pH-Dependent Molecular Behaviors of a New Potential Color Additive: 4{[6-(1-carboxyethyl)-2-hydroxy -1-naphthyl]diazenyl}-3,5-dinitro Benzoic Acid

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Abstract: Phenyl azo hydroxynaphthalene (PAHNP) is the pharmacophore in certain color additives (e.g. sunset yellow, FD & C yellow No. 6) approved for use in human foods, drugs and cosmetics. The azo compound, 4{[6-(1-carboxyethyl)-2-hydroxy -1-naphthyl] diazenyl}-3,5-dinitrobenzoic acid (AZ-03) is a new, non-genotoxic, PAHNP analog, in a mono-azo chemical library. Process understanding of pH dependent behaviors of the highly functionalized molecule, is a critical aid to its identification. Absorption spectra of a fixed concentration of AZ-03 [3.30 x 10^{-5} M] in buffer solutions of varied pH were recorded. Spectra overlay suggested isosbestic point exists around 520 nm. Absorbance measurements of solutions (1.10 x 10^{-5} M) of pH 7, 10 and 11 were taken at 522, 524, 526 and 528 nm. Relative contribuiton of variables to overall variance was; wavelength (7%, p = 0.16), pH of media (57%, p < 0.0001), interaction between pH and wavelength (7%, p = 0.46, 2-way ANOVA, Bonferroni test). The probability that signal difference across pH was due to chance was 4 times greater at 522 nm (p=0.29) relative to 524 nm (p=0.08, 1-way ANOVA). The orange, unionized specie and violet, ionized specie both exhibited intense absorption, with molar absorptivities of 14,600 (490 nm) and 12,800 (550 nm) [L mol^{-1} cm^{-1}] respectively. AZ-03 was degraded by *pseudo* first-order kinetics in alkaline medium by specific-base catalysis. It was shown with some statistical rigor that the dye exhibits isosbestic point at 522 nm . Base-catalyzed degradation gave a non-linear and diagnostic rate-pH profile. These findings could aid precise authentication of this potential color additive, and thus facilitate further understanding and studies of its solution chemistry.

Keywords: 4-carboxyl-2,6-dinitrophenylazo hydroxynaphthalenes, color additive, non-genotoxic, isosbestic point, rate-pH profile.

INTRODUCTION

The aromatic amino acid; 4-amino-3,5-dinitrobenzoic acid (ADBA) is a very weakly basic, and hence, an intractable amine. The successful design and development of the corresponding diazonium ion; 4-carboxyl-2,6-dinitrobenzene diazonium ion (CDNBD) as a chromophoric labeling agent was documented by Idowu and co-workers [1-4]. It has been amply demonstrated as a useful derivatizing reagent in instrumental drug analysis [5-16]. CDNBD was also applied as synthetic intermediate in the rational design of a small chemical library of functionalized potential non-toxic azo dyes [17]. The monoazo dye library consists of 4 dyes prepared by diazo coupling reaction of CDNBD with β-naphthol (AZ-01), α-naphthol (AZ-02), naproxen (AZ-03) and nabumetone (AZ-04).

The new dye series, 4-carboxyl-2,6-dinitrophenylazo hydroxynaphthalenes (CDNPAHNP) has the pharmacophore: "phenyl azo hydroxynaphthalene". This structural moiety is common to allura red (FD & C Red No. 40) and sunset yellow (FD & C yellow No. 6), two azo dyes, out of many color additives, subject to batch certification, approved for use in human foods, drugs and cosmetics by the United States Food and Drug Administration [18].

Idowu *et al*., [19] documented the lipophilicity profiling of the dye series on a reversed phase chromatographic platform, and predicted lipophilicity ranking of AZ-03 < AZ-02 < AZ-01 < AZ-04; showing AZ-03 as the least lipophilic congener. Further physicochemical characterization by Adegoke *et al*., documented the solvatochromic behaviors [20] and relative dominance of hydrazone-azo tautomers [21]. Recently, Adegoke *et al*., [22] documented the *in vitro* evaluation of the genotoxicity potential of the CDNPAHNP's. Comet assay revealed AZ-03 and AZ-04 were non-genotoxic, with AZ-03 being the safest, not showing any form of genotoxicity at all the concentrations tested. The toxicity profile correlates with the lipophilicity profile of AZ-03, while the most lipophilic of the series, AZ-04 which is aslo non-genotoxic shares the structural feature of AZ-03 in having additional C-6 substitutent unlike the other two congeners, AZ-01 and AZ-02, which exhibited genotoxicity. It therefore appears that structural alert, rather than overall compound lipophilicity is the critical predictor of toxicity in the series. The electronic code of Federal regulations for the approved colours contain entries on "identity" and "specifications" [23]. It is of

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utmost importance, therefore, to investigate and document the solution chemistry of a new potential color additive. The solution chemistry will supply the technical details for entries under "specifications" and "identification" in any monograph that may be prepared in due course to aid precise authentication of this new chemical entity.

This paper documents, for the first time, some aspects of the solution chemistry of AZ-03; 4{[6-(1-carboxyethyl)-2-hydroxy -1-naphthyl]diazenyl}-3,5-dinitro benzoic acid. In particular, behaviors of the molecule that are dependent on the pH of the aqueous solution are described. The existence of isosbestic point is reliably established, which underscores the existence of chemical equilibrium in the absorbing medium, in absorption spectroscopy. The multiple ionization steps involved in the chemical equilibrium is also attested to, unequivocally, by the distinctive rate-pH profile obtained, when the dye was subjected to base-catalyzed degradation.

MATERIALS AND METHODS

Chemicals and Reagents

The primary aromatic amine, 4-amino-3,5-dinitrobenzoic acid (ADBA) and the arenediazonium ion; 4-carboxyl-2,6-dinitro benzenediazonium ion (CDNBD) were synthesized in our laboratory as previously described [4] . Azo dye (AZ-03) was synthesized from CDNBD intermediate as previously described [17] , dimethylformamide, sodium hydroxide, potassium dihydrogen phosphate, boric acid, potassium chloride (BDH, U.K.).

Equipment

pH meter (Mettler, U.K), digital colorimeter (Jenway, Model 6051, U.K.), vortex mixer (Griffin and George, Ltd.) UV/visible spectrophotometer (UNICAM), thermostated water bath/sonicator (Langford ultrasonics, U.K.).

Preparation of Buffer Solutions

Buffer solutions ranging from pH 4-12 were prepared as described in the United States Pharmacopoeia [24].

Preparation of Azo Dye Stock Solution

10 mg of the dye (AZ-03) was weighed into a 10 mL volumetric flask, dissolved in a little amount of dimethylformamide (DMF) and the volume was made up to 10 mL with DMF (1 mg/mL).

Determination of Isosbestic Point

i) Spectral Recording

Aliquot of the stock solution of AZ-03 (0.15 mL) was taken, and each time made up to 10 mL with each of the buffer solution prepared, that is from pH 4 to pH 12 (3.30×10^{-5}M). The absorption spectra of the resulting solutions were then recorded immediately on a UV/visible spectrophotometer. The spectra were overlaid on each other, and the isosbestic point was determined by inspection and comparison of the actual absorbance value at the wavelength corresponding to spectra intersection point.

ii) Validation of the Point (Around 520 nm)

Standard solutions (1.10×10^{-5} M) of AZ-03 were prepared from the stock solution using buffers pH 7, 10 and 11. The solutions were prepared in triplicate in each buffer solution and the absorbance measurements were immediately taken at 522, 524, 526 and 528 nm.

Determination of Specific Absorbance and Molar Absorptivity

Standard solutions of known concentrations were prepared from the stock solution (1 mg/mL) to contain 2.5, 5, 10, 15, and 20 μg/mL by making up appropriate aliquots to 10 mL in a volumetric flask, with buffer solutions of pH 7 and pH 12 in separate series of dilution. The absorbance value of each solution was then recorded immediately, at 490 nm for pH 7 solutions and 550 nm for pH 12 solutions.

Determination of Degradation Rate – pH Profile

Aliquot of the AZ-03 stock solution (0.3 mL) was taken into several test tubes and the volume made up to 3 mL with neutral buffer (pH 7). The test tubes were incubated, in duplicate, in water bath maintained at 60^{o}C and removed after time intervals of 5, 10, 15, 20, 25 and 30 minutes and kept in ice bath. The reaction mixture was then made up to 10 mL in volumetric flask with buffer solution (pH 11). The absorbance values of the resulting violet colored solution were recorded at 540 nm. The dye incubation procedure was repeated using buffer solutions of other pH values; 9.5, 10, 11 and 12. Final test solution was prepared in all cases by making up to 10 mL with buffer solution of pH 11.

The absorbance readings of the violet colored solution were taken at 540 nm (AU_{540}) at each time interval in quadruplicate. Mean absorbance reading was transformed to kinetic data for curve fitting analysis. Initial absorbance reading was designated A_o. Absorbance reading of a sample left to decompose for 24h was designated A_∞. Instantaneous absorbance readings were designated A_t.

$$C_o = A_o - A_\infty,\ C = A_t - A_\infty$$

The data was fitted to first-order equations, and the model parameters were computed, with appropriate mathematical constraints (K>0, Span >0, plateau is set to a constant value and shared).

Mathematical Modeling and Statistical Analysis

The absorbance data obtained at the four wavelengths from recording sample solutions of the three pH, in turn, were compared for any statistical similarity, by 2-way analysis of variance (ANOVA). The two variables are wavelength and pH of media. Bonferroni post-test was performed after the analysis to identify where significant difference actually lies in the multiple comparison performed. 1-way ANOVA was afterwards performed to precisely locate the best wavelength, where signal difference across pH has the highest probability of occuring due to chance. P<0.05 was taken as significant in all the analyses.

Linear regression analysis was performed on the calibration data at 490 nm (pH 7 solutions) and at 550 nm (pH 12 solutions). Specific absorbance and molar absorptivity were computed from the slope of the lines.

Non-linear regression analysis (mono-exponential decay) was performed on the kinetic data obtained from base-catalyzed degradation at various pH values, with samples kept at 60°C. A plot of Log K versus pH (rate-pH profile) was made in order to elucidate possible mechanism of degradation.

All the analyses were performed by GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com, 2005).

RESULTS

Overlaid absorption spectra of AZ-03 in buffer solutions of varying pH revealing the existence of isosbestic point around 520 nm is shown in Figure **1**. The 2-way ANOVA parameters indicating the relative contribution of wavelength, pH and interaction between the two factors, to overall variance in absorbance measurements is diplayed in Table **1**. Figure **2** shows

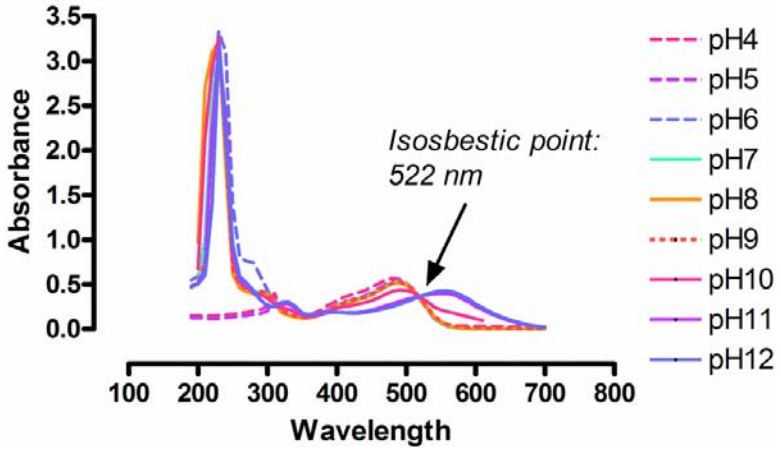

Figure 1: Overlaid absorption spectra of AZ-03 in buffer solutions of various pH showing isosbestic point at 522 nm.

Table 1: 2-Way ANOVA Parameters Indicating the Relative Contribution of Wavelength, pH and Interaction to the Overall Variance in the Absorption Spectroscopy Data

Source of variation	% of total variation	P value	Remark
Interaction	7%	0.46	Not significant
pH	57%	P <0.0001	Significant
Wavelength	7%	0.16	Not significant

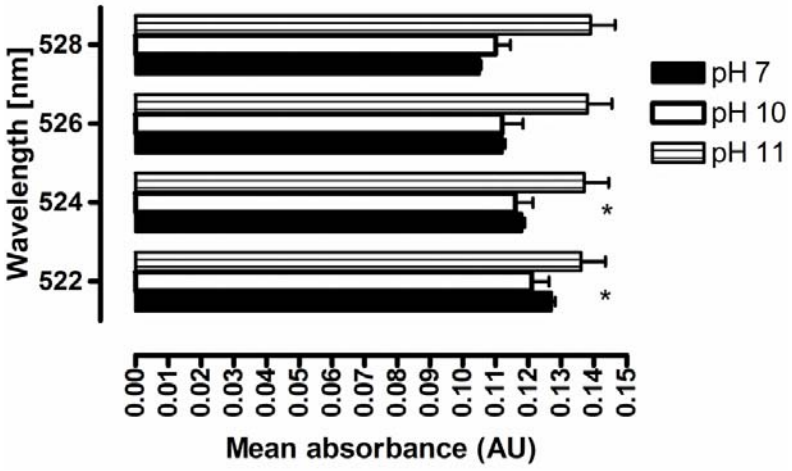

Figure 2: Graph showing the relative difference in absorptivity of dye solution in aqueous buffers at various wavelengths around 520 nm. Asterisk indicates the wavelengths at which absorptivity is statistically similar across the pH values of absorbing medium.

Table 2: Bonferroni Post-Test Showing that Absorptivity is Statistically Similar at 522 and 524 nm but Statistically Different at 526 and 528 nm Across the 3 pH Values

Wavelength, nm	Difference	t	P value	Summary*
pH 7 vs. pH 10				
522	-0.0060	0.79	P>0.05	NS
524	-0.0020	0.26	P>0.05	NS
526	0.00	0.00	P>0.05	NS
528	0.0050	0.66	P>0.05	NS
pH 7 vs. pH 11				
522	0.0090	1.2	P>0.05	NS
524	0.019	2.5	P>0.05	NS
526	0.026	3.4	P<0.01	Significant
528	0.034	4.5	P<0.001	Very significant

*NS = Not significant.

the relative difference in absorptivity of AZ-03 in aqueous buffers at various wavelengths around the nominal 520 nm. Table **2** shows the Bonferroni post-test analysis revealing where the significant difference actually lies and pointing to the direction of statistical similarity. Table **3** shows the wavelengths of interest and the associated p value, signifying the difference of absorptivity at 526 and 528 nm and similarity of absorptivity at 522 and 524 nm. Table **4** shows the calibration line data analysis. The regression equations of the best-fit lines and the corresponding molar absorptivities are displayed. Figure **3** displays the *pseudo* first-order degradation kinetics of AZ-03 when subjected to base catalyzed degradation at different pH values. The best-fit values of model paratmeters associated with the degradation experiments are displayed in Table **5**. A non-linear AZ-03 degradation rate-pH profile was obtained, as shown in Figure **4**. Figure **5** reveals the multiple ionization steps involved in the chemical equilibrium existing between the unionized and the fully ionized specie of the dye.

Table 3: 1-Way ANOVA of Absorbance Measurements of AZ-03 (1.10×10^{-5} M) Across pH 7, 10 and 11 Showing Absorptivity is Statistically Different at 526 and 528 nm but Similar at 522 and 524 nm

Wavelength, nm	P value*
522	0.29
524	0.08
526	0.04
528	0.01

*p<0.05 is taken as significant.

Table 4: Calibration Line Data for the Determination of Molar Absorptivity of AZ-03 in the Unionized (pH 7) and Fully Ionized Specie (pH 12)

pH	Wavelength	Specific absorbance*, A [1%, 1cm]	Molar absorptivity ε [L mol^{-1} cm^{-1}]
7	490	322.17	14,600
12	550	281.41	12,800

*Linear regression equation:
490 nm: y = 322.17x + 0.042, r^2 = 0.999; 550 nm: y = 281.41x + 0.018, r^2 = 0.992.

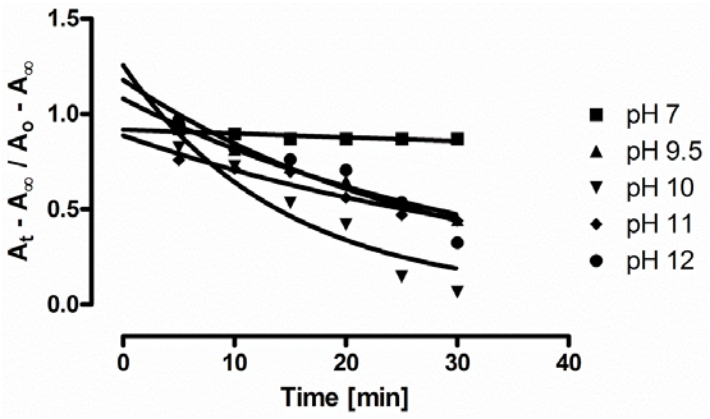

Figure 3: Degradation of AZ-03 in neutral and alkaline buffer solutions at 60°C by *pseudo* first-order kinetics.

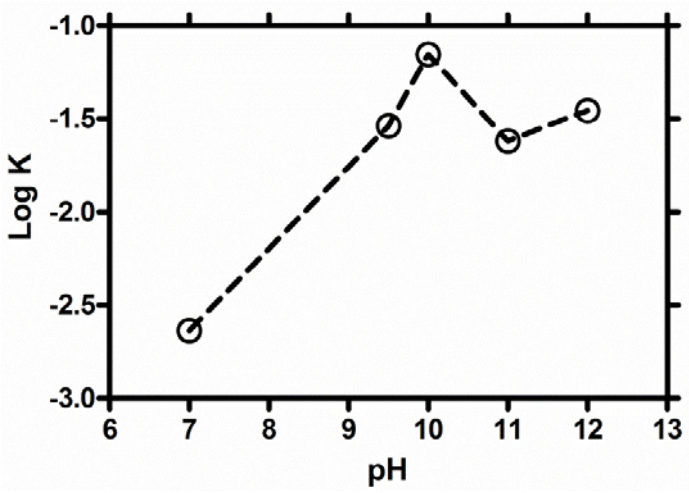

Figure 4: Non-linear rate-pH profile of AZ-03 at 60°C indicating the multiple ionization steps existing in the chemical equilibrium between the unionized specie (pH 7) and the fully ionized specie (pH 12).

Table 5: Best-Fit Values for the Degradation of AZ-03 by *pseudo* First-Order Kinetics at 60°C in Neutral and Alkaline Buffers of Various pH

pH	Half life, $t_{1/2}$ min	Span (A)	Plateau*	K, min^{-1}	Log K
7	295	0.88	0.04	0.0023	-2.64
9.5	24	1.0	0.04	0.029	-1.54
10	9.9	1.2	0.04	0.070	-1,16
11	29	0.85	0.04	0.024	-1,62
12	20	1.1	0.04	0.035	-1.46

*Constraints: K>0, Span >0, Plateau = constant value and shared.

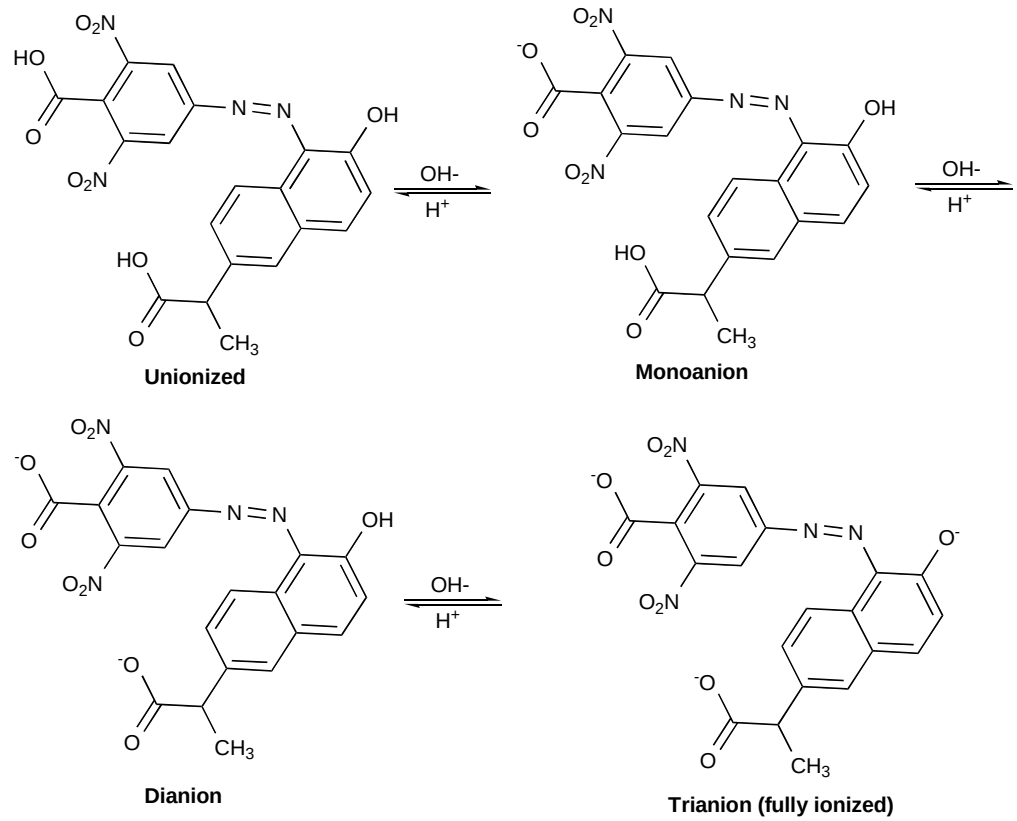

Figure 5: Schematic representation of multiple ionization steps in the chemical equilibrium between unionized specie (pH 7) and fully ionized specie (trianion, pH 12) of AZ-03.

DISCUSSION

Determination of Isosbestic Point

Spectra overlay of absorption spectra recorded at various pH as shown in Figure **1** is capable of revealing the existence of isosbestic point, which signifies the wavelength where the absorptivity of a system at equilibrium is constant, regardless of the pH of the recording medium [25]. Cursory inspection shows the point is around 520 nm. However, in other to be precise in this determination, measuremnts at 4 wavelengths in the region, namely; 522, 524, 526 and 528 nm were taken and evaluated statistically to ascertain the constancy of absorbance reading and hence absorptivity across 3 pH values; 7, 10 and 11. Figure **2** shows the relative difference in absorptivity across the two variables, while Table **1** shows the relative percentage contribution of the 2 factors and interaction between the factors; wavelength and pH, to the overall variance. Bonferonni post-test results displayed in Table **2** reveals that significant difference exists in the absorptivity of the dye between 526 and 528 and especially at pH 11 relative to pH 7 and 10. Performing 1-way analysis of variance at each of the wavelengths across the pH 7, 10 and 11 revealed the difference in probability of signal variation being due to chance at wavelength 522 and 524 nm. It was shown that the probability is 4 times higher at 522 nm (p=0.29) than at 524 nm (p =0.08). On the strength of this statistical evidence (Table **3**), it was therefore taken that isosbestic point exists at 522 nm for AZ-03.

Determination of Molar Absorptivity

The results of calibration lines constructed at pH 7 and pH 12 were used to compute the specific absorbance and molar absorptivity of the unionized and fully ionized specie respectively. The linear regression equations obtained is diplayed in Table **4**. The slope of the line, is the particular parameter used to compute the molar absorptivities. The results show the orange unionized specie exhibited more intense absorption than the violet fully ionized specie. The greater intensity of the acid colour and the acid stability of the dye are both good features for a potential color additive. It suggests that a very small concentration of the dye will produce sufficient intensity that satisfies the aesthetic value requirement for a color additive. A low concentration of the additive is in turn desirable for safety and toxicity considerations which are usually concentration-dependent.

Determination of Degradation Rate – pH Profile

Preliminary observation showed that the dye is more stable in the acidic environment than it is in alkaline environment. Other experimental data (not displayed) revealed that the base-catalyzed degradation in a fixed pH, at various temperatures followed *pseudo* first-order kinetics. This pattern is evident in the degradation at 60^{o}C in buffer solutions of varied pH shown in Figure **3**. The investigation reported here was an attempt to decipher the mechanism of degradation. The alkaline buffer composition comprises of hydroxyl ion as the prinicipal base, while additional anions like chloride and borate ions are similar in concentration across the pH range. The rise in rate of degradation from pH 7 to pH 9.5 suggests a clear case of specific base catalysis. A further rise from pH 9.5 peaks at pH 10, followed by a decrease at pH 11 and a gentle rise again at pH 12. Overall, a non-linear complex curve was obtained for the rate – pH profile. This kind of behavior has been reported for the hydrolysis of hydrochlorothiazide [26]. The hydrolytic reaction was found to be reversible, and the complex curve was said to indicate the multiple steps and an intermediate involved in the reaction.

This mechanistic explanation was adapted for the rate – pH profile obtained for the hydrolysis of AZ-03. The ionization of the unionized specie to the fully ionized specie proceeds with multiple steps as shown in Figure **5**. The chemical equilibrium shows that the mono-anion and di-anion species consititute intermediates before the formation of the fully ionized tri-anion specie. The apparent deviation from the linear rate – pH profile typical of specific base catalysis can therefore be accounted for. This distinctive profile can also be a diagnostic tool in the authentication of the dye. This is the more important, given the fact that other structurally related congeners in the chemical library [17] do not have the attractive safety profile of AZ-03 [22].

CONCLUSIONS

The potential color additive, AZ-03 has three ionizable protons. The pH dependent molecular behavior was therefore investigated to document the distinctive solution chemistry expected of such a functionalized molecule. It was shown with some statistical rigor that the dye exhibits isosbestic point at 522 nm. The orange, unionized specie, exhibits more intense absorption than the violet, ionized specie, with molar absorptivities of 14,600 (490 nm) and 12,800 (550 nm) [L mol^{-1} cm^{-1}] respectively. Base-catalyzed degradation gave a non-linear and diagnostic rate-pH profile. These findings could aid precise authentication of this promising, potential color additive, and thus facilitate further understanding and studies of its solution chemistry.

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