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# Multispectral UV Fluorescence Detection of a Dilute Constituent in an Optically Dense Matrix

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## Abstract

Multispectral UV fluorescence measurements were made of an optically dense medium (fetal bovine serum, FBS) spiked with sodium salicylate at concentrations from 0.2 to 500 $\mu$g/ml . Analysis of the spectra show that, depending on experimental conditions, reasonably good estimates of concentration can be obtained across the entire range of concentrations. Experimental conditions required for recovering these estimates are demonstrated. These results indicate that it is possible to detect and

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discriminate between organic compounds in optically dense media, such as undiluted blood plasma, by this method.

## 1. Introduction

Optical detection of chemicals and pathogens in a natural environment is complicated by the fact that the background often is optically dense. Under these conditions spectral information may be obscured by radiative transfer[1]. This process of self-absorption and re-emission of light determines the coherence length for information contained in the fluorescence spectral profile for a target molecule. For example blood serum contains some tens of milligrams per milliliter serum protein, which absorbs and fluoresces the extent that the spectral signature from trace chemicals would be obscured and distorted by the interaction of emitted light with the serum proteins. In addition, the linear mathematical methods normally used with such measurements may be of limited use since the response of the optically dense background to excitation is nonlinear[2–4].

In multispectral UV fluorescence spectroscopy, a sample is sequentially illuminated with monochromatic light at chosen wavelengths, $\lambda_{exc}$, in the ultraviolet spectrum, and then the fluorescence is measured over a finite bandwidth, $\lambda_{flr}$, thus generating a two-dimensional spectral signature. With this technique, spectra from unknown samples can be analyzed by multivariate methods to determine the presence and concentration of chemicals and proteins for which known database spectra have been acquired[2,4]. The only constraint on the multivariate analysis is that it is linear, so that it is expected to fail for samples which yields a nonlinear response to excitation (i.e. samples which are optically dense).

Multispectral ultraviolet fluorescence is a powerful technique and potentially useful in pharmacokinetic studies. The very nature of those studies requires measurement of drug compounds at low concentration against optically dense backgrounds (blood plasma or tissue). For compounds with sufficiently large fluorescence cross sections, multivariate techniques are sufficient to resolve the spectral differences between compounds and allow for estimates of concentration[5,6]. Furthermore, we have recently reported that MUFS could be used to detect $PrP^{Sc}$, a protein associated with a class of fatal neurodegenerative dissorders, prion diseases[6]. By defining the spectral signature of this protein, detection may be possible within optically dense backgrounds. The ability to readily analyse disease-specific markers within blood or tissue would be a major advancement in the diagnosis of these dissorders. However, the effect of nonlinear response of the supporting matrix on the spectra and thus determination of concentration is as yet unknown.

## 2. Materials and Methods

*Sample preparations.* Dilute solutions of sodium salicylate (Fischer Scientific) were prepared by first dissolving the salicylate in 95% ethanol (Fischer Scientific) at a final concentration of 10 mg/ml and then diluting this to 500 $\mu$g/ml with fetal bovine serum (FBS) (Gibco/BRL). Samples were prepared by two-fold serial dilutions in FBS yielding samples in the 0.24 to 500 $\mu$g/ml concentration range. Additional samples were prepared by dilution with Hanks' buffered saline (Gibco/BRL) for spectral comparison with respect to salicylate in FBS at similar concentrations. Samples were then loaded into either 0.1 mm optical path length (total 30 $\mu$l vol., Starna Cells Inc. 20/C-Q-0.1), or 10 mm path length (total 3.5 ml vol.,

Spectrocell Corp. RF-3010-T) UV fluorescence cells for measurement of spectra.

*Fluorometer and Spectral Measurements.* The excitation source for the system was a 150 watt high pressure UV Xe lamp (Oriel Model 68805 arc lamp with Model 6254 Xenon bulb). The white light from the lamp was focused into an F/4, 1/8 meter double monochromator (CVI Model 120, 200-400 nm range, 5 nm steps 7 nm bandwidth) in order to reduce the scattered light outside of the pass band of the monochromator. The light from the monochromator was then imaged into the center of a UV quartz sample cell. The scattered light and the fluorescence from the material in the cell were detected at 90 degrees to the incident excitation source. The light from the excitation region was imaged into the entrance slit of an F/4, 1/8 meter imaging spectrometer (200-700 nm range, 5 nm resolution, ISA Jobin-Yvon CP-200). At the image plane the light was detected by a 1024 element intensified reticon array (EG&G Princeton Applied Research, Model 1420). The resolution of the detection spectrometer was 5 nm. Measurements of absorption were performed using short path length cells and a Perkin-Elmer Lambda 9 UV/VIS/NIR spectrophotometer.

## 3. Results and Analysis

We have made a series of measurements of an optically dense medium, FBS, spiked with an aromatic compound, sodium salicylate, across a reasonably broad concentration range. The purpose of these measurements was to determine the effect of nonlinearities in the measurements on estimates of concentration of the salicylate obtained from linear multivariate analysis. Sodium salicylate was used for these measurements since it is an easily handled, well characterized compound with a reasonably large fluorescence cross section. The use of

sodium salicylate in these studies is of practical importance (from pharmacokinetic stand point) since the compound and its derivatives are commonly used as an analgesic, antipyretic, and anti-inflammatory agent. The purpose of these measurements was to check the degree of linearity of response of the spectral features associated with the compound.

Shown in Figure 1 are examples of multispectral signatures from FBS, sodium salicylate at 125.0 $\mu$g/ml in Hanks buffered saline, and sodium salicylate at 125.0 $\mu$g/ml in FBS. The first order elastically scattered light, which obeys $\lambda_{flr} = \lambda_{esc}$, typically saturates the detector in these measurements and hence carries no information. Therefore, we have masked out the first order elastically scattered light in Fig. 1. When the scattered light does not saturate the detector it contains information about the absorption characteristics of the sample and is of potential use in analysis. Fig. 1 indicates the relative positions of the main spectral features from the FBS and the salicylate. Both the FBS and the salicylate, at these concentrations, are sufficiently concentrated as to be optically dense. As can be seen, the spectral features associated with the salicylate are reasonably well separated from the main fluorescence peak associated with the FBS. The peak fluorescence for the FBS is near 337 nm at an excitation wavelength of 290 nm, and the peak fluorescence for the salicylate is near 405 nm at an excitation wavelength of 305 nm.

In order to establish the effect of the optically dense matrix on the fluorescence due to the salicylate, a series of spectra were taken consisting of FBS both unspiked and spiked with sodium salicylate in a range of concentrations 0.24 to 500.0 $\mu$g/ml . This range of concentrations of salicylate is sufficiently large that it spans concentrations which are optically thin at the low concentration end of the range and optically dense at the high concentration end of the range (see below). Measurements were taken using both short (0.1

mm) and long (10.0 mm) optical path length cells.

Estimates of the relative concentration of salicylate were computed using multivariate analysis (singular valued decomposition)[3,4]. The spectral signatures for FBS and salicylate at 7.8 $\mu$g/ml were used as standards in the analysis, the latter being at a concentration where the optical density due to the salicylate alone is negligible (see below). For our purposes the analysis was restricted to the fluorescence part of the spectrum alone (i.e. elastic scatter was masked out, consistent with the data shown in Fig. 1). Computations were performed using data sets taken with both short and long optical path length cells.

Figure 2 shows the results of our multivariate analysis. As can be seen, at concentrations below 20 $\mu$g/ml salicylate, we were able to recover reasonably good estimates of the concentration for both data sets. The deviation from linearity at the low end of the concentration range is due to the fact that we are losing sufficient signal for the integration time of the measurements. At the high end of the concentration range, the results for the long optical path length measurements deviate significantly from a linear response.

The only difference between data sets for the results shown in Figure 2 , is the difference in optical path length. To see why the results deviate at the high concentration end of the data, one needs to consider the effect of the salicylate on the optical density. As an estimate of the effect, we have determined the optical depth for FBS and FBS with salicylate at 125 $\mu$g/ml , using the short path length cells (0.1mm). It should be noted that as far as the multivariate analysis is concerned this is only an estimate of the effect since the multivariate technique is affected by total spectral distortion, not just absorption. Figure 3 shows the absorption ($A = -log(\frac{I}{I_0})$, where I is intensity) for the two cases, and their difference, which shows the contribution of the salicylate alone. At 305 nm the optical depth (i.e. the e-folding

length) of the FBS alone is 2.54 mm, while in the FBS with salicylate at a concentration of 125 $\mu$g/ml this decreases to 1.4 mm, thus almost half the absorption is due to the salicylate alone. The apparent reason for the deviation between results is that the concentration of the salicylate is contributing to the spectral distortion of the signature for the measurements with the long optical path length. We would not expect to see salicylate affect the short path length results until the concentration was at least an order of magnitude greater, since the path length for these measurements is a tenth of the optical depth.

**4. Discussion**

At first glance, it is surprising that one can obtain a linear response (and hence reasonable estimates of concentration) from a system which is optically dense. However, since changes of the concentration of the salicylate at these levels do not "dilute" the serum, the nonlinear distortion of the salicylate's spectral features (by the serum) is constant for all samples. Thus, as long as the optical path length of the measurement is less than the optical depth of the salicylate, the contribution of the salicylate to the overall spectrum scales linearly with concentration (distorted though it might be by the optically dense matrix).

The above result is important since it implies that the effect of the optical density of the matrix can be accounted for as part of the system response. Thus, if one can estimate the optical density of the matrix, its distortion of spectral signatures of known compounds can be accounted for, and concentrations can be calculated. Applying this method to natural backgrounds may still be problematic, in that there are several issues which need to be resolved. There are of course the obvious constraints that the fluorescence cross section for the tar-

get molecule is sufficiently large, and the spectral signature sufficiently distinguishable from the background fluorescence, as to account for sufficient signal to noise to make detection feasible[5]. In addition to this there are additional issues that need to be addressed which are due to the matrix itself. For example, in the case our system mimics, the detection of a drug compound in serum, we have not taken into account the variability of the spectral features of the serum itself, which may be altered with changes in the makeup of the population of serum proteins. We have also not accounted for how the variation in the concentration of serum protein (i.e. the optical density of the serum) would affect the spectral distortion. How these changes in the serum makeup and concentration change the manner in which the spectral signatures for the drug compound is distorted would need to be addressed for these techniques to be of practical use.

These results show promise for the use of optical detection methods in clinical applications. If the issues we discussed above can be resolved, these techniques have a number of potential applications including; therapeutic drug monitoring, pharmacokinetic-pharmacodynamic evaluation, disease state studies (i.e. how disease state alters serum protein content). In addition to this the method itself has the potential to greatly speed and simplify such work, as it requires no sample preparation (e.g. extraction or protein precipitation) and signatures can be acquired in a matter of seconds.

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# Captions

Fig. 1. Spectral signatures from FBS (upper left), and sodium salicylate in Hanks buffered saline to a final concentration of 125.0 $\mu$g/ml (upper right), and sodium salicylate in FBS at 125.0 $\mu$g/ml (lower center), first oder elastically scattered light has been masked. Note that there is reasonably good separation between peaks due to the serum and the salicylate.

Fig. 2. Comparison of multivariate estimates of concentrations computed from data sets using short (0.1 mm) optical path length, and long (10.0 mm) optical path length cells.

Fig. 3. Measured absorption coefficient "A" for FBS, FBS and sodium salicylate at a concentration of 125.0 $\mu$g/ml , and the difference between the two, showing the contribution to the optical density of the salicylate alone (measurements were taken with 0.1 mm path length cell).

## FIGURES

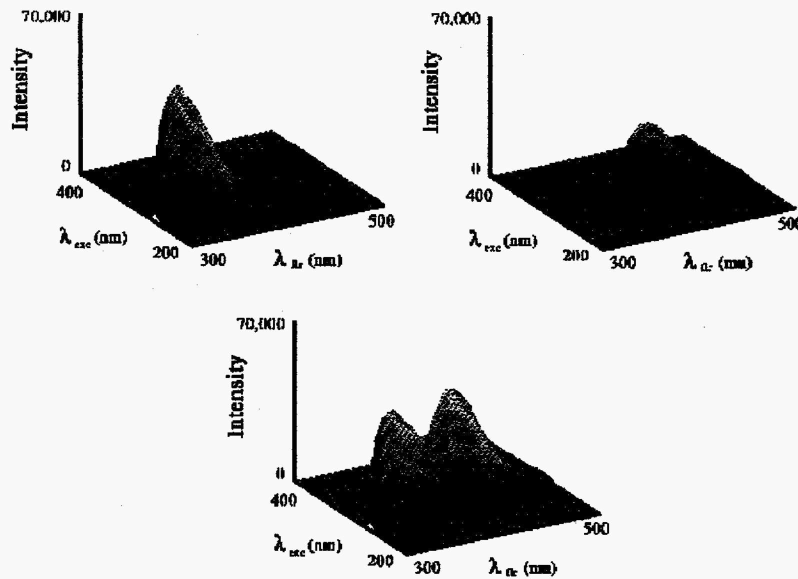
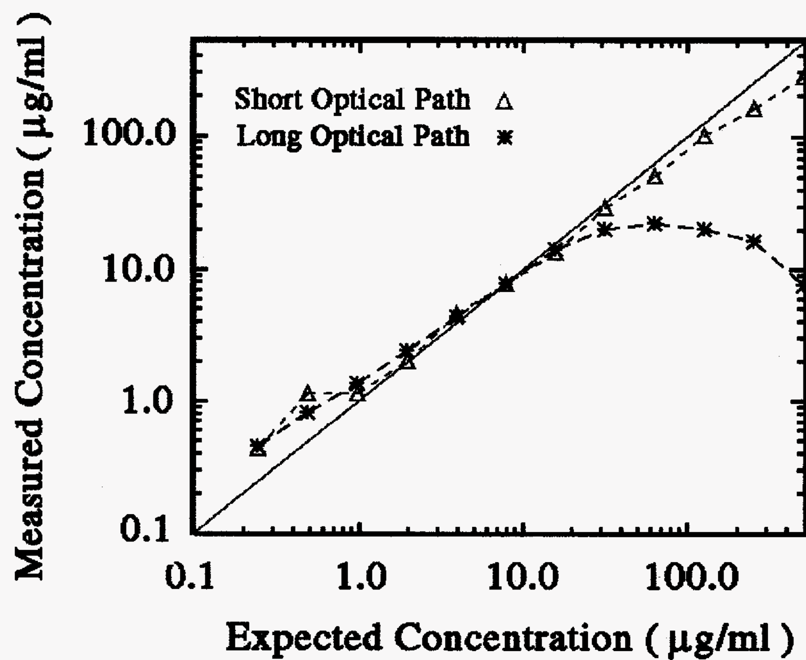



Fig. 1. Spectral signatures from FBS (upper left), and sodium salicylate in Hanks buffered saline to a final concentration of 125.0 $\mu$g/ml (upper right), and sodium salicylate in FBS at 125.0 $\mu$g/ml (lower center), first oder elastically scattered light has been masked. Note that there is reasonably good separation between peaks due to the serum and the salicylate.

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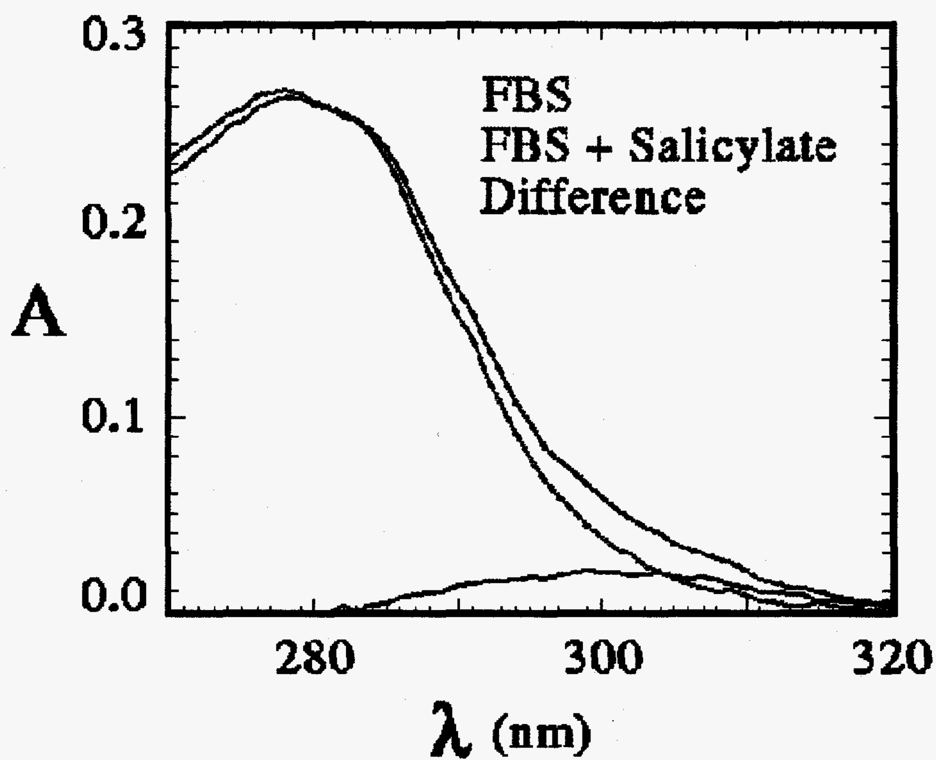


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