



Use of the Spectral Scanning Capabilities of the PowerWave™ 200 Microplate Spectrophotometer

Spectral analysis of samples can be used for a number of different requirements, including contaminant detection, peak absorbance determination, and product formation. Here we describe some of the uses of the spectral scanning capabilities of the PowerWave 200 scanning microplate spectrophotometer in conjunction with KC4 data reduction software.

Introduction

The spectral analysis of samples has a number of different purposes. The most obvious is the determination of the wavelength of light that is absorbed maximally by a particular solution. Similarly, absorbance profiles can be determined in order to ascertain wavelengths, while not necessarily maximal, that provide adequate absorbance for accurate determination. Contaminants in samples can be detected by the deviation from known patterns of spectral analysis. In many cases the contaminant can be determined by the new spectral scan. Newly synthesized or unknown compounds can also be identified using a spectral analysis of that compound in solution. Here we describe some of the applications for spectral analysis of compounds in solution.

Materials and Methods

The 96 well clear UV transparent microplates, catalogue number 3635, were purchased from Costar, (Cambridge, MA). Egg shade color yellow food coloring in solution was obtained from National Institutional Food Distributor Associates Inc. (Atlanta, GA). Sodium fluorescein, methylumbelliferone (7-hydroxy-4-methyl-coumarin), and propidium iodide were purchased from Molecular Probes (Eugene OR), and FD&C Blue No. 1 and Yellow No. 5 were purchased from Warner Jenkinson (St. Louis MO) as powders.

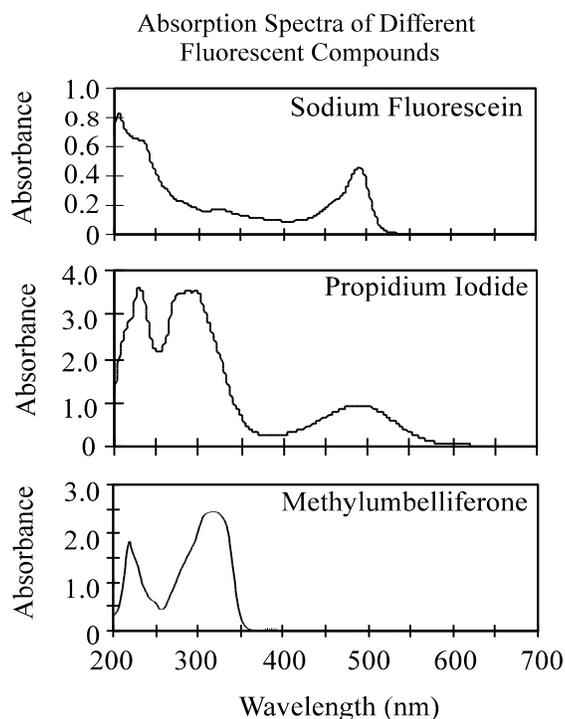


Figure 1. The absorption spectra from 200 nm to 700 nm for sodium fluorescein, propidium iodide, and methylumbelliferone. The absorbances at wavelengths ranging from 200 nm to 700 nm were determined in 1 nm increments using the PowerWave 200. Sodium fluorescein and propidium iodide samples were blanked on water, while methylumbelliferone was blanked on methanol.

Scanning spectrophotometric measurements were made using a PowerWave 200 scanning microplate spectrophotometer (Bio-Tek Instruments Winooski, VT) in Costar UV-transparent microplates. Data was captured using KC4 data reduction software to control reader function (Bio-Tek Instruments, Winooski, VT). In order to correct for the different background absorbance of the microplate at different wavelengths, the absorbance values were exported to Microsoft Excel and the absorbance at each wavelength of a water blank was subtracted from the experimental data. Thus, samples were blanked with water at each wavelength.

Peak Absorbance Determinations

The absorption spectra of several fluorescent compounds were examined. Stock solutions of, sodium fluorescein, propidium iodide, were prepared by dissolving the powders in distilled water, while methylumbelliferone was dissolved in methanol. For absorbance spectrum analysis 200 μ l of each solution was placed in a microplate well and scanned from 200 nm to 700 nm in 1 nm increments. As demonstrated by Figure 1, different compounds will exhibit different spectrum patterns of absorption. Sodium fluorescein in aqueous solution exhibits a peak in absorbance centered at 490 nm. Propidium iodide demonstrates three such peaks: 230 nm, 293 nm and 488 nm, while methylumbelliferone exhibits an absorbance peak at 321 nm.

Spectral scan comparison

Purified genomic herring sperm DNA was digested with EcoRI (Gibco-BRL, Gaithersburg, MD) followed by organic phenol/chloro-form/isoamylalcohol extraction and ethanol precipitation. A concentrated stock solution was made with subsequent rehydration to a 5 mg/ml final concentration. Prior to spectral analysis, the DNA stock solution was further diluted with distilled water to 300 mg/ml and 150 mg/ml. Following dilution, 200 μ l of the 300 mg/ml DNA solution was placed in a microplate well and scanned from 225 nm to 325 nm in 1 nm increments using a PowerWave 200 scanning microplate spectrophotometer. Comparison data was obtained using

a Spectronic Plus 1001 spectrophotometer (Milton Roy, Rochester, NY) to scan the 150 mg/ml DNA solution over the same wavelengths in 1 nm increments.

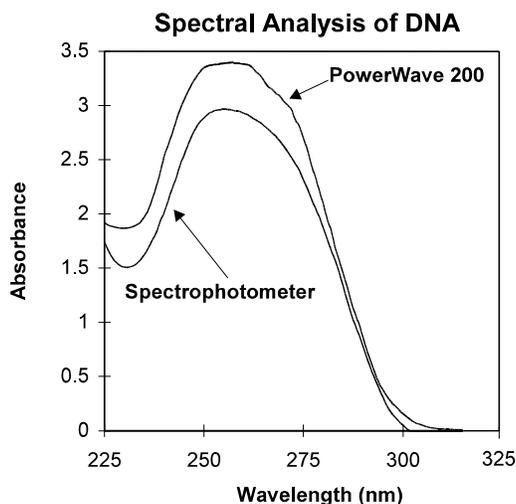


Figure 2. Comparison of absorbance spectrums produced by the PowerWave to a conventional spectrophotometer. DNA in solution was scanned from 225 nm to 325 nm in 1 nm increments using either a Spectronic 1001 spectrophotometer (Milton Roy Co. Rochester, NY) or a PowerWave 200 microplate reader (Bio-Tek Instruments, Winooski, VT). Note, the DNA concentration used for determinations with the spectrophotometer was approximately half that used for determinations using the PowerWave 200.

When the spectra for DNA in solution obtained using the PowerWave 200 is compared to that obtained from a conventional spectro-photometer, very similar peaks in absorbance are observed (Figure 2). Differences in the light path length require that different concentrations of DNA solution be scanned for equivalent absorbance comparison. Because the light path length in a cuvette is approximately two times that of 200 ml of solution in a flat bottom microplate well (1.0 cm vs. 0.55 cm), the concentration used with the spectrophotometer was half that used for the microplate reader. Although slight differences in the magnitude of the absorbance peak can still be observed as a result of incomplete compensation for light path length, the peak wavelength, as well as the general shape of the curve were observed to be the same.

Effect of concentration on absorbance spectra

In order to demonstrate the effect of concentration on absorbance with spectrum analysis of compounds, several dilutions of water soluble dye were prepared and scanned from 200 nm to 600 nm in 1 nm increments. Using a stock solution empirically determined to have an absorbance of approximately 4 at 450 nm, several dilutions of egg shade color yellow food coloring were made using various amounts of a stock solution of dye in each well diluted to a final volume of 300 ml per well with distilled water. Data was collected from 200 nm to 700 nm in 1 nm increments.

Figure 3 demonstrates the effect of concentration on the absorption spectrum of a solution, as well as the importance of proper sample dilution prior to the determination of absorbance. The sample with the highest concentration displays a two plateaus representing absorbance values above the range of the reader, while less concentrated dilutions do indeed demonstrate discrete peaks at those locations. If samples could not be diluted, the use of non maximal wavelengths could be used to quantitate the sample. For example, measuring the absorbance of the sample depicted in Figure 3 at 260 nm would allow the direct quantitation of the samples at concentrations that exceed the instrumental maximal levels if measured at the peak in absorbance at 428 nm.

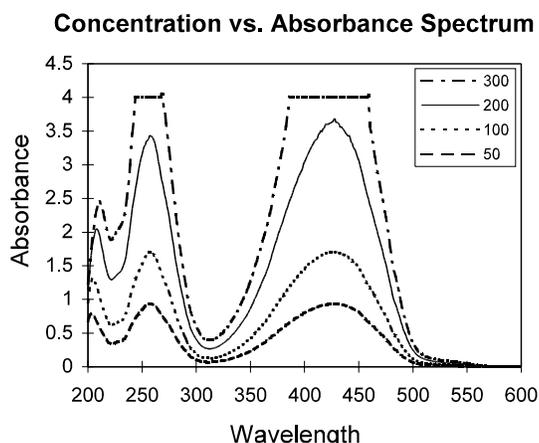


Figure 3. The effect of concentration of a solution on absorbance. The absorbance was determined for various dilutions of egg shade yellow dye using the PowerWave 200 from 200 nm to 600 nm in 1 nm increments. Indicated value represents the volume in microliters of a stock solution present in a flat bottom microplate well. Thus the sample marked 50 represents a six fold dilution of the sample marked 300. All wells were diluted to a final volume of 300 ml per well with distilled water prior to scanning.

Determination of components from a mixture

The ability to detect different compounds from a mixture was examined by mixing stock solutions of FD&C Blue No. 1 with FD&C Yellow No. 5. The stock solutions of both dyes, prepared as described above, were diluted empirically such that 100 ml of the blue or yellow solution had an absorbance at 630 nm or 450 nm of 3-4 ODs respectively. For analysis, 100 ml of each dye was placed in a single well (200 ml total volume), then scanned from 200 nm to 750 nm in 1 nm increments. For comparison, 100 ml of each dye were pipetted into separate wells, diluted to 200 ml with distilled water and scanned.

Identification of individual compounds from a mixture is demonstrated by Figure 4. When a solution containing a mix of FD & C Blue No. 1 and Yellow No. 5 is scanned (Figure 4A), the visually green solution is observed to have four discrete peaks in absorbance at 210 nm, 257 nm, 415 nm, and 629 nm. Comparison with known solutions can identify the peak in absorbance centered at 629 nm to be the result of the presence of FD&C Blue No. 1 (Figure 4B), while the three other peaks are the absorbance spectrum of FD&C Yellow No. 5 (Figure 4C).

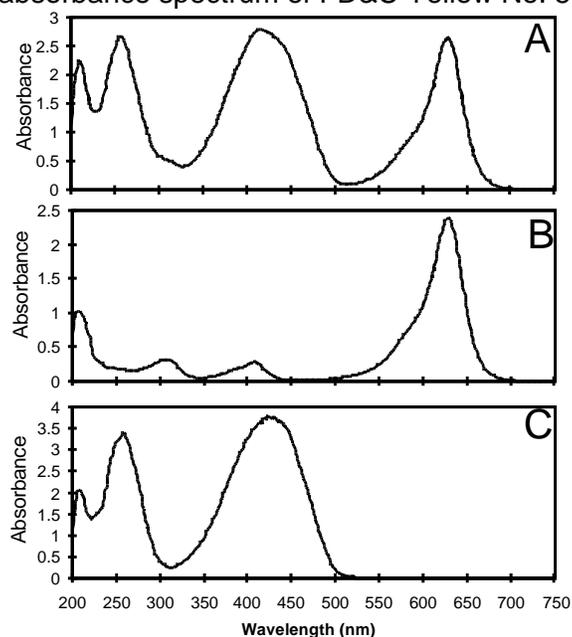


Figure 4. Determination of components from a mixture by comparison to the spectrum of known compounds. The absorbances of solutions containing a mix of FD&C Blue No. 1 and FD&C Yellow No. 5 dyes (A); FD&C Blue No. 1 dye only (B); and FD&C Yellow No 5 dye only (C) were determined using the scanning feature of the PowerWave 200 from 200 nm to 750 nm in 1 nm increments. Any absorbance generated by the microplate was corrected by subtracting the absorbance at each wavelength of a distilled water control.

Discussion

While the examples of using the scanning function of the PowerWave[®] 200 scanning microplate spectrophotometer are relatively simplistic, they do demonstrate the utility of the function. In the case of fluorescent compounds, finding a region, preferably a peak, in the absorption spectra that does not overlap the fluorescent emission spectrum is necessary for fluorescent determinations. Direct comparison of the absorption spectra of DNA suggests that the PowerWave 200 can be used in place of a standard spectrophotometer.

Using the KC4 data reduction package to capture the data has several features not presented with these examples. The KC4 data reduction software allows the user to automatically determine maximum and minimum values, along with their corresponding wavelengths. After scanning, the wavelength range to be examined can also be narrowed such that information concerning minor peaks can be obtained.

The parameters by which the spectrum scan is performed can influence the amount of time required to read the plate. The time required to scan a complete microplate (96 samples) from 200 nm to 800 nm in 1 nm increments is approximately 40 minutes. Naturally, scanning less than a complete plate will decrease the amount of time necessary for the scan. Because the reader reads multiple wells in the same 1 x 8 strip, placing the samples in the fewest number of strips will maximize throughput. It is important to note that one can identify specific strips to scan using KC4. The time can also be shortened considerably by either narrowing the wavelength range of the scan or by increasing the increment used.

In the past, such spectral scan determinations have been performed using a conventional spectrophotometer. This method usually entailed using matched cuvettes to perform the analysis resulting in a very low throughput. The ability to use the PowerWave[®] 200 scanning microplate reader to perform this type of analysis allows this routine procedure to be performed on 96 samples without user intervention in a matter of minutes leading to a tremendous increase in productivity and throughput.

Paul Held, Ph. D.
Senior Scientist & Applications Lab Manager

Rev. Date: 2/6/01