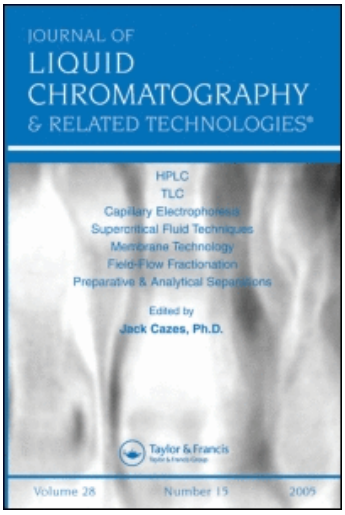


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# Resolution of the Non-Specific Spectra of Barbiturates by UV-Photodiode Array Detection

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# RESOLUTION OF THE NON-SPECIFIC SPECTRA OF BARBITURATES BY UV-PHOTODIODE ARRAY DETECTION

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## ABSTRACT

The procedure described uses a photodiode array detector for the qualitative determination of peak identity. The object was to determine the capability of a photodiode array detector to accurately discriminate among non-specific spectra. Ten barbiturates, all of which possessed highly similar non-specific spectra are analyzed and spectrally compared. This is accomplished by establishing a standard spectrum for each barbiturate sample, then comparing a sample spectrum of each barbiturate to the spectral library and noting the accuracy of the spectral match. In order to confirm the ruggedness of the technique, sample spectra from analyses performed with a different HPLC column and with fresh mobile phase were also compared to the standard spectra. In all ten cases, the photodiode array system accurately matched the sample spectra with the corresponding standard spectra.

## INTRODUCTION

The primary advantage of a photodiode array detector is the availability of spectral information throughout the course of the analysis. By providing such spectral information, peak identity can be accurately determined. This ability has made photodiode array detectors useful in a variety of industries. There are many examples

in the literature of pharmacology applications such as the toxicological screening of drugs and their metabolites [1-7]. Biomechanical applications include the detection and identification of peptides [8-9] and the determination of xanthines in amniotic fluid [10]. The technique has been used in the analysis of complex hydrocarbon mixtures in fossil fuels [11] and in the determination of chlorotriazines and their photolysis products [12].

Because UV spectra do not contain the information content provided by other techniques (such as mass spectroscopy), care must be taken in the generation of spectra. Spectra obtained can be influenced by a number of factors, such as background changes (due to baseline drift) that can be attributed to the HPLC column or the mobile phase. Wright discusses the optimization of such eluent parameter when using HPLC with photodiode array detection[13]. When comparing non-specific spectra, the most accurate standard spectra are those which are "spectral averages" of several separate analyses. Because the creation of accurate standard spectra can therefore be quite time consuming, the accuracy of the comparison of sample spectra to the standards should be consistent given normal changes experienced in the laboratory, such as fresh mobile phase or a column change. If this ruggedness is lacking, the technique is not suited for some analytical environments. Exclusion of irrelevant wavelengths is also critical in the creation of an accurate standard spectra, and the comparison of these standards to sample spectra. Because of these and other considerations, sophisticated software is necessary for the optimization of the spectra generated by photodiode array detection. Sheehan et al discusses more fully the software and capabilities of this particular system[14].



An ideal photodiode array spectra is achieved when the following conditions are met:

- Very large signal to noise ratio
- Ideal baselines

- Very low UV cutoff
- Good resolution
- Equivalent AU for all peaks
- Significant spectral differences

The last requirement is of specific interest for this study. Often in the pharmaceutical industry, a series of products may be derived from the same starting materials and utilize similar synthetic pathways. These products in turn have highly similar structures, such as a common ring element with different functionalities. This is the case with barbiturates, where the common element is a pyrimidinetrione ring with different functional groups at the number 5 position (see Figure 1). As expected, highly similar structures yield highly similar spectra. In the case of barbiturates, this is compounded by the fact that the spectra are non-specific, i.e., the absorbance is restricted to the short wavelength region of the UV spectra (190 - 240 nm). This work investigates the ability of a photodiode array system to accurately and reliably distinguish the non-specific spectra of barbiturates.



## MATERIALS AND METHODS

The equipment used was a Model 9010 pump, a Model 9095 autosampler and a Model 9065 photodiode array detector (all by Varian Instrument Division, Walnut Creek CA). System control was via the Series 9020 LC Star Workstation, Revision C (Varian Instrument Division). Spectral manipulation and comparison were performed using Polyview$^{TM}$ Revision D (Varian Instrument Division). The LC control and spectral processing software functions in a Windows$^{TM}$ environment (Windows Version 3.0, Microsoft Corporation, Redmond WA) on a 386 PC equipped with a math coprocessor. Separations were achieved using 300 x 3.9 mm uBondapak $C_{18}$ columns (Waters Division of Millipore, Milford MA). B&J Brand$^{TM}$ high purity acetonitrile

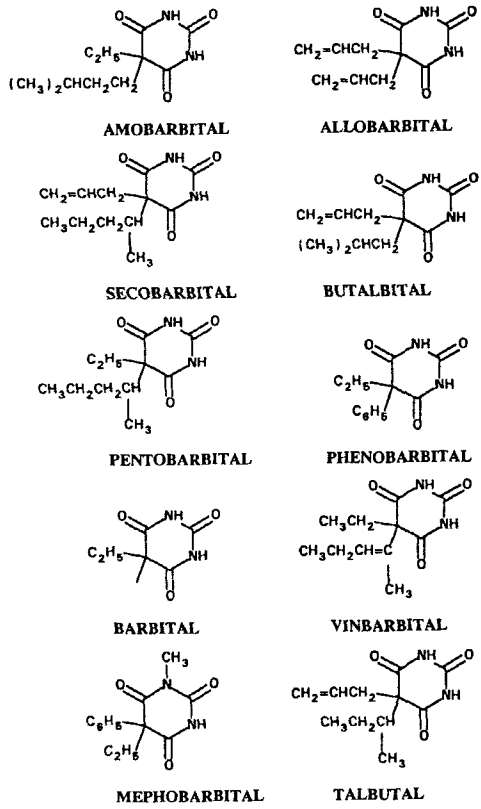


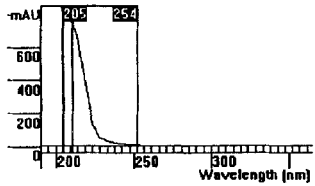

FIGURE 1

BARBITURATE STRUCTURES

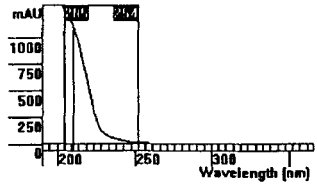


was purchased from Burdick and Jackson (Baxter Healthcare Corporation, Muskegon MI). Distilled de-ionized water was provided by a NANOpure II (Barnstead Thermolyne, Dubuque IA) water purification system. Barbiturate samples were provided by Ganes Chemicals Inc., (Pennsville NJ).

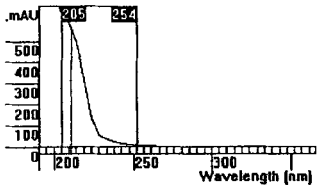
The mobile phase used was 60/40 0.1 M $KH_2PO_4$/ acetonitrile pH 3.0. After mixing and pH adjustment, the mobile phase was filtered and degassed by sonication



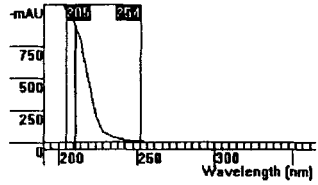

AVERAGE SPECTRUM OF AMOBARBITAL



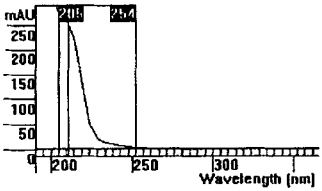

AVERAGE SPECTRUM OF ALLOBARBITAL



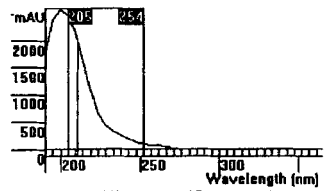

AVERAGE SPECTRUM OF SECOBARBITAL



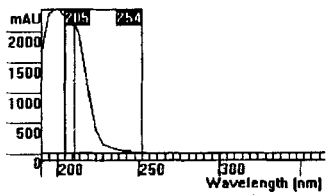

AVERAGE SPECTRUM OF BUTALBITAL



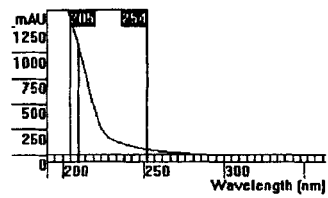

AVERAGE SPECTRUM OF PENTOBARBITAL



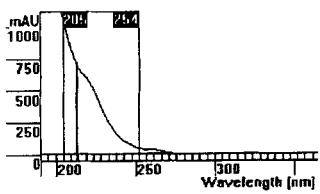

AVERAGE SPECTRUM OF PHENOBARBITAL



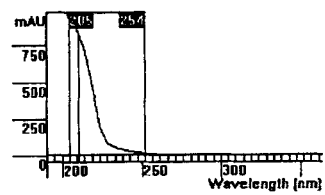

AVERAGE SPECTRUM OF BARBITAL




AVERAGE SPECTRUM OF VINBARBITAL



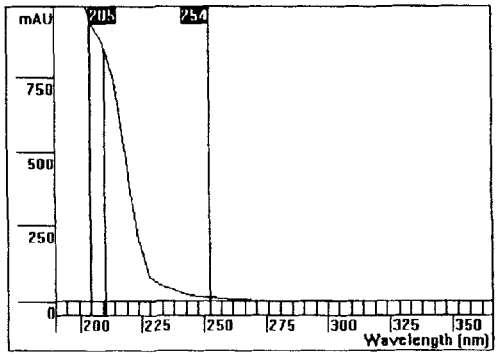

AVERAGE SPECTRUM OF MEPHOBARBITAL




AVERAGE SPECTRUM OF TALBUTAL

**FIGURE 2**

**STANDARD SPECTRA OF BARBITURATES**




STATISTICAL DATA FOR TALBUTAL STANDARD SPECTRUM

| No | Filename | tR (min) | Spectrum Type | Correction | PuP (205->254 nm) |
|---|---|---|---|---|---|
| 1 | 44b1073.run | 6.881 | Peak Apex | Baseline | 210.28 |
| 2 | 44b1074.run | 6.881 | Peak Apex | Baseline | 210.28 |
| 3 | 44b1075.run | 6.875 | Peak Apex | Baseline | 210.28 |
| 4 | 44b1076.run | 6.900 | Peak Apex | Baseline | 210.28 |
| 5 | 44b1077.run | 6.900 | Peak Apex | Baseline | 210.28 |
| 6 | 44b1078.run | 6.863 | Peak Apex | Baseline | 210.28 |
| 7 | 44b1079.run | 6.893 | Peak Apex | Baseline | 210.28 |

Best Correlation: 1 and 2 Sim: 1.000000 Dissim: 0.000000
Worst Correlation: 1 and 6 Sim: 1.000000 Dissim: 0.000245
Average PuP = 210.281 nm Standard Deviation = 0.001 nm

FIGURE 3

TALBUTAL STANDARD SPECTRUM

under vacuum. Barbiturate samples were prepared in mobile phase at a concentration of approximately 0.5 mg/ml. Each barbiturate was analyzed seven times. The seven spectra for each barbiturate were then averaged to provide a standard spectra for use with sample comparison. Analyses on the barbiturates were repeated and these sample spectra were compared to the standard spectra. At this point the column was changed and the barbiturate samples were analyzed and spectrally compared again. When this was complete, the initial mobile phase was discarded and replaced with a fresh batch, and final analyses using the fresh mobile phase and second column were performed.

**TABLE 1**

**SUMMARY OF BARBITURATE STANDARD SPECTRA**

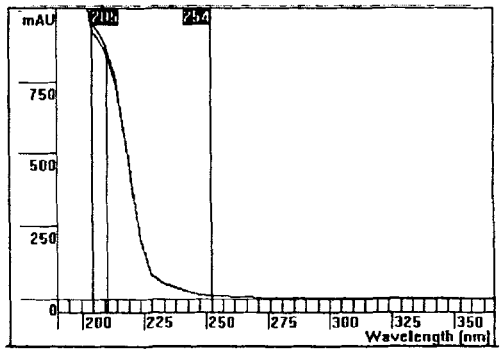
| STANDARD | SPECTRUM TYPE[1] | AVERAGE PuP (nm) | STANDARD DEVIATION (nm) |
|---|---|---|---|
| VINBARBITAL | PEAK APEX | 209.618 | 0.004 |
| AMOBARBITAL | PEAK APEX | 210.088 | 0.004 |
| BUTALBITAL | PEAK APEX | 210.118 | 0.002 |
| BARBITAL | PEAK APEX | 210.222 | 0.043 |
| PENTOBARBITAL | PEAK APEX | 210.267 | 0.007 |
| SECOBARBITAL | PEAK APEX | 210.270 | 0.002 |
| TALBUTAL | PEAK APEX | 210.281 | 0.001 |
| ALLOBARBITAL | PEAK APEX | 210.345 | 0.002 |
| PHENOBARBITAL | PEAK APEX | 210.454 | 0.035 |
| MEPHOBARBITAL | PEAK APEX | 212.768 | 0.009 |

1. Peak region where spectrum was taken.



## RESULTS

Because the spectra for these barbiturates are highly similar and non-specific (see Figure 2), the creation of the standard spectra for comparison was critical. The standard spectrum was the average of seven spectra for each barbiturate. Seven was not an arbitrarily chosen number, it is the maximum number of spectra that can be averaged at one time by this system. Figure 3 is the standard spectra for talbutal including the relevant statistical information. Table 1 is a summary of the statistical data for all of the standard spectra. Figure 4 is the library search report for talbutal under the initial conditions, Figure 5 is the search report for talbutal with the analyses




(spectra not normalized)

SEARCH REPORT

| Matches: | | Sim. | Dissim. | PuP(nm) | tR(min) | Lib # |
|---|---|---|---|---|---|---|
| AVERAGE SPECTRA OF | TALBUTAL | 1.00000 | 0.00049 | 210.281 | 0.000 | A 10 |
| AVERAGE SPECTRA OF | SECOBARBI | 1.00000 | 0.00276 | 210.270 | 0.000 | A 2 |
| AVERAGE SPECTRA OF | BUTALBITA | 0.99985 | 0.01710 | 210.110 | 0.000 | A 4 |
| AVERAGE SPECTRA OF | PENTOBARB | 0.99980 | 0.02009 | 210.267 | 0.000 | A 5 |
| AVERAGE SPECTRA OF | ALLOBARBI | 0.99979 | 0.02056 | 210.345 | 0.000 | A 1 |

Search Conditions
PuP Range: 285->254 nm PuP Interval: +/- 5.00 nm
Time Range: 0.000 - 1440.000 min

FIGURE 4

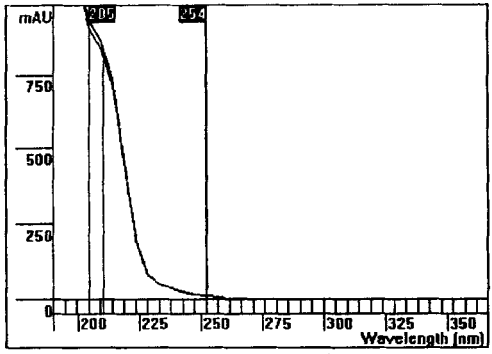
TALBUTAL: MATCH OF SAMPLE SPECTRUM WITH STANDARD SPECTRUM, INITIAL CONDITION



performed using a different column, and Figure 6 is the search report for talbutal with the analyses performed using the second column and fresh mobile phase. Table 2 is the summary of the search reports performed on all of the barbiturate samples with the corresponding analytical conditions.

## DISCUSSION

The primary means to distinguish different spectra using this system is the assignment of a Purity Parameter™ (abbreviated PuP). This value is defined as the average wavelength of a spectrum weighted by the square of the absorbance at each wavelength of the spectrum over the range that defines that purity[15]. For all of the




(spectra not normalized)

**SEARCH REPORT**

| Matches: | | Sim. | Dissim. | PuP(nm) | tR(min) | Lib # |
|---|---|---|---|---|---|---|
| AVERAGE | SPECTRA OF TALBUTAL | 1.00000 | 0.00035 | 210.281 | 0.000 | A 10 |
| AVERAGE | SPECTRA OF SECOBARBI | 1.00000 | 0.00307 | 210.270 | 0.000 | A 2 |
| AVERAGE | SPECTRA OF BUTALBITA | 0.99965 | 0.01742 | 210.110 | 0.000 | A 4 |
| AVERAGE | SPECTRA OF PENTOBARB | 0.99900 | 0.01985 | 210.267 | 0.000 | A 5 |
| AVERAGE | SPECTRA OF ALLOBARBI | 0.99970 | 0.02002 | 210.345 | 0.000 | A 1 |

Search Conditions:
PuP Range: 205->254 nm PuP Interval: +/- 5.00 nm
Time Range: 0.000 - 1440.000 min

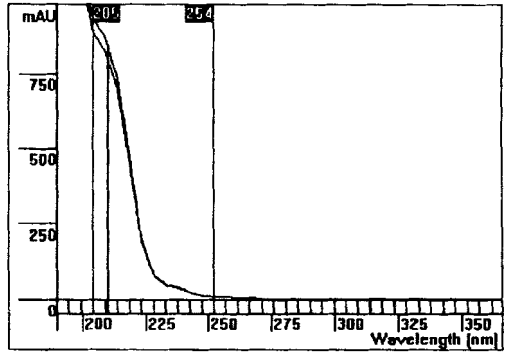
**FIGURE 5**

**TALBUTAL: MATCH OF SAMPLE SPECTRUM WITH STANDARD SPECTRUM, COLUMN CHANGE**



barbiturate samples analyzed, the defined range was 205 - 254 nm. Wavelengths above 254 were excluded because of negligent absorbance. By eliminating these irrelevant wavelengths, the accuracy of the spectral comparisons are greatly enhanced. In order to compare the spectrum, the similarity and dissimilarity are measured. Under these conditions, the dissimilarity is more sensitive to small changes than similarity. Similarity is the correlation coefficient expressed as:

$$Corr(\vec{A},\vec{B}) = \frac{\sum a_i b_i}{\sqrt{\sum a_i^2 \sum b_i^2}} = \cos\theta$$




(spectra not normalized)

SEARCH REPORT

| Matches: | Sim. | Dissim. | PwP(nm) | tR(min) | Lib # | |
|---|---|---|---|---|---|---|
| AVERAGE SPECTRA OF TALBUTAL | 1.00000 | 0.00156 | 219.291 | 0.000 | A | 10 |
| AVERAGE SPECTRA OF SECOBARBI | 0.99999 | 0.00316 | 219.270 | 0.000 | A | 2 |
| AVERAGE SPECTRA OF BUTALBITA | 0.99987 | 0.01600 | 219.118 | 0.000 | A | 4 |
| AVERAGE SPECTRA OF PENTOBARB | 0.99981 | 0.01937 | 219.267 | 0.000 | A | 5 |
| AVERAGE SPECTRA OF ALLOBARBI | 0.99977 | 0.02139 | 219.345 | 0.000 | A | 1 |

Search Conditions
PwP Range: 285->254 nm PwP Interval: +/- 5.00 nm
Time Range: 0.000 - 1440.000 min

FIGURE 6

TALBUTAL: MATCH OF SAMPLE SPECTRUM WITH STANDARD SPECTRUM, FRESH MOBILE PHASE



The dissimilarity is defined by the following expression:

$$Dissim(A,B) = \sin\theta = \sqrt{1-\cos^2} \quad = \sqrt{1-Corr^2(\vec{A},\vec{B})}$$

Where θ is the angle between an n dimensional vector for the sample spectra as compared to the reference spectra. In Figure 2 the similarity of the spectra of the barbiturate samples is readily observable. Only three spectra are unique; mephobarbital shows a small "hump" in the 210 - 230 nm range, while both vinbarbital and phenobarbital exhibit some absorbance above 254 nm. However, this absorbance can be discounted since it is outside the specified spectral range.

## TABLE 2

## SPECTRAL MATCH SUMMARY

| SAMPLE | INITIAL CONDITIONS[1] BEST MATCH (SIMILARITY) (DISSIMILARITY) | WITH DIFFERENT COLUMN[2] BEST MATCH (SIMILARITY) (DISSIMILARITY) | WITH FRESH MOBILE PHASE BEST MATCH (SIMILARITY) (DISSIMILARITY) |
|---|---|---|---|
| VINBARBITAL | VINBARBITAL (1.00000) (0.00060) | VINBARBITAL (1.00000) (0.00049) | VINBARBITAL (1.00000) (0.00176) |
| AMOBARBITAL | AMOBARBITAL (1.00000) (0.00179) | AMOBARBITAL (1.00000) (0.00109) | AMOBARBITAL (1.00000) (0.00120) |
| BUTALBITAL | BUTALBITAL (1.00000) (0.00129) | BUTALBITAL (1.00000) (0.00049) | BUTALBITAL (1.00000) (0.00085) |
| BARBITAL | BARBITAL (0.99999) (0.00532) | BARBITAL (0.99999) (0.00329) | BARBITAL (1.00000) (0.00237) |
| PENTOBARBITAL | PENTOBARBITAL (1.00000) (0.00077) | PENTOBARBITAL (1.00000) (0.00210) | PENTOBARBITAL (1.00000) (0.00179) |
| SECOBARBITAL | SECOBARBITAL (1.00000) (0.00183) | SECOBARBITAL (1.00000) (0.00176) | SECOBARBITAL (1.00000) (0.00085) |
| TALBUTAL | TALBUTAL (1.00000) (0.00049) | TALBUTAL (1.00000) (0.00035) | TALBUTAL (1.00000) (0.00154) |
| ALLOBARBITAL | ALLOBARBITAL (1.00000) (0.00283) | ALLOBARBITAL (1.00000) (0.00049) | ALLOBARBITAL (1.00000) (0.00129) |
| PHENOBARBITAL | PHENOBARBITAL (0.99991) (0.01369) | PHENOBARBITAL (0.99995) (0.00958) | PHENOBARBITAL (0.99999) (0.00520) |
| MEPHOBARBITAL | MEPHOBARBITAL (1.00000) (0.00035) | MEPHOBARBITAL (1.00000) (0.00124) | MEPHOBARBITAL (1.00000) (0.00060) |

1. Using the same mobile phase during the generation of the standard spectra, column SN P12771B 42.

2. Column SN P12461C 08.

From Table 1, all ten barbiturates have a PuP value within a range of just over 3 nm. If the extremes (vinbarbital and mephobarbital) are excluded, the remaining eight barbiturates have PuP values within a range of less than 0.5 nm. Mephobarbital was the easiest spectra to resolve, since the PuP value was at least 2 nm greater than that of any of the other barbiturates. The most difficult case would be the resolution of pentobarbital from secobarbital. The difference in PuP values for these two samples was only 0.003 nm, which is less than the standard deviation calculated in the creation of the pentobarbital standard spectra. The resolution of butalbital from barbital may actually be even more difficult. Although the difference in PuP values is 0.004 nm, the standard deviation calculated in the creation of the barbital standard was spectra 0.043 nm, the highest standard deviation observed. In the three experimental cases, the pentobarbital spectra was correctly discriminated from the secobarbital spectra. This was also the case with the discrimination of butalbital from barbital.



In Table 2, the poorest matching was with the phenobarbital samples. Yet the similarity values did improve from an initial low of 0.99991 to a final of 0.99999. Even more striking are the high dissimilarity values observed for phenobarbital. This may be indicative of a problem with the standard spectra, particularly in view of the high similarities and low dissimilarities observed with the other barbiturates. An inconsistency with the sample spectra would have probably yielded much wider variations, including a misidentification. Because the spectra for the barbiturate samples are highly similar, a similarity index of 1.00000 would be optimal in a match. Of the 30 matches listed on Table 2, only five are not 1.00000. The search reports listed in Figures 4, 5 and 6 indicate that the matching is not only accurate, but consistent throughout the course of the experiment. In all three cases, not only was talbutal listed correctly as the most likely candidate, the order of the other potential candidates (secobarbital, butalbital, pentobarbital and allobarbital) is identical. This was also true in the matches with the other nine samples. While the similarity of the

spectra is important, because all of the spectra are highly similar the dissimilarity becomes a more valuable means of determining the accuracy of the spectral match.

Because of the relatively high standard deviation observed with the phenobarbital and barbital standard spectra, this data was reviewed further. The standard spectra are both observed to have an absorbance in excess of 2 AU (Figure 2). This is above the linear range of the detector (1.5 AU). The negative deviation from linearity observed in the low wavelength range resulted in (A) a higher PuP value due to an incorrect weighting factor for the wavelengths and (B) higher imprecision for PuP values obtained for these two compounds. Recalling those conditions which are necessary in the generation of an ideal spectra, this is the explanation for the deviations observed for these samples.



## CONCLUSION

The photodiode array system was able to accurately and reliably distinguish among all ten of the barbiturate samples. The technique proved to be rugged enough for normal laboratory use, standard spectra that were compiled as the averages of seven runs were accurately matched to sample spectra over the course of the experiments. Given this reliability, a spectral library created for the identification of these drugs would provide correct matching over time. A simple system suitability test, such as analyses of a pair of highly similar spectra (secobarbital and pentobarbital) and the match of these sample spectra with the correct standard spectra would indicate that system accuracy has not been compromised. One factor that was not addressed in these experiments was the use of retention time as a second matching criteria. Although secobarbital and pentobarbital have highly similar spectra, their respective retention times differed by more than two minutes. This was not addressed since the objective was to evaluate the accuracy of the spectral matching. Although the separation system used did provide

adequate chromatographic resolution of some of the barbiturates, in a rapid assay (such as toxicological screening), high resolution chromatography may be unavailable. In such a case, the spectral matching would be the primary means of determining the compound identity. Also, this evaluation was limited to the photodiode array system of a single manufacture, and there are several systems on the market. Different systems may have different capabilities that would affect the results obtained in these experiments.

## ACKNOWLEDGEMENT

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