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Immunoassay, biosensors and other nonchromatographic methods

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1 Introduction

Nonchromatographic methods for residue detection consist of a wide variety of techniques. For illustrative purposes these may be divided into 'biological'- and 'physical'-based methods, based on whether or not biological reagents are involved. Biological techniques include immunoassays, biosensors, bioassays, enzyme assays and polymerase chain reaction (PCR). Among the physical techniques that fit this category are spectrophotometry and voltammetry. The focuses of this article are the 'biological' techniques, in particular immunoassays and PCR, with a brief introduction to biosensors.

2 Immunoassay for pesticides

The concept of immunoassay was first described in 1945 when Landsteiner suggested that antibodies could bind selectively to small molecules (haptens) when they were conjugated to a larger carrier molecule.[1] This hapten-specific concept was explored by Yalow and Berson in the late 1950s, and resulted in an immunoassay that was applied to insulin monitoring in humans.[2,3] This pioneering work set the stage for the rapid advancement of immunochemical methods for clinical use.

The first application of immunologically based technology to pesticides was not reported until 1970, when Centeno and Johnson developed antibodies that selectively bound malathion.[4] A few years later, radioimmunoassays were developed for aldrin and dieldrin[5] and for parathion.[6] In 1972, Engvall and Perlman introduced the use of enzymes as labels for immunoassay and launched the term enzyme-linked

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immunosorbent assay (ELISA).[7] In 1980, Hammock and Mumma[8] described the potential for ELISA for agrochemicals and environmental pollutants. Since then, the use of immunoassay for pesticide analysis has increased dramatically. Immunoassay technology has become a primary analytical method for the detection of products containing genetically modified organisms (GMOs).

The advantages of immunoassay technology relative to other analytical techniques have been discussed in several reviews,[8–12] and include the following:

- low detection limits
- high analyte selectivity
- high throughput of samples
- reduced sample preparation
- versatility for target analytes
- cost effectiveness for large numbers of samples
- adaptability to field use.

As is the case with every analytical method, immunoassay technology has limitations, including:

- interferences from sample matrices
- cross reactivity to structural analogs of the target analyte
- poor suitability for some multi-analyte applications
- low availability of reagents
- longer assay development time than some classical analytical methods
- a large number of anticipated samples required to justify the development of a new assay for an analyte of interest.

The immunoassay is clearly not the best analytical method for all analytes in all situations. For example, gas–liquid chromatography (GLC) remains the method of choice for the analysis of volatile compounds. However, immunoassay technology is important for the analyst because it complements the classical methods, thus providing a confirmatory method for many compounds and the only reasonable analytical choice for others.[13] Most immunoassays can be used to obtain quantitative results with similar or greater sensitivity, accuracy and precision than other analytical methods. They are generally applicable to the analysis of small molecules, including pharmaceuticals and pesticides, identification of pest and beneficial species, characterization of crop quality, detection of GMOs, product stewardship, detection of disease and even monitoring for bioterrorism.

2.1 Principles of immunoassays

Immunoassays are based on the reaction of an analyte or antigen (Ag) with a selective antibody (Ab) to give a product (Ag–Ab) that can be measured. The reactants are in a state of equilibrium that is characterized by the law of mass action (Figure 1).

Several types of labels have been used in immunoassays, including radioactivity, enzymes, fluorescence, luminescence and phosphorescence. Each of these labels has advantages, but the most common label for clinical and environmental analysis is the use of enzymes and colorimetric substrates.

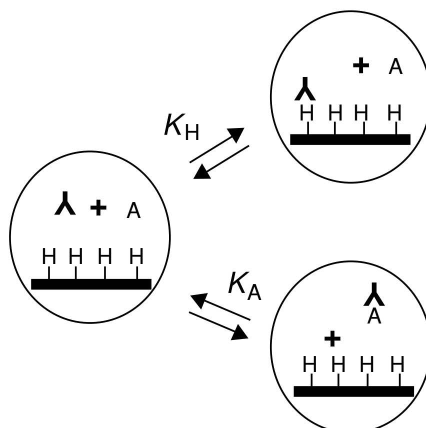

Figure 1 Schematic of the quasi-equilibria using heterologous haptens in coating antigen immunoassay formats. K_A represents the equilibrium constant for binding of antibody (**Y**) to target analyte (**A**). K_H is the equilibrium constant for the binding of antibody to hapten–protein conjugate (**H**–) immobilized on a solid phase

Enzyme immunoassays can be divided into two general categories: homogeneous and heterogeneous immunoassays. Heterogeneous immunoassays require the separation of bound and unbound reagents (antibody or antigen) during the assay. This separation is readily accomplished by washing the solid phase (such as test-tubes or microtiter plate wells) with a buffer system. Homogeneous immunoassays do not require a separation and washing step, but the enzyme label must function within the sample matrix. As a result, assay interference caused by the matrix may be problematic for samples of environmental origins (i.e., soil, water, etc.). For samples of clinical origin (human or veterinary applications), high target analyte concentrations and relatively consistent matrices are often present. Thus for clinical or field applications, the homogeneous immunoassay format is popular, whereas the heterogeneous format predominates for environmental matrices.

2.2 Immunoassay formats

The microplate ELISA test is conducted in standard 96-well microplates. A microplate consists of a 12×8 grid of wells for test solutions. The three most widely used ELISA formats are immobilized antigen competitive immunoassay, immobilized antibody competitive immunoassay and sandwich immunoassay.[14,15]

The following is a generic description of the immobilized antigen ELISA (Figure 2), commonly termed indirect competitive immunoassay, on a microtiter plate.

Preparation of microtiter plates. A constant amount of the coating antigen is bound to the surface of polystyrene microtiter plate wells by passive adsorption. After a predetermined incubation time, the plate is washed to remove unbound coating antigen.

Competitive inhibition. A constant amount of anti-analyte antibody (primary antibody) and a series of solutions containing increasing amounts of analyte are added to the prepared microtiter plate wells. During incubation, the free analyte and bound

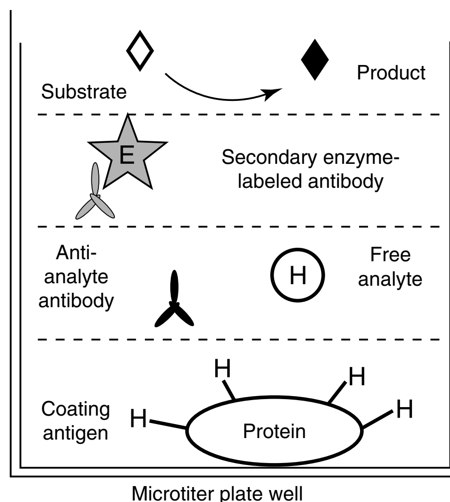

Figure 2 Immobilized antigen ELISA format. Antigen is immobilized to a solid phase by passive adsorption. Following removal of unbound antigen, analyte (free **H**) and antigen (**H**–protein) compete for a fixed number of primary antibody (**Y**) binding sites. Unbound materials are removed (dotted line). Secondary antibody–enzyme conjugate (**Y**–**E**) is added to bind to primary antibody followed by another wash step. Substrate (◇) for the enzyme is added to detect the bound enzyme. The amount of colored product (◆) detected is inversely proportional to the amount of analyte present

coating antigen compete for binding to antibodies in the mixture. Unbound reagents are washed out.

Secondary antibody and determination. A secondary antibody labeled with an enzyme is added which binds to the primary antibody that is bound to the coating antigen. If the primary antibody were produced in a rabbit, an appropriate secondary antibody would be goat anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP) (or another enzyme label). Excess secondary antibody is washed away. An appropriate substrate solution is added that will produce a colored or fluorescent product after enzymatic conversion. The amount of enzyme product formed is directly proportional to the amount of first antibody bound to the coating antigen on the plate and is inversely proportional to the amount of analyte in the standards.

Another commonly used ELISA format is the immobilized antibody assay or direct competitive assay (Figure 3). The primary anti-analyte antibody is immobilized on the solid phase and the analyte competes with a known amount of enzyme-labeled hapten for binding sites on the immobilized antibody. First, the anti-analyte antibody is adsorbed on the microtiter plate wells. In the competition step, the analyte and enzyme-labeled hapten are added to microtiter plate wells and unbound materials are subsequently washed out. The enzyme substrate is then added for color production. Similarly to indirect competitive immunoassay, absorption is inversely proportional to the concentration of analyte. The direct competitive ELISA format is commonly used in commercial immunoassay test kits.

Sandwich ELISAs (Figure 4) are the most common type of immunoassay used for the detection of proteins. A capture antibody is immobilized on the wells of a microplate. The solution containing the analyte is introduced and antibody–analyte

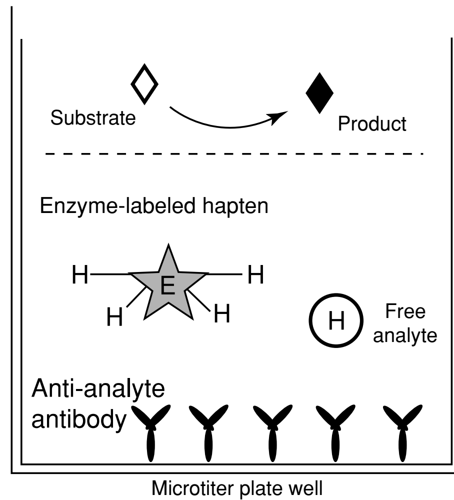

Figure 3 Immobilized antibody ELISA. Primary antibody (**Y**) is passively adsorbed to the surface of a polystyrene microtiter plate. Analyte (free **H**) and an enzyme-labeled hapten (**H**–**E**) compete for the fixed number of primary antibody binding sites. Following a wash step (dotted line), the substrate for the enzyme is added (◇) and a colored product formed (◆). The amount of product is inversely proportional to the amount of analyte present

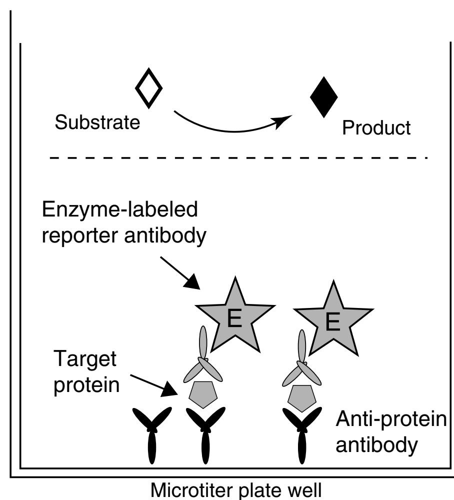

Figure 4 Sandwich immunoassay. A capture antibody (**Y**) is passively adsorbed on a solid phase. The target protein contained in the sample and the enzyme-labeled reporter antibody (**Y**–**E**) are added. Both the capture antibody and enzyme-labeled reporter antibody bind to the target protein at different sites, 'sandwiching' it between the antibodies. Following a wash step, the substrate (◇) is added and colored product (◆) formed. The amount of colored product is directly proportional to the amount of target protein captured

binding occurs. A second, analyte-specific, enzyme-labeled antibody is added and it also binds to the analyte, forming a sandwich. A substrate is added, producing a colored product. Unlike the competitive immunoassays described in Figures 2 and 3, the absorbance in the sandwich immunoassay is directly proportional to the concentration of the analyte in the sample solution.

A commonly used field-portable immunoassay format is the lateral flow device. Lateral flow devices are designed for threshold or qualitative testing. Advantages of this format are that the cost per test is low, it is field portable, it can be done at ambient temperature, it requires no specialized equipment and only minimal user training is required. Each immunoassay strip test (lateral flow device) is a single unit allowing for manual testing of an individual sample. The device contains a reporter antibody labeled with a colored particle such as colloidal gold or latex, which is deposited in a reservoir pad. An analyte-specific capture antibody is immobilized on the membrane. When the strip is placed into the test solution, the solution enters the reservoir pad and solubilizes the labeled reporter antibody, which binds to the target analyte. This analyte–antibody complex flows with the liquid sample laterally along the surface of the strip. When the complex passes over the zone where the capture antibody has been immobilized, the complex binds to the capture antibody and is trapped, accumulating and producing the appearance of a colored band at the capture zone on the strip. If the result is negative and no analyte is present in the test solution, only the control band appears in the result window. This band indicates that the liquid flowed properly up the strip. If the result is positive, two bands appear in the result window. A lateral flow strip test can provide a yes/no determination of the presence of the target analyte or a threshold (semi-quantitative) result, typically in 5–10 min.

Commercial test kits that use 96-well microtiter plates or test tubes have been available for some pesticides since the 1980s.[16] Several vendors have assays for analytes such as herbicides that appear in groundwater or runoff water, e.g., triazines, alachlor, diazinon and chlorpyrifos. More recent emphasis has been the production of kits for compounds of concern in developing countries (such as DDT) and for GMOs. When selecting a test kit, the user should determine the intended use, (i.e., as a screening method or a quantitative method) and whether the method will be used in the laboratory or the field. The cost per assay, assay sensitivity, cross-reactivity, availability of published validation by independent groups and the availability of technical support are important considerations in selecting a test kit. It is critical that the assay has been validated in the matrix of interest. If a kit or method intended for water is used for another aqueous media such as urine, inaccurate results may be obtained. Because the test kit must be validated in the matrix of concern, the sponsoring company will usually actively collaborate or assist with the validation. Several papers on test kit validations or comparisons of test kits from different manufacturers have been published.[16–19]

2.3 Data reduction

The absorbance values obtained are plotted on the ordinate (linear scale) against the concentration of the standards on the abscissa (logarithmic scale), which produces a sigmoidal dose–response curve (Figure 5). The sigmoidal curve is constructed by

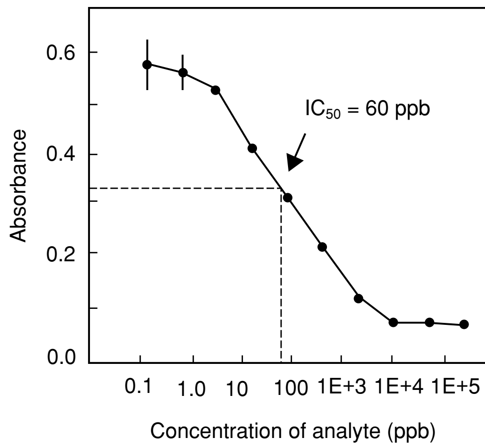

Figure 5 An example calibration curve. Absorbance is plotted against log (concentration of analyte). The competitive equilibrium binding process results in a sigmoidal curve that is fitted using a four-parameter fit. [20] The IC_{50} is defined as the concentration of analyte that results in a 50% inhibition of the absorbance

using the four-parameter logistic curve regression of the known concentration of the standard calibration solutions and their subsequent absorbance.[20]

Assay sensitivity is defined here as the concentration of analyte that inhibits the observed absorbance by 50% or the IC_{50}. The lower limit of detection (LLD) is the lowest analyte concentration that elicits a detector response significantly different from the detector response in the absence of analyte. In some cases, the LLD is defined as three standard deviations from the mean of the zero analyte control. In other cases, the LLD is defined empirically by determining the lowest concentration of analyte that can be measured with a given degree of accuracy. Readers are referred to Grotjan and Keel[21] for a simplified explanation and to Rodbard[22] for the complete mathematics on the determination of LLD.

The concentration of analyte in the unknown sample is extrapolated from the calibration curve. To obtain an accurate and precise quantitative value, the optical density (OD) for the sample solutions must fall on the linear portion of the calibration curve. If the sample OD is too high, the sample solution must be diluted until the OD falls within the quantitative range of the assay. The concentration of the analyte in the original sample is calculated by correcting for any dilution factor that was introduced in preparing the sample for application to the microplate.

2.4 Sample collection and preparation

Once the immunoassay that meets the study objectives has been identified, sample collection begins. Proper sampling is critical in order to obtain meaningful results from any type of analytical assay. An appropriate sampling scheme will support the objective of the test. For example, a plant breeder may take a single leaf punch to determine quickly whether a specific protein has been expressed in an experimental plant. A more complex sampling regime would be used to determine the expression

profile of a specific protein in corn grain, leaves and stalks for a regulatory study. These regulatory field studies are often modeled after crop residue studies for chemical pesticides. The protocol typically describes sampling from representative plants, tissues, growth stages and geographical sites.

Sampling has the potential to introduce significant uncertainty and error into a measurement; therefore, a proper plan should be devised with the assistance of a qualified statistician. Grain sampling is a routine practice and standard methods for taking samples from static lots – such as trucks, barges and railcars – and for taking samples from grain streams can be found in the United States Department of Agriculture Grain Inspection Protection Service (USDA GIPSA) 'Grain Inspection Handbook, Book 1, Grain Sampling'.[23] Ultimately, the optimum sampling strategy is a balance between sensitivity, cost and confidence.

Sample preparation techniques vary depending on the analyte and the matrix. An advantage of immunoassays is that less sample preparation is often needed prior to analysis. Because the ELISA is conducted in an aqueous system, aqueous samples such as groundwater may be analyzed directly in the immunoassay or following dilution in a buffer solution. For soil, plant material or complex water samples (e.g., sewage effluent), the analyte must be extracted from the matrix. The extraction method must meet performance criteria such as recovery, reproducibility and ruggedness, and ultimately the analyte must be in a solution that is aqueous or in a water-miscible solvent. For chemical analytes such as pesticides, a simple extraction with methanol may be suitable. At the other extreme, multiple extractions, column cleanup and finally solvent exchange may be necessary to extract the analyte into a solution that is free of matrix interference.

The protein analyte is extracted from the plant material by adding a solvent and blending, agitating or applying shearing or sonic forces. Typical solvents used are water or buffered salt solutions. Sometimes detergents or surfactants are added. As with chemical pesticide extraction methods, the protein extraction procedure must be optimized for the specific sample matrix. Processed samples may have been subjected to processes resulting in protein precipitation and/or denaturation. These factors can influence protein extraction efficiency. The problem can often be overcome by changing the buffer composition and the extraction procedure.

Because the protein analyte is endogenous to the plant, it can be difficult to demonstrate the efficiency of the extraction procedure. Ideally, an alternative detection method (e.g., Western blotting) is used for comparison with the immunoassay results. Another approach to addressing extraction efficiency is to demonstrate the recovery of each type of protein analyte from each type of food fraction by exhaustive extraction, i.e., repeatedly extracting the sample until no more of the protein is detected.[24]

Some examples are given below to illustrate extraction procedures for proteins that have been optimized for different matrices and testing strategies.

Neomycin phosphotransferase II (NPTII) extraction from cotton leaves and cottonseed. The extraction buffer consists of 100 mM Tris, 10 mM sodium borate, 5 mM magnesium chloride, 0.2% ascorbate and 0.05% Tween 20 at pH 7.8. The frozen leaf sample is homogenized in cold (4 °C) buffer. An aliquot of the homogenate is transferred to a microfuge tube and centrifuged at 12 000 *g* for 15 min. The supernatant is diluted and assayed directly by ELISA.

The extraction procedure for cottonseed samples is the same, except that the cottonseed samples are crushed before the buffer is added for homogenization.[25]

5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS) extraction from processed soybean fractions. The extraction buffer consists of 0.138 M NaCl, 0.081 M Na_2HPO_4, 0.015 M KH_2PO_4, 0.027 M KCl and 2% sodium dodecyl sulfate (SDS) at pH 7.4. Aqueous buffers are inadequate to extract EPSPS efficiently from processed soybean fractions owing to protein precipitation and the denaturation that occurs throughout soybean processing. Efficient extraction is achieved through the use of detergent in an aqueous buffer, mechanical tissue disruption and heating.[25]

Bt11 endotoxin extraction from corn grain. The following example is a description of a commercial kit procedure for extraction of the Cry1A (b) and Cry1A (c) from corn grain for analysis with an immunoassay strip test (lateral flow device). It is important to note that for the Bt11 event the endotoxin is expressed in seed (grain) and plant tissue. However, corn plants from the Bt176 event do not express detectable quantities of the *Bacillus thuringiensis* (Bt) endotoxin in grain, and therefore a negative result in a corn grain sample does not necessarily mean the sample does not contain genetically modified material.

Reagents A and B are supplied with the kit, but the composition of these solutions is not described. A sample (25 g) of corn grain is weighed into a 4-oz glass Mason jar. Using a Waring blender, the sample is ground for 10 s on the low-speed setting. Buffered water (40 mL), consisting of 200 mL of Reagent A in 1 gal of distilled water, is added to the ground corn. The jar is capped and shaken vigorously for at least 30 s. The solids are allowed to settle and the supernatant is withdrawn with a transfer pipet. Six drops of the supernatant are dispensed into the reaction tube and three drops of Reagent B are added. The reaction tube is capped and mixed by inverting it three times. The sample is analyzed with the lateral flow device.[26]

2.5 *Development of pesticide immunoassays*

The development of sensitive and inexpensive immunoassays for low molecular weight pesticides has been an important trend in environmental and analytical sciences during the past two decades.[8,10,27–29] To design an immunoassay for a pesticide, one can rely on the immunoassay literature for agrochemicals,[30–32] but many of the innovations in clinical immunoanalysis are also directly applicable to environmental analysis.[11,33,34] Conversely, the exquisite sensitivity required and difficult matrices present for many environmental immunoassay applications have forced the development of technologies that are also useful in clinical immunoassay applications. In the following discussion we will describe widely accepted procedures for the development of pesticide immunoassays.

The major steps in the development of an immunoassay are as follows:

- design and synthesis of haptens
- conjugation of haptens to antigenic macromolecular carriers
- immunization of host animals and subsequent generation of antibodies

- characterization of antibodies
- assay optimization
- assay validation.

2.5.1 Basic analysis of the target analyte structure

In general, immunoassays are more readily developed when the target analyte is large, hydrophilic, chemically stable and foreign to the host animal.[8] In theory, the sensitivity and selectivity of an immunoassay are determined by the affinity of the antibody to the analyte, and hence immunogen design and antibody production are of fundamental importance to assay development. For a molecule to be immunogenic it must have a molecular mass of at least 2000 Da and possess a complex and stable tertiary structure. Low molecular weight antigens (less than 2000 Da), a size that includes most pesticides, are not directly immunogenic. Such nonimmunogenic molecules are termed 'haptens'. Haptens possess no, or very few, epitopes that are recognizable by immune systems of host animals. As a consequence, they must be linked to larger molecules in order to become immunogenic to host animals.

Factors an analyst should consider when designing a hapten–immunogen system are outlined in Table 1. The immunizing hapten should be designed to mimic closely the target analyte. Ideal haptens have close chemical similarity to the target analyte and possess a functional group to allow coupling to carrier molecules; coupling to carrier antigens usually occurs through a 'linker,' 'spacer' or 'handle' molecule (discussed below). Retention of the unique functional groups of the analyte, especially ionizable groups or groups that form hydrogen bonds, are critical for the production of high-affinity antibodies. Also important are the ease of hapten synthesis, hapten solubility, and the nature of the method to be used for conjugation to proteins.

2.5.2 Design of the immunizing hapten

(1) Position of spacer arm. The position of the linker group on the target analyte that connects it to the immunogen has a profound influence on the selectivity and sensitivity of the subsequent assays. The handle should be attached as far as possible from the unique determinant groups, allowing maximum exposure of the important

Table 1 Guidelines for the design and synthesis of an immunogen hapten

1. Position of handle on target molecule
Distal to hapten determinant groups
Avoid attachment to functional groups
2. Handle selection
Length of handle
Avoid functional groups in handle (unless used to increase exposure or improve solubility)
3. Coupling of haptens
Type of coupling reaction
Compatibility of reaction with target molecule functional groups
4. Stability of hapten under coupling conditions and subsequent use
5. Ease of synthesis
6. Characterization of conjugates and determination of hapten/protein ratio

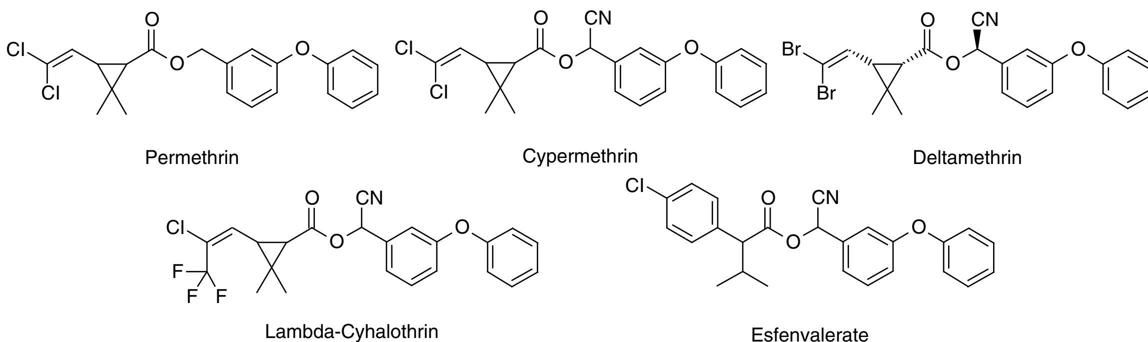

Figure 6 Structures of some major use pyrethroids

structural features of the analyte to the immune system. Presentation of unique features of the target analyte is particularly important for ensuring selectivity to a single chemical structure within a chemical class. For example, we attempted to develop compound-specific immunoassays for the major pyrethroids esfenvalerate, permethrin and cypermethrin. As shown in Figure 6, these pyrethroids have similar or identical alcohol moieties, while containing relatively unique acyl substituents. If a carrier protein was linked through the acid portion, leaving the common phenoxybenzyl group unchanged, the resulting antibodies generated from such an immunogen would be expected to recognize many pyrethroids. In order to develop a compound-specific assay, we retained the relatively unique acid substituents, and attached the linkers to the aromatic phenoxy benzyl groups (Figure 7). Using this strategy, sensitive and selective assays for permethrin and esfenvalerate were developed.[35,36] Another design option was to modify the α-cyano group to support a linker for protein conjugation (Figure 8). In this case, nearly the whole pyrethroid is unchanged; antibodies developed based on this strategy were specific for the target compounds.[37,38]

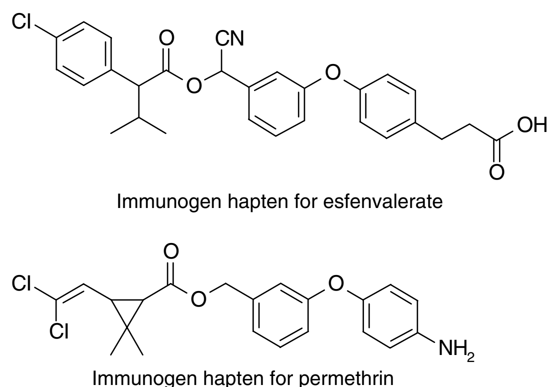

Figure 7 Structure of the haptens used in the immunogen for the development of antibodies that recognize pyrethroid insecticides, esfenvalerate and permethrin. The esfenvalerate hapten was coupled to proteins through the carboxylic acid group and the permethrin hapten was coupled to proteins through the amine group. Because antibody recognition of the structure is greatest most distal to the point of attachment to the protein, the antibodies were selective for the acid portions of the pyrethroid molecules resulting in highly selective assays for esfenvalerate and permethrin, respectively

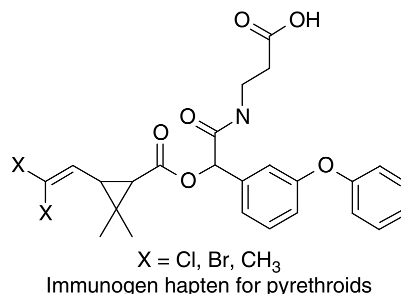

Figure 8 Structure of immunogen haptens for pyrethroids with spacer arm attachment at the α-position of the alcohol moiety. Since the whole pyrethroid molecule is available for recognition by the antibody, assays resulting from these immunogens were selective for the parent pyrethroids

However, if a class-selective assay is desirable (for multi-analyte assays), the handle should be located at or near a position that differentiates members of the class and exposes features common to the class. Using the pyrethroid example, an ideal immunogen should retain the phenoxybenzyl moiety and link the protein from the distal acid end (Figure 9). Using such an immunogen hapten, a class-specific immunoassay was developed that was highly cross-reactive with the type I pyrethroids permethrin, phenothrin, resmethrin and bioresmethrin.[39]

For small molecules, the retention of each determinant group identity is very important. Attaching the handle to a determinant group should be avoided because this alters the target molecule's structure, geometry and electronic properties relative to the parent compound. Some target analytes may contain acid, amino, phenol or alcohol groups that can be directly conjugated. Because hydrogen bonding is often the major force for interaction between an antigen and an antibody, such groups are very important determinants for antibody affinity and specificity. A good example of functional group importance is the immunoassay for phenoxybenzoic acid (PBA), a major metabolite of some pyrethroids. To develop an antibody against PBA, two options were used to design and conjugate haptens to the carrier protein. Phenoxybenzoic acid was directly conjugated with the antigenic protein using its −COOH group (Figure 10, site 2). This reaction could be accomplished using relatively simple chemistry for conjugation, but would likely result in poor antibody specificity because the phenoxybenzyl moiety is present in many parent pyrethroids. In addition,

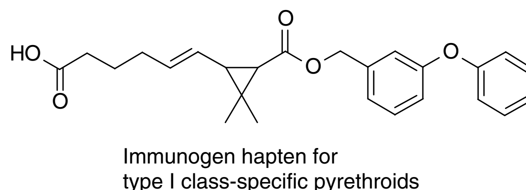

Figure 9 Structure of the immunogen hapten used to generate antibodies for a type I pyrethroid class-selective assay. Pyrethroids lacking an α-cyano group are generally termed type I. This hapten exposed the features most common to type I pyrethroids, the phenoxybenzyl group, the cyclopropyl group and the lack of a cyano group, resulting in antibodies that recognized permethrin, phenothrin, resmethrin and bioresmethrin, but not cypermethrin

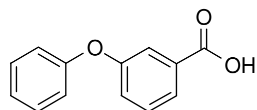

Figure 10 Structure of the target analyte phenoxybenzoic acid (PBA). The arrows point to the ideal sites for conjugation of the molecule to proteins for optimum recognition. Use of site 2 for conjugation to protein resulted in antibodies that recognized free PBA poorly

the lack of hydrogen bonding elements and reduced solubility of conjugates would likely significantly influence subsequent antibody affinity and specificity. Alternatively, we designed a hapten that left the −COOH group unchanged by attaching to the distal aromatic benzene, site 1, a linker containing a terminal aldehyde group that was used to conjugate to protein (Figure 11). The resulting antibodies had a high binding affinity and resulted in the development of a highly sensitive and selective assay [($IC_{50} = 1\ \mu g\ L^{-1}$ (ppb)] that was about 1000 times more sensitive than the assay developed from an antibody raised against an immunogen conjugated at site 2. No cross-reactivity to any other parent pyrethroid or their metabolites was measured for the antibody resulting from site 1 conjugation. Although some structural change in the target molecule is usually unavoidable, when selecting a handle for the immunogen hapten the original steric and electronic characteristics of the target molecule should be preserved as much as practical. Especially electronic features including electron density around important atoms, net charge at important atoms and hybridization of electronic orbitals of characteristic groups should be preserved.

(2) Handle selection. For small molecules (including most pesticides), the selection of a spacer or linker arm is important. Omitting the spacer arm from the structure of immunogen may result in assays with poor sensitivity and/or weak recognition of the portion of the target molecule near the attachment to the carrier protein. Generally, the optimal linking group has a chain length of about four to six atoms.[40–42] For hydrophobic haptens such as pyrethroids and dioxins, the role of the spacer may be of critical importance because the hapten may fold back on the protein surface or within the protein core after conjugation. The antibody resulting from such an immunogen will have low affinity and poor selectivity. A hapten with a rigid spacer can overcome such hydrophobic interactions. A double bond-containing spacer for the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) immunogen hapten (Figure 12) resulted in a highly sensitive immunoassay with an IC_{50} of 240 ng L^{-1}.[43,44] In contrast, when a flexible hexanoic acid spacer was used for development of an ELISA

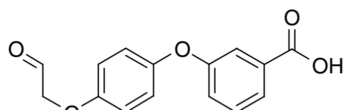

Figure 11 Structure of the phenoxybenzoic acid (PBA) immunogen hapten. Conjugation to the protein through the aldehyde resulted in an immunogen that generated antibodies selective and sensitive for PBA

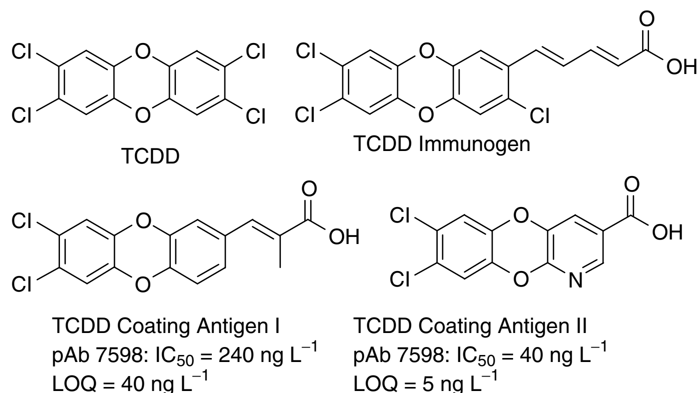

Figure 12 Structures of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), immunogen and coating haptens. The immunogen was synthesized with a rigid spacer so the lipophilic hapten would not fold back into the hydrophobic core of the protein preventing recognition by the immune system. The affinity of the antibody for coating antigen II is less than for coating antigen I owing to structural changes, hence the assay using coating antigen II is more sensitive for TCDD

for polychlorinated biphenyls (PCBs), a modestly successful assay with an IC_{50} of 100 μg L^{-1} resulted.[45]

In concept, a lipophilic hapten can be attached to glycoprotein linkers to prevent the hapten from folding into the protein. However, the use of glycoprotein linkers may lead to the recognition of the handle. In general, the spacer arm should not include polar, aromatic or bulky groups; at a minimum, these moieties should not be linked directly to the target structure. An aliphatic straight-chain linker is preferred.[46]

2.5.3 Haptens for coating antigens and tracers

Careful design of coating haptens should take into consideration the reversible antibody/analyte equilibrium competition with an antibody/hapten–protein conjugate that is illustrated in Figure 1. Assuming that no analyte (**A**) is present, only the K_H, which is variable by changing hapten structure, for coating hapten–protein (**H**) is in operation between antibody (**Y**) and coating antigen (**H**), and a maximum signal from the **Y–H** is observed. On the addition of analyte (**A**), this equilibrium is shifted towards the formation of antibody–analyte (**Y–A**), described by K_A. Formation of **Y–A** dramatically reduces the amount of **Y–H** and hence the tracer signal decreases. Thus, for a fixed quantity of antibody; the lowest IC_{50} (or sensitivity) is observed when the affinity of the antibody for the analyte is greater than the affinity of the antibody for the coating-hapten ($K_A \gg K_H$). Therefore, with a fixed K_A for **Y–A**, one can shift the equilibrium by selecting a coating hapten with decreased relative affinity for the antibody; lower analyte concentrations may compete with these reagents under equilibrium conditions, resulting in assays with greater sensitivities. This competition is the rationale for improving assay sensitivity through use of heterologous haptens[47] and is employed extensively in our laboratory for triazine herbicides,[41,48] arylurea herbicides,[46,49] pyrethroid insecticides[35,36,39] and dioxins.[44,50] Guidelines for obtaining this heterology are outlined in Table 2.

Table 2 Guidelines for design of coating/tracer haptens

1. Heterology of hapten structure
Position of handle
Composition of handle
Conjugation chemistry
2. Alterations in target molecule structure
Use of partial structure
Change of key determinants
3. Cross-reactivity data of hapten structures (or derivatives)
4. Determination of hapten/protein ratio

Hapten heterology, site heterology, linker heterology, geometric heterology and the use of different conjugation techniques (discussed later) are useful tools to improve assay performance for both coating-antigen and enzyme tracer formats. In the development of TCDD immunoassays, our first assay employed a heterologous hapten **I** containing a short linker that lacked chlorine at position 2; a sensitive immunoassay resulted.[44] To improve the sensitivity, a new coating antigen (hapten **II**) was designed by replacing the benzene ring proximal to the linker with a pyridine ring (Figure 12). The resulting assay was five times more sensitive than the original assay having an IC_{50} of 40 ng L^{-1} and a limit of quantitation (LOQ) of 5 ng L^{-1}.[50]

Immunoassays for diuron (Figure 13) are another example of improved assay performance using heterologous assay conditions. One antibody was derived from a hapten that extended the dimethylamine side chain of diuron with methylene groups.

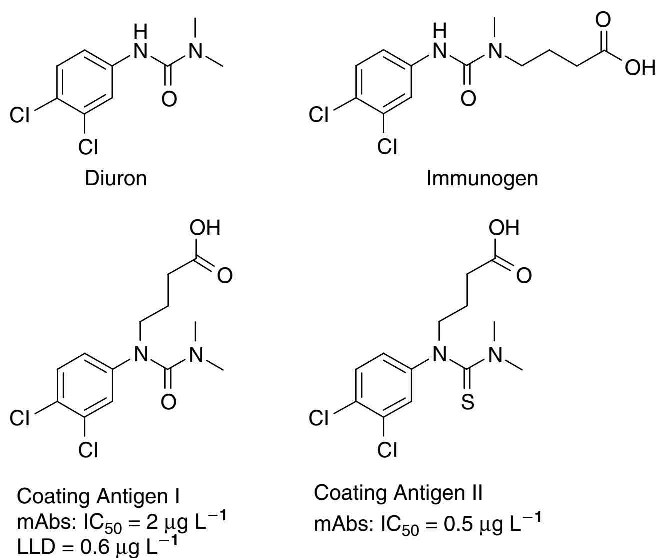

Figure 13 Structures of haptens used for immunizing and coating antigens in a monoclonal antibody-based immunoassay for diuron. A sensitive assay was developed using coating hapten I that had the handle in a position different from the immunogen hapten. When the oxygen in the urea moiety of hapten I was replaced with a sulfur (hapten II), increasing the heterology, even greater sensitivity was achieved

The best coating antigen of three evaluated consisted of an isomer in which the butyric acid handle was attached to the dichloroaniline nitrogen. The IC_{50} was 2 μg L^{-1} with an LOQ of 0.6 μg L^{-1}.[49] Using the rationale that a coating hapten with a lower affinity for the antibody was desirable, we replaced the oxygen of the diuron immunogen hapten with a sulfur to make a thiourea coating antigen. The resulting assay had an IC_{50} of 0.5 μg L^{-1} for diuron.[46] Sulfur, being larger than oxygen, probably did not fit well in the anti-diuron antibody pocket and there would be a substantially lower affinity owing to the loss of hydrogen bonding between the thiocarbonyl and antibody.

For chiral haptens, the use of enantiomers or diastereoisomers as the coating hapten may significantly improve the assay sensitivity. This was the case in the development of the permethrin immunoassay. The antibody was raised against a *trans*-permethrin hapten (Figure 14). Use of the corresponding *cis*-permethrin hapten as a coating antigen resulted in a sensitive and selective assay with an IC_{50} of 2.5 μg L^{-1} and an LOQ of 0.4 μg L^{-1}, which is about 200 times more sensitive than the homologous system in which the *trans*-permethrin hapten was the coating antigen.[35]

There are tradeoffs with developing assays based on assay heterology. For example, the highest titer of antibody is normally identified with a coating hapten that is very similar to the immunizing hapten. Rabbit antisera raised against acylurea insecticide haptens had high titers for the acylurea haptens that were similar to the immunizing structure. However, the target acylurea insecticide could not inhibit these assays because the antibodies bound to the coating hapten with greater affinity than to the acylurea insecticide. Changing the coating hapten to one containing a different handle than used for the immunizing hapten resulted in a decrease in antibody titer, demonstrating that the antibody bound with less affinity to the new coating antigen. However, the affinity for the target analyte was improved and a very sensitive assay for the acylurea insecticides resulted.[47] The benefit of careful design of a heterologous assay normally is greater with small haptens and spacers (primary or secondary amines compared with tertiary amines and amides) that are readily distinguished by the immune system than it is with large haptens.

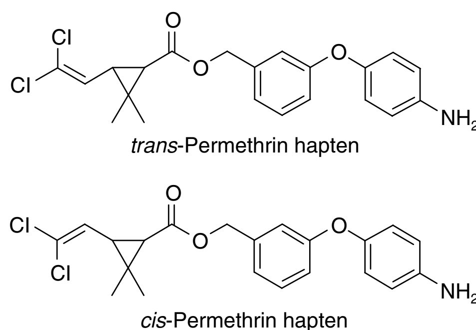

Figure 14 Permethrin immunogen and coating antigen haptens. Using enantiomers or diastereoisomers is a strategy to provide hapten heterology. Assays using antibodies raised to the *trans*-permethrin hapten were more sensitive when the *cis*-permethrin hapten was used instead of the *trans*-permethrin hapten for the coating antigen

2.5.4 Hapten conjugation

In order to elicit a satisfactory immune response, haptens must first be covalently attached to a carrier protein, which is usually foreign to the animal being immunized. In addition, the hapten used for immunization and other similar haptens are conjugated to enzymes and (or) other proteins for use in the assay. For hapten–protein conjugates, protein solubility, the presence of functional groups and stability under reaction conditions are important variables to consider during immunoassay development. Many conjugation methods are available[14,51–53] and the selection of an appropriate method is ultimately dependent on the functional group available in the hapten.

(1) Carrier protein. A wide variety of proteins are available for the synthesis of immunogens or antigens including bovine serum albumin (BSA) and human serum albumin (HSA), ovalbumin, thyroglobulin, keyhole limpet hemocyanin (KLH) or horseshoe crab hemocyanin (LPH), and the synthetic polypeptides poly-L-lysine and polyglutamic acid. Among these, KLH is often the first choice as an immunogen carrier protein because it is large (approximately 10^6 Da) and is highly immunogenic. In addition, KLH contains an abundance of functional groups available for conjugation, including over 2000 lysine amines, over 700 cysteine sulfhydryls and over 1900 tyrosine residues. It should be noted that KLH requires a high-salt buffer (at least 0.9 M NaCl) to maintain its stability and solubility. In solutions with NaCl, concentrations lower than 0.6 M KLH will precipitate and denature, and maintaining solubility after hapten conjugation can be difficult. Hence conjugation reactions using KLH should be carried out under high-salt conditions to preserve the solubility of the hapten–carrier complex.

Thyroglobulin has been increasingly used as an immunogenic carrier protein owing to its excellent water solubility. Another frequently used protein in immunoassay is BSA. Although BSA is immunogenic, it is mostly used as a coating antigen carrier. Advantages of BSA include its wide availability in relatively pure form, its low cost and the fact that it is well characterized. BSA has a molecular weight of 64 000 and it contains 59 primary amino groups, one free cysteine sulfhydryl, 19 tyrosine phenolate residues and 17 histidine imidazolides. It is also relatively resistant to denaturation and is suitable for some conjugation procedures that involve organic solvents. Moreover, BSA conjugates are usually readily soluble, which makes their isolation and characterization easier. Although a general rule states that large and phylogenetically foreign proteins make the best antigenic proteins, we have obtained antibodies when smaller proteins such as fetuin were used as carriers.[54]

(2) Conjugation methods. The selection of conjugation method is dependent on the functional group on the hapten (e.g., carboxylic acid, amine, aldehyde). A hapten with a carboxylic acid group can conjugate with a primary amino group of a protein using the carbodiimide, activated *N*-hydroxysuccinimide (NHS) ester or mixed anhydride methods. Haptens with free amines can be coupled to proteins using glutaraldehyde condensation or diazotization. Haptens that have been designed to contain spacers may be linked directly to the protein with methods such as the mixed anhydride, whereas haptens lacking a spacer should be coupled using methods that insert a linker between the hapten and the protein such as with glutaraldehyde. Typical procedures

Table 3 Conjugation of a carboxyl-containing hapten to a protein using a carbodiimide method

Materials
- BSA (Sigma, Fraction V or similar)
- Hapten
- EDC[a]
- Phosphate buffer (0.1 M, pH 6): prepared from KH_2PO_4 (3.025 g), Na_2HPO_4 (0.39 g) and water (250 mL)

Method
1. Dissolve the hapten (0.04 mmol) in phosphate buffer containing 50 mg of BSA
2. Add 150 mg (0.78 mmol) of EDC to the buffer solution. Stir the mixture at room temperature to allow all the reagents to dissolve
3. React at room temperature for 24 h
4. Purify conjugate by gel filtration, dialysis or ethanol precipitation

[a] EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl.

are provided below for methods that have been successfully used in this laboratory or for which extensive literature is available.

(3) Haptens with free carboxylic acids. Methods for linking hapten carboxyl groups to amine groups of antigenic proteins include activation by carbodiimides, isobutyl chloroformate or carbonyldiimidazole. In the widely used carbodiimide method, the carbodiimide activates the carboxylic acid to speed up its reaction with the amine. Acidic conditions catalyze the formation of the active *O*-acylurea intermediate while the protein is more reactive at higher pH, when the lysine amino groups are unprotonated. Therefore, as a compromise, a pH near 6 is used. The choice of carbodiimide is dependent on the reaction conditions. For example, dicyclohexylcarbodiimide (DCC) is used in nonaqueous media with nonpolar, water-insoluble haptens where the carrier protein, in aqueous solution, is added to the activated hapten in a two-step reaction. For more water-soluble haptens, water-soluble derivatives of DCC such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) or 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC or Morpho CDI) are used in one-step reactions (Table 3, Figure 15). However, EDC will react directly with protein, and some antibodies are certain to be

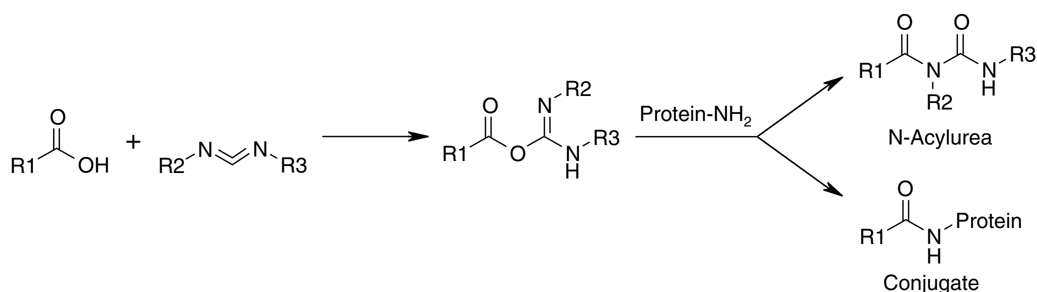

Figure 15 Conjugation of a carboxylic acid and an amine using the carbodiimide method. The carbodiimide activates the carboxylic acid to speed up the reaction to the amine. Carbodiimides can be used with nonpolar or polar solvents, including water. Undesirable urea complexes may form as by-products. Details of the reaction are given in Table 3

Table 4 Conjugation of a carboxyl-containing hapten to a protein using *N*-hydroxysuccinimide

Materials
- BSA (Sigma, Fraction V or similar)
- Hapten
- DCC
- NHS
- DMF[a]
- Phosphate buffer (0.1 M, pH 7.4): prepared from KH_2PO_4 (0.67 g), Na_2HPO_4 (0.285 g) and distilled water (250 mL)

Method
1. Dissolve the hapten (0.04 mmol) in DMF (0.5 mL)
2. Add DCC (15 mg, 0.15 mmol) followed by NHS (20 mg, 0.17 mmol)
3. React at room temperature for 3.5 h
4. Remove the precipitate, dicyclohexylurea, by centrifugation
5. Add the supernatant to phosphate buffer (∼5 mL) containing 50 mg of BSA
6. React at room temperature for 2 h
7. Purify conjugate by gel filtration, dialysis or ethanol precipitation

[a] DMF = dimethylformamide (>99%, from Aldrich).

generated to the resulting highly immunogenic protein–urea complex. Formation of these antibodies is not a drawback as long as a different coupling chemistry is used to prepare coating antigens.

Activated NHS esters of carboxylic acids are prepared by reacting the acid with NHS in the presence of DCC (Table 4, Figure 16). *N*-Hydroxysuccinimide esters are stable when kept under anhydrous and slightly acidic conditions, and they react rapidly with amino groups to form an amide in high yield.

Like the carbodiimide method, the mixed anhydride method[55,56] results in an amide complex (Table 5, Figure 17). The acid-containing hapten is dissolved in a dry, inert, dipolar, aprotic solvent such as *p*-dioxane, and isobutyl chloroformate is added with an amine catalyst. The activated mixed anhydride is chemically stable and can be isolated and characterized. The aqueous protein solution is added to the activated acid and the pH is maintained at around 8.5. A low temperature (around 10 °C) is necessary during the reaction to minimize side reactions.

(4) Haptens with an amino group. Amine groups in haptens, carrier proteins or both can be modified for conjugation through homo- or heterobifunctional cross-linkers such as acid anhydrides (e.g., succinic anhydride), diacid chlorides (e.g.,

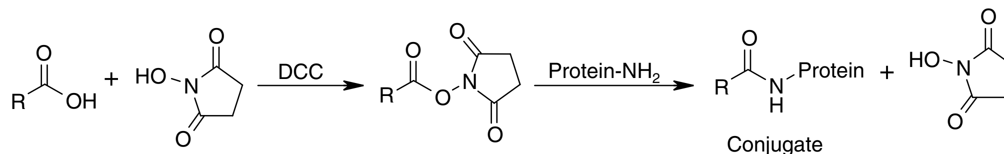

Figure 16 Conjugation of an amine and a carboxylic acid via the *N*-hydroxysuccinimide (NHS)-activated ester method. NHS esters may be isolated and characterized and are stable to long term storage as the powder. Alternatively, the NHS esters may be used immediately upon formation without isolation. Details of the reaction are given in Table 4

Table 5 Conjugation of a carboxyl-containing hapten to a protein using the mixed anhydride procedure

Materials
BSA (Sigma, Fraction V or similar)
Hapten
Isobutyl chloroformate
1,4-Dioxane (>99%, from Aldrich)
Tributylamine
Method
1. Dissolve the hapten (0.04 mmol) in dioxane (5 mL) in a small tube and cool to 10 °C
2. Add tributylamine (11 μL, 0.044 mmol) to the solution followed by isobutyl chloroformate (6 μL, 0.044 mmol)
3. React at 10 °C for 60 min to activate the carboxylic acid
4. Add BSA solution (50 mg of BSA dissolved in 5 mL of distilled water and adjusted to pH 9 with NaOH) and stir for 4 h
5. Monitor the solution pH over the period and maintain it at 8.5 by the addition of dilute NaOH
6. Purify conjugate by gel filtration, dialysis or ethanol precipitation

succinyl chloride) or dialdehydes (e.g., glutaraldehyde). Glutaraldehyde condensation (Table 6) has been used widely to produce protein–protein and hapten–protein conjugates. The glutaraldehyde reagent should not have undergone polymerization. To check for polymerization, add a few drops of water to an aliquot of stock glutaraldehyde solution; a white precipitate is indicative of polymerization whereas unpolymerized reagent will not precipitate.

A disadvantage of the glutaraldehyde condensation method is that dimers of the hapten and polymers of carrier protein may also form. To overcome this problem, the reaction time is limited to 2–3 h, or an excess of an amine-containing compound, e.g., lysine or cysteamine hydrochloride, is added. A two-step approach also minimizes dimerization.[57]

Aromatic amine-containing haptens are converted to diazonium salts with ice-cold nitrous acid. Diazonium salts can then react with a protein at alkaline pH (around 9) through electrophilic attack of the diazonium salt at histidine, tyrosine and(or) tryptophan residues of the carrier protein (Table 7).

(5) Other reactions. Other reactions can also be used to couple haptens to proteins. The periodate oxidation is suitable for compounds possessing vicinal hydroxyl groups such as some sugars. Schiff's base method has been used for conjugating aldehyde-containing haptens to primary amino groups of carrier proteins. *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) is a heterobifunctional reagent that will cross-link a free amine at one end and a free thiol at the other. Heterobifunctional

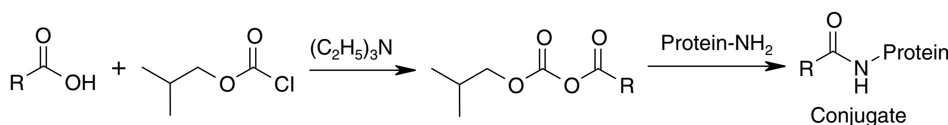

Figure 17 Conjugation of an amine and a carboxylic acid via the mixed anhydride method. Although the activated mixed anhydride is stable, it is usually used without purification. Use of low-temperature reactions will limit undesirable side products. Details of the reaction are given in Table 5

Table 6 Conjugation of an amino-containing hapten to a protein using the glutaraldehyde method

Materials
- BSA (Sigma, Fraction V or similar)
- Hapten
- Glutaraldehyde solution (0.2%, 0.02 M) in buffer
- Lysine monohydrochloride (1 M) in water
- Phosphate buffer (0.1 M, pH 7): prepared from KH_2PO_4 (1.40 g), Na_2HPO_4 (2.04 g) and distilled water (250 mL)

Method
1. Dissolve the hapten (0.03 mmol) and BSA (40 mg) in phosphate buffer
2. Add the glutaraldehyde solution (2 mL) dropwise over a period of 30 min
3. React at room temperature for 90 min. During this period the reaction mixture should turn yellow
4. Add the lysine solution to quench the reaction and stir for 60 min
5. Purify conjugate by gel filtration, dialysis or ethanol precipitation

reagents are commercially available but their use for immunizing antigens may lead to extensive handle recognition. A more complete discussion of other cross-linking and conjugation reagents can be found in Hermanson.[51]

2.5.5 Characterization of conjugates

Hapten density is important for both immunization and assay performance, and hence the extent of conjugation or hapten density should be confirmed by established methods. A characteristic ultraviolet (UV) or visible absorbance spectrum that distinguishes the hapten from the carrier protein or use of a radiolabeled hapten can be used to determine the degree of conjugation. If the hapten has a similar λ_{max} to the protein, the extent of incorporation can still be estimated when the concentration of the protein and the spectral characteristics of the hapten and protein are known. The difference in absorbance between the conjugate and the starting protein is proportional to

Table 7 Conjugation of an amino-containing hapten to protein using the diazotization method

Materials
- BSA (Sigma, Fraction V or similar)
- Hapten
- DMF (>99%, from Aldrich)
- Sodium nitrite (0.2 M) in water
- Phosphate buffer (0.1 M, pH 8.8): prepared from KH_2PO_4 (1.40 g), Na_2HPO_4 (2.04 g) and distilled water (250 mL)

Method
1. Dissolve the hapten (0.10 mmol) in 4 drops of ethanol and treat with 1 mL of 1 N HCl
2. Stir the solution in an ice-bath while adding 0.5 mL of 0.20 M sodium nitrite
3. Add 0.4 mL of DMF dropwise to give a homogeneous solution
4. Dissolve 45 mg of BSA in 5 mL of 0.2 M borate buffer (pH 8.8) and 1.5 mL of DMF
5. Add the activated hapten solution dropwise to the stirred protein solution. Stir in an ice-bath for 45 min
6. Purify conjugate by gel filtration, dialysis or ethanol precipitation

the amount of hapten conjugated.[41] Hapten density can also be determined indirectly by measuring the difference in free amino groups between conjugated and unconjugated protein using trinitrobenzenesulfonic acid.[58] These methods are at best rough estimates because the process of conjugation usually alters the apparent number of amine or sulfhydryl groups on the protein. Careful titration of reactive groups on very large proteins is particularly difficult.

Alternatively, competitive ELISA can be used to estimate the hapten density if an antibody that specifically recognizes the hapten is available.[59] At first observation this approach seems circular because the immunoassay developed is used to determine hapten density on proteins used for immunization. However, if a small molecule mimic of the protein conjugate is used as a standard, the method can be accurate. For example, a hapten containing a carboxylic acid can be coupled to phenethylamine or tyramine, its structure confirmed and the material used to generate a calibratron curve to estimate hapten density.

Advanced mass spectrometry (MS) techniques offer a new way of determining the hapten density of protein conjugates. For example, matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) detects covalently bound haptens.[60] Increasingly powerful instruments allow higher resolution of conjugates. However, large proteins cannot be analyzed by MS. Protein heterogeneity and some post-translational modifications, particularly glycosylation, will obscure the results and lower resolution instruments cannot distinguish among desired conjugates and unwanted reaction by-products. It is possible, however, to measure hapten density on small peptides unequivocally by MS techniques and extrapolate to proteins such as KLH and thyroglobulin that are too large and/or heterologous for MS analysis.

Hapten density, and also the common positions where haptens are bound, can also be estimated by cyanogen bromide or enzymatic cleavage of the protein and either MALDI-MS or separation of the components by reversed-phase ion-pair chromatography and electrospray or electrospray time-of-flight (TOF) analysis.

Conjugates with a broad range of hapten/protein or hapten/enzyme ratios of about 1–30 have been used successfully to elicit antibody production or as enzyme tracers.[29,61,62] The optimum hapten ratio may depend on the study objectives, the nature of the antigen, immunization protocol, etc. A general rule of thumb is to target high hapten ratios for immunogens and low hapten ratios for coating antigens or enzyme tracers. For immunogens, a high hapten ratio implies greater exposure of the immune system to the hapten; for coating antigens or enzyme tracers, a lower hapten density implies fewer haptens to compete with the analyte in the assay. Optimum hapten density is often determined empirically with checkerboard titration procedures. Such procedures are very rapid and are normally adequate to optimize ELISAs without knowing the exact hapten density. With the development of more sophisticated biosensors, the determination of exact hapten densities may become increasingly important.

2.5.6 Antibody production

Essentially any vertebrate can be used as a source of antibodies. Rabbits are easy to care for, and produce a moderate amount of serum, often with high antibody titers.

Goats or sheep also produce high-quality antiserum in larger amounts. Antibodies derived from serum consist of a population of antibodies that recognize a variety of antigenic determinants with varying degrees of specificity and affinity and are thus termed polyclonal. Although two antisera are rarely identical, even if they come from the same rabbit at different times, it is simple to evaluate each antiserum for specificity and affinity.

In contrast, monoclonal antibodies are obtained from a murine cell line ultimately traceable to a single cloned cell. If carefully screened and selected, the monoclonal antibody will recognize a single antigenic determinant with constant affinity and specificity. The hybrid cell line comes ultimately from spleen lymphocytes (from a previously immunized animal) that have been fused to an immortal myeloma cell line. This fusion ensures that the cell line will continue to produce the selected antibody while it grows and replicates. Although it is attractive to have a permanent supply of antibody with constant specificity and affinity, these cell lines may contain an unstable chromosome complement and their immortality depends upon proper storage and maintenance. The advantages, disadvantages, and production of monoclonal antibodies have been discussed.[63–65]

Immunization procedures and schedules vary depending on the laboratory.[66,67] Usually an initial series of injections is followed by booster injections some weeks later. Animals are generally bled 7–14 days after each booster injection and the characteristics of the serum determined. Serum may be collected or pooled following numerous booster injections and(or) the animal may be exsanguinated.

For long-term storage, antibodies are best stored frozen either in solution or as a lyophilized powder. Similarly to most biological materials, repeated freeze–thaw cycles are detrimental to antibodies, and hence antibodies should be stored in clearly labeled aliquots. A single vial may be used for a set of experiments extending over several months. Antibodies can be kept in solution containing 0.1% sodium azide (to prevent growth of microorganisms) in a refrigerator for up to a year. Solutions can also go through freeze–thaw cycles several times without alarming loss of activity. Although antibodies are relatively hardy proteins, the concentration should be kept above 1 mg mL^{-1} during storage, solutions should be frozen quickly in liquid nitrogen before placing in a standard freezer, and for long-term storage antibodies should be lyophilized and the container sealed under dry nitrogen.

Building on the monoclonal antibody technology and the advent of molecular biology techniques, it is now possible to isolate antibodies from combinatorial libraries and express them in a variety of expression systems. Efficient systems for the cloning and expression of antibody genes in bacteria were developed in the late 1980s.[68] The discovery of PCR simplified the cloning of monoclonal antibody genes from mouse monoclonal cell lines. These functional recombinant antibody fragments could be expressed in bacteria for use.[69] To take advantage of recombinant technology, efficient, large-scale screening techniques must be used. A variety of techniques have been reviewed by Maynard and Georgiou.[70] The ability to engineer antibodies for therapeutic uses, such as neutralizing toxins (antivenoms), cancer therapy and imaging of tumors, is attractive. For environmental residue analysis, the most likely use of recombinant antibodies is as detector molecules in biosensors, where engineering could provide useful surface linkage chemistry, unique labels or improved robustness of the sensor. A few recombinant

antibodies for pesticides have been developed and at least one applied to a sensor format.[71–75]

2.5.7 Assay optimization

Assay optimization involves determining the optimum coating antigen/hapten–enzyme conjugate and anti-pesticide antiserum concentrations using a checkerboard titration. Using a 96-well plate, the coating antigen concentration is varied by row and the antibody concentration is varied by column so that each well has a different combination of antigen and antibody concentrations. By plotting the resulting absorbance values versus either reagent concentration an estimate can be made of the concentrations that will yield a reasonable signal and at which the system is not saturated.[76]

Using the optimum reagent concentrations, the assay is tested for inhibition by the target analyte. If a useable IC_{50} is obtained, then further optimization is conducted. This second stage of optimization includes determining the optimum assay temperature and incubation times and the effect of potential interferences (e.g., solvent, salt, pH, matrix). When evaluating immunoassays, it is important to remember that the law of mass action applies and interferences affect the equilibrium condition. For example, assays are conducted with reagents that have been equilibrated to room temperature. If room temperature is not constant (within 3–5 °C), then assays should be conducted using a forced-air incubator. Shaking the plate periodically during incubation may improve precision because reactions occur at the surface of the microtiter plate, causing a localized concentration of reactants. For immunoassays utilizing 30-min or longer incubation periods, the reactants have likely come nearly to equilibrium, and precise timing of the incubation period is less critical than for nonequilibrium immunoassays. Each of these variables should be evaluated and controlled if necessary in order to improve the precision of the measurements.

2.5.8 Validation

Consistent with other analytical methods, immunoassays must be validated to ensure that assay results are accurate. Initial validation involves an evaluation of the sensitivity and specificity of the immunoassay, while later validation includes comparison with a reference method. Because a goal of immunoassays is to minimize sample preparation, validation also includes testing the effects of sample matrices and(or) sample cleanup methods on results. The final steps in validation involve testing a limited number of samples containing incurred residues to determine if the method provides reliable data.

Structurally related compounds may cross-react with the antibody, yielding inaccurate results. In screening for the herbicide alachlor in well water by immunoassay, a number of false positives were reported when compared with gas chromatography (GC) analysis. A metabolite of alachlor was found to be present in the samples and it was subsequently determined that the cross-reactivity by this metabolite accounted for the false-positive results.[77] On the other hand, cross-reactivity by certain structural analogs may not be an issue. For example, in an assay for the herbicide atrazine, cross-reactivity by propazine is 196%;[78] because of atrazine and propazine field use

patterns, they are not usually found together. Conversely, this assay also cross-reacts with simazine by 30% and simazine is expected to be present. Hence, if the sample is positive and the presence of simazine is expected, another method of analysis would be necessary to determine the relative contribution of each triazine.

The second phase of validation involves comparing the immunoassay with an established method with a known accuracy using an identical same sample set. For most pesticides, reference methods are based on gas chromatography/mass spectrometry (GC/MS) or high-performance liquid chromatography (HPLC). When comparing two methods, it is important to be aware of the strengths and weaknesses of each. For example, many pesticide immunoassays require minimum sample cleanup before analysis, relative to the corresponding GC/MS or HPLC methods. Thus, immunoassay data may reflect higher values if there are losses occurring during further sample workup for GC/MS. On the other hand, the immunoassay data may be higher because a cross-reacting species is present that the GC/MS differentiates by chromatography. Comparison of immunoassay results with results obtained from a validated method will determine if the immunoassay is accurate.

For pesticide residue immunoassays, matrices may include surface or groundwater, soil, sediment and plant or animal tissue or fluids. Aqueous samples may not require preparation prior to analysis, other than concentration. For other matrices, extractions or other cleanup steps are needed and these steps require the integration of the extracting solvent with the immunoassay.[79] When solvent extraction is required, solvent effects on the assay are determined during assay optimization. Another option is to extract in the desired solvent, then conduct a solvent exchange into a more miscible solvent. Immunoassays perform best with water-miscible solvents when solvent concentrations are below 20%. Our experience has been that nearly every matrix requires a complete validation. Various soil types and even urine samples from different animals within a species may cause enough variation that validation in only a few samples is not sufficient.

Matrix effects are determined by running calibration curves in various dilutions of matrix and comparing the results with those for corresponding calibration curves run in buffer. Overlapping curves indicate no effect of matrix. Parallel curves are an indication that a matrix interference is binding the antibody in the same manner as the analyte. Nonparallel curves are indicative of nonspecific matrix interferences. Grotjan and Keel[21] described parallelism tests, similarity of curves and the corresponding statistics. A second test for matrix effects is to analyze a sample before and after a known amount of analyte has been added (test of additivity). If the values for the ‘before’ and ‘after’ samples are not additive, a matrix effect is presumed. If matrix effects are present, then adjustment of the immunoassay method, such as running the calibration curve in the matrix or further sample preparation, is necessary.

2.5.9 Quality control (QC) and troubleshooting

Unlike GC/MS methods, internal standards are not appropriate for immunoassays. Internal standards that would react with the antibody but would not interfere with the assay are nonexistent. In the place of internal standards, external QC must be maintained.

One strategy is to use appropriately stored batch QC samples that are analyzed with each assay because intra- and interassay variability are easily tracked. Various types of QC samples can be employed to demonstrate the performance of the assay. A blank sample such as an empty well or buffered solution can indicate any background response that can be subtracted from the sample and standard responses. A negative control sample (i.e., matrix extract solution known to contain no analyte) can reveal whether a nonspecific response or matrix effect is occurring. A positive control or matrix extract fortified with a known amount of the analyte can determine accuracy. Precision can be determined using standards and samples run in replicate. Blanks, negative controls, positive controls, fortified sample extracts standardized reference material extracts and replicates are typically run on each microplate to control for plate-to-plate variation.[80] Recording assay accuracy and precision and maximum (no analyte present) and minimum (completely inhibited) absorbances over time will provide a warning of deteriorating assays.[81,82]

If an assay does not meet performance criteria, there are a variety of corrective measures (Table 8). The most frequent immunoassay performance problem is a high coefficient of variation for replicates or spurious color development. Plate washing and pipetting techniques are the greatest sources of this error.[76,83] A decrease in the maximum absorbance can be attributed to loss of enzyme activity or hapten conjugate degradation. To check enzyme activity, dilute the enzyme–conjugate about 2–5 times greater than normal for the assay. For example, if the method calls for a 1:2500 dilution of the enzyme label, then make dilutions of 1:5000 to 1:10 000, or greater. Add the substrate solution to the enzyme dilution and incubate for the time indicated in the method. Color development should be similar to that obtained in the assay when it is performing according to specifications. If the color development is lower, the enzyme label reagent should be replaced. Hapten–conjugate degradation can only be remedied by replacing the reagent.

Another important factor for QC is temperature. Reagents should be used at room temperature and plates should be protected from wide fluctuations in temperature while conducting the immunoassay. If an incubator is used or the ambient temperature is high, uneven heating of the wells may occur. Variations in final absorbances may be manifested in what is called an ‘edge effect’, in which greater variation occurs among the wells on the edges of the plate. Use of a forced-air incubator can reduce this problem. Detailed immunoassay troubleshooting information has been presented by Schneider *et al*.[84]

2.6 Applications

Pesticide immunoassays have been developed for a variety of pesticides and, more recently, GMOs, and have been used for matrices such as surface water, groundwater, runoff water, soil, sediment, crops, milk, meat, eggs, grain, urine and blood.[85–90] Table 9 is a partial list of immunoassays for chemical pesticides developed since 1995 and includes notations on the matrices studied. A fairly comprehensive list of pesticide immunoassays developed prior to 1994 was provided by Gee *et al*.[91]

Table 8 Troubleshooting the optimized immunoassay

Symptom	Cause	Remedy
Poor well to well replication	Poor pipetting technique	Check instrument, practice pipetting, calibrate pipet
	Poor binding plates	Check new lot, change manufacturer
	Coating antigen or antibody is degrading	Use new lot of coating reagent or antibody
	Coated plates stored too long	Discard plates, coat a new set, decrease storage time
	Poor washing	Wash plates more, or more carefully, remake buffer
	Uneven temperature in the wells	Deliver reagents at room temperature, avoid large temperature fluctuations in the room
	Sample carryover	Watch for potential carryover in pipetting and washing steps
Low or no color development	Loss of reagent integrity	Systematically replace or check reagents, including buffers and beginning with the enzyme label
	Incubation temperature too cold	Lengthen incubation time or increase temperature by using a circulating air-temperature controlled incubator
	Sample matrix effect	Dilute matrix if possible, check pH of matrix, increase the ionic strength of the buffer, re-evaluate matrix
Color development too high	Incubation too long or temperature too high	Decrease incubation time or temperature
	Matrix effect	Dilute matrix or re-evaluate matrix effects
Change in calibration curve parameters	Degradation of reagents	Systematically check or replace reagents, including buffers

2.6.1 Human exposure monitoring

The immunoassay is one of the most promising methods for the rapid monitoring and assessment of human exposure. The great specificity and sensitivity of immunoassays allow their use for monitoring pesticide exposure levels by determining parent compound, key metabolites[92] or their conjugates in human urine, blood,[93] and(or) saliva.[94] Recently, several immunoassays have been developed to assess human exposure to alachlor,[95,96] atrazine,[97,98] metolachlor,[99] and pyrethroids.[100] In the case of the herbicide atrazine, the mercapturic acid conjugate excreted in human urine[101] is a specific biomarker for exposure. A sensitive immunoassay has been developed for this metabolite[97] that can be detected at 0.1 $\mu g\ L^{-1}$ in urine. The great advantage of the immunoassay over chromatographic methods is high throughput, which is

Table 9 Immunoassays developed since 1995

Class	Name, matrix	Reference
Herbicide	Chlorpropham, food	139
	Isoproturon, water	140
	Metsulfuron-methyl, water	141, 142
	Bensulfuron-methyl, water	143
	Chlorsulfuron	144
	Fluometuron, soil	145
	Trifluralin, soil, water, food	146, 147
	Cyclohexanedione	148, 149
	Triazines, water, food	19, 150, 151
	Dichlobenil	152
	Propanil, water	153
	Dichlorprop methyl ester	154
	Hexazinone, water	155
	Fluroxypyr, triclopyr, soil	156
Insect growth regulator	Fenoxycarb	157, 158
	Flufenoxuron, soil, water	159
Insecticide	Hexachlorocyclohexane, water, soil	160
	Azinphos-methyl, water	161
	Carbofuran, food	162–164
	Chlorpyrifos, water	165, 166
	Chlorpyrifos-ethyl	74
	Pymetrozine, plants	167
	Azinophos-methyl, water	161, 168
	Pyrethroids	37, 39, 169
	Allethrin	170
	Esfenvalerate, water	36
	Flucythrinate, soil, water, food	171
	Permethrin, air, water	35, 172
	Organophosphates	112, 173, 174
	Fenitrothion, food, water	175, 176
	DDT, soil, food	177–179
	Etofenprox	180
	Phosalone	181
	Spinosyn A, water	182
	Spinosad, food, water, sediment	89, 183
	Imidacloprid, water, food	13, 175, 184
	Acetamiprid, water, food	175
	Azadirachtin, food, formulations	185
	Oxamyl, food	186
	Propoxur	187
Fungicide	Myclobutanil, soil, water, food	188
	Procymidone, food	189
	Benalaxyl, food, water	190
	Thiram, food	191, 192
	Chlorothalonil, water, plant residues, food	193–195
	Tebuconazole, food	196, 197
	Thiabendazole, food	198–200
	Imazalil, food	201
	Tetraconazole	197, 202
	Myclobutanil, water, soil, food	188, 202
	Hexaconazole, formulations	203
	Didecyldimethylammonium chloride	204
	Methyl 2-benzimidazolecarbamate, soil, food	205, 206
	Captan, food, water	207

particularly suitable for screening large numbers of samples generated during human exposure studies.

2.6.2 Immunoassay in agricultural biotechnology

Agricultural biotechnology providers include agricultural biotechnology companies, seed companies, food companies and other research organizations. Technology providers use qualitative, quantitative and threshold immunoassays during all stages of the research and development of biotech crops, the choice depending on the specific application. Immunoassays are used for gene discovery, event selection, screening, transformant identification, line selection, plant breeding and seed quality control. Agricultural biotechnology companies also use immunoassays for product support, product stewardship and intellectual property protection.

Technology providers use quantitative immunoassays to determine expression data of field material for regulatory submissions. Regulatory authorities require that expression levels of introduced proteins in various plant parts be determined by quantitative, validated methods. Immunoassays are also used to generate product characterization data, to assess food, feed and environmental characteristics, to calculate concentrations for toxicology studies and to obtain tolerance exemption or establish tolerances for pesticidal proteins.

Immunoassays are also useful in the food handling and distribution system. Threshold assays are most commonly used to test agricultural commodities entering the food distribution channel to ensure compliance with relevant labeling regulations.[102] Immunoassays can be applied to raw, fresh and or lightly processed foods. The protein analyte can be denatured during processes such as heating. This creates potential difficulties in the analysis of heavily processed finished food products.

2.6.3 Flow injection immunoassay (FIIA)

In FIIA, antibodies are immobilized to form an affinity column and analyte is pumped over the column. The loading of the antibodies with analyte is followed by pumping over the column enzyme tracers that compete with the pesticide for the limited binding sites of the antibodies. Generally, the indirect format produces a result inversely proportional to the pesticide concentration. FIIA can be used with electrochemical, spectrophotometric, fluorimetric and chemiluminescence detection methods. Conventional UV visible spectrophotometry is also suitable for the FIIA detection of bioligand interactions.[103] FIIA has been used for the detection of diuron and atrazine in water.[104] The method was developed as a cost-effective screen for determining compliance with the European drinking water directive. One analysis for either atrazine or diuron, including column regeneration, took about 50 min using the system that is shown schematically in Figure 18. The column material was regenerated up to 1600 times over a 2.5 month period. FIIA is a powerful analytical tool for semi-continuous, high sample throughput applications and may serve as an alternative or complementary technique to solid-phase immunoassay by providing real-time monitoring data.[105] In addition, the continuous flow system is easier to automate than assays using tubes or microplates. More rapid results and sensitive detection will be possible by miniaturizing the column and fluid handling and

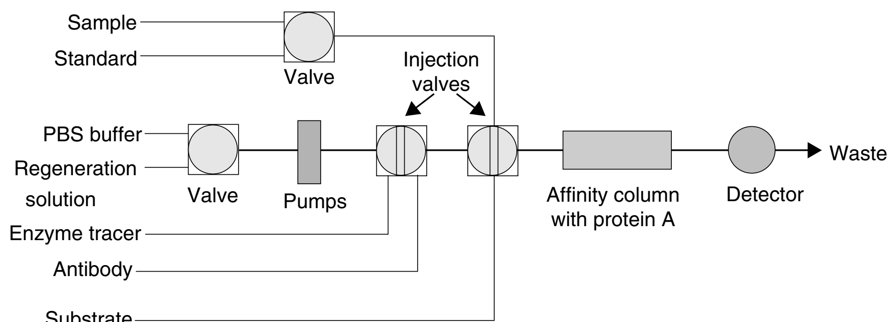

Figure 18 Flow chart of the automated on-line flow injection immunoassay (FIIA). Six steps are involved in each cycle: (1) addition of antibody and incubation; (2) addition of analyte (or standard) and incubation; (3) addition of enzyme–tracer and incubation; (4) addition of substrate and incubation; (5) downstream measurement of fluorescence; (6) regeneration of affinity column

with the development of sensors that can detect antibody–antigen binding events directly.

2.6.4 Multi-analyte analysis

Immunoassays traditionally have been used as a single-analyte method, and this is often a limitation of the technology. However, several approaches are possible to overcome this limitation. A simple approach is to have highly selective assays in different wells of a single microtiter plate, as was demonstrated for the sulfonylureas.[106] A more elegant approach than using a microtiter plate is to use a compact disk (CD)-based microarray system.[107] A microdot system was developed that utilized inkjet technology to 'print' microdots on a CD. The CD was the solid phase for immunoassay, and laser optics were used to detect the near-infrared fluorescent label. The advantage of the CD system is the ability both to conduct assays and to record and/or read data from the same CD. Since the surface of a single CD can hold thousands of dots, thousands of analyses can be made on a single sample simultaneously. Such high-density analyses could lead to environmental tasters where arrays of immunosensors are placed on chips[108,109] or high-density plates. Because the CD format has the potential for high-density analyses, there will be the opportunity for easily generating multiple replicates of the same sample, including more calibration standards, thus improving data quality.

The development of class-selective antibodies is another approach to multi-analyte analysis. The analyst may design haptens that will generate antibodies that recognize an epitope common to several compounds, as explained above for the analysis of pyrethroids by measuring PBA. Other examples of class-selective immunoassays that have been developed are mercapturates,[110] glucuronides,[111] pyrethroids,[37,39] organophosphate insecticides,[112] and benzoylphenylurea insecticides.[113]

Rather than have one antibody that can detect a class, a third approach is to analyze a sample using multiple immunoassays, each with a known cross-reactivity spectrum, and determine the concentration of the analytes and confidence limits mathematically.[114–116] A drawback to using class-selective assays or assays with known cross-reactivity is that for a given antibody, the sensitivity for each analyte

will vary, and the sensitivity for some analytes may not be sufficient, hence selection of well-characterized antibodies will be a critical step.

2.6.5 Future prospects

Immunoassays designed for environmental applications are mostly sold as some variation of the ELISA format. ELISA-like formats dominate the field because they are inexpensive and because they provide high sensitivity and precision without requiring complex instrumentation. The basic ELISA format supports both field and laboratory-based applications but is limited by multiple steps and inadequate sensitivity for some applications, excessive variability and sometimes long analysis times. Some of the other formats discussed in this article may replace the ELISA for selected applications; however, because many laboratories are familiar with the ELISA technology, there will be a significant delay before alternative formats are widely accepted.

In the near term, to improve throughput, the 96-well ELISA is likely to be replaced by higher density arrays. For example, plates, readers and robotic systems are being developed for high-throughput screening in the pharmaceutical industry in 384-, 768-, and 1536-well formats. Other high-throughput formats will utilize inkjet printing technology on CD surfaces or FIIA-like systems, which offer advantages for sequential analysis as discussed above. Biosensor technology will also likely be integrated with ELISAs to generate improved formats.

It is critical to keep in mind that existing reagents can be used for multiple formats. For example, polyclonal antibodies dominate the environmental field because they generally provide greater sensitivity and specificity for small molecules at a much lower cost than do monoclonal or recombinant antibodies. With some biosensors monoclonal or engineered antibodies or recombinant binding proteins may offer advantages.

3 PCR for products of agricultural biotechnology

The recent introduction of genetically modified crops has changed both the agriculture and food industries. United States Department of Agriculture (USDA) surveys report that 25% of corn, 61% of cotton and 54% of soybean acreage grown in the USA in 2000 were genetically modified.[117]

Agricultural biotechnology involves inserting a novel gene [deoxyribonucleic acid (DNA) sequence] into plants or animals using recombinant DNA techniques. These techniques even allow the transfer of DNA from a donor organism to a recipient organism that is not genetically related, a feat not possible using conventional breeding techniques. The novel DNA codes for the expression of a specific protein that confers a new trait or characteristic to the plant or animal. Most traits are described as either input or output traits. Input traits are useful for crop production and include commercial biotech crops that contain herbicide tolerance or resistance to insect pests or diseases. Output traits offer valuable quality enhancements such as improved nutritional value or improved handling or processing characteristics.

Since the commercial introduction of biotech crops, a need has emerged for analytical methods capable of detecting the novel DNA sequences introduced into the plant genome and also methods for detecting the protein products expressed by the

plant. PCR is a powerful tool for the amplification and detection of defined DNA sequences. This section describes the basic principles of agricultural biotechnology and covers principles of both conventional and real-time PCR for DNA analysis. Examples of how these techniques are currently used for analytical testing of raw agricultural commodities and finished food are presented.

3.1 Basic principles of agricultural biotechnology

Within the nuclei of plant cells, chromosomal DNA provides instructions for the cells to replicate themselves and to carry out vital functions. Individual, unique DNA sequences (genes) code for the production of individual, unique proteins. With the tools of modern biotechnology, it is possible to introduce novel DNA sequences that instruct plant cells to synthesize or over-express proteins that confer new traits to the plant. It is also possible to 'down-regulate' or turn off a native gene, thereby suppressing or eliminating the synthesis of a native protein, which can also produce a new trait. Plants that have been transformed in these ways have been called transgenic, genetically modified (GM), genetically engineered (GE), biotech plants and(or) genetically modified organisms (GMOs).

There are several methods that can be used to introduce foreign genes into plant cells, a process called, in general, transformation. Among the most common plant transformation methods are biolistics and exposure to *Agrobacterium tumefaciens.*

Biolistics involves bombarding plant cells with tiny (4-µm) microprojectiles made of gold or tungsten. These microprojectiles are coated with DNA and are propelled at high velocity from a particle gun or 'gene gun' into plant tissue or cells. In this method, the projectile penetrates the cell wall and carries the transgene into the cell nucleus.

A. tumefaciens is naturally able to transform a wide variety of plant species. Mature differentiated plant tissue (an explant) is exposed to *A. tumefaciens* bacteria harboring a 'foreign' gene. The bacterial infection results in foreign DNA from the bacterium being transferred into the genome of the host plant, and results in a crown gall tumor. This naturally occurring process can easily be exploited to produce a transgenic plant.

Plasmids are often used as vectors to transfer DNA into plant cells. In particular, the tumor-inducing (Ti) plasmid of *A. tumefaciens* is a common vector. Plasmids are extrachromosomal, autonomously replicating, circular double strands of DNA that can occur in high copy number in a bacterial cell. It is possible to construct a recombinant Ti plasmid by inserting an effect gene, regulatory sequences (such as transcriptional promoters and terminators), along with a selectable marker gene (such as antibiotic or herbicide resistance) into the circular plasmid.

After the recombinant plasmid has been constructed using *in vitro* methods, leaf disks or protoplasts are infected with recombinant *A. tumefaciens* cells. The infection process incorporates the foreign gene and other genetic elements into the host-plant genome. The host cells are then regenerated from undifferentiated callus tissue into a transgenic plant in tissue culture. Only some of the cells receive the gene of interest, so it is necessary for explants to be grown up in a selective medium.[118]

In order for any gene to synthesize a protein, it must contain certain genetic elements such as promoter and terminator sequences. These regulatory regions signal where the DNA sequence that encodes a product (i.e., a gene) begins and ends. The recombinant

DNA construct will often contain an effect gene and a selectable marker gene (such as antibiotic or herbicide resistance), both of which are bracketed by promoter and terminator sequences. A plasmid vector carries this cassette of genetic information into the plant genome by one of the above methods.

Multiple or ‘stacked’ traits are sometimes introduced into a single plant. These could include resistance to multiple viruses, fungal resistance, etc. Each of these stacked-trait genes usually has an associated promoter and terminator sequence. Obtaining information about particular gene constructs, including marker and regulatory sequences, is vital for PCR testing to detect GMOs in a crop or food sample. The required sequence information can be inferred by restriction mapping of the recombinant plasmid or, more commonly, by DNA sequencing.

GMO screening often relies on the common genetic elements that are present in many commercial GMOs. Many genetically modified plants use common regulatory sequences and/or marker genes, which makes it possible to simultaneously screen for many GMOs by detecting these sequences. The cauliflower mosaic virus (CaMV) 35S-promoter and the *A. tumefaciens nos*-terminator are examples of two DNA sequences that are present in many commercial GMOs.

A positive result for one of these sequences does not necessarily indicate that the test sample contains GM material. Since the 35S-promoter comes from a virus that infects cauliflower, positive results from plants that belong to the genus *Brassica* would need to be carefully evaluated. Likewise, the *nos*-terminator originated in *A. tumefaciens* and this soil bacterium has a broad spectrum of potential hosts. *Nos*-positive results must be confirmed to rule out bacterial contamination. Testing for these common genetic elements only serves as a GMO screening; it is necessary to apply a specific test to determine which GMO is present in the sample. The following list gives some genetic elements that are commonly detected in GMO screening tests:

- *CaMV 35S* promoter: a promoter sequence from the CaMV
- *nos* terminator: nopaline synthase, a terminator sequence from *A. tumefaciens*
- *bar* gene: a herbicide resistance selectable marker from *Streptomyces hygroscopicus* that encodes phosphinothricin acetyltransferase
- *pat* gene: phosphinothricin acetyltransferase, a herbicide resistance selectable marker
- *npt II*: neomycin phosphotransferase, an antibiotic resistance selectable marker.[119]

For PCR analysis of a specific GMO, it is necessary to have sequence information about the gene construct, so primers can be designed to be specific to a gene or to a sequence that bridges genetic elements of the specific construct. An example is the specific test for the genetic modification in Roundup Ready soybeans. The target sequence is the transition that links the transit peptide gene from petunia to the 35S promoter region. This transition DNA sequence is specific to Roundup Ready soybeans.

Table 10 lists United States Food and Drug Administration (FDA) submissions in 2000 for commercial GMOs, including the food, gene, source and intended effect.[120]

Table 10 Commercial GMOs

Food[a] Company/year	Gene, gene product, or gene fragment	Source	Intended effect
Corn* DowAgro/2000	Cry1F protein, phosphinothricin acetyltransferase (PAT)	*Bacillus thuringiensis*, *Streptomyces viridochromogenes*	Resistance to certain lepidopteran insects; tolerance to the herbicide glufosinate
Corn Monsanto/2000	5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS)	*Agrobacterium* sp. strain CP4	Tolerance to the herbicide glyphosate
Corn Aventis/1999	Barnase, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Male sterility, tolerance to glufosinate
Rice Aventis/1999	PAT	*Streptomyces hygroscopicus*	Tolerance to the herbicide glufosinate
Canola Rhone-Poulenc/1999	Nitrilase	*Klebsiella ozaenae* subsp. *ozaenae*	Tolerance to the herbicide bromoxynil
Cantaloupe Agritope/1999	*S*-Adenosylmethionine hydrolase	*Escherichia coli* bacteriophage T3	Delayed fruit ripening due to reduced ethylene synthesis
Canola BASF/1997	Phytase	*Aspergillus niger* van Tieghem	Degradation of phytate in animal feed
Canola AgrEvo/1998	Barnase, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Male sterility, tolerance to glufosinate
Canola AgrEvo/1998	Barstar, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Fertility restorer, tolerance to glufosinate
Sugar beet Monsanto and Novartis/1998	EPSPS	*Agrobacterium* sp. strain CP4	Tolerance to the herbicide glyphosate
Soybean AgrEvo/1998	PAT	*Streptomyces viridochromogenes*	Tolerance to the herbicide glufosinate
Tomato* Calgene/1997	CryIAc protein	*Bacillus thuringiensis* subsp. *kurstaki* (Btk)	Resistance to certain lepidopteran insects
Corn Monsanto/1997	Modified EPSPS	Corn	Tolerance to the herbicide glyphosate
Flax University of Saskatchewan/1997	Acetolactate synthase (csr-1)	*Arabidopsis*	Tolerance to the herbicide sulfonylurea
Potato* Monsanto/1997	CryIIIA, PVY coat protein	*Bacillus thuringiensis* subsp. *tenebrionis* (Btt), potato virus Y (PVY)	Resistance to Colorado potato beetle and PVY
Potato* Monsanto/1997	CryIIIA, PLRV replicase	*Bacillus thuringiensis* subsp. *tenebrionis* (Btt), potato leafroll virus (PLRV)	Resistance to Colorado potato beetle and PLRV
Cotton* Calgene/1997	Nitrilase, Cry1Ac protein	*Klebsiella pneumoniae* subsp. ozaene, *Bacillus thuringiensis* var. *kurstaki* (Btk)	Tolerance to the herbicide bromoxynil, resistance to certain lepidopteran insects
Corn* AgrEvo/1998	Cry9C protein, PAT	*Bacillus thuringiensis* subsp. *tolworthi* (Bt), *Streptomyces hygroscopicus*	Resistance to several lepidopteran insects, tolerance to the herbicide glufosinate

Sugar beet AgrEvo/1998	PAT	*Streptomyces viridochromogenes*	Tolerance to the herbicide glufosinate
Corn Pioneer Hi-Bred/1998	DNA adenine methylase (DAM), PAT	*Escherichia coli*, *Streptomyces viridochromogenes*	Male sterility, tolerance to glufosinate
Canola AgrEvo/1997	PAT	*Streptomyces viridochromogenes*	Tolerance to the herbicide glufosinate
Radicchio Bejo Zaden/1997	Barnase, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Male sterility, tolerance to glufosinate
Squash* Seminis Vegetable Seeds/1997	Coat proteins from CMV, ZYMV and WMV2	Cucumber mosaic virus (CMV), zucchini yellow mosaic virus (ZYMV) and watermelon mosaic virus 2 (WMV2)	Resistance to the viruses CMV, ZYMV and WMV2
Papaya* University of Hawaii/1997	PRV coat protein	Papaya ringspot virus (PRSV)	Resistance to PRSV
Corn* Dekalb Genetics/1996	CryIAc	*Bacillus thuringiensis* subsp. *kurstaki* (Btk)	Resistance to European corn borer
Soybean DuPont/1996	GmFad2-1 gene to suppress endogenous GmFad2-1 gene, which encodes delta-12 desaturase	Soybean	High oleic acid soybean oil
Corn* Monsanto/1996	CryIAb protein, EPSPS, glyphosate oxidoreductase	*Bacillus thuringiensis* subsp. *kurstaki* (Btk), *Agrobacterium* sp. strain CP4, *Ochrobactrum anthropi*	Resistance to European corn borer, tolerance to the herbicide glyphosate
Corn Monsanto/1996	CryIAb protein	*Bacillus thuringiensis* subsp. *kurstaki* (Btk)	Resistance to European corn borer
Potato* Monsanto/1996	CryIIIA protein	*Bacillus thuringiensis* var. *tenebrionis* (Btt)	Resistance to Colorado potato beetle
Oilseed rape Plant Genetic Systems/1995	Barnase, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Male sterility, tolerance to glufosinate
Oilseed rape (Canola) Plant Genetic Systems/1995	Barstar, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Fertility restorer, tolerance to glufosinate
Oilseed rape Plant Genetic Systems, America/1996	Barnase, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Male sterility, tolerance to glufosinate
Cotton Dupont/1996	Acetolactate synthase (ALS)	*Nicotiana tabacum* cv. *Xanthi* (tobacco)	Tolerance to the herbicide sulfonylurea
Corn Dekalb Genetics/1995	PAT	*Streptomyces hygroscopicus*	Tolerance to the herbicide glufosinate
Corn* Monsanto/1995	CryIAb protein	*Bacillus thuringiensis* subsp. *kurstaki* (Btk)	Resistance to European corn borer
Corn* Northrup King/1995	CryIAb protein	*Bacillus thuringiensis* subsp. *kurstaki* (Btk)	Resistance to European corn borer

Table 10—*Continued*

Food[a] Company/year	Gene, gene product, or gene fragment	Source	Intended effect
Tomato Agritrope/1996	*S*-Adenosylmethionine hydrolase	*Escherichia coli* bacteriophage T3	Delayed fruit ripening due to reduced ethylene synthesis
Corn AgrEvo/1995	PAT	*Streptomyces viridochromogenes*	Tolerance to the herbicide glufosinate
Cotton Monsanto/1995	EPSPS	*Agrobacterium* sp. strain CP4	Tolerance to the herbicide glyphosate
Oilseed rape (Canola) Calgene/1992	12:0 Acyl carrier protein thioesterase	*Umbellularia californica* (California Bay)	High-laurate canola oil
Corn* Ciba-Geigy/1995	CryIAb protein	*Bacillus thuringiensis* subsp. *kurstaki* (Btk)	Resistance to European corn borer
Oilseed rape (Canola) AgrEvo/1995	PAT	*Streptomyces viridochromogenes*	Tolerance to the herbicide glufosinate
Oilseed rape (Canola) Monsanto/1995	EPSPS, glyphosate oxidoreductase (GOX)	*Agrobacterium* sp. strain CP4, *Achromobacter* sp. strain LBAA	Tolerance to the herbicide glyphosate
Cotton* Monsanto/1994	CryIAc protein	*Bacillus thuringiensis* subsp. *kurstaki* (Btk)	Resistance to cotton bollworm, pink bollworm and tobacco budworm
Tomato DNA Plant Technology/1994	A fragment of the gene encoding aminocyclopropanecarboxylic acid synthase (ACCS) to suppress the endogenous ACCS enzyme	Tomato	Delayed ripening due to reduced ethylene synthesis
Squash* Asgrow/1994	ZYMV and WMV2 coat proteins	ZYMV and WMV2	Resistance to ZYMV and WMV2
Potato* Monsanto/1994	CryIIIA protein	*Bacillus thuringiensis* subsp. *tenebrionis* (Btt)	Resistance to Colorado potato beetle
Cotton Calgene/1994	Nitrilase	*Klebsiella ozaenae*	Tolerance to the herbicide bromoxynil
Tomato Zeneca/1994	A fragment of the polygalacturonase (PG) gene to suppress the endogenous PG enzyme	Tomato	Delayed softening due to reduced pectin degradation
Tomato Monsanto/1994	1-Aminocyclopropane-1-carboxylic acid deaminase (ACCD)	*Pseudomonas chloraphis*	Delayed softening due to reduced ethylene synthesis
Soybean Monsanto/1994	EPSPS	*Agrobacterium* sp. strain CP4	Tolerance to the herbicide glyphosate
Tomato Calgene/1991	Antisense PG gene to suppress the endogenous PG enzyme	Tomato	Delayed softening due to reduced pectin degradation

[a] An asterisk indicates that the modified plant produces a pesticidal substance that is regulated by the United States Environmental Protection Agency (USEPA).

3.2 Basic principles of the PCR

DNA is the molecule that encodes genetic information. DNA is a double-stranded molecule with two sugar–phosphate backbones held together in the shape of a double helix by weak hydrogen bonds between pairs of complementary nitrogenous bases. The four nucleotides found in DNA contain the nitrogenous bases adenine (A), guanine (G), cytosine (C) and thymine (T). A base sequence is the order of nucleotide bases in a DNA molecule. In nature, base pairs (bp) form only between A and T and between G and C; hence the base sequence of each single strand can be deduced from that of its complementary sequence.

The PCR is a method for amplifying a DNA base sequence in vitro using a heat-stable DNA polymerase and two primers, complementary to short sequences flanking the target sequence to be amplified. A primer is a short nucleotide chain, about 20 bp in length, which anneals to its complementary sequence in single-stranded DNA. DNA polymerase, an enzyme that aids in DNA replication, adds new deoxyribonucleotides to the extensible ($3'$) end of the primer, thereby producing a copy of the original target sequence. Taq polymerase (isolated from a thermophilic bacterium called *Thermus aquaticus*) is the most common heat-stable DNA polymerase used in the PCR.

A PCR cycle involves DNA denaturation, primer annealing and strand elongation. Because the newly synthesized DNA strands can subsequently serve as additional templates for the same primer sequences, the PCR produces rapid and highly specific amplification of the target sequence. Repeated rounds of thermal-cycling result in exponential amplification of the target sequence. Theoretically, 2^n copies of the target can be generated from a single copy in n cycles. There is therefore a theoretical quantitative relationship between number of cycles and starting copy number. This will be covered in more detail in the discussion of real-time PCR.

3.2.1 Isolation and purification of the template DNA

The quantity, quality and purity of the template DNA are important factors in successful PCR amplification. The PCR is an extremely sensitive method capable of detecting trace amounts of DNA in a crop or food sample, so PCR amplification is possible even if a very small quantity of DNA is isolated from the sample. DNA quality can be compromised in highly processed foods such as pastries, breakfast cereals, ready-to-eat meals or food additives owing to the DNA-degrading action of some manufacturing processes. DNA purity is a concern when substances that inhibit the PCR are present in the sample. For example, cocoa-containing foodstuffs contain high levels of plant secondary metabolites, which can lead to irreversible inhibition of the PCR. It is important that these substances are removed prior to PCR amplification. Extraction and purification protocols must be optimized for each type of sample.

Several standard DNA isolation kits are commercially available, including the QIAamp DNA Stool Mini Kit and the DNeasy Plant Mini Kit made by Qiagen. Both of these products are based on silica gel membrane technology and allow for the extraction of total DNA from processed foods and raw foodstuffs, respectively. In

both methods, the cellular components of the samples are first lysed; next the isolated DNA is bound to a membrane gel matrix and washed thoroughly. DNA is then eluted. The DNA Stool Mini Kit includes an extra pre-purification step to remove PCR inhibitors.[121]

Classical approaches to plant DNA isolation aim to produce large quantities of highly purified DNA. However, smaller quantities of crudely extracted plant DNA are often acceptable for PCR analysis. Another efficient method for preparation of plant DNA for PCR is a single-step protocol that involves heating a small amount of plant tissue in a simple solution. Several factors influence nucleic acid release from tissue: salt, EDTA, pH, incubation time and temperature. These factors must be optimized for different sample substrates. EDTA in the sample solution binds the Mg^{2+} cofactor required by the Taq polymerase in the PCR, so the EDTA concentration in the solution, or the Mg^{2+} concentration in the PCR, must be carefully optimized.

An optimized single-step protocol for the extraction of leaf tissue or seed embryos is given here. The template preparation solution (TPS) contains:

100 mM Tris–HCl, pH 9.5
1 M KCl
10 mM EDTA

1. To a sterile 1.7-mL microcentrifuge tube containing 20 μL of TPS, add a maximum of a 2-mm^2 piece of leaf or 0.5-mg piece of embryo and incubate at 95 °C for 10 min.
2. Add a 1-μL portion of the supernatant (or dilution thereof, if inhibitors are present) to the 50-μL PCR reaction.

Making sure that the sample size does not exceed the maximum area or weight is important to minimize the amounts of interfering substances that are coextracted. If the leaf sample is larger than 2 mm^2, coextractive substances can inhibit the PCR assay. Regardless of which extraction method is used, it is important that the PCR assay is evaluated for coextractive interferences or inhibitors.[122]

3.2.2 Components of a PCR

The components necessary for a PCR are assembled in what is known as a mastermix. A PCR mastermix contains water, buffer, $MgCl_2$, dNTPs, forward and reverse primers and DNA polymerase (enzyme). After the mastermix has been assembled, template DNA is added.

1. *Water*: The water used in the assay should be deionized, ultrafiltered and sterile.
2. *Buffer*: The PCR buffer is usually provided as a 10-fold solution and is designed to be compatible with the enzyme. Common buffer components are: 500 mM KCl; 100 mM Tris–HCl, pH 9.3; 1–2% Triton X-100; 0.1% Tween.
3. *$MgCl_2$*: 0.5–3.5 mM $MgCl_2$ salt must be added to the assay, as Mg^{2+} is required as a cofactor for the DNA polymerase.
4. *dNTPs*: Deoxynucleoside triphosphates (dATP, dTTP, dCTP, dGTP) are the nucleotide building blocks for the synthesis of new DNA. The dNTPs are sensitive to repeated freeze–thaw cycles and are usually stored in small aliquots (10 mM pH 7.0); concentrations of 20–200 mM are needed in the assay; too high a

concentration can lead to mispriming and misincorporation of nucleotides. All four nucleotides must have the same concentration in the assay.

5. *Primers*: The primers are short (15–30) oligonucleotide sequences designed to base pair or anneal to complementary sequences that flank the DNA target sequence to be amplified. The primers are added at 0.1–1 μM in the assay.
6. *Enzyme*: Taq polymerase (or some other enzyme) adds new deoxyribonucleotides during strand elongation. Taq is added to the assay at 1 unit per 50 μL of reaction mixture.
7. *DNA*: The template DNA is isolated from cells by some sort of extraction procedure. This is usually the last thing added to the reaction before the tube is placed in the thermal cycler.[123]

3.2.3 Contamination control

Because the PCR exponentially copies the target molecule or molecules, amplicon contamination in the laboratory is a serious concern. It is recommended that the mastermix is prepared in an isolated area, such as a PCR station equipped with a UV light. This work area should be exposed to UV radiation after use to destroy any DNA contaminants. The use of dedicated pipets and filtered pipet tips is also recommended. The template DNA should be prepared and added to the reaction in an area that is isolated from the mastermix preparation hood. The thermal cycling and gel electrophoresis should be conducted in a third work area and care should be taken not to introduce amplified PCR products into the mastermix or template preparation work areas.

3.2.4 Thermal cycling

Once the reaction tube has been placed in the thermal cycler, there are normally three steps in a PCR cycle:

1. *Denaturation step*. This step separates the double-stranded DNA into complementary single strands. Also called melting, this usually occurs at a temperature of about 95 °C for 30 s or 97 °C for 15 s.
2. *Annealing step*. The second step is primer annealing, where the forward and reverse primers find their complementary sequences and bind, forming short double-stranded segments. The annealing temperature (T_a) can be estimated from the melting temperature (T_m) by the following equations:

$$T_a = T_m - 5\,^{\circ}\mathrm{C} \tag{1}$$

$$T_m = (A + T) \times 2 + (\mathrm{C} + \mathrm{G}) \times 4 \tag{2}$$

3. *Elongation step.* The third step is strand elongation, where the DNA polymerase synthesizes new DNA strands starting at the primer sequences. Under optimum conditions, approximately 60 bp are synthesized per second. Typically, elongation takes place at about 72 °C.

The number of PCR cycles depends on the number of source molecules. For 10^5 source molecules, 25–30 cycles are required; for 10^4 source molecules, 30–35 cycles; and for 10^3 source molecules, 35–40 cycles. Running more than 40 cycles can cause the formation of unspecific fragments and does not normally yield any more of the target sequence.[123]

3.2.5 Gel electrophoresis

After amplification, it is necessary to visualize the PCR products. Agarose gel electrophoresis is a technique for separating DNA fragments by size. Purified agar (isolated from seaweed) is cast in a horizontal slab. The agarose slab is submerged in a buffer solution and samples are loaded into wells in the gel. An electric current is applied to electrodes at opposite ends of the gel to establish an electrical field in the gel and the buffer. Because the sugar–phosphate DNA backbone is negatively charged, the fragments migrate by size through the pores in the agarose toward the positive electrode. The addition of an intercalating dye such as ethidium bromide causes bands on the gel to fluoresce under UV radiation.

3.2.6 Multiplex PCR

It is possible to amplify and detect multiple DNA sequences in a single reaction tube by using multiple primer pairs, which recognize and bind to the flanking regions of different specific target sequences. Since the PCR products (amplicons) are separated and visualized according to fragment size, it is important to be sure that the fragments produce bands that can be resolved on a gel during electrophoresis. It is also important to design primers that are not likely to compete or bind to each other to form primer dimers.

3.2.7 Results and data interpretation

Smaller nucleic acid fragments migrate more rapidly than larger ones, hence migration distance can be related to fragment size by comparing bands in sample lanes with a molecular marker containing reference DNAs of known lengths run on the same gel. Solutions are loaded into wells at the top of the gel and the migration distance from the well to the band front is related to the size of the DNA fragment.

The gel photograph in Figure 19 shows seven lanes of data. The 100-bp molecular marker was loaded into lane 1. Sample solutions after PCR were loaded into lanes 2–6. These plant samples were assayed to determine transgenic status. In this multiplex PCR assay, three primer sets were used to amplify three target DNA sequences: top band – species-specific endogenous gene; middle band – introduced effect gene (transgene); bottom band – selectable marker gene (transgene).

The presence of the band for the species-specific endogenous gene in all sample lanes demonstrates that the PCR amplification was successful. It is clear that the plant sample in lane 3 is negative for the transgene of interest, because the only band present is the endogenous species-specific gene. It is clear that the plant samples in lanes 2, 4, 5 and 7 are all positive for the transgene of interest because all three of the target sequences are visible on the gel.

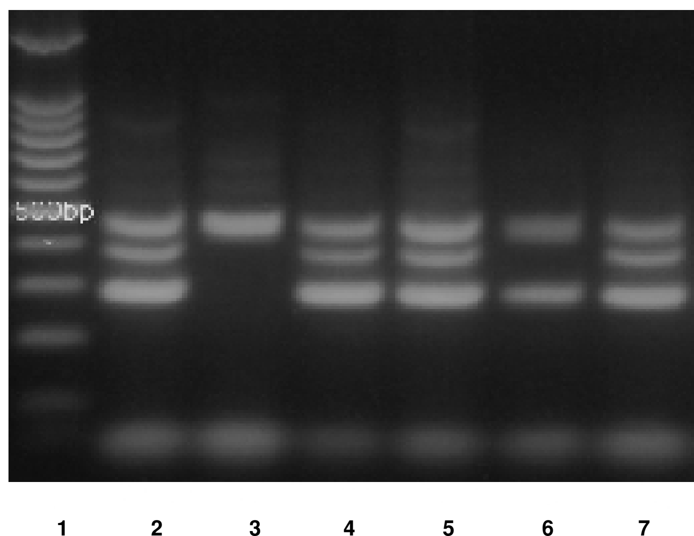

Figure 19 Sample gel of the results of a PCR. Lane 1 is a 100-bp molecular marker; lanes 2–6 are samples. The presence of the top bands (the species-specific endogenous gene) demonstrates that the PCR amplification was successful. Lack of the middle band (the introduced effect gene) and the bottom band (the selectable marker gene) in lane 3 indicates that sample is negative for the effect gene. Presence of all three bands in the remaining lanes indicates the samples are positive for the effect gene

The plant sample in lane 6 is also positive for the transgene of interest. Because the band for the effect gene (middle band) is typically fainter than the band for the selectable marker gene (bottom band), it appears that for lane 6, the PCR product amplification for the effect gene is below the assay detection threshold. Because the selectable marker is clearly present and the PCR amplification worked, lane 6 can be interpreted as a positive result for the transgene of interest.

3.2.8 PCR controls

There are three types of PCR controls, endogeneous reference genes and negative and positive controls. Primers that amplify a species-specific endogenous reference gene are used as internal controls in the PCR. For example, in a soybean assay, the soy lectin gene may be used as the species-specific reference gene (Table 11).[121] Maize invertase can be used as the endogenous reference gene in corn (Table 12).[121]

Table 11 Primer sequences for PCR analysis of Roundup Ready (RR) Soy

Primer	Sequence (5′–3′)[a]	Length of amplicon (bp)
Lectin	GACGCTATTGTGACCTCCTC	
Lectin	GAAAGTGTCAAGCTTAACAGCCGACG	318
EPSPS RR Soy-specific	TGGCGCCCAAAGCTTGCATGGC	356
EPSPS RR Soy-specific	CCCCAAGTTCCTAAATCTTCAAGT	

[a] Standard one-letter amino acid abbreviation (see list of Abbreviations and Acronyms).

Table 12 Primer sequences for PCR analysis of Bt corn[a]

Primer	Sequence (5′–3′)[a]	Length of amplicon (bp)
Invertase	CCGCTGTATCACAAGGGCTGGTACC	
Invertase	GGAGCCCGTGTAGAGCATGACGATC	226
Cry1A(b)	ACCATCAACAGCCGCTACAACGACC	
Cry1A(b)	TGGGGAACAGGCTCACGATGTCCAG	184

[a] Standard one-letter amino acid abbreviation (see list of Abbreviations and Acronyms).

These reference genes demonstrate that the DNA isolated was of sufficient quality and quantity for PCR amplification. It is assumed that in the course of food processing, the species-specific reference gene and the transgene are degraded in a similar manner. It is also assumed that effects of the matrix on PCR amplification will be similar. The reduced amplification efficiency of both genes presumably has no effect on the ratio of their amounts, which reflects the ratio of modified and unmodified DNA.

Negative controls demonstrate the absence of laboratory contamination or sample cross-contamination. DNA extracts from nontransgenic plants, clean buffer and mastermix with no template DNA added are common negative controls that are run concurrently with the test samples in the PCR.

Positive controls demonstrate adequate amplification and may be used to quantify the sensitivity of the reaction. One approach is to add known amounts of reference material [e.g., soybean and corn powder containing 0.1% (w/w) genetically altered material] to the standard PCR and to run these concurrently with the test samples. Plant genomic DNA and GMO genomic DNA may also be used as positive controls in the PCR.

3.2.9 Primer design

Primer design is one of the most important aspects of a robust PCR assay. In general, primers should be designed such that they are not able to form secondary structures such as stemloop or hairpin configurations. A primer must not be complementary at the 3′ end, as this will cause primer dimers to form. All primers should have similar melting temperatures and should not contain stretches of individual nucleotides. There are software programs available to assist in primer design, but it is crucial that primers are tested in the assay, especially in a multiplex system.

3.2.10 PCR confirmatory techniques

Presented below are four increasingly stringent confirmatory techniques for PCR and a brief discussion of considerations, limitations and advantages of each. These four techniques are agarose gel electrophoresis, restriction analysis, Southern blotting and sequencing.

Agarose gel electrophoresis can be used to determine whether the PCR amplicon is the expected size. The density of the gel should be chosen to ensure resolution of

the amplicon, and the molecular weight marker should be chosen to encompass the expected size range of the amplicon. A limitation to this approach is that it gives an indication only of the size of the amplification product, not its identity. An advantage is that the technique is quick and easy, allowing for screening of many samples within a short period of time.

Restriction analysis utilizes known restriction enzyme cleavage sites within the DNA sequence of interest. Knowing the sequence of the target PCR product, one can cleave the DNA with appropriate restriction enzymes and separate those fragments by agarose gel electrophoresis. As with agarose gel electrophoresis, the density of the gel and molecular weight markers must be chosen to appropriately resolve and identify the size of the resultant DNA fragments. This type of analysis will give an indirect indication of the identity of the amplicon based solely on common restriction sites and size. Using the known restriction enzyme cleavage sites gives more conclusive data than simple gel electrophoresis, because the recognition site must be present to produce a DNA fragment of the predicted size. Restriction analysis is easily performed on a large number of samples in a short period of time.

Southern blotting consists of agarose gel electrophoresis of the PCR product followed by transfer of the DNA to a solid support matrix, and hybridization with a labeled DNA probe. This technique allows for the determination of the amplicon size and infers specificity related to the DNA probe. As with agarose gel electrophoresis, the density of the gel and molecular weight markers must be chosen appropriately for the size of amplicon being analyzed. It is important that the DNA probe be adequately characterized to ensure its specificity to the targeted DNA sequence. The Southern blotting technique is a lengthy process, but this technique allows for the confirmation of reactivity to a specific DNA probe, giving more confidence about the identity of the PCR product.

Sequencing the amplicon is the most conclusive confirmatory technique. The main consideration is that the DNA must be appropriately purified to achieve unambiguous sequencing data. However, sequencing requires expensive laboratory equipment that may not be available in all labs. Sequencing does not depend upon the specificity of a probe, or restriction enzyme, but gives a direct identification of the amplicon of interest.

3.3 Basic principles of real-time PCR

Real-time quantitative PCR offers an approach to DNA detection by monitoring the accumulation of PCR products as they are generated. A single copy of a target DNA sequence can yield 2^n copies after n cycles. Hence, theoretically, there is a relationship between starting copy number and amount of PCR product at any given cycle (Figure 20, line A). In reality, replicate reactions often yield widely different amounts of PCR product (Figure 20, line B). This is due to reagents and enzyme activity limiting the reaction. It is difficult to quantify the starting amount of target DNA based on the endpoint. Real-time PCR has the potential to decrease the variability of the measurement by using kinetic rather than endpoint analysis of the PCR process.

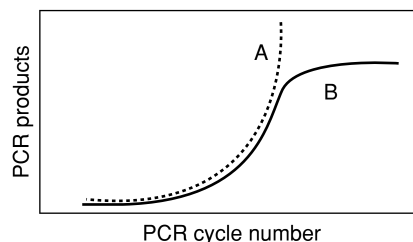

Figure 20 Plot of PCR products produced against the number of amplification cycles. (A) Theoretical PCR product amplified and (B) actual PCR product amplified

3.3.1 Intercalating dyes

The first real-time systems detected PCR products as they were accumulating using DNA binding dyes, such as ethidium bromide.[124,125] UV radiation was applied during thermal cycling, resulting in increasing amounts of fluorescence, which was captured with a charge-coupled device (CCD) camera. The increase in fluorescence ($\Delta_n R$) was plotted against cycle number to give a picture of the kinetics of the PCR process rather than merely assaying the amount of PCR product that had accumulated at a fixed endpoint. These binding dyes are nonspecific, because a fluorescent signal is generated for any double-stranded DNA present. The presence of double-stranded DNA could be due to mispriming or the formation of primer dimer artifacts rather than specific amplification of the target sequence. Nonetheless, DNA binding dyes are very useful in real-time PCR when specificity is not a concern. Examples of commonly used intercalators are ethidium bromide and SYBR Green.[126]

3.3.2 Fluorogenic probes

With fluorogenic probes, it is possible to detect specifically the target sequence in real-time PCR because specific hybridization is required to generate fluorescence. A typical fluorogenic probe is an oligonucleotide with both a reporter and a quencher dye attached. The probe typically binds to the target sequence between the two primers. The proximity of the quencher in relation to the reporter molecule reduces the Forster resonance energy transfer (FRET) of the fluorescent signal emitted from the reporter. There are also a wide range of fluorophores/quenchers and several different hybridization probe strategies available (Table 13).

The three main categories of hybridization probes for real-time PCR are (1) cleavage based assays such as TaqMan, (2) displaceable probe assays such as Molecular Beacons and (3) probes which are incorporated directly into primers such as Scorpions.

Table 13 Common fluorophores/quenchers

DABCYL	4-(4-Dimethylaminophenylazo)benzoic acid
FAM	Fluorescein
TET	Tetrachloro-6-carboxyfluorescein
HEX	Hexachloro-6-carboxyfluorescein
TAMRA	Tetramethylrhodamine
ROX	Rhodamine-X

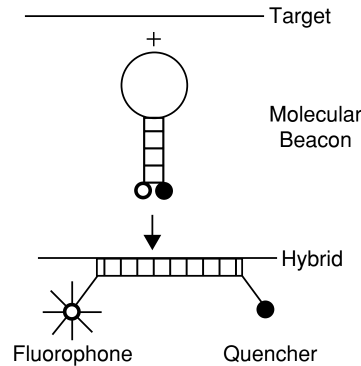

Figure 21 Schematic of the Molecular Beacon

3.3.3 Examples of fluorescent PCR systems

The TaqMan system is also called the fluorogenic 5′ nuclease assay. This technique uses the 5′ nuclease activity of Taq polymerase to cleave an internal oligonucleotide probe. The probe is labeled with both a fluorescent reporter dye and a quencher. The assay results are detected by measuring changes in fluorescence that occur during the amplification cycle as the fluorescent probe is cleaved, uncoupling the dye and quencher labels. The increase in the fluorescent signal is proportional to the amplification of target DNA.

The Molecular Beacons system uses probes that are configured in the shape of a stem and loop. In this conformation, the probe is 'dark' (background level fluorescence) because the stem hybrid keeps the fluorophore in close proximity to the quencher. When the probe sequence in the loop hybridizes to its target, forming a rigid double helix, a conformational reorganization occurs that separates the quencher from the fluorophore, resulting in increased fluorescence proportional to the amplification of target DNA (Figure 21).

The Scorpions system combines a primer, a specific hybridization probe, fluorophore and quencher in a single molecule. When the Scorpions primer is in a stem and loop conformation, the fluorophore and quencher are in close proximity. The initial heating step denatures the template and also the stem of the Scorpions primer. The primer anneals to the template and strand elongation occurs, producing a PCR amplicon. This double-stranded DNA is denatured and the specific hybridization probe (sequence originally within the loop of the stem/loop) reaches back and hybridizes to the PCR product, binding to the target in an intramolecular manner. The new conformation separates the fluorophore and quencher, resulting in an increase in the fluorescent signal that is proportional to the amplification of target DNA.[127]

3.3.4 Quantitative results/data interpretation

A method for quantitation of the amount of target involves measuring threshold cycle (C_T) and use of a calibration curve to determine starting copy number. The parameter C_T is defined as the fractional cycle number at which the fluorescence passes a fixed threshold. A plot of the log of initial target copy number for a set of standards versus C_T is a straight line (Figure 22).[125] Thus, when the percentage of GMOs in the sample

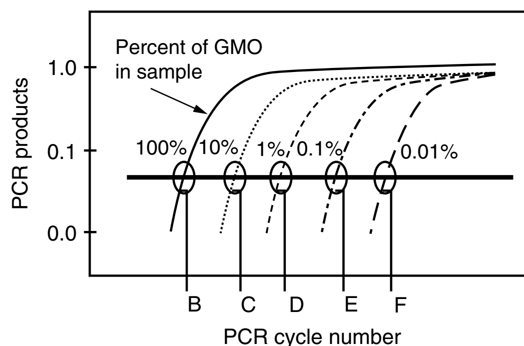

Figure 22 Real-time quantitation of PCR products. The straight line represents the threshold fluorescence value. Each curved line is a plot of the PCR products formed against the number of cycles for different samples. For samples containing 100% GMO, only *B* cycles are required to reach the threshold fluorescence. Samples containing 0.01% GMO will require *F* cycles before the threshold is attained

is 100%, the threshold fluorescence will be reached after only *B* cycles, whereas the sample containing 0.01% of GMO will reach the threshold after *F* cycles.

The use of C_T values also expands the dynamic range of quantitation because data are collected for every PCR cycle. A linear relationship between C_T and initial DNA amount has been demonstrated over five orders of magnitude, compared with the one or two orders of magnitude typically observed with an endpoint assay.[126]

3.4 Applications of PCR to agricultural biotechnology

3.4.1 Research and development

The PCR technique is very useful during all stages of the research and development of biotech crops. PCR analysis is used for gene discovery, event selection, screening, transformant identification, line selection and plant breeding. Quantitative real-time PCR is used to determine the number of transgene copies inserted in experimental plants.

3.4.2 Regulatory submissions

PCR is used to support regulatory submissions. For example, a petition for nonregulated status for a biotech crop must contain the following information:

- rationale for development of product
- description of crop
- description of transformation system
- the donor genes and regulatory sequences
- genetic analysis and agronomic performance
- environmental consequences of introduction
- adverse consequences of introduction
- references.

PCR analysis is one of the techniques used to generate data for the genetic analysis requirement.

3.4.3 Food and commodity testing

There are commercial testing laboratories that offer PCR testing of commodities and food for GMO content. Testing of bulk commodities such as corn grain requires a large sample size. A 2500-g sample is required to have a 99.9% probability of detecting 0.1% GMO content in a sample. The sampling strategy must produce a statistically valid sample for the test results to be meaningful. The entire 2500-g sample would typically be ground, and duplicate 10-g subsamples of raw corn or soy would be extracted. For processed or mixed foods, duplicate 2-g subsamples would typically be extracted.

These PCR laboratories often offer GMO screening, specific tests for certain commercial GMOs and real-time quantitative testing. The different approaches vary widely in cost and the choice would depend on the testing objective.

3.5 Recent advances in nucleic acid amplification and detection

Many nucleic acid detection strategies use target amplification, signal amplification or both. Invader, branched DNA (bDNA) and rolling circle amplification (RCA) are three approaches.

Invader is a signal amplification approach. This cleavage-based assay uses two partially overlapping probes that are cleaved by an endonuclease upon binding of the target DNA. The Invader system uses a thermostable endonuclease and elevated temperature to evoke about 3000 cleavage events per target molecule. A more sensitive homogeneous Invader assay exists in which the cleaved product binds to a second probe containing a fluorophore and quencher. The second probe is also cleaved by endonuclease, generating 10^7 fluorescence events for each target molecule, which is sensitive enough to detect less than 1000 targets.[128]

bDNA achieves signal amplification by attaching many signal molecules (such as alkaline phosphatase) to a DNA dendrimer. Several tree-like structures are built in each molecular recognition event. The Quantiplex bDNA assay (Chiron) uses a dioxetane substrate for alkaline phosphatase to produce chemiluminescence.[127]

The linear RCA method can use both target and signal amplification. A DNA circle (such as a plasmid, circular virus or circular chromosome) is amplified by polymerase extension of a complementary primer. Up to 10^5 tandemly repeated, concantemerized copies of the DNA circle are generated by each primer, resulting in one single-stranded, concantemerized product.[129]

4 Biosensors: immunosensors

The development of immunosensors is one of the most active research areas in immunodiagnostics. A large number of immunosensors, which combine the sensitivity and specificity of immunoassays with physical signal transduction, have been developed

in recent years for pesticide analysis. A classical biosensor consists of three components, including a receptor (an antibody or binding protein), a transducer (e.g., an optical fiber or electrode) and signal processing electronics. The receptor is usually immobilized to the transducer surface, which enables it to detect interaction with analyte molecules. In contrast to immunoassays, immunosensors commonly rely on the reuse of the same receptor surface for many measurements. Direct signal generation potentially enables real-time monitoring of analytes, thus making immunosensors suitable tools for continuous environmental monitoring.

There are several classes and subclasses of immunosensors, each with advantages for environmental analysis. Piezoelectric sensors (including bulk acoustic and surface acoustic wave) use an external alternating electric field to directly measure the antibody–antigen interaction. Electrochemical sensors (including potentiometric, amperometric, capacitative and conductimetric) may offer inexpensive analytical alternatives for effluent monitoring.[130,131] Optical sensors (including fiber-optic, evanescent wave biosensors and Mach–Zehnder interferometer sensors) measure the absorption or emission of a wavelength of light and base detection on fluorescence, absorbance, luminescence or total internal reflectance fluorescence.[132,133]

Surface plasmon resonance (SPR) is an optical electronic technique in which an evanescent electromagnetic field generated at the surface of a metal conductor is excited by light of a certain wavelength at a certain angle. An immunosensor has been developed for the detection of atrazine using SPR.[134] Moreover, a grating coupler immunosensor was evaluated for the measurement of four *s*-triazine herbicides.[135] One could detect terbutryn in the range 15–60 nM using this biosensor. Because antibody-based biosensors have no associated catalytic event to aid in transduction, they are far more complex than enzyme-based biosensors. In addition, they do not release their ligand quickly, leading to a slow response. Theoretically, biosensors are capable of continuous and reversible detection, but reversibility is difficult to achieve in practice because sensitive antibody–antigen interactions have high affinity constants. Because cost and time are critical factors in environmental monitoring, it is likely that the development of small-probe antibody-based biosensors yielding continuous readouts of an analyte at low concentration will not be rapid. However, research in the sensor field is certain to give improvements in many aspects of immunoassay technology, and antibody–hapten and receptor–ligand binding assays are being coupled to biological and physical transducers in many ingenious ways.

4.1 Biological transducers

With enzymes, binding proteins or receptors, it is attractive to use biological transduction. A simple example is acetylcholinesterase for the detection of organophosphate and carbamate insecticides. Binding of these materials to the enzyme inhibits it, thus blocking substrate turnover. Similar approaches can be used for herbicide detection. Coupling a receptor to its natural responsive element also can provide a valuable biosensor. This could be induction of natural proteins such as vitellogenen by estradiol or the responsive element could be moved upstream of luciferase, a fluorescent protein or other easily detected biological molecules.[136]

5 Conclusion

As described by Hammock and Mumma,[8] there are many unique applications for immunodiagnostics in pesticide chemistry. Such uses include human monitoring, field monitoring, analysis of chirality, analysis of complex molecules and analytical problems where large numbers of samples must be processed quickly. Such applications are expanding as we see the development of more complex and nonvolatile pesticide chemicals and the need to monitor polar metabolites, environmental degradation products and GMOs. However, other analytical technologies are improving. For example, liquid chromatography/mass spectrometry (LC/MS) technologies increasingly can handle complex molecules and, like immunoassay, tandem mass spectrometry (MS/MS) technologies avoid the need for many cleanup steps. Hence, many of the traditional applications of immunoassay will be replaced by other technologies if immunochemistry remains static. Active research on new formats and new applications of immunoassays argues for a continued place for the technology in the repertoire of environmental chemists. Coupled immunochemical techniques are promising where, for example, antibodies are used as sensitive, selective detection systems for HPLC[137] or for immunoaffinity procedures preceding MS[138] or other analyses.

Although immunoassays can compete effectively with other technologies in the analysis of small molecules, a major strength of the technology is in the analysis of peptides and proteins. With the expanded use of GMOs in agriculture, all of which to date are expressing novel proteins, there is a new and important application for immunoassay. The technology will be important for GMO development, product stewardship and quality control. With some public concern over the safety of GMOs, there is a commercial need for high-throughput and for field analysis of food products for GMO content. High throughput and field analysis are two major strengths of immunoassay technology, making it an ideal technology for monitoring indicators of food quality. Food quality monitoring, then, represents a major market for this technology.

6 Abbreviations

A	adenine
Ab	antibody
ACCD	1-aminocyclopropane-1-carboxylic acid deaminase
ACCS	aminocyclopropane carboxylic acid synthase
Ag	antigen
ALS	acetolactate synthase
bDNA	branched DNA
bp	base pairs
BSA	bovine serum albumin
Bt	*Bacillus thuringiensis*
C	cytosine
CaMV	cauliflower mosaic virus
CCD	charge-coupled device
CD	compact disk

CMC	1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (same as Morpho CDI)
CMV	cucumber mosaic virus
C_T	threshold cycle
DAM	DNA adenine methylase
DCC	dicyclohexylcarbodiimide
DMF	dimethylformamide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
FDA	Food and Drug Administration
FIIA	flow injection immunoassay
FRET	Forster resonance energy transfer
G	guanine
GC	gas chromatography
GC/MS	gas chromatography/mass spectrometry
GE	genetically engineered
GLC	gas–liquid chromatography
GM	genetically modified
GMO	genetically modified organism
GOX	glyphosate oxidoreductase
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
HSA	human serum albumin
I_{50}	the concentration of analyte that inhibits the immunoassay by 50%
IgG	immunoglobulin G
K_A	equilibrium binding constant for the binding of analyte to antibody
K_H	equilibrium binding constant for the binding of hapten to antibody
KLH	keyhole limpet hemocyanin
LC/MS	liquid chromatography/mass spectrometry
LLD	lower limit of detection
LOQ	limit of quantitation
LPH	horseshoe crab hemocyanin
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
MBS	*m*-maleimidobenzoyl-*N*-hydroxysuccinimide
Morpho CDI	1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (same as CDI)
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NHS	*N*-hydroxysuccinimide
NPTII	neomycin phosphotransferase II

OD	optical density
PAT	phosphinothricin acetyltransferase
PBA	phenoxybenzoic acid
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
PG	polygalacturonase
PRSV	papaya ringspot virus
QC	quality control
RCA	rolling circle amplification
SDS	sodium dodecyl sulfate
SPR	surface plasmon resonance
T	thymine
T_a	annealing temperature
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
T_m	melting temperature
Ti	tumor-inducing
TOF	time-of-flight
TPS	template preparation solution
USDA	United States Department of Agriculture
USDA GIPSA	United States Department of Agriculture Grain Inspection Protection Service
USEPA	United States Environmental Protection Agency
UV	ultraviolet
UV/VIS	ultraviolet/visible
WMV2	watermelon mosaic virus2
ZYMV	zucchini yellow mosaic virus
λ_{max}	wavelength of maximum absorption

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