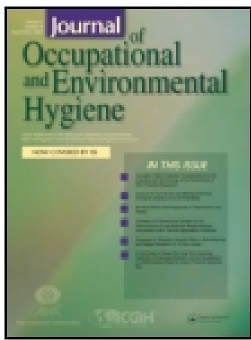

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Analytical Performance Criteria
The Use of Immunochemical and Biosensor Methods for Occupational and Environmental Monitoring. Part II: Immunoassay Data Analysis and Immunobiosensors

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BACKGROUND

This is the second of a two-part series on the use of immunochemical and biosensor methods for occupational and environmental monitoring. In Part I (Introduction to Immunoassays), a general overview of the immunology and types of immunoassays was discussed. The major types of enzyme-linked immunosorbent assays (ELISAs), radioimmunoassays (RIA), and lateral flow techniques were described. In the present column, how data are reduced from immunoassays is addressed and some state-of-the-art immunobiosensors are introduced.

IMMUNOASSAY DATA ANALYSIS

Standard curves for enzyme-linked immunosorbent assays (ELISAs) are normally prepared by adding purified analyte to the matrix that is going to be used in the analysis. If the analyte is only slightly water soluble, small amounts of organic solvents may be added to the standards to enhance solubility after first determining that the solvent has no effect on the assay.[1] In some cases when measuring conjugated urinary metabolites of a chemical exposure, it is useful to enzymatically remove the conjugated portion of the molecule before analysis (e.g., removal of the glucuronide from the 3,5,6 trichloro-2-pyridinol metabolite of chlorpyrifos with β-glucuronidase).[2] Again, control experiments to determine the effect of the enzymatic treatment on the assay have to be performed.

Data can be treated from ELISA experiments where a set of known standard concentrations of analyte are measured and a relationship between the standard concentration and the ELISA system's response is analyzed. Many mathematical models have been used to construct ELISA standard curves including logit-log transforms,[3] log-log transforms,[4] four-parameter logistic-log curves, etc.[5] The four-parameter logistic-log model (4-PL) is given by:

$$y = \frac{a - d}{1 + \left(\frac{X}{c}\right)^{b}} \quad (1)$$

where y is the measured response (optical density, median fluorescence intensity, etc.), X is the analyte concentration; a and d are the estimated responses at zero and infinite concentration, respectively; c is the concentration giving 50% response (C_{50}); and b is the slope of the tangent at c[6] (these parameters are diagrammed in Figure 1A).

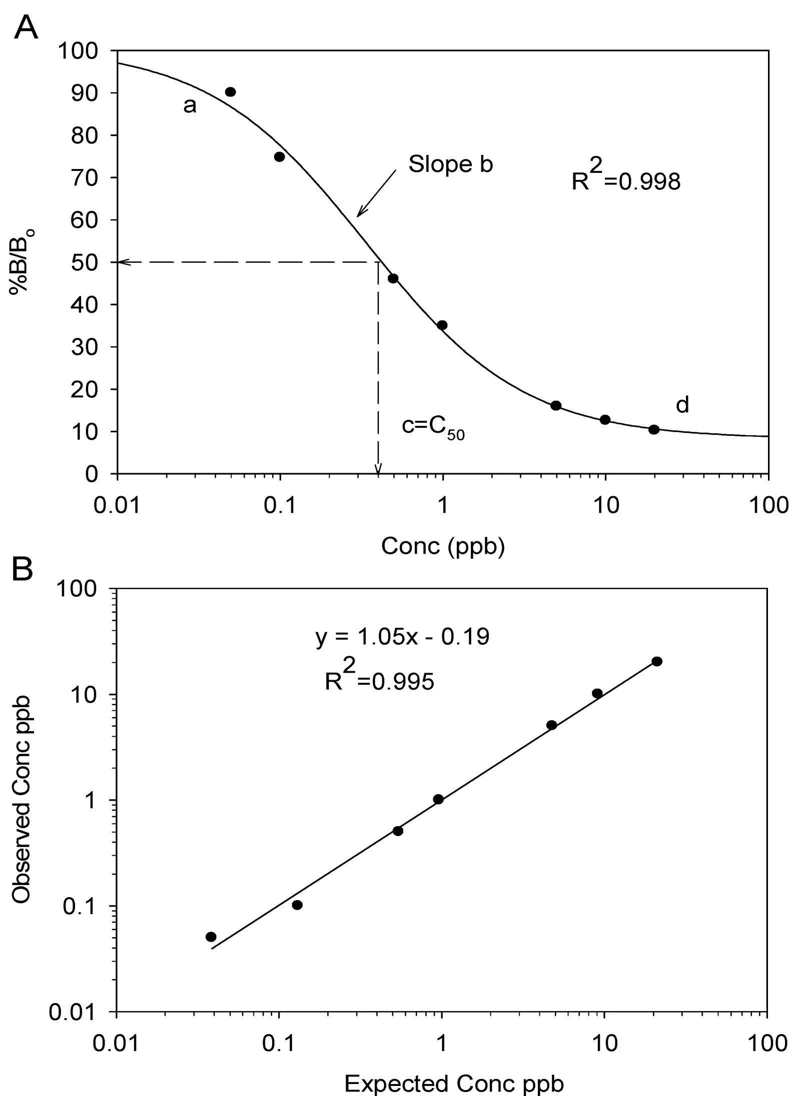

FIGURE 1. Calibration curves: (A) Percent B/Bo plotted against log (concentration of analyte). Data were fitted using a using a four-parameter logistic model (a and d are the estimated responses at zero and infinite concentration, respectively; c is the concentration giving 50% response; and b is the slope of the tangent at c). C_{50} is defined as the estimated concentration at 50% B/Bo. (B) Linear regression of the observed interpolated results from the 4-PL fit compared with analyte concentrations added (expected).

The 4-PL fit has been shown to be superior to log-log and other fits for immunoassays even when R^2 values are high (>0.97).[7] The 4-PL fit extends the range of the assay, thus providing a more precise measurement of analyte concentrations.[7]

It has been suggested that a five-parameter logistic-log model (5-PL) where g is an asymmetry factor:

$$y = \left[\frac{a - d}{1 + \left(\frac{x}{c}\right)^b}\right]^g \tag{2}$$

may improve curve fits in some situations.[8] A practical method for assessing the quality of a standard curve fit is to calculate the concentrations of the standards after the regression has been completed.[9,10] This procedure is known as standards recovery (Figure 1B) and is performed by calculating the concentration of each standard from the 4-PL fit and then comparing it with the actual concentration. This method yields information about the relative error in the calculation of samples. It is most desirable to have each standard fall between 70% and 130%[11] of the actual value, although more stringent ranges may be applied if greater accuracy is desired. The limitation of using back calculation as the sole method of evaluating goodness of fit is the existence of a bias toward the concentrations of the standards. More specifically, only the standard concentrations are used to assess the quality of the fit; the portions of the curve between each of the standard points are ignored.[9]

Spike recovery may also be used to assess the overall accuracy of an assay.[12] This method incorporates variables in assay preparation as well as the regression analysis. Samples

are spiked with known concentrations of analyte and analyzed to determine the closeness of the calculated value to the actual value. The chosen concentrations are usually between the concentrations of the standards. The results are assessed in the same manner as the standards recovery, using the formula above. A spike recovery value between 80% and 120%[11] is considered acceptable. The disadvantage of this method is that it is affected by variables other than curve fitting. Errors in sample preparation or assay preparation (e.g., pipetting, adding reagents) may affect overall recovery. In addition, it is difficult to accurately spike low levels of analyte into samples due to the relative imprecision of pipets that deliver small volumes.[13]

The linear dynamic range of an immunoassay (range of concentrations where interpolated concentrations can be determined accurately) can be calculated by evaluating standard concentrations that return observed vs. expected values of 70–130% or by systematically removing data pairs from both ends of the observed vs. expected regression line in such a way as to maximize the R^2 value.[14]

Estimates of least detectable doses (LDD) and minimum detectable concentrations (MDC) have also been calculated using numerous methods, including graphically from the intersection of the asymptote of a 4-PL regression's 95% confidence interval (CI) with the regression line,[15] or as a multiple of standard deviations of the blank response.[16,17] In competitive assays where the response is expressed as $\%B/B_o$ data, where $B =$ the response of a standard and $B_o =$ the mean optical density measured for the blank, 90% B/B_o is routinely used as the LDD.[2]

Specificity is an important characteristic of any laboratory test that describes its ability to identify the analyte in the presence of potential interferents. With immunoassay methods, interferences that affect specificity can be categorized into two major classes: (1) those that affect the binding event between the antibody and an antigen in a general way, such as pH or ionic strength; or (2) those substances that affect binding of antigen by competing for the specific binding site on the antibody. These "specific" interferences are often referred to as "cross-reactants." In the analysis of pesticides and/or pesticide metabolites, it is often desirable to have high levels of cross-reactivity with related compounds and metabolites of the parent compounds so that broad screening can be performed.

The specificity of an immunoassay may be characterized by adding increasing amounts of a potential cross-reacting substance to a sample and measuring the response in the immunoassay. The results of this experiment can be reported several ways. One method of representing the comparative reactivity of these compounds is to determine the concentration of each compound required to displace the same amount of labeled antigen from antibody. For example, one commonly calculates cross-reactivity using the concentrations required to displace 50% of the label or 50% $B/B_{o.}$ The concentration is called the EC_{50} (estimated concentration at 50% B/B_o). A ratio of the resulting concentrations can be referred to as the "percent cross-reactivity at the EC_{50}."

Cross-reactivity can also be calculated at other levels of displacement, such as 20% (EC_{20}). Depending on the slope and shape of the response curve, the percent cross-reactivity may be different at different displacement levels. If one chooses the lowest level of displacement that can be reliably distinguished from zero displacement, the resulting concentrations could be represented as a least detectable dose (LDD) for each cross-reactant. Evaluation of cross-reactivity in poorly defined biological samples may be very complex.[18]

The analytical specificity of the assay for the analyte being measured can be evaluated by competitive inhibition. For example, in an indirect immunoassay for *Bacillus anthracis* protective antigen (PA) specific IgG in serum, preincubation of positive and control sera with soluble PA is performed. The soluble PA binds to the anti-PA IgG antibody, essentially removing its ability to react with the immobilized antigen during the subsequent anti-PA IgG assay. Percent inhibition is calculated by the following expression:

$$\%\text{inhibition} = 100 \times \left(\frac{(\text{non-inhibited assay response}) - (\text{inhibited assay response})}{\text{non-inhibited assay response}}\right) \quad (3)$$

Percent inhibition above ~95% is indicative of the specificity of the assay.[15,19]

Numerous extraneous factors can be present in a sample that may influence antigen-antibody binding, including pH; ionic strength; endogenous components such as enzymes, immunoglobulins, bile and salts; and exogenous substances such as drugs, polymers and detergents.[20] These factors contribute to matrix effects defined as follows: (i) the influence of a sample property, other than analyte, on the measurement; and (ii) the physicochemical effect(s) of the matrix on the analytical method's ability to accurately measure an analyte.[20]

To insure the integrity of immunoassay data, analytical quality control measures are essential. Each analyst must take an independent responsibility for ensuring that the analytical quality control system works. This can be accomplished by using known spiked samples that closely simulate the samples being measured with regard to concentration and interferences. Analytical quality control in immunoassays can also be evaluated by comparison with reference classical instrumental methods.[1] There are consensus protocols to evaluate the performance characteristics of immunoassays published by the Clinical and Laboratory Standards Institute (formerly NCCLS). Some of these protocols address the evaluation of over all assay performance characteristics,[21] precision,[22] assay linearity,[23] the evaluation of bias,[24] and matrix effects.[25]

IMMUNOBIOSENSORS

Recent advances in solid state electronics and the availability of inexpensive computing power have allowed for the development of immunoassay systems other than those that are based on enzyme detection systems. These systems are collectively identified as immunobiosensors (i.e., analytical

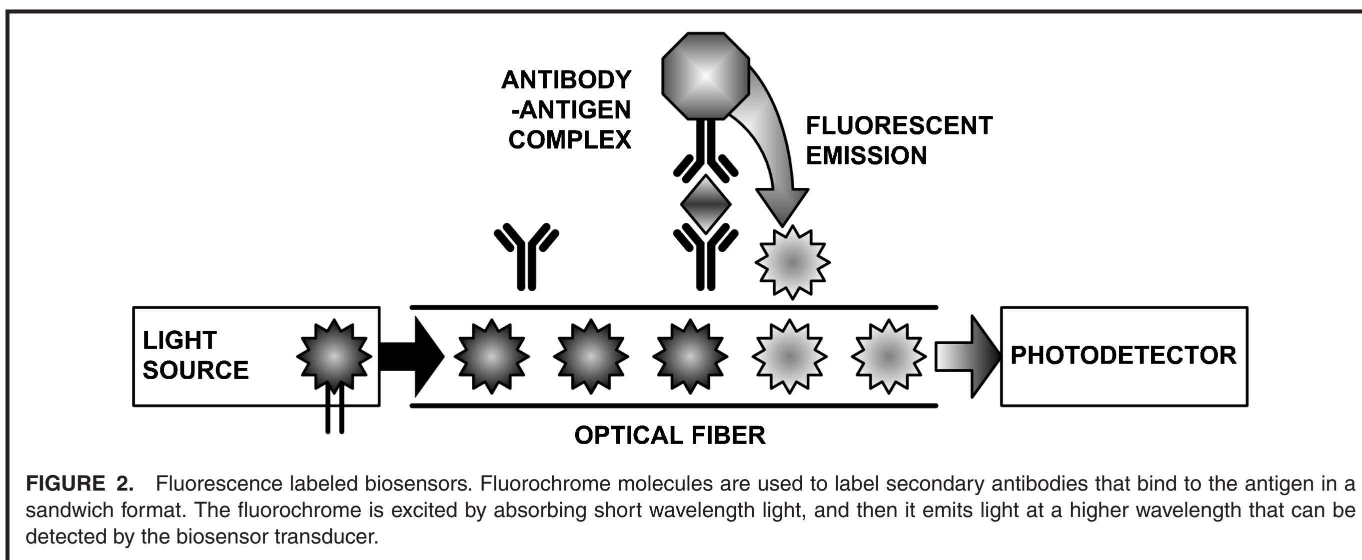

FIGURE 2. Fluorescence labeled biosensors. Fluorochrome molecules are used to label secondary antibodies that bind to the antigen in a sandwich format. The fluorochrome is excited by absorbing short wavelength light, and then it emits light at a higher wavelength that can be detected by the biosensor transducer.

devices with the potential for portability that combines the specificity of antigen-antibody interaction with a transducer that produces a signal proportional to the target analyte concentration).[26] The antigen-antibody complex is in close contact with a signal transducer (e.g., optical, electro-analytical, or acoustic) coupled to a data acquisition and processing system.[27] Immunobiosensors, because of their specificity, fast response times, low cost, portability, ease of use, and a continuous real time signal, present some distinct advantages over alternative methods of analyses.[28]

The primary optical characteristics that are exploited in the development of immunobiosensors include fluorescence, chemiluminescence, and refractive index change. These optical effects can be measured by surface plasmon resonance (SPR) or evanescent wave effects.[29,30] For example, fluorescent fiber optic biosensors (Figure 2) have optical fiber probes, each coated with an antibody specific for a particular analyte. Samples flow over the probes, followed by exposure to another antibody to which a fluorescent dye has been attached. If the fluorescent antibody binds to the captured agent, a fluorescent signal is generated at the surface of the probe.

Refractive index can also be exploited to measure antigen-antibody interactions. The manner in which light interacts at an interface can be exploited to measure changes in surface condition occurring when antigens bind to antibodies on a surface, and may be measured by surface plasmon resonance (SPR).[30,31] An SPR immunobiosensor (Figure 3) is composed of an SPR transducer and a biological recognition element (e.g., antibody) that can interact with the targeted analyte. The biomolecular recognition element is immobilized on the SPR transducer surface.

When a liquid sample is brought into contact with the sensor surface, the interaction between the biomolecular recognition element and the analyte occurs, which produces a change in the refractive index at the sensor surface. This change results in a change in the propagation constant of a surface plasmon excited at the sensor surface and is eventually measured by measuring a change in one of the characteristics of light interacting with the surface plasmon-resonant wavelength, resonant angle, intensity, phase, and polarization.

Piezoelectric immunobiosensors (Figure 4) are based on a quartz crystal resonator, consisting of a disk with electrodes plated on its surface. Application of an external oscillating electric potential across the device induces an acoustic wave that propagates through the crystal. The frequency of the

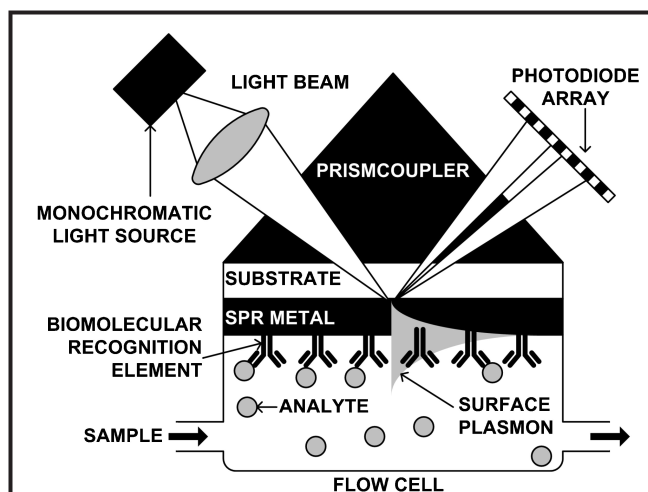

FIGURE 3. Surface Plasmon Resonance (SPR). A narrow-band convergent beam from a light-emitting diode is launched into a prism coupler and made incident onto a thin metal (gold) film. The angular component of light that fulfills the coupling condition excites a surface plasmon wave at the outer boundary of the metal film. The coupling produces a narrow dip in the angular spectrum of the reflected light; the precise angular position is determined using a computer-controlled position sensitive photodetector. When a solution containing analyte molecules is injected into the flow cell, analyte molecules in the sample bind to the biomolecular recognition elements immobilized on the SPR sensor surface, producing a shift in the position of the dip in the angular spectrum of reflected light. The shift can be correlated with the concentration of analyte in the sample.

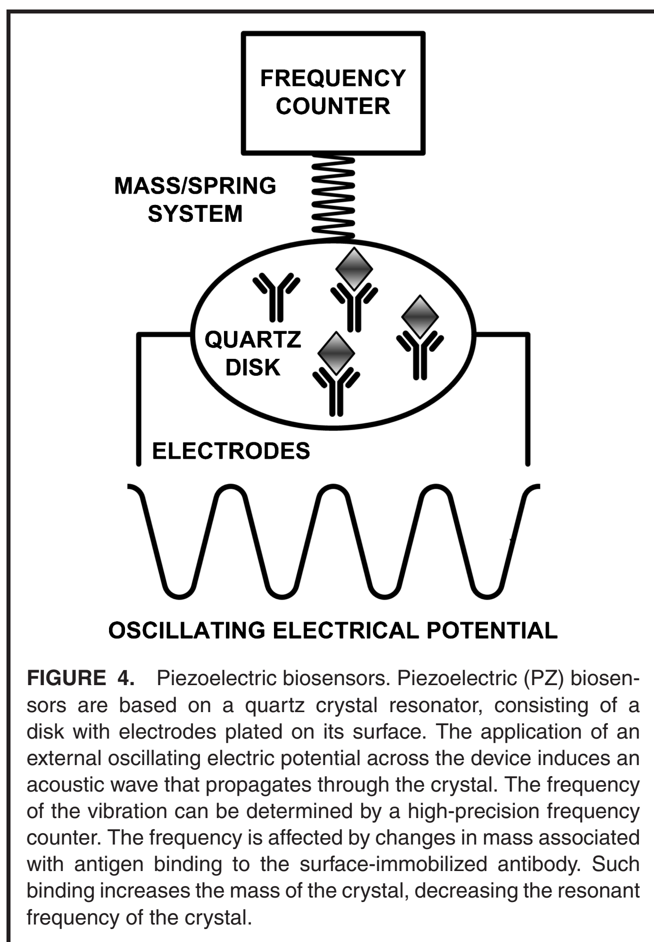

FIGURE 4. Piezoelectric biosensors. Piezoelectric (PZ) biosensors are based on a quartz crystal resonator, consisting of a disk with electrodes plated on its surface. The application of an external oscillating electric potential across the device induces an acoustic wave that propagates through the crystal. The frequency of the vibration can be determined by a high-precision frequency counter. The frequency is affected by changes in mass associated with antigen binding to the surface-immobilized antibody. Such binding increases the mass of the crystal, decreasing the resonant frequency of the crystal.

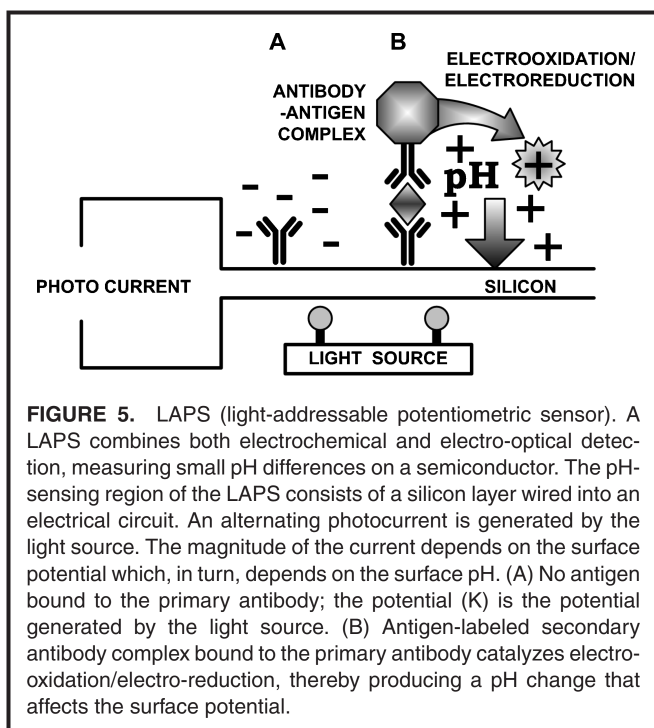

FIGURE 5. LAPS (light-addressable potentiometric sensor). A LAPS combines both electrochemical and electro-optical detection, measuring small pH differences on a semiconductor. The pH-sensing region of the LAPS consists of a silicon layer wired into an electrical circuit. An alternating photocurrent is generated by the light source. The magnitude of the current depends on the surface potential which, in turn, depends on the surface pH. (A) No antigen bound to the primary antibody; the potential (K) is the potential generated by the light source. (B) Antigen-labeled secondary antibody complex bound to the primary antibody catalyzes electro-oxidation/electro-reduction, thereby producing a pH change that affects the surface potential.

vibration can be determined by a frequency counter and is affected by minute changes in mass associated with antigen binding to the surface-immobilized antibody. This binding increases the mass of the crystal, decreasing the resonant frequency which can be measured.[30,32]

Light-addressable potentiometric sensors (LAPS) combine both electrochemical and electro-optical detection (Figure 5), measuring small pH differences (∼0.01 pH units) on a semiconductor. The pH-sensing region of the instrument consists of a conductive silicon layer wired into an electrical circuit. A LAPS measures an alternating photocurrent generated when a light source such as a light emitting diode (LED) flashes rapidly. The current magnitude depends on the surface potential, which in turn depends on the surface pH.[30,33]

CONCLUDING REMARKS

The advantages of immunoassays and immunobiosensors such as high specificity, portability, small required sample volumes, high sample throughput with simultaneous analysis of multiple samples, reduced sample preparation, reduction in the use of chemicals and production of waste, ease of automation and, in some cases, better accuracy, precision and sensitivity[5,30,34] than traditional chemical/instrumental methods (CIM) far outweigh their limitations. Comparison of ELISA and CIM (usually GC/MS) has shown linear slopes of essentially unity for numerous low molecular weight analytes (triazine herbicides, chlorpyrifos metabolite, dioxins, etc).

Recent and future developments in the development of antibody mimics and other receptors, molecular imprinting, microarrays, improved detection systems, immunoaffinity sample preparations, multiplexed immunoassays, and sensors for unattended and real-time analysis will make the use of bioanalytical immunoassays even more attractive. Bioanalytical immunoassay methods are not a panacea, but they should be considered when they can serve as adjuncts to CIM due to their advantages. Yet, current immunoassays, because they use the products of biological systems, are subject to biological variability, sometimes leading to antibody cross-reactivity and other salient binding peculiarities.[1]

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