平成 26 年度　厚生労働科学研究費補助金（食品の安全確保推進研究事業）

検査機関の信頼性確保に関する研究

分担研究報告書

食品衛生外部精度管理調査用適正試料の作製検討と信頼性確保に関する研究（その 5）
—カビ毒検査のための適正調査試料の作製検討—

主任研究者　渡辺　卓穂　(一財) 食品薬品安全センター　秦野研究所　食品衛生事業部長
協力研究者　三宅　司郎　(公財) 京都高度技術研究所　主幹研究員
平川　由紀　(公財) 京都高度技術研究所　研究員
山崎　朋美　(公財) 京都高度技術研究所　研究員
岩佐　精二　豊橋技術科学大学　環境・生命工学系　教授

研究要旨

カビ毒アフラトキシン（AF）は、カビが産生する二次代謝産物で、ヒトや動物に発ガン性を示し、肝臓への障害などをもたらす。食品中の AF 汚染は、その実態解明が進み、2011 年 10 月から新たに総 AF（AFB1, B2, G1, G2）を対象とした規格が施行された。施行に伴い、抗体を利用したイムノアフィニティーカラムが HPLC による測定時の試料調製方法として採用されたが、より簡便に現場で総 AF の測定が可能な ELISA キットの適用が求められている。そこで、イムノアフィニティーカラムにも採用されている、AF にグループ特異的に反応するマウスモノクローナル抗体を利用して、直接競合 ELISA キットの構築を試みた。まず、キット材料であるマウスモノクローナル抗体をマウス腹水から硫安分画法により精製した。また、AFB1 をオキシム化し、活性エステル法により西洋ワサビペルオキシダーゼに結合させた。これらを用いて、直接競合 ELISA を構築した。構築した直接競合 ELISA は、AFB1 と 6.2-120 pg/mL、B2 と 8.5-240 pg/mL、G1 と＜6.3-100 pg/mL、G2 と 6.5-250 pg/mL の測定範囲で測定可能であった。さらに、市販の輸入ピーナッツバターを用いて、AF の添加回収試験を実施した。結果は、110％－200%の回収率とやや高かったが、スクリーニング試験には適用可能と考えられた。今後、開発した ELISA キットを改良し、総 AF の迅速・簡便検査法としてより適した性能を付与できるよう、キット構築条件の検討を継続する必要がある。

A. 研究目的

Aspergillus 属のカビが産生するアフラトキシン（AF）は、国際ガン研究機関 IARC による発ガン性リスクの分類において、グループ 1（ヒトに対する発ガン性が認められる）と評価されており、継続的な摂取によ

る肝臓がん発症リスクの増加や、大量摂取による肝機能障害の発症を引き起こすことが知られている。2002年には、ケニアで患者数317名のうち125名が死亡する、AFによる食中毒が発生している。規制に関しては、食品中のAF汚染の実態解明が進み、2011年10月から新たに総AF(AFB1, B2, G1, G2)を対象とした規格が施行された(厚生労働省医薬食品局食品安全部長通知, 食安発0331第5号(平成23年3月31日))。これによると、総AFを10 μg/kgを超えて検出する食品は、食品衛生法第6条第2号に違反する。規格の施行に伴い、抗体を利用したイムノアフィニティーカラムがHPLCによる測定時の試料調製方法として採用された(厚生労働省医薬食品局食品安全部長通知, 食安発0816第1号(平成23年8月16日))。今後は、免疫測定法の需要が増加すると見込まれ、多種類のキットが市場に投入されている。

我々は、AF類に対してグループ特異的に反応するマウスモノクローナル抗体の作製に成功し、この抗体を用いて通知法に収載されたイムノアフィニティーカラムを開発した(*J. Agric. Food Chem.* 57, 8728-8734 (2009))。このカラムは、堀場製作所が製造・販売している。また、農薬測定用の直接競合ELISAキットを20種類開発し、同様に堀場製作所が製造・販売している。

そこで本年度は、このモノクローナル抗体を応用した直接競合ELISAキットの開発を試みた。さらに、ピーナッツバターにAFを添加し、試作した直接競合ELISAキットによる添加回収試験を試みた。

B. 研究方法

1. マウスモノクローナル抗体の調製

AFとグループ特異的に反応するマウスモノクローナル抗体(MoAb2-3)は、「知の拠点あいち」重点研究プロジェクトを通して、堀場製作所製からマウス腹水として提供された。抗体は、33%飽和度の硫安により不溶化させ、腹水中から分画した。この抗体分画をPBS(10mmol/L リン酸ナトリウム、150mmol/L 塩化ナトリウム; pH 7.0)に溶解し、さらにPBSを用いて透析を行い、抗体標品を得た。純度は、SDS-PAGEにより90%以上であることを確認した。

2. AFB1のオキシム化

AFB1 20 μmolをピリジン:メタノール:蒸留水(1:4:1; v/v) 5.0 mLに溶解し、アミノオキシ酢酸ヘミ塩酸塩 72 μmolを加えて混合した。この混合液を加熱しながら2時間環流させることにより、AFB1のカルボニル基にアミノオキシ酢酸ヘミ塩酸塩を結合させ、オキシム化を行った。

シリカゲルカラムクロマトグラフィー(1 g)(展開溶媒; クロロホルム:メタノール(9:1; v/v))を用いて、上記反応液からオキシム化AFB1を含むフラクションを分画し、さらに、エバポレーターを用いて減圧濃縮を行い、オキシム化AFB1を得た。

3. HRP標識AFB1の調製

オキシム化AFB1 20 μmolと、これと等量(モル当量)のN-ヒドロキシスクシンイミド及び、EDCを脱水したDMSO 2.0 mL中で混合した。この混合溶液を、室温で1.5時間静置し、オキシム化AFB1のカルボキシル基にN-ヒドロキシスクシンイミドを結合し、活性エステル化した。

HRP 10 mg を PBS 1 mL に溶解し、上記の活性エステル 440 μL を加えた。室温で 1.5 時間ゆっくり撹拌しオキシム化 AFB1 のカルボキシル基と HRP に存在するリジン残基のεアミノ基をアミド結合させた。

さらに、ゲル濾過カラムクロマトグラフィー（担体：Sephadex G-25 Super-Fine (GE Healthcare) φ15×500 mm、展開溶媒：PBS）により HRP 標識 AFB1 を精製した。

4. 直接競合 ELISA の構築

96 ウェルマイクロタイタープレートに 5 μg/mL 抗マウス IgG/10 mM PBS を 100 μL/ウェルで添加後、4℃で一晩静置し固相した。その後、0.4% BSA/10 mM PBS を 300 μL/ウェルで添加後、室温で 30 分静置し、ブロッキングを行った。そこに、0.2% BSA/10 mM PBS で 15 ng/mL に希釈した MoAb2-3 を 100 μL/ウェルで添加後、室温で 1 時間静置した。反応後、0.02% Tween20/10 mM PBS でウェルを洗浄し、0.2% BSA/10 mM PBS で 15 ng/mL に希釈した HRP 標識 AFB1 と測定対象 AF との等量混合液を 100 μL/ウェルで添加し、室温で 1 時間静置して、競合反応させた。反応後、ウェルを洗浄し、HRP に対する基質（テトラメチルベンジジン）を加えて室温で 10 分間発色させ、0.5 mol/L 硫酸で発色を停止させた後、分光光度計 (BIO-RAD 製 X-Mark) を用いて 450 nm における各ウェルの吸光度を測定した。

4 種 AF (B1, B2, G1, G2) に対する反応性の確認においては、各 AF を 400、200、100、50、25、12.5、6.25 、0（ブランク）pg/mL となるように 10%メタノールで希釈し、上記の測定対象 AF として直接競合 ELISA に供試した。

5. 添加回収試験

市販の輸入ピーナッツバター（原材料名：ピーナッツ、砂糖、植物油、食塩）を用いて、AF の添加回収試験を実施した。ピーナッツバター2.5 g に対して、NaCl 0.25 g、80%メタノール 10 mL を加え、ピーナッツバター調整液とした。メタノールで 1 μg/mL に希釈した各 AF4 種、及びそれらの等量混合物（各 0.25 μg/mL）をそれぞれ、ピーナッツバター調整液に 25 μL ずつ添加した（試料中最終 AF 量:10ng/g)。これらを 30 分間室温で振とうし、遠心分離により得られた上清を 10%メタノール相当となるように蒸留水で希釈した。さらに、上記で構築した直接競合 ELISA の測定範囲に収めるために、10%メタノールで 15 倍希釈した。これらの希釈液を上記の測定対象 AF として直接競合 ELISA に供試した。

（倫理面への配慮）

AF は毒性が高いため、使用後は直ちに自亜塩素酸溶液に浸漬して分解、無毒化した。

C. D. 結果および考察

1. 直接競合 ELISA における各 AF の反応性

AF を加えないブランクの反応性を 20%阻害する濃度（IC_{20} 値）を測定下限、80%阻害する濃度（IC_{80} 値）を測定上限とした場合に、図 1 に示した通り、AFB1 と 6.2-120 pg/mL、AFB2 と 8.5-240 pg/mL、AFG1 と< 6.3-100 pg/mL、AFG2 と 6.5-250 pg/mL の範囲で測定できることが分かった。この結果から、MoAb2-3 を用いて構築した直接競合 ELISA は、非常に高感度であり、4 種類の AF において比較的同等の反応性で測定できることが判った。このような総 AF の測

定に適した抗体は、文献を調査しても他に存在しない。この条件は、今後さらに検討することで、より好適な性能に改善する可能性がある。

2. 添加回収試験

AF 未添加のブランク試料について、G1 及び B2 の検量線から AF 量を読み取った結果、4.5 及び 8.4 ng/g であった（表 1)。今回構築した直接競合 ELISA は、G1 が最も感度が高く、B2 が低い（図 1)。これらの結果から、購入したピーナッツバター中には、AF が 4.5 -8.4 ng/g 含まれていたことになる。そこで、添加回収試験では、実際の測定値と、この値から AFG1 及び AFB2 の検量線で得られたブランク値を引いた値を示した（A： 測定値-ブランク値（AFG1)、B: 測定値-ブランク値（AFB2))。即ち、A 条件による回収率は 156%-201%、B 条件による回収率は 111%-156%となった。総 AF の検査においては偽陰性が出ないことが求められる。今回の結果は、構築した直接競合 ELISA が真値よりやや高い濃度を示すことを示唆したが、偽陰性は生じにくく総 AF 検査に適していることを示唆した。

E 結論

より迅速・簡便・高感度な直接競合 ELISA の開発を目指した。その結果、＜6.3-250 pg/mL と極めて高感度で、AFB1, B2, G1, G2 間においてほぼ同等に測定可能であり、総 AF 検査に適する直接競合 ELISA を開発することができた。添加回収試験の結果は、回収率が 111-201%の範囲であり、スクリーニング検査として十分に実用に耐える性能を持つことが示された。また、実試料を測定に供したところ、規制値に近い AF を検出した。国内の市場には、これらの汚染食品が実際に多く流通している可能性がある。今後は、構築した直接競合 ELISA を用いたスクリーニングを進めることで、その実用性能を確認していきたい。

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

なし

2. 学会発表

なし

H. 知的所有権の取得状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

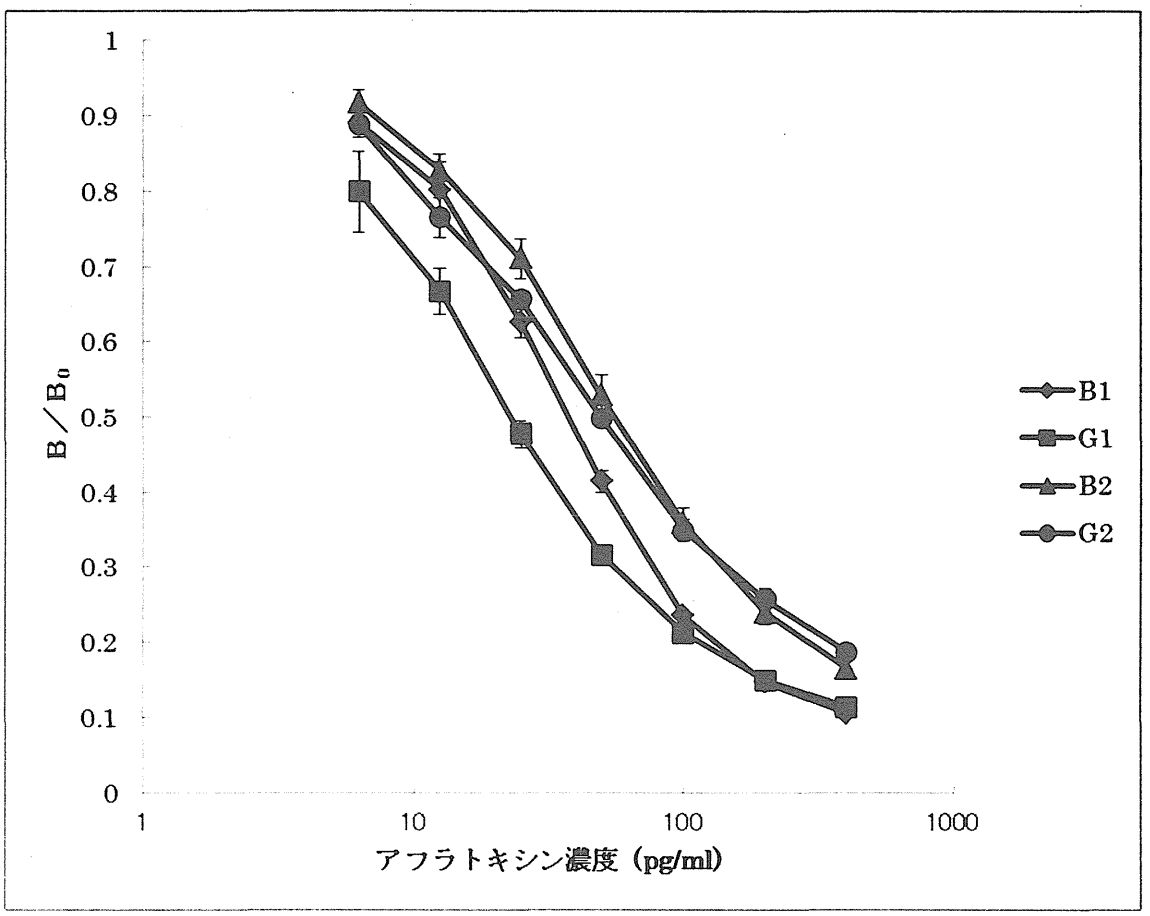

図１．直接競合 ELISA による各 AF（AFB1, AFB2, AFG1, AFG2）の標準曲線
結果は、全て別個に３回測定した平均値

表１．AF を添加したピーナッツバターの直接競合 ELISA による測定結果

	ブランク値（未添加） ng/g	測定値 ng/g	測定値－ブランク値（AFB2）		測定値－ブランク値（AFG1）	
			ng/g	回収率(%)	ng/g	回収率(%)
AFB1	6.7	21.4	13.0	130	16.9	169
AFB2	8.4	24.4	15.6	156	20.1	201
AFG1	4.5	20.4	12.1	121	15.9	159
AFG2	7.3	24.0	15.6	156	19.4	194
混合 AF		19.9	11.1	111	15.6	156

AF の添加濃度は、全て 10 ng/g

結果はすべて 2 回測定した平均値（単位: ng/g）

厚生労働科学研究費補助金（食品の安全確保推進研究事業）

検査機関の信頼性確保に関する研究

平成 26 年度

研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
なし							

誌上発表

発表者氏名	論文タイトル名	発表雑誌名	巻号	ページ	出版年
鎗田孝： Takashi Yarita, Takamitsu Otake, Yoshie Aoyagi, Takayoshi Kuroiwa, Masahiko Numata, Akiko Takatsu	Proficiency testing for determination of pesticide residues in soybean: Comparison of assigned values from participants' results and isotope-dilution mass spectrometric determination.	Talanta	132	269-277	2015
渡辺卓穂： 渡辺卓穂	食品分析の信頼性を確保するための外部精度管理について(計測標準フォーラム講演会)	*計測標準と計量管理*	63 (4)	6-11	2014

学会発表

発表者氏名	タイトル名	発表学会名	出版年
尾花裕孝： 高取聡、北川陽子、福井直樹、山口聡子、吉光真人、小阪田正和、梶村計志、尾花裕孝、	残留農薬分析におけるマトリックス効果補正法の比較	第51回全国衛生化学技術協議会年会（大分）	2014
山口聡子、高取聡、福井直樹、北川陽子、吉光真人、小阪田正和、梶村計志、尾花裕孝、	妥当性評価ガイドラインを踏まえた残留農薬検査における大阪府の取り組み	第51回全国衛生化学技術協議会年会（大分）	2014
鎗田孝： 鎗田孝、大竹貴光、青柳嘉枝、高坂典子、鈴木達也、渡辺卓穂	同位体希釈質量分析法による食品衛生学部精度管理調査試料（残留農薬分析用）の分析	2014年度AOAC International 日本セッション年次大会（東京）	2014
鎗田孝、大竹貴光、青柳嘉枝	安定同位体標識内部標準を用いた残留農薬分析におけるマトリックス効果の影響	第37回農薬残留分析研究会(仙台)	2014

大竹貴光、鎗田孝、青柳嘉枝、沼田雅彦、高津章子	農薬残留玄米試料を用いた技能試験結果の概要	第37回農薬残留分析研究会(仙台)	2014
渡辺卓穂： 梅津麻実、米澤夏岐、鈴木達也、渡辺卓穂	特定原材料の外部精度管理用調査試料の作製検討　―落花生 ELISA キットの測定に影響を及ぼす原材料についての検討―	第 108 回日本食品衛生学会学術講演会（金沢）	2014
安達玲子、酒井信夫、有馬優美、山本智之、佐久間恵、最上（西巻）知子	新規抽出液を用いて調製した特定原材料定量検査法標準品に関する検討	第 108 回日本食品衛生学会学術講演会（金沢）	2014
Reiko Adachi, Shinobu Sakai, Tomoko Nishimaki-Mogami	Food allergen labeling regulation in Japan and recent topics.	8th Workshop on Food Allergens Methodologies	2014

厚生労働科学研究費補助金（食品の安全確保推進研究事業）

検査機関の信頼性確保に関する研究

平成 26 年度

研究成果の刊行物・別刷

論文発表

Talanta 132 (2015) 269–277

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Proficiency testing for determination of pesticide residues in soybean: Comparison of assigned values from participants' results and isotope-dilution mass spectrometric determination

Takashi Yarita*, Takamitsu Otake, Yoshie Aoyagi, Takayoshi Kuroiwa, Masahiko Numata, Akiko Takatsu

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

ARTICLE INFO

Article history:
Received 1 August 2014
Received in revised form
30 August 2014
Accepted 2 September 2014
Available online 16 September 2014

Keywords:
Proficiency testing (PT)
Candidate reference material
Certified reference material (CRM)
Organophosphorus pesticide
Pyrethroid pesticide
Assigned value
z-Score
Primary method of measurement

ABSTRACT

Proficiency testing (PT) for the determination of pesticide residues in soybean samples was organized by the National Metrology Institute of Japan (NMIJ). The candidate certified reference material, NMIJ CRM 7509-a, that was prepared from the raw soybeans containing target pesticides (diazinon, fenitrothion, chlorpyrifos, and permethrin) was used as the test sample. Forty participants submitted two sets of analytical results along with the details of the analytical method and conditions they applied. Two types of assigned values were established for each target pesticide: one was derived from the analytical results of the participants, and the other was provided from the analytical results by isotope-dilution mass spectrometry (IDMS). The latter values were 7.4–16% higher than the former values, plausibly because the analytical values from the IDMS measurements were not affected by the recovery ratio of the target pesticides during the analytical process. Thus, two kinds of *z*-scores were calculated for individual participants using the corresponding assigned values: one (z_1-score) showed the relative performance score for the present PT and the other (z_2-score) could be used for evaluation of the trueness of their analytical methods.

1. Introduction

Various pesticides are used worldwide to protect foods against pests and diseases [1]. However, high levels of residual pesticides in foods may result in adverse effects on human health. In Japan, the Positive List System for Agricultural Chemicals Remaining in Foods was introduced in 2006 to prohibit the distribution of foods that contain agricultural chemicals above a certain level, even if the maximum residue limits (MRLs) have not been established [2]. Under this system, analysis of a wide variety of residual pesticides in foods that are under quarantine or in the market is routinely performed.

Analytical protocols for determining the presence of pesticide residues in food samples usually involve complex extraction of the target pesticides, multi-step clean-up of the obtained extracts, and sensitive and selective quantification via a chromatographic technique [3–5]. Ensuring the reliability of the results is crucial to control the risk associated with pesticide residues. Proficiency testing (PT) is one of the key elements in the implementation of an appropriate quality assurance program and performance monitoring procedure for chemical analysis laboratories [6,7]. Consequently, a number of PT programs for the determination of pesticide residues in food samples have already been organized [8–15].

The National Metrology Institute of Japan (NMIJ) has recently undertaken the development of crop certified reference materials (CRMs) for the validation/verification and quality assurance of pesticide residue analysis. The candidate materials used for development of the CRMs were prepared from raw crops containing the target pesticides. Characterization of the target pesticides was carried out by isotope-dilution mass spectrometry (IDMS), which has the potential to be a primary method of measurement [16–19]. To date, we have issued five kinds of CRMs in this field [20–23].

Another recent undertaking of the NMIJ is the organization of PTs for inorganic-constituent analysis of food samples to support skill upgrading of food analysis laboratories [24,25]. In 2012, the NMIJ initiated another PT program for pesticide-residue analysis of food samples. The first round PT was carried out using the candidate soybean powder material, NMIJ CRM 7509-a [23], as a test sample. In this PT, two kinds of assigned values were

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

http://dx.doi.org/10.1016/j.talanta.2014.09.001
0039-9140/

established: one was derived from the analytical results of the participants, and the other was provided from the analytical result used in the certification of CRM 7509-a. In this article, the process and the results of the PT are reported and the difference between the two assigned values is discussed.

2. Experimental

2.1. Test samples

An outline of the preparation of the test samples is presented as follows: the raw soybeans (*Glycine max*, cv. Enrei) were cultivated so as to contain *O,O*-diethyl-*O*-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate (diazinon), *O,O*-dimethyl-*O*-4-nitro-*m*-tolyl phosphorothioate (fenitrothion), *O,O*-diethyl-*O*-3,5,6-trichloro-2-pyridyl phosphorothioate (chlorpyrifos), and 3-phenoxybenzyl (1*RS*, 3*RS*, 1*RS*, 3*RS*)-3-(2, 2-dichlorovinyl)-2, 2-dimethylcyclopropanecarboxylate (permethrin). The soybeans were air-dried, freeze pulverized, mixed, bottled into amber glass bottles (10 g each), sterilized by γ-irradiation (15 kGy), and then the prepared samples were stored at ca. −80 °C until use. The homogeneity of the prepared samples was evaluated by the Japan Food Research Laboratories (Tokyo, Japan) by quantifying the target pesticides. The relative standard uncertainties related to the inhomogeneity were evaluated according to ISO Guide 35 [26] with values of 1.89% for diazinon, 4.00% for fenitrothion, 3.10% for chlorpyrifos, and 3.16% for permethrin. The details of the preparation and the homogeneity evaluation of the test samples have been described elsewhere [23].

2.2. Determination of pesticides in the test samples by NMIJ

The analytical results used in the certification of NMIJ CRM 7509-a were utilized in the present PT. These results were obtained by three analytical methods (Method 1, Method 2, and Method 3) conducted at NMIJ, the flow diagrams and the corresponding target pesticides of which are shown in Fig. 1. These methods consisted of extraction and clean-up processes that were based on the Analytical Methods for Residual Compositional Substances of Agricultural Chemicals, Feed Additives, and Veterinary Drugs in Food [3] and IDMS quantification by GC/MS measurements. Specifically, the initial analytical results (0 month) of the long-term stability assessment that were obtained by Method 1, and the analytical results of the characterization that were obtained by Method 2 or Method 3 were used to obtain the assigned values 2 (described in Section 2.4.). These analyses were performed in Nov. 2011, which was about four months prior to distribution of the samples to the participants. Additionally, the analytical results of the short-term stability monitoring that were obtained by Method 1 were used to evaluate the stability of the PT samples. These analyses were performed two weeks before distribution of the sample (April 2012) and three weeks after the deadline for submission of the participant results (August 2012).

2.3. Analysis by the participants

This PT round was announced to food manufacturers, agricultural producers, testing laboratories, public research institutes, etc., in Japan and was subscribed by 43 participants. On May 8, 2012, the test samples (2 bottles) and working instructions were sent to each participant by a delivery company using a refrigerated transport. The participants were asked to store the samples at −30 to −20 °C in the dark, and then to perform duplicate analysis of the target pesticides (the target pesticides to be determined were selectable by each participant). The participants were also asked to report the details of the analytical method and quantification conditions (such as the purity or concentration of primary calibrants, linear range of the calibration curves, the extraction and clean-up procedure, and the quantification method and its conditions) as well as the analytical results with observed chromatograms using a prescribed sheet in Excel format. The deadline was set to July 31, 2012.

2.4. Establishment of assigned values

For each target pesticide, two kinds of assigned values were established. The assigned value 1 (X_m) was obtained from the analytical results of the participants as follows: the outliers of the results were excluded by Cochran's test and Grubbs' test, and the median of the included results was then calculated. The normalized interquartile range (*NIQR*) was also calculated by multiplying the interquartile range (the difference of quartile 3 and quartile 1) of the included analytical results by 0.743. The assigned value 2 (X_{NMIJ}) was the weighted mean of the NMIJ analytical results obtained by the corresponding analytical methods (Method 1 and either Method 2 or Method 3). Simultaneously, the predicted reproducibility standard deviation ($PRSD_R$), which is the expected inter-laboratory precision for each target pesticide, was calculated using the modified Horwitz equation [27].

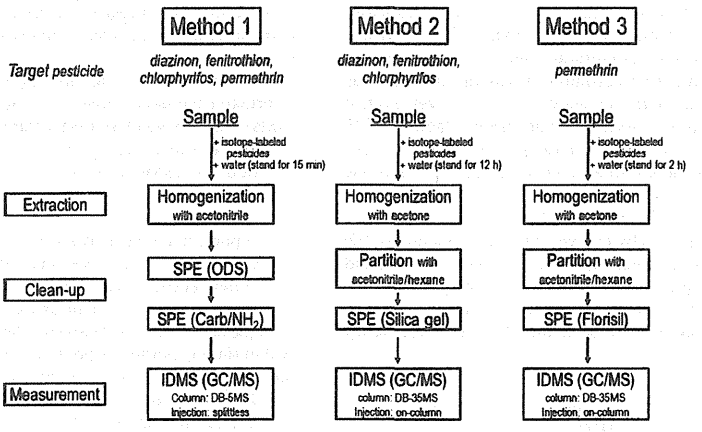

Fig. 1. Flow diagrams of the analytical methods for respective target pesticides used by NMIJ.

2.5. Calculation of z-scores

The performance of each participant was evaluated via the z-score approach in accordance with the ISO/IEC 17043 [28], ISO 13528 [29], and the International Harmonized Protocol for the Proficiency Testing of Analytical Chemistry Laboratories [7] methods. Because two kinds of assigned values were established in this PT, the corresponding z-scores (z_1 and z_2) were calculated using the following equations:

$$z_1 = \frac{x_i - X_m}{NIQR} \quad (1)$$

$$z_2 = \frac{x_i - X_{NMIJ}}{PRSD_R} \quad (2)$$

where x_i represents the analytical result for the target pesticide of the individual participants.

3. Results and discussion

3.1. Stability assessment of the test samples

The target pesticides of the test samples were assayed on three occasions (in November 2011, April 2012, and August 2012) by the NMIJ using Method 1. A one-way analysis of variance (ANOVA) test ($p < 0.05$) indicated that for each target pesticide, the difference in these three data points were not significant. These results suggested that the concentrations of the target pesticides in the PT samples stored at NMIJ remained unchanged during the period from the measurements to obtain assigned value 2 to the deadline for submission of the analytical results by the participants. Furthermore, the pesticide concentrations should remain constant if the participants stored the samples under the instructed conditions (at −30 to −20 °C in the dark). The relative standard deviation of the concentration for permethrin during the stated period was 6.87%, whereas the standard deviation for the other target pesticides was calculated to be below 0.

3.2. Analytical methods and results of the participants

Analytical results were submitted by 40 participants. Answers to the questionnaire on the analytical method and quantification conditions (matrix-matching of the calibration solutions, collection of the observed analytical values with the recovery ratio) that the participants applied are summarized in Fig. 2. More than half of the participants utilized the Multiresidue Method for Agricultural Chemicals by GC/MS (Agricultural Products) (hereafter referred to as "Multiresidue Method") as the extraction and clean-up method; this is an analytical method established by the Analytical Methods for Residual Compositional Substances of

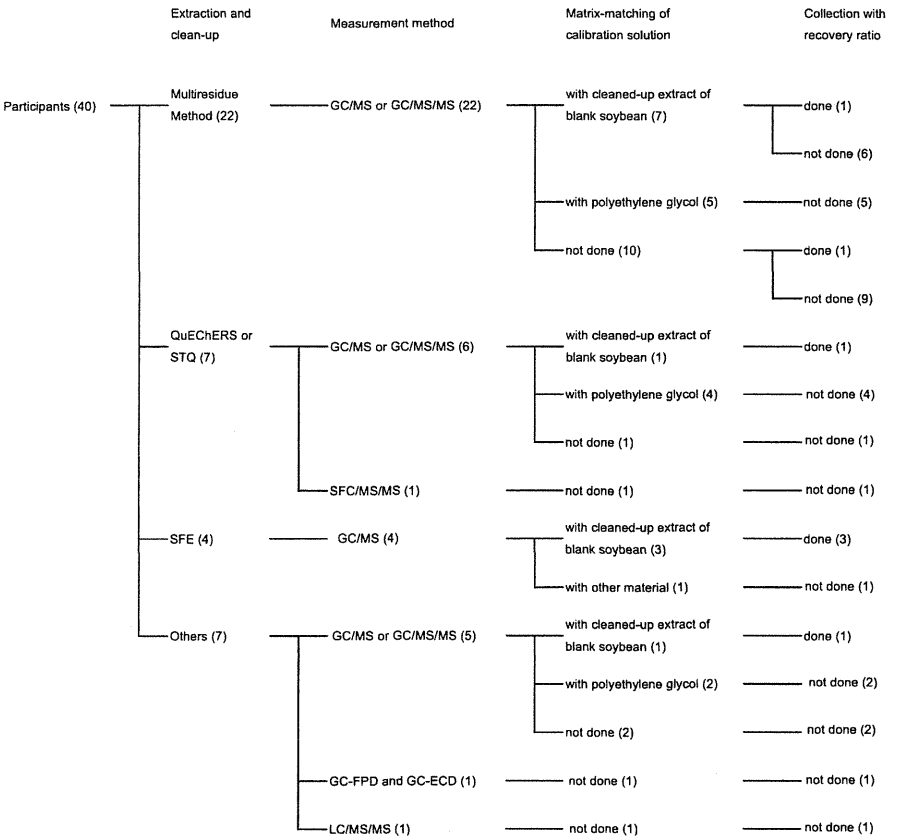

Fig. 2. Summary of the analytical methods and quantification conditions used by the participants. Numbers in parentheses indicate the frequencies of the participants.

Agricultural Chemicals, Feed Additives, and Veterinary Drugs in Food [3]. This method was verified by the Ministry of Health, Labour, and Welfare, Japan, and is intended for use in the Positive List System for Agricultural Chemical Residues in Foods [2]. The procedure is almost the same as that of Method 1, in which the target pesticides are extracted with acetonitrile followed by clean-up using SPE (see Fig. 1). In a spike and recovery test, as a criterion, the recovery ratio of each target pesticide should be in the range 70–120% [30]. If this criterion is met, the observed analytical results from the "Multiresidue Method" can be used without collection of the data based on the corresponding recovery ratio. Therefore, most participants who used this method in the present PT did not collect their analytical values. On the other hand, seven participants applied either the QuEChERS method [5,31] or the STQ [32] method; in both methods, acetonitrile was used as the extraction medium. The latter method consisted of extraction of the target pesticides by the QuEChERS method and clean-up of the extracts using small-size SPE cartridges. Supercritical fluid extraction (SFE), which can shorten the analytical time in comparison with the classical solvent extraction [5,33], was used by four participants. In all cases, carbon dioxide was used as the extraction medium without any modifier. Three participants cleaned up the extracts using SPE cartridges. Moreover, three participants collected the observed analytical values with the recovery ratios of the target pesticides, given that the trapping efficiency of some target pesticides by the collection solvent, which was placed at the end of the system, was not close to 100%.

The results of analysis of the concentration of fenitrothion were submitted by all the participants, whereas those for diazinon and

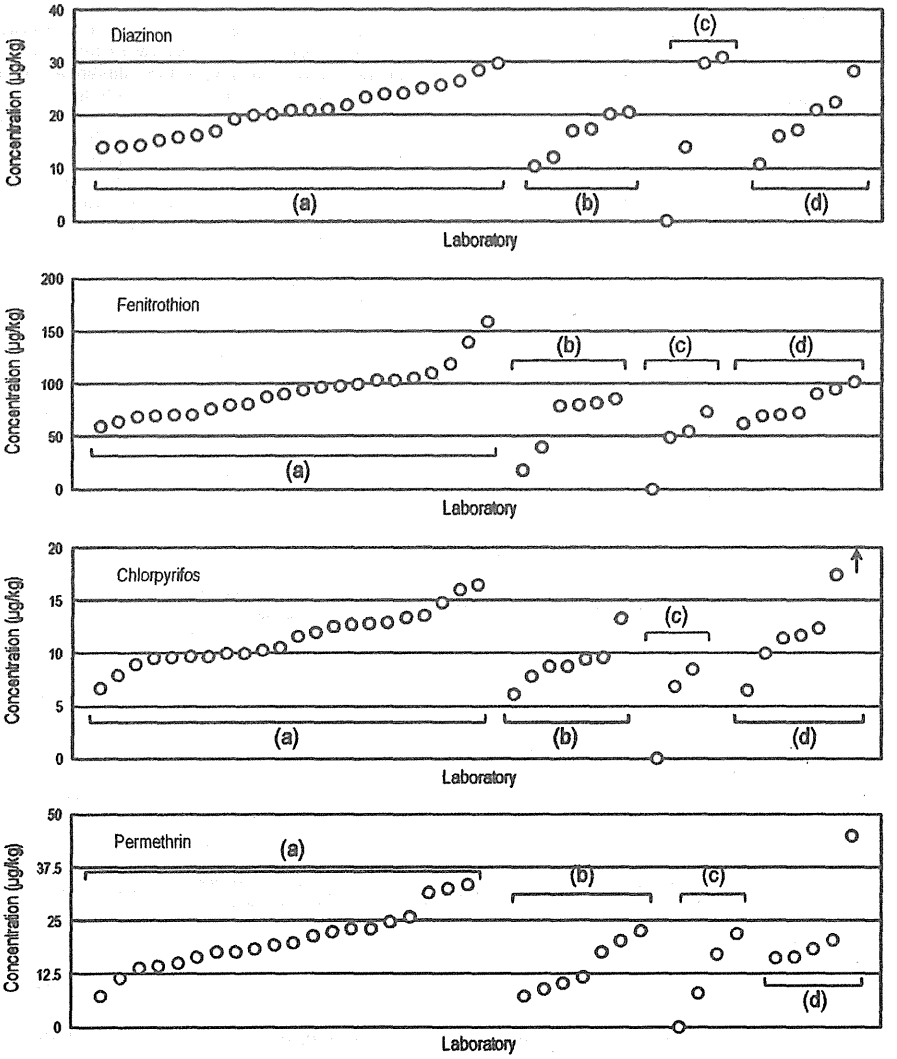

Fig. 3. Effect of the extraction and clean-up method on the analytical results of the participants. (a) Multiresidue Method for Agricultural Chemicals by GC/MS (Agricultural Products), (b) QuEChERS method or STQ method, (c) SFE method, and (d) other methods.

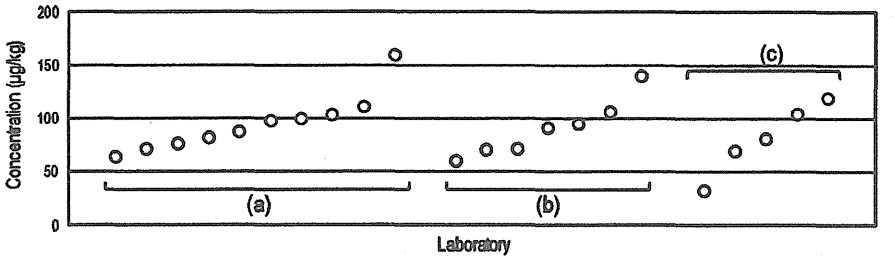

Fig. 4. Effect of matrix-matching of the calibration solution on the analytical results of the participants for assay of fenitrothion. The results obtained by the Multiresidue Method for Agricultural Chemicals by GC/MS (Agricultural Products) are indicated. (a) Without matrix-matching, (b) with matrix-mating using cleaned-up extract of blank soybean, and (c) with matrix-matching using polyethylene glycol.

Table 1
Assigned value 1 and other fundamental statistics.

Pesticide	Quartile 1	Assigned value 1 (median)	Quartile 3	*NIQR*
Diazinon	16.0	20.3	24.1	6.0
Fenitrothion	69.5	80.7	98.3	21.3
Chlorpyrifos	8.9	10.0	12.9	2.9
Permethrin	14.5	18.1	22.5	6.0

The unit of the values is μg/kg.

chlorpyrifos were submitted by 39 participants, and that for permethrin was submitted by 37 participants. Fig. S1 shows histograms of the analytical results. For each target pesticide, an unimodal distribution was obtained for the relationship between the analytical concentration and the number of participants (frequency). A Kolmogorov–Smirnov test ($p > 0.05$) was performed to evaluate the distribution of the analytical results, in which the normality of the distribution was supported for all target pesticides.

The analytical results were plotted based on the method employed, as shown in Fig. 3. One participant that applied SFE obtained results below 0.05 μg/kg for all of the target pesticides, probably due to an error in the conversion of the units. Fig. 3 indicates that even with the use of the same analytical method, there was considerable variation of the observed analytical values among the laboratories. Thus, the effect of the analytical method on the results was not significant. Similar consideration was given to the effect of the matrix-matching of the calibration solution on the analytical values. It is well known that the response of analyte of the sample solution (which was prepared from a real food sample herein) in GC/MS measurement is sometimes higher than that of the calibration solution [34–36]. To avoid this effect, 12 participants used calibration solutions that were matrix-matched using the cleaned-up extract of the blank soybean, and the other 11 participants employed calibration solutions containing polyethylene glycol. As an example, the analytical results of fenitrothion obtained by the Multiresidue Method were plotted based on the type of calibration solution, as shown in Fig. 4. The effect of matrix-matching of the calibration solution on the observed values was much less significant than the variation of the values among laboratories. Note that both the target pesticides and matrix used in the present PT were quite limited, further investigation is required for a more general evaluation of these effects.

3.3. Evaluation of the participants' performance using assigned value 1

3.3.1. Assigned value 1

The median of the analytical results of the participants was used as the X_m value given that this approach is considered robust and has been widely adopted by Japanese PT providers [37]. Initially, the analytical result from the participant that had made an error in the conversion of the units was rejected as an outlier. Subsequently, Cochran's test and Grubbs' test were carried out, in which one result was rejected for diazinon and chlorpyrifos, and three results were rejected for permethrin in the latter test. Using the remaining results, the fundamental statistics (including the X_m and *NIQR* values) were obtained, as shown in Table 1. When compared with the MRLs [38], the X_m values were 20% for diazinon, 40% for fenitrothion, 3.3% for chlorpyrifos, and 36% for permethrin, expressed as relative concentrations. These concentrations were deemed suitable for evaluation of the performance of the individual participants.

3.3.2. Assessment using z_1-scores

For each participant, the z_1-scores for the respective target pesticides were calculated using Eq. (1). The distribution of the participants' z_1-scores is shown in Fig. 5. Based on the ISO/IEC 17043 protocol, the participants' performance was ranked into the following classes:

(1) $|z_1| \leq 2.0$, "satisfactory" performance,
(2) $2.0 < |z_1| < 3.0$, "questionable" performance, and
(3) $3.0 \leq |z_1|$, "unsatisfactory" performance.

The number of participants in the individual classes is summarized in Table 2. If the participants' $|z_1|$ values follow the normal distribution, the ratio of the participants having $|z_1|$ values ranking in the second or third classes should comprise almost 5% of the entire participants. The observed ratios in the present PT were 5% for diazinon, 13% for fenitrothion, 13% for chlorpyrifos, and 16% for permethrin, where the ratio was slightly higher for target pesticides other than diazinon.

3.4. Evaluation of the participants' performance using assigned value 2

3.4.1. Assigned value 2

For each target pesticide, the weighted mean of the analytical results obtained by Method 1 and either Method 2 or Method 3 was calculated, and the result was used as the X_{NMIJ} value. The analytical methods used here were based on IDMS, and independent extraction and clean-up procedures were applied to avoid any possible analytical bias. The uncertainty corresponding to the Assigned value 2 was then estimated according to the ISO Guide 35 by combining the standard uncertainty of the analytical results and those corresponding to the inhomogeneity and instability of the PT samples. The details of these processes are described in the Supplementary material, and the results are summarized in

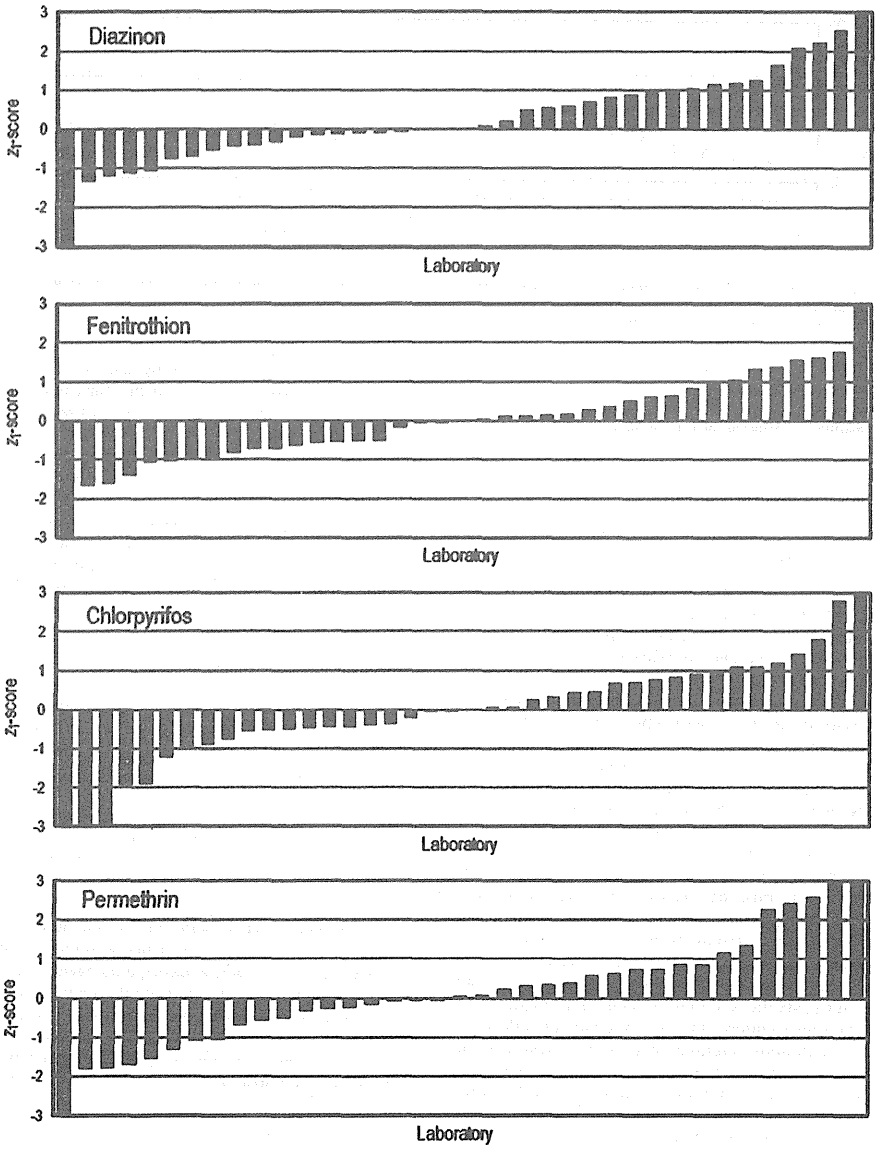

Fig. 5. Distribution of the participants' z_1-scores.

Table 2
Classification of the participants' z_1- and z_2-scores.

Pesticide	Number of participants	$\|z_1\| \le 2.0$	$2.0 < \|z_1\| < 3.0$	$3.0 \le \|z_1\|$	$\|z_2\| \le 2.0$	$2.0 < \|z_2\| < 3.0$	$3.0 \le \|z_2\|$
Diazinon	39	37	0	2	34	3	2
Fenitrothion	40	35	3	2	33	3	4
Chlorpyrifos	39	34	3	2	35	2	2
Permethrin	37	31	3	3	26	7	4

Table 3
Assigned value 2 with corresponding combined standard uncertainty and $PRSD_R$.

Pesticide	Assigned value 2	Combined standard uncertainty of assigned value 2	$PRSD_R$
Diazinon	21.8	0.6	4.8
Fenitrothion	87.9	5.6	19.3
Chlorpyrifos	11.6	0.6	2.5
Permethrin	19.9	1.9	4.4

The unit of the values is μg/kg.

Table 3. The strategy to obtain the Assigned value 2 and the corresponding uncertainty is the same as that used to certify the NMIJ CRMs [39], and the obtained X_{NMIJ} values seemed highly reliable and traceable to SI units.

3.4.2. Assessment using z_2-scores

If the participants submitted the analytical results with the corresponding uncertainties, their performance could be assessed using Assigned value 2 via the E_n-number approach [28]. However, at present, it is not easy for most food-analysis laboratories in Japan to estimate the uncertainties of their analytical results. In the present PT, therefore, participants were asked only to submit two sets of the analytical results without uncertainties. Thus, the performance of the participants using Assigned value 2 was also assessed by the *z*-score approach. For this purpose, the $PRSD_R$ value was calculated for each target pesticide using the modified Horwitz equation. Table 3 shows the results, where the relative

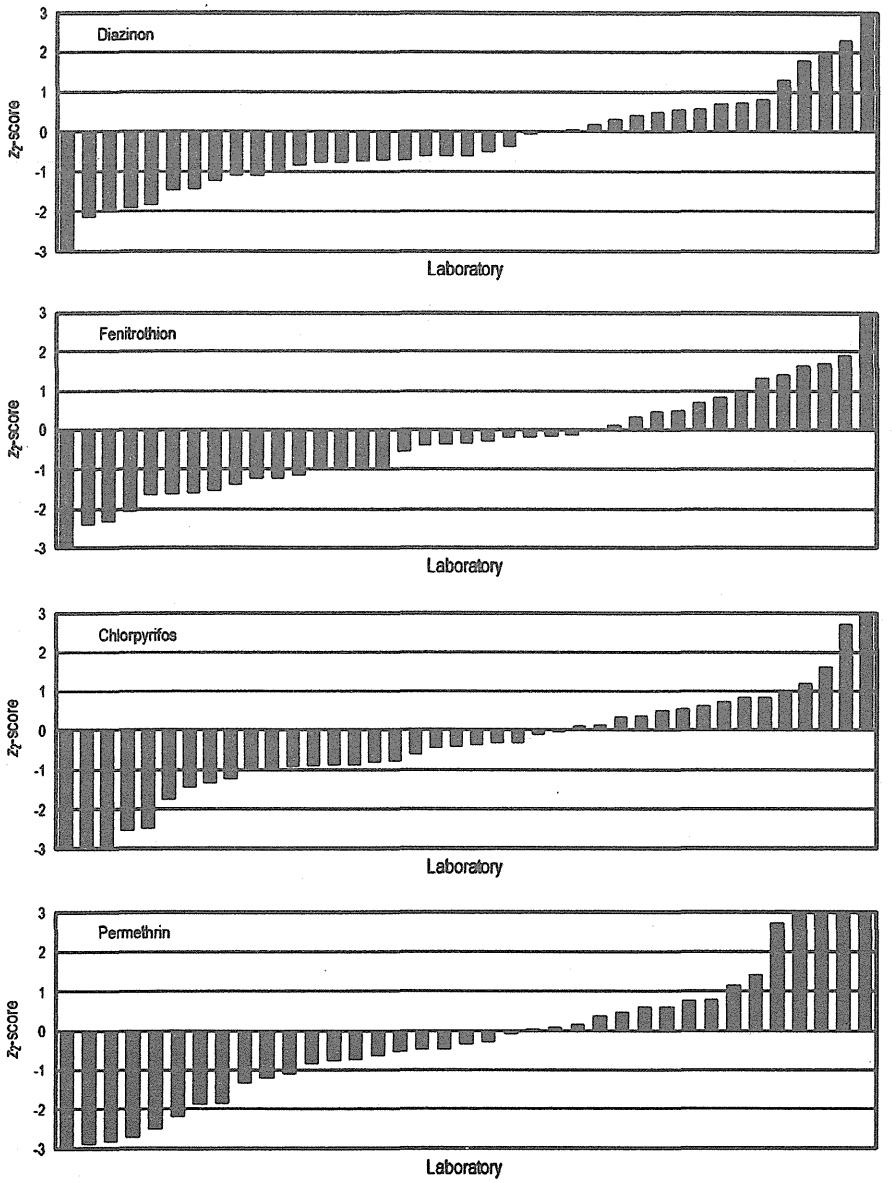

Fig. 6. Distribution of the participants' z_2-scores.

percentage of the respective $PRSD_R$ values was 22% in relation to the corresponding X_{NMIJ} values, and the $PRSD_R$ was much larger than the combined standard uncertainty of the Assigned value 2.

The z_2-scores for the individual target pesticides were then calculated for each participant using Eq. (2). Fig. 6 shows the distribution of the participants' z_2-scores. The performance of the participants was ranked into the following classes:

(1) $|z_2| \leq 2.0$, "satisfactory" performance,
(2) $2.0 < |z_2| < 3.0$, "questionable" performance, and
(3) $3.0 \leq |z_3|$, "unsatisfactory" performance.

The number of participants in the individual classes is also summarized in Table 2. The ratio of the participants having $|z_2|$ values ranking in the second or third classes was 13% for diazinon, 18% for fenitrothion, 10% for chlorpyrifos, and 30% for permethrin. Most participants quantified permethrin according to the following process: *cis*- and *trans*- isomers were individually quantified using corresponding calibrants and the observed concentrations were then summed. This might result in a higher ratio of permethrin in comparison with the other pesticides.

3.5. Comparison of two assigned values

Because assigned value 1 was obtained using the analytical results of the participants, this value possibly depended on the analytical method and quantification conditions used by the participants, the participants' skill, etc. Therefore, the analytical methods and quantification conditions of all participants were listed in the final report of the present PT. On the other hand, assigned value 2 was obtained by the analytical methods based on IDMS at NMIJ. The observed X_{NMIJ} values were 7.4–16% higher than the corresponding X_m values. As described above, most participants did not correct their observed analytical values with the recovery ratios of the target pesticides during the analytical process. At NMIJ, the recovery ratios of isotope-labeled pesticides spiked onto the samples were monitored. The recovery ratios were 79–91% in Method 1. In the IDMS measurement, the recovery ratios of the target pesticides did not affect the analytical values if equilibrium between the target and isotope-labeled pesticides was achieved. The differences between the two assigned values may be due to the difference in the impact of the recovery ratio during the two processes. In previous PTs for the determination of pesticide residues in food samples, the assigned values (concentration) obtained using the participants' analytical results were lower than the spiked concentrations employed for preparation of the test samples [15,40]: these results support our previous conclusion that the differences between the two assigned values may be due to the difference in the impact of the recovery ratio during the two processes.

Because assigned value 2 was higher than the corresponding assigned value 1, the z_2-score tended to be higher than the corresponding z_1-score (if the *NIQR* and $PRSD_R$ values were not significantly different). This difference between the two scores might be confusing to the participants; therefore, the participants were advised that the z_1-scores showed the relative performance score for the present PT. They were also advised to use the z_2-scores in their evaluation of the trueness of their analytical methods.

4. Conclusion

A new PT program for pesticide-residue analysis of food samples was initiated, and the first round of the program was carried out using the candidate soybean material, NMIJ CRM 7509-a, as the test sample. Two kinds of assigned values were established using either the participants' analytical results or the NMIJ's IDMS analytical results, and the corresponding *z*-scores were calculated for individual participants. The participants could select the appropriate score according to the purpose of their assessment. The effects of the analytical method and quantification conditions on the participants' analytical results were also cursorily considered; further investigation is required to evaluate these effects more generally.

Acknowledgments

The authors thank KANSO Technos for preparing the test samples and Japan Food Research Laboratories for evaluating their homogeneity. Gratitude is extended to the Korea Research Institute and the National Institute of Metrology, China, for analysis of test samples to ensure the analytical results of the NMIJ. This work was partly supported by the Research and Development Projects for Application in Promoting New Policy of the Agriculture Forestry and Fisheries (No. 21044) of the Ministry of Agriculture, Forestry and Fisheries, Japan, and the Health and Labour Science Research Grant of the Ministry of Health, Labour, and Welfare, Japan.

Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.09.001.

References

[1] F.P. Carvalho, Environ. Sci. Policy 9 (2006) 685–692.
[2] Ministry of Health, Labour and Welfare, Japan, Introduction of the Positive List System for Agricultural Chemical Residues in Foods, (http://www.mhlw.go.jp/english/topics/foodsafety/positivelist060228/introduction.html), 2006.
[3] Ministry of Health, Labour and Welfare, Japan, Annex of the Director Notice (Syoku-An No 124001) Analytical Methods for Residual Compositional Substances of Agricultural Chemicals, Feed Additives, and Veterinary Drugs in Food, 2005 (Amendment in 2006) (in Japanese).
[4] B. Jin, L. Xie, Y. Guo, G. Pang, Food Res. Int. 46 (2012) 399–409.
[5] L. Zhang, L. Shaowen, X. Cui, C. Pan, A. Zhang, F. Chen, Cent. Eur. J. Chem. 10 (2012) 900–925.
[6] I. Taverniers, M. De Loose, E. Van Bockstaele, Trends Anal. Chem. 23 (2004) 535–552.
[7] M. Thompson, S.R. Ellison, R. Wood, Pure Appl. Chem. 78 (2006) 145–196.
[8] M.E. Poulsen, H.B. Christensen, S.S. Herrmann, Accredit. Qual. Assur. 14 (2009) 477–485.
[9] F.G.M. Violante, M.H.W.M. Cardoso, J.M. Rodrigues, A.V. Gouvèa, C.N. Borges, P.R. da, F. Santos, D. da, S. Santos, H.C. de A Góes, V. Souza, A. de S. José, R.D.C. C. Bandeira, V. Cunha, A. Nóbrega, J. Chromatgr. Sci. 47 (2009) 833–839.
[10] K.M. Chan, S.T.C. Cheung, Y.-L. Wong, A.L.S. Cheng, C.-s. Mok, W.-w. Wong, D.W. Tholen, Y.-c. Wong, Trends Anal. Chem. 29 (2010) 562–576.
[11] P. Medina-Pastor, M. Mezeua, C. Rodríguez-Torreblanca, A.R Fernández-Alba, Anal. Bioanal. Chem. 397 (2010) 3061–3070.
[12] M. Okihashi, M. Osakada, K. Uchida, H. Nagayoshi, T. Yamaguchi, K. Kamimoto, Y. Nakayama, H. Obana, Shokuhin Eiseigaku Zasshi, J. Food Hyg. Soc. Jpn. 51 (2010) 253–257 (in Japanese).
[13] B. Kim, S. Ahn, Y. Mitani, Accredit. Qual. Assur. 16 (2011) 499–505.
[14] T. Watanabe, Shokuhin Eiseigaku Zasshi, J. Food Hyg. Soc. Jpn. 52 (2011) J315–J320 (in Japanese).
[15] M. Sykes, M. Thompson, S. Reynolds, J. Agric. Food Chem. 61 (2013) 4205–4209.
[16] T.J. Quinn, Metrologia 34 (1997) 61–65.
[17] W. Richter, Accredit. Qual. Assur. 2 (1997) 354–359.
[18] P. De Bièvre, H.S. Peiser, Fresenius J. Anal. Chem. 359 (1997) 523–525.
[19] M.J.T. Milton, T.J. Quinn, Metrologia 38 (2001) 289–296.
[20] T. Otake, N. Itho, Y. Aoyagi, M. Matsuo, N. Hanari, S. Otsuka, T. Yarita, J. Agric. Food Chem. 57 (2009) 8208–8212.
[21] T. Otake, T. Yarita, Y. Aoyagi, Y. Kuroda, M. Numata, H. Iwata, K. Mizukoshi, M. Nakamura, M. Watai, H. Mitsuda, T. Fujikawa, H. Ota, J. Agric. Food Chem. 59 (2011) 8568–8574.
[22] T. Otake, T. Yarita, Y. Aoyagi, Y. Kuroda, M. Numata, H. Iwata, M. Watai, H. Mitsuda, T. Fujikawa, H. Ota, Food Chem. 138 (2013) 1243–1249.
[23] T. Yarita, T. Otake, Y. Aoyagi, Y. Kuroda, M. Numata, H. Iwata, M. Watai, H. Mitsuda, T. Fujikawa, H. Ota, Talanta 119 (2014) 255–261.

[24] Y. Zhu, T. Kuroiwa, T. Narukawa, K. Inagaki, K. Chiba, Trends Anal. Chem. 34 (2012) 152–160.
[25] S. Miyashita, K. Inagaki, S. Naito, K. Shindoh, T. Narukawa, Y. Zhu, T. Kuroiwa, A. Hioki, T. Fujimoto, K. Chiba, Anal. Bioanal. Chem. 405 (2013) 8347–8362.
[26] ISO Guide 35, Reference Materials – General and Statistical Principles for Certification, International Organization for Standardization, Geneva, 2006.
[27] M. Thimpson, Analyst 125 (2000) 385–386.
[28] ISO/IEC 17043, Conformity Assessment – General Requirements for Proficiency Testing, International Organization for Standardization, Geneva, 2010.
[29] ISO 13528, Statistical Methods for Use in Proficiency Testing by Interlaboratory Comparison, International Organization for Standardization, Geneva, 2005.
[30] Ministry of Health, Labour and Welfare, Japan, Annex of the Director Notice (Syoku-An No 1224-1) Guidelines for the Validation of Analytical Methods for Agricultural Chemical Residues in Food, 2010 (in Japanese).
[31] A. Wilkowska, M. Bizink, Food Chem. 125 (2011) 803–812.
[32] S. Hiramatsu, K. Nishiyama, S. Tokuhashi, T. Ashida, A. Kageyama, M. Takamiya, A. Nakamura, N. Takuma, K. Nishimori, Rep. Publ. Health Kochi, 59 (2013) 47–52 (in Japanese).
[33] K. Uranishi, H. Yamashita, K. Yamamoto, Shokuhin Eiseigaku Zasshi, J. Food Hyg. Soc. Jpn. 53 (2012) 63–74 (in Japanese).
[34] K. Sugitate, S. Nakamura, N. Orikata, K. Mizukoshi, M. Nakamura, A. Toriba, K. Hayakawa, J. Pestic. Sci. 37 (2012) 156–163.
[35] K. Sugitate, M. Saka, T. Serino, S. Nakamura., A. Toriba, K. Hayakawa, J. Agric. Food Chem. 60 (2012) 10226–10234.
[36] S. Walorczyk, Talanta 120 (2014) 106–113.
[37] M. Kubota, Y. Takata, K. Koizumi, Y. Ishibashi, R. Matsuda, Y. Matsumoto, K. Shikakume, A. Ono, M. Sakata, K. Kakita, Bunseki Kagaku 57 (2008) 393 (in Japanese).
[38] The Japan Food Chemical Research Foundation, Maximum Residue Limits (MRLs) List of Agricultural Chemicals in Foods, ⟨http://www.m5.ws001.squarestart.ne.jp/foundation/search.html⟩, 2014.
[39] T. Yarita, A. Takatsu, K. Inagaki, M. Numata, K. Chiba, K. Okamoto, Accredit. Qual. Assur. 12 (2007) 156–160.
[40] Hatano Research Institute, Food and Drug Safety Center, Summary Report of the External Quality Control for Food Hygiene 2013, Hatano, 2014, pp. 67–101 (in Japanese).

特集　計測標準フォーラム講演会（1）

食品分析の信頼性を確保するための外部精度管理について

一般財団法人 食品薬品安全センター 秦野研究所
食品衛生事業部　渡　辺　卓　穂

1 はじめに

食品分析を行う機関にとっての分析値は食品の安全性を考える上で信頼性を確保して提示することが重要である。その信頼性を確保するための一般的な手法は、内部精度管理と外部精度管理であると考えられる。たとえば、理化学検査において、同じ検体を複数の機関で分析したとき、試験検査機関の分析能力の違いから、各機関で異なる分析結果を出していたのでは、行政処分等の対策もとれない。食品分野においては、平成8年にGLP (Good Laboratory Practice) が導入され、厚生省（現厚生労働省）は「食品衛生検査施設等における検査等の業務の管理の実施について」(平成9年4月1日) を通知し、内部精度管理と外部精度管理を規定して、食品の分析データの信頼性を確保している。このGLPはデータの信頼性確保のためのきわめて有用なシステムであり、検査結果の信頼性を保証するためには不可欠である。そこで、平成9年度より財団法人食品薬品安全センター秦野研究所（現　一般財団法人食品薬品安全センター秦野研究所）が厚生省（現　厚生労働省）の適合性の確認を受けて、検疫所、衛生研究所、保健所、食品衛生登録検査機関や自治体等のその他の公的検査機関を対象とした食品衛生外部精度管理調査（理化学、微生物学）を開始している。これに加え、平成12年度からは民間の食品分析機関を対象とした食品衛生精度管理比較調査も行っている。また、食品衛生法の改正により、平成18年5月ポジティブリスト制度が導入され、約800種類の農薬、動物用医薬品に対して、一律に残留基準値が設定された。これに伴い、分析対象物質が大幅に増え、一段と分析値に対する信頼性が要求されることになった。

このように、試験検査機関の分析値の信頼性を確保するために外部精度管理調査は非常に重要と考えられる。一方で、WTO（世界貿易機関）の貿易の技術的障害に関する一般協定（TBT協定）においては、一つの分析試験所で得られた分析値が世界で受け入れられるようなシステムの構築が施行されており、分析値の同等性が求められている。そのため、分析試験所に対して、分析値の信頼性を確保するためのシステムと一定の能力が求められ、各国の認定制度を同じ基準で運用することが不可欠になる。試験所認定は、試験所においての測定されたデータの信頼性を確保するため、試験所が一定の基準を満たし、特定の分野の試験を行う能力があることを第三者の認定機関が認定する制度であり、認定を行う機関がそれぞれ相互認証協定を結ぶことで試験データの共有を実現しようとしている。そのための規格として、ISO/IEC 17025（試験所認定）が現在種々の分野において用いられている。この要求事項は15項目の管理上の要求事項、10項目の技術的要求事項があり、使用する分析法は妥当性が確認されていなければならない。その確認方法の一つに試験所間比較（技能試験）があり、試験所認定を受けるために必須となっている。食品の輸出入に係る試験所は (1) ISO/IEC 17025に適合している。(2) 適切な技能試験に参加している。(3) 妥当性確認された方法を用いる。(4) 内部精度管理を実施している。これら4要件がガイドライン（CAC/GL 27-1997）に示され満たすように求められている。

以上の背景より、食品関連の検査施設には分析値の信頼性を確保するために技能試験、言い換えれば外部精度管理調査への参加が非常に重要であると考えられる。本稿では食品衛生法に基づいて公的機関等を対象として実施されている食品衛生外部精度管理調査について紹介する。