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Review Paper

Dot Elisa: Immunological Technique for Pesticide Residue Analysis

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Abstract

Pesticides are used globally for enhancing crop yields. However, their excessive use/misuse, especially in the developing countries, results in widespread food and environmental contamination. Therefore current methods such as gas chromatography and high-performance liquid chromatography have been used successfully, with great sensitivity and reliability, for analysis of many pesticides. However, these classical methods require a high capital expenditure and skilled analysts including time-consuming sample preparation steps. Therefore, there is a growing demand for more rapid and economical methods for determining pesticide residues. Immunoassays have recently been emerging as an alternative to traditional methods to meet such demands of fast, sensitive and cost-effective tool for pesticide residue analysis. The dot ELISA is a qualitative ELISA test, which can be performed more quickly without the need of equipments or technical expertise. Dot ELISA is a micro ELISA, utilizing antigen "dotted" onto nitrocellulose filter discs that has been used for more than 25 years. Because of its relative speed and simplicity, the dot ELISA is an attractive alternative to standard ELISA .This technique can even detect at nano-gram scale among targeted compounds in situ.

Keywords: Dot ELISA, GC, HPLC, Immunochemical technique, Pesticide residue.

Introduction

The term pesticide covers a wide range of compounds including insecticides, fungicides, herbicides, rodenticides, molluscicides, nematicides, plant growth regulators and others. Among these, organophosphate (OP) insecticides, are being used successfully for controlling a number of pests. Pesticides are used globally for enhancing crop yields. However, their excessive use/misuse, especially in the developing countries, results in widespread food and environmental contamination. The presence of pesticide residues in food and environment has posed a serious threat to human health and caused a great concern. In order to keep human from being affected from pestides, analytical and monitoring system of pesticide residues in food and environment must be developed. Environmental contamination (inorganic and organic) is recognized as a worldwide problem. Many contaminants can be analyzed using sometimes highly sophisticated analytical techniques. Much effort has been put into research concerning the development of new and improved existing methods for contaminant/pesticide analysis. The methods generally used to measure contaminants are HPLC and GC. These methods require extensive purification, experienced technicians, expensive equipment and reagents. As a consequence, attention has been directed to new methods like immunoassay which seems to be a good alternative, at least for screening purposes. The immunoassay is not new, because it has been used for many years in clinical chemistry as a reliable, sensitive, and selective method to determine low concentrations of organic compounds in, for example, blood, urine, tissue extracts, etc[1,2,3]. In addition, the conventional methods usually require a lot of complex pre-treatment of samples. Therefore, convenient and rapid pesticide detection system is urgently needed. Immunoassay (IA) technology is such an analysis system with simple, rapid and cost-effective characteristics and widely used in pesticides detection.

An "immunoassay" is a quantitative or qualitative method of analysis for a substance which relies on an antibody (Ab), or mixture of antibodies, as the analytical reagent. Antibodies are a class of proteins with the unique ability to bind with high specificity to one or a very limited group of molecules. A molecule that binds to an antibody is called an antigen (Ag). In addition to binding specificity, another important feature of immunoassays is its ability to produce a measurable signal in response to a specific binding. Most immunoassays today depend on the use of an analytical reagent that is associated with a detectable label, such as radioactive elements, enzymes and so on. Immunoassay has a rather long history and has become a widely accepted technique, particularly in the clinical area. The phenomenon of competition between labelled and unlabeled insulin among the diabetic patient was firstly described by using radioimmunoassay and this work was considered as the beginning of modern immunoassay [4].

The use of enzymes for labels in immunochemical reactions dates back to the mid sixties when enzyme-labeled antibodies were applied to the identification and localization of pesticide. The use of enzyme labels in an immunoassay was first published in 1971 by a Dutch group[4].

Development of an Immunoassay (ELISA)

ELISA is a common example of an immunoassay using an enzyme tracer. A test tube or well in a 96-well plastic microtiter plate is coated with a known amount of pesticide (conjugated to the carrier molecule) and so the pesticide is immobilized on the surface of the tube or well. Immunoassays are generally termed as assays that employ antibodies to detect and quantify antigens. In environmental analysis the potential of immunoassays for quick and large-scale screening of contaminants has been recognized and an increasing number of modification of assays have been developed [5, 6]. The main component of immunoassays is the antibody that specifically binds a target molecule. The antibodies used in immunoassays belong to the immunoglobulin's gamma i.e IgG [7, 8]. The antibodies can be derived from antiserum – polyclonal antibodies (PAb) or from hybridomas monoclonal antibodies (MAb) [9-13].

Molecules with both immunogenicity and reactogenicity are called as “complete antigens”, while molecules that only possess reactogenicity are defined as “incomplete antigens” or “haptens”. Pesticide molecules are typical haptens that can induce the immune system to produce antibody only when attached to a large carrier such as a protein. Antibody is the key regent of immunoassay, and can be produced by animal immunization, hybridoma technique or recombinant antibody technique. As to the specific procedure of immunoassay, many kinds of immunoassay format have been developed. However, the competitive immunoassay is the most frequently used one in pesticide immunoassay.

Immunogen/Hapten Preparation

The first step of pesticide immunoassay development is to design and prepare a rational techniques based on the principle of competition between the analyte and a radiolabeled tracer with the same antigenic properties as the analyte. These two components would compete to occupy a limited number of antibody molecules. The amount of analyte present in the sample could be determined when the system reached equilibrium by separating the bound and unbound fractions of the tracer, and measuring the latter for antibody production. The typical procedure to prepare immunogen in pesticide immunoassay system development is to link the pesticide molecules to a carrier protein by a coupling spacer /linker (figure 1).

Glossary

Antibodies	A class of proteins known as immunoglobulines which are produced in response to a foreign substance.
Antigen (or immunogen)	A foreign compound which can be bound by antibodies and simulate their formation, it can be biological and synthetic
Hapten	Small foreign molecules do not induce an immune response but recognize by some antibodies
Monoclonal Antibodies (MAb)	A homogenous antibody population produces by a hybridoma cell line
Polyclonal antibodies (PAb)	A serum sample that contain hetrogenious population of antibodies, varying in specifity and affinity
Recombinal antibodies (RAb)	Antibodies produced by in vitro synthesis (cloning and recombination of DNA)
Hybridoma	The product of the fusion of two different parental cells that contains genomic material from both cells. This cell combination (hybridoma) can produce a single type of antibodies.
Epitope	A specific chemical domain on an antigen that is recognized by an antibody, also called antigen determinant.

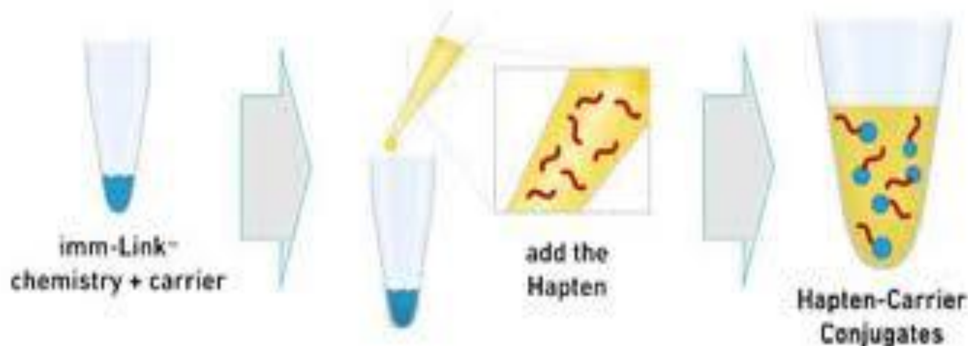

Figure-1
Immunogen preparation

The site of coupling to the carrier, the length of coupling spacers, the selection of optimized carriers, the coupling procedure as well as the number of haptens bound to one carrier molecule can be of major importance for the sensitivity and selectivity of the resulting antibody [14]. Pesticides are usually made up of molecules too small to induce the production of antibodies. Therefore, pesticides must first be conjugated to a larger carrier molecule, often a protein. Once attached to the carrier molecule, the pesticide is called a hapten. The chemical synthesis of the hapten-carrier conjugate is generally considered to be the most important factor in obtaining useful antibodies for analytical use, and the chemistry involved is a major factor in the cost of immunoassay development [15]. The hapten carrier conjugate is then injected into a vertebrate, e.g. a rodent or rabbit, or for large amounts of antibodies, a sheep or goat. The animal will produce an array of antibodies, some will bind to the carrier molecule, some to the hapten-carrier conjugate, and some to the hapten. Only the last of these is useful for developing an immunoassay to detect the pesticide. These antibodies will be heterogeneous because different B lymphocytes may produce antibodies that bind to slightly different sites on the hapten. These antibodies are known as polyclonal because they are produced from a number of different B lymphocyte clones in the animal. They need to be characterized for their affinity for the hapten (the strength of their binding interaction with the hapten) and their specificity (whether they bind only to the hapten or to other related chemicals as well). For most immunoassays the greater the affinity, then the greater the sensitivity of the analysis [16].

Hapten- Protien Cojutation

The desired haptens should be one that hapten-carrier conjugates can induce specific immune response and produce high quality hapten-specific antibodies. While designing/selecting haptens, the most important thing is to ensure the final chemical structure and stereochemistry to be identical or similar with the original haptens (analytical targets). If haptens contain active groups such as -COOH, -NH2 and -OH, they can be directly coupled with the carrier proteins. Otherwise, derivatives of the haptens should be prepared to introduce reactive groups into the structure. In addition, the haptens themselves should possess complicated chemical structures.

Generally, most of these desired haptens are characterized by the following aspects: i. amino group or carboxyl group or both; ii. aromatic compounds; iii. high branch; iv. heteroatom rings. The carrier protein is not only to simply increase the molecular weight of the hapten-carrier conjugate, it can also affect the quality and quantity of immune responses. Bovine serum albumin (BSA), ovalbumin (OVA), keyhole limpet hemocyanin (KLH), and human serum albumin (HSA) are usually used as carriers for the preparation of immunogen. Among these proteins, BSA is the popular one because its physical and chemical stability is higher, not expensive, easily available, having more lysine residues and more amino groups. In addition, BSA can also present excellent solubility under various pH value and ionic strength and react with the targeted haptens in organic solvents such as pyridine and N, N, dimethylformamide (DMF) [17]. Generally, the carrier should be heterogenous with the experimental animals, since it is easier to induce strong immune response and to produce high-titer and hapten-specific antibody.

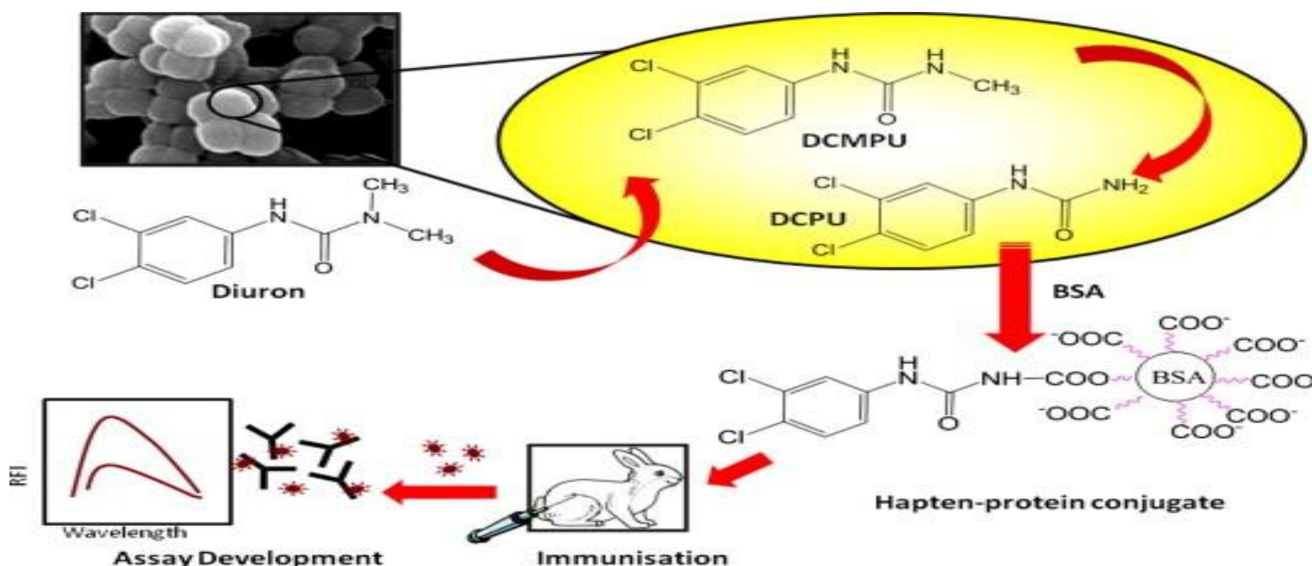

Figure- 2
Hapten protien conjugation

Conjugate Purification

After the artificial hapten-protein conjugate is prepared, a purification step is usually proceeded, since the unreacted hapten molecules, salts and other impuriiesy affect the quality of antibody. Usually, dialysis and chromatography are employed. Comparing the two techniques, dialysis takes long time (usually 2 days or more). However, it can obtain well purified conjugate and the process is simple, which is suitable in various laboratories The conjugate is separated from the uncoupled haptens by dialysis since it results in a well purified antigen and a simple process. Dialysis has earlier been successfully used for separation of hapten-protein conjugates from uncoupled haptens. Bromophos hapten-protein conjugates were separated from the uncoupled haptens by gel filtration (Sephadex G-25), the same has been applied for the separation of chlorpyrifos-protein conjugates. Reports are also available describing the separation of Cyanophos-protein conjugates by gel filtration followed by Dialysis [18.] After purification, it is necessary to carry out an identification procedure with two purposes to confirm whether the desired haptens have been successfully connected on the carriers and how many haptens have been bound to one carrier molecule

Antibody Production

Monoclonal antibody production: The production of monoclonal antibodies can offer some benefits over polyclonal antibodies, but some tradeoffs exist. Monoclonal antibodies are produced through a fusion of mouse or rat B lymphocyte spleen cells with myeloma tumor cells to produce hybridoma cells, a small percentage of which will produce the desired antibody. The spleen is normally taken from an animal that has first successfully produced useful polyclonal antibodies. This process takes a minimum of 3 months before large quantities of antibodies can be produced [19]. Hybridomas can live almost indefinitely and can produce an unlimited amount of homogeneous monoclonal antibodies without interfering antibodies that may exist with polyclonal antibodies and like polyclonal antibodies, hybridomas can be stored in liquid nitrogen and easily distributed between laboratories. Monoclonal antibodies, however, are not necessarily the better choice for a specific immunoassay. Polyclonal-based immunoassay may be adequate for an immunoassay, and in some cases, they are the more sensitive of the two [20]. But for other pesticides, monoclonal may be necessary [20]. Production of monoclonal antibodies requires more time, labor, equipment, and training than polyclonal antibodies and can add 25 percent to development costs [21].

Immunoassay is based upon the specific reaction between an antibody and its corresponding antigen. Antibodies are serum glycoprotein of the immunoglobulin class (Ig) produced by the immune system against foreign materials such as pathogens or xenobiotics, and bind the target substance with high selectivity and affinity. Although there are five distinct classes of antibody in most higher mammals (IgA, IgD, IgE, IgG, IgM), IgG accounts up approximately 80% of the total Ig in human serum. In reality, most immunoassay systems rely upon IgG as the major antibody[21]. (figure.3)

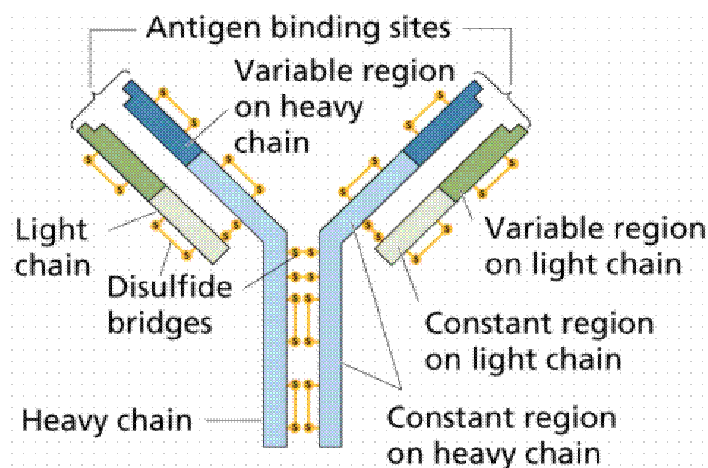

Figure-3
Basic structure of Ag-Ab Immunoglobulin molecule

Ig consists of two identical heavy (H) chains and two identical light (L) chains stabilized and linked by inter- and intra-chain disulfide bonds. The H- and L-chains are organized into variable and constant regions. The antigen binding site (combining site) is formed by the association of parts of the variable regions of the H- and L-chains, located at the amino terminal end. The variable regions of both chains are organized into three hypervariable or complementary determining regions (CDRs) separated by four framework regions. The greatest amino acid sequence variation occurs within the CDRs whereas the framework regions are more conserved. It is assumed that the association of the CDR regions forms the combining site. The lower part of the molecule contains the last heavy chain domains (crystallizable fragment, Fc) which is responsible for some important biological effector functions such as complement fixation and is not necessary for antigen binding. The whole of the Ig molecule or antibody fragments, F(ab)2 and Fab (antibody fragments containing the antigen binding site(s)) can be used in immunoassay. Moreover, recent research results exhibited that the recombinant antibody fragment, scFv (single-chain Fv fragment) can form intact antigen binding site and can also used for immunoassay[21].

Polyclonal antibody production: Antibody production is conveniently carried out in warm blood animals, e.g. rabbits, sheep, mice or chickens. In this procedure, polyclonal antibodies (pAbs) are obtained from the serum and comprise a mixture of different antibody populations.

Response (Immunization)

Rabbits are the species almost exclusively used for pesticide pAbs production, principally because they are of a size capable

of producing adequate amounts of antibodies, have a relatively long life span, and are relatively easy to handle. Usually, adjuvant is used to improve the immune response to the immunogen. After emulsifying the immunogen by adjuvant, this combination can be injected subcutaneously on the back at least 4 sites per rabbit with a maximum of 0.25ml in each site and with the total not to exceed 1ml. The immunization schedule has a decisive influence on the result, and must be rationally designed. Specific recommendations for the interval between primary and booster immunizations are usually not cited. In general, a booster can be considered after the antibody titre has plateaued or begun to decline and 2 to 3 weeks is recommended between each booster injections. In most cases, the endpoint of pAb production should be judged when the antibody titre has reached an acceptable level. This should usually occur after 3 to 5 boosters .Then, the blood of the immunized rabbit can be collected, and serum can be separated for immunoassay development [22].

Dot Elisa: The dot ELISA, a qualitative ELISA test, can be performed more quickly with the end detection done visually. Dot ELISA is a micro ELISA utilizing antigen "dotted" onto nitrocellulose filter discs that has been used from many decades. Because of its relative speed and simplicity, the dot ELISA is an attractive alternative to standard ELISA

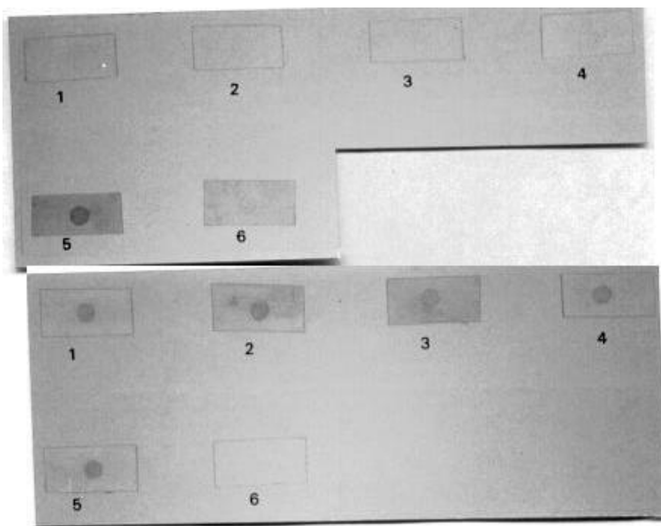

Figure-4
Detection of anti-*Trichinella spirallis* and anti- *Ascaris suum* antibodies in pig sera by Dot-ELISA Pappas *et al* **(1988)**

The dot enzyme-linked immunosorbent assay (Dot-ELISA) is a highly versatile solid-phase immunoassay for antibody or antigen detection. The assay uses minute amounts of reagent dotted onto solid surfaces such as nitrocellulose and other paper membranes which binds proteins. After incubation with antigen-specific antibody and enzyme-conjugated anti-antibody, the addition of a precipitable, chromogenic substrate causes the formation of a colored dot on the solid phase which is visually read. The Dot-ELISA has been used extensively in the detection of human and veterinary protozoan[23]. (figure. 4) The dot enzyme-linked immunosorbent assay (Dot-ELISA), standard ELISA and the complement fixation (CF) tests were compared in the serodiagnosis of African visceral leishmaniasis (kala-azar). Assay sensitivity was determined using sera from 44 patients with parasitologically confirmed kala-azar. [24]. The dot-ELISA assay may be more suitable for routine detection of SRBSDV (southern rice black-streaked dwarf virus) because it is fast, inexpensive and user-friendly. This is the first report that has used a dot-ELISA to diagnose SRBSDV infection. With this method, SRBSDV could be detected in several milligrams of infected rice tissue within 3 hours. Based on our detection data, we found that 55 districts out 61 sampled had an outbreak of SRBSDV disease, indicating the seriousness of the blight to China [25].

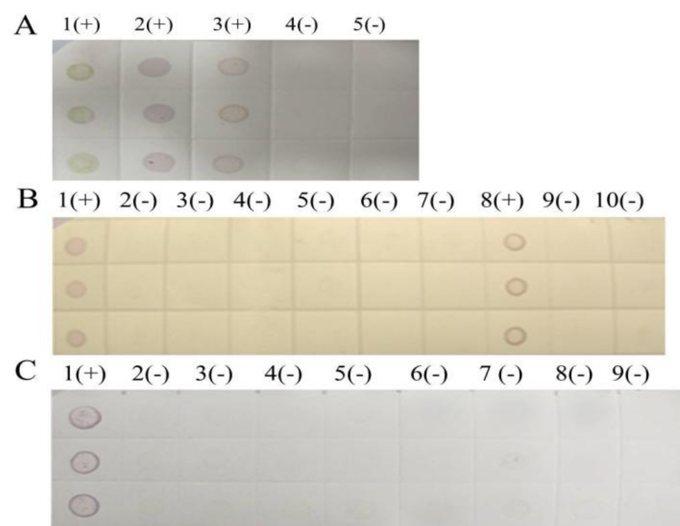

Figure-5
Dot-ELISA test (NC membrane) results of suspected rice plants from Yunnan and Sichuan provinces Zhenchao *et al* (2012)

Comparison of Dot-Elisa with Standard-Elisa

In case of Dot ELISA an unknown amount of antigen is affixed to a surface while a specific antibody linked with an enzyme is washed over the surface allowing it to bind to the surface antigen. A substance is added that triggers a detectible signal from the enzyme. While standard ELISA utilizes a microtiter plate format and require a relatively high amount of reagent and sample, heating, repetitive reagent dispensing, agitation, and spectrophotometers for enzyme detection. Dot ELISA attempt for its qualitativeness and antigens surface treatments with various viscosities. It has positive and negative controls with varying incubation times and it attempt to reduction of noise. Performance of a newly developed Dot-ELISA with that of a previously described Sandwich-ELISA to detect parasite antigens in sera from patients with better sensitivity limits. The same monoclonal antibody and the same sera were used in both tests. In the Dot-ELISA, 67 of 70 sera from microfilaremic donors were deemed to contain filarial antigens when screened at a dilution of 1:50. End titers were 1:80-1:1280. With the

Sandwich-ELISA, 64 of the same sera were positive at a dilution of 1:10 and 42 were positive at a dilution of 1:50. End titers were 1:10-1:320. The specificity of both assays was greater than 95%, but their sensitivity was remarkably different. The Dot-ELISA could detect as little as 0.055 ng/ml microfilarial antigen added to normal human sera, whereas the lower limit with the Sandwich-ELISA was 10 ng/ml parasite antigen. Additionally, the Dot-ELISA does not require radioactivity or sophisticated equipment and, therefore, can be performed in virtually all filariasis-endemic areas [26].

Limitations: Bubble Formation in Channels caused by difference in fluid properties of buffers: i. Non-uniform distribution of reagents, ii. Causes faint lines in results, iii. Reduction in colorimetric signal

Non-specific binding: i. Binding of secondary or non-target molecules on reaction surface, ii. Low signal-to-noise ratio, iii. Better immobilization of monoclonal antibody, iv. Surface modification

Dot ELISA only qualitative

Conclusion

Dot ELISA can be simpler to use than conventional techniques, require less skilled personnel, and require minimal instrumentation time and comparatively inexpensive equipment as well immunoassay can be more portable and simpler to use, they may be adaptable to field use for food. The actual costs of an immunoassay used on food for pesticide analysis versus a conventional method have not been compared but the research is under progress in this area. Despite these advantages, the use of immunoassay for monitoring pesticide residues in food has been constrained by a number of factors. Immunoassays may not be as sensitive for some compounds as conventional methods, and they can have lower levels of reproducibility. Because immunoassays are compound-specific, they are not suitable for multi residue analysis sometime. Therefore, while they may analyze more samples in a given time than multiresidue methods so becoming an important part of immunoassay techniques for pesticide residue detection.

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