Acid-Base Behavior of Fluorescein Isothiocyanate in Aqueous Media and in Micellar Surfactant Solutions

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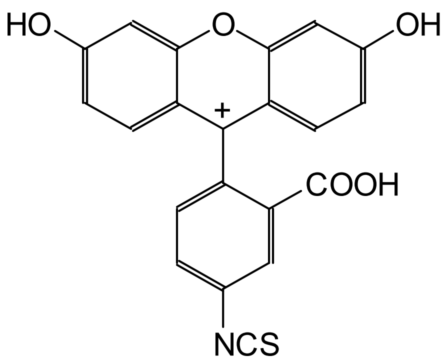
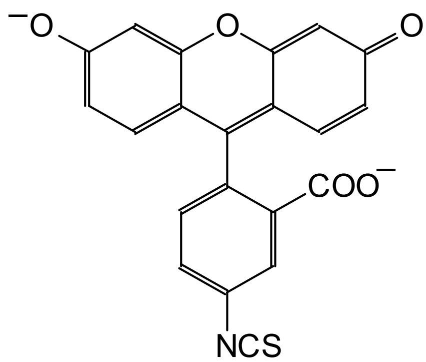
Abstract: The experimental data characterizing the acid-base behavior of fluorescein isothiocyanate (FLITC) in water and in micellar solutions of a cationic surfactant N-cetylpyridinium chloride are presented. Absorption spectra are tabulated within a wide pH region at ionic strength 0.05 M (298 K). The pK_a values of stepwise dissociation of FLITC ($H_3R^+ \rightleftarrows H_2R \rightleftarrows HR^- \rightleftarrows R^{2-}$) are calculated.

1. Introduction

Fluorescein isothiocyanate (FLITC) belongs to hydroxyxanthene dyes, which are widely used owing to their unique optical properties. Protolytic (acid-base and tautomeric) equilibria of fluorescein dyes have been intensively investigated for decades.[1-21] However, FLITC still was not studied in details, notwithstanding its wide utilization in fluorosensors and for other purposes.[22-24] Therefore, it is of interest to clarify the behavior of FLITC not only in water, but also in organized solutions. Thus, in the present communication we report the data characterizing the protolytic equilibria of FITC in water and in micellar solutions of a cationic surfactant, N-cetyl pyridinium chloride, CPC. The cationic and dianionic structures of the dye, H_3R^+ and R^{2-}, respectively, are shown below.





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Preliminary Vis-spectroscopic studies revealed, in accord with our expectations, that for this dye, ionization in aqueous solutions occurs stepwise:

$$H_3R^+ \rightleftharpoons H_2R + H^+ \;, \quad K_{a0} \tag{1}$$

$$H_2R \rightleftharpoons HR^- + H^+ \;, \quad K_{a1} \tag{2}$$

$$HR^- \rightleftharpoons R^{2-} + H^+ \;, \quad K_{a2} \tag{3}$$

The spectrophotometric method was used to determine dissociation constants, K_a.

2. Experimental

Fluorescein-5-isothiocyanate ('Isomer I'), $C_{21}H_{11}NO_5S$, 389.38, Spiro[isobenzofuran-1(3H),9'-(9H)xanthen]-3-one, 3',6'-dihydroxy-5-isothiocyanato- from Sigma (approx. 98%, HPLC) was kindly provided by Dr. Sergei A. Eremin, Department of Chemical Enzymology, Faculty of Chemistry, M.V. Lomonosov Moscow State University. CPC (Merk, 98%) was used as received.

To create the required pH values, analytical-grade hydrochloric acid, sodium hydroxide, acetic and phosphoric acids and sodium chloride were used. The ionic strength (I, M) of the solutions was, as a rule, constant: in the buffer solutions appropriate amounts of NaCl stock solutions were added to maintain the total I = 0.05 M, except solutions with higher HCl concentrations. In CPC solutions, the 'true' ionic strength was somewhat higher due to the presence of the surfactant. However, such small deviations from I = const display practically no influence on the equilibrium state.[17] The spectra of the H_3R^+ species were obtained in 2 and 3 M HCl solutions. The standard aq. sodium hydroxide solution was prepared using CO_2 –free water and kept protected from the atmosphere. The pH values of solutions were checked by means of potentiometry by using cells with a liquid junction (1 M KCl), glass electrode ESL-63-07, and a silver / silver chloride reference electrode, according to the compensation scheme on a potentiometer P 363/3 and pH meter-millivoltmeter pH-121. All the solutions were prepared and pH measurements performed at 298.0 ± 0.1 K. Standard buffer solutions (pH 1.68, 3.56, 4.01, 6.86, and 9.18) were used for cell calibration. The experimental uncertainty in the measured pH value did not exceed 0.02 pH unit (standard deviation). The spectra of the dye solutions were measured by using SP-46 apparatus (of USSR origin). The working dye concentrations were as a rule ca. 2×10^{-6} M, and in several cases somewhat higher. All the spectrophotometric experiments were performed at 298 ± 1 K.

3. Results

3.1 Determination of pK_a *values*

3.1.1 Aqueous solutions

The studies were carried out with a series of 38 solutions within the wavelength range λ = 400-550 nm and the acidity range from 3 M HCl to pH 12; dye concentration was 1.84×10^{-6} M, optical path length 5 cm. Most of the data are presented in Table 1. At fixed λ the dependence of absorbance, A, vs. pH can be described by eq. (4):[16-21]

$$A = \frac{A_{H_3R^+}(a_{H^+})^3 + A_{H_2R}(a_{H^+})^2 K_{a0} + A_{HR^-}a_{H^+}K_{a0}K_{a1} + A_{R^{2-}}K_{a0}K_{a1}K_{a2}}{(a_{H^+})^3 + (a_{H^+})^2 K_{a0} + a_{H^+}K_{a0}K_{a1} + K_{a0}K_{a1}K_{a2}} \quad (4)$$

($a_{H^+} = 10^{-pH}$). In this case only the $A_{H_3R^+}$ and $A_{R^{2-}}$ values can be measured directly at the appropriate acidity. The band of cation H_3R^+ has λ_{max} = 441 nm, with molar absorptivity $E_{H_3R^+} = 59.8\times10^3$ M^{-1} cm^{-1}, while the band of dianion R^{2-} is characterized by λ_{max} = 488 nm and $E_{R^{2-}}$ value, equal to 86.5×10^3 M^{-1} cm^{-1}.

As a first approximation, the fluorescein dissociation constants, K_{a0}, K_{a1}, and K_{a2}, were obtained by using the procedure of successive iterations, described earlier.[16] The spectrum at pH 3.78 was taken as the H_2R spectrum, and the pK_{a0} value was calculated within the pH range 1.31-2.47 (I = 0.05 M), at λ = 435, 440, and 445 nm: pK_{a0} = 2.04 ± 0.04. Hence, at pH ≥ 4 the concentration of cationic species is negligible. Then, the pK_{a1} and pK_{a2} values were calculated jointly with the A_{HR^-} values at pH 3.96-6.68 (I = 0.05 M) and 20 wavelengths, from λ = 420 nm to 515 nm, by using the CLINP program.[18,19,20,21] The next step was the refinement of A_{HR^-} values, by using eq. 5:

$$A_{HR^-} = A + (A - A_{H_2R})a_{H^+}K_{a1}^{-1} + (A - A_{R^{2-}})(a_{H^+})^{-1}K_{a2} \quad , \quad (5)$$

Table 1. Absorbances (A) of FLITC in aqueous solutions; 298 K, I = 0.05 M (at pH > 1.3).

pH	λ, nm																			
	420	425	430	435	440	445	450	455	460	465	470	475	480	485	490	495	500	505	510	515
H_3R^+	0.297	0.349	0.407	0.466	0.502	0.469	0.375	0.244	0.137	0.072	0.037	0.021	0.013	0.011	0.009	0.008	0.007	0.007	0.007	0.007
1.13	0.281	0.333	0.389	0.440	0.469	0.435	0.349	0.227	0.129	0.069	0.036	0.021	0.020	0.011	0.011	0.009	0.009	0.007	0.005	0.005
1.31	0.277	0.325	0.380	0.428	0.451	0.417	0.328	0.220	0.119	0.067	0.034	0.022	0.015	0.012	0.010	0.007	0.006	0.004	0.003	0.003
1.49	0.274	0.323	0.379	0.423	0.438	0.400	0.312	0.206	0.117	0.067	0.036	0.026	0.021	0.018	0.015	0.012	0.009	0.007	0.007	0.004
1.65	0.273	0.317	0.369	0.407	0.412	0.374	0.292	0.192	0.112	0.067	0.042	0.032	0.026	0.022	0.021	0.017	0.014	0.011	0.010	0.009
1.82	0.267	0.312	0.359	0.393	0.395	0.354	0.272	0.184	0.109	0.069	0.048	0.039	0.032	0.031	0.027	0.021	0.017	0.014	0.012	0.010
1.99	0.266	0.307	0.349	0.377	0.374	0.328	0.253	0.173	0.109	0.074	0.054	0.048	0.044	0.037	0.035	0.028	0.023	0.022	0.016	0.015
2.23	0.250	0.287	0.325	0.350	0.342	0.294	0.228	0.155	0.101	0.070	0.055	0.049	0.045	0.042	0.032	0.027	0.020	0.016	0.014	0.011
2.47	0.242	0.277	0.308	0.330	0.319	0.276	0.212	0.147	0.101	0.076	0.062	0.058	0.052	0.047	0.040	0.034	0.025	0.019	0.014	0.013
3.78	0.185	0.208	0.223	0.231	0.224	0.213	0.194	0.174	0.158	0.152	0.149	0.148	0.140	0.119	0.093	0.064	0.042	0.028	0.019	0.015
3.96	0.182	0.203	0.220	0.225	0.227	0.220	0.209	0.192	0.178	0.171	0.172	0.171	0.159	0.138	0.106	0.073	0.047	0.030	0.020	0.015
4.00	0.177	0.197	0.213	0.227	0.235	0.238	0.237	0.224	0.213	0.208	0.209	0.210	0.199	0.166	0.125	0.084	0.052	0.032	0.021	0.014
4.15	0.178	0.198	0.213	0.224	0.231	0.231	0.223	0.213	0.199	0.195	0.195	0.195	0.179	0.153	0.116	0.078	0.051	0.031	0.019	0.015
4.36	0.175	0.199	0.213	0.225	0.238	0.244	0.244	0.235	0.223	0.220	0.222	0.220	0.204	0.175	0.133	0.087	0.056	0.035	0.021	0.016
4.60	0.180	0.201	0.218	0.234	0.250	0.265	0.268	0.262	0.252	0.249	0.252	0.250	0.234	0.194	0.148	0.097	0.061	0.039	0.025	0.018
4.82	0.179	0.197	0.215	0.233	0.256	0.273	0.282	0.278	0.270	0.269	0.273	0.270	0.254	0.213	0.161	0.105	0.064	0.039	0.023	0.015
5.00	0.174	0.194	0.211	0.231	0.256	0.277	0.290	0.287	0.278	0.279	0.286	0.286	0.265	0.223	0.169	0.112	0.069	0.040	0.024	0.015
5.21	0.171	0.189	0.206	0.229	0.257	0.282	0.294	0.294	0.290	0.288	0.297	0.298	0.278	0.242	0.182	0.120	0.074	0.042	0.024	0.013
5.48	0.167	0.185	0.203	0.225	0.253	0.282	0.298	0.300	0.295	0.298	0.308	0.312	0.298	0.264	0.209	0.143	0.089	0.048	0.028	0.018
5.60	0.162	0.179	0.199	0.220	0.249	0.277	0.294	0.298	0.297	0.298	0.308	0.317	0.307	0273	0.218	0.155	0.097	0.055	0.030	0.017
5.80	0.159	0.177	0.195	0.218	0.246	0.278	0.297	0.304	0.303	0.308	0.321	0.335	0.333	0.307	0.297	0.180	0.116	0.064	0.035	0.020
5.97	0.156	0.175	0.192	0.215	0.246	0.276	0.297	0.305	0.307	0.314	0.330	0.347	0.354	0.331	0.277	0.204	0.129	0.068	0.037	0.021
6.00	0.160	0.176	0.194	0.216	0.244	0.277	0.298	0.312	0.316	0.325	0.347	0.369	0.380	0.367	0.316	0.238	0.153	0.085	0.045	0.028
6.22	0.146	0.162	0.178	0.201	0.231	0.261	0.288	0.303	0.312	0.326	0.354	0.385	0.415	0.418	0.375	0.286	0.190	0.108	0.054	0.032
6.58	0.135	0.148	0.166	0.187	0.215	0.246	0.278	0.301	0.316	0.334	0.372	0.418	0.469	0.492	0.458	0.355	0.235	0.133	0.072	0.038
6.68	0.126	0.138	0.153	0.173	0.199	0.234	0.265	0.294	0.316	0.339	0.379	0.440	0.504	0.548	0.523	0.417	0.270	0.150	0.078	0.041
R^{2-}	0.073	0.082	0.093	0.105	0.128	0.163	0.213	0.266	0.312	0.354	0.413	0.520	0.664	0.777	0.796	0.684	0.466	0.258	0.128	0.060

Note. The H_3R^+ spectrum is measured in solutions with I > 0.05 M: C (HCl) = 1 and 2 M.
The R^{2-} spectrum is measured at pH = 9.58; 10.00; 11.79; 11.95.

Table 2. Absorbances (A) of FLITC in CPC solutions; 298 K, I = 0.05 M (at pH > 1.3).

pH	λ, nm																		
	435	440	445	450	455	460	465	470	475	480	485	490	495	500	505	510	515	520	530
H_3R^+	0.509	0.550	0.541	0.452	0.325	0.211	0.135	0.087	0.066	0.055	0.049	0.047	0.046	0.046	0.046	0.046	0.046	0.046	0.045
1.15	0.369	0.395	0.374	0.297	0.197	0.117	0.063	0.036	0.024	0.019	0.016	0.015	0.012	0.011	0.010	0.008	0.009	0.009	0.009
1.33	0.312	0.330	0.308	0.242	0.162	0.099	0.057	0.032	0.024	0.017	0.017	0.015	0.013	0.012	0.009	0.009	0.007	0.007	0.007
1.59	0.206	0.213	0.196	0.153	0.102	0.061	0.037	0.024	0.019	0.017	0.015	0.013	0.011	0.007	0.007	0.007	0.004	0.004	0.004
1.87	0.115	0.117	0.107	0.084	0.059	0.039	0.027	0.021	0.017	0.014	0.014	0.015	0.012	0.012	0.008	0.006	0.004	0.004	0.003
3.78	0.064	0.066	0.072	0.079	0.085	0.085	0.079	0.074	0.073	0.075	0.077	0.072	0.058	0.044	0.027	0.019	0.011	0.007	0.005
3.89	0.089	0.093	0.104	0.115	0.121	0.121	0.112	0.108	0.108	0.112	0.112	0.104	0.085	0.063	0.041	0.023	0.018	0.012	0.007
4.10	0.121	0.129	0.143	0.159	0.172	0.169	0.158	0.149	0.148	0.154	0.156	0.147	0.122	0.093	0.061	0.040	0.025	0.017	0.010
4.15	0.114	0.120	0.132	0.148	0.156	0.155	0.144	0.138	0.140	0.141	0.144	0.137	0.115	0.085	0.058	0.038	0.026	0.018	0.011
4.34	0.129	0.138	0.153	0.171	0.184	0.182	0.171	0.160	0.161	0.166	0.172	0.162	0.137	0.101	0.068	0.042	0.027	0.016	0.010
4.57	0.150	0.159	0.177	0.202	0.216	0.213	0.203	0.192	0.192	0.199	0.206	0.195	0.166	0.128	0.090	0.059	0.035	0.022	0.010
4.78	0.180	0.192	0.211	0.235	0.258	0.250	0.237	0.227	0.227	0.239	0.243	0.234	0.203	0.162	0.123	0.085	0.054	0.039	0.022
5.00	0.190	0.203	0.224	0.258	0.270	0.270	0.258	0.246	0.250	0.261	0.270	0.264	0.235	0.196	0.150	0.109	0.069	0.044	0.021
5.20	0.192	0.206	0.227	0.256	0.274	0.276	0.265	0.257	0.262	0.276	0.286	0.282	0.261	0.231	0.186	0.135	0.088	0.053	0.023
5.52	0.186	0.199	0.222	0.249	0.270	0.276	0.269	0.266	0.274	0.290	0.305	0.314	0.308	0.286	0.243	0.178	0.117	0.068	0.025
5.65	0.169	0.180	0.199	0.227	0.249	0.261	0.264	0.265	0.277	0.295	0.321	0.345	0.366	0.367	0.334	0.257	0.166	0.094	0.027
5.90	0.148	0.159	0.177	0.204	0.229	0.247	0.260	0.273	0.287	0.310	0.345	0.390	0.440	0.472	0.452	0.361	0.237	0.135	0.034
6.10	0.139	0.150	0.166	0.192	0.216	0.240	0.258	0.277	0.295	0.321	0.362	0.423	0.498	0.544	0.523	0.424	0.278	0.160	0.042
6.19	0.126	0.138	0.155	0.177	0.203	0.231	0.253	0.278	0.298	0.323	0.369	0.435	0.523	0.582	0.577	0.465	0.319	0.179	0.044
6.44	0.110	0.120	0.135	0.155	0.182	0.215	0.246	0.281	0.303	0.334	0.385	0.469	0.582	0.658	0.662	0.545	0.375	0.204	0.049
6.79	0.094	0.105	0.117	0.138	0.166	0.206	0.243	0.286	0.312	0.347	0.400	0.498	0.635	0.740	0.750	0.633	0.440	0.248	0.063
R^{2-}	0.063	0.073	0.085	0.102	0.128	0.172	0.223	0.274	0.308	0.341	0.400	0.509	0.672	0.801	0.854	0.762	0.535	0.312	0.072

Note. The H_3R^+ spectrum is measured in solutions with I > 0.05 M: C (HCl) = 1 and 2 M.
The R^{2-} spectrum is measured at pH = 8.11; 9.00; 9.94.

where A is absorption at the current pH value. The A_{H_2R} values were, in turn, calculated by using eq. 6:

$$A_{H_2R} = A + (A - A_{H_3R^+})a_{H^+}K_{a0}^{-1} + (A - A_{HR^-})(a_{H^+})^{-1}K_{a1} \quad . \tag{6}$$

Finally, the CLINP program was used once more; the data for 28 working solutions with various pH and 20 wavelengths, from λ = 420 nm to 515 nm, were utilized in the calculations with the result, referring to I = 0.05 M: $pK_{a0} = 2.05 \pm 0.03$; $pK_{a1} = 4.35 \pm 0.02$; $pK_{a2} = 6.62 \pm 0.01$.

3.1.2 Apparent dissociation constants in micellar CPC solutions

In 0.003 M CPC solutions, at bulk ionic strength I = 0.05 M, the absorption of 28 working solutions of FLITC (2.056×10^{-6} M) with different pH were measured at 19 wavelength, λ = 435-530 nm (Table 2). The determination of pK_{a1}^{a} and pK_{a2}^{a} values was made by the aforementioned procedure. The pK_{a0}^{a} value of FITC was obtained isolated from the other steps, at λ = 435, 440, and 445 nm, within the pH range 1.33–1.87 (HCl + NaCl), because in a more acidic region the ionic strength would be higher than 0.05 M. The final results are as follows: $pK_{a0}^{a} = 1.36 \pm 0.18$; $pK_{a1}^{a} = 4.31 \pm 0.01$; $pK_{a2}^{a} = 6.00 \pm 0.01$.

3.2 Molar absorptivities of ionic and molecular species

Having the K_{a1} and K_{a2} values (or pK_{a1}^{a} and pK_{a2}^{a} – in the case of micellar solutions), it was then possible to calculate the molar absorptivities of HR^- at various wavelengths, in such a way obtaining the spectra of these species (eq. 5). The interval $pK_{a1} \le pH \le pK_{a2}$ was used for this purpose; such procedure can be regarded as an additional step in the procedure of pK_a and A_{HR^-} iterative calculations described above. Molar absorptivity at each wavelength was calculated as $E_{HR^-} = A_{HR^-}c^{-1}l^{-1}$, where c is the working dye concentration, l is the optical path length.

The refinement of E_{H_2R} values of FLITC ($E_{H_2R} = A_{H_2R}c^{-1}l^{-1}$) was carried out at $pK_{a0} \le pH \le pK_{a1}$, with the help of equation (6), to avoid any influence of traces of intensely colored ions (H_3R^+ and HR^-) on the spectra of the neutral forms. The spectra were measured at different c values near to 1×10^{-5} M.

Thus obtained spectra of FLITC ionic and molecular species are depicted in Fig. 1, 2.

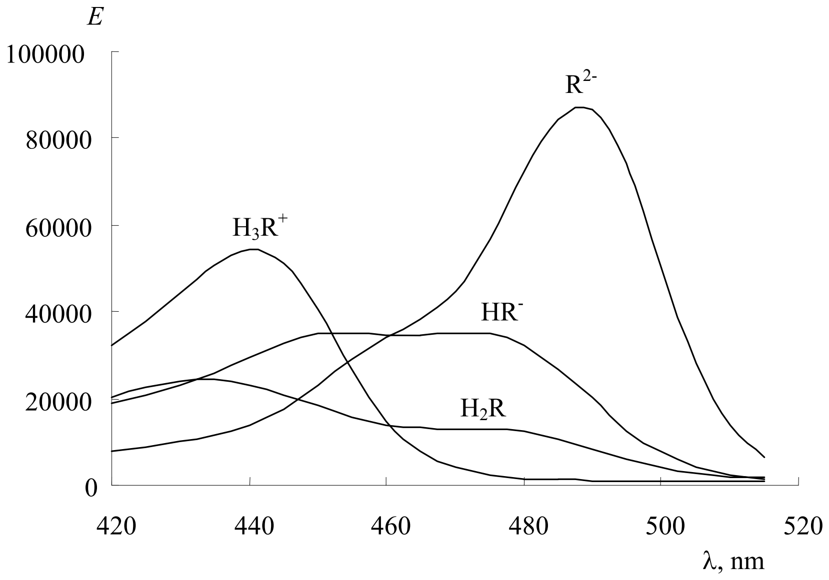

Fig.1. Absorption spectra of FLITC species in aqueous solutions, I = 0.05 M (except the cationic spectrum).

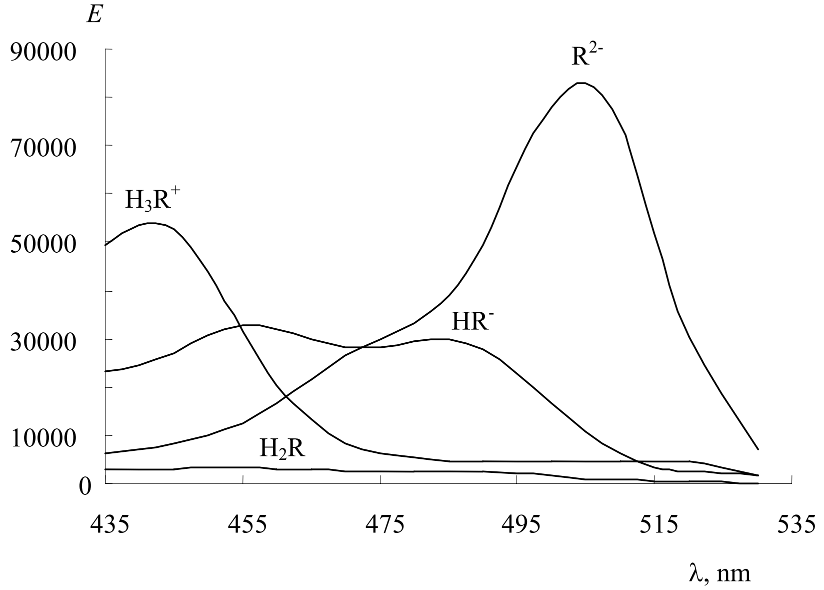

Fig.2. Absorption spectra of FLITC species in CPC micellar solutions, I = 0.05 M (except the cationic spectrum).

The detailed analysis of the protolytic (acid-base and tautomeric) equilibria of the dye in solutions will be published in the nearest future.

4. Conclusions

1) The pK_a values of FLITC in aqueous solutions at 298 K and I = 0.05 M are determined: $pK_{a0} = 2.05 \pm 0.03$; $pK_{a1} = 4.35 \pm 0.02$; $pK_{a2} = 6.62 \pm 0.01$.

2) The apparent pK_a^a values of FLITC in 0.003 M CPC solutions at 298 K and I = 0.05 M are determined: $pK_{a0}^a = 1.36 \pm 0.18$; $pK_{a1}^a = 4.31 \pm 0.01$; $pK_{a2}^a = 6.00 \pm 0.01$.

3) Molar absorptivities of ionic and molecular species of the dye are evaluated in the two aforementioned solvent systems.

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