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Analytical evaluation of enzyme-linked immunosorbent assay for neonicotinoid dinotefuran for potential application to quick and simple screening method in rice samples

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ABSTRACT

The analytical performance of a kit-based enzyme-linked immunosorbent assay (ELISA) for the determination of a neonicotinoid insecticide dinotefuran residue in rice samples is addressed. The sensitivity (I_{50} value) was 5.4 ng/mL, with the limit of detection, 0.6 ng/mL and the dynamic range from 1.0 to 30 ng/mL. The ELISA showed substantially high specificity toward dinotefuran besides clothianidin (184%). For rice samples, dinotefuran was extracted with methanol and the extracts were directly determined with the ELISA because of no significant matrix interference. Good recoveries were observed and ranged from 92.5% to 113.2% with coefficients of variation below 10%. The results obtained with the ELISA correlated well with those by the HPLC method for rice samples ($r > 0.98$). These findings strongly indicate that the evaluated and validated ELISA has a potential utility in a quick, simple, and reliable residue analysis, especially a screening method before shipment contributing to food safety.

1. Introduction

Dinotefuran, (*RS*)-1-methyl-2-nitro-3-(tetrahydro-3-furylmethyl)guanidine, which was selected as a target pesticide in this work, is a new furanicotinyl insecticide, which represents the third generation of neonicotinoid insecticides. Although the insecticide acts as an agonist of insect nicotinic acetylcholine receptors by binding to postsynaptic nicotinic receptors in the insect central nervous system [1], it is postulated that dinotefuran affects the nicotinic acetylcholine binding in a mode that differs from other neonicotinoid insecticides [2]. On the basis of its action, the insecticide is widely used for the control of various harmful pest species such as plant bug, plant hopper, green rice leafhopper, and so on [2], and especially shows prominent effect on pest insects resistant to conventional insecticides such as organophosphates, carbamates, and pyrethroids.

In Japan, dinotefuran was registered for application to several agricultural products in 2004, and then the MRLs were established by the Ministry of Health, Labour and Welfare [3] based on the acceptable daily intake (0.22 mg/kg body weight/day),which was estimated by the Food Safety Commission [4]. The insecticide is applied to wide agricultural products, and especially has been frequently used in rice cultivation by nursery box application, unmanned helicopter application, or aerial application [2]. Due to its high insecticidal activity at very low application rates, and its safety for humans, mammals, fowls, and the environment [2], dinotefuran has been attracting interest as a promising insecticide.

Current analytical methods for the determination of dinotefuran residues are high-performance liquid chromatography (HPLC) equipped with ultraviolet (UV), diode array (DAD) [5] or highly sensitive mass spectrometric (MS) detector [6–10]. Generally, these chromatographic methods are suitable for determinations of pesticide residue in various matrices at trace levels because of its high accuracy and sensitivity. However, it is no exaggeration to say that the residue analysis for dinotefuran in agricultural samples has not been well-established yet because some of the previous developed methods would be insufficient to utilize as the means for the determination of dinotefuran due to the low sensitivity [7] or the unsatisfactory recovery from spiked agricultural samples [8,9] besides a few systematic determinations for neonicotinoid insecticides [5,10] and a simultaneous multiresidue determination for various types of pesticides [6].

Unlike these chromatographic methods, enzyme-linked immunosorbent assay (ELISA) methods offer another effective approach to pesticide residue analysis because of several advantages, (1) high analyte selectivity (specificity), (2) high throughput

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of samples, (3) reduced need for sample pre-treatments, and (4) increased cost-effectiveness for large numbers of samples [11]. Moreover, no complicated and sophisticated instrumentation is required. Therefore, it is expected that ELISA methods display great ability as a quick and simple screening method for pesticide residues in agricultural products. However, there are also several disadvantages that ELISA methods are susceptible to interferences from organic solvents and sample matrices [11].

The present work aims to evaluate the analytical performance of the newly developed kit-based ELISA for determination of dinotefuran residues and the analytical reliability using rice as a model sample, and to suggest the potential utility in a quick and simple screening method of agricultural products.

2. Experimental

2.1. Chemicals and materials

Pesticide-grade dinotefuran with a purity of 99.9% (by HPLC) was purchased from Hayashi Pure Chemical Ind., Ltd. (Osaka, Japan). Other neonicotinoid analogues (clothianidin, thiamethoxam, imidacloprid, acetamiprid, nitenpyram, thiacloprid, and thiacloprid-amide) for cross-reactivity studies were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Kanto Chemical Co., Inc. (Tokyo, Japan). Three kinds of dinotefuran metabolites (1-methyl-2-nitroguanidine, MNG (99.5%), 1-methyl-3-(tetrahydro-3-furylmethyl)guanidine, DN (99.5%) and 1-methyl-3-(tetrahydro-3-furylmethyl)urea, UF (99.7%)) were gifts from Mitsui Chemicals Agro, Inc. (Tokyo, Japan). Stock solutions of dinotefuran (1 mg/mL) were prepared in methanol for ELISA analysis and in acetonitrile for HPLC analysis, and were kept at 4 °C when not in use. Working standard solutions of various concentrations were prepared every time by appropriate dilutions of stock aliquots in water/methanol (9:1, v/v) for ELISA analysis and water for HPLC analysis. HPLC-grade and pesticide residue analysis-grade organic solvents were supplied from Wako Pure Chemical Industries, Ltd. HPLC-grade water was produced with a Milli-Q water purification system (Millipore, Bedford, MA). Chem Elut solid-phase extraction (SPE) cartridges packed with about 17 g of diatomaceous earth material were purchased from Varian (Harbor City, CA). Envi-Carb/NH_2 SPE cartridges packed with 500 mg of graphitized carbon black and 500 mg of aminopropyl silica gel were from Supelco (Bellefonte, PA). The kit-based ELISA for dinotefuran (SmartAssay Series) was from Horiba, Ltd. (Kyoto, Japan).

2.2. Samples

After rice (unpolished) samples obtained from a local supermarket were smashed with a Rotor-Speed Mill P-14 (Fritsch GmbH, Idar-Oberstein, Germany) equipped with sieve ring (0.08 mm), the absence of dinotefuran and potential cross-reactant residues had been beforehand verified by HPLC analysis.

Spiked rice samples were prepared by adding aliquots of diluted standard solutions in methanol for ELISA analysis and in acetonitrile for HPLC analysis, and allowed to set at ambient temperature for 30 min prior to extraction.

2.3. Sample pre-treatment procedures for ELISA analysis

After 5 g of sample was soaked in 5 mL of water for 30 min, 25 mL of methanol was added and then the sample mixtures were vigorously shaken for 30 min. Next, they were centrifuged at 3000 rpm for a few minutes, and to 1 mL of the supernatants were added 7.5 mL of water to adjust methanol concentration to 10%. If necessary, the prepared extracts were additionally 2-fold diluted with water/methanol (9:1, v/v).

2.4. ELISA analysis

Working standard solutions and diluted sample extracts were analyzed according to the following procedure: 150 μL of either working standard solution or sample extract was added to borosilicate glass tubes, followed by 150 μL of horseradish peroxidase (HPR)-labeled dinotefuran (HRP-conjugate) solution. After the well-mixed solutions (100 μL/well) were added to the ELISA plate pre-coated with an anti-dinotefuran monoclonal antibody in duplicates at least, the wells were covered with plate seal to minimize evaporation and incubated at ambient temperature for 1 h. After incubation, the seal was removed, and the wells were washed with a washing solution containing detergent using a Nunc-Immuno Wash eight-microplate washer (Roskilde, Denmark) four times and tapped dry. The color was developed by adding 100 μL/well of a color solution containing the substrate, H_2O_2 and the chromogen, 3,3′,5,5′-tetramethylbenzidine. After 10 min at ambient temperature, the enzymatic reaction was stopped by adding 100 μL/well of 0.5 M sulfuric acid, and the absorbance at 450 nm was read by using a PerkinElmer Wallac ARVO HTS 1420 multilabel counter (Turku, Finland). Sample concentrations were calculated by multiplying results estimated from the standard curve by the appropriate dilution factor.

2.5. Sample pre-treatment procedures for HPLC analysis

Sample pre-treatments for HPLC analysis was performed according to the method described by Watanabe et al. [5] with slight modifications. Rice sample (10 g),which was soaked in 20 mL of water for 30 min before extraction was homogenized with 100 mL of acetonitrile for 3 min by means of a Polytron PT2100 homogenizer (Kinematica, Lucerne, Switzerland). After homogenization, the resulting mixture was filtered through a funnel by suction, and then the residue on the funnel was similarly treated with 50 mL of acetonitrile. Both extracts were accurately made up to 200 mL with acetonitrile in a volumetric flask. A 50 mL aliquots of the extract, equivalent to 2.5 g of sample, was concentrated to about 5 mL, and then 10 mL of water was added to the concentrated extract. After the aqueous extract was applied to a Chem Elut SPE cartridge, it stood for 10 min. The cartridge was washed with 100 mL of *n*-hexane, and then dinotefuran was eluted with 200 mL of ethyl acetate. The eluate was concentrated to about 1 mL, and then the residue reconstituted with 5 mL of acetonitrile/toluene (3:1, v/v) was applied to an Envi-Carb/NH_2 SPE cartridge preconditioned with 10 mL of acetonitrile/toluene (3:1, v/v). After dinotefuran was eluted with 20 mL of acetonitrile/toluene (3:1, v/v), the eluate was concentrated to about 1 mL, and then evaporated to dryness by a gentle nitrogen stream. The residue was reconstituted with 1 mL of water, and then the solution was filtered with a PTFE membrane syringe-driven filter unit (0.45 μm, Millipore, Billerica, MA).

2.6. HPLC analysis

The HPLC system consisted of an Agilent 1100 series equipped with a quaternary pump, an autosampler, a column oven, and a DAD. The detection wavelength was 270 nm. The column used was a SunFire C18 (250 mm × 4.6 mm, 5 μm particle size, Waters, Milford, MA) reversed-phase column used in conjunction with a security guard column (20 mm × 4.6 mm, 5 μm particle size). The column oven temperature was kept at 40 °C, and the sample injec-

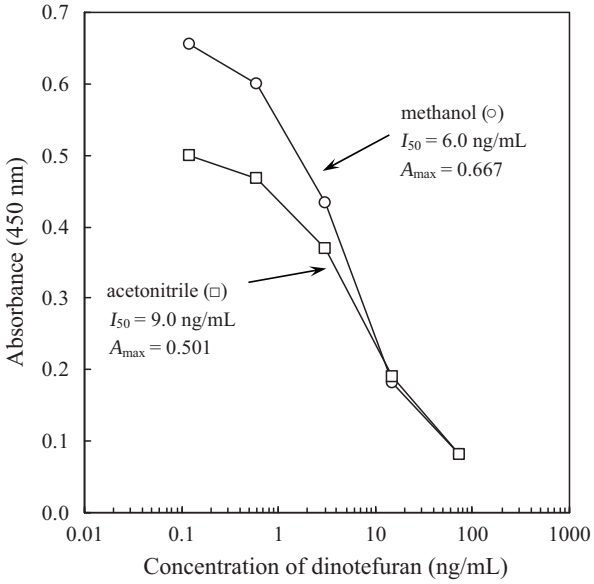

Fig. 1. Selection of most suitable organic solvents for the ELISA, and their influence on the sensitivity of the ELISA and the color development.

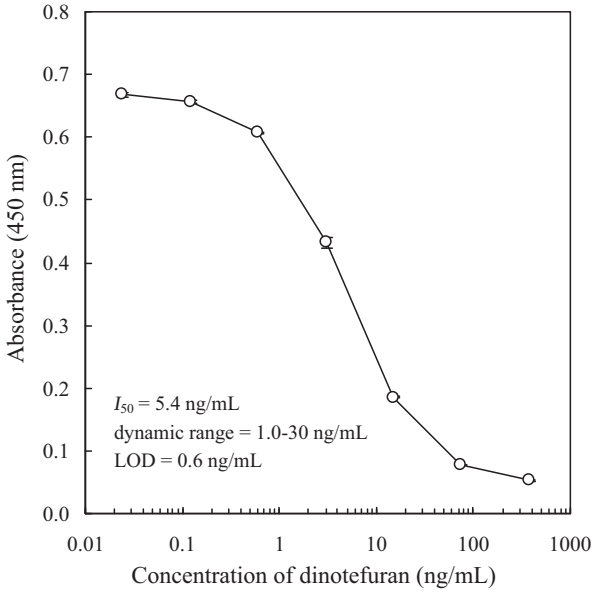

Fig. 2. Standard curve prepared in water/methanol (9:1, v/v) for dinotefuran. Each point is the mean of triplicate determinations.

tion volume was 20 μL. The mobile phase was acetonitrile/water (1:9, v/v), and the flow rate was 0.8 mL/min.

3. Results and discussion

3.1. Selection of best organic solvent for ELISA analysis

Water-miscible organic solvents must be used to quantitatively extract pesticides from agricultural samples. Acetonitrile is often used as a suitable extractant for dinotefuran in HPLC analysis [6–10]. However, because acetonitrile could influence on sensitivity of ELISA for pesticide residue analysis, commonly methanol is often used as a best extractant [12–16].

So, the influence of acetonitrile and methanol on the assay performance was evaluated by preparing standard curves using water containing 5% (v/v) of each solvent as their final concentration in the well. As shown in Fig. 1, the influence of acetonitrile was rather stronger than that of methanol both the sensitivity (I_{50} value) and color development (A_{max} value). Accordingly ethanol, which has little influence on them at 5% (v/v) final concentration, was selected as the most suitable cosolvent for the ELISA. Furthermore, the extraction efficiency of methanol by shaken for 30 min from rice samples spiked with 2 mg/kg of dinotefuran was also investigated by comparing with that of acetonitrile by homogenization twice. The results suggested that methanol (96.2 ± 3.7%, coefficient of variation (CV) = 3.9%) has satisfied extraction efficiency as well as acetonitrile (88.8 ± 0.02%, CV = 0.02%).

3.2. Analytical parameters of ELISA

A seven-point standard curve for dinotefuran prepared with serial working standard solutions in water/methanol (9:1, v/v) based on triplicate determinations is shown in Fig. 2. The I_{50} value, which expresses the sensitivity of the ELISA was 5.4 ng/mL. The dynamic range of the ELISA established between concentrations producing 20% and 80% inhibition was 1.0–30 ng/mL, and the limit of detection (LOD) defined as the lowest detectable analyte concentration based on 10% inhibition was 0.6 ng/mL [17].

Taking account of the required total dilution factor (102-fold) consisted of extraction (6-fold), adjustment of methanol concentration with water (8.5-fold) and equal mix with HRP-conjugate solution (2-fold) described in the Section 2, the dynamic range in agricultural samples converted from the factor was 0.10–3.1 mg/kg. Since the MRL for rice is 2 mg/kg [3], the ELISA can adequately determine the adjacent concentration levels to the MRL.

3.3. Cross-reactivity assay

As shown in Table 1, cross-reactivity of the ELISA was estimated as the percentage obtained by calculating the ratio of the I_{50} value of dinotefuran to that of the given analogue. The results showed that although clothianidin, one of nitroguanidine analogues exhibited higher cross-reactivity (I_{50} = 3.8 ng/mL, 184%) than dinotefuran, the antibody showed no cross-reaction against chloronicotinyl analogues such as imidacloprid and acetamiprid, and other nitroguanidine analogues including major metabolites of dinotefuran in plant, MNG, DN and UF [4]. These suggested that the results obtained from the ELISA show false positive only when remaining clothianidin. It is necessary to distinguish dinotefuran and clothianidin with HPLC, if the analytical data of a sample in which the residue compound was unknown was positive result.

3.4. Matrix effect assessment

In order to evaluate possible matrix interference from rice sample in the ELISA, the standard curves of dinotefuran were run in no

Table 1
Cross-reactivity of the ELISA for dinotefuran toward other structurally related neonicotinoid analogues and metabolites.

Analogue	I_{50} (ng/mL)	Cross-reactivity (%)[a]
Dinotefuran	7	100
Clothianidin	3.8	184
MNG	7100	0.1
DN	>10,000	<0.1
UF	>10,000	<0.1
Thiamethoxam	>10,000	<0.1
Imidacloprid	>10,000	<0.1
Acetamiprid	>10,000	<0.1
Nitenpyram	>10,000	<0.1
Thiacloprid	>10,000	<0.1
Thiacloprid-amide	>10,000	<0.1

[a] Cross-reactivity (%) = (I_{50} value of dinotefuran/I_{50} value of other analogue) × 100.

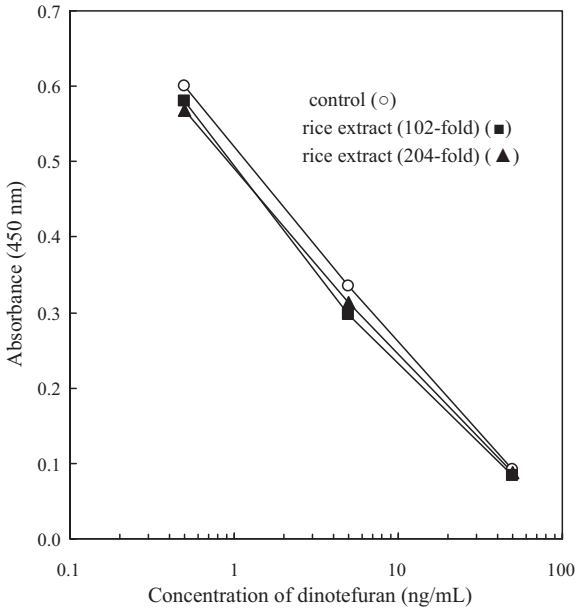

Fig. 3. Influence of rice matrix on the standard curves.

diluted extract and additionally 2-fold diluted extract. The influence of the rice matrix on the standard curves is shown in Fig. 3. Both standard curves prepared in both rice extracts substantially agreed with the control curve done in water/methanol (9:1, v/v), that is, the matrix interference of rice samples were fairly diminished without dilution and with diluted by water/methanol (9:1, v/v) if necessary. The results suggested that dinotefuran could be directly determined in rice samples only giving simple sample pretreatment procedures described in the Experimental section.

3.5. Accuracy of ELISA

A precision study using rice samples spiked with dinotefuran at 2 mg/kg was performed by determination five times in duplicates on five different days. The within- and between-day variations were calculated based on analysis of variance [18]. The CV values within- and between-days were less than 3%. The total CV ($n = 25$) was 3.0%.

The accuracy of the ELISA was investigated by means of mimic samples spiked with dinotefuran at levels ranging from 0.1 to 2.5 mg/kg. The results of these determinations are summarized in Table 2. Repeatability of the ELISA may be considered very good for examined spiked levels, with CV below 10% and the mean recovery values ranging from 92.5% to 113.2%. Therefore, the ELISA investigated here can be considered to quantitatively and reliably determine dinotefuran, and could be used to judge whether a rice sample violates the MRL.

Table 2
Recovery of dinotefuran from spiked rice samples with the ELISA.

Spiked concentration (mg/kg)	Mean recovery (%, $n = 3$)	CV (%)
0.1	113.2	7.7
0.2	98.6	7.9
0.5	99.3	9.3
1.0	97.6	5.4
1.5	96.3	2.0
2.0	101.3	1.2
2.5	92.5	5.1

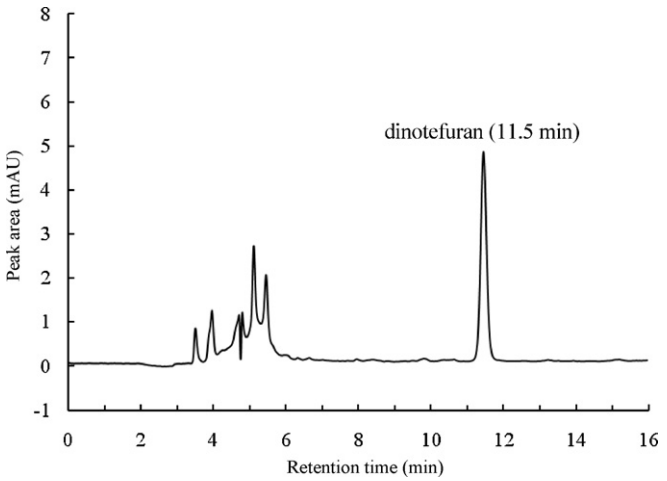

Fig. 4. HPLC chromatogram at 270 nm for an extract of rice sample spiked with dinotefuran at 2 mg/kg.

3.6. Comparison between proposed ELISA and HPLC

Fig. 4 shows a typical chromatogram acquired by HPLC analysis of an extract of rice sample spiked with dinotefuran at 2 mg/kg. Rice matrix does not present interference compounds in the chromatographic area of interest, thus allowing an optimal determination of dinotefuran.

In addition, the results obtained from the ELISA correlated well with the HPLC method ($r = 0.987$) (Fig. 5). The only significant discrepancy between results were the slope of the linear regression analysis of rice samples, which were >1.0. The discrepancy probably is due to dinotefuran losses as a consequence of multistage sample pre-treatment procedures prior to the HPLC analysis rather than overestimation by the ELISA analysis, because the mean recovery values were 100.8% for the ELISA and 89.7% for the HPLC.

The DAD used as the reference method showed a good linearity ($r = 0.9999$) and a high sensitivity (LOD = 0.004 μg/mL, signal-to-noise ratio of 3) in the range from 0.005 to 1 μg/mL. Therefore, it seems that the reason for the unsatisfactory recoveries of dinotefuran could be greatly attributed to the losses during multistage

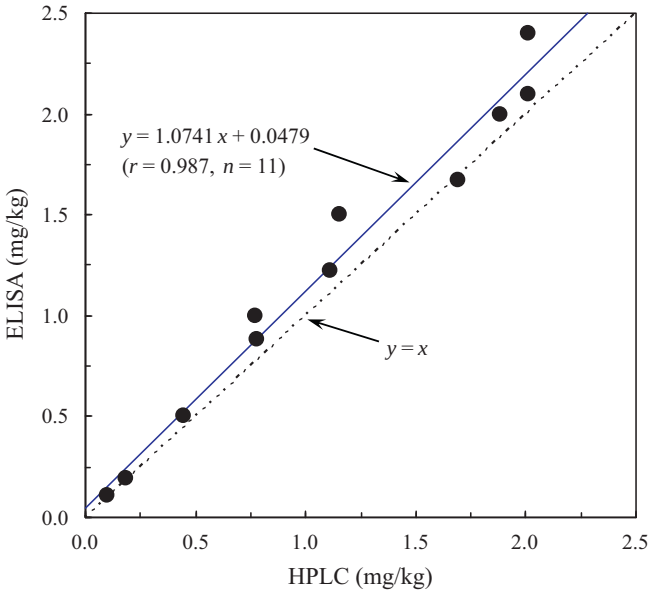

Fig. 5. Correlation between the ELISA and the HPLC methods.

sample pre-treatment procedures rather than the problem in determination with HPLC due to low sensitivity [7]. Accordingly, it is important to secure the accurate recovery by selection of suitable sample pre-treatment procedures for dinotefuran.

We weighed the analytical characteristics of the proposed ELISA with them of the HPLC methods. For the HPLC, 2–3 h are required for the sample pre-treatment. On the other hand, since the pre-treatment for the ELISA analysis finishes only extraction and dilution of sample extracts, about 40 min are required per one sample. The ELISA analysis has practically no burden to environment and health hazard to analysts because of cut in approximately 95% on consumption of organic solvents. Furthermore, the ELISA is possible to cut in approximately 90% on required time for acquirement of analytical results when 40 samples are simultaneously handled as an example. Therefore, the proposed ELISA fulfills the analytical requirements for screening methods for pesticide residues before shipment of agricultural products.

4. Conclusions

Results in the present work clearly prove that the kit-based ELISA is able to determine dinotefuran in rice samples at the adjacent concentration levels to the MRL with accuracy and precision comparable to those obtained with the HPLC. It does not basically require multistage sample pre-treatment procedures such as concentration and clean-up with SPE cartridges prior to the determination because of no interference from rice matrix, and therefore dinotefuran residue in rice samples can be directly determined only giving extraction with methanol and adjustment of methanol concentration in a sample with water. However, due to showing significant cross-reaction toward clothianidin, it is essential to pay enough attention to a false positive caused by the insecticide residue. As mentioned-above, there are little reports on development and evaluation of reliable chromatographic methods for dinotefuran residue in agricultural samples. Therefore, from this point of view the ELISA would be applied in routine analysis for the determination of dinotefuran residue, especially a means for screening method before shipment of agricultural products to markets.

Anyway, we think that ELISAs for determinations of pesticide residues should be ranked as complementary methods to chromatographic analyses. By way of parenthesis, work is in progress to extend the application of the ELISA to further agricultural samples.

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