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Research Article

Simultaneous Determination of B_1, B_2, B_3, B_6, B_9, and B_{12} Vitamins in Premix and Fortified Flour Using HPLC/DAD: Effect of Detection Wavelength

Y. Albawarshi,[1] **A. Amr**,[2] **K. Al-Ismail**,[2] **M. Shahein**,[2] **M. Majdalawi**,[1] **M. Saleh**,[2] **A. Khamaiseh**,[2] **and B. El-Eswed**[1]

[1]*Department of Medical Allied Sciences, Zarqa College, Al-Balqa Applied University, Zarqa, Jordan*
[2]*Department of Nutrition & Food Technology, The University of Jordan, Amman, Jordan*

Correspondence should be addressed to Y. Albawarshi; yanalbawarshi@bau.edu.jo

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Simultaneous determination of water-soluble B vitamins is a troublesome analytical procedure since they have greatly variable structures and acid-base properties which imposed difficulties on eluting them in short time and selecting wavelength of detection. The aim of the present study was to develop a simple method that overcomes these difficulties. The method was successful in simultaneous determination of B_1, B_2, B_3, B_6, B_9, and B_{12} in premix and fortified flour by extracting vitamins with 0.1% (w/v) of ascorbate and ethylene diamine tetra-acetic acid (EDTA) solution followed by eluting using gradient mobile phase consisting of 0.03% trifluoroacetic acid aqueous solution (pH 2.6) and acetonitrile on high performance liquid chromatography (HPLC) instrument coupled with diode array detector (DAD). Elution of vitamins was completed within 9.3 min, and the lowest values obtained for limit of quantification (LOQ) for B_1, B_2, B_3, B_6, B_9, and B_{12} were 0.6, 0.2, 0.8, 0.3, 0.5, and 0.7 μg/mL, respectively, at four wavelengths of 361, 280, 265, and 210 nm. In general, variation of wavelength of detection in the range from 210 to 361 nm affects sensitivity but had a marginal effect on the linearity and LOQ of the developed method and its application for determining B vitamins in premix and fortified flour. The 210 nm wavelength exhibited the highest sensitivity though resulted in higher values of B vitamins in fortified flour with respect to 265, 280, and 361 nm. Noteworthy, determination of B_2 and B_{12} in the premix at 361 nm had relatively high RSD values compared to the lower wavelengths. Thus, wavelengths in the range from 265 to 280 may be more favorable over 210 and 361 nm. The method reported in the present work does not require any sample cleanup/preconcentration steps, and chromatographic elution was achieved in 9.3 min without the need for ion-pairing reagents.

1. Introduction

More than two billion people have micronutrient deficiencies in the world due to dietary deficiency of vitamins and minerals. The prevalence of vitamin deficiencies has serious effects, especially on those suffering from poverty, food insecurity, and lack of health knowledge [1, 2]. Unfortunately, young children and pregnant women are the most affected sector by these deficiencies which cause health problems in the growth, resistance to infection, and development of cognition in fetal and child [3, 4].

Since most of wheat vitamins (thiamine B_1, riboflavin B_2, niacinamide B_3, pyridoxine B_6, and tocopherol E vitamins) are concentrated in the outer layer of the wheat grain, their concentrations in flour are reduced significantly after milling and purification [5]. Consequently, fortification of flour with vitamins "premix" was adopted to compensate for this loss and thus to improve the nutritional quality of flour [6]. The Food Fortification Initiative [7] defines premix as a powdery blend of vitamins, minerals, antioxidants, calcium carbonate, and carriers such as modified starch, rice hulls, and wheat middling [8]. However, the homogeneity of these solid components in commercial premixes is rarely fulfilled [9, 10], which affects the efficiency of fortification. Depending on quality assurance data from 20 national

Table 1: Summary of reported methods for simultaneous determination of B vitamins in multivitamin supplements, energy drinks, and pharmaceuticals.

Sample (reference)	Method	Stationary phase	Mobile phase	Elution time	Sequence of elution time (detection wavelength nm)
Pharmaceuticals [12]	HPLC-UV	C18	Gradient A: 10%v/v propanol in 0.01 M H_3PO_4 with 0.03 M sodium dodecyl sulfate. B: same as A but 60% v/v propanol	12 min	B_2(270), B_3(264), B_9(264), B_6(290), and B_1(245)
Baby foods [13]	HPLC-UV	Amide C16	Gradient A: 0.01 M KH_2PO_4 (pH 6). B: acetonitrile	30 min	B_3(266), B_6(326), B_1(266), B_9(266), B_{12}(361), and B_2(266)
Multivitamin dietary supplement [14]	LC-UV/MS	C18	Gradient A: 0.1% formic acid in water. B:0.1% formic acid in acetonitrile	18 min	B_1(260), B_3(260), B_6(292), B_9(280), and B_2(270)
Pharmaceuticals and premix [15]	HPLC-DAD	C18	Gradient A: 0.6% H_3PO_4 (pH 1.7) B: acetonitrile	30 min	B_1(244), B_6(292), B_9(297), and B_2(270)
Infant formula, cereal, and multivitamin pill [16]	HPLC-DAD	C30	Gradient A: 0.02 M KH_2PO_4 (pH 3) B: 50%v/v in 0.02 M KH_2PO_4 in acetonitrile	50 min	B_1, B_3, B_6, B_9, B_{12}, and B_2 (275 for all)
Energy drink [17]	HPLC-DAD	C18	Gradient A: methanol. B:0.05 M NaH_2PO_4 and 0.05 M hexanesulfonic acid (pH 3)	30 min	B_3(260), B_6(290), B_1(245), B_9(280), B_{12}(207, 360), and B_2(450)
Multivitamin dietary supplement [18]	HPLC-UV	C18	A: 0.005 M sodium hexanesulfonic acid, 0.02 M H_3PO_4, 0.016 M triethylamine (pH 3.0). B: acetonitrile 75:25 (v/v)	60 min	B_3, B_6, B_1, B_9, B_2, B_{12} (210 for all)
Veterinary premix [19]	HPLC-UV	C18	Gradient A: 0.025 M KH_2PO_4 and 0.005 M sodium hexanesulfonate (pH 4). B: 0.005 M sodium hexanesulfonate in methanol	25 min	B_6, B_1, B_2 (278), and B_{12} (361)
Plasma and urine [20]	HPLC-UV	C18	Gradient A: 0.05 M KH_2PO_4 and 0.005 M sodium heptanesulfonate (pH 3). B: acetonitrile	22 min	B_3, B_6, B_2, and B_1 (240)

fortification programs in 12 countries, Luthringer et al. [11] found that only less than half of the samples were adequately fortified. This necessitates the development of a simple analytical method for routine determination of vitamins in premix and fortified flour.

Few studies were reported for simultaneous determination of B vitamins (Table 1) in energy drinks [17], pharmaceuticals [12], multivitamin dietary supplements [14, 18], low-calorie food and multivitamin pill [16], and baby foods [13]. All these studies (Table 1) employed gradient elution on HPLC-DAD or UV instruments with nonpolar stationary phase such as C18 or C30 except the work of Vinas et al. [13] which used amide C16. Almagro et al. [12], Gliszczynska-Swiglo and Rybicka [17], and Sasaki et al. [18] added sodium dodecyl sulfate or sodium hexane sulfate to the mobile phase to reduce the retention times of vitamins by forming reversed phase micelles. Relatively highly acidic phosphoric acid buffer was used in these studies (except Vinas et al. [13]), which resulted in lower retention times for pyridinic-based B vitamins (Figure 1). We have noticed that these studies suffer from some difficulties. The first is that simultaneous determination of B vitamins, especially in short retention times, was a challenging issue since the retention times of B_1, B_3, and B_6 increase with decreasing pH of mobile phase. On the hand, the retention time of B_9 (folic acid) decreases with increasing pH, and the retention times of B_2 and B_9 are independent on pH [15]. The second difficulty is that these reported methods varied greatly in detection wavelengths as shown in Table 1, which explicate a question about the importance of detection wavelength in the simultaneous determination of B vitamins, especially if we consider the difference among these vitamins in conjugation structure and chromophores (Figure 1).

To the limit of our knowledge, there are two previous studies (Bendryshev et al. [15, 19]) that were directed to simultaneous determination of B vitamins in premix (Table 1). Bendryshev et al. [15] reported a simultaneous method for analysis of B_1, B_2, B_6, and B_9 in premix using HPLC-DAD, C18, and gradient mobile phase composed of 0.6% phosphoric acid and acetonitrile (pH 1.7) in a run time of about 30 min. Extraction of B vitamins from the premix was carried out using 1% solution of phosphoric acid (pH 1.5). Hosain et al. [19] reported a method that depends on extracting B vitamins from premix using distilled water followed by simultaneous analysis of B_1, B_2, B_6, and B_{12} using HPLC-UV, C18, and gradient mobile phase composed of 0.025 M KH_2PO_4, 0.005 M sodium hexanesulfonate, and methanol (pH 4) in a run time of about 25 min. The aim of the present work is to develop a method for simultaneous chromatographic analysis of six water-soluble vitamins including B_1, B_2, B_3, B_6, B_9, and B_{12} in premix and fortified

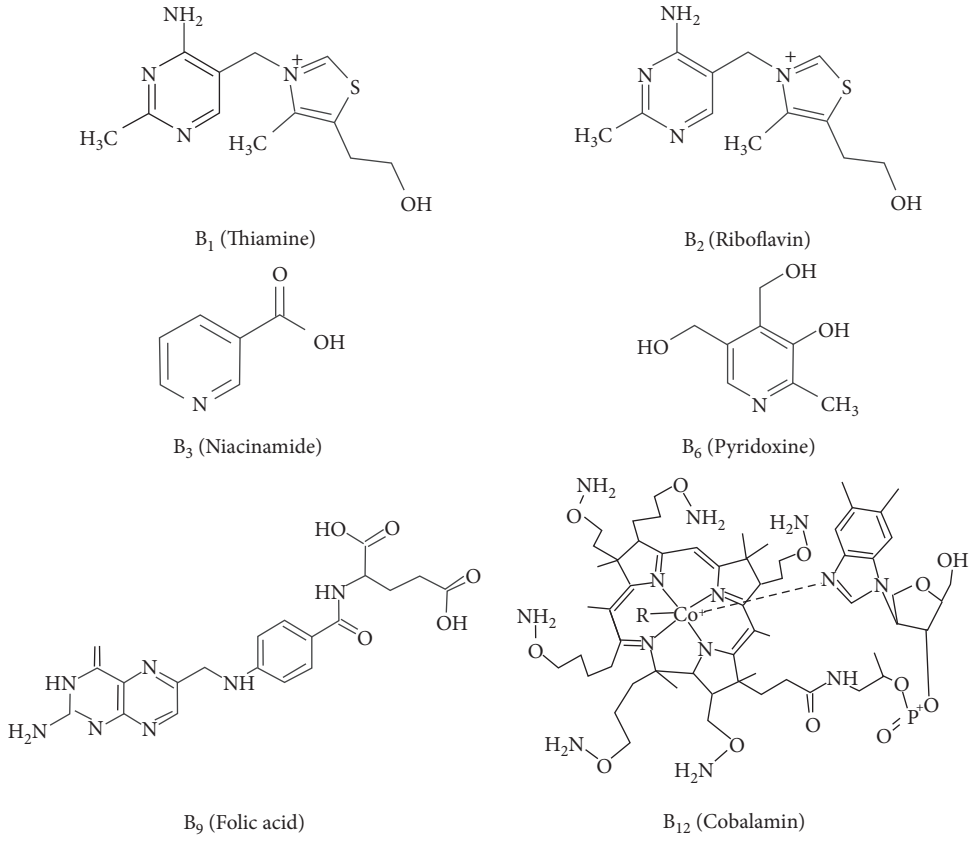

Figure 1: Chemical structures of vitamins.

Table 2: Gradient elution programme.

Time	% A (0.03% TFA in water)	% B (acetonitrile)
0	100	0
2	100	0
4.5	83	17
9.5	83	17
9.6	100	0
13.5	Equilibration	

flour using HPLC/DAD, C18, and gradient mobile phase consisting of 0.03% trifluoroacetic acid (TFA) in water (pH 2.6) and acetonitrile. Special attention was given for eluting vitamins in short time and studying the effect of wavelengths of detection (210, 265, 280, and 361 nm), which are usually used in the literature for detection of B vitamins (Table 1).

2. Materials and Methods

2.1. Materials. All vitamins used were obtained from Sigma-Aldrich (USA). HPLC grade water, methanol, acetonitrile, trifluoroacetic acid (TFA), EDTA, and sodium hydroxide were from Merck (Geneva, Switzerland). Polyvitaminated premix was provided by Jordanian Ministry of Health, and straight flour was obtained from Jordan Silos and Supply Company. Fortified flour was prepared by homogenizing 1 kg with 250 mg premix.

2.2. Preparation of Standards. Stock solutions (100 μg/mL) of B_1 (CAS 70–16–6), B_3 (CAS 98–92–0), B_6 (CAS 65–23–6), and B_{12} (CAS 68–19–9) were prepared by dissolving 5 mg of each vitamin in 50 mL HPLC grade water. On the other hand, stock solutions of B_2 (CAS 83–88–5) and B_9 (CAS 59–30–3) were prepared by dissolving 5 mg of these vitamins in 50 mL of 0.025% sodium hydroxide solution and then mixed with former solution to prepare a stock solution that contains all the B vitamins. The stock solution was kept in capped amber vials and stored at −18°C to avoid degradation. Working standards ranging from 0.1 to 20 μg/mL were prepared daily by diluting stock solution of vitamins using HPLC grade water.

2.3. Extraction of Vitamins from Premix and Fortified Flour. 0.1 g of premix or fortified flour sample, weighed to the nearest 0.0001 g, was mixed with 10 mL solution containing 0.1% (w/v) of each of ascorbate (antioxidant) and EDTA (chelating agent) in 25 mL conical centrifuge tube, followed by vortexing for 1 minute, shaking for 15 minutes in water bath at 50°C (Memmert WB 14, Germany), and centrifuging (Hermle Z 206 A, Germany) at 6000 rpm for 10 minutes. After centrifugation, the supernatant was collected, and the precipitate was re-extracted with 5 mL of the extracting solution. The supernatants were combined and finally filtered through a 0.45 μm nylon membrane and delivered for HPLC analysis. All extraction steps were performed under

subdued light conditions to prevent vitamins from degradation.

2.4. Chromatographic Conditions. The standard solutions and extracts of vitamins were analyzed using Thermo Scientific Dionex UltiMate®3000 HPLC instrument consisting of an LPG 3400 SD pump, ACC-3000 autosampler, DAD detector, and controlled with Chromeleon® 6.80 Chromatography Data System (CDS) software. Reverse phase-HPLC with ACEC18-AR (250 × 4.6 mm; 5 μm) column and gradient elution (Table 2) was applied using mobile phase consisting of 0.03% trifluoroacetic acid in water at pH 2.6 (A) and acetonitrile (B). The injection volume was 20 μl, the flow rate was 0.9 mL/min, and the column temperature was 35°C. The signal (peak area) of each vitamin was recorded at four wavelengths of 361, 280, 265, and 210 nm.

2.5. Method Validation. Analytical method developed in the present work was validated by measuring the basic parameters of the validation process such as system suitability, precision, linearity, limits of detection (LOD), limits of quantification (LOQ), and recovery. The validation parameters were evaluated according to the guidelines of the International Conference on Harmonization (ICH) [21].

2.5.1. System Suitability. The system suitability was evaluated by injecting 9 replicates of 20 μl of a standard mixture solution of B vitamins (5.0 μg/ml for each vitamin). The acceptance criteria of the relative standard deviation (RSD) for retention time and peak area (PA) are less than 2%, the number of theoretical plates (TP) is more than 2000, tailing factor (TF) is less than 2, and peak resolution (RS) is greater than 2.

2.5.2. Precision. Precision of the method was determined by repeatability and intermediate precision. A concentration of 4 μg/mL of B vitamins mixture was analyzed in 6 independent series during the same day (repeatability) and over 7 consecutive days (intermediate precision).

2.5.3. Linearity. The linearity of the method was evaluated by injecting 7 standard mixtures of B vitamins which ranged from 0.1 to 20 μg/mL. Calibration curves with regression equations and R^2 were obtained for these vitamins.

2.5.4. LOD and LOQ. The limit of detection (LOD = 3.3 multiplied by the error in Y intercept divided by the slope of calibration curve) and limit of quantification (LOQ = 3 multiplied by LOD) were calculated from the calibration curves according to the guidelines of ICH [22]. This approach may be considered as the most robust evaluation criteria [23].

2.5.5. Recovery. In the present work, spiking blank matrix with vitamins was not possible in the case of premix which contains large amounts of B vitamins. However, fortification of flour using premix may be considered as a kind of spiking. Thus, the measured per claimed % of B vitamins in premix and flour was adopted in the present work as an estimation of recovery.

3. Results and Discussion

3.1. Method Validation. In the present work, simultaneous elution of B vitamins was conducted in short time (<9.3 min) as shown in Figure 2. The elution times of reported methods are generally high, though they used ion pairing agents (25-60 min), except the work of Almagro et al. [12] which had 12 min. elution time (Table 1). Bendryshev et al. [15] reported that increasing the pH of mobile phase resulted in an increase in the retention time of B_1, B_3, and B_6 vitamins, decrease of the retention time of B_9, and small effect on B_2 and B_{12}, reflecting the difficulty of eluting these vitamins in short time.

As shown in Figure 2, the retention times obtained for B_1 (4.42 min), B_3 (5.03), B_6 (6.33), B_9 (8.04), B_{12} (8.33), and B_2 (9.28) are relatively low. This could be explained by the fact that the low pH (2.6) of the mobile (0.03% trifluoroacetic acid) phase causes protonation of B_1, B_3, and B_6, which contain basic pyridine moiety and have relatively lower molar mass than B_2, B_9, and B_{12}. Protonation of the pyridinic moiety results in an increase of solubility of vitamins in the mobile phase and consequently decreases their retention time. As shown in Table 1, the same order of elution was reported by Bendryshev et al. [15], Suh et al. [16], and Chen and Wolf [14] using acidic mobile phases (pH 1-3). However, different elution orders of B vitamins were reported when ion-pairing agents were employed [12, 17, 18], as shown in Table 1.

The system suitability parameters (Table 3) showed that the percentage of RSD for retention time and peak area (PA) ≤ 2%, the number of theoretical plates (TP) is more than 2000, tailing factor (TF) ≤ 2, and resolution (RS) > 2, which reflected that the values are within the specified limits of the ICH validation guidelines [22]. Precision (RSD % for peak area) under repeatability conditions ($n = 6$) and intermediate precision ($n = 7$) was ≤2 as shown in Table 4. From the linearity of Figure 3 and Table 5, it is found that all the vitamins maintain excellent linearity ($R^2 > 0.999$) within the concentration range of 0.1-20 μg/mL. The lowest values obtained for limit of quantification (LOQ) of B_1, B_2, B_3, B_6, B_9, and B_{12} were 0.6, 0.2, 0.8, 0.3, 0.5, and 0.7 μg/mL (Table 5).

3.2. Effect of Detection Wavelength on Sensitivity, LOD, and LOQ of B Vitamins Standards. The calibration curves (equation and R^2), sensitivity (slope of calibration curve), limit of detection, and limit of quantification of B vitamins at different wavelengths of detection are presented in Figure 3 and Table 5. All the calibration curves exhibit $R^2 > 0.997$ (except B_2 at 210 nm), suggesting that the developed method exhibits good linearity (Figure 3) at all wavelengths of detection. The highest sensitivity (slope of calibration curve)

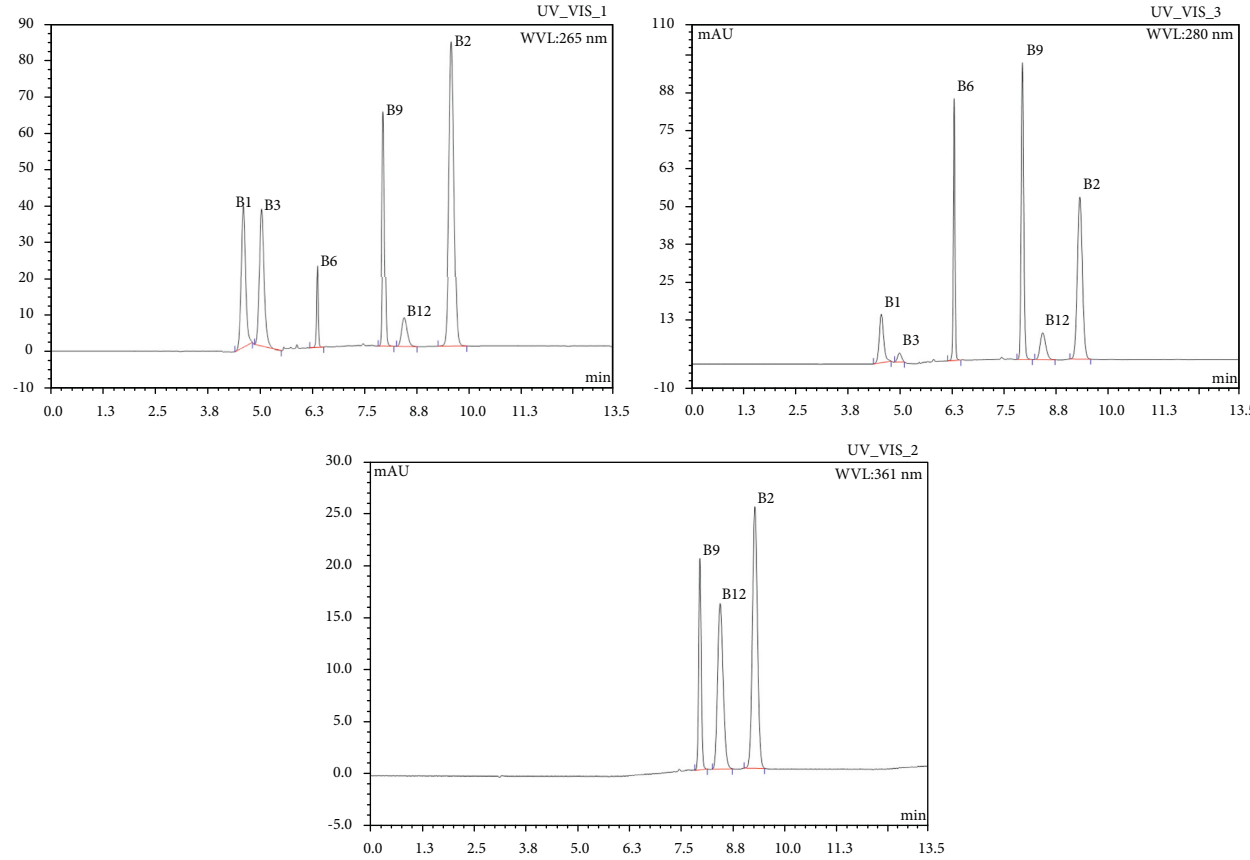

Figure 2: Chromatograms for standard solution of a mixture of B vitamins at 280, 265, and 361 nm.

Table 3: System suitability test results for the HPLC method for determination of B vitamins (9 injections of aqueous standard solution containing 5 mg/L).

Vitamin	Wavelength (nm)	RT (min)[a]	% RSD of RT	PA	% RSD of PA	Capacity factor (K)	Selectivity (α)	Resolution (RS)	Tailing factor (TF)	TP
B_1	265	4.37	1.40	2.65	0.79	0.73	1.32	3.41	1.23	12020
	280	4.32	1.45	1.13	1.55	0.72	1.33	3.45	1.23	11896
B_3	265	4.96	1.31	2.49	0.63	0.97	1.54	9.96	1.41	10815
	280	4.92	1.52	0.24	2.30	0.95	1.57	10.72	1.29	10940
B_6	265	6.29	0.39	0.67	2.43	1.50	1.46	21.65	1.85	117192
	280	6.29	0.39	2.16	1.37	1.50	1.46	22.15	1.21	130915
B_9	265	8.02	0.24	1.42	1.38	2.18	1.05	3.08	1.15	137022
	280	8.02	0.24	2.1	0.92	2.18	1.05	3.04	1.15	143606
	361	8.02	0.23	0.46	2.44	2.18	1.05	3.06	1.16	142637
B_{12}	265	8.30	0.18	0.94	0.74	2.29	1.16	8.05	1.11	103467
	280	8.30	0.18	1.06	0.89	2.29	1.16	7.94	1.12	99293
	361	8.30	0.21	1.91	1.45	2.29	1.16	8.03	1.11	107767
B_2	265	9.23	0.37	5.47	1.33	2.66	—	—	1.12	78402
	280	9.23	0.37	3.65	2.35	2.66	—	—	1.12	80602
	361	9.23	1.37	1.68	2.54	2.65	—	—	1.11	80869

[a] The retention time of unretained peak is 2.52 min. RT: retention time, RSD: relative standard deviation, PA: peak area, and TP: theoretical plates.

for determination of B_1 and B_2 was at wavelength of 265 nm and that of B_3, B_6, B_9, and B_{12} was at 210 nm (Table 5). The high absorbance at 210 nm may be due to $n-\pi^*$ transition which has high molar absorptivity. The lowest LOQ values for B_1, B_2, B_3, B_6, B_9, and B_{12} were 0.6 μg/mL (280/265 nm), 0.2 μg/mL (265/280/361 nm), 0.8 μg/L (265 nm), 0.3 μg/mL (210 nm), 0.5-0.6 μg/L (210/265/280/361 nm), and 0.2 μg/mL (210 nm), respectively (Table 5). Although the wavelength of 210 nm exhibits the lowest LOQ for B_6 and B_{12}, it gives the highest LOQ in the case of B_1, B_2, and B_3. Thus, good performance was found at 210 nm although it is close to the cutoff frequency of the mobile phase which contains

Table 4: Precision (RSD % for peak area) under repeatability conditions ($n = 6$). Intermediate precision ($n = 7$).

Vitamin		265 nm	361 nm	280 nm	210 nm
B_1	Repeatability	0.78		1.27	2.14
	Intermediate	0.79		1.55	2.25
B_2	Repeatability	0.75	1.79	1.70	0.75
	Intermediate	1.33	2.54	2.35	1.33
B_3	Repeatability	1.16		1.83	1.95
	Intermediate	0.63		2.30	1.86
B_6	Repeatability	1.76		1.15	0.53
	Intermediate	2.22		1.37	0.70
B_9	Repeatability	1.08	2.31	0.84	1.23
	Intermediate	1.38	2.44	0.92	1.39
B_{12}	Repeatability	0.86	1.30	0.86	1.05
	Intermediate	0.74	1.45	0.89	1.35

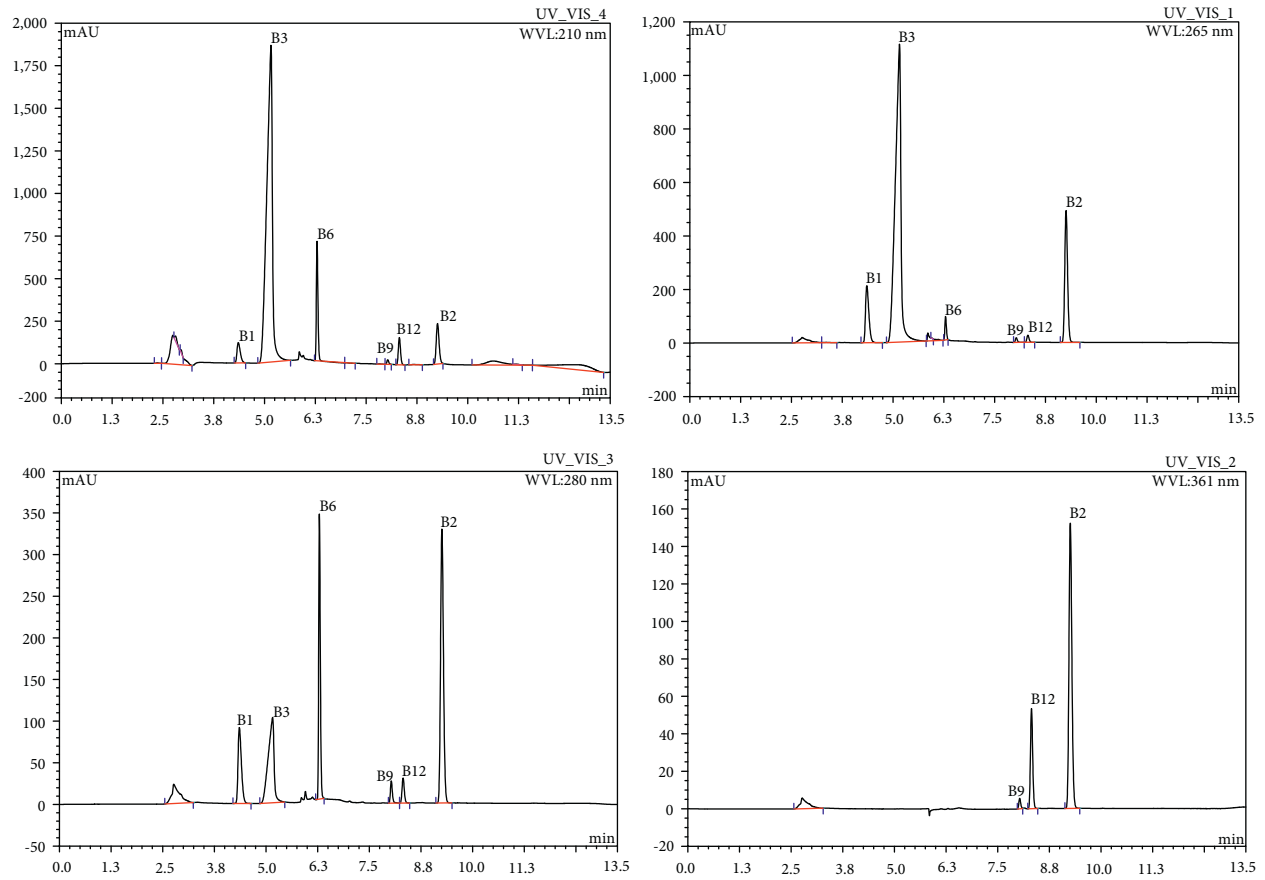

Figure 3: Chromatograms of the B vitamins extracted from fortified flour (peak identity was specified depending on retention time with respect to standard vitamins solution).

acetonitrile. In agreement with these findings, Sasaki reported simultaneous determination of B_1, B_2, B_3, B_6, B_9, and B_{12} in multivitamin dietary supplements using HPLC–UV at 210 nm [18]. On the other hand, higher wavelengths were preferred by other workers as shown in Table 1.

3.3. Determination of Vitamins in the Premix. Figure 4 showed typical chromatograms of B vitamins extracted from the premix using mild extractant containing 0.1% (w/v) ascorbate (antioxidant) and EDTA (chelating agent). B vitamins are commonly extracted from premix by strong acids (HCl, H_2SO_4, and trichloroacetic acid) [24, 25] and highly toxic sodium cyanide [26] which prevents binding of metal ions (present in the premix) to the vitamins.

The measured and labeled (claimed) values of B vitamins in premix are given in Table 6. The percentage of measured per claimed values (M/C %) of B_2, B_3, and B_6 ranges from 85-103%, indicating that the determined values are close to the labeled ones. In general, there was no significant effect of

Table 5: Linearity of calibration curves, sensitivity, limit of detection (LOD), and limit of quantification (LOQ) for vitamins.

Vitamin	λ (nm)	Slope (sensitivity)	Y-intercept	R^2	Linear range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
B_1	265	0.7242	−0.0231	0.9999	0.117-14.9	0.179	0.596
	280	0.3122	−0.0159	0.9999		0.175	0.585
	210	0.4023	−0.0553	0.9970		0.854	2.847
B_2	265	1.4615	0.0464	0.9999	0.122-7.73	0.066	0.219
	361	0.4485	0.0006	1.0000		0.046	0.154
	280	0.9746	0.0132	1.0000		0.048	0.159
	210	0.8165	0.6794	0.9645		1.842	6.141
B_3	265	0.6777	0.0214	0.9998	0.113-14.4	0.243	0.811
	280	0.0690	−0.0050	0.9995		0.371	1.236
	210	1.0842	0.0762	0.9970		0.882	2.942
B_6	265	0.1616	0.0416	0.9995	0.107-13.6	0.354	1.181
	280	0.6143	0.0792	0.9978		0.708	2.360
	210	1.2621	0.0443	1.0000		0.081	0.270
B_9	265	0.6889	−0.0407	0.9998	0.158-10	0.181	0.605
	361	0.2270	−0.0126	0.9997		0.196	0.654
	280	1.0625	−0.0533	0.9998		0.155	0.516
	210	1.0771	−0.0504	0.9997		0.187	0.622
B_{12}	265	0.1813	0.0089	0.9999	0.156-20.6	0.206	0.685
	361	0.3645	0.0182	0.9999		0.197	0.657
	280	0.2050	0.0104	0.9999		0.206	0.686
	210	1.0820	0.0225	1.0000		0.067	0.223

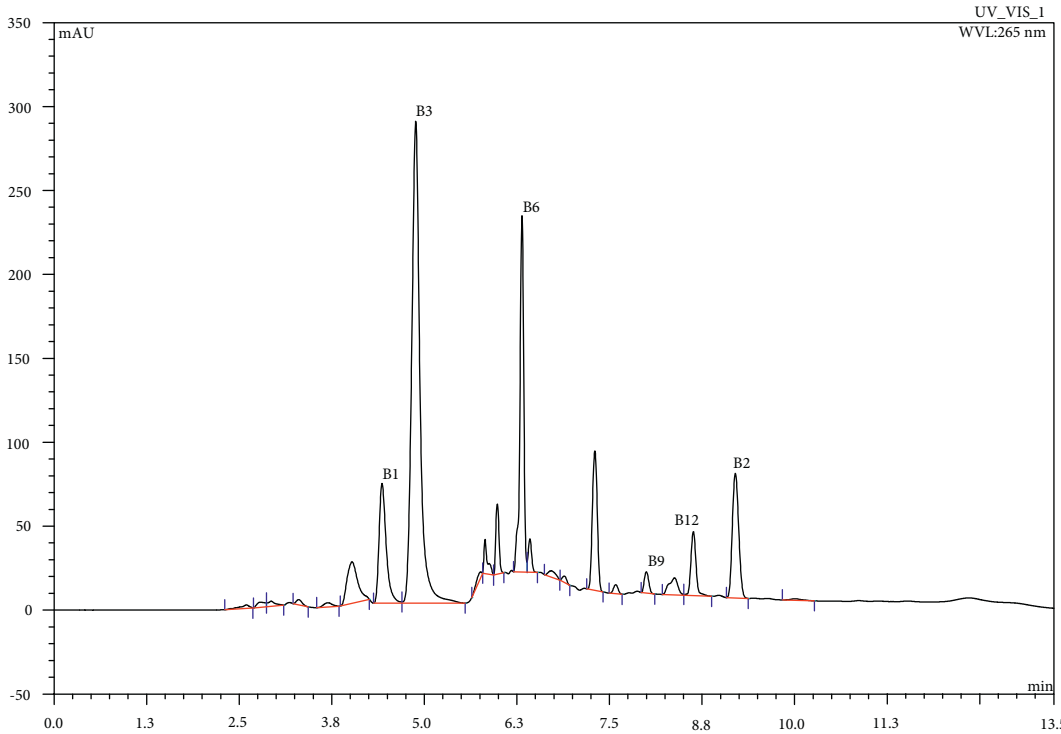

Figure 4: Chromatograms of the B vitamins extracted from premix.

wavelength on the M/C % values of vitamins. Remarkably, the M/C % of B_9 was between 14-17% due to the limited solubility of folic acid (B_9) in the extractant. Thus, basic medium may be necessary to extract folic acid. The M/C % of B_1 and B_{12} was 124-125% and 140-158%, respectively. Since the M/C % values were not affected by wavelength of detection, it seems that the premix contains more B_1 and B_{12} than the claimed or labeled values. Noteworthy, determination of B_2 and B_{12} in the premix at 361 nm had relatively high RSD values compared to the lower wavelengths.

Table 6: Determination of B vitamins in premix and the percentage of measured per claimed values (M/C %).

Vitamin	λ (nm)	Labeled (mg/kg*)	Determined (mg/kg)	RSD (%)	(M/C %)
B_1	265	11592	14374	3.6	124
	280		14374	3.3	124
	210		14490	3.9	125
B_2	265	14400	14688	3.6	102
	361		13248	16.1	92
	280		14688	3.7	102
	210		12240	4.5	85
B_3	265	140000	138600	3.4	99
	280		133000	4.5	95
	210		144200	3.7	103
B_6	265	14480	13322	3.9	92
	280		13177	3.9	91
	210		13032	3.9	90
B_9	265	6064	970	29.3	16
	361		849	39.5	14
	280		1031	31.0	17
	210		970	33.3	16
B_{12}	265	30.50	47.88	5.1	157
	361		42.70	16.8	140
	280		44.53	4.7	146
	210		48.19	7.6	158

*The labeled values were obtained from the Ministry of Health–MOH, Jordan (2021).

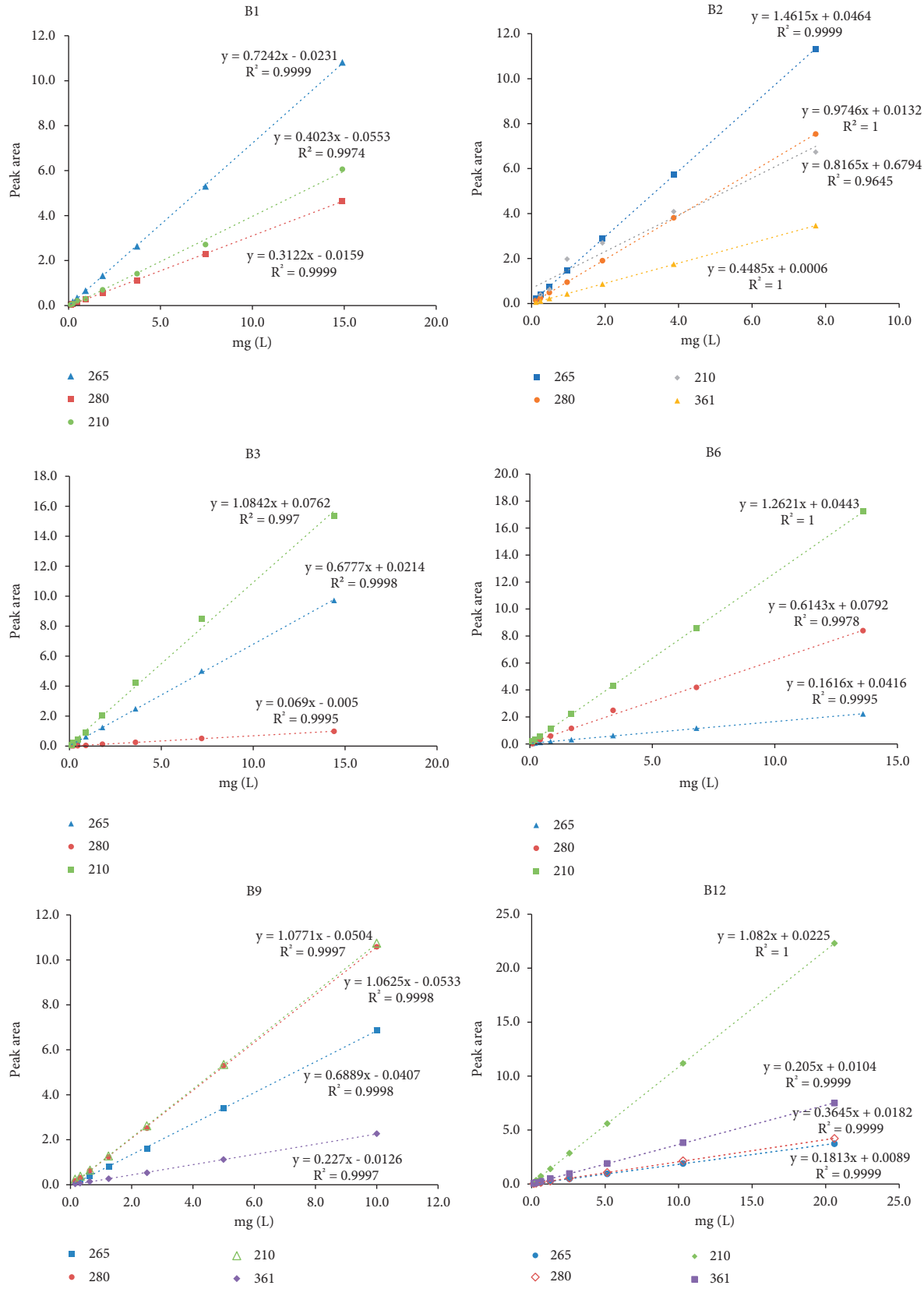

Figure 5: Calibration curves of the B vitamins in their standard mixture at different wavelengths.

Table 7: Determination of B vitamins in fortified flour, and the percentage of measured per claimed values (M/C %). The claimed values were calculated depending on the mixing proportions of premix and flour.

Vitamin	λ (nm)	(M/C %)	RSD (%)
B_1	265	91	1.7
	280	91	1.7
	210	103	3.9
B_2	265	61	8.3
	361	62	7.5
	280	61	8.7
	210	95	13.4
B_3	265	75	2.1
	280	75	3.2
	210	72	6.2
B_6	280	118	6.8
	210	139	5.6
B_9	265	18	4.5
	361	15	8.8
	280	17	4.7
	210	17	5.0
B_{12}	265	135	6.8
	361	105	3.3
	280	109	5.6
	210	120	5.9

3.4. Determination of Vitamins in Fortified Flour. Figure 5 showed typical chromatograms of B vitamins extracted from the fortified flour, and the percentage of measured per claimed (M/C %) values of B vitamins in fortified flour is given in Table 5. The M/C % values of B_1, B_2, B_3, B_6, B_9, and B_{12} in fortified flour were 91-103, 61-95, 72-75, 118-139, 15-18, and 105-135, respectively. Thus, it was possible to get values that are close to the claimed values except in the case of B_9 which has low solubility in the extractant. Using 210 nm wavelength of detection, the determined amounts of B_1, B_2, B_6, and B_{12} in fortified flour were higher than those obtained at the other wavelengths (Table 7), indicating some interference effect from the components of flour. This effect was not observed in the case of premix, reflecting the simplicity of premix matrix compared to flour. Despite the 210 nm, there was no significant effect of other detection wavelengths (265, 280, and 361 nm) on the measured values of B vitamins in fortified flour.

4. Conclusion

Fortification of flour with premix is necessary to compensate for the loss of vitamins arising from processing of wheat grain. To ensure adequate fortification, routine simple analytical methods must be developed to determine the amount of B vitamins in premix and fortified flour. Simultaneous HPLC determination of water-soluble B vitamins is a troublesome analytical procedure in the literature since they have greatly variable structures and acid-base properties which imposed difficulties on eluting them in short time and selecting wavelength of detection. In the present work, a simple method was developed for simultaneous determination of B_1, B_2, B_3, B_6, B_9, and B_{12} in premix and fortified flour by extracting vitamins with 0.1% (w/v) of ascorbate and EDTA solution followed by eluting using HPLC-DAD, C18, and gradient mobile phase consisting of 0.03% trifluoroacetic acid aqueous solution (pH 2.6) and acetonitrile. Elution of vitamins was completed within 9.3 min with detection limits ranging from 0.2-0.8 μg/L. In general, variation of wavelength of detection in the range from 210 to 361 nm affects sensitivity but had a marginal effect on the linearity and LOQ of the developed method and its application for determining B vitamins in premix and fortified flour. The 210 nm wavelength exhibited the highest sensitivity though resulted in higher values of B vitamins in fortified flour with respect to 265, 280, and 361 nm. Furthermore, determination of B_2 and B_{12} in the premix at 361 nm had relatively high RSD values compared to the other wavelengths.

Data Availability

Data available will be available on request by the corresponding author.

Additional Points

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or nonfinancial interest in the subject matter or materials discussed in this manuscript. This research does not involve human participants and/or animals.

Consent

The authors declare that informed consent was not applicable.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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