Development of a direct competitive enzyme-linked immunosorbent assay for determination of the fungicide mepanipyrim and its metabolite

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A direct competitive enzyme-linked immunosorbent assay (dc-ELISA) was developed for determination of anilinopyrimidine fungicide mepanipyrim in vegetables. Two derivatives of mepanipyrim and mepanipyrim propanol type metabolite which carried carboxy acid were synthesized and conjugated with keyhole limpet hemocyanin. BALB/c mice were immunized to prepare anti-mepanipyrim monoclonal antibodies (MoAbs) by obtained conjugates. The dc-ELISAs based on the prepared MoAbs, MPP107 and MPP204, showed working ranges between 0.12 and 1.8 ng/mL with mepanipyrim for MPP107, 0.12 and 2.4 ng/mL with mepanipyrim for MPP204, and 0.2 ng/mL and 5.7 ng/mL with the mepanipyrim propanol type for MPP204. The dc-ELISAs showed the sufficient sensitivity to determine the mepanipyrim residues for the MRLs of 1–15 mg/kg among the majority of vegetables and fruits in Japan. Recovery and/or correlation results from HPLC suggested that the dc-ELISAs would be applicable to the residue analysis of mepanipyrim and its propanol type in vegetables.

Keywords: analysis, mepanipyrim, residue, antibody, immunosorbent.

Electronic supplementary material: The online version of this article contains supplementary material (Supplemental information), which is available at http://www.jstage.jst.go.jp/browse/jpestics/

Introduction

Mepanipyrim, N-(4-methyl-6-prop-1-ynylpyrimidin-2-yl) aniline, is one of the anilinopyrimidine fungicides reported by Nagata et al.10 It is widely used for the control of gray mold (Botrytis cinerea) infecting vegetables, fruits, and flowers.20 After mepanipyrim is applied to garden crops, it is gradually metabolized to 1-(2-anilino-6-methylpyrimidin-4-yl)-2-propanol, called mepanipyrim propanol type.3,4 Mepanipyrim and its propanol type are major residues in vegetables and fruits. The maximum residue limits (MRLs) were therefore defined as the total concentration of them in Japan: 1–15 mg/kg among the majority of vegetables and fruits.5 When detecting only mepanipyrim is the objective, enzyme-linked immunosorbent assays (ELISAs) are also useful methods.8–14 ELISAs are simple, rapid, and inexpensive compared to the above chromatography techniques, as they do not require expensive instruments or sophisticated techniques.15 ELISAs are used especially for on-site residue analysis by farmers.

Some ELISAs were developed for the detection of mepanipyrim and its related anilinopyrimidine fungicides.16–19 They were sufficiently sensitive for mepanipyrim detection but their reactivity with the propanol type was not considered. This study re-

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ports that monoclonal antibodies (MoAbs) reacted with mepanipyrim and/or its propanol type were prepared by improving the hapten design and that the direct competitive (dc)-ELISAs based on their MoAbs could be applied to residue analysis in vegetables.

Materials and Methods

1. Materials
Mepanipyrim, cyprodinil, pyrimethanil, chlorothalonil, and boscalid that were of analytical grade for pesticide residue analysis, as well as keyhole limpet hemocyanin (KLH) were purchased from Fujifilm Wako Pure Chemical Co. (Osaka, Japan). Analytical-grade mepanipyrim propanol type was purchased from Hayashi pure chemical Ind., Ltd. (Osaka, Japan). Bovine serum albumin (BSA; Prod. No. A7888) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Ninety-six-well microtiter plates, a horseradish-peroxidase (HRP)-labeled antirabbit IgG (H+L) antibody from a rabbit, and an anti-mouse IgG (H+L) antibody from a goat were purchased from Thermo Fisher Scientific K. K. (New York, NY, USA). HRP was purchased from Toyobo Co., Ltd. (Osaka, Japan). All other chemicals and reagents used were of analytical grade and were purchased from Fujifilm Wako Pure Chemical Co. or Nacalai Tesque, Inc. (Kyoto, Japan).

2. Preparation of hapten–protein conjugate
The two derivatives in which the carboxy group was introduced to the R1 site of mepanipyrim and its propanol type were synthesized as described in the supplemental information. Their carboxy groups were covalently bound with the primary amine of the lysine residues in each of the proteins (KLH, BSA, and HRP) by the conventional method described previously.13) The prepared hapten–KLH conjugate was used for the immunization of BALB/c mice. The hapten–BSA conjugate was used for constitution of the indirect competitive (ic)-ELISA. The hapten–HRP conjugate was used for constitution of the dc-ELISA.

3. Preparation of antibodies
Polyclonal antibodies (PoAbs) and MoAbs were prepared as described previously.13) In brief, BALB/c mice (7-week-old females) purchased from Japan SLC, Inc. (Shizuoka, Japan) were immunized with 100 µL of the hapten–KLH conjugate (100 µg/mouse). After 1 month, the booster immunization was performed twice at 2-week intervals with 100 µL of the hapten–KLH conjugate (25 µg/mouse). Serum was prepared from blood of the mouse tail vein 1 week after the second immunization. The serum was used as the PoAb.

Three days after the last immunization, spleen cells from the mice were fused with P3-X63-AG8.653 myeloma cells using a polyethylene glycol solution. The fused cells were cultured at 37°C for 7–10 days, and the hybridoma cells secreting anti-mepanipyrim MoAbs were screened using the ic-ELISA. The cultured fluids were used as the MoAb solutions.

The mouse experiments were performed under the guidelines of the Animal Experiment Committee of Kyoto Women’s University following the bulletin (No. 71, 2006) of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

4. ic-ELISA
An ic-ELISA was constituted as described previously.13) A hapten–BSA conjugate (1.0 µg/mL) dissolved in phosphate-buffered saline (PBS; 10 mmol/L phosphate buffer, 150 mmol/L NaCl, pH 7.0) was adsorbed to each well of a 96-well microtiter plate by adding 100 µL of its solution and by incubation at 4°C for overnight. After the solution was removed, 0.4% BSA was added for the blocking. After the microtiter plate was incubated at 25°C for 1 hr, the solution was discarded. Standard solutions of mepanipyrim dissolved in 10% methanol (0.024–10,000 ng/mL) were mixed with an equal volume of each of the PoAbs or the MoAbs, of which final concentrations derived the half of the maximum absorbance in this ic-ELISA, in PBS modified with 0.2% BSA (PBS–BSA). The mixture was added to each well at 100 µL/well. The microtiter plate was incubated at 25°C for 1 hr. After the wells were washed 3 times with PBS modified with 0.02% tween20 (PBS–tween), 100 µL of HRP-labeled anti-mouse IgG antibodies diluted to 2000-fold with PBS–BSA was added to the wells. This was incubated at 25°C for 1 hr. The wells were washed 3 times with PBS–tween, and then 100 µL of the HRP substrate solution (3.3’,5,5’-tetramethylbenzidine 100 µg/mL and 0.006% H2O2 dissolved in 100 mmol/L sodium acetate buffer; pH 5.5) was added to them. This was incubated at 25°C for 10 min. The reaction was stopped by adding 100 µL of sulfuric acid (0.5 mol/L), and the absorbance was measured at 450 nm in an xMark microplate reader (Bio-Rad Laboratories, CA, USA).

5. dc-ELISA
A dc-ELISA was constituted as described previously.13) An anti-mouse IgG antibody from a goat was diluted to 5 µg/mL in PBS. This was adsorbed onto each well of a 96-well microtiter plate by adding 100 µL of the solution and by incubation at 4°C overnight. Each well was blocked, as well as the ic-ELISA. The MoAb solutions were diluted to 25-fold for MoAb MPP107 and 100-fold for MoAb MPP204 in PBS–BSA, of which final concentrations derived the half of the maximum absorbance in this ic-ELISA. The solution was added to each well at 100 µL/well. These were incubated at 25°C for 1 hr to immobilize the MoAbs by an antigen–antibody interaction. The plate was washed once with PBS-tween. Standard solutions of mepanipyrim (0.006–50 ng/mL) or its related chemicals (10 µg/mL) were dissolved in 10% methanol, and the measurement samples were mixed with an equal volume of each of the hapten–HRP conjugates (200 ng/mL for MPP107 and 50 ng/mL for MPP204) in PBS–BSA. One hundred microliters of the mixture was added to the wells and allowed to stand at 25°C for 1 hr. After washing the wells 3 times with PBS-tween, the color development and absorbance measurement were performed as described for the ic-ELISA.
6. Sample preparation for recovery and correlation examinations

Eggplants, cucumbers, lettuce, and green onions were purchased from a market in Kyoto for recovery examination. Each of them was homogenized in a blender (7011HB, Waring, CT, USA). Mepanipyrim or its propanol type was added to the homogenized sample (5.0 g) at a concentration range from 1 to 5 mg/kg in a 50 mL screw-cap tube.

Eggplants, cucumbers, tomatoes, and green peppers were cultivated in an experimental farm at the Institute for Agro-Environmental Sciences for correlation examination. Each (200 g) was harvested 1 day and 7 days after applying 2000-fold diluents of a mepanipyrim formulation containing 40% mepanipyrim (Furupica flowable: Kumiai Chemical Industry Co., Ltd.). These were homogenized and kept at −80°C until use.

For the dc-ELISAs, samples (5.0 g) were combined with methanol (25 mL), vigorously shaken for 30 min and then centrifuged (3000 rpm) at 25°C for 10 min. Obtained supernatant was diluted with distilled water by 8.5-fold to prepare a 10% methanol equivalent solution. The solutions were used as the measurement samples.

For the HPLC analysis, 25 mL of acetone was added to the samples (10 g). The mixtures were extracted by vigorous shaking for 30 min. The supernatant was prepared by centrifugation (3000 rpm) at 25°C for 10 min. The remaining precipitate was then re-extracted with 10 mL of acetone. Both of the extracts were mixed in the flask, and the volume was filled up to 50 mL with acetone. Twenty milliliters of the extract (equivalent to 4 g of the sample) was concentrated to about 5 mL using a rotary evaporator at 40°C. The residue was dissolved with 2 mL of acetonitrile/toluene (3:1, v:v) and then applied to a Supelclean ENVI-Carb/SA (500 mg +500 mg/6 mL; Supelco, Bellefonte, PA, USA). This was eluted with 20 mL of acetonitrile/toluene (3:1, v:v). The eluent was concentrated to about 0.5 mL by using a rotary evaporator and then evaporated to dryness by a gentle nitrogen stream. The dried residue was re-dissolved with 0.8 mL of acetonitrile/water (6:4, v:v). After filtration through a membrane (0.45 µm), a portion of the solution (20 µL) was subjected to HPLC.

7. HPLC analysis

The HPLC system consisted of an Agilent 1100 series equipped with a quaternary pump, an autosampler, a column oven, and a diode array detector (Agilent Technologies Inc., Waldbronn, Germany). The separation of mepanipyrim was performed on a Cadenza CD-C18 (150 mm × 4.6 mm I.D., 3 µm particle size, Imtakt Co., Kyoto, Japan) with a guard cartridge (5 mm × 2 mm I.D., 3 µm particle size). Acetonitrile/distilled water (60:40, v:v) was used in the mobile phase. The flow rate was 0.8 mL/min. The column oven was set at 40°C. Mepanipyrim was detected at a wavelength of 270 nm.

Results and Discussion

1. Hapten design

Derivatives of mepanipyrim and its propanol type, called hapten in this study, are necessary for the conjugation with KLH being prepared for immunization. The carboxy group for the conjugation had been introduced to the R2 site (hapten A) and R3 site (hapten B) by Esteve-Turrillas et al., as shown in Fig. 1, to prepare the specific antibody recognizing the characteristic propyne structure of the R1 site.17) The carboxy group had also been introduced to the R1 site (hapten C) by the same research group, so that the prepared antibody recognized the common structure of the anilinopyrimidine fungicides.19) Their previous

<table>
<thead>
<tr>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>ref.</th>
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<td>mepanipyrim</td>
<td>=C(CH3)</td>
<td>H</td>
<td>H</td>
<td>1</td>
</tr>
<tr>
<td>mepanipyrim propanol type</td>
<td>CH2CH(CH3)OH</td>
<td>H</td>
<td>H</td>
<td>3,4</td>
</tr>
<tr>
<td>cyprodinil</td>
<td>CH2C</td>
<td>H</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>pyrimethanil</td>
<td>CH2C</td>
<td>H</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>hapten A</td>
<td>=C(CH3)</td>
<td>(CH2)2COOH</td>
<td>H</td>
<td>17</td>
</tr>
<tr>
<td>hapten B</td>
<td>=C(CH3)</td>
<td>H</td>
<td>(CH2)2COOH</td>
<td>17</td>
</tr>
<tr>
<td>hapten C</td>
<td>(CH2)2COOH</td>
<td>H</td>
<td>H</td>
<td>19</td>
</tr>
<tr>
<td>hapten 1</td>
<td>=C(CH2OCH2)COOH</td>
<td>H</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>hapten 2</td>
<td>CH2C(CH2)NOCH2COOH</td>
<td>H</td>
<td>H</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Structure of anilinopyrimidine fungicides, mepanipyrim metabolite, and mepanipyrim hapten.
results suggested that the R₁ site, rather than R² and R³, might be a better site for linking with KLH to prepare a group-specific antibody for mepanipyrim and its propanol type. Two kinds of haptns were therefore designed by mimicking each of the R₁ structures in mepanipyrim and its propanol type, as shown in Fig. 1. Hapten 1 was designed by mimicking the characteristic propyne structure of mepanipyrim. Hapten 2 was designed by mimicking the isopropyl structure of the mepanipyrim propanol type. It was expected that the antibody against hapten 2, rather than the one against hapten 1, might also react with mepanipyrim, not only its propanol type, because the isopropanol structure is bulky compared to the propyne structure.

2. Preparation of antibodies reacted with mepanipyrim

PoAbs were prepared by using hapten 1 and hapten 2. As shown in Fig. 2-A, the PoAb raised against hapten 1 reacted with mepanipyrim for which 20%, 50%, and 80% of the inhibition concentration (IC₂₀, IC₅₀, and IC₈₀) values were 2.7 ng/mL, 47 ng/mL, and 1200 ng/mL, respectively, in the ic-ELISA. The PoAb raised against hapten 2 also reacted with mepanipyrim, although the reactivity was 79-fold lower than the PoAb against hapten 1, as expected from the concept of their hapten design.

MoAbs were prepared from the same mouse as for the above PoAbs. The MoAbs from hapten 1 were screened based on the reactivity with mepanipyrim in the ic-ELISA. Among the prepared MoAbs, MPP107 had the highest reactivity with mepanipyrim. As shown in Fig. 2-A, MPP107 showed 43-fold higher reactivity than the hapten 1 PoAb. The phenomenon in which MoAb showed higher reactivity than the PoAb was often experienced, as reported in previous study. The IC₂₀, IC₅₀, and IC₈₀ values of MPP107 were 0.1 ng/mL, 1.1 ng/mL, and 11 ng/mL, respectively. On the other hand, the MoAbs from hapten 2 were also screened based on their reactivity with mepanipyrim, but not its propanol type. It was expected that with this screening procedure, the prepared MoAb from hapten 2 would react with mepanipyrim, not only the propanol type. Among the prepared MoAbs from hapten 2, MPP204 showed the highest reactivity with mepanipyrim, 22-fold higher than the hapten 2 PoAb. The IC₂₀, IC₅₀, and IC₈₀ values of MPP204 were 32 ng/mL, 172 ng/mL, and 780 ng/mL, respectively. However it seemed that MPP204 reactivity with mepanipyrim was too low to apply to the residue analysis of mepanipyrim in vegetables.

3. Reactivity of MoAbs with mepanipyrim and its propanol type in dc-ELISA

Both MPP107 and MPP204 were used for constitution of the dc-ELISA, which is generally applied to the detection of pesticide residues. As shown in Fig. 2-B, the IC₂₀, IC₅₀, and IC₈₀ values of MPP107 with mepanipyrim were 0.12 ng/mL, 0.46 ng/mL, and 1.8 ng/mL, respectively, a range similar to that of the ic-ELISA above. On the other hand, the IC values of MPP204 with mepanipyrim were drastically improved to 0.12 ng/mL, 0.46 ng/mL, and 2.4 ng/mL, which showed 370-fold higher reactivity according to the IC₅₀ value compared to the above ic-ELISA.

The reactivity of MPP204 with mepanipyrim changed to almost the same as that of MPP107, different from the results of the above ic-ELISAs. Such a drastic change had also been found in the ic-ELISA and dc-ELISA for aflatoxin detection. MoAbs were immobilized at the well surface in the 96-well microtiter plate in the dc-ELISA but were dissolved in the liquid phase in the ic-ELISA. It seemed that the immobilization might produce minor structural changes to MPP204 that effectively changed its binding ability.

4. Cross-reactivity of the MoAbs with mepanipyrim and its related chemicals in the dc-ELISA

The IC₅₀ values of mepanipyrim propanol type and the other anilinopyrimidine fungicides were compared to the IC₅₀ value of mepanipyrim in the dc-ELISAs. As described in Table 1, MPP107 was not almost reacted with mepanipyrim propanol type at only 3% of the cross-reactivity but was reacted with cyprodinil and pyrimethanil at 41% and 21%, respectively. The mepanipyrim propanol type isopropanol group has the bulkiest structure among the examined mepanipyrim related chemicals and has a different polarity. This would explain why mepanipy-
Development of dc-ELISA for determination of mepanipyrim

5. Cross-reactivity of MoAbs with mepanipyrim and its structurally related compounds in dc-ELISA

<table>
<thead>
<tr>
<th>Compounds</th>
<th>CR (%)</th>
<th>MPP107</th>
<th>MPP204</th>
<th>CR (%)</th>
<th>MPP107</th>
<th>MPP204</th>
</tr>
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<tbody>
<tr>
<td>Mepanipyrim</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
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<tr>
<td>Mepanipyrim propanol type</td>
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<td>42</td>
<td>&lt;0.005</td>
<td>&lt;0.007</td>
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<td></td>
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<tr>
<td>Chlorothalonil</td>
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<td>&lt;0.007</td>
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</tr>
<tr>
<td>Cyprodinil</td>
<td>41</td>
<td>128</td>
<td></td>
<td></td>
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<tr>
<td>Pyrimethanil</td>
<td>21</td>
<td>73</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boscalid</td>
<td>&lt;0.005</td>
<td>&lt;0.007</td>
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</tbody>
</table>

a) CR (%) shows the reactivity ratio compared to IC₅₀ value of mepanipyrim. Each data is the mean of 3 replicates in independent examinations.

5. Recovery examinations of mepanipyrim and its propanol type

Recovery examinations of mepanipyrim and its propanol type have the lowest reactivity.

On the other hand, the MPP204 prepared from hapten 2 reacted to cyprodinil and pyrimethanil at 128% and 73%, respectively, and to mepanipyrim propanol type at 42%. The structurally unrelated fungicides chlorothalonil and boscalid did not show any cross-reactions. The type and/or the configuration of the amino acids associated with the binding would be different between MPP107 and MPP204.

5. Recovery examinations of mepanipyrim and its propanol type in the dc-ELISAs

It was found that the dc-ELISA based on MPP204 would be applicable to the residue analysis of the total concentration of mepanipyrim and its propanol type. It was further considered that the combination of both of the dc-ELISAs on MPP107 and MPP204 may be the best way to achieve a precise determination.

For confirmation of the applicability to the MRLs of 1–15 mg/kg, as described in Table 2, mepanipyrim was added to the eggplant, cucumber, lettuce, and green onion of which the genus was different. The results showed satisfactory recoveries at a range within 71–120% for almost all of the sample conditions in both of the dc-ELISAs except for green onion, and mepanipyrim was slightly higher (121–129%). Mepanipyrim propanol type was added to the eggplant and cucumber, and both showed satisfac-

Table 2. Recovery of mepanipyrim and its propanol type spiked in vegetables by dc-ELISAs

<table>
<thead>
<tr>
<th>MoAb</th>
<th>Spiked mepanipyrim Type</th>
<th>Eggplant MRL: 5 mg/kg Rec (%)</th>
<th>RSD (%)</th>
<th>Cucumber MRL: 1 mg/kg Rec</th>
<th>RSD</th>
<th>Lettuce MRL: 3 mg/kg Rec</th>
<th>RSD</th>
<th>Green onion MRL: 10 mg/kg Rec</th>
<th>RSD</th>
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<tr>
<td>MPP107 Mepanipyrim 1</td>
<td>83</td>
<td>2.2</td>
<td>93</td>
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<td>80</td>
<td>12</td>
<td>123</td>
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<tr>
<td>2</td>
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<td>10</td>
<td>84</td>
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<td>92</td>
<td>4.6</td>
<td>123</td>
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</tr>
<tr>
<td>MPP204 Mepanipyrim 1</td>
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<td>9.6</td>
<td>112</td>
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<td>102</td>
<td>10.6</td>
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</table>

a) Rec shows recovery (%). b) RSD shows relative standard deviation (%). Each data point is the mean of 3 replicates in independent examinations.

Fig. 3. Correlation results between 2 kinds of dc-ELISAs on MPP107 and on MPP204 (A), between HPLC and the dc-ELISA on MPP107 (B), and between HPLC and the dc-ELISA on MPP204 (C), for determination of the residual mepanipyrim in the incurred samples: eggplant (○), cucumber (▲), tomato (□), and green pepper (◇). Each data point is the mean of 3 replicates; error bars indicate ± S.D.
6. Correlation results among the dc-ELISAs based on MPP107 and MPP204, and the HPLC

Eggplants, cucumbers, tomatoes, and green peppers were harvested after a mepanipyrim formulation was applied. Residual mepanipyrim and its propanol type were determined by using both of the dc-ELISAs. However, the results of the dc-ELISA on MPP107 and the dc-ELISA on MPP204 were almost consistent, as shown in Fig 3-A. The results suggested that the mepanipyrim propanol type did not exist in the examined vegetables samples. As shown in Fig 3-B and -C, the results of the dc-ELISAs on MPP107 and MPP204 highly correlated with the results of HPLC. The results showed that both of the dc-ELISAs developed were at least applicable for detection of mepanipyrim residues in vegetables, and the residual mepanipyrim concentrations in all of the samples were not over the MRLs.

Applicability of the dc-ELISA on MPP204 could not be confirmed by using the incurred samples containing mepanipyrim propanol type from the correlation results. However, it was suggested that the dc-ELISA would be applicable to the residue analysis of mepanipyrim and its propanol type in vegetables based on the recovery results.

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