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Review

Pesticide Immunoassays: Experience and Future Perspective in Pakistan

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Abstract. An overview on the use of immunochemical technology for pesticide residue analysis has been described. Pioneering work on the development of pesticide immunoassays (IA) using ELISA technique in Pakistan has been carried out and described in detail. Dieldrin, atrazine and DDT IAs were performed using Enzyme-Linked Immunosorbent Assay (ELISA) kits to determine residual levels of these pesticides in soil and water. ELISA was found to be successful for dieldrin, atrazine and DDT residue analysis. A highly sensitive in-house ELISA has now been developed for atrazine residues determination. Polyclonal antibodies were raised in rabbits by immunization with an atrazine-BSA conjugate and optimized with atrazine-peroxidase (POD) conjugate. It showed middle of the test (50% B/Bo) at 90 ng/l and lowest detection limit (LDL) at 1ng/l in water. For environmental samples, 50% B/Bo was at 75 ng/l and LDL at 4 ng/l. No cross-reactivities were shown by terbutryn, ametryn, des-isopropylatrazine, de-ethylatrazine except terbuthylazine (17%) and hydroxyatrazine (30%). Data obtained by ELISA, significantly correlated with those obtained by HPLC (r = 0.990). It required no clean-up for water samples and less clean-up steps (than HPLC) in soil/coloured extracts, but showed matrix effect. Validation showed good accuracy and precision thereby suggesting that this test can be applied accurately for atrazine detection in water. This IA experience demands future IAs development in Pakistan for commonly used pesticides.

Keywords: pesticides, immunoassay, HPLC

Introduction

Environmental contamination is recognized as a worldwide problem. Part of this problem is caused by the application of pesticides that are being used in agriculture, public health, forestry and animal husbandry. Pesticides are important in modern farming and remain inevitable for the foreseeable future in order to feed such a large world population applying safe agricultural practices throughout the food chain (Dankwardt, 1999; FAO/IAEA Training and Reference Centre). However, food quality is of equal importance to food quantity. Inherently, pesticides show a certain degree of toxicity and especially the less degradable, more persistent compounds such as organochlorine pesticides pose hazardous problems in the environment (Meastroni *et al*., 2001; 1998; Hock *et al*., 1994; Hussain *et al*., 1994a, b, c, 1995; Dreher and Podratzki, 1988; Fenske and Sternback, 1987; Louis and Kisselbach, 1987; Brook, 1974). Over the past few years, there has been an increasing concern about their pollution potential because of their effects on non-target organisms (Moore and Waring, 2001; Dankwardt *et al*., 1998a; Taguchi and Yakushiji, 1988). This concern is expressed in established regulations, norms, standards, monitoring programmes, acts, etc. (EPA, USA, 2008; GEMS/Food, 1997). The concern seems valid since the huge amount of data, generated over the recent years, showed significant influences on the whole ecosystem including commodities like water, soil, plant and animal tissues and more specifically food (EPA, USA, 2008; Ibitayo and Monosson, 2007; Frank and Wilson, 2004; Dankwardt and Hock, 1997, 1993; Jourdan *et al*., 1996; Lawrence *et al*., 1996; Leavitt *et al*., 1991; Chamberlain, 1990; Bushway *et al*., 1989). Widespread occurrence of pesticides in the evironment requires periodic monitoring during seasonal applications (Greenfield *et al*., 2004; WHO, 1997). However, in Pakistan, which is an agricultural country and a variety of pesticides are being continuously sprayed on a variety of crops to maintain agricultural productivity, no documentation regarding routine environmental monitoring for pesticide residues is made. Only pesticide dosages are standardized according to the targeted pests attack and recommended to the end-users (Saleem and Haq, 2003).

Need to develop an ELISA technique. Keeping in view the persistence, toxicity and common use of pesticides, an efficient methodology for their routine monitoring in water, soil, agricultural produce and other samples, indirectly related to these commodities, is of utmost importance. Much effort is therefore, required in the research and investigation concerning development of the new and improvement of the existing methods for pesticide analysis. Conventional analytical methods used by pesticide residue analysts worldwide involve multi-step sample clean-up procedures followed by

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gas chromatography (GC), gas chromatography-mass spectrometry (GC/MS) or high-performance liquid chromatography-mass spectrometry (HPLC-MS) (Aysal *et al*., 2007; Giorgi *et al*., 2005; Aguera *et al*., 2002; Brady *et al*., 1995; Schews *et al*., 1993; Chamberlain, 1990; Ferris and Haigh, 1987; Vermeulen *et al*., 1982). However, these methods are expensive, sophisticated, time consuming, require highly purified gasses and relatively large volume of solvents involving extraction of large volumes of water, expensive instrumentation, extensive purification, and often derivatization is required. For a developing country like Pakistan, these factors become the major problem for routine environmental monitoring, which involves large number of samples. These methods do not cope with the volume of work if strict check on permissible maximum residual limits (MRL) in the environment is required (Dankwardt, 1999). For this purpose, a sensitive, rapid and most important cost-effective technology should be explored. As a consequence, attention has been directed to newer methods and immunoassay (IA) is likely the best option (Kim *et al*., 2007; Ahn *et al*., 2007a, 2004; Hennion and Barcelo, 1998; Jones *et al*., 1997; Knopp, 1995; Meulenberg *et al*., 1995; Aga *et al*., 1994; Sherry, 1992; Hammock *et al*., 1990).

Immunoassay is not a new technique. In the field of clinical chemistry, it was developed for the measurement of insulin in blood in 1960. Since that time, it had been used for many years in clinical chemistry as a reliable, sensitive, and selective method to determine low concentration of organic compounds, for example, in diabetes, HIV, hepatitis, for analyzing blood, urine, cerebrospinal fluid, saliva, and tissue extracts. Rapid field tests are required to achieve semi quantitative or qualitative results and ELISA offers applicability because immunoassay methods of analysis need equipment that are significantly less complex and less expensive than the chromatographic methods. It is also being used for diagnosis of different animal diseases (e.g., haemorrheagic septicemia) and plants diseases e.g., curl viruses of cotton leaf (CLCV) and potato leaf (PLCV).

As IAs are based on the selective and sensitive antibody-antigen (Ab-Ag) reaction, these have become a valuable tool in the field of environmental analysis; especially for screening a large number of samples within a short time (Hall *et al*., 1997; Jones *et al*., 1997; Kramer and Schmid, 1991; Hammock *et al*., 1990, 1987; Hammock and Mumma, 1980; Kemney and Challacombe, 1989). These assays may require less sample clean-up steps and scale because these are specific tests. IAs are being used for determination of different environmental pollutants like xenostrogens, pesticides or mercury (Schollhorn *et al*., 2000; Marx *et al*., 1998; Marx and Hock, 1998; Oosterkamp *et al*., 1997; Mapes *et al*., 1992). The possibility to adapt IAs for environmental studies was recognized more than a decade ago. Since that time, numerous assays have been developed, of which a great deal refers to pesticides. IAs are becoming more acceptable monitoring tools to monitor pesticide contamination. (Ahn *et al*., 2007b, 2006; Meastroni *et al*., 2001, 1998; Matsui *et al*., 2000; Yazynina *et al*., 1999; Haupt *et al*., 1998; Hennion and Barcelo, 1998; Dankwardt *et al*., 1998a, b; Dankwardt and Hock, 1997).

Principle of ELISA methods. Mostly, pesticide immunoassays are based on competitive ELISA where, antibodies (Abs) are adsorbed to a solid phase (e.g., the cavities of a microtiter plate), in a coating step. Immobilization in this case is based on the passive adsorption of the Abs to a plastic surface e.g., polystyrol. After washing in order to remove unbound molecules, hapten (standard or sample) and enzyme tracer are added and incubated for the immune reaction. Hapten and enzyme tracer both compete for the available Abs-binding sites. Therefore, the less hapten is available in the assay, the more enzyme tracer is bound by the Abs. This competing situation becomes apparent after the washing step that follows. After the subsequent addition of enzyme substrate, the rate of substrate conversion is determined, which is proportional to the concentration of the bound tracer and therefore, inversely proportional to the applied hapten concentration. The concentration of the hapten of unknown samples, therefore, can be determined by means of the calibration curve (Maqbool *et al*., 2008, 2002).

For pesticide immunoassays, polyclonal antibodies (pAb), monoclonal antibodies (mAb) and recombinant antibodies (rAb) have been used so far. Polyclonal antibodies (pAb) are obtained from the serum and comprise a mixture of different Ab populations (Garrett *et al*., 1997; Kramer and Hock, 1996a, b; Hock *et al*., 1994). Monoclonal antibodies consist of a single monospecific Ab population. These Abs are produced in cell culture by single hybridoma cell derived from fusion of B-lymphocytes with myeloma cells (Bryne *et al*., 1996; Kohler and Milestein, 1975). The hydridoma cells can then be propagated almost indefinitely in culture and will continue to produce the Ab of the lymphocyte parent. Since an individual lymphocyte produces only a single Ab type, all of the Ab molecules produced by a hybridoma cell line derived from a single hybrid cell, are identical and have same binding properties. Therefore, the hybridoma technology guarantees the unlimited production of mAb with constant characteristics (Hock *et al*., 1995). Due to great labour required for mAb production, many IA are still employing pAb. During the last years a third possibility to create Ab has emerged, i.e. recombinant Ab (rAb) techniques (Kramer and Hock, 1996a, b;

Chaudhary *et al*., 1995). Here, immunoglobulin genes can be cloned, introduced and expressed in expensive and relatively simple host systems. Although several non-mammalian host systems (yeast, plant and insect cells) have been used to produce rAb, the most common vehicle is *E. coli* (Hall *et al*., 1997; Lee *et al*., 1995).

Nevertheless, ELISA technique has not been used widely for pesticide residue analysis. The reasons include, concerns about interference from substances co-extracted with the pesticide (the so-called matrix effects), cross-reactivity to compounds within a group of related chemicals and doubts that it gives sufficiently quantitative results. But immunoassays (IA) offer advantages over chromatographic methods in terms of cost and simplicity and speed where large number of similar samples are involved. Very sensitive competitive immunoassays have also been developed with detection limits between 1 and 50 mg/l for example for the triazine and urea herbicides (Pichon *et al*., 1995; Schneider *et al*., 1994; Giersch, 1993).

Development of ELISA technique for pesticides. ***Pesticide immunoassays*****.** Commercial ELISA kits are relatively expensive (US $ 300-500 per kit). If single rather than triplicate analyses are carried out, this would give up to 90 samples at a cost of about US $ 5 per sample. But very careful pipetting has to be carried out in order to obtain valid data with single replicates. The current cost of a GC analysis is about US $ 150 per sample. However, the price of a kit may still be quite expensive for developing countries like Pakistan (Dankwardt, 1999). If no kits are commercially available or sample numbers are too great to be screened using costly commercial kits, other sources of antibodies and immuno-reagents have to be found. In this regard, these reagents can be produced at the local laboratory scale. A variety of IA has been developed for pesticides.

Commercial as well as in-house developed assays, had been tested in field as well as in laboratory experiments, by several groups. Different groups working in different parts of the world have used ELISA for monitoring of different classes of pesticides like organochlorinates, organophosphates, carbamates, and pyrethyroid (Kim *et al*., 2007; Gao *et al*., 2006; Park *et al*., 2004; Lee *et al*., 2003, 2002; Watanabe *et al*., 2001; Schneider *et al*., 1994; Francis and Craston, 1994; Itak *et al*., 1993; Gee *et al*., 1988). There may be research groups from universities, which have produced surplus antibodies. An example of this approach is one group in Germany (Hock *et al*., 1994; Kramer *et al*., 1994) which has explored immunoassays for pesticides monitoring. Similarly, an American group of scientists has also developed a variety of ELISAs for a variety of pesticides (Van Emon *et al*., 2008; Ahn *et al*., 2007a, b, 2006, 2004; Mak *et al*., 2005; Shan *et al*., 2004; 2000; Lucas *et al*., 1995; 1993; Schneider *et al*., 1992; Hammock *et al*., 1990, 1987, 1980; Gee *et al*., 1988). Even, in India, a group has developed antibodies for cyclodienes, DDT, DDA, carbendazin/benomyl and others (Skerritt and Rani, 1996).

s-Triazine immunoassays**.** Extensive work on ELISA development for detection/quantification of triazine group of herbicides has also been done (Wittmann and Schmid, 1994; Wittmann and Hock, 1993, 1990; Wust and Hock, 1992; Goh *et al*., 1992). In all of these studies, they have used ELISA using polyclonal or monoclonal antibodies with varying detection limits ranging from 1 µg/l to a maximum of 40 µg/l. Field samples were analyzed for cross-reactivity measurements. Many of the developed ELISAs are also commercially available (Dankwardt *et al*., 1998a; Hennion and Barcelo, 1998).

Atrazine immunoassays**.** Typical laboratory analysis for atrazine involves lengthy solvent extraction followed by gas-liquid chromatography (GLC) (Lee and Chau 1983; Sirons *et al*., 1973) or high performance liquid chromatography (HPLC) analysis (Dicorcia *et al*., 1987; Ferris and Haigh, 1987; Vickery *et al*., 1980). Immunoassay techniques have been developed for atrazine, which are relatively inexpensive and can be quickly conducted in the laboratory or at field locations (Dankwardt *et al*., 1998b; Jiang *et al*., 1995; Muldoon *et al*., 1993; Wust and Hock, 1992; Weller *et al*., 1992; Schneider and Hammock, 1992; Van Beveren and Noji, 1991; Wittmann and Hock, 1990, 1989; Bushway *et al*., 1989, 1988; Schlaeppi *et al*., 1989).

Present status in Pakistan. At present, herbicides are extensively used to control weeds in crops. For these herbicides, many ELISA tests have been developed in the environmental monitoring programme. Interest in the use and development of IA techniques for pesticide residue analysis has grown worldwide over the years, but in Pakistan a little work has been done and IAs have yet to be utilized to their full potential (Maqbool *et al*., 2008, 2002, 1998a, b; Maqbool, 2003; Maqbool and Qureshi, 1994). Pioneer studies were initiated to use commercial ELISA kits for pesticide residue analysis. These kits performed successful standardization and residue analysis of dieldrin, DDT/DDE and atrazine in soil and water (Maqbool *et al*., 1998a, b; Maqbool and Qureshi, 1994). These studies lead to thinking that this cost-effective technique should be developed under local environmental conditions, so that all the virtues of this technology can be fully utilized. Although, commercial ELISA tests are cheaper than GC or HPLC, they are still quite costly for developing countries. Production of polyclonal antibodies against pesticides in a developing country like Pakistan can remarkably reduce the price of ELISA kits (Skerritt and Rani, 1996; Dankwardt *et al*., 1995).

For this purpose, studies were initiated to develop and standardize an in-house ELISA kit for *s*-triazine (specifically atrazine) detection and determination at the residue levels in different environmental samples (Maqbool *et al*., 2002). Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) is a *s*-triazine herbicide which prevents the growth of susceptible weed species by inhibition of photosynthesis. Depending on the weed infestation, atrazine may be used alone or mixed with other herbicides to control *s*-triazine resistant weeds or grasses. In Pakistan, atrazine is used in combination with different other herbicides like ametryn [2,4-diamine, N-ethyl- N'-(1-methylethyl)-6-(methylthio)-1,3,5, triazine], cyanazine 2-[(4-chloro-6(ethylamino)-1,3,5-triazine-2yl) amino)-methyl-] and metolachlor [2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) (Saleem and Haq, 2003). Atrazine is degraded by different mechanisms in water, soil, plant, food and environment and immunoassay has been used for its determination in different types of matrices (Dankwardt *et al*., 1998b, 1994; Hardy and Hurburg, 1994; Wittmann and Hock, 1993; 1990; Winklemann and Klain, 1991; Dunbar *et al*., 1985).

The need for validation of newly developed ELISAs either by HPLC or GC/MS is recognized (Dankwardt *et al*., 1997; Brady *et al*., 1995; Dankwardt *et al*., 1995; Mountfort *et al*., 1994; Thurman *et al*., 1990; Penegelley, 1985). This in-house developed ELISA was also validated by comparing it with ELISA itself, (internal validation) and by HPLC (external validation) for its sensitivity, robustness and reproducibility with HPLC for atrazine residue determination in different environmental samples. The details of these studies are as under:

In-house development of ELISA for atrazine residue determination in Pakistan. A highly sensitive enzyme immunoassay has been developed, optimized and evaluated at the laboratory scale for the detection of atrazine residues. The hapten (atrazine derivative) was conjugated to bovine serum albumin (BSA) to obtain an immunizing antigen and to horseradish peroxidase enzyme (POD) to have enzyme tracer. The formation of these conjugations was confirmed by UV spectroscopy as well as by gel-electrophoresis. Coupling was evaluated by the change in UV absorbance i.e., in conjugate, shifting of the peak to a different position as compared with atrazine derivative and BSA. The absorbance increase depends upon the hapten density after coupling. The molar ratios of the conjugate were determined by UV spectra, assuming that the absorbances of BSA and modified hapten were additive. This conjugation procedure yielded 29-atrazine residues/BSA molecule. With this atrazine-BSA conjugate, polyclonal antibodies were raised in rabbits by immunization. An ELISA on microtitration plates was optimized with peroxidase-atrazine conjugate. The middle of the test (50% B/Bo) was found to be at 90 ng/l, which is well below the maximum concentration permitted by the EC guidelines for drinking water. Detection limits for atrazine of about 1 ng/l could be reached. The assay did not require concentration or clean up steps for drinking or ground water samples. Validation experiments showed a good accuracy and precision. No cross-reactivities were shown by other *s*-triazines like terbutryn, ametryn, terbuthylazine, desisopropylatrazine, de-ethylatrazine except terbuthylazine (17%) and hydroxyatrazine (30%) (Maqbool *et al*., 2002).

Internal (by ELISA itself) as well as external validations (by HPLC) of this developed ELISA showed good accuracy and precision for reproducibility. In-house developed ELISA was standardized to monitor atrazine residues in different environmental samples. The standard curve was linear, indicating an increase in log concentration with decrease in absorbance (%B/B0 = 1.075-0.042 Log C; r = -0.966). The middle of the test was at 75 ng/l and the lowest detection limit at 4 ng/l. ELISA significantly correlated with the high performance liquid chromatography (HPLC) (r = 0.990). Internal validation showed good accuracy and precision. Maximum atrazine residues were present in Jehlum River water/sediments and maize/sugarcane plant roots. Most of the food samples were found contaminated. ELISA required less clean-up steps than HPLC, but showed matrix effect in soil/coloured extracts (Maqbool *et al*., 2008).

It is concluded that the newly developed ELISA is sensitive, reproducible, cheaper and require fewer clean-up steps than HPLC for atrazine monitoring in water and low-coloured sample extracts. It significantly correlated with HPLC estimates and detected appreciable amounts of atrazine residues in water, sediment, soil, plant portions and food samples. Water samples were analyzed directly. For low-coloured extract, an increase in settling period, and one-step filtration improved ELISA performance, but for highly coloured matrices, repeated clean-up steps and matrix effect calibrations have to be applied. In contrast, HPLC required filtration, centrifugation, and solid-phase extraction for all types of matrices including water. The running cost of in-house developed ELISA is US$ 3 per sample for triplicate analysis. On this cost/benefit basis, an integrated approach is suggested to use ELISA first for screening and only positive samples should be subjected to instrumental techniques like GC, HPLC or GC/MS. Based on these results, it is suggested that this test can be applied to obtain fairly accurate results for atrazine concentration in water samples from different sources.

Recommendations for future. The strength of immunochemical methods lies in the screening of a large number of samples

within a short time at low costs. Therefore, they can be valuable supplements to conventional analytical methods. Important applications are seen in the analysis of ground and drinking water, where matrix effects are seldom observed. Also, food commodities that turn over quickly are ideal targets for IA measurements. Due to the low costs of one analysis, more replicates from one site can be measured or special sites can be sampled more often, enabling more information about variations of analyte concentrations over sites and seasons (Dankwardt, 1999).

Some restrictions are imposed by the fact that IA is single analyte method. However, new approaches for multianalyte measurements are being undertaken, such as the integration of IA with liquid chromatography (LC). Here, Abs are used in conjunction with LC, e.g., to concentrate an analyte from a large volume of sample and separate it from an interfering matrix (Katmeh *et al*., 1997; Lawrence *et al*., 1996; Kramer *et al*., 1994). In this case, LC uses an immunoadsorbent column before analysis. The immunoadsorbent column contains immobilized specific Abs, which bind the analyte, while interfering substances pass through. The analyte can be eluted by using a pH gradient or an organic solvent (Marx and Geirsch, 1995; Pichon *et al*., 1995). Therefore, large sample volumes with low concentrations of the analyte can be reduced to small volumes with sufficiently high concentrations without co-extracting interfering substances like humic acids or food compounds. This raises the effective sensitivity of the analysis. Antibody mixtures can be used to bind substances from different compound classes, e.g., the phenyl urea herbicides and the triazines. Immunoadsorbents are now commercially available.

When cross-reacting Abs are applied in IA, the obtained signal is not only related to the analyte, but also to similar compounds. This problem can be circumvented by the use of LC prior to the IA. LC-IA was applied by Kramer *et al*. (1994), to determine 4-nitrophenols. The nitrophenols were separated with different LC-systems and determined by IA. LC-IA was about 8-10 times more sensitive than LC with UV detection. Therefore, the integration of LC with IA combines the high separation quality of the LC and the sensitivity of an IA (Lucas *et al*., 1995; Frutos and Regnier, 1993). Another approach is seen in the performance of a homogeneous IA without tracer followed by the separation of the bound and the free reaction partners and the quantification of the bound and the free Ab fraction immuno-LC (Dankwardt, 1999; Hock *et al*., 1995). Hybrid LC/MS systems allow the subsequent identification of the bound analyte. The problem of cross-reactivities inherent to all IA is therefore, resolved by the structural analysis of any cross-reacting compound.

Furthermore, multi-analyte systems are under development. One concept is the microspot IA (Ekins *et al*., 1990), which uses many microspots with fluorescence-labelled Abs of different selectivity immobilized on a ship. After incubation with the analyte (antigen or hapten) a fluorescence-labelled tracer Ab is added. The tracer Ab is either directed against the antigen or consists of an anti-idiotype Ab directed against the binding site of the capture antibody. Sensor and tracer Ab carry different fluorescence labels. Therefore, it is possible to determine the amount of analyte bound to the sensor Ab with optical scanning methods by measuring the signal ratio (radiometric assay). Lately, a variety of non-competitive and competitive microspot analysis systems have been developed, mainly related to the medical field (Chu *et al*., 1997), but are clearly of particular importance in areas such as environmental monitoring. Another possibility is the use of cross-reacting Abs for multianalyte detection. Known cross-reactivities of different Abs can be used to calculate various concentrations of different analytes in a sample containing several contaminants (Muldoon *et al*., 1993). The estimation of the individual concentration is carried out by complex calculating procedures, e.g. by neural networks or iterative procedures (Jones *et al*., 1997; Wittmann *et al*., 1997).

Immunochemical analysis is a fast developing field with numerous possibilities for further improvement. Much effort is put into the development of continuous measurements, such a flow injection immunoanalysis (FIIA) and immunosensors (Rigo and Hock, 1997; Kramer, 1996). A quasi-continuous FIIA of pesticides was developed by Kramer and Schmid (1991) on the basis of a competitive IA. Here, the Abs are immobilized on a membrane. The reaction takes place in the membrane reactor, the central part of the flow injection system. All reagents are sequentially added to the reactor and the product is assayed with the aid of a flow fluorimeter. The measuring range of the flow injection analysis almost equals that of the EIA. Wittmann and Schmid (1994) used an Ab column reactor filled with polystyrene or glass beads with the Ab, immobilized via the avidin/biotin system. This system showed a stable Ab activity for a minimum of 500 measuring cycles. Detection limits for atrazine of about 1 ng/l with pAb and 30 ng/l with mAb could be reached.

Important progress is to be expected in the field of immunosensors where the detectors are based on Ab (Rigo and Hock, 1997). Some relatively simple devices are dipsticks or dot-blots and immunofiltration test. The test principle is the same as for the microtiter plate tests but the reaction time is much shorter due to the high surface area of the membrane and the short distance between reaction partners. Application of remission measurements yields a proportional relationship between

analyte and remitted light. By using a pocket reflectometer, this set-up is ideally suited for field-monitoring purposes (Niessner, 1994). An interesting development is liposome-amplified immunomigration strips (Seibert *et al*., 1995; Reeves *et al*., 1995a). They employed liposome-encapsulated markers which act as single-enhancers of the competitive binding reaction instead of enzymes. These devices have been used for the determination of alachlor (Wittman and Schmid, 1994). If a pesticide of interest is conjugated to a lipid, it can also be incorporated into the liposome structure, leading to a competitive liposome IA (Reeves *et al*., 1995b).

In more complicated systems, the immunological recognition system is immobilized in the direct vicinity of a transducer, an electrochemical, and optical or gravimetric device. They respond to chemical compounds or ions and yield electrical signals, which depend on the concentration of the analyte. Immunosensors with piezoelectric crystals as physical sensors are in a relatively advanced state of development (Minnunni *et al*., 1995). They function as microbalances on to which Ab are immobilized. Other physical sensors use optical systems such as surface plasmon resonance (SPR), interferometry or grating couplers (Brecht *et al*., 1995; Bier and Schmid, 1994). A biosensor employing SPR was used for the determination of atrazine. Bier and Schmid used a grating coupler immunosensor for the determination of terbutryn, a triazine herbicide. A detection limit of 15 nmol/l was established.

Also new strategies for Ab production are being developed. Genetically engineered monoclonal Abs appear very attractive because their selectivity and affinity can be tailored by site directed mutations without requiring new immunizations (Barbas and Burton, 1996). Methods are now provided to rapidly isolate desired clones from Ab libraries and to manipulate individual recombinant antibodies (rAb) to match specific demands of environmental analysis. Binding proteins derived from Ab but consisting only of a part of their light or heavy chain and recombinant Ab fragments (Fab) directed against different *s*-triazines, diuron and parathion have been produced (Bryne *et al*., 1996; Ward *et al*., 1993). In several cases, the detection limit of the rAb was the same as with the parent mAb (Garrett *et al*., 1997; Kramer and Hock, 1996a, b; Karu *et al*., 1994). A promising goal is completely synthetic production of binding proteins or other synthetic receptors, which are fitted to the structure of the analyte by molecular design. The use of libraries guarantees to overcome the bottleneck in Ab production. Also, Ab with special properties such as resistance to matrix effects or organic solvent stability can be selected from the libraries, providing an important contribution to the analysis of water, soil and food samples.

All this review of literature strongly recommends that immunoassays have great potential to play a vital role in environmental protection issues. Such new trends are direly needed in a developing country like Pakistan, where the persons directly engaged with practical pesticide use, are commonly illiterate. They are using these toxicants continuously without any proper training. Sometimes, their illiteracy results in misuse of these toxicants ultimately polluting the whole ecosystem. ELISA development studies have great potential to be explored for most commonly used pesticides such as pyrethroids (extensively used for cotton crop production) and herbicides in drinking water. This type of research has been done extensively in the developed countries (Van Emon *et al*., 2008; Ahn *et al*., 2007a, b, 2006, 2004; Mak *et al*., 2005; Shan *et al*., 2004; 2000; Lucas *et al*., 1995, 1993; Schneider *et al*., 1992; Hammock *et al*., 1990, 1987, Gee *et al*., 1988; Hammock and Mumma, 1980) but no work has been done in Pakistan. This is an area to be explored and researched in future.

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