A pH determination method suitable for high-throughput approaches based on spectral absorbance

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- **Spectral absorbance data for bromothymol blue, collected on the SPECTROstar^{Nano} exhibit pH dependence**
- **pH changes of both basic and neutral forms of bromothymol blue can be used to create standard curves**

Introduction

Accurate determination of pH is important due to its effect on enzyme activity and other biological and chemical applications[1]. However, data collection using electrodes is not compatible with high throughput approaches which employ microplate readers.

Here we describe a spectrometer-based method that employs the SPECTROstar^{Nano}. We employed the phenol-based dye bromothymol blue, which is ideally suited for a colorimetric assay to study pH in the physiological range. This method allows for rapid and accurate pH measurement which can be easily placed in line with Hudson's SOLO Automated Pipettor and Micro 10X Dispenser for plate preparation. Complete automation can be attained by including the PlateCrane EX Robotic Arm from Hudson.

Assay Principle

Bromothymol blue is a phenol-based dye that acts as a weak acid in solution. Under basic conditions it is deprotonated, and this ring-opened form is blue and absorbs light at around 620 nm (Figure 1; right side). The neutral, ring-closed form is yellow and exhibits absorbance maxima between 400 and 430 nm (Figure 1; left side).

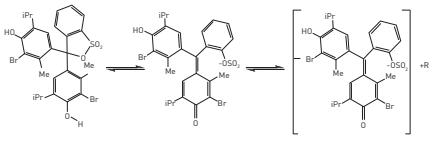

Fig. 1: Transitions between forms of bromothymol blue. Structure of the neutral (yellow) form and basic (blue) form are depicted.

Materials & Methods

- SOLO Automated Pipettor and 10 X Dispenser (Hudson)
- 384-well, flat bottom plates (UV transparent, Costar)
- SPECTROstar^{Nano}
- Bromothymol blue and pH standard buffers obtained from commercial sources

Experimental Procedure

Plates were prepared using the SOLO Automated Pipettor and Micro 10X Dispenser. First 20 µl of bromothymol blue was added to each well. Subsequently, 80 µl of each standard was added. Five replicates were used for each standard, representing a range from pH 6 to 7.8. Plates were read with the SPECTROstar^{Nano} using the following settings.

Instrument Settings

Optic settings	Absorbance, spectrum	
	Wavelength range (step-width) nm	220-1000 (1)
General settings	Number of flashes	200
	Settling time	1.0 s

Data analysis was performed using BMG LABTECH's MARS software.

Results & Discussion

Figure 2 shows the results of spectral measurements for the pH 7 standard. In this sample you can see the absorbance maxima for both the basic and neutral forms of bromothymol blue at 618 nm and 420 nm respectively.

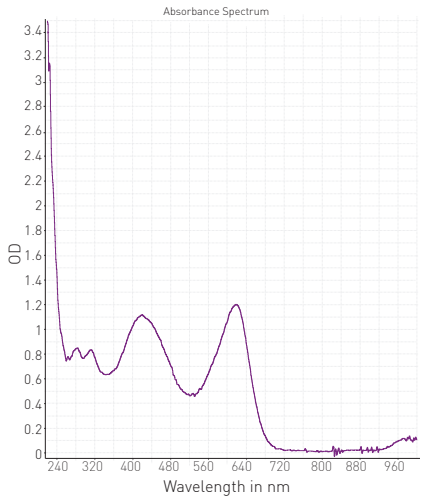

Fig. 2: Absorbance spectrum for bromothymol blue at pH = 7

For the basic form of bromothymol blue, plotting the OD at the maxima (618 nm) versus the pH value results in a standard curve that corresponds well to a 4-parameter fit (Figure 3).

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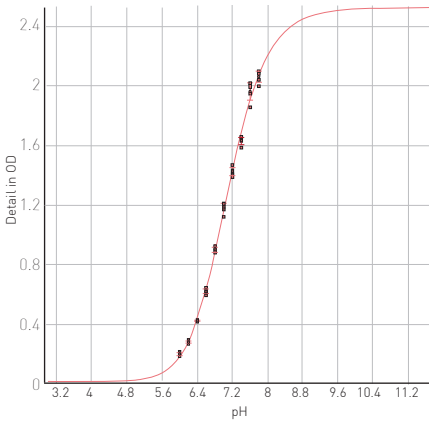

Fig. 3: Plot of pH dependence of the absorbance of the basic form of bromothymol blue
4-parameter fit; OD at maxima (618 nm) – OD at baseline (750 nm). R^2=0.99.

Many researchers use the absorbance of the basic form of bromothymol blue to determine pH of unknowns. However, any variations that change the focal length can introduce errors into the measured OD. For that reason, we explored the possibility of using the changes seen in the neutral form of bromothymol blue.

Figure 4 shows an overlay of spectra obtained between 320 and 500 nm. As pH increases the maximum OD decreases as does the wavelength at which the maxima is observed.

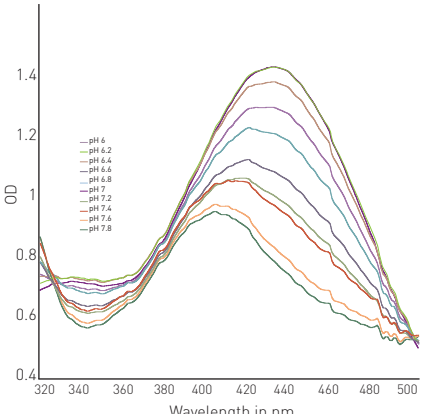

Fig. 4: Absorbance spectra of the neutral form of bromo. thymol blue at varied pH.
Absorbance from 320-500 nm is shown. Absorbance maxima are observed between 405 nm and 434 nm in this spectral range.

Figure 5 shows that plotting the max absorbance for the neutral form versus pH is also suitable for pH prediction.

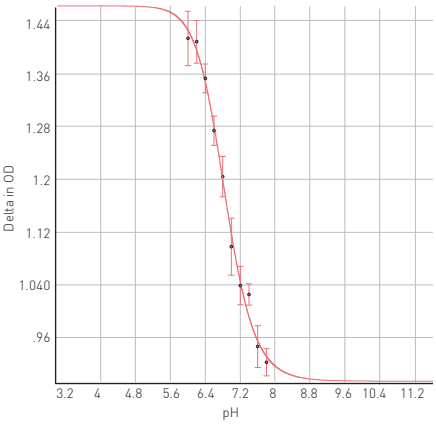

Fig. 5: Plot of pH dependence of the absorbance of the neutral form of bromothymol blue
4-parameter fit; OD at maxima – OD at baseline (750 nm). R^2=0.993

Conclusion

Using either the basic form or the neutral form of bromothymol blue provides equivalent prediction of the pH of unknown samples. Preliminary data (not shown) indicate that using the neutral form improves the %CV of the calculated pH values. The SPECTROstar^{Nano} and MARS data analysis make the possibility of analyzing the neutral form both fast and easy.

References

1. Wang, J et al. A graphical method of analyzing pH dependence of enzyme activity. Biochim. Biophys. Acta (1999) 1435: 177-183.