Application Note

Food & Beverage Testing

The Analysis of Vitamin B12 in Infant Formula

Using the Agilent 1260 Infinity II Prime LC System with diode array detection

Author

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Abstract

Free vitamin B12 at low ppb levels in infant formula can be analyzed confidently within 3.5 minutes on an Agilent 1260 Infinity II Prime LC coupled with diode array detector (DAD). The experimental setup also featured an Agilent InfinityLab Max-Light cartridge flow cell with a 60 mm flow path and an immunoaffinity cleanup column.

Introduction

Vitamin B12, also known as cobalamin, is a water-soluble vitamin that predominantly exists in the form of 5'-deoxyadenosylcobalamin and methylcobalamin in living tissues. Hydroxocobalamin, which is formed due to the disruption of the carbon-cobalt bond in 5'-deoxyadenosylcobalamin and methylcobalamin by the exposure to light, is present in minute amounts. Vitamin B12 is an essential nutrient for human growth, cell development, metabolism of amino acids, and DNA synthesis. However, vitamin B12 is produced solely by prokaryotic microorganisms. Therefore, the only natural sources of vitamin B12 for humans are diets of animal origin. Several food products, such as cereals and adult nutritional formulae, are fortified with cyanocobalamin, the most pharmacologically stable form of vitamin B12, to supplement daily intake. The structures of different forms of vitamin B12 are shown in Figure 1.

Conventionally, vitamin B12 in food products is determined through microbiological assays due to its high sensitivity, but this methodology is reported to have low selectivity and precision. High-performance liquid chromatography (HPLC) with ultraviolet (UV) detection is one of the alternative approaches for vitamin B12 analysis. In general, the protein-bound forms of vitamin B12 in food products first need to be released through acid or enzymatic hydrolysis, followed by conversion into their cyano forms in the presence of heat and cyanide ions before analysis. The concentration of vitamin B12 in food products is normally low, and hence an immunoaffinity cleanup column is incorporated into the sample preparation workflow to further improve selectivity and sensitivity. This enhanced workflow has been published in AOAC Official Method 2014.02.

In this study, a rapid analysis method has been developed for vitamin B12 content in infant formula with reference to AOAC Official Method 2014.02. Fortified/free vitamin B12 content was evaluated without the use of cyanide compounds (i.e., sodium or potassium cyanide). The 1260 Infinity II Prime LC enables the use of small particle size columns, owing to its high, 800-bar pressure limit, to achieve excellent peak shape and sensitivity. The Beer-Lambert Law states that the path length of the detection flow cell is proportional to the light absorption of the analyte species. Hence, to further enhance the detection sensitivity, an InfinityLab Max-Light cartridge flow cell with a 60 mm path length was used in the Agilent 1290 Infinity II DAD instead of the standard 10 mm path length flow cell. This combination provides a robust solution for vitamin B12 determination.

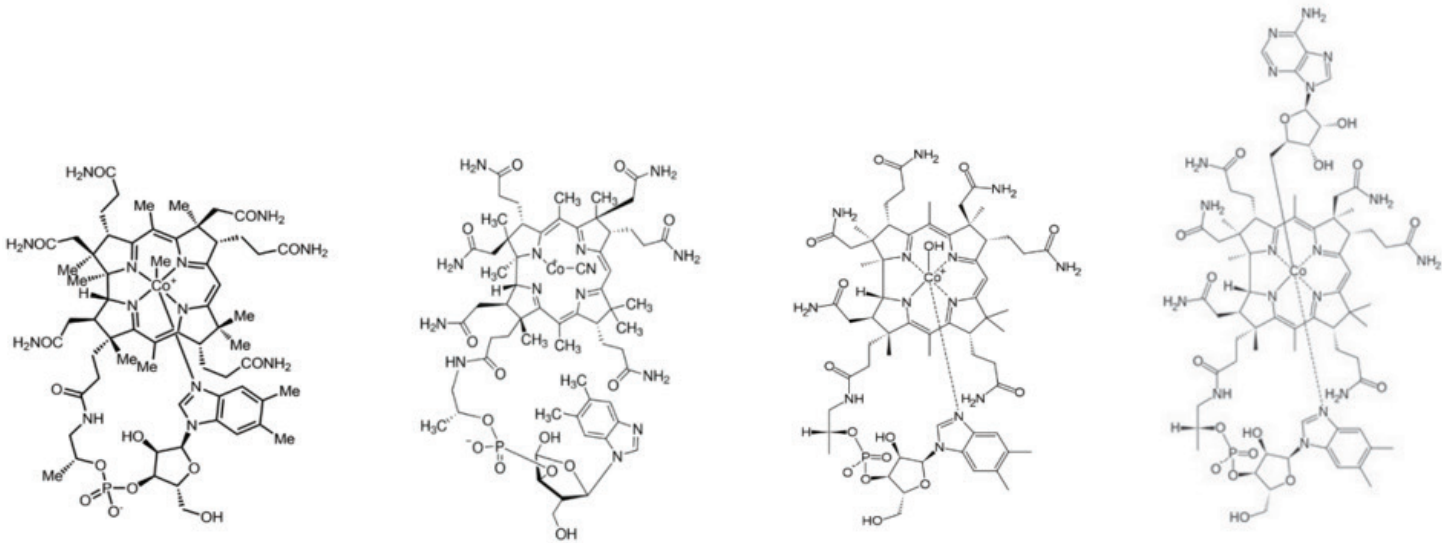

Figure 1. Structures of various forms of vitamin B12.

Experimental

Instrumentation

The 1260 Infinity II Prime LC system comprises the following modules:

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

Chromatographic conditions

The analysis conditions are described below:

Parameter	Value
Column	Agilent InfinityLab Poroshell 120 SB-Aq, 3.0 × 100 mm, 2.7 µm (p/n 685975-314)
Mobile Phase	A) 0.0125% Formic acid in water B) 0.0125% Formic acid in acetonitrile
Gradient	0 minute – 15% B 2.5 minutes – 45% B 2.51 minutes – 70% B 3.49 minutes – 70% B 3.5 minutes – 15% B
Flow Rate	0.5 mL/min
Stop Time	3.5 minutes run time and 3.5 minutes re-equilibration
Injection Volume	35 µL, sample temperature was kept at 8 °C
DAD Wavelength	361 nm
Column Temperature	40 °C Agilent InfinityLab Quick Connect heat exchanger, large id (p/n G7116-60051)

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 are as follows:

- Acetonitrile (HPLC grade, purchased from Aik Moh Paints & Chemicals Pte. Ltd.)
- Formic acid (LC/MS grade, purchased from Aik Moh Paints & Chemicals Pte. Ltd.)
- De-ionized water (Milli-Q water)
- Cyanocobalamin (analytical grade)
- Sodium acetate (ACS grade, purchased from Sigma-Aldrich Pte. Ltd.)
- Acetic acid (ACS grade, purchased from Sigma-Aldrich Pte. Ltd.)

Samples

Two samples were used in this work:

- Infant formula certified reference material (NIST1869)
- Infant formula purchased from a local supermarket

Sample preparation consumables

EASI-EXTRACT VITAMIN B12 (LGE) Immunoaffinity Column (IAC) from R-Biopharm was used for the sample matrix cleanup.

Typical sample preparation procedures

The sample preparation procedures for infant formula are summarized in the Figure 2.

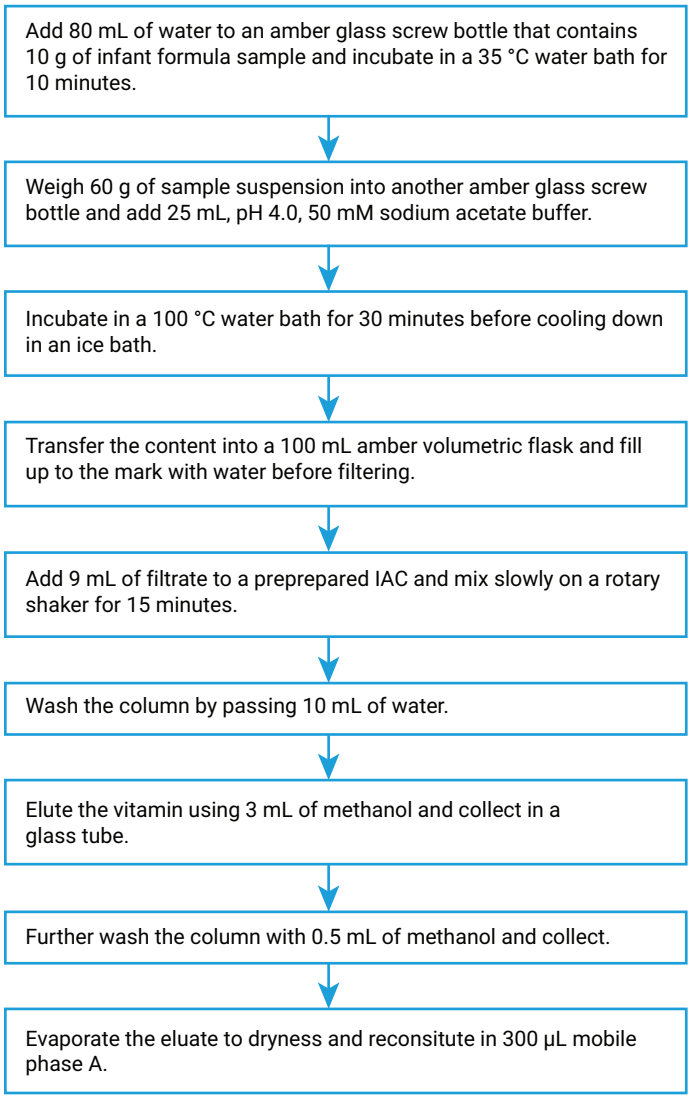

Figure 2. Sample preparation flowchart.

Results and discussion

Method validation

Limit of quantification (LOQ): The LOQ of an analyte is often defined as the concentration at which the signal-to-noise (S/N) ratio of the analyte is observed to be 10. In this study, the LOQ, established from the lower linearity level of the vitamin B12 standard of 3 ng/mL, was 1.2 ng/mL.

Linearity: A series of vitamin B12 standards of different concentrations (i.e., 1, 5, 10, 25, 50, 100, and 250 ng/mL) was injected to construct the calibration curve, which showed excellent linearity, with a correlation coefficient (R^2) of close to 1. Figure 3 shows the calibration curve of vitamin B12 standards.

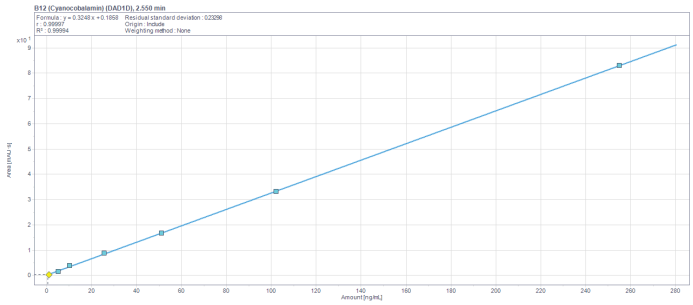

Figure 3. Calibration curve of vitamin B12 in standard solution (1, 5, 10, 25, 50, 100, and 250 ng/mL).

Repeatability

A total of six consecutive injections of 3 ng/mL vitamin B12 standards were performed to evaluate the standard repeatability based on the relative standard deviation (RSD) of retention time and peak area (Figure 4). The repeatability was excellent, with a retention time RSD of less than 0.1% and peak area RSD of 1.16%. Detailed information is given in Table 1.

Table 1. Data related to six consecutive injections of 3 ng/mL vitamin B12 standards, demonstrating good repeatability.

n	Retention Time (min)	Peak Area
1	2.542	1.095
2	2.545	1.074
3	2.545	1.074
4	2.543	1.059
5	2.542	1.065
6	2.542	1.080
Average	2.543	1.075
RSD (%)	0.06	1.16

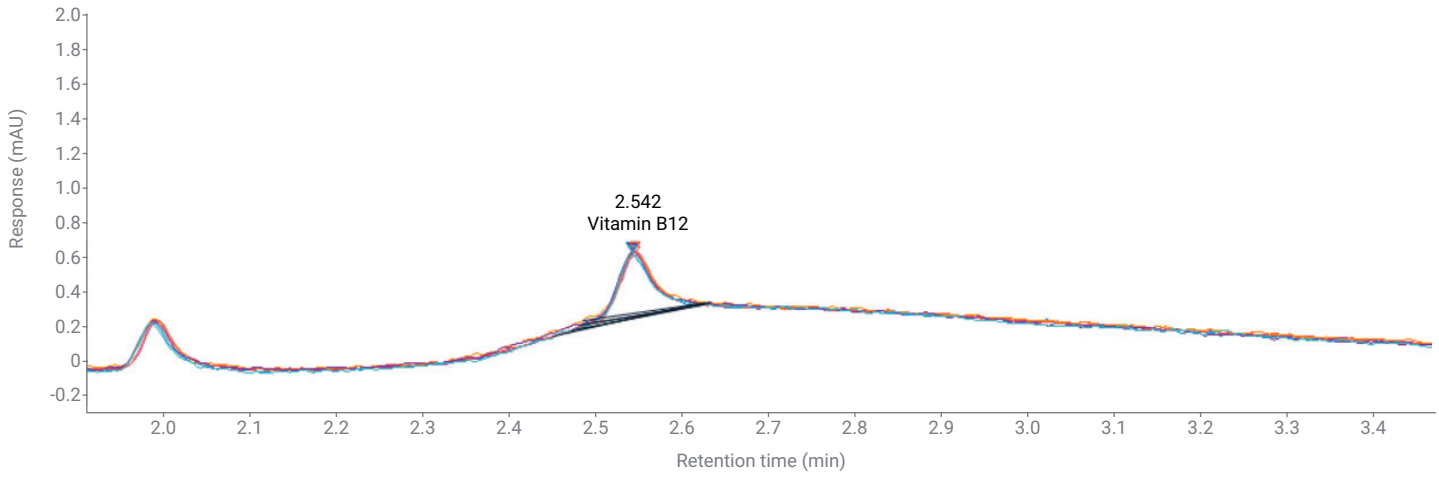

Figure 4. Overlay of chromatograms of six consecutive standard injections.

Recovery

Vitamin B12 content in NIST1869 certified reference material was analyzed to investigate the recovery of this analytical method. By comparing the recovered vitamin B12 from the NIST1869 sample with the reference value provided in the certificate of analysis, the recovery of 88.8% is in line with the recovery data provided by the IAC supplier, which ranged from 70% to 110%. Detailed analysis results are tabulated in Table 2 and the chromatogram is shown in Figure 5.

Table 2. Vitamin B12 content in NIST1869 certified reference material.

Sample	Analyte	Analysis Results (ng/g)	Reference Value (ng/g)	Recovery (%)
NIST1869	Vitamin B12	39.7	44.7	88.8

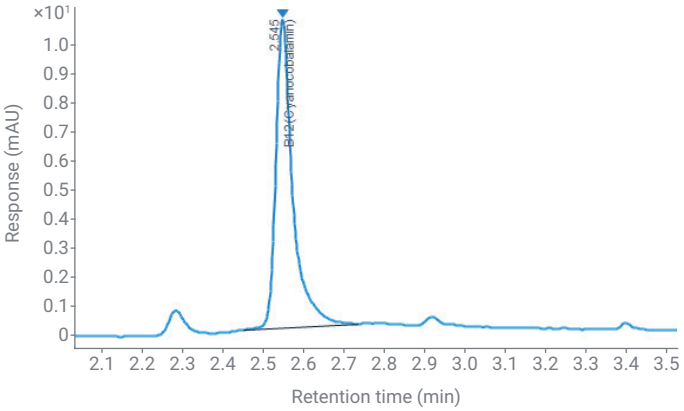

Figure 5. Chromatogram of NIST1869 infant formula sample.

Sample analysis

An infant formula was purchased from a local supermarket, and its vitamin B12 content was determined successfully. The chromatogram and vitamin B12 content are shown in Figure 6.

Vitamin B12 is known to be present in small amounts in infant formula, and this adds to the difficulty in its detection aside from the complexity of the infant formula matrix. The use of an immunoaffinity column is essential to improve the detection sensitivity of vitamin B12 in infant formula by removing possible matrix interference. The concentration step before the sample injection also contributed to the enhancement in detection sensitivity.

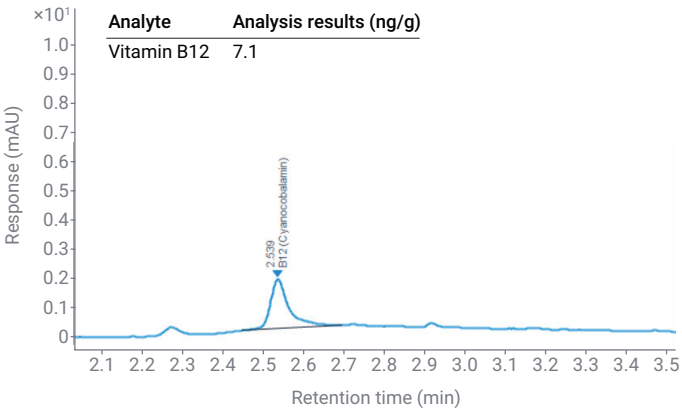

Figure 6. Chromatogram of infant formula from local supermarket.

Conclusion

A fast analysis method has been developed and validated to analyze vitamin B12 content in infant formula on the Agilent 1260 Infinity II Prime LC. Together with the Agilent InfinityLab Poroshell 120 SB-Aq column and 60 mm Agilent InfinityLab Max-Light cartridge flow cell, the method has achieved high sensitivity and great recovery in determining vitamin B12 in infant formula.

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