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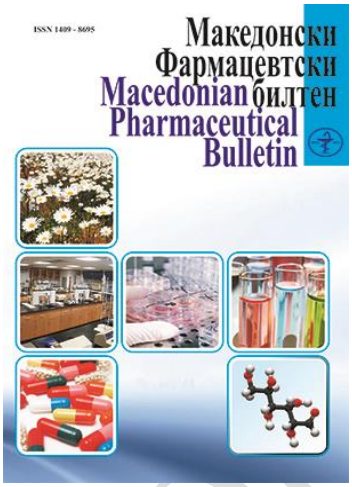

Title: UV-VIS spectrophotometric-assisted chemometric calibration models for simultaneous determination of thiamine and pyridoxine vitamins in powdered infant formula

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UV-VIS spectrophotometric-assisted chemometric calibration models for simultaneous determination of thiamine and pyridoxine vitamins in powdered infant formula

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Abstract

Thiamine and pyridoxine, which are hydrophilic B vitamins, are compounds that play an important role in many biochemical events for the health of infants and adults. Recently, because of the complexity of food and pharmaceutical composition, chemometric models are preferred to obtain relevant information from multivariate measured data. The aim of this study was to determine the simultaneous quantitative analysis of thiamine and pyridoxine in powdered infant formula and pharmaceutical formulations by the UV-visible spectrophotometric method coupled with chemometric calibration models (partial least squares (PLS) and principal component regression (PCR)). These vitamins were extracted by addition of trichloroacetic acid (precipitation of proteins) after samples of powdered infant formula was dissolved in Milli-Q water. Factorial design was used to prepare the calibration (25 samples) and validation (8 samples) sets containing mixture of thiamine and pyridoxine within their linear range (2.5-25 μg/mL). Spectra of obtained mixtures and samples were recorded between wavelengths of 200-360 nm with the intervals $\Delta\lambda$=1 nm. In regression models created by using PLS and PCR algorithm, the results had acceptable recovery ≤98.14% for both compounds. These chemometric models predict the quantification of both, thiamine and pyridoxine in powdered infant formula samples via interpreting the UV-Vis spectrum. The average thiamine and pyridoxine contents of powdered infant formula samples were found to be 5.02±0.065 μg/g and 4.32±0.086 μg/g for PLS model and 4.98±0.078 μg/g and 4.03±0.091 μg/g for PCR model, respectively.

Keywords: thiamine, pyridoxine, infant formula, factorial design, chemometrics

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Introduction

All humans require several organic compounds to maintain metabolic reactions in organism. Vitamins are one of these substances that have to be provided from food. Vitamins have several biological activities in living organisms. Among these, they work as cofactors in production of several proteins (enzymes) that are critical in major metabolic pathways in the body, such as glycolysis (Emden-Meyerhof-Parnas pathway), citric acid cycle (Szent Györgyi-Krebs cycle) and pentose phosphate pathway (PPP). Glycolysis and citric acid cycle are mostly associated with ATP synthesis, and the latter (PPP) is pivotal in accordance with the enforcement of the antioxidant balance of the organism.

Thiamine, B1, is one of these vitamins participating in several biochemical reactions and a vital molecule for all living organisms. Vitamin B1 has two main functional groups which are pyrimidine (4-amino-2-methylpyrimidine) and thiazolium (4-methyl-5-(2-hydroxyethyl)-thiazolium). Thiamine is a water soluble compound (Chawla and Kvarnberg, 2014). Many research studies have contributed to monitor the importance of thiamine in humans (Tylicki et al., 2018). The first detection was by Kanehiro who was general surgeon in the Japanese navy in 1884 by rejecting the notion that beriberi was an infectious disease. He recommended that barley, meat, milk and bread had to be added to white rice for improving health and sanitary conditions. By this manner, beriberi disease was eliminated during a 9-month sea voyage. In 1901 the associate "G. Grijns to Eijkman C" revealed the correct relation between polished rice and beriberi explaining the loss of thiamine. Eijkman was awarded the Nobel Prize for Physiology or Medicine due to his discovery of thiamine. Its structure was completely determined in 1934 by Robert R. Williams (Bettendorff, 2013).

Pyridoxine which is also known as B6 has three main forms as pyridoxamine, pyridoxine and pyridoxal (Zeng et al., 2021). These molecules were metabolized in living organism as pyridoxal phosphate and pyridoxamine phosphate which took a crucial part in metabolism of several amino acids, carbohydrates, fatty acids, lipids, nucleic acids hemoglobin and even gamma butyric acid neurotransmitters (Calderón-Ospina and Nava-Mesa, 2020). Due to its importance, B6 is an essential micronutrient for humans. Vitamin B6 was firstly determined during the investigation of pellagra disease which is a severe and mortal illness (main symptoms: severe dermatitis diarrhea and demans) (Harsa et al., 2019). Scientists mostly administered treatment on pellagra by B1 and B2 vitamins. However, they observed that the disease has been vanished after adding yeast in their diet. An unknown molecule cured the disease. They tried to isolate this unknown substance. In

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1938, pyridoxine was firstly isolated and its structure was firstly illuminated in next year. Pyridoxal and pyridoxamine were also purely isolated in 1944 (Rosenberg, 2012).

Thiamine and pyridoxine are formulated in combined pharmaceutical forms due to their synergistic effect especially on the nervous system, by participating in several different biochemical reactions. Because of that reason, several spectroscopic and chromatographic methods were developed and validated in order to determine their concentrations in either *in vivo* or *in vitro* media (Marszałł et al., 2005; Porter and Lodge, 2021; Woollard and Indyk, 2002). Several studies were performed in order to detect thiamine and pyridoxine in powdered infant formula via chromatographic separation (Woollard and Indyk, 2002). However, there is no study associated with measuring both vitamins by chemometrics approach. Chemometrics algorithm were defined as using multivariate statistical methods and calibration models which are based on the spectrum of samples that have been applied to overcome overlapping spectra of mixtures in any different media (Albayrak et al., 2019; Dinç and Baleanu, 2002; El-Gindy et al., 2007; Mohamed and Salem, 2005). Multivariate data analysis could give analytical information and have important advantages which minimize chemical consumption and sample preparation, provide improvement on total run time of analysis, good accuracy and precision values without chromatographic elution. In this study, the main aim was development and validation of an alternative method for simultaneous quantification of pyridoxine and thiamine via different chemometrics algorithms as Partial Least Square (PLS), Principle Component Regression (PCR) in powdered infant formula.

Materials and methods

Instrument and software

All spectrophotometric analysis was performed via Thermo Scientific Multiscan GO 51119300 model UV-Visible instrument. 1 nm of spectral bandwidth was selected and the wavelength scanning speed was settled to be 1000 nm/min. PLS and PCR models were carried out by using MATLAB R2019b (PLS-Toolbox software version 8.5.1). Calibration and validation data sets were generated by Design-Expert 12.0 (Stat-Ease Inc., Minneapolis, MN, USA) software. Microsoft Excel 2018 and SPSS 11.5 software program (SPSS, Chicago, IL, USA) were used for statistical tests.

Materials and samples

Thiamine and pyridoxine were purchased from Sigma-Aldrich (Germany). Hydrochloric acid (Merck, Germany), trichloroacetic acid (TCA) (Merck, Germany) and double de-ionized water

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(Milli-Q water) were of analytical grade. Powdered formula samples have been purchased in Turkey, Bilecik markets.

For both compounds, stock solutions (1 mg/mL) were prepared by dissolving them in 0.1 M HCl. Linearity range for both vitamins was set between 2.5 and 25 µg/mL. The concentration profiles of calibration (2 categories at 5 levels) and validation (2 categories at 3 level) solutions were generated with factorial design.

Preparation of powdered infant formula

Three conventionally used standard infant formulas were kindly obtained from local pharmacy stores. All analyzed brands were initial formula-based formulas generated for infant consumption during the first six months. The infant formulas were prepared regarding to the instructor manual in order to attain vitamins concentrations as given on the label (as thiamine and pyridoxine (mg) per 100 mL of prepared infant formula. An amount of sample (20 g) was accurately weighed, dissolved in 10 mL fresh Milli-Q water. Afterwards, sample was boiled and cooled to 70 ºC and sonicated for 10 min. Finally, 0.1 g of TCA was added to 1 mL aliquot of the freshly prepared infant formula samples, mixed on a magnetic stirring plate for 10 min and centrifuged for 10 min at 4000 rpm. Liquid phase was separated, filtered through a 0.45 µm filter and the volume was completed to 2 mL with 0.1 M HCl. Samples were stored at 4°C and in the dark until analysis.

Results

Absorption spectra of thiamine with pyridoxine and Experimental Design

To determine the optimum conditions of individual spectra for thiamine and pyridoxine, different polar solvents (water, methanol and glycerin) were evaluated at low pH because these molecules are insoluble in organic solvents (chloroform, benzene, acetone and ether). It was reported in the literature that water soluble vitamins get higher absorbance in acidic medium (Özdemir and Dinc, 2004; Zafra-Gómez et al., 2006). According to these information, it was observed that thiamine and pyridoxine completely dissolved in 0.1 M HCl solution and get the highest absorbance compared to the other solvents. In addition to this, no interference was encountered.

After determining the optimum spectrophotometric conditions, individual absorption spectra of thiamine and pyridoxine vitamins were obtained between 200-360 nm wavelength range with 1 nm intervals ($\Delta\lambda$) in 0.1 M HCl solution. For standard thiamine and pyridoxine, it was shown that the maximum absorbances were 245 and 285 nm (Fig. 1a), respectively. Individual linear working range was determined for each vitamin as 2.5-25 µg/mL. The spectra of combined form

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could be overlapped between 200-360 nm as shown Fig. 1b, which could not be easily resolved with classical spectroscopic approaches. Due to this reason, this method needs a separation method in order to obtain an accurate and precise quantification. Recently, spectrum of binary vitamins have overlapped and they were successfully quantitated by both, chemometric calibration models.

Fig. 1

In addition to this, a new PLS and PCR chemometrics calibration model were developed for simultaneous quantitative analysis of each drug. In order to maintain a chemometrics analysis, calibration and validation data matrix were prepared. For this purpose, Calibration sets including 25 binary mixtures (Fig. 2a) were prepared by using a five-level factorial design considering the linear range individually selected concentrations (2.5, 10, 15, 20 and 25 µg/mL). Validation sets including 8 binary mixtures (Fig. 2b) was prepared by using a three-level factorial design containing especially low, medium and high concentrations in the linear range (2.5, 10, 15, 20 and 25 µg/mL).

Fig. 2

PLS and PCR models development, validation and comparison for simultaneous determination of thiamine with pyridoxine vitamins

For PLS chemometric calibration algorithm, absorbance values of spectrum (X-matrix) and concentration of standards (Y-Matrix) were established. PCR algorithm evaluate X-scores to clarify the maximum proportion of factor variation (Sorouraddin et al., 2011) In this study, both models were developed by evaluating absorbance and concentration matrices at 161 absorbance values between 220–360 nm for created sets (calibration and validation). All values were exported into MATLAB 2017a PLS-toolbox 8.3 software. Variance-covariance matrices of the X and Y scores of calibration set was calculated in accordance with the both models. For calibration data set, the PLS and PCR calibrations were obtained by the mathematical relationship between the concentrations after the absorbances were subjected to the composition process in the variance-covariance matrix. In order to determine the factor numbers for both models, a cross validation (25 calibration spectra) was evaluated. In this process, one calibration solution was measured as a sample at a time. This process was iterated for 25 times. PLS and PCR calibrations were plotted using 24 calibration spectra. In each sample, the estimated concentrations of analytes proposed by each model were compared with their actual concentrations to evaluate the estimation power of the chemometrics model. For both algorithms, the optimum factor numbers were selected to get better

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prediction performance coupled with the lowest bias. The optimum factor numbers which are called latent variables (LV) for both models were determined with the Prediction Error Sum of Squares (PRESS) for the cross validation results. The predicted concentrations of the infant formula in each sample were statistically evaluated with the actual concentrations in these calibration samples. Root-mean-square error of cross-validation (RMSECV) was determined for both models. Furthermore, the root-mean standard error of calibration prediction (RMSEP) were determined for predictions of the standard variation for each calibration in infant formula. In addition to this, correlation-coefficient were determined for both models. Both vitamins with each models were explained the 3 LV (Fig. 3).

Fig. 3

Predictivity power of both algorithms were evaluated by plotting the actual concentrations versus the predicted concentrations graph by using the concentrations versus absorbance data matrix. In addition, the validation set, containing eight samples, was analyzed by the proposed procedure. The predicted values for simultaneous analysis of thiamine and pyridoxine vitamins both PLS and PCR chemometric calibration models were given the Table 1.

Table 1

To validate the developed chemometric models, validation set was monitored and tested (Fig. 2b). The validation set was analyzed with the same procedure of calibration data set. Similarly, the statistical values were summarized in Table 1. The precision and accuracy of models were calculated as the Relative Standard Deviation. (RSD%: standard deviation/mean) x 100) and Recovery (R%: (Cact-Cpred) x 100), respectively. The prediction results for PLS and PCR calibration models were exhibited in Table 2. For the PLS and PCR models, the RSD% was found to be ≤2.40 and ≤2.31 for thiamine with ≤0.92 and ≤2.09 for pyridoxine, respectively. To determine the accuracy, the R% values for the PLS and PCR models were determined as ≤ 98.55% and ≤98.22% for thiamine with ≤98.63% and ≤98.14% for pyridoxine.

In addition to this, limit of detection (LOD:3$\|\delta_r\|)\|b_k\|$)) was calculated to determine sensitivity of chemometric calibration models. (b_k: the vector of regression coefficients, $\|\delta_r\|$: noise) and found as 0.89 µg/mL and 0.94 µg/mL for tiamine and 0.59 µg/mL and 0.63 µg/mL for pyridoxine for PLS and PCR calibration models, respectively. In addition, extraction recoveries (ER %) were determined by spiking of concentrations 2.5, 10, 15, 20 and 25 µg/mL of the thiamine

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and 2.5, 10, 15, 20 and 25 μg/mL of pyridoxine with necessary dilutions in powdered infant formula. The ER% of both PLS and PCR models were found as ≤98.37% for thiamine and ≤98.21 % for pyridoxine. indicating good accuracy.

Table 2

Correlation matrix and p-values obtained from PLS and PCR models were compared with Pearson method. Correlation matrix for both, thiamine and pyridoxine were found as ≤0.998 and *p* values were found to be <0.0001. According to the Pearson test, any statistical difference was found between both models.

Simultaneous determination of thiamine and pyridoxine in powdered infant formula with chemometric calibration models

The above-described PLS and PCR chemometric calibrations were successfully applied to best-selling powdered infant formula on Local Market in Turkey in order to simultaneous determination of the thiamine and pyridoxine content. Ten replicates determination were made. The obtained results showed acceptable recovery values for each vitamin (Table 3).

Table 3

Discussion

There are different chemical or microbiological techniques, which are difficult and time consuming for determination of water-soluble vitamins. In this study, we recommend a very reproducible and simple protein precipitation method via TCA. However, it has poor extraction capacity for some vitamins, such as niacin, ascorbic acid, pantothenic acid and folic acid. This provides a great advantage to determine thiamine and pyridoxine without any interference caused by other vitamin B species. (Zafra-Gómez et al., 2006). So, TCA was used primarily to precipitate proteins in sample preparation step. However, this is not enough because the simultaneous determination of thiamine and pyridoxine in real samples is not possible via conventional regression analysis due to the resolution of the binary mixture in the presence of the other components. Chemometrics models are based on the application of mathematical and statistical methods to design optimum conditions and to afford maximum information through analysis of chemical data (El-Kosasy et al., 2016). In such analysis, chemometric models can be obtained without

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chromatographic separations. Generally, chemometrics approaches are very trustworthy algorithm for simultaneous analysis of binary mixture where spectrum of each compound intercepted leads to an interference that cause a great challenge for quantitative analysis of each compound via conventional methods.

From different chemometric models, firstly, experimental design was used in generating calibration and validation mixture. Experimental design techniques take all factors together. When experiments are made by creating different combinations of different levels of these factors, the effects of the factors can be obtained in a way that includes the effects of other factors at different levels. An effective design provides the most information with the least amount of work. One of the experimental designs is factorial design (Erdal, 2007). Full factorial design is beneficial in low number of factors as this study. Selecting 5 or 6 factors in experimental design requires at least 2^6 experiments and it is time consuming. To maintain two levels and 'k' factors of full factorial design, '2k' number of experiments are needed. Increasing the number of independent variables lead to higher factors and 2-fold higher experiment. Partial factorial design eliminates the insignificant factors and lowered the number of experiment to explain the whole analysis. In order to carry out a partial factorial design several concentrations were determined for each analyte and highly correlated data were taken into account. By this way model prediction is achieved by lower number of experiments compared to full factorial design. Partial factorial design makes chemometrics model possible for application of real samples. In this study, the 25 calibration samples (2 categories at 5 levels) and 8 validation samples (2 categories at 3 levels) were construed by using the factorial design. By this way, lower number of experiments were performed to perform PLS and PCR data sets to predict the concentration of each compound.

In the proposed study, chemometric calibrations (PLS and PCR) in spectral analysis and experimental design were obtained. All spectra were evaluated to get accurate and precise results instead of plotting a calibration curve for only one wavelength as conventional linear regression analysis (Ni and Gong, 1997).

PLS is a chemometric model that generate data matrices and reduce multivariate variables into two dimensions via latent variables. PCR is integration of two different chemometric models which are principal component analysis (PCA) and inverse least square (ILS). PLS is a robust and reproducible algorithm for quantitative prediction of binary mixtures. PCR algorithm calculates response independent variables from factors underlying the predictive variables of PCA. The number of factors selected to construct a predictive model is important for accurate and precise analysis in PLS calibration. Suggested criteria for finding the optimum factors are the minimum PRESS and RMSECV and satisfactory correlation coefficient values and the F-statistic for

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calibration and RMSEC for validation both PLS and PCR calibration models (Table 1). Predictive capabilities of PLS and PCR calibration models were evaluated by comparing the UV-Vis spectra of reference standards. According to the results, good accuracy and precision values were obtained for each vitamin (Table 2). In addition, this, both chemometric calibration models (PLS and PCR) show acceptable accuracy and there is no statistical variation between each algorithm in accordance with Pearson test. But, correlation matrix proved that each model has very small difference for prediction whereas results of PLS were slightly better than PCR in accordance with actual concentration. Thus, each models could be trustfully applied for simultaneous determination of both vitamins in powdered infant formula (Table 3).

Conclusion

A novel chemometrics assisted spectrophotometric method was developed and validated for simultaneous determination of thiamine and pyridoxine in powdered infant formula. These proposed models could be an accurate and precise alternative to chromatographic methods that are used in routine quality control samples without using chromatographic elution. In addition to this, quantitative analysis of B1 and B6 vitamins were performed without any derivatization or ratio spectra models. Moreover, the chemometric calibration models make it easier to analyze complex spectra in a short time with accurate and precise predictions. The received results showed that the proposed models could be applicable as an accurate alternative for simultaneous quantitation of thiamine and pyridoxine in routine quality control analysis in food industries.

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Резиме

UV-VIS спектрофотометриски и хемометриски калибрациони модели за истовремено одредување на тиамин и пиридоксин во формула за бебиња

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Клучни зборови: тиамин, пиридоксин, формула за доенчиња, факторијален дизајн, хемометрија

Тиамин и пиридоксин, кои се група хидрофилни витамини Б, се соединенија кои играат важна улога во многу биохемиски процеси вклучени во здравјето на децата и возрасните. Поради сложеноста на составот на храната и фармацевтските производи, хемометриските модели се употребуваат со цел да добијат соодветни информации од мултиваријантни измерени податоци. Целта на оваа студија беше истовремена квантитативна анализа на тиамин и пиридоксин во формула за бебиња со UV-VIS спектрофотометриска метода, заедно со хемометриски модели за калибрација (парцијални најмали квадрати (PLS) и регресија на главната компонента (PCR) Овие витамини беа екстрахирани со трихлорооцетна киселина (таложење на протеини) откако примероците од формулата за бебиња беа растворени во вода Milli-Q. Факторијален дизајн беше искористен за подготовка на сетови за калибрација (25 примероци) и валидација (8 примероци) кои содржат смеси на тиамин и пиридоксин во рамките на нивните линеарни опсези (2,5-25 μg/mL). Спектрите на добиените смеси и примероци беа снимени на бранови должини од 200-360 nm со интервали $\Delta\lambda = 1$ nm. Во регресионите модели креирани со употреба на PLS и PCR алгоритми, резултатите имаа задоволително recovey ≤98,14% за двете соединенија. Со овие модели може да се идентификуваат и квантифицираат тиамин и пиридоксин во формула за бебиња. Просечната содржина на тиамин и пиридоксин во формулите за бебиња беше 5,02 ± 0,065 μg/g и 4,32 ± 0,086 μg/g за моделот PLS и 4,98 ± 0,078 μg/g и 4,03 ± 0,091 μg/g за PCR моделот, соодветно.

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Table 1. Statistical parameters for simultaneous quantitation of thiamine and pyridoxine vitamins by chemometric calibration models (PLS and PCR)

Models	*PLS*		*PCR*	
Concentration (µg/mL)	2.5-25		2.5-25	
Spectral region (nm)	200-360		200-360	
Cross-validation Results	Thiamine	Pyridoxine	Thiamine	Pyridoxine
Optimum number of factors	3	3	3	3
Calibration curves	1.0103x- 0.0907	0.9998x-0.0763	1.0131x-0.0843	1.0082x-0.03
R^2	0.9999	0.9999	0.9998	0.9997
RMSE-CV (µg/mL)	0.3374	0.4423	1.88499	1.70553
RMSEC	0.2836	0.4423	1.52774	1.22065
Validation Results				
Slope	0.9851	1.0123	0.9668	1.0007
Intercept	0.1240	-0.0275	0.3139	0.0625
R^2	0.9998	0.9998	0.9999	0.9997
RMSEC	0.283	0.4420	0.2447	0.6920
Prediction Bias	1.77×10^{-15}	0.12×10^{-20}	0.00888	0.13067

PRESS=$\sum$(Cact-Cpred)^2, RMSECV=$\sqrt{}$(PRESS/n)), (RMSEC=n$\sum$(Cact-Cpred)(2n-1), Cpred: predicted concentrations, Cact: actual concentrations, n: analysis number.

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Table 2. The prediction results obtained with validation set for PLS and PCR calibration models

MODEL	MIXTURE (µg/mL)		FOUND (µg/mL)		RECOVERY %		RSD%	
	Thiamine	Pyridoxine	Thiamine	Pyridoxine	Thiamine	Pyridoxine	Thiamine	Pyridoxine
PLS	10	10	9.98±0.08	10.27±0.05	99.80±0.79	102.6±0.46	0.79	0.45
	10	20	10.14±0.04	20.10±0.01	101.4±0.38	100.5±0.07	0.38	0.07
	15	25	14.88±0.34	25.33±0.12	99.18±2.30	101.3±0.47	2.32	0.46
	15	2.5	15.03±0.08	2.54±0.02	100.1±0.51	101.6±0.47	0.53	0.78
	20	10	19.78±0.47	9.86±0.09	98.91±2.37	98.63±0.9	2.40	0.92
	20	20	19.50±0.17	19.95±0.11	97.51±0.87	99.75±0.54	0.89	0.54
	25	15	24.69±0.36	15.25±0.05	98.74±1.43	101.6±0.30	1.45	0.30
	2.5	15	2.53±0.01	15.23±0.05	98.55±0.25	101.2±034	0.40	0.33
PCR	10	10	10.04±0.11	10.24±0.06	100.36±1.13	102.36±0.57	1.13	0.55
	10	20	10.28±0.24	20.02±0.42	102.79±3.43	100.10±2.09	2.31	2.09
	15	25	14.79±0.25	24.99±0.26	98.61±0.15	99.96±076	1.71	1.04
	15	2.5	14.82±0.06	2.52±0.02	98.82±0.06	98.39±0.03	0.42	0.79
	20	10	19.69±0.13	9.94±0.02	98.46±0.13	100.80±0.02	0.66	0.21
	20	20	19.69±0.38	19.62±0.28	98.45±0.78	98.14±1.41	1.93	1.44
	25	15	24.55±0.28	15.10±0.31	98.22±0.03	100.66±0.68	1.15	2.05
	2.5	15	2.53±0.02	15.07±0.13	101.20±0.02	100.49±0.13	0.47	0.88

RSD%: Relative standard deviation.

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Table 3. Simultaneous determination of thiamine and pyridoxine in the powdered infant formula

Model	Powdered infant formula	Mean* (µg/g)±SD	%Recovery	%RSD
PLS	Thiamine	5.02±0.065	100.4	1.294
	Pyridoxine	4.32±0.086	103.8	1.990
PCR	Thiamine	4.98±0.078	99.60	1.566
	Pyridoxine	4.03±0.091	96.87	2.258

* In µg of vitamins per g of prepared infant formula

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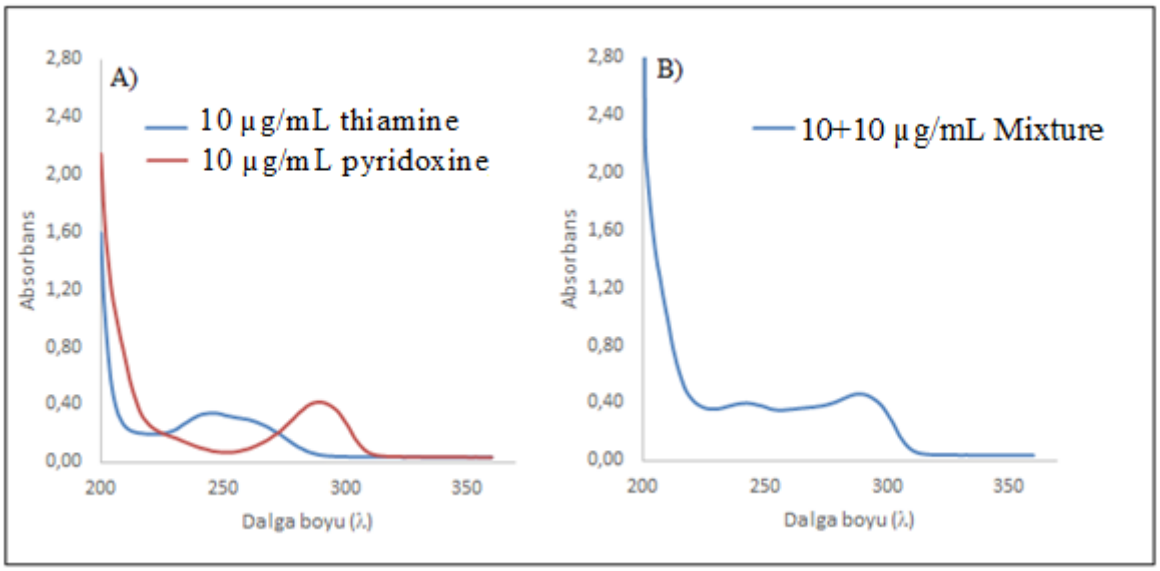

Fig. 1. UV absorption spectrum of a) Thiamine (10 µg/mL) and Pyridoxine (10 µg/mL); b) 10 µg/mL Thiamine + 10 µg/mL Pyridoxine mixture.

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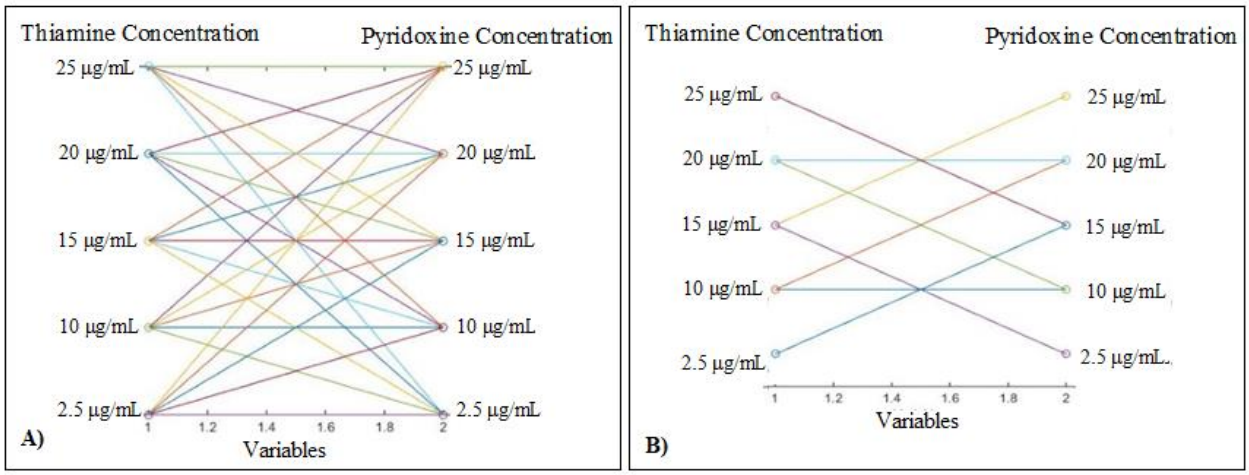

Fig. 2. Sets of containing different concentrations of each vitamin a) Calibration; b) Validation

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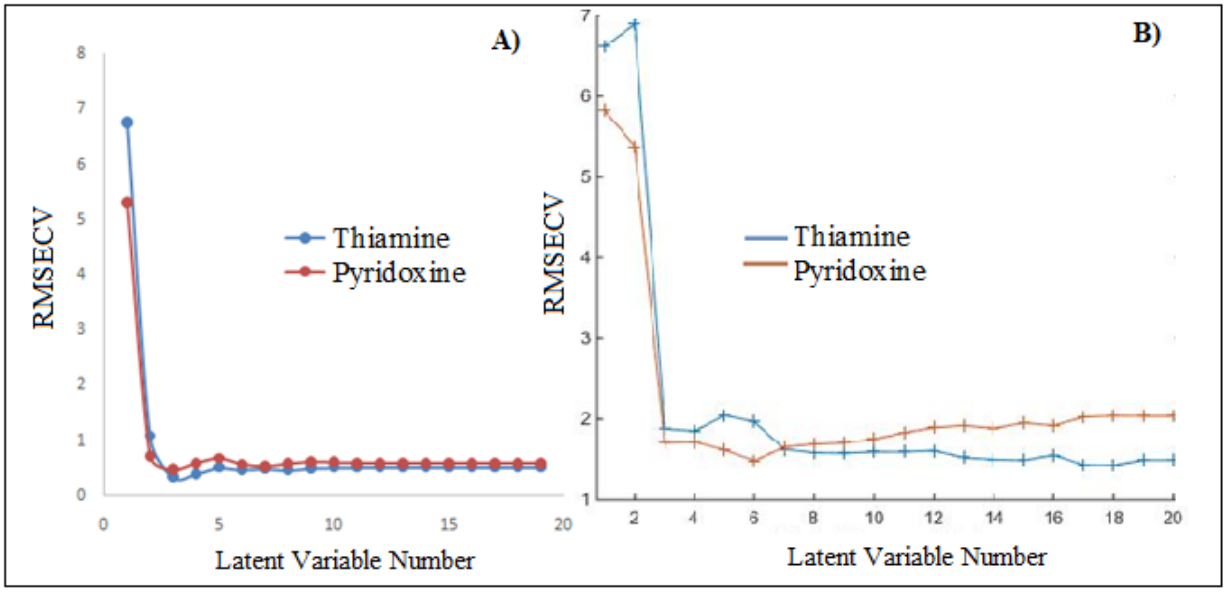

Fig. 3. a) RMSECV-LV curves each vitamin for PLS model; b) RMSECV-LV curves both vitamin for PCR model.

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