# 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

ABSTRACT

A soybean certified reference material for pesticide residue analysis was developed by the National Metrology Institute of Japan. Three organophosphorus (diazinon, fenitrothion, chlorphyrifos) 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 $21.7 \pm 3.2$ µg/kg for diazinon, $88 \pm 21$ µg/kg for fenitrothion, $11.1 \pm 3.2$ µg/kg for chlorpyrifos, and $20.1 \pm 4.3$ 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).

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)