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Author	Jung Min Park, Jong Ho Koh, Jin Man Kim
Affiliation	Konkuk University, Seoul, Korea Korea Polytechnic University, Korea
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ORCID (All authors must have ORCID) https://orcid.org	Jung Min Park (https://orcid.org/0000-0002-8817-2856) Jong ho Koh (https://orcid.org/0000-0002-9727-8273) Jin Man Kim (https://orcid.org/0000-0002-2887-8195)
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7
8
9 **CORRESPONDING AUTHOR CONTACT INFORMATION**

For the corresponding author (responsible for correspondence, proofreading, and reprints)	JIN MAN KIM
First name, middle initial, last name	First name: JIN MAN, last name: KIM
Email address – this is where your proofs will be sent	jinmkim@Konkuk.ac.kr

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Secondary Email address	
Postal address	05029
Cell phone number	82-10-4011-3541
Office phone number	82-2-450-3688
Fax number	82-2-455-1044

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12 **Abstract** Vitamin B_{12} deficiency may lead to serious health issues in both infants and
13 adults. A simple analytical method involving sample pretreatment with enzyme, followed by
14 cyanide addition under acidic conditions; separation on an immunoaffinity column; and high-
15 performance liquid chromatography was developed for the rapid detection and quantitation of
16 vitamin B12 in powdered milk. Detection limit and powdered milk recovery were determined
17 by quantitative analysis. The limits of detection and quantitation were 2.71 and 8.21 μg/L,
18 respectively. Relative standard deviations of the intra-day and inter-day precisions varied in the
19 ranges of 0.98–5.31% and 2.16–3.90%, respectively. Recovery of the analysis varied in the
20 range of 83.41–106.57%, suggesting that the values were acceptable. Additionally, vitamin B_{12}
21 content and recovery in SRM 1849a were 54.10 μg/kg and 112.24%, respectively. Our results
22 suggested that the analytical method, including the sample pretreatment step, was valid. This
23 analytical method can be implemented in many laboratory-scale experiments that seek to save
24 time and labor. Therefore, this study shows that immunoaffinity–high-performance liquid
25 chromatography/ultraviolet is an acceptable technique for constructing a reliable database on
26 vitamin B_{12} in powdered milk containing starch as well as protein and/or fat in high amounts.

27 **Keywords** vitamin B_{12}, powdered milk, HPLC, analytical method

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Introduction

Vitamin B_{12} is a water-soluble vitamin belonging to a family of compounds called cobalamins. Amongst the cobalamins, cyanocobalamin, hydroxycobalamin, adenosylcobalamin, and methylcobalamin are the major forms of vitamin B_{12} (Pakin et al., 2005; Anatol et al., 2019; Cho et al., 2019). Vitamins are produced by microorganisms and are accumulated in the liver. Thus, they are found in animal products such as meat, fish, egg, and milk products but are present in vegetables in very low concentrations (ng/g). The recommended daily intake of vitamin B_{12} is 2.4 kg/day for a Korean adult and 2.6 kg/day for pregnant and lactating women (Choi et al., 2008; Jang et al., 2014; Moon et al., 2018). Although the recommended value is very low, vitamin B_{12} deficiencies have been shown to affect neurodevelopment in infants. Additionally, vitamin B_{12} deficiency may lead to megaloblastic anemia, nervous system disorders, and/or improper synthesis of DNA (Cho et al., 2019).

The recent advances in this field have drawn the consumers' attention to minor nutrients, such as vitamins. However, there are only limited reliable databases for vitamin B_{12} for the evaluation of national nutrition in South Korea. The complex structure and multiple possible vitamers render the analysis of vitamin B_{12} particularly challenging (Fang et al., 2017).

Vitamin B_{12} has been analyzed using several methods, including spectrophotometry, microbiological methods, and high-performance liquid chromatography (HPLC) (Esteve et al., 2002; Guggisberg et al., 2012). Microbiological assays and chromatographic approaches are the most suitable methods for determining the vitamin B_{12} content in food (Szterk et al., 2012). Microbiological assays are the oldest assay method and the most commonly used technique for vitamin B_{12} detection. Although such assays are highly sensitive, they lack specificity as inactive cobalamins in some food matrices may interfere with the microorganism growth. These methods are also time-consuming, as they involve steps such as tissue culture and

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53 preservation of strain. Moreover, these methods lack sensitivity and have low precision
54 (O'Broin and Kelleher, 1992).
55 Numerous methods for the analysis of vitamin B_{12} have been described by Karmi et al. (2011).
56 Among them, HPLC–mass spectrometry is probably the most frequently used technique for
57 determining vitamin B_{12} in food and biological samples. To overcome the low sensitivity of the
58 existing techniques, which is a limitation, an attempt was made to obtain food samples with
59 low concentrations of vitamin and analyze them through pretreatment methods such as sample
60 concentration using solid phase extraction or immunoaffinity columns (Iwase and Ono, 1997;
61 Heudi et al., 2006; Sun et al., 2016; Jie et al., 2019). Vitamin B_{12} exists in free and bound forms
62 in foods. It can be extracted from protein-rich foods using proteolytic enzymes. However,
63 information on the extraction of vitamin B_{12} from powdered milk is very limited. Especially,
64 powdered milk add starch as well as protein and/or fat to improve its nutrition value (Seo et al.,
65 2018). The presence of these additional components renders the analysis of vitamin B_{12}
66 extremely difficult (Lee et al., 2015; Bito et al., 2016). Hence, the analysis of vitamin B_{12} in
67 powder milk must include a pretreatment step. Currently, the methods validated by the Ministry
68 of Food and Drug Safety (MFDS) apply to infant formula, baby formula diet, and milk formulas;
69 however, powdered milk containing starch is not included in this list. In this study, a
70 chromatographic approach involving a pretreatment step and immunoaffinity column
71 purification during the sample preparation of powdered milk containing starch was adopted to
72 remove interfering matrix components and enrich the sample with the target analyte to ease
73 quantitation. This analytical method involving a pretreatment step coupled with
74 immunoaffinity purification and HPLC/Ultraviolet (UV) was validated and applied for the
75 determination of total vitamin B_{12} content in powdered milk containing starch.
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Materials and Methods

Standard, sample, and reagent

The powdered milk used in this study was purchased from a local market and kept at 4°C for further use. An powdered milk standard reference material, SRM 1849a (National Institute of Standard and Technology, USA), which is a certified reference material, was used in the recovery tests. Vitamin B_{12} content in SRM 1849a was 48.2±8.5 μg/kg. Sodium acetate was purchased from Junsei Chemical (Japan), while the enzyme, amylase, was purchased from ANKOM (catalogue TAHTL-NC24). HPLC grade water and acetonitrile were purchased from Merck (Germany).

Preparation of standards

Vitamin B_{12} in the form of cyanocobalamin (Cat. No. 1152009), with a purity of 1.04% (10.4 μg/mg), was bought from US Pharmacopeial Convention (USP, USA) to be used as the reference standard. The standard material (100 mg) was dissolved in water in a 100 mL volumetric flask to prepare a 10 mg/L stock solution. This stock solution was serially diluted with water to prepare 25, 50, 100, 250, and 500 μg/L working solutions.

Development of sample preparation

A previously reported sample preparation method (Kirchner et al., 2012; Moon et al., 2018) was used to remove protein, fat, and starch from the sample, after a slight modification of the method. Five grams of the cereal infant formula sample was placed on a 55 mL screw cap tube and dissolved in 49 mL of 0.2 M sodium acetate. The pH of the sample solution was adjusted to 4.0 to remove casein, which comprises ~80% of the milk protein fraction. Lowering the pH beyond 4.0 (isoelectric point of casein) resulted in isoelectric precipitation. Following this, 0.5

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101 mL of 1% sodium cyanide was added and mixed, and the sample was extracted ultrasonically
102 at 25°C for 10 min. After the addition of 0.5 mL of α-amylase, the sample was incubated for
103 30 min at 40°C and then for 30 min at 100°C in an incubator to initiate the reaction. Next, 20
104 mL of the above solution was filtered by a Whatman paper and transferred to an immunoaffinity
105 column (Easi-Ex-tract Vitamin B_{12}, r-Biopharm, Glasgow, UK). The column was washed with
106 10 mL water and injected with 40 mL of air by syringe to dry it. The loaded sample was eluted
107 with 3 mL of methanol. The eluate was volatilized to dryness and then reconstituted in 0.5 mL
108 of water. This was used as the test sample.
109

110 **Chromatography parameters**

111 Chromatographic conditions were determined based on previously reported analogous
112 methods that used LC–UV. A Shimadzu HPLC system (Shimadzu, Japan) equipped with a
113 Shiseido Capcell Pak C18 UG 120 column (4.6 mm x 250 nm, 5 μm) was used for the analysis
114 of vitamin B_{12}. Water and acetonitrile were used as the mobile phases for gradient elution. A
115 flow rate of 1.0 mL/min and a column temperature of 35°C were maintained, and the injection
116 volume was 50 μL. HPLC grade solvents were filtered through a 0.45 μm membrane and
117 ultrasonically degassed prior to use. The specific chromatography conditions are A (water): B
118 (acetonitrile) gradient system 0 - 3.4 min (100:0), 3.5 - 10.9 min (75:25), 11.0 - 18.9 (65:35),
119 19 - 20 min (90:10), and 20 - 26 min (100:0).
120

121 **Method validation**

122 Selectivity for vitamin B_{12} detection was determined by comparing the chromatographic
123 peaks of the test sample with those of the standard solutions. Linearity was assessed by
124 injecting 25 to 500 μg/L of vitamin B_{12} solutions in duplicate. Qualitative parameters were

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125 determined by comparing the retention times of the standard solution with those of the samples.
126 The analyte was quantified from the calibration plot equations calculated by the least-squares
127 method. Precision was calculated in terms of intra-day (n = 3) and inter-day repeatabilities (n
128 = 3) by analyzing spiked cereal infant formula samples and was evaluated by calculating the
129 relative standard deviation (RSD). Accuracy of the method was determined by calculating the
130 recovery and appropriate standard deviation (SD) in cereal infant formula samples spiked with
131 different amounts of vitamin B_{12}. Detection limits were assessed in terms of limit of detection
132 (LOD, signal-to-noise ratio (S/N) = 3) and limit of quantitation (LOQ, S/N = 10).
133

134 ## Results and Discussion

135 ### Development of pretreatment method

136 Analysis using the current method proposed by the MFDS is complex; moreover, it does not
137 yield a desirable peak resolution in the analysis of powdered milk samples. In addition, the
138 MFDS has not yet provided an appropriate method for the analysis of powder milk containing
139 starch. Although the reason for the low peak resolution is not clear, the unstable nature of starch,
140 proteins, and fats during sample treatment has been assumed to be a limitation in this
141 conventional method. In addition, it is difficult to detect vitamin B_{12} in some food samples
142 using only one pretreatment method
143 (http://foodsafetykorea.go.kr/foodcode/01_03.jsp?idx=324). Since vitamin B_{12} exists in
144 different forms at very low concentrations in powdered milk containing cereal, the sample
145 preparation methodology is extremely crucial (Lee et al., 2015). In this study, individual
146 pretreatment methods were developed by modifying the Association of Official Analytical
147 Chemists (AOAC) method to detect vitamin B_{12} in powdered milk containing starch in high
148 amounts. In the modified AOAC method, samples were purified using an immunoaffinity

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column and then subjected to HPLC to quantitate vitamin B_{12} in the samples. Sodium cyanide and α-amylase were used to remove starch, as mentioned in the experimental section. The pre-treatment involving clean-up and concentration using an immunoaffinity column enabled the efficient separation of trace amounts of vitamin B_{12} from powdered milk samples. As a result of the sample pretreatment, vitamin B_{12} was eluted at 9.3 min in the HPLC run, suggesting its efficient separation from the degradation products. This method allowed the separation and detection of vitamin B_{12} within 10 min (Fig. 1). Detection using this approach under the described experimental conditions was slightly more rapid compared to that under the experimental conditions employed in a previous study (Heudi et al., 2006).

Method validation

The specificity of the proposed technique was ensured by employing the well-established method of using highly selective immunoaffinity column for sample preparation (Nakos et al., 2017; Anatol et al., 2019). Detection limit and powdered milk containing starch recovery were determined by quantitative analysis, and certified reference material, SRM 1849a, was used to validate our analytical HPLC method. The amount of vitamin B_{12} recovered in the SRM 1849a reference was 54.10 μg/kg. Compared with value of 48.20 μg/kg (given SRM 1843a certified value), the test represented recovery of the authentication value of 112.24%. The external calibration curve of vitamin B_{12} standard solutions was linear in the range of 25–500 μg/L, with $r^2 > 0.9999$. The equation of the calibration curve was $y = 53.806x - 150.44$, where y represents the peak area of the curve obtained through UV detection, and x is the concentration (μg/L) of vitamin B_{12}. It is evident that the correlation coefficients were greater than 0.9999, which indicated a good correlation between the concentration and peak area of the investigated compounds. Accuracy was assessed by adding a known amount of the analyte, followed by

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173 calculating the recovery using standards. Accuracy of the method was satisfactory, ranging
174 from 83.41% to 106.57%, which was well within the recovery range reported for other food
175 matrices (Zironi et al., 2013; Chamlagain et al., 2015). Intra-day and inter day variations were
176 used to determine the precision of the established method. As shown table 1, RSD of intra-day
177 and inter-day variations for compound was less than 5.31% and 3.90, respectively. The LOD
178 and LOQ were 2.71 and 8.21 μg/L, respectively (Table 2). These results suggest that the HPLC
179 method involving sample pretreatment, immunoaffinity column separation is precise, accurate
180 and sensitive for quantitative determination of active compounds in powdered milk containing
181 starch.

182

183 **Monitoring test cereal infant formulas**

184 Four different powdered milk containing starch samples, of which two were manufactured
185 in South Korea and two were manufactured in USA, were analyzed using the method developed
186 in this study. The sample pre-treatment was repeated three times for each sample; the results
187 are presented in Table 2. It is evident from Table 2 that the vitamin B_{12} content in powdered
188 milk products was in the range of 11.03 – 42.18 μg/kg. As determined from the HPLC analysis,
189 all the products contained trace nutrients that were higher than those displayed on the content
190 labels. Therefore, the vitamin B_{12} content displayed in powdered milk packaging available in
191 the South Korean markets was well verified.

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Conclusion

The nutrition labeling system of foods is being strengthened to provide appropriate information to consumers while choosing a food product. Therefore, there is an increasing need for scientifically established analytical techniques to strengthen the national management of foods with high nutritional components. In this work, sample pretreatment, immunoaffinity column separation, and HPLC were employed in combination to develop an analytical method for the extraction of vitamin B_{12}. In the proposed method, starch was removed using a small quantity of α-amylase, unlike the traditional methods. The validation results indicated high sensitivity and good accuracy and precision. The recovery and relative standard deviations were in the acceptable range. Additionally, the value obtained for the certified reference material (SRM 1849a) was within the range of certificated values. The developed method based on HPLC and sample pretreatment for the detection of vitamin B_{12} could reduce the analysis time and manual labor, thereby proving to be an appropriate alternative to conventional analytical methods. Although, there are several methods for the detection of vitamin B_{12} in dairy products, powdered milk etc., this is the first study to attempt the rapid detection of vitamin B_{12} in powdered milk containing starch. Moreover, a beginner can be expected to easily perform this analytical procedure because of its simplicity. This method for the analysis of vitamin B_{12} may be utilized in industries for micronutrient analysis in dairy products, functional foods, as well as powdered milk.

Conflicts of Interest

The authors declare no potential conflict of interest.

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219

220 ## Author Contributions

221 Conceptualization was Jin Man Kim, investigation, experiment and writing was Jung Min
222 Park, and review & editing was Jong Ho Koh and Jin Man Kim.

223

224 ## Ethics approval

225 This paper does not require IRB/IACUC approval because there are no human and animal
226 participants.

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294 products. J Dairy Sci 96:2832-2836.
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296 **Table 1. Liquid chromatography (LC) conditions for vitamin B_{12}**

Parameter	Condition			
Column	UG 120 C18 4.6 X 250nm, 5 µm, Shimadzu			
Detector	UV 361 nm			
Mobile phase	A: water B: Acetonitrile Gradient system	Time (min)	Solvent (A) %	Solvent (B) %
		0	100	0
		3.5	75	25
		11.0	65	35
		19.0	90	10
		20.0	100	0
		26.0	100	0
Flow rate	1.0 mL/min			
Column temperature	35°C			
Run time	25 min			
Injection volume	50 µL			

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298 **Table 2. Inter-day and inter-day precision of vitamin B_{12}**
299 All values are mean SD of three replicates.

Precision	Recovery (%)	SD	RSD[1)] (%)
Intra-day precision	103.72	5.51	5.31
	98.96	5.08	5.13
	93.61	3.94	4.21
	84.69	0.83	0.98
Inter-day precision	106.57	2.53	2.37
	95.02	2.05	2.16
	89.59	3.49	3.90
	83.41	2.01	2.40

300 [1)] RSD, relative standard deviation.
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302 **Table 3. Validation factors and monitoring test for vitamin B_{12} in certified reference material**
303 **(SRM 1849a)**

		Tested value (μg/kg)		RSD[1)] (%)	Recovery (%)
SRM 1849a		54.10±0.84		1.88	112.24±2.11
Samples		Tested value (μg/kg)			
Cereal infant formula		T-1	T-2	T-3	T-4
		11.93±2.08	11.03±0.16	42.18±1.57	16.65±1.18
r2	0.999			Linear Regression	y= 53.806x-150.44
LOD[2)]	2.71 μg/L			Range	25-500 μg/L
LOQ[3)]	8.21 μg/L				

304 All values are mean SD of three replicates.
305 [1)] RSD, relative standard deviation; [2)] LOD, limit of detection; [3)] LOD, limit of quantitation.
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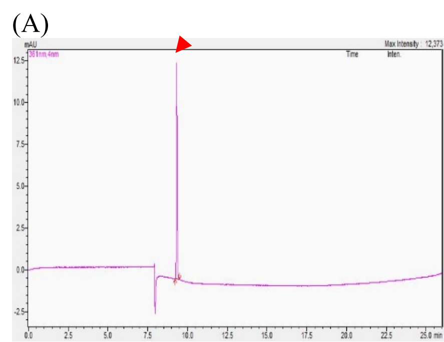
(A)



(B)

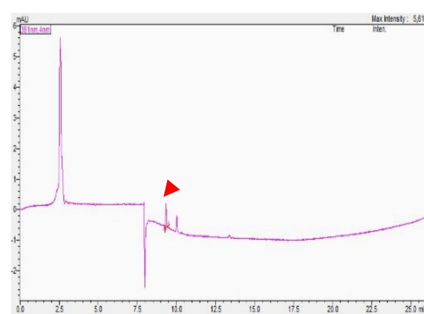


Fig. 1. Chromatogram of vitamin B_{12}, (A) Standard of vitamin B_{12}, (B) Powdered milk containing starch

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