J. Pestic. Sci., **33**(1), 51–57 (2008)
DOI: 10.1584/jpestics.G07-21

Original Article

Application of commercially available fenitrothion-ELISA kit for soil residue analysis

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(Received July 4, 2007; Accepted October 1, 2007)

A commercially available fenitrothion-ELISA kit, designed for residue analysis of crop samples, was applied in a method development study using 10 different soils spiked with the target pesticide. Recoveries determined by gas chromatography (GC) were compared to those in ELISA analysis. Recoveries in ELISA were biased high in six soil samples which had low pH and high sand content. The range of recoveries in 10 soils was from 87 to 163% while, in contrast, GC recovery was 72–86%. Soil matrix, such as high-molecular-weight organics and divalent cations, influenced the ELISA reaction to cause an overestimation of recovery. © Pesticide Science Society of Japan

Keywords: ELISA, fenitrothion, soil matrix, pesticide residue monitoring.

Introduction

Pesticide application in agriculture for controlling and managing pest problems is a common practice among many users to produce crops of good quality. Because their extensive usage constitutes an important risk for non-target species, the importance of pesticide residue monitoring in the environment has been advocated to ascertain levels of pesticide residues.

Soil remains a depository ground for various pesticides, and receives most of the applied pesticides intended for crops.[1)] In all situations, the use of pesticides could become a serious problem with concerns for environmental safety; therefore, regular monitoring of pesticide residues in soil environments is deemed necessary.

Fenitrothion is a widely used insecticide in agriculture. As an organophosphate, fenitrothion is considered a cholinesterase inhibitor. This compound effectively controls penetrating, chewing, and sucking insect pests on various crops, fruits, cotton, and is also used in public health programs.[2)]

Pesticide monitoring is a simple task, but it is tedious and requires a lot of time for analysis. The conventional method of analysis is the use of gas chromatography (GC) and/or high performance liquid chromatography (HPLC); however, a complicated cleanup method is required to achieve good results with the instruments. Enzyme Linked Immunosorbent Assay (ELISA) for pesticide quantification in environmental samples is an evolving non-chromatographic technique in the field of pesticide residue analysis. ELISA is highly regarded by many researchers for its high precision and good recovery results.[3–6)] The methodological work using commercially produced ELISA kits is simple and requires no complicated clean-up steps, utilizes less harmful solvents, and is feasible for many samples. ELISA assay is considered to be valuable as a screening tool for identifying and categorizing large numbers of samples before decisive GC and HPLC analyses.

Commercially available ELISA kits have been advancing in the field of pesticide residue monitoring activities mainly for crop and water samples. The main objective of this research is to apply a commercially available fenitrothion-ELISA kit, designed for residue analysis of crop samples, to soil samples. We compared the results with those obtained by a standard GC method, and examined the effects of soil matrix on the ELISA reaction.

Materials and Methods

1. *Chemicals and reagents*

An analytical standard (98% purity) of fenitrothion (*O*,*O*-dimethyl *O*-3-methyl-4-nitro-phosphorothioate) was purchased from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan).

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Millipore water was used in the preparation of 10% methanol and for the dilution of soil extracts. An fenitrothion-ELISA kit was purchased from HORIBA, Ltd. (Kyoto, Japan). The kit is in the form of a competitive immunoassay, sensitivity range of 1.5–15 ppb and is exclusively for the analysis of crop samples. The kit uses a 96-well microplate coated with antibody in an 8×12-well format.

2. Soil samples

Ten soils of different types from different sampling points were used for method validation. The soil samples were used after air drying and sieving through 2 mm mesh. The identity and corresponding physico-chemical characteristics of the soil samples are listed in Table 1. Total carbon and total nitrogen contents were analyzed by an N–C analyzer, Sumigraph NC-90A (Sumika Chemical Analysis Service, Ltd., Osaka, Japan). Soil pH and EC were measured by a pH meter, TPX-90i (Toko Kagaku Co., Ltd., Hiroshima, Japan) and a conductivity meter, D24 (HORIBA), respectively. The pH of the soil-methanol extract was measured after tenfold dilution with water. Ca^{2+} and Mg^{2+} ions were measured by Inductively Coupled Plasma–Atomic Emission Spectroscopy, ICPS 200 (Shimadzu, Kyoto, Japan). Particle size distribution in soil was analyzed by the pipette method.[7)]

3. Soil preparation and extraction

Fenitrothion-spiked soil samples were prepared in triplicate by adding fenitrothion working standard solution in acetone at 0.26 μg/g dry soil to each of the 10 g soil samples in a 50 ml polyethylene test tube. The spiked samples were flushed gently with N_2 gas to expel the solvent. The soil samples were thoroughly mixed by gentle agitation, and then placed inside the draft chamber where the solvent was completely evaporated. The extraction of soil samples was followed the method of Suzuki *et al.*[8)] with some modifications. Samples were extracted with 20 ml acetone or methanol by vigorously shaking for 5 min with a vertical mechanical shaker at 280 rpm, and sonicated in an ultrasonic water bath at 38 kHz for 5 min. The former solvent was from the above referred protocol, while the latter was for ELISA and for comparison of recoveries with acetone extraction. The extracts were centrifuged at 10,000 rpm for 10 min, and filtered with a 0.5-μm glass microfiber filter (GC-50, Advantec, Tokyo, Japan). This extraction was repeated 3 times until 60 ml of soil extracts was collected. The soil-methanol extract was fractioned for ELISA and GC analyses. An aliquot was diluted with Millipore water to attain 10% methanol for ELISA to reach the target concentration. The remaining methanol extracts as well as acetone extracts were concentrated on a rotary evaporator to 10 ml, followed by liquid–liquid clean-up twice with 20 ml dichloromethane in 5% NaCl solution. The total organic layer was filtered through qualitative filter paper with Na_2SO_4, followed by concentration to about 1 ml in a rotary evaporator, and dried under an N_2 gas stream. The residue was reconstituted with acetone/hexane; 5 : 95 (v/v), and further cleaned-up with a Sep-Pak silica mini column (Nihon Waters K. K., Tokyo, Japan). The sample was loaded onto the pre-conditioned column and elution of fenitrothion was followed by adding 10 ml×2 of the same acetone/hexane mixture. The cleaned extract was evaporated again and acetone was added, and then transferred to a vial and kept at 4°C until GC analysis.

4. Gas chromatographic analysis

The amounts of fenitrothion recovered in the soil extracts were determined by gas chromatograph with a flame thermoinic detector (Shimadzu GC-14B, Kyoto, Japan). The GC system was equipped with a Rtx-5 column (30 m×0.53 mm i.d., Restek, USA). Parameters were as follows: 250°C (detec-

Table 1. Soil samples and their physico-chemical properties

Soil	Classification[a)]	TC (%)	TN (%)	pH (H_2O)	pH (extract)[b)]	EC (mS/cm)	Ca^{2+} [c)]	Mg^{2+} [c)]	Clay[d)]	Silt[d)]	Sand[d)]
Inbe A	Andosols	8.8	0.38	5.1	5.7	0.32	0.11	0.02	41	22	37
Inbe B	Andosols	2.8	0.22	4.6	5.8	0.76	0.01	n.d.	21	27	52
Inbe C	Andosols	4.6	0.30	5.4	5.8	0.38	n.d.	n.d.	22	33	45
Honjo A	Acrisols	1.2	0.13	5.8	5.5	1.82	0.03	0.01	28	32	40
Honjo B	Acrisols	1.7	0.15	6.2	5.2	0.75	0.19	0.03	18	25	57
Izumo	Arenosols	1.3	0.12	7.5	4.8	0.31	0.45	0.06	2	10	88
Koryo	Arenosols	10.1	1.02	6.4	4.1	5.02	2.84	0.98	5	5	90
Jinzai	Arenosols	3.7	0.31	4.9	4.9	0.20	3.06	0.37	2	10	88
Takano A	Andosols	8.0	0.56	5.3	3.0	0.47	0.12	0.03	4	32	64
Takano B	Andosols	5.6	0.45	5.5	3.8	0.80	0.19	0.04	4	38	58

[a)] Soil classification based on FAO.[16)] [b)] pH of tenfold diluted soil-methanol extracts with water. [c)] ppm in soil-methanol extracts. [d)] Particle size distribution in relative percentage (%).

tor temp), 220°C (column temp), and 230°C (injector temp), and the carrier gas flow was 20 ml/min. The injection volume of the cleaned soil extracts was 2 μl, and the detection limit was 0.05 ppm.

5. ELISA analysis

The analysis of fenitrothion in the ELISA plate was performed according to the manufacturer's instructions. The 15 ppb standard solution was further diluted to 7.5, 3.75, and 1.5 ppb for the preparation of a standard calibration line. Each diluted soil-methanol extract and the fenitrothion standards were mixed with the conjugate solution at 1 : 1 ratio (v/v), then 100 μl of the mixture was loaded in duplicate into the wells. After incubation for 1 hr at 25°C, the solution was removed, the plate was washed 3 times with washing solution, and then 100 μl of substrate solution was added, and kept again at 25°C for another 10 min for color development. Finally, 100 μl of the stopping solution was added, and then kept for 15 min before measuring the absorbance with an UV-VIS microplate reader (SLT Spectra, WAKO) at 450 nm.

6. Soil matrix effect on ELISA analysis

6.1 Soil matrix interferences

Four soil samples, Jinzai, Koryo, Honjo-A, and Izumo, were chosen and further evaluated for their possible matrix interferences. Fenitrothion-unspiked soil samples were extracted with methanol following the same procedure described above. The soil extracts were diluted to 1/10 and 1/100 with 10% methanol and used for the preparation of standard fenitrothion solutions at 3, 6, and 9 ppb. Changes in absorbance in ELISA were plotted as a function of fenitrothion log concentration. The experiment was conducted in duplicate.

6.2 Effect of pH

In addition to measuring the pH of the diluted soil-methanol extracts, the effect of pH on ELISA was examined by adjusting pH from 4 to 6 using fenitrothion standard solutions prepared with 10% methanol. Final concentrations of fenitrothion were set at 3, 6, and 9 ppb, and the experiment was conducted in duplicate.

6.3 Effect of soil humus

The diluted soil-methanol extracts were filtered with High-Performance Centrifugal Concentrators (Apollo 9 kDa, Orbital Bioscences, Topsfield, USA) to remove high-molecular-weight soil organic matter. Fenitrothion standard solutions were prepared using filtered as well as unfiltered extracts. The samples were applied to ELISA to examine changes in absorbance together with a control. The final concentration of fenitrothion was set at 3 ppb, and the experiment was conducted in duplicate.

6.4 Effect of Ca^{2+} and Mg^{2+} ions

To further examine the sensitivity of ELISA, standard solutions of calcium and magnesium ions were prepared in 10% methanol individually. The solutions were used to prepare fenitrothion standard and checked for the change in absorbance together with a control. Concentrations of co-extracted soluble calcium and magnesium ions in the soil-methanol extracts were determined using Inductively Coupled Plasma–Atomic Emission Spectroscopy (ICPS 2000, Shimadzu). The final concentration of fenitrothion was set at 7.5 ppb, and the experiment was conducted in duplicate.

Results and Discussion

1. Recovery of fenitrothion in GC and ELISA analyses

The ELISA kit was assessed by comparing the recovery with the standard GC method. The analysis of fenitrothion in 10 soil samples gave adequate results (Table 2). The percentage recoveries of fenitrothion at 0.26 μg/ġ dry soil were within the acceptable range (70–120%) by methanol and acetone extractions analyzed by GC. In contrast, the percentage recoveries by ELISA were fairly high compared with the GC results. Six out of ten samples in ELISA showed recoveries ranging from 123–163%, which were considered to be an overestimation. The coefficient of variation (CV) values were greater in ELISA (5-26) than in GC (1-12), but there were no differences in CV between samples with and without the overestimation. As the same methanol soil extracts were used for ELISA and GC, the difference in variation seemed to result from methodological reasons.

The high recovery values were probably due to the effects of components in the soil extract which influenced the ELISA antigen-antibody reaction during the course of analysis. The light yellow color of the soil extracts indicated the presence of humus-like compounds in the extracts. Since ELISA is based on antibody binding with enzymes, signal amplification, and colorimetric determination, the presence of humus-like compounds could change the ELISA response. Concerning the effects of humic substances, the ELISA standard curve was

Table 2. Recovery of fenitrothion from spiked soil samples[a)]

Soil	Recovery (%)[b)]		
	GC-acetone	GC-MeOH	ELISA-MeOH
Inbe A	88 (1)[c)]	83 (3)	87 (15)
Inbe B	95 (7)	86 (9)	90 (13)
Inbe C	89 (3)	86 (4)	101 (23)
Honjo A	86 (2)	86 (4)	116 (5)
Honjo B	90 (1)	72 (8)	127 (18)
Izumo	85 (8)	84 (2)	145 (8)
Koryo	85 (3)	79 (4)	161 (12)
Jinzai	92 (3)	79 (1)	163 (26)
Takano A	88 (3)	78 (2)	149 (19)
Takano B	113 (12)	83 (6)	123 (8)

[a)] Fenitrothion was spiked at 0.26 μg/ġ dry soil. [b)] Average values (n=3). [c)] Coefficient of variation.

highly affected by the prepared humic acid.[9] In addition, the effects of pH on ELISA[3,10,11] and soluble inorganic ions[12–14] have been reported.

To clarify the causes of the overestimation, the relationship between the recovery values and several soil parameters was examined. The pH values of the soil-methanol extracts were negatively correlated (Fig. 1a), and sand and clay contents were positively and negatively correlated, respectively (Fig. 1b, c). The Ca^{2+} content appeared positively correlated, but the correlation was inconclusive due to uneven distribution of the Ca^{2+} content (Fig. 1d). Other factors, such as TC, TN, EC and Mg^{2+} in the soil-methanol extracts, showed no significant correlation (data not shown).

2. *Matrix interferences in ELISA*

The overestimation of fenitrothion recovery was further investigated by using four soil samples. In immunoassays, antigen-antibody interactions are generally more susceptible to interference by various soil components such as lignin, lipids, soluble ions, clay minerals, and humic materials. The disadvantage is that the analyte-antibody interaction is governed by weak molecular interactions and is subject to nonspecific interferences *i.e.* matrix effect.[12] Shifting of the absorbance to high or low demonstrates the matrix effect in analysis. Changes in the optical density of prepared standard curves with the four soil extracts revealed that all tested soils caused interference, having lower absorbance than no soil extract (Fig. 2). As usually reported in immunoassays, this peculiar shift is caused by either non-specific alterations of the analyte (antigen)-binding sites of the antibody or adsorption of soil matrix with antibody.[15] Honjo A and Izumo soil extracts showed higher matrix interferences than Koryo and Jinzai. The change in absorbance was reduced by dilution from 1/10 to 1/100 in Koryo at a lower concentration of fenitrothion. Based on the theoretical fenitrothion concentration of 4.4 ppb in diluted soil extracts and on the calibration line at 1/10 dilution in Fig. 2, overestimation of up to 165% was calculated. These figures are comparable to the actual values of the recovery overestimation. All the tested soils indicated various soil matrix interferences able to cause the overestimation.

3. *Effect of pH on ELISA*

Matrix interferences in some samples include not only organic materials co-extracted during analysis but some soluble ions and pH of the extracts could influence the assay reaction.[12] It was noticed for most samples (Izumo, Koryo, Jinzai, and Takano A and B) having a low pH in the soil-methanol extracts that the recovery was overestimated (Table 1). Kim *et al.* reported the effect of altering the pH of the buffer on ELISA, and presented the inhibition of the antibody-antigen recognition site and thus alteration of the absorbance below pH 4.5.[10] The ELISA kit used in this study, however, showed no significant change in absorbance at various pH from 6 to 4, as shown in Fig. 3. It was suggested that pH did not directly affect the reaction and some indirect pH-dependent reactions occurred with soil matrices.

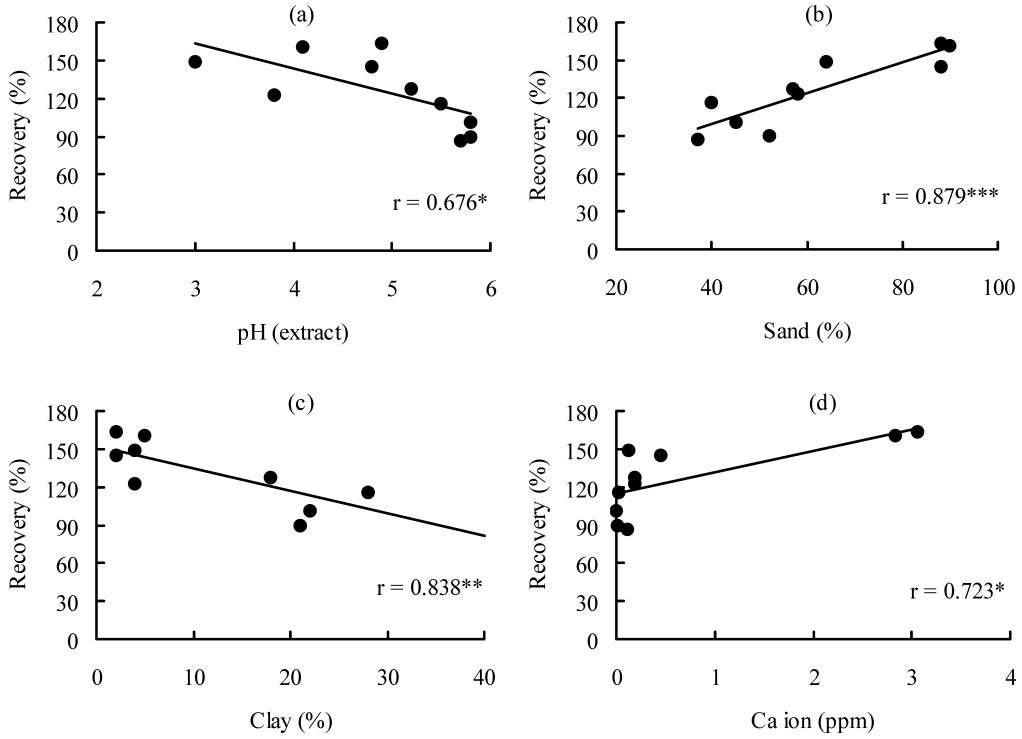

Fig. 1. Correlation of recoveries in fenitrothion-ELISA kit with different possible matrix interferants. *, **, ***: significant at 0.05, 0.01, and 0.001 levels, respectively.

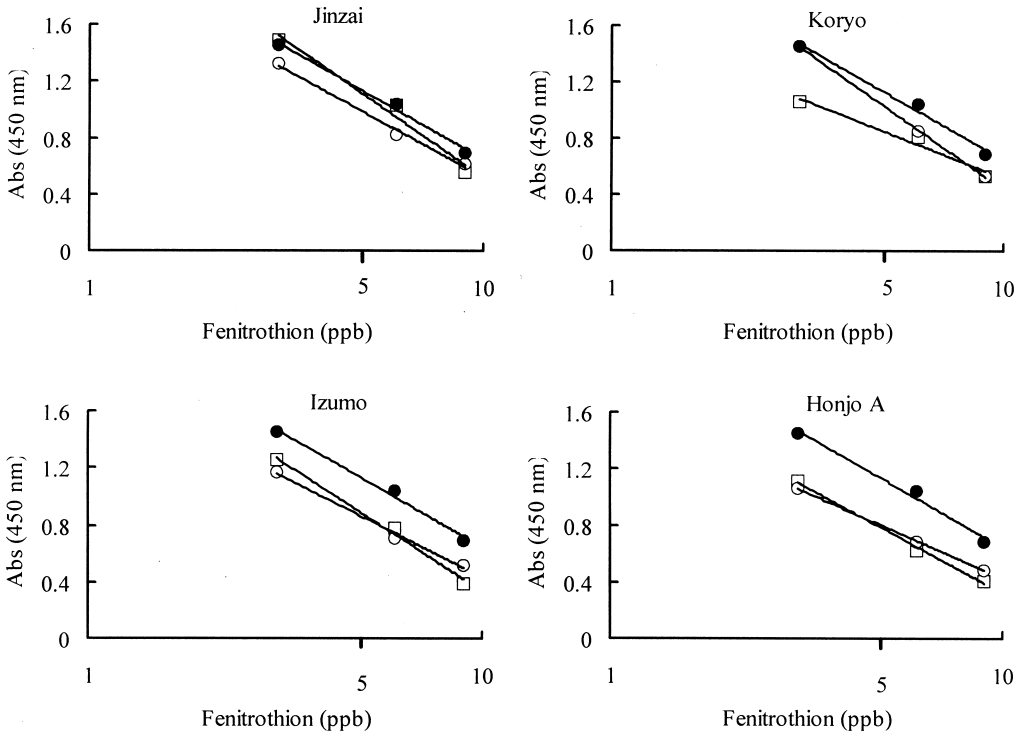

Fig. 2. Matrix effect of four soils in fenitrothion-ELISA kit. Soil matrix interferences of four soils after 1/10 (□) and 1/100 (○) dilution of the original methanol soil extracts (●) to 10% methanol.

4. Effect of ultrafiltration on ELISA

ELISA is susceptible to the presence of various organic substances that may have interfered in the assay performance. Toscano *et al.*[9] explained that humic substances like humic or fulvic acid can influence the antibody, creating more favorable binding with hydrophobic compounds; therefore, filtration of the extracts to remove some high-molecular-weight organic interferants before analysis was considered. Although Koryo had the highest carbon content, the ultrafiltration cut off above 9 kDa showed minimal improvement compared to the unfiltered control, while in Honjo A, some improvement in absorbance reduction of 14% compared to 53% in the unfiltered control was observed (Fig. 4). It was assumed that the effect could be due to the removal of humic substances and/or some clay minerals which were higher in this sample. Differ-

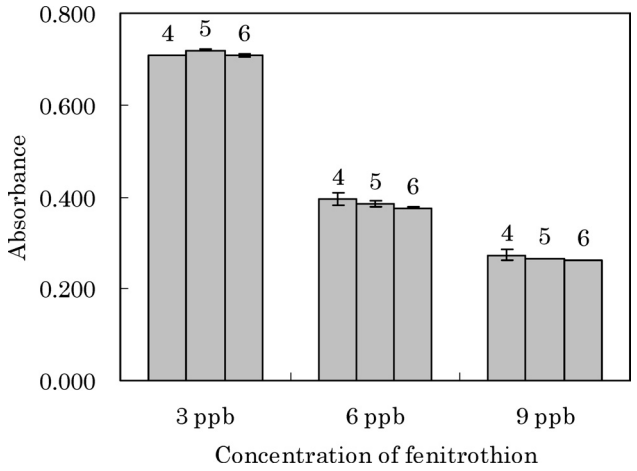

Fig. 3. The effect of pH in fenitrothion-ELISA kit. Adjusted pH values of standard solutions are above bars. Bars indicate the range of data (n=2).

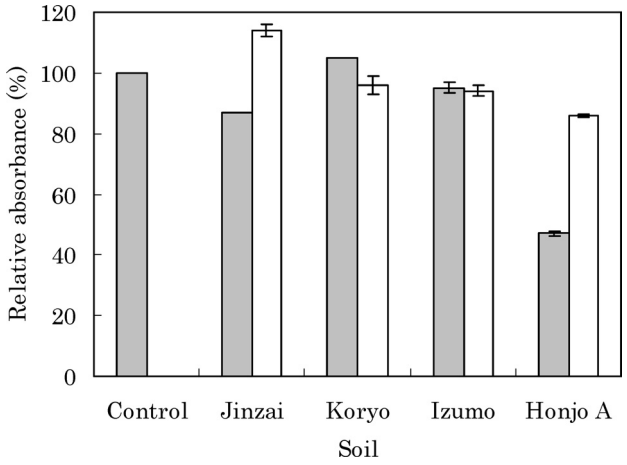

Fig. 4. Changes in the sensitivity of fenitrothion-ELISA kit with soil matrices added at 3 ppb of fenitrothion before (closed bars) and after (open bars) filtration by 9 kDa. Bars indicate the range of data (n=2).

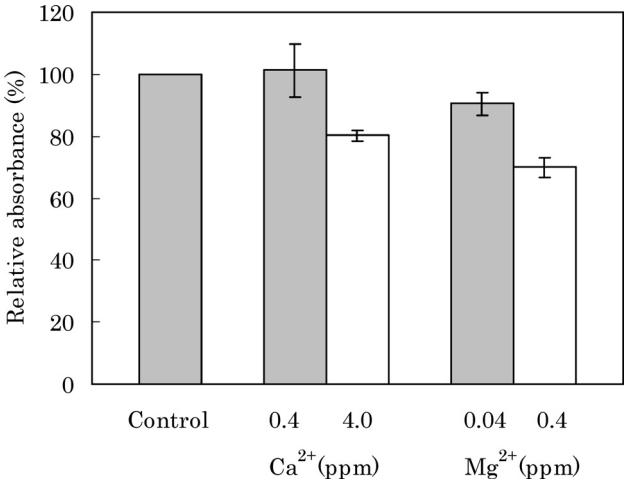

Fig. 5. The effect of Ca^{2+} and Mg^{2+} ions in fenitrothion-ELISA kit. Standard fenitrothion (7.5 ppb) was prepared with 10% methanol in control, and with 10% methanol containing Ca^{2+} at 0.4 and 4.0 ppm, and Mg^{2+} at 0.04 and 0.4 ppm. Bars indicate the range of data (n=2).

ent improvement among soils may be due to the different chemical properties of the extracted humic substances from the soil samples.

5. *Effect of Ca^{2+} and Mg^{2+} ions on ELISA*

The presence of some soluble divalent cations in the extracts might have also affected the performance of the kit. This influence, possibly modifying immunoassay characteristics, was mentioned by several authors.[12–14] The relative recoveries of fenitrothion in 10 soils and the amount of detected ions in the extracts could explain the overestimation in some samples. About 3 ppm of Ca^{2+} ion was found in Koryo and Jinzai soil extracts, in which the recoveries were overestimated and were above the 120% cut-off range for acceptable recovery (Tables 1 and 2), followed by Takano A and Izumo, in which concentrations of Ca^{2+} were 0.12 and 0.45 ppm, respectively. The concentration of Mg^{2+} ion in the two former soils was 0.98 and 0.37 ppm, respectively, while in Takano A and Izumo, the Mg^{2+} ion concentration was 0.03 and 0.06 ppm, respectively. The ELISA absorbance of the extracts with added Ca^{2+} and Mg^{2+} was reduced, particularly with higher amounts of the two ions compared to no addition. This effect could be easily recognized, especially with Mg^{2+} ion with 10 and 30% reduction at 0.4 and 0.04 ppm, respectively, compared to Ca^{2+} ion with 20% reduction at 4.0 ppm (Fig. 5). On the one hand, Schneider *et al.*[12] mentioned that the effect of the ions on ELISA was not clear because it may affect not only binding to the enzyme tracer, coating antigen, or analyte to the antibody but also ionization of the analyte, and that the matrix effect is difficult to conclude. From our results in this study, it is suggested that overestimation of up to 30% can be predicted if the ions are present in considerable amounts.

Conclusion

A commercially available fenitrothion-ELISA kit, designed for analysis of crop samples, was applied to soil samples and showed good performance in some samples. No recoveries below 70% in all samples were achieved; however, for sandy soils the recoveries were biased high (>120%). This overestimation can be explained by soil matrix interferences, such as pH, humic substances, and soluble Ca^{2+} and Mg^{2+} ions. Based on this evaluation, the fenitrothion-ELISA kit can be applied for monitoring or pre-screening soil samples contaminated with fenitrothion pesticide, except for sandy samples, in which Ca^{2+} and Mg^{2+} ions are sometimes high. The burden of extensive and rigorous cleanup of extracts for gas chromatographic analysis can be omitted in the ELISA test. This expands the advantage of ELISA in the analysis of many samples in a short time.

Acknowledgment

We would like to thank Dr. H. Miyake of HORIBA, Ltd. for his technical advice.

References

1) H. M. G. van der Werf: *Agric. Ecosyst. Environ.* **60**, 81–96 (1996).
2) C. D. S. Tomlin (ed.): "The Pesticide Manual 12th Edition," British Crop Protection Council, Surrey, pp. 389–390, 2000.
3) J. J. Manclus and A. Montoya: *J. Agric. Food Chem.* **44**, 4063–4070 (1996).
4) A. Abad and A. Montoya: *J. Agric. Food Chem.* **45**, 1495–1501 (1997).
5) M. J. Moreno, A. Abad, R. pelegri, M. Martinez, A. Saez, M. Gamon and A. Montoya: *J. Agric. Food Chem.* **49**, 1713–1719 (2001).
6) E. Watanabe, K. Baba, H. Eun, T. Arao, Y. Ishii, M. Ueji and S. Endo: *J. Chromatogr. A* **1074**, 145–154 (2005).
7) P. R. Day: "Method of Soil Analysis," Part I, ed. By C. A. Black, D. D. Evans, L. E. Ensminger, J. L. White and F. E. Clark, American Society of Agronomy, Madison, Winconsin, U.S.A., 1982.
8) T. Suzuki, K. Yaguchi, S. Suzuki and T. Suga: *J. Environ. Qual.* **30**, 18–23 (2001).
9) I. Toscano, J. Gascon, M. P. Marco, J. C. Rocha and D. Barcelo: *Analysis* **26**, 130–134 (1998).
10) H. J. Kim, S. Liu, Y. S. Keum, E. C. Hwang and Q. X. Li: "Environmental Fate and Effects of Pesticides," ed. by J. R. Coats and H. Yamamoto, ACS Symposium series 853, pp. 30–45, 2003.
11) E. Watanabe, H. Kubo and H. Nakazawa: *Anal. Chim. Acta* **460**, 99–110 (2002).
12) D. P. Schneider, S. J. Gee, S. B. Kreissig, A. S. Harris, P. Kramer, M. P. Marco, A. D. Lucas and B. D. Hammock: "Troubleshooting during the development and use of immunoassay for environmental monitoring," New Frontiers in Agrochemical Immunoassay, pp. 103–121, 1995.
13) J. J. Manclus and A. Montoya: *J. Agric. Food Chem.* **44**, 3710–

3716 (1996).
14) P. L. Del Valle, M. T. Muldoon, J. S. Karns, J. O. Nelson and W. W. Mulbry: *J. Agric. Food Chem.* **44**, 945–952 (1996).
15) A. J. Krotzky and B. Zeeh: *Pure Appl. Chem.* **67**, 2065–2088 (1995).
16) "World Reference Base for Soil Resources 2006," Food and Agriculture Organization of the United Nations, Rome, 2006.