

Research Article

Intermolecular Interactions between Eosin Y and Caffeine Using $^1\text{H-NMR}$ Spectroscopy

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DETECHIP has been used in testing analytes including caffeine, cocaine, and tetrahydrocannabinol (THC) from marijuana, as well as date rape and club drugs such as flunitrazepam, gamma-hydroxybutyric acid (GHB), and methamphetamine. This study investigates the intermolecular interaction between DETECHIP sensor eosin Y (DC1) and the analyte (caffeine) that is responsible for the fluorescence and color changes observed in the actual array. Using $^1\text{H-NMR}$, $^1\text{H-COSY}$, and $^1\text{H-DOSY}$ NMR methods, a proton exchange from C-8 of caffeine to eosin Y is proposed.

1. Introduction

DETECHIP, a highly selective and sensitive molecular sensor that produces color and fluorescence changes in the presence of analytes, was recently developed. A quick, sensitive, and selective detection system is required for many applications, such as alerting security officers to the presence of explosives or their precursors, preincident monitoring/screening for homeland security purposes [1] such as weapons of mass destruction, and detection and quantification of doping compounds in competitive sports [2–5]. The method that is currently most widely used for the detection of such substances is gas chromatography-mass spectrometry (GC-MS) [6, 7]. However this method requires a skilled operator and cannot be easily miniaturized. Current screening reagents for abused narcotics like flunitrazepam (often used for date rape, assault, or theft) [8], methylephedrine, caffeine, nicotine, and others include immunoassays [9], ion trap mobility spectrometry [9–11], wet colorimetric assays [12–14], spot tests such as Marquis [13], Scott Drug Testing Company drug tests (<http://www.scottcompany.com>), or the b-Glucuronidase Drug Analysis Bundle (Sigma-Aldrich) and Magnotech technology testing [15].

DETECHIP uses an array of sensors that can be used for identification of drugs and other molecules by fluorescence and color changes [16–18]. This method relies on

molecular interactions between the analyte molecules and the DETECHIP sensors [18]. Unlike other color tests which provide a single “yes” or “no” response, DETECHIP gives multiple simultaneous responses in the form of color and fluorescent changes using two different buffers, allowing users to quickly characterize suspect materials. Figure 1 shows an example of a 96-well plate DETECHIP assay. Eight sensors (DC1–DC8) are added to the rows in the plate in two different buffers (A + B), and the analytes are tested alongside a control in the columns of the plate.

While DETECHIP has worked well for many analytes, little is known regarding the intermolecular interaction between the sensors and the analytes. As further development of DETECHIP is in progress, it is important to determine the reasons behind the color or fluorescence changes observed. Understanding the underlying mechanism of sensor interactions with analytes allows for the selection and preparation of better sensors for future DETECHIP prototypes. Color or fluorescence changes may be associated with structural changes of the sensor. Structural changes of the sensors may be due to tautomerism, conformational changes, or the formation of a more complex supramolecular structure.

Xanthene dyes, such as eosin Y, exhibit different tautomeric structures with different protolytic forms, either proton “on” or “off” depending on pH as illustrated in

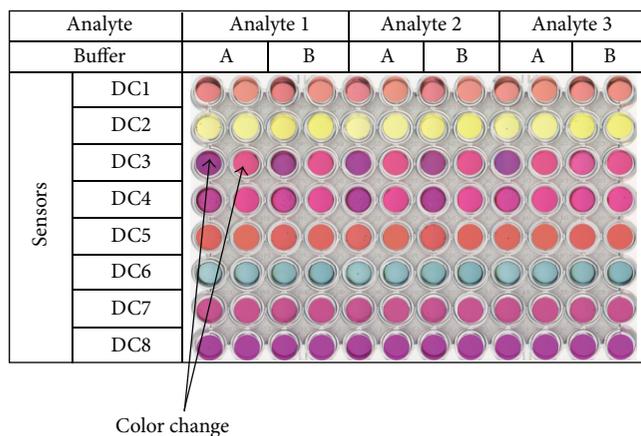


FIGURE 1: Setup of a typical DETECHIP assay showing presence or absence of color changes of the sensors in presence of analytes compared to control wells.

Figure 2 [19]. The color associated with the dye derives from the quinoid structure of the anionic form [20, 21]. The pK_a of 2.02 in Figure 2 is associated with the phenolic proton. The pK_a values may vary as reported by Bartistela et al. [19]. At $pH = 7$, the most prevalent form of eosin Y is the di-ionic form at a pK_a of 3.8 [19, 20]. In DETECHIP assays, the pH is buffered at $pH = 7$, and therefore the color changes must be a function of the chemical environment due to the presence of the analyte. Caffeine exhibits strong color and fluorescence changes with eosin Y. Eosin Y is therefore a good candidate for the investigation of the sensor-analyte intermolecular interaction. Figure 3 shows the structures of eosin Y in its di-ionic form and caffeine.

We used proton nuclear magnetic resonance (1H -NMR) to investigate the intermolecular interaction between the sensor, eosin Y, and the analyte, caffeine. NMR is one of the most effective tools for the structural elucidation of complex organic molecules [22, 23]. Structure determination and intermolecular bonding interactions studies can be achieved by use of 1H -NMR and two-dimensional proton correlation spectroscopy (1H -COSY) NMR. A COSY pulse-sequence is usually used to identify spins of the same isotope that are coupled to each other. It consists of a single RF pulse ($p1$) followed by the specific evolution time ($t1$) followed by a second pulse ($p2$) followed by a measurement period (t). The two-dimensional spectrum that results from the COSY experiment shows the frequencies for the isotope under study, most commonly hydrogen (1H) along both axes. This method allows us to determine which protons on the analyte are correlated to the protons of the analyte.

Experiments determining the presence of multiple components in a solution have recently gained popularity by the determination of the diffusion coefficient (D) using two-dimensional diffusion ordered spectroscopy (DOSY) NMR [24, 25]. The measurement of diffusion is carried out by observing the attenuation of the NMR signals during a pulsed-field gradient experiment. The degree of attenuation is a function of the magnetic gradient pulse amplitude and

occurs at a rate proportional to the diffusion coefficient (D) of the molecule. A “normal” NMR spectrum is obtained in the horizontal axis, while in the vertical axis, the diffusion coefficient is obtained. Since components of different sizes have unique diffusion coefficients, the vertical axis provides diffusion peaks which can help determine how many components are in a mixture. DOSY allows us to evaluate if more than two components are in solution when the analyte is added to the sensor, such as the presence of products that form as a result of a chemical reaction.

2. Experimental Section

2.1. Reagents and Materials. The sensors and caffeine were purchased from Sigma-Aldrich, St Louis, MO. Dibasic potassium phosphate (K_2HPO_4) was purchased from VWR International, West Chester, PA. The NMR deuterated solvent (D_2O) and standard sodium-3-trimethylsilylpropionate d_4 (TSP) were purchased from Cambridge Isotope Laboratories, Cambridge, MA. All reagents were used as received.

2.2. NMR Measurements. All measurements were performed on a 400 MHz Bruker Avance III spectrometer equipped with a 5 mm z -gradient probe at room temperature. Chemical shifts (δ) are reported relative to the methyl signal of TSP (0 ppm).

The 1D 1H spectra were acquired with presaturation to reduce the residual water. COSY data was acquired using gradient pulses for selection, purge pulses, and presaturation to reduce the water signal. DOSY experiments were acquired with a stimulated echo sequence incorporating a longitudinal eddy delay (LED), bipolar gradient pulses, and two spoil gradients.

2.3. Sample Preparation. An NMR buffer solution was prepared at 400 mM of dibasic potassium phosphate (K_2HPO_4) in deuterium oxide (D_2O) at $pH = 7$ using deuterium chloride (DCl) solution. The NMR standard used was 0.1% TSP. Using the buffer solution, 12.5 mM caffeine and 20 mM eosin Y solutions were prepared, from which the NMR samples were made. The eosin Y and caffeine samples were each used without further dilution. The final concentrations in the eosin Y-caffeine mixture sample were 6.25 mM caffeine and 10 mM eosin Y.

2.4. 1H -NMR Spectra Predictions. 1H -NMR spectra were calculated using ChemBioDraw 12.0 software (CambridgeSoft, Cambridge, UK) and used in the peak assignments in the experimental spectra.

To determine the change in spectral intensity, the 1H -NMR spectra were fitted using Peakfit program (Seasolve Software, Framingham, MA).

2.5. Ultraviolet-Visual Spectroscopy Experiments. Absorbance measurements were carried out in a Varian Cary 50 ultraviolet-visual spectrometer (UV-Vis). Both samples were made using 400 mM dibasic potassium phosphate buffer solution at $pH = 7$. A 20 μM solution sample of eosin Y was made

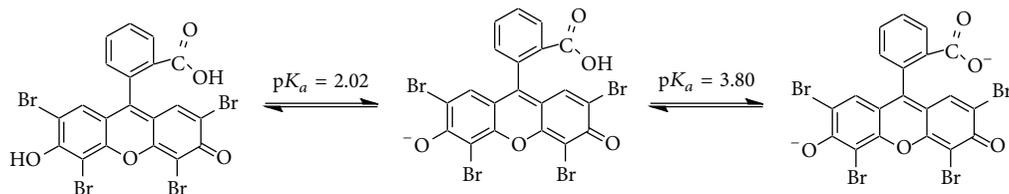


FIGURE 2: Protolytic equilibria of eosin Y with the most representative tautomeric structure.

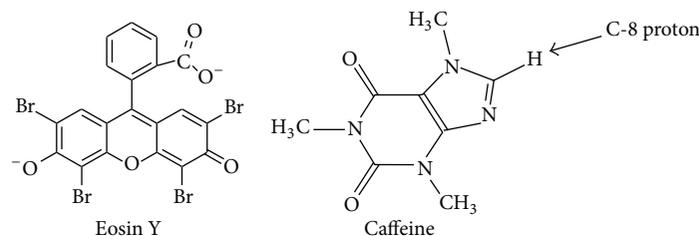
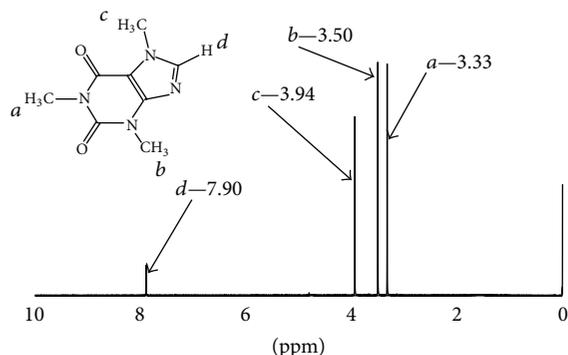


FIGURE 3: Structures of the eosin Y and caffeine.

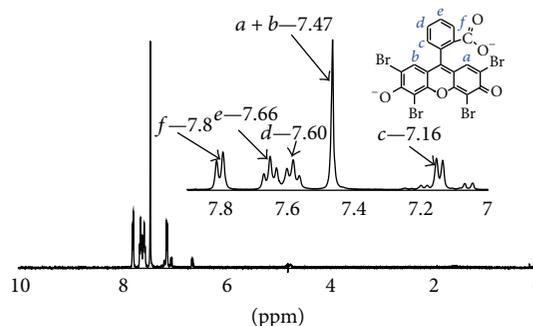
FIGURE 4: $^1\text{H-NMR}$ spectrum of 12.5 mM caffeine in deuterated phosphate buffer, indicating the peak assignments.

by a 1000-fold dilution of 20 mM eosin Y. A 1:1 mixture of 20 mM eosin Y and 12.5 mM caffeine was diluted 1000-fold to make the mixture sample.

3. Results and Discussion

$^1\text{H-NMR}$ spectra were obtained for caffeine, eosin Y, and their mixture. Figure 4 shows the $^1\text{H-NMR}$ spectrum of caffeine. The peaks corresponding to the N-methyl protons are labeled *a*, *b*, and *c* at 3.33, 3.50, and 3.94 ppm, respectively. The C8 proton is labeled *d* and shows a peak at 7.90 ppm. Changes in position and/or intensity of these peaks, would signify changes in the structure of caffeine and were monitored in the caffeine-eosin Y mixture sample. Similarly, changes were monitored on the peaks of the eosin Y.

The $^1\text{H-NMR}$ spectrum of eosin Y is given in Figure 5. The corresponding proton peaks are labeled *a* and *b* at 7.47 (s), *c* at 7.17 (*d*), *d* at 7.60 (*t*), *e* at 7.66 (*t*), and *f* at 7.80 (*d*) ppm.

FIGURE 5: $^1\text{H-NMR}$ spectrum of 12.5 mM eosin Y with an expansion (inset) showing peak assignments.

The $^1\text{H-NMR}$ spectrum of the caffeine-eosin Y mixture is shown in Figure 6. There is a slight downfield shift in the proton peaks for eosin Y: *a + b* from 7.49 to 7.47 ppm and *c* 7.30 to 7.19 ppm.

Table 1 shows the chemical shifts for the eosin Y protons in the eosin Y sample and in the eosin Y-caffeine mixture sample. The downfield shift in the eosin Y proton signals is due to a reduction in electron density, caused by the transfer of a proton from caffeine. The peak for caffeine *d* proton (C-8), 7.90 ppm, in the caffeine spectrum is shifted to 7.67 ppm and is drastically reduced in intensity in the eosin Y-caffeine mixture spectrum. The eosin Y-caffeine mixture spectrum was fitted using Peakfit software to enable comparing the area ratio of the peak at 7.90 ppm to the one at 3.35 ppm in the caffeine spectrum and the area ratio of the peak at 7.69 ppm to that at 3.39 ppm in the caffeine-eosin Y mixture spectrum. A 5-fold reduction is observed, implying that there is loss of a C-8 proton from caffeine.

The chemical shifts for the caffeine protons in the two samples are given in Table 2 and can be seen in Figure 7, where the upfield shifts of N-methyl proton peaks for caffeine

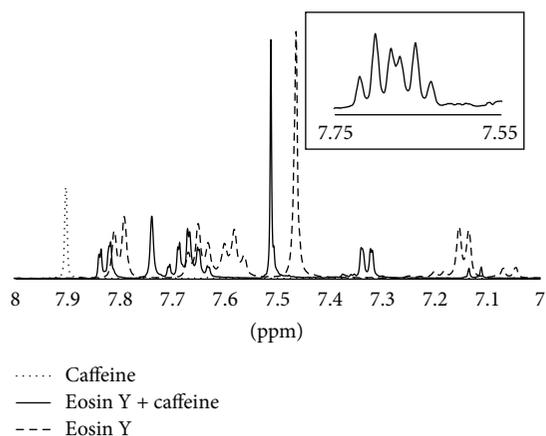


FIGURE 6: An overlay of $^1\text{H-NMR}$ spectra of 12.5 mM caffeine, 60 mM eosin Y, and a mixture of 10 mM eosin Y and 6.25 mM caffeine showing the region 7–8 ppm. A slight downfield shift on the eosin Y proton peaks is evident in the presence of caffeine. Inset: $^1\text{H-NMR}$ spectrum (expansion) for eosin Y at pH = 4 showing a pentet-like signal.

TABLE 1: The $^1\text{H-NMR}$ chemical shifts (δ) in ppm of eosin Y aromatic protons in the samples of eosin Y alone compared to eosin Y-caffeine mixture.

Sample	H_a	H_b	H_c	H_d	H_e	H_f
Eosin Y	7.47	7.47	7.16	7.60	7.66	7.80
Eosin Y + caffeine	7.51	7.51	7.32	7.67	7.74	7.83

TABLE 2: The $^1\text{H-NMR}$ chemical shifts (δ) in ppm of the caffeine protons in the samples of caffeine alone and in a mixture of caffeine and eosin Y.

Sample	H_a	H_b	H_c	H_d
Caffeine	3.34	3.50	3.95	7.90
Eosin Y + caffeine	3.19	3.39	3.77	7.67

in the eosin Y-caffeine mixture are evident. This is characteristic of an increase in electron density around the protons due to the formation of a caffeine anion.

A potential mechanism for the abstraction of the caffeine C-8 proton to eosin Y is given in Figure 8. The negatively charged carboxylate deprotonates the C-8 hydrogen which results in the protonated eosin Y and the deprotonated caffeine.

The COSY $^1\text{H-NMR}$ spectrum in Figure 9 shows off-diagonal peaks for the C-8 proton in caffeine correlated with “b” proton peaks. While this shows that the C-8 protons are still attached to the caffeine molecules, the reduction in intensity confirms that some of the protons have been transferred.

To test for the presence and identity of different components in the mixture such as new products formed due to a chemical reaction between eosin Y and caffeine, or dimerization between eosin Y molecules [26], a DOSY NMR experiment was obtained. The horizontal axis shows the normal proton NMR spectrum. The vertical axis represents

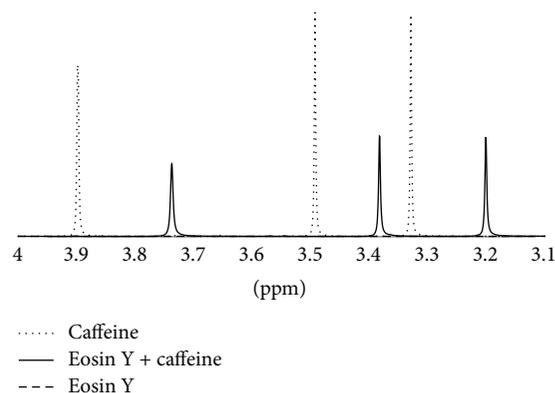


FIGURE 7: An overlay of $^1\text{H-NMR}$ spectra of 12.5 mM caffeine, 20 mM eosin Y, and a mixture of 10 mM eosin Y and 6.25 mM caffeine showing the region 3.1–4.0 ppm. The upfield shifts of the N-methyl proton peaks for caffeine in presence of eosin Y are evident.

the diffusion constant: each component diffuses at a certain rate thus, the separation.

Results from the DOSY NMR, Figure 10, show that the mixture comprises 3 components: water, eosin Y, and caffeine. This argues that a supramolecular complex with hydrogen bonding or dimerization may not have occurred under these experimental conditions.

Based on the COSY results in Figure 9 and the DOSY results in Figure 10, caffeine and eosin Y are not reacting to form a new molecule. No new diffusion peak appeared in the DOSY spectrum which would indicate that a product was formed due to a chemical reaction between caffeine and eosin Y. However, as seen in Figure 7, the proton labeled “d” on C-8 disappears when eosin Y is added to caffeine. This disappearance of the peak at 7.9 ppm can be explained by the following: (1) the peak shifted and overlaps with eosin Y peaks and is not visible anymore, (2) some of the caffeine molecules lost the proton which transferred to eosin Y, or (3) there is a hydrogen bond interaction between the caffeine and eosin Y. At pH = 4, eosin Y exists as dianion in equilibrium with the monoanion protonated at the carboxylic group as shown in Figure 2 [19]. In order to investigate this proton transfer, $^1\text{H-NMR}$ spectra were obtained at lower pH values. The spectrum observed at pH = 4 shows a pentet-like signal at approximately 7.6 ppm that is similar to the one observed in the eosin Y-caffeine mixture spectrum in Figure 6. This illustrates that in the mixture, eosin Y exists in the protonated form.

UV-Vis spectra were obtained at 0.75 μM eosin Y and mixtures of 0.75 μM eosin Y and caffeine at various concentrations (from 0.42 mM to 11.25 mM) in 400 mM dibasic potassium phosphate solution are shown in Figure 11. In the presence of caffeine, the λ_{max} is red shifted by up to 8 nm depending on caffeine concentration. This is consistent with the color change observed in the 96-well plate DETECHIP assay (Figure 1). This red shift increases with increasing caffeine concentration, very low eosin Y concentrations (less than 10 mM). Dimerization of eosin Y is not expected [26]

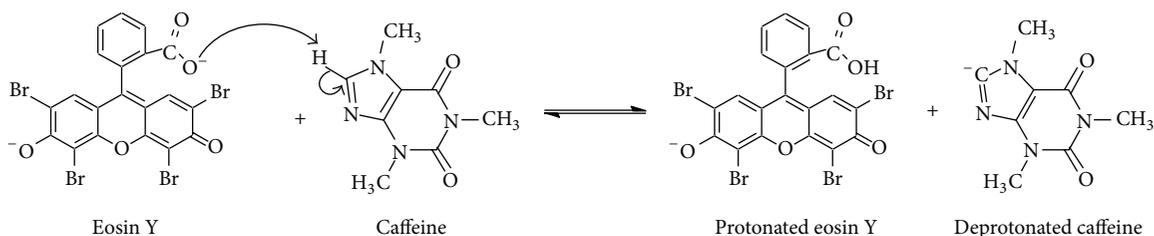


FIGURE 8: Mechanism for proton transfer from caffeine to eosin Y.

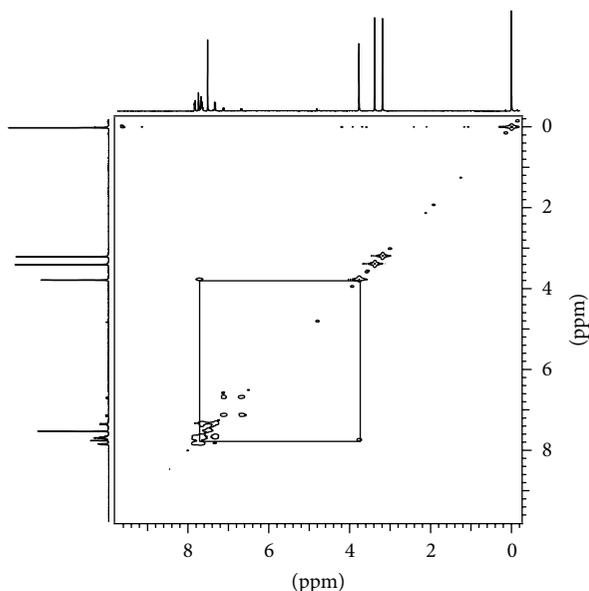


FIGURE 9: COSY NMR spectrum for the eosin Y-caffeine mixture showing residual C-8 still attached to the caffeine molecule.

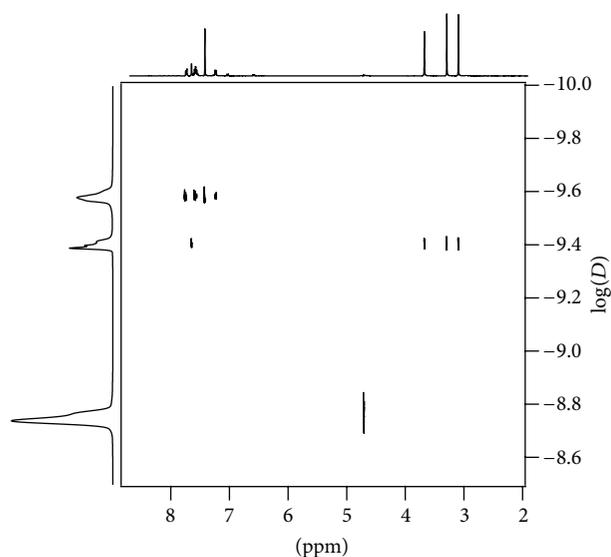
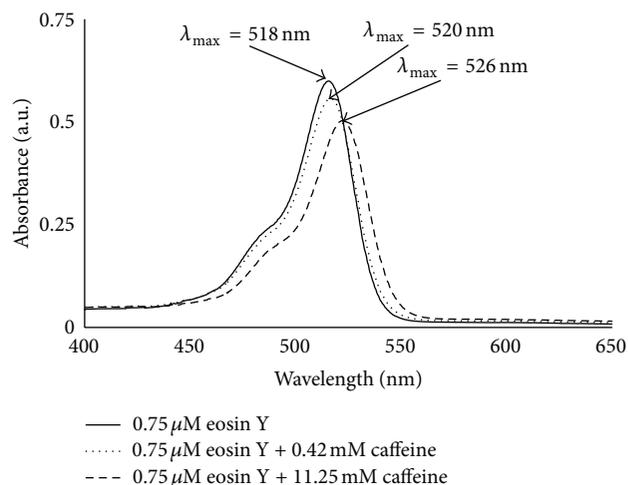


FIGURE 10: DOSY NMR spectrum of eosin Y-caffeine mixture showing 3 distinct components of the mixture.

FIGURE 11: UV-Vis spectra of 0.75 μM eosin Y and mixtures of 0.75 μM eosin Y and 0.42 mM and 11.25 mM caffeine showing a shift in λ_{max} from 518 to 520 and 526 nm, respectively.

at these concentrations of eosin Y and a proton transfer mechanism between caffeine and eosin Y is feasible.

$^1\text{H-NMR}$ results indicated that the negatively charged oxygen on eosin Y may deprotonate C-8 of caffeine. This potentially created a negatively charged caffeine intermediate and the change of conjugation in eosin Y led to a visible color change. The literature reports $\text{p}K_a$ values for the proton on C-8 in caffeine between 10.4 [27] and 14.0 [28]. Thus, this proton is very labile and can be abstracted easily at $\text{pH} = 7$. Similar hydrogen exchange reactions in nucleic acids and similar heterocycles have been observed in previous studies [29].

4. Conclusion

DETECHIP is based on fluorescence and color changes from which a unique identification has been developed for many drugs. The results in this study have shown that some of the color and fluorescence changes observed in DETECHIP assays may be a consequence of a proton transfer reaction between the sensors and the analyte molecules or donor-acceptor hydrogen bond kind of interaction. Further detailed studies are in progress for the interaction between other DETECHIP sensors and other analytes.

Acknowledgments

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