Supporting Information

Rapid Determination of Ionization Constants (pK_a) by UV Spectroscopy Using 96-well Microtiter Plates

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1) Preparation of buffer solutions

The pH of the buffers was measured with a glass electrode (HANNA HI-2210 pH-meter) at 30 ºC. All the solutions were prepared with distilled water. The 0.1 M standard solution of HCl was titrated with K_2CO_3 (C = 0.108 M). The 0.1 M standard solution of NaOH was sonicated during 10 min and titrated with potassium phthalate (C = 0.102 M). The ionic strength of each buffer solution was calculated using equation 1 and the amount of KCl needed to adjust the ionic strength to 0.1 M was calculated for each buffer (Table S1).

$$I = \frac{1}{2}\sum_{i=1}^{n} c_i z_i^2 \qquad (1)$$

where c_i is the molar concentration of ion i, z_i is the charge number of that ion, and the sum is taken over all ions in the solution.[1]

a) Acetic acid/sodium acetate buffers (AcOH/AcONa) covering the pH range 3.0 to 5.0.

100 mL of 0.1 M sodium acetate solution was prepared by dissolving sodium acetate (820.3 mg, 10 mmol) in 100 mL of distilled water.

50 mL of each buffer solution (pH 3.0, 4.0 and 5.0) was prepared as follows: 25 mL of 0.1 M sodium acetate solution was stirred at 30 ºC. The pH was adjusted to the required value by adding 0.1 M HCl standard solution with a burette. The volume was adjusted to 50 mL with distilled water. Finally, the ionic strength of the buffer solutions was calculated using equation 1 and adjusted to 0.1 M by adding KCl (see Table 1).

b) Potassium hydrogen phosphate/dipotassium hydrogen phosphate (KH_2PO_4/K_2HPO_4) buffers covering the pH range 6.0 to 8.0.

1 L of 0.05 M potassium hydrogen phosphate solution was prepared by dissolving KH_2PO_4 (6.8 g, 50 mmol) in 1 L of distilled water.

50 mL of each buffer solution (pH 6.0 to 8.0) was prepared as follows: 25 mL of 0.1 M KH_2PO_4 was stirred at 30 ºC in an erlenmeyer flask. The pH was adjusted to the required value by adding 0.1 M HCl or 0.1 M NaOH standard solution with a burette. The volume was adjusted to 50 mL with distilled water. The ionic strength calculated for these buffer solutions (Eq 1) were in the range 0.027 – 0.068 M. Hence, the ionic strength was adjusted to 0.1 M by adding the necessary quantity of KCl (see Table S1).

c) Sodium tetraborate decahydrate (Borax)/boronic acid ($Na_2B_4O_7/H_3BO_3$) buffers covering the pH range 8.2 to 10.8

1 L of 0.025 M borax solution was prepared by dissolving borax decahydrate (9.54 g, 25 mmol) in 1 L of distilled water.

50 mL of each buffer solution (pH 8.2 to 9.0) was prepared as follows: 25 mL of 0.025 M borax solution was stirred at 30 ºC. The pH was adjusted to the required value by adding 0.1 M HCl. The volume was adjusted to 50 mL with distilled water. The ionic strength calculated for these buffer solutions were in the range of 0.053 – 0.061 M. Hence, the ionic strength was adjusted to 0.1 M by adding the necessary quantity of KCl (see Table S1).

50 mL of each buffer solution (pH 9.2 to 10.8) was prepared using 25 mL of 0.025 M borax ($Na_2B_4O_7.10H_2O$) stirred at 30 ºC. The pH was adjusted to the required value by adding 0.1 M NaOH. Finally, the ionic strength was adjusted to 0.1 M by adding KCl (See Table Sl).

d) Disodium hydrogen phosphate/sodium phosphate (Na_2HPO_4/Na_3PO_4) buffers covering the pH range 10.9 to 12.

1 L of 0.05 M disodium hydrogen phosphate solution was prepared by dissolving Na_2HPO_4 (7.098 g, 50 mmol) of in 1L of distilled water.

25 mL of a solution of 0.05 M Na_2HPO_4 was stirred at 30 ºC. The pH was adjusted to the required value by adding 0.1 M NaOH. The volume was adjusted to 50 mL with distilled water. The ionic strength calculated for these buffer solutions were in the range 0.077 – 0.088 M. Hence, the ionic strength was adjusted to 0.1 M by adding the necessary quantity of KCl (see Table 1).

e) Glycine/NaOH buffer (pH 12.6)

This buffer was commercially available (Fluka 33552).

Table S1. Preparation of buffer solutions of constant ionic strength (I = 0.1 M)

buffer	Target pH	Measured pH	Conc. of salt solution (M)	Vol. of salt solution (mL)	Vol. of 0.1 M* HCl (mL)	Vol. of 0.1 M** NaOH (mL)	Vol. H_2O (mL)	Calculated Ionic strength (M)	Conc. of KCl required (M)	Mass of KCl (mg)
NaOAc/HOAc	3.00	**2.91**	0.1	25	22.8		2.2	0.05	0.05	186.38
	4.00	**3.78**			19.9		5.1	0.05	0.05	186.38
	5.00	**4.99**			8.8		16.2	0.05	0.05	186.38
KH_2PO_4/K_2HPO_4	6.00	**6.04**	0.05	25		0.73	24.27	0.028	0.072	268.38
	6.20	**6.22**				1.12	23.88	0.030	0.07	260.93
	6.40	**6.41**				1.68	48.32	0.032	0.068	253.47
	6.60	**6.60**				2.47	47.53	0.035	0.065	242.29
	6.80	**6.78**				3.50	46.5	0.039	0.061	227.38
	7.00	**6.99**				4.75	45.25	0.044	0.056	208.74
	7.20	**7.30**				6.14	43.86	0.05	0.05	186.38
	7.40	**7.37**				7.53	42.47	0.056	0.044	164.01
	7.60	**7.56**				8.78	41.22	0.061	0.039	145.37
	7.80	**7.77**				9.80	40.2	0.065	0.035	130.46
	8.00	**8.03**				10.59	39.41	0.068	0.032	119.28
Borax	8.20	**8.23**	0.025	25	5.73		19.27	0.06	0.040	149.10
	8.40	**8.42**			5.46		44.54	0.059	0.041	152.83
	8.60	**8.61**			5.09		44.91	0.058	0.042	156.56
	8.80	**8.80**			4.89		45.11	0.056	0.044	164.01
	9.00	**8.98**			3.97		46.03	0.053	0.047	175.19
	9.20	**9.19**				2.98	47.02	0.05	0.050	186.38
	9.40	**9.41**				3.69	46.31	0.05	0.050	186.38
	9.60	**9.58**				4.35	45.65	0.05	0.050	186.38
	9.80	**9.80**				4.9	45.1	0.05	0.050	186.38
	10.00	**9.99**				5.33	44.67	0.05	0.050	186.38
	10.20	**10.24**				5.63	44.37	0.05	0.050	186.38
	10.40	**10.41**				5.85	44.15	0.05	0.050	186.38
	10.60	**10.61**				5.99	44.01	0.05	0.050	186.38
	10.80	**10.81**				6.08	43.92	0.05	0.050	186.38
Na_2HPO_4/Na_3PO_4	11.00	**11.02**	0.05	25		2.2	22.8	0.077	0.023	85.73
	11.20	**11.22**				3.1	46.9	0.077	0.023	85.73
	11.40	**11.39**				4.1	45.9	0.079	0.021	78.28
	11.60	**11.58**				6.4	43.6	0.081	0.019	70.82
	11.80	**11.76**				9.1	40.9	0.084	0.016	59.64
	12.00	**12.00**				12.9	37.1	0.088	0.012	44.73

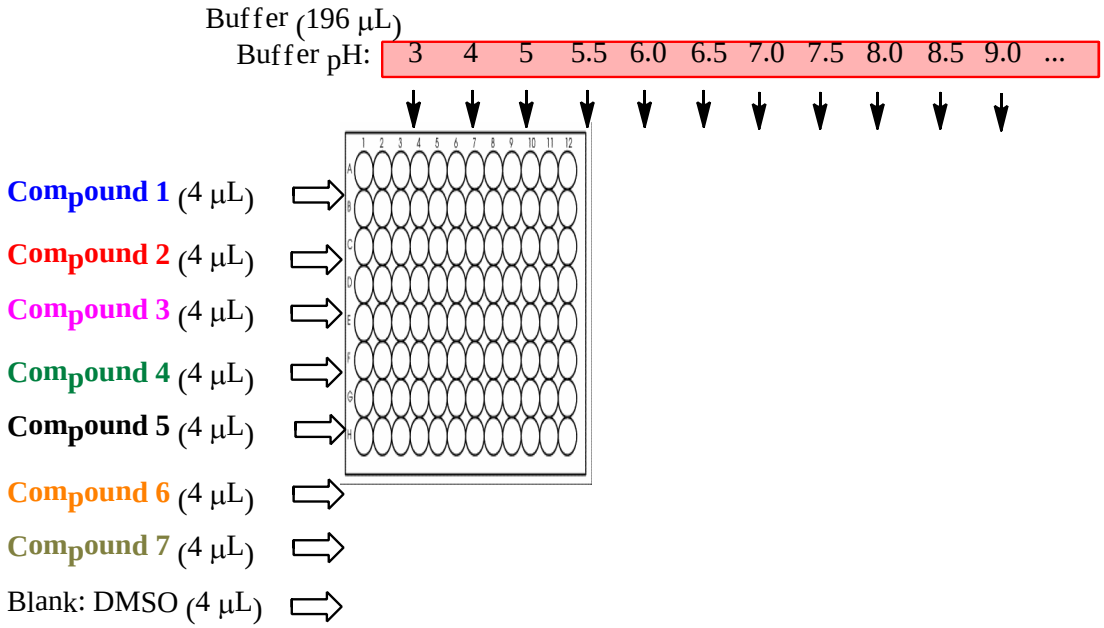
* C = 0.108 M ** C = 0.102 M

2) Experimental procedure

A small quantity (1–2 mg) of each compound was weighted in an eppendorf tube using a high precision analytical balance (Mettler Toledo XS105). The compound was dissolved in DMSO to a concentration of 10 mM (stock solution). The UV transparent 96-well microplate (Thermo Scientific Nunc) was loaded as shown in Figure S1: each line of the plate was loaded with 196 µL of buffer solutions of increasing pH (pH ranging from 3 to 12.6). Then, 4 µL of the compound stock solutions were added to each well with a micropipette (the resulting analyte solution was premixed with the micropipette). The final concentration of the analyte compound in each well was 200 µM. One blank solution was prepared for each buffer by adding 4 µL of DMSO to 196 µL of the corresponding buffer solution (i.e., free of analyte compounds) in the well. The 96-well plate was loaded into the UV-spectrophotometer (THERMO Multiskan Spectrum apparatus), incubated at 30 ºC and shaken for 10 min before the reading was performed. UV-spectra scans were recorded between 210 nm and 400 nm (2 nm resolution).

It should be noted that the number and range of buffer solutions needed to determine the pK_a (e.g., every 0.2, 0.5 or 1 pH units) was adjusted depending on the compound tested. In general, a first screening with 12 buffers ranging from 3 to 12 should give an approximate pK_a value which can be refined when repeating the experiment using buffers within ± 2 pH units of the pK_a value.

Figure S1. Example of loading of the 96-wells microtiter plate



3) Data Analysis

In the present study we have used a similar data analysis as described by Tomsho et al. for the determination of ionization constants by spectral analysis.[2]

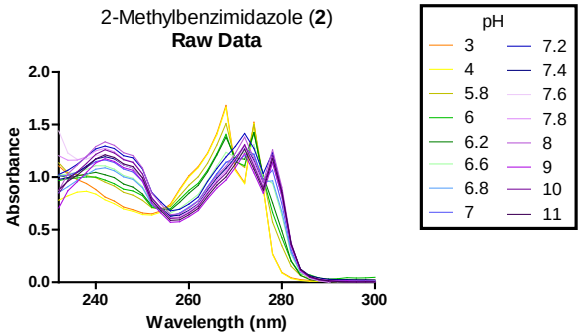
The raw UV-spectra scans were imported to the Excel program and processed as follows: 1) UV-spectra of the analyte compounds were corrected by subtracting the UV-spectra of the blank solutions; 2) The raw scans were normalized ($Abs_{400\ nm} = 0$); 3) The spectral difference between the acid spectra and the spectra at every other pH was plotted; 4) The wavelengths of maximum positive and negative absorbance were determined graphically from the spectral difference plot; 5) The total absorbance difference at the chosen wavelengths (that is: the sum of the absolute values of the maximum positive and negative absorbance in the spectral difference plot) was plotted against the pH. 6) These data were imported to the Prism program and the pK_a values were worked out by non linear regression using equation 1:

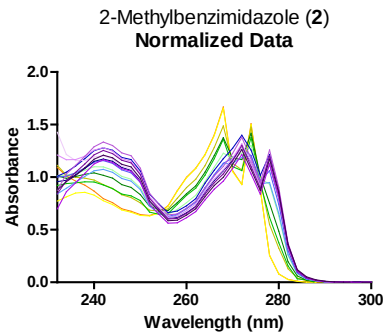
$$Absorbance\ total = \frac{\varepsilon_{HA} - \varepsilon_{A^-} * [10^{(pH - pKa)}]}{1 + 10^{(pH - pKa)}} * [S_t] \qquad \text{Eq. 1}$$

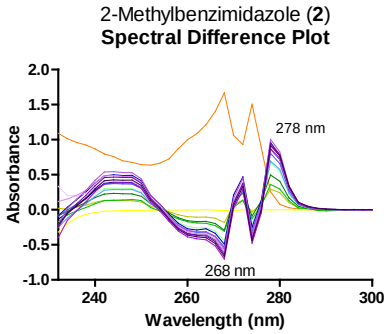
ε_{HA} and ε_{A^-} are the extinction coefficients of the acid and base forms of the compound, respectively (i.e., the minima and maxima of the absorbance difference curve, respectively), and $[S_t]$ is the total compound concentration.

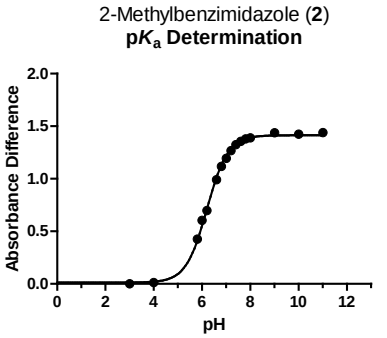
4) Spectra and pK_a determination plots for compounds 2–8

- 2-Methylbenzimidazole (**2**)

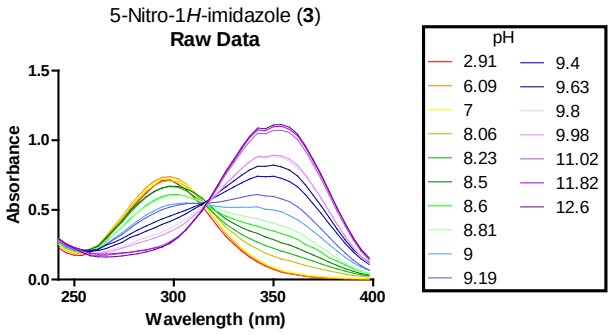


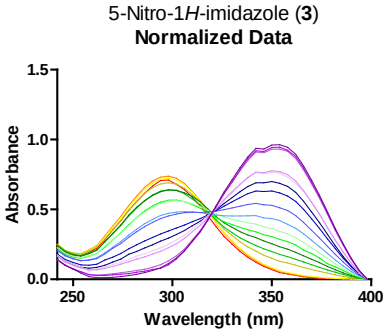


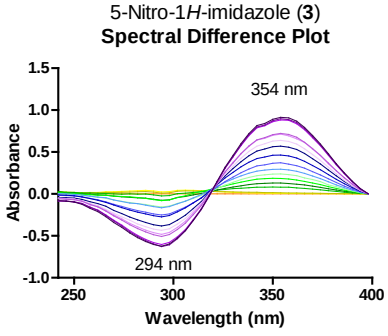


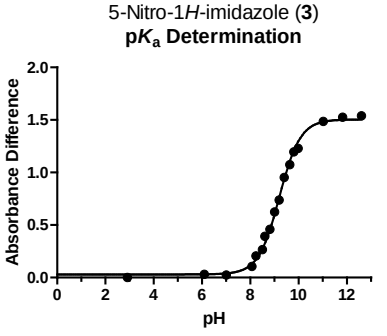


- 5-Nitro-1*H*-imidazole (**3**)









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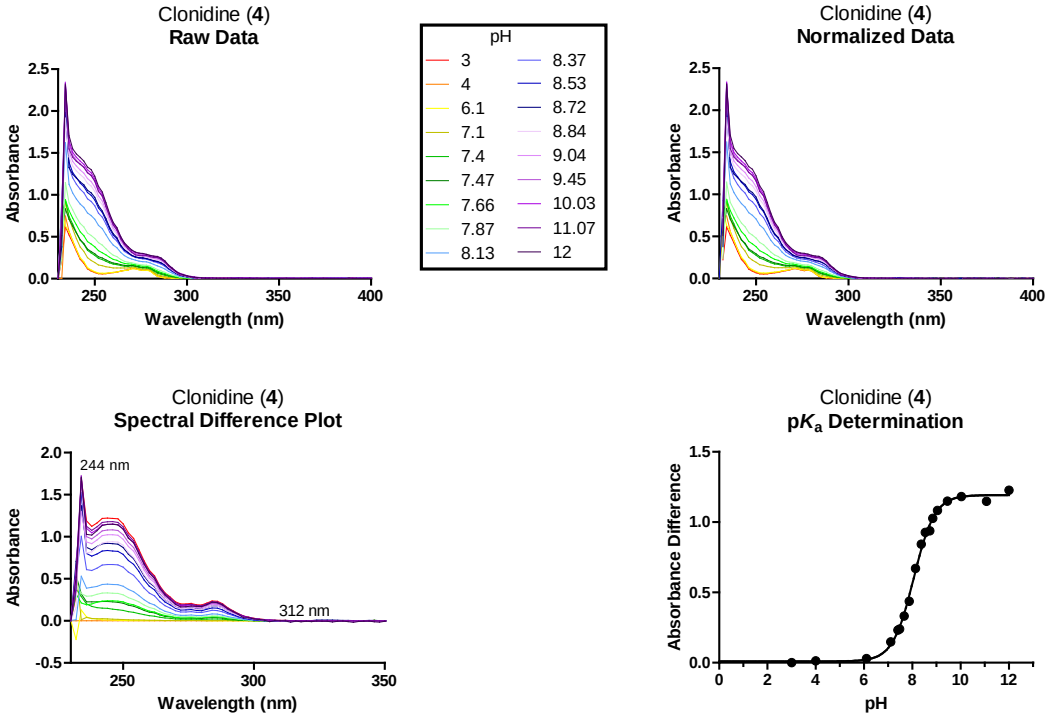
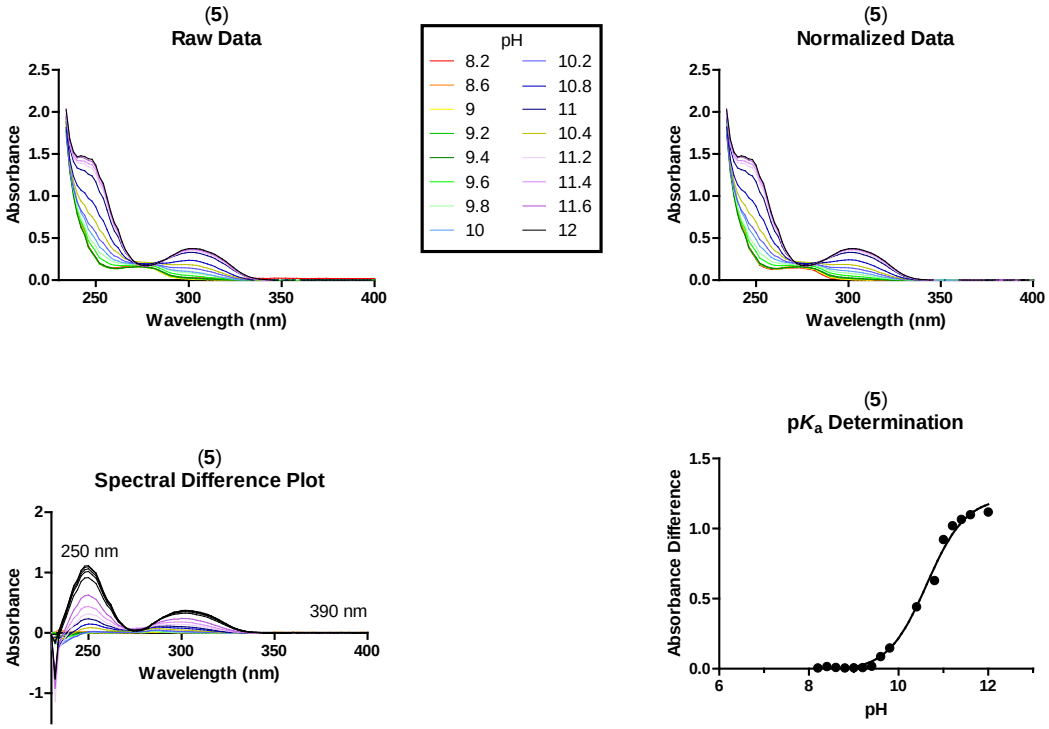


o Oxybis(4,1-phenylene) dicarbamimidate (**5**)



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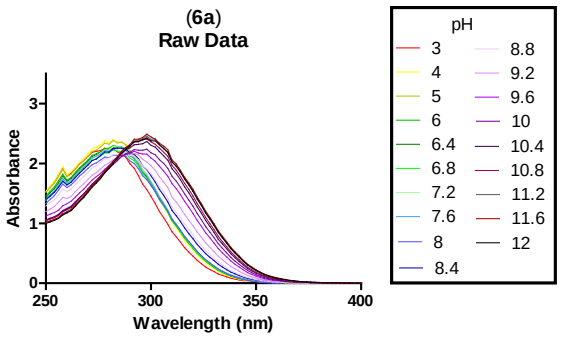

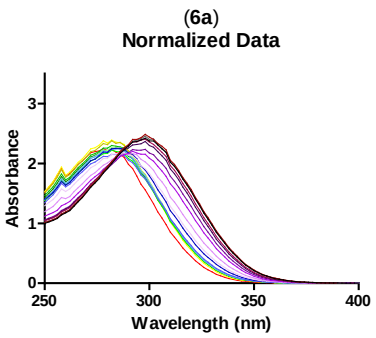

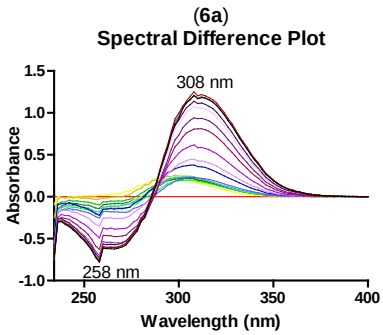

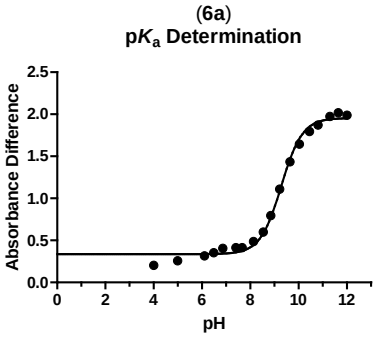

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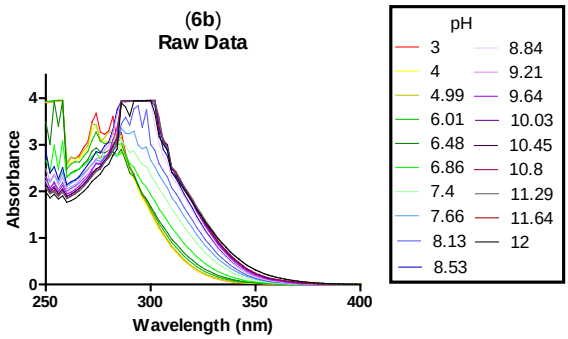

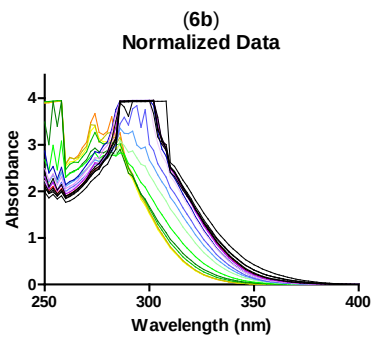

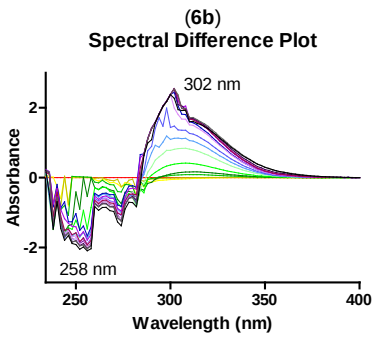

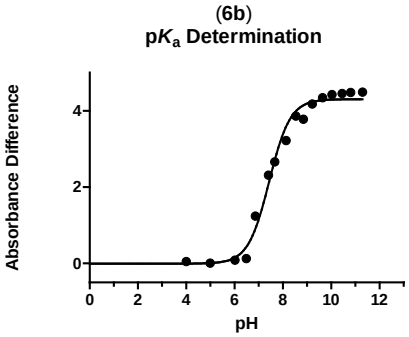

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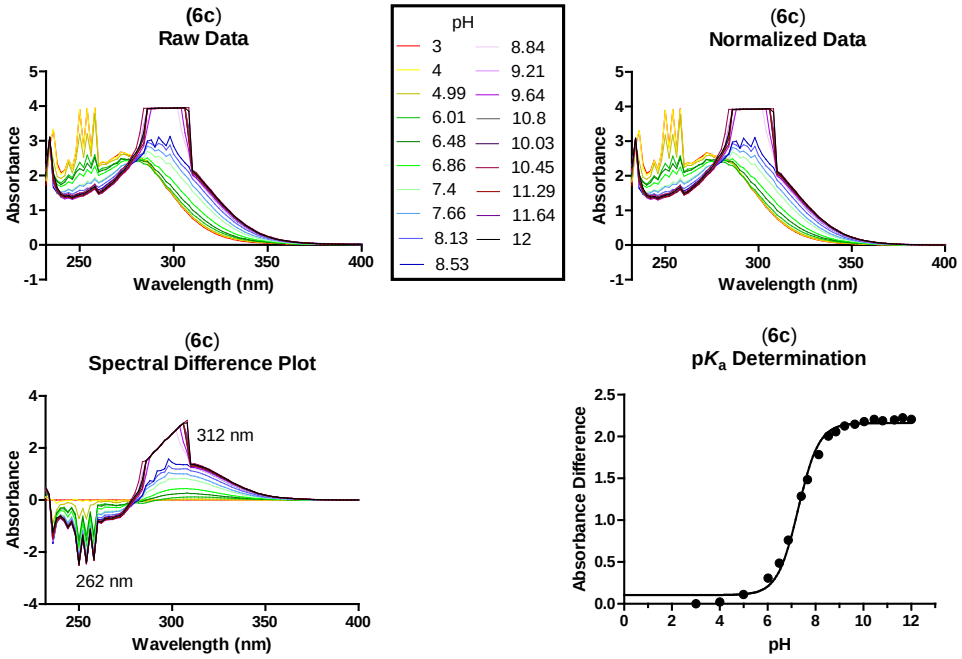

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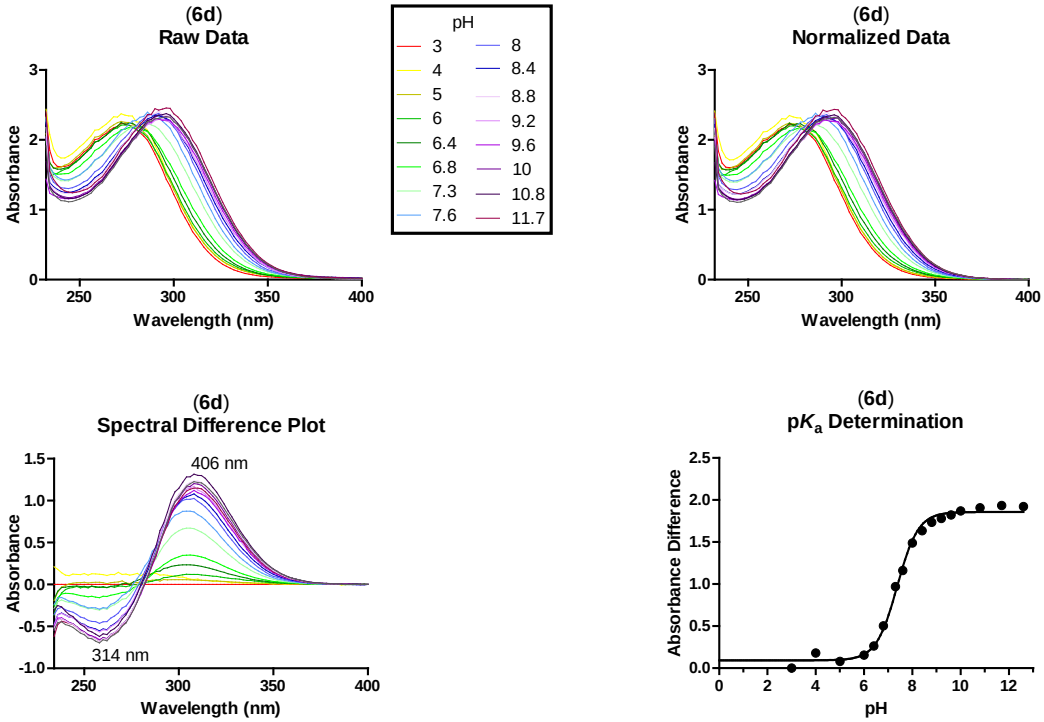

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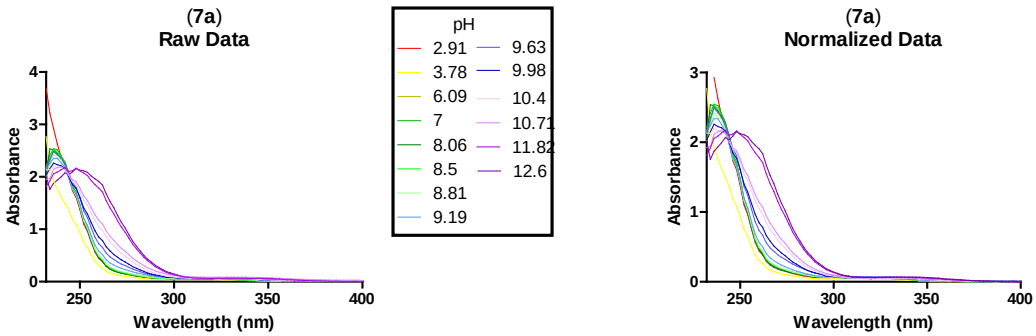

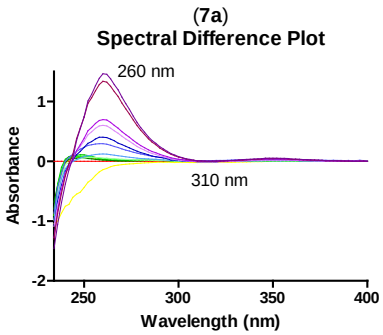

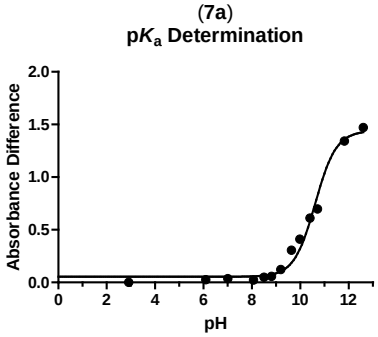

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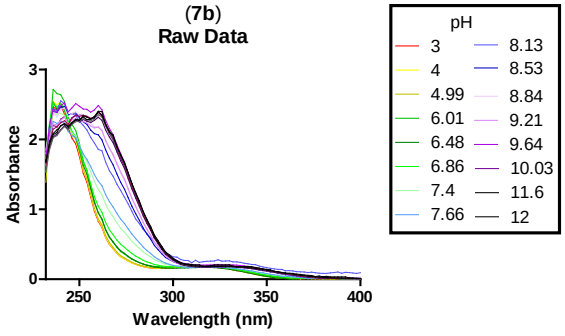

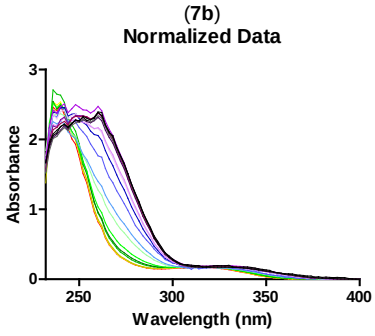

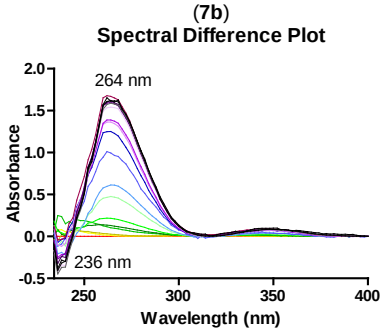

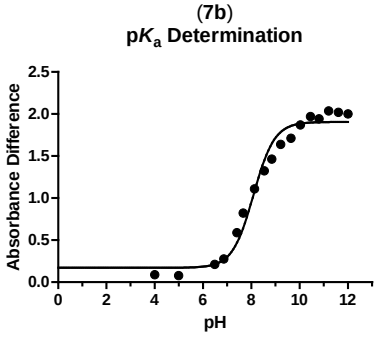

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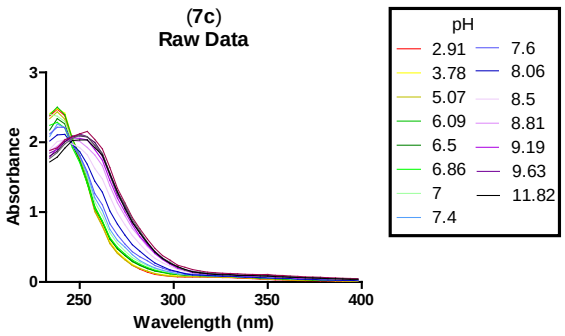

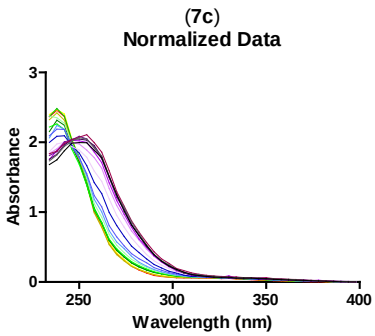

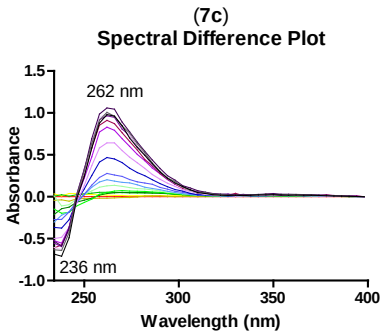

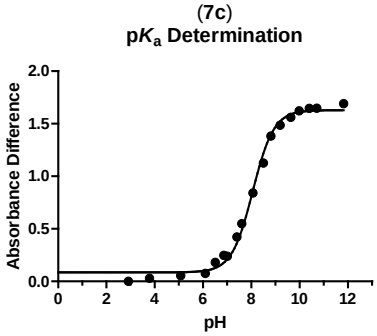

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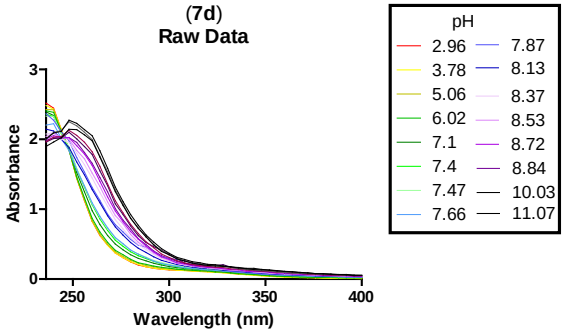
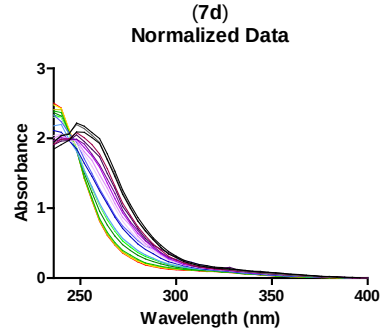
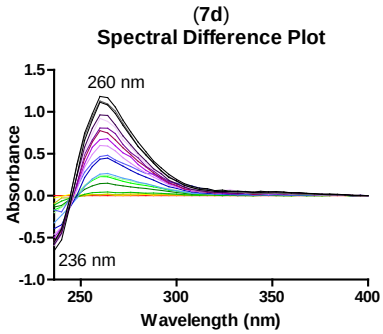
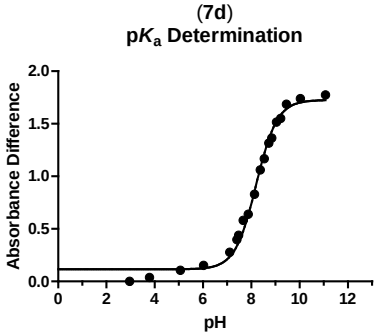
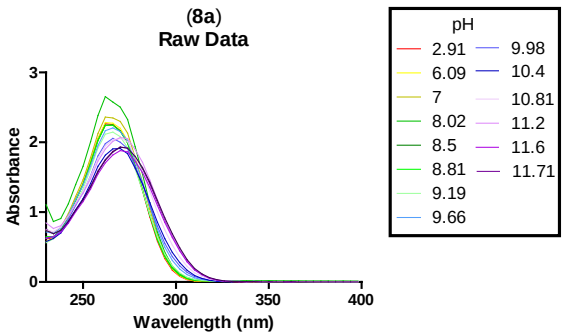


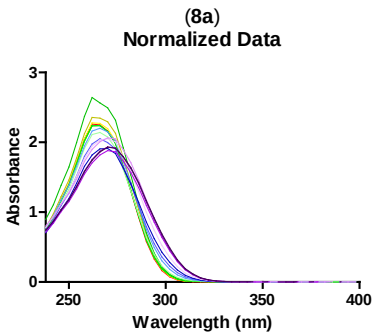


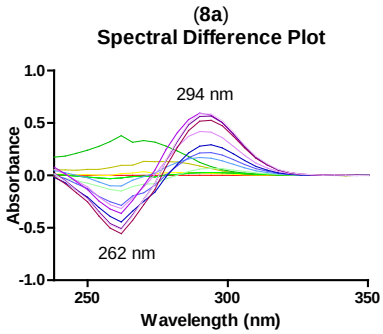


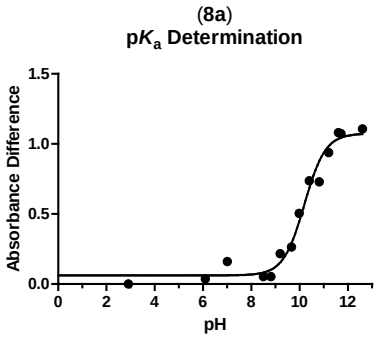


- Compound **8a**

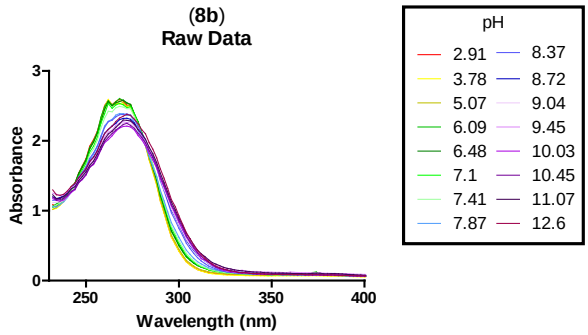


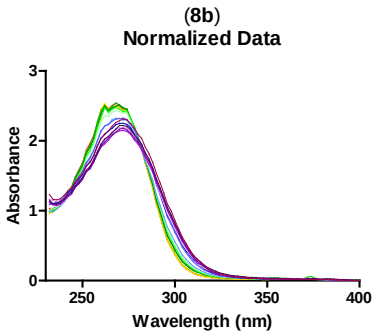


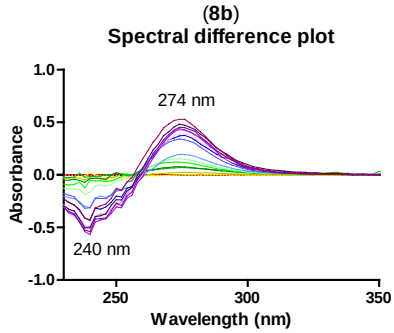


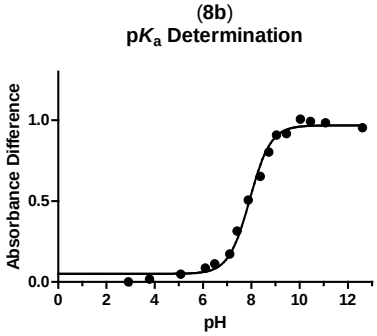


- Compound **8b**









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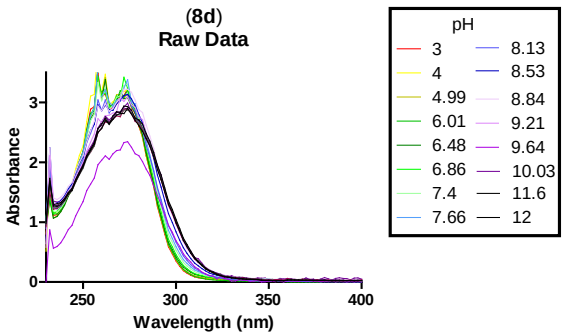

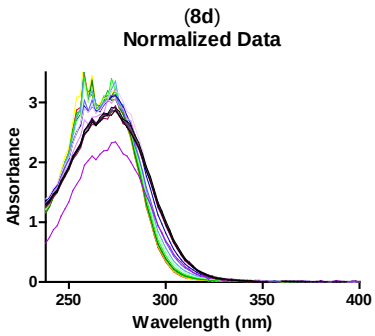

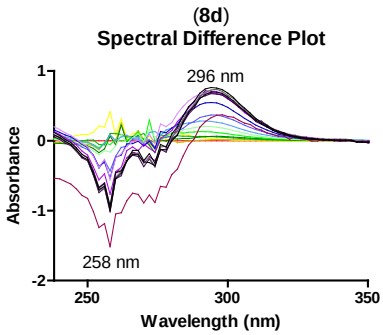

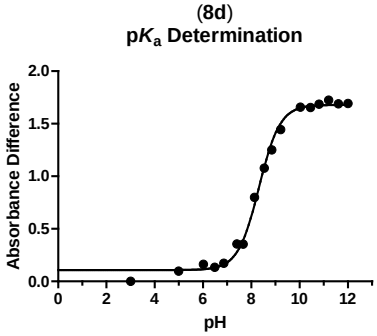

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