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# Modeling systematic errors: polychromatic sources of Beer–Lambert deviations in HPLC/UV and nonchromatographic spectrophotometric assays

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**Abstract**

It is well established that the use of polychromatic radiation in spectrophotometric assays leads to excursions from the Beer–Lambert limit. This Note models the resulting systematic error as a function of assay spectral width, slope of molecular extinction coefficient, and analyte concentration. The theoretical calculations are compared with recent experimental results; a parameter is introduced which can be used to estimate the magnitude of the systematic error in both chromatographic and nonchromatographic spectrophotometric assays. It is important to realize that the polychromatic radiation employed in common laboratory equipment can yield assay errors up to $\sim 4\%$, even at absorption levels generally considered 'safe' (i.e. absorption $< 1$). Thus careful consideration of instrumental spectral width, analyte concentration, and slope of molecular extinction coefficient is required to ensure robust analytical methods. 

*Keywords:* Beer's Law deviation; Polychromatic radiation; Systematic errors in spectrophotometric assays; Nonexponential power attenuation

## 1. Introduction

### 1.1. Motivation

The determination of analyte concentration via light absorption, i.e. spectrophotometric assay, enjoys widespread application in production and research settings for some very good reasons (1) the assay can be applied either prior to or following separation, as determined by the required accuracy; (2) electromagnetic radiation from the UV to the audio range can be used, as dictated by the signal strength from the analyte with respect to other species present; (3) the required spectrophotometric equipment can be relatively inexpensive, portable, and/or rugged, and (4) the Beer–Lambert law, also known as Beer's Law [1], provides a simple expression relating the transmitted electromagnetic power to the concentration of the analyte. Because of the extensive application of Beer–Lambert analysis to spectrophotometric

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assay, it is critical for scientists to understand the physical and chemical sources of Beer–Lambert deviations. This paper models the magnitude of systematic error due to a common source of Beer–Lambert deviation, polychromatic radiation. It is important to realize that this bias, present in common laboratory equipment, can result in systematic errors up to $\sim$ 4% (see below); as this error is independent of absorption magnitude, the deviation may exist, at high value, even in attenuation regimes generally considered 'safe' (i.e. absorption $<1$).

All spectrophotometric devices, including benchtop UV/VIS spectrophotometers, detection modules of HPLC/UV instruments, and magnetic resonance systems, employ an assay beam of nonzero spectral width; this width is variously known as the slit width, bandpass, or bandwidth. While Beer–Lambert deviation due to polychromatic radiation has been an established topic in the optical literature for over 70 years [2–7], and it has been shown that for a fixed cell length $l$, the absorbance per concentration is a decreasing function of the spectral width $\Gamma$ [7], the magnitude of the systematic error has not been modeled as a function of $\Gamma$, analyte concentration, and properties of the molecular extinction coefficient. This calculation, with comparison to experimental results, is the subject of this paper. It is hoped the resulting model will aid the assignation of cause in instrument–instrument assay discrepancies and associated interlaboratory robustness issues.

### 1.2. Description of model

A necessary condition of the Beer–Lambert law is the exponential decay of radiative power along the probe beam of a spectrophotometric assay. When the power decay over the absorbing medium is nonexponential, a deviation from the Beer-Lambert limit is observed. One source of such deviation is the use of polychromatic radiation to sample molecular spectral regions with a nonzero $d\varepsilon/d\omega$, that is, changes in the molecular extinction coefficient with respect to radiation frequency. As each component of the radiation beam is attenuated by the molecular decay constant of that frequency, the distribution of decay constants yields nonexponential power decay over the medium.

In spectrophotometric assays using common laboratory equipment, suitable diffraction optics direct a slit transfer function of spectral width $\Gamma$ through the sample of interest. Following the interaction of the radiation with the absorbing medium, the beam is directed to a single photodetector, which produces a voltage, $V_{\text{sample}}$, proportional to the radiative power. This voltage is then used to calculate the absorbance of the medium with respect to a reference value, $\log_{10}(V_{\text{ref}}/V_{\text{sample}})$, either during data acquisition by using a balanced photocircuit, or following data acquisition by recording the reference and sample photovoltages in memory.

It is important to recognize the difference between this detection configuration, in which the entire power of the slit transfer function is transduced by a single photodetector, versus a spectrophotometric assay performed with multichannel detection systems such as a diode array, in which each photodetector sees only a fraction of $\Gamma$. In this latter detector configuration, absorbance calculations are made using the response of a single detection element or combinations of elements according to the software algorithm chosen by the user. The model below describes only the experiment in which single channel detection is employed to transduce the entire probe and reference beam.

With respect to concentration of the absorbing species, the model addresses two common experimental configurations of spectrophotometric assay, (1) static absorption, in which the analyte concentration is constant during data acquisition, and (2) chromatographic absorption, in which the analyte concentration is changing during data acquisition, such as HPLC separation followed by UV–Vis detection. An expression for percent recovery, as a function of a simple, dimensionless parameter, which incorporates the experimental and molecular sources of nonexponential power decay, is introduced to allow estimation of the resultant deterministic error.

## 2. Experimental

### 2.1. Derivation of model static absorption

Our goal is to generate an analytical expression of Beer's Law absorption which includes an assay beam, $\Pi_0(\omega)$, of nonzero spectral width, and a linear change in the extinction coefficient $d\varepsilon(\omega)/d\omega$ over the spectral width of the assay beam. Consider the slit transfer function $\Pi_0(\omega)$, modeled as a Gaussian in frequency $\omega$, with a full width at half-maximum (FWHM) of $\Gamma$:

$$\Pi_0(\omega) = P_0 e^{-4 \ln 2(\omega-\omega_0)^2/\Gamma^2} \quad (1)$$

where $P_0$ is the beam power at the center frequency $\omega_0$. (Extensions of the model to express non-Gaussian slit transfer functions are straightforward.) Experimental values of $\Gamma$ in contemporary laboratory equipment are typically 6 nm or less. Over this spectral width, the Napierian molecular extinction coefficient $a(\omega)$ can be expanded in the vicinity of the center radiation frequency $\omega_0$, and truncated following the linear term:

$$a(\omega) = a_0 - \frac{\Delta a}{\Gamma}(\omega-\omega_0) \quad (2)$$

where the extinction coefficient $a(\omega)$ is written as an explicit function of angular frequency, $a_0$ is the extinction coefficient at the center frequency $\omega_0$, and $\Delta a/\Gamma$ is the slope of the molecular extinction coefficient with respect to $\omega$. Eq. (2) describes a region of the molecular response where $a(\omega)$ is approximately linear over $\Gamma$; experiments at absorption maxima should be modeled with Gaussian or Lorentzian $a\ (\omega-\omega_0)$.

The reference detector transduces $\Pi_0(\omega)$ by integrating over the frequency components,

$$V_{\text{ref}} = R\int_0^\infty d\omega \Pi_0(\omega) \quad (3)$$

and producing a photovoltage, $V_{\text{ref}}$, proportional to the total reference power. The proportionality constant, $R$, is the responsivity of the photodetector. While this quantity is a function of the photon energy and in general would be included in the integrand, over the $\sim 6$ nm maximum spectral width of our model, $R$ is constant. In a sample of low optical density, each spectral component $\omega_k$ of $\Pi_0(\omega)$ is attenuated by $\exp(-a(\omega_k)cl)$. The experimental detector observes the sum of these interactions

$$\sum_k \Pi(\omega_k) = \sum_k \Pi_0 e^{-a(\omega_k)cl} \quad (4)$$

where $c$ is the concentration of the absorbing species, and $l$ is the optical path length. As the assay beam $\Pi_0(\omega)$ is a continuous function with respect to $\omega$, the discrete sum can be replaced by an integral. The resultant photovoltage, $V_{\text{sample}}$, is

$$V_{\text{sample}} = R\int_0^\infty d\omega \Pi_0(\omega) e^{-a(\omega)cl} \quad (5)$$

and the absorption is calculated[1]:

$$\begin{aligned}\text{absorbance} &= \log_{10}\frac{V_{\text{ref}}}{V_{\text{samp}}} \\ &= \log_{10}\frac{\int_0^\infty d\omega \Pi_0(\omega)}{\int_0^\infty d\omega \Pi_0(\omega)\, e^{-a(\omega)cl}} \\ &= \varepsilon_0 cl - \frac{(\Delta\varepsilon cl)^2}{16(\log_{10} e)(\ln 2)},\end{aligned} \quad (6)$$

where $\varepsilon_0$ is the decadic extinction coefficient at the center frequency $\omega_0$, and $\Delta\varepsilon$ is the change in the decadic extinction coefficient over the slit transfer function FWHM $\Gamma$:

$$(\Delta\varepsilon)^2 = \left\{\varepsilon\left(\omega_0+\frac{\Gamma}{2}\right)\right\} - \left\{\varepsilon\left(\omega_0-\frac{\Gamma}{2}\right)\right\}^2 \quad (7)$$

Note as $\Delta\varepsilon \to 0$, Eq. (6) goes to the Beer-Lambert limit $\varepsilon_0 cl$, as expected. Also note the sign in the extinction coefficient expansion (Eq. (2)), does not effect the sign in the absorbance expression; as the slit transfer function is symmetric, it is inconsequential which half of the beam is more strongly attenuated. The term responsible for the deviation from the Beer–Lambert limit is proportional to $(cl)^2$ as each spectral component of the slit transfer function $\Pi_0(\omega)$ is attenuated by $\exp(-a(\omega_k)cl)$, the divergence between the Beer–Lambert limit and the experimentally recorded absorption increases as the attenuation of the probe light increases. When $(cl)$ gets very large,

[1] Part of Eq. (6) appears, unsolved, in [7].

the expression for absorption goes negative. This nonphysical result is a consequence of truncating the expansion of the molecular extinction coefficient, Eq. (2), after the linear term. The region of model applicability can be estimated by determining the maximum in Eq. (6):

$$\frac{\partial \text{Absorption}}{\partial(cl)} = \varepsilon_0 - \frac{2\Delta\varepsilon^2(cl)_{\text{limit}}}{\alpha} = 0 \tag{8}$$

so

$$(cl)_{\text{limit}} = \frac{2.4\varepsilon_0}{\Delta\varepsilon^2} \tag{9}$$

where $\alpha = 16$ ($\log_{10} e$) (ln 2). The applicability of Eq. (6) is restricted to sample and standard concentrations such that $(c_{\text{sample, standard}} \times l) \ll (2.4\varepsilon_0)/(\Delta\varepsilon)^2$. The discrepancy between the Beer-Lambert limit and the experimentally recorded absorption expressed by Eq. (6) is conveniently summarized by writing the Beer–Lambert divergence, $\delta$:

$$\delta = \frac{((e0\text{cl}) - \{((\Delta\varepsilon)^2(\text{cl})^2)/\alpha\})}{\varepsilon_0(\text{cl})} \tag{10}$$

as a function of the dimensionless parameter, $\chi_{\text{SA}} = cl\Delta\varepsilon^2/\varepsilon_0$, (SA, static absorption):

$$\delta(\chi_{\text{SA}}) = 1 - \frac{\chi_{\text{SA}}}{\alpha} \tag{11}$$

Note when $\chi_{\text{SA}}$ is 0.1, which is $\ll 2.4$, the deviation from the Beer–Lambert limit is $\sim 2\%$.

In a laboratory spectrophotometric assay, however, it is not the absolute deviation from the Beer–Lambert limit that is of primary interest. More typically, after consideration of the sample concentration range to be measured, a standard concentration is chosen from within this range. The concentration of the standard may be in the center of the concentrations to be measured, or it may at one of the concentration extremes, as in a measurement of a pharmaceutical dissolution profile using the 100% release concentration as the molecular standard. The analytical problems arise not primarily from the absolute deviation from the Beer–Lambert limit, but because samples with $c_{\text{SAMPLE}} > c_{\text{STANDARD}}$ deviate more than the standard, while $c_{\text{SAMPLE}} < c_{\text{STANDARD}}$ deviate less. For a given $\Gamma$, the largest systematic errors due to polychromatic radiation are seen when $|c_{\text{SAMPLE}} - c_{\text{STANDARD}}|$ is large.

Spectrophotometric assays are performed by calculating the relative response factors for the sample and a standard. The percent recovery calculated from a static absorption measurement can be written in terms of $\chi_{\text{SA}} = cl\Delta\varepsilon^2/\varepsilon_0$:

$$\text{Percentage recovery} = \left(\frac{\text{Absorbance}_{\text{SAMPLE}}}{(\text{cl})_{\text{SAMPLE}}}\right)\left(\frac{\text{Absorbance}_{\text{STANDARD}}}{(\text{cl})_{\text{STANDARD}}}\right)^{-1} \times 100\% = \frac{\alpha - \chi_{\text{SA}}^{\text{SAMPLE}}}{\alpha - \chi_{\text{SA}}^{\text{STANDARD}}} 100\% \tag{12}$$

Fig. 1 displays the result of using Eq. (12) to estimate the magnitude of static absorption deterministic errors for assay methods with nonzero $\Delta\varepsilon$. Due to the narrow spectral assay width of benchchtop spectrophotometers ($\sim 1$ nm), deterministic errors described by Eq. (12) are typically small with respect to the indeterminate error ac-

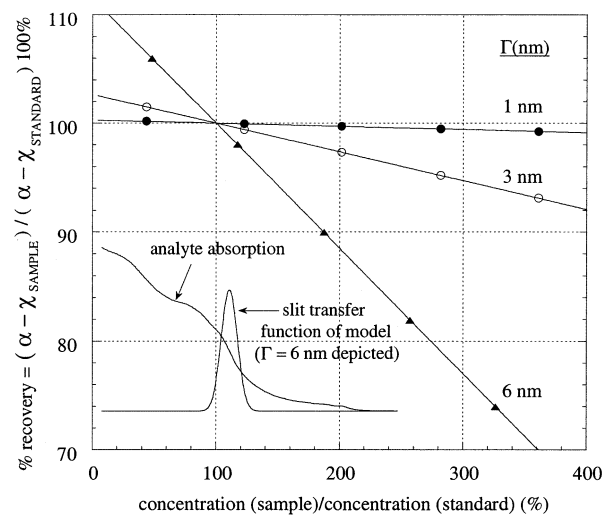


Fig. 1. Solid lines, percent recovery, static absorption (Eq. (12)), plotted as a function of concentration (sample)/concentration (standard percent). Solid circles, $\Gamma = 1$ nm; open circles, $\Gamma = 3$ nm; solid triangles, $\Gamma = 6$ nm. $\Delta\varepsilon/\Gamma = 2$ ml/mg cm nm (obtained by linear fit, centered at 245 nm, of analyte absorption spectrum); $\varepsilon_0 = 32$ ml/mg cm; concentration (standard) = 0.11 mg/ml; $\alpha = 16$ ($\log_{10} e$) (ln 2). Insert, absorption spectrum of analyte. Gaussian model of slit transfer function with FWHM of 6 nm.

companying a standard preparation ( ~ 1%). Such may not be the case, however, for spectrophotometric methods employing HPLC separation with UV–Vis detection.

## 3. Results/discussion

### 3.1. Application of the model

In a recent pharmaceutical method validation, the concentration of the analyte ranged from 0.005 to 0.5 mg/ml; 100% release, 0.11 mg/ml, was chosen as the standard concentration. The analytical method called for quantitation via HPLC/UV spectrophotometric assay. To demonstrate spectrophotometric accuracy and linearity, it was considered sufficient to test the region where $(cl)_{\mathrm{SAMPLE}} \geq (cl)_{\mathrm{STANDARD}}$. As the chromatographic conditions of the assay resulted in a dilution of greater than fivefold of the method concentration, absorbance spectra for concentrations ranging from 0.11 to 0.017 mg/ml were recorded on a benchtop spectrophotometer; the experimental absorption values were all less than 0.4. Accuracy was determined by using the 0.033 mg/ml response factors, the absorbance at each wavelength divided by the concentration, to calculate the percent recovery of the samples. In the wavelength region of interest, results ranged from 99.7 to 100.5% recovery over the sample concentration range; there was no observed correlation between percent recovery and concentration. Linearity was demonstrated as the experimental response factors were independent of concentration and assay wavelength. With this data supporting the spectrophotometric component of the method, trial testing began, incorporating HPLC/UV analysis at 245 nm. The accuracy and linearity samples, ranging in method concentration from 0.005 to 0.5 mg/ml, failed miserably (see solid circles, Fig. 2).

The failure of the spike and recovery assay was unexpected because of three erroneous assumptions, (1) deviations from the Beer–Lambert limit occur only at the high end of a concentration range where $c_{\mathrm{SAMPLE}} > c_{\mathrm{STANDARD}}$, (2) it is sufficient to investigate accuracy and linearity on any reliable spectrophotometer and (3) as HPLC is a dilution

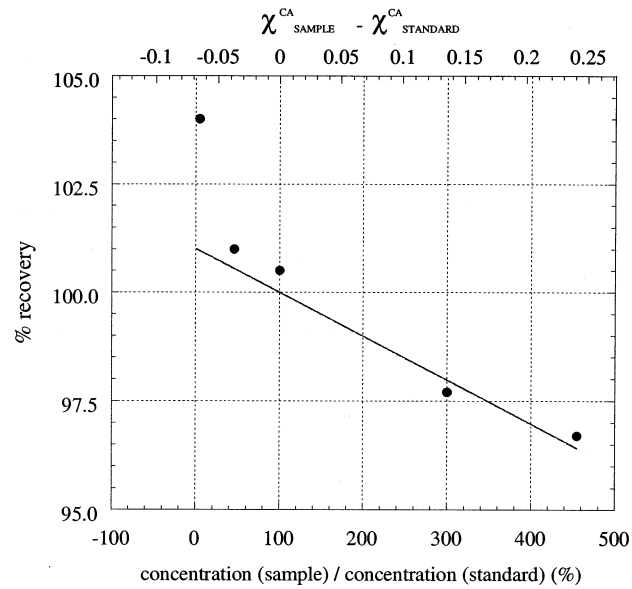


Fig. 2. Solid circles, Experimental results of spike and recovery. Recovery quantitated via HPLC/UV assay with following conditions: standard concentration 0.11 mg/ml, sample concentration shown on upper X-axis; $\lambda_{\mathrm{detected}} = 245$ nm; column, Phenomenex Prodigy ODS-3, 50 × 4.6 mm; 5 um particle size; mobile phase, 68:32 20 mM $KH_2PO_{4(aq)}$ pH 6.0 to acetonitrile; diluent, 100 mM $KH_2PO_{4(aq)}$ pH 3.0, 0.6% NaCl. Solid line, Eq. (15) (see text) after direct insertion of experimental values, $\varepsilon_0 = 32$ ml/mg cm, $\Delta\varepsilon/\Gamma = 12$ ml/mg cm over 6 nm, $D_{\mathrm{minimum}} = 0.088$, standard concentration = 0.11 mg/ml.

technique, it is sufficient to investigate and document the spectrophotometric properties of the analyte at the highest concentration to be recorded during the assay. Erroneous assumption 1 is certainly not true when polychromatic radiation is used in the assay; we have already seen that when $\Delta\varepsilon \neq 0$, samples in which $c_{\mathrm{SAMPLE}} > c_{\mathrm{STANDARD}}$ will assay low, while samples where $c_{\mathrm{SAMPLE}} < c_{\mathrm{STANDARD}}$ will assay high. While high analyte concentrations can yield nonexponential decay of the probe beam power when $\Delta\varepsilon = 0$, this phenomena is not related to polychromaticity[2]. Erroneous assumption 2 is probably the most

[2] dP/P must equal $\kappa \mathrm{d}l$ for $0 < \mathrm{d}l <$ optical path length. $\kappa$ cannot be a function of $l$ ($P$ is optical power, $l$ is optical path length, $\kappa$ is an attenuation constant with units of $l^{-1}$, see, for example, D. Skoog and D. West, *Principles of Instrumental Analysis*, Saunders College/Holt, Rinehart and Winston, Philadelphia, PA. (1980).

consequential for the failure at hand. As $\delta(\chi)$ is proportional to $\Delta\varepsilon^2$, a change in slit transfer width from a typical value for a benchtop spectrophotometer of $\sim 1$ to the 6 nm width employed in the HPLC/UV assay in question, will result in a larger deviation from the Beer–Lambert limit for a given $|(cl)_{\mathrm{SAMPLE}} - (cl)_{\mathrm{STANDARD}}|$. Erroneous assumption 3 is a bit subtle, as $\delta(\chi)$ is a function of the analyte concentration during data acquisition. In HPLC/UV assays, the analyte concentration, the standard concentration, and therefore the value of $\delta(\chi)$ are changing as the molecular zone moves through the detection optics. How deterministic error via polychromatic radiation is manifested in chromatographic absorption assays can be examined by extending the expression for static absorption, Eq. (6), to describe an HPLC/UV assay.

### 3.2. Extension of model: chromatographic absorption

The spectrophotometric optics and detection circuitry used to derive Eq. (6) are also applicable to chromatographic absorption. The concentration of the absorbing species can be modeled as a Gaussian in time, centered at $t_{\mathrm{R}}$, with FWHM of $\gamma$:

$$c(t) = c_{\mathrm{initial}} D_{\mathrm{minimum}}\, e^{-4\ \ln\ 2(t - t_R)^2/\gamma^2} \tag{13}$$

where $c_{\mathrm{initial}}$ is the injected concentration, and $D_{\mathrm{minimum}}$ is the minimum dilution observed. $D_{\mathrm{minimum}}$ is measured by calculating the maximum concentration observed in the chromatogram. It is approximately equal to the injected volume $V_{\mathrm{injected}}$ divided by the product of the mobile phase flow rate $F$ and the peak width $\gamma$ ($D_{\mathrm{minimum}} \sim V_{\mathrm{injected}}/F\gamma$). After directly substituting this concentration expression into Eq. (6), integration over time allows calculation of the chromatographic response factor $R(c_{\mathrm{initial}})$:

$$R(c_{\mathrm{initial}}) = \frac{l D_{\mathrm{minimum}} \pi^{1/2} \gamma}{2(\ln\ 2)^{1/2}} \left( \varepsilon_0 - \frac{\Delta\varepsilon^2 l c_{\mathrm{initial}} D_{\mathrm{minimum}}}{2^{1/2}\alpha} \right) \tag{14}$$

Note as $\Delta\varepsilon \to 0$, the response factor goes to the concentration independent Beer–Lambert limit. For chromatographic absorption, the discrepancy from the Beer–Lambert limit is proportional to the dimensionless parameter $\chi_{\mathrm{CA}} = c_{\mathrm{initial}} D_{\mathrm{minimum}} l \Delta\varepsilon^2/\varepsilon_0$ (CA, chromatographic absorption). The expression for the integrated area (response factor times the initial concentration) goes negative at large $(cl)$; applicability of Eq. (14) is restricted to sample and standard concentrations such that $\chi_{\mathrm{CA}} \ll 3.4$.

HPLC/UV assays are performed by calculating the relative response factors for the sample and a standard. Eq. (14) can be rewritten in terms of $\chi_{\mathrm{CA}}$ to express percent recovery:

$$\text{Percent recovery} = \left( \frac{R_{\mathrm{SAMPLE}}(c_{\mathrm{initial}})}{R_{\mathrm{STANDARD}}(c_{\mathrm{initial}})} \right) \times 100\% = \frac{\beta - \chi_{\mathrm{CA}}^{\mathrm{SAMPLE}}}{\beta - \chi_{\mathrm{CA}}^{\mathrm{STANDARD}}} \times 100\% \tag{15}$$

where $\beta = 2^{1/2}\alpha = 2^{1/2}16\ (\log_{10}\ \mathrm{e})\ (\ln\ 2)$; note Eq. (15) is in the limit that the chromatographic peak shape is not a strong function of analyte concentration. The solid circles in Fig. 2 show the experimental results, average of three trials, of the dissolution method validation using HPLC/UV assay; all the peak heights were such that absorbance $< 0.7$. The solid line in Fig. 2 is Eq. (15) after direct substitution of the analyte and assay properties (see Fig. 2 caption for details). Note the solid line equals 100% recovery at the standard concentration of 0.11 mg/ml (i.e. $\chi_{\mathrm{CA}}^{\mathrm{SAMPLE}} - \chi_{\mathrm{CA}}^{\mathrm{STANDARD}} = 0$). Also note $|\chi_{\mathrm{CA}}^{\mathrm{SAMPLE}} - \chi_{\mathrm{CA}}^{\mathrm{STANDARD}}|$, which can be calculated for any HPLC/UV assay, must be $< 0.2$ to keep the systematic error due to polychromatic radiation sources less than $\pm 3\%$ recovery.

The agreement between Eq. (15) and the experimental results is good save for the lowest sample concentration of 0.005 mg/ml. As this data point is not colinear with the point (0.11 mg/ml, 100%) and the remaining data points, it will not be fit with a linear model. The experimental results for percent recovery show some positive curvature with respect to initial

concentration, indicating an additional deterministic error not expressed herein.

## 4. Conclusions

This note has attempted to illustrate the physical phenomena responsible for the deviations from the Beer–Lambert limit when polychromatic radiation is used in spectrophotometric assays. The key contributors to the de1viation have been identified as $\Delta\varepsilon/\Gamma$ and $|c_{\mathrm{SAMPLE}} - c_{\mathrm{STANDARD}}|$; the nonexponential attenuation of the probe beam is the source of their contribution[3]. The dimensionless parameter $\chi_{\mathrm{SA}}$ or $\chi_{\mathrm{CA}}$, as appropriate to the analytical method, can be calculated and used to estimate the magnitude of the resultant systematic error. The systematic error due to polychromatic radiation is minimized by reducing $\Delta\varepsilon$, $\Gamma$, and $|c_{\mathrm{SAMPLE}} - c_{\mathrm{STANDARD}}|$.

[3] Additional analytical problems arise when using the extinction properties, $\varepsilon_0^{\iota}$ and $\Delta\varepsilon^{\iota}$, of one molecule (e.g. the active ingredient in a pharmaceutical formulation) to quantitate the amount present of another molecule with extinction properties, $\varepsilon_0^{\kappa}$ and $\Delta\varepsilon^{\kappa}$, (e.g. a degradate of the active ingredient). Also consider that if the degradate is present at 0.1%, $\chi^{\kappa} \ll \chi^{\iota}$.

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