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Brief Report

Assessment of the long-term stability of pesticide residues under freezing conditions in brown rice and soybean certified reference materials

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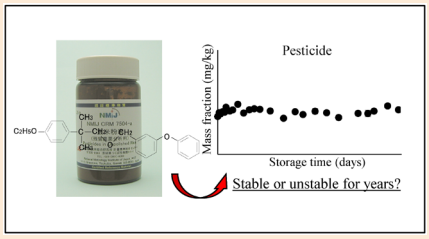
The objective of this study was to assess the long-term stability of pesticide residues in brown rice and soybean. The long-term stability of pesticide residues in brown rice and soybean was assessed for 5415 days (over 14 years) and 1801 days (about 5 years), respectively. The samples—certified reference materials (CRMs) 7504-a (brown rice) and 7509-a (soybean) —were prepared by freeze-pulverization. Two target pesticides (etofenprox and fenitrothion) were selected for brown rice and four (chlorpyrifos, diazinon, fenitrothion, and permethrin) for soybean. Our analytical results for long-term stability based on highly reliable isotope dilution mass spectrometry were in the range of expanded uncertainty ($k=2$) for the certified values of each CRM. The concentration showed a decreasing trend in none of the target pesticides when the samples were stored at temperatures between −20 °C and −30 °C, which indicated that the target pesticides were stable for the tested long terms.



Keywords: long-term stability, pesticide residue, brown rice, soybean, certified reference material.

Introduction

It is important to analyze the residue levels of pesticides in foods to monitor for contamination and to investigate the relationship between exposure and health risks. The analysis of pesticides in food includes complex pretreatments of samples as well as highly selective instrumental analyses; thus, quality control is required to obtain accurate analytical results and to ensure the validity of the applied method.[1)] For this purpose, the use of certified reference materials (CRMs) is indispensable.[1)] The National Metrology Institute of Japan (NMIJ) has developed food CRMs, *e.g.*, brown rice and soybean, for the quantification of pesticide residues.[1–3)] The assessment of CRM stability has been performed during storage to confirm the quality of the target analytes, and this assessment is particularly important for pesticide residues that may not be stable, unlike such analytes as polychlorinated biphenyls (PCBs).

The objectives of the present study were to assess the long-term stability of pesticide residues in brown rice (CRM 7504-a) and soybean (CRM 7509-a) stored at temperatures between −20 °C and −30 °C according to the certificates of CRM 7504-a and CRM 7509-a.[4,5)] The long-term stability (ranging from one to several years) of organic contaminants and pesticides in food samples has also been assessed in previous studies.[6–10)] For example, cypermethrin in green tea,[6)] hexachlorobenzene, dicofol, *p,p′*-DDE, and *o,p′*-DDT in tea,[10)] *alpha*-endosulfan, *beta*-endosulfan, and endosulfan sulfate in tea,[8)] and diazinon, fenitrothion, cypermethrin, and permethrin in apple powder[7)] were assessed for about one year. In addition, Schantz *et al.*[9)] assessed the stability of polycyclic aromatic hydrocarbons (PAHs), PCBs, and chlorinated pesticides in frozen mussel tissue (*Mytilus edulis*) Standard Reference Materials (SRMs) stored at −80 °C over 25 years for SRM 1974, 20 years for SRM 1974a, and 12 years for SRM 1974b, and the results indicated that the concentrations of the target analytes were stable over the storage times. However,

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to the best of our knowledge, there are no studies that assessed the long-term stability (several years) of pesticide residues in brown rice and soybean. In the present study, isotope dilution mass spectrometry (IDMS), which has potential to be a primary measurement method,[11–14] was applied to ensure a reliable analysis for an accurate assessment of long-term stability. If we can accurately assess the long-term stability, the obtained results will be useful for sample management, *e.g.*, sample storage method and period until the analysis. In addition, the food samples available for long periods of time will be helpful for quality control of analysis.

Materials and methods

1. Samples used for long-term stability assessment

NMIJ CRMs 7504-a (brown rice powder) and 7509-a (soybean powder) stored at temperatures between −20 °C and −30 °C in the dark were used for the long-term stability assessment, as shown in Table 1. The raw materials (brown rice and soybean) used for the CRMs were grown and sprayed with various target pesticides.[1–3] After harvesting, the materials were freeze-pulverized, homogenized, placed in clean glass bottles, and sterilized with ^{60}Co γ-radiation (15 kGy). Additional details of the sample preparation and evaluation are described in previous papers.[1–3] As shown in Table 1, two pesticides, 2-(4-ethoxyphenyl)-2-methylpropyl 3-phenoxybenzyl ether (etofenprox) and *O*,*O*-dimethyl-*O*-4-nitro-*m*-tolyl phosphorothioate (fenitrothion), were selected as the target analytes for brown rice (CRM 7504-a) because these are certified pesticides.[4] Similarly, four pesticides, *O*,*O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate (chlorpyrifos), *O*,*O*-diethyl *O*-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate (diazinon), fenitrothion, and 3-phenoxybenzyl (1*RS*, 3*RS*; 1*RS*, 3*RS*)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate (permethrin), were selected for soybean (CRM 7509-a).[5]

2. Chemicals

Acetonitrile, acetone, hexane, toluene, anhydrous sodium sulfate (for pesticide residue and PCB analysis grade), and sodium chloride (reagent grade) were purchased from Kanto Chemical Co., Tokyo, Japan. A 0.5 mol/L phosphate buffer solution (pH 7.0) was prepared from dipotassium hydrogen phosphate (reagent grade; Kanto Chemical), potassium dihydrogen phosphate (reagent grade; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and purified water (Puric α, UP-0090α-TU1, Organo Corp., Tokyo, Japan). Purified water (Organo) was also used for the water-soaking process.

3. Preparation of surrogate and syringe spike solutions

The surrogate solutions were gravimetrically prepared by dissolving isotope-labeled pesticides in acetone. Etofenprox-d_5 and fenitrothion-d_6 (Hayashi Pure Chemical, Osaka, Japan) were used for brown rice (CRM 7504-a), and chlorpyrifos-d_{10}, diazinon-d_{10}, fenitrothion-d_6 (Hayashi Pure Chemical), *cis*-permethrin-$^{13}C_6$, and *trans*-permethrin-$^{13}C_6$ (Cambridge Isotope Laboratories, Andover, MA) were used for soybean (CRM 7509-a). The syringe spike solution was also gravimetrically prepared by dissolving 2-chloro-2′,6′-diethyl-*N*-(methoxymethyl) acetanilide (alachlor; GL Sciences, Tokyo, Japan) in acetone.

4. Preparation of calibration solutions

The calibration solutions were prepared by gravimetric mixing as follows: the pesticide solutions were prepared by mixing the individual pesticide reagents with acetone, followed by a combination of the solutions. Etofenprox and fenitrothion (TraceSure grade; FUJIFILM Wako Pure Chemical) were used for brown rice (CRM 7504-a), and chlorpyrifos (Traceable Reference Material (TRM) grade; FUJIFILM Wako Pure Chemical), diazinon, fenitrothion, *cis*-permethrin, and *trans*-permethrin (TraceSure grade; FUJIFILM Wako Pure Chemical) were used for soybean (CRM 7509-a). The calibration solutions for the quantification of pesticides in brown rice (CRM 7504-a) were gravimetrically prepared by mixing this mixed pesticide solution with surrogate and syringe spike solutions. For the quantification of pesticides in soybean (CRM 7509-a), the matrix-matched calibration solutions were prepared by mixing the final mixed solution with cleaned-up extracts of blank soybean (confirmed to have no target pesticides detectable). These solutions were prepared in a way to match as closely as possible with the final concentration of each pesticide in the cleaned-up extract of brown rice (CRM 7504-a) or soybean (CRM 7509-a).

5. Analytical method with IDMS

The analyses were carried out in accordance with a published paper.[2,3] The brown rice (3 g, CRM 7504-a) or soybean (5 g, CRM 7509-a) sample was weighed in a glass vial, and the surrogate solution and purified water (10 mL) were added to this

Table 1. Samples, target pesticides, and evaluation period for long-term stability assessment

Matrix	NMIJ CRM No.	Target pesticides	Evaluation period for stability (days)
Brown rice	7504-a	Etofenprox Fenitrothion	5415
Soybean	7509-a	Chlorpyrifos Diazinon Fenitrothion Permethrin	1801

sample. After 15 min, the samples were homogenized for extraction in acetonitrile. The obtained crude extracts were cleaned up by solid phase extraction (SPE) cartridges (octadecylsilanized silica gel (1 g); Bond Elut MEGA BE-C18 1GM; Agilent Technologies, Santa Clara, CA, USA, and graphite carbon/aminopropyl-silanized silica gel layered cartridge (500 mg/500 mg); ENVI-Carb/LC-NH_2; Supelco, Division of Sigma-Aldrich, St. Louis, MO, USA). The cleaned-up samples were measured by using a gas chromatograph with a mass spectrometer (GC/MS; Agilent 7890A GC equipped with a DB-5 ms column (30 m ×0.25 mm i.d., 0.25 μm film thickness; Agilent Technologies) and a 5975C MSD). GC/MS measurement was performed by using the on-column (for brown rice, CRM 7504-a) or splitless (for soybean, CRM 7509-a) injection mode, and the injection volume was 0.5 μL (for brown rice, CRM 7504-a) or 1.0 μL (for soybean, CRM 7509-a). Helium was used as the carrier gas (1.0 mL/min) and the injector temperature was 220 °C for splitless injection. The GC oven was programmed to remain at 50 °C for the initial 1 min, then increase to 125 °C at 25 °C/min, further increase to 300 °C at 10 °C /min, and then hold for 6.5 min. Quantitative analysis was conducted by SIM mode and the monitoring ions for quantification were as follows: chlorpyrifos, 314; chlorpyrifos-d_{10}, 324; diazinon, 304; diazinon-d_{10}, 314; etofenprox, 163; etofenprox-d_5, 168; fenitrothion, 277; fenitrothion-d_6, 283; *cis*- and *trans*-permethrin, 183; *cis*- and *trans*-permethrin-$^{13}C_6$, 189; and alachlor, 160. The pesticides were quantified by IDMS.[2,3]

6. Stability assessment

The concentrations of the target pesticides were monitored periodically (one or more times about once a year according to the NMIJ quality management system based on ISO 17034[15]) for 5415 days (over 14 years) and 1801 days (about 5 years) for brown rice (CRM 7504-a) and soybean (CRM 7509-a), respectively. Quantification of the target pesticides was performed as described above.

7. Moisture content measurement of brown rice (CRM 7504-a) and soybean (CRM 7509-a)

The sample moisture was evaluated by using a portion of the CRM (about 1 g) and drying it at 105 °C for 24 hr in a drying oven (Ettas Series ONW-450S; As One, Osaka, Japan). The sample moisture was calculated based on the mass difference before and after the drying process.

Results and discussion

The results for the long-term stability of the pesticide residues are given in Fig. 1 for (A) brown rice (CRM 7504-a) and (B) soybean (CRM 7509-a), respectively. The first assessment was defined as day 0. As shown in Fig. 1, all the analytical results were in the range of expanded uncertainty (k=2) for the certified values of each CRM,[4,5] indicating the reliability of our long-term stability results obtained by IDMS, which generally delivers higher accuracy and precision[16] compared with external or internal standard methods if the analysis (including extraction, cleanup, and instrumental measurement) is carried out under adequate conditions.

The slopes of line b_1 in Fig. 1 were calculated in accordance with ISO Guide 35[17] by using Eq. 1:

$$b_1 = \frac{\sum_{i=1}^{n}(X_i - \bar{X})(Y_i - \bar{Y})}{\sum_{i=1}^{n}(X_i - \bar{X})^2} \quad (1)$$

where X_i and Y_i represent the storage time (days) and the concentration at day i, respectively, and $\bar{X}$ and $\bar{Y}$ represent the average of X_i and that of Y_i, respectively. The standard deviation of b_1 ($s(b_1)$) was calculated by using Eq. 2[17]:

$$s(b_1) = \frac{s}{\sqrt{\sum_{i=1}^{n}(X_i - \bar{X})^2}} \quad (2)$$

The s and the intercept b_0 were calculated by using Eqs. 3 and 4, respectively[17]:

$$s^2 = \frac{\sum_{i=1}^{n}(Y_i - b_0 - b_1 X_i)^2}{n-2} \quad (3)$$

$$b_0 = \bar{Y} - b_1\bar{X} \quad (4)$$

The significance of the instability of the target pesticides was tested by Eq. 5[17]:

$$|b_1| < t_{0.95,n-2} \times s(b_1) \quad (5)$$

where $t_{0.95,n-2}$ represents Student's t coefficient at a 95 % confidence level. The calculation results are shown in Table 2. Based on these results, there were no significant changes in the concentration of etofenprox in brown rice (CRM 7504-a) or in that of chlorpyrifos, diazinon, and fenitrothion in soybean (CRM 7509-a) because the requirement of Eq. 5 was satisfied. Although the results for fenitrothion in brown rice (CRM 7504-a) and permethrin in soybean (CRM 7509-a) did not satisfy the requirement of Eq. 5, a decreasing trend in the concentration was not observed (the reason for the increasing trend in these concentrations was not clear and requires further investigation). Our CRM samples were sterilized with ^{60}Co γ-radiation (15 kGy) and stored at temperatures between −20 °C and −30 °C in the dark as described earlier. Therefore, hydrolysis (not photolysis or microbial degradation) is considered to be the main degradation factor when the concentrations of target pesticides show a decrease. The data of degradation half-lives of target pesticides in hydrolysis is shown in Table 3.[18] Based on this data, etofenprox and permethrin is considered to be more stable than the other target pesticides for hydrolysis. However, it is necessary to monitor the concentrations to confirm the actual behavior in brown rice and soybean sample. Our results indicated that the target pesticides in brown rice (CRM 7504-a) and soybean (CRM 7509-a) were not hydrolyzed for 5415 days (over 14 years) and 1801 days (about 5 years), respectively, because there was no decreasing trend in the concentration of any of the target pes-

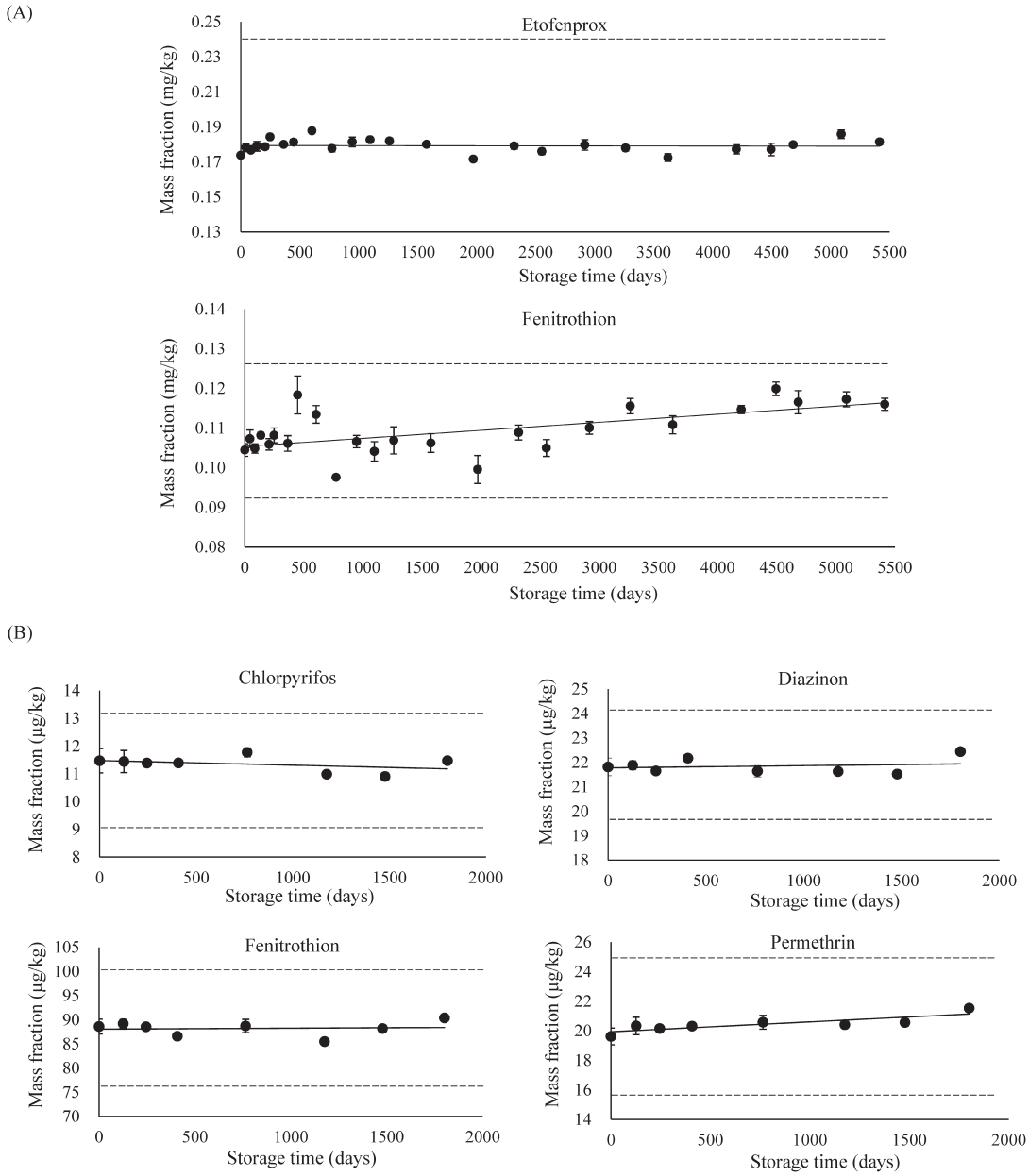

Fig. 1. Long-term stability of (A) etofenprox and fenitrothion in brown rice (CRM 7504-a) and (B) chlorpyrifos, diazinon, fenitrothion, and permethrin in soybean (CRM 7509-a) Plots and error bars represent the mean values and standard deviations, respectively. Each solid line denotes the regression line. The dotted lines denote the range of expanded uncertainty (k=2) for the certified values of (A) CRM 7504-a and (B) CRM 7509-a; the first assessment was defined as day 0; total assessments were (A) 25 plots and n=4 for each plot and (B) 8 plots and n=4 for day 0, 126, 764; standard deviations for (B) are not shown for the results of day 245, 408, 1176, 1478, and 1801 because these assessments were performed using n=2.

Table 2. Results for the statistical analysis of long-term stability assessment

Matrix	NMIJ CRM No.	Target pesticides	b_1	b_0	s	$s(b_1)$	$t_{0.95,\ n-2}\times s(b_1)$
Brown rice	7504-a	Etofenprox	−0.0000001	0.1794	0.0039	0.0000004	0.000001
		Fenitrothion	0.000002	0.1054	0.0047	0.000001	0.000001
Soybean	7509-a	Chlorpyrifos	−0.00017	11.4776	0.2773	0.0002	0.00038
		Diazinon	0.00009	21.7787	0.3350	0.0002	0.00046
		Fenitrothion	0.00019	88.0798	1.6412	0.0009	0.00227
		Permethrin	0.00067	19.9343	0.3199	0.0002	0.00044

Table 3. Degradation half-lives of target pesticides in hydrolysis[18)]

Target pesticides	Degradation half-lives in hydrolysis
Chlorpyrifos	72 days (pH of 5 and 7; 25 °C), 16 days (pH of 9; 25 °C)
Diazinon	7 days (pH of 5; 25 °C), 93 days (pH of 7; 25 °C), 65 days (pH of 9; 25 °C)
Etofenprox	Over a year (pH of 5, 7, and 9; 25 °C)
Fenitrothion	57 days (pH of 7.1, 30 °C)
Permethrin	Over a year (pH of 4 and 7; 25 °C), 43.5 days (pH of 9; 20 °C)

ticides. The moisture content was 13.1 ± 0.08 % for brown rice (CRM 7504-a; n=50 (n=2 for each assessment)) and 16.2 ± 0.14 % for soybean (CRM 7509-a; n=4), which are comparable to those of commercially available brown rice and soybean.[19)] Thus, it is considered that a similar trend in hydrolysis may be observed in commercially available brown rice and soybean for our target pesticides. The sample for CEN Standard Method EN 15662 of the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method, which has been widely applied to various compounds in the world, is supposed to be prepared by freeze-pulverization.[20)] Therefore, our results are useful for the sample management, *e.g.*, storage period until analysis, of brown rice and soybean for QuEChERS analysis. In addition, our brown rice (CRM 7504-a) and soybean (CRM 7509-a) available for long periods of time are helpful for quality control of analysis.

Conclusions

The long-term stability of pesticide residues in brown rice (CRM 7504-a) and soybean (CRM 7509-a) was assessed for 5415 days (over 14 years) and 1801 days (about 5 years), respectively. The samples were prepared by freeze-pulverization, and accurate analyses for long-term stability assessments were performed using the IDMS method. No decreasing trends were observed for the concentrations of any of the target pesticides when the samples were stored at temperatures between −20 °C and −30 °C. Since frozen storage at temperatures between −20 °C and −30 °C generally inhibits microbial activities,[21)] microbial degradation of pesticides in brown rice and soybean samples may be inhibited even without ^{60}Co γ-radiation. Therefore, although the types of pesticide residues in brown rice and soybean assessed in this study are limited, our results will be useful for managing the sample storage method and period until the analysis is conducted. In addition, brown rice and soybean samples available for long periods of time are helpful for quality control of analysis.

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