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The Proficiency Testing of Determination of Dioxins in Food

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Introduction

Food intake is the main route of human dioxin exposure, making the determination of dioxins in food indispensable for risk assessment and risk management of dioxins. The uncertainty of analytical results, however, can be very great because of the low concentration of the analytes and complicated cleanup procedures. The risk assessment of dioxins based on analytical results also suffers from a similar degree of uncertainty. The Ministry of Health, Labor and Welfare of Japan has published "Guideline for the Determination of Dioxins in Food" to standardize the analytical procedures. The guideline contains the quality assurance procedures to obtain reliable analytical results and recommends participation in the relevant proficiency testing scheme. The proficiency testing provides the fair evaluation of the analytical results. The central science laboratory in England and the food and drug safety center in Japan offer the proficiency testing on food. The National Institute of Health Sciences of Japan (NIHS) also has carried out proficiency testing of dioxins in food since 1998 to assure the quality of analytical results for dioxins. In this presentation we will show the results of 5 rounds of proficiency testing.

Methods and Materials

Samples The samples used in the proficiency testing are listed in Table 1. Table 1 also shows the number of participants and the TEQ of each sample.

BCR CRM607 and BCR RM 534 were prepared by the European Commission's Institute for Reference Materials and Measurements. Eleven certified value for PCDDs and PCDFs were given to CRM607. Eleven values were assigned for PCDDs and PCDFs in RM534 although not certified. CARP-1 was prepared by the National Research Council of Canada. Eighteen concentrations are certified, including PCBs. The custom-prepared standard solutions containing native PCDDs,

PCDFs and PCBs were prepared by Wellington Laboratories (Canada).

Other samples (freeze-dried fish and freeze-dried spinach) were prepared by the Japan Food Research Laboratories. The homogeneity of the samples was verified by the Japan Food Research Laboratories and the NIHS.

Analytical methods All participants determined dioxins by HRGC/HRMS as stipulated in the "Guideline for the Determination of Dioxins in Food".

Statistical analysis The mean and the standard deviation (SD) of the concentrations reported for each compound from the participants were calculated. There was the possibility of outlyers, but the application of tests for outlyers such as the Grubbs test was not advisable due to the small number of participants. The robust mean and the robust SD were then calculated using algorithm A[1]. The RSDs of TEQ in Table 1 were calculated from the robust mean and the robust SD. Examples of the statistical results are shown in Table 2. One participant reported a very high concentration of OCDD. This outlying high value lead to the high mean (2.85 pg/g) and the large SD (6.33 pg/g). The robust mean and SD of the same data were 0.67 pg/g and 0.25 pg/g, respectively, after the effect of the outlyer was eliminated. The z-Score of each participant was calculated using the robust mean and robust SD. The techniques of participants who gave a z-score of more than 3 or less than -3 were regarded as unsatisfactory, and review of their analytical procedures was recommended.

Results and Discussion

Year 1998 A CRM was used to verify the trueness of the results. The participants used the same standard solution, provided by the NIHS. The mean values of the results reported for two isomers were out of the confidence intervals of the certified values. All the results reported by two participants fell within the 95% confidence interval of the certified value. The other 4 participants reported results outside the 95% confidence interval but *the number of the outlying results was only 1-3. Reproducibility calculated from the 6 participants was 2.8-48 % RSD for each isomer and 6.6 % RSD for total TEQ.

Year 1999 The same CRM was used to compare the results with those in 1998. Many reports suggested that fish is the main route of dioxin intake, making the reliability of analysis of dioxins in fish crucial[2]. CARP-1 was then included in the proficiency testing. One plausible reason for poor reproducibility was the difference

among the standard solutions used by the participants. Mixed standard solutions of PCDDs, PCDFs and PCBs were used to estimate the variation in standard solutions among the participants.

For 6 isomers, the mean of participants was outside the confidence intervals of the certified values. The reproducibility for CRM607 (TEQ) was 11% RSD and larger than that in 1998. The decline in analytical performance probably arose from the difference between standard solutions. In 1998, all participants performed the determinations using the same standard solution. In 1999, each participant used their own standard solution. The number of participants increased to 15 in 1999, and inexperienced laboratories were included. This explains the increase in RSD.

The difference in the mean of the reported value for the mixed standard solution sample and the stated concentration was below 10%. The reproducibility of the standard solution sample was 8-15 RSD %. Bavel reported the RSDs of reported values of participants in proficiency testing in which a standard solution was used[3]. The RSDs after removing the outlyers were, with one exception, 10-17%. These results are similar to ours. The analysis of the solution required no cleanup procedure and the results were expected to represent the variability of the standard solutions of participants. According to the manufacturer's statement, the range of standard solution concentration is ± 5%, corresponding to an RSD of 2.9%. The higher reproducibility suggested other causes, such as the change in the concentration of the internal standards due to unsuitable storage conditions.

The mean of the reported values for CARP-1 was within the confidence interval of the certified value. The reproducibility of TEQ was 8.0% RSD. The TEQ of CARP-1 was 79 pg/g and was fairly large compared with the CRM607 (3.3). The large TEQ of CARP-1 led to its small reproducibility RSD.

Year 2000 Another RM and a standard solution with different isomer concentrations were used. The mean of the reported value for the RM was lower than the reference value for all compounds with reference values. The reproducibility of RM534 (TEQ) was 18% RSD. The reason for this poor reproducibility was not clear. The bias and reproducibility of the mixed standard solution sample were comparable to those in 1999. Differences in the standard solution used by the participants could not explain the large negative bias or large RSD.

Year 2001 As mentioned above, dioxin intake from marine fish is of great concern, and the use was requested of samples from wild polluted marine fish. The TEQ of CARP-1 is higher than that of wild fish, so it did not seem appropriate for proficiency testing aiming at the assurance of quality for analysis of common foods. Because no appropriate samples made of marine fish were available, we attempted the preparation of our own samples. Since 1998, no vegetable samples had been used in the proficiency testing, in spite of public concern about the contamination of leaf vegetables by dioxins.[4] For assurance of the performance of the vegetable analysis, a sample made of spinach was also prepared. Both samples were confirmed to be homogeneous and were thus suitable for proficiency testing. The reproducibilities of TEQ for the fish sample and spinach sample were 10% and 30%, respectively. The TEQ of the spinach sample was quite low (0.34 pg/g) at 1/20 of that of the fish sample. The large RSD was not extraordinary taking the low TEQ into consideration.

Year 2002 Another marine fish sample was prepared from grey mullet. Grey mullet contain more fat than sea bass and require further cleanup procedures. The results are likely to represent the actual analytical performance. The reproducibility was 7.1% RSD and comparable to the result of CARP-1.

The results of 5 rounds of proficiency testing revealed several problems with the determination of dioxins in foods. The variability of the standard solution is of major importance. Periodical confirmation of the validity of the standard by the use of CRM or by participation in proficiency testing is strongly recommended.

Although the TEQ of sea bass or grey mullet samples was about 1/10 of that of CARP-1, the reproducibility RSDs were comparable. These results show that repeated participation in proficiency testing improves the analytical skills of the laboratories. It is clear that for proficiency testing, the use of samples representing actual foods is preferable. Our attempted production of samples led to sufficiently homogeneous samples of fish and vegetables that could be prepared by freeze-drying. This technique opens the possibility of preparing samples from a variety of foods, leading to enhanced the effectiveness of proficiency testing.

Acknowledgements

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Table 1 Samples used in the proficiency testing and their reproducibility

Year	No of Participants	Samples		TEQ (pg/g)	Reproducibility RSD%
1998	6	BCR CRM607	spray-dried milk	3.3	6.6
1999	15	BCR CRM607	spray-dried milk	3.6	11
		CARP-1	homogenized fish	79	8.0
		Nonane solution of standards		23	8.7
2000	10	BCR RM534	spray-dried milk	4.6	18
		Nonane solution of standards		16	9.0
2001	9	Sea bass	freeze-dried	6.1	11
		Spinach	freeze-dried	0.32	31
2002	8	Grey mullet	freeze-dried	7.3	7.1

Table 2 Results of proficiency testing in 2001 Sample: Sea bass

Analyte			Normal statistics			Robust statistics		
	max (pg/g)	min (pg/g)	mean (pg/g)	SD (pg/g)	RSD (%)	mean (pg/g)	SD (pg/g)	RSD (%)
2,3,7,8□TCDD	0.31	0.18	0.24	0.05	19	0.24	0.05	22
1,2,3,7,8□PeCDD	1.08	0.70	0.84	0.13	15	0.84	0.13	16
1,2,3,4,7,8□HxCDD	0.28	0.12	0.18	0.05	28	0.17	0.04	25
1,2,3,6,7,8□HxCDD	0.63	0.46	0.55	0.06	12	0.55	0.07	13
1,2,3,7,8,9□HxCDD	0.13	0.07	0.09	0.02	24	0.09	0.03	28
1,2,3,4,6,7,8□HpCDD	0.70	0.20	0.33	0.16	48	0.29	0.08	26
, ụ ḅ ç c	18.50	0.37	2.85	6.33	222	0.67	0.25	38
2,3,7,8□TCDF	1.93	1.27	1.50	0.22	15	1.49	0.22	15
1,2,3,7,8□PeCDF	0.69	0.34	0.46	0.12	26	0.45	0.12	26
2,3,4,7,8□PeCDF	2.49	1.51	1.87	0.28	15	1.84	0.22	12
1,2,3,4,7,8□HxCDF	0.35	0.14	0.20	0.07	35	0.18	0.04	20
1,2,3,6,7,8□HxCDF	0.19	0.14	0.17	0.02	14	0.17	0.03	16
1,2,3,7,8,9□HxCDF	1.04	-	-	-	-	-	-	-
2,3,4,6,7,8□HxCDF	0.33	0.26	0.30	0.03	9	0.30	0.03	10
1,2,3,4,6,7,8□HpCDF	0.57	0.06	0.19	0.17	92	0.14	0.06	45
1,2,3,4,7,8,9□HpCDF	0.14	0.01	0.07	0.05	77	0.07	0.06	87
, ụ ḅ ç e	0.29	0.01	0.18	0.11	61	0.18	0.13	69
3,3',4,4'□TCB	75.5	43.7	53.7	9.8	18	52.48	8.02	15
3,4,4',5□TCB	3.32	1.69	2.43	0.53	22	2.43	0.60	25
3,3',4,4',5□PeCB	38.7	20.7	30.0	5.4	18	30.0	6.0	20
3,3',4,4',5,5'□HxCB	14.6	9.4	11.2	1.8	16	11.10	1.71	15
2,3,3',4,4'□PeCB	1180	827	983	106	11	979	112	11
2,3,4,4',5 □PeCB	134.7	51.0	77.9	23.2	30	73.7	12.1	16
2,3',4,4',5□PeCB	3589	2741	3214	310	10	3214	351	11
2',3,4,4',5□PeCB	73.9	33.2	47.4	12.5	26	46.1	11.0	24
2,3,3',4,4',5□HxCB	491	391	435	35	8	435	39	9
2,3,3',4,4',5'□HxCB	140	97	114	14	12	113.2	14.4	13
2,3',4,4',5,5'□HxCB	246	205	221	16	7	221	18	8
2,3,3',4,4',5,5'□HpCB	61.7	45.5	54.9	4.7	9	55.2	4.6	8
TEQ	6.90	5.19	6.19	0.60	10	6.19	0.68	11

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Enzyme-Linked Immunosorbent Assay for Monitoring Toxic Dioxin Congeners in Milk Based on a Newly Generated Monoclonal Anti-Dioxin Antibody

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Enzyme-Linked Immunosorbent Assay for Monitoring Toxic Dioxin Congeners in Milk Based on a Newly Generated Monoclonal Anti-Dioxin Antibody

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To develop an enzyme-linked immunosorbent assay (ELISA) for monitoring the toxicity due to polychlorinated dibenzo-*p*-dioxins and dibenzofurans contaminated in human breast milk, we have generated novel monoclonal antibodies using some haptenic derivatives linked to bovine serum albumin via the C-1 or C-2 position on the dioxin skeleton. BALB/c or A/J mice were repeatedly immunized with the immunogen, and spleen cells were fused with P3/NS1/1-Ag4-1 myeloma cells. After five fusion experiments, a hybridoma clone was established that secretes an antibody D9-36 group specifically recognizing the major toxic congeners, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin, and 2,3,4,7,8-pentachlorodibenzofran. An ELISA is developed on the basis of the competitive and labeled-antigen format. The toxic congeners extracted from butter or milk specimens by a novel extraction cartridge and a peroxidase-labeled dioxin analogue were sequentially reacted with a fixed amount of D9-36 in the presence of Triton X-100. The bound fraction was captured on a microtiter plate, immobilizing a second antibody, and the enzyme activity was colorimetrically determined. This ELISA afforded a practical sensitivity (measurable range, 1−100 pg/assay; detection limit, 1.0 pg/assay as 2,3,7,8-TCDD equivalent). The assay values for milk and butter samples were in reasonable accordance with the sum of the toxicity-equivalent quantity of each congener, which had been determined by a high-resolution gas chromatography/high-resolution mass spectrometry method.

Environmental chemical contaminants have been concerns of the public and the government for many years. Particularly, polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) are persistent pollutants because of their high lipophilicity that exhibit potential teratogenic, reproductive, immunotoxic, and carcinogenic effects. Therefore, PCDDs and PCDFs (PCDD/Fs) are the subject of surveillance by regulatory agencies. To evaluate the toxicity to newborn children, it is particularly important to monitor the trace amount of dioxins that may contaminate human breast milk. Gas chromatography/mass spectrometry (GC/MS) has conventionally been used as the reference method of determining these compounds. This method offers high sensitivity and specificity and has the potential for the simultaneous determination of multiple isomers having very similar structures. However, the GC/MS method requires complicated and time-consuming sample cleanup procedures, resulting in a poor performance of treating a number of samples. Furthermore, the equipment is available at relatively few research institutes and requires well-trained operators. Any feasible and quicker method which is therefore suitable for the routine analysis of PCDD/Fs in all laboratories is in demand, particularly for large-scale epidemiological studies or long-term monitoring.

Enzyme-linked immunosorbent assay (ELISA) is expected to be suitable for this purpose when the incorporated antibody shows enough affinity and specificity to target dioxin congeners. Several immunoassay procedures for PCDD/Fs have already been reported;[1−9] however, most of them have required a large amount of environmental samples because of insufficient sensitivity that

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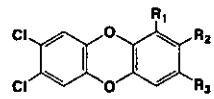
is attributed to lack of antibody affinity. We recently established a practical ELISA system for PCDD/Fs using a rabbit polyclonal antibody that is group-specific to some toxic dioxin congeners (unpublished data). Modern immunoassays, however, require the use of monoclonal antibodies, which are obtainable by hybridoma technology.[10] These monoclonal antibodies can be constantly supplied while maintaining well-defined and unique binding properties and are consequently able to standardize assay performance. From these points of view, we generated novel monoclonal antibodies, one of which was group-specific to the toxic dioxin congeners, which enabled the development of an ELISA system for monitoring the toxicity of dioxin contaminants in milk and butter samples. This ELISA provided assay values that are in good accordance with the toxicity-equivalent quantity (TEQ) of PCDD/Fs, which had been determined by a high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) method.

MATERIALS AND METHODS

Reagents. 1,2,3,4-Tetrachlorodibenzo-*p*-dioxin was purchased from AccuStandard (New Haven, CT). 2,7-Dichrolo-, 2,3,7-trichrolo-, 1,3,6,8-tetrachloro-, 1,2,4,6,8-/1,2,4,7,9-pentachloro-, and 1,2,3,4,6,7-hexachlorodibenzo-*p*-dioxins, polybrominated dibenzo-*p*-dioxins/dibenzofrans, and polychlorinated biphenyls (PCBs) were obtained from Cambridge Isotope Laboratories (Andover, MA). All the other PCDD/Fs, including 2,3,7-trichloro-8-methyldibenzo-*p*-dioxin (TMDD) and 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47), were purchased from Wellington Laboratories (Ontario, Canada). Bovine serum albumin (BSA), horseradish peroxidase (HRP; EC 1.11.1.7, type IV) (250–330 U/mg), and Triton X-100 were obtained from Sigma Chemical Co. (St Louis, MO). Freund's complete adjuvant (FCA) and incomplete adjuvant (FIA) were purchased from DIFCO (Detroit, MI). AffiniPure rabbit anti-mouse IgG + IgM antibody (the second antibody in the following ELISA) was obtained from Jackson ImmunoResearch (West Grove, PA). Protein G Sepharose 4 Fast Flow Lab Packs were purchased from Amersham Biosciences (Piscataway, NJ). 96-Well EIA/RIA plates (No. 3590) and plastic cell culture wares were obtained from Costar (Cambridge, MA). Reagents for the cell culture, including media, fetal bovine serum, hybridoma cloning factor (HCF), and poly(ethylene glycol) (PEG) were previously described.[11,12] Benzene, $CHCl_3$ (for PCB analysis), MeOH, EtOH, *n*-hexane (for dioxin analysis), and Presep phthalocyanine immobilized silica gel cartridges (Presep cartridges) were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other reagents and solvents were of analytical grade.

Haptenic Derivatives and Their BSA Conjugates. Five kinds of dioxin haptens used for preparing the immunogenic conjugates or enzyme-labeled antigens were those having a chemical linker on the C-1 (Ia, Ib, Ic) or C-2 (IIa, IIb) position of the dioxin skeleton[13] (Figure 1). The synthesis of haptens Ib[1], Ic[7], and IIb[7] has already been reported. The BSA conjugates of the haptens Ia, Ib, Ic, and IIa were prepared according to a reported method.[13] The hapten/BSA molar ratios in the conjugates were 11 (Ia), 16 (Ib), 11 (Ic), and 14 (IIa).



Haptenic derivative	R_1	R_2	R_3
Ia	$NHCO(CH_2)_3COOH$	H	Cl
Ib	$NHCO(CH_2)_4COOH$	H	Cl
Ic	$CH{=}CHCOOH$	Cl	Cl
IIa	H	$O(CH_2)_3COOH$	Cl
IIb	H	$CH{=}CHCOOH$	Cl

Figure 1. Structures of haptenic derivatives of dioxin used for antibody production and enzyme-labeling.

Buffers. The following were the buffers used: buffer A, 0.05 M sodium phosphate buffer (pH 7.4); buffer B, buffer A containing 0.9% NaCl; and buffer C, buffer B containing 0.1% gelatin.

Preparation of Enzyme-Labeled Antigen. The *N*-succinimidyl ester of the dioxin hapten Ib, Ic, or IIb was synthesized according to the reported method[14] and was reacted with HRP (4 mg) in 5 mM $NaHCO_3$ (200 μL) at room temperature for 30 min. The molar ratio of the activated ester to the enzyme in this reaction was adjusted to 5. To remove the unreacted haptens, the reaction mixture was submitted to gel filtration chromatography using Sephadex G-25, and the effluent showing the HRP activity was collected and stored at 4 °C for a few days. After the solution had become cloudy due to the remaining unreacted dioxins, the solution was centrifuged (1000*g*, 10 min), and the supernatant (1 mL) was washed three times with $CHCl_3$ (each 3 mL). After centrifugation, the resulting aqueous solution containing the enzyme-labeled antigen was diluted with buffer B and stored at 4 °C until use.

Immunization. Female BALB/c and A/J mice (each 8 weeks of age; 5 each) (Japan SLC; Hamamatsu, Japan) were immunized at three-week intervals with one of the hapten-BSA conjugates. The conjugate (50 μg) was subcutaneously injected with an emulsion of FCA (primary immunization) or FIA (booster immunizations) and sterile saline (1:1; 0.2 mL) into the footpads and at multiple sites on the back. Seven days after the fifth booster injection, blood was collected from the retrobulbar plexus, and the binding ability of the serum antibodies (diluted to 1:25 000) (50 μL) to the HRP-labeled dioxin (12.5 ng; 50 μL) was determined by the ELISA procedure (see below). The BSA conjugate (50 μg) in sterile saline (0.5 mL) was intraperitoneally injected into three BALB/c mice and two A/J mice that showed a high immune response, from which spleen cells were prepared 3 days later.

Monoclonal Antibody Production. The cell fusion experiment was performed according to previous papers.[11,12] Briefly, the immune spleen cells (1×10^8 cells) and 1/5 numbers of P3/NS1/1-Ag4-1 myeloma cells[15] were fused with 0.4 g/mL PEG 4000 in

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a sterile phosphate-buffered saline containing 10% (v/v) DMSO and 10 μg/mL poly-L-arginine·HCl solution (1 mL). The fused cells were suspended in a HAT medium supplemented with 10% HCF and cultured in 96-well cluster dishes (100 μL/well) overnight under 5% CO_2/95% air at 37 °C. After further culture for approximately 2 weeks in HAT medium, the hybridoma supernatants were submitted to screening by the ELISA described below. The antibody-secreting hybridomas were expanded in HT medium, cloned by limiting dilution, and were grown until confluence was reached. These hybridomas were inoculated into pristane-treated male BALB/c or BALB/c nude mice (8 weeks of age), and the relevant monoclonal antibodies were prepared on a large scale as ascitic fluids. The contained antibodies were purified using the Protein G column, and were used in the following ELISA.

ELISA Procedure for Hybridoma Screening. A solution of the second antibody diluted with buffer A (1.0 μg/mL; 100 μL) was distributed into each well of the EIA/RIA plates, which were left overnight at 4 °C. After washing twice with buffer B, the wells were blocked with a 5 mg/mL BSA solution in buffer B (200 μL) at room temperature for 2 h. The wells were washed twice with buffer B, to which the HRP-labeled hapten (20 ng/well) and the culture supernatant (1:5 dilution), each of which had been diluted with buffer C (each 50 μL), were then added. After incubation at room temperature for 1 h, the solutions were aspirated off, and the wells were washed three times with buffer B. The bound enzyme activity was colorimetrically measured using a substrate solution (100 μL) containing 0.05% *o*-phenylenediamine·2HCl and 0.01% H_2O_2. After incubation at room temperature for 1 h, the enzyme reaction was terminated by the addition of 3 M H_2SO_4 (50 μL), and the absorbance at 490 nm was measured using a BL 312e microplate reader (Bio-Tek Instruments; Winooski, VT).

Cleanup Procedure of Milk and Butter Samples for ELISA. A commercially available and ordinal butter or milk to which a known amount of dioxin [2,3,7,8-TCDD, 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (1,2,3,7,8-PeCDD), or 2,3,4,7,8-pentachlorodibenzofuran (2,3,4,7,8-PeCDF)] had been added was suspended in 5-fold (milk) or 10-fold (butter) volumes of EtOH, and saponified with KOH (final KOH concentration, 1 M) at 30−40 °C over 2 h. The resulting mixture was diluted with an equal volume of water and extracted three times with *n*-hexane (10 mL each). The organic layer was washed with H_2SO_4 (1 mL per each round) until the H_2SO_4 layer became clear. The organic solvent was evaporated and the residue was dissolved in H_2SO_4 (1 mL), which was left at room temperature for more than 1 h. This mixture was extracted with *n*-hexane (3 mL), and the organic layer was washed with 5% $NaHCO_3$ and water (each 3 mL) and then applied to the Presep cartridge. The cartridge was washed with *n*-hexane (3 mL) and *n*-hexane/benzene (9:1) (1 mL), then eluted with *n*-hexane/benzene (7:3) (1 mL). This solution was submitted to the ELISA as shown below.

Optimized ELISA Procedure for Determination of Dioxins in Milk and Butter Samples. Calibration curves were mainly obtained by using 2,3,7,8-TCDD. In some experiments, a nontoxic analog TMDD[7] was used as a "surrogate standard antigen," because this shows almost the same reactivity against the antibody D9-36 as 2,3,7,8-TCDD. The standard solution (2,3,7,8-TCDD or TMDD) (0−12.5 ng/mL in MeOH) (100 μL) or the effluent containing dioxins recovered from the fat or milk (see above) (1 mL) was mixed with 0.05% Triton X-100 in MeOH (50 μL), and the solvent was evaporated off. Buffer C containing the antibody D9-36 (0.5 ng/assay; 125 μL) was added to the residue, mixed, and left at room temperature for 30 min. An aliquot of the resulting solution (50 μL) and buffer C containing the HRP-labeled hapten Ic (20 ng/assay; 50 μL) were added to the second antibody-coated microtiter plate prepared as above, mixed using a plate mixer, and left overnight at 4 °C. The solution was then aspirated, and the plate was washed three times with buffer B. The bound HRP activity was measured as described above. Using the software installed in the microplate reader, sigmoidal dose−response curves were fitted to the following four-parameter logistic equation,

$$y = \{(A - D)/[1 + (x/C)^B]\} + D$$

where A is the maximum absorbance at zero concentration, B is the curve slope at the inflection point, C is the x analyte concentration giving 50% inhibition, and D is the minimum absorbance (background signal) at infinite concentration.[16]

HRGC/HRMS Analysis. HRGC/HRMS was carried out on a Hewlett-Packard (Palo Alto, CA) 5890-II gas chromatograph equipped with a Supelco 2331 column (60 m × 0.25 mm i.d.) combined with a JEOL (Tokyo, Japan) JMS-700 mass spectrometer (electron ionization) at a resolution R = 10 000 in the selected ion monitoring mode. Details of the conditions were as follows: (HRGC) column temperature program, 130 °C (2 min), 130−200 °C (15 °C/min), 200−260 °C (3 °C/min), 260 °C (30 min); carrier gas, He; column head pressure, 168 Kpa; injection temperature, 270 °C; injection volume, 2 μL (splitless): (HRMS) ion source temperature, 270 °C; ionizing current, 600 μA; ionizing energy, 38 eV; and accelerating voltage, 10 KV.

Safety Considerations. As is well-known, some dioxin congeners are highly toxic and we should consider the probability that any dioxin-related compound might be revealed to be toxic in the future. On the basis of such recognition, all of the handling of the dioxin-related compounds was performed in a draft chamber by well-trained researchers who used extreme caution as listed below. The researchers wore protective gloves, a laboratory coat, safety glasses, and a protective mask containing a carbon powder layer. The laboratory glassware was decontaminated by treatment with KPEG reagent (potassium poly(ethylene glycol)ate) at 150 °C for 12 h to remove chlorine atoms from the dioxin skeleton. Any contaminated site on the laboratory bench was cleaned up by a thorough wiping using wet cloths with an adequate organic solvent (acetone, toluene, or methoxymethanol) followed by UV irradiation to degrade the dioxin-related compounds.[17,18]

RESULTS

Monoclonal Antibody Production. To enlarge the opportunity for obtaining antibody-secreting hybridomas, we im-

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Table 1. Cross-Reactivity of Monoclonal Antibodies D2-37, D9-36, and D35-42 with Various PCDD/F Congeners in the ELISA[a]

congener[b]	cross-reactivity D2−37	D9−36	D35−42	TEF
2,3,7,8-TCDD	1.00	1.00	1.00	1.0
1,2,3,7,8-PeCDD	0.19	0.48	0.96	1.0
1,2,4,6,8-PeCDD		0.008		
1,2,4,7,9-PeCDD		0.008		
1,2,3,4,6,7-HxCDD		0.003		
1,2,3,4,7,8-HxCDD	0.10	0.07	0.0001	0.1
1,2,3,6,7,8-HxCDD	0.07	0.04	0.0002	0.1
1,2,3,7,8,9-HxCDD	0.12	0.06	0.0014	0.1
1,2,3,4,6,7,8-HxCDD	0.08	0.006	0.0001	0.01
1,2,3,4,6,7,8,9-OCDD	0.03	<0.0001	<0.0001	0.0001
2,3,7,8-TCDF	0.16	0.14	0.0001	0.1
1,2,3,7,8-PeCDF	0.08	0.03	0.0003	0.05
2,3,4,7,8-PeCDF	0.16	0.17	0.0004	0.5
1,2,3,4,7,8-HxCDF	0.06	0.02	0.0002	0.1
1,2,3,6,7,8-HxCDF	0.13	0.08	0.0001	0.1
1,2,3,7,8,9-HxCDF	0.05	0.07	0.002	0.1
2,3,4,6,7,8-HxCDF	0.07	0.06	0.0001	0.1
1,2,3,4,6,7,8-HpCDF	0.04	0.002	0.0001	0.01
1,2,3,4,7,8,9-HpCDF	0.03	0.002	0.0001	0.01
1,2,3,4,6,7,8,9-OCDF	0.02	<0.0001	0.0001	0.0001
2,7-DiCDD		0.05		
2,3,7-TrCDD		0.16		
1,2,3,4-TCDD	<0.001	0.0002	<0.001	
1,3,6,8-TCDD		0.0004		
TMDD	0.74	0.87	0.61	

[a] ELISA conditions are described in Figure 3. [b] Abbreviations: DiCDD, dichlorodibenzo-*p*-dioxin; TrCDD, trichlorodibenzo-*p*-dioxin; HxCDD, hexachlorodibenzo-*p*-dioxin; HpCDD, heptachlorodibenzo-*p*-dioxin; OCDD, octachlorodibenzo-*p*-dioxin; HxCDF, hexachlorodibenzofuran; HpCDF, heptachlorodibenzofuran; OCDF, octachlorodibenzofuran.

munized five BALB/c mice that are the commonest spleen donors in cell fusion and also five A/J mice using one of four kinds of haptens (Ia, Ib, Ic, and IIa) (Figure 1) conjugated with BSA. Thus, a total of forty mice, each of which was numbered, were immunized. The A/J mouse strain was selected here because it often shows a better immune response than the BALB/c strain against some lipophilic small molecules, such as steroids (e.g., the active form of vitamin D_3[11]). The haptens used here are the derivatives of 2,3,7,8-TCDD having a chemical bridge at the C-1 or C-2 position on the dioxin skeleton. The relationship between the number of mice and the strain/immunogen used is summarized in Figure 2. After the fifth booster immunization, small amounts of serum were collected from all these mice, and the titer of the anti-dioxin antibody was compared in the ELISA using the HRP-labeled hapten Ib, Ic, or IIb. The selection of these three HRP-labeled antigens was intended to avoid a false negative due to the inadequate combination of the haptenic derivatives used for immunization and enzyme labeling.[19] In site heterologous combination, for example, the combination such as an antibody derived from the hapten Ia and the enzyme-labeled antigen prepared with the hapten IIa, the anti-hapten antibody sometimes recognizes the difference in the position of the bridge and consequently does not show enough binding to the labeled antigen.

The sera affording significant positive signals (from mice nos. 2, 7, 9, 10, 14, 17, 18, 21, 26, 29, and 35) (Figure 2) were further assessed for their affinity to dioxins by comparing the inhibition of the bound enzyme activity when fixed amounts (1, 10, and 100 pg) of TMDD were added (data not shown; the cross-reactivity of TMDD to the antibodies, see Table 1). On the basis of these results, mice nos. 2, 9, 14 (the BALB/c mice immunized with the hapten Ia, Ib, and Ic, respectively), 26, and 35 (A/J mice immunized with the hapten Ic and IIa) were selected as spleen donors. Five fusion experiments were performed using the spleen cells prepared from each of these mice. About 10 days after the cell fusion, hybridomas were observed in over 95% of the microwells. The ELISA screening of the anti-dioxin antibody-secreting hybridomas and subsequent cloning by limited dilution allowed establishing three hybridoma clones, D2-37, D9-36, and D35-42. Antibodies D2-37 and D9-36 were each derived from the BALB/c mouse no. 2 (immunized with the hapten Ia) and the BALB/c mouse no. 9 (immunized with the hapten Ib), respectively; and D35-42 was from the A/J mouse no. 35 (immunized with the hapten Ic). These monoclonal antibodies were prepared as ascitic fluids, purified using the protein G column, and used in the following ELISA.

Characterization of Monoclonal Antibodies and Optimization of ELISA Conditions. The isotypes of the heavy and light chains of each monoclonal antibody were as follows: D2-37 (γ2a, κ), D9-35 (γ1, κ), and D35-42 (γ2a, κ). The binding characteristics of these antibodies were investigated by the competitive ELISA system using the HRP-labeled hapten Ic that showed satisfactory reactivity against all these antibodies. Because of the very strong hydrophobicity of the dioxins, we predicted that a suitable solubilizing agent (carrier proteins, detergents, or organic solvents) must be contained in the assay buffer. After several examinations (data not shown), we found that Triton X-100 works effectively for this purpose. Tween 20 and poly(vinyl alcohol) were also tested but were much less effective. A defined amount of Triton X-100 was added to the solution of the standard compounds or the extract fat or milk, and then the solvent was evaporated. The resulting residue [the mixture of dioxin(s) and Triton X-100] was dissolved in the assay buffer containing the monoclonal antibody. This procedure had to be done in a glass tube (not plastic tube or plate), and was important in order to increase the immunoreactivity of the dioxin(s) in the aqueous buffer.

To compare the assay sensitivity obtainable using each antibody, dose−response curves were constructed for 2,3,7,8-TCDD in combinations with the HRP-labeled hapten Ic (Figure 3). These assays have been done in the assay buffer containing 0.01% (v/v) Triton X-100 as the final concentration (the procedure for adding Triton X-100 is as described above). The optimum dilution rate of the antibody was arbitrarily determined as the dilution ratio affording the B_0 enzyme activity corresponding to 0.6−0.8 absorption units by a 1-h enzyme reaction. Although every antibody afforded a dose−response curve having an acceptable sensitivity, the curves of D2-37 and D9-36 were obviously more sensitive than D35-42.

We separately confirmed that the final concentration of Triton X-100 employed above (0.01%) is optimum by comparing the dose−response curves obtained at various concentrations of this detergent (Figure 4). In these experiments, antibody D9-36 was used employing TMDD as the competitor instead of 2,3,7,8-TCDD because TMDD provided dose−response curves almost equal to

(19) Hosoda, H.; Yoshida, H.; Sakai, Y.; Miyairi, S.; Nambara, T. *Chem. Pharm. Bull.* **1980**, *28*, 3035−3040.

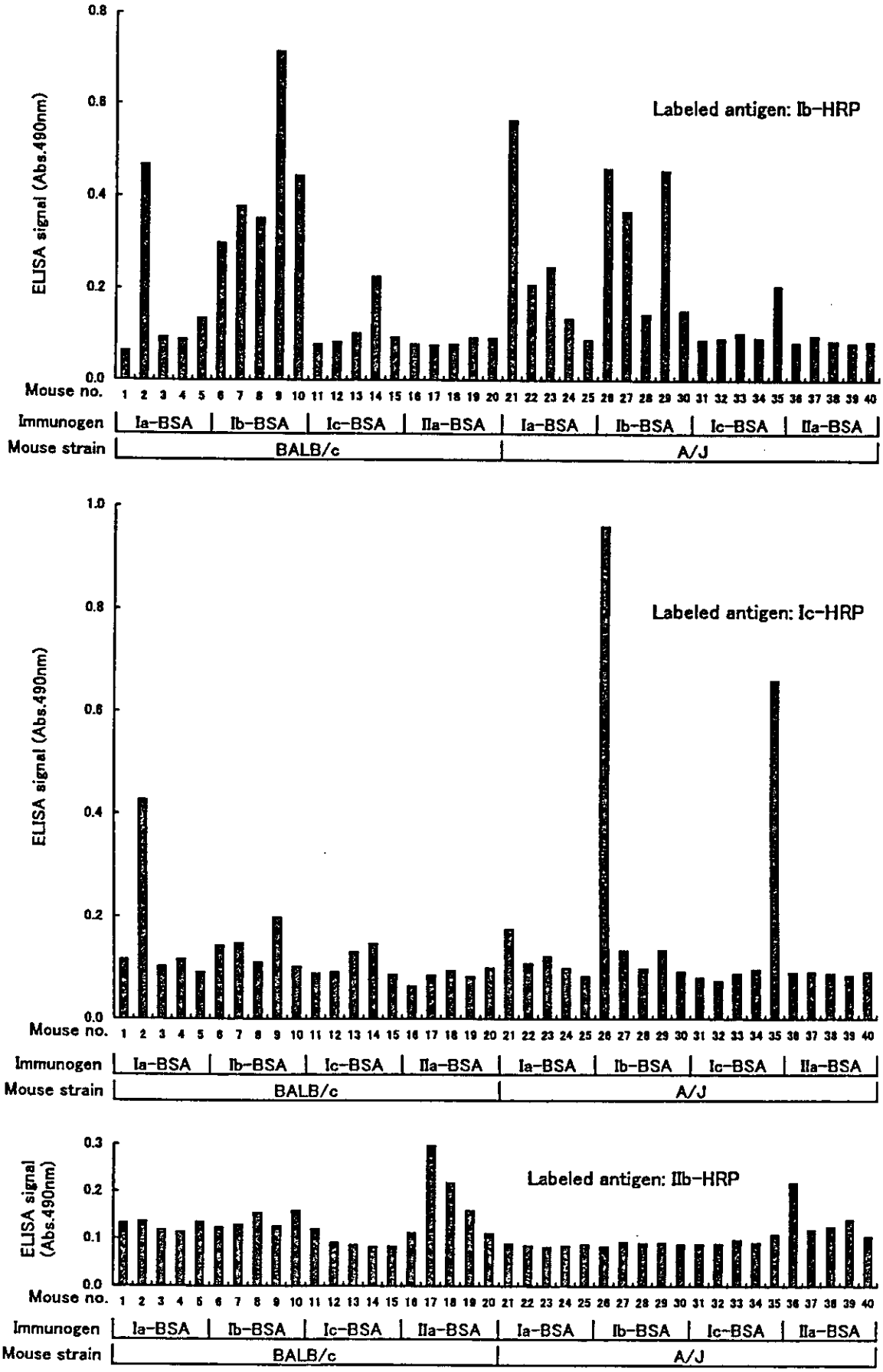

Figure 2. Comparison of the titer of anti-dioxin antibodies in the serum (1:25 000 dilution) obtained from the BALB/c or A/J mice after the fifth immunization with various immunogens (the BSA conjugate of haptenic derivative Ia, Ib, Ic, or IIa). Each bar shows the bound enzyme activity in the ELISA using the HRP-labeled hapten Ib, Ic, or IIb (25 ng/assay).

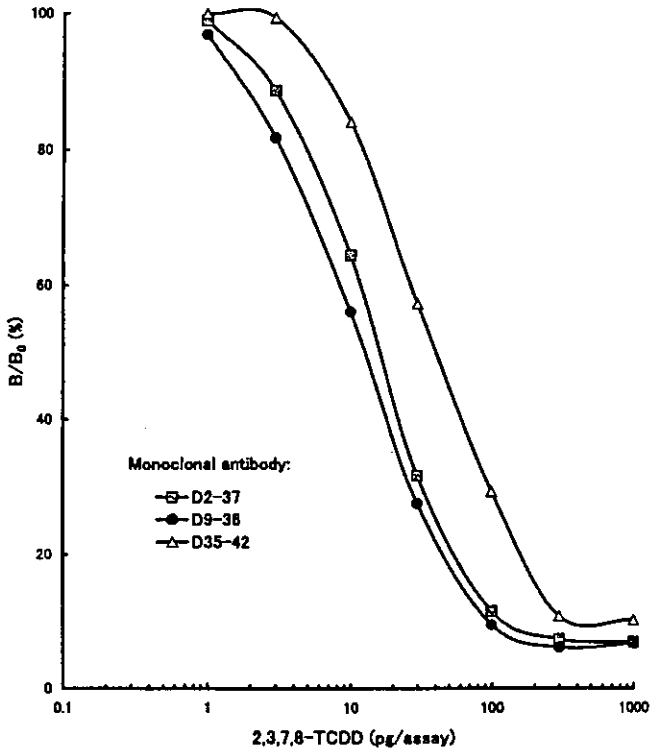

Figure 3. Dose–response curves for 2,3,7,8-TCDD in the ELISA systems using the monoclonal antibody D2-37 (4.0 ng/assay), D9-36 (0.4 ng/assay), or D35-42 (4.0 ng/assay) in combination with the enzyme-labeled hapten Ic (20 ng/assay) at a final Triton X-100 concentration of 0.01%.

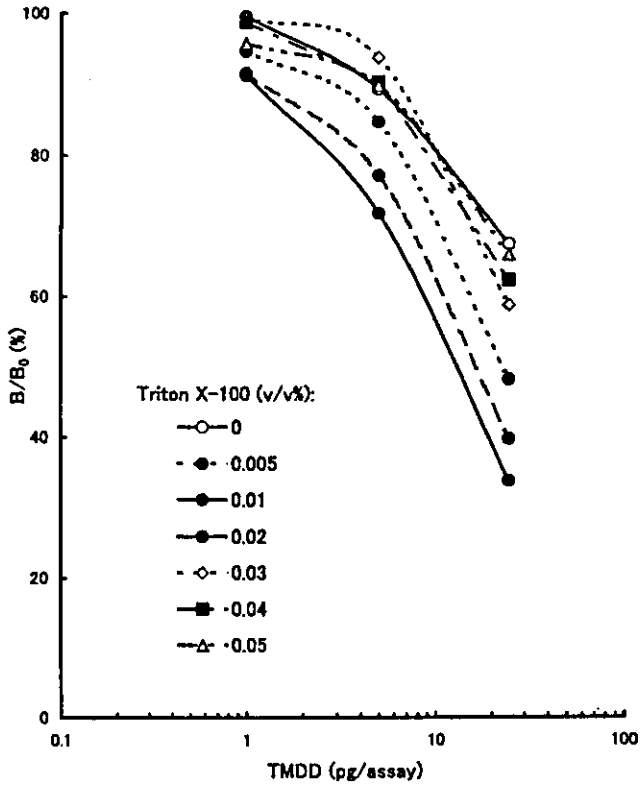

Figure 4. Effect of final concentration of Triton X-100 added to the assay medium on the assay sensitivity. ELISA was performed using the antibody D9-36 (1.0 ng/assay) in combination with the enzyme-labeled hapten Ic (20 ng/assay) at various concentrations of Triton X-100 [0, 0.005, 0.01, 0.02, 0.03, 0.04, or 0.05% (v/v)].

those obtained with 2,3,7,8-TCDD for antibody D9-36 (cross-reactivity of TMDD, 0.87; see Table 1). Therefore, this much less toxic congener is available as the standard compound instead of 2,3,7,8-TCDD, if necessary. Figure 4 also shows that the addition of Triton X-100 clearly increased the assay sensitivity and that the optimum concentration is 0.01%.

The cross-reactivity with various congeners of PCDD/F was then determined by the 50% displacement method[20] with a modification (Table 1), making it convenient to compare it with the toxicity-equivalent factor (TEF). TEF is defined to be the relative toxicity of a congener where the toxicity of 2,3,7,8-TCDD is taken to be 1.0, and TEQ is defined as the mass of a congener multiplied by TEF.[21,22] Namely, the cross-reactivity with each congener was expressed as the reciprocal of the ratio of its amount that is required for 50% displacement of the bound enzyme activity (midpoint), where the midpoint of 2,3,7,8-TCDD was taken to be 1.00 (e.g., the cross-reactivity value of 0.50 means that the congener required twice the amount as 2,3,7,8-TCDD). To establish an ELISA system for monitoring the sum of the toxicity of dioxins in breast milk, the antibody should possess a cross-reaction profile that is similar to the profile of TEF, particularly for the following major congeners detected in milk: 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, and 2,3,4,7,8-PeCDF. Antibody D2-37 exhibited a poor cross-reactivity (0.19) with 1,2,3,7,8-PeCDD, although this congener exhibits toxicity equal to that of 2,3,7,8-TCDD. Antibody D35-42 reacted with neither 2,3,7,8-tetrachlorodibenzofuran (2,3,7,8-TCDF) nor 2,3,4,7,8-PeCDF, both showing a considerable toxicity (TEF 0.1 and 0.5), although the cross-reactivity with 1,2,3,7,8-PeCDD was quite favorable. On the other hand, antibody D9-36 had a practically acceptable recognition pattern for these congeners, as shown by the cross-reactivity with 2,3,7,8-TCDF (0.14), 1,2,3,7,8-PeCDD (0.48) and 2,3,4,7,8-PeCDF (0.17). The relationship between the cross-reactivity of this antibody and TEF is schematically illustrated in Figure 5. The cross-reactivity of D9-36 with various derivatives of polychlorinated biphenyl (PCB), polybrominated dibenzo-*p*-dioxins and dibenzofrans, and BDE-47 (the polybrominated flame retardant which is most abundant in the environment) was also determined and found to be almost negligible, being in good correlation with the TEF values of these compounds (Table 2).

On the basis of these results, we choose antibody D9-36 for developing the ELISA system in combination with the HRP-labeled hapten Ic. This is a bridge heterologous system[19] because D9-36 is derived from the hapten Ib.

A typical dose–response curve for 2,3,7,8-TCDD obtained using the purified antibody D9-36 that covered the 1–100 pg/assay is shown in Figure 6. The midpoint was 15 pg/assay, and the detection limit was determined to be 2SD below the average B_0 (n = 10) was 1.0 pg/assay.

Application of ELISA to Milk and Butter Samples. The direct measurement of the dioxins in biological specimens containing considerable amounts of lipids, not only breast milk but also blood specimens, is almost impossible for immunoassays.

(20) Abraham, G. E. *J. Clin. Endocrinol. Metab.* **1969**, *29*, 866–870.
(21) WHO fact sheet no. 225, June, **1999**.
(22) Van den Berg, M.; Peterson, R. E.; Schrenk, D. *Food Addit. Contam.* **2000**, *17*, 347–358.

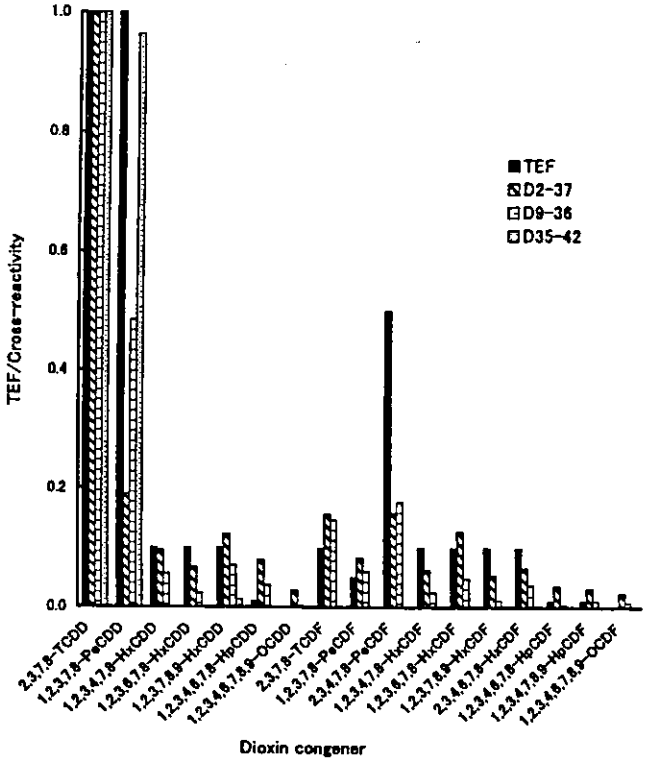

Figure 5. Schematic representation of the relationship between TEQ and the cross-reactivity of the monoclonal antibody D2-37, D9-36, or D35-42 for various dioxin congeners. Cross-reactivity was determined in the ELISA system using the enzyme-labeled hapten Ic (20 ng/assay) at a final Triton X-100 concentration of 0.01%. Abbreviations: HxCDD, hexachlorodibenzo-*p*-dioxin; HpCDD, heptachlorodibenzo-*p*-dioxin; OCDD, octachlorodibenzo-*p*-dioxin; HxCDF, hexachlorodibenzofuran; HpCDF, heptachlorodibenzofuran; OCDF, octachlorodibenzofuran.

Because dioxins are highly lipophilic compounds, it is essential to separate these compounds from the lipids that are abundant in these body fluids. Unless this has been achieved, accompanying lipids would interact with the dioxins by a strong hydrophobic attraction to make a kind of complex and consequently prohibit the dioxins from being captured by the hydrophilic antibody molecules.

Taking into account these difficulties and our goal, that is, the development of an ELISA system for the monitoring of the dioxin-contamination in human breast milk, we investigated a new cleanup procedure that is applicable to the lipid-abundant biological samples. To evaluate the efficiency of removing interfering lipids, we used the milk and butter samples, to which a known amount of toxic PCDD/F congeners (2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, or 2,3,4,7,8-PeCDF) had been added. Initially, these samples were submitted to saponification with potassium hydroxide, followed by the extraction of the dioxins with *n*-hexane. After a thorough washing with sulfuric acid, the *n*-hexane layer was further submitted to solid-phase extraction using the Presep cartridge which allows efficient isolation of the dioxins. As described above, the addition of Triton X-100 to the effluent from the cartridge was essential to afford the reasonable immunoreactivity of the recovered dioxins. The analytical recovery rates (mean ± SD) of 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, and 2,3,4,7,8-PeCDF were 64 ± 3.7, 75 ± 6.7, and 85 ± 8.2, respectively.

Table 2. Cross-Reactivity of Monoclonal Antibody D9-36 with Various PBDD/F Congeners and PBCs in the ELISA[a]

congener[b]	cross-reactivity	TEF
2,3,7,8-TBDD	0.39	
1,2,3,7,8-PeBDD	0.04	
1,2,3,6,7,8-HxBDD	0.003	
1,2,3,4,6,7,8,9-OBDD	0.0007	
2,3,4,7-TBDF	0.16	
2,3,4,7,8-PeBDF	0.11	
3,3′,4,4′-TCB (PCB-77)	0.0008	0.0001
3,4,4′,5-TCB (PCB-81)	0.0009	0.0001
2,3,3′,4,4′- PeCB (PCB-105)	<0.0001	0.0001
2,3,4,4′,5- PeCB (PCB-114)	<0.0001	0.0005
2,3′,4,4′,5- PeCB (PCB-118)	<0.0001	0.0001
2′,3,4,4′,5- PeCB (PCB-123)	<0.0001	0.0001
3,3′,4,4′,5-PeCB (PCB-126)	0.0005	0.1
2,3,3′,4,4′,5-HxCB (PCB-156)	<0.0001	0.0005
2,3,3′,4,4′,5′-HxCB (PCB-157)	<0.0001	0.0005
2,3′,4,4′,5,5′-HxCB (PCB-167)	<0.0001	0.00001
3,3′,4,4′,5,5′-HxCB (PCB-169)	<0.0001	0.01
2,2′,3,3′,4,4′,5-HpCB (PCB-170)	<0.0001	
2,2′,3,4,4′,5,5′-HpCB (PCB-180)	<0.0001	
2,3,3′,4,4′,5,5′-HpCB (PCB-189)	<0.0001	0.0001
2,2′,4,4′-TBDE (BDE-47)	<0.0001	

[a] ELISA conditions are described in Figure 3. [b] Abbreviations: TBDD, tetrabromodibenzo-*p*-dioxin; PeBDD, pentabromodibenzo-*p*-dioxin; HxBDD, hexabromodibenzo-*p*-dioxin; HpCDD, heptabromodibenzo-*p*-dioxin; OBDF, octabromodibenzo-*p*-dioxin; TBDF, tetrabromodibenzofuran; PeBDF, pentabromodibenzofuran; TCB, tetrachlorobiphenyl; PeCB, pentachlorobiphenyl; HxCB, hexachlorobiphenyl; HpCB, heptachlorobiphenyl; TBDE, tetrabromodiphenyl ether.

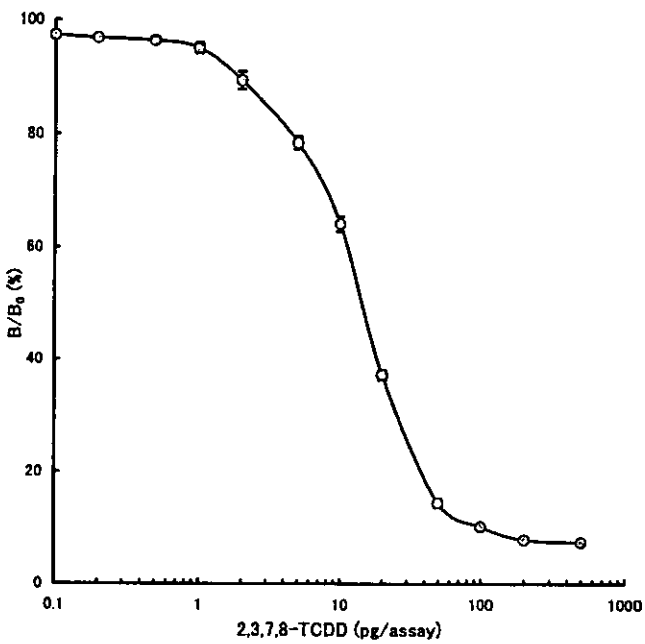

Figure 6. Typical dose–response curve for 2,3,7,8-TCDD in the optimized ELISA system using the monoclonal antibody D9-36 (0.50 ng/assay) in combination with the enzyme-labeled hapten Ic (20 ng/assay) at a final Triton X-100 concentration of 0.01%. Vertical bars denote SD of quintuplicate measurements.

The milk and butter samples containing the various amounts of the dioxin(s) were then extracted by this procedure and submitted to the ELISA. These assay values (2,3,7,8-TCDD equivalent in picograms) were in good correlation ($Y = 0.4182X + 3.5687$, $r = 0.9043$) with the sum of the TEQ (pg) of these

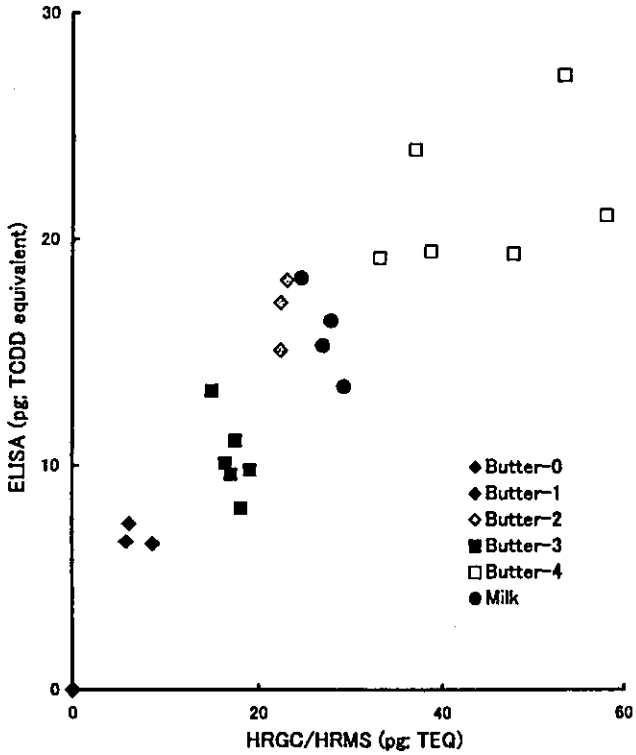

Figure 7. Correlation between the assay values (2,3,7,8-TCDD equivalent) obtained by the ELISA and the values (TEQ) obtained by the HRGC/HRMS method for milk or butter samples to which various amounts of toxic PCDD/F congener(s) were added as follows: milk, 10 mL of ordinal milk + 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, and 2,3,4,7,8-PeCDF (each 30 pg) (TEQ, 7.5 pg/mL); butter-0, ordinal butter only (TEQ, 0 pg/g); butter-1, the butter 1 g + 2,3,7,8-TCDD (10 pg) (TEQ, 10 pg/g); butter-2, the butter 1 g + 2,3,7,8-TCDD (30 pg) (TEQ, 30 pg/g); butter-3, the butter 1 g + 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, and 2,3,4,7,8-PeCDF (each 10 pg) (TEQ, 25 pg/g); butter-4, the butter 1 g + 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, and 2,3,4,7,8-PeCDF (each 30 pg) (TEQ, 75 pg/g).

congeners that had been separately determined by the HRGC/HRMS (Figure 7). Thus, the ELISA values are consistently ~40% of the TEQ values, results which are reasonable when one takes into account the imperfect coincidence between the cross-reactivity of the antibody D9-36 and the TEQ of the three kinds of dioxins (see Figure 5). Satisfactory parallelism was observed for the butter samples between the assay values and the amount of fat extract used in the assay (Table 3), indicating that the interfering materials have practically been removed. Mean analytical recovery rates of the three butter samples (1, 2, 3; Table 3) were 76, 70, and 69%, respectively.

Table 3. Determination of Toxic TCDD/F Congeners Contained in Butter Samples after Purification with the Present Cleanup Method and Serial Dilution

butter sample[a]	fat amount (g/assay)[b]	2,3,7,8-TCDD equivalent[b] pg/assay	pg/g fat
1	0.2	4.01	20.1
	0.4	7.50	18.8
	0.6	10.7	17.8
2	0.2	3.61	18.1
	0.4	7.11	17.8
	0.6	10.1	16.8
3	0.2	3.66	18.3
	0.4	6.79	17.0
	0.6	9.87	16.5

[a] Butter samples containing a known amount of 2,3,7,8-TCDD were prepared and purified in triplicate (for samples 1, 2, and 3) as follows: an ordinal butter (3.0 g) to which 2,3,7,8-TCDD (75 pg) had been added was saponified and extracted with the Presep cartridge according to the procedure described in the Experimental Section. [b] Aliquots of the effluent from the cartridge (0.25, 0.50, and 0.75 mL out from the initial 1.5 mL effluent) [each of which finally corresponds to 0.2, 0.4, and 0.6 g fat (extracted)/assay] were taken separately and mixed with 0.05% Triton X-100 in MeOH (50 μL). After the solvent was evaporated off, the resulting residue was submitted to the ELISA, as described in the Experimental Section.

DISCUSSION

Since Albro and co-workers reported the first immunoassay for dioxins using a polyclonal antibody and a ^{125}I-labeled dioxin analogue,[1] considerable effort has been made to develop a practical dioxin immunoassay system. However, two kinds of problems have been mainly hampering the need to establish a suitable assay system providing enough sensitivity and specificity. One of these problems is the difficulty in producing a practical antibody that group-specifically recognizes the limited kinds of toxic congeners from a family of PCDD/Fs that are composed of 75 kinds of congeners having very similar structures. Another problem has arisen from the highly hydrophobic nature of the dioxins. Because immunoassays rely on the antigen−antibody reaction that proceeds in aqueous media, we have to dissolve the hydrophobic dioxin molecules in the media and preserve their reactivity against the antibody. When an enzyme-aided immunoassay system (containing ELISA) is preferable, such difficulty even increases, because a large difference in the solubility between the highly lipophilic free dioxins and the dioxins labeled with a hydrophilic enzyme will prohibit a desirable competitive reaction against an anti-dioxin antibody.

Recently, Sugawara et al. succeeded in developing an ELISA system that is applicable to biological samples that uses a rabbit polyclonal antibody elicited against a hapten−carrier conjugate in which 2,3,7,8-TCDD had been linked to BSA via the 2-position.[8] This success would be due to the somewhat unusual nature of the antibody used in the assay: namely, the antibody maintained the antigen-binding ability even in an assay medium containing up to 37.5% dimethyl sulfoxide, which worked as a powerful solubilizing agent. This ELISA provided a much higher sensitivity (the midpoint was 12 pg/assay) than those of the previous dioxin immunoassays. It should also be considered that the ELISA system employed the competitive reaction based on the antigen-coated format (and using an enzyme-labeled second antibody as a detector) that could avoid the problem due to the difference in solubility between the labeled and unlabeled haptens mentioned above.

The use of monoclonal antibodies, however, should be desirable, because these antibodies can be constantly supplied by maintaining a well-defined and unique binding property and consequently enable an assay standardization. Although a few attempts have been made to generate monoclonal anti-dioxin antibodies, no one showing practical affinity and specificity to dioxin congeners has been reported so far. The most successful assay based on a monoclonal antibody would be the one reported by Harrison and Carlson.[6] They used a monoclonal antibody showing a practical specificity that had been elicited by Stanker et al.[4] The detection limit of this assay (reported to be 25 pg/

well), however, did not reach a "sub-parts-per-trillion sensitivity" that is required for analysis of dioxins in body fluids.

Taking advantage of the previous experience for producing some monoclonal antibodies against such hydrophobic small molecules as steroids or vitamin D metabolites,[11,12,23,24-26] we arranged a systematic immunization schedule combining two strains of mice and four haptenic derivatives in order to enhance the chance of obtaining a suitable spleen donor showing a stronger humoral response against dioxins. This strategy afforded several candidates as the donor, which allowed us five times the cell fusion experiments, after which we finally succeeded in establishing a hybridoma cell line secreting an antibody D9-36 having a practical recognition profile. Another important factor that led us to the present ELISA system was the use of Triton X-100 for solubilizing dioxins, the usefulness of which has previously been suggested.[1,5] The phenyl group in this detergent might have been a crucial factor for dissolving the dioxins (by forming a somewhat "loose" complex) and further let dioxins be immunoreactive in the aqueous solution. In the presence of the optimized amount of this detergent, excellent assay sensitivity [the midpoint, 15 pg/assay and the detection limit, 1.0 pg (corresponding to 3.1 fmol) per assay for 2,3,7,8-TCDD] was achieved. This sensitivity is comparable to that of the assay reported by Sugawara et al.[8] (see above) and much higher than the previous dioxin immunoassays using monoclonal antibodies. This successful high sensitivity, however, should be due to not only the effective solubilization of the target hapten but also a sufficient binding affinity of the antibody D9-36 to these congeners. Although we have not determined the *Ka* value of the antibody to 2,3,7,8-TCDD because of the unavailability of the corresponding radio-labeled compound, we expect that the value would be in the range of $\sim 10^9$ to 10^{10} (M^{-1}) taking into account our previous studies.[11,12,23,24,26]

It should also be emphasized that the present ELISA is the successful "labeled-antigen format" providing femtomole-range sensitivity that should be difficult to apply to such a hydrophobic target molecule as dioxins. To achieve the maximum sensitivity in this format, the very careful preparation of enzyme-labeled hapten is essential. Namely, in addition to the common consideration of the hapten/enzyme molar ratio[27] and combination of haptens used in the labeling and antibody production,[19,28] exhaustive removal of unreacted hapten-related molecules is required. The usual dialysis or gel chromatography is not effective enough for such hydrophobic haptens (see Experimental Section), because the unreacted haptens are noncovalently adsorbed on hydrophobic portions in enzyme molecules by a strong hydrophobic interaction and comigrate with the desirable enzyme-labeled haptens. We found that washing of the labeling reaction mixture with chloroform effectively removed the excess free dioxins with negligible loss of the enzyme activity [HRP activity after the treatment, 85 ± 3.7% (n = 6) of the initial activity]. This simple procedure should be widely applicable to a workup in the enzyme labeling of various hydrophobic haptens and helpful for improving assay sensitivity. A systematic study of this procedure will be reported elsewhere. One more important factor for obtaining a high sensitivity is the use of the purified monoclonal antibody. When the culture supernatant was used as the source of the antibody, much less sensitivity was obtained. Some components in the supernatant, for example, BSA derived from fetal calf serum supplied to the culture medium, might have become bound to the dioxins with a significant affinity (probably by hydrophobic interactions) and, consequently, might have decreased their reactivity against the antibodies.

We also established a new cleanup procedure that is essential for avoiding the problem inherent in the hydrophobic dioxins (mentioned above). To achieve this, the dioxins in biological fluids must be thoroughly separated from the accompanying lipids before the reaction with antibodies. Even after saponification of the samples, a trace amount of interfering lipids would be extracted together with dioxins, forming a kind of complex by hydrophobic interaction and, consequently, prohibit the access of antibodies. The usual normal-phase and reversed-phase chromatographies were not effective enough for removing such interferences. Fortunately, we found that the Presep cartridge is suitable for separating aromatic dioxin-related compounds from such an interference. Further optimization is now ongoing in our laboratories to improve the analytical recovery of all the target congeners. Affinity chromatography using a solid-phase immobilizing of the present antibody would be another promising way for isolating the dioxins from the lipids, because this method is based on a very specific molecular recognition toward the target compounds.

As shown in Figure 7, the present ELISA provides the assay values as the 2,3,7,8-TCDD equivalent that corresponds to ~40% of the total TEQ values of the important toxic congeners, 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, and 2,3,4,7,8-PeCDF. Although a new antibody showing perfect group-specificity to these congeners would afford almost the same values as the sum of the TEQ and would be even preferable, a satisfactorily good correlation (r = 0.9043; Figure 7) allows us to evaluate the present ELISA to be useful enough for routine surveillance of dioxin contamination. Very recently, we found that an improved enzyme-labeled antigen, in which the 3-hydrogen (instead of chlorine atom) analogue of the hapten Ic (which is expected to be less toxic than compound Ic itself) is linked to HRP, is also available in the ELISA system without altering the assay performance described so far.

In conclusion, the present "monoclonal ELISA" for dioxins is selective, has a sufficient sensitivity, is feasible, and consequently, does not require skillful operation or an expensive facility. This assay system will be particularly useful for the first screening of a number of breast milk specimens prior to the second examination for each congener by HRGC/HRMS.

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食品衛生外部精度管理調査研究の概要
遺伝子組換えトウモロコシ(CBH351)および遺伝子組換えジャガイモ(NewLeaf Plus and NewLeaf Y)の検知用試料の作製と調査成績

An Outline of External Quality Assessment Research for Food Hygiene (First Report)
On the Preparation of Test Sample and the Investigation Results for Detection of Genetically Modified Maize (CBH351) and Potato (NewLeaf Plus and NewLeaf Y)

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I　はじめに

近年，遺伝子組換え(GM)食品の開発が急速に進んでおり，わが国においてもダイズ，トウモロコシ，ジャガイモ等のGM食品ならびにそれらを原料とする加工食品が流通するようになっている。わが国においては平成12年の厚生省告示第232号ならびに第233号により，安全性の確認されていないGM食品が国内で流通しないよう，食品衛生法の規格基準を改正し，平成13年4月以降，GM食品の安全性審査ならびに表示を法的に義務付けた[1,2]。さらに，流通ならびに表示の科学的検証を行うため，「組換えDNA技術応用食品の検査方法について」[3,4]を通知し，GM食品の検査方法を定めている。この検査方法により安全性審査の終了していないGM食品が定性的に検知された場合は，積戻しあるいは廃棄の対象となる。そのため，当検査方法を用いて得られる測定結果の信頼性を確保することが大変重要であり，精度管理が不可欠であると考えられる。

精度管理は「内部精度管理」と「外部精度管理(技能試験)」に分類され，前者は機関内での精度の均一化を目的とし，後者は機関間での均一化を目的とする。一般に機関間にみられる試験結果のばらつきは機関内でのばらつきに比べ大きい。したがって外部精度管理調査を実施することにより，一定の検査方法における機関間のばらつきの程度ならびにその要因を把握すること，さらには検査担当者が自己の技術を客観的に認識し，検査技術の維持向上を図ることは極めて重要と思われる。

GM食品検査法の外部精度管理調査に関しては，検査法が分子生物学的技術を要するために，食品衛生外部精度管理調査事業としてではなく，調査研究的な要素を含んだ形態で平成13年度より実施されている[5]。

本稿では平成13年度に実施したGMジャガイモおよびトウモロコシの定性検査法における外部精度管理試験調査研究の内容を紹介し，分析結果の相互比較を通じて，検査機関によるばらつきの程度ならびにその要因について詳細な検討を行った。

Ⅱ　調査試料調製と均一性確認

1　調査試料の調製法

試験対象として選定した安全性審査が終了していないGMトウモロコシのStarlink®(CBH351，以下CBHと略)，GMジャガイモのNewLeaf Plus®(RBMT 21-350系統，Russet Burbank種，以下NL-Pと略)，GMジャガイモNewLeaf Y®(SEMT 15-15系統，Shepody種，以下NL-Yと略)，および疑似混入試料調製時のマトリックスとして使用した非遺伝子組換え(non-GM)トウモロコシ試料(ブラジル産トウモロコシ)は，厚生労働省医薬局食品保健部監視安全課(現厚生労働省医薬食品局食品安全部監視安全課)を通じて入手した。Non-GMジャガイモには，世田谷区内のスーパーマーケットで購入した国内産ジャガイモ(男爵)を使用した。

入手したすべてのトウモロコシおよびジャガイモ試料を凍結乾燥処理した後に粒径が500 μm(トウモロコシ)，あるいは200 μm(ジャガイモ)以下となるよう粉砕し，その後，GM試料の混入率が重量換算で0.1, 1.0, 0%となるようトウモロコシ試料，ジャガイモ試料のそれぞれについて混合した。混合法は，Trapmannら[6]ならびに栗原ら[7]が報告しているGMトウモロコシのダイズ疑似混入試料調製法を参考にし，一部改変した。まず，均一に粉砕した試料を再度凍結乾燥処理した。その後，上記重量比となるようマトリックス(non-GM試料)とGM試料を正確に秤量し全量を50 gとして，プラスチック製の袋に量り採った。袋中で十分な混合を行った後，ふるいにかけ，再び袋中で混合を繰り返した。この混合操作は合計3回行った。混合操作後の試料を粉砕器で再度粉砕した後，凍結乾燥処理した。疑似混入試料調製後，均一性試験を3機関[国立医薬品食品衛生研究所(国立衛研)，食品薬品安全センター秦野研究所，東京大学大学院]で行った。また，安定性試験は，国立衛研において調製し測定した各試料を−20℃の条件で1カ月間保存後，再度測定することで実施した。

2　調査試験の実施

均一性の確認された試料2 g(トウモロコシ)または200 mg(ジャガイモ)を，それぞれ50 mL容遠沈管または15 mL容遠沈管に正確に秤量分注し，乱数表を用い同一試料2本を1組として無作為に番号を付した上で試験検体とし，盲検独立並行試験として参加機関に送付した。なお，試料名称および1機関当たりの試料数および内容は以下の通りである。トウモロコシ：試料名称；トウモロコシ粉砕物，試料数；3組6試料，内容；0.1%，1.0% CBHおよび0%それぞれ2試料，ジャガイモ：試料名称；ジャガイモ凍結乾燥粉末，試料数；5組10試料，内容；0.1%，1.0% NL-P，0.1%，1.0% NL-Yおよび0%それぞれ2試料，また，試料送付時には，諸注意事項を含む実施要領，平成13年食発第158号[4]に準じ作成した試験マニュアル，調査項目ならびに試験結果についての報告方法を規定した各種報告様式を同送した。

調査項目としては，検査全般についての経験年数，遺伝子組換え食品の検査実績，検査実施環境および実験機器，器具共用の有無，各種機器のメーカー名，使用したDNA抽出法，プライマーのメーカーおよびグレード，電気泳動条件，染色方法を取り上げ，検査全般にわたって詳細な調査が行えるよう配慮した。試験結果については，抽出されたDNAの吸光度(230，260，280ならびに320 nm)と収量，さらに各種プライマー対を用いたPCRにおいて遺伝子増幅バンドが検出されたか否かアガロースゲル電気泳動の写真を添えて試料ごとに記載の上，1組2試料の検査結果から厚生労働省通知「組換えDNA技術応用食品の検査方法について」[3,4]に従って組ごとにGM含有の有無およ

び含有するGMの種類結果を判定し，報告するものとした。これら試験方法の作成に当たってはThompsonらによる報告[8]ならびにAssociation of Official Agricultural Chemists(AOAC)のマニュアル[9]を参考にした。

Ⅲ　調査試料の確認結果と調査成績

1　各種調査試料検体の均一性の確認

トウモロコシおよびジャガイモ各疑似混入試料を対象とした均一性試験結果のアガロースゲル電気泳動像を，図1～3に示す。試験は，それぞれの試料についてn＝3でDNA抽出，PCR増幅を行い実施した。CBHを対象とした試験においては，図1に示すように，対照プライマーを用いた試験で0,0.1,1.0％試料ともほぼ均一な濃さの増幅バンドが得られた。さらに検出用，確認用両プライマー対を用いた試験では，0.1％疑似混入試料での増幅バンドに濃淡は認められるものの0.1％，1.0％試料のすべてにおいて，それぞれの増幅バンドが得られた。したがって試験に供したすべての試料においても均一性が確認できたものと考えられた。NL-P，NL-Yを対象とした試験では，対照プライマーを用いた試験ではすべての試料について得られた増幅バンドの濃淡に明確な差が認められなかった。また図2に示すように，NL-Pの検出用，確認用両プライマー対を用いた試験では，0.1％，1.0％試料のすべてにおいて，それぞれ対応する増幅バンドが得られた。さらに図3に示すように，NL-Yの検出用，確認用両プライマー対を用いた試験では，0.1％，1.0％試料のすべてにおいて，それぞれ対応する増幅バンドが得られた。したがってジャガイモ試料においても試験に供したすべての試料において均一性が確認できたものと考えられた。

しかしNL-P検出用プライマー対と確認用プラ

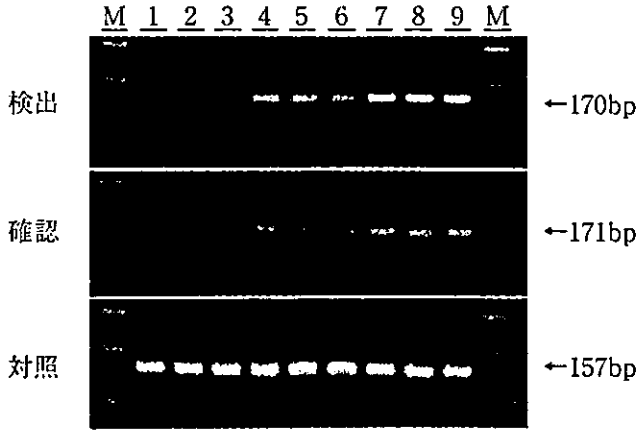

図1　CBH351トウモロコシ検体の均一性試験
1-3：　0%　試料から抽出されたDNAのPCR増幅産物
4-6：0.1%　試料から抽出されたDNAのPCR増幅産物
7-9：1.0%　試料から抽出されたDNAのPCR増幅産物
M：標準DNAラダー

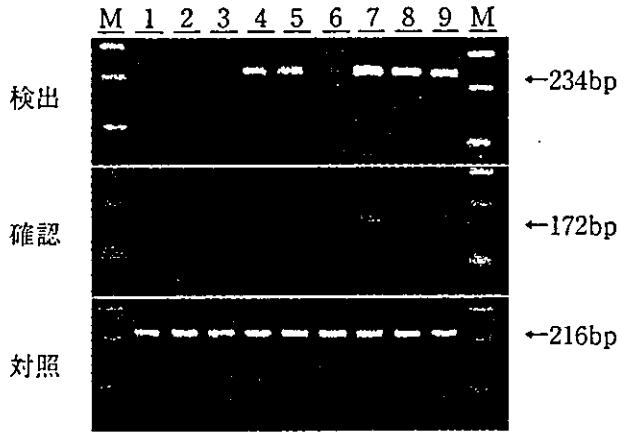

図2　NewLeaf Plusジャガイモ検体の均一性試験
1-3：　0%　試料から抽出されたDNAのPCR増幅産物
4-6：0.1%　試料から抽出されたDNAのPCR増幅産物
7-9：1.0%　試料から抽出されたDNAのPCR増幅産物
M：標準DNAラダー

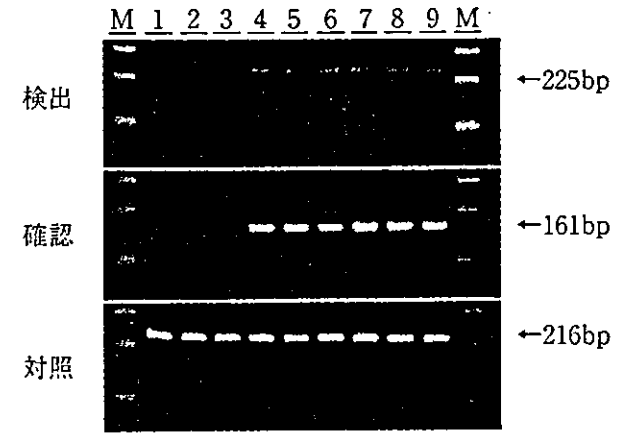

図3　NewLeaf Yジャガイモ検体の均一性試験
1-3：　0%　試料から抽出されたDNAのPCR増幅産物
4-6：0.1%　試料から抽出されたDNAのPCR増幅産物
7-9：1.0%　試料から抽出されたDNAのPCR増幅産物
M：標準DNAラダー

表1　トウモロコシ検体の各機関における吸光度比およびDNA濃度(シリカゲル膜タイプキット法)

		O. D. 260/280			DNA濃度(ng/μL)		
		Non G	0.1%	1%	Non G	0.1%	1%
A	抽出A	1.780	1.780	1.730	70.5	77.5	98.0
	抽出B	1.720	1.760	1.700	76.5	72.0	81.5
B	抽出A	1.785	1.730	1.692	108.0	109.0	112.5
	抽出B	1.800	1.797	1.730	85.5	106.0	86.5
C	抽出A	1.569	1.818	1.808	215.0	120.0	151.0
	抽出B	1.783	1.824	1.769	140.0	114.0	141.5
D	抽出A	1.591	1.536	1.618	122.5	137.5	123.0
	抽出B	1.612	1.513	1.543	133.0	121.0	142.0
E	抽出A	1.638	1.673	1.683	116.0	133.0	120.0
	抽出B	1.671	1.679	1.669	122.0	71.0	141.0
F	抽出A	1.890	2.000	1.840	49.0	45.1	56.9
	抽出B	1.760	1.890	1.740	69.5	62.7	63.6
G	抽出A	1.670	1.660	1.630	110.0	125.0	116.0
	抽出B	1.620	1.640	1.660	121.0	122.0	105.0
I	抽出A	1.830	1.830	1.830	162.0	115.5	105.0
	抽出B	1.850	1.860	1.860	112.0	103.0	97.5
J	抽出A	1.720	1.770	1.730	171.7	151.2	139.1
	抽出B	1.770	1.730	1.770	154.1	155.7	155.7
L	抽出A	1.789	1.771	1.768	127.0	135.5	148.5
	抽出B	1.806	1.779	1.750	130.0	132.5	157.5
M	抽出A	1.715	1.709	1.721	165.5	149.5	163.5
	抽出B	1.722	1.729	1.676	145.5	153.0	171.0
平均値		1.731	1.749	1.724	123.0	114.2	121.6

イマー対の結果を比較すると，確認用プライマーにおける増幅バンドの濃さが薄く，検出用プライマー対と確認用プライマー対とで増幅効率が異なることが示唆された。また，NL-Pを対象とした試験結果とは逆に，図3に示すように，NL-Yを対象とした試験では，確認用プライマー対に比べ，検出用プライマー対により得られる増幅バンドの濃さが薄かった。この結果もまた，検出用プライマー対と確認用プライマー対とで増幅効率が異なっていることを示唆していると考えられた。

2　調査結果における抽出DNA収量および精製度

トウモロコシ検体について，各機関におけるDNA抽出液の吸光度比(O.D.260nm/O.D.280nm)，およびそのDNA濃度についての結果を表1に示す。それぞれトウモロコシ検体を対象にシリカゲル膜タイプキット法を用いた機関のみの結果である。トウモロコシ検体からのDNA抽出の結果は各機関で大差なく，良好な精製度および収量を示した。食発第158号[4]中では，トウモロコシからのDNA抽出法としてシリカゲル膜タイプキット法に加え，CTAB法が併記されており，3機関においてCTAB法が使用されていたが，2法の分析結果