Hindawi
Journal of Food Quality
Volume 2022, Article ID 7649228, 7 pages
https://doi.org/10.1155/2022/7649228

Research Article

Determination of Vitamin B_{12} in Milk and Dairy Products by Isotope-Dilution Liquid Chromatography Tandem Mass Spectrometry

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Received 13 April 2022; Accepted 23 July 2022; Published 12 August 2022

Academic Editor: Ying Hu

An isotope-dilution liquid chromatography tandem mass spectrometry method was established for the determination of vitamin B_{12} in milk and dairy products. The samples were spiked with stable isotope-labeled vitamin B_{12} and digested by pepsin and amylase. The various forms of cobalamin were transformed to cyanocobalamin by potassium cyanide after they were released from the enzymatically digested samples. Cyanocobalamin was extracted and purified by an immunoaffinity SPE cartridge and then measured in multiple reaction monitoring mode (MRM). The linear correlation coefficient (R^2) of this method was greater than 0.999 in the range of 2–100 ng/mL. The detection limit and the quantification limit were 0.5 μg/kg and 1.0 μg/kg, respectively. The spiked recoveries ranged from 92.0% to 99.4% at the three spiked levels with the relative standard deviation (RSD) between 1.89% and 4.51%. The measured results of NIST SRM1849a and NIST SRM1869a by the current method are all within the reference value range. The Z value was 0.8 during participating in the FAPAS proficiency test using the developed method in 2021. The method is simple, rapid, accurate, and sensitive, and it is suitable for the determination of vitamin B_{12} in different types of milk and dairy products such as whey powder, whole milk powder, pure milk, fermented milk, infant formula, and prescription food for special medical purposes.

1. Introduction

Vitamin B_{12} (VB_{12}) is also known as cobalamin or cobamide, and it has at least five chemical variants with similar molecular structure [1] (Figure 1). The naturally occurring forms of VB_{12} in food are hydroxocobalamin, 5′-deoxyadenosylcobalamin, methylcobalamin, sulphitocobalamin, and a small amount of cyanocobalamin. Among them, cyanocobalamin has the most stable chemical structure, and other chemical variants of VB_{12} are photolabile [2, 3]. The amount of VB_{12} required by the human body is very small. However, VB_{12} is an essential nutrient for humans, which plays an important role in the formation of normal red blood cells and the maintenance of the normal function of myelinated nerve cells in the human body. VB_{12} deficiency can cause anemia, nervous system disorders, and other symptoms [4–6]. The recommended intake for adults is 2 μg/d and the adequate intake (AI) for infants at 0–6 months and 6–12 months is 0.6 μg/d [7]. VB_{12} cannot be synthesized by humans de novo and it must be acquired through dietary intake of animal-based foods rich in VB_{12} such as milk and dairy products [8]. VB_{12} is usually presented as a protein-bound form in milk and dairy products, whereas cyanocobalamin is the main form of VB_{12} used in human dietary supplements. The low content of VB_{12} in food usually has various photolabile forms, which make it difficult for quantitative detection. It is urgent to establish an effective detection method for the determination of VB_{12} in various foods including milk and dairy products.

The existing reported methods for determination of VB_{12} in infant formula mainly include microbiological assays [9, 10], high-performance liquid chromatography (HPLC) [11–19], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [20–26], and liquid chromatography and

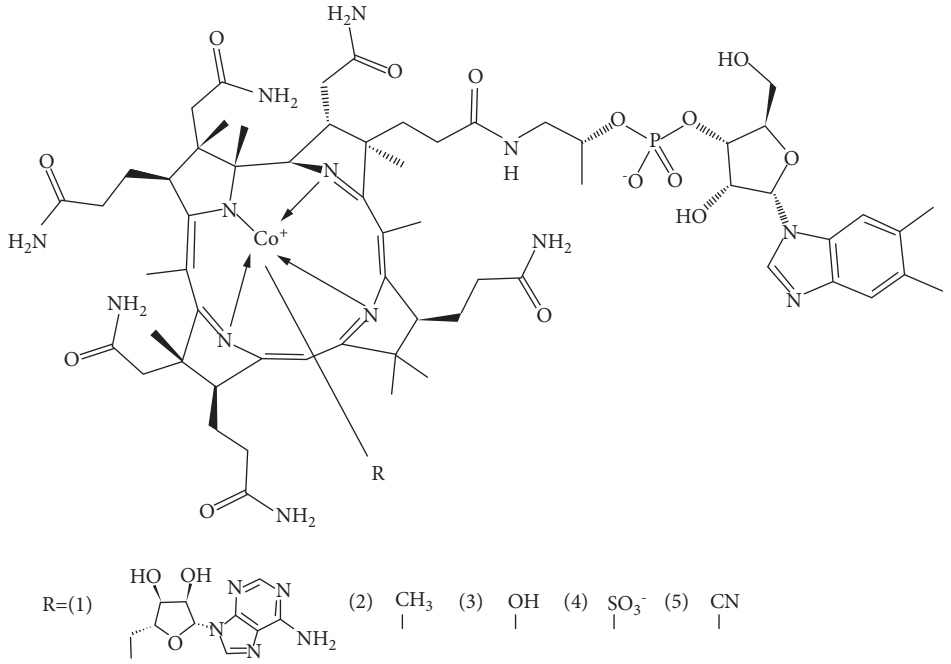

Figure 1: Structure of vitamin B_{12}: (1) 5′-deoxyadenosylcobalamin; (2) methylcobalamin; (3) hydroxocobalamin; (4) sulphitocobalamin; (5) cyanocobalamin.

inductively coupled plasma-mass spectrometry (LC-ICP-MS) [27–29]. The microbiological assays usually have a long measurement time and complicated operation. The strains need to be frequently resurrected and stored until analysis. Multiple dilutions need to be prepared to optimize a suitable linear range for the determination of samples with unknown contents. It is difficult for unskilled inspectors to carry out the inspection tests. HPLC methods for determination VB_{12} usually employ immunoaffinity purification and online solid phase extraction (SPE) followed by column switching techniques. Although HPLC methods have the advantages of simple operation, efficient separation, and high degree of automation, the methods based on UV detectors are not sensitive enough to detect VB_{12} in non-fortified food. It takes a long time to transform VB_{12} to fluorescent compounds by derivatization reaction using a fluorescence detector as VB_{12} itself does not emit fluorescence. In recent years, LC-MS/MS has been used to detect the content of VB_{12} in food. The trace level VB_{12} content in food can be detected by LC-MS/MS due to its high selectivity and sensitivity. However, the sample preparation using pepsin and potassium cyanide has not been comprehensively optimized in the existing reports. Based on our research of vitamins [30–32], in this study, an isotope-dilution ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method was established to detect VB_{12} content in milk and dairy products. This study focused on the effects of pepsin and potassium cyanide solution during sample preparation on the determination results of VB_{12} in milk and dairy products. The sample preparation procedure, quality control, and chromatographic and mass spectrometry conditions were systematically optimized for the first time. The immunoaffinity SPE cartridge and stable isotope-labeled VB_{12} were applied for sample pretreatment and MS analysis to ensure high specificity and good accuracy of the developed method. The method has the characteristics of high sensitivity, good repeatability, and high accuracy, and it can be applied and popularized in food testing laboratories.

2. Materials and Methods

2.1. Chemical Reagents. HPLC grades of acetonitrile (ACN), ethanol (EOH), acetic acid, and ammonium acetate were purchased from Fisher Chemical (Canada). Analytical grade sodium acetate and sodium hydroxide were purchased from Shanghai Lingfeng Chemical Reagent Co. Ltd. (China). Ultrapure water was obtained from a Milli-Q water purification system (Millipore, USA). Vitamin B_{12} (cyanocobalamin, 1.000 ± 0.006 mg/mL) was purchased from SIGMA (USA). [$^{13}C_7$]-vitamin B_{12} (0.995 ± 0.030 μg/mL) was purchased from Beijing Manhage Biotechnology Co. Ltd. (China). Immunoaffinity SPE cartridge was purchased from R-Biopharm (Germany). Pepsin from porcine gastric mucosa (250 U/mg, 400 U/mg, 600–1800 U/mg, 2500 U/mg) and Takadiastase (100 U/mg) were purchased from SIGMA (USA). Milk and dairy products were purchased from local supermarkets in Hangzhou, China.

2.2. Analytical Instrumentation. The analyte chromatographic separation was performed on an Waters BEH C_{18} column (10 cm × 2.1 mm i. d.; 1.7 μm), and the mobile phase consisted of water containing 2.5 mmol/L ammonium acetate (A) and water/acetonitrile (10 : 90 V/V) (B), for a total run time of 7 min and column temperature of 40°C, with a sample injection volume of 2 μL. The chromatographic

Table 1: LC/MS/MS parameters.

Compound (m/z)	Precursor ion (m/z)	Daughter ion	Cone voltage (V)	Collision energy (eV)
Vitamin B_{12}	678.6	147.1*	32	37
		359.2		23
[^{13}C7]-vitamin B_{12}	681.5	153.9*	32	37
		365.8		23

*means qualifier ion.

gradient was operated at a flow rate of 0.4 mL/min starting from 0 min: 2% B; 0–1 min: 2% B; 1–4 min: 2%–90% B; 4–5 min: 90% B; 5–7 min: 2% B.

The mass spectrometer was XEVO TQ-S, a triple quadrupole instrument equipped with an ESI ionization source (Waters Corp.). All analyses were conducted in positive ESI mode using multiple reaction monitoring on the 2 main product ions; the optimized mass spectrometer conditions for both analytes are reported in Table 1. The capillary voltage was 3.0 kV, source temperature was 150°C, and desolvation temperature was 650°C. Nitrogen was used as desolvation gas (1000 L/h) and cone gas (50 L/h), whereas the collision gas was argon (flow rate of 0.25 mL/min). Data acquisition processing was performed using MassLynx 4.1 software (Waters Corp.).

2.3. Preparation of Standard Solutions. Vitamin B_{12} intermediate standard (20.0 μg/mL) was prepared by diluting 1 mL of vitamin B_{12} stock standard solution (cyanobalamine, 1.000 ± 0.006 mg/mL) to 50 mL with water. Vitamin B_{12} working standard (200 ng/mL) was prepared by diluting 1 mL of vitamin B_{12} intermediate standard (20.0 μg/mL) to 100 mL with water. [$^{13}C_7$]-vitamin B_{12} working standard (50 *n*g/mL) was prepared by diluting 2.5 mL of [$^{13}C_7$]-vitamin B_{12} stock standard solution (0.995 ± 0.030 μg/mL) to 50 mL with water. A set of standard solutions containing vitamin B_{12} in the concentration range of 2–100 ng/mL was prepared, containing 5 ng/mL of [$^{13}C_7$]-vitamin B_{12}.

2.4. Sample Preparation-Extraction and Purification. Weigh, to the nearest 0.01 g, about 30 g of solid milk and dairy products into a 500 mL flask, dissolve the sample in 180 g of warm water (40°C to 45°C) and mix until homogeneous. The liquid milk can be weighed directly after shaking. Reconstituted milk or liquid milk was accurately weighed into a 50 mL centrifuge tube to withstand high temperatures of 100°C. 100 μL of [$^{13}C_7$]-vitamin B_{12} working standard solution (50 ng/mL), 25 mL of sodium acetate solution, 10 mg of Takadiastase, and 1 mL of potassium cyanide (1%) were added, respectively, under agitation and the solution was incubated at 37°C for 30 min in thermostatic oscillator. The hydrolysates were transferred to a water bath at 100°C for 30 min. Cooling down to room temperature, the solution was shaken fully and centrifuged at 8000 r/min for 10 min. The supernatant was filtered through a 1.6 μm glass fiber filter paper before purification.

All filtrate was loaded onto an immunoaffinity column, using a suitable glass adapter. The column was washed with 10 mL of water and then completely dried by passing through at least 10 mL of air. Vitamin B_{12} was eluted into a 10 mL glass tube with 1 mL of methanol for 3 times by complete denaturation of the antibody. The eluate was concentrated to dryness at 60°C under slow nitrogen gas flow and reconstituted in 1 mL of mobile phase and filtered through a 0.22 μm membrane filter before HPLC analysis.

3. Results and Discussion

3.1. Optimization of the MS/MS Conditions. Structural information to confirm the identity of analytes and the optimization of the instrumental sensitivity were obtained by performing a preliminary fragmentation study and MS/MS experiments. Full scans under the positive ESI mode for vitamin B_{12} and [$^{13}C_7$]-vitamin B_{12} were acquired for the selection of the precursor ions according to the relative intensities of multiple charged ions. $[M + 2H]^{2+}$ at m/z 678.1 and 681.8 were the precursor ions selected for vitamin B_{12} and [$^{13}C_7$]-vitamin B_{12} as it gave the most intense peak in the mass spectrum. Product ion scan mass spectra are shown in Figure 2. Collision-induced dissociation (CID) of vitamin B_{12} produced two product ions at m/z 147 and 359, and [$^{13}C_7$]-vitamin B_{12} produced two product ions at m/z 154 and 366, also shown by the fragmentation pattern in Figure 2. For each analyte, two among all available MRM transitions were chosen on the basis of the best chromatographic signal-to-noise (S/N) ratio, to perform quantitative and confirmative analysis on the selected food matrices.

3.2. Optimization of the LC Separation Conditions. Acetonitrile was selected as an organic modifier for its chromatographic selectivity, and ammonium acetate was added to the mobile phase, it could provide the best ionization contions. The best compromise in terms of sensitivity and analyte separation was afforded by the 2.5 mmol/L concentration of ammonium acetate as mobile phase A and water/acetonitrile (10 : 90 V/V) as mobile phase B.

3.3. Optimization of Extraction Solvent. Vitamin B_{12} can dissolve in strong polar solvents, such as water, methanol, and ethanol, but not in organic solvents with medium polarity, such as acetone, chloroform, and ether. Thus, strong polar solvent was suitable for extraction in the sample process. There are other kinds of naturally occurring cobalamin in infant formula, prepared milk powder, and instant grain food including fortified cyanocobalamin. These cobalamins coordinated with the protein to form stable large molecular compounds. The cobalamin must dissociate from

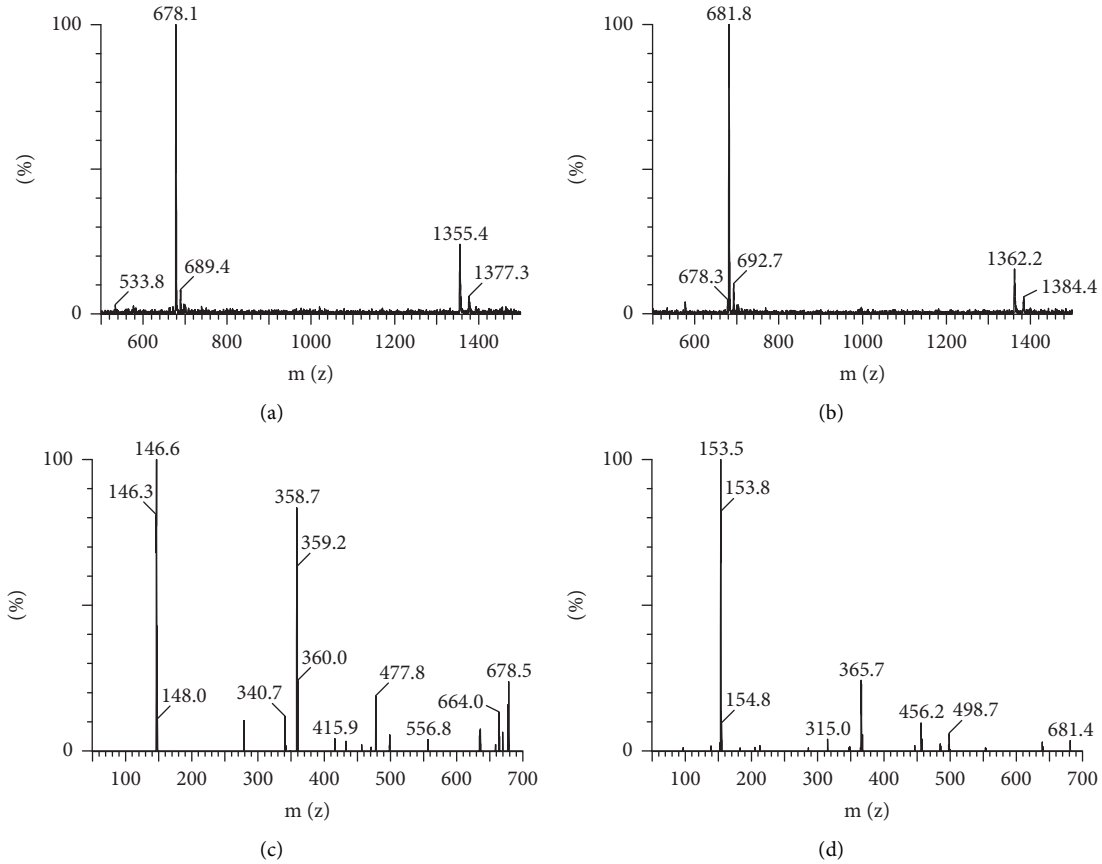

Figure 2: Mass spectrum of full scan and daughter scan of VB_{12} and isotope-labeled VB_{12}.(a) MS scan of VB_{12} (b) Full MS scan of $^{13}C_7$-VB_{12} (c) Daughter scan of VB_{12} (d) Daughter scan of $^{13}C_7$-VB_{12}.

Table 2: Comparison of results by adding potassium cyanide or not.

Samples	Adding potassium cyanide		Not adding potassium cyanide		t-Test
	Value (μg/kg)	SD (μg/kg)	Value (μg/kg)	SD (μg/kg)	P value
FAPAS-1	19.90	2.13	8.75	0.65	<0.05
FAPAS-2	16.49	3.12	9.97	0.48	<0.05
NIST SRM 1849a	47.17	4.06	42.12	0.51	0.814 > 0.05
Infant formula-1	39.88	2.76	10.09	1.4	<0.05
Infant formula-2	32.76	0.57	23.08	0.94	<0.05
Non-fat milk powder	28.82	2.79	5.54	0.38	<0.05
Demineralized whey	23.81	2.06	15.83	0.6	<0.05
Whole milk powder	16.73	1.81	3.23	0.17	<0.05
Pure milk	4.44	0.53	1.14	0.23	<0.05

combined compounds and transform into cyanobalamin prior to determination, and the reaction was proceeded in the condition of aqueous solution. Enzymatic hydrolysis of diastase can also react in aqueous solution. Therefore, water was chosen as the extraction solvent.

3.4. Necessity of Potassium Cyanide. There are many speciations of vitamin B_{12} with physiological activity, such as cyanocobalamin, aquacobalamin, adenosylcobalamin, and mecobalamin in natural food. Cyanocobalamine was the most stable speciation among them, and other speciation of vitamin B_{12} must be transformed into cyanobalamin prior to determination. In this study, measured results of 9 milk and dairy products were compared by adding potassium cyanide or not. As shown in Table 2, there was a significant difference between the two groups, most of the p values were less than 0.05, excepting sample of NIST SRM 1849a, wich was 0.814. The measured values adding potassium cyanide are 1.1–14.9 times more than the results without potassium cyanide. Measured values would significantly lower if potassium cyanide was not used in the determination of vitamin B_{12} in milk and dairy products and would cause determination deviation.

3.5. Optimization of Enzymatic Hydrolysis Conditions. Pepsin is often used coupled with diastase in the determination of vitamin B_{12} [11–14]; in the meanwhile, there are background values of vitamin B_{12} in pepsin. To further evaluate the influence of background value, several pepsins were purchased with different activities of 250 U/mg, 400 U/mg, 600 U/mg, and 2500 U/mg, respectively. The concentration of vitamin B_{12} was determined following the optimization method in 1 g sample with 0.2 g pepsin and 0.05 g diastase added. As shown in Figure 3, the background value of vitamin B_{12} in pepsin increased with the activity of pepsin. The background value of vitamin B_{12} in pepsin cannot be neglected.

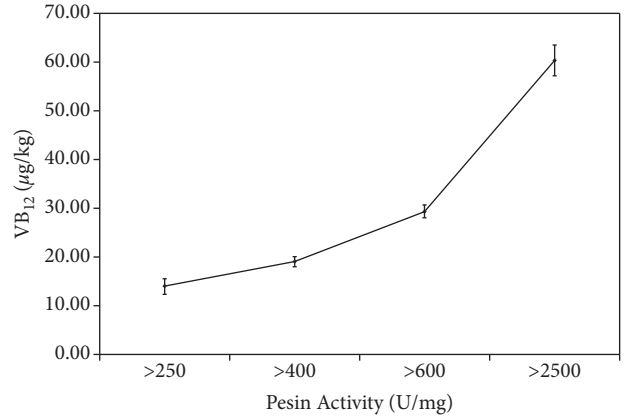

Figure 3: Relationship of background value of vitamin B_{12} and pepsin activity.

3.6. Choice of Internal Standard. In most cases, the concentrations of vitamin B_{12} are not very high in foods, although it widely exists in dairy products. It is necessary to purify and concentrate in the determination to achieve ideal detection sensitivity. Two studies [21, 22] reviewed vitamin B_{12} loss during cooking treatment with contradictory results. The absolute recovery of immunoaffinity SPE cartridge for vitamin B_{12} is not very well; meanwhile, quality differences between batches would influence the accuracy of determination. Luo [24] developed an analytical method for the determined of vitamin B_{12} in food products and multivitamin multimineral tablets by HPLC-ESI-MS, and ginsenoside Re was used as an internal standard (I.S.). Ginsenoside Re is not a perfect internal standard of vitamin B_{12} for the obvious contrast between the molecular structure of ginsenoside Re and vitamin B_{12}. In this study, to achieve the purpose of accurate quantification, [$^{13}C_7$]-vitamin B_{12} isotope-labeled vitamin B_{12} was used as an internal standard in the whole analysis.

3.7. Matrix Effect. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has become a "gold standard" in many fields of analytical chemistry. One of the most common interference types in the case of LC-MS/MS analyses is the alteration of ionization efficiency (usually ionization suppression) due to co-eluting compounds, called matrix effect (ME). Matrix effect results in differing analyte peak areas while comparing the sample and standard with the same analyte concentration. There are many fats, proteins, and sugars naturally present in milk, which would produce interfering substances after enzymatic hydrolysis and heating process. These interfering substances would cause certain matrix effects in the LC-MS/MS analyses if purification process was less efficient. In common, matrix effect was calculated as a ratio of the slope of the matrix matching the standard curve and the slope of the solvent standard curve. There is no obvious matrix effect if the ratio is between 85% and 115%. It is difficult to find a blank matrix of vitamin B_{12} for vitamin B_{12} is widespread in milk and dairy products, so the isotope of vitamin B_{12} was used to evaluate the matrix effect of infant formula, non-fat milk powder, fermented milk, and cheese samples instead of vitamin B_{12} in this study. The matrix effect (ME%) can be quantitatively expressed by (1), where A_{matrix} and $A_{standard}$ are the peak areas of the equal amount of [$^{13}C_7$]-vitamin B_{12}, respectively, in presence and in the absence of possibly interfering compounds.

$$\mathrm{ME\%} = \frac{\mathrm{Amatrix}}{\mathrm{As\,tan\,dard}} \times 100\%. \quad (1)$$

This technique is also called post-extraction spiking: the analytical signal of a blank sample extract spiked with [$^{13}C_7$]-vitamin B_{12} (A_{matrix}) is compared with the signal of the equal amount of [$^{13}C_7$]-vitamin B_{12} in pure solvent ($A_{standard}$).

ME% ($n = 6$) of infant formula, non-fat milk powder, fermented milk, and cheese was $99.51\% \pm 1.71$, $102.51\% \pm 6.12$, $102.15\% \pm 4.65$, and $102.18\% \pm 1.66$, respectively. The results have shown that the purification effect of immunoaffinity SPE cartridge was efficient, and the interfering substance almost can be removed after purification treatment with immunoaffinity SPE cartridge.

3.8. Method Validation. The standard series solutions with the concentration of 2, 5, 10, 25, 50, and 100 ng/mL were prepared, and the standard curves were drawn according to the corresponding peak areas. The correlation coefficients were all greater than 0.999, and the standard curves had good linearity. The limit of quantitation (LOQ) and the limit of detection (LOD) were investigated according to 10 times and 3 times of signal-to-noise ratio, respectively. When 1 g of infant formula was taken for determination, the limit of quantitation and the limit of detection were 1.0 μg/kg and 0.5 μg/kg, respectively.

The whole milk powder (the background value was 16.7 μg/kg $\pm$ 1.8 μg/kg) was selected as the sample. Each standard addition concentration was determined 6 times in parallel. The recoveries of vitamin B_{12} were 92.0%~99.4%. The RSD of six repeated determination was 3.03%~5.76%.

For further confirmation of the accuracy of the established method, two standard reference materials (SRMs),

Table 3: Results of vitamin B_{12} in NIST SRM1849a and NIST SRM1869 ($n = 6$).

SRM	Measured value (μg/kg)	RSD%	Reference value (μg/kg)
NIST SRM1849a	47.2	4.82	48.2 ± 8.5
NIST SRM1869	44.8	4.69	43.5 ± 6.5

NIST SRM1849a and NIST SRM1869 infant formula, were selected as verified objects. The results are shown in Table 3.

The present method was also applied to determine vitamin B_{12} in the infant formula of FAPAS Proficiency Test in 2021. The measured value was 19.9 ± 1.0 μg/kg, and Z value was 0.8, and the result is satisfied.

4. Concluding Remarks

An isotope-dilution liquid chromatography tandem mass spectrometry method was established for the determination of vitamin B_{12} in milk and dairy products. The sample preparation procedure using pepsin and potassium cyanide, quality control, and chromatographic and mass spectrometry conditions were systematically optimized for the first time. The various forms of cobalamin were transformed to cyanocobalamin by potassium cyanide after they were released from the enzymatically digested samples. Cyanocobalamin was extracted and purified by immunoaffinity SPE cartridge before UPLC-MS/MS. The measured results of NIST SRM1849a and NIST SRM1869a by the current method are all within the reference value range. The Z value was 0.8 during participating in the FAPAS proficiency test using the developed method in 2021. All validation results showed that the method is simple, accurate, and sensitive, and it is suitable for the determination of vitamin B_{12} in different types of milk and dairy products.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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