DOI:10.1021/jf901758n

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Development of Certified Reference Material for Quantification of Two Pesticides in Brown Rice

Takamitsu Otake,* Nobuyasu Itoh, Yoshie Aoyagi, Mayumi Matsuo, Nobuyasu Hanari, Satoko Otsuka, and Takashi Yarita

National Metrology Institute of Japan (NMIJ) and National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1, Umezono, Tsukuba, Ibaraki 305-8563, Japan

Brown rice powder certified reference material, NMIJ CRM 7504-a, for the analysis of pesticide residues was developed by the National Metrology Institute of Japan, part of the National Institute of Advanced Industrial Science and Technology. Brown rice sample was harvested to contain the pesticides such as etofenprox and fenitrothion, and that was collected from a field in Ibaraki Prefecture in Japan. The certification was carried out using multiple analytical methods such as pressurized liquid extraction, homogenization, and solid−liquid extraction (shaking); the values of target pesticides were obtained by isotope dilution mass spectrometry. Certified values were 0.19 ± 0.05 mg/kg and 0.109 ± 0.017 mg/kg for etofenprox and fenitrothion, respectively.

KEYWORDS: Quality assurance/quality control; certified reference material; certification; pesticide; brown rice

INTRODUCTION

In Japan, a Positive List System for Agricultural Chemical Residues in Foods (PL) has been enforced since May 2006 to prohibit the distribution of foods that contain agricultural chemicals, viz. pesticides, feed additives, and veterinary drugs, above a certain level (*1*); then, maximum residue limits (MRLs) are stipulated. The pesticides are extensively used to protect foods against pests and diseases (*2*). However, if pesticides remain in foods more than MRLs, they may be ingested by humans through the food and may cause some adverse effects (*3*−*5*). Thus, it is important to analyze and monitor the pesticides in foods to investigate the relationship between exposure and health risks. For accurate assessment of exposure level and health risks, accurate analytical results are needed.

The analysis of pesticides in food includes complex pretreatments of the samples as well as highly selective instrumental analyses; thus, a quality control is required. In ensuring the reliability of the analytical results, the validation for method performance of pesticide analysis as written in some reports and studies (*6*−*8*) is essential. In validation of an analytical method, matrix certified reference materials (CRMs) are one of the key elements. Testing recovery by spiking surrogates to food matrix samples is widely used for the evaluation of an analytical method in a lot of testing laboratories; however, this may be insufficient because there are solute−matrix interactions for native compounds. Even if the recovery yields of spiked compounds are satisfactory, native compounds may not be extracted adequately.

The National Metrology Institute of Japan (NMIJ) has developed brown rice powder CRM (NMIJ CRM 7504-a) for the validation of pesticide residue analysis, and the certification of target pesticides in NMIJ CRM 7504-a is described in this paper. Certification of NMIJ CRM 7504-a was carried out by isotope dilution mass spectrometry (IDMS), which has a potential as the primary method of measurement (*9*−*11*). To ensure the reliability of certification, certified values were decided from the analytical results obtained by three independent procedures with three measurement methods.

*To whom correspondence should be addressed. Tel: +81-29-861-4271. Fax: +81-29-861-6865. E-mail: t-ootake@aist.go.jp.

EXPERIMENTAL PROCEDURES

Preparation of Candidate Reference Material. A brown rice sample was harvested to contain the pesticides. The three pesticides, that is, 2-(4-ethoxyphenyl)-2-methylpropyl 3-phenoxybenzyl ether (etofenprox), *O*,*O*-dimethyl-*O*-4-nitro-*m*-tolyl phosphorothioate (fenitrothion), and diisopropyl 1,3-dithiolan-2-ylidenemalonate (isoprothiolane), were selected as the candidates for certification since they are registered pesticides available for (brown) rice and widely used in the rice fields in Japan. The pesticides were applied to brown rice samples twice at the milk-ripe stage and once at 7 days before harvest by a sprayer. This brown rice was collected from a field in Ibaraki Prefecture, Japan. The brown rice was freeze-pulverized, homogenized, and then placed into clean glass bottles. The samples were sterilized with ^{60}Co γ-radiation (15 kGy) and stored at about −30 °C until analysis.

Chemicals. Acetonitrile, hexane, ethyl acetate, toluene, anhydrous sodium sulfate (for pesticide residue and PCB analysis grade), sodium chloride, and hydrochloric acid (reagent grade) were purchased from Kanto Chemical (Tokyo, Japan). Methanol [liquid chromatography/mass spectrometry (LC/MS) analysis grade] was purchased from Wako Pure Chemical (Osaka, Japan). Phosphate buffer solution (pH7.0) was prepared from dipotassium hydrogen phosphate (reagent grade; Kanto Chemical), potassium dihydrogen phosphate (reagent grade; Wako Pure Chemical), and purified water (Milli-Q gradient A10 Elix; Millipore, MA). Purified water (Milli-Q gradient A10 Elix; Millipore) was also used for the water-soaking process in homogenization and shaking extraction.

Preparation of Surrogate and Syringe Spike Solutions. Surrogate solutions were gravimetrically prepared by dissolution in acetone from

 Published on Web 08/24/2009

Table 1. Monitoring Ions of Target Pesticides for GC/MS and LC/MS[a]

	monitoring ion (*m*/*z*)			monitoring ion (*m*/*z*)	
pesticides	GC/MS	LC/MS (cone voltage)	internal standards	GC/MS	LC/MS (cone voltage)
etofenprox	163, 135	358.9, 176.9 (30 V)	etofenprox-d_5	168, 136	363.9, 181.9 (30 V)
fenitrothion	277, 125	276.8, 259.8 (−10 V)	fenitrothion-d_6	283, 131	282.9, 265.7 (−10 V)
isoprothiolane	162, 290	290.8, 230.8 (20 V)	isoprothiolane-d_4	166, 294	294.8, 234.8 (20 V)
alachlor	160, 188	269.8, 237.8 (10 V)			

[a] The underlined ions were used for quantification except isoprothiolane and isoprothiolane-d_4 for GC/MS with shaking extraction (method #5 in **Figure 1**) since coexisting matrices interfered with the GC chromatogram. The other ion (not underlined) was used for isoprothiolane and isoprothiolane-d_4 of method #5 in **Figure 1**. Values in parentheses for LC/MS indicate positive mode ($APPI^+$) or negative mode ($APPI^-$) for cone voltage.

deuterated pesticides: etofenprox-d_5 (Hayashi Pure Chemical, Osaka, Japan), fenitrothion-d_6 (C/D/N Isotopes, Quebec, Canada), and isoprothiolane-d_4 (Hayashi Pure Chemical). A syringe spike solution was also gravimetrically prepared by dissolution in acetone from 2-chloro-2′,6′-diethyl-N-(methoxymethyl) acetanilide (alachlor; GL Sciences, Tokyo, Japan).

Preparation of Calibration Solution. The pesticide solutions were gravimetrically prepared by dissolution in acetone from pesticide reagents: etofenprox, fenitrothion (Wako Pure Chemical), and isoprothiolane (Hayashi Pure Chemical), and these were mixed with each other. This mixed pesticide solution was further mixed gravimetrically with surrogate and syringe spike solutions as prepared above, and this was used as a calibration solution. The calibration solution was prepared to be almost the same with the final concentration of each pesticide in the cleaned up extract.

Assay for Purity of Pesticide Reagents. The purities of pesticide reagents were evaluated using a gas chromatograph with a flame ionization detector (GC-FID; GC-2010, Shimadzu, Kyoto, Japan) and a high-performance liquid chromatograph with an ultraviolet detector (HPLC-UV; LC-10 system, Shimadzu). The mass fraction of water was measured by a Karl Fischer (KF) titrator (MKC-510, Kyoto Electronics Manufacturing, Kyoto, Japan). The purities of the pesticide reagents were as follows: 99.10 ± 0.66% for etofenprox, 98.85 ± 0.33% for fenitrothion, and 99.73 ± 0.14% for isoprothiolane (means ± combined standard uncertainties).

Conversion to Dry Mass Basis. The certified values in the CRM 7504-a are given on a dry mass basis. A dry mass correction factor for sample moisture was evaluated by using a portion of the material (ca. 1 g) and drying it at 95 °C for 12 h. The dry mass correction factor at the time of the certification was 0.8672 ± 0.0002 [mean ± standard deviation (SD), four independent bottles].

Pressurized Liquid Extraction (PLE). The brown rice sample (3 g) was weighed in an extraction cell (stainless steel; volume, 11 mL), and anhydrous sodium sulfate was added. After the contents were mixed by shaking, the surrogate solution was added into the cell. The sample was extracted with acetonitrile (ca. 20 mL) by using a PLE system (ASE 200; Dionex, CA) and an extracting program preset to two cycles and 10 MPa at 130 °C for 10 min in each cycle (*12*).

Sodium chloride (7 g) and hexane saturated with acetonitrile (50 mL) were added to the crude extracts, and they were shaken in a separatory funnel for 15 min. The lower (acetonitrile) layer was collected, and the upper (hexane) layer was re-extracted with 50 mL of acetonitrile. The acetonitrile layers were combined, and anhydrous sodium sulfate was added. This crude extract was concentrated and dried by a rotary evaporator and nitrogen gas stream.

After the addition of 5 mL of toluene/ethyl acetate (1:4, v/v) to the extract residue, this was cleaned up by using a solid-phase extraction (SPE) cartridge [graphite carbon/aminopropylsilanized silica gel layered cartridge (500 mg/500 mg); ENVI-Carb/LC-NH_2, Supelco, Division of Sigma-Aldrich, United States; conditioned with 10 mL of toluene/ethyl acetate (1:4, v/v)]. Pesticides were eluted with toluene/ethyl acetate (1:4, v/v; 25 mL) followed by concentration and drying processes using a rotary evaporator and nitrogen gas stream. Then, a syringe spike solution (0.5 mL) was added to this cleaned up extract.

Homogenization. This was based on a previous method (*13*), and we partly modified it. The surrogate solution and purified water (10 mL) were added to the weighed brown rice sample (3 g). After 15 min, this sample was homogenized in acetonitrile (25 mL) and filtrated with cellulose filter (diameter, 60 mm; no. 5A, Kiriyama glass, Tokyo, Japan). The residues on filter paper were re-extracted by homogenization with acetonitrile (10 mL), and filtrates were combined. This crude extract was shaken with sodium chloride (10 g) and 0.5 mol/L phosphate buffer solution (pH7.0, 20 mL) in a separatory funnel for 10 min. The acetonitrile layer was passed through a SPE cartridge [octadecylsilanized silica gel (1 g); Bond Elut MEGA BE-C18 1GM, Varian, CA; conditioned with 10 mL of acetonitrile]. This crude extract was dried by a rotary evaporator after adding anhydrous sodium sulfate and filtration, and then, 2.0 mL of toluene/acetonitrile (1:3, v/v) was added.

The crude extract was cleaned up by a SPE cartridge [ENVI-Carb/LC-NH_2; conditioned with 10 mL of toluene/acetonitrile (1:3, v/v)]. Pesticides were eluted with toluene/acetonitrile (1:3, v/v; 20 mL) followed by concentration and drying processes using a rotary evaporator and nitrogen gas stream. Then, a syringe spike solution (0.5 mL) was added to this cleaned up extract.

Shaking (Solid−Liquid Extraction). This method was referred to a previous study (*14*), and we partly modified it. The surrogate solution, 1 M HCl (0.75 mL), and purified water (29.75 mL) were added to the weighed brown rice sample (3 g). After 2 h, this sample was shaken for 30 min with acetone (50 mL) and filtered with Celite (Wako Pure Chemical). The residues on Celite were re-extracted by shaking with acetone (50 mL) for a short time, and filtrates were combined. The saturated sodium chloride aqueous solution (100 mL) and hexane (100 mL) were added to this crude extract, and they were shaken in a separatory funnel for 5 min. The upper (hexane) layer was collected, and the lower (water) layer was re-extracted with 50 mL of hexane. After dehydration by an anhydrous sodium sulfate, this crude extract was shaken with hexane saturated with acetonitrile (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 crude extract was concentrated and dried by a rotary evaporator and nitrogen gas stream.

After the addition of 9 mL of hexane to the sample, this was cleaned up by using a SPE cartridge [Florisil cartridge (1 g); Bond Elut MEGA BE-FL 1GM, Varian; conditioned with 10 mL of acetone and 10 mL of hexane]. Pesticides were eluted with hexane (5 mL) and ethyl acetate/hexane (3:7, v/v; 25 mL) followed by concentration and drying processes using a rotary evaporator and nitrogen gas stream. Then, a syringe spike solution (0.5 mL) was added to this cleaned up extract.

Gas Chromatograph with Mass Spectrometer (GC/MS) for On-Column Injection. An Agilent Technologies 6890 GC equipped with a DB-5MS column (30 m × 0.25 mm i.d.; Agilent Technologies, CA) and a 5975 MSD was used. The analysis was performed by using on-column injection mode, and the injection volume was 0.5 μL. A deactivated fused silica capillary (length, 1 m; i.d., 0.25 mm; Agilent Technologies) was placed as a retention gap between the injector and the DB-5MS column using a fused silica union (F.S. Union Universal, two-way; Agilent Technologies) to eliminate peak broadening of the chromatogram. Helium was used as the carrier gas (1.0 mL/min), and the inlet temperature was set as oven track mode. The GC oven was programmed to remain at 50 °C for the initial 2 min, ramped at 20 °C/min to 160 °C, then raised to 300 °C at a rate of 7 °C/min, and held for 10 min.

Quantitative analysis was conducted by SIM mode, and monitor ions are shown in **Table 1**. The pesticides were quantified by IDMS.

GC/MS for Splitless Injection. An Agilent Technologies 6890 GC equipped with a DB-35MS column (30 m × 0.25 mm i.d.; Agilent Technologies) and a 5973N MSD was also used. The analysis was

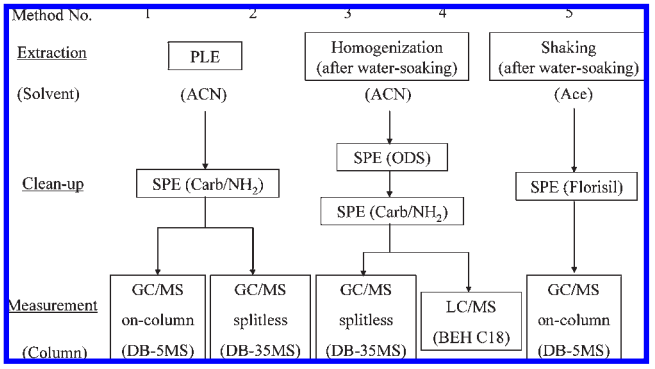

Figure 1. Analytical scheme for the certification of NMIJ CRM 7504-a. Carb/NH_2, graphite carbon/aminopropylsilanized silica gel; ODS, octadecylsilanized silica gel; ACN, acetonitrile; and Ace, acetone.

performed by using the splitless injection mode, and the injection volume was 1.0 μL. Helium was used as the carrier gas (1.0 mL/min), and the injector temperature was 220 °C. The GC oven was programmed to remain at 50 °C for the initial 2 min, ramped at 20 °C/min to 180 °C, then rasied to 300 °C at a rate of 5 °C/min, and held for 10 min. The other conditions were the same with GC/MS for on-column injection.

The matrix-matched calibration standards were prepared by mixing with calibration solution and cleaned up extracts of blank brown rice (confirmed to have no target pesticides detectable) and were used for quantification with splitless injection of GC/MS.

Quantification of Pesticides by LC/MS. A Waters (MA) UPLC/SQD system equipped with an Acquity BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm spherical porous particles; Waters) was used. The LC separation was carried out at 30 °C with a linear gradient from 50% methanol (MeOH) in water (held for 0.5 min) to 100% MeOH over 10 min and followed by an isocratic hold for 24.5 min. The flow rate was 0.2 mL/min, and the injection volume was 2 μL. Pesticides were ionized by atmospheric pressure photoionization (APPI). Another pump (LC-20AD, Shimadzu) was used to deliver acetone as a dopant during APPI, and the LC mobile phase was mixed with the dopant in a four-direction SQD valve in combined mode (as dopant-assisted APPI). The APPI conditions were as follows: desolvation gas flow, 800 L/h; cone gas flow, 50 L/h; source temperature, 150 °C; and probe temperature, 400 °C. The monitoring ions, the cone voltage, and positive/negative mode are shown in **Table 1**. The pesticides were quantified by IDMS.

Stability Assessment. The short-term stability assessment was performed after the preservation at 5 °C for 1 month by using brown rice samples that the target pesticides were spiked. The condition was assumed when the sample was transported.

The concentration was monitored for long-term stability assessment on a periodic basis until 248 days after the analysis for certification. The bottles were stored at about −30 °C in the dark. The analysis was performed by homogenization-GC/MS as described above.

Homogeneity Assessment. The between-bottle homogeneity of the CRM was assessed by quantifying etofenprox and fenitrothion in three subsamples taken from 10 bottles randomly selected from 330 bottles. Target pesticides were analyzed by PLE-GC/MS as described above. Analysis of variance (ANOVA) was used for the analysis of differences of concentration between bottles.

RESULTS AND DISCUSSION

Analytical Methods for Certification. Some extraction techniques, cleanup procedures, instruments, GC injection techniques, and GC columns were applied for certification to avoid the bias associated with a certain analytical method. Analytical methods for certification of the target pesticides are shown in **Figure 1**.

The extraction is an especially critical operation in ensuring the reliability of the analytical results. For pesticide analysis, homogenization and shaking (solid−liquid extraction) are conventional and well-validated methods (*13*, *14*). Furthermore, as efficient alternatives, the condition for PLE was optimized for

Table 2. Recovery Yields (%) of Surrogates Obtained by Five Analytical Methods[a]

	method 1	method 2	method 3	method 4	method 5
etofenprox-d_5	85 ± 6	82 ± 7	91 ± 9	92 ± 9	84 ± 2
fenitrothion-d_6	99 ± 8	89 ± 7	93 ± 9	86 ± 9	101 ± 3

[a] The values represent the means ± SDs. The method numbers correspond to **Figure 1**. Methods 1 and 2, n = 8; methods 3−5, n = 5.

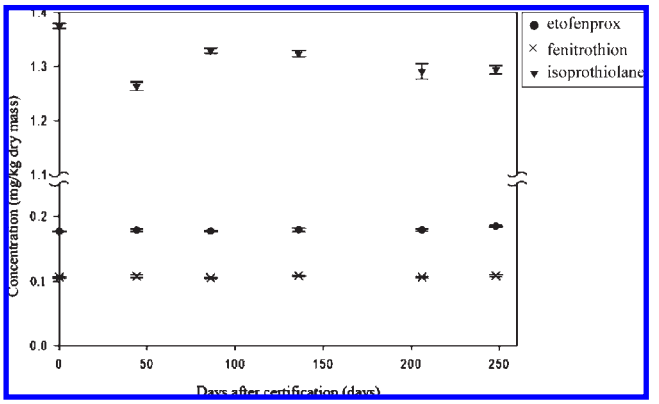

Figure 2. Trend of the concentration of the target pesticides during long-term stability assessment. Plots and error bars represent the mean values and SDs, respectively. Day 0 means the day that the analysis for certification was carried out; n = 4.

pesticide analysis in brown rice (*12*). These methods, viz. PLE, homogenization, and shaking (solid−liquid extraction), were applied for certification as the methods that have different extraction principles.

The cleanup methods (graphite carbon/aminopropylsilanized silica gel, octadecylsilanized silica gel, and Florisil) were validated for eliminating the coexisting matrices in the brown rice interfering with GC and LC separation of the target pesticides and for obtaining sufficient recovery yields of the analytes. As shown in **Table 2**, recovery yields of surrogates were satisfactory (> 82% as mean value) according to the method of positive list since our results were in the range of 70−120% (*15*). This result indicates that the cleanup methods were sufficiently optimized and can be applied for certification.

Because the degradation of thermolabile pesticides can be caused in the hot GC injection port, full attention should be paid for accurate analytical results. Therefore, an on-column injection technique (*16*) and LC approach (*17*, *18*), which have been considered to be recommended techniques for thermolabile pesticides, were applied for certification. Furthermore, in pesticide analysis, it is suggested that the occurrence of matrix effects has a major effect on the quantitative value. Matrix effects can cause an enhancement or suppression in the observed chromatographic response for pesticide residues in a matrix extract as compared with the same concentration in a matrix-free solution (*18*). In fact, it was observed that there was a matrix effect for fenitrothion in our study. It is suggested that using a matrix-matched standard is effective for preventing matrix effects (*18*); thus, these were applied for certification.

Long-Term Stability Assessment. To our knowledge, no similar CRM has been available at present. Thus, to determine if the target pesticides are stable or not, the concentrations had to be regularly monitored. The monitoring was performed after certification analysis to 248 days, and the stability was assessed for etofenprox, fenitrothion, and isoprothiolane. The results are shown in **Figure 2**. There was a significant decrease only for the concentration of isoprothiolane (about 6% decrease; ANOVA,

Table 3. Analytical Results for Certified Pesticides in CRM 7504-a[a]

	method 1	method 2	method 3	method 4	method 5
etofenprox	0.195 ± 0.016	0.190 ± 0.015	0.174 ± 0.001	0.230 ± 0.003	0.178 ± 0.005
fenitrothion	0.115 ± 0.008	0.113 ± 0.009	0.104 ± 0.002	0.110 ± 0.003	0.106 ± 0.003

[a] The values represent the mean concentrations ± SDs, and the unit of values is mg/kg dry mass. The method numbers correspond to **Figure 1**. Methods 1 and 2, $n = 8$; methods 3−5, $n = 5$.

$p < 0.05$) even when the samples are stored about −30 °C in the dark; thus, this pesticide has been removed from certification. The reason for the decrease of the concentration of isoprothiolane was not clear, although this pesticide may not be stable. There were no significant decreases for the other pesticides, and this result indicates that etofenprox and fenitrothion were stable during long-term stability assessment. Because the expiration date of each pesticide reagent is 3−5 years, that of CRM 7504-a for certified pesticides (etofenprox and fenitrothion) was provisionally decided to be 3 years after the date of certification if it is stored at about −30 °C in the dark. The stability, of course, must be further evaluated at regular intervals because the behavior for the degradation of pesticide reagents may be different from being in brown rice.

The SDs between bottles (s_{bb}) were calculated by using the following eq 1, and the results were used as the uncertainty of instability: etofenprox, 1.5% relative; and fenitrothion, 1.1% relative.

$$s_{bb} = \sqrt{\frac{MS_{among} - MS_{within}}{n}} \quad (1)$$

where MS_{within} and MS_{among} represent the mean squares within a group and among groups, respectively.

Homogeneity Assessment. The homogeneity was assessed for etofenprox and fenitrothion as described above. No statistically significant differences for their concentration values between bottles were observed. This result indicates that the material is homogeneous for etofenprox and fenitrothion analysis.

For the calculation of inhomogeneity uncertainty, the s_{bb} was calculated by using eq 1. In the case of large variability of measurement, the influence of analytical variation on the SD between units (u_{bb}) was calculated and used as the estimate for the inhomogeneity instead of s_{bb} (*19*). The u_{bb} was calculated by using the following eq 2:

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

where $\nu_{MS_{within}}$ represents the number of degrees of freedom of MS_{within}. The s_{bb} and u_{bb} were calculated by using eqs 1 and 2, respectively, and the results were used as the uncertainty of inhomogeneity: etofenprox, 2.3% relative; and fenitrothion, 1.6% relative.

Analytical Results and Certified Values. The analytical results in CRM 7504-a for two certified pesticides (etofenprox and fenitrothion) were obtained by the methods in **Figure 1**, and the results by each method are summarized in **Table 3**. The certified values were calculated based on the obtained analytical results, and they were 0.19 ± 0.05 mg/kg and 0.109 ± 0.017 mg/kg for etofenprox and fenitrothion, respectively. The concentration of target pesticides was calculated by the following equation:

$$C = F_{ext} \times \left(\frac{R_{sample}}{R_{cal}} - \frac{R_{blank}}{R_{cal}} \right) \times F_{cal} \times \frac{M_{cal} \times C_{cal} \times M_{spike(sample)}}{M_{sample} \times F_{dry} \times M_{spike(cal)}} \quad (3)$$

where C is a concentration of analyte in the sample, F_{ext} is a factor concerning extraction and a cleanup step, R_{sample} is a ratio of the peak area of analyte/surrogate observed for the sample solution, R_{blank} is a ratio of the peak area of analyte/surrogate observed for the blank solution, R_{cal} is a ratio of the peak area of analyte/surrogate observed for the calibration solution, F_{cal} is a correction factor of preparing the calibration solution, M_{cal} is a weight of the standard solution of analytes taken for preparation of the calibration solution, C_{cal} is a concentration of analyte in the calibration solution, $M_{spike(sample)}$ is a weight of the surrogates solution added to the sample, M_{sample} is a weight of the sample taken for analysis, F_{dry} is a correction factor concerning the moisture content in the sample, and $M_{spike(cal)}$ is a weight of the surrogates solution taken for preparation of the calibration solution.

In **Table 3**, the concentrations for fenitrothion obtained by five different methods were in good agreement with each other; however, those for etofenprox were not. In particular, the results with LC/MS (method 4 in **Figure 1**) for etofenprox were significantly higher than those of other methods measured with GC/MS. The separation and chromatogram for etofenprox were not interfered with by the coexisting matrices of both GC and LC. Furthermore, as described above, recovery yields of surrogates were satisfactory (**Table 2**), and there were not significant differences for recovery yields of etofenprox-d_5 among five methods in **Figure 1**. Therefore, the obtained values of both GC/MS and LC/MS were included for the calculation of a certified concentration for etofenprox. The reason for higher value of LC/MS was not clear, although the results might be attributed to the difference of ionization mechanism between GC/MS and LC/MS.

When compared with certified values and MRLs in PL (*20*), certified values were about half of MRLs (MRLs were 0.5 and 0.2 mg/kg for etofenprox and fenitrothion, respectively). In the validation guideline for the testing method of agricultural chemicals in food (*8*), it is established that a spiking test can be performed with two concentration levels for agricultural chemicals in principle. Because one of those spiking levels is mentioned as MRLs or half of MRLs (*8*), it is considered that our CRM was prepared in an adequate concentration level, and NMIJ CRM 7504-a will be useful for quality assurance/quality control of pesticides (etofenprox and fenitrothion) analysis in brown rice.

Uncertainties of the Certified Values. The uncertainties were calculated according to the Guide to the Expression of Uncertainty in Measurement (*21*). The uncertainty budget is summarized in **Table 4**. The ISO guide 35 (*22*) specifies that uncertainty of CRMs is estimated from standard uncertainty due to characterization, u(char); standard uncertainty due to long-term instability, u(lts); short-term instability (instability during transportation), u(sts); and inhomogeneity of the material, u(bb). The u(char) was estimated from $u(C_{ind})$, $u(C_{com})$, and $u(C_{bm})$. The uncertainty combined as $u(C_{ind})$ associated with each analytical method was obtained from the uncertainty of R_{sample}, R_{blank}, R_{cal}, F_{ext}, M_{sample}, F_{dry}, and $M_{spike(sample)}$ in eq 3. The $u(C_{com})$ that is common to analytical methods was estimated from the uncertainty of F_{cal}, M_{cal}, C_{cal}, and $M_{spike(cal)}$ in eq 3 (the uncertainty of C_{cal} was combined the uncertainty for purity of neat pesticides and for weighing). The uncertainty for between-method variance [$u(C_{bm})$] was calculated by performing an ANOVA on the result obtained from the analytical method in **Figure 1**. The u(lts) was included for the uncertainties by using the result of long-term

Table 4. Uncertainty Budget for the Certified Values of CRM 7504-a[a]

uncertainty component	source	values	
		etofenprox	fenitrothion
u(char)	uncertainty due to characterization	0.025	0.0083
u(lts)	uncertainty due to long-term instability	0.003	0.0012
u(sts)	uncertainty due to short-term instability	not included	not included
u(bb)	uncertainty for inhomogeneity of the material	0.004	0.0017
combined standard uncertainty, u_c		0.026	0.0086
expanded uncertainty, U (k = 2)		0.05	0.017

[a] The unit of values is mg/kg.

stability assessment as described above; however, we did not include the uncertainties for u(sts) in the uncertainty of certified values because no systematic change during short-term stability test for two certified pesticides was detected. The u(bb) derived from the inhomogeneity of the material was estimated in homogeneity assessment as described above. The expanded uncertainty U of the certified value is equal to ku_c, where u_c is the combined standard uncertainty with a coverage factor $k = 2$, corresponding to a 95% confidence interval.

The NMIJ CRM 7504-a (pesticides in brown rice), which had been certified for two pesticides (etofenprox and fenitrothion), was issued by NMIJ. This CRM is a useful tool to validate the analytical methods, and it enables the traceability of routine methods to the national standards.

LITERATURE CITED

(1) Ministry of Health, Labour and Welfare. Introduction of the Positive List System for agricultural chemical residues in foods, **2006**; http://www.mhlw.go.jp/english/topics/foodsafety/positivelist060228/introduction.html (accessed May 25, **2009**).

(2) Carvalho, F. P. Agriculture, pesticides, food security and food safety. *Environ. Sci. Policy* **2006**, *9*, 685–692.

(3) Tamura, H.; Maness, S. C.; Reischmann, K.; Dorman, D. C.; Gray, L. E.; Gaido, K. W. Androgen receptor antagonism by the organophosphate insecticide fenitrothion. *Toxicol. Sci.* **2001**, *60*, 56–62.

(4) Beard, J. DDT and human health. *Sci. Total Environ.* **2006**, *335*, 78–89.

(5) Perry, M. J.; Venners, S. A.; Barr, D. B.; Xu, X. Environmental pyrethroid and organophosphorus insecticide exposures and sperm concentration. *Reprod. Toxicol.* **2007**, *23*, 113–118.

(6) Food and Agriculture Organization of the United Nations. Validation of analytical methods for food control, FAO Food and Nutrition Paper 68, **1997**; ftp://ftp.fao.org/docrep/fao/007/w8420e/w8420e00.pdf (accessed May 25, **2009**).

(7) Thompson, M.; Ellison, S. L. R.; Wood, R. Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report). *Pure Appl. Chem.* **2002**, *74*, 835–855.

(8) Ministry of Health, Labour and Welfare. Validation guideline for testing method of agricultural chemicals in food, **2007**; http://www.mhlw.go.jp/topics/bukyoku/iyaku/syoku-anzen/zanryu3/dl/071115-1.pdf (accessed May 25, **2009**, in Japanese).

(9) De Bievre, P; Peiser, H. S. Basic equations and uncertainties in isotope-dilution mass spectrometry for traceability to SI of values obtained by this primary method. *Fresenius J. Anal. Chem.* **1997**, *359*, 523–525.

(10) Quinn, T. J. Primary methods of measurement and primary standards. *Metrologia* **1997**, *34*, 61–65.

(11) Milton, M. J. T.; Quinn, T. J. Primary methods for the measurement of amount of substance. *Metrologia* **2001**, *38*, 289–296.

(12) Otake, T.; Aoyagi, Y.; Matsuo, M.; Itoh, N.; Yarita, T. Evaluation of pressurized liquid extraction for the analysis of four pesticides in unpolished rice. *J. Environ. Sci. Health, Part B* **2008**, *43*, 390–394.

(13) Ministry of Health, Labour and Welfare. Analytical methods for residual compositional substances of agricultural chemicals, feed additives, and veterinary drugs in Food, **2006**; http://www.mhlw.go.jp/english/topics/foodsafety/positivelist060228/dl/060526-1a.pdf (accessed May 25, **2009**).

(14) Study Group on Analytical Method for Pesticide Residues. *The Analytical Method for Pesticide Residues*, revised ed.; Chuohoki Publishers Co., Ltd.: Tokyo, Japan, 2006 (in Japanese).

(15) Ministry of Health, Labour and Welfare. The results of multiresidue method for agricultural chemicals by GC/MS and LC/MS (agricultural products), and those of multiresidue method for agricultural chemicals by GC/MS (animal and fishery products), **2005**; http://www.mhlw.go.jp/topics/bukyoku/iyaku/syoku-anzen/positivelist/dl/040806-1l1.pdf (accessed May 25, **2009**, in Japanese).

(16) Zheng, P.; Hu, Y.-Y.; Sheng, X.; Zhang, L.; Sun, H.; Sheng, G.-P. Multiresidue determination of thermolabile insecticides in cereal products by gas chromatography-mass spectrometry: Evaluation with on-column injection and conventional hot splitless injection. *J. Sep. Sci.* **2007**, *30*, 2719–2726.

(17) Fialkov, A. B.; Gordin, A.; Amirav, A. Extending the range of compounds amenable for gas chromatography-mass spectrometric analysis. *J. Chromatogr. A* **2003**, *991*, 217–240.

(18) Poole, C. F. Matrix-induced response enhancement in pesticide residue analysis by gas chromatography. *J. Chromatogr. A* **2007**, *1158*, 241–250.

(19) Linsinger, T. P. J.; Pauwels, J.; van der Veen, A. M. H.; Schimmel, H.; Lamberty, A. Homogeneity and stability of reference materials. *Accredit. Qual. Assur.* **2001**, *6*, 20–25.

(20) The Japan Food Chemical Research Foundation. Maximum residue limits (MRLs) of agricultural chemicals in foods, **2006**; http://www.ffcr.or.jp/zaidan/FFCRHOME.nsf/pages/MRLs-p (accessed May 25, **2009**).

(21) International Organization for Standardization. *Guide to the Expression of Uncertainty in Measurement*, 1st ed.; ISO: Geneva, Switzerland, 1993.

(22) International Organization for Standardization. *Guide 35: Reference Materials—General and Statistical Principles for Certification*, 3rd ed.; ISO: Geneva, Switzerland, 2006.

Received May 25, 2009. Revised manuscript received August 5, 2009. Accepted August 7, 2009. A part of this work was supported by the SME Intellectual Foundation Construction Project of the Japanese Ministry of Economy, Trade and Industry.