

PDA-CAD method for the determination of magnesium, pyridoxine and thiamine in a dietary supplement supported by analytical quality by design methodology

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Abstract

In this research, Analytical Quality by Design (AQbD) methodology was used to develop the HILIC-PDA-CAD method for the determination of magnesium, pyridoxine and thiamine content in a dietary supplement. Experiments were conducted on the Dionex Ultimate 3000 HPLC system with PDA and CAD detectors. Separations were performed on the ZIC-HILIC PEEK column (100 × 4.6 mm, 5 μm) with a mixture of acetonitrile, acetone and ammonium acetate/ammonium formate aqueous solution (48:12:40, v/v/v) as the mobile phase. The detection was performed at 220 nm. The optimal settings of CAD were defined with the one-factor-at-a-time approach: evaporation temperature 50°C, filter constant 10 s, pressure gas 60 psi. The influence of qualitative (salt type) and quantitative factors (pH and salt concentration) on the magnesium retention factor, k_{Mg} and the selectivity factor between pyridoxine and chloride anion, α_{B6/Cl^-} , was tested using the *I*-optimal design. The design space was defined by Monte Carlo simulations and model coefficients' errors were propagated with the aim of identifying the conditions that meet the following criteria: $k_{Mg} < 4$ and $\alpha_{B6/Cl^-} > 3.5$, with the probability $\pi = 95\%$. From the derived 2D-Design Space graph, 95 mM of ammonium formate pH 4.4 was selected as the optimal composition of the aqueous phase. The method was validated and its reliability in routine application was confirmed.

Keywords: HILIC-PDA-CAD, magnesium, pyridoxine, thiamine, AqbD

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Introduction

Dietary supplements are defined as orally applied products that might contain vitamins, minerals, amino acids, fiber, enzymes, herbs or botanicals, algae or macroscopic fungi. They are available as pills, tablets, capsules, powders, energy bars, and liquids and must be labeled as dietary supplements. They are used as compensation for diets, medical conditions, or eating habits that limit the intake of essential vitamins and nutrients, to boost energy or to get a good night's sleep, also to counter a sudden drop in estrogen levels at postmenopausal women (1). The regulatory framework on dietary supplements in the Republic of Serbia is provided by the Food Safety Law (2) and Rules on the health suitability of dietary products (3). They are regulated as foods rather than as pharmaceuticals, and consequently are not as closely monitored as it may be necessary. Therefore, the quality, efficacy and safety of the marketed dietary supplements can be questionable.

The combination of magnesium, thiamine and pyridoxine in a dietary supplement is recommended in cases of cramps and muscle pain, problems with constipation due to lazy bowels, increased exercise in athletes, fatigue and weakness, nervous tension and irritability, postmenopausal syndrome, migraine and other types of headaches (4). A comprehensive literature survey indicated that the analytical method for the simultaneous analysis of such solute mixture had not been previously published. The aim of this research was the development and validation of the first HILIC-PDA-CAD method for the estimation of magnesium, thiamine and pyridoxine content in dietary supplements.

A non-chromophore dependent detection technique, such as Charged Aerosol Detector (CAD), was the reasonable choice, since magnesium cation lacks UV absorption. CAD is a mass-dependent detector, suitable for all non-volatile and the majority of semi-volatile analytes, that generates the response unrestricted with spectral or physicochemical properties of the detected solute (5). Since nebulization and evaporation processes precede detection in CAD, it is of utmost importance that only volatile constituents are combined in the mobile phase. It is desirable to use large amounts of organic solvents, like acetonitrile, that will abate mobile phase viscosity and have a favorable effect on the nebulization, resulting in a greater number of particles. Furthermore, the evaporation of the formed aerosol will be facilitated, producing a large number of dry particles and increasing response intensity and detector sensitivity (6-8). When necessary, volatile mobile phase additives, such as the formic acid, acetic acid, trifluoroacetic acid, ammonium acetate or ammonium formate can also be used. Hydrophilic Interaction Liquid Chromatography (HILIC) is a useful novel approach for the effective separation of compounds exhibiting high polarity characteristics. Polar stationary phases (silica, amino, diol, zwitterionic or cyano) are employed along with RP-HPLC similar mobile phases. A desirable mobile phase comprises more than 70% (v/v) of organic solvent, mainly acetonitrile, and a low content of water or aqueous solution of volatile buffer, providing better sensitivity and adequate retention for polar ionic compounds. The high volatility of HILIC mobile phases facilitates evaporation and makes this chromatographic technique particularly suitable for coupling with CAD (9, 10).

Analytical Quality by Design (AQbD) is a cost-effective strategy to obtain robust and reliable analytical methods in line with the set of performance criteria and quality requirements adopted in the Analytical Target Profile (ATP). The outcome of implementing quality and risk assessment in the method development phase is the possibility of flexible moving of method parameters within the computed Design Space (DS) (11, 12). The AQbD approach is based on detailed understanding of the analytical procedure and all the potential risks leading to method failure. The focus of AQbD is the definition of the DS within which Critical Method Attributes (CMAs) meet the defined criteria with an acceptable level of probability (π), while small, intentional drifts of Critical Method Parameters (CMPs) have no significant effect on them. Consequently, the analytical method is not defined by one single combination of method parameters, but with the set of experimental conditions within the DS.

Experimental

Materials and chemicals

HPLC grade acetonitrile and acetone (Fluka, Sigma-Aldrich, Germany), ammonium formate (Fluka, Sigma-Aldrich, Germany), ammonium acetate (Fluka, Sigma-Aldrich, Germany) and water filtered through Simplicity 185 (Millipore, Billerica, MA) were utilized for the mobile phases' preparation. The aqueous phase pH was adjusted within the 4.25- 5.75 range with formic acid (98%, Fluka, Sigma Aldrich, Germany) or glacial acetic acid (Fluka, Sigma-Aldrich, Germany). For the preparation of stock solutions for method validation magnesium oxide (99% purity, Sigma-Aldrich, USA), reference standards of thiamine HCl and pyridoxine HCl (LGC standards, UK), as well as *Magnezijum 375 Plus B6*[®] capsules (Inpharm d.o.o, Serbia), were used. One capsule of *Magnezijum 375 Plus B6*[®] contains magnesium (375 mg magnesium oxide - MgO), pyridoxine (1.4 mg pyridoxine HCl) and thiamine (1.1 mg thiamine HCl).

Apparatus and experimental conditions

All the experiments were conducted on the Dionex Ultimate 3000 HPLC system (Thermo Scientific, USA) with PDA (Photodiode Array Detector) and CAD (Corona Veo-Charged Aerosol Detector) detector (Thermo Scientific, USA). Separations were performed on the SeQuant ZIC- HILIC (100 × 4.6 mm, 5 μ m) (Merck, Germany) column. The mobile phase flow rate was set at 1 mL min⁻¹. The injection volume was 10 μ L. During the optimization of chromatographic conditions, CAD parameters were set at the following values: evaporation temperature 50°C, gas pressure 60 psi, data collection rate 10 Hz, filter constant 10 s and function 1. PDA detector was set at 220 nm. On the basis of preliminary experiments, acetonitrile, acetone and aqueous phase ratio in the mobile phase was kept constant (48: 12: 40, v/v/v). Salt concentration in the aqueous phase (x_1), pH of the aqueous phase (x_2) and salt type (x_3) were varied according to the plan of experiments (Table I) defined by the I-optimal design using Design-Expert[®] software. The experimentally obtained results were analyzed by Microsoft[®] Excel 2013 (Microsoft Corporation, USA) and Matlab[®]R2018 (Mathworks, USA).

Table I Plan of experiments and experimentally obtained response values**Tabela I** Plan eksperimenata i eksperimentalno dobijene vrednosti odgovora sistema

<i>N₀</i>	<i>x₁</i>	<i>x₂</i>	<i>x₃</i>	<i>α_{B6/Cl}</i>	<i>k_{Mg}</i>
1	50	4.25	AF	3.445	3.71
2	100	4.25	AF	2.125	4.46
3	50	5.00	AF	4.520	3.91
4	75	5.00	AF	3.342	3.20
5	75	5.00	AF	3.348	3.18
6	75	5.00	AF	3.407	3.16
7	100	5.00	AF	2.441	4.83
8	50	5.75	AF	3.982	4.07
9	75	5.75	AF	3.344	3.84
10	100	5.75	AF	3.013	3.81
11	50	4.25	AA	3.666	2.98
12	75	4.25	AA	2.740	3.37
13	75	4.25	AA	2.740	3.29
14	50	5.00	AA	5.015	2.88
15	100	5.00	AA	2.486	3.66
16	100	5.00	AA	2.472	3.71
17	75	5.75	AA	4.925	2.55
18	75	5.75	AA	4.929	2.57

x₁ - salt concentration in the aqueous phase (mM), *x₂* - pH of the aqueous phase,

x₃ - salt type (AF - ammonium format, AA - ammonium acetate)

k_{Mg} - retention factor of Mg²⁺, *α_{B6/Cl}* - selectivity factor between pyridoxine HCl and Cl⁻

Preparation of solutions for method development and validation

Solutions for the Design Space definition

Stock solutions of MgO, thiamin HCl and pyrodoxine HCl were prepared in the concentration of 1 mg mL⁻¹ by dissolving respective amounts of MgO in the 0.25% aqueous solution of formic acid, while thiamin HCl and pyrodoxine HCl were dissolved in HPLC grade water. Working solutions were prepared from the stock solution. The appropriate amounts of stock solutions were transferred to the volumetric flasks and filled up with acetonitrile-water mixture (80:20, v/v) to attain the concentration of 200 µg mL⁻¹ for MgO and 100 µg mL⁻¹ for both vitamins.

Preparation of the mobile phase for method validation

The aqueous solution of ammonium format in the concentration of 95 mM was prepared by weighing 5.985 g of salt, which was transferred by 600 mL of HPLC water to a 1000 mL volumetric flask. After sonication in the ultrasonic bath for 10 minutes, the solution was filled to the mark with HPLC water. The pH value of the 95 mM ammonium format aqueous solution was adjusted to 4.4 with formic acid. The mobile phase was prepared by mixing 480 mL of acetonitrile, 120 mL of acetone and 400 mL of 95 mM ammonium format aqueous solution.

Solutions for selectivity evaluation

Selectivity was evaluated using chromatograms of colloidal silica (Fargon, Rotterdam, The Netherlands) and anhydrous lactose (Fargon, Rotterdam, The Netherlands) solutions, a solution containing 200 $\mu\text{g mL}^{-1}$ of MgO, as well as a sample solution with 80 $\mu\text{g mL}^{-1}$ of pyridoxine HCl and 80 $\mu\text{g mL}^{-1}$ of thiamin HCl. Colloidal silica and anhydrous lactose solutions were prepared in the final concentration of 100 $\mu\text{g mL}^{-1}$ by treating them in the same way as the samples used for the precision evaluation of the method.

Solutions for linearity testing

Stock solution of MgO was prepared in the 0.25% aqueous solution of formic acid in the concentration of 1 mg mL^{-1} . Working solutions were prepared in the concentrations of 100 $\mu\text{g mL}^{-1}$, 150 $\mu\text{g mL}^{-1}$, 200 $\mu\text{g mL}^{-1}$, 250 $\mu\text{g mL}^{-1}$ and 300 $\mu\text{g mL}^{-1}$ by dilution of stock solution with 95 mM ammonium format - acetone -acetonitrile mixture (20:16:64, v/v/v).

Stock solutions of thiamin HCl and pyridoxine HCl were prepared in acetonitrile - water (50:50, v/v) in the concentration of 1 mg mL^{-1} . Working solutions were prepared in the concentrations of 40 $\mu\text{g mL}^{-1}$, 60 $\mu\text{g mL}^{-1}$, 80 $\mu\text{g mL}^{-1}$, 100 $\mu\text{g mL}^{-1}$ and 120 $\mu\text{g mL}^{-1}$ by dilution of stock solutions with 95 mM ammonium format - acetone -acetonitrile mixture (20:16:64, v/v/v).

Solutions for accuracy testing

The mixture of *Magnezijum 375 Plus B6*[®] capsule content (corresponding to 25 mg of MgO) and 25 mg of MgO was placed into a 25 mL volumetric flask and extracted with the 0.25% aqueous solution of formic acid in the ultrasonic bath for 20 minutes. The volumetric flask was filled up to the volume with the previously mentioned solvent. The obtained solution was filtered through Syringe Nylon Filters, 25 mm, with 0.45 μm pore size (Waters, Milford, USA). The solvent consisting of 95 mM ammonium format - acetone -acetonitrile mixture (20:16:64, v/v/v) was further used for dilution. Three series of three solutions corresponding to 80% (160 $\mu\text{g mL}^{-1}$), 100% (200 $\mu\text{g mL}^{-1}$) and 120% (240 $\mu\text{g mL}^{-1}$) were used for accuracy estimation.

The mixture of *Magnezijum 375 Plus B6*[®] capsule content (corresponding to 5 mg of pyridoxine HCl or thiamin HCl), 5 mg of thiamine HCl or 5 mg pyridoxine HCl was

placed into 25 mL volumetric flasks and extracted with the solvent acetonitrile–water (50:50, v/v) in the ultrasonic bath for 20 minutes. The volumetric flasks were filled up to the volume with the previously mentioned solvent. The obtained solutions were filtered through Syringe Nylon Filters, 25 mm, with 0.45 µm pore size (Waters, Milford, USA). The solvent consisting of 95 mM ammonium format - acetone -acetonitrile mixture (20:16:64, v/v/v) was further used for dilution. Three series of three solutions corresponding to 80% (64 µg mL⁻¹), 100% (80 µg mL⁻¹) and 120% (96 µg mL⁻¹) for each vitamin were used for accuracy estimation.

Solutions for precision testing

Magnezijum 375 Plus B6[®] capsule powder corresponding to 25 mg of MgO was weighed and placed into a 25 mL volumetric flask, extracted with the 0.25% aqueous solution of formic acid in the ultrasonic bath for 20 minutes. The volumetric flask was filled up to the volume with the previously mentioned solvent. The obtained solution was filtered through Syringe Nylon Filters, 25 mm, with 0.45 µm pore size (Waters, Milford, USA). Six working solutions were obtained from the stock solution, containing 200 µg mL⁻¹ of MgO, and were prepared in 95 mM ammonium format - acetone -acetonitrile mixture (20:16:64, v/v/v).

The capsule powder was weighed to attain 5 mg of pyridoxine HCl or thiamin HCl and was transferred into a 25 mL volumetric flask. The acetonitrile–water (50:50, v/v) solvent was used for extraction in the ultrasonic bath for 20 minutes. The volumetric flask was filled up to the volume with the same solvent, and then the solution was filtered through Syringe Nylon Filters, 25 mm, with 0.45 µm pore size (Waters, Milford, USA). From that stock solution, six solutions containing 80 µg mL⁻¹ of pyridoxine HCl and thiamin HCl were prepared in 95 mM ammonium format - acetone - acetonitrile mixture (20:16:64, v/v/v).

***The analysis of Magnezijum 375 Plus B6*[®] capsules**

Ten *Magnezijum 375 Plus B6*[®] capsule contents were pulverized. From the obtained powder, capsule content corresponding to 25 mg of MgO was weighed, placed into a 25 mL volumetric flask, and extracted with the 0.25% aqueous solution of formic acid using the ultrasonic bath for 20 minutes. The volumetric flask was filled up to the volume with the same solvent. Afterwards, the solution was filtered through Syringe Nylon Filters, 25 mm, with 0.45 µm pore size (Waters, Milford, USA) and used for the preparation of working solutions. Stock solution was diluted with 95 mM ammonium format - acetone - acetonitrile mixture (20:16:64, v/v/v), and six solutions containing 200 µg mL⁻¹ of MgO were obtained in this way.

Ten *Magnezijum 375 Plus B6*[®] capsule contents were pulverized, a quantity of capsule content corresponding to 5 mg of pyridoxine HCl or thiamin HCl was placed into a 25 mL volumetric flask and extracted with the acetonitrile–water mixture (50:50, v/v) using the ultrasonic bath for 20 minutes. The volumetric flask was filled to the mark with the same solvent, and the solution was filtered through Syringe Nylon Filters, 25 mm,

with 0.45 μm pore size (Waters, Milford, USA). Diluting that stock solution with 95 mM ammonium format - acetone - acetonitrile mixture (20:16:64, v/v/v), six solutions containing 80 $\mu\text{g mL}^{-1}$ of pyridoxine HCl and thiamin HCl were prepared.

Results and discussion

Within the registry of dietary supplements (13), various products comprising merely magnesium or its combination with the B complex vitamins are listed. Considering the growing dietary supplement commercial market and the safety concerns, quality and efficacy of these products, as well as a precise and accurate assay of active compounds in these products, are of utmost importance. Therefore, the main goal of this research was to develop the Hydrophilic Interaction Liquid Chromatography - Photodiode array - Charged Aerosol Detector (HILIC-PDA-CAD) method for the determination of magnesium, thiamine and pyridoxine in *Magnezijum 375 Plus B6* marketed dietary supplement following the Analytical Quality by Design (AQbD) principles.

In the first step, the influence of salt concentration in the aqueous part of the mobile phase (x_1), pH value of the aqueous part of the mobile phase (x_2) and salt type (x_3) were investigated by I-optimal experimental design towards retention behavior of the abovementioned analytes. These parameters were denoted as critical method parameters (CMPs) according to the QbD terminology. Under the experimental conditions presented in Table I, pyridoxine was eluting first, followed by chloride anion (Cl^-) and thiamine, while magnesium cation (Mg^{2+}) was the last eluting compound. Two critical peak pairs, pyridoxine / Cl^- and Cl^- / thiamine, were distinguished by CAD. Consequently, selectivity factors of the defined critical peak pairs ($\alpha_{\text{B6/Cl}}$, $\alpha_{\text{Cl/B1}}$) and Mg^{2+} retention factor (k_{Mg}) were originally adopted as critical method attributes (CMAs). However, the analysis of solutions for selectivity evaluation indicated co-elution of thiamine and lactose that is excipient with an intensive CAD response. Therefore, we decided to employ PDA for the thiamine determination at 220 nm, and to use Design Space (DS) computation for $\alpha_{\text{B6/Cl}}$ and k_{Mg} as the CMAs. The experimentally obtained CMA values are provided in Table I.

Logarithmic transformation of k_{Mg} and power transformation of $\alpha_{\text{B6/Cl}}$ preceded the mathematical model construction. For $\ln k_{\text{Mg}}$ and $\alpha_{\text{B6/Cl}}^{-2.89}$, a linear model with two factor interactions and quadratic model were obtained, respectively. The ANOVA test, lack-of-fit test and determination coefficients (R^2 , adjusted R^2 - adj. R^2 and predicted R^2 - pred. R^2) confirmed the adequacy of the obtained models. The mathematical model coefficients were calculated for coded factor values and their values, statistical significance, lack-of-fit, R^2 , adj. R^2 and pred. R^2 are presented in Table II. From the derived mathematical models for each level of qualitative factor salt type (ammonium format (AF) - lower level, ammonium acetate (AA) - upper level), separate mathematical models were computed. The equations 1 - 2 and 3 - 4 relate CMPs and CMSs when AF and AA are used, respectively.

$$\alpha_{\text{B6/Cl}}^{-2.89} = 0,0312 - 0,0024 x_1 - 0,0016 x_2 + 0,0044 x_1 x_2 - 0,0133 x_1^2 \quad (1)$$

$$\ln k_{\text{Mg}} = 3,2668 - 0,7278 x_1 + 0,3003 x_2 \quad (2)$$

$$\alpha_{B6/Cl}^{-2,89} = 0,0493 - 0,0139 x_1 + 0,0165 x_2 + 0,0044 x_1 x_2 - 0,0133 x_1^2 \quad (3)$$

$$\ln k_{Mg} = 3,7637 - 1,2150 x_1 + 1,1376 x_2 \quad (4)$$

Table II Coefficients of estimated models calculated for coded factor values and their statistical significance

Tabela II Koeficijenti modela izračunati za kodirane vrednosti faktora i statistički značajni parametri dobijeni analizom varijanse

	$\alpha_{B6/Cl}^{-2,89}$		$\ln k_{Mg}$	
	coefficient	p^*	coefficient	p^*
b_0	0.0403	-	3.5152	-
b_1	-0.0082	0.0005*	-0.9714	< 0.0001*
b_2	0.0074	0.0009*	0.7190	< 0.0001*
b_3	0.0091	< 0.0001*	0.2485	0.0007*
b_1b_2	0.0044	0.0842	/	/
b_1b_3	-0.0058	0.0048*	-0.2436	0.0074*
b_2b_3	0.0091	0.0002*	0.4186	0.0001*
b_1^2	-0.0133	0.0002*	/	/
b_2^2	/	/	/	/
R^2	0.9469		0.9549	
$adj. R^2$	0.9098		0.9361	
$pred. R^2$	0.7921		0.8893	

* statistically significant coefficients for $p < 0.05$

b_0 is the intercept. b_1 , b_2 and b_3 are the coefficients of main effect terms x_1 , x_2 and x_3 respectively, b_{12} , b_{13} and b_{23} are coefficients of the interaction terms. b_1^2 , b_2^2 and b_3^2 are coefficients of quadratic terms

k_{Mg} , $\alpha_{B6/Cl}$ - same as in Table I caption

The negative sign of the coefficients corresponding to x_1 suggests a decrease in both the separation of the critical peak pair and retention of Mg^{2+} , with an increase in salt concentration in the aqueous phase. Conversely, the positive sign of the coefficients corresponding to x_2 indicates the opposite effect of pH of the aqueous phase, leading to a prolonged chromatographic run and better separation at higher pH values. The absolute values of the calculated coefficients suggest that the influence of salt concentration in the aqueous phase is more profound on both k_{Mg} and $\alpha_{B6/Cl}$ compared to the pH of the aqueous phase.

For the computation of 2D-DS, the limits were set for the selected CMAs $\alpha_{B6/Cl} > 3.5$ and $k_{Mg} < 4$ with the probability $\pi=95\%$. Experimental space was gridded by the factor value discretization in the following manner: the salt concentration [50:2.5:100] and the pH value [4.25:0.075:5.75].

In the next step, Monte Carlo simulations with 5000 iterations in each of 21 points for salt concentration \times 21 points for pH of the aqueous phase = 441 grid points were performed. The distribution of $\alpha_{B6/Cl}$ and k_{Mg} for each grid point was obtained by the adding of the uniform error distribution equal to the calculated standard error to the model coefficient estimates. Eventually, the region of experimental domain, comprising the values of $\alpha_{B6/Cl}$ and k_{Mg} that met the defined criteria with the desired quality level ($\pi \geq 95\%$), was determined and illustrated in Figure 1A for AF and Figure 1B for AA. From the results obtained for AF, a larger DS was constructed covering a wider range of chromatographic conditions, and it was used for working point selection. CMPs corresponding to working point were 95 mM AF in the aqueous phase with pH adjusted at 4.4. A verification experiment was conducted and the experimentally obtained CMAs were $\alpha_{B6/Cl} = 3.74$ и $k_{Mg} = 2.36$. The corresponding chromatogram is given in Figure 2.

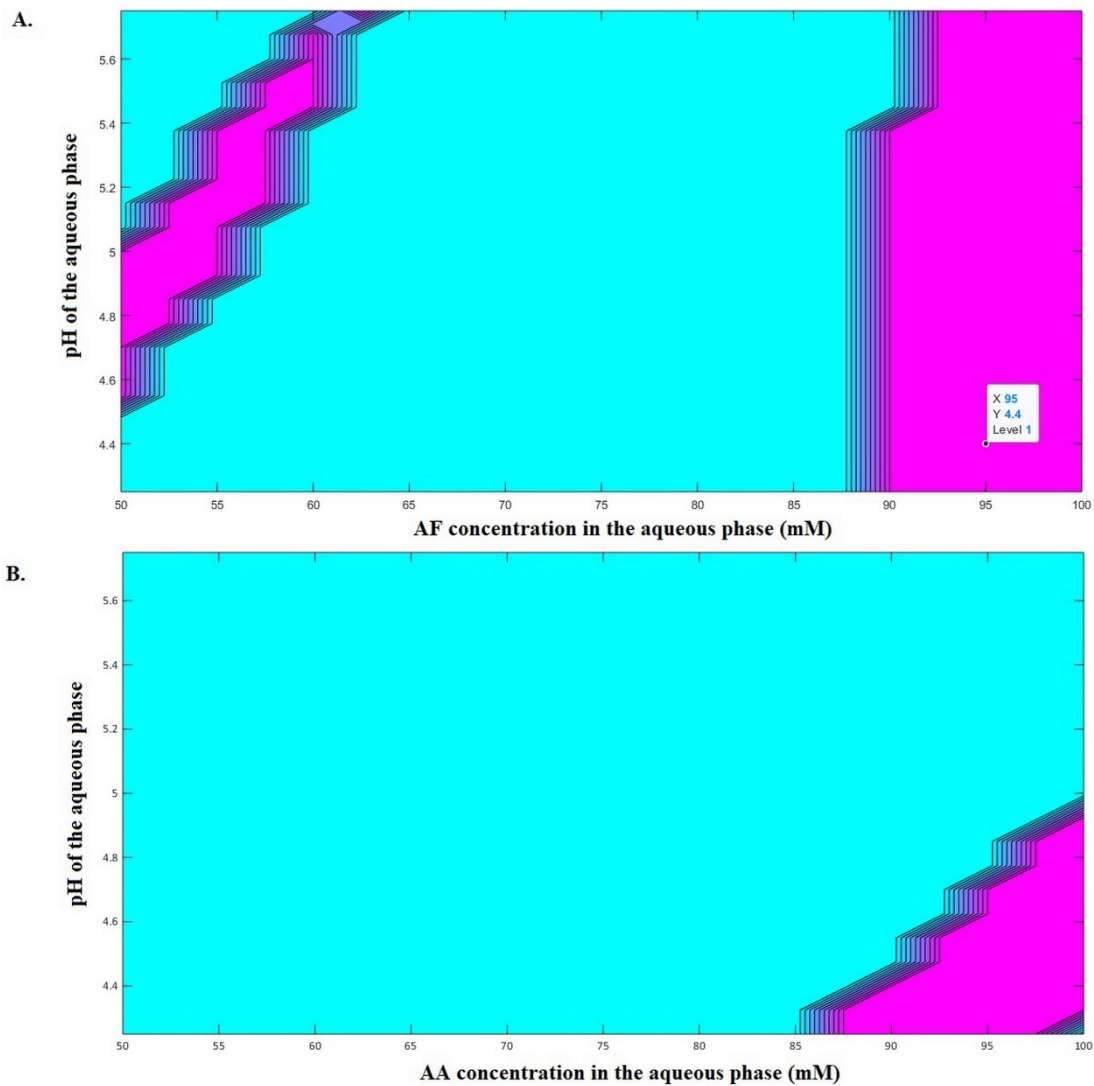


Figure 1. 2D representation of DS for predefined CMAs ($\alpha_{B6/Cl} > 3.5$ and $k_{Mg} < 4$) achieved with probability $\pi \geq 95\%$. The pink part represents the region of design space where the working point should be selected from.

A. AF in the aqueous part of mobile phase, B. AA in the aqueous part of mobile phase

Slika 1. 2D prikaz DS za utvrđene CMAs ($\alpha_{B6/Cl} > 3,5$ and $k_{Mg} < 4$) konstruisan za verovatnoću $\pi \geq 95\%$. Region roze boje odgovara delu dizajna prostora iz koga treba odabrati radnu tačku.

A. AF u vodenoj fazi, B. AA u vodenoj fazi

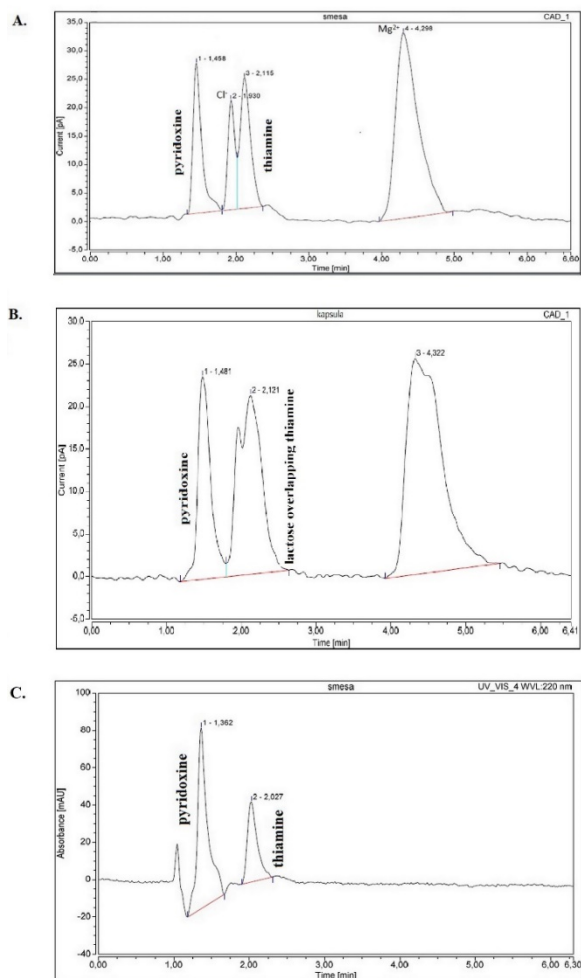


Figure 2. A. CAD chromatogram of mixture containing 200 µg/mL of *MgO* and 100 µg mL⁻¹ of both vitamins: *MgO* (*tr* = 4.298 min.), pyridoxine (*tr* = 1.458 min) and thiamine (*tr* = 2.115 min), B. CAD chromatogram of sample solution containing 80 µg mL⁻¹ of both vitamins *MgO* (*tr* = 4.322 min.), pyridoxine (*tr* = 1.481 min) and lactose overlapping thiamine (*tr* = 2.121 min) and C. UV chromatogram of mixture containing 100 µg mL⁻¹ of both vitamins at 220 nm: pyridoxine (*tr* = 1.362 min) and thiamine (*tr* = 2.027 min) recorded under conditions given by the working point: 95 mM AF in the aqueous phase with pH adjusted at 4.4

Slika 2. A. CAD hromatogram smeše koja sadrži 200 µg/mL of *MgO* i 100 µg mL⁻¹ oba vitamina: *MgO* (*tr* = 4,298 min.), piridoksin (*tr* = 1,458 min) i tiamin (*tr* = 2,115 min), B. CAD hromatogram rastvora uzorka koji sadrži 80 µg mL⁻¹ oba vitamina *MgO* (*tr* = 4,322 min.), piridoksin (*tr* = 1,481 min) i laktoza koeluirana sa tiaminom (*tr* = 2,121 min) i C. UV hromatogram smeše koja sadrži 100 µg mL⁻¹ oba vitamina na 220 nm: piridoksin (*tr* = 1,362 min) i tiamin (*tr* = 2,027 min) snimljeni pod uslovima definisanim radnom tačkom: 95 mM AF u vodenoj fazi čija je pH vrednost podešena na 4.4

Finally, the one-factor-at-a-time approach was applied to the maximization of pyridoxine and Mg^{2+} CAD response. The influence of evaporation temperature was evaluated at 35°C and 50°C level and it led to the conclusion that the higher temperature provides a more intense response. Experiments were also conducted at various levels of filter constant (0,1 s, 5 s and 10 s), gas pressure (40 psi, 50 psi and 60 psi) and data collection rate (1 Hz, 10 Hz and 100 Hz) as well. The response increases with the rise in gas pressure, base line is smoother at higher values of filter constant, while the change of data collection rate from 10 Hz to 100 Hz did not lead to improvement of peak shape or enlargement of the peak area. Eventually, CAD parameters were set at the following values: evaporation temperature 50°C, gas pressure 60 psi, data collection rate 10 Hz and filter constant 10 s.

Method performance qualification was performed by testing the suitability of the developed method via selectivity, linearity, accuracy and precision evaluation. The exact list of excipients in *Magnezijum 375 Plus B6*[®] was not available, since the manufacturer only vaguely reported the presence of an anti-caking agent. Therefore, for the purpose of selectivity testing, the influence of silicon dioxide was investigated (colloidal silicon dioxide or colloidal silica) because it is frequently used in solid dosage forms as an anti-caking agent, adsorbent, disintegrant or glidant to allow powder to flow freely when tablets and capsules are processed. Furthermore, anhydrous lactose, which is widely used as a filler or diluent in tablets and capsules, was also tested. The interference of colloidal silica was not evidenced with either CAD or PDA. On the other hand, the lactose had an intensive CAD response and co-eluted with thiamine, while no interference was observed with PDA detection at 220 nm. The values for calculated regression parameters and recovery values (Table III) are in agreement with the criteria for linearity and accuracy, respectively (14,15). For an estimation of precision, RSDs were calculated: MgO (1.22%), thiamine (1.79%) and pyridoxine (1.70%) and the obtained values met the defined criteria of RSD < 2% for active ingredients (14, 15). The content of MgO in the analyzed capsules was 108.5% (406.87 mg/ caps), that of thiamine hydrochloride 75.07% (0.83 mg/ caps) and pyridoxine hydrochloride 101.98% (1.43 mg/ caps).

Table III Validation parameters: linearity and accuracy of the proposed HILIC-PDA-CAD method**Tabela III** Validacioni parametri: linearnost i tačnost predložene HILIC-PDA-CAD metode

Substance	Linearity			Accuracy		
	Concentration range ($\mu\text{g mL}^{-1}$)	a	b	r	Concentration level ($\mu\text{g mL}^{-1}$)	Recovery (%)
<i>MgO</i>	100 – 300	102.8	7142.8	0.9995	160	99.24
					200	98.98
					240	100.01
Pyridoxine HCl	40 – 120	31.465	303.32	0.9981	64	100.50
					80	101.47
					96	99.68
Thiamine HCl	40 – 120	66.194	748.62	0.9987	64	100.09
					80	101.21
					96	99.27

a – slope, b – intercept, r – correlation coefficient (acceptance value >0.9900)

Conclusion

The Analytical Quality by Design approach was selected as the most appropriate in the development of the HILIC-PDA-CAD method for determination of magnesium, pyridoxine and thiamine in a dietary supplement. The organic modifier mixture comprising acetonitrile and acetone was selected as the most suitable while CMPs (salt type, concentration of the salt in the aqueous part of the mobile phase and pH value of the mobile phase) were optimised by the I-optimal experimental design. CMAs were the two responses distinguished as the most relevant ($\alpha_{B6/Cl}$, k_{Mg}). Mathematical models were obtained for each response, and their validity was confirmed according to ANOVA test, lack-of-fit and determination coefficients values. The 2D-DS plots were constructed for CMAs $\alpha_{B6/Cl} > 3.5$ and $k_{Mg} < 4$ with the probability $\pi=95\%$. The grid point search and Monte Carlo simulations with 5000 iterations contributed to optimal condition selection according to the defined criteria. The selected working point included 95 mM AF as part of the aqueous phase, while the pH value was set to 4.4. The CAD parameters that complied with the best responsiveness of pyridoxine and Mg^{2+} involved: evaporation temperature 50°C, gas pressure 60 psi, data collection rate 10 Hz and filter constant 10 s. The determination of thiamine by the HILIC-CAD method was prevented due to lactose signal interference. This was resolved employing PDA detection at 220 nm, giving consideration to chromophores lacking lactose structure, resulting in no response generation in the UV wavelength range. The developed method met all the defined validation criteria set by the ICH Q2(R1).

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Razvoj HILIC-PDA-CAD metode za određivanje sadržaja magnezijuma, piridoksina i tiamina u dijetetskom suplementu primenom metodologije ugradnje kvaliteta u analitičku metodu

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Kratak sadržaj

U ovom istraživanju korišćena je metodologija ugradnje kvaliteta u metodu (AQbD) u cilju razvoja HPLC-PDA-CAD metode za određivanje magnezijuma, piridoksina i tiamina u dijetetskom suplementu. Eksperimenti su izvedeni na Dionex Ultimate 3000 HPLC sistemu sa PDA i CAD detektorima. Razdvajanje je vršeno izokratskim eluiranjem mobilnom fazom koja je smeša acetonitrila, acetona i vodenog rastvora amonijum acetata/amonijum formijata (48:12:40, V/V/V) na ZIC-HILIC PEEK koloni (100 × 4.6 mm, 5 µm). Talasna dužina detekcije bila je 220 nm. Optimalna podešavanja CAD su definisana jednofaktorskim pristupom i obuhvaju: temperaturu isparavanja 50°C, filter konstantu 10 s, pritisak gasa 60 psi. Uticaj kvalitativnih (vrsta soli) i kvantitativnih faktora (pH vrednost i koncentracija soli) na retencioni faktor magnezijuma (k_{Mg}) i faktor selektivnosti piridoksina i hloridnog anjona ($\alpha_{B6/Cl}$) ispitan je primenom *I*-optimizacionog dizajna. Prostor dizajna je definisan Monte Karlo simulacijom i propagiranjem greške koeficijenata dobijenih matematičkih modela sa ciljem definisanja hromatografskih uslova kod kojih su zadovoljeni sledeći kriterijumi: $k_{Mg} < 4$ i $\alpha_{B6/Cl} > 3,5$, sa verovatnoćom $\pi = 95\%$. Iz dobijenog 2D-grafika prostora dizajna izabran je optimalni sastav vodene faze koji je obuhvatao 95 mM amonijum formijat čija je pH vrednost podešena na 4,4. Metoda je validirana i dokazana je njena pouzdanost u rutinskoj primeni.

Ključne reči: HILIC-PDA-CAD, magnezijum, piridoksin, tiamin, AQbD
