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Nature-friendly chemometrics-assisted UV-spectrophotometric method for the simultaneous determination of vitamins B9 and B12 in syrup formulation: Net analyte signal (NAS) and partial least squares (PLS) approaches

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Abstract

In this study, the UV-spectrophotometric technique by applying chemometrics methods, namely Net analyte signal (NAS) and partial least squares (PLS) was carried out for the simultaneous determination of vitamin B9 and vitamin B12 in synthetic mixtures and the commercial syrup sample. In the net analyte signal method, the standard solutions had the limit of detection (LOD) and limit of quantitation (LOQ) of 0.2321, 0.2322 and 0.4351, 0.5402 µg/mL for vitamin B9 and vitamin B12, respectively. In addition, the mean recovery values related to the test set were obtained at 98.89% and 99.08% for vitamin B9 and vitamin B12, respectively. The root mean square error (RMSE) was found to be 0.2868 and 0.2319 for B9 and B12 vitamins, respectively. Cross-validation was used to select the optimum number of components in the PLS approach. The number of components was equal to 2 with the mean squared prediction error (MSPE) of 4.6908 and 5.9534 for B9 and B12, respectively. The mean recovery values of the test sets in this method were reported 99.48% and 99.54% for B9 and B12 vitamins, respectively. The one-way analysis of variance (ANOVA) test at the 95% confidence level was implemented on a pharmaceutical sample (Feroglobin syrup). The results did not show any significant difference between the methods. The proposed methods are simple, fast, low-cost, and accurate compared to the high-performance liquid chromatography (HPLC) technique. Also, it does not require separation steps and the use of expensive and environmentally polluting solvents.

Keywords: *Spectrophotometry, Net analyte signal, Partial least squares, Water-soluble vitamins*

1. Introduction

One of the groups of organic molecules is vitamins. They are necessary in small amounts for normal growth and some body processes [1]. Regulation of metabolic procedure is carried out by vitamins that play the role of coenzymes or precursors of coenzymes [2]. Direct production of energy by vitamins is not possible or they are not used as building blocks of polymers. Except for vitamin D, their production mostly does not occur in the body and they are supplied through diet [3]. The principal sources of vitamins are fruits and vegetables. Vitamins are divided into two classes: fat-soluble (vitamins A, D, E, and K), and water-soluble (vitamins B1, B2, B3, B5, B6, B7, B9, B12, and C) [4]. Because water-soluble vitamins play an important role in the metabolic process, inadequate intake may deleteriously affect health outcomes and the progression of heart failure (HF). It has been reported that patients with severe of dietary micronutrient deficiencies had nearly double the risk of 1-year hospitalization or death. Vitamins B are significant for preserving health due to their role in energy metabolism and antioxidant activity [5].

Vitamin B9 or folic acid (FA) or folate is a crucial vitamin for synthesizing DNA and protein, as well as its essential role in the adaptive immune response, has been reported [6]. It is known as a main nutrient for preventing neural tube defects in the fetus in pregnant women. The shortage of vitamin B9 may lead to various illnesses, such as megaloblastic anemia, cancer, hypertension, some psychiatric disorders, coronary heart disease, diabetes, and metabolic syndromes [7].

Vitamin B12 also called cyanocobalamin has the metal element cobalt in its structure, and its cellular metabolic function is integrated with folate [8]. The function of vitamin B12 is as a cofactor or coenzyme in some necessary biochemical processes such as the synthesis of DNA, myelin maintenance, and cell metabolism. Meat, fish, shellfish, and dairy products are sources of vitamin B12 for humans [9]. Vitamin B12 deficiency causes nervous system disorder, reversible megaloblastic anemia, dysfunction of DNA synthesis, and disruption of mitochondrial metabolism [10,11].

Several techniques like high-performance liquid chromatography (HPLC) [12,13], reversed-phase high-performance liquid chromatography (RP-HPLC) [14,15], high-performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) [16], and so on have been studied for the concurrent estimation of vitamin B9 and vitamin B12. Although these methods are accurate and sensitive, they are time-consuming, costly in terms of instruments and maintenance, and need a professional operator [17,18]. In addition, the use of separation and preconcentration methods is necessary for the low concentration of drugs in complex biological matrices. Two common techniques are solid-phase extraction (SPE) and liquid-phase extraction (LLE), which have been used in this case. The mentioned approaches possess some disadvantages [19,20]. Chemometrics makes different analytical techniques more feasible for the simultaneous analysis of multiple components. The interference of components with each other, complicated overlapping of spectra, failure of resolving power, and incapacity to use entire spectral data led to analysis failure using conventional spectrophotometry [21]. Chemometrics-assisted UV-Vis spectrophotometric method can solve these problems [22,23]. NAS and PLS require no additional sample preparation. Therefore, they can be powerful and substituted approaches in comparison with HPLC for analysis of multiple components in facile steps with high precision and accuracy. The benefits of simultaneous determination using the mentioned chemometrics methods in that they can avoid time-consuming extraction and

separation, and minimize the usage of expensive reagents [24]. Chemometric tools possess the potential to be applied in analytical technique development to minimize the process's environmental effect. In these ways, consumption of solvents or reagents can be minimized [25]. In contrast to the chromatography methods, which consume a large amount of environmentally polluting solvents, the spectrophotometric method uses less solvent, which can be said to be environmentally friendly [26].

One family of multivariate methods is based on the concept of net analyte signal (NAS) [27]. At first, Lorber defined the NAS calibration for a multivariate problem [28]. According to Lorber's description, the NAS based on spectroscopic techniques for any analyte is that part of the spectrum related to the analyte, which is placed orthogonally to the space included by the spectra of all components except the analyte (all interfering components). Because of showing the NAS vector in a direction that is only affected by variations in analyte concentration, it can act as a highly selective analyte determination [27]. Besides, the NAS presents the possibility of estimation of the analytical figures of merit and method efficiency, sensitivity, and selectivity for each constituent [28].

The other multivariate method is partial least squares (PLS). In the beginning, Wold introduced the nonlinear iterative partial least squares (NIPALS) in the 1960s [29,30] and developed by Lohmöller a few decades later [31]. A measurement model and a structural model are two components of the PLS route model. The measurement model or outer model, indicates the unilateral predictive relationships between each latent and its correlated remarked indicator variables. The association of indicator variables is always with a single latent construct. Reflective and formative ones are two various measurement models that the PLS makes a distinction between them. There are relationships between the unobserved or latent constructs, which are represented by the structural model (inner model). In this model, a difference occurs between exogenous and endogenous constructs [32]. Compared to the other multivariate approach named principal component regression (PCR), the PLS and NAS methods also work with small data. In contrast, in the PCR method, dimension reduction is done and it does not give results for small data.

Ghasemi and Abbasi reported simultaneous spectrophotometric determination of group B vitamins (B1, B2, B6, B9) using parallel factor analysis [33]. Monakhova et al. used an advanced independent component analysis algorithm (MILCA) for the simultaneous chemometric determination of fat- and water-soluble vitamins (C, B6, A, E) in complex mixtures (multivitamin drugs, food additives, and energy drinks) [34].

In this work, two multivariate calibration approaches along with the UV spectrophotometric method, including NAS and PLS were applied to determine vitamin B9 and vitamin B12 in the dietary supplement syrup. These methods are uncomplicated, affordable, precise, reliable, and available. The proposed method is a green way for the simultaneous analysis of vitamins B9 and B12 in a Feroglobin plus syrup without any separation procedure and without the consumption of pollutant solvents. These methods were compared with HPLC as a reference method using analysis of variance (ANOVA). It can be said that the suggested method is simpler, more economical, and faster than the HPLC technique.

2. Experimental Section

2.1. Apparatus and software

A double-beam UV-Vis spectrophotometer (Varian, Cary 100 bio, Australia) was utilized to record the absorbance of stock solutions, standard solutions, pharmaceutical samples, and spike solutions. Waters Alliance 2695 HPLC was utilized to analyze the syrup sample. MATLAB (R2021a) was applied to write and run the programs of NAS and PLS approaches. Microsoft Office Excel 2018 was used to plot diagrams and calculations.

2.2. Materials

Vitamins B9 and B12 with pharmaceutical purity (98.9%) were prepared by Jalinous Pharmaceutical Company and Daroo Salamat Pharmed Company, respectively. Feroglobin plus syrup containing 0.3 mg of vitamin B9 and 0.01 mg of vitamin B12 was purchased from Vitabiotics.

2.3. Preparation of standard solutions

Stock solutions of vitamins B9 and B12 were individually prepared by dissolving 0.01 g of each component in 100 mL of distilled water in a volumetric flask. Standard solutions (dilute solutions) were provided by accurate dilution from the stock solutions in the range of 5-40 μg/mL for both components. Afterward, their absorbance was recorded in the range of 200-400 nm.

2.4. Preparation of mixtures

20 mixtures containing different concentrations of each component (vitamin B9 and vitamin B12) were prepared from stock solutions. Then, the absorbance of each mixture was recorded. The prepared actual values (laboratory concentrations) are given in Table 1.

Table 1. Synthetic samples in different concentration ranges for the two components of vitamin B9 and vitamin B12

Mixture	B12 (µg/mL)	B9 (µg/mL)
1	5	8
2	7	10
3	9	12
4	10	14
5	13	17
6	18	20
7	16	22
8	21	24
9	23	26
10	25	29
11	27	31
12	29	33
13	31	35
14	33	37
15	35	40
16	39	42
17	40	44
18	42	46
19	45	48
20	47	50

2.5. Preparation of pharmaceutical sample

1 mL of Feroglobin plus syrup was made up to the volume in a 50 mL volumetric flask with distilled water. To achieve acceptable absorption, dilution was done. Finally, the absorbance of the sample was recorded in the range of 200-400 nm.

2.6. Preparation of spike solutions

The spike method was performed to evaluate the interference or non-interference of other components in the sample matrix with the analyte signal. Spike samples for each component were prepared separately by adding different amounts of stock solutions to the real sample and their absorption spectra were recorded.

2.7. Chromatographic conditions

Atlantis T3-C18 (3 µ, 2.1×150 mm) column with a temperature of 35 was used. The mobile phase includes A: acetonitril+formic acid 0.1% and B: water+formic acid 0.1% with a flow rate of 0.2 mL/min. 2 µL was considered as injection volume [14].

3. Results and discussion

3.1. Spectral features

The absorption spectra of solutions of vitamins B9 and B12 are illustrated in Fig 1 over the wavelength range of 200-400 nm. Normal absorption spectra of these components revealed complete overlap. Therefore, the concurrent determination of these two analytes without prior separation is impossible. In such cases, the spectrophotometric technique along with NAS and PLS as chemometrics methods can be useful for the simultaneous estimation of multicomponent formulations. These approaches allow the quantitative determination of one component that exists beside another component, as well as with excipients without their interference.

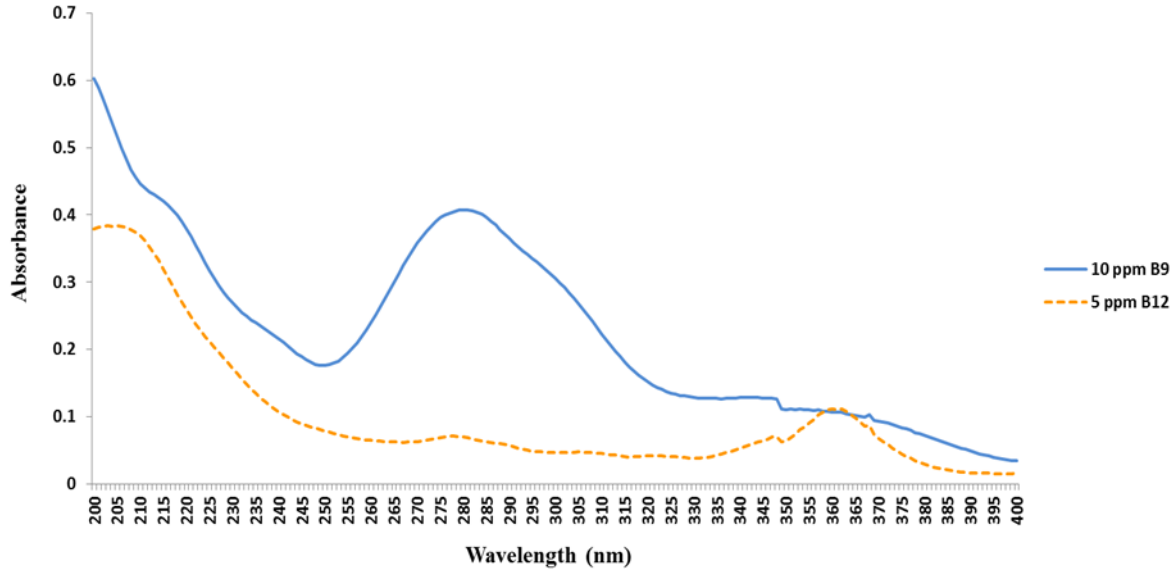


Fig 1. The UV absorption spectra of B9 and B12

3.2. NAS results

It is possible to survey the establishment of linear behavior of the B9 and B12 in the presence of each other by checking the summability of the absorption spectra. The main reason for implementing this step is that the NAS method is based on a linear relationship between the device response (in this case absorbance) and the concentration. Also, the non-establishment of summability between two spectra causes a large error in the calculation of the regression vector. Hence, the necessity of implementing this step is understandable. Based on this, it is necessary that the spectrum of vitamin B9 and vitamin B12 does not interact with each other and the presence of one next to the other does not change or affect its spectrum.

First, the mathematical summability of the spectra was investigated and a comparison was made with the spectrum of the mixture of vitamins. When two spectra are equal, there is a summability property. The existence of a summability property between vitamin B9 and vitamin B12 is exhibited in Fig 2. The equality of the spectrum of the mixture of vitamins with the sum of the spectrum of the individual components can be observed, so it can be said that the two components have no spectral interaction.

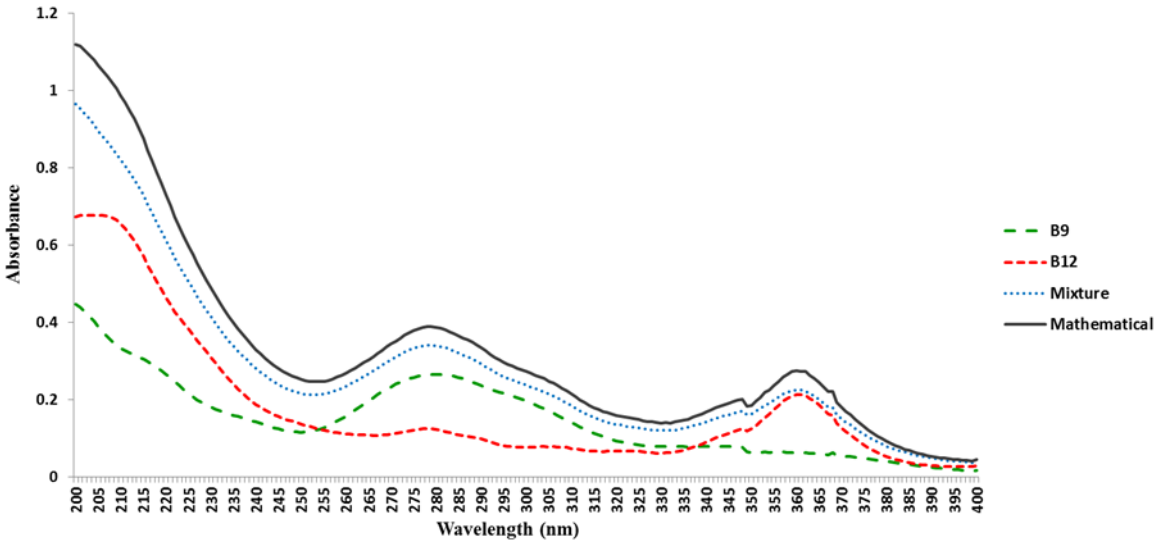


Fig 2. The mathematical sum of the absorption spectra of vitamin B9 and vitamin B12, mixture of components, and spectrum of B9 and B12 individually

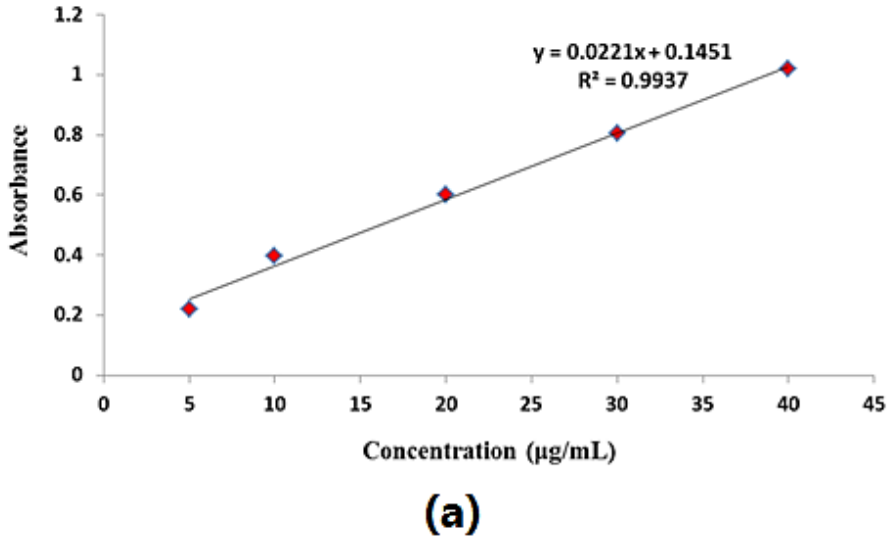
Determining the linear range is very important in multivariate calibration methods such as NAS. Therefore, the calibration graphs (absorbance versus concentration) related to vitamin B9 and B12 at λ_{max} equal to 200 nm and 202 nm were evaluated, respectively (Fig 3). The limit of detection (LOD) and limit of quantification (LOQ) were estimated based on equations (1) and (2), respectively.

$$LOD = y_B + 3s_B \quad (1)$$

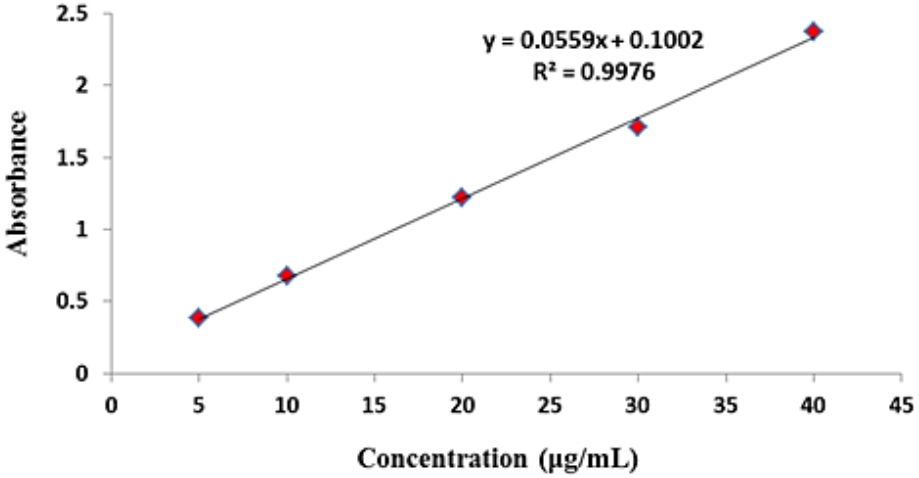
$$LOQ = y_B + 10s_B \quad (2)$$

The blank signal (y_B) and standard deviations (s_B) are specified in these equations [35].

LOD and LOQ values were found to be 0.2321, 0.2322 µg/mL and 0.4351, 0.5402 µg/mL, respectively (Table 2). In addition, the coefficient of determination (R^2) and Pearson correlation (r) values are given in Table 2. R^2 was equal to 0.9937 for B9 and 0.9976 for B12, as well as r values were obtained at 0.9968 and 0.9988 for B9 and B12, respectively. These results give an acceptable efficiency of the NAS method.



(a)



(b)

Fig 3. Calibration graphs of (a) vitamin B9 and (b) vitamin B12 at λ_{max}=200 nm and at λ_{max}=202 nm, respectively

Table 2. The statistical results of calibration standard solutions graphs obtained by the NAS method

Parameter	B9	B12
Wavelength (nm)	200	202
Liner range (µg/mL)	5-40	5-40
Slope	0.0221	0.0559
Intercept	0.1451	0.1002
Coefficient of determination (R^2)	0.9937	0.9976
Correlation (r)	0.9968	0.9988
LOD (µg/mL)	0.2321	0.2322
LOQ (µg/mL)	0.4351	0.5402

The implementation of the NAS technique was performed using two sets related to the 20 mixtures, including calibration (training) (65%) and test (validation) (35%). Also, the absorption of 20 mixtures was created as a matrix in an Excel file. The prediction error sum of squares (PRESS) versus the number of components was plotted to acquire the optimum number of components for B9 and B12 (Fig 4). The lowest value of error indicated the optimal value. Eq (3) is represented the PRESS.

$$PRESS = \sum_{i=1}^{n}\sum_{j=1}^{m}(x_i - \hat{x}_i)^2 \quad (3)$$

Herein the predicted and standard concentrations are denoted by x_i and $\hat{x}_i$, respectively. Furthermore, "n" and "m" are the total number of samples and the total number of components, respectively. Fig 4 displays that the number of optimum components for vitamins B9 and B12 is equal to 2 with the PRESS of 1.242 and 2.794, respectively.

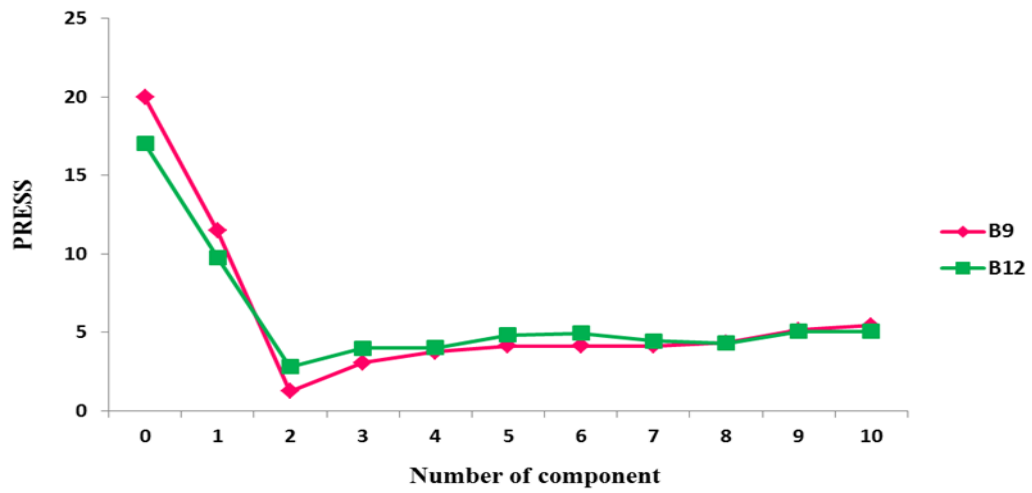


Fig 4. PRESS diagram versus the number of components for B9 and B12

The recovery percentage and the mean recovery percentage, which expresses the accuracy of the method, were calculated based on the test series (35%, i.e. 8 points) (Table 3). The recovery percentage was between 96.24% to 100.36% and 97.60% to 100.29% for B9 and B12, respectively. Moreover, the average recovery values were 98.89% and 99.08% for vitamins B9 and B12, respectively. The closeness of the recoveries to 100% demonstrates that the accuracy of the method is acceptable and it has a good performance for prediction. The root mean square error (RMSE) (Eq 4) as a parameter for showing the error was found to be 0.2868 and 0.2319 for B9 and B12, respectively (Table 3). This also confirms that the NAS method is suitable for predicting the concentration of both components. To represent the closeness of the predicted values to the actual values, the coefficient of determination (R^2) of each component was investigated by drawing their corresponding graph (predicted values versus actual values) (Fig 5). The R^2 equal to 0.9998 for vitamin B9 and 0.9999 for vitamin B12 indicates a high matching of the predicted values with the experimental data.

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(y_{pred} - y_{obs})^2}{n}} \quad (4)$$

Where y_{pred} and y_{obs} are the determined concentration and the real value of the concentration, respectively. The number of samples is demonstrated by n [36].

Table 3. Obtained recovery, mean recovery, and RMSE related to the test series for B9 and B12 in NAS method

Mixtures	**Actual ($\mu g/ml$)**		**Found ($\mu g/ml$)**		**Recovery (%)**	
	B9	**B12**	**B9**	**B12**	**B9**	**B12**
1	10	7	9.624	6.857	96.24	97.96
2	20	18	19.54	17.56	97.72	97.60
3	29	25	28.83	24.83	99.41	99.35
4	31	27	30.74	26.68	99.16	98.82
5	35	31	35.12	30.88	100.36	99.64
6	40	35	39.67	34.79	99.19	99.40
7	42	39	41.75	38.83	99.41	99.58
8	46	42	45.81	42.12	99.59	100.29
Mean Recovery (%)					98.89	99.08
RMSE					0.2868	0.2319

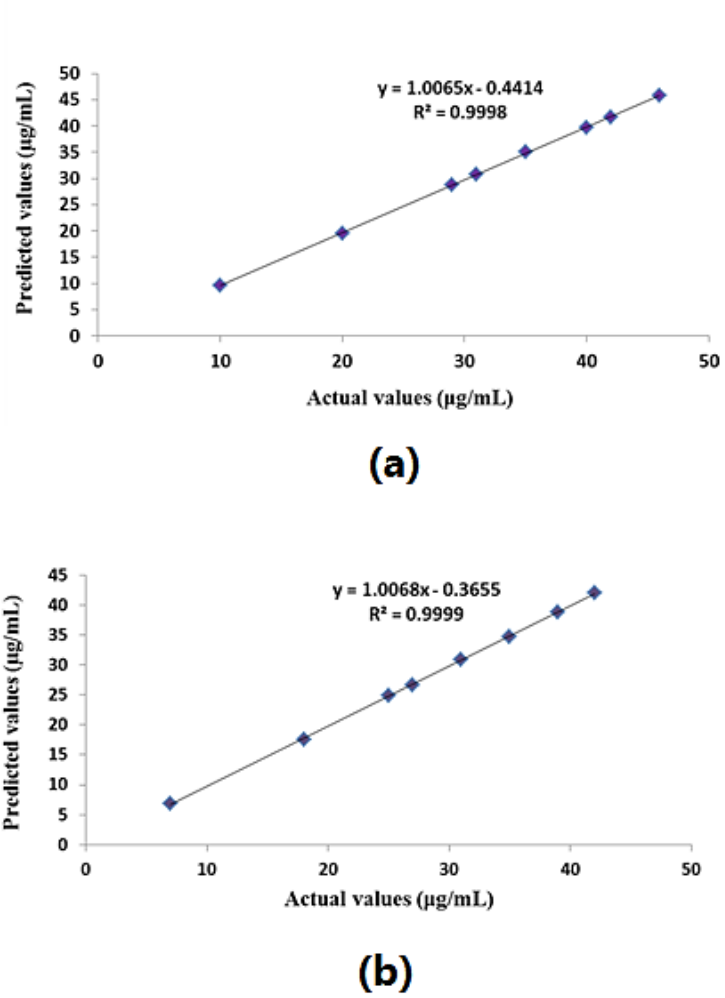


Fig 5. Predicted values versus actual values of test series for (a) B9 and (b) B12 in NAS method

3.3. PLS results

In order to construct the PLS model, the absorption spectra of the mixtures were selected in the wavelength range of 200-400 nm and then analysis of the PLS was implemented. A cross-validation method, using k-fold cross-validation, was applied to choose the optimum number of principal components (PCs) based on the minimum mean square prediction error (MSPE) (Fig 6). The optimum PCs were 2 with an error of 4.6908 for vitamin B9 and 2 with an error of 5.9534 for vitamin B12. In k-fold cross-validation, the sample is randomly divided into k equal-sized subsamples, which are named "folds". From these k subsamples, a single subsample is maintained as the validation data for testing the model, and the k−1 remaining subsamples can be used for training set. Then, this process is repeated k times, with each of the k subsamples used once as the validation data.

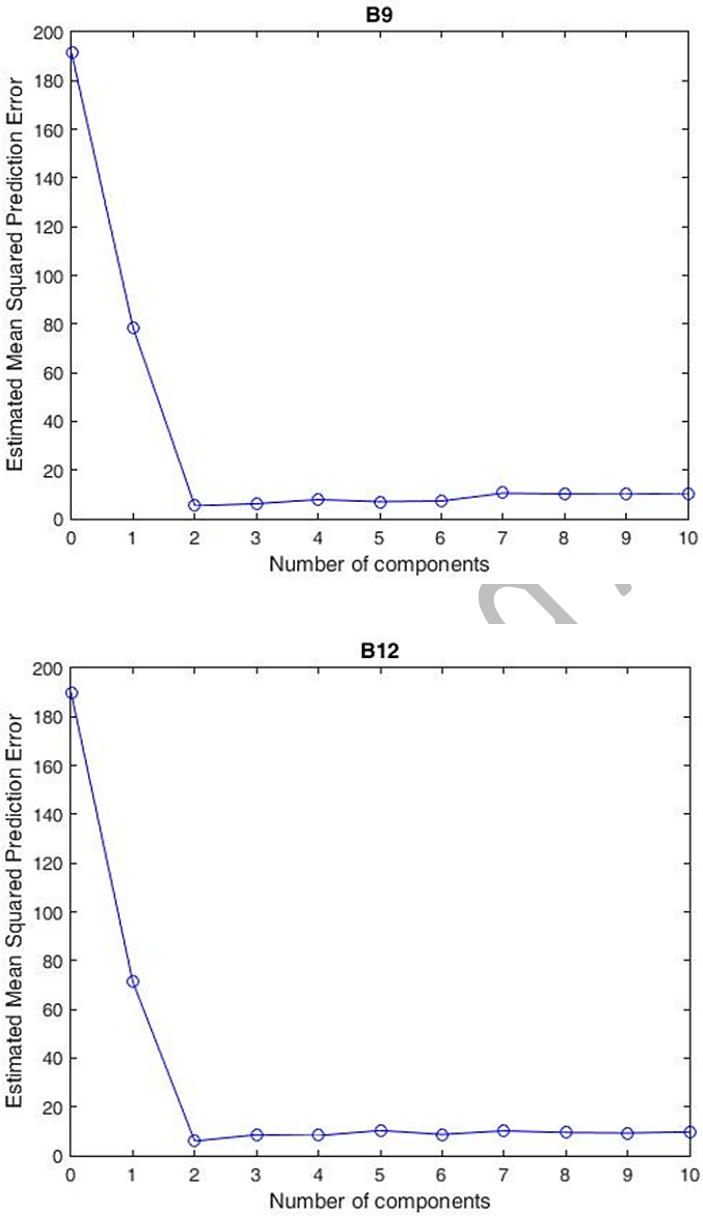


Fig 6. MSEP versus number of components of B9 and B12 in PLS method

20 mixtures were divided into two series of training (12 mixtures) and test (8 mixtures), and the validity of the method was checked with 8 mixtures of the test set. The predicted values of these 8 mixtures and the recovery percentage, the average recovery percentage, and errors related to the mixtures are presented in Table 4. The percentage range of recoveries was acceptable (98.13% to 100.13% for vitamin B9 and 98.05% to 100.01% for vitamin B12). Also, the average recovery was reported to be nearly 100 (99.48 for B9 and 99.54 for B12). On the other hand, the RMSE was less than 0.2 for both components. All these results are proof of the high efficiency of this method for the prediction of concentration. Also, the high prediction ability can be studied by drawing a graph of the predicted values against the actual values (Fig 7). The placement of the points on a straight line and their

non-dispersion with R^2 equal to 1 for vitamin B9 and 0.9999 for vitamin B12 prove that the PLS approach has the necessary efficiency for prediction.

Table 4. Recovery percentage, mean recovery percentage, and RMSE results of the test set for the PLS method

No	Actual values (µg/mL)		Predicted values (µg/mL)		Recovery (%)	
	B9	B12	B9	B12	B9	B12
1	10	7	9.813	6.969	98.13	99.56
2	20	18	19.73	17.64	98.67	98.05
3	29	25	28.91	24.95	99.69	99.83
4	31	27	30.83	26.77	99.45	99.15
5	35	31	35.04	30.99	100.13	99.99
6	40	35	39.98	34.95	99.95	99.85
7	42	39	41.98	38.95	99.97	99.89
8	46	42	45.92	42.004	99.83	100.01
Mean recovery (%)					99.48	99.54
RMSE					0.1370	0.1507

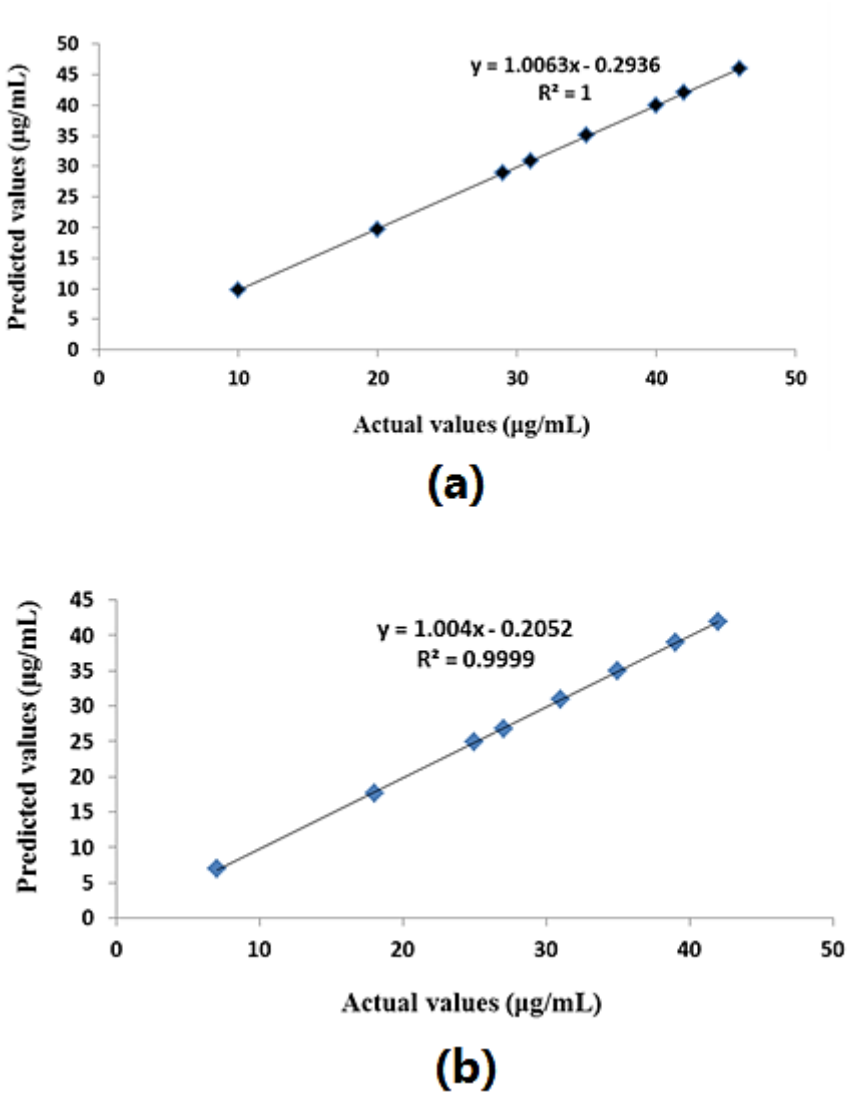


Fig 7. Actual concentrations versus predicted concentrations of (a) B9 and (b) B12 by PLS method

Fig 8 shows the residual values for this method. The dispersion of the values around the zero point (-0.05 to 0.3 for vitamin B9 and -0.05 to 0.35 for vitamin B12) shows the acceptability of the method.

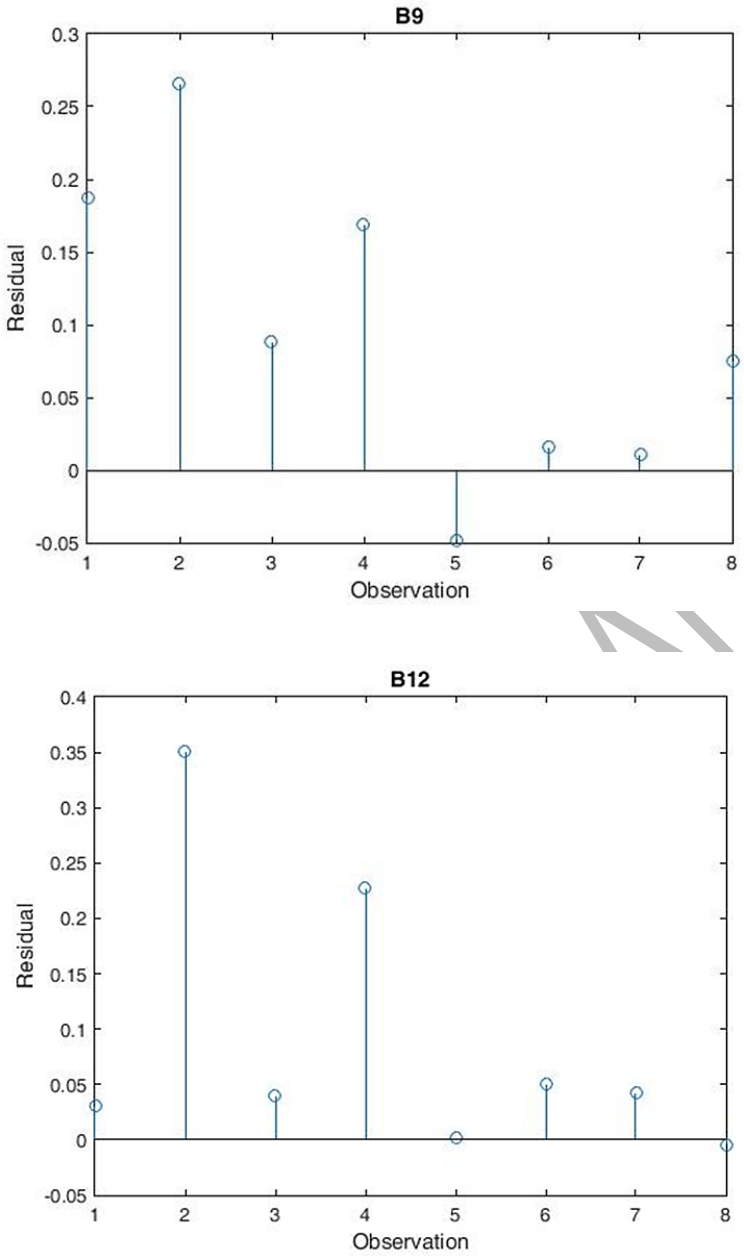


Fig 8. Residual of prediction by PLS method for B9 and B12

3.4. HPLC results

The chromatogram related to the analysis of the syrup sample is shown in Fig 9. Its retention time was 1.88 min and 1.74 min for B9 and B12, respectively. The method validation studies were evaluated by determining basic parameters such as accuracy, LOD, and LOQ. The accuracy of B9 and B12 was found to be 98.04% and 100.30%, respectively. The LOD and LOQ values were 1.06, 1.19 µg/mL and 3.23, 4.83 for B9 and B12 µg/mL, respectively. The excipients present in the syrup did not have any interference with B9 and B12 peaks because no excipient peaks appeared in the chromatogram of the pharmaceutical formulation [37].

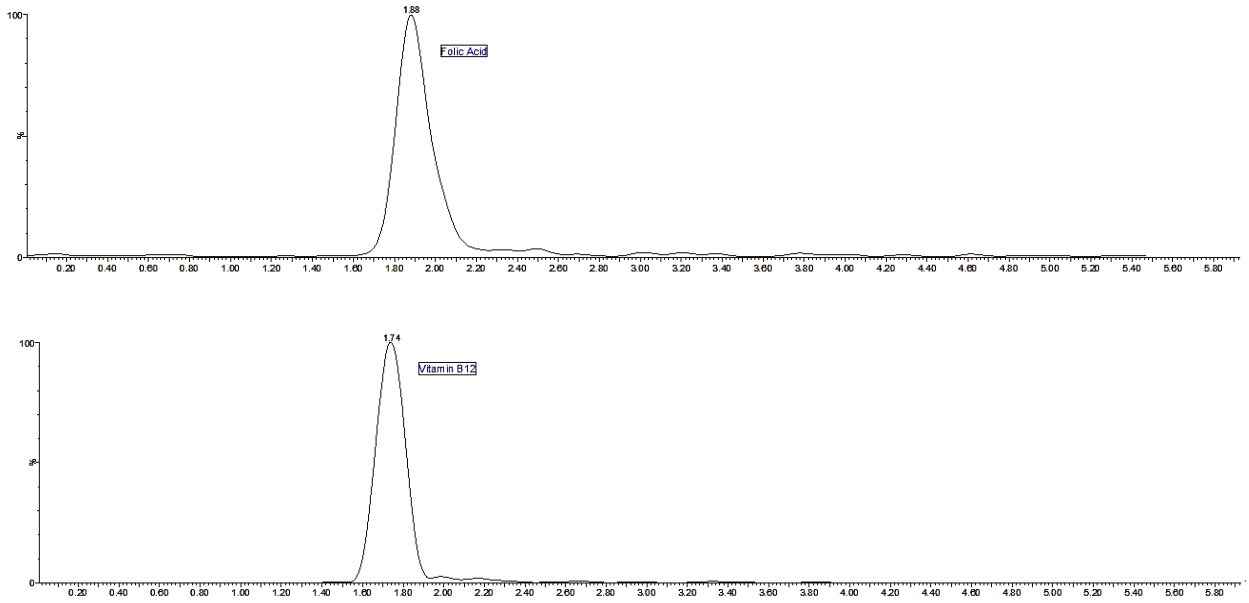


Fig 9. HPLC chromatogram of the Feroglobin plus syrup containing vitamin B9 and vitamin B12

3.5. Syrup sample analysis

Acceptable results of the application of the NAS and PLS methods in the syrup formulation assay were obtained (Table 5), demonstrating the high efficiency of these approaches for the analysis of B9 and B12 in quality control laboratories. Mean recovery percentage and relative standard deviation (RSD) were found to be >94% and <2.2% for the suggested methods, indicating their good accuracy and precision. The results are close to the label-claimed concentration and HPLC. It should be also noted that excipients did not interfere with the estimation of both components. Comparison between chemometric methods and HPLC reveals good coincidence. Although the HPLC technique is more specific than the chemometrics coupled with spectrophotometry it needs high-cost equipment and materials like HPLC-grade solvents and columns. On the other hand, chemometric approaches are less expensive and they do not need complicated instrumentation and any prior separation procedure. These methods require software for the resolution and determination of the drugs in the mixture.

Table 5. Results of analyzing syrup by the proposed and reference methods (0.3 mg B9 and 0.01 mg B12 in syrup formulations)

	B9			**B12**		
	NAS	PLS	HPLC	NAS	PLS	HPLC
Found[a] (mg)	0.286±0.0041	0.287±0.0040	0.293±0.0057	0.0094±0.0002	0.0095±0.0001	0.0096±0.0001
Mean recovery (%)	95.44	95.88	97.77	94.00	95.66	96.00
RSD (%)	1.433	1.393	1.945	2.127	1.157	1.041

[a] Mean value of the three measurements

3.6. Statistical analysis

The results obtained from the analysis of the real sample using the proposed methods and the chromatography method were compared through the ANOVA test to determine whether there is a significant difference or not.

According to the calculated F values (vitamin B9 = 3.921985816 and vitamin B12 = 2.866666667) and critical F, it can be proved that there is no significant difference at the 95% confidence level between the methods (Table 6).

Table 6. Statistical analysis of the NAS, PLS, and HPLC methods using an ANOVA test

Source of variation	SS[a]	df[b]	MS[c]	F Calculated	F Critical
Between groups					
B9	0.000122889	2	6.14444E-05	3.921985816	5.14325285
B12	9.55556E-08	2	4.77778E-08	2.866666667	5.14325285
Within groups					
B9	9.4E-05	6	1.56667E-05		
B12	1E-07	6	1.66667E-08		
Total					
B9	0.000216889	8			
B12	1.95556E-07	8			

[a] Sum of squares

[b] Degree of freedom

[c] Mean squares

3.7. Spike results

The average recovery for vitamin B9 in NAS and PLS methods was 94.44%, 94.76% and 103.66%, 94.19% for concentrations of 30 and 40 µg/ml, respectively. Also, mean recoveries greater than 93% and 94% were observed in concentrations of 10 and 40 µg/ml vitamin B12 in NAS and PLS methods, respectively. On the other hand, the RSD values were less than 0.3% and 0.09% for the NAS and PLS methods, respectively (Table 7). The tremendous efficiency of these methods for the concurrent analysis of B9 and B12 in the matrix without considerable error was confirmed.

Table 7. The results of the standard addition for vitamins B9 and B12 in syrup

NAS, B9

No.	Added (µg/mL)	Found (µg/mL)	Mean recovery (%)	RSD (%)
1	30	28.03	93.44	0.267
2	40	41.46	103.66	0.014

PLS, B9

No.	Added (µg/mL)	Found (µg/mL)	Mean recovery (%)	RSD (%)
1	30	28.43	94.76	0.028
2	40	37.67	94.19	0.013

NAS, B12

No.	Added (µg/mL)	Found (µg/mL)	Mean recovery (%)	RSD (%)
1	10	9.25	92.54	0.081
2	40	37.58	93.95	0.018

PLS, B12

No.	Added (µg/mL)	Found (µg/mL)	Mean recovery (%)	RSD (%)
1	10	9.34	93.41	0.077
2	40	37.79	94.49	0.017

3.7. Effect of interfering substances

The effect of foreign species (several ion species) was assessed to determine B9 and B12 (Table 8). The results indicate that interfering ions did not have a significant effect on the measurement of the desired vitamins. The permissible concentration of these interfering substances is higher than the concentration of B9 and B12, indicating a proper selectivity between vitamins and other substances. Mentioned ions can be allowed at relatively high concentrations.

Table 8. Interfering effect of several species for the determination of B9 and B12

Species	Tolerance limit [X]/ [B9]	Tolerance limit [X]/ [B12]
Na^+, Cl^-, K^+, NO^{3-}, CO_3^{2-}	1000	1000
Ni^{2+}, Fe^{3+}	100	50
Fe^{2+}, SO_4^{2-}	200	400
Mg^{2+}, Zn^{2+}	300	500

3.8. Comparison with other methods

The proposed method was compared to the chromatographic technique in terms of LOD, LOQ, and linear range (Table 9). It can be concluded that the results of this study are almost close to the other techniques. On the other hand, the spectrophotometric method is easier and less expensive than the chromatographic methods. Also, the analysis time with the proposed methods is less than the chromatographic methods. The solvent used in this research is distilled water, while the solvents used in the chromatography methods are a combination of environmentally polluting solvents.

Table 9. Comparison between suggested methods and other techniques

Method	Sample	LOD (µg/mL)		LOQ (µg/mL)		Linear range (µg/mL)		Ref.
		B9	B12	B9	B12	B9	B12	
RP-HPLC	Capsule	---	---	---	---	---	4.6-13.9	[14]
RP-HPLC	Supplement	1.06	8.38	3.22	25.39	2-540	20-350	[15]
HPLC	Fortified Flour	0.155	0.206	0.5	0.7	0.15-10	0.15-20	[38]
HPLC	Food	0.0099	0.0032	0.03	0.0097	0.05-	0.01-600	[39]
RP-HPLC	Tablet	---	0.0625	---	0.125	250	0.5-1.5	[40]
UV-Vis+NAS	Syrup	0.2321	0.2322	0.4351	0.5402	---	4-40	Present
UV-Vis+PLS	Syrup	---	---	---	---	4-40	4-40	study
						4-40		Present
								study

4. Conclusion

Analytical techniques with good precision and accuracy, as well as the ability to accomplish an analysis of multicomponent formulations within very less time, have been always a concern of researchers. In this study, this was obtained when the spectrophotometry method was coupled with chemometrics tools (NAS and PLS). The aid

of chemometrics to the spectrophotometry approach makes it robust and efficient for the concurrent estimation of drugs in multi-component formulations. A comparison was performed between the use of chemometric-assisted UV spectrophotometry and HPLC for the simultaneous determination of vitamin B9 and vitamin B12 in syrup formulation by using one-way ANOVA. The calculated F-value indicated that there are no significant differences among the NAS, PLS, and HPLC. Hence, the suggested methods can be applied to analyze the pharmaceutical formulation. Chemometrics methods like NAS and PLS along with UV-Vis spectrophotometry were found to be economical, rapid, and simple in comparison with HPLC because they do not need prior separation and expensive instruments and materials.

References

[1] Lieberman, S., Bruning, N., The real vitamin & mineral book. New York: Avery Group; 1990.

[2] McDonald, A., Natow, A., Heslin, J.A., Smith, S.M., editors. Complete book of vitamins and minerals. Publications International, Ltd; 2000.

[3] Stillwell, W. Lipid-Soluble Vitamins. An Introduction to Biological Membranes (Second Edition)., Chapter 21, (2016), PP. 479-498.

[4] Mellidou, I.; Georgiadou, E.C.; Kaloudas, D.; Panagiotis, K.; Fotopoulos, V.; Kanellis, A.K. Vitamins. Postharvest Physiology and Biochemistry of Fruits and Vegetables., Chapter 17, (2019), PP. 359-383.

[5] Suk Lee, K.; Moser, D.K.; Park, J.H.; Lennie, T.A. The association of deficiencies of water‐soluble vitamin intake with health‐related quality of life and prognosis in patients with heart failure. *Quality of Life Research*. **30**: 1183–1190 (2021).

[6] Kumar, P.; Kumar, M.; Bedi, O.; Gupta, M.; Kumar, S.; Jaiswal, G.; Rahi, V.; Gangaram Yedke, N.; Bijalwan, A.; Sharma, S.; Jamwa, S. Role of vitamins and minerals as immunity boosters in COVID‐19. *Inflammopharmacology*. **29**: 1001–1016 (2021).

[7] Paulo Winiarski, J.; Rampanelli, R.; Carlos Bassani, J.; Zambelli Mezalira, D.; Luisa Jost, C. Multi-walled carbon nanotubes/nickel hydroxide composite applied as electrochemical sensor for folic acid (vitamin B9) in food samples. *Journal of Food Composition and Analysis*. **92**: 103511 (2020).

[8] Qin, Y.; Shi, Y.; Yu, Q.; Yang, S.; Wang, Y.; Dai, X.; Li, G.; Cheng, Z. Vitamin B12 alleviates myocardial ischemia/reperfusion injury via the SIRT3/AMPK signaling pathway. *Biomedicine & Pharmacotherapy*. **163**: 114761 (2023).

[9] Vinueza Veloz, A.F.; Valeria Carpio Arias, T.; Sthefania Vargas Mejía, J.; Carolina Tapia Veloz, E; Piedra Andrade, J.S.; Nicolalde Cifuentes, T.M.; Heredia Aguirre, S.I.; Fernanda Vinueza Veloz, M.

Cognitive function and vitamin B12 and D among community-dwelling elders: A cross-sectional study, Clinical Nutrition ESPEN. **50**: 270-276 (2022).

[10] Green, R.; Allen, L.H.; Bjørke-Monsen, A.L.; Brito, A.; Guéant, J.L.; Miller, J.W.; Molloy, A.M.; Nexo, E.; Stabler, S.; Toh, B.H.; Magne Ueland, P.; Yajnik, C. Vitamin B12 deficiency. *Nature Reviews Disease Primers*. **3**: 17040 (2017).

[11] Stabler, S.P. Vitamin B12 Deficiency, *The New England Journal of Medicine*. **368**: 149-160 (2013).

[12] Amin, M.; Reusch, J. High-performance liquid chromatography of water-soluble vitamins. Part 3. Simultaneous determination of vitamins B1, B2, B6, B12 and C, nicotinamide and folic acid in capsule preparations by ion-pair reversed-phase high-performance liquid chromatography, Analyst. **112**: 989-991 (1987).

[13] Mohamed, G.G.; Fekry, A.M.; Abou Attia, F.M.; Ibrahim, N.S.; Azab, S.M. Simultaneous determination of some antidepressant drugs and vitamin B12 in pharmaceutical products and urine sample using HPLC method. *Journal of Chromatography B.* **1150**: 122178 (2020).

[14] Poongothai, S.; Ilavarasan, R.; Karrunakaran, C.M. simultaneous and accurate determination of vitamins B1, B6, B12 and alphalipoic acid in multivitamin capsule by reverse-phase high performance liquid chromatography method. *International Journal of Pharmacy and Pharmaceutical Sciences*. **2**: 133-139 (2010).

[15] Farag, A.M.; Rizk, M.S.; El-Bassel, H.A.; Youssif, M.H. Fast and Efficient RP-HPLC Method for Simultaneous Determination of Water-soluble Vitamins in Some Nutraceutical Supplements. *Egyptian Journal of Chemistry*. **62**: 2195– 2211 (2019).

[16] Zheng, X.H.; Jiang, L.Y.; Zhao, L.T.; Zhang, Q.Y.; Ding, L. Simultaneous quantitation of folic acid and 5-methyltetrahydrofolic acid in human plasma by HPLC–MS/MS and its application to a pharmacokinetic study. *Journal of Pharmaceutical Analysis*. **5**: 269–275 (2015).

[17] Chen, C.; Huo, J.; Yin, J.; Li, Y.; Mao, H.; Zhuo, Q.; Jia, X. High-Throughout Simultaneous Detection of Vitamin B12 and Folate using Intrinsic Factor and Folate Binding Protein. *International Journal of Electrochemistry*. **15**: 5262 –5276 (2020).

[18] Kong, D.; Liu, L.; Song, S.; Kuang, H.; Xu, C. Development of Sensitive, Rapid, and Effective Immunoassays for the Detection of Vitamin B12 in Fortified Food and Nutritional Supplements. *Food Analytical Methods*. **10**: 10-18 (2017).

[19] Goudarzi, N., Farsimadan, S., Arab Chamjangali, M., Bagherian, G.A. Optimization of modified dispersive liquid–liquid microextraction coupled with high-performance liquid chromatography for the

simultaneous preconcentration and determination of nitrazepam and midazolam drugs: An experimental design, Journal of Separation Science. **38**: 1673–1679 (2015).

[20] Goudarzi, M., Farsimadan, S., Arab Chamjangali, M., Ali Bagherian, G., Development of coupled ultrasound-assisted and reversed-phase dispersive liquid–liquid microextraction before high-performance liquid chromatography for the sensitive determination of vitamin A and vitamin E in oil samples, Journal of Separation Science. **38**: 3254–3261 (2015).

[21] Palur, K.; Charan Archakam, S.; Koganti, B. Chemometric assisted UV spectrophotometric and RP-HPLC methods for simultaneous determination of paracetamol, diphenhydramine, caffeine and phenylephrine in tablet dosage form, *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*. 243: 118801 (2020).

[22] Raeis Farshid, B.; Sohrabi, M.R.; Davallo, M.; Raeis Farshid, S. Green spectrophotometric method for the spectral pattern recognition based on fuzzy inference system compared to continuous wavelet transform for the quantitative determination of anti-psoriasis drugs in commercial skin ointment formulation: Comparison with HPLC. *Sustainable Chemistry and Pharmacy*. **33**: 101107 (2023).

[23] Tawakkol, S.M.; El-Zeiny, M.B.; Hemdan, A.; Full spectrum and selected spectrum based chemometric methods for the simultaneous determination of cinnarizine and dimenhydrinate in laboratory prepared mixtures and pharmaceutical dosage form. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*. **173**: 892-896 (2017).

[24] Upadhyay, Y., Sharma, N., Sarma, G.S., Rawal, R.K., Application of RP–HPLC method in dissolution testing and statistical evaluation by NASSAM for simultaneous estimation of tertiary combined dosages forms, Journal of Pharmaceutical Analysis. **5**: 307-315 (2015).

[25] Kalinowska, K., Bystrzanowska, M., Tobiszewski, M., Chemometrics approaches to green analytical chemistry procedure development, Current Opinion in Green and Sustainable Chemistry. **30** 100498 (2021).

[26] Noroozi, R.; Sohrabi, M.R.; Davallo, M. A simple and rapid spectrophotometric method coupled with intelligent approaches for the simultaneous determination of antiepileptic drugs in pharmaceutical formulations, biological, serological, and breast milk samples. *Chemometrics and Intelligent Laboratory Systems*. 228: 104633 (2022).

[27] Mirmohseni, A.; Abdollahi, H.; Rostamizadeh, K. Net analyte signal-based simultaneous determination of ethanol and water by quartz crystal nanobalance sensor. *Analytica Chimica Acta*. **585**: 179–184 (2007).

[28] Lorber, A.; Faber, K.; Kowalski, B.R. Net Analyte Signal Calculation in Multivariate Calibration. *Analytical Chemistry*. **69**: 1620-1626 (1997).

[29] Wold, H. 1966. Estimation of principal components and related models by iterative least squares. In: Krishnaiah, P.R. (Ed.), International Symposiumon Multivariate Analysis, 391–420. AcademicPress, Dayton, OH.

[30] Arabzadeh, A., Sohrabi, M.R., Goudarzi, N., Davallo, M., Using artificial neural network and multivariate calibration methods for simultaneous spectrophotometric analysis of Emtricitabine and Tenofovir alafenamide fumarate in pharmaceutical formulation of HIV drug, Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. **215**: 266-275 (2019).

[31] Lohmöller, J.B., 1989. Latent Variable Path Modeling with Partial Least Squares. Physica-Verlag, Heidelberg, Germany.

[32] Kaufmann, L.; Gaeckler, J. A structured review of partial least squares in supply chain management research. *Journal of Purchasing and Supply Management*. **21**: (2015) 259-272.

[33] Ghasemi, J., Abbasi, B., Simultaneous Spectrophotometric Determination of Group B Vitamins Using Parallel Factor Analysis: PARAFAC, Journal of the Chinese Chemical Society. **52**: 1123-1129 (2005).

[34] Monakhova, Y.B., Mushtakova, S.P., Kolesnikova, S.S., Astakhov, S.A., Chemometrics-assisted spectrophotometric method for simultaneous determination of vitamins in complex mixtures, Analytical and Bioanalytical Chemistry. **397**: 1297–1306 (2010).

[35] J. N. Miller, J. C. Miller, Statistics and Chemometrics for Analytical Chemistry, Sixth ed. 2010, ISBN- 978-0-273-73042-2.

[36] Elmi, Z., Faez, K., Goodarzi, M., Goudarzi, N., Feature selection method based on fuzzy entropy for regression in QSAR studies, Molecular Physics. **107**: 1787–1798 (2009).

[37] Maher, H.M., Sultan, M.A., Olah, I.V., Development of validated stability-indicating chromatographic method for the determination of fexofenadine hydrochloride and its related impurities in pharmaceutical tablets, Chemistry Central Journal. **5**: 76 (2011).

[38] Albawarshi, Y., Amr, A., Al-Ismail, K., Shahein, M., Majdalawi, M., Saleh, M., Khamaiseh, A., El-Eswed, B., Simultaneous Determination of B1, B2, B3, B6, B9, and B12 Vitamins in Premix and Fortified Flour Using HPLC/DAD: Effect of Detection Wavelength, Journal of Food Quality. **2022**: 1-11 (2022).

[39] Antakli, S., Sarkees, N., Sarraf, T., Determination of water-soluble vitamins B1, B2, B3, B6, B9, B12 and c on C18 column with particle size 3 µm in some manufactured food products by hplc with uv-dad/fld detection, International Journal of Pharmacy and Pharmaceutical Sciences. **7**: 219-224 (2015).

[40] Amidzic, R., Broboric, J., Cudina, O., Vladimirov, S., RP-HPLC Determination of vitamins B1, B3, B6, folic acid and B12 in multivitamin tablets, Journal of the Serbian Chemical Society. **70**: 1229–1235 (2005).