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Spectrophotometric Determination of Acidity Constants of Group B Vitamins in Different Ionic Strengths at 25±0.1 °C

J. Ghasemi[†,#*], S. Ghobadi[‡], B. Abbasi[†], and M. Kubista[‡,§]
[†]*Department of Chemistry, Faculty of Science, Razi University, Kermanshah, Iran*
[‡]*Department of Biology, Faculty of Science, Razi University, Kermanshah, Iran*
[#]*Department of Chemistry and Biosciences, Chalmers University of Technology, Gothenburg, Sweden*
[§] *MultiD Analyses AB, Gothenburg, Sweden*
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J. Ghasemi[†,#*], S. Ghobadi[‡], B. Abbasi[†], and M. Kubista[‡,§]
[†]*Department of Chemistry, Faculty of Science, Razi University, Kermanshah, Iran*
[‡]*Department of Biology, Faculty of Science, Razi University, Kermanshah, Iran*
[#]*Department of Chemistry and Biosciences, Chalmers University of Technology, Gothenburg, Sweden*
[§] *MultiD Analyses AB, Gothenburg, Sweden*
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요 약. 25 °C±0.1에서 이온세기를 달리하면서 네 가지 수용성 비타민 - 엽산(비타민 B_9 또는 B_o), 티아민(비타민 B_1), 리보플라빈(비타민 B_2) 및 피리독신(비타민 B_6)의 겉보기 산해리상수를 분광도법으로 결정하였다. 다른 이온세기에서 산도상수를 결정하기 위하여, 계량화학 개념에 기초한 정교하고 정교한 방법을 분광학적 적정 데이터에 적용하였다. 다른 이온세기에서 225-500 nm 영역에서 스펙트럼을 기록하였다. 다양한 이온세기에서 pH-흡광도 데이터를 일-, 이- 및 삼양성자 약산의 질량균형방정식에 컴퓨터 핏팅하는 방법을 이용하여 모든 비타민의 산도 상수를 계산하였다. 컴퓨터 프로그램 DATAN을 사용하여 스펙트럼 데이터로부터 원하는 정보를 추출하였다. 핏팅 과정에서 산도 상수, 순수한 형태의 스펙트럼 프로파일, 본포 도표 및 기타 인자분석 데이터를 얻었다. 산도상수에 대한 이온세기의 영향을 고찰하였다.

주제어: Vitamin B Group, Acidity Constants, DATAN, Distribution Diagrams, pK_a Values, Ionic Strength

ABSTRACT. The apparent acid dissociation constants of four water-soluble vitamins, folic acid (vitamin B_9 or B_o), thiamine (vitamin B_1), riboflavin (vitamin B_2) and pyridoxal (vitamin B_6) were determined spectrophotometrically in different ionic strengths at 25 °C±0.1. An accurate and sophisticated method based on chemometrical concepts was applied in order to determine acidity constants at different ionic strengths. For this purpose, spectral titration data were used. The spectra were recorded in the range 225-500 nm at different ionic strengths. The acidity constants of all vitamins at various ionic strengths were calculated by means of computer fitting of the pH-absorbance data with appropriate mass balance equations according to mono-, di- or triprotic acids. The computer program DATAN was used to extract the desired information from the spectral data. The outputs of the fitting processes were acidity constants, spectral profiles of pure forms, distribution diagrams, and other factor analysis data. The effect of ionic strength on the acidity constants is discussed.

Keywords: Vitamin B Group, Acidity Constants, DATAN, Distribution Diagrams, pK_a Values, Ionic Strength

INTRODUCTION

Acid dissociation constants are useful physico-chemical parameters describing the extent of ionization of functional groups with respect to pH. These parameters are important in research areas such as pharmaceutical drug development, where knowledge of the ionization state of a particular functional group often is crucial in order to understand its pharmacokinetics and pharmacodynamics.[1]

B vitamins form a wide organic-compound group that cannot be synthesized by humans. Since these compounds are necessary for the tropism of human beings, they need to be part of our daily intake. Vitamin B_1 (Thiamine), vitamin B_2 (Riboflavin), vitamin B_6 (Pyridoxal) and vitamin B_0 (folic acid) occur in living cells as essential substances for growth. Any deficiency of these in human nutrition will have adverse effects. Therefore, B vitamins are often supplemented to the diet as composite vitamin B tablets.[2]

Folic acid, also known as vitamin B_0, is also referred to as folacin or folate, while its chemical name is pteroylglutamic acid.[3] Folic acid is required for DNA synthesis and cell growth to take place, and it is important for the formation of red blood cells, for energy production, for the formation of amino acids. It is also required in protein metabolism and in treating folic acid anemia.[4] Deficiency of folic acid is a common nutritional problem of worldwide importance. Long-term deficiency may result in anemia and later in osteoporosis, as well as cancer of the bowel and cervix.[5]

Thiamine, also called vitamin B_1, assits in a great many bodily functions. It plays a key metabolic role in the cellular production of energy, primarily in glucose metabolism.[6-7] Thiamine is unstable at high pHs,[8-9] and in food it degrades by cooking of under mildly basic conditions.[10] Deficiency of vitamin B_1 leads to beriberi,[11] a nutritional disease characterized by degenerative changes in the nervous system, including multiple peripheral neuritis; accompanied by generalized edema and serous effusions, with a tendency to cardiac hypertrophy and dilation.[11]

Riboflavin (vitamin B_2) is the prosthetic group of flavin enzymes, which are of great importance in general metabolism and particularly in metabolism of proteins. Vitamin B_2 is required for the health of the mucous membranes in the digestive tract and aidsin the absorption of iron and vitamin B_6.[3] It is needed especially during periods of rapid growth, but also when protein intake is high. It is highly beneficial to the skin, hair and nails.[3] A shortage of this vitamin may manifest itself as cracks and sores at the corners of the mouth, eye disorders, inflammation of the mouth and tongue, and skin lesions.[6]

Vitamin B_6 (pyridoxal) is a common cofactor in enzymes that support amino acids metabolism. It controls the absorbtion, metabolism and conversion of amino acids into neurotransmitters, antibodies, digestive enzymes, muscles and tissues in the body.[12] Deficiency of vitamin B_6 may make patients prone to nerve or blood disorders, and may cause convulsions in children.[13]

In the present work, the protonation constants of vitamin B group in different concentrations of KNO_3 were determined spectrophotometrically at 25 °C. The investigation of ionic strength influence on the acid-base behavior of simple organic compounds may contribute to a better understanding of the properties of complex substances such as natural organic matters.

THEORY

The theory and application of the physical constraints method have been thoroughly discussed by Kubista *et al.*[14-22] In the following, the general principal will be outlined briefly.

Spectra of each vitamin at different pH values and at different ionic strengths are digitized and arranged in a data matrix $\boldsymbol{A}$, which is decomposed into an orthonormal basis set by NIPALS or any equivalent method:[14]

$$\mathbf{A} = \mathbf{TP}' + \mathbf{E} \approx \mathbf{TP}' = \sum_{i}^{r} t_i p_i' \qquad (1)$$

The orthogonal target vectors t_i and orthonormal projection vectors p_i' are mathematical constructs that cannot be directly related to component spectra

and concentrations. The symbol r represents the number of independent spectroscopic components, which corresponds to the number of light-absorbing chemical species. It is determined by visual inspection of the t and p' vectors, or by applying statistical methods, such as the χ2-test.[23-25] E is an error matrix.

By assuming linear responses, the spectra in matrix A are linear combinations of the concentrations, C, and spectral responses, V, of the chemical components.

$$\mathbf{A} = \mathbf{CV} + \mathbf{E} \approx \mathbf{CV} \quad (2)$$

If the spectral profiles of the components are known, the concentration of each component can be calculated easily, for example, by least squares minimization. If standards are not available, it is generally assumed that the components' spectral responses cannot be separated, which precludes their identification. This is due to ambiguity in determining the rotation matrix, $\boldsymbol{R}$, in the following equations; from Eqs. (1) and (2) follows that there is a square matrix $\boldsymbol{R}$ ($r \times r$) that satisfies

$$\boldsymbol{T} = \boldsymbol{CR} \quad (3)$$

$$\boldsymbol{P'} = \boldsymbol{R}^{-1}\boldsymbol{V} \quad (4)$$

scince $\boldsymbol{A}=\boldsymbol{CV}=\boldsymbol{C}(\boldsymbol{RR}^{-1})\boldsymbol{V}=\boldsymbol{CR}(\boldsymbol{R}^{-1}\boldsymbol{V})=\boldsymbol{TP'}$. If $\boldsymbol{R}$ can be determined, the spectral responses $\boldsymbol{V}$ and concentrations $\boldsymbol{C}$ of the components can be calculated from the target $\boldsymbol{T}$ and projection $\boldsymbol{P'}$ matrices:

$$\boldsymbol{C}=\boldsymbol{TR}^{-1} \quad (5)$$

$$\boldsymbol{V}=\boldsymbol{RP'} \quad (6)$$

The thermodynamic expressions that describes the concentration of the components is the main constraint used to determine $\boldsymbol{R}$, from which thermodynamic parameters, acidity constants, and components spectral responses and concentrations of all species are calculated. So, according to these facts, the strategy for determining the rotation matrix $\boldsymbol{R}$ is as follows. The concentrations of the chemical species are calculated, using equilibrium expressions and various trial values of the acidity constants, and fitted to the calculated target vectors according to Eq. (3). The accuracy of this fiting depends crucially on the trial values of the acidity constants, and the best fit determines their values and the elements of matrix $\boldsymbol{R}$.

EXPERIMENTAL

Reagents

All the chemicals were of analytical reagent grade. Four given solutions(as working solutions, folic acid, thiamine, riboflavin and pyridoxal) were prepared in 100 mL volumetric flasks by dissolving 2.00, 2.00, 2.50 and 3.00 mg of each compound in water, respectively, and the solutions were used for pH titration. Titration of each vitamin was carried out at five fixed ionic strengths with NaOH solution. The starting points of pH titrations were pH 2.00, which were set using concentrated solutions of HCl and NaOH. The concentrated NaOH solution was also used for titrations, to avoid dilution of the working solutions.

To maintain the ionic strencgth at a desired value a high concentrated solution of KNO_3 was used for all titrations. All experiments were carried out at 25 ºC. For all of the above-mentioned solutions, doubly distilled water used throughout and the solutions were kept in brown volumetric flasks to protect from light.

Apparatus and software

The pH values were measured by model 300 HANA pH-meter using a combined glass electrode. The glass electrode was calibrated on the basis of the proton concentration at each constant ionic strength according to the procedure described elsewhere.[26] The calibration was repeated at each ionic strength. The calibration procedure was as recommended by the IUPAC for glass electrodes.[27]

Absorption spectra were measured on an Agilent 8453 UV-Visible Diode-Array spectrophometer using the Agilent UV-Visisble ChemStation Software for data acquisition. A cell of 10 mm optical path was used for all measurements.

The data were preprocessed using MATLAB software, version 6.5 (Mathworks, Natick, USA) and the deconvolution of the obtained data matrix

was performed using DATAN version 3.1.

RESULTS AND DISCUSSION

The electronic absorption spectra of group B vitamins were recorded in different ionic strengths and at various pH values. Sample spectra of each vitamin at different pH values and at five ionic strengths are shown in *Figs*. 1-4. The principal component analysis of all absorption data matrices obtained at various pH values shows the different number of factors for each vitamin. The number of factors could be attributed to the number of dissociation equilibria of each vitamin. The pK_a values of group B vitamins were investigated spectrophotometrically at 25 °C in five different ionic strengths. The acidity constants of these vitamins in several ionic strengths were evaluted by the DATAN program using the corresponding spectral absorption-pH data. From inspection of the experimantal spectra, it is hard to guess even the number of protolytic species involved. The number of calculated projection vectors with clear spectral features, as compared to noise, shows the presence of four, three, two and three spectroscopically distinguishable components for folic acid, thiamine, riboflavin and pyridoxal, respectively.

The output of DATAN comprises pK_a values, the number of principal components, projection vectors(loadings), diagrams of the concentration distribution, and the spectrum of each assumed species. The obtained pK_a values are listed in *Table* 1. The pKa values correspond to the pH dependent variation of absorption spectra in all ionic strengths. One of the most important outputs of the program is the calculated spectrum of different forms of each vitamin at each ionic strength. The most important features of the distribution diagrams are the pH limits of the evolving and disappearing of components. Some typical distribution diagrams are shown in *Fig*. 5.

Consider the cationic form of pyridoxal (*Scheme* 1), which has two dissociable protons bound to distinctly different sites, the phenolic oxygen and the ring nitrogen.

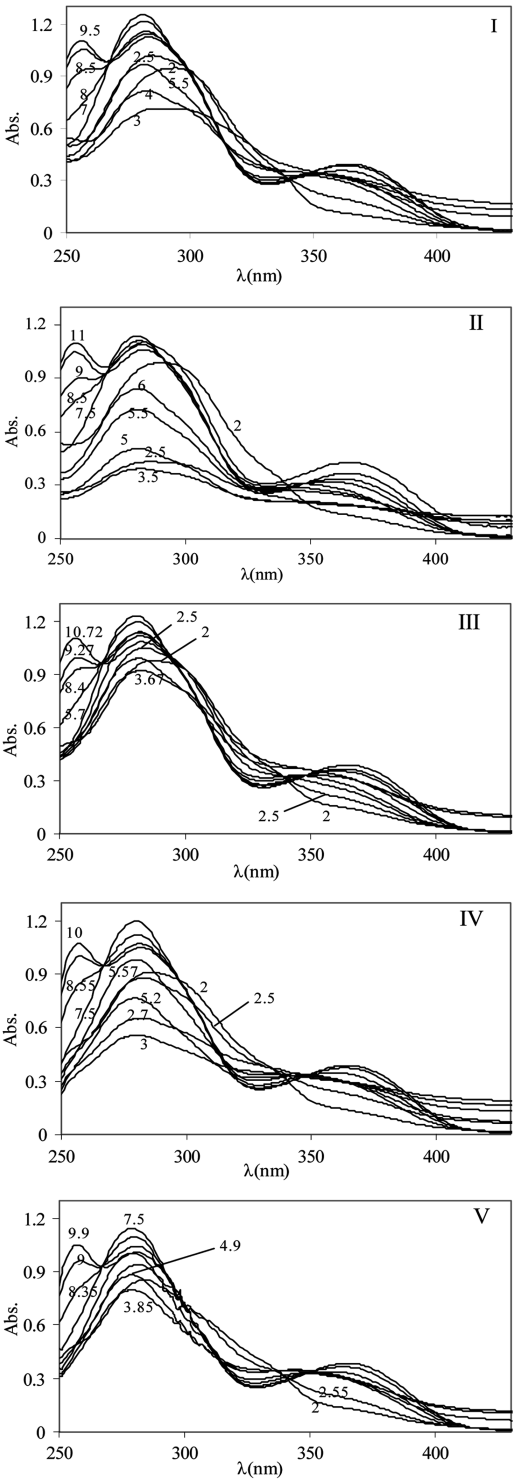


Fig. 1. Absorption spectra of Folic Acid, in different concentrations of KNO_3: (I) 0.00, (II) 0.01, (III) 0.05, (IV) 0.10, (V) 0.30.

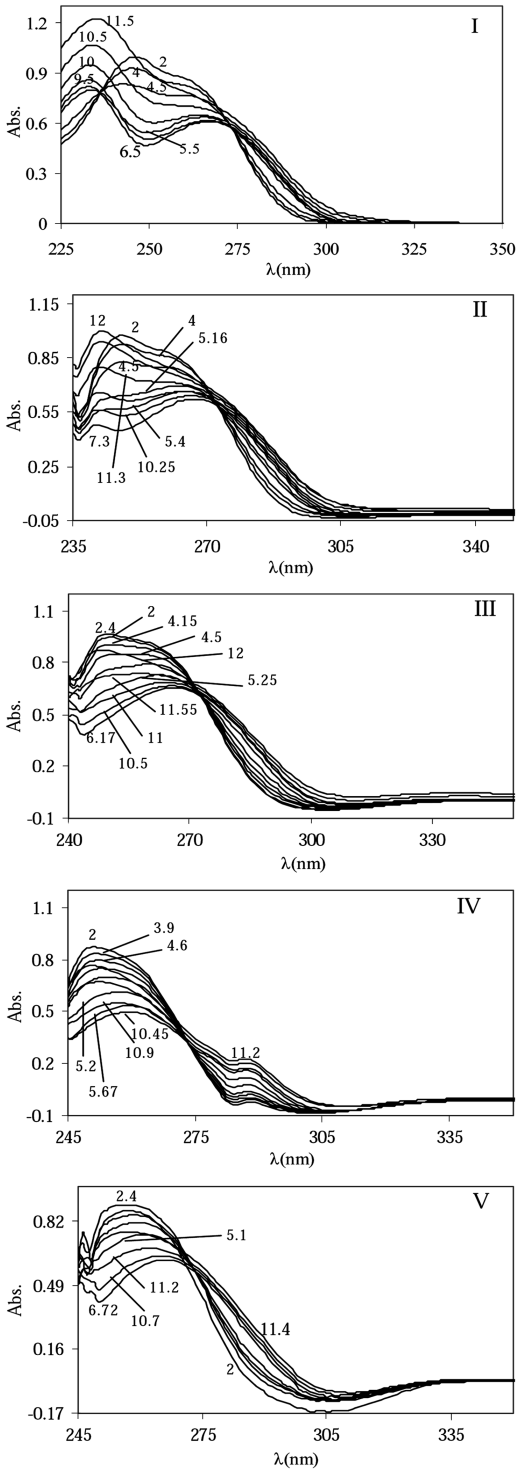


Fig. 2. Absorption spectra of Thiamine in different concentrations of KNO_3: (I) 0.00, (II) 0.01, (III) 0.05, (IV) 0.10, (V) 0.30.

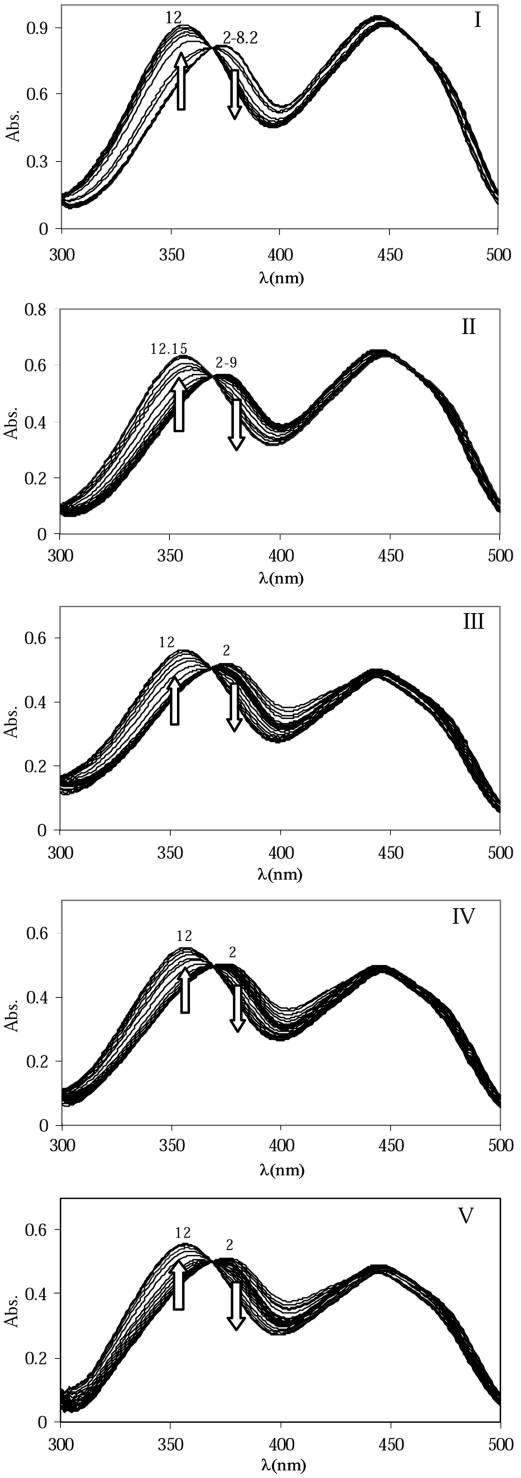


Fig. 3. Absorption spectra of Riboflavin in different concentrations of KNO_3: (I) 0.00, (II) 0.01, (III) 0.05, (IV) 0.10, (V) 0.30.

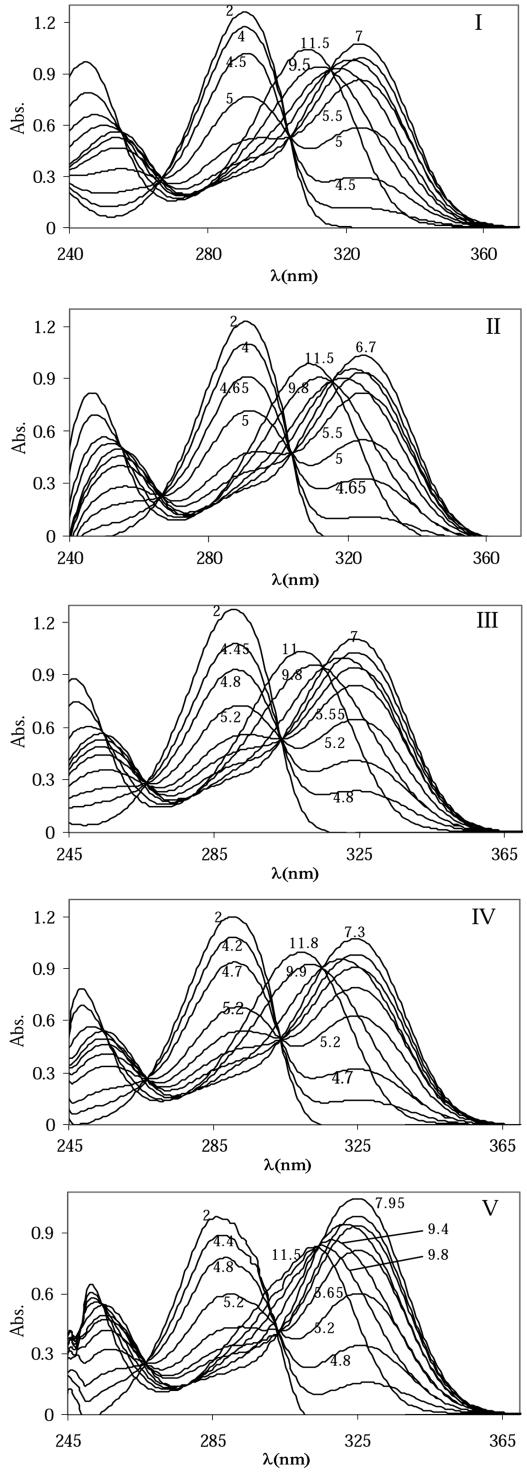


Fig. 4. Absorption spectra of Pyridoxal, in different concentrations of KNO_3: (I) 0.00, (II) 0.01, (III) 0.05, (IV) 0.10, (V) 0.30.

Either of two protons could dissociate first as the pH is raised. However, the two microscopic dissociation constants are distinctly different.[28] At 25 ºC in the neutral (monoprotonated) form 80% of the molecules carry a proton on the N, while the remaining 20% are protonated on the less basic-O^-.[28] The obtanied pK_1 and pK_2 by computer fitting of spectral data are listed in *Table* 1. The previously reported values of pK_1 and pK_2 at pure water are 4.64 and 8.89, respectively.[23]

As it is clear from *Table* 1, the pK_a's are dependent on the ionic strength and this in turn is due to the dependence of the activity factors on the ionic strength. The ionic strength due to vitamin and also buffer solution constituents is negligible, so essentially all ionic strength is due to KCl salt. This clearly shows that the reported values and the obtained values are more or less the same, within the experimental and instrumental errors.

Riboflavin consists of a hetrocyclic isoalloxazine ring attached to the sugar alcohol, ribitol. It is stable to heat but extremely sensitive to light. One of the products of photolysis is lumichrome.[28] Freshly prepared solution of this vitamin was used as a titration solution to determine the corresponding acidity constant, avoiding thereby the photolysis of riboflavin. As is clear from the structural scheme of riboflavin (*Scheme* 2), which has a similar group to phthalimide, it has a dissociable proton bound to the ring nitrogen. The pK_a value obtained in this work (*Table* 1) for the similar functional group is between 9.5 and 11,[29] and the previously reported pK_a value for riboflavin was 10.2. Since the change of ionic strength is associated with change of pK_a values[30] in the case of riboflavin. These variations are small and probably a more sensitive probe such as fluorescence spectroscopy is needed to determine the dependence of the pK_a's on the ionic strength.

The weakly basic portion of thiamine (*Scheme* 3) or of its coenzyme form is protonated at low pH, largely on N-1 of the pyrimidine ring.[31-33] The reported pK_a value is ~4.9.[28]

The hydrogen atom in the 2-position of the thiazolium ring, between the sulfur and the nitrogen atoms, dissociates as H^+ during catalysis and the

Table 1. The acidity constants of vitamins at different ionic strengths at 25 °C

KNO_3(M)	Pyridoxal		Riboflavin	Thiamine		Folic acid		
	pK_{a1}	pK_{a2}	pK_{a1}	pK_{a1}	pK_{a2}	pK_{a1}	pK_{a2}	pK_{a3}
0.00	4.92	9.19	10.64	4.76	10.30	2.46	5.47	8.28
0.01	4.96	9.42	10.64	4.98	11.05	2.63	5.76	8.78
0.05	5.05	9.46	10.62	5.05	11.41	3.56	4.88	8.69
0.10	5.06	9.45	10.58	5.07	11.04	2.40	5.35	8.48
0.30	5.12	9.44	10.57	5.23	11.29	3.23	5.13	8.39

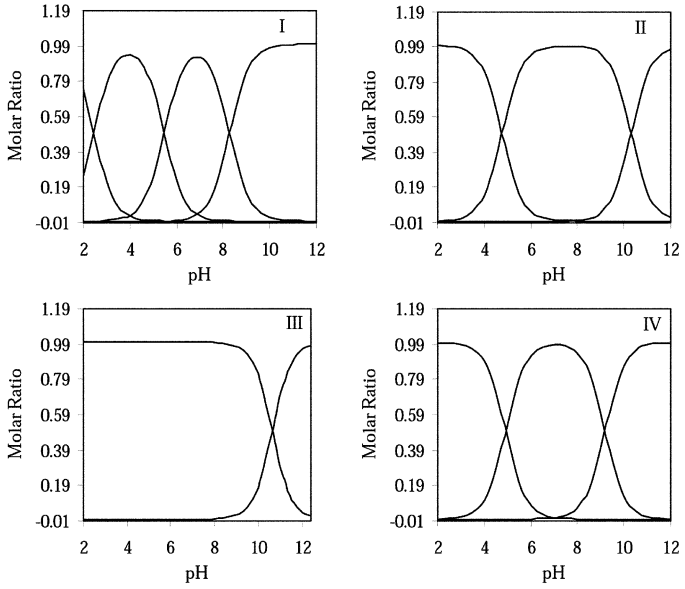
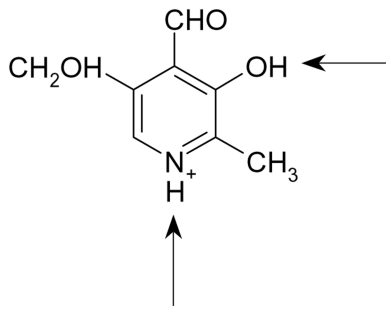
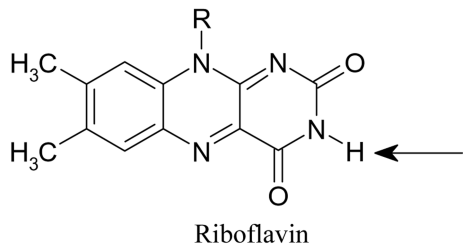


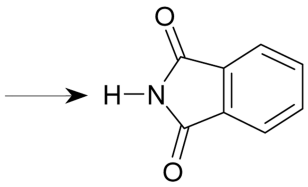
Fig. 5. Concentration distribution diagrams of (I) Folic Acid, (II) Thiamine, (III) Riboflavin and (IV) Pyridoxal, in aqueous solution at 25 °C and zero ionic strength.



Scheme 1.



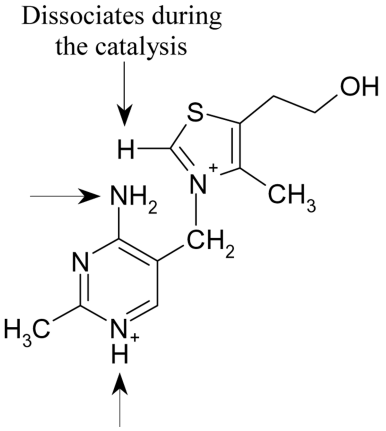
Riboflavin



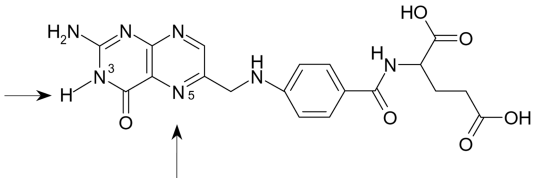
Phthalimide

Scheme 2.

pK_a value of this proton has been estimated as ~18, which means that it cannot be as a acidic proton.[34] The portion that can be protonated next to the NH_2 group is the pyrimidine ring. The pK_{a2} value that we obtained in this report (*Table* 1) is comparable with



Scheme 3.



Scheme 4.

previously reported values.[28]

The pK_{a1} and pK_{a2} values have shown a fair dependence to ionic strength. As it can be seen from *Table* 1 the higher ionic strength the higher pK_a values.

The folic acid, as shown in *Scheme* 4, has a complicated structure and allocates the obtained acidity constants to specific groups. The previously reported pK_a values are 4.82 (related to N-5 site) and 10.5 (related to N-3 site, transfering from O-position to N-position during tautomerism).[28]

Three pK_a values were obtained, which are listed in *Table* 1. As the structural scheme shows, folic acid possesses two carboxylic groups apart from the two acidic positions, as discussed above. It can be assumed that the obtained pK_{a1} value relates to one of the two carboxylic groups, and the other two pK_a values can be compared with reported values, 4.82 and 10.5 for pK_{a1} and pK_{a2}, respectively.[23] It is surprising, to note that, the dependence of the pK_a's values of the folic acid on the ionic strength did not show a regular pattern. This, unlikely, is related to the no systematic changing of the absorption spectra in the course of titration, *Fig*. 1. And in turn these irregularities return to this fact that folic acid solution has not enough stability and as it cleared by the manufacturer companies, the solution and the pure compound must be kept in cool and dark place.

As shown by *Table* 1 and *Figs*. 1-4, changes in ionic strengths have more or less observable effects on the spectral data of the four vitamins, which means that the acidity constants for pK_{a1} and pK_{a2} change mildly by changing ionic strength. Of course, they show some irregular variations, which may be due to experimental and instrumental errors in some cases. So, variation in ionic strength which have some effects on the acidity constants of these vitamins can influence on the ionic state of these compounds as well as on the functionality of the enzymes which benefits the catalytic properties of these molecules as coenzymes.

CONCLUSION

The dissociation constants of the group B vitamins were calculated with spectrophotometric titrations using a chemometric method. The striking advantage of the proposed method is using of the whole spectral information in the computation process which enable us to have more precise and accurate thermodynamics constants in comparison to the classical methods such as single wavelength approach. The effect of the ionic strength on the acidity constants is investigated and it reveals the complex relations of the dissociation constants to the ionic strength. The results show good consistency with the previous reported results.

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