Modeling and Analysis of the Uncertainty of Enzyme Measurement Processes in Clinical Laboratories

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Abstract

Clinical laboratory measurement processes inform every stage of the medical decision-making process, and measurement of the activity levels of enzymes such as alkaline phosphatase, alanine aminotransferase, etc. provide vital information regarding various body functions such as the liver and gastrointestinal tract. The uncertainty associated with the result of these enzyme assays describes the quality of the measurement process, and therefore methods to improve the quality of the measurement process require minimizing the measurement uncertainty of the enzyme assay. In this paper, we develop a physics-based mathematical model of the alkaline phosphatase (ALP) measurement process, with uncertainty introduced into its components to represent the sources of variation in the measurement process. The Monte Carlo method is then utilized to describe the long-term behavior of the model and estimate the uncertainty associated with the result of the measurement. An uncertainty profile used to generate estimates of uncertainty at different patient sample activity levels is constructed. We then utilize the model to quantify the contributions of the individual sources of uncertainty to the net measurement uncertainty, and also quantify the effect of uncertainty within the calibration process on the net measurement uncertainty.

Keywords
Measurement uncertainty, Enzyme assays, Clinical measurement simulation

1. Introduction

The uncertainty associated with the result of a measurement quantifies the quality of the measurement process. This information is vital for clinical measurements, as they are used to inform every stage of the medical decision-making process, from diagnostic and prognostic assessment of patients to determining drug dosage prescription. This need has been recognized by the United States Congress in its passage of the Clinical Laboratory Improvement Amendments Act in 1988, which mandates the utilization of estimates of measurement uncertainty in establishing valid quality control systems in the clinical laboratory. In this paper we estimate the uncertainty associated with the measurement of the level of activity of the alkaline phosphatase (ALP) enzyme. We develop a mathematical model of the measurement process, and introduce uncertainty into the process by characterizing the parameters of the model as random variables. The Monte Carlo method is used to estimate the uncertainty associated with the result of the measurement.

A clinical laboratory measurement process, also referred to as an assay, consists of three stages: the pre-analytical stage, encompassing all activities performed before the patient sample is analyzed on the instrument, including patient sample collection, transportation, preparation, etc.; the analytical stage, which involves calibration of the instrument and the analysis of the patient sample on the calibrated instrument; and finally, the post-analytical stage, which involves recording, reporting and interpreting the measurement result [1]. In this paper, we model only the analytical stage of the...
measurement process, as identifying and quantifying the variation of the numerous sources of uncertainty associated with the pre-analytical stage would require a separate study in itself. The uncertainty of the post-analytical stage is largely attributable to human error, and hence is beyond the scope of this study.

The ALP assay is performed on the Roche Diagnostics P-Modular Analytics measurement platform. The assay consists of conducting a chemical reaction between reactants contained in two reagents. The chemical reaction is catalyzed by the enzyme in the patient sample, and the amount of enzyme in the patient sample is characterized by its effect on the rate of the reaction. The rate of the reaction is a function of the amount of the reaction product that is formed per unit time, and the amount of product formed at a given point in time is proportional to the optical absorbance of the reaction mixture measured at that point in time. The optical absorbance of the reaction mixture is measured at regular intervals of time in order to estimate the rate of the reaction. A calibration function then converts the measured rate of the reaction into the “activity level” of the enzyme, which represents the amount of enzyme in the patient sample. The commonly used unit of enzyme activity is enzyme unit per liter (denoted as U/L), which is defined as the amount of enzyme that catalyzes the conversion of 1 micro mole of the substrate into the reaction product per minute.

The concept of measurement uncertainty and the analytical rules for estimating the uncertainty associated with the measurement process were formalized in 1993 in the ISO/BIPM/OIML/IUPAC Guide to the Expression of Uncertainty in Measurement [2], and then revised in subsequent editions [3]. Uncertainty is defined in the GUM as “any parameter that characterizes the dispersion of the distribution of the values that can be attributed to the result of a measurement”. In this study, the parameter used is the standard deviation, since the distributions of the sources of uncertainty operating within the measurement system are modeled as Gaussian distributions, and the distribution of the measurement result is also found to be Gaussian. The quantity to be measured, level of enzyme activity, will henceforth be referred to as the measurand in this paper.

Several studies in the literature estimate the uncertainty associated with specific clinical laboratory measurement processes [4–7]; however, there is very limited literature regarding the estimation of measurement uncertainty associated with the spectrophotometric determination of enzyme activity levels in general, and in particular, our search of the literature did not yield any studies involving the estimation of the uncertainty of the ALP assay. The application of a systems engineering methodology to model and estimate the uncertainty associated with clinical assays has been suggested by Aronsson et al. in 1974 [8], and by Krouwer in 2002 [9]. Ramamohan et al. [10] apply such a systems engineering perspective and develop a physics-based mathematical model of the uncertainty associated with the measurement of cholesterol concentration in human blood serum, and then utilized the Monte Carlo method to conduct simulation experiments to optimize various calibration protocols in terms of minimizing the uncertainty associated with the assay. The cholesterol assay modeled in [10] belongs to a class of assays known as endpoint substrate assays, wherein a single optical absorbance measurement directly estimates the amount of the cholesterol in the sample. The ALP assay modeled in this paper belongs to a class of significantly more complex assays wherein the rate of the reaction is measured using multiple optical absorbance measurements, and is then converted to enzyme activity level by a linear calibration function.

In this paper, we present a similar methodology involving the development of a mathematical model of the measurement process of the activity level of the enzyme alkaline phosphatase that describes the biochemistry of the catalytic process. The Monte Carlo method is applied to estimate the uncertainty associated with model, and also estimate the contributions of the individual components of the measurement system to the net measurement uncertainty. The use of the Monte Carlo method to estimate measurement uncertainty is appropriate if any of the following conditions apply to the model: a.) the model of the measurement system is non-linear; b.) the estimation of the degrees of freedom of the sources of uncertainty in the measurement system is not possible, which is typically the case when their variation is characterized by an ad-hoc method (also known as a Type B method); and c.) the distribution of the measurement result or any of the sources of uncertainty is not Gaussian [11]. The first two conditions apply in the case of our model. The mathematical model developed, as will be shown in the sections below, is nonlinear; and the variation of the sources of uncertainty within the model are characterized by an ad-hoc Type B method. Further, the use of the Monte Carlo method allows for conducting simulation experiments with the model, and therefore facilitates the extraction of information about the measurement system that would otherwise require controlled experimentation in the laboratory.

The estimates of measurement uncertainty obtained from the model are based on performance specifications for sub-components of the instrument provided by the manufacturer, and can hence be used to aid efforts to bridge the disparity between model estimates of uncertainty and those observed in the clinical laboratory. The model is then used to estimate the contributions of individual components of the instrument and the process to the net measurand uncertainty. This study was carried out in collaboration with the Roche Diagnostics Corporation in Indianapolis, IN (USA).
2. Model Development

In this section, we describe the ALP enzyme measurement process in additional detail, and then describe the development of the assay uncertainty model. The chemical reaction forming the basis of the ALP assay is given below:

\[ p\text{-nitrophenyl phosphate} + \text{H}_2\text{O} \xrightarrow{\text{ALP}} p\text{-nitrophenol} + \text{phosphate} \]  

(1)

The chemical reaction above yields the product of interest, p-nitrophenol, and the measured optical absorbance is directly proportional to the concentration of the p-nitrophenol.

Two reagents are added as part of the ALP assay: \( R_1 \), containing the metal ion buffer for the reaction, and \( R_2 \), containing the reactant p-nitrophenyl phosphate. The patient sample supplies the ALP enzyme that catalyzes the reaction. In the following description of the model, we use the term ‘substrate’ to refer to the p-nitrophenyl phosphate supplied by the reagent \( R_2 \) and the term ‘reagent’ to refer to the metal ion buffer supplied by the reagent \( R_1 \). The corresponding volumes of the reagent and the substrate are denoted by \( V_{r1} \) and \( V_{r2} \). The volume of the patient sample is denoted by \( V_x \). The concentration of any species is denoted by enclosing its symbol within square brackets.

The ALP activity level is directly proportional to the rate of the reaction (rate of change of absorbance with time), and is estimated using the calibration equation, specified below:

\[ [E]_x = K(m_x - m_b) \]  

(2)

Here \([E]_x\) represents the ALP activity level in the patient sample; \( K \) is the calibration parameter and \( m_b \) represents the rate of the reaction (absorbance/min) for a blank sample. The value of \( m_b \) is typically zero. Further, any property (absorbance, activity level, etc) of the patient sample with unknown enzyme activity level will be denoted using the subscript \( x \) in this document.

\( K \), the calibration parameter, is calculated as follows:

\[ K = \frac{[E]_2 - [E]_1}{m_2 - m_1} \]  

(3)

Here \([E]_1\) and \([E]_2\) represent the desired lower and higher activity levels of the ALP enzyme in the calibrators 1 and 2 respectively. \( n \) absorbance readings, recorded at equal intervals of time and denoted by \( A_1, A_2, ..., A_n \), are used to fit a linear relationship between absorbance and time. The rate of the reaction \( m \) is estimated by fitting a linear model between these \( n \) absorbance readings and time, i.e:

\[ m = \frac{\sum_i t_i A_i - \frac{1}{n} \sum_i t_i \sum_i A_i}{\sum_i t_i^2 - \frac{1}{n} (\sum_i t_i)^2} \]  

(4)

2.1 Calibration Phase

In the calibration phase, the values of \( K \) and \( m_b \) are established. Since the sample activity level is a linear function of the rate of change of absorbance, a two-point calibration is performed. Three sources of uncertainty are identified as changing the value of the calibrator activity level: calibrator set point uncertainty \( (u_e) \), vial to vial variability \( (u_v) \) and calibrator reconstituted stability \( (u_c) \). The first, calibrator set point uncertainty, refers to the uncertainty in the calibrator activity level during manufacture and prior to its use in the laboratory. The second source of calibrator uncertainty, vial-to-vial variability, involves the uncertainty introduced in the sample activity in the laboratory while preparing different vials of the calibrator from the batch supplied by the manufacturer. The third source of uncertainty, calibrator reconstituted stability, describes the deterioration (percentage decrease in activity per day) of the sample when the calibrator vial is stored and reconstituted after each use. When these are introduced into the model, the values of \( E_1 \) and \( E_2 \) change according to the following equation:

\[ [E]_1' = [E]_1 (1 + u_e) (1 + u_v) \prod_{i=1}^{N} (1 + u_c) \]  

(5a)

\[ [E]_2' = [E]_2 (1 + u_e) (1 + u_v) \prod_{i=1}^{N} (1 + u_c) \]  

(5b)
The variation of these sources of uncertainty, along with the others identified as operating within the measurement process, are characterized by fitting appropriate distributions to the specifications provided by the instrument manufacturer for each source of uncertainty. As an example, specifications for vial-to-vial variability were provided by the instrument manufacturer in the form of a coefficient of variation (CV) of 1.5%. After discussion with the manufacturers, a Gaussian distribution with a mean of 0% and a standard deviation of 1.5% was assumed to describe the variation in the calibrator activity levels due to vial-to-vial variability. The mean of the distribution was assumed to be 0%, as systematic errors in the calibrator manufacturing process were ruled out based on the manufacturer’s recommendation. Therefore, at a desired (error-free) calibrator enzyme activity level of 200 units/liter, the activity level in practice would be described by a Gaussian distribution with a mean of 200 U/L and a standard deviation of 3 U/L.

The activity values of the calibrator also change due to sources of instrument uncertainty. Three sources of uncertainty operate within the instrument: sample pipetting uncertainty, reagent pipetting uncertainty and photometer uncertainty. Sample and reagent pipetting uncertainty describe the uncertainty in the volumes of the sample and reagents that are pipetted into the reaction cell, and hence result in a change in the total volume of the reaction mixture and the number of enzyme and reactant molecules in the reaction mixture before the reaction begins. Therefore, their effect on the measurement process occurs at time $t = 0$. However, photometer uncertainty results in a change in each of the 15 optical measurements recorded during the course of the reaction. The variation of these sources of uncertainty are also characterized in a manner similar to that of the sources of calibrator uncertainty, and hence are also described by Gaussian distributions. The parameters of the distributions used to characterize the sources of uncertainty are provided in Table 1. The derivation of the effect of sample and reagent pipetting uncertainty on each optical absorbance measurement recorded during the reaction is explained below. In the derivation below, the concentration of a reacting species is denoted by enclosing its symbol within square brackets.

Table 1: Characterization of sources of uncertainty

<table>
<thead>
<tr>
<th>Source of uncertainty</th>
<th>Distribution</th>
<th>Mean (%)</th>
<th>SD (%)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator set-point uncertainty</td>
<td>Gaussian</td>
<td>0.00</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>Vial-to-vial variability</td>
<td>Gaussian</td>
<td>0.00</td>
<td>1.50</td>
<td></td>
</tr>
<tr>
<td>Reconstituted stability</td>
<td>Gaussian</td>
<td>-1.25</td>
<td>0.42</td>
<td>Decrease of enzyme activity per day</td>
</tr>
<tr>
<td>Sample pipetting uncertainty</td>
<td>Gaussian</td>
<td>0.00</td>
<td>1.50</td>
<td></td>
</tr>
<tr>
<td>Reagent pipetting uncertainty</td>
<td>Gaussian</td>
<td>0.00</td>
<td>4.00</td>
<td></td>
</tr>
<tr>
<td>Photometer uncertainty</td>
<td>Gaussian</td>
<td>0.00</td>
<td>0.15</td>
<td></td>
</tr>
</tbody>
</table>

We begin with the assumption that the optical absorbance at time $t$, denoted by $A_t$, is proportional to the concentration of the product p-nitrophenol in the reaction mixture at time $t$ (denoted by $[P]_t$). That is:

$$A_t = k[P]_t + A_0(t)$$

Here $k$ is the molar extinction coefficient, $A_0(t)$ represents the absorbance at time $t$ when the p-nitrophenol concentration at time $t$ is zero. It is reasonable to assume that the optical absorbance at time $t$ at zero p-nitrophenol concentration is equal to the optical absorbance at time $t = 0$ at zero p-nitrophenol concentration. The p-nitrophenol concentration will be zero if the chemical reaction does not take place; in other words, it can correspond to a configuration wherein the sample is not added, or a configuration wherein just a water blank is used. In either case, the optical absorbance will be independent of time, and the measurements recorded at regular time intervals will remain constant. Therefore, we replace the intercept term $A_0(t)$ by a more general intercept term $A_0$ to denote optical absorbance of a sample with zero enzyme activity. Since water blanks are commonly used to represent a sample with zero enzyme activity in the calibration of the instrument in the clinical laboratory, the term $A_0$ refers to the optical absorbance of the reaction mixture with a water blank added in lieu of the sample. Equation 6 can then be rewritten as:

$$A_t = k[P]_t + A_0$$

In order to express the concentration $[P]_t$ of p-nitrophenol as a function of time and the initial concentration of the substrate $[S]_0$, we consider the fact that reaction 1 belongs to the class of enzyme reactions that follow the reaction mechanism given below:
Here $E$ refers to the enzyme ALP, $S$ to the substrate p-nitrophenyl phosphate, $ES$ to the enzyme-substrate complex that is formed during the reaction, and $P$ to the product, p-nitrophenol. The rate constants for the forward reactions are denoted by $k_1$ and $k_2$, and the rate constant for the reverse reaction is $k_{-1}$. We recall here that the patient sample supplies the enzyme $E$, reactant $R_2$ supplies the substrate $S$ and the product $P$ refers to p-nitrophenol.

The initial velocity of the reaction is defined as the rate of formation of the p-nitrophenol. Briggs and Haldane developed a framework in 1925 [12], based on the work by Michaelis and Menten in 1910 [13], for the analysis of enzyme kinetics that expressed the initial velocity of the reaction as a function of the initial enzyme concentration as well as the initial substrate concentration (at time $t=0$). They derived the following expression for the initial velocity of the reaction:

$$
\frac{d\left[P\right]}{dt} = \frac{k_2 k_1 \left[E\right]_0 \left[S\right]_0}{k_{-1} + k_2 + k_1 \left[S\right]_0}
$$

Upon dividing the right-hand side of the above equation by $k_1$, the above equation can be rewritten as following:

$$
\frac{d\left[P\right]}{dt} = \frac{v_{max} \left[S\right]_0}{K_m + \left[S\right]_0}
$$

Here we write $\frac{k_2 + k_{-1}}{k_1}$ as $K_m$, and replace the term $k_2 \left[E\right]_0$ by $v_{max}$. $K_m$ is also known as the Michaelis constant, and $v_{max}$ is the maximum rate - the reaction velocity at which all the enzyme molecules are in the enzyme-substrate complex form. Now, when $\left[S\right]_0 = K_m$, we find that the rate of the reaction, as defined by equation 10 is exactly equal to $\frac{1}{2}v_{max}$, and therefore the Michaelis constant represents the value of the initial substrate concentration at which the reaction attains half its maximum velocity.

We can use equation 10 to determine the relationship between product concentration $[P]$ and time as follows:

$$
\int_0^{[P]_t} d\left[P\right] = \int_0^t \frac{v_{max} \left[S\right]_0}{K_m + \left[S\right]_0} dt
$$

That is,

$$
[P]_t = \left(\frac{v_{max} \left[S\right]_0}{K_m + \left[S\right]_0}\right) t
$$

Substituting the above expression for $[P]_t$ in equation 11 we obtain the relationship between optical absorbance and the initial enzyme and substrate concentrations and time.

$$
A_t = k \left(\frac{v_{max} \left[S\right]_0}{K_m + \left[S\right]_0}\right) t + A_0
$$

In order to maintain economy of notation in the rest of the derivation, we express the product concentration at time $t$ as a function $f$ of the initial substrate and enzyme concentrations and time $t$ as follows:

$$
[P]_t = f([S]_0, [E]_0, t)
$$

Therefore, equation 13 now becomes:

$$
A_t = k f([S]_0, [E]_0, t) + A_0
$$

Now, at $t = 0$, substrate concentration in the reaction mixture can be written as the ratio of the number of moles of the substrate $N_{S(0)}$ to the volume of the reaction mixture $V$. That is, the above equation can be written as:

$$
A_t = k f\left(\frac{N_{S(0)}}{V}, [E]_0, t\right) + A_0
$$
Further, the number of moles of the substrate \( N_{S(0)} \) can also be written as the product of the substrate concentration \([S]_2\) in \( R_2\) and its volume \( V_{r_2}\). The distinction between the terms \([S]_0\) and \([S]_2\) must be emphasized here: the former refers to the desired substrate concentration in the reaction mixture at time \( t = 0\), and the latter to the desired substrate concentration in the reagent \( R_2\) before it is added to the reaction mixture. Therefore, the above equation can be written as:

\[
A_t = k f \left( \frac{[S]_2 V_{r_2}}{V}, [E]_0, t \right) + A_0 \tag{16}
\]

The total volume of the reaction mixture \( V\) is the sum of the sample and reagent volumes \( V_s, V_{r_1}\) and \( V_{r_2}\). We now introduce pipetting uncertainty into the model. Let the fractional change in sample volume due to sample pipetting uncertainty be \( x\), the fractional change in reagent volumes due to reagent pipetting uncertainty be \( y_1\) and \( y_2\), and the fractional change in total reaction mixture volume be denoted by \( z\). Then,

\[
V_s + \delta V_s = V_s(1 + x) \tag{17a}
\]

\[
V_{r_1} + \delta V_{r_1} = V_{r_1}(1 + y_1) \tag{17b}
\]

\[
V_{r_2} + \delta V_{r_2} = V_{r_2}(1 + y_2) \tag{17c}
\]

\[
V + \delta V = V(1 + z) \tag{17d}
\]

Now, using the fact that \( V = V_s + V_{r_1} + V_{r_2}\), we have:

\[
V + \delta V = V_s(1 + x) + V_{r_1}(1 + y_1) + V_{r_2}(1 + y_2) \tag{18a}
\]

That is,

\[
V + \delta V = V_s + V_{r_1} + V_{r_2} + xV_s + y_1V_{r_1} + y_2V_{r_2} \tag{18b}
\]

and

\[
\delta V = xV_s + y_1V_{r_1} + y_2V_{r_2} \tag{18c}
\]

Uncertainty in the instrument can also manifest itself as an error in the time at which the absorbance measurement is recorded. This in turn can change the extent to which the reaction has occurred, and therefore the optical absorbance measured ostensibly at time \( t\) refers to this uncertainty in time of measurement as clock uncertainty. We denote this error in the time of measurement as \( \delta t\) and the fractional change in the desired time of measurement \( t\) as \( u_t\). If we denote the change in optical absorbance measured at time \( t\) as \( \delta A_t\), then the optical absorbance after the incorporation of pipetting and clock uncertainty can be written as:

\[
A_t + \delta A_t = k f \left( \frac{[S]_2 (V_{r_2} + \delta V_{r_2})}{(V + \delta V)}, [E]_0, (t + \delta t) \right) + A_0 \tag{19}
\]

Using equations \(17a\) through \(17d\) we obtain:

\[
A_t + \delta A_t = k f \left( \frac{[S]_2 V_{r_2} (1 + y_2)}{V (1 + z)}, [E]_0, t (1 + u_t) \right) + A_0 \tag{20}
\]

Subtracting equation \(16\) from equation \(20\) we obtain the change in absorbance due to instrument uncertainty.

\[
\delta A_t = k \left[ f \left( \frac{[S]_2 V_{r_2} (1 + y_2)}{V (1 + z)}, [E]_0, t (1 + u_t) \right) - f \left( \frac{[S]_2 V_{r_2}}{V}, [E]_0, t \right) \right] \tag{21}
\]

We denote the fractional change in optical absorbance at time \( t\) due to pipetting and clock uncertainty, \( \frac{\delta A_t}{A_t} \), by the term \( u_{pc(t)}\). Now, equation \(21\) denotes the change in absorbance at time \( t\) from the desired value that occurs before the measurement is performed. When the measurement is performed, the uncertainty due to the photometer changes the absorbance further by the fractional amount \( u_{pc(t)}\). Therefore, the final expression for optical absorbance after incorporating pipetting and clock uncertainty into the model is given below:

\[
A'_t = A_t (1 + u_{pc(t)})(1 + u_{pc(t)}) \tag{22}
\]
The above expression denotes the value of absorbance after all sources of uncertainty affecting the optical absorbance measurement have been incorporated into the model. This process is repeated for all absorbance measurements recorded during the chemical reaction. Therefore, the corresponding rate of the reaction is estimated as follows:

$$m_{int} = \frac{\sum t_i A_i^1 - \frac{1}{n} \sum t_i \sum A_i}{\sum t_i^2 - \frac{1}{n}(\sum t_i)^2}$$

(23)

Now, since the patient sample supplies the enzyme ALP that catalyzes the assay reaction, a change in the volume of the patient sample due to sample pipetting uncertainty changes the number of enzyme molecules available to catalyze the reaction. The change in the rate of the reaction due to a change in sample volume is linearly proportional to the change in volume; therefore, an \(x\)% change in the sample volume would cause the same \(x\)% change in the rate of the reaction. Therefore, the final rate of the reaction after all the sources of uncertainty operating within the calibration process is given by:

$$m' = m_{int}(1 + x)$$

(24)

This process of incorporating uncertainty into the calibration process is applied to both calibrators \(E_1\) and \(E_2\), and their corresponding desired reaction rates \(m_1\) and \(m_2\). Therefore, after incorporating the uncertainty introduced by the calibration process, this results in the estimation of the calibration factor as:

$$K' = \frac{[E_2' - E_1']}{m_2 - m_1}$$

(25)

In the case of most clinical enzyme assays, one of the calibrators is a water blank, and hence only the slope of the calibration line, the calibration factor, is estimated.

### 2.2 Measurement Phase

Once the value of the calibration factor is estimated, the process moves into the measurement phase. In terms of the calibration line, the uncertainty associated with this phase is estimated to be associated with the independent variable \(m\); that is, the absorbance measurements recorded as part of the analysis of the patient sample. Instrument uncertainty is the primary source of uncertainty operating within this phase of the process.

Instrument uncertainty has been dealt with in the previous section, and we denote the fractional change in optical absorbance at time \(t\) due to pipetting uncertainty and clock uncertainty in the measurement phase as \(u_{pc(t,x)}\). If we denote the 'true' enzyme activity level of the sample as \([E]_t\), and the corresponding absorbance at time \(t\) as \(A_{s(t)}\), the absorbance obtained after the incorporating sample and instrument uncertainty is expressed as:

$$A'_{s(t)} = A_{s(t)}(1 + u_{pc(t,x)})(1 + u_{p(t,x)})$$

(26)

Here \(A'_{s}\) represents the absorbance after the uncertainty of the measurement phase is introduced into the process. This process of incorporating instrument uncertainty is followed for each absorbance measurement, and the rate of the reaction corresponding to the patient sample (denoted by \(m_{int(x)}\)) is estimated as the following:

$$m_{int(x)} = \frac{\sum t_i A'_{s(t)} - \frac{1}{n} \sum t_i \sum A'_{s(t)}}{\sum t_i^2 - \frac{1}{n}(\sum t_i)^2}$$

(27)

This value of the rate of the reaction is further changed due to sample pipetting uncertainty as follows:

$$m'_x = m_{int(x)}(1 + x)$$

(28)

The term \(m'_x\) represents the value of the rate of the reaction after all sources of uncertainty operating within the measurement process are incorporated into the model. When this value is input into the calibration line, we get the system output- the ALP activity of the sample - as:

$$[E]_t' = K'm'_x$$

(29)

The uncertainty associated with this model is estimated by generating patient sample activity levels (in the current implementation, 1000, since it corresponds to the average number of tests conducted on the P-modular analytics platform in a day in a hospital) for different sets of realizations of the sources of uncertainty, and then computing the standard deviation of these 1000 activity levels. We note here that while the model accounts for clock uncertainty, it was decided not to include it in the implementation of the model upon the recommendation of the manufacturer, as it is negligible in practice.
3. Results and Analysis
The model was implemented computationally using the Python programming platform. Values of the rate constant \( k_2 \) (34/s) and the Michaelis constant \( K_m \) (4.48 mmol/L) were obtained from the BRENDA Comprehensive Enzyme Information System. The measurement uncertainties (estimated as coefficients of variation (CV)) for a range of patient sample activity levels between 120 U/L - 360 U/L varied from 3.63% - 2.11%. In order to estimate the measurement uncertainty at different activity levels, the simulation model was used to construct an empirical function, referred to as the uncertainty profile, that generates an estimate of measurement uncertainty at a given activity level. The uncertainty profile is constructed by generating uncertainty estimates at different enzyme activity levels in the possible range of patient sample activity levels, and then finding the function that is the best statistical fit to the data. The sample activity level (in U/L) is the independent variable, and the standard deviation (in U/L) of the distribution of the measurement result is the dependent variable. The uncertainty profile for the ALP assay is shown in Figure 1. A sample activity level range of 120 U/L - 360 U/L, traversed in increments of 5 U/L, was used in constructing the uncertainty profile.

Figure 1: Uncertainty profile for alkaline phosphatase assay

One of the principal uses of such an uncertainty model is to estimate the contribution of each individual source of uncertainty in both the calibration and measurement phase of the measurement process. The contribution of a given source of uncertainty in a given phase is estimated by setting its variation to zero (mean and standard deviation of its distribution to zero), and then re-estimating the net measurement uncertainty. The difference between the value of the measurement uncertainty estimated without the source under consideration and the value estimated with all sources of uncertainty represents its contribution to the net measurement uncertainty.

First, the contributions of the sources of uncertainty operating within the measurement phase are estimated. Only the sources instrument uncertainty operate within the measurement phase, and their individual contributions to the net measurement uncertainty within the measurement phase are summarized in Table 2. It is clear that the photometer and the sample pipette are the largest contributors to the net measurement uncertainty, and that reducing the imprecision in their operation would lead to a substantial decrease in net measurement uncertainty. As an illustration, a decrease in 50% of photometer uncertainty (from an SD of 0.15% to 0.075%) reduces the net measurement uncertainty by approximately 20%.

Next, the contributions of the sources of calibrator uncertainty are quantified. These sources, as is evident, operate within the calibration phase, and hence their contribution is estimated not by studying the effect of nullifying their variation on the net measurement uncertainty, but by studying the distribution of the calibration parameter \( K' \). The effect of these sources of uncertainty on the distribution of the calibration parameter \( K' \) is estimated by setting the variation of these sources of uncertainty to zero in the calibration phase alone, and then re-estimating the net measurement uncertainty.

Uncertainty in the calibration parameter \( K' \) shifts the expected value of the distribution of the measurement result. We refer to this shift in the expected value of the measurand distribution as bias, and quantify the worst-case bias of the measurand due to each source of uncertainty operating within the calibration phase. We define the worst case bias of the measurand as the absolute value of the percentage deviation in the expected value of the measurand distribution from the desired error-free enzyme activity level, when the calibration parameter is at \( \pm 3 \) standard deviations from...
Table 2: Contribution of individual sources of uncertainty to net system uncertainty

<table>
<thead>
<tr>
<th>Source of uncertainty</th>
<th>Net system uncertainty with all sources operating (CV, %)</th>
<th>Net system uncertainty with source removed (CV, %)</th>
<th>% contribution of source to net system uncertainty</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample pipetting uncertainty</td>
<td>2.50</td>
<td>2.02</td>
<td>19.20</td>
</tr>
<tr>
<td>Reagent pipetting uncertainty</td>
<td>2.50</td>
<td>2.31</td>
<td>7.60</td>
</tr>
<tr>
<td>Photometer uncertainty</td>
<td>2.50</td>
<td>1.78</td>
<td>28.80</td>
</tr>
</tbody>
</table>

its expected value. Since the measurand distribution is found to be Gaussian, and therefore symmetric and unimodal, the choice of whether the calibration parameter is at plus or minus three standard deviations from its expected value is immaterial in quantifying the worst-case bias. The estimates of the worst-case bias due to each source of uncertainty are provided in Table 3.

Table 3: Contribution of individual sources of uncertainty in the calibration phase

<table>
<thead>
<tr>
<th>Source of uncertainty removed</th>
<th>Mean of calibration parameter K’ (U·s/L)</th>
<th>CV of calibration parameter K’ (%)</th>
<th>Worst-case bias (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.002094</td>
<td>2.560%</td>
<td>7.269</td>
</tr>
<tr>
<td>Calibrator set-point uncertainty</td>
<td>0.002093</td>
<td>2.553%</td>
<td>7.169</td>
</tr>
<tr>
<td>Vial-to-vial variability</td>
<td>0.002093</td>
<td>2.537%</td>
<td>7.093</td>
</tr>
<tr>
<td>Reconstituted stability</td>
<td>0.002093</td>
<td>2.480%</td>
<td>6.970</td>
</tr>
<tr>
<td>All calibrator sources</td>
<td>0.002095</td>
<td>2.506%</td>
<td>7.077</td>
</tr>
<tr>
<td>Sample pipetting uncertainty</td>
<td>0.002093</td>
<td>2.088%</td>
<td>5.924</td>
</tr>
<tr>
<td>Reagent pipetting uncertainty</td>
<td>0.002093</td>
<td>2.341%</td>
<td>6.616</td>
</tr>
<tr>
<td>Photometer uncertainty</td>
<td>0.002093</td>
<td>1.796%</td>
<td>5.185</td>
</tr>
</tbody>
</table>

It is seen from Table 3 that the sources of uncertainty operating within the calibration phase do not have a significant effect on the expected value of the calibration parameter. This is primarily due to the fact that the expected values of the sources of uncertainty are all assumed to be zero except for reconstituted stability. Also, it is evident that the sources of calibrator uncertainty, including reconstituted stability, do not have a significant effect on the distribution of $K'$ in general. Among the sources of calibrator uncertainty, reconstituted stability has the largest effect on the worst-case bias, with a reduction of 4.11% when its variation is set to zero. It is clear from Table 3 that the effect of the sources of instrument uncertainty dominate that of the sources of calibrator uncertainty on the calibration parameter, and therefore on the measurand distribution. Once again, it is evident that photometer uncertainty has the largest effect on the worst-case bias, with a reduction of approximately 30% in the CV of $K'$ and a reduction of approximately 29% in the worst-case bias as compared to the case when all sources of uncertainty are operating within the measurement system. The dominance of photometer uncertainty in its effect on the measurand distribution is explained by the fact that multiple absorbance measurements are made within the assay analysis process - 15 within each phase in the current configuration of the ALP assay.

4. Conclusions
Experimental verification of the model assumptions was not possible due to limitations of access to experimental equipment. However, a minimum level of validation was performed by comparing estimates of measurement uncertainty obtained from the model with uncertainty estimates provided by the instrument manufacturer. Estimates of uncertainty for the ALP assay were provided by the instrument manufacturer in the form of an upper bound of 4% for the CV for ALP activity levels greater than 75 U/L. As can be seen from the uncertainty profile in Figure 1, this condition is satisfied for all enzyme activity levels greater within the range of enzyme activity levels considered in the model. While this comparison is not an adequate substitution for validation via controlled experimentation, it serves to indicate the model provides estimates of uncertainty comparable to that seen in the laboratory.
In conclusion, the primary advantage of developing such uncertainty models lies in their use to extract information about the measurement procedure that would otherwise necessitate experimentation in controlled laboratory conditions. Further, we have introduced a method to estimate the effect of uncertainty within the calibration phase on the distribution of the calibration parameters, and therefore on the measurement result. The use of the model to estimate the contributions of individual sources of uncertainty in both phases of the measurement process provides instrument manufacturers with guidance as to which component of the instrument they should focus their design efforts on.

This methodology can be extended to general linear as well as nonlinear measurement systems. The calibration function provides a convenient starting point for developing the model of measurement uncertainty. Uncertainty within the calibration process can be incorporated into the parameters of the calibration function, and the uncertainty associated with the measurement of the sample property can be incorporated into the independent variable. Once the mathematical model is developed and the sources of uncertainty are characterized, the model can be used to simulate and optimize the measurement process in terms of minimizing its uncertainty.

References