



Validation of High-Performance Liquid Chromatography Method for Determination of Vitamin B1 in Powder Milk

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Abstract

Vitamin B1 plays an important role in the co-enzymatic reactions for energy-rich compounds called ATP (Adenosine Tri Phosphate). Therefore, it should be added to various food products, for example, milk powder. One method that can be used to determine vitamin B1 is SNI number 3751: 2009, but the method is intended for wheat flour. If the method is to be used for the analysis from other samples, such as milk powder, optimization, and validation, are needed. This experiment was carried out using HPLC, C18 column, and UV detector with a wavelength of 254 nm. The mobile phase used is methanol: acetic acid: bi-distilled water = 32:1:67 (v/v/v), flow rate = 1 mL/minute, isocratic, and reverse phased technique. Method validation parameters include tests of system suitability, linearity, the limit of detection, the limit of quantitation, precision (repeatability), and accuracy. The results showed that the system suitability test was obtained relative standard deviations (% RSD) for retention time and peak area, tailing factor, resolution, separation factor was 0.297%, 1.476%, 1.113, 6.693, and 4.406 respectively. The validation test gets a correlation coefficient (R) of 0.9996, the limit of detection and limit of quantitation were 0.0122 mg/100 mL and 0.0244 mg/100 mL, respectively. The precision test obtained Horwitz's ratio of 0.27%. Accuracy test using CRM obtained % recovery of 93.79–97.77%. All these results meet the requirements of method validation, so it can be concluded that the method of SNI number 3751: 2009 is valid for the determination of vitamin B1 in milk powder and can be used for routine analysis procedure.

1. Introduction

Milk powder is one product that is produced from processed fresh milk. This milk is in the form of powder with minimal moisture content. According to the BSN (2015) [1], powder milk is a dairy product obtained by reducing a large portion of water through the drying process of fresh milk with or without the addition of vitamins, minerals, and other food additives.

Vitamins are one of the micronutrients, which are usually added to milk powder. Vitamins are complex organic compounds that are needed in large quantities for the metabolic functions of the human body [2]. Vitamins can be grouped into two main groups, i.e., fat-soluble vitamins which include vitamins A, D, E, and K and water-soluble vitamins consisting of vitamin C and vitamin B. Vitamin B1 (or thiamine) is a vitamin that acts as a coenzyme in reaction to carbohydrates to produce energy

by forming energy-rich compounds called ATP (Adenosine Triphosphate) in the human body. Vitamin B1 can only be stored in limited quantities in the body, such as in the liver, kidneys, heart, brain, and muscles. Vitamin B1 cannot be made in the human body, so it needs to be supplied from food consumed, such as milk [3].

There is much method for vitamin B1 determination such as method developed by Gámiz-Gracia and Luque de Castro [4], Rada *et al.* [5], Yantih *et al.* [6] for pharmaceutical product and Haşlımoğlu and Ghodke [7] for veterinary consumption. We choose the SNI method because wheat flour and milk powder have the same characteristics. Both of them are powder and food products. According to Eurachem [8], a method should be validated when non-standard methods, laboratory-designed/developed methods, standard methods used outside their intended scope, and amplifications and

modifications of standard methods. SNI method number 3751: 2009 is for wheat flour, so it is needed to be validated when we want to apply to milk powder as wheat flour and milk powder has different matrices.

The purpose of this experiment is to validate the testing method of vitamin B1 content milk powder and ensure that the analytical method is accurate, specific, and reliable so that this method can be used as a routine method for analyzing vitamin B1 in milk powder samples.

2. Material and Methods

This experiment consists of three stages, namely the preparation, testing, and processing of data. The preparation stage includes the preparation of reagents, mobile phase solutions, standard series, sample preparation, and conditioning of high-performance liquid chromatography (HPLC). The testing stages include a test of system suitability, linearity test, the limit of detection and limit of quantitation (LOD & LOQ), precision (repeatability), and accuracy [7]. The data processing stage is done by calculating the results of the validation data and estimating the uncertainty of the test results and then comparing the results of the validation method with terms of acceptance. The method is valid and can be used as a routine analysis method if all method validation parameters meet the acceptability limit [8, 9].

2.1. Equipment and Materials

The equipment used in this experiment was high-performance liquid chromatography (Waters e2695) equipped with Empower 3 Chromatography software, analytical balance (Mettler Toledo AL 204), water bath (Mettler WNB 14), ultrasonic bath (Elma S100H), magnetic stirrer (Cimarec II), pH meter (Toledo MP 220), beaker glass (50 and 500) mL, graduated cylinder (50 and 500) mL, volumetric pipette (0.5; 1; 2; 5; 10) mL, volumetric flask (50 and 100) mL, the vial of 2 mL, syringe (3 and 5) mL and filter membrane 0.45 μm .

The materials used in this experiment consisted of chocolate powder milk Certified Reference Material (ERM BD600) thiamin 4.5 ± 0.6 mg/kg, hydrochloric acid (Merck) 0.1 N, sodium acetate (Merck) 2 M, standard vitamin B1 (Merck), glacial acetic acid (Merck), methanol (Merck), double distilled water, and hexane-1-sulfonic acid sodium salt (Merck).

2.2. Preparation of a standard solution of Vitamin B1

The standard vitamin B1 was weighed 0.0100 g into a 50 mL beaker glass, then added 50.00 mL of 0.1 N hydrochloric acids quantitatively. This standard solution is stirred with a magnetic stirrer, then heated in a water bath with a temperature of 95–100°C for 30 minutes. This standard solution is cooled at room temperature ($\pm 25^\circ\text{C}$) then the pH of the solution is adjusted to pH 4.5 with sodium acetate 2M solution. This standard solution is put into a 100 mL volumetric flask and diluted with double distilled water to the mark, then homogenized and filtered with a filter membrane of 0.45 μm . A standard solution of 10 mg/100 mL of vitamin B1 was taken as much as 5.00 mL and then put into a 50 mL volumetric flask to make a standard solution of 1 mg/100 mL. The standard

solution of 1 mg/100 mL of vitamin B1 was taken (0.5; 1; 2; 6; 12) mL then put into 50 mL volumetric flask, respectively to make concentration (0.01; 0.02; 0.04; 0.12; 0.24) mg/100 mL. This standard solution is diluted with double distilled water and then homogenized.

2.3. Preparation of milk powder samples

Milk powder weighed 5.5000 g into a 50 mL beaker glass and added 0.1 N hydrochloric acid as much as 50.00 mL quantitatively. The sample solution is stirred with a magnetic stirrer then heated with a water bath at a temperature (95–100)°C for 30 minutes. The sample solution is cooled at room temperature ($\pm 25^\circ\text{C}$) then the pH of the solution is adjusted to reach pH 4.5 with a 2 M sodium acetate solution. The sample solution is put into a 100 mL volumetric flask and diluted with double distilled water to the mark and then homogenized. The sample solution is filtered with Whatman filter number 42 and then filtered again with a filter membrane 0.45 μm .

2.4. Preparation of mobile phase solution

The mobile phase solution consists of methanol: acetic acid: water (32: 1: 67, v/v/v) prepared as much as 500 mL. 5 mL of acetic acid and 335 mL of double-distilled water (1:67) were put into a 500 mL beaker glass and then added 0.4718 g hexane-1-sulfonic acid sodium salt. The solution is stirred until dissolved then filtered with filter membrane 0.45 μm . The acetic acid solution and the water are treated for 20 minutes then put into the mobile phase container. Methanol is prepared and put into other mobile phase containers. The mobile phase solution is mixed in the HPLC instrument with a ratio of methanol and a mixture of acetic acid–water = 32:68 (v/v)

2.5. Validation parameters

Validation of the analytical method deals to ensure that the developed method is acceptable and reliable for its deliberate purpose. As per ICH guidelines, the developed HPLC method was validated for different validation characteristics such as system suitability, selectivity, linearity, precision, accuracy, LOD, and LOQ.

2.5.1. System suitability

The system suitability ensures the validity and specificity of the developed method. System suitability test was performed by measuring chromatographic parameters such as peak area, retention time, theoretical plates as well as tailing factor and by determining the standard deviation (RSD) for each parameter. The percent RSD of peak area (< 2), tailing factor (< 2), and theoretical plates ($N > 2000$) were within the acceptable limits [10, 11].

2.5.2. Specificity

The specificity of a method is the ability of a method to measure certain substances, carefully and thoroughly, in the presence of other components that might be in the sample matrix. For the selectivity test, the substance to be tested must be determined first. Then made a standard solution, test solution and the blank solution is then tested by the method, whether the method can separate the analyte from the sample matrix

2.5.3. Linearity

Linearity is the ability of the analytically-developed procedure to check the result of the sample analytes, whether it is linear to its concentration. The linearity of the method was tested by analyzing different concentrations range of the analytes. The regression analysis data for the calibration curve shows a linear relation over this concentration range for analytes. The correlation coefficient value was found to be $R > 0.995$ [9, 12], which suggested excellent correlation and good linearity for the optimized method.

2.5.4. Precision (Repeatability)

The precision was carried out by analyzing samples of 7 replications using HPLC. From the seven replications, the value of % RSD was calculated and compared with % RSD (Horwitz), so the Horwitz ratio was obtained. Horwitz ratio can be accepted if the value < 2 .

2.5.5. Accuracy (%Recovery)

The accuracy test is done by analyzing the Certified Reference Material for milk powder four times. % recovery can be obtained by comparing the CRM measurement results and CRM concentration on the certificate.

$$\% \text{ recovery} = \frac{\text{measurement concentration}}{\text{theoretical concentration}} \times 100\%$$

2.5.6. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ values are obtained from statistical calculations through linear regression from the calibration curve. According to (NATA) [13], LOD and LOQ can be calculated using the formula:

$$\text{LOD} = (3 \times \text{Sy/x}) / b$$

$$\text{LOQ} = (10 \times \text{Sy/x}) / b$$

Notes:

Sy/x: residual standard deviation from a calibration curve
 b : slope

2.6. Determination of Vitamin B1 content in powder milk

HPLC should be conditioned first before used.

Table 1. Parameters of High-Performance Liquid Chromatography conditions

Instrument	HPLC Waters e2695 with Separations Module
Column	InertSustain C18 4.6x150 mm, 5µm
Detector	UV Detector with a wavelength of 254 nm
Flow rate	1.0 mL/minute
Time	15 minutes
Technique	Isocratic
Injection volume	20 µL

The content of vitamin B1 in powdered milk can be obtained by plotting the peak area of the sample solution into the calibration curve so that the measured concentration is obtained, and then the vitamin B1

content in the test sample can be calculated based on the weight and volume of the solution.

$$C \left(\frac{\text{mg}}{100\text{mL}} \right) = \frac{y - a}{b}$$

$$\text{The content of vitamin B1} \left(\frac{\text{mg}}{100\text{g}} \right) = C \times \frac{V_s}{W}$$

Notes:

C: measured concentration (mg/100 mL)

Vs: volume of sample solution (mL)

W: sample weight (g)

3. Results and Discussion

3.1. System suitability test

Before conducting the analysis, we need to do a system suitability test to ensure that the HPLC is functioning properly. The standard solutions of vitamin B1 were measured to know the retention time of the thiamin. After we know the retention time of the thiamin, the sample was injected eight times to determine the repeatability of the area and retention time. From the chromatogram in Figure 1, we can get the system suitability data as follows:

Table 2. System suitability parameters

Parameters	Data obtained	%RSD	Requirement [10, 11]
Peak area	22932+339	1.476	RSD < 2
Retention time	8.624+0.026	0.297	RSD < 2
Tailing factor	1.113+0.021		T ≤ 2
Resolution	6.693+0.257		Rs > 2
Retention factor	4.406+0.012		k ≥ 2

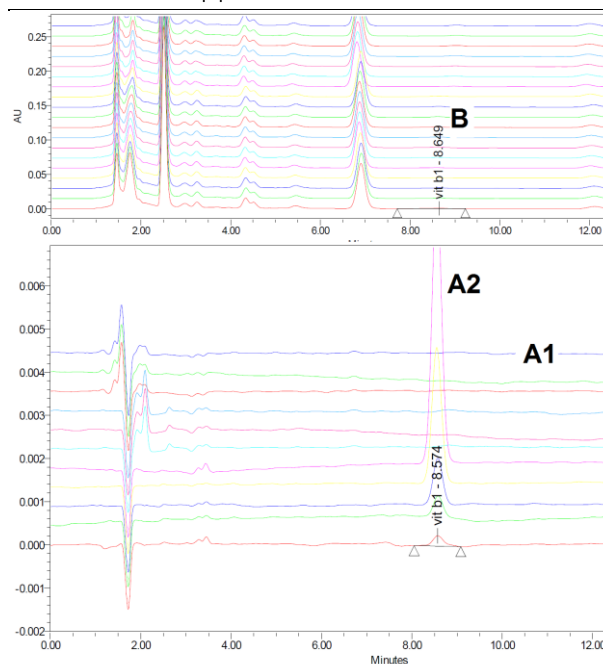


Figure 1. Chromatogram of blank (A1), standard thiamin (A2) and sample thiamin (B), measured using HPLC with the mobile phase methanol: acetic acid-water (32:68), column C18 4.6 x 150 mm 5µm, flow rate 1ml/minute and wavelength 254 nm

3.2. Specificity

Based on the chromatogram in Figure 1, it can be concluded that a retention time of 8.624 is specific for thiamin. It means that this method has good selectivity because the retention time of analyte is far from the other peak ($R = 6.693$), and the retention time of the analyte is not disturbed by the sample matrix.

3.3. Linearity

Linearity tests were carried out by measuring the peak area of a standard series of vitamin B1 at concentrations (0.0100; 0.0200; 0.0400; 0.1200; 0.2400) mg/100 mL. The standard series concentrations are made in the range (50-150)% of the estimated or expected concentration of the analyte in the test sample. Standard series solutions were prepared at five different concentrations with solution preparation three times repetition. The chromatogram and calibration curve for standard vitamin B1 can be seen in Figure 2.

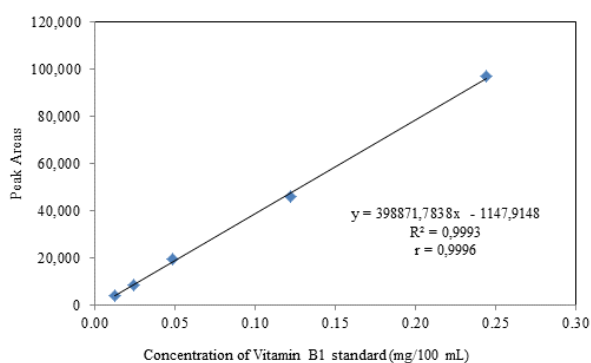


Figure 2. Calibration Curve of Vitamin B1 standard

The curve shows the relationship between concentration and peak area. The line connecting the points on the curve above is called the regression line or correlation line. The parameter that states the relationship between two variables (on the x-axis and y-axis) is called the correlation coefficient. The magnitude of the correlation coefficient (r) on the calibration curve above is 0.9996. This value meets the requirements of $r > 0.9950$ [9, 12]. The slope value on the calibration curve can be used to see sensitivity. The slope value on the curve above is 398871.78 indicating high sensitivity. Intercept is the intersection point at y when x is 0. Ideally, the intercept is 0, but the intercept on the curve above is negative. When y is 0, and the intercept is interpolated to the regression equation, an x value of 0.002878 mg/100 mL is obtained.

3.4. Precision (repeatability)

Precision is useful to see the repeatability of the analytical method carried out under the same conditions of the tool, place, and analyst, in the next test period. Measurements were made with seven replications. The results of the vitamin B1 precision test can be seen in Table 3.

Table 3. Results for precision test for determination of Vitamin B1.

No.	Area	Weight of sample (g)	Content of Vitamin B1 (mg/100 g)	Requirement [11]
1	22959	5.5018	1.126	
2	22995	5.5022	1.128	
3	22697	5.5022	1.114	
4	22788	5.5028	1.118	
5	23373	5.5052	1.145	
6	22260	5.5015	1.093	
7	22375	5.5018	1.098	
Total	159447.0	38.518	7.822	
Average	22778.1	5.5030	1.117	
SD			0.02	
% RSD			1.61	
% RSD(Horwitz)			6	
Horwitz ratio = (%RSD/%RSD(Horwitz))			0.27	< 2

Based on precision results, the Horwitz ratio of the sample is 0.27, it is still less than 2, so the precision is still within the acceptable limit.

3.5. Accuracy

Accuracy testing is performed to determine the closeness between the measured value and the accepted real value, which is expressed as percent recovery. Accuracy testing is carried out using a Certified Reference Material, whose purity is known. CRM is treated according to the test sample preparation method. The accuracy testing results can be seen in Table 4.

Table 4. Results for accuracy test for determination of Vitamin B1

CRM of Vitamin B1							
No.	Certified value (mg/kg)	Uncertainty	Peak Areas	Weight of CRM (g)	Measured value (mg/kg)	% Recovery	Requirements [10, 11]
1	4.5	0.6	6965	4.7262	4.40	97.77	92 - 105%
2	4.5	0.6	7044	4.7079	4.46	99.07	
3	4.5	0.6	6779	4.7079	4.32	95.98	
4	4.5	0.6	6591	4.7079	4.22	93.79	

Based on Table 4, the obtained accuracy is in the range of 93.79-99.07%. The accuracy of this method is good, indicated by recovery values that are close to 100%

3.6. Limit of detection (LOD) and limit of quantitation (LOQ)

The theoretical LOD and LOQ are obtained from statistical calculations from the calibration curve data. From the theoretical LOD and LOQ, the next step was confirmed by making and analyzing standards with a

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