQ (ng)	Linearity range (ng) on-column	R^2 value	Total levels, replicates = 6
1	Menadione	0.025	0.05	0.05–200	0.99995	10
2	Linolenic acid	0.5	1	1–500	0.99990	7
3	Retinoic acid	0.025	0.05	0.05–200	0.99999	10
4	Retinal	0.05	0.1	0.1–200	1.00000	9
5	vitamin K2	0.05	0.1	0.1–500	0.99999	10
6	Cholecalciferol	0.05	0.1	0.1–500	0.99998	10
7	Tocopherol	0.5	1	1–500	0.99966	7
8	vitamin K1	0.1	0.25	0.25–500	0.99990	9

Table 5
LOD and LOQ values derived from the UHPLC method using Agilent 1290 Infinity LC system.

Conclusion

Nine fat soluble vitamins were separated and quantified using an Agilent Poroshell 120 EC-C18 column. Using the Agilent 1260 Infinity LC System, a robust, 15 minute long, HPLC gradient method was developed. The method can be used successfully to quantify vitamins like menadione, Linolenic acid, various form of vitamin A, D, E, and K. The linearity range of the current study is broad to accommodate various possible concentrations of these vitamins in many food matrices. Gradient conditions ensured better chromatographic resolution improved sensitivity, and less matrix interference. The method is simple, specific, sensitive, rapid, and also provides good precision, linearity and recovery values. Efficient use of this method was demonstrated by quantifying vitamin D from infant formula and multivitamin tablet matrix. Later, this method was transferred to a short 3 minute UHPLC method using the Agilent 1290 Infinity LC System which in turn saves 66% of analysis time and solvent. These methods using the Agilent 1260 Infinity LC and Agilent 1290 Infinity LC systems can be applied for accurate routine nutrition labeling analysis of fat soluble vitamins.

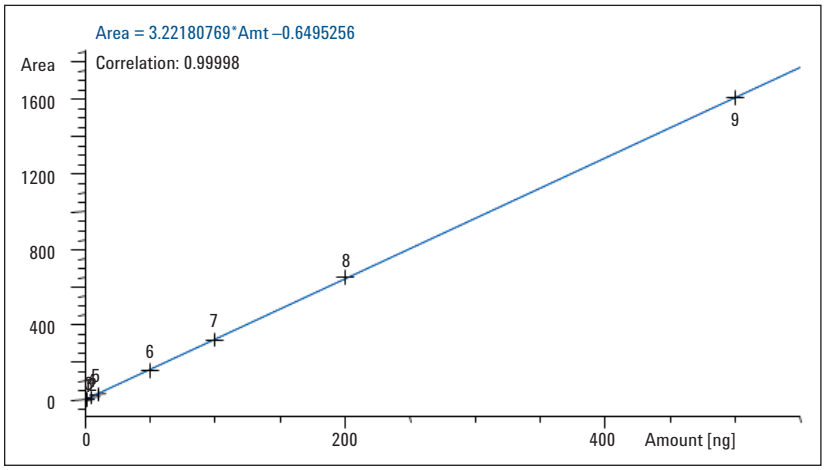

Figure 7
Linearity of vitamin-D from 0.05 ng to 500 ng showing a correlation of 0.99998 (9 levels and six replicates). Injection volume is 1 µL.

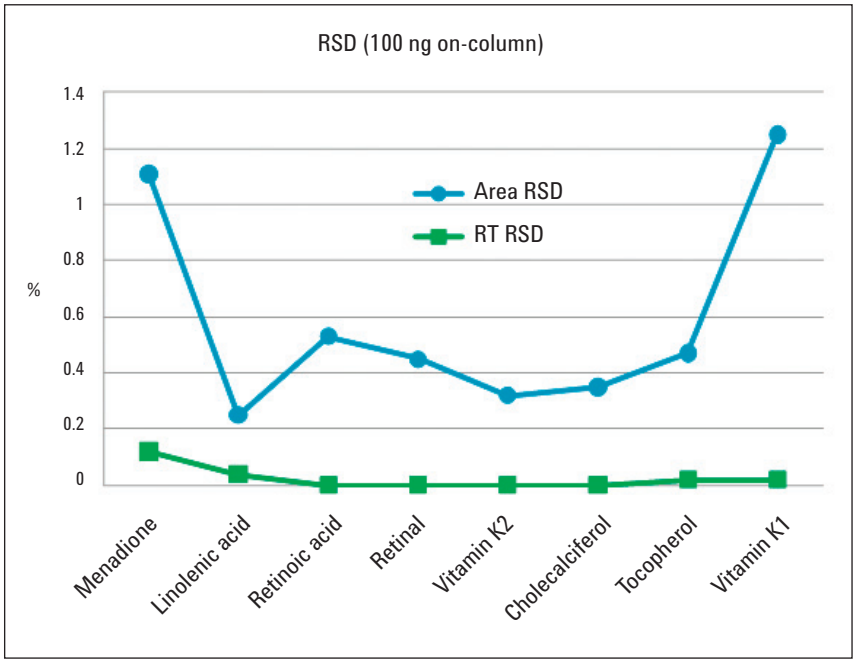

Figure 8
Area and RT RSD values from UHPLC results for all vitamins except retinol at an on-column concentration of 100 ppm. Injection volume is 1 µL and six replicates.

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1.
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