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Research Paper

Optimization of a Modified QuEChERS Method of Experimental Design for Vitamin B_2, B_3, B_6 and B_9 in Powder Formula Milk by HPLC/DAD

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Abstract
The purpose of this work is to use HPLC/DAD to ascertain the best extraction and cleanup conditions for various forms of vitamin B in powder formula milk. The QuEChERS method's key variables were optimized using a Box-Behnken design with 3-level 4-variable and Minimum-Run Resolution IV Screening Design. Following the selection of the best experimental setup, the suggested approach was applied to quantify four vitamins B (B_2, B_3, B_6, and B_9) in powder formula milk matrix by HPLC/PDA in accordance with AOAC and ICH guidelines. When taking into account the performance standards given in this guideline, the approach presented recovery between 83.55 and 108.43 for 4 B vitamins, which is adequate. The intra-day and inter-day percentage relative standard deviations were 1.66 to 2.75% and 0.47 to 4.78%, respectively. Furthermore, the technique enabled the determination of low detection limits in less than 25 minutes of analytical time. The proposed method's excellent accuracy, precision, and efficiency made it acceptable for regular B vitamin analysis when applied to powder formula milk samples. The findings confirmed that the modified QuEChERS technique is appropriate for routinely assessing B vitamin levels in powder formula milk matrix.

Keywords
QuEChERS, B Vitamins, Powder Formula Milk, HPLC/DAD

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1. INTRODUCTION

The body requires extremely little amounts of vitamin B on a daily basis. Although vitamins are abundant in many foods, including cereal grains, bread, meat, chicken, eggs, milk, vitamin deficiencies are common and can have detrimental impacts, particularly for people who are poor, food insecure, or lack health information. These days, a wide range of vitamin-supplementing goods are available on the market, including milk formulas, nutritional powders, and dietary supplements in the form of vitamin B pills or vitamin mixtures. Among these, formula milk is regarded as a vital source of nutrients, particularly for young children, those in school, and the elderly. It can be ingested raw or in the form of dairy products. The national technical rules for formula nutritional goods (Ministry of Health, 2012a; Ministry of Health, 2012c; Ministry of Health, 2012b) that control the composition and techniques of nutrient analysis, including B vitamins, have now been released by the Vietnamese government. Therefore, several nations have milk monitoring programs for vitamin B.

Vitamin-enriched mixes are made up of many vitamins, and it is necessary to find out how much of each vitamin is in both the original combination and the food items that are supplemented with these mixtures. Because it can detect many substances at once, high-performance liquid chromatography (HPLC) is one of the most widely used techniques for determining water-soluble vitamins. Reverse phase HPLC is utilized most of the time (Almagro et al., 2002; Viñas et al., 2003; Chen and Wolf, 2007; Bendryshev et al., 2010; Suh et al., 2011; Gliszczyńska-Świgło and Rybicka, 2015; Mateeva et al., 2023). It is challenging to determine all of the water-soluble vitamins simultaneously in an isocratic manner. Either gradient elution or ion pair reagents can be used to tackle such an issue. Ion pair chromatography is the standard technique for determining many vitamins at once (Suh et al., 2011; Gliszczyńska-Świgło and Rybicka, 2015). Nevertheless, it is sometimes not possible to achieve full resolution of the peaks of several vitamins with matrix components or each other. There aren't many opportu-

nities in this instance for the ion-pair variation to increase the resolution of the nearby peaks.

The Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method is a simple extraction technique that involves partitioning first, followed by extract clean-up using dispersive solid-phase extraction (d-SPE). At the 4th European Pesticide Residue Workshop in Rome in 2002, Anastassiades, Lehotay, Stajnbaher, and Schenck first presented the technique (Anastassiades and Lehotay, 2002). Later, in 2003, the thorough method was published (Anastassiades et al., 2003). Originally developed to remove pesticide residues from fruits and vegetables, the QuEChERS technology gained popularity due to its comprehensive ability to extract analytes from a wide range of matrices. Natural agricultural goods and other non-fatty food samples are the main applications for the QuEChERS technique. It has not been optimized to utilize the QuEChERS technique with fatty or emulsified meals. For determining multiclass pesticide residues in fatty meals, a number of quick and simple techniques have been devised (Lehotay et al., 2001). The QuEChERS technique was originally developed for usage in fruits, vegetables, and herbal products (González-Curbelo et al., 2015; Nguyen et al., 2022), but it was later adapted to analyze the lipid matrices in eggs, cake, milk, and avocados (Vela-Soria et al., 2018; Zhou et al., 2018; Guo et al., 2018; Targuma et al., 2021; Mnyandu and Mahlambi, 2021). Using acetonitrile as the extraction solvent, this approach then adds sodium chloride and magnesium sulfate to facilitate partitioning. Owing to the method's efficiency, several changes have been made and it has been applied to other analyte types and matrices (Holmes et al., 2015; Zhang et al., 2017; Choi et al., 2015; Kolberg et al., 2011). In recent years, several QuEChERS technique modifications have been proposed for various purposes. The dispersive solid-phase extraction for clean-up was changed to cartridge-based solid-phase extraction instead of dispersive solid-phase extraction (Rejczak and Tuzimski, 2017), and only primary secondary amine was claimed to be employed for clean-up in the QuEChERS update.

In this work, a two-level fractional factorial screening design called Minimum-Run Resolution IV Screening Design was used to optimize a number of factors influencing the extraction efficiency in order to obtain the best extraction result from the QuEChERS method. Subsequently, the modified QuEChERS condition was optimized for the simultaneous HPLC/DAD detection of four vitamins B (B_2, B_3, B_6, and B_9) in formula powder milk using the Box-Behnken design.

2. EXPERIMENTAL SECTION

2.1 Reagents and Chemicals

The National Institute of Drug Quality Control (Vietnam) provided the riboflavin (98.6%), nicotinamide (99.9%), pyridoxine hydrochloride (99.8%), and folic acid (91.8%) that were purchased. Sigma-Aldrich (Steinheim, Germany) provided the HPLC quality methanol, acetonitrile (≥99.9%), and salts needed to make the buffer solution. Throughout the studies, water from a Millipore system was utilized.

2.2 Buffer Preparation

To make the buffer, three grams of sodium dihydrogen phosphate were dissolved in 500 milliliters of distilled water. The orthophosphoric acid was used to adjust the pH to three. The buffer was filtered through a membrane filter (0.20 μm) using a Millipore glass filter holder.

2.3 Preparation of Standards

The stock solutions contained vitamin B_3 (1000 μg/mL) and B_6 (1000 μg/mL), which were prepared by dissolving precisely weighed amounts of reference standards in methanol. Methanol was used to dilute the B_2 stock solutions (200 μg/mL) to a ratio of 0.1% formic acid to water (70:30, v/v). In order to create B_9 stock solutions (1000 μg/mL), 500 μL of NaOH 1N were dissolved in methanol. Then, using distilled phosphate buffer (pH = 6.6) to dilute exact amounts of each of the four different stock solutions, 10 μg/mL of vitamins B_2, B_3, and B_6 and 50 μg/mL of vitamin B_9 were obtained.

2.4 Sample Preparation

2.4.1 Extraction

The formula's milk powder samples came from a number of shops in Can Tho City, Vietnam. The process of extraction was completed: One gram of milk product sample was put into a 15 mL centrifuge tube, dissolved in boiling water at 40°C, and then spiked with a mixture of vitamin standards at a concentration of 10 μg/mL. The tube was filled with 4 mL of ACN containing 1% (v/v) acetic acid, and the liquid was shaken arduously for 30 seconds. Then, 0.4 g of $MgSO_4$ and 0.1 g of NaCl were added, mixed for 30 seconds in a vortex, then centrifuged at 3000 rpm for 5 minutes.

2.4.2 Clean-up

Subsequently, a 15-mL centrifuge tube was filled with 1 mL of the supernatant, 150 mg of $MgSO_4$, and 25 mg of PSA. After a 30-second shaking, the tube was centrifuged for five minutes at 3000 rpm. A vial containing 1000 μL of the upper phase was filled in advance of the LC injection after this cleanup.

2.5 Optimization of the Modified QuEChERS Method by Experimental Design

Firstly, Minimum-Run Resolution IV Screening Design was employed to assist filter out variables that have negligible to no effect on extraction efficiency in order to discover the key elements. Resolution IV designs will enable main effect estimate. Two-factor interactions will be aliased by higher and other two-factor interactions. Good designs to reduce the number of runs in the unlikely case that there are interactions. Table 1 shows the Screening Design matrix generated by the Design Expert 12 tool for 10 variables and 20 experimental runs. The variables included in the analysis were the following: Acetonitrile volume (A), percent of acetic acid (B), amounts of $MgSO_4$ (C), amounts of NaCl (D), and amount of sample (E); vortex time (F), centrifugation time (G), centrifugation speed (J), amount of $MgSO_4$ clean-up (I), and amount of PSA clean-up (K). Table

Table 1. Minimum-Run Resolution IV Screening Design Factors and Levels of Design of Experiments

Variables	Code Levels	
	Low (-1)	High (+1)
Acetonitrile volume (mL) (A)	3	5
Percent of acetic acid (%) (B)	1	3
Amounts of $MgSO_4$ (g) (C)	0.4	0.8
Amounts of NaCl (g) (D)	0.1	0.2
Amounts of sample (g) (E)	0.5	1
Vortex time (min) (F)	1	2
Centrifugation time (min) (G)	5	10
Centrifugation speed (rpm) (H)	3000	5000
Amounts of $MgSO_4$ clean-up (mg) (I)	150	300
Amounts of PSA clean-up (mg) (J)	25	50

Table 2. Experimental Design Factors and Levels of Design of Experiments

Independent Variable	Units	Experimental Value	
		Low (-1)	High (+1)
Acetonitrile volume (A)	mL	3	5
Amounts of $MgSO_4$ (C)	g	0.4	0.8
Amounts of sample (g) (E)	g	0.5	1
Amounts of $MgSO_4$ clean-up (I)	mg	150	300

1 also includes the components and their respective amounts. The response was calculated using the analytes' mean recovery attained (%).

Following the screening design, the updated QuEChERS technique was optimized by considering the four most important factors. These variables contribute the highest percentage to the recovery of the four vitamins tested. Four quantitative variable levels were developed for this purpose: 3-5 ml of ACN volume (A), 0.4-0.8 g of $MgSO_4$ (C), 1-2 g of milk sample (E), and 150-300 mg of $MgSO_4$ clean-up (I). BBD was then conducted with these values in mind. Table 2 displays the design matrix that was employed, along with the variables that were evaluated and their respective levels in the optimization process. At the center point, 25 tests were carried out in duplicate at random.

2.6 Chromatographic Conditions

Using a UFLC Shimadzu (LC-20AD) system equipped with an autosampler, quaternary pump, injection system, and DAD detector, four vitamins (B_2, B_3, B_6, and B_9) were detected and measured in milk samples. The LabSolution software was used to control the system. Chromatographic separation was performed using a reversed-phase Phenomenex C18 column (250 mm × 4.6 mm i.d., 5-μm particle size) (USA) at 30°C. Twenty-μL samples were injected using the full loop injection option. The mobile phase, which included 0.05 M NaH2PO4 containing orthophosphoric acid, pH 3.0 (solvent D), and HPLC-grade methanol (solvent B), was utilized to elute the column at a steady flow rate of 1 mL/min. The following was the gradient elution program: 10% B and 90% D for 0–7 min; 40% B and 60% D for 15 min; and 10% B and 90% D for 25 min. Two wavelengths of 270 nm (Vitamin B_2 and B_3) and 290 nm (Vitamin B_6 and B_9) were selected for the DAD detector.

2.7 Method Validation

By assessing the fundamental validation process parameters such as system suitability, accuracy, linearity, limits of detection (LOD), limits of quantification (LOQ), and recovery of the analytical technique described in this study was verified. The International Conference on Harmonization (ICH) criteria European Medicines Agency (2005) were followed in the evaluation of the validation parameters.

2.7.1 System Suitability

To evaluate the applicability of the system, six duplicates of a standard mixed solution of B vitamins (10 μg/ml for each vitamin) were injected. The theoretical number of plates (TP) must be greater than 2000, the peak resolution (RS) must be greater than 2, the tailing factor (TF) less than 2, and the relative standard deviation (RSD) for the retention time and peak area (PA) less than 2%.

2.7.2 Specificity

The specificity was tested by analyzing the milk sample using the UFLC method. It was evaluated by comparing the peaks found during extract analysis with the RT and UV absorption spectra of each component in reference solutions. The peak purity of the analytes was > 99.9%, according to the spectrum overlaid on the three-point purity detection plots.

2.7.3 Linearity

The method's linearity was assessed by injecting five standard B vitamin combinations, ranging in concentration from 0.5 to 20 μg/mL. For these vitamins, calibration curves with regression equations and R^2 were obtained.

2.7.4 LOD and LOQ

In following the ICH guidelines, the calibration curves were utilized to calculate the limit of detection (LOD = 3.3 multiplied by the error in Y intercept divided by the slope of the calibration curve) and the limit of quantification (LOQ = 3 multiplied by LOD) (European Medicines Agency, 2005).

2.7.5 Precision

Repeatability and intermediate precision dictated the method's precision. Six independent series were performed on the same day (intra-day) and over the course of three successive days (inter-day) to examine a combination of B vitamins at a concentration of 10 μg/mL.

2.7.6 Accuracy

Since milk includes a lot of B vitamins, it was not possible to spike the blank matrix with vitamins in the current investigation. On the other hand, adding milk to flour fortification might be seen as a kind of doping. In the current investigation, the estimated percentage of recovery was determined using the measured per content of B vitamins in powder formula milk.

2.8 Application

HPLC/DAD was used to analyze the B vitamins in seven powder formula milk samples using the improved QuEChERS technique and confirmed quantification.

3. RESULTS AND DISCUSSION

3.1 Optimization of the Modified QuEChERS Method

3.1.1 Experimental Design Approach and Optimization Process Screening by Minimum-Run Resolution IV Screening Design

Preliminary research was crucial in determining the extraction parameters for efficient sample preparation and, consequently, good recoveries for all analytes. Because of the B vitamins' chemical makeup, the organic solvents that are utilized have a direct impact on the extraction efficiency. In this regard, the effects on extraction efficiency of acetonitrile and its various mixtures including varying ratios of acetic acid (AA) were studied. The analytes with the maximum recovery were found to be obtained when acetonitrile containing AA was used. The significance of ten factors influencing the modified QuEChERS technique for the B vitamin content was ascertained by using the Minimum-Run Resolution IV Screening Design. The experimental results generated by the Minimum-Run Resolution IV Screening Design were displayed as a mean recovery value (%) in Table 3. The new QuEChERS approach was optimized by taking into account the four most significant aspects after the screening design. The recovery of the four vitamins under test is most influenced by these factors.

Table 4 shows that, within the range of investigated levels of variables, all factors were significant in the extraction and clean-up phases, with the exception of ACN volume (A), $MgSO_4$ amounts (C), sample amounts (E), and amounts of $MgSO_4$ clean-up (I). It is important to note that using diatomaceous earth, $MgSO_4$, and PSA as a clean-up sorbent for milk products has resulted in purer chromatograms when compared to those who did not use it. It appears to have no discernible effect on the extraction efficiency since its levels, or more specifically, the amounts used in the sample preparation were enough. NaCl and PSA did not have any notable impact either, but they are all necessary for a thorough cleanup. However, in order to shorten the experimental run, the variables centrifugation speed (H), centrifugation duration (G), vortex time (F), and percent of AA (B) are not included in the optimization stage.

3.1.2 Box-Behnken Experimental Design

BBD used a 3-level approach to optimize the four most important variables in the screening design: ACN volume (A), amounts of $MgSO_4$ ((C), amounts of sample (E), and $MgSO_4$ clean-up amounts (I). A total of 25 trials were conducted. Table 5 summarizes the experimental settings and the observed responses as B vitamins content.

The statistical summary of quadratic model suggested by the software is presented in Table 6. The relationship between four B vitamin contents and the four chosen factors is shown in Equations 3.1.2-3.1.2:

Vitamin B_3 (Y_1)

$$Y_1 = -8.28 - 10.07A - 33.70C - 10.42E - 30.65I + 1.6AC + 2.25AE - 10.76AI - 7.61CE + 1.65CI \; 13.72EI - 3.4A^2 - 32.25C^2 - 1.6E^2 - 13.08I^2 \quad (1)$$

Vitamin B_6 (Y_2)

$$Y_2 = -5.99 - 15.55A - 7.17C - 33.06E - 31.00I - 1.92AC - 0.4170AE + 1.1AI - 2.26CE - 10.62 \; CI + 7.29EI - 7.54A^2 + 0.6750C^2 - 34.95E^2 - 26.52I^2 \quad (2)$$

Vitamin B_9 (Y_3)

$$Y_3 = 13.71 - 7.1A + 21.77C - 5.71E + 21.87I - 1.74 \; AC - 6.39AE + 1.19AI + 4.03CE - 3.29CI + 4.1 \; EI + 5.68A^2 + 18.47C^2 - 6.39E^2 + 21.77I^2 \quad (3)$$

Table 3. Minimum-Run Resolution IV Screening Design matrix with Ten Independent Variables Investigated

Run	A	B	C	D	E	F	G	H	I	J	Mean
1	5	1	0.4	0.1	0.5	2	10	5000	300	50	30.39
2	3	3	0.4	0.1	1	2	5	5000	300	25	20.04
3	5	3	0.8	0.2	0.5	2	5	5000	150	25	16.00
4	5	1	0.8	0.1	1	1	5	5000	150	50	7.73
5	5	3	0.4	0.1	1	2	10	3000	150	50	40.76
6	5	1	0.4	0.2	1	2	5	3000	300	50	50.24
7	5	1	0.8	0.1	1	2	10	3000	300	25	26.18
8	5	1	0.8	0.2	0.5	1	10	3000	150	50	41.52
9	3	1	0.8	0.2	1	2	10	5000	150	50	67.30
10	3	1	0.4	0.1	1	1	10	3000	300	50	14.53
11	3	1	0.4	0.1	0.5	2	5	3000	150	25	94.39
12	3	3	0.8	0.1	0.5	2	5	3000	300	50	39.98
13	3	3	0.8	0.2	1	1	5	3000	150	25	26.76
14	3	3	0.4	0.2	0.5	2	10	3000	300	25	25.97
15	3	1	0.8	0.2	0.5	1	5	5000	300	25	40.39
16	5	3	0.8	0.2	1	1	10	5000	300	50	34.05
17	3	3	0.8	0.1	0.5	1	10	5000	150	25	67.88
18	5	1	0.4	0.2	1	1	10	5000	150	25	90.91
19	3	3	0.4	0.2	0.5	1	5	5000	150	50	118.56
20	5	3	0.4	0.1	0.5	1	5	3000	300	25	32.60

Table 4. The Result of Percentage Contribution of Ten Independent Variables Investigated for Minimum-Run Resolution IV Screening Design

	A	B	C	D	E	F	G	H	I	J
Vitamin B_3	10.7	0.02	4.91	4.01	2.23	2.42	0.06	4.83	13.52	0.03
Vitamin B_6	1.14	0.12	13.11	0.08	2.14	2.69	0.18	3.25	17.75	3.61
Vitamin B_9	5.4	3.57	2.36	1.86	6.91	0.001	0.68	5.83	0.36	4.2
Vitamin B_2	17.08	0.66	1.31	8.19	11.22	0.25	0.02	0.51	24.83	0.25
Sum percentage contribution	34.32	4.37	21.69	14.14	22.5	5.361	0.94	14.42	56.46	8.09

Vitamin B_2 (Y_4)

$$Y_4 = -19.39 - 13.83A - 51.06C - 17.03E - 47.11I - 1.53AC - 0.0142AE - 18.09AI - 15.53CE + 2.09CI - 20.38EI - 2.32A^2 - 44.91C^2 + 4.42E^2 - 18.78I^2 \quad (4)$$

The analysis of variance (ANOVA) results (Table 6) demonstrate that the proposed response model characterizes the extraction very well, with an $R^2 > 0.8$ at a 95% confidence level. Moreover, the significance of the suggested model is indicated by its F-value (F model $> F14, 12 = 2.64$) and probability value ($p < 0.05$). Moreover, the estimated lack of fit for the model is smaller than the computed F value. Consequently, it is also possible to state the statistical significance of the model.

3.1.3 Response Surface Graphs by BBD and Optimal Values

The relationships between the B vitamin concentration and these four variables are shown in Figure. 1A-D (a-f). Each graph displays the effects of four variables within the examined ranges, with one variable at the optimal level. A fuller picture of how each variable tends to influence the quantity of B vitamins is given by the response surface. The nonlinear viewpoint of the graphs supports the evidence of their interactions, which leads us to believe that significant parameters influence the recoveries achieved during the extraction process, particularly in relation to Figure 1A-D.

Using the software's optimization tool, the ideal parameters for the modified QuEChERS method of the analytes were determined to be acetonitrile volume (A) of 4.688 ml, $MgSO_4$ amount (C) of 0.621 g, sample amount (E) of 0.753 g, and $MgSO_4$ clean-up amount (I) of 153.338 mg. When the following parameters were selected: 3%, 0.2 g, 1 minute by vortex, 5 minutes and 5000 rpm by centrifugation, and 50 mg, respectively, for the working values of acetic acid percentage (B), NaCl quantity (D), centrifugation duration (G), centrifugation speed (H), and PSA clean-up amount (J). The equivalent B vitamin contents under these ideal circumstances

Table 5. Rotatable Central Composite Design Setting in the Original and Coded Forms of the Independent Variables (A,C,E,I) and Experimental Results of B Vitamins Content

Run	Independent Variables				Responses			
	A	C	E	I	Vitamin B_3 (μg/mL)	Vitamin B_6 (μg/mL)	Vitamin B_9 (μg/mL)	Vitamin B_2 (μg/mL)
1	4	0.4	0.5	225	4.56	7.71	1.75	2.53
2	3	0.6	0.5	225	13.66	5.24	1.57	18.89
3	4	0.8	0.5	225	13.66	7.84	1.32	18.89
4	4	0.6	0.5	300	16.04	4.14	1.87	16.29
5	4	0.6	0.75	225	15.66	20.42	1.17	16.63
6	4	0.4	0.75	300	4.33	12.57	9.43	0.86
7	4	0.6	1	150	14.60	4.51	4.33	17.12
8	3	0.6	0.75	300	11.99	3.50	9.49	16.98
9	5	0.8	0.75	225	4.42	13.50	10.93	1.42
10	5	0.6	0.5	225	8.10	6.00	8.17	19.37
11	4	0.4	1	225	5.28	14.71	6.34	2.27
12	4	0.8	0.75	150	2.98	20.72	11.89	1.99
13	4	0.4	0.75	150	8.02	14.96	13.23	4.61
14	3	0.8	0.75	225	2.49	16.93	12.46	1.18
15	5	0.4	0.75	225	4.11	13.30	10.43	3.66
16	3	0.6	0.75	150	2.59	4.70	21.51	1.16
17	5	0.6	1	225	11.78	2.70	3.04	14.41
18	4	0.6	0.5	150	7.29	11.75	5.27	4.40
19	3	0.4	0.75	225	5.37	12.88	8.47	0.38
20	4	0.6	1	300	9.62	4.19	5.03	8.64
21	3	0.6	1	225	12.85	2.77	9.22	13.96
22	5	0.6	0.75	150	17.48	5.61	15.19	20.73
23	4	0.8	0.75	300	0.94	7.71	11.38	0.32
24	5	0.6	0.75	300	5.37	6.63	5.55	0.38
25	4	0.8	1	225	6.76	12.57	9.95	3.10

Table 6. ANOVA for the Effect of the Independent Variables (A,C,E,I) on the B Vitamins Content Using a Quadratic Response Surface Model

Term	Df	Vitamin B_3		Vitamin B_6		Vitamin B_9		Vitamin B_2	
Mode		F-ratio	p-value	F-ratio	p-value	F-ratio	p-value	F-ratio	p-value
Model	14	3.46	0.0273	3.72	0.0213	3.36	0.0301	5.54	0.005
X_1	1	0.0449	0.8364	0.0211	0.8873	0.6944	0.4241	0.2799	0.6083
X_2	1	0.0015	0.9698	0.0709	0.7955	0.5388	0.4798	0.8032	0.3912
X_3	1	0.0484	0.8303	0.011	0.9184	2.54	0.1424	2.21	0.1681
X_4	1	0.1821	0.6786	3.98	0.074	6.46	0.0293	0.2178	0.6507
X_1X_2	1	0.255	0.6245	0.3198	0.5842	0.2866	0.6041	0.1417	0.7145
X_1X_3	1	0.5047	0.4937	0.015	0.9048	3.85	0.0782	0	0.9973
X_1X_4	1	11.57	0.0068	0.1055	0.7521	0.1333	0.7227	19.93	0.0012
X_2X_3	1	1.45	0.2566	0.1105	0.7464	0.3832	0.5498	3.67	0.0844
X_2X_4	1	0.0677	0.8	2.44	0.1494	0.2553	0.6243	0.0666	0.8016
X_3X_4	1	4.7	0.0553	1.15	0.3088	0.3966	0.543	6.32	0.0307
X_1^2	1	3.27	0.1009	13.87	0.004	8.58	0.0151	0.9213	0.3598
X_2^2	1	18.34	0.0016	0.007	0.9352	5.68	0.0384	21.67	0.0009
X_3^2	1	0.0454	0.8355	18.64	0.0015	0.6784	0.4293	0.21	0.6566
X_4^2	1	3.02	0.1129	10.73	0.0083	7.88	0.0186	3.79	0.0802
R^2		0.8289		0.8389		0.8246		0.8858	
Adj R^2		0.5893		0.6135		0.5790		0.7258	

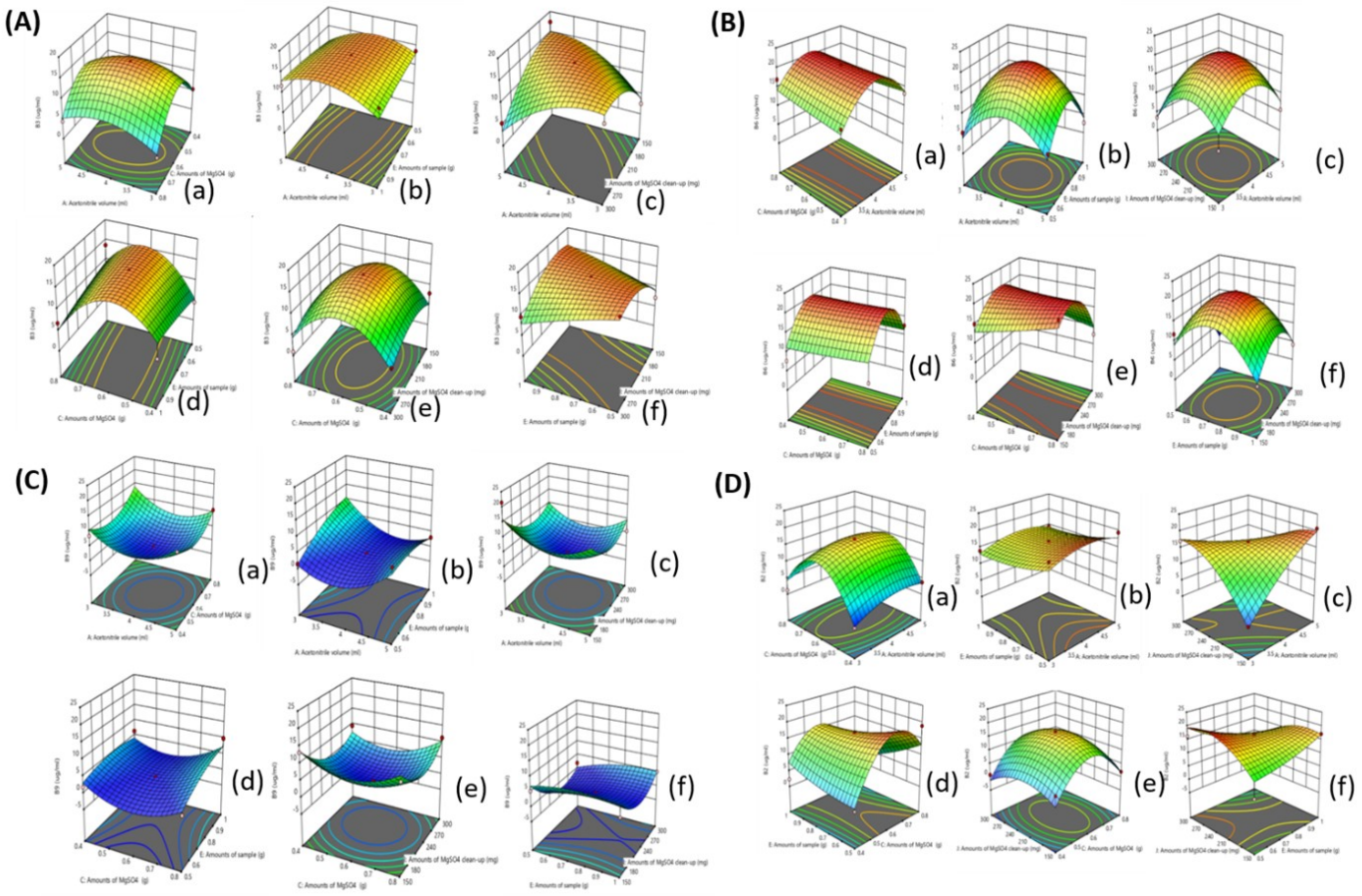

Figure 1. 3D Response Surface Curve Showing the Influences of Independent Variables on the Vitamin B_3 Content (a-f) (A); on the Vitamin B_6 Content (a-f) (B); on the Vitamin B_9 Content (a-f) (C) and on the Vitamin B_2 Content (a-f) (D)

Table 7. Precision, Recovery, Calibration Parameters, LOD and LOQ

Substance	Calibration Curve		Precision (n=6)		Recovery (%)				
	Regression Equation	R^2	Intra-Day RSD (%)	Inter-Day RSD (%)	Low-Level	Mid-Level	High-Level	LOD (μg/ mL)	LOQ (μg/ mL)
Vitamin B_3	$y = 12602x + 319.97$	0.9996	2.75	2.43	97.16	105.70	100.75	0.9	2.8
Vitamin B_6	$y = 27230x - 1229.9$	0.9998	1.66	3.46	85.00	92.23	85.57	0.5	1.2
Vitamin B_9	$y = 39576x + 10831$	0.9996	2.03	0.47	83.55	108.43	98.88	0.16	0.5
Vitamin B_2	$y = 49838x - 6008.9$	0.9999	1.91	4.78	92.55	103.39	105.70	0.5	1.6

were 15.077 μg/mL for Vitamin B_3, 12.601 μg/mL for Vitamin B_6, 10.14 μg/mL for Vitamin B_9, and 18.054 μg/mL for Vitamin B_2. The HPLC/PDA analytical technique was approved and utilized to determine the B vitamin content of actual samples.

3.2 Method Validation

The mobile phase, which included 0.05 M NaH_2PO_4 containing orthophosphoric acid, pH 3.0 (solvent D) and HPLC-grade methanol (solvent B), was utilized to elute the column at a steady flow rate of 1 mL/min. Vitamin solubility increases in the mobile phase upon protonation of the pyridinic moiety, hence reducing vitamin retention time. A. Bendryshev et al. (2010)'s study employed acetonitrile and 0.6% H_3PO_4 (pH 1.7) in the mobile phase to analyze B_1, B_6, B_9, and B_2 in premixes and medicines. Ion-suppressed analytes exhibit better retention than ionized analytes, which is crucial to keep in mind when developing methods utilizing ionizable analytes. Select a low-mobile-phase buffered pH for acidic analytes to prevent ionization. The analytes' pKa allows for an efficient selection of the pH of the mobile phase. Avoid extremely high or low pH values while utilizing silica columns to avoid reducing column life. As a result, our study chose a pH 3 mobile phase that would both safeguard the C18 column and be appropriate

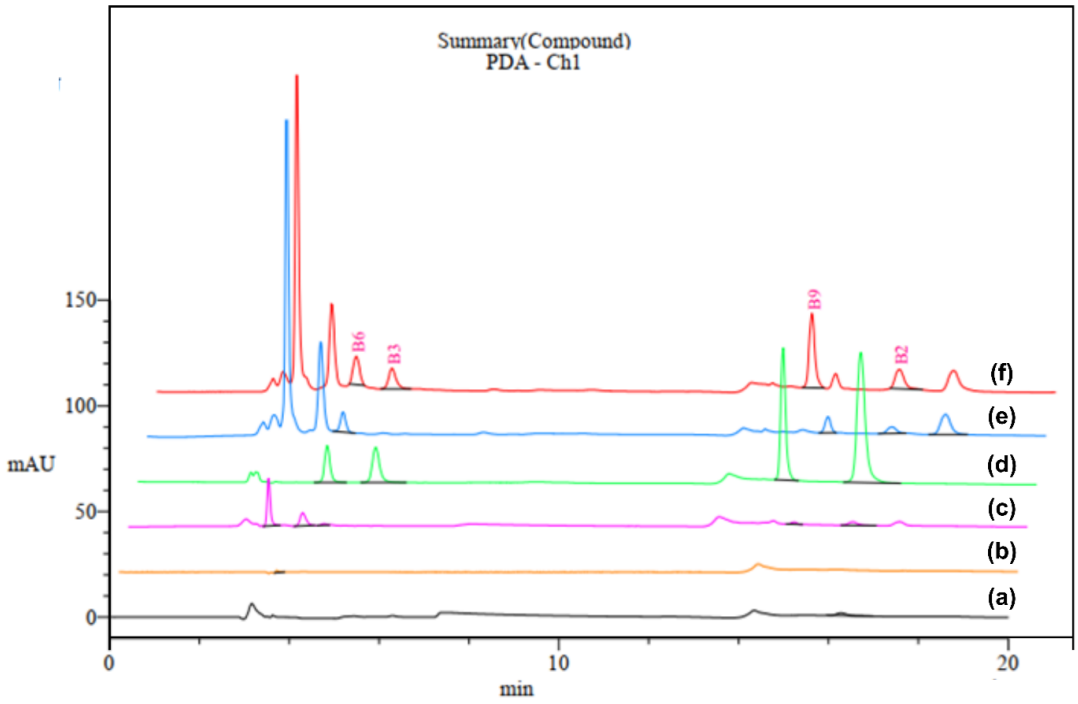

Figure 2. HPLC Chromatography of B Vitamins in Milk Sample (a: Solute Sovent; b: Mobile Phase solvent; c: Blank Sample; d: Mixed Standards Solution; e: Milk Sample; f: Spiked Sample)

Table 8. The Application Quantification B Vitamins in Formula Milk Powder on Purchased in Vietnam Market

No.	Vitamin B_3			Vitamin B_2			Vitamin B_6			Vitamin B_9		
	Label (mg)	Found (mg)	Rec (%)	Label (mg)	Found (mg)	Rec (%)	Label (mg)	Found	Rec (%)	Label (mg)	Found (mg)	Rec (%)
1	0.47	0.42	112.96	1.25	1.23	101.23	0.42	0.43	97.68	0.06	0.063	107.19
2	6.69	8.4	79.66	0.94	1.1	85.48	0.19	0.17	110.41	0.15	0.15	97.14
3	3..65	3.41	107.14	0.49	0.47	103.94	0.56	0.54	103.30	0.14	0.105	75.82
4	5.25	5.36	97.91	0.85	0.86	98.73	0.23	0.24	95.83	0.13	0.115	88.56
5	8.55	8.5	100.54	1.01	1.06	95.73	0.33	0.3	108.37	0.19	0.18	95.70
6	4.65	3.41	136.49	0.62	0.67	91.86	0.58	0.54	107.26	0.19	0.195	103.03
7	11.63	11.3	102.93	0.61	0.65	94.23	1.95	1.8	108.15	0.23	0.271	115.53

*Rec (%): The Percentage of Recovery

for four analytes.

As seen in Figure 2, the simultaneous elution of B vitamins was carried out in the current study in a brief amount of time (20 min). With the exception of the study of Almagro et al. (2002), which had a 12-minute elution time, the described techniques' elution periods are often lengthy even though they utilized ion pairing agents (25 – 60 minutes) (Viñas et al., 2003; Suh et al., 2011; Sasaki et al., 2020) in various matrixes such as baby meals, medications, premix, infant formula, cereal, and multivitamin pills. According to Bendryshev et al. (2010), raising the mobile phase's pH increased the retention time of vitamins B_3, B_6, and B_9 while having no influence on B_2, indicating how difficult it is to elute these vitamins quickly. The retention periods found for B_6 (5.19 min), B_3 (6.61 min), B_9 (16.78 min), and B_2 (19.55 min) are rather short (Figure 2).

The system suitability parameters revealed that the values for the tailing factor (TF) < 2, the resolution (RS) > 2, the percentage of RSD for retention time and peak area (PA) ≤2%, and the number of theoretical plates (TP) exceeding 2000 fall within the specified bounds of the ICH validation guidelines (European Medicines Agency, 2005). Table 7 shows that for both intermediate precision (n = 6) and repeatability conditions (n = 6), accuracy (RSD% for peak area) was less than 6. All vitamins show good linearity (R^2 > 0.999) across the concentration range of 0.1–20 μg/mL, according to the linearity.

The LOQ values for B_2, B_3, B_6 and B_9 were 1.6 μg/mL

(270 nm), 2.8 μg/mL (270 nm), 1.2 μg/mL (290 nm) and 0.5 μg/mL (290 nm), respectively. The limit of detection (LOD) for B_2, B_3, B_6 and B_9 were 0.5 μg/mL (270 nm), 0.9 μg/mL (270 nm), 0.5 μg/mL (290 nm) and 0.16 μg/mL (290 nm), respectively (Table 7). In the study of Albawarshi et al. (2022), the LOQ values for B_2, B_3, B_6 and B_9 in premix and fortified flour were 0.2 μg/mL (265/280/361 nm), 0.8 μg/mL (265 nm), 0.3 μg/mL (210 nm), and 0.5-0.6 μg/mL (210/265/280/361 nm), respectively. In the research of Mateeva et al. (2023), the LOQ of B_2, B_3, B_6, B_9 in food supplements and brewer's yeast were 13.45 μg/mL, 0.08 μg/mL , 19.14 μg/mL , and 4.2 μg/mL. The LOQ and LOQ of research depend on different matrices and analysis equipment with different sensitivities. In our research, the LOD and LOQ were moderately sensitive to the analysis of four B vitamins in formula milk powder in Vietnam market.

3.3 Application

The quantity of four vitamins in formula milk powder on the label ranges from B_3 (79.66% - 136.49%), B_2 (85.48% - 103.94%), B_6 (95.83% - 110.41%), and B_9 (75.82% - 115.53%) after using the proposed technique to concurrently measure four B vitamins (Table 8). The bulk of B vitamins listed on the label are completely expressed in powdered milk samples that can be bought off the shelf, and the quantity after measurement is almost the same as what is listed on the label.

Because the matrix of milk powder is so complex and contains large amounts of lipids and proteins, it is sometimes necessary to separate and prepare the sample thoroughly before conducting an instrumental analysis in order to identify any trace residues or impurities. A preparation technique that is easy to use, requires less time, and is reasonably priced is perfect. It requires less harmful organic solvent usage and improves recovery (Ma et al., 2014).

The increasingly popular sample preparation approach known as QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) has been widely employed in recent years due to its advantages such as simplicity, minimal stages, and efficacy in cleaning up complicated samples (Anastassiades et al., 2003). The QuEChERS approach consists of two steps: The first stage of extraction involves using salting-out extraction to evenly divide an aqueous layer and an organic layer. The second phase, known as a dispersive SPE, entails further cleanup with $MgSO_4$ in conjunction with other sorbents, such as C18 or primary and secondary amines (PSA), in order to eliminate any interfering materials. This sample treatment has been used for the study of multiclass veterinary medications and pesticide residues in animal tissue, eggs, and milk in previous research. These investigations all combined MS detection with LC or UHPLC. Pesticide residue analysis was the main use of the QuEChERS technique in milk (Martínez Vidal et al., 2010; Manav et al., 2019). To the best of our knowledge, no research has determined the levels of vitamins B_2, B_3, B_6, and B_9 in milk using the QuEChERS technology. The reason our study was successful was that we were able to apply the Box-Behnken Experimental Design and the Minimum-Run Resolution IV Screening Design to improve the modified QuEChERS technique. When applied to the market's milk powder matrix, the improved QuEChERS condition had a high recovery rate.

4. CONCLUSION

To determine the levels of four vitamin B in formula milk simultaneously using HPLC and DAD, a dependable modified QuEChERS technique was created. The ideal experimental parameters for the recommended extraction method were found using the Box-Behken experimental design with the goals of optimization and screening. The Minimum-Run Resolution IV Screening Design came next. Using the software's optimization function, the ideal parameters for the modified QuEChERS method of the analytes were determined to be the following: acetonitrile volume (A) of 4.688 ml, $MgSO_4$ amount (C) of 0.621 g, sample amount (E) of 0.753 g, and $MgSO_4$ clean-up amount (I) of 153.338 mg. For the percent of acetic acid (B), the amount of NaCl (D), the vortex time (F), the centrifugation length (G), the centrifugation speed (H), and the PSA clean-up (J), the working values of 3%, 0.2 g, 1 min by vortex, 5 min and 5000 rpm by centrifugation, and 50 mg were chosen, respectively. The approach showed good validation parameters and recovery values ranging from 83.55 to 108.43%, with relative standard deviations ranging from 1.11% to 4.58%, for all real samples spiked at three different amounts of B vitamin. The AOAC and ICH recommendations were satisfied by these outcomes. In conclusion, the results confirmed that the modified QuEChERS method is suitable for regularly determining the amounts of B vitamins in samples of powdered formula milk.

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