Video Article

Qualitative Identification of Carboxylic Acids, Boronic Acids, and Amines Using Cruciform Fluorophores

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Abstract

Molecular cruciforms are X-shaped systems in which two conjugation axes intersect at a central core. If one axis of these molecules is substituted with electron-donors, and the other with electron-acceptors, cruciforms' HOMO will localize along the electron-rich and LUMO along the electron-poor axis. This spatial isolation of cruciforms' frontier molecular orbitals (FMOs) is essential to their use as sensors, since analyte binding to the cruciform invariably changes its HOMO-LUMO gap and the associated optical properties. Using this principle, Bunz and Miljanić groups developed 1,4-distyryl-2,5-bis(arylethynyl)benzene and benzobisoxazole cruciforms, respectively, which act as fluorescent sensors for metal ions, carboxylic acids, boronic acids, phenols, amines, and anions. The emission colors observed when these cruciform are mixed with analytes are highly sensitive to the details of analyte's structure and - because of cruciforms' charge-separated excited states - to the solvent in which emission is observed. Structurally closely related species can be qualitatively distinguished within several analyte classes: (*a*) carboxylic acids; (*b*) boronic acids, and (*c*) metals. Using a hybrid sensing system composed from benzobisoxazole cruciforms and boronic acid additives, we were also able to discern among structurally similar: (*d*) small organic and inorganic anions, (*e*) amines, and (*f*) phenols. The method used for this qualitative distinction is exceedingly simple. Dilute solutions (typically 10^{-6} M) of cruciforms in several off-the-shelf solvents are placed in UV/Vis vials. Then, analytes of interest are added, either directly as solids or in concentrated solution. Fluorescence changes occur virtually instantaneously and can be recorded through standard digital photography using a semi-professional digital camera in a dark room. With minimal graphic manipulation, representative cut-outs of emission color photographs can be arranged into panels which permit quick naked-eye distinction among analytes. For quantification purposes, Red/Green/Blue values can be extracted from these photographs and the obtained numeric data can be statistically processed.

Video Link

The video component of this article can be found at http://www.jove.com/video/50858/

Introduction

Molecular cruciforms are defined as X-shaped cross-conjugated molecules in which two conjugation circuits intersect at a central core.[1,2,3] With appropriate donor-acceptor substitution, these molecules can *spatially localize* their frontier molecular orbitals (FMOs), so that the highest occupied molecular orbital (HOMO) resides dominantly along the electron-rich axis of the molecule, while the lowest unoccupied molecular orbital (LUMO) has the bulk of its density positioned along the electron-poor arm of the molecule. Such spatial isolation of FMOs is essential in the applications of these cruciforms as sensors for small molecules, since analyte binding to the cruciform invariably changes its HOMO-LUMO gap and the associated optical properties. This behavior has been demonstrated in cruciforms based on 1,4-distyryl-2,5-bis(arylethynyl)benzene,[1] 1,2,4,5-tetrakisethynylbenzene,[4] and benzobisoxazole[5,6] structural motifs. Since all three classes of molecules are inherently fluorescent, this methodology allowed their use as small-molecule sensors. In all three examples, cruciforms were substituted with Lewis basic pyridine and dialkylaniline groups and were thus responsive to Lewis acidic analytes, such as protons and metal ions.[1,4,5,7,8,9]

In 2011, Bunz and coworkers have shown[10] that the fluorescence responses of 1,4-distyryl-2,5-bis(arylethynyl)benzene cruciforms 1 - 3 (**Figure 1**) dramatically varied depending on the structure of the carboxylic acid used to induce protonation of the cruciform. Subsequently, Miljanić *et al.* demonstrated that benzobisoxazole cruciforms such as 4 (**Figure 1**) also show highly specific fluorescence emission responses to structurally related carboxylic acids, and that similar distinction can be seen among very similar organoboronic acids, too.[11] Origins of this highly selective emission color changes are at present unclear, and are most likely complex - as fluorescence quenching by electron poor analytes, residual analyte fluorescence, and protonation-induced shifting of cruciforms' emission maxima all presumably play a role. Nevertheless, the ability to discriminate among structurally related analytes is significant, especially since statistically relevant distinction can be obtained without the need to perform exhaustive UV/Vis absorption or fluorescence characterization of the optical response of cruciforms to analytes. Instead, simple photographs of emission color are sufficiently distinct to allow the discrimination among structurally closely related analytes, especially if the photographs are taken in different solvents or using more than one cruciform sensor. Using this quick methodology, dozens of analytes can be

quickly analyzed in an afternoon (see panels in **Figures 3-5**), whereas the same analysis would require weeks if rigorous spectroscopy was employed. Furthermore, since boronic acids are dynamic species that can coordinate nucleophiles through boron's empty *p*-orbital, Miljanić used this feature to develop hybrid sensors composed of benzobisoxazole cruciform 4 and simple non-fluorescent boronic acid additives B1 and B5 (**Figure 4**).[11,12] This methodology operates as follows: cruciform 4 and boronic acids complex into a transient complex 4·*n*B1 (or 4·*n*B5); the precise structure of this complex is at present unknown, but its fluorescence differs from that of the pure cruciform. If this solution is exposed to Lewis basic analytes, they can replace one or both -OH groups on the boronic acid,[13] thus significantly altering the electronic properties of boron and, in turn, the fluorescence of the entire complex. Using this "vicarious sensing" methodology, sensing of phenols, organic amines and ureas, as well as of small organic and inorganic anions, could be achieved.

In this paper, we present a tutorial on the use of both direct and vicarious sensing methodology to quickly qualitatively distinguish between structurally related (*a*) carboxylic acids (**Figure 3**), (*b*) boronic acids (**Figure 4**), and, vicariously, (*c*) organic amines (**Figure 5**). To illustrate the broad applicability of the reported protocols, Bunz's cruciforms were used to detect carboxylic acids, while Miljanić's compounds were employed to detect boronic acids, and, through a hybrid sensor, small organic amines. We presume that these sensors could be readily interchanged without major consequences to the quality of analyte discrimination.

Protocol

1. Detection of Carboxylic Acids Using Distyrylbis(arylethynyl)benzene Cruciforms

1. Prepare a fresh stock solution of cruciforms 1-3 with a concentration of 1.0 x 10^{-3} mol/L in DCM. It is not necessary to use spectroscopic quality solvents; ACS reagent grade purity is sufficient.
2. Using the stock solutions from 1.1 prepare 100 ml each of 2.0 x 10^{-6} M solution of 1-3 in dichloromethane (DCM), ethyl acetate (EtOAc), acetonitrile (AN), *N,N*-dimethylformamide (DMF), isopropyl alcohol (*i*PrOH) and methanol (MeOH). It is not necessary to use spectroscopic quality solvents; ACS reagent grade purity is sufficient.
3. Weigh out 0.65 mmol (88.2-124.2 mg) of the analyte carboxylic acid A1 - A10 in 5 ml dram vials, add 5 ml of the solutions prepared in 2.1 and shake the vial. If heterogeneous, the corresponding solution should be left to settle (filtration is unnecessary).This leads to a total concentration of 0.13 M (31 g/L) of the carboxylic acid.
4. Capture digital photographs of the fluorescence in a dark room in the absence of ambient light. The photographic setup (**Figure 2**) includes a digital camera (Canon EOS 30D) equipped with an objective (EFS 18-55 mm zoom lens) and two UV-lamps (excitation wavelength 365 nm). The uncapped vials should be positioned under the two UV-lamps for maximum exposure with a distance of 60 cm between camera lens and sample vials. Exposure times were varied for each solution to produce images reflecting the color of emission (0.25 - 15 sec).

2. Detection of Boronic Acids Using Benzobisoxazole Cruciforms

1. Prepare a 1.0 x 10^{-4} M solution of cruciform 4 in DCM. It is not necessary to use spectroscopic quality solvent; ACS reagent grade purity is sufficient.
2. Prepare five individual solutions for each boronic acid analyte, by dissolving 50 mg (0.24-0.41 mmol) of the analyte in 3 ml each of acetonitrile (AN), 1,2,4-trichlorobenzene (TCB), dichloromethane (DCM), cyclohexane (CH), and chlorobenzene (CB). This should result in approx. 16.7 g/L solutions with respect to each analyte. It is not necessary to use spectroscopic quality solvents; ACS reagent grade purity is sufficient.
3. Transfer 1.8 ml of each of the analyte solutions prepared in 2.2 into five separate 10 x 10 mm quartz cuvettes (commonly used for UV/Vis spectroscopy). Then, add 20 µl of the cruciform solution prepared in 2.1 into each of the five cuvettes, and stir the two solutions to homogenize. If any precipitation is observed, the corresponding solution should simply be left to settle (filtration is unnecessary).
4. Place all five cuvettes onto a glass plate and irradiate them by a handheld UV lamp (365 nm) from the top. The UV lamp should be positioned in a fashion that ensures equal irradiation to all five vials.
5. Ensure that the room is dark (turn off lights, block windows and other sources of natural and artificial light) and immediately take a digital photograph of the emission colors of the solutions. Miljanić *et al.* have used two digital camera models: FujiFilm FinePix S9000 and Canon EOS Rebel T3i, with a 45 cm distance between the camera lens and the sample cuvettes. Shutter speed was 0.5 sec.

3. Detection of Amine Analytes Using Benzobisoxazole Cruciform/Boronic Acids Hybrid Sensing System

1. Prepare (at least) 80 ml each of 1.0 x 10^{-6} M solutions of cruciform 4 in acetonitrile (AN), 1,2,4-trichlorobenzene (TCB), cyclohexane (CH), dichloromethane (DCM), and chloroform (CF).
2. Dissolve B1 (152.6 mg, 0.80 mmol) in 40 ml of each of the solutions prepared in 3.1.
3. Dissolve B5 (97.6 mg, 0.80 mmol) in 40 ml of each of the solutions prepared in 3.1.
4. Immediately after the solutions described in 3.2 and 3.3 are prepared, use them (2 ml each) to dissolve the desired amine analyte (40 mg, 0.19-0.47 mmol). For each amine analyte, ten solutions should be prepared: five with B1 and five with B5 as additives. It is not necessary to use spectroscopic quality solvents; ACS reagent grade purity is sufficient.
5. For each analyte, transfer aliquots of the ten prepared analyte/boronic acid/cruciform 4 solutions into ten separate quartz cuvettes. Place these two five-cuvette sets (one for 4/B1, one for 4/B5) onto a glass plate, irradiate at 365 nm by a handheld UV lamp, and immediately photograph using the settings described in 2.5 above.

4. Image Processing and Numeric Analyte Discrimination

1. Using Adobe PhotoShop or a similar image-processing program, cut out a representative square segment from digital photographs of the emission colors of each photographed vial. Organize these cut-outs into panels similar to those in **Figures 3B, 4,** and **5**. These panels in many cases allow rapid naked-eye discrimination among analytes.
2. If quantification of differences in emission color is desired, R/G/B values can be extracted from panels in 4.1 and then statistically treated. Freely downloadable Colour Contrast Analyzer[14] can be used for this purpose. To obtain relative standard deviations of emission colors of one analyte relative to another (*e.g.* compounds B1 and B2, **Figure 4**), the following equation is used:

$$\sigma'_{B1@B2} = \sqrt{\frac{\sum_{solv}^{i}(R_{B1} - R_{B2})^2 + (G_{B1} - G_{B2})^2 + (B_{B1} - B_{B2})^2}{3 * i}}$$

3. The equation from 4.2 is also used to identify unknown carboxylic acid analytes. Therefore every deviation is determined between the unknown analyte to all substances of the calibration data set. The smallest deviation indicates the corresponding substance.

Representative Results

To illustrate the potential of cruciform fluorophores in sensing and discriminating closely related analytes, three classes of results are presented. First, 1,4-distyryl-2,5-bis(arylethynyl)benzene cruciforms 1-3 (**Figure 1**) are used to discriminate among structurally related carboxylic acids A1-A10 shown in **Figure 3**. Then, benzobisoxazole-based cruciform 4 (**Figure 1**) has been used to analyze boronic acids B1-B9 (**Figure 4**). Finally, cruciform 4 is used in combination with boronic acids B1 and B5 to analyze amine analytes shown in **Figure 5**.

Using cruciform fluorophores 1-3 in six different solvents, the fluorescent responses were found to be dependent upon the concentration and the structural identity of a carboxylic acid. **Figure 3B** shows the digitally recorded emission color of all fluorophore, solvent, carboxylic acid combinations. This array exhibits 18 characteristic emission colors per analyte, which can be used to uniquely characterize an analyte. Using the R/G/B values of the emission color, all analytes can be discerned relative to carboxylic acids A1-A10 and identified as shown in the autocorrelation plot in **Figure 3C**.

Using a completely analogous procedure, boronic acids B1-B9 are readily discriminated from each other using cruciform 4, as evidenced by the emission color panel and the correlation graph shown in **Figure 4**.

Analysis of amines is achieved using *in situ* formed complexes of cruciform 4 with a large excess of boronic acid additives B1 and B5. At present, the structure of these complexes are unknown, although they likely involve either coordinative N-B bond, or some kind of hydrogen bonding between the boronic acids and the nitrogen atoms in the cruciform. These complexes - whose emission colors are different from those of the pure cruciform - can respond to amine analytes in two ways. Amines more basic than pyridine (compounds N1-N3 in **Figure 5**) displace cruciform 4 from its complexes with boronic acid additives, thus regenerating the emission colors of pure uncomplexed cruciform 4. On the other hand, less basic species (*i.e.* aniline derivatives and substituted ureas, N4-N12 in **Figure 5**), appear to bind to the 4·*n*ArB(OH)$_2$ complex without destroying it and this event results in the modulation of complex's fluorescence emission. Therefore, vicarious sensing methodology is characterized by a leveling effect, wherein analytes above certain threshold of basicity can no longer be discriminated from each other.

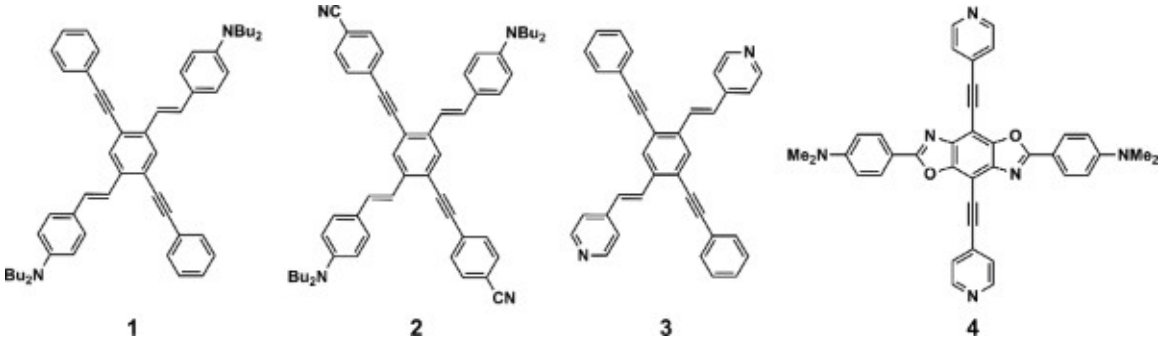

Figure 1. Cruciform fluorophores 1-4, based on 1,4-distyryl-2,5-bis(arylethynyl)benzene (1-3) and benzobisoxazole (4) nuclei, can be used to qualitatively distinguish structurally related carboxylic acids, boronic acids, amines, and other analytes. Click here to view larger figure.

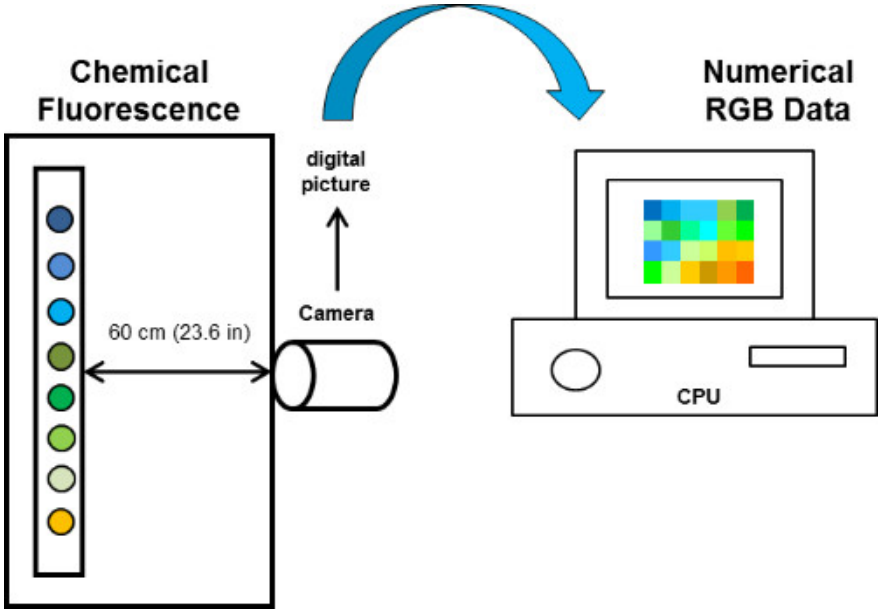

Figure 2. Setup for taking digital pictures of emission color and transformation into R/G/B values.

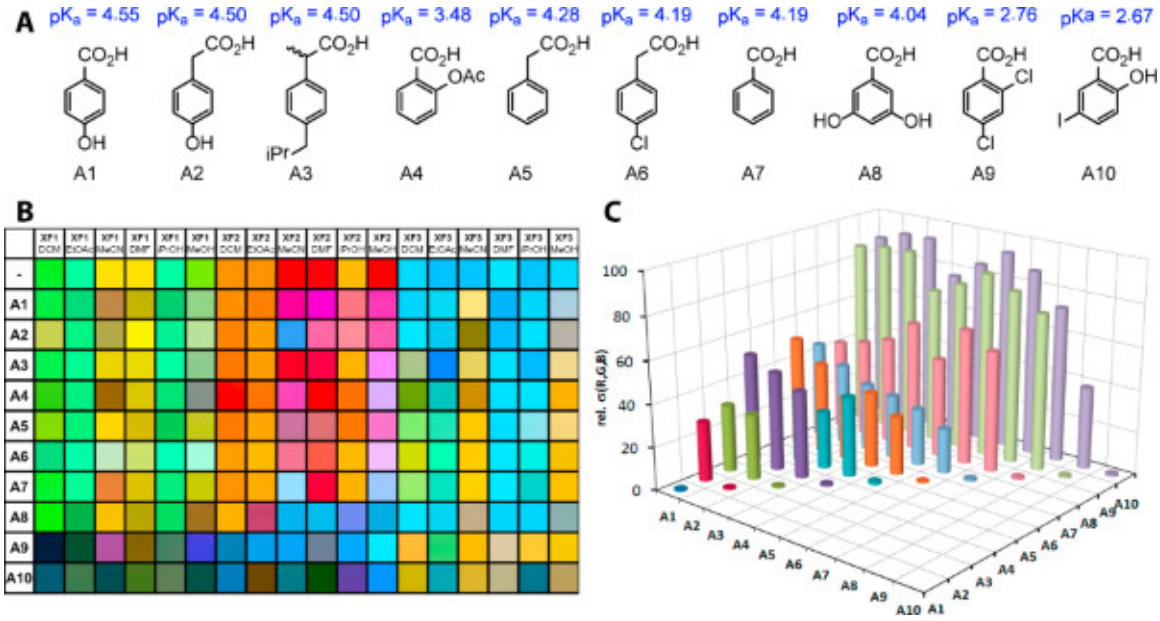

Figure 3. (A) Investigated carboxylic acids. **(B)** Array of digital photographs formed by XF 1-3 using six different solvents and ten different carboxylic acids (- = reference; A1 = 4-hydroxybenzoic acid; A2 = 4-hydroxyphenylacetic acid; A3 = ibuprofen; A4 = aspirin; A5 = phenylacetic acid; A6 = 4-chlorophenylacetic acid; A7 = benzoic acid; A8 = 3,5-dihydroxybenzoic acid; A9 = 2,4-dichlorobenzoic acid; A10 = 5-iodosalicylic acid); digital photographs were taken under black light irradiation (excitation wavelength 365 nm). **(C)** Autocorrelation plot formed from the fluorescent responses (encoded in R/G/B values) of the carboxylic acids A1-A10 from the array on the left side. The *z*-axis represents the relative standard deviation of R/G/B values to carboxylic acid A1. Click here to view larger figure.

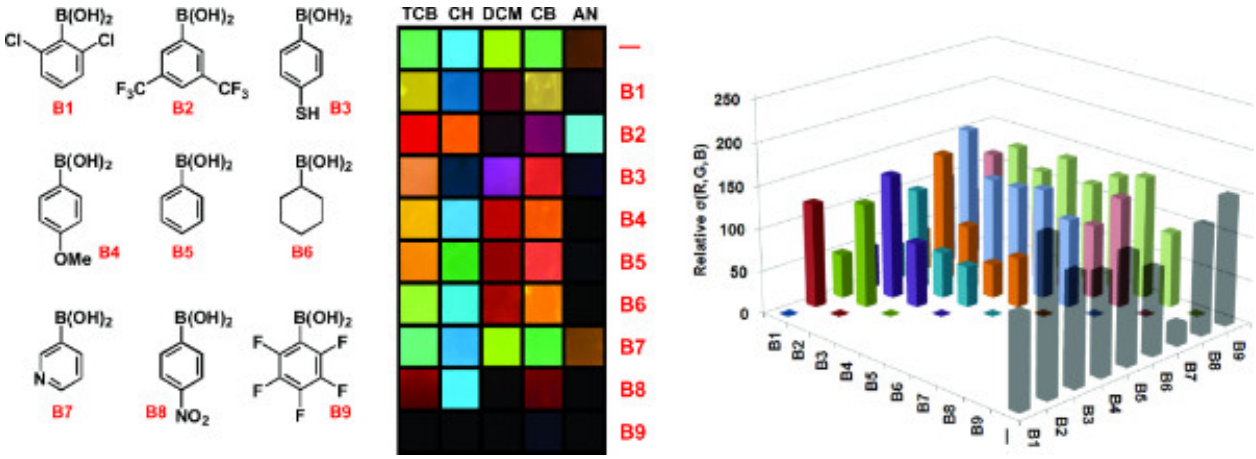

Figure 4. Discrimination of structurally closely related organoboronic acids B1-B9 (left) can be achieved using the solutions of cruciform 4 in various solvents (central panel; TCB = 1,2,4-trichlorobenzene; CH = cyclohexane; DCM = dichloromethane; CB = chlorobenzene; AN = aceto-nitrile). On the right, correlation diagram of various analytes' R/G/B values. Click here to view larger figure.

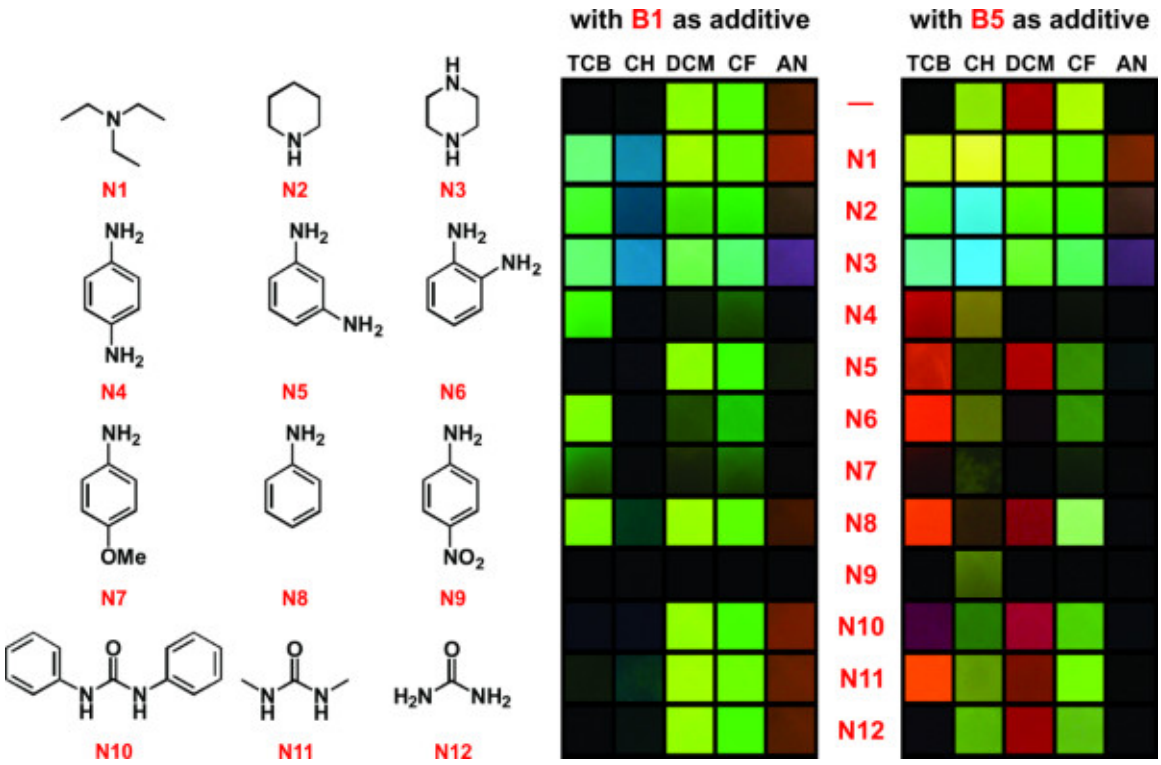

Figure 5. Discrimination of organic amines and ureas N1-N12 using a hybrid sensing system composed of cruciform 4 and boronic acid additives B1 and B5.

Discussion

The protocols for qualitative discrimination described in this paper and video hold significant potential in routine quality analyses, where even a minimally trained operator could discern the differences in composition, or deviations from a well-defined formula. Practicality of this technique could be further enhanced by using simple cellphone cameras, which, in combination with pattern- and image-recognition software such as Google Goggles, could match the recorded emission colors to the database of known compositions. Simple photography of emission colors is approximately two orders of magnitude faster that the rigorous fluorescence emission spectroscopy analysis, and in many cases can match spectroscopy in its ability to discern among different analytes.

While the presented protocols are highly selective in discerning structural differences among analytes, they are not very sensitive. Typically, analyte concentrations of several grams per liter are required to modulate cruciforms' emission colors. Thus our methods are unlikely to play a

role in analyses of trace ingredients. However, their strength lies in analyzing species that are available in large quantities but are sensitive to decomposition or counterfeiting: pharmaceuticals, food additives, basic chemicals, or alcoholic beverages.

Nevertheless, fluorophores 1-4 could in principle be rendered more sensitive by enhancing the binding affinities for analytes. As their pyridine and amine functionalities are basic in nature, the modification of the pyridine ring or the aniline to more specific or alkaline functionalities would be a promising start to decrease the detection limits. For example, 2-methylpyridine and 2,6-dimethylpyridine are more alkaline than pyridine and therefore the interaction of acidic analytes and fluorophore should improve. Another way to improve the detection would be the usage of a guanidine functionality instead of the alkylated amine. Finally, the sensitivity of the self-assembled 4/boronic acid sensing system could be improved by switching from boronic acid to a more electrophilic boron source, such as $PhBF_2$, which would increase the complexation constant for the complex. Several of these synthetic routes are underway in our laboratories.

Caveat exists: the analysis of fluorescent signal recorded with a digital camera is dependent on the color space and shutter speed, according to our recent findings.[15] Therefore, the RGB values of an emission color differ somewhat, depending on the camera adjustments. The following adjustments can be made on most cameras: white balance, shutter speed, film sensitivity, focal aperture, data format (RAW files or JPEG files), and color space (*i.e.* sRGB, Adobe RGB, or ProPhotoRGB). The best strategy for ensuring the constancy of the emission color response is to keep the white balance, the film sensitivity, and the focal aperture constant and only vary the shutter speed. Most of the issues are resolved when transforming the RGB values into coordinates of the CIE LUV color space and use only the hue coordinates *u'v'* without any brightness information. For this transformation it is important to know the color space of the recorded image. Using brightness-removed color coordinates, the identification of an unknown analyte is greatly improved when having recorded images with different RGB intensities.

Disclosures

We have nothing to disclose.

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