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Protolytic fluorescein species evaluated using chemometry and DFT studies

Vagner Roberto Batistela [a], Jaime da Costa Cedran [b], Hueder Paulo Moisés de Oliveira [c], Ieda Spacino Scarminio [d], Leonardo Tsuyoshi Ueno [e], Antonio Eduardo da Hora Machado [f], Noboru Hioka [b,*]

[a] Centro de Tecnologia, Universidade Estadual de Maringá, Umuarama, Paraná, Brazil
[b] Departamento de Química, Universidade Estadual de Maringá, Av, Colombo, 5.790; CEP 87.020-900 Maringá, Paraná, Brazil
[c] Universidade Camilo Castelo Branco, S. José dos Campos, São Paulo, Brazil
[d] Departamento de Química, Laboratório de Quimiometria em Ciências Naturais, Universidade Estadual de Londrina, Londrina, Paraná, Brazil
[e] Faculdade de Ciências Integradas do Pontal, Laboratório de Química Teórica Computacional, Universidade Federal de Uberlândia, Ituiutaba, Minas Gerais, Brazil
[f] Instituto de Química, Laboratório de Fotoquímica, Universidade Federal de Uberlândia, Uberlândia, Minas Gerais, Brazil

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ABSTRACT

Fluorescein (C.I. Solvent Yellow 94) contains three acid-base groups that lead to a protolytic equilibrium involving four species namely, dianionic, monoanionic, neutral and cationic. Since these species display superimposed bands in their electronic absorption spectra and close pKa values, the determination of pKa using traditional methods is complex. By applying chemometric tools, principal component and Q factor analysis followed by varimax and Imbrie oblique rotations, pKa values in water, $pKa_1 = 2.5$, $pKa_2 = 3.8$ and $pKa_3 = 6.1$, were obtained. Geometric parameters secured using Density Functional Theory combined with a polarized continuum model that simulates the surrounding water molecules showed that the predominant neutral fluorescein structure is quinoid, while the monoanion is carboxylic. Time-Dependent Density Functional Theory predicted the origin of electronic transitions of each species, which agreed with the spectra generated using chemometrics.

1. Introduction

C.I. Solvent Yellow 94 (Fluorescein; FSC) enjoys widespread use in many areas of science, technology and medicine [1–4]; the dye has been employed, for example, as a fluorescent probe in detecting eye diseases [5–7]. The many applications of fluorescein are related to its high absorption in the visible region, namely high fluorescence emission, low toxicity and strong interaction with biomolecules [8]. In contrast, the dye is prone to photobleaching and has a complex protonation equilibrium in the ground state. The number of acidic groups in the dye suggests that pH plays an important role in its behavior, including light absorption and emission properties, which permits fluorescein to act as a probe for specific cell environments and biological targets [9]. Fluorescein is a xanthene dye, with the central carbon bonded to the position 2- of a benzoic acid (Fig. 1).

The commercial form of fluorescein is typically either the dianion (sodium salt) or neutral form. Its protolytic equilibrium involves four species namely the dianion (F^{2-}), the monoanion (FH^-), the neutral (FH_2) form, and the cation (FH_3^+), so that three acid-base equilibria are expected. These pKa have been assigned the approximate pH values of 2, 4, and 6 [10,11].

$$FH_3^+ + H_2O \rightleftharpoons FH_2 + H_3O^+ \quad pKa_1$$
$$FH_2 + H_2O \rightleftharpoons FH^- + H_3O^+ \quad pKa_2$$
$$FH^- + H_2O \rightleftharpoons F^{2-} + H_3O^+ \quad pKa_3$$

FH_3^+ and F^{2-} are the prevalent forms at extreme pH, the first one at low pH and the dianion at high or neutral pH (Fig. 1) [10,11]. The dianion molar absorptivity in water at pH ~ 9 is high; at 490 nm, in the present work the value of 88,000 L mol^{-1} cm^{-1} was used [11–13], although a value of 76,900 L mol^{-1} cm^{-1} have been quoted [10]. This species predominates in the range applicable to human physiological media (pH ~ 7.3) [10,11]. As pH decreases, protonation leads to the monoanion FH^- ($pKa_3 \sim 6$), which exist as two possible tautomers, the monoanionic carboxylate (MAC) and the monoanionic phenolate (MAF) [10] (Fig. 1). Subsequent decrease in pH produces the neutral form, FH_2, that exhibits three possible structures namely a lactone (NEL), a zwitterion (NEZ) and a quinoid (NEQ). The proposition of the existence of various tautomers for

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[*] Corresponding author. Tel.: +55 44 32613654; fax: +55 44 32614125.
E-mail address: nhioka@uem.br (N. Hioka).

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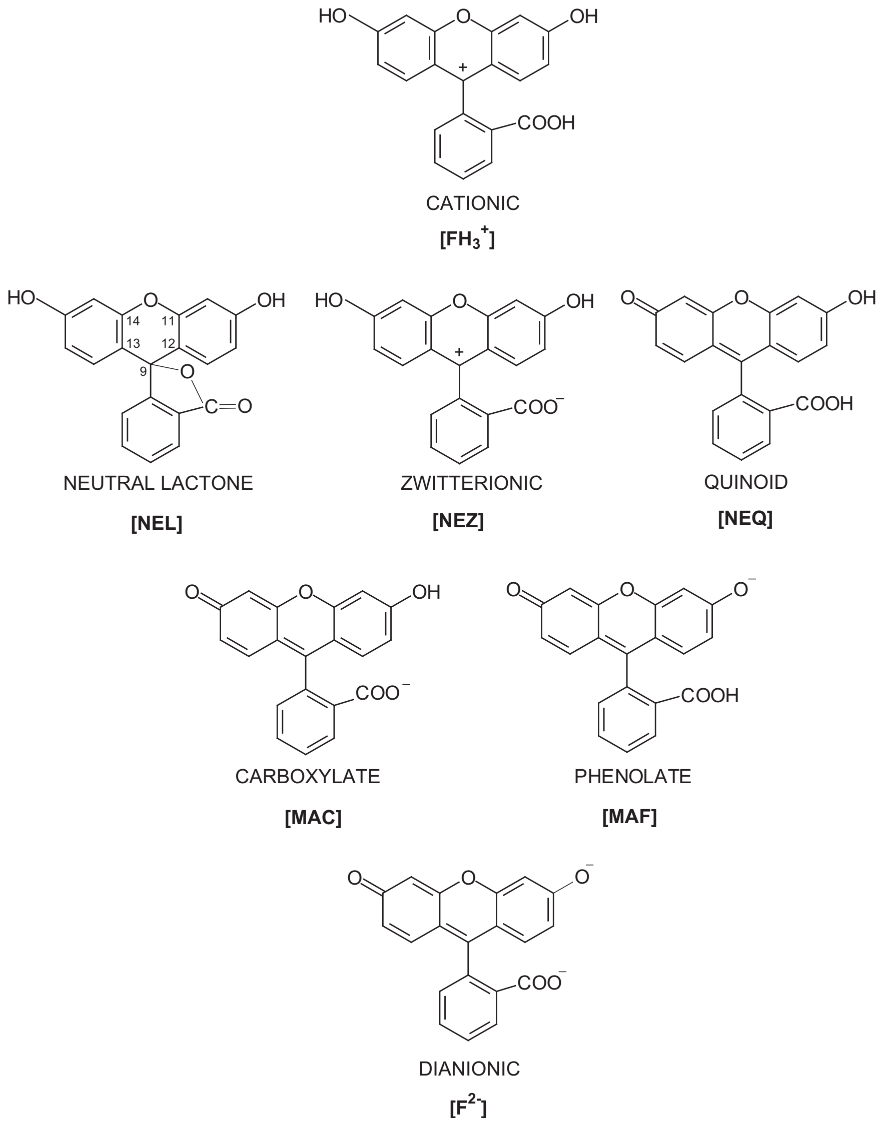

Fig. 1. FSC protolytic forms.

FH_2 is based on solid state studies, which determined that these structures show distinct diffraction patterns and IR spectra [14,15]. Additionally, the existence of the lactone (NEL) in DMSO is based on the IR detection of C=O stretching of lactones, $\nu_{max} = 1755\ cm^{-1}$ [16]. This structure was already isolated from frozen 1,4-dioxane anhydrous solutions [14]. The zwitterionic structure (NEZ) was proposed from solid Fluorescein (yellow) by IR data similarities with pyrylium salts [14,15]. However, the predominance of each tautomer in solution depends on the solvent effect.

As previously shown, the validity of pKa determination depends on the analytical method employed, including the mathematical treatment, and involves several experimental parameters, such as solvent, concentration, ionic strength, and temperature [17]. The analysis of the protolytic species of FSC by UV–Vis absorption spectrophotometry is difficult due to extensive spectral band overlapping and close pKa values. Classically, pKa estimations by UV–Vis are based on application of the univariate method in which absorption is monitored at one (or two) single wavelength(s) (the analytical wavelength(s)). However this method is recommended only in systems that presents low spectral superposition and pKa differences higher than 3 pH units ($\Delta pKa > 3$) [17]. Due to the importance of reliable pKa values for FSC, the chemometric approach could potentially be useful [10,18,19]. The chemometric approach involves analysis of all wavelengths simultaneously (full spectra), searching for the individual contributions of each species at each pH, and using many data points instead of a single analytical wavelength.

Although quantum chemical studies on protolytic FSC structures have been reported [20–27], these concern only a limited number of tautomers of each protolytic species and detailed aspects of structural parameters were unresolved. An important contribution was that of Tamulis et al. [20] who used Time-Dependent Density Functional Theory (TD-DFT) and suggested that the increased fluorescence of F^{2-} was attributable to its extensive molecular symmetry. Protonation decreases molecular symmetry and increases the number of allowed electronic states for the mono-anion (only MAC was considered), favoring the non-radiative deactivation of the excited state.

In the present work, pKa values for fluorescein were estimated using UV–Vis spectrophotometry and chemometric tools. The predominant tautomer of each protolytic species in water and vacuum were studied using several techniques based on Density Functional Theory and their electronic transition properties were characterised.