Selected Article

Development of Immunoassay Test Kit "SmartAssay Series" for Pesticide Analysis
イムノアッセイによる農薬測定キット「SmartAssayシリーズ」の開発

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Twenty one kinds of direct competitive ELISA test kits were developed for checking insecticides and fungicides. The pesticides are applied just before harvests. Low molecular compounds including pesticides do not have an antibody by producibility themselves. However, some of them produce antibody by binding covalently to the surface of immunogenics protein to innoculate. These compounds are called hapten and anti-pesticide antibodies were also prepared by this behavior. In the basic examinations of hapten design and antibody preparation, it was found that monoclonal antibodies showed higher reactivity to the pesticides than polyclonal antibodies and efficient reaction property to pesticide analysis such as tolerance to organic solvent. Pesticide residue analysis test kits "SmartAssay series" developed based on such a knowledge have been evaluated by researchers as applicable to residual pesticide analysis in farm products. The kits are used for pesticide test before shipment.

農産物の収穫直前に散布される主な農薬(殺虫剤と殺菌剤)21種類について，直接競合ELISAを利用した測定キットを開発した。農薬などの低分子性物質は，それ自体に抗体産生能がないが，免疫原性を持つタンパク質表面に共有結合し接種することで抗体を産生する場合がある。このような物質をハプテンといい，農薬に対する抗体もこの性質を利用して調製した。まずハプテン設計や抗体調製の基礎的検討を行い，モノクローナル抗体が対象農薬と高い反応性を示すほか，有機溶媒耐性など農薬分析に有効な反応特性を示すことを見出した。これらの知見に基づいて開発した農薬測定キット「SmartAssay(スマートアッセイ)シリーズ」は，農産物中の残留農薬測定に適用可能との評価を得，農産物の出荷前検査に利用されている。

Introduction

In Japan where it is hot and humid, various kinds of agricultural pests appear. Especially, if they appear in a harvest period, they reduce the commercial value of farm products. Therefore, it is indispensable to control pests using insecticides or fungicides. All pesticides are used under the registration system and adequate usages for the users are provided by Agricultural Chemicals Regulation Act. However, it is difficult to dispel the cases in which the pesticide residues exceed the standard values and the cases in which unregistered pesticides are detected. On the other hand, in Food Sanitation Act, Positive List System for Agricultural Chemical Residues in Foods has come into operation since May 2006 and a standard value of pesticide residue is set for each farm product per agricultural chemical. Thus, the regulatory environment of pesticide residue in farm products was completed. That leads a high requirement for acceptance inspection for pesticide residue by growers.

For pesticide residue analysis, instrument analysis using

Table 1 SmartAssay Serices Line up

Measurement Item (Insecticides)	Measuring Range (ppb)		Measurement Item (Fungicides)	Measuring Range (ppb)	
	Lower Limit	Limit		Lower Limit	Limit
Acetamiprid	0.30	4.0	Iprodione	1.5	30
imidacloprid	2.0	100	Isoprothiolane	6.0	100
Fenitrothion	0.15	2.0	Myclobutanil	0.2	2.0
Isoxathion	1.0	20	Enilconazole	5.0	50
Chlorfenapyr	2.0	10	Flutolanil	1.0	8.0
Malathion	15	250	Bitertanol	9.0	50
Carbaryl	1.5	30	Triflumizole	2.0	20
Clothianidin	1.5	15	Chlorothalonil	0.15	1.5
Dinotefuran	1.5	30	Azoxystrobin	10	200
Emamectin	0.30	3.0			
Thiamethoxam	0.30	3.0			
Nitenpyram	5.0	100			

gas chromatography (GC) or high-performance liquid chromatography (HPLC) is generally used. Instrument analysis is superior in sensitivity and accuracy, and has the advantage of analyzing simultaneously many compounds in combination with a mass spectrometer. However, as it requires expensive instruments, dedicated installation sites and high analytical technique including the pretreatment of farm products, it was not considered to be suitable for an acceptance inspection in a production site.

Compared with instrument analysis, immunoassay (collective term of analysis method using antibody) using antibody is known as a rapid, easy and economical method. It is used for a routine inspection work in the healthcare field and contributes in diagnosing the clinical condition of patients. Although it was developed also for pesticides, it was used for environmental analysis such as groundwater contamination and was rarely used for the acceptance inspection. This arises from inaccurate results of existing immunoassay caused by matrix influence.

In such a condition, HORIBA, Ltd. found that a direct competitive ELISA (abbreviation of Enzyme-Linked ImmunoSorbent Assay: an immunoassay technique or an analysis method, in which one side of antigen or antibody is bound to solid phase and the liquid phase antigen or antibody is enzyme-labeled for antigen antibody reaction. By washing unreacted substances for removal after the reaction, the enzyme activity bound to solid phase is detected for highly sensitive analysis.), using monoclonal antibody was insusceptible to matrix and was applicable to the inspection of farm products. The 21 kinds of pesticide residue test kits developed on the basis of the knowledge (Refer to Table 1) have been spreading into the acceptance inspection of farm products by growers. In this article, I state the preparation method of antibody for pesticides, development of direct competition ELISA, commercialization of kits and applicability to analysis samples.

Preparation of Antibody

Low-molecular substances such as pesticides are only metabolized and excreted, and never generate any antibody even if they are inoculated into animals. However, if they are covalently-bound to the surface of immunogenic protein before inoculation (immunity), not only the protein but the antibody for the pesticide part may be generated. Such a substance is called hapten and the antibodies against pesticides are also prepared using this behavior. Some pesticides have the functional group that enables a direct binding with protein, however, in many cases, the design of hapten such as newly introducing a functional group (example: carboxyl group) is required. The successful example of preparing the antibody that has high reactivity for subject pesticides by devising the design of hapten is shown in Figure 1. Although the design of hapten differs depending on pesticides, there are several effective designs: (1) like the case of oxamyl, the linker chain in calboxyl group introduction part is stretched to inhibit the interaction with the surface of protein, (2) like the case of malathion, in the living body, an unstable chemical structure is modified and stabilized and (3) like pyrethroid insecticides that have chrysanthemic acid in common, in preparation of group specific antibody, common structure of structural related substance group is bound to protein.[2-4]

Antibody is divided into polyclonal antibody (PoAb) and monoclonal antibody (MoAb). PoAb is the purified antibody component of prepared serum from an immunized animal blood and consists of multiple antibody molecules that have diverse reactions for subject pesticides. On the other hand, MoAb is the prepared antibody derived from an antibody producing-cell in a living body and consists of antibody molecules of which the reactivity to subject pesticides is uniform. As a result of comparing the reactivity of PoAb and MoAb in several pesticides, MoAb always showed higher reactivity than

PoAb. The reason is considered that in PoAb, antibody molecules are distributed, mainly around its 50% inhibition concentration to show the proportional reactivity, on the other hand, in MoAb, the antibodies that show a high reactivity to at the low concentration range could be constantly selected. Additionally, as many pesticides are hydrophobic compounds, it is effective to use for analysis the antibodies that show a tolerance to organic solvents. In PoAb, it is difficult to prepare highly tolerant antibodies because the tolerance varies among antibody molecules, on the other hand, in MoAb, by the screening that uses the tolerance for organic solvents as index, it was possible to select the antibody molecule.[1] From these results, it was decided to use the monoclonal antibody that has high reactivity and tolerance for organic solvents to develop direct competitive ELISA. It was expected that pesticides could be analyzed in a relatively stable way, by the characteristic of monoclonal antibody, under the existence of complex and large quantity of matrix derived from farm products.

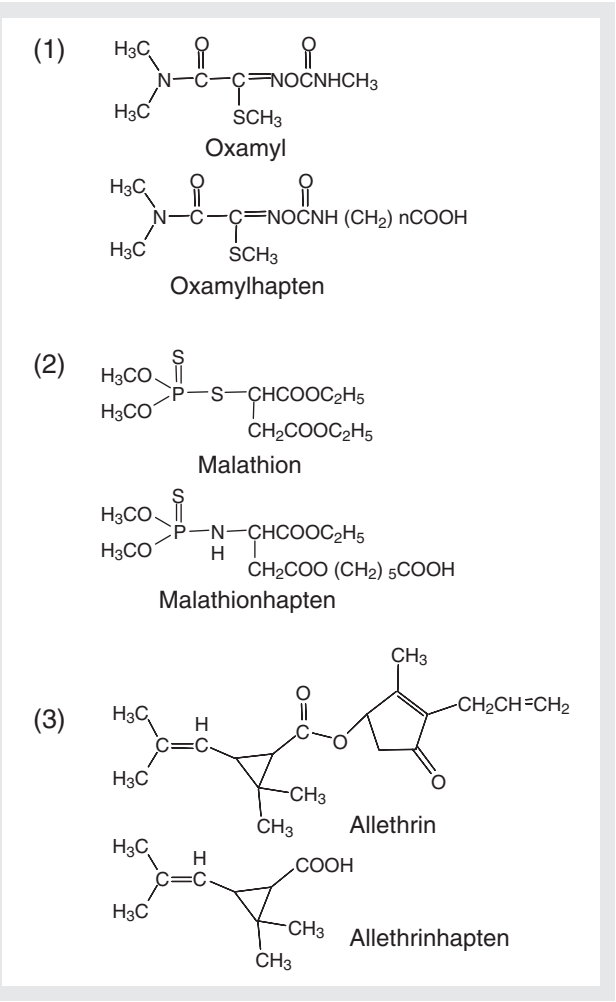

Figure 1 The Structure of Pesticides and Haptens

Direct Competitive ELISA

Direct competitive ELISA determines the concentration of pesticides, using enzyme activity of enzyme labeled hapten bound to antibody after the subject substance and the conjugate (enzyme-labeled hapten) to its hapten and horseradish peroxidase (enzyme) competitively react with the antibodies for them. As the number of reaction steps at analysis is small and it is highly sensitive and accurate, it is used for analyzing diverse low molecule substances. Immunoassay kits for pesticide analysis were also developed by applying the direct competitive ELISA. As shown in Figure 2, the upper analysis flow is the case that any pesticide is contained in the sample and the lower analysis flow is the case that excess pesticide is contained

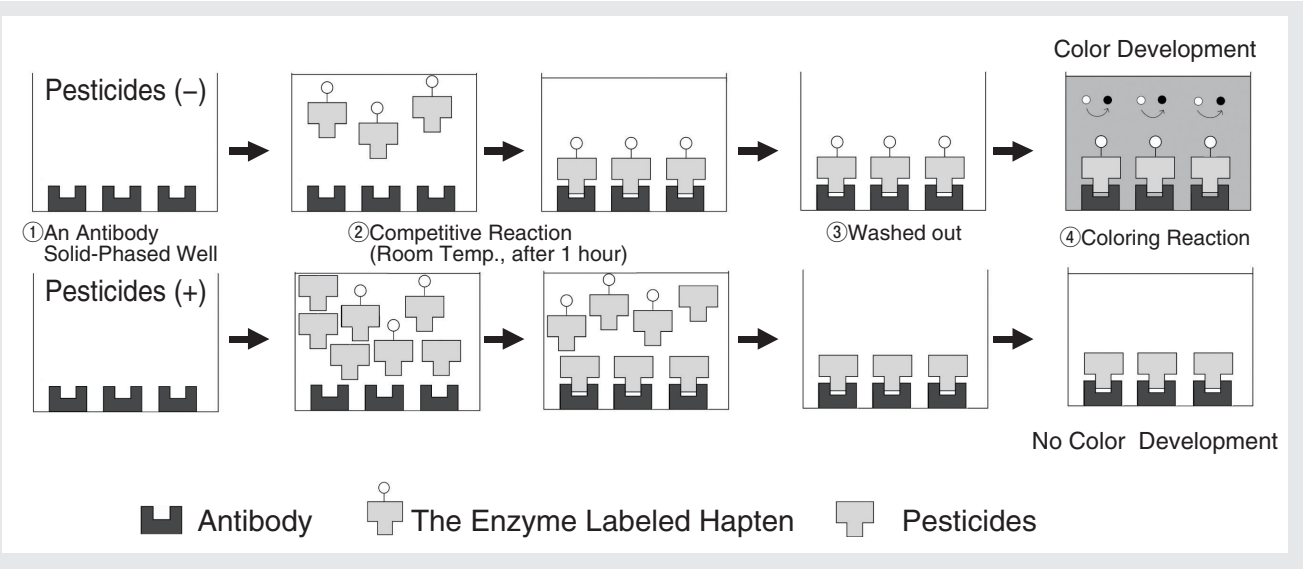

Figure 2 Measuring flow of Direct Competitive ELISA

in the sample. One of the characteristics of direct competitive ELISA is that the coloring level is low depending on the concentration of pesticide if it is within the determination range. The steps of the analysis flow are explained as follows.

A subject pesticide and 10% methanol solution of enzyme-labeled hapten are added in each well of an antibody solid-phased 96 well micro-titer plate for competitive reaction at room temperature for one hour. The concentration of antibody is preliminarily adjusted so that required determination range and absorbance are obtained. The determination range usually depends on the reactivity of antibody and the absorbance depends on the concentration of enzyme-labeled hapten.

After competitive reaction, the wells are washed with normal saline 3 times to remove the enzyme-labeled hapten and the pesticide that did not bind to the solid-phase antibody. Via the antibody, the solution including tetramethylbenzidine is added as the chromogenic substrate of the enzyme bound to the solid phase of the wells. After the chromogenic reaction at room temperature for 10 minutes, 0.5M sulfuric acid is added to stop the reaction. At this time, enzyme is inactivated by diluted sulfuric acid, the degradation of pH changes the color of chromogenic agent from blue to yellow and then the chromogenic reaction stops.

The absorbance after the stop of chromogenic reaction is determined with the dedicated spectrophotometer for micro plate. If the concentration of the pesticide contained in the sample is high, the quantity of the enzyme-labeled hapten bound to the antibody is small and the level of chromogenic reaction becomes low. If the concentration of the pesticide is low, the chromogenic reaction is strong. A calibration curve is drawn with standard pesticide concentration on the x-axis and the corresponding absorbance on the y-axis, and the absorbance of the sample is applied to calculate the pesticide concentration. In Figure 3, the calibration curve (2 point calibration) of dinotefuran test kit and the standard curve by 5 point calibration are illustrated by examples. For a standard curve that is, in general, a sigmoid curve, multiple point calibration is used in most of the cases. However, in a pesticide analysis test kit “SmartAssay Series”, the determination range is the linearity range by 2 point calibration limited to the higher linearity. This is for the purpose of minimizing the number of wells used for calibration curve and increasing the number of samples for analysis. As clarified in the Figures, 2 point calibration ensures the practical accuracy.

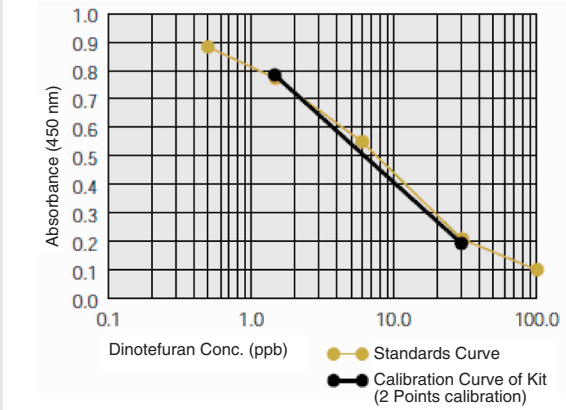

Figure 3 Calibration Curve (Dinotefuran Test Kit)

Commercialization of Kits

If the direct competitive ELISA is made in a laboratory and used for analysis, many preparations are required before analysis. First of all, an antibody diluted to the specified concentration is dispensed to the wells of a 96 well-micro titer plate and the plate is left at 4˚C for a night so that the antibody is solid phased and an antibody plate is formed via blocking. That requires, in general, a day. Concerning enzyme-labeled hapten, concentrated one by 100 times is dispensed into vials and frozen. For analysis, it is fused and diluted. The pesticide standard solution and chromogenic substrate used for drawing calibration curve are easily degraded and should be prepared for each analysis. Moreover, the difference between preparations is often large, which causes inaccurate results.

The kits (See Figure 4) are commercialized for the purpose of omitting these preparations and also stabilizing the reagent. Using the kits, pesticide residue is inspected without a cumbersome preparation for analysis. As the product performance is guaranteed in the specified range, the difference between preparations is considered

Figure 4 SmartAssay Series Nitenpyram Test Kit

in the guaranteed range. Therefore, the accuracy is ensured by verifying the technique of person in charge of the analysis, the analyzer and the analysis condition.

Sample Analysis

Unlike instrument analysis, immunoassay does not basically require a cleanup in the pretreatment of farm products. In the pretreatment condition for vegetables and fruits, first of all, samples are chopped and ground by a mixer for approximately one minute for homogenization. Five gram of the sample is taken to a centrifuge tube, 25mL of methanol is added and the mixture is shaken for 30 minutes for extraction. The extract is filtered and the filtrate is diluted to 7.5 folds with purified water. By this operation, the methanol in the sample is diluted to 10% equivalent. This diluted solution is normally used as an analysis sample for pesticide residue in farm products.

Watanabe and others examined the correlativity with instrument analysis concerning several kits of SmartAssay Series. As the result, a slight difference with HPLC, r=0.913 for green peppers was shown in Imidacloprid test kit. However, the correlativity higher than r=0.979 was shown for cucumbers, eggplants and lettuces.[5] In chlorfenapyr test kit, the reactivitiy higher than r=0.989 was shown for apples and for pears.[6] On the other hand, in chlorothalonil test kit, although the slope of regression line and the GC/MS result of the combination of GC and the mass spectrometer placed a disproportionate emphasis on the immunoassay side, it showed a high correlativity (r=0.986). The reason why the slope placed a disproportionate emphasis is considered that as chlorothalonil is an unstable pesticide in farm products developed, the degradation developed in the pretreatment of GC that consists of many processes.[7] Watanabe and others consider that immunoassay is a promising means as easy and rapid screening test.

On the other hand, even in Smart Assay Series, the cases in which the farm products derived matrix extracted with pesticides interrupts a normal reaction have been reported in scientific meetings. The effects and how to avoid them are written in detail in the technical description. The effective methods are (1) dilute the sample again with 10% methanol, (2) filter the sample by ultrafiltration membrane and (3) clean up the sample with activated carbons or a mini column. Amano summarizes that the better use after understanding the features enables the kits to maximize the potential. Smart Assay Series have been developed using the monoclonal antibody that shows the high reactivity and organic solvent tolerance. As a result, pesticide residue analysis in farm products has been realized by inhibiting the effects of the farm products-derived matrix. However, it is true that some combinations of kits and farm products are affected by the matrix. The users have to verify the effects and how to avoid them beforehand.

Conclusion

Immunoassay, compared with instrument analysis, has the advantages such as (1) Expensive instruments are not required., (2) Its pretreatment is easy and rapid., (3) An advanced knowledge is not required. (4) Many samples are simultaneously analyzed. Therefore, it is effective for the pesticide residue test in the consolidating and shipping areas where many samples are tested before shipment of farm products. In fact, tests before shipment using Smart Assay Series have been diffused in the places, mainly agricultural cooperatives or wholesale markets. In addition, the products have started to be used in the fields where the target pesticides are clarified, such as in efficacy test of pesticides, residual efficacy test or environment monitoring.

As above, the hapten design, antibody preparation together with the development of kits by the direct competitive ELISA and its applicability are outlined. It is a pleasure of the author that this article makes understand the readers that pesticides are rapidly and easily determined at high sensitivity and is informative for the users to use the kits for pesticide analysis.

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