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SPECTROPHOTOMETRIC INVESTIGATION OF THE ACIDITY CONSTANTS OF FLUORESCEIN IN VARIOUS WATER-ORGANIC SOLVENT MEDIA

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Spectrophotometric Investigation of the Acidity Constants of Fluorescein in Various Water-Organic Solvent Media

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We use a new procedure for estimating acid ionization constants of fluorescein using the spectrophotometric method at 25°C and at the constant ionic strength I = 0.1 M (KNO₃) in pure water as well as in aqueous media containing variable percentages (10–50% w/v) of organic solvents. The used organic solvents were methanol, ethanol, N,N-dimethyl formamide, dimethyl sulfoxide, acetonitrile, and dioxane. The obtained results indicated that acidity constants decrease as the content of an organic solvent in the medium increases. The acidity constants of all related equilibria are estimated using the whole spectral fitting of the collected data. The DATAN program was applied for determination of acidity constants. The effects of various solvents on absorption properties and acidity constants of each component are also discussed.

Keywords Acidity constant; DATAN; Fluorescein; Organic solvent

Introduction

Fluorescein dye is probably the most common fluorescent probe today. Its very high molar absorptivity at the wavelength of the argon laser (488 nm), large fluorescence quantum yield, and high photostability makes it a very useful and sensitive fluorescent label. Fluorescein is commercially available in many derivatives, such as fluorescent isothiocyanate and fluorescein succinimidyl ester, that can be covalently attached to macromolecules and to amino acids. The labeled molecules can be detected with very high sensitivity, which is useful in, for example, capillary electrophoresis. The emission spectrum of fluorescein overlaps extensively the absorption spectrum of tetra methyl rhodamine, which is a related strongly fluorescent dye, making this pair very suitable for energy transfer experiments to determine distance within and between labeled macromolecules. Fluorescein (Figure 1) in aqueous solution occurs in cationic, neutral, anionic, and dianionic forms, making its

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Figure 1. Chemical structures of fluorescein.

absorption and fluorescence properties strongly dependent on the medium such as pH and organic solvents. In order to use fluorescein in different solvent media, understanding its pK_a values in each medium is necessary. The protolytic constants relating the concentrations of the protolytic forms have been difficult to determine, because their spectra overlap substantially and the different pK_a values are quite close (Biswas et al., 1999; Sjoback et al., 1995).

Dissociation constants are important parameters to indicate the extent of ionization of molecules in solution at different pH values. The acidity constants of organic reagents play a fundamental role in many analytical procedures such as acid-base titration, solvent extraction, complex formation, and ion transport. It has been shown that the acid-base properties affect the toxicity, chromatographic retention behavior, and pharmaceutical properties of organic acids and bases. Much of the theoretical foundation of modern organic chemistry is based on the observation of the effects on acid-base equilibrium of changing molecular structure (Safavi and Abdollahi, 2001; Ghasemi et al., 2003; Beltran et al., 2003). Various methods for the determination of dissociation constants, such as potentiometric titration, spectrophotometric determination, conductometry, and spectroscopic methods, have been reported. Of these, potentiometric titration and spectrophotometric determination

are the most useful and widely used. For potentiometric titration, the dissociation constants of extremely acidic or basic compounds cannot be accurately determined because of their instability in an extreme pH range and because of the limitations of pH meters. Another essential requirement of this method is that the initial concentration of the samples must be accurately determined, that is to say, the samples must be pure and dry.

Mixed solvents are interesting, because two solvents mixed together produce a solvent with quite different properties, both physically (dielectric, density, and viscosity) and chemically (acid-base and donor-acceptor properties). As far as the acid-base properties are concerned, an important feature is that the nature of the solvent is crucial for the strength of acids and bases. In particular, the proton affinity is important, or in other words, the proton-donating and proton-accepting properties of a solvent as well as its polarity. In addition, the ionization degree of a solute depends on its dielectric constant. Media of high dielectric constants are strongly ionizing, whereas those of low dielectric constants ionize to a lesser extent. By mixing solvents of different polarities in proper ratios, the dielectric constant of the medium can be varied and, at the same time, the strength of dissolved acids and bases. It should also be emphasized that solvent mixtures can be more convenient than individual solvents owing to enhanced solubilizing efficiency, increased sharpness of color change of indicators during titration, and more manageable shape of acid-base titration curves (Azab et al., 2004; Durust et al., 2002; Rived et al., 2001).

Spectrophotometric methods are in general highly sensitive and are as such suitable for studying chemical equilibria solutions. If the components involved can be obtained in pure form, or if their spectral responses do not overlap, such analysis is, in general, trivial. For many systems, particularly those with similar components, this is not the case, and these have been difficult to analyze (Alimasifar et al., 1997; Shamsipur et al., 1993). Data analysis was carried out by the DATAN package developed by the Kubista group (Kubista et al., 1993, 1995, 1999; Scarminio and Kubista, 1993; Nygren et al., 1996), called the physical constraints approach, which provides a unique solution by requiring that the calculated concentrations obey an assumed equilibrium expression and demonstrates its applicability by determining the acidity constants of two and four protolytic forms of fluorescein. A possible advantage of the Kubista and coworkers method is that it mixes a soft modeling approach with a hard modeling approach. This might be the best more general strategy, since it can handle different situations with only a partial knowledge of the chemistry of the system. The physical constraints method calculates spectral profiles, concentrations, and equilibrium constants by utilizing equilibrium expressions that relate the components. The theory and application of the physical constraints method was discussed by several workers (Ghasemi et al., 2002, 2004, 2005; Niazi et al., 2006a,b,c).

In this study, we applied the UV-visible spectrophotometric method and physical constraints approach to determine the acidity constants of fluorescein in pure water and in different binary water-organic mixtures at 25°C and an ionic strength of 0.1 M (KNO₃). The used organic solvents were the amphiprotic (methanol, ethanol), dipolar aprotic (*N,N*-dimethyl formamide, dimethyl sulfoxide), low basic aprotic (acetonitrile), and low basic nonpolar (dioxane). The analysis is readily performed with the DATAN programs. The obtained pK_a values are discussed in terms of both the content and nature of the organic solvent.

Experimental Section

Materials

Fluorescein, methanol, ethanol, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), acetonitrile, dioxane, hydrochloric acid, sodium hydroxide, and potassium nitrate were analytical-grade commercial products from Merck. These reagents were used without further purification. Standard stock solution of 1.15×10^{-4} M of fluorescein was prepared by dissolving appropriate amounts of fluorescein in water. All the solutions were prepared in deionized water.

Instrumentation and Software

A Scinco (SUV-2120) spectrophotometer controlled by a computer and equipped with a 1 cm path length quartz cell was used for UV-visible spectra acquisition. Spectra were acquired between 300 and 550 nm. A HORIBA M-12 pH meter furnished with a combined glass-saturated calomel electrode was calibrated with at least two buffer solutions at pH 3.00 and 9.00. All absorption spectra where digitized at five data points per nanometer in the wavelength 300–550 nm and transferred (in ASCII format) to an AMD 2000 XP (256 Mb RAM) computer for subsequent analysis by Excel software, version 2003 (Microsoft), and for processing by the DATAN program.

Spectrophotometric Titrations

For the fluorescein (2.3×10^{-5} M) in pure water, water-methanol, water-ethanol, water-DMF, water-DMSO, water-acetontrile, and water-dioxane mixture titrations, absorption spectra were measured with a titration setup consisting of a computer interfaced to a spectrophotometer. To 5 mL of an approximately 1.15×10^{-4} M fluorescein solution an appropriate volume of solvent and 2.5 mL of 1 M KNO$_3$ were added. The pH of this solution was adjusted to the desired value by the addition of dilute sodium hydroxide or 5 mL of universal buffer (acetic, phosphate, and boric); the solution was then diluted to the mark in a 25 mL volumetric flask. A portion of the solution was transferred to a quartz cell. The cell was placed in the holder at 25°C in the spectrophotometer, and the spectra were recorded. The pH values in various solvent-water mixtures were corrected using the equation $pH^* = pH(R) - \delta$, where pH^* is the corrected reading and $pH(R)$ is the pH meter reading obtained in a partially aqueous organic solvent, determined by Douheret (1967, 1968).

Results and Discussion

Figure 1, which describes the interconversions of protolytic species and was discussed in previous reports (Mchedlov-Petrossyan and Kleshchevnikova, 1994), implies low acidity for different groups (Sjoback et al., 1995). The protolytic constants relating the chemical activity of the cation, neutral form, anion, and dianion are pK_{a1} = 2.08, pK_{a2} = 4.31, and pK_{a3} = 6.43. Song et al. (2000) and Biswas et al. (1999) investigated the absorption and fluorescence spectra of fluorescein in different micellar media. Also, Mchedlov-Petrossyan et al. (1994, 1999) and Mchedlov-Petrossyan (2003) have reported on fluorescein ionization in mixed and nonaqueous
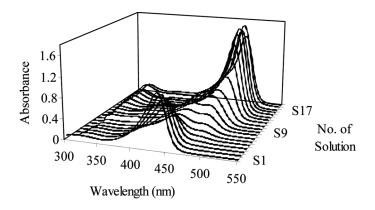
Figure 2. Absorption spectra of fluorescein (2.3×10^{-5} M) in pure water at 0.1 M KNO$_3$ at different pH values: (1) 1.84, (2) 2.34, (3) 2.84, (4) 3.33, (5) 3.84, (6) 4.33, (7) 4.83, (8) 5.33, (9) 5.83, (10) 6.36, (11) 6.87, (12) 7.25, (13) 7.83, (14) 8.37, (15) 8.95, (16) 9.52, (17) 10.51.

solvents. The difference between the results of this study and reported results might be because of difference in experimental conditions.

The absorption spectra of fluorescein in binary solvent mixtures at various pH values in 300–550 nm intervals were recorded. Sample spectra of fluorescein in water with pH values ranging from 1.0 to 12.1 and 50% (w/v) of different solvents to water at 0.1 M KNO$_3$ with pH values ranging from 1.0 to 13.0 are shown in Figures 2–8. Singular value decomposition (SVD) analysis performed on all absorption data matrices obtained at various pH values for fluorescein gives the number of components that best represent the system. SVD is the preferred algorithm because it is most stable under the widest range of applications (Malinowoski, 1991). The SVD algorithm decomposes an arbitrary data matrix, **A**, with more rows than columns, into three matrices, $\mathbf{A} = \mathbf{USV}^T$. The columns of matrix **U** (which has the same dimensions as **A**), constitute the so-called basis spectra of **A**. SVD has the very useful

Figure 3. Absorption spectra of fluorescein (2.3×10^{-5} M) in 50% (w/v) methanol to water at 0.1 M KNO$_3$ at different pH values: (1) 1.54, (2) 2.00, (3) 2.50, (4) 3.04, (5) 3.51, (6) 4.01, (7) 4.51, (8) 5.01, (9) 5.51, (10) 5.95, (11) 6.51, (12) 7.03, (13) 7.53, (14) 8.03, (15) 8.53, (16) 9.04, (17) 9.56, (18) 10.10, (19) 10.53, (20) 11.03, (21) 11.34, (22) 11.89.

Figure 4. Absorption spectra of fluorescein (2.3×10^{-5} M) in 50% (w/v) ethanol to water at 0.1 M KNO$_3$ at different pH values: (1) 1.44, (2) 2.09, (3) 2.66, (4) 3.24, (5) 3.91, (6) 4.56, (7) 5.02, (8) 5.36, (9) 5.87, (10) 6.31, (11) 6.81, (12) 7.11, (13) 7.85, (14) 8.36, (15) 8.85, (16) 9.58, (17) 10.00, (18) 10.46, (19) 11.15, (20) 11.43, (21) 11.92.

property of compressing the information contained in **A** into the first few columns of **U**, such that these columns are mutually orthogonal (linearly independent) and their importance decreases as rapidly as possible after the first column. The importance of each column of **U** in representing **A** least squares sense is given by the squares of nonnegative diagonal values (singular values) of **S**. **V**^{T} (where T means the transpose) describes the composition of **A** in terms of basis spectra. **V** and **S** have dimensions equal to the number of columns of **A**. Four significant factors are also supported by the statistical indicators of Elbergali et al. (1999). These factors could be attributed to the three dissociation equilibria of a triprotic acid such as fluorescein. The pK_a values of fluorescein were investigated in seven different solvent-water binary mixtures spectrophotometrically at 25°C and an ionic strength of 0.1 M.

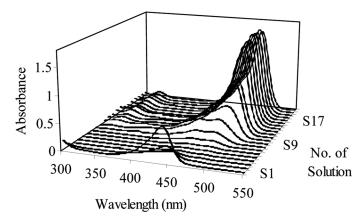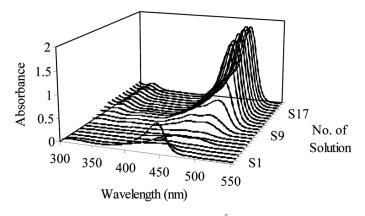**Figure 5.** Absorption spectra of fluorescein (2.3×10^{-5} M) in 50% (w/v) DMF to water at 0.1 M KNO$_3$ at different pH values: (1) 1.11, (2) 1.65, (3) 2.11, (4) 2.68, (5) 3.24, (6) 3.89, (7) 4.10, (8) 4.63, (9) 5.02, (10) 5.63, (11) 6.12, (12) 6.63, (13) 7.21, (14) 7.76, (15) 8.03, (16) 8.51, (17) 8.91, (18) 9.32, (19) 10.21, (20) 10.65, (21) 11.02, (22) 11.48.

Figure 6. Absorption spectra of fluorescein (2.3×10^{-5} M) in 50% (w/v) DMSO to water at 0.1 M KNO$_3$ at different pH values: (1) 1.05, (2) 1.77, (3) 2.17, (4) 2.88, (5) 3.37, (6) 3.91, (7) 4.47, (8) 4.87, (9) 5.05, (10) 5.54, (11) 6.06, (12) 6.52, (13) 7.04, (14) 7.66, (15) 8.01, (16) 8.52, (17) 9.00, (18) 9.51, (19) 10.00, (20) 10.55, (21) 11.03, (22) 11.65, (23) 12.02.

Acidity constants of fluorescein in several mixtures were evaluated with the DATAN program using the corresponding spectral absorption-pH data. The obtained pK_a values are listed in Table I. The pK_a values cited in Table I indicate that the acid ionization constants are dependent on both the proportion and the nature of the organic solvent. Generally, the acidity constants are decreased by increasing the mole percentage of all organic solvents used. However, some pK_a values could not be determined in solutions containing high mole fraction of applied organic solvents (Table I), because in high mole fraction of organic solvents, only at high pH, we observed the absorption spectra. According to the pH changes in the region of H$_2$F production, absorption is not observed. It can be thought that the species H$_2$F, which has the lactone form, is formed in solution and tautomeric

Figure 7. Absorption spectra of fluorescein (2.3×10^{-5} M) in 50% (w/v) acetonitrile to water at 0.1 M KNO$_3$ at different pH values: (1) 1.01, (2) 1.51, (3) 2.01, (4) 2.51, (5) 3.01, (6) 3.51, (7) 4.01, (8) 4.51, (9) 5.01, (10) 5.51, (11) 6.02, (12) 6.52, (13) 7.02, (14) 7.52, (15) 8.02, (16) 9.02, (17) 9.52, (18) 10.20, (19) 10.52, (20) 11.02, (21) 11.52, (22) 12.03, (23) 12.32.
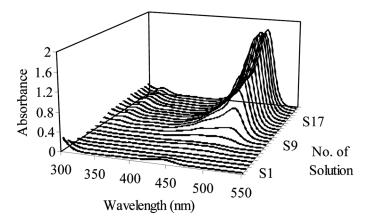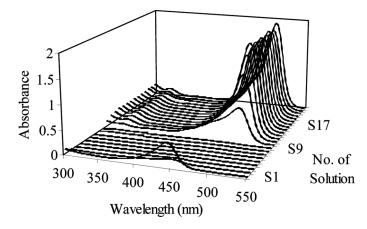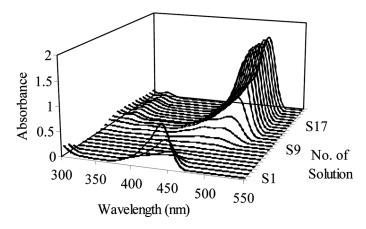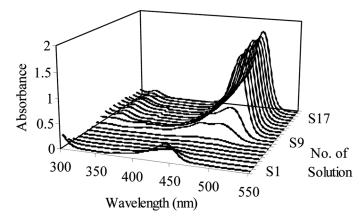
Figure 8. Absorption spectra of fluorescein $(2.3 \times 10^{-5} \text{M})$ in 50% (w/v) dioxane to water at 0.1 M KNO$_3$ at different pH values: (1) 1.51, (2) 2.05, (3) 2.66, (4) 3.19, (5) 3.61, (6) 4.41, (7) 4.79, (8) 5.23, (9) 5.91, (10) 6.45, (11) 7.13, (12) 7.86, (13) 8.34, (14) 8.67, (15) 9.21, (16) 9.76, (17) 10.83, (18) 11.41, (19) 11.95.

reaction does proceed. So, we could not obtain pK_a at mole fractions higher than 50% for different solvents.

Increasing the mole fraction of different solvents in the medium leads to an increase in the pK_a for fluorescein. The acid ionization constant in a pure aqueous medium ($K_{a(w)}$) is related to that in a partly aqueous medium ($K_{a(s)}$) by the relation (Ahmed et al., 2004):

$$K_{a(w)} = K_{a(s)}(\gamma_A^- \gamma_H^+ / \gamma_{HA})$$

where γ is the activity coefficient of the respective species in a partly aqueous medium relative to that in pure water. The electrostatic effect resulting from the change in the relative permittivity of the medium operators depends on the activity coefficient of any charged species. Generally, by increasing the mole percentage of different solvents in the aqueous medium, the relative permittivity of the medium is lowered. This will increase both the γ_A^- and γ_H^-, yielding a decrease in the acid ionization constants (i.e., high pK_a values). This is consistent with the results obtained for the acid ionization constants (pK_a) of fluorescein in various water-solvents mixtures. However, in the light of the relation (Ahmed et al., 2004) p$K_a = e^2/(2.303 \, aKT(\varepsilon)_m)$, where e, a, K, and T correspond to electronic charge, specified constant, Boltzmann constant, and absolute temperature, which correlates the variation of pK_a with the relative permittivity of the medium $(\varepsilon)_m$ (Akadov, 1969), points on the plots of pK_a values against $\{1/(\varepsilon)_m\}$ for different solvents, where linear regression analysis was used to obtain the best fit correlation, deviate from linearity (Table I). The relative permittivity of the medium $(\varepsilon)_m$ is obtained from the relation (Table I) (Ahmed et al., 2004):

$$(\varepsilon)_m = \varepsilon_{(w)}X_{f(w)} + \varepsilon_{(s)}X_{f(s)}$$

where ε and X_f are respectively the relative permittivity and mole fraction and the subscripts w and s refer to water and organic solvent. The same trend has already been reported for various organic molecules in different solvent mixtures (Ghasemi et al., 2002, 2003; Azab et al., 2004; Rived et al., 2001; Alimasifar et al., 1997; Shamsipur et al., 1993; Niazi et al., 2006b). It has been reasonably assumed that preferential

Table I. pK_a values for fluorescein in different water-organic solvent mixtures at 25°C and at the constant ionic strength $I = 0.1\,M$ (KNO$_3$)

% (W/V) of organic solvent	$(\varepsilon)_m$	$\{1/(\varepsilon)_m\}10^2$	pK_1	pK_2	pK_3
0	78.40	1.28	2.11 ± 0.02	4.31 ± 0.03	6.39 ± 0.05
				Methanol	
10	73.82	1.35	2.14 ± 0.02	4.57 ± 0.04	6.41 ± 0.05
20	69.24	1.44	2.28 ± 0.02	4.90 ± 0.03	6.44 ± 0.05
30	64.66	1.55	2.36 ± 0.03	5.15 ± 0.03	6.47 ± 0.07
40	60.08	1.66	2.44 ± 0.04	5.41 ± 0.04	6.50 ± 0.06
50	55.50	1.80	2.49 ± 0.03	5.75 ± 0.05	6.54 ± 0.05
				Ethanol	
10	72.99	1.37	2.29 ± 0.03	4.97 ± 0.03	6.42 ± 0.04
20	67.58	1.48	2.38 ± 0.02	5.29 ± 0.03	6.46 ± 0.05
30	62.17	1.61	2.44 ± 0.02	5.48 ± 0.04	6.49 ± 0.06
40	56.76	1.76	2.51 ± 0.03	5.65 ± 0.06	6.53 ± 0.06
50	51.35	1.95	2.56 ± 0.04	5.81 ± 0.04	6.58 ± 0.06
				DMF	
10	74.23	1.35	2.18 ± 0.02	4.39 ± 0.02	6.39 ± 0.05
20	70.06	1.43	2.25 ± 0.01	4.46 ± 0.03	6.40 ± 0.06
30	65.89	1.52	2.31 ± 0.02	4.53 ± 0.03	6.41 ± 0.06
40	61.72	1.62	2.37 ± 0.03	4.62 ± 0.05	6.44 ± 0.06
50	57.55	1.74	2.41 ± 0.03	4.64 ± 0.05	6.47 ± 0.07
				DMSO	
10	75.45	1.33	2.12 ± 0.03	4.51 ± 0.05	6.44 ± 0.05
20	72.50	1.38	2.16 ± 0.03	4.76 ± 0.04	6.51 ± 0.06
30	69.55	1.44	2.19 ± 0.04	4.96 ± 0.04	6.59 ± 0.06
40	66.60	1.50	2.21 ± 0.03	5.09 ± 0.04	6.65 ± 0.06
50	63.65	1.57	2.23 ± 0.05	5.24 ± 0.06	6.70 ± 0.07
				Acetonitrile	
10	74.16	1.35	2.14 ± 0.04	4.54 ± 0.04	6.48 ± 0.05
20	69.92	1.43	2.18 ± 0.05	4.66 ± 0.04	6.54 ± 0.05
30	65.68	1.52	2.25 ± 0.05	4.78 ± 0.05	6.67 ± 0.06
40	61.44	1.63	2.30 ± 0.05	4.83 ± 0.06	6.77 ± 0.06
50	57.20	1.75	2.33 ± 0.06	5.05 ± 0.06	6.81 ± 0.07
				Dioxane	
10	70.77	1.41	2.15 ± 0.04	4.69 ± 0.06	6.42 ± 0.06
20	63.14	1.58	2.20 ± 0.04	4.88 ± 0.06	6.46 ± 0.07
30	55.51	1.80	2.24 ± 0.04	5.23 ± 0.05	6.51 ± 0.06
40	47.88	2.09	2.30 ± 0.05	5.39 ± 0.06	6.55 ± 0.07
50	40.25	2.48	2.36 ± 0.05	5.59 ± 0.05	6.60 ± 0.07

solvation of the charged particles by water is mainly responsible for such a monotonic dependence of acidity constants of fluorescein on the solvent composition.

It was recognized that solvent effects such as hydrogen bonding and solvent basicity as well as dispersion forces and proton-solvent interactions exert a profound influence on the ionization process of weak acid in the presence of organic solvent.

The effective density of dispersion centers in the organic solvent used is higher than in pure water. Accordingly, one can expect higher stabilization of the conjugate base A^- from each step of ionization by dispersion forces, which are established between the delocalized oscillator dipole of the solvent. Furthermore, the proton is expected to be highly stabilized in aqueous mixtures by its interactions with organic solvent and water molecules (proton-solvent interaction) compared with water molecules alone in pure water. Consequently, both A^- and H^+ become highly stabilized upon increasing the mole fraction of solvent in aqueous medium, i.e., γ_{A^-} and γ_{H^+} decrease. Thus, the acid ionization constants of the studied fluorescein increase (pK_a decrease) with increasing solvent content in the medium. However, this is not the case, as is evident from the data cited in Table I. Therefore, one can conclude that both the dispersion forces and proton-solvent interaction effects do not have significant roles in the ionization processes of fluorescein.

On the other hand, water molecules are characterized by a high tendency to act as hydrogen donors compared with other solvent molecules. Therefore, the conjugate base A^- is expected to be less stabilized by hydrogen-bonding interaction with solvent molecules as the mole fraction of solvents increase (i.e., γ_{A^-} increase). This will tend to increase the pK_a values of all steps in the fluorescein system. Accordingly, the observed increase in the pK_a values of fluorescein upon increasing mole fraction of different organic solvents in aqueous mixtures can be ascribed, in addition to the electrostatic effect, to the hydrogen-bonding interaction between the conjugate base A^- and different solvents.

Conclusion

In this work, the behavior of acidity constants of fluorescein in solvent-water mixtures at 25°C and an ionic strength of 0.1 M is studied by multi-wavelength spectrophotometric and chemometric methods. The precision and accuracy of the method are rather low in comparison with that provided by classical procedures, but these results are satisfactory for many applications. The organic solvents used were methanol, ethanol, *N,N*-dimethyl formamide, dimethyl sulfoxide, acetonitrile, and dioxane. The shifts in acidity constants of fluorescein in the mixtures were derived. There is a linear relationship between acidity constants and the mole fraction of different solvents in the solvent mixtures. The effect of solvent properties on acid-base behavior is discussed. This indicates that the acid ionization constants of fluorescein obtained in various percentages of different solvent-water mixtures are governed by electrostatic effects.

Acknowledgment

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