Developing a Spectrophotometric Quantitative Assay for $p$-Nitrophenol

The insecticide parathion ($O,O$-diethyl-$o$-$p$-nitrophenyl phosphorothioate) undergoes a well-defined pathway of biodegradation. In the first step, bacteria present in soil hydrolyze parathion, producing diethylthiophosphoric acid and $p$-nitrophenol (PNP).

$$\text{parathion} \xrightarrow{\text{bacteria}} \text{PNP}$$

Over time, PNP decomposes to 4-nitrocatechol, and, in series of steps, to $\text{CO}_2$ and $\text{NO}_3^-$.

Because of PNP’s environmental toxicity, methods for determining its concentration are necessary. Solutions of PNP are yellow in color, making it relatively easy to determine its concentration spectrophotometrically. In this experiment you will develop a quantitative spectrophotometric assay for PNP using an external standardization, and evaluate its accuracy. The steps to be completed are (a) preparing a set of external standards, (b) selecting a wavelength for the analysis, (c) constructing an external standard calibration curve and verifying that it obeys Beer’s law, (d) validating the external standardization using a standard solution with a known concentration of PNP, and, finally, (e) analyzing a real sample for which the concentration of PNP is unknown.

Safety Considerations. In solid form, $p$-nitrophenol poses a moderate health hazard. Acute inhalation or ingestion of PNP may lead to headaches, drowsiness, nausea, and cyanosis (blue color in lips, ears, and fingernails). Contact with eyes may cause irritation and PNP may be absorbed through the skin. At the solution concentrations used in this experiment, however, the hazards associated with PNP are minimal. You should, of course, use appropriate care when handling any chemicals, wearing safety glasses, being careful to clean up spills, to keep chemicals off of your clothing, to minimize contact with your skin, and to wash your hands when finished. Protective gloves are available in the lab.

Preparing a Set of External Standards. A stock solution with a nominal concentration of 25 mg/mL PNP is available (be sure to record the solution’s exact concentration). Using this stock solution, prepare 25 mL each of four standards with PNP concentrations between 1 and 10 mg/mL. Calculate the concentration of PNP in each external standard.

Selecting a Wavelength for the Analysis. Using your highest concentration standard, obtain its visible spectrum using distilled water for the reference. Examine your spectrum and select a wavelength for further work.

Constructing an External Standard Calibration Curve. Set the spectrophotometer to the wavelength you selected and measure the absorbance of each external standard, using distilled water as a reference. Prepare an external standard calibration curve by plotting absorbance vs. mg/mL PNP and verify that Beer’s law is obeyed. Determine the equation for your calibration curve using a linear regression analysis.

Validating Your External Standardization. To validate your external standardization, analyze the provided calibration standard for which the concentration of PNP is known. Note that the concentration of PNP in the calibration standard exceeds that of your highest con-
centration external standard. You will, therefore, need to dilute the calibration standard so that its absorbance falls within the range of your calibration curve. Measure the absorbance for your diluted calibration standard using distilled water as the reference, calculate the concentration of PNP in the calibration standard (don’t forget to account for the dilution), and evaluate the accuracy of your analysis.

**Analyzing a Real Sample.** Having completed and validated your external standardization, you are ready to analyze a real sample for which the concentration of PNP is unknown. A sample taken from a kinetic study of PNP’s biodegradation is available. Using your external standardization, determine the concentration of PNP in this sample.

**Questions to Ponder.** We will discuss these questions as a group; be prepared to share your answers.

1. Why is it necessary to dilute the standard solution before measuring its absorbance?
2. What is meant by the phrase external standards?
3. Why is distilled water used as a reference?
4. How confident are you in your analysis of the real sample?
Using a Spike Recovery to Validate Results

Even though an external standardization provides acceptable results when analyzing a standard solution, it may provide inaccurate results when used to analyze samples. For example, your determination of the concentration of PNP in a sample may be in error if the sample’s matrix influences the analyte’s absorbance; this is known as a “matrix effect.”

The possibility of a matrix effect raises an interesting question. If you don’t know an analyte’s true concentration in a sample, then how do you know if your reported results are accurate? One way to solve this problem is to analyze the sample using a second, independent analytical method. If both methods give the same result, then the original analysis is probably valid; if not, then at least one of the two analyses must suffer from a determinate error. One limitation to this approach is the time needed to carry out a second, independent analysis.

Another solution, which is simpler and quicker, is to use a spike recovery. In this approach we first analyze the sample, obtaining a concentration for the analyte of \( C_{\text{sample}} \). Next, we add a known concentration of analyte to the sample, \( C_{\text{added}} \). Upon reanalyzing the sample, we obtain the analyte’s new concentration, \( C_{\text{spiked}} \). The spike recovery, \( \%R \), is defined as

\[
\%R = \frac{C_{\text{spiked}} - C_{\text{sample}}}{C_{\text{added}}} \times 100
\]

If the external standard calibration curve is valid for the sample (i.e. there are no significant matrix effects), then the percent recovery should be near 100%. A percent recovery that deviates significantly from 100% strongly suggests that the sample’s matrix cannot be ignored. If this is the case, then we must reject the external standardization.

Safety Considerations. The same safety considerations mentioned in the first portion of this lab still apply.

Performing the Spike Recovery. To test the utility of a spike recovery, you will use it to validate your analysis for both the calibration standard, for which the concentration of PNP is known, and the sample, for which the concentration of PNP is unknown. Transfer exactly 5-mL each of the calibration standard and the sample to separate 10-mL volumetric flasks and dilute to volume with distilled water. Analyze these solutions using your previously determined external standards calibration curve, determining \( C_{\text{sample}} \) for each. Next, prepare the spiked samples by transferring exactly 5-mL each of the calibration standard and the sample to separate 10-mL volumetric flasks. Using a digital pipet, add exactly 0.4 mL of the nominally 25 mg/mL PNP stock solution to each volumetric flask. Dilute each mixture to volume using distilled water. Analyze the resulting spiked solution and determine \( C_{\text{spiked}} \) for each. Calculate the spike recovery for the calibration standard and the sample.

Exploring the Effect of the Sample’s Matrix. Using the nominally 25 mg/mL PNP solution with a distilled water matrix, prepare a set of external calibration standards using one of the available buffer solutions. Measure the absorbance of each external standard and prepare a suitable calibration curve.

Questions to Ponder. We will discuss these questions as a group; be prepared to share your answers.
1. Why might we reasonably conclude that an analysis is accurate if a second, independent method gives the same result?

2. Explain why a recovery significantly greater than 100% or significantly less than 100% is possible.

3. What conclusion did you reach about the appropriateness of your external standardization for the standard solution and the sample?

4. If an external standardization is valid for a sample (i.e. there are no matrix effects), then a spike recovery should be near 100%. Obtaining a percent recovery of 100%, however, does not guarantee that an analysis is accurate. Speculate on why this is possible.

5. Considering the structure of \( p \)-nitrophenol, which is shown below, speculate on why the sample’s pH affects PNP’s absorbance, and why this produces a significant matrix effect.

6. Obtain calibration curves from other groups using pH levels different than yours. Plot the slope of the calibration curve vs. pH and explain the shape of the resulting graph.

\[
\begin{align*}
\text{HO} & \text{-} \\
& \text{NO}_2 \\
\text{PNP}
\end{align*}
\]
Quantitative Analysis for PNP Using the Method of Standard Addition

A matrix effect occurs when a difference between the matrices of the external standards and samples leads to a determinate error in an analysis. Why might a difference in matrices cause a problem?

Consider, for example, a spectrophotometric analysis for an acid–base indicator that obeys Beer’s law

\[ A = abC \]

where \( A \) is the sample’s absorbance, \( a \) is the indicator’s absorptivity (in mL mg\(^{-1}\) cm\(^{-1}\)), \( b \) is the pathlength (in cm), and \( C \) is the indicator’s concentration (in mg/mL). An acid–base indicator exists in two forms: an acid form, HIn, that predominates at more acid pH levels, and a base form, In\(^-\), that predominates at more basic pH levels. Because the indicator’s two forms differ in color, solutions containing the same concentration of indicator, but at different pH levels, will have different absorbances; clearly this means that

\[ a_{\text{HIn}} \neq a_{\text{In}} \]

Any experimentally determined value for \( a \), therefore, must fall within the limits of \( a_{\text{HIn}} \) and \( a_{\text{In}} \), with the actual value depending on the solution’s pH.

Now we can appreciate the source of a potential matrix effect for solutions containing acid–base indicators. When we perform an external standardization, the slope of the calibration curve, which is equivalent to \( ab \), provides an experimental estimate of the indicator’s absorptivity. If the external standards are at a different pH than the sample, then the experimentally determined \( a \) for the standards may be significantly different than that for the sample. The result is a determinate error due to the matrix, or a “matrix effect.”

There are two approaches to completing a quantitative analysis suffering from a matrix effect. The first is to carefully match the matrix of the standards to that of the samples. For example, if we are analyzing samples for the concentration of an acid–base indicator and know that the samples are buffered to a pH of 6.47, then we can prepare standards with the same pH. When it isn’t possible (or desirable) to match the matrices of the standards and samples, then we can use the method of standard addition.

A spike recovery is the simplest example of a standard addition. Recall that there are two absorbance measurements in a spike recovery: one for the sample before adding the spike, for which

\[ A_{\text{sample}} = abC_{\text{sample}} \left\{ \frac{V_{\text{sample}}}{V_{\text{total}}} \right\} \]

and one after adding the spike, for which

\[ A_{\text{spike}} = abC_{\text{spike}} = ab \left\{ C_{\text{sample}} \left( \frac{V_{\text{sample}}}{V_{\text{total}}} \right) + C_{\text{standard}} \left( \frac{V_{\text{spike}}}{V_{\text{total}}} \right) \right\} \]

where \( V_{\text{sample}}, V_{\text{spike}}, \) and \( V_{\text{total}} \) are, respectively, the volume of sample, the volume of standard solution taken as a spike, and the final volume to which both solutions are diluted, and \( C_{\text{sample}} \) and \( C_{\text{standard}} \) are the analyte’s concentration in the sample and the standard solution used for the spike. If \( V_{\text{spike}} \ll V_{\text{sample}} \) then the addition of the spike usually does
not significantly alter the sample’s matrix and we may assume that \( a \) is the same for both solutions; thus

\[
\frac{A_{\text{sample}}}{C_{\text{sample}} \left( \frac{V_{\text{sample}}}{V_{\text{total}}} \right)} = \frac{A_{\text{spike}}}{C_{\text{sample}} \left( \frac{V_{\text{sample}}}{V_{\text{total}}} \right) + C_{\text{standard}} \left( \frac{V_{\text{spike}}}{V_{\text{total}}} \right)}
\]

which is solved for \( C_{\text{sample}} \). A spike recovery, therefore, is identical to a 1-point standard addition.

To minimize the contributions of indeterminate errors, a standard addition usually involves the preparation of several spikes using increasingly greater volumes of the standard solution. The absorbances for these solutions are

\[
A = abC_{\text{sample}} \left( \frac{V_{\text{sample}}}{V_{\text{total}}} \right) + abC_{\text{standard}} \left( \frac{V_{\text{spike}}}{V_{\text{total}}} \right)
\]

A plot of \( A \) vs. \( C_{\text{standard}} \left( \frac{V_{\text{spike}}}{V_{\text{total}}} \right) \) is a straight-line with a slope of \( ab \), a \( y \)-intercept of \( abC_{\text{sample}} \left( \frac{V_{\text{sample}}}{V_{\text{total}}} \right) \), and an \( x \)-intercept of \( -C_{\text{sample}} \left( \frac{V_{\text{sample}}}{V_{\text{total}}} \right) \). The analyte’s concentration in the original sample, therefore, is easy to obtain from the \( x \)-intercept.

**Safety Considerations.** The same safety considerations mentioned in the first portion of this lab still apply.

**Preparing the Standard Additions.** Place 5.00 mL of the calibration standard in each of five 10-mL volumetric flasks. Add 0, 0.1, 0.2, 0.3, and 0.4 mL spikes of the nominally 100 mg/mL PNP standard to the separate flasks, diluting each to volume with distilled water. Repeat using 5.00 mL of the sample in place of the calibration standard.

**Constructing the Standard Addition Calibration Curve.** Set the spectrophotometer to the wavelength determined in the earlier experiment and zero using distilled water as a reference. Measure the absorbance for each solution (a total of 10 absorbance measurements). Construct standard addition calibration curves for the calibration standard and the sample, and determine the concentration of PNP in each.

**Questions to Ponder.** We will discuss these questions as a group; be prepared to share your answers.

1. Calculate the concentration of PNP in the calibration standard and the sample using your data from the spike recovery. How do these results compare to the results of your standard addition calibration curves? Be sure to explain any discrepancies in your results.

2. Suppose the solution used to zero the spectrometer doesn’t correct for the absorbance of a species in the sample other than the analyte. How might this affect your results?

3. Briefly discuss the advantages and disadvantages of using an external standardization and compare to the advantages and disadvantages of using a standard addition.

4. Suppose that you need to develop a quantitative spectrophotometric method for PNP. How would you design an external standardization so that matrix effects are minimized and sensitivity is maximized? Please provide your answer as a set of written instructions suitable for a laboratory handout.