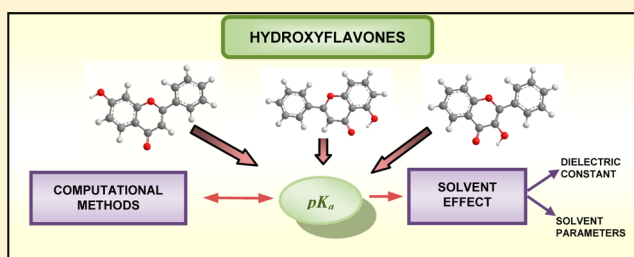


Solvent Effects on the Dissociation Constants of Hydroxyflavones in Organic–Water Mixtures. Determination of the Thermodynamic pK_a Values by UV–Visible Spectroscopy and DFT Calculations

Yamina A. Dávila, Matias I. Sancho, María C. Almandoz, and Sonia E. Blanco*

Área de Química Física, Facultad de Química Bioquímica y Farmacia, Instituto Multidisciplinario de Investigaciones Biológicas (IMIBIO-SL) CONICET, Universidad Nacional de San Luis, Chacabuco 917, 5700 San Luis, Argentina

ABSTRACT: The acid dissociation constants (pK_a) of 7-hydroxyflavone, 3-hydroxyflavone and 5-hydroxyflavone, which are practically insoluble compounds in water but of great biological and physicochemical interest, were determined by UV–visible spectroscopy in ethanol–water and acetonitrile–water solutions, varying the solvent relative permittivity in the interval of 61 to 75, at constant ionic strength ($0.050 \text{ mol}\cdot\text{kg}^{-1}$) and temperature (298.15 K). The pK_a values of the compounds increase as the permittivity of the reaction medium decreases. Correlations were established between the pK_a values and empirical parameters of the solvents, such as the relative permittivity and hydrogen-bond donor capacity. These equations allow the interpretation of the solvent effect on the acid–base equilibria and the determination of pK_a values at 298.15 K in pure water. The ordering of the pK_a values was 7-hydroxyflavone (7.28) < 3-hydroxyflavone (8.68) < 5-hydroxyflavone (11.75). In addition, the dissociation constants were also calculated by means of DFT methods (B3LYP/6-311+G(2d,p) level of theory), employing several thermodynamic cycles. The solvent effect on the optimized structures in the gas-phase was evaluated using the polarizable continuum model. A good agreement was observed between the theoretical and experimental pK_a values. Finally, the experimental ordering in the acidity of the hydroxyflavones was explained using natural bond orbital analysis.



1. INTRODUCTION

The acidity constant, pK_a , of a compound is an important property in analytical and biological chemistry. The pK_a , solubility, and lipophilicity are key factors determining the absorption and bioavailability of the molecule. A wide range of methodologies can be applied for the pK_a determination of organic compounds, such as capillary electrophoresis,^{1,2} solubility measurements,^{3,4} potentiometry,^{5,6} and UV–vis absorption spectroscopy.^{7–9} The selection of a suitable analytical method can be done taking into account the properties of the studied compounds. In the particular case of practically insoluble molecules in water, the determination of pK_a in aqueous–organic mixtures using UV–vis spectroscopy represents a convenient methodology of work. The cosolvents most frequently employed for this aim are methanol,^{10,11} ethanol,^{9,11} dimethylsulfoxide,⁵ *N,N*-dimethylformamide,⁹ and acetonitrile.⁸ Among the very low aqueous solubility compounds, the flavonoids are highly significant due to their interesting physicochemical and biological properties.^{12–14} Because of the mentioned low aqueous solubility, the determination of their pK_a values in pure water is not always possible. Only a limited number of studies related with pK_a values of flavonoids are found in the literature.^{2,5,15–22} The values reported for these compounds do not always agree, because of the low solubility, stability, or the methodology used for its determination. For instance, M. Herrero-Martínez et al. have determined the acidity constants of morin, fisetin, and

quercetin in methanol–water by potentiometry. The values reported for quercetin are $pK_1 = 7.59$, $pK_2 = 9.33$ and $pK_3 = 11.56$.²¹ While, S. Ramešová et al. have determined for quercetin the values of $pK_1 = 5.87$ and $pK_2 = 8.48$ by means of alkalimetric titration and UV–vis spectrophotometry under deaerated conditions.²²

In this paper we analyze the solvent effect on the dissociation constants of three monohydroxyflavones, as part of a research project focused on the study of relevant physicochemical properties of flavonoids and benzophenones.^{12,19,20,23–25} The pK_a values of 7-hydroxyflavone (7(OH)Fl), 3-hydroxyflavone (3(OH)Fl), and 5-hydroxyflavone (5(OH)Fl) were determined in ethanol–water (EtOH-W) and acetonitrile–water (ACN-W) solutions by UV–vis spectroscopy, varying the solvent permittivity between 61 and 75, at 298.15 K and constant ionic strength. Despite the importance of these compounds, the determination of the dissociation constant in ACN-W mixtures has not been reported yet. The solvent effect on the acidity constants was analyzed by means of linear relationships between these values and solvation parameters of the medium. These equations were used to calculate the pK_a values of the flavones in pure water. In addition, the dissociation constants of

Received: February 7, 2013

Accepted: May 1, 2013

Table 1. Description of the Hydroxyflavones Employed

abbreviated name	IUPAC name	source	initial mass fraction purity	purification method	final mass fraction purity	analysis method
7(OH)Fl	7-hydroxy-2-phenyl-4H-chromen-4-one	Sigma	≥ 0.98	recrystallization	0.995	HPLC ^a
5(OH)Fl	5-hydroxy-2-phenyl-4H-chromen-4-one	Sigma	≥ 0.97	recrystallization	0.99	HPLC ^a
3(OH)Fl	3-hydroxy-2-phenyl-4H-chromen-4-one	synthesis		recrystallization	0.998	HPLC ^a

^aAnalyzed using a HPLC Gilson 322 series pump and a Gilson 152 UV-vis detector, equipped with a Luna (C₁₈ (2), 250 mm×4.6 mm, 5 μm) column.

the studied flavones were also calculated by means of DFT methods, employing several thermodynamic cycles reported in the literature^{26,27} and an alternative method proposed by us. Finally, the experimental ordering in the acidity of the flavones is explained using natural bond orbital (NBO) analysis.

2. EXPERIMENTAL SECTION

2.1. Reagents. Information about the studied hydroxyflavones is shown in Table 1, and the structure and chemical numbering system of these compounds are depicted in Figure 1.

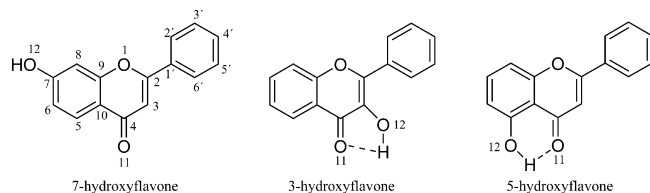


Figure 1. Molecular structure and numbering system of the studied flavones.

3(OH)Fl was obtained in our laboratory following the Algar–Flynn–Oyamada synthesis reaction,²⁸ and then, it was purified according to a previously reported procedure.²⁵ 5(OH)Fl and 7(OH)Fl were purchased from Sigma. The purity control of the compounds was chromatographically determined (HPLC). Ethanol (EtOH) (spectroscopic grade), acetonitrile (ACN) (HPLC grade), and all the reagents (analytical grade) employed in the preparation of the buffer solutions were purchased from Merck. Double-distilled water purified with a Super Q Millipore System was used to prepare the solvent mixtures.

The solutions employed in the present study were (a) HCl–KCl, pH 2.0; (b) NaOH–KCl, pH 13.0; (c) NaH₂PO₄–Na₂HPO₄ (1.0·10⁻² mol·kg⁻¹), from pH 6.20 to 8.00, with a variation of 0.20 ± 0.01 pH units; (d) H₃BO₃–NaOH (3.5·10⁻² mol·kg⁻¹), from pH 8.00 to 9.20 and Na₂HPO₄–NaOH (5.0·10⁻³ mol·kg⁻¹), from pH 10.5 to 12.0.

The ionic strength (0.050 mol·kg⁻¹) of the buffer solutions was fixed by adding KCl, and the corrected pK_a values for this ionic strength were considered in the calculations for the preparation of the solutions.²⁹ Several EtOH–W and ACN–W mixtures (from *w* = 0.10 to *w* = 0.30, where *w* is the organic solvent fraction (*w*/*w*)) were employed as solvents. The relative permittivity (*D*) range of these solvent mixtures at 298.15 K goes from 74.8 to 60.7.

2.2. Procedure. **2.2.1. EtOH–W Mixtures.** “A stock solution of 7(OH)Fl 9.38·10⁻³ mol·kg⁻¹ was prepared in EtOH. Standard solutions were prepared adding appropriate amounts of stock solution to 5.00 ± 0.02 g of phosphate buffers (pH between 6.20 and 8.00) in EtOH–W (*w*_{EtOH} = 0.0946 and *D* = 73.22). The same procedure was followed at pH 2.0 and 13.0. The analytic concentration of these standard solutions was

3.79·10⁻⁵ mol·kg⁻¹. Series of similar solutions were prepared using phosphate buffers at the same pH interval, with the following fractions of EtOH (*w*_{EtOH} = 0.138, 0.179, 0.216, 0.281) and the corresponding permittivity values (*D* = 70.67, 68.26, 66.08, 62.25).³⁰ All these series of solutions were placed in UV cells, hermetically closed, and thermostated at 298.15 K for 15 min. After this, the UV-vis spectra were recorded on a Cary 50 UV-visible spectrophotometer, between 200 and 450 nm. The corresponding absorbance values were determined at wavelengths of maximum absorption.³¹

2.2.2. ACN–W Mixtures. A similar procedure as that described above was followed for these mixtures. Now, the stock solution of 7(OH)Fl was prepared in ACN and the buffer solutions of pH 6.20 to 8.0, pH 2, and pH 13 were prepared in ACN–W mixtures (*w*_{ACN} = 0.118, 0.136, 0.175, 0.214 and 0.280). The relative permittivity values of these solutions were 74.12, 73.39, 71.67, 69.85 and 66.61,³² respectively. The analytic concentration of 7(OH)Fl in these standard solutions was 3.29·10⁻⁵ mol·kg⁻¹.

For 3(OH)Fl and 5(OH)Fl, the procedure described for 7(OH)Fl in EtOH–W and ACN–W was followed. To analyze these compounds, the buffer solutions H₃BO₃–NaOH (pH 8.00 to 9.20) for 3(OH)Fl and Na₂HPO₄–NaOH (pH 10.5 to 12.0) for 5(OH)Fl were employed. The spectra were recorded in the 200 to 500 nm interval.

It is well-known that the pH of aqueous buffer solutions slightly changes when a small amount of organic solvent is added. For this reason, the methodology and notation employed by Rosés et al.³³ and adopted by us in previous studies²³ was followed. The pH measurements (Orion SA 520 pH-meter) were performed directly on the mixed aqueous–organic buffers, using the electrode previously calibrated with aqueous standard buffers. “Such pH values are denoted as (^S*w*pH) and the respective pK_a values obtained from data expressed in the (^S*w*pH) scale are also expressed in the same scale (^S*w*pK_a)”.²³

3. COMPUTATIONAL DETAILS

The initial geometries of the molecules were fully optimized with the hybrid DFT functional B3LYP^{34,35} at the 6-311+G(2d,p) level. The corresponding vibrational frequencies of all the chemical species were calculated in order to obtain the thermodynamic parameters and to make sure that the optimized structures were true minima. Then, the Gibbs energy in the gas phase (*G*₂₉₈⁰) was calculated for all the analyzed structures using the following equation:

$$G_{298}^0 = E + ZPVE + H_{\text{corr}} - TS_{298}^0 \quad (1)$$

where *E* is the gas phase total energy, ZPVE is the zero-point vibrational energy, *H*_{corr} is the thermal correction to enthalpy, *T* is the temperature, and *S*₂₉₈⁰ is the entropy. The solvent effect on the optimized structures in the gas-phase was analyzed by performing a geometry optimization using the polarizable continuum model

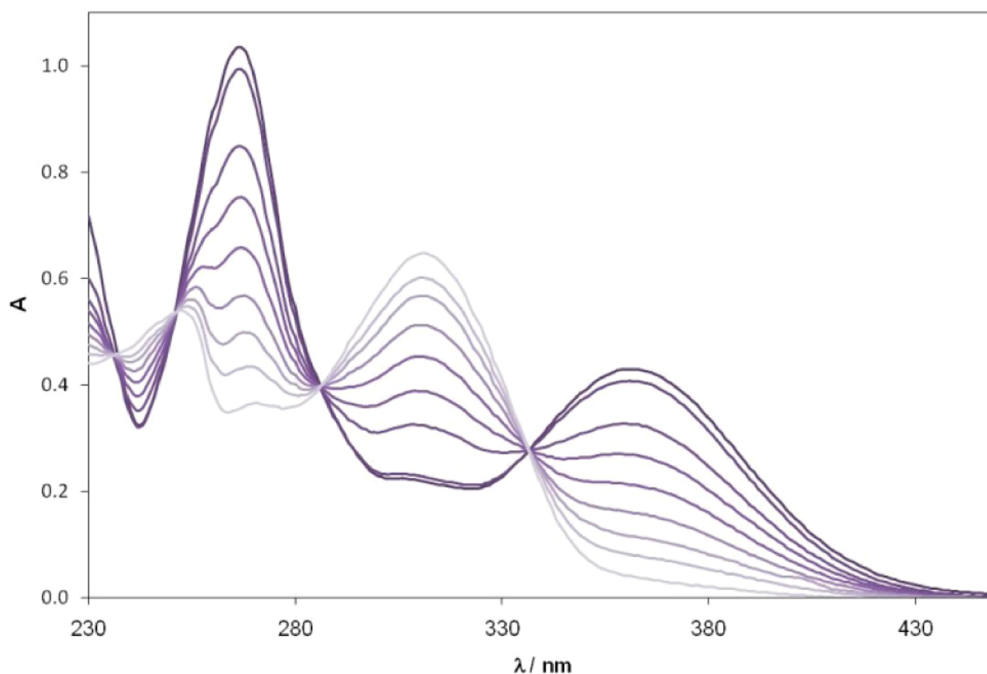


Figure 2. Absorption spectra of 7-hydroxyflavone ($3.23 \cdot 10^{-5} \text{ mol} \cdot \text{kg}^{-1}$) at different ^spH values in acetonitrile–water ($w_{\text{ACN}} = 0.214$), $T/\text{K} = 298.15$. Run and ^spH : (1) 2.17; (2) 7.20; (3) 7.42; (4) 7.63; (5) 7.85; (6) 8.06; (7) 8.27; (8) 8.70; (9) 13.12 (in the order from bottom to top at $\lambda_{\text{max}} 362.5 \text{ nm}$).

with the integral equation formalism (IEF-PCM).³⁶ The UAHF radii sets were employed to build the solvent cavity. The solvation Gibbs energies ($\Delta_{\text{solv}}G$) and Gibbs energies in solution (G_s) were calculated for each structure using the expressions:

$$\Delta_{\text{solv}}G = E_{\text{PCM}} - E \quad (2)$$

$$G_s = G_{298}^0 + \Delta_{\text{solv}}G \quad (3)$$

In addition, a natural bond orbital³⁷ (NBO) analysis was carried out on the flavones using the B3LYP/6-311+G(2d,p) level of theory. The second order perturbation theory was employed to estimate the energy of donor–acceptor interactions. The stabilization energy associated with the delocalization of orbital (i)→orbital (j) was calculated as follows:

$$\Delta E_{ij} = \frac{q_i F_{ij}}{(\epsilon_j - \epsilon_i)} \quad (4)$$

where q_i is the donor orbital occupancy, ϵ_i and ϵ_j are the energies of interacting orbitals, and F_{ij} is the off-diagonal NBO Fock matrix element. All the calculations were performed with Gaussian 03 program packages.³⁸

4. RESULTS AND DISCUSSION

4.1. Determination of pK_a . The determination of ionization constants by UV–vis spectroscopy is an appropriate method when a substance is sparingly soluble in water or when its pK_a value is particularly low or high.³⁹ The UV-absorption spectra of the analyzed hydroxyflavones show mainly two bands (Figures 2 to 4). The absorption bands corresponding to the nonionized flavones (shorter wavelengths) are located at a 312.0 nm (7(OH)Fl), 344.0 nm (3(OH)Fl), and 336.0 nm (5(OH)Fl), while the bands of the ionized species (longer wavelengths) are observed at 362.5 nm (7(OH)Fl), 407.0 nm (3(OH)Fl), and 382.0 nm (5(OH)Fl). In Figures 2 to 4 the spectral changes of these compounds with the pH of the medium can be observed. The pK_a of the compounds were

determined at both wavelengths of maximum absorption and the numerical values were practically coincident.

The analyzed dissociation reaction can be schematically represented with the simple equation,



where AH is 7(OH)Fl, 3(OH)Fl, or 5(OH)Fl, A^- is the corresponding anion, HS is the solvent, and H_2S^+ is the protonated solvent. Taking into account that low percentages of organic solvent were employed in the mixtures and the high solvation ability of ions by water, the simplification of replacing HS and H_2S^+ by H_2O and H_3O^+ , respectively, can be made in eq 5. Then, from the equilibrium constant of this reaction (eq 5) the known Henderson–Hasselbach equation can be obtained. This equation is very useful in the determination of pK_a values^{31,40–42} and it can be formulated in the following way

$$^s\text{pH} = ^s\text{pK}_a + \log \frac{(A - A_a)}{(A_b - A)} \quad (6)$$

where A_a and A_b are the absorbance of the neutral (AH) and the ionized (A^-) form measured in HCl and NaOH solutions, respectively, A is the absorbance due to the presence of both A^- and AH in the buffer solutions, and $^s\text{pK}_a$ is the apparent acidity constant.

4.2. Data Analysis. The data collected from a typical experiment in the determination of the $^s\text{pK}_a$ value are presented in Table 2 for 7(OH)Fl at 362.5 and 312.0 nm in ACN-W ($w_{\text{ACN}} = 0.136$). Figure 5 shows a graphical representation of eq 6 for the three analyzed compounds in ACN-W. The slope and intercept values of eq 6 were calculated by means of linear regression analysis, and from the intercept the corresponding $^s\text{pK}_a$ values were obtained. In Table 3 the parameters of the Henderson–Hasselbach equation determined for 7(OH)Fl (362.5 nm), 3(OH)Fl (407.0 nm), and 5(OH)Fl (382.0 nm) are listed in different EtOH-W and ACN-W mixtures. A good correlation coefficient is observed in all the

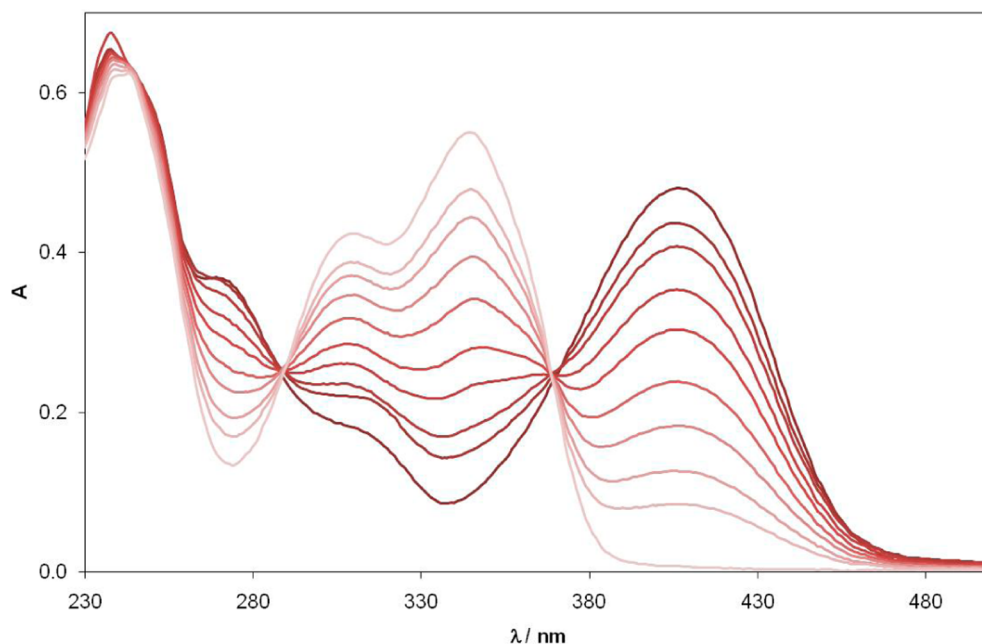


Figure 3. Absorption spectra of 3-hydroxyflavone ($3.74 \cdot 10^{-5} \text{ mol} \cdot \text{kg}^{-1}$) at different $S_{\text{w}}\text{pH}$ values in acetonitrile–water ($w_{\text{ACN}} = 0.196$), $T/\text{K} = 298.15$. Run and $S_{\text{w}}\text{pH}$: (1) 2.17; (2) 8.64; (3) 8.85; (4) 9.06; (5) 9.26; (6) 9.47; (7) 9.68; (8) 9.88; (9) 10.09; (10) 13.12 (in the order from bottom to top at λ_{max} 407.0 nm).

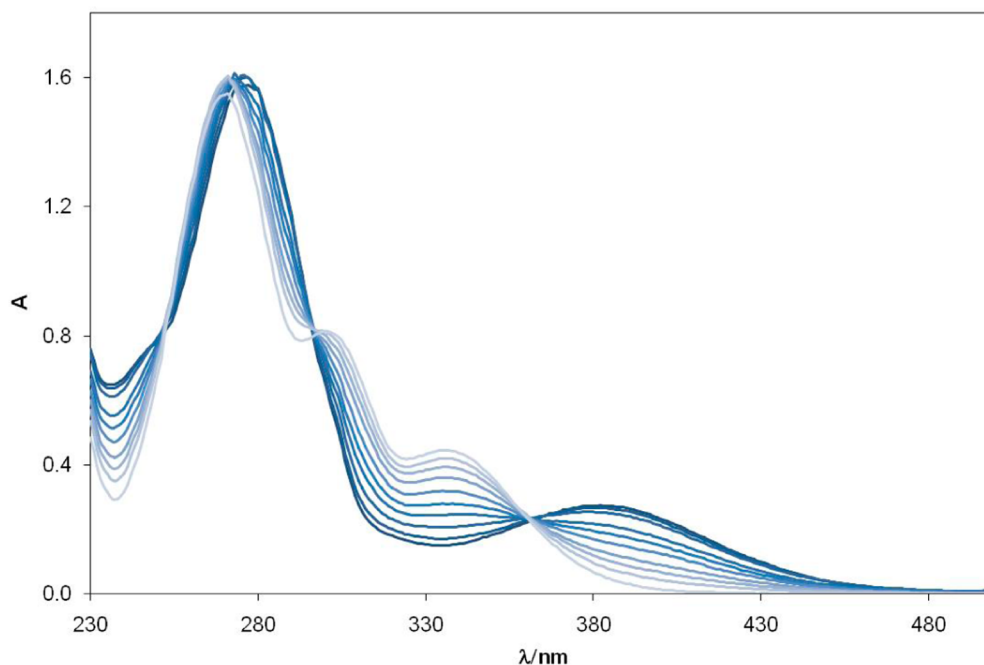


Figure 4. Absorption spectra of 5-hydroxyflavone ($8.07 \cdot 10^{-5} \text{ mol} \cdot \text{kg}^{-1}$) at different $S_{\text{w}}\text{pH}$ values in acetonitrile–water ($w_{\text{ACN}} = 0.195$), $T/\text{K} = 298.15$. Run and $S_{\text{w}}\text{pH}$: (1) 2.17; (2) 11.00; (3) 11.18; (4) 11.37; (5) 11.55; (6) 11.73; (7) 11.91; (8) 12.27; (9) 12.64; (10) 13.13 (in the order from bottom to top at λ_{max} 382.0 nm).

experiments and the slope values are close to unity, which is the predicted value of the Henderson-Hasselbach equation. The $S_{\text{w}}\text{pK}_a$ values of 3(OH)Fl in EtOH-W were previously determined with this methodology¹⁹ and are reported here with a comparative purpose.

4.3. Solvent effects. When a given compound is poorly soluble in water, the pK_a determination is usually done in several organic–water mixtures, and the obtained $S_{\text{w}}\text{pK}_a$ values are extrapolated to the aqueous medium.^{5,21,23,31} In the present study, low fractions of EtOH or ACN (between $w = 0.095$ and

0.32) were employed in the $S_{\text{w}}\text{pK}_a$ determinations. Under these conditions, the pK_a values obtained from this indirect method will be more accurate and their values will be closer to the measured ones if the compound would be water-soluble. Table 3 shows the $S_{\text{w}}\text{pK}_a$ values for the studied compounds at 298.15 K, determined in different EtOH-W and ACN-W mixtures. At constant ionic strength ($0.050 \text{ mol} \cdot \text{kg}^{-1}$) it can be observed that the $S_{\text{w}}\text{pK}_a$ of the compounds increase when the permittivity of the reaction medium decreases. These $S_{\text{w}}\text{pK}_a$ values were plotted against $1/D$ according to Kirkwood's equation⁴³ applied

Table 2. Data for Determination of Apparent Acidity Constant (${}^S_w pK_a$) of 7-Hydroxyflavone in Acetonitrile–Water ($w_{ACN} = 0.136$) at 298.15 K^a

${}^S_w pH$	$A_{362.5\text{ nm}}$	$(A - A_a)/(A_b - A)$	$\log((A - A_a)/(A_b - A))$	$A_{312.0\text{ nm}}$	$(A_a - A)/(A - A_b)$	$\log((A_a - A)/(A - A_b))$
6.52	0.037	0.089	-1.049	0.580	0.113	-0.946
6.87	0.068	0.200	-0.699	0.549	0.209	-0.679
7.06	0.098	0.331	-0.480	0.513	0.345	-0.463
7.27	0.134	0.531	-0.275	0.472	0.541	-0.267
7.49	0.174	0.839	-0.076	0.424	0.858	-0.066
7.66	0.217	1.346	0.129	0.375	1.353	0.131
7.84	0.260	2.239	0.350	0.331	2.093	0.321
8.02	0.284	3.112	0.493	0.304	2.832	0.452
8.24	0.312	5.000	0.699	0.272	4.346	0.638

^aNotation: w , organic solvent fraction (w/w); A_a = absorbance of the acid form (AH) in HCl+KCl pH = 2; A_b = absorbance of the conjugate form (A^-) in NaOH + KCl pH = 13; A = absorbance due to the species A^- and AH in the buffer solution; and ${}^S_w pH$ is the apparent pH in the acetonitrile–water mixture. Analytic concentration of 7-hydroxyflavone = $3.29 \cdot 10^{-5}$ mol·kg⁻¹; A_a at 362.5 nm = 0.007; A_b at 362.5 nm = 0.373; A_a at 312.0 nm = 0.624; A_b at 312.0 nm = 0.191. The standard uncertainties u are $u(w) = 0.001$, $u(pH) = 0.01$, $u(A) = 0.0005$.

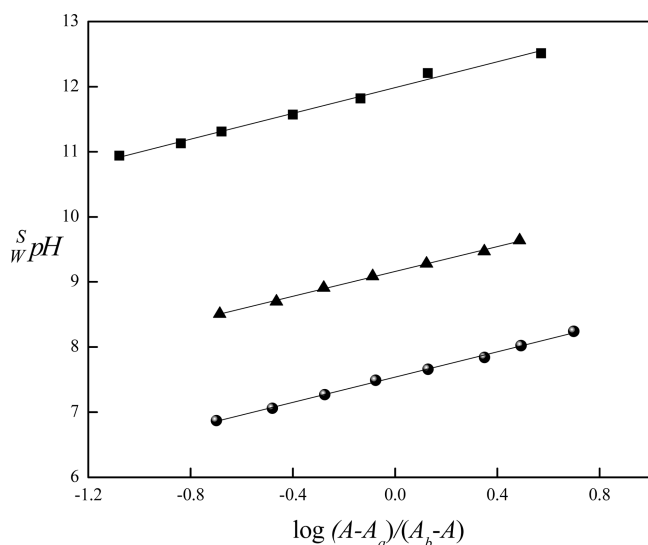


Figure 5. Determination of the ${}^S_w pK_a$ of hydroxyflavones in acetonitrile–water ($w_{ACN} = 0.14$) at 298.15 K. Graphical representation of eq 6: ■, 5-hydroxyflavone; ▲, 3-hydroxyflavone; ●, 7-hydroxyflavone; and solid lines are the result of linear regression using eq 6.

to acid–base equilibrium,³¹ and the following expressions were obtained (the values in parentheses are the standard deviations): 7(OH)Fl

$${}^S_w pK_a(\text{EtOH-W}) = 158.49(3.1) \frac{1}{D} + 5.245(0.045)$$

$$R^2 = 0.9989 \quad SD = 0.0057 \quad (7)$$

$${}^S_w pK_a(\text{ACN-W}) = 466.52(11) \frac{1}{D} + 1.174(0.16)$$

$$R^2 = 0.9983 \quad SD = 0.014 \quad (8)$$

3(OH)Fl

$${}^S_w pK_a(\text{EtOH-W}) = 294.59(6.4) \frac{1}{D} + 4.991(0.11)$$

$$R^2 = 0.9981 \quad SD = 0.022 \quad (9)$$

$${}^S_w pK_a(\text{ACN-W}) = 375.25(11) \frac{1}{D} + 4.021(0.17)$$

$$R^2 = 0.9972 \quad SD = 0.017 \quad (10)$$

5(OH)Fl

$${}^S_w pK_a(\text{EtOH-W}) = 199.67(7.7) \frac{1}{D} + 9.008(0.11)$$

$$R^2 = 0.9955 \quad SD = 0.016 \quad (11)$$

$${}^S_w pK_a(\text{ACN-W}) = 266.47(12) \frac{1}{D} + 8.354(0.17)$$

$$R^2 = 0.9940 \quad SD = 0.020 \quad (12)$$

From Table 3 it is evident that in all the EtOH-W mixtures with lower D values than the corresponding ACN-W the acid dissociation of the flavones is higher. This comparison can be made quantitatively using eqs 7 to 12. For example, ${}^S_w pK_a$ values of 7(OH)Fl in a mixture with $D = 65$ obtained by means of eqs 7 and 8 were 7.68 ± 0.02 and 8.35 ± 0.05 , respectively. These differences in the ${}^S_w pK_a$ values might be explained taking into consideration the different solute–solvent intermolecular forces that take place in the dissociation reaction. Electrostatic interactions as well as specific interactions play significant role in this process. It is known that the solvation of anions is effective in protic solvents where hydrogen bond may be formed between the proton of the solvent and the lone pairs of the anions.⁴⁴ This kind of interactions can be analyzed with the empirical parameter α , which measures the hydrogen bond donating (HBD) ability of a solvent. The α values for the ethanol and acetonitrile aqueous mixtures (Table 3) were obtained by interpolation of experimental data available in the literature.⁴⁵ The ${}^S_w pK_a$ values reported in Table 3 were plotted against the α parameters and the following expressions were obtained (the values in parentheses are the standard deviations): 7(OH)Fl

$${}^S_w pK_a(\text{EtOH-W}) = -2.889(0.14)\alpha + 10.75(0.15)$$

$$R^2 = 0.9931 \quad SD = 0.014 \quad (13)$$

$${}^S_w pK_a(\text{ACN-W}) = -5.192(0.080)\alpha + 13.31(0.09)$$

$$R^2 = 0.9993 \quad SD = 0.0088 \quad (14)$$

3(OH)Fl

$${}^S_w pK_a(\text{EtOH-W}) = -8.395(0.48)\alpha + 18.40(0.48)$$

$$R^2 = 0.9869 \quad SD = 0.059 \quad (15)$$

Table 3. Parameters of Henderson–Hasselbach Equation. Apparent Acidity Constants (${}^S_w\text{p}K_a$) of Hydroxyflavones at 298.15 K in Ethanol–Water and Acetonitrile–Water Solution^a

w_{EtOH}	α^b	D^c	slope	intercept (${}^S_w\text{p}K_a$)	R^2	w_{ACN}	α^b	D^d	slope	intercept (${}^S_w\text{p}K_a$)	R^2
7-Hydroxyflavone						7-Hydroxyflavone					
0.0946	1.16	73.22	1.04 (0.02)	7.41 (0.01)	0.9964	0.118	1.13	74.12	0.97 (0.02)	7.45 (0.01)	0.9973
0.138	1.13	70.67	0.96 (0.02)	7.49 (0.01)	0.9975	0.136	1.11	73.39	0.97 (0.01)	7.54 (0.007)	0.9988
0.179	1.10	68.26	1.00 (0.03)	7.56 (0.02)	0.9918	0.175	1.08	71.67	1.00 (0.003)	7.69 (0.001)	0.9999
0.216	1.07	66.08	1.05 (0.01)	7.65 (0.008)	0.9985	0.214	1.05	69.85	0.97 (0.03)	7.86 (0.02)	0.9949
0.281	1.03	62.25	1.00 (0.01)	7.79 (0.01)	0.9992	0.280	0.99	66.61	0.98 (0.01)	8.17 (0.01)	0.9986
3-Hydroxyflavone ^e						3-Hydroxyflavone					
0.203	1.08	66.84		9.39		0.146	1.10	72.95	0.95 (0.02)	9.16 (0.007)	0.9983
0.263	1.04	63.31		9.63		0.196	1.06	70.72	0.97 (0.01)	9.32 (0.005)	0.9992
0.303	1.01	60.96		9.83		0.256	1.01	67.81	1.02 (0.04)	9.58 (0.02)	0.9927
0.352	0.99	58.07		10.10		0.278	0.99	66.70	0.95 (0.02)	9.64 (0.007)	0.9984
0.406	0.96	54.89		10.36		0.324	0.96	64.28	0.98 (0.02)	9.85 (0.01)	0.9988
0.451	0.93	52.25		10.61							
5-Hydroxyflavone						5-Hydroxyflavone					
0.122	1.14	71.61	0.97 (0.03)	11.78 (0.016)	0.9942	0.102	1.14	74.76	1.01 (0.02)	11.91 (0.01)	0.9978
0.140	1.13	70.55	1.06 (0.01)	11.86 (0.009)	0.9985	0.137	1.11	73.35	0.99 (0.04)	11.98 (0.02)	0.9938
0.180	1.10	68.20	0.99 (0.01)	11.93 (0.009)	0.9984	0.195	1.06	70.76	1.03 (0.03)	12.15 (0.02)	0.9959
0.246	1.05	64.31	1.08 (0.02)	12.11 (0.008)	0.9983	0.255	1.01	67.85	0.98 (0.04)	12.27 (0.03)	0.9917
0.308	1.01	60.66	1.04 (0.02)	12.30 (0.01)	0.9945	0.310	0.97	65.02	0.97 (0.04)	12.45 (0.02)	0.9915

^aNotation: w , organic solvent fraction (w/w); α , hydrogen-bond donor capacity of the solvent mixtures. ^bReference 45; D , solvent relative permittivity at 298.15 K. ^cReference 30. ^dReference 32. ^eReference 19 and R^2 is the square regression coefficient of the least-squares linear fit. The standard uncertainties u are $u(w) = 0.001$, $u(T) = 0.01$ K. Values in parentheses are standard fitting deviations at the 95 % confidence level.

$${}^S_w\text{p}K_a(\text{ACN-W}) = -4.840(0.24)\alpha + 14.47(0.27)$$

$$R^2 = 0.9909 \quad \text{SD} = 0.030 \quad (16)$$

5(OH)Fl

$${}^S_w\text{p}K_a(\text{EtOH-W}) = -3.777(0.23)\alpha + 16.10(0.25)$$

$$R^2 = 0.9886 \quad \text{SD} = 0.026 \quad (17)$$

$${}^S_w\text{p}K_a(\text{ACN-W}) = -3.115(0.18)\alpha + 15.45(0.19)$$

$$R^2 = 0.9902 \quad \text{SD} = 0.025 \quad (18)$$

In the analyzed aqueous–organic mixtures, the acid–base dissociation of hydroxyflavones is enhanced when the polarity and the HBD of the reaction media increases. Solvation by polarity affects both anions and cations, whereas the solvation of anions is effective in protic solvent (HBD). Although there is a dipole–dipole interaction between the polar hydroxyflavones and the polar solvents, these are less important than the aforementioned specific interaction. Finally it is important to notice the HBD ability reflects the interaction of a solute with its immediate surroundings, while the dielectric constant is a bulk property of the mixture as a whole.

Equations 7 to 12 and 13 to 18 describe the solvent effects on the acid–base equilibria and allow the determination of $\text{p}K_a$ values at 298.15 K in pure water ($D = 78.39$, $\alpha = 1.24$) and at ionic strength $0.050 \text{ mol}\cdot\text{kg}^{-1}$ (${}^W_w\text{p}K_{aI=0.05}$). Table 4 summarizes these values in aqueous solution, and the final reported ${}^W_w\text{p}K_{aI=0.05}$ is taken as an average of the values listed in this Table. These results reflect the different intermolecular forces acting between the solute and the solvent molecules:

7(OH)Fl

$${}^W_w\text{p}K_{aI=0.05} = 7.11 \pm 0.17$$

Table 4. Estimated Acidity Constants ($\text{p}K_a$) Values in Water at 298.15 K and Ionic Strength $0.050 \text{ mol}\cdot\text{kg}^{-1}$ (${}^W_w\text{p}K_{aI=0.05}$), from eqs 7 to 12 and 13 to 18, Together with Error Limits Using the 95 % Confidence Level. In Parentheses Are the Numbers of These Equations

${}^W_w\text{p}K_{aI=0.05}$		
7-hydroxyflavone	3-hydroxyflavone	5-hydroxyflavone
7.27 ± 0.02 (7)	8.75 ± 0.08 (9)	11.56 ± 0.05 (11)
7.13 ± 0.05 (8)	8.81 ± 0.07 (10)	11.75 ± 0.06 (12)
7.17 ± 0.06 (13)	8.00 ± 0.32 (15)	11.42 ± 0.11 (17)
6.87 ± 0.04 (14)	8.47 ± 0.18 (16)	11.59 ± 0.10 (18)

3(OH)Fl

$${}^W_w\text{p}K_{aI=0.05} = 8.51 \pm 0.37$$

5(OH)Fl

$${}^W_w\text{p}K_{aI=0.05} = 11.58 \pm 0.14$$

The ${}^W_w\text{p}K_{aI=0.05}$ of 7(OH)Fl indicates that this compound is sensitive to deprotonation at physiological pH (7.4). On the other hand, in 3(OH)Fl and 5(OH)Fl the intramolecular hydrogen bond hinders deprotonation. Then, their $\text{p}K_a$ values are above the physiological pH range. This factor is very important when the antioxidant activity of flavonoids at this pH is studied.

To obtain the thermodynamic acid dissociation constants ${}^W_w\text{p}K_{aI=0}$ of these compounds, the ${}^W_w\text{p}K_{aI=0.05}$ values were corrected by means of the Debye–Hückel equation (eq 19), valid for ionic strength values up to 0.1. The activity coefficients of the neutral molecules were assigned a value of unity, whereas the activity coefficients of the ions were estimated as

$$\log \gamma_i = -\frac{Az_i^2\sqrt{I}}{(1 + a_iB\sqrt{I})} \quad (19)$$

where z_i is the charge of the ionic species, A and a_iB are parameters of this equation and can be calculated by using the density and dielectric constant of water at 298.15 K and by adopting the Bates-Guggenheim convention, which considers a constant value of 4.56 Å for the Debye radius (a_i).³⁹ According to this, the activity coefficients of a cation or an anion in aqueous medium, can be expressed as

$$\log \gamma_i = -\frac{z_i^2 0.51 \sqrt{I}}{(1 + 1.5 \sqrt{I})} \quad (20)$$

The ${}^W pK_{a,I=0}$ values obtained by eq 20 were

7(OH)Fl

$${}^W pK_{a,I=0} = 7.28 \pm 0.17$$

3(OH)Fl

$${}^W pK_{a,I=0} = 8.68 \pm 0.37$$

5(OH)Fl

$${}^W pK_{a,I=0} = 11.75 \pm 0.14$$

The thermodynamic acid dissociation constants of the three analyzed flavones in pure water and at zero ionic strength have not been reported. In Table 5, the experimental conditions

Table 5. Experimental Acidity Constants (${}^W pK_{a,I=0}$) Values Determined in This Work, Available from the Literature and Predicted with SPARC Online Calculator in Aqueous Medium

compound	${}^W pK_{a,I=0}$	pK_a from literature	pK_a predicted with SPARC ^e
7-hydroxyflavone	7.28 ± 0.17	8.48 ^a	7.30
		8.12 ^b	
		7.19 ^c	
3-hydroxyflavone	8.68 ± 0.37	8.52 ^d	9.10
		10.34 ^a	
		9.6 ^b	
5-hydroxyflavone	11.75 ± 0.14	11.44 ^a 11.56 ^b	9.21

^aReference 15. Experimental conditions: potentiometric titration, 25 °C, 1,4-dioxane-water 50 % (v/v), $I = 0.1$. ^bReference 16. Experimental conditions: spectrophotometer procedure, 21 °C, EtOH–water (1:1), $I \sim 0.1$. ^cReference 17. Experimental conditions: spectrophotometer procedure, 25 °C, aqueous solution with DMSO ~ 0.5 %, $I = 0.1$. ^dReference 19. Experimental conditions: spectrophotometer procedure, 25 °C, EtOH–water, $I = 0.05$. pK_a extrapolated to pure water. ^eReference 46. Error limits in ${}^W pK_{a,I=0}$ are the average uncertainties of ${}^W pK_{a,I=0.05}$ of Table 4.

informed in the literature regarding with the determination of the pK_a of these three compounds are summarized.^{15–17,19} The same Table also displays predicted values in aqueous medium with the SPARC online calculator.⁴⁶ It is important to notice that any comparison between pK_a values obtained by different experimental methods makes sense if the experimental conditions were the same during the measurements. Thus, some differences can be observed between the previously reported pK_a values and the obtained values in the present study,

especially for 7(OH)Fl and 3(OH)Fl. In this work, the same experimental conditions were adopted for a proper comparison of the pK_a values of the studied flavones. Several measurements were made in aqueous–organic mixtures, with low percentages of organic solvent. In these water-rich mixtures, the extrapolation method (${}^S pK_a$ vs $1/D$) leads to good results. From the solvent effect analysis, specific and nonspecific solute–solvent interactions were considered. For this reason, the ${}^W pK_{a,I=0}$ reported in this work can be considered as consistent and suitable for our purpose.

4.4. Molecular Modeling. To estimate an acidity constant (K_a) value from DFT calculations, a reaction scheme must be proposed in the first place. The choice of an appropriate reaction model is very important, since the numerical values of the calculated constants will strongly depend on the selected scheme. In the present work, five different schemes have been analyzed and they are depicted in Table 6, along with the

Table 6. Reaction Schemes Employed for the Calculations of the Acidity Constants (pK_a) Using the Thermodynamic Cycle Methodology^a

scheme	reaction	pK_a
A	$HA \leftrightarrow H^+ + A^-$	$\Delta G_s/RT \ln(10)$
B	$HA + H_2O \leftrightarrow H_3O^+ + A^-$	$\Delta G_s/RT \ln(10) - \log [H_2O]$
C	$HA + 2H_2O \leftrightarrow H_3O^+ + A^- (H_2O)$	$\Delta G_s/RT \ln(10) - 2 \log [H_2O]$
D	$HA + \text{Ref}^- \leftrightarrow \text{HRef} + A^-$	$\Delta G_s/RT \ln(10) + pK_a(\text{HRef})$
E	$HA + OH^-(3H_2O) \leftrightarrow A^-(H_2O) + 3H_2O$	$\Delta G_s/RT \ln(10) + 14 + 3 \log [H_2O]$

^a ΔG_s , Gibbs energy change in solution; HA, neutral hydroxyflavones; A^- , ionized form of hydroxyflavones; HRef and Ref^- , neutral 4'-hydroxyflavone and the corresponding anion.

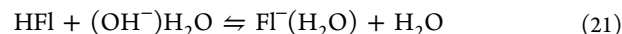
equations employed for the calculation of the pK_a values. The ΔG_s values necessary to obtain the pK_a have been calculated using a strategy based on thermodynamic cycles.²⁶ It can be seen from this Table that scheme A corresponds to the simplest way to describe an acid–base dissociation. In spite of its simplicity, this scheme has a major drawback; the experimental solvation free energy ($\Delta_{\text{solv}}G$) of the proton is required. There is an important variation in the reported experimental values of this magnitude, introducing a source of error in the calculation of the pK_a . In this work the $\Delta_{\text{solv}}G(H^+) = -1112.48 \text{ kJ}\cdot\text{mol}^{-1}$ and $G_{298}^0(H^+) = -18.36 \text{ kJ}\cdot\text{mol}^{-1}$ are used, following some previous recommendations.^{27,47} An alternative method to avoid experimental values of $\Delta_{\text{solv}}G(H^+)$ is the use of water molecules as a coreactant (schemes B and C). Scheme D corresponds to an isodesmic reaction method, where HRef is an appropriate hydroxylated flavone with experimentally known pK_a value. A serious problem of this approach is that its result is highly dependent on the chosen reference acid (HRef). However, it gives very good results since the number and the kind of charged species are conserved on both sides of the chemical equation.^{27,48} In this work 4'-hydroxyflavone is chosen as a reference compound ($pK_{a,\text{exp}} = 8.28$).⁴⁹ Finally, scheme E also conserves the number and kind of charged species; but it is independent from experimental values and it has been proved that it reproduces very well the experimental pK_a values of large phenolic derivatives.²⁷ The thermodynamic parameters calculated for all the chemical species considered in schemes A to E are given in Table 7. From these parameters the ΔG_s values are obtained using eqs 1 to 3.

Table 7. Calculated Thermodynamic Parameters of the Studied Compounds, Together with All the Chemical Species Involved in the Equilibria Depicted in Table 6 and in eqs 21 and 23^a.

	7(OH)Fl	7O ⁻ Fl	7O ⁻ Fl(H ₂ O)	SOHFl	SO ⁻ Fl	SO ⁻ Fl(H ₂ O)	SOHFl	3O ⁻ Fl	3O ⁻ Fl(H ₂ O)	4 ⁻ O ⁻ Fl	H ₂ O	OH ⁻ (H ₂ O)	H ₃ O ⁺	OH ⁻ (3H ₂ O)
<i>E</i> /Hartree	-803.529242	-802.9807233	-879.4751529	-803.5425736	-802.9740553	-879.459673	-803.532458	-802.9801175	-879.464472	-803.5300951	-76.4595255	-152.3341619	-76.7316321	-305.320427
<i>E</i> /kJ·mol ⁻¹	-2109971.17	-2108528.08	-2309393.00	-2110003.43	-2108510.57	-2309352.35	-2109976.87	-2108526.49	-2309364.95	-2109970.67	-200773.26	-400010.67	-201487.78	-801733.68
ZPVE/kj·mol ⁻¹	555.69	520.40	584.06	557.90	517.98	583.78	558.32	519.30	584.95	519.76	55.92	80.60	90.34	217.83
<i>H</i> _{corr} /Hartree	0.2263	0.212385	0.240127	0.226579	0.211847	0.240027	0.226911	0.212057	0.240125	0.212306	0.025078	0.035879	0.038256	0.093714
<i>H</i> _{corr} /kJ·mol ⁻¹	594.15	557.70	630.54	594.97	556.28	630.28	593.84	556.84	630.54	557.49	65.85	94.21	100.46	246.08
<i>S</i> ^o /J·K ⁻¹ ·mol ⁻¹	491.25	489.39	570.58	481.51	497.59	571.74	488.64	484.22	553.13	492.72	188.66	256.89	202.44	414.36
<i>T</i> · <i>S</i> ^o /kJ·mol ⁻¹	146.47	145.91	170.12	143.56	148.36	170.47	145.69	144.37	164.92	146.90	56.25	76.59	60.36	123.54
<i>G</i> ^o /kJ·mol ⁻¹	-2108967.79	-2107595.90	-2308348.51	-2108994.13	-2107584.66	-2308308.75	-2108968.40	-2107594.72	-2308314.37	-2108967.70	-200707.74	-399912.45	-201357.34	-801393.31
<i>E</i> _{PCM} /Hartree	-803.5546574	-803.0844666	-879.5633653	-803.5563902	-803.0724556	-879.5515088	-803.5454886	-803.0691261	-879.548214	-803.5550036	-76.4710683	-152.4564216	-76.9062421	-305.426317
<i>G</i> _o /kJ·mol ⁻¹	-2109031.79	-2107868.32	-2308580.14	-2109030.41	-2107843.05	-2308549.90	-2109002.61	-2107828.45	-2308534.27	-2109033.11	-200738.05	-400233.49	-201815.85	-801671.37

^aNotation: *E*, gas phase total energy; ZPVE, zero-point vibrational energy; *H*_{corr}, thermal correction to enthalpy; *S*^o₂₉₈, entropy; *T*, temperature; *G*^o₂₉₈, Gibbs energy in gas phase; *E*_{PCM}, total energy in solution; *G*_o, Gibbs energy in solution; 7OHFl, SOHFl, 3OHFl, and 4⁻O⁻Fl, neutral form of the hydroxyflavones; 7O⁻Fl, SO⁻Fl, 3O⁻Fl, and 4⁻O⁻Fl, ionized form of the hydroxyflavones; 7O⁻Fl(H₂O), SO⁻Fl(H₂O), 3O⁻Fl(H₂O), ionized form of the hydroxyflavones hydrated with one water molecule; OH⁻(H₂O), hydroxyl ion hydrated with one water molecule; OH⁻(3H₂O), hydroxyl ion hydrated with three water molecules.

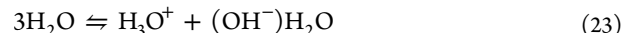
The calculated *pK*_a of the studied flavones using these schemes are listed in Table 8. It can be seen from this Table that the closer values to experimental results are obtained with schemes A and D. The average error associated with the first model is higher than the isodesmic model. This may be attributed to the uncertainty in the experimental $\Delta_{\text{sol}}G(H^+)$ value, estimated in 8.4 kJ·mol⁻¹,²⁶ while scheme D has the advantage of partial error cancellation. The inclusion of water molecules (B and C) does not improve the theoretical results; on the contrary, it makes them worse. Both schemes involve the generation of anions and cations from neutral molecules; and the errors in energy calculations of ionic species by continuum solvation methods are very high, typically around 17 kJ·mol⁻¹.²⁶ In addition, the proton is solvated by only one molecule of water in schemes B and C. A better calculation of the proton solvated energy should require a more realistic representation, that is, the H⁺ solvated by a large aqueous cluster. In this representation the charge found on H⁺ will delocalize across local networks of hydrogen-bonded water molecules.⁵⁰ Scheme E yields *pK*_a values of intermediate accuracy, better than B and C but worse than A and D. As mentioned above, Scheme E conserves the number and kind of charged species, resulting in error cancellation, and it does not require any experimental value. These results suggest that experimental quantities are necessary to obtain theoretical *pK*_a values of hydroxyflavones from thermodynamic cycles with a reasonable accuracy (± 2 units of *pK*_a). For this reason, and taking into account that the presence of water molecules is critical in the acid–base equilibrium, an alternative procedure to estimate the *pK*_a values from DFT results is proposed. This procedure assumes the reaction of the neutral acid with a hydrated hydroxyl to give the corresponding solvated anion and a water molecule.



To simplify the discussion the approximation of replacing the activities in equilibrium constants for the molar concentrations will be apply in this analysis. Then, for this reaction the equilibrium constant, *K*_C, can be defined as follows:

$$K_C = \frac{[\text{Fl}^-(\text{H}_2\text{O})][\text{H}_2\text{O}]}{[\text{HFl}][(\text{OH}^-)\text{H}_2\text{O}]} \quad (22)$$

In addition, the dissociation of water molecules (autoprotolysis) in aqueous solutions must be considered as well. For a convenient representation of the autoprotolysis of water, in this work, the following reaction is considered:²⁰



The equilibrium written on eq 23 considers that both H⁺ and OH⁻ ions are solvated with one water molecule. For this reaction, the equilibrium constant *K*_N can be defined:

$$K_N = \frac{[\text{H}_3\text{O}^+][(\text{OH}^-)\text{H}_2\text{O}]}{[\text{H}_2\text{O}]^3} \quad (24)$$

Furthermore, the autoprotolysis constant of water *K*_W can be formulated as

$$K_W = [\text{H}_3\text{O}^+][(\text{OH}^-)\text{H}_2\text{O}] \quad (25)$$

The constant *K*_N can be related to *K*_W by combining eqs 24 and 25,

Table 8. Calculated Acidity Constants (pK_a) Values of Hydroxyflavones in Water at B3LYP/6-311+(2d,p) Level of Theory Using Different Reaction Schemes. The Unsigned Errors Are Indicated in Parentheses

compound	reaction schemes					exp
	A	B	C	D	E	
7-hydroxyflavone	5.72 (1.56)	13.26 (5.98)	16.12 (8.84)	7.81 (0.53)	3.27 (4.01)	7.28
3-hydroxyflavone	7.59 (1.09)	15.14 (6.46)	19.04 (10.36)	9.69 (1.01)	6.19 (2.49)	8.68
5-hydroxyflavone	9.90 (1.85)	17.45 (5.70)	21.17 (9.42)	12.00 (0.25)	8.32 (3.43)	11.75

$$K_N = \frac{K_W}{[H_2O]^3} \quad (26)$$

where $[H_2O]$ is the molar concentration of water. Therefore, at 298.15 K the numerical value of K_W^{51} is $1.008 \cdot 10^{-14}$ and then $K_N = 5.88 \cdot 10^{-20}$. Finally, combining eqs 22 and 24, the equilibrium constant for the global reaction can be expressed as:

$$K_C K_N = \frac{[F^-(H_2O)][H_3O^+]}{[HF][H_2O]^2} = \frac{K_a}{[H_2O]^2} \quad (27)$$

$$K_a = K_C K_N [H_2O]^2 \quad (28)$$

If the K_C value is calculated, the pK_a of the compound can be obtained by using eq 28. The K_C values are estimated from the known expression $\ln K_C = -\Delta G_s/(RT)$, and the ΔG_s are the corresponding to the reaction describe on eq 21. In Table 9 the different equilibrium constants (K_C , K_a) proposed for this methodology are listed along with the pK_a values calculated for the studied flavones. It can be seen from these results, that the

Table 9. Gibbs Energy Change in Solution (ΔG_s) and equilibrium constants K_C and K_a Calculated from eqs 22 and 28, Respectively

	7-hydroxyflavone	5-hydroxyflavone	3-hydroxyflavone
$\Delta G_s/\text{kJ}\cdot\text{mol}^{-1}$	-52.912	-24.046	-36.211
K_C	$1.880 \cdot 10^9$	$1.640 \cdot 10^4$	$2.250 \cdot 10^6$
K_a	$3.411 \cdot 10^{-7}$	$2.976 \cdot 10^{-12}$	$4.082 \cdot 10^{-10}$
pK_{aDFT}	6.47	11.53	9.39
pK_{aexp}	7.28 ± 0.17	11.75 ± 0.14	8.68 ± 0.37

errors are comparable with the ones obtained using the scheme of reaction D, but the experimental pK_a (HRef) is no longer necessary. In addition, a careful examination to eqs 21 and 23 demonstrates that the global acid–base dissociation reaction considered in this method is coincident with the reaction depicted on scheme C (Table 6). However, in the case of hydroxyflavones, the predicted pK_a values obtained with this alternative methodology are closer to the experimental values than the pK_a values predicted with scheme C.

4.5. NBO Analysis. By looking at Tables 8 and 9, it is important to notice that the ordering of the pK_a values is coincident with the experimental one regardless of the method used, this is to say, pK_a 7(OH)Fl < pK_a 3(OH)Fl < pK_a 5(OH)Fl. This behavior can be explained in terms of the intramolecular H bond that these molecules present. As can be seen from Figure 1, 7(OH)Fl do not form an intramolecular H-bond. The other two flavones, 3(OH)Fl and 5(OH)Fl, present this H-bond, resulting in a five member and a six member closed ring, respectively. To quantify the strength of this interaction a NBO analysis was performed on these flavonoids. For this, the interaction among the lone pair (LP) of the electron donor, the carbonylic oxygen, and the antibonding (σ^*) orbitals of the hydroxyl group was examined. The results of this treatment have been summarized in Table 10. It can be seen that the stabilization energy (ΔE_{ij}) due to the formation of the H-bond is higher in 5(OH)Fl than in 3(OH)Fl. Moreover, the two LP orbitals of 5(OH)Fl interact with the σ^* orbital of the OH, while in 3(OH)Fl only one of the donor orbitals (LP_2) exhibits this interaction. A low energy difference ($\epsilon_j - \epsilon_i$) between the donor and the acceptor orbital favors hyperconjugation, and the strength of the H-bond is increased. The results in Table 10

Table 10. Results of NBO Analysis for 3-Hydroxyflavone (3(OH)Fl) and 5-Hydroxyflavone (5(OH)Fl) at B3LYP/6-311+G(2d,p) Level of Theory^a.

flavone	Φ_i	Φ_j	$\Delta E_{ij}/\text{kJ}\cdot\text{mol}^{-1}$	$\epsilon_j - \epsilon_i/\text{au}$	F_{ij}/au
3(OH)Fl	$LP_1 (O_{11})$	$\sigma^* (OH)$			
	$LP_2 (O_{11})$	$\sigma^* (OH)$	13.97	0.67	0.043
5(OH)Fl	$LP_1 (O_{11})$	$\sigma^* (OH)$	11.97	1.07	0.050
	$LP_2 (O_{11})$	$\sigma^* (OH)$	90.83	0.70	0.112
	orbital		energy	s/p character/%	
3(OH)Fl	$LP_1 (O_{11})$		-0.71154	59.5/40.5	
	$LP_2 (O_{11})$		-0.28158	0.2/99.8	
	$\sigma^* (OH)$		0.38831	H 100/0	
		(23.1 % O + 76.9 % H)		O 22/77.7	
5(OH)Fl	$LP_1 (O_{11})$		-0.68687	59.5/40.5	
	$LP_2 (O_{11})$		-0.31754	4.1/95.7	
	$\sigma^* (OH)$		0.38338	H 100/0	
		(22.1 % O + 77.9 % H)		O 24.5/75.2	

^aNotation: Φ_i and Φ_j , donor and acceptor orbitals, respectively; LP, lone pair orbitals; σ^* , antibonding orbitals; ΔE_{ij} , second order stabilization energy; $\epsilon_j - \epsilon_i$, energy difference of the interacting orbitals; F_{ij} , off-diagonal NBO Fock matrix element.

show that $\epsilon_j - \epsilon_i$ between LP_2 and $\sigma^*(OH)$ orbitals presents very similar values for 5(OH)Fl and 3(OH)Fl. For this reason, the larger ΔE_{ij} value of 5(OH)Fl can be explained in terms of the significantly larger F_{ij} , indicating a higher overlapping ability between LP_2 and $\sigma^*(OH)$. In addition, the interaction between LP_1 and $\sigma^*(OH)$ is notably lower than the corresponding one between LP_2 and $\sigma^*(OH)$ due to a large value of $\epsilon_j - \epsilon_i$. This result may be explained in terms of the lower energy of LP_1 and the mixed s/p character of this orbital (LP_2 is almost a pure p orbital), which reduces (5(OH)Fl) and prevents (3(OH)Fl) the hyperconjugation with the σ^* orbital. Taking into account that 7(OH)Fl does not present the intramolecular H-bond, and that this bond is stronger in 5(OH)Fl than in 3(OH)Fl, the proton dissociation is easier in 7(OH)Fl, then in 3(OH)Fl, and finally in 5(OH)Fl. These NBO results regarding the H-bond strength of the analyzed flavones are consistent with the ordering observed for the experimental and calculated pK_a values.

5. CONCLUSIONS

The acidity constants of hydroxyflavones, sparingly soluble compounds in water, were determined in diverse EtOH-W and ACN-W solutions using a UV-visible spectroscopic method based on the Henderson-Hasselbalch equation. The differences in the ${}^s_w pK_a$ values for the same compound, in EtOH-W and ACN-W mixtures, were explained considering that the dissociation process is ruled by electrostatic interaction as well as by specific solute-solvent interactions. In the analyzed aqueous-organic mixtures, the acid-base dissociation of hydroxyflavones is enhanced when the polarity and the HBD of the reaction media increases. Using linear relationships between these solvent parameters and the ${}^s_w pK_a$ measured in aqueous-organic mixtures, the ${}^s_w pK_a$ values in pure water were obtained with enough accuracy. The dissociation constants were also calculated by means of DFT methods (B3LYP/6-311+G(2d,p) level of theory), employing five thermodynamic cycles reported in the literature and an alternative method proposed by us. The latter method takes into account the solvation of anions by one water molecule, in the partial neutralization of the hydroxyflavone and in the autoprotolysis of water reactions. The obtained results with this method are in very good agreement with the experimental values, with errors lower than 1 pK_a unit. Finally, the experimental ordering in the acidity of the hydroxyflavones indicates that the *p*-OH group ionizes more easily than the *o*-OH group. From the natural bond orbital (NBO) analysis, it can be seen that the stabilization energy due to the formation of the H-bond is higher in 5(OH)Fl than in 3(OH)Fl.

AUTHOR INFORMATION

Corresponding Author

*Tel.: +54-266-4424689. Fax: +54-266-4430224. E-mail: sblanco@unsl.edu.ar.

Funding

This work was supported by grants from National University of San Luis and by the Consejo Nacional de Ciencia y Tecnología (CONICET) project PIP 11220100100151 (Argentine Republic).

Notes

The authors declare no competing financial interest.

REFERENCES

- (1) Fuguet, E.; Rafols, C.; Roses, M. A Fast High Throughput Method for the Determination of Acidity Constants by Capillary Electrophoresis. 3. Basic Internal Standards. *J. Chromatogr. A* **2011**, *1218*, 3928–3934.
- (2) Herrero-Martínez, J. M.; Sanmartín, M.; Rosés, M.; Bosch, E.; Ràfols, C. Determination of Dissociation Constants of Flavonoids by Capillary Electrophoresis. *Electrophoresis* **2005**, *26*, 1886–1895.
- (3) Grbic, S.; Parojcic, J.; Malenovic, A.; Djuric, Z.; Maksimovic, M. A Contribution to the Glimepiride Dissociation Constant Determination. *J. Chem. Eng. Data* **2010**, *55*, 1368–1371.
- (4) Antonilli, M.; Bottari, E.; Festa, M. R.; Gentile, L. Coulometry: A Fine Procedure to Determine Acidity Constants of Slightly Soluble Acids. *J. Chem. Eng. Data* **2010**, *55*, 3373–3378.
- (5) Jabbari, M.; Gharib, F. Solute-Solvent Interaction Effects on Protonation Equilibrium of Some Water-Insoluble Flavonoids. *J. Solution Chem.* **2011**, *40*, 561–574.
- (6) Meloun, M.; Ferenčíková, Z.; Vrána, A. Determination of the Thermodynamic Dissociation Constant of Capecitabine Using Spectrophotometric and Potentiometric Titration Data. *J. Chem. Thermodyn.* **2011**, *43*, 930–937.
- (7) Benvidi, A.; Heidari, F.; Tabaraki, R.; Mazloum-Ardakan, M. Application of Principal Component-Wavelet Neural Network in Spectrophotometric Determination of Acidity Constants of 4-(2-thiazolylazo)-resorcinol. *Spectrochim. Acta A* **2011**, *78*, 1380–1385.
- (8) Şanlı, S.; Şanlı, N.; Alsancak, G. Spectrophotometric Determination of Acidity Constants of Some Macrolides in Acetonitrile-Water Binary Mixtures. *Acta Chim. Slov.* **2010**, *57*, 980–987.
- (9) Ebead, Y. H. The Role of the Medium on the Acid Dissociation Constants of Some Azo Dyes in View of Experimental and Theoretical Data. *J. Mol. Struct.* **2010**, *982*, 100–106.
- (10) Shoghi, E.; Romero, L.; Reta, M.; Ràfols, C.; Bosch, E. Enthalpies and Constants of Dissociation of Several Neutral and Cationic Acids in Aqueous and Methanol/Water Solutions at Various Temperatures. *J. Pharm. Biomed. Anal.* **2009**, *49*, 923–930.
- (11) Hamborg, E. S.; van Aken, C.; Versteeg, G. F. The Effect of Aqueous Organic Solvents on the Dissociation Constants and Thermodynamic Properties of Alkanolamines. *Fluid Phase Equilib.* **2010**, *291*, 32–39.
- (12) Sancho, M. I.; Almandoz, M. C.; Blanco, S. E.; Castro, E. A. Spectroscopic Study of Solvent Effects on the Electronic Absorption Spectra of Flavone and 7-Hydroxyflavone in Neat and Binary Solvent Mixtures. *Int. J. Mol. Sci.* **2011**, *12*, 8895–8912.
- (13) Masuoka, N.; Matsuda, M.; Kubo, I. Characterisation of the Antioxidant Activity of Flavonoids. *Food Chem.* **2012**, *131*, 541–545.
- (14) Cushnie, T. P. T.; Lamb, A. J. Recent Advances in Understanding the Antibacterial Properties of Flavonoids. *Int. J. Antimicrob. Agents* **2011**, *38*, 99–107.
- (15) Thompson, M.; Williams, C. R.; Elliot, G. E. P. Stability of Flavonoid Complexes of Copper(II) and Flavonoid Antioxidant Activity. *Anal. Chim. Acta* **1976**, *85*, 375–381.
- (16) Tyukavkina, N. A.; Pogodaeva, N. N. Ultraviolet Absorption of Flavonoids II. Ionization Constants of 7- and 4'-hydroxy Derivatives of Flavone and Flavonol. *Chem. Nat. Prod.* **1971**, *7*, 8–11.
- (17) Costantino, L.; Rastelli, G.; Albasini, A. A Rational Approach to the Design of Flavones as Xanthine Oxidase Inhibitors. *Eur. J. Med. Chem.* **1996**, *31*, 693–699.
- (18) Sauerwald, N.; Schwenk, M.; Polster, J.; Bensch, E. Spectrometric pK_a Determination of Daphnetin, Chlorogenic Acid and Quercetin. *Z. Naturforsch., B: J. Chem. Sci.* **1998**, *53*, 315–321.
- (19) Gasull, E. I.; Blanco, S. E.; Ferretti, F. H. Variation of 4-Hydroxychalcone and 3-Hydroxyflavone pK_a with Solvent Permittivity. *An. Asoc. Quím. Argent.* **1999**, *87*, 73–82.
- (20) Castro, G. T.; Ferretti, F. H.; Blanco, S. E. Determination of the Overlapping pK_a Values of Chrysin Using UV-vis Spectroscopy and ab Initio Methods. *Spectrochim. Acta A* **2005**, *62*, 657–665.
- (21) Herrero-Martínez, J. M.; Repollés, C.; Bosch, E.; Rosés, M.; Ràfols, C. Potentiometric Determination of Aqueous Dissociation Constants of Flavonols Sparingly Soluble in Water. *Talanta* **2008**, *74*, 1008–1013.
- (22) Ramešová, S.; Sokolová, R.; Degano, I.; Bulíčková, J.; Zabka, J.; Gál, M. On the Stability of the Bioactive Flavonoids Quercetin and

Luteolin Under Oxygen-free Conditions. *Anal. Bioanal. Chem.* **2012**, *402*, 975–982.

(23) Sancho, M. I.; Jubert, A. H.; Blanco, S. E.; Castro, E. A.; Ferretti, F. H. Determination of Dissociation Constants of *p*-hydroxybenzophenone in Aqueous Organic Mixtures. Solvent Effects. *Can. J. Chem.* **2008**, *86*, 462–469.

(24) Sancho, M. I.; Gasull, E. I.; Blanco, S. E.; Castro, E. A. Inclusion Complex of 2-Chlorobenzophenone with Cyclomaltoheptaose (β -cyclodextrin): Temperature, Solvent Effects and Molecular Modeling. *Carbohydr. Res.* **2011**, *346*, 1978–1984.

(25) Dávila, Y. A.; Sancho, M. I.; Almandoz, M. C.; Blanco, S. E. Structural and Spectroscopic Study of Al (III)-3-hydroxyflavone Complex. Determination of the Stability Constants in Water-Methanol Mixtures. *Spectrochim. Acta A* **2012**, *95*, 1–7.

(26) Ho, J.; Coote, M. L. A Universal Approach for Continuum Solvent pK_a Calculations: Are We There Yet? *Theor. Chem. Acc.* **2010**, *125*, 3–21.

(27) Rebolgar-Zepeda, A. M.; Campos-Hernández, T.; Ramírez-Silva, M. T.; Rojas-Hernández, A.; Galano, A. Searching for Computational Strategies to Accurately Predict pK_a s of Large Phenolic Derivatives. *J. Chem. Theory Comput.* **2011**, *7*, 2528–2538.

(28) Dean, F. M.; Podimuang, V. The Course of the Algar–Flynn–Oyamada (A.F.O.) Reaction. *J. Chem. Soc.* **1965**, 3978–3987.

(29) Segel, I. H. *Cálculos de Bioquímica*, 2nd ed.; Acibria: Spain, 1982.

(30) Akerlöf, G. Dielectric Constants of Some Organic Solvent–Water Mixtures at Various Temperatures. *J. Am. Chem. Soc.* **1932**, *54*, 4125–4138.

(31) Castro, G. T.; Giordano, O. S.; Blanco, S. E. Determination of the pK_a of Hydroxy-Benzophenones in Ethanol–Water Mixtures. Solvent Effects. *J. Mol. Struct. (Theochem)* **2003**, *626*, 167–178.

(32) Naderi, F.; Farajtabar, A.; Gharib, F. Protonation of Tetrakis(4-sulfonaphenyl)porphyrin in Aqueous Solutions of Acetonitrile and Dioxane. *J. Solution Chem.* **2012**, *41*, 1033–1043.

(33) Rosés, M.; Bosch, E. Influence of Mobile Phase Acid–Base Equilibria on the Chromatographic Behaviour of Protolytic Compounds. *J. Chromatogr. A* **2002**, *982*, 1–30.

(34) Becke, A. Density-Functional Exchange-Energy Approximation with Correct Asymptotic Behavior. *Phys. Rev. A* **1988**, *38*, 3098–3100.

(35) Lee, C.; Yang, W.; Parr, R. G. Development of the Colle–Salvetti Correlation-Energy Formula into a Functional of the Electron Density. *Phys. Rev. B* **1988**, *37*, 785–789.

(36) Cancès, E.; Mennucci, B.; Tomasi, J. A New Integral Equation Formalism for the Polarizable Continuum Model: Theoretical Background and Applications to Isotropic and Anisotropic Dielectrics. *J. Chem. Phys.* **1997**, *107*, 3032–3041.

(37) Reed, A. E.; Curtiss, L. A.; Weinhold, F. Intermolecular Interactions From a Natural Bond Orbital, Donor–Acceptor Viewpoint. *Chem. Rev.* **1988**, *88*, 899–926.

(38) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, J. A. Jr.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, I.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M. P.; Gill, M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; Pople, J. A. *Gaussian 03*, Revision B.01; Gaussian, Inc.: Pittsburgh, PA, 2003.

(39) Albert, A.; Serjeant, E. P. *The Determination of Ionization Constants*, 3rd ed.; Chapman and Hall: New York, 1984.

(40) Tiwari, S.; Ghosh, K. K.; Marek, J.; Kuca, K. Spectrophotometric Determination of the Acidity Constants of Some Oxime-Based α -Nucleophiles. *J. Chem. Eng. Data* **2010**, *55*, 1153–1157.

(41) İslamoğlu, F.; Kahveci, B.; Akyüz, E. Determination of Protonation Constants of Some 3-Alkyl(aryl)-4-(*p*-*tert*-butyl(benzyl/benzylidene)amino)-4,5-dihydro-1*H*-1,2,4-triazole-5-one Derivatives with Spectrophotometric Method. *J. Chem. Pharm. Res.* **2011**, *3*, 822–830.

(42) Ferreira, Q.; Gomes, P. J.; Ribeiro, P. A.; Jones, N. C.; Hoffmann, S. V.; Mason, N. J.; Oliveira, O. N.; Raposo, M. Determination of Degree of Ionization of Poly(allylamine hydrochloride) (PAH) and Poly[1-[4-(3-carboxy-4-hydroxyphenylazo)-benzene sulfonamido]-1,2-ethanediy], sodium salt] (PAZO) in Layer-by-Layer Films using Vacuum Photoabsorption Spectroscopy. *Langmuir* **2013**, *29*, 448–455.

(43) Laidler, K. J. *Chemical Kinetics*, 3rd ed.; Harper Collins: New York, 1987.

(44) Ruff, F.; Csizmadia, I. G. *Organic Reactions. Equilibria, Kinetic and Mechanism*; Elsevier: London, 1994.

(45) Park, J. H.; Jang, M. D.; Kim, D. S.; Carr, P. W. Solvatochromic Hydrogen Bond Donor Acidity of Aqueous Binary Solvent Mixtures for Reversed-Phase Liquid Chromatography. *J. Chromatogr.* **1990**, *513*, 107–116.

(46) Sparc On-Line Calculator, <http://ibmlc2.chem.uga.edu/sparc> (accessed January 2013).

(47) Camaioni, D. M.; Schwerdtfeger, C. A. Comment on “accurate experimental values for the free energies of hydration of H⁺, OH⁻, and H₃O⁺”. *J. Phys. Chem. A* **2005**, *109*, 10795–10797.

(48) Matulis, V. E.; Halauko, Y. S.; Ivashkevich, O. A.; Gaponik, P. N. CH Acidity of Five-Membered Nitrogen-Containing Heterocycles: DFT Investigation. *J. Mol. Struct. (Theochem)* **2009**, *909*, 19–24.

(49) Lemańska, K.; Szymusiak, H.; Tyrakowska, B.; Zieliński, R.; Soffers, A. E. M. F.; Rietjens, I. M. C. M. The Influence of pH on Antioxidant Properties and the Mechanism of Antioxidant Action of Hydroxyflavone. *Free Radical Biol. Med.* **2001**, *31*, 869–881.

(50) Keith, J. A.; Carter, E. A. Quantum Chemical Benchmarking, Validation, and Prediction of Acidity Constants for Substituted Pyridinium Ions and Pyridinyl Radicals. *J. Chem. Theory Comput.* **2012**, *8*, 3187–3206.

(51) Atkins, P. W. *Physical Chemistry*, 6 ed.; Oxford University Press: England, 1998.