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Chapter 24

Immunochemical Methods of Pesticide Residue Analysis

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Immunochemical methods are rapidly gaining acceptance as analytical techniques for pesticide residue analysis. Unlike most quantitative methods for measuring pesticides, they are simple, rapid, precise, cost effective, and adaptable to laboratory or field situations. The technique centers around the development of an antibody for the pesticide or environmental contaminant of interest. The work hinges on the synthesis of a hapten which contains the functional groups necessary for recognition by the antibody. Once this aspect is complete, immunochemical detection methods may take many forms. The enzyme-linked immunosorbent assay (ELISA) is one form that has been found useful in residue applications. This technique will be illustrated by examples from this laboratory, particularly molinate, a thiocarbamate herbicide used in rice culture. Immunoassay development will be traced from hapten synthesis to validation and field testing of the final assay. Emphasis will be placed on the justification of and the resources required for the successful incorporation of immunochemical technology into an existing analytical laboratory. Special attention will be given to aspects of immunochemical and related technology not covered in other recent reviews. Present use of immunoassay for pesticide analysis will be described and future potential applications and problems will be discussed.

Some basic immunology and definition of a few common terms will allow understanding of the central concepts of immunoassay. Antibodies are serum proteins which bind to specific molecules, called antigens, due to a complementarity of chemical structure between antibody and antigen. Immunization with an antigen preferentially induces the production of antibodies specific for

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that antigen. While most antigens are naturally occurring proteins or complex carbohydrates, the extraordinary genetic flexibility of the immune system allows for the production of antibodies against virtually any chemical structure, including synthetic antigens. Antigens below a poorly defined size threshold of approximately 1000 daltons will not stimulate the production of specific antibodies. However, small molecules, called haptens, can elicit specific antibodies when attached to larger carrier molecules such as proteins. The phenomenon of immune response to haptens has been studied for over fifty years with the successful production of antibodies to an astounding range of natural and synthetic chemical structures. Thus the potential clearly exists for the development of immunoassays for nearly any compound.

An occasional misconception is that immunoassays are bioassays because of their use of biologically derived antibodies. It is important to realize that although immunoassays are dependent upon antibodies, these antibodies obey the Law of Mass Action just as reagents in any other ligand binding assay and are therefore well suited to quantitative analysis. Theoretical understanding of quantitative immunoassays has evolved significantly in the last three decades through extensive clinical and research use (26). An indication of the massive commercial support for immunochemical techniques is the degree of sophistication of data analysis available in numerous commercial software packages which exploit these developments (HP-Genenchem, Molecular Devices, Dynatech, and many others). This support illustrates the maturity of immunochemical techniques and technology, much of which can be directly appropriated for residue analysis by immunoassay.

The development of immunochemical methods for analysis of haptens such as drugs and steroids (4,11) occurred during the dominance of the pesticide market by highly nonpolar organochlorines, which are easily analyzed by gas chromatography with ion selective detectors due to their lipophilicity and halogenation. There is a long history of extensive clinical and research use of immunoassays for analysis of drugs and hormones, including steroids, peptides, and others (4,11,24,25). The reasons for the lack of use of immunochemical technology in pesticide residue analysis seem to be primarily historical. Present technology makes it feasible to analyze for nearly all pesticides in current use by immunochemical methods.

Reasons for Applying Immunochemical Technology to Pesticides

Since the use of immunochemical technology for pesticide residue analysis was first reviewed by Ercegovich in 1971 (9) several helpful reviews of this application have been published (13,14,23,33). Despite the potential demonstrated during this period, few researchers and regulators apply immunochemical technology to their own problems in pesticide residue analysis.

Immunochemical technology for residue analysis offers the existing analytical laboratory many advantages. Sensitivity and specificity are generally comparable to existing techniques with large improvements in speed and cost. The general applicability of immunochemical technology is also a valuable asset. Immunoassays can be modular; by substituting two reagents, analysis for a

different analyte can be performed using the same instrumentation. Multianalyte procedures can be developed through parallel processing. The factors limiting the present use of immunochemical technology for residue analysis are primarily lack of acceptance and poor antibody availability rather than the difficulty of developing antibodies to target compounds. This situation appears to be changing as the advantages of immunoassay for environmental analysis are recognized by analytical chemists. One reason for this recognition is that immunoassay is the best choice among present technologies for the analysis of the large number of samples needed for thorough environmental analysis.

It can be shown by sampling theory that a large number of samples must be analyzed to obtain high confidence estimates of low contamination rates (Figure 1). With immunochemical technology available, the analytical chemist no longer need be dismayed by the prospect of analyzing such large sample loads. The technology for automation of some types of immunoassays is advancing rapidly and sample processing rates are increasing dramatically. The application of robotic systems to the residue laboratory has been discussed (22) and an automated ELISA system using commercially available robots and automated extraction systems has been described which can analyze 10,000 or more seed and plant samples per day for viral and bacterial diseases (34).

Applications of immunoassay to pesticide chemistry have been described which address some difficult problems in analysis by classical methods. These include stereospecific analysis of optically active compounds such as pyrethroids (38), analysis of protein toxins from Bacillus thuringiensis (5,37), and compounds difficult to analyze by existing methods, such as diflubenzuron (35) and maleic hydrazide (15; also Harrison, R.O.; Brimfield, A.A.; Hunter, K.W.,Jr.; Nelson, J.O. J. Agric. Food Chem, submitted). An example of the excellent specificity possible is seen in assays for parathion (10) and its active form paraoxon (3). Some immunoassays can be used directly for analysis without extensive sample extraction or cleanup, dramatically reducing the work needed in typical residue analysis. An example of this is given in Figures 2 and 3, comparing the direct ELISA analysis of molinate in rice paddy water to the extraction required before GC analysis.

While the use of immunoassay for residue analysis should continue to expand, it is not the answer to all problems in environmental analysis. Immunochemical technology should serve best as a complement to existing methods rather than a replacement for them. It is especially important to recognize the potential of immunoassay for impact in environmental screening through the developments which will be discussed later in this chapter. It is in this area that we expect immunochemical technology to make its greatest contribution to environmental analysis.

Resources Required for Immunoassay Development

Perhaps the most important distinction to be drawn in this chapter is that between the development of immunochemical residue methods and their implementation for routine use. The labor and resources required for developing a specific assay are significant, as will be shown in this section. However, the routine use of validated assays

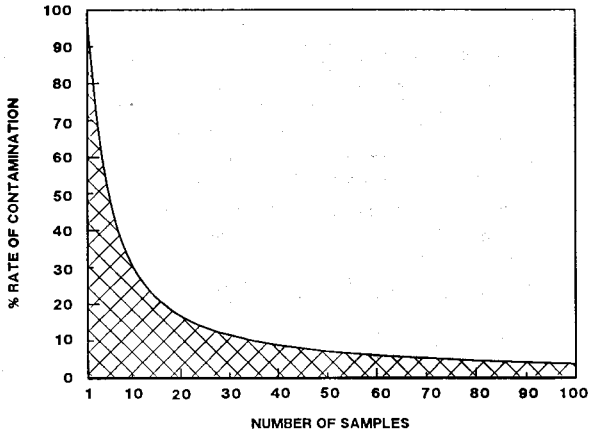

Figure 1. The shaded area represents the 95% confidence interval for true contamination rate, assuming random distribution of contaminated samples and given a constant 0% detected contamination rate (adapted from ref. 1). The upper boundary of the curve represents the upper 95% confidence limit for true contamination rate. Thus, if ten samples were analyzed with no contamination detected, the true contamination rate 95% confidence interval would be between 0% and 30.8%.

requires little expertise, equipment, or supplies not already found in most analytical labs. A summary of the resources needed for immunoassay development is given in Table 1.

TABLE 1 Resources needed for immunoassay development

Organic chemistry: hapten synthesis and conjugation to carriers
Biology: antibody production
Immunochemistry: immunoassay design and optimization
Analytical and residue chemistry: sample preparation, data analysis, assay validation and application

This table illustrates one of the major impediments to the rapid assimilation of immunochemical technology into pesticide residue analysis labs. Because of the amount and variety of work involved, new method development costs may be high when compared to routine chromatographic methods. However, the low cost per run allows for rapid recovery of the initial investment with sufficiently high sample loads. For example, the cost of reagents and supplies for an ELISA for diflubenzuron was estimated to be $0.20/sample as compared with $4 for HPLC or $11 for GC (35). In addition to the lower reagent and supply costs, the major economic advantage of immunoassay is the dramatic decrease in labor costs.

Steps in the Development of Pesticide Immunoassays

The steps involved in the development of pesticide immunoassays have been described previously (14) and numerous reports of pesticide immunoassay development now illustrate this process (3,10,12,15,19,32,35,36,38). These steps will be reiterated here to reinforce salient points and to emphasize the distinction between the development and implementation of immunoassays.

Hapten synthesis. The general synthetic approach chosen will be dictated by the desired assay specificity. Compound or class specific antibodies can be produced depending on which part of the molecule is used for conjugation. Antibody specificity is generally highest for the part of the molecule furthest from the carrier. The importance of carefully planned and rational hapten synthesis cannot be overemphasized. The chosen synthetic route should preserve as much structure as possible of the molecule to be analyzed and must provide a functional group for conjugation to a carrier molecule (usually protein). The optimum hapten structure for antibody production may however lack an apparently essential moiety and this is a tricky area for the non-immunochemist to predict. For example, preservation of the nitro group of parathion by conjugation through the normally unsubstituted aromatic ring positions led to the production of antisera which did not recognize free parathion (31), while parathion specific antisera were produced against conjugates of aminoparathion (10). Conjugation position was also important in the production of antibodies to maleic hydrazide (15; also Harrison, R.O.; Brimfield, A.A.; Hunter, K.W.,Jr.; Nelson, J.O. J. Agric. Food Chem, submitted). Immunization with N-conjugates of maleic hydrazide led to maleic hydrazide specific and hapten specific antibodies, while immunization with O-conjugates of maleic hydrazide

led to only hapten specific antibodies. There is experimental evidence that haptens are more immunogenic when separated from the carrier by a spacer arm. Presentation of the hapten to receptors on cells of the immune system is less likely to be sterically hindered if the hapten is on a spacer arm several atoms long.

The production of haptens for conjugation is the last point in the assay development process where rigorous structural analysis is possible. Because the assay specificity is determined by the structure(s) of the conjugated haptens, it is critically important for these compounds to be pure and structurally well characterized before conjugation renders this impossible.

Conjugation to carriers. A wide range of proven methods are available for conjugation of haptens to their carriers, most of them using common commercially available reagents. These have been summarized previously (11,14) and many examples of their use in pesticide immunoassay development exist (3,10,12,15,19,32,35,36,38).

Verification of conjugation. As noted above, structural analysis is difficult or impossible after conjugation, but it is possible to verify conjugation even though the structure of the conjugated molecules is unknown. If the hapten has an appropriate UV absorption spectrum, difference spectra between conjugates and original carriers can be used for both qualitative demonstration of conjugation and estimation of the molar ratio of hapten to carrier, often called the hapten density. This method has been applied to both UV absorbing parent compounds (15) and haptens containing a UV absorbing spacer group (3). Alternative methods include quantitation of free primary amino groups and conjugations using radiolabelled haptens.

Antibody production. Antibodies used for immunoassay can be either polyclonal, contained in serum from rabbits or other animals, or monoclonal, produced by mouse lymphocytes immortalized for *in vitro* culture (called hybridomas). The advantages of each have only recently begun to be examined systematically in the context of quantitative hapten analysis (2). Both monoclonal (3,15) and polyclonal (5,10,12,19,32,35-38) antibodies have proven effective for pesticide analysis, although most reported immunoassays have used rabbit sera. Production of monoclonal antibodies is labor intensive and may not warrant the extra effort for the planned application. For standardized methods which will be widely used, monoclonal antibodies may be superior to polyclonal sera because of their biochemical uniformity and defined affinity and specificity. Stable hybridoma cell lines provide a continuous source of their monoclonal antibodies as long as they can be maintained in culture. Monoclonal antibodies will also be useful in the discrimination of related multivalent antigens such as those produced by related strains such as *Bacillus thuringiensis israelensis* and *Bacillus thuringiensis kurstaki* or genetically engineered organisms. The specificity of monoclonal antibodies for single protein determinants will likely also prove useful for distinguishing active and inactive forms of protein toxins, such as from *Bacillus thuringiensis*.

Antibody characterization. A useful description of some important considerations in antibody characterization has been given by Hammock and Mumma (14). Specificity is generally tested using some form of competitive binding, such as a competitive ELISA. An estimation of the average affinity constant may be useful in the comparison of different antibodies and in later optimization of the assay. This value can be conveniently estimated from antiserum dilution curves (27) for selection of the best antibodies in a screening process. This requires the choice of an assay format (see later section on assay formats), such as ELISA, preferably using a different carrier to eliminate carrier crossreactivity. It is important that specificity and sensitivity be evaluated at this point and be deemed acceptable before investing further resources in assays based on inadequate antibodies.

Assay optimization. An optimization step not always taken, but nonetheless important to the success of any immunochemical method of analysis is the selection of materials, such as test tube or plastic plates, which maximize assay performance. An example of this is the selection of 96-well microtiter plates for enzyme-linked immunosorbent assay (ELISA) which give maximum protein binding capacity and minimum interwell variability (28,30; also Harrison, R.O.; Nelson, J.O. J. Immunoassay, submitted). The type of microtiter plate may be the most important single determinant of ELISA performance and this selection should not be made carelessly. Significant error may also occur in the reading of assays performed in 96-well microtiter plates, due to alignment errors of automatic plate readers undetected in normal use (Harrison, R.O.; Nelson, J.O. J. Immunoassay, submitted; Harrison, R.O.; Hammock, B.D. J. Assoc. Off. Anal. Chem., submitted), and a plate reader test should allow further reduction of error. These steps should be taken before a major investment in time, effort, or money is made in an assay system which may later be found to be less than acceptable.

Once developed, any assay system should be optimized to provide the best possible sensitivity. One important variable affecting ELISA performance is the selection of coating antigens. Wie and Hammock (36) showed that prudent choice of coating antigens can significantly improve sensitivity. A useful empirical approach to optimization is described by Hunter and Bosworth (17), consisting mainly of identifying reagent concentrations which provide the highest signal to noise ratio below binding saturation.

Assay application. At this point major differences appear between the historical use of clinical immunoassays and the potential applications of environmental and pesticide immunoassays. Most clinical assays have been applied to simple or well defined and consistent matrices such as urine or serum. In contrast, most matrices likely to be analyzed for pesticides are more complex, less well defined, and more variable. The potential for serious problems with matrix effects in the environmental field is far greater than most clinical immunoassays have encountered. The application of immunoassays to environmental analysis requires sampling strategies, cleanup procedures, and data handling fundamentally similar to those presently in use in any good analytical lab. The critical factor in the success of immunochemical technology will likely be competence

in analytical and residue chemistry rather than immunochemical expertise. There are several considerations unique to immunoassay, such as the tolerance of antibodies for organic solvents or variations in pH or ionic strength, and stability of biological reagents in storage. Sample presentation to the reagent antibody is an important consideration. Immunoassay of lipophilic materials in a lipophilic environment is not likely to suceed without some means of presenting the lipophilic analyte to the hydrophilic antibody. This can be done in most cases using water miscible organic cosolvents or detergents to induce micelle formation. Additionally, the effect of the matrix being analyzed on the antibody used for analysis must be evaluated. If the matrix effects are minimal with little or no sample cleanup, then analysis can be done directly. In addition to the analysis of molinate in rice field water (Figures 2 & 3), immunoassays for several other compounds have been reported which require little or no sample cleanup, including parathion (10), chlorsulfuron (19), paraquat (32), and maleic hydrazide (15).

The sample workup necessary for pesticide residue analysis will vary with each combination of analyte and antibody, each of which may have a different tolerance for the matrix and other factors. The effects of these factors must be considered as with the development of any other analytical technique. Matrix effects for one ELISA system are summarized in Figure 4. While the effect of the matrix on the antibodies in Figure 4 is different for each antibody-solvent-matrix combination, the competitive ELISA standard curves for most of these combinations are similar when expressed as percent of the appropriate control. Some systems may not require extensive adjustment, but this must be tested with each individual system. For example, our molinate assay performs equally well in a variety of water types at high concentrations of molinate (Figure 5). The small difference seen between the buffer and water standard curves in Figure 5 was eliminated by the addition of small amounts of concentrated buffer to water samples to equalize them to the buffer composition.

Assay validation. The validation procedure consists primarily of comparison of the immunoassay method to an existing method and verification of the statistical reliability of the new method according to well established principles in analytical chemistry. A general approach to the problem of validation has been summarized in a useful form by Horwitz (16). Participation of groups such as the Association of Official Analytical Chemists (AOAC) and the U.S. Environmental Protection Agency (EPA) in the official validation process should lead to well defined validation protocols suitable for general immunoassay use. These protocols must deal realistically with matrix effects such as those shown in Figure 4. Demonstration of standard curves in the matrix of interest (such as shown in Figure 5) is important, but is not sufficient to predict success of the method. Biotransformation and variability of matrix effects among samples must also be evaluated by the analysis of field treated samples.

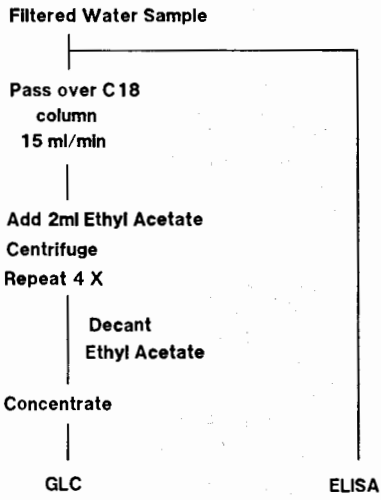

Figure 2. Procedure for extraction and analysis of molinate from water.

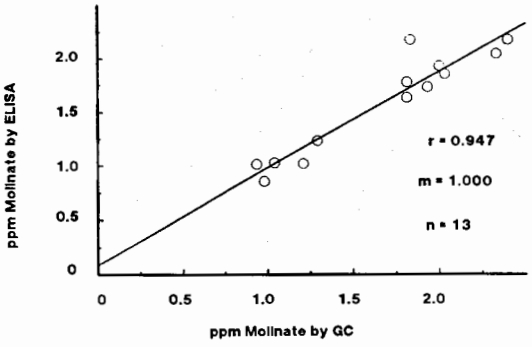

Figure 3. GC vs. ELISA correlation for analysis of molinate field water samples.

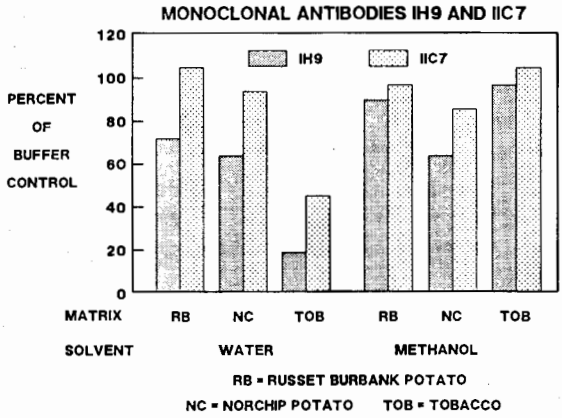

Figure 4. Matrix effects in ELISA of aqueous and methanolic extracts of potato and tobacco using purified anti-maleic hydrazide monoclonal antibodies (modified from ref. 15).

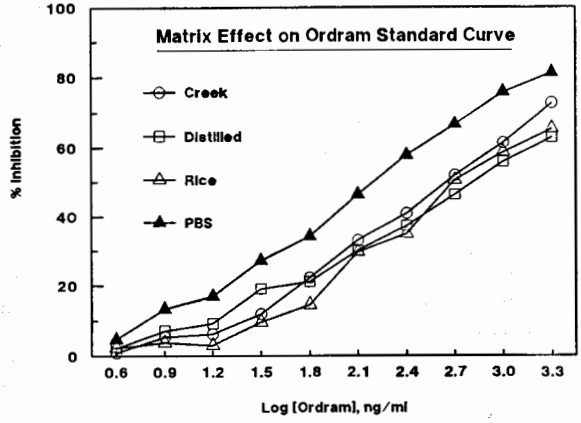

Figure 5. ELISA standard curves for molinate in water from different sources.

Assay formats

Haptens and anti-hapten antibodies are reagents which can be used in a wide variety of assay formats. One fundamental division among immunoassay formats is based on the type of label or tracer which is ultimately detected. Two of the most common types of label are radioisotopes and enzymes, used respectively in radioimmunoassay (RIA) and enzyme immunoassay (EIA). RIA is the older of the two and remains very important and widely used in clinical and research situations. However, the use of radioisotopes carries with it possible health hazards, the need for special handling precautions, and mandatory regulatory oversight. Because EIA avoids these problems, it is gaining popularity rapidly among users of immunoassays.

The format presently favored for pesticide immunoassay is the competitive ELISA or enzyme-linked immunosorbent assay. This is a heterogeneous assay, which is based upon competition between an unknown and variable amount of soluble analyte (the sample) and a small fixed amount of the analyte immobilized on the solid phase, for binding a small amount of soluble antibody. The concentration of analyte in the sample is indirectly measured by the quantitation of bound antibody after it is separated from the free antibody. In contrast, homogeneous assays require no separation step and so can be performed in a single step in one tube. An example of this as applied to pesticides is the Emit analysis of dieldrin and 2,4-D (12). Both heterogeneous and homogeneous assay systems have been used extensively in the clinical field (24,25). Useful comprehensive reviews of the various assay formats have been published (21,24). The choice between these two major classes of assay format is important for several reasons. Homogeneous formats are generally less sensitive, but have fewer steps and are easier, thus lending themselves to field use. Heterogeneous assays are becoming more versatile with the development of many different choices of equipment. There are many different format and equipment possibilities for ELISA alone and selection from among these depends on the needs and budget of the user. Tests can be performed in single tubes or in 96 well microtiter plates using a variety of manual or semiautomatic single-tip or multichannel pipettors. Manual or automatic microtiter plate readers may be used or fully automated systems can be set up which can process thousands of samples per day. A variety of equipment is available for any desired degree of automation.

Assay implementation

The list above is not intended to be comprehensive, but it will serve as a general guide to immunoassay development. We must emphasize again the difference between development and implementation. Assays which work in the laboratory generally require modification before they can be used to analyze field samples. Implementation of assays that have been fully validated for field samples may require little additional commitment by the user, other than analyst training. For the near future, there may be considerable pressure to transfer immunoassay methods to the analytical lab as soon as possible after development, and the

analytical chemist may need to do additional work to adapt and fully validate many assays. This will demand that the analytical chemist develop some immunochemical expertise. Many of the implementation problems likely to be encountered will best be solved with traditional analytical chemistry expertise tempered by an understanding of practical immunochemistry.

Present Use

The use of immunochemical technology for pesticide residue analysis has now advanced beyond the potential stage. Some groups are now using ELISA for real residue data. Dupont has submitted cyanazine (Bladex) ELISA data to EPA for product registration (Sharp, J., Dupont, personal communication, 1987) and the California Department of Food and Agriculture is initiating a program of monitoring rice paddy water for molinate and thiobencarb by ELISA.

Some commercial kits are available now, including ELISA tests for atrazine, simazine, and propazine, and for carbofuran, sold by ImmunoSystems, Inc. for $15 per test in a package of ten tests. These kits offer cost and sensitivity competitive with other methods and can be used quantitatively in the field with a portable spectrophotometer. Other companies have immunoassay kits in various stages of development (20,23).

Several recent events indicate the intense interest in immunoassay for analysis of pesticides and other environmental contaminants. Special symposia on immunochemical technology have been or are being presented by the Association of Official Analytical Chemists at their 1986, 1987, and 1988 annual meetings and by the International Union of Pure and Applied Chemistry at the quadrennial Pesticide Chemistry Congress in Ottawa in 1986 (13,23). Several government agencies have recently solicited proposals for development of immunoassay methods for product or environmental analysis, including the U.S. Food and Drug Administration (8), the Food Safety and Inspection Service of the U.S. Department of Agriculture (7), and the U.S. Army (6). The U.S. Environmental Protection Agency is performing a validation study of an immunoassay method for analysis of pentachlorophenol in water, developed by Westinghouse Bioanalytic Systems of Rockville, MD (18).

Future use

One estimate of the size of the market for non-traditional immunoassays is $24 million for plant diagnostics, $180 million for hazardous chemicals, and $126 million for food testing by the year 2000, totaling $330 million (20). For certain pesticides and hazardous chemicals, the market for analysis may be larger than the market for use. Assays for new compounds will continue to be developed, especially including pesticides which are difficult to analyze by traditional methods. Other environmental contaminants and biotechnology products, including genetically engineered pesticides, will also be excellent candidates for immunoassay. We expect that even if limited to only the present level of technology, immunoassays in some form will soon have a large impact on the analysis of pesticides, hazardous chemicals, industrial by-products, and natural toxins in products and the environment. Novel assay

formats will be developed which offer greater opportunity for field testing, including rapid or instantaneous procedures, such as described by Stanbro et al. (29). Such new technology has great potential for development of multianalyte methods by merely combining multiple antibodies and haptens on a single probe. The use of monoclonal antibodies for these and other immunoassay applications will increase because of specificity, regulatory, patent, or economic considerations. Increasing automation of the entire immunoassay process will occur even in the absence of radically new technology, due to the increasing availability and sophistication of laboratory robots and dedicated automatic immunoassay aids.

Acceptance of immunochemical technology for pesticide residue analysis will become more widespread as more commercial immunoassay products reach the market and more programs utilizing immunochemical technology begin producing tangible results. Increasing realization of the need for high sample loads for thorough environmental analysis will increase the demand for low cost methods of analysis. Official validation of immunoassay methods will accelerate as analytical chemists become familiar with immunoassay and gain experience with these methods in the validation process. This new experience will undoubtedly be accompanied by better control of assay sources of error and better instrumentation specifically designed for quantitative immunoassay. Greater scientific understanding of underlying principles will be gained as a direct result of the need to deal with matrix effects in the analysis of complex samples and this understanding will facilitate the application of immunochemical technology to new analytical problems. All immunoassays, no matter how novel, will continue to depend on the production of specific antibodies as decribed above. This process in turn depends upon rational antigen synthesis, from hapten design and synthesis through conjugation, to novel methods of antibody production, as outlined in this chapter. Regardless of the exact configuration of the final immunoassay, the principles outlined above will continue to be important for the forseeable future.

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