Application Note

Food & Beverage Testing

Simultaneous Analysis of Vitamins B7, B9, and B12 in Infant Formula

Using an Agilent 1260 Infinity II Prime LC system with diode array detection

Authors

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Abstract

Infant formula typically contains the essential vitamins B7, B9, and B12 in small quantities, with B12 often present in particularly low levels compared to other B vitamins. The small concentrations and the complexity of the infant formula matrix make these vitamins challenging to detect and measure. This application note describes the simultaneous analysis of vitamins B7, B9, and B12 in infant formula using an Agilent 1260 Infinity II Prime LC coupled with diode array detection. The analytical method was developed with the assistance of the 60 mm Agilent InfinityLab Max-Light cartridge flow cell, which enhances the detection sensitivity of these three B vitamins. The method was demonstrated to be robust, showing excellent recovery of B vitamins from National Institute of Standards and Technology (NIST) standard reference material (SRM).

Introduction

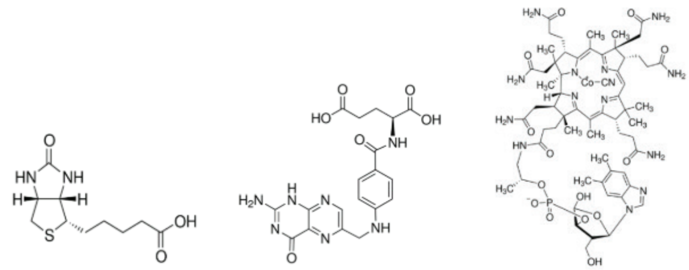
Vitamins B7, B9, and B12 are essential B-complex vitamins that play crucial roles in various bodily functions. Vitamin B7 (biotin) is vital for the metabolism of carbohydrates, fats, and proteins. Vitamin B9 (folic acid) is important for the synthesis and repair of DNA. Vitamin B12 (cyanocobalamin) is involved in the production of red blood cells. However, the human body cannot produce these vitamins in sufficient quantities on its own, necessitating their intake from dietary sources of animal origin or through supplementation. Deficiencies in these vitamins can negatively impact health. Therefore, their concentrations are highly regulated in foods such as infant formula. The U.S. Food and Drug Administration (FDA) has published a reference guide outlining the daily values for each B vitamin, as shown in Table 1.[1] Robust analytical techniques are imperative for accurately quantifying these essential compounds.

Table 1. FDA daily values for B vitamins.

Nutrient	Daily Value (mg)
Thiamin (Vitamin B1)	1.2
Riboflavin (Vitamin B2)	1.3
Niacin (Vitamin B3)	16
Pantothenic Acid (Vitamin B5)	5
Pyridoxine (Vitamin B6)	1.7
Biotin (Vitamin B7)	0.03
Folic acid (Vitamin B9)	0.4
Cyanocobalamin (Vitamin B12)	0.0024

In the past, microbiological assays were commonly used to measure the concentration of B vitamins in foods due to their high sensitivity. However, with the advancement of technologies, high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection has emerged as an alternative analysis approach.[2]

In this study, a single analytical method has been developed for the simultaneous analysis of vitamin B7, B9, and B12 content in infant formula using an immunoaffinity column (IAC) as a matrix cleanup tool. The structures of vitamin B7, B9, and B12 are depicted in Figure 1. The 1260 Infinity II Prime LC system, with a high-pressure limit of 800 bar, is an excellent choice for coupling with a small particle size column to achieve exceptional resolution and sensitivity. The use of a 60 mm InfinityLab Max-Light cartridge flow cell in a diode array detector (DAD) further enhances the detection of vitamin B7, B9, and B12, which are typically present in lower amounts compared to other B vitamins.



Biotin (B7) Folic acid (B9) Cyanocobalamin (B12)

Figure 1. Structure of vitamins B7, B9, and B12.

Experimental

Instrumentation

The 1260 Infinity II Prime LC system comprises the following modules, shown in Table 2.

Table 2. Agilent 1260 Infinity II Prime LC system instrumentation.

Part Number	Instrument
G7104C	1260 Infinity II flexible pump
G7167A	1260 Infinity II multisampler
G7116A	1260 Infinity II multicolumn thermostat
G7117B	1290 Infinity II DAD with 60 mm InfinityLab Max-Light cartridge flow cell (part number G4212-60007)

Chromatographic conditions

Table 3 describes the analysis conditions.

Table 3. Chromatographic conditions of infant formula analysis.

Parameter	Value
Mobile Phase	A) 0.0125% Formic acid in water B) 0.0125% Formic acid in acetonitrile
Gradient	Time (min) %B 0.00 8 8.00 17 8.01 70 9.49 70 9.50 8
Flow Rate	0.5 mL/min
Stop Time	9.5 min Run time and 4.5 min re-equilibration
Injection Volume	35 µL, Sample temperature was kept at 8 °C
Column	Agilent InfinityLab Poroshell 120 SB-Aq, 3.0 × 100 mm, 2.7 µm (p/n 685975-314)
Column Temperature	40 °C
Heat Exchanger	Agilent InfinityLab Quick Connect heat exchanger, large id (part number G7116-60051)
DAD Wavelength	205 nm (Vitamin B7) 268 nm (Vitamin B9) 361 nm (Vitamin B12)
Data Rate	10 Hz

Software

Agilent OpenLab CDS version 2.7 was used for data acquisition and interpretation.

Chemicals and solvents

The chemicals and solvents that were used in the analysis were as follows:

- Acetonitrile (HPLC grade, purchased from Aik Moh Paints & Chemicals, Singapore)
- Methanol (HPLC grade, purchased from Aik Moh Paints & Chemicals, Singapore)
- Formic acid (LC/MS grade, purchased from Aik Moh Paints & Chemicals, Singapore)
- De-ionized water (Milli-Q, purchased from MilliporeSigma, Burlington, MA)
- Sodium L-ascorbate (analytical grade, purchased from Sigma-Aldrich, St. Louis, MO)
- Ammonium hydroxide (ACS grade, purchased from Sigma-Aldrich, St. Louis, MO)
- Pepsin (purchased from Sigma-Aldrich, St. Louis, MO)
- Sodium phosphate monobasic dihydrate (analytical grade, purchased from Sigma-Aldrich, St. Louis, MO)
- Sodium phosphate dibasic anhydrous (analytical grade, purchased from Sigma-Aldrich, St. Louis, MO)
- Phosphate buffered saline (PBS) (purchased from Sigma-Aldrich, St. Louis, MO)
- Biotin (analytical grade)
- Folic acid (analytical grade)
- Cyanocobalamin (analytical grade)

Samples

Two samples were used in this study:

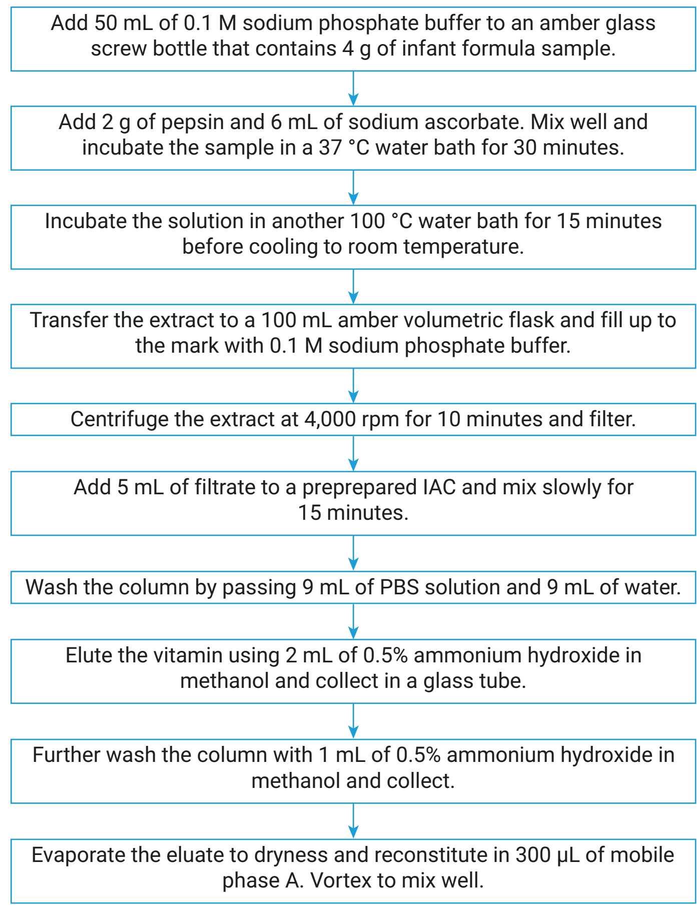
- Infant formula NIST 1869 SRM
- Infant formula purchased from a local grocery store

Sample preparation consumables

EASI-EXTRACT MULTI-VIT B (LGE) IAC from R-Biopharm was used for the sample matrix cleanup.[3,4,5]

Typical sample preparation procedure

The sample preparation procedure for infant formula is shown in Figure 2.



Note: Infant formula sample size, final volume of extract in 0.1 M sodium phosphate buffer, and volume of filtrate loaded into the IAC were adjusted based on the expected vitamin B content.

Figure 2. Sample preparation procedure for infant formula.

Results and discussion

Method validation

Limit of quantification (LOQ): The LOQ of an analyte is commonly defined as the concentration at which the signal-to-noise ratio (S/N) of the analyte is observed to be 10. In this study, the LOQ, estimated from the lower concentration of the B vitamin standard solution, is tabulated in Table 4.

Table 4. LOQ of each B vitamin.

Vitamin	LOQ (ng/mL)
B7	36.1
B9	0.3
B12	1.5

Linearity: A series of standard solutions with varying concentrations of B vitamins were injected to construct the calibration curves. These curves demonstrated excellent linearity, with correlation coefficients (R^2) of 1. Table 5 details the calibration parameters for each B vitamin.

Table 5. Calibration parameters of each B vitamin.

Vitamin	Calibration Level (ng/mL)	R^2
B7	50 to 5,000	1.000
B9	50 to 5,000	1.000
B12	5 to 500	1.000

Repeatability: Six consecutive injections of a B vitamin standard solution were performed to assess injection repeatability, evaluated based on the relative standard deviation (RSD) of retention time (RT) and peak area. The repeatability was remarkable, with an RT RSD of less than 0.1% and a peak area RSD of less than 2%. Detailed results, including the injected concentration of each B vitamin, are provided in Table 6. Figure 3 presents the representative chromatogram of each B vitamin.

Table 6. Repeatability data (n = 6) of each B vitamin.

	Vitamin B7 (50 ng/mL)		Vitamin B9 (0.5 ng/mL)		Vitamin B12 (3 ng/mL)	
n	RT (min)	Peak Area	RT (min)	Peak Area	RT (min)	Peak Area
1	4.586	15.526	5.608	0.501	7.975	1.073
2	4.588	15.255	5.616	0.502	7.981	1.062
3	4.589	15.026	5.611	0.510	7.984	1.075
4	4.591	15.580	5.614	0.526	7.984	1.077
5	4.595	15.801	5.620	0.512	7.989	1.062
6	4.595	15.775	5.619	0.506	7.989	1.079
Average	4.591	15.494	5.615	0.510	7.984	1.071
RSD (%)	0.08	1.95	0.08	1.79	0.07	0.70

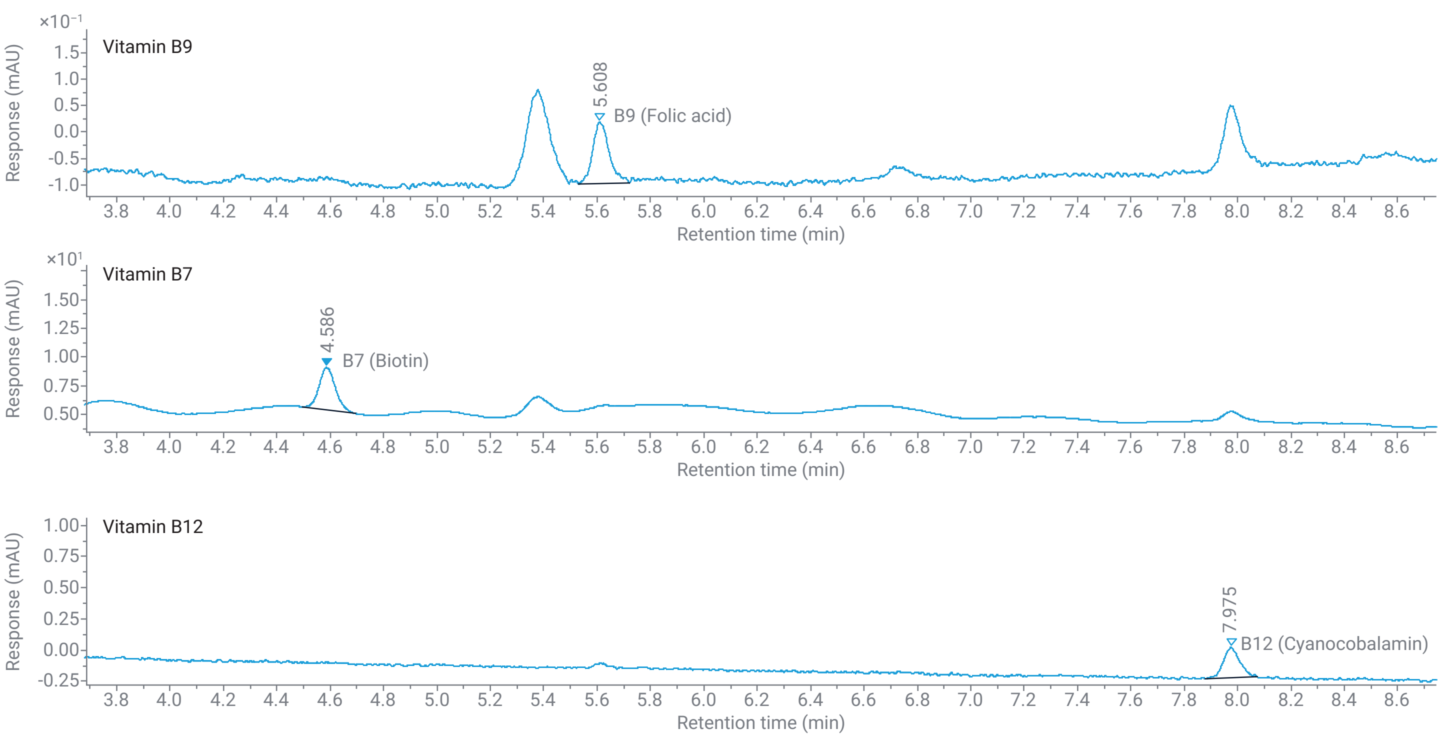


Figure 3. Representative chromatograms of vitamins B7, B9, and B12.

Recovery: Vitamin B7, B9, and B12 content in NIST 1869 SRM was analyzed to assess the method recovery. The recovery specification provided by the vendor for these three B vitamins using the IAC was between 80 and 110%. Detailed analysis results are presented in Table 7. The recovery of vitamins B9 and B12 from NIST 1869 SRM falls within the specification range, except for vitamin B7, which achieved a recovery of 71%. Nevertheless, the recovery of vitamin B7 aligns with the data provided in the validation report (infant formula NIST 1849a SRM), where the recovery ranges from 70 to 85%. Figure 4 shows the chromatograms of B vitamins in NIST 1869 SRM.

Table 7. Vitamin B content in NIST 1869 SRM.

Vitamin	Analysis Results (µg/g)	Reference Value (µg/g)	Recovery (%)
B7	1.34	1.89	70.90
B9	2.07	2.24	92.41
B12	0.0361	0.0447	80.76

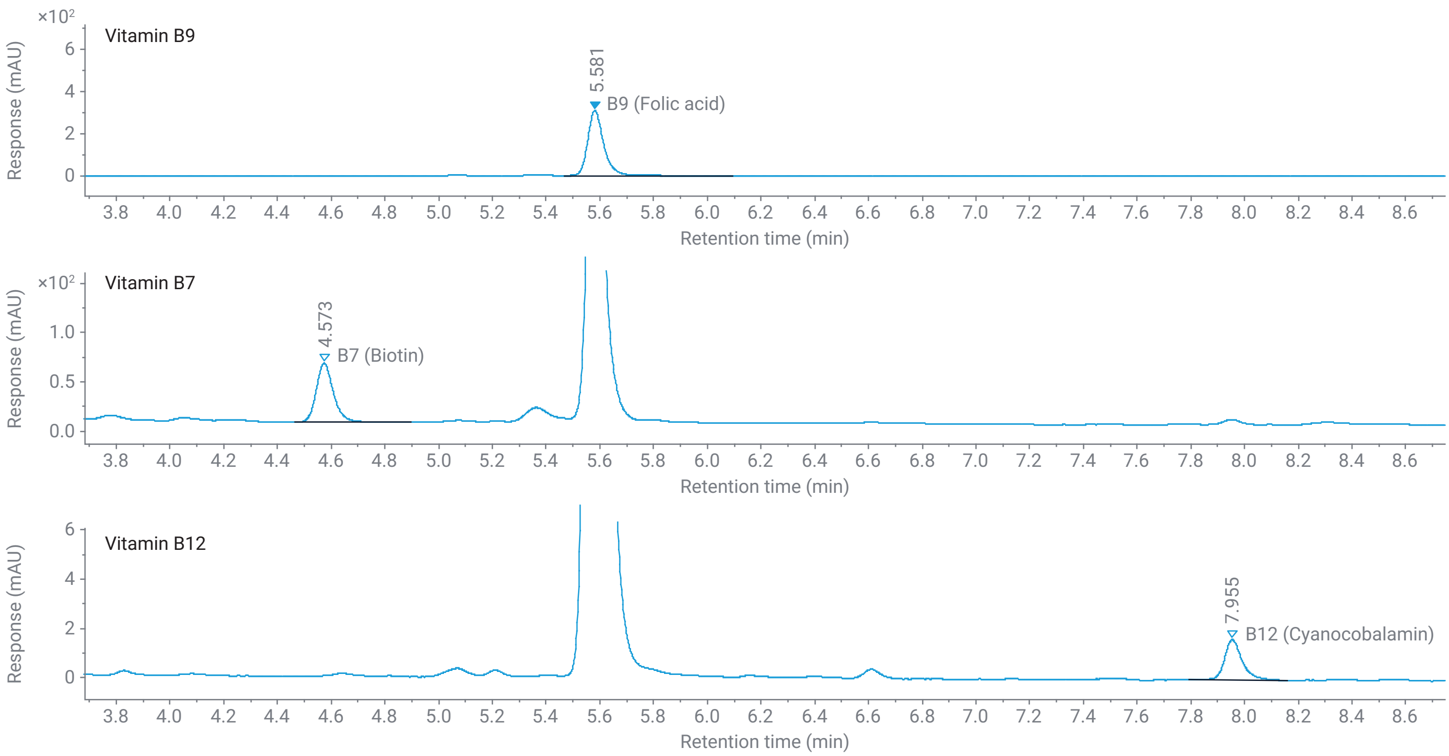


Figure 4. Chromatograms of B vitamins in NIST 1869 SRM.

Sample analysis

The vitamin B7, B9, and B12 content of an infant formula obtained from a local grocery store was successfully determined. The analysis results are summarized in Table 8 and the chromatograms illustrating the three B vitamins are shown in Figure 5.

Table 8. Vitamin B content in infant formula sample.

Vitamin	Analysis Results (µg/g)
B7	0.14
B9	1.00
B12	0.0068

Vitamin B7, B9, and B12 are typically found in smaller quantities in infant formula, with vitamin B12 often present in particularly low levels compared to other B vitamins. This adds to the challenge of their detection, aside from the complexity of the infant formula matrix. The use of an IAC is essential to enhance the detection sensitivity of these B vitamins in infant formula by eliminating potential matrix interference. Additionally, the concentration step prior to sample injection also plays a role in enhancing detection sensitivity, as does using a detector flow cell of longer path length.

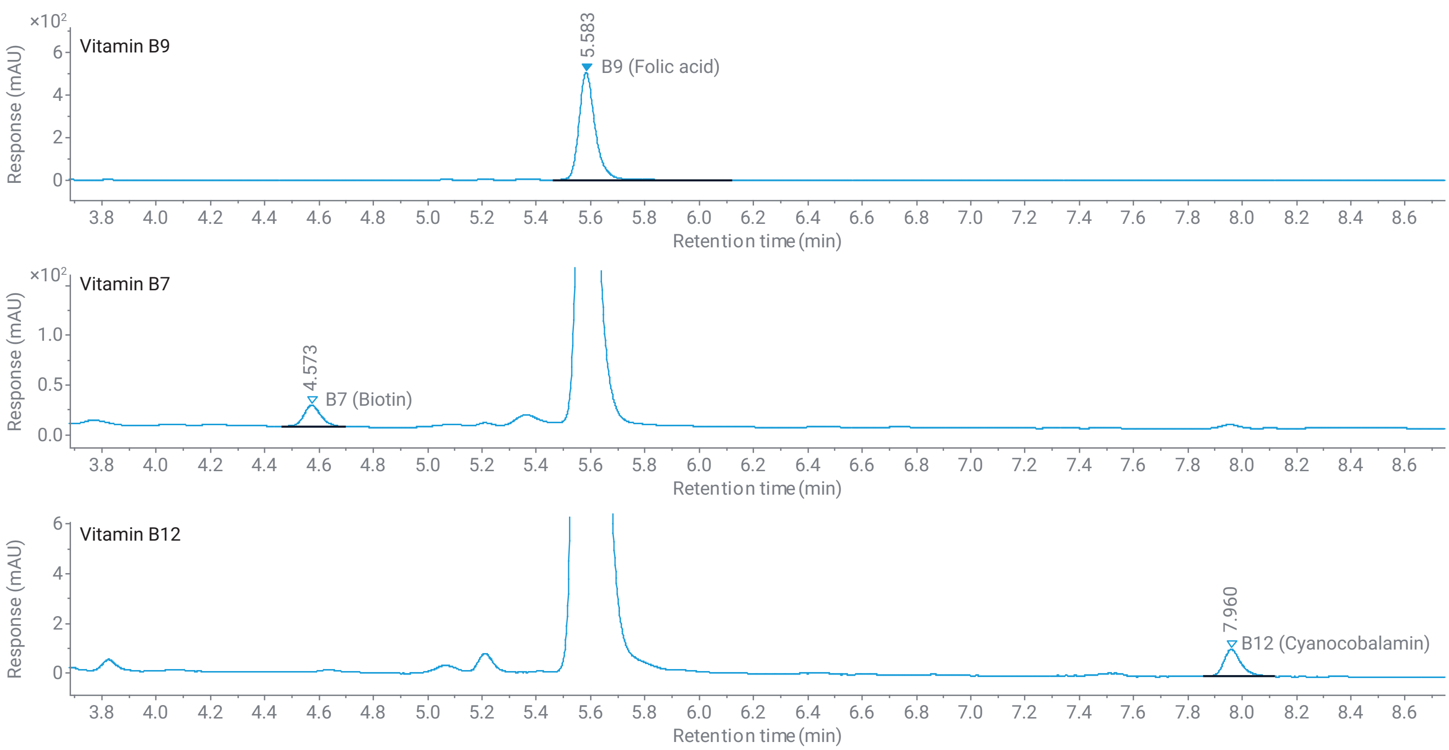


Figure 5. Chromatograms of vitamins B in infant formula sample.

Conclusion

A robust method was developed and validated for the simultaneous analysis of vitamin B7, B9, and B12 content in infant formula using the Agilent 1260 Infinity II Prime LC system together with the Agilent InfinityLab Poroshell 120 SB-Aq column and a 60 mm Agilent InfinityLab Max-Light cartridge flow cell. The method demonstrated high sensitivity and yielded satisfying recovery rates for the targeted B vitamins.

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