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Development of soybean certified reference material for pesticide residue analysis

Takashi Yarita [a,*], Takamitsu Otake [a], Yoshie Aoyagi [a], Youko Kuroda [a], Masahiko Numata [a], Hitoshi Iwata [b], Masatoshi Watai [b], Hitoshi Mitsuda [c], Takashi Fujikawa [c], Hidekazu Ota [c]

[a] National Metrology Institute of Japan (NMIJ), National Institute of Advanced Industrial Science and Technology (AIST), AIST Tsukuba Central 3, Umezono, Tsukuba, Ibaraki 305-8563, Japan
[b] Japan Food Research Laboratories, 6-11-10, Nagayama, Tama, Tokyo 206-0025, Japan
[c] The General Environmental Technos Co., Ltd. (KANSO Technos), 3-1-1, Higashikuraji, Katano, Osaka 576-0061, Japan

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ABSTRACT

A soybean certified reference material for pesticide residue analysis was developed by the National Metrology Institute of Japan. Three organophosphorus (diazinon, fenitrothion, chlorpyrifos) and one pyrethroid (permethrin) pesticides were sprayed on soybeans three times before harvest. These soybeans were freeze pulverized, homogenized, bottled, and sterilized by γ-irradiation to prepare the candidate material. Three isotope-dilution mass spectrometric methods that varied in terms of the solvents used for extraction of the target pesticides, the clean-up procedure, and the injection techniques and columns used for quantification via gas chromatography/mass spectrometry were applied to the characterization. Each target pesticide was quantified by two of these analytical methods, and the results were in good agreement. Homogeneity and stability assessment of the material demonstrated that the relative standard uncertainties due to the inhomogeneity and the instability for an expiry date of 55 months were 1.89–4.00% and 6.65–11.5%, respectively. The certified pesticide concentrations with expanded uncertainties (coverage factor $k=2$, approximate 95% confidence interval) calculated using the results of the characterization and the homogeneity and stability assessment were 21.7 ± 3.2 μg/kg for diazinon, 88 ± 21 μg/kg for fenitrothion, 11.1 ± 3.2 μg/kg for chlorpyrifos, and 20.1 ± 4.3 μg/kg for permethrin (as the sum of the constituent isomers).

1. Introduction

Various pesticides are used to protect foods against pests and diseases [1]. However, high levels of residual pesticides in food may result in adverse effects on human health. For example, organophosphorus pesticides, which are currently used on a wide range of crops, are toxic when absorbed by humans because of the resulting acetyl-cholinesterase deactivation [2]. Moreover, pyrethroid pesticides that are generally considered safe for humans and have increasingly replaced previously used pesticides, such as organochlorine and organophosphorus pesticides, have nevertheless been found to be neurotoxic to mammals due to their effect on voltage-sensitive sodium channels [3]. In 2006, the Positive List System for Agricultural Chemicals Remaining in Foods was introduced in Japan to prohibit the distribution of foods that contain agricultural chemicals above a certain level, even if maximum residue limits (MRLs) have not been established [4]. Under this system, analysis of a wide variety of residual pesticides in foods that are under quarantine or on the market is routinely performed.

Analytical methods to determine the presence of pesticide residues in crops usually involve complex extraction of the target pesticides, multi-step clean-up of extracts, and sensitive and selective quantification via a chromatographic technique [5–7]. Ensuring the reliability of the results is crucial to control the risk associated with pesticide residues. Therefore, the validation and quality assurance of the pesticide residue analysis is of great importance [8,9]. Certified reference materials (CRMs) are a key element for the validation/verification of analytical methods, as well as for quality assurance in individual laboratories. However, due to the general instability of some pesticides, no CRM is currently available [10]. In recent years, the National Metrology Institute of Japan (NMIJ) has issued rice [11], green onion [12], cabbage [12], and apple [13] CRMs for pesticide residue analysis. In the development of these CRMs, the materials were prepared from raw crops containing the residual target pesticides. Characterization of the target pesticides was then carried out by isotope-dilution mass spectrometry (IDMS), which has the potential to be a primary method of measurement [14–17], with independent

* Corresponding author. Tel.: +81 29 861 9416; fax: +81 29 861 6866.
E-mail address: t-yarita@aist.go.jp (T. Yarita).

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extraction and clean-up procedures for each analysis. Other National Metrology Institutes are also working on the development of similar CRMs [10,18,19]. Thus, the development of crop CRMs for pesticide residue analysis is of great interest to both the users and suppliers of CRMs.

In the Guidelines for the Validation of Analytical Methods for Agricultural Chemical Residues in Food [9] (hereinafter referred to as "guidelines"), beans are classified as one of the matrix types of crop samples, and the use of at least one type of bean is required to validate analytical methods for this class of samples. NMIJ recently issued a soybean CRM (NMIJ CRM 7509-a) for validation/verification of analytical methods and the quality assurance of pesticide residue analysis of bean samples. This paper reports the development of this CRM including preparation of the candidate material, certification analyses, homogeneity and stability assessment, determination of the certified value, and estimation of the corresponding uncertainties.

2. Experimental

2.1. Preparation of candidate reference material

Three organophosphorus pesticides, *O*,*O*-diethyl *O*-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate (diazinon), *O*,*O*-dimethyl-*O*-4-nitro-*m*-tolyl phosphorothioate (fenitrothion), and *O*,*O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate (chlorpyrifos), and a pyrethroid pesticide, 3-phenoxybenzyl (1*RS*, 3*RS*; 1*RS*, 3*RS*)-3-(2, 2-dichlorovinyl)-2, 2-dimethylcyclopropanecarboxylate (permethrin), were selected as target analytes for the certification. The MRLs in soybean are 0.1 mg/kg for diazinon, 0.2 mg/kg for fenitrothion, 0.3 mg/kg for chlorpyrifos, and 0.05 mg/kg for permethrin, respectively [20]. Soybeans (*Glycine max*, cv. Enrei) were cultivated in the Niigata Prefecture, Japan and sprayed with a mixture of the target pesticides 21, 14, and 7 days before harvest. The candidate CRM was prepared using these soybeans, as follows: the soybeans were air dried, freeze pulverized, mixed using an Aichi Electric (Kasugai, Japan) RM-10-2 rocking mixer, bottled into 208 amber glass bottles (10 g each), sterilized by γ-irradiation (15 kGy), and the prepared samples were then stored at ca. −80 °C to the point of use. Preparation of the candidate CRM was carried out in cooperation with NMIJ and KANSO Technos.

2.2. Chemicals

The following chemicals were used for the characterization and stability assessment. High-purity standards of the target pesticides were obtained from Wako Pure Chemical Industries (Osaka, Japan). The purities (mass fractions) of the target pesticides were determined by NMIJ in a previous study [12] to be as follows (mean ± combined standard uncertainty): diazinon (99.74 ± 0.15)%, fenitrothion (99.53 ± 0.17)%, chlorpyrifos (99.48 ± 0.25)%, *cis*-permethrin (99.85 ± 0.12)%, and *trans*-permethrin (99.73 ± 0.15)%. High-purity standards of diazinon-d_6, fenitrothion-d_{10}, and chlorpyrifos-d_{10} were obtained from Hayashi Pure Chemical Ind. (Osaka, Japan). Standard solutions of *cis*-permethrin-$^{13}C_6$ and *trans*-permethrin-$^{13}C_6$ (50 μg/mL each, solvent: nonane) were obtained from Cambridge Isotope Laboratories (Andover, MA). 2-Chloro-2′, 6′ -diethyl-*N*-(methoxymethyl) acetanilide (alachlor), which was used as a syringe spike, was obtained from GL Sciences (Tokyo, Japan). Acetonitrile, acetone, toluene, hexane, ethyl acetate, diethyl ether, and anhydrous sodium sulfate, all of which were of Pesticide Residue and PCB Analysis grade, were obtained from Kanto Chemical (Tokyo, Japan). Reagent grade sodium chloride, dipotassium hydrogenphosphate, and potassium dihydrogen phosphate, and diatomaceous earth were also obtained from Kanto Chemical. The water used for sample preparation was prepared with a Millipore (San Jose, CA) Milli-Q Gradient system at an output of 18.2 MΩ cm.

Chemicals used for homogeneity assessment are described in the Electronic Supplementary Material.

2.3. Preparation of surrogate solution, syringe spike solution, and calibration solutions

The surrogate solution was gravimetrically prepared by dissolving the high-purity standards of diazinon-d_6, fenitrothion-d_{10}, and chlorpyrifos-d_{10}, and the standard solutions of *cis*-permethrin-$^{13}C_6$ and *trans*-permethrin-$^{13}C_6$ in acetone. The syringe spike solution was also gravimetrically prepared by dissolving alachlor in acetone.

The calibration solutions were prepared by gravimetric mixing as follows: the pesticide solutions were prepared by mixing the individual high-purity standards with acetone, followed by combination of the solutions. This pesticide-standard solution was further mixed with the surrogate and syringe spike solutions. Three batches of the calibration solution were prepared by two experimenters (two batches by one experimenter and one batch by the other experimenter), and they were cross-checked by GC/MS under the conditions described in Section 2.4.1.

Then, matrix-matched calibration solutions were prepared by mixing the prepared solutions with cleaned-up extracts of blank soybean (confirmed to have no detectable target pesticides). The final concentrations of the target and isotope-labeled pesticides in these solutions were adjusted to be in 70–110% (relative) to those in the sample solutions that were prepared for GC/MS analysis.

2.4. Analytical methods used for characterization

NMIJ applied the following three analytical methods to the characterization of the target pesticides. The extraction and clean-up protocols of these methods were based on the Analytical Methods for Residual Compositional Substances of Agricultural Chemicals, Feed Additives, and Veterinary Drugs in Food [5] and were partially modified. Each target pesticide was characterized using two of these analytical methods.

2.4.1. Method 1

This method was applied to the characterization of all target pesticides. The soybean sample (5 g) was weighed, and the surrogate solution (750 μL) was added to it. After more than 3 h, water (20 mL) was added, and the sample was allowed to stand for 15 min. The mixture was then homogenized with acetonitrile (50 mL) for 2 min using a Kinematica (Lucerne, Switzerland) Polytron PT 1200E homogenizer equipped with a PT-DA 12/2EC-E157 dispersing aggregate, and filtered with a cellulose filter (diameter: 60 mm; retentive particle size: 1 μm) obtained from Kiriyama Glass Works (Tokyo, Japan). The residue on the filter was re-extracted with acetonitrile (20 mL) for 2 min and the pesticide-containing filtrates were combined. An approximately 40 mL aliquot of the crude extract was fractionated in a separatory funnel, and shaken with sodium chloride (10 g) and 0.5 mol/L phosphate buffer solution (pH 7.0, 20 mL) for 10 min. A series of the upper (acetonitrile) layer and acetonitrile (2 mL) was passed through an Agilent Technologies (Palo Alto, CA) Bond Elut C18 cartridge (1 g) that was conditioned in advance with acetonitrile (10 mL), and the entire volume of the eluate was collected. After addition of anhydrous sodium sulfate (approximately 10 g) and filtration with quartz wool, the extract was concentrated and dried using a rotary evaporator and a nitrogen gas stream, respectively; an acetonitrile/toluene mixture (3:1, *v*/*v*; 2 mL) was then added. The extract was further purified using a Supelco (Bellefonte, PA)

ENVI-Carb/LC-NH2 cartridge (500 mg/500 mg) that was conditioned in advance with an acetonitrile/toluene mixture (3:1, *v/v*; 10 mL). An acetonitrile/toluene mixture (3:1, *v/v*; 20 mL) was then passed through the cartridge and the eluate was subsequently concentrated and dried as described above. The dried residue was re-dissolved using the weighed syringe spike solution (0.5 mL).

The target pesticides in this sample solution were quantified using an Agilent Technologies 7890A/5975C GC/MS system. An Agilent Technologies DB-5MS capillary column (30 m × 0.25 mm, film thickness: 0.25 μm) was used as the separation column. The GC system was operated under the following conditions: mobile phase: helium; flow rate: 1.0 mL/min; oven temperature: 50 °C for 1 min, 25 °C/min to 125 °C, 10 °C/min to 300 °C for 6.5 min; injection mode: splitless; injection temperature: 220 °C; injection volume: 1 μL. The MS ionization conditions were as follows: ionization: electron ionization; electron energy: 70 eV. MS data were obtained in the selected ion monitoring mode. The monitor ions (*m/z*) were as follows: diazinon: 304; diazinon-d_{10}: 314; fenitrothion: 277; fenitrothion-d_6: 283; chlorpyrifos: 314; chlorpyrifos-d_{10}: 324; *cis*- and *trans*-permethrin: 183; *cis*- and *trans*-permethrin-$^{13}C_6$: 189; alachlor: 160.

2.4.2. Method 2

This method was applied to the characterization of diazinon, fenitrothion, and chlorpyrifos. The soybean sample (2 g) was weighed, and the surrogate solution (300 μL) was added to it. After more than 3 h, water (20 mL) was added and the sample was allowed to stand for 2 h. The mixture was homogenized with acetone (100 mL) for 3 min using the same homogenizer as in Method 1, and filtered using a cellulose filter (diameter: 60 mm; retentive particle size: 1 μm) obtained from Kiriyama Glass Works with diatomaceous earth. The residue on the diatomaceous earth was re-extracted with acetonitrile (50 mL) for 3 min and the pesticide-containing filtrates were combined. Acetone in the crude extract was removed using a rotary evaporator, and the aqueous residue was shaken with an ethyl acetate/hexane mixture (1:4, *v/v*; 100 mL) and saturated sodium chloride aqueous solution (100 mL) in a separatory funnel for 5 min. The upper (organic) layer was collected in an Erlenmeyer flask, and the lower (aqueous) layer was re-extracted with an ethyl acetate/hexane mixture (1:4, *v/v*; 50 mL). The upper layers were combined, dehydrated with anhydrous sodium sulfate (approximately 10 g), and filtered with quartz wool. The inner wall of the flask and the anhydrous sodium sulfate residue were washed with hexane (20 mL) twice, and the obtained solutions were added to the filtrate. The filtrate was then concentrated using a rotary evaporator, and shaken with hexane (30 mL) and acetonitrile saturated with hexane (30 mL). The lower (acetonitrile) layer was collected, and the upper hexane layer was re-extracted with 30 mL of acetonitrile saturated with hexane; this operation was performed twice. The obtained lower layers were combined and concentrated, and then dried as described above. The residue was dissolved with a hexane/acetone mixture (1:1, *v/v*; 5 mL), and cleaned-up using an Agilent Technologies Bond Elut SI cartridge [5 g, anhydrous sodium sulfate (5 g) was added on top of this cartridge] that was conditioned in advance with a hexane/acetone mixture (1:1, *v/v*; 10 mL). A hexane/acetone mixture (1:1, *v/v*; 100 mL) was then passed through the cartridge and then the eluate was concentrated and dried as described above. The dried residue was re-dissolved using the weighed syringe spike solution (0.5 mL).

Diazinon, fenitrothion, and chlorpyrifos in this sample solution were quantified using the same GC/MS system described in Method 1, except for replacement of the separation column with an Agilent Technologies DB-35MS capillary column (30 m × 0.25 mm, film thickness: 0.25 μm). The on-column injection was performed by setting the inlet temperature to oven track mode, and the injection volume was set to 0.5 μL. The oven temperature was ramped as follows: 50 °C for 1 min, 25 °C/min to 125 °C, 10 °C/min to 320 °C for 10 min. The other operating conditions were the same as in Method 1.

2.4.3. Method 3

This method was applied to the characterization of permethrin. The soybean sample (2 g) was weighed, and the surrogate solution (300 μL) was added to it. After more than 3 h, water (20 mL) was added and the sample was allowed to stand for 2 h. The mixture was homogenized, as described in Method 2. After concentration to about 30 mL using a rotary evaporator, the crude extract was shaken with hexane (100 mL) and 10% sodium chloride aqueous solution (mass fraction, 100 mL) in a separatory funnel for 5 min. The upper (hexane) layer was collected in an Erlenmeyer flask, and the lower (aqueous) layer was re-extracted with hexane (50 mL). The upper layers were combined, and the resulting mixture was dehydrated using anhydrous sodium sulfate (approximately 10 g) and filtered with quartz wool. The inner wall of the flask and the anhydrous sodium sulfate residue were washed with hexane (20 mL) twice, and the obtained solutions were added to the filtrate. The filtrate was then concentrated by rotary evaporation, and shaken with hexane (30 mL) and acetonitrile saturated with hexane (30 mL). The lower (acetonitrile) layer was collected, and the upper (hexane) layer was re-extracted with 30 mL of acetonitrile saturated with hexane; this operation was conducted twice. The obtained lower layers were combined, concentrated, and dried. The residue was dissolved in hexane (5 mL), and the solution was cleaned-up using an Agilent Technologies Bond Elut FL cartridge (5 g, anhydrous sodium sulfate (5 g) was added on top of this cartridge) that was conditioned in advance with hexane (10 mL). Permethrin was then eluted with hexane (50 mL) and a hexane/diethyl ether mixture (3:1, *v/v*; 150 mL), and the eluate was concentrated and dried. The dried residue was re-dissolved using the weighed syringe spike solution (0.5 mL).

The GC/MS measurements were performed for quantification of permethrin in this sample; the conditions were the same as used in Method 2.

2.5. Homogeneity assessment

The homogeneity of the CRM was evaluated by quantifying the target pesticides in two sub-samples taken from 10 bottles randomly selected from the stratified 208 bottles. The analysis was performed by the Japan Food Research Laboratories. The analytical method used is described in the Electronic Supplementary Material.

2.6. Stability assessment

The stability of the target pesticides in the CRM at −80 °C (storage condition of this CRM) was assessed on a periodic basis for about 13 months. In parallel, the stability at −30 °C was also assessed over the course of 4 months. In both cases, NMIJ monitored the concentrations using Method 1.

3. Results and discussion

3.1. Analytical methods used for the characterization

In the development of the matrix type CRM at NMIJ, analytical bias is prevented by using two or more analytical methods, which are principally based on the IDMS technique, for the characterization [21]. In the case of CRM 7509-a, different extraction and clean-up procedures, GC injection techniques, and GC columns

were applied. A spike and recovery analysis of these methods was first performed using two types of blank soybeans: the obtained analytical values were in 96–104%, and the repeatability was satisfactory (RSD < 3.2% for most target pesticides and methods). Thus, no large analytical bias was observed for any target pesticides in the spike and recovery analysis.

3.2. Analytical results of the characterization

The concentrations of the target pesticides were calculated by using Eq. (1). One-point calibration method was applied, because the linearity was good in the range of the sample solutions tested prior to the sample analyses.

$$C = F_{ext} \times \left(\frac{R_{sample}}{R_{cal}} - \frac{R_{blank}}{R_{cal}} \right) \times \frac{F_{cal} \times M_{cal} \times C_{cal} \times M_{spike(sample)}}{M_{sample} \times M_{spike(cal)}} \quad (1)$$

where C is the concentration of the target pesticide in the sample, F_{ext} is the precision factor for the extraction and clean-up process, R_{sample} is the ratio of the peak area of the target pesticide/surrogate observed for the sample solution, R_{blank} is the ratio of the peak area of the target pesticide/surrogate observed for the blank solution, R_{cal} is the ratio of the peak area of the target pesticide/surrogate observed for the calibration solution, F_{cal} is the precision factor for preparation of the calibration solution, M_{cal} is the mass of the pesticide-standard solution used for preparation of the calibration solution, C_{cal} is the concentration of the target pesticide in the pesticide-standard solution, $M_{spike(sample)}$ is the mass of the surrogate solution added to the sample, M_{sample} is the mass of the sample used for analysis, and $M_{spike(cal)}$ is the mass of the surrogate solution used for preparation of the calibration solution.

Table 1
Analytical results (μg/kg) for the determination of the target pesticides in CRM 7509-a [a].

Pesticides	Method 1	Method 2 or 3
Diazinon	21.5 ± 0.1	22.3 ± 1.1
Fenitrothion	87.6 ± 0.5	87.4 ± 5.6
Chlorpyrifos	10.9 ± 0.3	12.2 ± 1.3
cis-Permethrin	8.16 ± 0.35	9.34 ± 0.46
trans-Permethrin	11.6 ± 0.3	10.3 ± 1.3

[a] Each value represents the mean concentration ± standard deviation (n=5 for Method 1, and n=4 for Methods 2 and 3).

Table 2
Uncertainty budget for quantification of *cis*-permethrin by Method 3.

Source of uncertainty	x_i	$u(x_i)$	Unit	$C_i \left(=\frac{\partial f}{\partial x_i}\right)$	$\lvert C_i \rvert u(x_i)$
F_{ext}	1	0.022	–	9.34	0.20
R_{sample}/R_{cal}	0.8424	0.038	–	11.1	0.42
R_{blank}/R_{cal}	0	–	–	−11.1	–
F_{cal}	1	0.024	–	9.34	0.23
M_{cal}	0.13049	0.00006	g	71.6	0.0040
C_{cal}	2052.4	9.6	μg/kg	0.00455	0.044
$M_{spike(sample)}$	0.235	0.00042	g	39.7	0.017
M_{sample}	2.0	0.00014	g	−4.67	0.0007
$M_{spike(cal)}$	2.83877	0.00006	g	−3.29	0.0002
C and $u_c(C)$	9.34	0.52	μg/kg		

The explanation for each parameter is described in the text.

The analytical quantification of the pesticides in CRM 7509-a is summarized in Table 1. The concentrations determined using the various protocols were in good agreement. Statistical analysis of the difference between the analytical results obtained from the various methods via a one-way analysis of variance (ANOVA) test ($p < 0.05$) demonstrated that the differences were not significant, except in the case of *cis*-permethrin.

For each target pesticides, the standard uncertainty of the parameters included in Eq. (1) was estimated. In principle, the IDMS method has the highest accuracy among the quantification methods for matrix samples. In the analysis of solid samples, however, equilibrium between the native pesticide and isotope-labeled surrogates may not be realized [22]. Therefore, the repeatability of the analytical values was obtained as the standard uncertainty of F_{ext}. On the other hand, note that the ratio of R_{sample} to R_{cal} (R_{sample}/R_{cal}) were evaluated as a single factor, given that all of the sample solutions were analyzed at the same time using the calibration solution during the GC/MS measurement, and the R_{sample}/R_{cal} values were calculated in each case. The ratio of R_{blank} to R_{cal} (R_{blank}/R_{cal}) was treated similarly.

As an example, the uncertainty budget for the analytical results of *cis*-permethrin obtained by Method 3 is shown in Table 2. Here, F_{ext}, R_{sample}, R_{blank}, R_{cal}, $M_{spike(sample)}$, and M_{sample} depended on the corresponding analytical method (method-dependent factors), whereas F_{cal}, M_{cal}, C_{cal}, and $M_{spike(cal)}$ are common for all methods (method-common factors). For each target pesticide, the combined standard uncertainty of the method-dependent factors (u_i, where i is the method number) was calculated, and the results are shown in Table 3.

The weighted mean (C_{wmean}) of the analytical results from two corresponding methods (Methods 1 and 2 for diazinon, fenitrothion, and chlorpyrifos, and Methods 1 and 3 for *cis*-permethrin and *trans*-permethrin) was obtained using the following

Table 3
Uncertainty budget for the weighted means of the target pesticides in CRM 7509-a [a].

Source of uncertainty	Pesticides					
	Diazinon	Fenitrothion	Chlorpyrifos	*cis*-Permethrin	*trans*-Permethrin	(Permethrin) [b]
$u(C_{com})$	0.496	4.77	0.575	2.49	2.91	
$u(C_{ind})$ [c]	1.29	1.12	3.15	3.38	3.17	
(u_1	1.13	0.900	2.82	4.59	2.72)	
u_2 or u_3	4.85	6.44	10.6	4.93	12.7	
$u(C_{bm})$	1.98	0	7.53	9.37	7.38	
$u(C_{wmean})$ [d]	2.42	4.90	8.19	10.3	8.54	(6.57)

The explanation for each uncertainty is described in the text.

[a] Each value is represented as relative (%) to the mean value of the analytical results.
[b] Sum of *cis*- and *trans*- isomers.
[c] $u(C_{ind})$ was estimated from u_1 and either u_2 or u_3.
[d] $u(C_{wmean})$ was estimated from $u(C_{com})$, $u(C_{ind})$, and $u(C_{bm})$.

weight (w_i):

$$w_i = \frac{1/u_i}{1/u_1 + 1/u_{2(or3)}} \quad (2)$$

The weighted means of the pesticide concentrations were 21.7 μg/kg for diazinon, 87.6 μg/kg for fenitrothion, 11.1 μg/kg for chlorpyrifos, 8.72 μg/kg for *cis*-permethrin, and 11.4 μg/kg for *trans*-permethrin, respectively.

The combined standard uncertainty associated with the weighted mean of the pesticide concentration [$u(C_{wmean})$] was estimated as follows. First, $u(C_{com})$ was calculated from the standard uncertainty of method-common factors. Also, $u(C_{ind})$ associated with the method-dependent factors was also obtained by using the following equation:

$$u(C_{ind}) = \sqrt{w_1^2 u_1^2 + w_{2(or3)}^2 u_{2(or3)}^2} \quad (3)$$

Besides, the inter-method variance obtained from the above-mentioned ANOVA test was obtained, and it was used as the uncertainty corresponding to the variance between the analytical results of the different methods [$u(C_{bm})$]. The value of $u(C_{wmean})$ was then obtained by combining $u(C_{com})$, $u(C_{ind})$, and $u(C_{bm})$, and the results are summarized in Table 3.

Table 4
Results of homogeneity assessment [a].

Pesticides	s_{bb} [b]	u_{bb} [c]
Diazinon	1.89	1.45
Fenitrothion	–[d]	4.00
Chlorpyrifos	1.79	3.10
Permethrine	–[e]	3.16

[a] Each value is represented as relative (%) to the mean value of the analytical results.
[b] The standard deviations between bottles.
[c] The uncertainties due to possible inhomogeneity that can be masked by the method repeatability.
[d] Result of MSwithin - MSwithin < 0.
[e] Sum of *cis*- and *trans*- isomers.

The MRL for permethrin is based on the sum of the residues of the constituent isomers [20], thus, the C_{wmean} for the sum of these isomers was calculated as 20.1 μg/kg. The $u(C_{wmean})$ value for permethrin was also estimated by combining $u(C_{wmean})$ values for these isomers to be 6.57% (relative).

3.3. Homogeneity assessment

A one-way ANOVA test of the analytical results obtained from the homogeneity assessment ($p < 0.05$) indicated that the inhomogeneities of the pesticides between bottles were not significant. For evaluation of the uncertainty due to the inhomogeneity, the standard deviations between bottles (s_{bb}) were calculated by using Eq. (4):

$$s_{bb} = \sqrt{\frac{MS_{among} - MS_{within}}{n}} \quad (4)$$

where n represents the number of measurements per bottle, and MS_{within} and MS_{among} represent the mean squares within a group and between groups, respectively [23]. Furthermore, the uncertainties due to possible inhomogeneity that can be masked by the method repeatability (u_{bb}) were calculated by using the following equation:

$$u_{bb} = \sqrt{\frac{MS_{within}}{n}} \sqrt[4]{\frac{2}{\nu_{MS_{within}}}} \quad (5)$$

where $\nu_{MS_{within}}$ represents the number of degrees of freedom of MS_{within} [23]. Comparison of the s_{bb} and u_{bb} values, shown in Table 4, demonstrates that the u_{bb} values were larger for fenitrothion, chlorpyrifos, and permethrin. Moreover, these u_{bb} values were slightly higher than those obtained for the rice CRM (NMIJ CRM 7504-a, 1.6% for fenitrothion) which we developed previously by similar preparation procedure [11], plausibly because the residual concentration of pesticides is lower in the present CRM.

The uncertainty due to inhomogeneity was treated by using the larger of the values obtained for either s_{bb} or u_{bb}. In this homogeneity assessment, 1 g portion of the soybean sample was used for each analysis. Therefore, the use of more than 1 g CRM 7509-a is recommended for a single analysis so that the uncertainty due to the inhomogeneity may not exceed the estimated values.

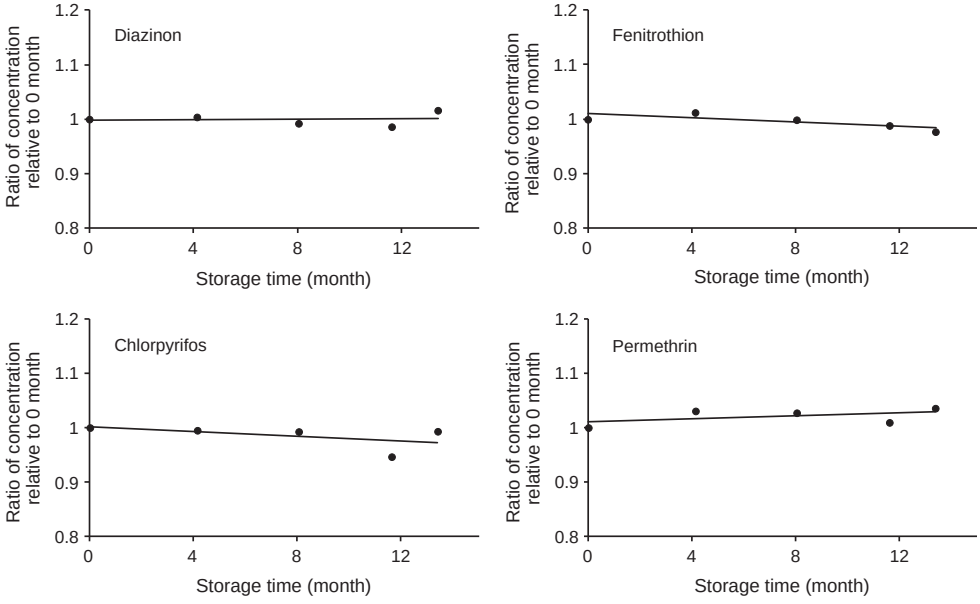

Fig. 1. Variation of the concentration of the target pesticides in CRM 7509-a with storage time. Storage temperature: −80 °C.

3.4. Stability assessment

3.4.1. Stability under long-term storage conditions

To determine whether the target pesticides were stable under storage at a temperature of ca. −80 °C, stability monitoring was performed. Fig. 1 shows the change in the concentration of the target pesticides with elapse of storage time. The results were evaluated in accordance with the ISO Guide 35 [23], as shown in Table 5. Here, the slope (b_1) and intercept (b_0) of the regression lines in Fig. 1 were calculated by using the following equations:

$$b_1 = \frac{\sum_{i=1}^{n}(X_i-\overline{X})(Y_i-\overline{Y})}{\sum_{i=1}^{n}(X_i-\overline{X})^2} \quad (6)$$

$$b_0 = \overline{Y} - b_1\overline{X} \quad (7)$$

where X_i and Y_i represent the elapsed time (months) and the relative concentration at i month to that at 0 month, respectively, and $\overline{X}$ and $\overline{Y}$ 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 the following equation:

$$s(b_1) = \frac{s}{\sqrt{\sum_{i=1}^{n}(X_i-\overline{X})^2}} \quad (8)$$

where, s was determined using the following equation:

$$s^2 = \frac{\sum_{i=1}^{n}(Y_i-b_0-b_1X_i)^2}{n-2} \quad (9)$$

For all target pesticides, the absolute values of b_1 were smaller than the corresponding values of $t_{0.95,n-2}\times s(b_1)$ ($t_{0.95,n-2}=3.18$, with 3 degrees of freedom), indicating that there were no statistically significant decreases in the concentration of the target pesticides when the CRM was stored at about −80 °C.

Table 5
Results of stability assessment at the storage temperature of −80 °C.

Pesticides	b_1	b_0	$s(b_1)$	$t_{0.95,n-2}\times s(b_1)$ [a]
Diazinon	0.0002	0.9984	0.0012	0.0039
Fenitrothion	−0.0019	1.0095	0.0009	0.0028
Chlorpyrifos	−0.0021	1.0012	0.0020	0.0063
Permethrin[b]	0.0014	1.0102	0.0014	0.0044

[a] $t_{0.95,n-2}=3.18$.
[b] Sum of *cis*- and *trans*- isomers.

3.4.2. Expiry date of the CRM

The uncertainty due to instability during storage [u(lts)] has a high correlation with the expiry date of CRMs, and thus was calculated based on the following equation [23]:

$$u(lts) = t \times s(b_1) \quad (10)$$

where t represents the expiry date (months). In the case where the absolute value of b_1 was larger than the corresponding absolute value of $s(b_1)$, the results obtained from Eq. (11) were used as the u (lts) to prevent underestimation attributed to the sample size used for the stability assessment.

$$u(lts) = t \times |b_1| \quad (11)$$

The results of this calculation demonstrate that at storage periods shorter than 55 months, the u(lts) almost satisfies the half level of the accuracy requirement (70–120%) specified in the guidelines [9]. Thus, the u(lts) values calculated for an expiry date of 55 months were used as the uncertainties due to instability during storage. The calculated results (relative) were as follows: 6.65% for diazinon, 10.3% for fenitrothion, 11.5% for chlorpyrifos, and 7.65% for permethrin.

3.4.3. Stability of CRM under storage conditions of user

Storage of the developed CRM at −80 °C may be impractical for most users. Thus, the stability at −30 °C was also investigated and compared with that at −80 °C. Fig. 2 shows the observed concentrations (relative) of the target pesticides after storage for 4 months. The error bars indicate the combined uncertainty associated with the measurement, which was calculated from the standard uncertainties of the method-dependent factors in Eq. (1) and coverage factor ($k=2$). The concentrations determined after storage under these conditions agreed well within the limit of uncertainty, which indicates that there was no significant decrease in the concentrations of the target pesticides at −30 °C within this storage period. Thus, the expiration and the storage temperature of this CRM for users were set to 3 months after the shipping date and about −30 °C, respectively.

3.5. Certified values and their uncertainties

The weighted means of the analytical results introduced above were adopted as the certified values of CRM 7509-a. The corresponding combined standard uncertaintiy (u_c) was estimated by combining the standard uncertainty due to characterization [$u(C_{wmean})$], the standard uncertainty due to inhomogeneity of the material (using the larger value of s_{bb} or u_{bb}), and the standard uncertainty due to instability of the material [u(lts)]; the uncertainty budget is presented in Table 6. The expanded uncertainty of the certified value (U) is equal to ku_c, where k is the coverage

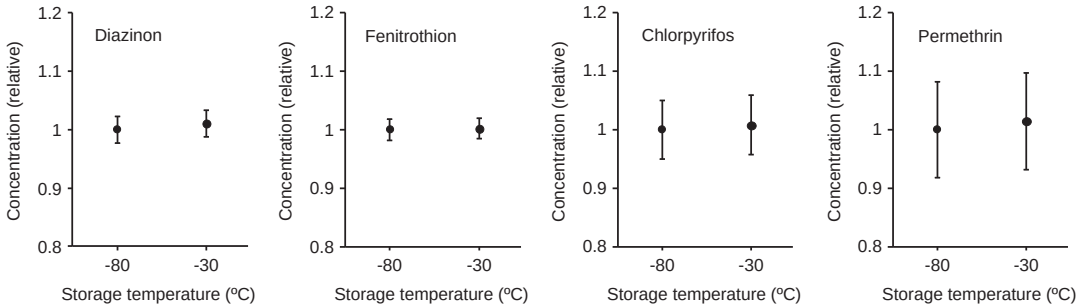

Fig. 2. Comparison of the concentrations of the target pesticides in CRM 7509-a after storage for 4 months at −80 °C and −30 °C. Error bars show combined uncertainty calculated from the standard uncertainties associated with F_{ext}, R_{sample}/R_{cal}, R_{blank}/R_{cal}, $M_{spike(sample)}$, and M_{sample} in Eq. (1) and the coverage factor of $k=2$.

Table 6
Uncertainty budget for the certified values of CRM 7509-a.

Source of uncertainty (relative, %)	Pesticides			
	Diazinon	Fenitrothion	Chlorpyrifos	Permethrin [a]
$u(C_{wmean})$	2.42	4.90	8.19	6.57
s_{bb} or u_{bb} [b]	1.89	4.00	3.10	3.16
u(lts)	6.65	10.3	11.5	7.65
Combined standard uncertainty of the certified values, u_c (relative, %)	7.33	12.1	14.4	10.6
Certified values and their expanded uncertainties, U (k=2, μg/kg)	21.7 ± 3.2	88 ± 21	11.1 ± 3.2	20.1 ± 4.3

[a] Sum of *cis*- and *trans*- isomers.
[b] Larger value of either s_{bb} or u_{bb} was adopted.

factor (=2), corresponding to an approximate 95% confidence interval.

When compared with the MRLs [20], the certified concentrations were 22% for diazinon, 44% for fenitrothion, 3.7% for chlorpyrifos, and 40% for permethrin, expressed as relative concentrations. These concentrations were deemed suitable for assessment of the analytical methods and processes. Moreover, the relative expanded uncertainties of the certified values were within 14–29%, which roughly corresponds to the accuracy requirement stated in the guidelines [9].

4. Conclusion

A new CRM, NMIJ CRM 7509-a, was developed by NMIJ. This is the first bean CRM in which the certified values traceable to the International System of Units (SI) were provided for four pesticides by using multiple IDMS methods. This CRM should be a useful tool for the validation/verification of analytical methods and for quality assurance in pesticide residue analysis of soybean and similar matrices.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.11.011.

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