Application Note

Food & Beverage Testing

Simplified Analysis Workflow – Determination of Vitamins B1, B2, B3, B5, and B6 in Infant Formula

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

Authors

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Abstract

This study developed and validated an efficient workflow for analyzing vitamins B1, B2, B3, B5, and B6 in infant formula. The workflow employed a straightforward sample preparation protocol, using trichloroacetic acid (TCA) for protein precipitation. For enhanced detection sensitivity, the analysis was performed using the Agilent 1260 Infinity II Prime LC system, equipped with a 60 mm Agilent InfinityLab Max-Light cartridge cell in the diode array detector (DAD). The developed methods demonstrated robustness and reliability in quantifying B vitamins in infant formula, ensuring accurate nutritional labeling and compliance with regulatory standards.

Introduction

B vitamins are essential for numerous bodily functions, such as supporting aerobic metabolism of glucose for energy production and maintaining immune function. Deficiencies in B vitamins can adversely affect human health. Since the human body cannot produce these vitamins in sufficient quantities, it is necessary to obtain them from dietary sources of animal origin or through supplementation.[1] Foods such as infant formula are allowed to be fortified with vitamins to meet infants' nutritional requirements, subject to strict regulatory control. Table 1 lists the forms of B vitamins that are commonly used in food fortification. Robust analysis techniques are imperative for accurately quantifying these essential compounds. The quantification of vitamin B7, B9, and B12 in infant formula has been detailed in previously published application notes.[2,3]

Table 1. Forms of B vitamins for food fortification.[4]

Vitamin	Forms for Food Fortification
B1	Thiamine hydrochloride
B2	Riboflavin hydrochloride
B3*	Nicotinamide
B5	Calcium or sodium pantothenate
B6	Pyridoxine hydrochloride
B7	Biotin
B9	Folic acid
B12	Cyanocobalamin

* **Note**: Nicotinic acid and nicotinamide are different forms of niacin (vitamin B3).

Acidic hydrolysis and enzymatic digestion are generally used to release protein-bound and phosphorylated B vitamins. However, in infant formula, the vitamin content is declared based on the amount added during the manufacturing. This means that the extraction protocol and the vitamin forms to be determined can be simplified and minimized. Traditionally, microbiological assays were employed to measure the B vitamin concentrations due to their high sensitivity. With technological enhancements, HPLC with ultraviolet (UV) detection has emerged as a more rapid and reliable quantification tool.[5]

In this study, a unified sample preparation workflow using TCA as protein precipitation agent was developed for: (1) the simultaneous analysis of vitamins B1, B2, B3, and B6, and (2) the analysis of vitamin B5 in infant formula. Each uses distinct HPLC analytical conditions. The structure of vitamins B1, B2, B3, B5, and B6 are shown in Figure 1. The 1260 Infinity II Prime LC, with a high-pressure limit of 800 bar, is ideally suited for coupling with a small particle size column, ensuring exceptional resolution and sensitivity. Additionally, the use of a 60 mm Max-Light cartridge cell in the DAD further enhances the detection of B vitamins, particularly, vitamin B5, which lacks chromophore.

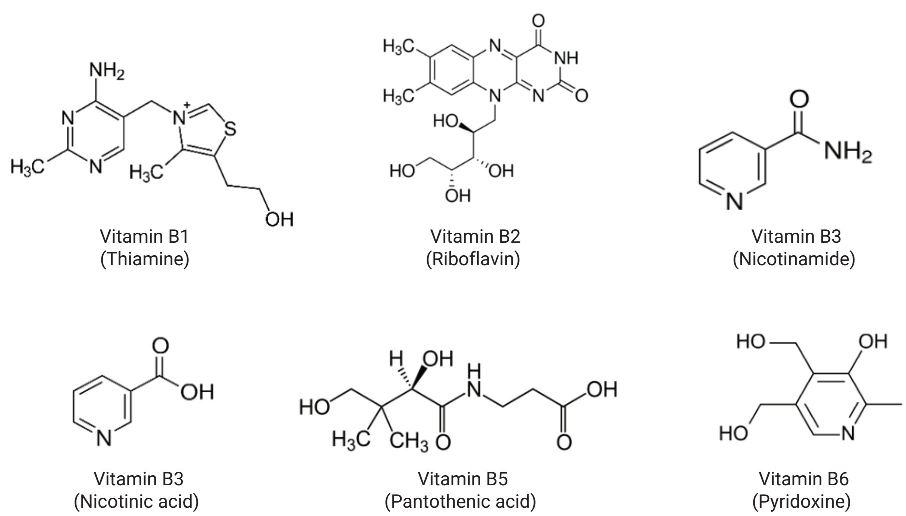

Figure 1. The structure of vitamins B1, B2, B3, B5, and B6.

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 cell (p/n G4212-60007)

Chromatographic conditions

Table 3 describes the analysis conditions for the simultaneous analysis of vitamins B1, B2, B3, and B6. Table 4 describes the conditions for the analysis of vitamin B5 in infant formula.

Software

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

Table 3. Chromatographic conditions when analyzing vitamins B1, B2, B3, and B6.

Parameter	Value
Mobile Phase	A) 0.1% Sodium dodecyl sulfate in water with 0.2% formic acid, pH 2.8 B) Acetonitrile
Gradient	Time (min) %B 0.0 5 1.00 10 4.50 36 6.00 36 6.01 43 8.30 43 9.00 80 10.00 80
Flow Rate	0.7 mL/min
Stop Time	10.00 min
Postrun	3.50 min
Injection Volume	5 µL, Sample temperature was kept at 8 °C
Column	Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm (p/n 695975-302)
Column Temperature	35 °C
Heat Exchanger	Agilent InfinityLab Quick Connect heat exchanger, large id (p/n G7116-60051)
DAD Wavelength	268 nm (Vitamins B1, B2, and B3) 296 nm (Vitamin B6)
Data Rate	10 Hz

Table 4. Chromatographic conditions when analyzing vitamin B5.

Parameter	Value
Mobile Phase	A) 25 mM Potassium phosphate monobasic (KH_2PO_4) in water, pH 2.8 B) Methanol
Gradient	Time (min) %B 0.0 4 8.00 4 8.01 70 10.00 70
Flow Rate	0.5 mL/min
Stop Time	10.00 min
Postrun	5.00 min
Injection Volume	2 µL, Sample temperature was kept at 8 °C
Column	Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm (p/n 695975-302)
Column Temperature	40 °C
Heat Exchanger	Agilent InfinityLab Quick Connect heat exchanger, large id (p/n G7116-60051)
DAD Wavelength	205 nm
Data Rate	10 Hz

Chemicals and solvents

The chemicals and solvents used in the analysis are as follows:

- Acetonitrile (HPLC grade, purchased from Aik Moh Paints & Chemicals Pte. Ltd., Singapore)
- Methanol (HPLC grade, purchased from Aik Moh Paints & Chemicals Pte. Ltd., Singapore)
- Formic acid (LC/MS grade, purchased from Aik Moh Paints & Chemicals Pte. Ltd., Singapore)
- De-ionized water (Milli-Q, purchased from Millipore Sigma, Burlington, MA)
- Sodium dodecyl sulfate (ACS reagent grade, purchased from Sigma-Aldrich, St. Louis, MO)
- Potassium phosphate monobasic (ACS reagent grade, purchased from Sigma-Aldrich, St. Louis, MO)
- TCA (reagent grade, purchased from Sigma-Aldrich, St. Louis, MO)
- Hydrochloric acid, 1 N (purchased from Sigma-Aldrich, St. Louis, MO)
- Sodium hydroxide (reagent grade, purchased from Sigma-Aldrich, St. Louis, MO)
- Vitamin B1/thiamine hydrochloride (analytical grade)
- Vitamin B2/riboflavin (analytical grade)
- Vitamin B3/nicotinamide (analytical grade)
- Vitamin B3/nicotinic acid (analytical grade)
- Vitamin B5/calcium pantothenate (analytical grade)
- Vitamin B6/pyridoxine (analytical grade)

Samples

Two samples were evaluated in this study:

- Infant formula NIST 1869 standard reference material (SRM)
- Infant formula purchased from a local grocery store

Sample preparation procedures

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

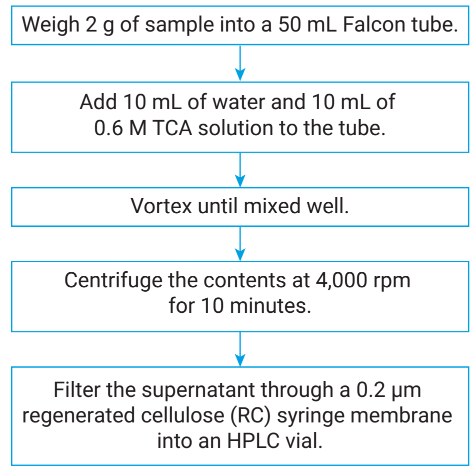

Figure 2. Sample preparation procedure for infant formula.

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) is observed to be 10. In this study, the LOQ, estimated using a lower concentration of standard solution for B vitamins, is presented in Table 5. Vitamin B5 has a higher LOQ compared to other B vitamins due to its lack of chromophore.

Table 5. LOQ of each B vitamin.

Vitamin	LOQ (µg/mL)
B1	0.055
B2	0.014
B3 (Nicotinamide)	0.093
B3 (Nicotinic Acid)	0.088
B5	1.040
B6	0.006

Linearity: A series of standard solutions with varying concentrations of B vitamins were injected into the LC system to construct the calibration curves. These curves exhibited excellent linearity, with correlation coefficients (R^2) of 1. Table 6 details the calibration parameters for each B vitamin. Figures 3 and 4 show the representative chromatograms of the B vitamins.

Table 6. Calibration parameters of each B vitamin.

Vitamin	Calibration Range (µg/mL)	R^2
B1	0.05 to 20	1.000
B2	0.05 to 20	1.000
B3 (Nicotinamide)	0.05 to 20	1.000
B3 (Nicotinic Acid)	0.05 to 20	1.000
B5	0.5 to 50	1.000
B6	0.05 to 20	1.000

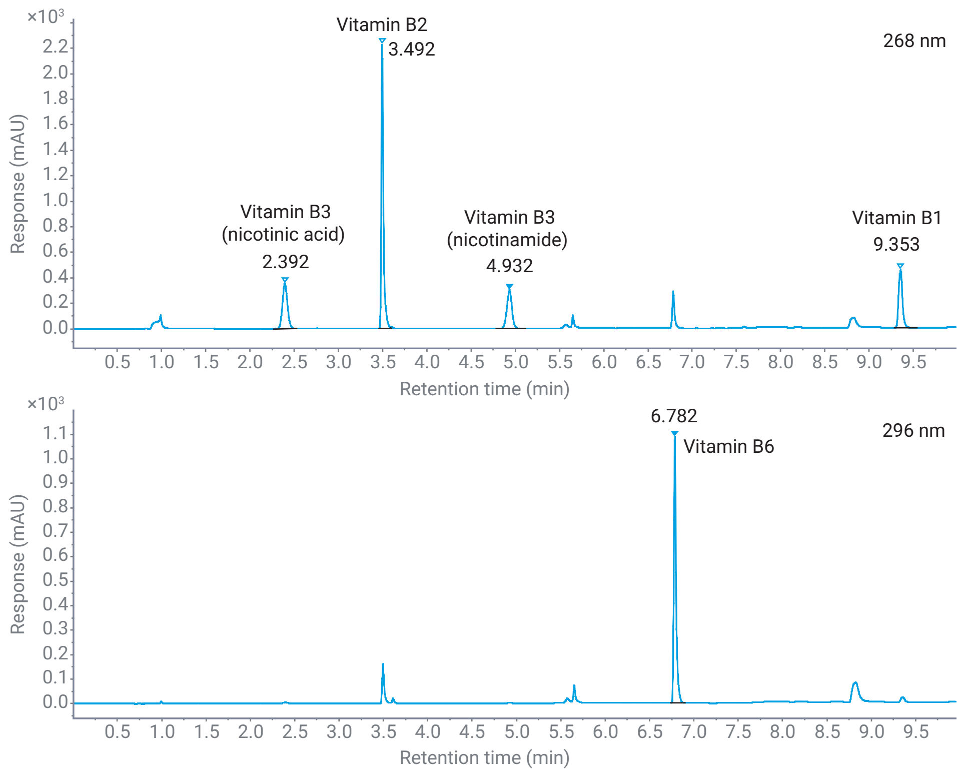

Figure 3. Representative chromatograms of vitamins B1, B2, B3 (nicotinamide and nicotinic acid), and B6 at 20 µg/mL.

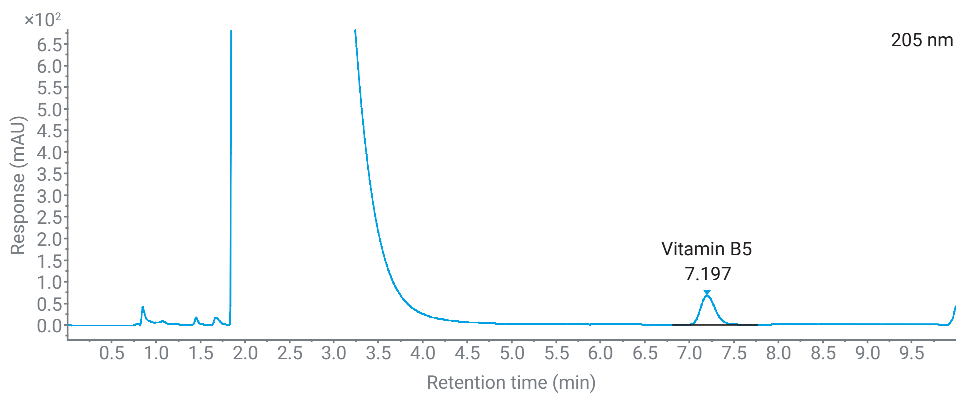

Figure 4. Representative chromatogram of vitamin B5 at 50 µg/mL.

Repeatability: Five consecutive injections of B vitamin standard solution were performed to assess injection repeatability. This was evaluated based on the relative standard deviation (RSD) of retention time (RT) and peak area. Overall, the repeatability was excellent, with an RT RSD of less than 0.3% and a peak area RSD of less than 1%, except for vitamin B5, which had a peak area RSD of 3.94%. This higher RSD for vitamin B5 can be attributed to baseline fluctuations at the acquisition wavelength of 205 nm. The repeatability study data are provided in Table 7.

Recovery: The analysis of vitamin B content in NIST 1869 SRM was conducted to assess the method's recovery. The analysis results are summarized in Table 8. The findings indicate that the recovery rates for B vitamins from NIST 1869 SRM fall within the ± 20% range, demonstrating the robustness and reliability of the analytical method used. Nicotinic acid was not detected in the analysis, which aligns with expectations as nicotinic acid is not used for food fortification. Figures 5 and 6 present the chromatograms of B vitamins in NIST 1869 SRM.

Table 8. Vitamin B content in NIST 1869 SRM.

Vitamin	Analysis Results (µg/g)	Reference Value (µg/g)	Recovery (%)
B1	15.77	13.36	118.04
B2	12.50	13.60	91.91
B3[1]	90.24	98.40	91.71
B3[2]	ND	NA	NA
B5	59.60	64.90	91.83
B6	15.00	13.09	114.59

[1] Nicotinamide
[2] Nicotinic acid
ND = Not detected
NA = Not available

Table 7. Repeatability data (n = 5) of each B vitamin.

Vitamin	Injected Concentration (µg/mL)	Average RT (min)	RT RSD (%)	Average Area	Area RSD (%)
B1	0.10	9.326	0.10	6.323	0.88
B2	0.025	3.489	0.04	4.902	0.70
B3 (Nicotinamide)	0.15	4.924	0.21	11.694	0.62
B3 (Nicotinic Acid)	0.15	2.420	0.19	10.882	0.60
B5	1.50	7.123	0.21	23.145	3.94
B6	0.015	6.783	0.11	1.591	0.56

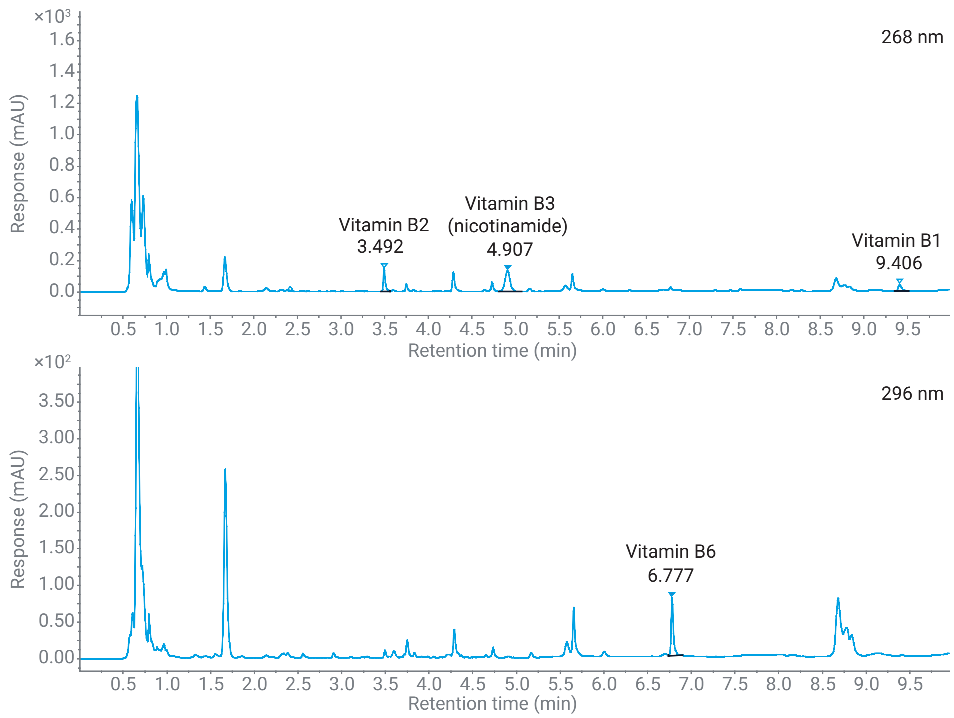

Figure 5. Chromatogram of vitamins B1, B2, B3 (nicotinamide), and B6 in NIST 1869 SRM.

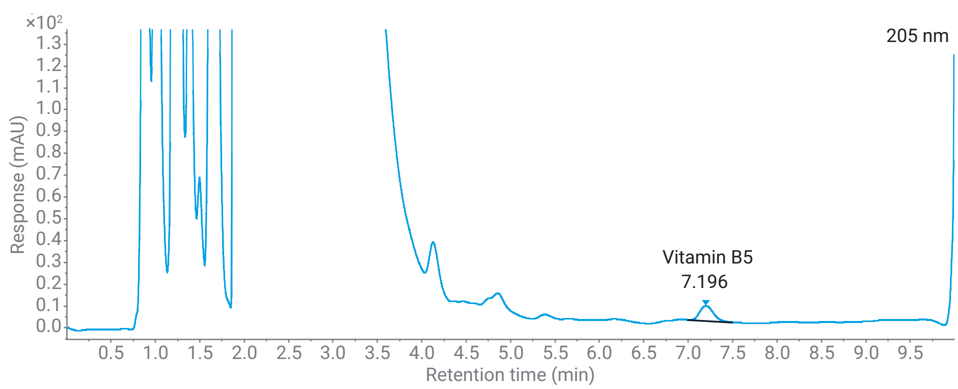

Figure 6. Chromatogram of vitamin B5 in NIST 1869 SRM.

Sample analysis

The vitamin B content of the infant formula obtained from a local grocery store was successfully determined. The analytical results are summarized in Table 9 and the chromatograms of B vitamins are shown in Figures 7 and 8.

The 60 mm InfinityLab Max-Light cartridge cell has a longer path length, enabling more interaction between the light and the sample. This improves the S/N and enhances the detection limits of compounds with weak UV absorbance, such as vitamin B5.

Table 9. Vitamin B content in infant formula sample.

Vitamin	Analysis Results (µg/g)
B1	5.82
B2	10.73
B3 (Nicotinamide)	38.51
B3 (Nicotinic Acid)	Not detected
B5	38.84
B6	3.96

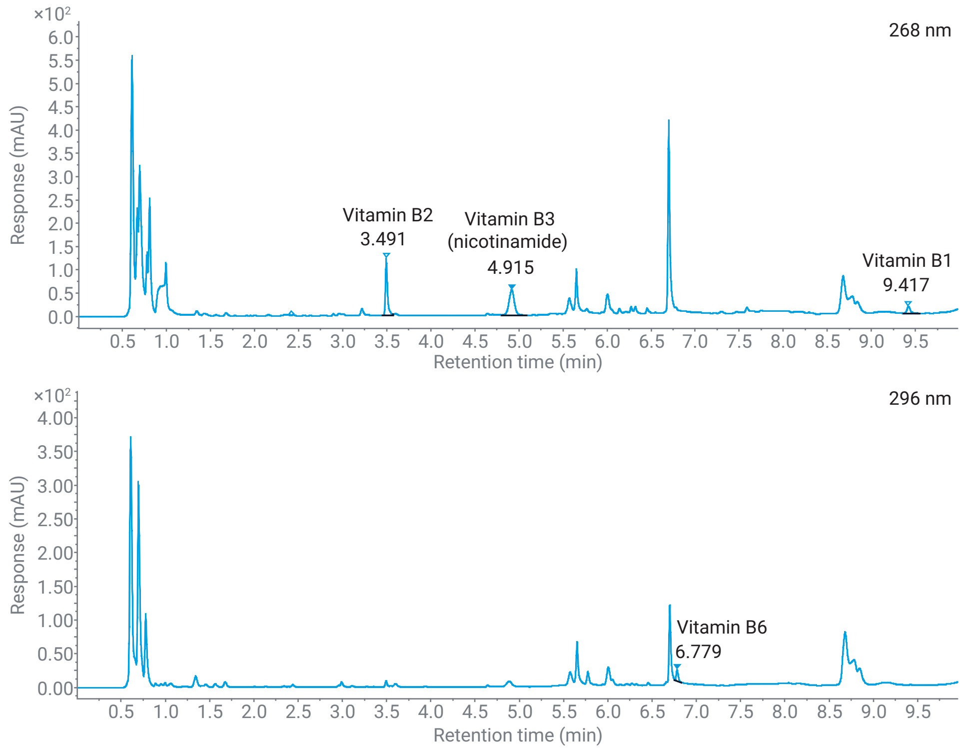

Figure 7. Chromatogram of vitamins B1, B2, B3 (nicotinamide), and B6 in infant formula sample.

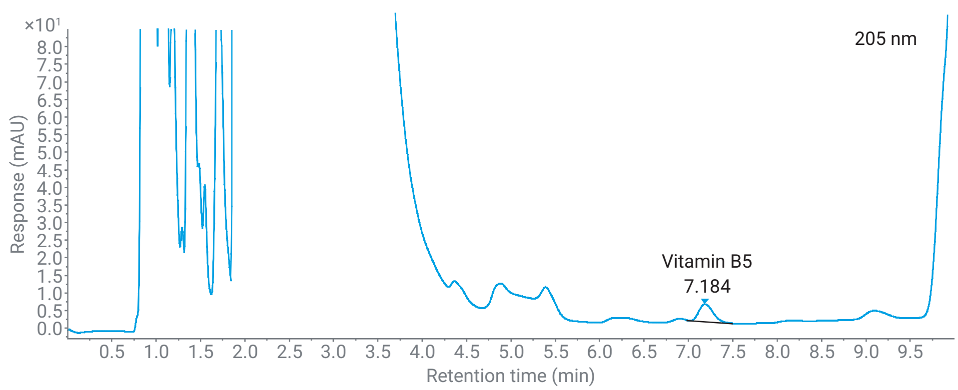

Figure 8. Chromatogram of vitamin B5 in infant formula sample.

Conclusion

This study successfully developed and validated an efficient workflow for quantifying B vitamins in infant formula. The workflow uses a simple and straightforward sample preparation protocol to precipitate proteins in the formula. Despite its simplicity, the study demonstrated excellent linearity, repeatability, and recovery, providing reliable measurements of vitamins B1, B2, B3, B5, and B6. The use of the Agilent 1260 Infinity II Prime LC system, paired with a 60 mm Agilent InfinityLab Max-Light cartridge cell in the DAD, significantly enhanced detection sensitivity.

References

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