DETERMINATION OF POLYBROMINATED DIBENZO-*p*-DIOXINS, Co-PXBS AND BROMINATED FLAME RETARDANT IN FISH

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Abstract

The levels of brominated flame retardants (BFRs) and related compounds were determined in market fish from Japan. We collected 12 fish samples from 3 regions (Tyubu, Tyugoku-Shikoku and Kyushu) in Japan and analyzed brominated dioxins, polychlorinated/ brominated biphenyls (Co-PXBs), polybrominated diphenyl ethers (PBDEs) and polybrominated biphenyls (PBBs) to determine levels of contamination. We found the brominated dioxins 2, 3, 7, 8 - TeBDD and 2, 3, 7, 8 - TeBDF in conger eels, but not at serious levels (total concentrations ranged from 0.009 to 0.015 pgTEQ/g ww). Mono-bromo polychlorinated dibenzo-*p*-dioxins/dibenzofurans (MoBrPCDD/DFs) and Co-PXBs were not detected in any samples. We detected PBDE congeners in all samples at 0.016 - 0.818 ng/g ww. The highest PBDEs concentration was found in conger eels and mackerel. For PBBs, we detected tetra-hexa brominated compounds in 9 samples at 0.105 - 2.24 pg/g ww, and the detected congeners were 2,2',5,5'-TeBB (#52), 2,2',4,5'-TeBB (#49), 2,2',4,5,5'-PeBB (#101), 2,2',4,4',6,6'-HxBB (#155) and 2,2',4,4',5,5'-HxBB (#153). The highest concentration was found in conger eels and mackerel, the same as the case with PBDEs. The most abundant congener was #155, followed by #52.

Introduction

Brominated flame retardants (BFRs) such as polybrominated diphenyl ethers (PBDEs) and polybrominated biphenyls (PBBs) have been widely used in plastics and textile coatings throughout the world. The major commercial products made with PBDEs were penta-BDE, octa-BDE and deca-BDE products. In Japan, although the usage of low brominated PBDEs has decreased, deca-BDE is currently in use. PBDEs are additives to polymers such as polystyrene and are not chemically bound to the polymer. Therefore, they are easily released into the environment from waste products. For PBBs, the commercial products are mixtures contain hexa-BB, octa-BB, nona-BB and deca-BB. Products made with PBBs have not been produced in Japan, but PBBs have been detected in environment samples in Japan[1)]. It is suspected that the contaminant came from imported products or impurities of other BFRs. Furthermore, *de novo* synthetic compounds related to BFRs, such as polybrominated dibenzo-*p*-dioxins, dibenzofurans (PBDD/DFs) and coplanar polychlorinated/brominated biphenyls (Co-PXBs) have been founded in market fish[2)3)] and human samples[4) 5)]. PBDD/DFs are pollutants generated by the manufacture of brominated flame retardants (BFRs) such as brominated diphenyl ethers (PBDEs) and are formed by the combustion of substances containing BFRs. Although the toxicity of these brominated dioxins is unclear, some studies have shown that the toxicity of 2, 3, 7, 8-TBDD is comparable to that of 2, 3, 7, 8-TCDD [6)]. Co-PXBs may also be formed from BFRs and have toxicity similar to that of Co-PCBs due to their similarity of structures.

It is important that we investigate levels of these brominated organic compounds in foods and estimate the influence on humans. Fish samples should take precedence over other food samples because they seem to be major contributors to dietary exposure of persistent compounds. In the present study, we analyzed brominated dioxins, Co-PXBs, PBDEs and PBBs in fish samples from 3 regions (Tyubu, Tyugoku-Shikoku and Kyushu) in Japan.

Materials and Methods

Sampling.

Table 1 shows fish samples analyzed in this study and their details. The fish samples were purchased from fish

markets in each of 3 regions (Tyubu, Tyugoku-Shikoku and Kyushu) in Japan from 2007 to 2008. The edible parts of fish samples were blended using a food processor. The food mixtures were kept below -20°C until analysis.

Analytical Methods and Instrumentation.

The PBDD/DFs (tetra-octa) and Co-PXBs (4'-Br-2,3',4,5-TeCB, 4'-Br-2,3,3',4-TeCB, 4'-Br-3,3',4,5-TeCB, 4'-Br-2,3,3',4,5-PeCB, 4'-Br-3,3',4,5,5'-PeCB, 3',4',5'-Br-3,4-DiCB) analytical standards were purchased from Cambridge Isotope Laboratories (Cambridge, MA). The PBDEs (tri-deca) analytical standards were purchased from Wellington Laboratories (Guelph, Ontario). The PBBs (tri-deca) analytical standards were purchased from Wellington Laboratories and AccuStandard, Inc. (New Haven, CT). Dichloromethane, *n*-hexane and toluene used for extraction and cleanup were of dioxins analysis grade (Kanto Chemicals, Tokyo). Silica gel (Wako Pure Chemical Industries, Ltd., Tokyo) was heated for 3 h at 130°C. Florisil (Kanto Chemicals) was heated for 3 h at 130°C and deactivated with 1% water.

The concentrations of brominated dioxins, Co-PXBs, PBDEs and PBBs in fish samples were determined using high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS). The analytical conditions of HRGC/HRMS are shown in Table 2. For measurements of PBDEs, PBBs and Co-PXBs, SLB-5MS was used for the GC column, and satisfactory separations and intensities were obtained. For measurements of brominated dioxins, especially for high brominates congeners, satisfactory intensities were obtained using DB-5.

Table 1 Details of fish samples

No.	Fish	Place of production	Sample type	Number of fish pooled	Approx. length of fish (cm)	Approx. weight of fish (g)
1	sea bream-1	Tyubu	nature	1	42	1250
2	sea bream-2	Tyubu	nature	1	43	1300
3	sea bream-3	Tyugoku-Shikoku	nature	1	43	1060
4	sea bream-4	Kyushu	nature	2	33	664
5	conger eel-1	Tyubu	nature	7	35	102
6	conger eel-2	Tyugoku-Shikoku	nature	9	43	120
7	horse mackerel	Kyushu	nature	4	32	360
8	sand borer	Tyugoku-Shikoku	nature	10	22	89
9	mackerel	Kyushu	nature	3	34	573
10	sardine	Kyushu	nature	28	16	47.5
11	shrimp	Kyushu	nature	58	9.3	9.7
12	flatfish	Tyugoku-Shikoku	nature	3	28	313

Table 2 Analytical conditions of HRGC/HRMS

	Column	Injection temp.	Injection type /volume	Oven temp.	HRMS conditions
PBDD/DFs MoBrPCDD/DFs	DB-5 (Agilent) 30 m, 0.25 mm (i.d.), 0.1 μm film	280°C	Splitless 1 μL	130°C - (20°C/min) - 240°C - (5°C/min) 320°C (7.5 min)	Electron energy 38eV Filament current 750 μA Ion source temp. 270° C Resolution 10,000
PBDEs	SLB-5MS (SUPELCO) 30 m, 0.25 mm (i.d.), 0.1 μm film	260°C	Splitless 1 μL	125°C (1 min) - (20°C/ min) - 200°C - (10°C/ min) - 330°C (5.2 min)	
PBBs	SLB-5MS (SUPELCO) 30 m, 0.25 mm (i.d.), 0.1 μm film	280°C	Splitless 1 μL	120°C (1 min) - (20°C /min) - 300°C (8 min)	
Co-PXBs	SLB-5MS (SUPELCO) 30 m, 0.25 mm (i.d.), 0.1 μm film	280°C	Splitless 1 μL	120°C (1 min) - (20° C/min) -200°C (10 min) - (10°C/min) - 330° C (2 min)	

Sample Preparation.

We analyzed the brominated dioxins, Co-PXBs, PBDEs and PBBs simultaneously using accelerated solvent extraction (ASE). Each 50 g sample was freeze dried using a model AD 2.0ES-BC (Virtis, Gardiner, NY) freeze dryer, and then dried samples were extracted with 10% (v/v) dichloromethane/*n*-hexane using an accelerated solvent extractor ASE300 (Dionex, Sunnyvale, CA). The temperature of extraction was 100°C; the time was 10

min. Extracts were treated with sulfuric acid three times and applied to a silica gel column. The column was prewashed with 100 mL *n*-hexane, and PBDD/DFs and PBDEs were eluted with 150 mL of 10% (v/v) dichloromethane/*n*-hexane. The eluate was evaporated and dissolved in *n*-hexane. and then loaded onto a Florisil (5 g) column. The PBDEs, PBBs and Co-PXBs were obtained by elution with 150 mL of *n*-hexane (fraction 1), and the PBDD/DFs fraction was obtained by elution with 200 mL of 60% (v/v) dichloromethane/*n*-hexane (fraction 2). The fraction 1 was treated with a DMSO/*n*-hexane partition to remove the matrix and then concentrated to a final volume of approximately 25 μL. The fraction 2 was loaded on an active carbon column, which in advance had been washed with 50mL of 10% (v/v) dichloromethane/*n*-hexane, eluted with 200 mL of toluene. The fractions were concentrated to a final volume of approximately 15 μL, and these samples were analyzed by HRGC/HRMS.

Results and Discussion

We analyzed brominated dioxins (a total of 18 congeners of PBDD/DFs and MoBrPCDD/DFs), Co-PXBs (7 congeners), PBDEs (23 congeners) and PBBs (18 congeners) in 12 fish from 3 regions in Japan. In our study, the limits of detection (LODs) of PBDD/DFs were 0.01 pg/g ww for tetra and penta, 0.05 pg/g ww for hexa, 0.1 pg/g ww for hepta and 1 pg/g ww for octa. The LODs of PBDEs were 0.1 pg/g ww for tetra-hepta, 0.2 pg/g ww for octa, 0.5 pg/g ww for nona and 1 pg/g ww for deca. TheLODs of Co-PXBs were 0.05 pg/g ww. The LODs of PBBs were 0.1 pg/g ww for tri-penta, 0.2 pg/g ww for hepta-nona and 0.5pg/g ww for deca.

Table 3 shows analyzed data for the total concentrations of brominated dioxins, Co-PXBs PBDEs and PBBs from fish samples in this study.

PBDD/DFs were detected in two samples of conger eel: 2,3,7,8-TeBDF was detected in conger eel-1 at 0.09 pg/g ww, and 2,3,7,8-TeBDD and 2,3,7,8-TeBDF were detected in conger eel-2 at 0.01pg/g ww and 0.05 pg/g ww, respectively. MoBrPCDD/DFs congeners were not detected in any fish. Regarding the estimation of toxicities by brominated dioxins, the World Health Organization stated that using the same TEF values for PBDD/DF or PXDD/DF congeners as the chlorinated analogues appears to be justified. Using TEFs of chlorinated dioxins, we calculated the concentrations of total brominated dioxins in these fish to be 0.009 - 0.015 pgTEQ/g ww. For Co-PXBs, it is reasonable to calculate TEQ values using TEFs of Co-PCBs in the same way as brominated dioxins. However, no Co-PXB congeners were detected from fish samples in this study.

The amount of daily fish consumption by an average Japanese person was estimated to be 82 g in an investigation conducted by the Ministry of Health, Labour and Welfare of Japan. Under this assumption, the daily intakes from fish in the case of 50 kg of body weight were calculated to be 0.015-0.025 pgTEQ/kg/day, estimated to be within Japanese TDI (4 pg TEQ/kg body weight/day).

PBDE congeners were detected in all samples at 0.016 - 0.818 ng/g ww. The most abundant congeners were #49, #154 and #209 (Figure 1). The highest PBDEs concentration was found in conger eel-1 at 0.818 ng/g ww, followed by mackerel at 0.617 ng/g ww and conger eel-2 at 0.414 ng/g ww. In our previous reports[3], a high concentration of PBDEs was found in fatty fish, and these fish had a higher fat content compared with other fish.

For PBBs, tetra-hexa brominated compounds were detected in 9 samples at 0.105-2.24 pg/g ww. The highest concentration was found in conger eels and mackerel, the same as the case with PBDEs. The levels of total PBBs were much lower than total PBDEs, however. The detected congeners were 2,2',5,5'-TeBB (#52), 2,2',4,5'-TeBB (#49), 2,2',4,5,5'-PeBB (#101), 2,2',4,4',6,6'-HxBB (#155) and 2,2',4,4',5,5'-HxBB (#153). The most abundant congener was #155, followed by #52 (Figure 2). In the reports about PBBs in Japan, #153 was mainly detected in incinerable waste, bulky waste, automobile shredder residue and office dust, and it was suspected that the imported PBBs or products containing PBBs were the sources[1]. Interestingly, the conger patterns of PBBs in fish seem to be different from those of environment samples. More information about food or environment samples must be collected.

In a recent report, the lowest observed adverse effect level (LOAEL) value suggested as reasonable for compounds or mixtures belonging to the PBDE group was 1mg / kg body weight / day [7]. For PBBs, it was suggested that the total daily intake should be less than 0.15 μg/kg/day, extrapolating from a no observed adverse effect level (NOAEL) of a positive carcinogenicity study, using an uncertainty (safety) factor of 1000[8]. The estimated intake values from fish with maximum concentration of PBDEs and PBBs in this study were 1.34 ng/kg/day and 3.67 pg/kg/day, respectively (assuming daily fish consumption is 82 g). Compared with

these values, the levels of these brominated compounds in fish were not considered a serious problem. However, it is important to collect more data about brominated dioxins and BFRs in food, because little information is available regarding the levels of these brominated compounds.

Table 3 Daily intake of brominated dioxins and PBDEs in Japan

No.	Fish	Fat content (%)	Brominated dioxins (pgTEQ/g ww)	Total Co-PXBs pg/g ww	Total PBDEs ng/g ww	Total PBBs pg/g ww
1	sea bream-1	0.48	ND	ND	0.100	0.230
2	sea bream-2	2.77	ND	ND	0.247	0.813
3	sea bream-3	0.42	ND	ND	0.018	ND
4	sea bream-4	0.19	ND	ND	0.016	0.105
5	conger eel-1	11.8	0.009	ND	0.818	2.24
6	conger eel-2	9.88	0.015	ND	0.406	1.83
7	horse mackerel	4.88	ND	ND	0.334	1.43
8	sand borer	0.60	ND	ND	0.095	0.299
9	mackerel	12.2	ND	ND	0.617	1.98
10	sardine	1.73	ND	ND	0.167	0.827
11	shrimp	0.12	ND	ND	0.033	ND
12	flatfish	1.10	ND	ND	0.044	ND

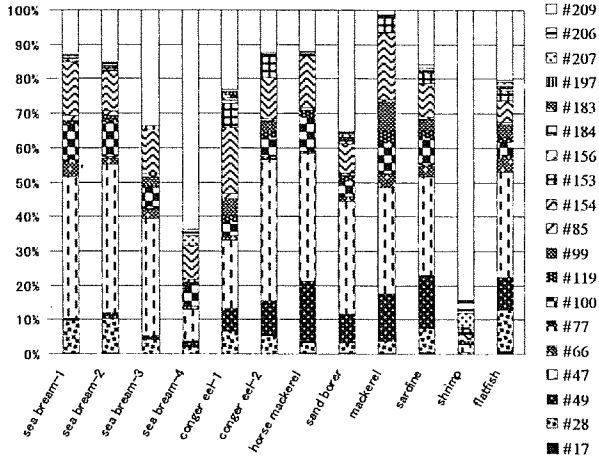

Figure 1 Congener patterns of PBDEs in fish

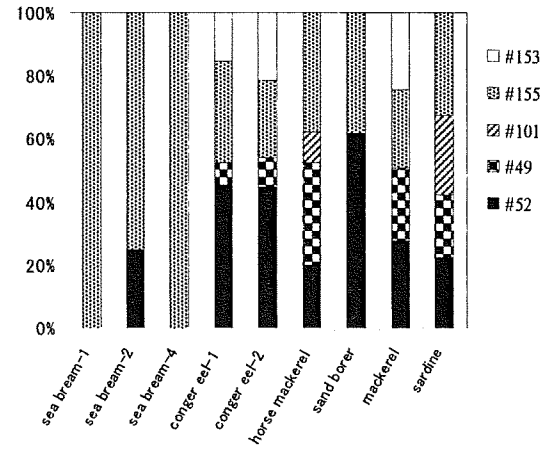

Figure 2 Congener patterns of PBBs in fish

Acknowledgement

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A NEW REPORTER GENE ASSAY FOR DIOXINS USING GREEN FLUORESCENT PROTEIN: INCREASED RESPONSIVENESS USING AMPLIFICATION OF THE DIOXIN RESPONSIVE ELEMENT

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Abstract
A highly responsive green fluorescent protein (GFP) reporter gene assay for dioxins has been newly developed. Initially, we constructed three aryl hydrocarbon receptor (AhR)-responsive GFP reporter vectors containing an increasing number of dioxin responsive elements (DREs) to confer high responsiveness to dioxins. The 5-36 cell line stably transfected with a pZs7.5 vector containing the maximum 20 DREs had the highest GFP responsiveness to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Induction of GFP expression at a low concentration of TCDD (10 pM) in this cell line was nearly 4-fold higher than that in the 1-34 cell line transfected with pZs7.1 containing the minimum 4 DREs. In addition, as compared with the H4G1.1c2 cell line (currently the most widely used AhR-responsive GFP reporter gene assay), the 5-36 cell line had nearly 4-fold higher GFP induction at 10 pM TCDD. The 5-36 cell line generally responded to selected dioxins in line with the level of their toxic equivalent factors, indicating that our assay may be suitable to determine toxic equivalent concentrations of dioxins. This newly developed assay has advantages including rapidity and ease of use; as the result, it will provide a useful high-throughput screen for relatively low concentrations of dioxins.

Introduction
Reporter gene assays, such as the chemical-activated luciferase gene expression (CALUX) assay, are currently considered to be the best screening method for polychlorinated dibenzo-*p*-dioxins, dibenzofurans and dioxin-like polychlorinated biphenyls (PCBs), which are collectively known as dioxins. The CALUX assay detects dioxin-like compounds on the basis of their activation of the aryl hydrocarbon receptor (AhR), which in turn increases expression of the luciferase reporter gene. Although the CALUX assay is very sensitive, its requirement for a substrate (luciferin) to measure luciferase activity makes it laborious. In contrast, a reporter gene assay using green fluorescent protein (GFP) as a reporter is suitable for high-throughput screening analysis because GFP requires no exogenous cofactors or independent substrates for activity and is significantly less costly. Because GFP has lower sensitivity as compared with luciferase, detection requires that its expression must be driven by strong promoters or other regulatory elements. However, the extreme stability of the GFP protein allows significant accumulation of reporter gene product and significantly elevated activity over time compared to luciferase. In the CALUX assay, expression of the luciferase gene under the control of the mouse mammary tumor virus (MMTV) promoter is regulated by dioxin responsive elements (DREs, the DNA recognition site of dioxin-bound AhR)[1]. Previously, we successfully developed CALUX reporter plasmids containing increasing numbers of DREs that dramatically improved sensitivity and reporter gene responsiveness to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)[2]. Here, we report the development of a new reporter gene assay for dioxins using GFP reporter vectors containing increasing numbers of DREs to improve assay responsiveness to dioxins.

Materials and Methods
Construction of GFP reporter vectors: The new GFP reporter vectors were created by excising the XhoI-HindIII fragment (800 – 3,000 bp) from pGL7.1, pGL7.3 and pGL7.5[2]. These fragments contain, respectively, 4, 12 and 20 DREs inserted upstream of the MMTV promoter, which confers dioxin responsiveness upon the MMTV promoter and adjacent reporter gene. These fragments were subcloned into the XhoI-HindIII site immediately upstream of the GFP (ZsGreen1) gene in the plasmid pZsGreen 1-1 (Clontech). ZsGreen1, a reef coral protein, is currently the brightest commercially available GFP. The resulting plasmids were designated pZs7.1, pZs7.3 and pZs7.5 and contained 4 DREs, 12 DREs and 20 DREs, respectively.

Stable transfections: The rat hepatome (H4IIE) cell line was maintained in alpha minimum essential medium (MEM) containing 10% fetal bovine serum (non-selective media) in a 24-well plate. The cells (approximately 90% confluent) were transfected with the constructed plasmids by using lipofectamineTM 2000 transfection reagent (Invitrogen) according to the manufacturers recommendations. After growth in non-selective media for 24 h, the transfected cells were split 1 to 10, replated into MEM with 10% FBS and incubated for 24 hr. Selective medium (MEM with 10% fetal bovine serum containing 500 μg/ml of G418) was added on the following day, and the cells were further cultured for about 2 weeks. Resistant clones were isolated and screened for the induction of GFP expression by treatment with 1 nM TCDD for 24 h. Clones exhibiting the highest ratio of inducible to constitutive GFP expression were further characterized.

Chemical treatment and measurement of GFP: The established cell lines were characterized and used to optimize for a 96-well microplate high-throughput analysis of GFP in intact cells. The cells were maintained in selective media. Cells were plated into black clear-bottomed 96-well microplates at 150,000 cells per well and allowed to attach for 20-24 h. The media was then replaced with non-selective media containing various concentrations of the dioxins to be tested (the final DMSO concentration was 1% in the media). After 24-26 hours of incubation at 33°C or 37°C, GFP levels were measured by using a Tecan F200 microplate reader with excitation and emission wavelengths of 485 nm and 535 nm, respectively.

Results and Discussion

We constructed three cell lines stably transfected with pZs7.1, pZs7.3 or pZs7.5 containing 4 DREs, 12 DREs or 20 DREs, respectively. The TCDD concentration response curves for the cell lines are shown in Figure 1 (a). The highest induction of GFP was observed in the 5-36 cell line transfected with pZs7.5 containing the maximum number of DREs. The induction response at the low concentration of TCDD (10 pM) in this cell line was nearly 4-fold higher than that in the 1-34 cell line transfected with pZs7.1 containing the minimum number of DREs. Slightly higher background (containing no TCDD) was observed in the 5-36 cell line. In addition, as compared with the H4G1.1c2 cell line using enhanced GFP (EGFP; a variant derived from the jellyfish *Aequorea Victoria*), which is currently the most widely used AhR-responsive GFP reporter gene assays, the 5-36 cell line had nearly 4-fold higher GFP induction at 10 pM TCDD. The 3-28 cell line transfected with pZs7.3 did not amplify the GFP induction response, although it contained 12 DREs. The transfection experiment using pZs7.3 might have not worked well, because the TCDD-responsive clones recovered after transfection were few in number (data not shown). Figure 1 (b) shows the overall fold induction by TCDD (comparison of background and TCDD-induced GFP) in the cell lines. The 5-36 cell line not only showed the highest level of GFP induction but also the highest fold induction at 10 pM TCDD. The 5-36 cell line had a minimal detection limit of ~3 pM, an EC50 of 10 pM, and maximal induction at ~100 pM (Figure 1 (a)). Although the detection limit was nearly identical to those of previously developed AhR-responsive GFP reporter gene assays[3,4,5], the significant increase in GFP expression makes our assay useful to distinguish between background and positive samples containing relatively low concentrations of dioxins.

Previous studies have reported that EGFP is temperature-sensitive, with stably transfected cells grown at 33°C exhibiting higher fluorescence than those grown at 37°C[3,4,5]. Therefore, we also tested the incubation temperature for the 5-36 cell line that had the highest level of TCDD-inducible GFP expression at 37°C. Figure 2 (a) shows induction of GFP expression by TCDD in the 5-36 cell line, as well as in the H4G1.1c2 cell line at 33°C and 37°C. Interestingly, the induction of GFP expression in the 5-36 cell line grown at 33°C was not significantly different from that at 37°C. In contrast, induction of EGFP expression in H4G1.1c2 grown at 33°C was significantly higher than that at 37°C, as reported previously[3,4,5]. These differences likely represent subtle species differences in the GFP reporter. The overall fold induction by TCDD is also shown in Figure 2 (b). The 5-36 cell line grown at 37°C had the highest fold induction at 10 pM TCDD.

We next examined the ability of selected dioxins to induce GFP expression in the 5-36 cell line (Figure 3). 1,2,3,7,8- Pentachlorodibenzo-*p*-dioxin (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and PCB 126, which are assigned relatively high toxic equivalent factors (TEFs) (>0.1), showed strong GFP induction, whereas octachlorodibenzo-*p*-dioxin (OCDD) and PCB 118, which are assigned relatively low TEFs (< 0.003), showed little or no GFP induction. Thus, the induction of GFP expression appeared to be reflected by the level of TEFs

assigned to dioxins tested. Thus, our assay will be suitable as a screening method to determine the toxic equivalent concentrations of dioxins.

In summary, development of GFP reporter vectors containing increasing numbers of DREs (pZs7.5) has enabled us to develop a reporter gene assay with significantly improved GFP expression in response to TCDD. Food has been generally recognized as the main route of dioxin uptake in humans. However, the concentration of dioxins in foodstuffs is relatively low as compared with other environmental samples. Our assay is rapid for measurement (less than 2 minute per a 96-well microplate) and easy to use, and has no reagent cost (compared to the CALUX assay) and as such, it has application for use in the preliminary screening of food samples prior to conventional instrumental analyses.

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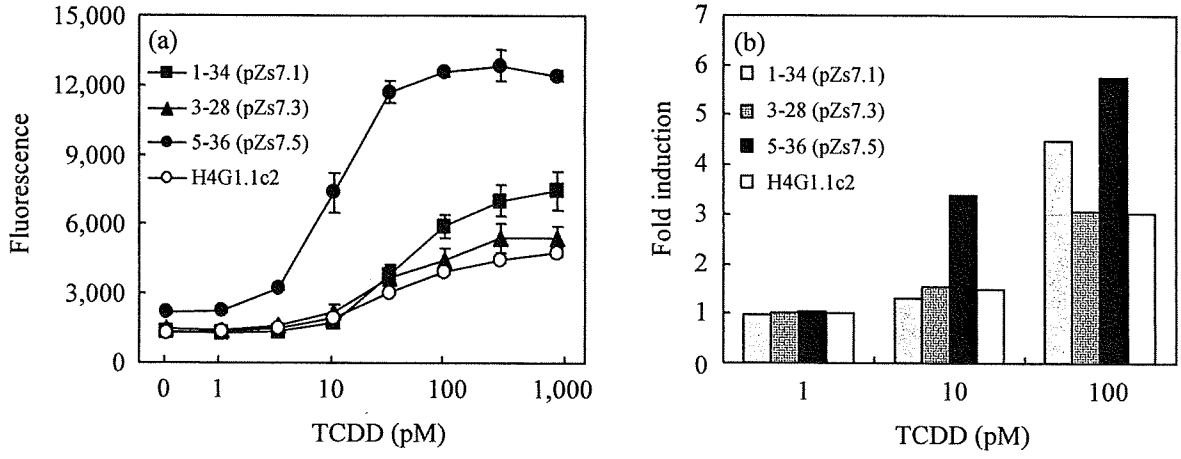

Figure 1. Dose-response curves for the induction of GFP expression (a) and fold induction (b). The three cell lines constructed (1-34, 3-28 and 5-36) and H4G1.1c2 were incubated with the indicated concentrations of TCDD at 37°C. (a) Values represent the mean ± S.D. of triplicate determinations. (b) Fold induction was calculated as the ratio between background (DMSO) and TCDD-induced fluorescence.

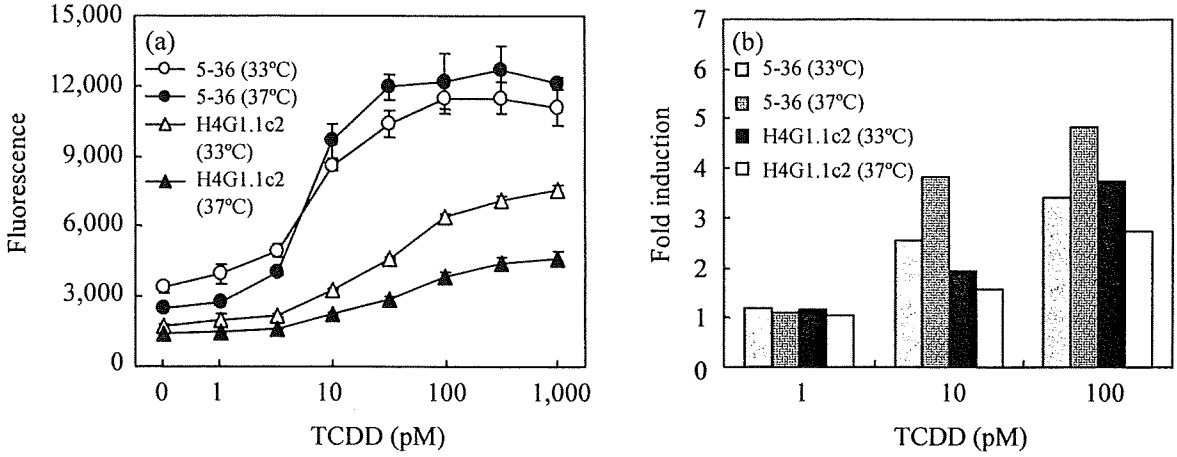

Figure 2. Induction of GFP expression (a) and fold induction (b) by TCDD in cells grown at different temperatures. The 5-36 and H4G1.1c2 cell lines were incubated with the indicated concentrations of TCDD at 33°C and 37°C. (a) Values represent the mean ± S.D. of triplicate determinations. (b) Fold induction was calculated as the ratio between background (DMSO) and TCDD-induced fluorescence.

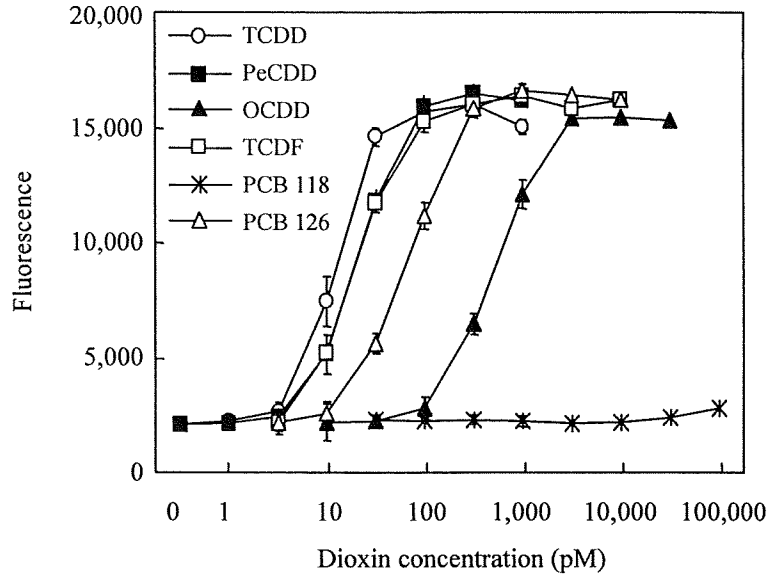

Figure 3. Dose-response curves for the induction of GFP expression by the selected dioxins. The 5-36 cell line was incubated with the indicated concentrations of dioxins at 37°C. Values represent the mean ± S.D. of triplicate determinations.

Simultaneous determination of dioxins and all PCB isomers in food samples using accelerated solvent extraction and gel permeation chromatography

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Abstract

We developed a simultaneous rapid analysis method for determination of 17 kinds of toxic PCDD/F isomers and 209 kinds of PCB isomers in food samples. This method makes it possible to evaluate total TEQ and total PCB concentration with a single extract operation. The method consists of four processes, as follows: (i) rapid and high-efficiency extraction using accelerated solvent extraction (ASE), (ii) sample cleanup using modified multi-layer silica gel column chromatography and active carbon column chromatography, (iii) high-efficiency cleanup by gel permeation chromatography (GPC), (iv) isomer specific determination using high-resolution gas chromatography coupled with high-resolution mass spectrometry (HRGC/HRMS). In this study, we examined the conditions needed for multi-layer silica gel column chromatography and GPC to remove fat and other matrices from extracts effectively. We evaluated the performance of the method using data from a standard recovery test and a quantitative reproduction test using a fish sample purchased in a market. The mean recovery rates for a labeled standard for 26 kinds of PCBs ranged from 46% to 117%, which is within the range officially recommended for dioxins. RSD (%) values for PCB concentrations are below 20%.

Introduction

The main source of human intake of PCDD/Fs and PCBs is food, especially fish in the case of the Japanese general population. Fish and other animal-origin food contamination levels of these chemicals have been observed closely under official regulations, and these data are also significant for the evaluation of human health effects caused by these chemicals. A method for the simultaneous determination of PCDD/Fs and PCBs is expected to improve the efficiency of laboratory performance, because these compounds are usually measured individually, and each conventional method requires considerable labor and time to complete the analysis. We previously developed a highly sensitive method for determining dioxin concentrations in food using combined ASE and HRGC/HRMS system equipped with a solvent cut large volume (SCLV) injection system [1) 2)]. We found that the extraction efficiency for dioxins (PCDD/Fs and 12 kinds of DL-PCBs) between ASE and conventional shaking extraction was confirmed to be similar in our extraction conditions. In this study, ASE was applied to rapid isomer specific determination of PCDD/Fs and all 209 kinds PCB isomers simultaneously in food samples.

Materials and Methods

^{13}C-labeled standards for cleanup-spiking solutions used in this study were products manufactured by Wellington Laboratories (Canada): NK-LCS-F for determination of PCDD/Fs and 68A-LCS for determination of PCBs. Both labeled standards were simultaneously spiked into weighed fish homogenates (25 g) prior to extraction. No detectable impurities were identified in either standard solution that affected the quantitative accuracy of the other solution when each solution was directly injected to HRGC/HRMS (data not shown)[3]. The conditions of GPC and multi–layer silica gel column chromatography were examined by using extract obtained from dried milk powder on the market. Concentrations and recovery rates of 17 kinds of 2,3,7,8-chlorine substituted PCDD/Fs and 26 kinds of PCBs were evaluated using the homogenized edible parts of amberjack purchased at a fish market in Japan. Automated extraction was performed using an ASE-300 (Dionex, CA) under conditions of 1,500 psi, extraction solvent of acetone/hexane (1:1). The GPC system was comprised of an MSpak GF-310 4D GPC column (150 x 4.6 mm i.d., Showa Denko Co.), a MIDAS automated sampler, a GL-7410 LC pump, a GL-7430 column oven, a GL-7451 UV-VIS detector (GL Science Co.), and an FC203B fraction collector (Gilson, WI). Acetone was used as the GPC mobile phase, and its flow rate was set to 0.1 mL/min. Isomer specific determination of PCDD/Fs and PCBs was performed with a model 6890 gas chromatograph (Agilent Technologies, CA) coupled to an Autospec-Ultima mass spectrometer (Micromass, UK). The operating conditions for the HRGC/HRMS for measurement of PCDD/Fs were based on the Japan Industrial Standards (JIS).

Results and Discussion

We developed operating conditions for the HRGC/HRMS for PCB measurements by use of an HT8-PCB capillary column [4]. Our method made it possible to determine all 209 kinds of PCB isomers with one-time sample injection and about thirty minutes of analysis time. As shown in Figure 1, an initial column temperature of 100°C was employed to obtain symmetrical and high- response peaks on monoCB chromatograms. The time course of MS data collection was divided into five sections.

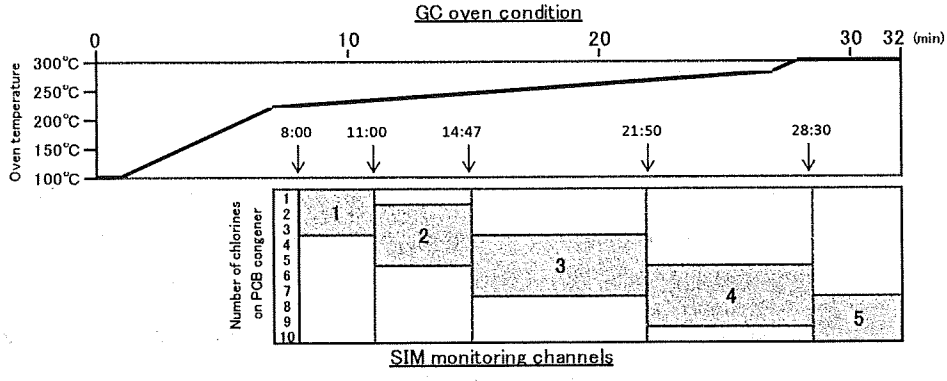
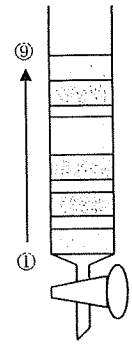


Fig.1 Operating condition of HRGC/HRMS on PCBs measurement

The multi-layer silica gel column chromatography specified in this method was designed to collect analytes with appropriate recovery rates and fine purification of animal-origin food extract obtained by ASE. As shown in Figure 2, the modified column was prepared with one-third quantity of each adsorbent mentioned in the Japanese official guideline of food dioxin analysis ("*Guideline*") to avoid recovery of low-chlorinated PCBs. Table 1 shows standard recovery rates of 26 kinds of ^{13}C-labeled PCBs from the modified column. When 10% (v/v) dichloromethane/hexane was used as the elution solvent, the recovery rates of mono-, di-, and triCBs ranged from 67% to 93%: the rates were improved more than 10% in comparison with when the other solvents used.



(Column upper side)
⑨ Sodium sulfate anhydrous 2.0 g
⑧ 10% silver nitrate-impregnated silica gel 1.0 g
⑦ Silica gel 0.3 g
⑥ 22% Sulfuric acid- impregnated silica gel 2.0 g
⑤ 44% Sulfuric acid- impregnated silica gel 1.5 g
④ Silica gel 0.3 g
③ 2% Potassium hydroxide- impregnated silica gel 1.0 g
② Silica gel 0.3 g
① Sodium sulfate anhydrous 2.0g
(Column bottom side)

Fig. 2 Scheme of multi-layer silica gel column chromatography

Table 1 Standard recovery rates on modified multi-layer silica gel column chromatography

Compounds	Column recovery (%)											
	hexane				5 %(v/v)dichloromethane/hexane				10%(v/v) dichloromethane/hexane			
	0-50mL	50-100mL	100-150mL	total	0-50mL	50-100mL	100-150mL	total	0-50mL	50-100mL	100-150mL	total
^{13}C- 2-CB(#1)	54	1	0	55	57	0	0	57	67	0	0	67
^{13}C- 4-CB(#3)	59	1	0	60	57	0	0	57	72	0	0	72
^{13}C- 2,2'-diCB(#4)	62	0	0	62	66	0	0	66	77	0	0	77
^{13}C- 4,4'-diCB(#15)	72	1	1	74	70	0	0	70	82	0	0	82
^{13}C- 2,2',6-triCB(#19)	73	1	1	75	69	1	0	70	81	0	0	81
^{13}C- 3,4,4'-triCB(#37)	83	0	1	84	87	1	0	88	93	0	0	93
^{13}C- 2,2',6,6'-tetraCB(#54)	78	0	1	79	71	1	0	72	83	0	0	83
^{13}C- 3,3',4,4'-tetraCB(#77)	93	0	0	93	94	0	0	94	95	0	0	95
^{13}C- 3,4,4',5-tetraCB(#81)	95	0	0	95	95	0	0	95	101	0	0	101
^{13}C- 2,2',4,6,6'-pentaCB(#104)	95	0	1	96	79	0	0	79	91	0	0	91
^{13}C- 2,3,3',4,4'-pentaCB(#105)	102	1	1	104	98	1	1	100	100	1	1	102
^{13}C- 2,3,4,4',5-pentaCB(#114)	96	0	0	96	94	0	0	94	98	0	0	98
^{13}C- 2,3',4,4',5-pentaCB(#118)	96	0	0	96	94	0	0	94	97	0	0	97
^{13}C- 2',3,4,4',5-pentaCB(#123)	95	0	0	95	94	0	0	94	97	0	0	97
^{13}C- 2,2',4,4',6,6'-hexaCB(#155)	85	0	0	85	82	0	0	82	92	0	0	92
^{13}C- 2,3,3',4,4',5-hexaCB(#156)	94	2	2	98	96	2	2	100	103	2	2	107
^{13}C- 2,3,3',4,4',5'-hexaCB(#157)	93	1	0	94	97	1	0	98	102	0	0	102
^{13}C- 2,3',4,4',5,5'-hexaCB(#167)	94	0	0	94	96	0	0	96	106	0	0	106
^{13}C- 3,3',4,4',5,5'-hexaCB(#169)	93	0	0	93	97	0	0	97	101	0	0	101
^{13}C- 2,2',3,4',5,6,6'-heptaCB(#188)	105	0	0	105	95	0	0	95	97	0	0	97
^{13}C- 2,3,3',4,4',5,5'-heptaCB(#189)	99	0	0	99	101	0	0	101	100	0	0	100
^{13}C- 2,2',3,3',5,5',6,6'-octaCB(#202)	99	0	0	99	97	0	0	97	99	0	0	99
^{13}C- 2,3,3',4,4',5,5',6-octaCB(#205)	101	0	0	101	99	0	0	99	98	0	0	98
^{13}C- 2,2',3,3',4,4',5,5',6-nonaCB(#206)	100	0	0	100	99	0	0	99	100	0	0	100
^{13}C- 2,2',3,3',4,5,5',6,6'-nonaCB(#208)	106	0	0	106	101	0	0	101	98	0	0	98
^{13}C- decaCB(#209)	92	0	0	92	84	0	0	84	88	0	0	88

When extract from 20 g of milk powder was applied to the modified column and eluted by 100 mL of 10% (v/v) dichloromethane/hexane, a colorless eluate was obtained and stained material remained in the column adsorbent, that is, column overload was not observed. However, a small amount of oily material was observed in the glass test tube when the eluate was concentrated to several hundred microliters, so additional cleanup was necessary prior to HRGC/HRMS analysis. We found that a large part of the matrices was separated with analytes by means of GPC cleanup: a fraction (1.0 mL) was taken as separated analytes between 22.0 min and 32.0 min after injection.

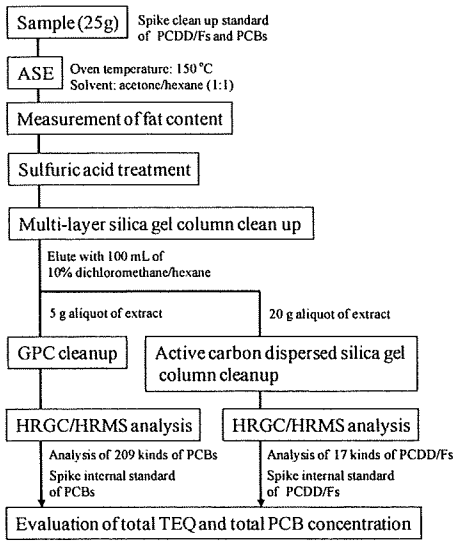


Fig. 3 Flow chart for simultaneous analysis of PCDD/Fs and PCBs

Table 2 Recovery rates and concentration values of PCB isomers in fish sample

Compounds	Recovery* Range (%)	Mean (%)	RSD (%)	Concentration Range (pg/g)	Mean (pg/g)	RSD (%)
2-monoCB(#1)	41 - 52	46	12	1.2 - 1.7	1.6	18
4-monoCB(#3)	47 - 54	50	7	1.3 - 1.6	1.5	12
2,2'-diCB(#4)	57 - 70	62	11	5.5 - 6.5	5.8	9
4,4'-diCB(#15)	58 - 95	75	25	ND - ND	-	-
2,2',6-triCB(#19)	87 - 91	89	2	5.6 - 6.4	6.0	7
3,4,4'-triCB(#37)**	103 - 131	117	12	(3.6) - (6.6)	(5.1)	(35)
2,2',6,6'-tetraCB(#54)	60 - 69	66	7	5.0 - 5.9	5.4	8
3,3',4,4'-tetraCB(#77)	79 - 91	84	8	55 - 56	56	1
3,4,4',5-tetraCB(#81)	71 - 85	77	9	9.5 - 12	10	10
2,2',4,6,6'-pentaCB(#104)	49 - 74	62	20	4.9 - 5.3	5.1	4
2,3,3',4,4'-pentaCB(#105)	84 - 94	88	6	300 - 350	325	8
2,3,4,4',5-pentaCB(#114)	84 - 98	90	7	22 - 30	26	16
2,3',4,4',5-pentaCB(#118)	81 - 92	86	7	960 - 1100	1000	8
2',3,4,4',5-pentaCB(#123)	88 - 102	96	7	20 - 21	21	5
2,2',4,4',6,6'-hexaCB(#155)	53 - 63	59	9	200 - 230	220	7
2,3,3',4,4',5-hexaCB(#156)	50 - 61	57	11	68 - 74	71	5
2,3,3',4,4',5'-hexaCB(#157)	70 - 77	74	5	36 - 48	42	15
2,3',4,4',5,5'-hexaCB(#167)	64 - 73	70	7	76 - 99	87	14
3,3',4,4',5,5'-hexaCB(#169)	54 - 68	63	13	3.5 - 3.9	3.6	6
2,2',3,4',5,6,6'-heptaCB(#188)	48 - 62	55	13	6.4 - 7.2	6.8	7
2,3,3',4,4',5,5'-heptaCB(#189)	53 - 64	60	11	19 - 22	21	8
2,2',3,3',5,5',6,6'-octaCB(#202)	54 - 69	62	13	44 - 53	49	10
2,3,3',4,4',5,5',6-octaCB(#205)	69 - 81	76	9	10 - 12	11	10
2,2',3,3',4,4',5,5',6-nonaCB(#206)	75 - 90	84	9	32 - 38	35	9
2,2',3,3',4,5,5',6,6'-nonaCB(#208)	47 - 68	59	18	16 - 18	17	9
decaCB (#209)	74 - 92	83	11	44 - 53	49	9

*Recovery rates were evaluated for each ^{13}C-labeled isomer.
**Relatively high value of method blank was found, so concentrations and statistical values were shown in parentheses for reference.

Table 2 shows the results for PCBs' whole recovery rates, concentrations, and reproducibility on fish homogenates using the designed method shown in Figure 3 (n=3; data for PCDD/Fs are not shown here). After multi-layer silica gel column cleanup, a concentrated 5-g aliquot of fish extract was applied to GPC cleanup for the determination of PCBs, while another concentrated 20-g aliquot was applied to active carbon dispersed column chromatography for the determination of PCDD/Fs.

As a result, the mean recovery rates for 26 kinds of PCB labeled analogs were in agreement with the range recommended in the "*Guideline*" (40-120%). RSD (%) values for recovery rates ranged from 2% to 25%, and those for concentrations were below 20%. These results were similar to the results obtained from our previous tests using the standard alkaline digestion method for dioxins [2]. Our results suggest that the present method is available for simultaneous determination of PCDD/Fs and PCBs in animal-origin food samples with a short extraction time and a small volume of extraction solvent compared to the conventional alkaline digestion extraction. The applicability of the presented methodology has been continuously verified in comparison to the individual analysis method for PCBs.

Acknowledgement

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(別紙)

食品中のダイオキシン類の測定方法
暫定ガイドライン

平成 20 年 2 月

第１章　概論

はじめに

ダイオキシン類は、食品汚染物質の中でも社会的関心の高い化学物質であり、健康影響の未然防止の観点から、早急な対策が必要となっている。

本ガイドラインは、食品に係るダイオキシン類 の検査の信頼性を確保するため、既存の知見を踏まえ、一般的な技術手法を示したものである。

また、今後、科学的知見の集積等によって、本ガイドラインの改定があり得るものである。

1. 分析対象

本ガイドラインでは、食品試料中のポリ塩化ジベンゾパラジオキシン（PCDDs）とポリ塩化ジベンゾフラン（PCDFs）とコプラナーポリ塩化ビフェニル（コプラナーPCBs）を分析対象物質としている。

2. 目標検出下限

本ガイドラインにおいては、検出下限や操作ブランク値等の許容性を判断する基準として、「目標検出下限」を導入した。目標検出下限は、分析の目的、試料の種類及び採取可能な試料量等に照らして決定されるが、本ガイドラインにおいては原則として、表1-1に示すとおりとした。

表 1-1　ダイオキシン類の目標検出下限

	PCDDs及びPCDFs			コプラナーPCBs	
	四～五塩化物	六～七塩化物	八塩化物	ノンオルト体	モノオルト体
食　品	0.01pg/g	0.02pg/g	0.05pg/g	0.1pg/g	1pg/g
水（注1）	0.2pg/L	0.5pg/L	1pg/L	1pg/L	10pg/L

3. 用語・略語の定義

ダイオキシン類：ポリ塩化ジベンゾパラジオキシン（PCDDs）とポリ塩化ジベンゾフラン（PCDFs）及びコプラナーポリ塩化ビフェニル（コプラナーPCBs）を合わせた総称。

コプラナーPCBs：PCBsの中で、PCDDs及びPCDFsと同様の毒性をもつ化合物又はその一群をいう。ただし、本ガイドラインでは毒性等価係数が与えられているオルト位(2,2',6及び6')に置換塩素を持たない（ノンオルト：non-ortho）４種類の化合物、オルト位に置換塩素を１個もつ（モノオルト：mono-ortho）８種類の化合物を示す。なお、コプラナーPCBsはダイオキシン様PCBsとも呼ばれる。

異性体：異性の関係にある化合物。ここでは、同一の化学式を持ち、塩素の置換位置が異なった化合物を指す（Isomer）。

同族体：同族列に属する塩素の置換数又は置換位置を異にする一群の化合物を指す(Congener)。

PCDDs：ポリ塩化ジベンゾパラジオキシン(Polychlorinated dibenzo-*p*-dioxins)

PCDFs：ポリ塩化ジベンゾフラン(Polychlorinated dibenzofurans)

PCBs：ポリ塩化ビフェニル(Polychlorinated biphenyls)

TCDDs：四塩化ジベンゾパラジオキシン(Tetrachlorodibenzo-*p*-dioxins)

PeCDDs：五塩化ジベンゾパラジオキシン(Pentachlorodibenzo-*p*-dioxins)

HxCDDs：六塩化ジベンゾパラジオキシン(Hexachlorodibenzo-*p*-dioxins)

HpCDDs：七塩化ジベンゾパラジオキシン(Heptachlorodibenzo-*p*-dioxins)

OCDD：八塩化ジベンゾパラジオキシン(Octachlorodibenzo-*p*-dioxin)

TCDFs：四塩化ジベンゾフラン(Tetrachlorodibenzofurans)

PeCDFs：五塩化ジベンゾフラン(Pentachlorodibenzofurans)

HxCDFs：六塩化ジベンゾフラン(Hexachlorodibenzofurans)

HpCDFs：七塩化ジベンゾフラン(Heptachlorodibenzofurans)

OCDF：八塩化ジベンゾフラン(Octachlorodibenzofuran)

TCBs：四塩化ビフェニル(Tetrachlorobiphenyls)

PeCBs：五塩化ビフェニル(Pentachlorobiphenyls)

HxCBs：六塩化ビフェニル(Hexachlorobiphenyls)

HpCBs：七塩化ビフェニル(Heptachlorobiphenyls)

PFK：ペルフルオロケロセン(Perfluorokerosenes)

TEF：毒性等価係数 (2,3,7,8－TCDD Toxic Equivalency Factor)

TEQ：毒性当量(2,3,7,8－TCDD Toxic Equivalent Quantity)

HRGC：高分解能ガスクロマトグラフィー(High Resolution Gas Chromatography)
又は高分解能ガスクロマトグラフ(High Resolution Gas Chromatograph)

HRMS：高分解能質量分析法(High Resolution Mass Spectrometry)
又は高分解能質量分析計(High Resolution Mass Spectrometer)

SIM：選択イオン検出(Selected Ion Monitoring)

RRF：相対感度係数 (Relative Response Factor)

μg：10^{-6} g

ng：10^{-9} g

pg：10^{-12} g

4. 分析方法

4.1 概要

食品中ダイオキシン類の分析には、①試料採取、②抽出、③クリーンアップ、④同定及び定量の各工程が含まれる。

各工程にはいくつかの手法があるので、本ガイドラインでは、複数の方法を示す。これらの手法は、4.2 分析方法の要件を満たす方法として一般に用いられている方法である。

分析者は、試料の種類等に応じて手法を選択して使用することができる。

また、本ガイドラインに示した以外の手法も、4.2 分析方法の要件を満たしていれば、使用することができる。

本ガイドラインの構成は、図1-1 の通りである。

4.2 分析方法の要件

新規に開発されたり、本ガイドラインには採用されていないが、一般に用いられており、実証試験を行い、本ガイドラインに示した分析方法と同等あるいはそれ以上の性能を有する方法は、有効に活用することができる。その際、少なくとも以下に示す事項について十分に検討し、本ガイドラインに示す分析精度が確保される必要がある。

さらに、複数の機関による検証試験が実施され、ダイオキシン類に対する分析方法として充実されていくことが望ましい。

①前処理（抽出）

a)様々な状況に応じて抽出効率が安定した方法であるか。

②前処理（クリーンアップ）

a)試薬・器具のブランク値は低いか。

b)各クリーンアップの溶出画分は安定しているか。

c)確実で効果的にクリーンアップできるか。

d)実試料で検出される可能性のある妨害成分の影響を、分離・除去できるか。

③GC-MS分析

a)分析対象物質の異性体特異分析（Isomer specific analysis）を行うことができるか。

b)キャピラリーカラムの異性体分離能は良いか。

c)GC-MS装置の校正、試料の濃度範囲と定量可能範囲（検量線）の応答性が十分であるか。

d)装置の検出下限は目標検出下限を達成できるか。

e)装置の感度の変動（ドリフト）が十分少ないか。

f)高分解能質量分析計（HRMS）の使用分解能（M/△M, 10%谷）が 10,000 以上であるか。

④同定・定量

a)操作ブランク値が十分低いか。

b)検出下限は、目標検出下限と同等か。

c)同一試料についての再現性があるか。

⑤分析法の性能

a)添加した内標準物質の回収率は40～120%か。

b)標準試料が正しく分析できるか。

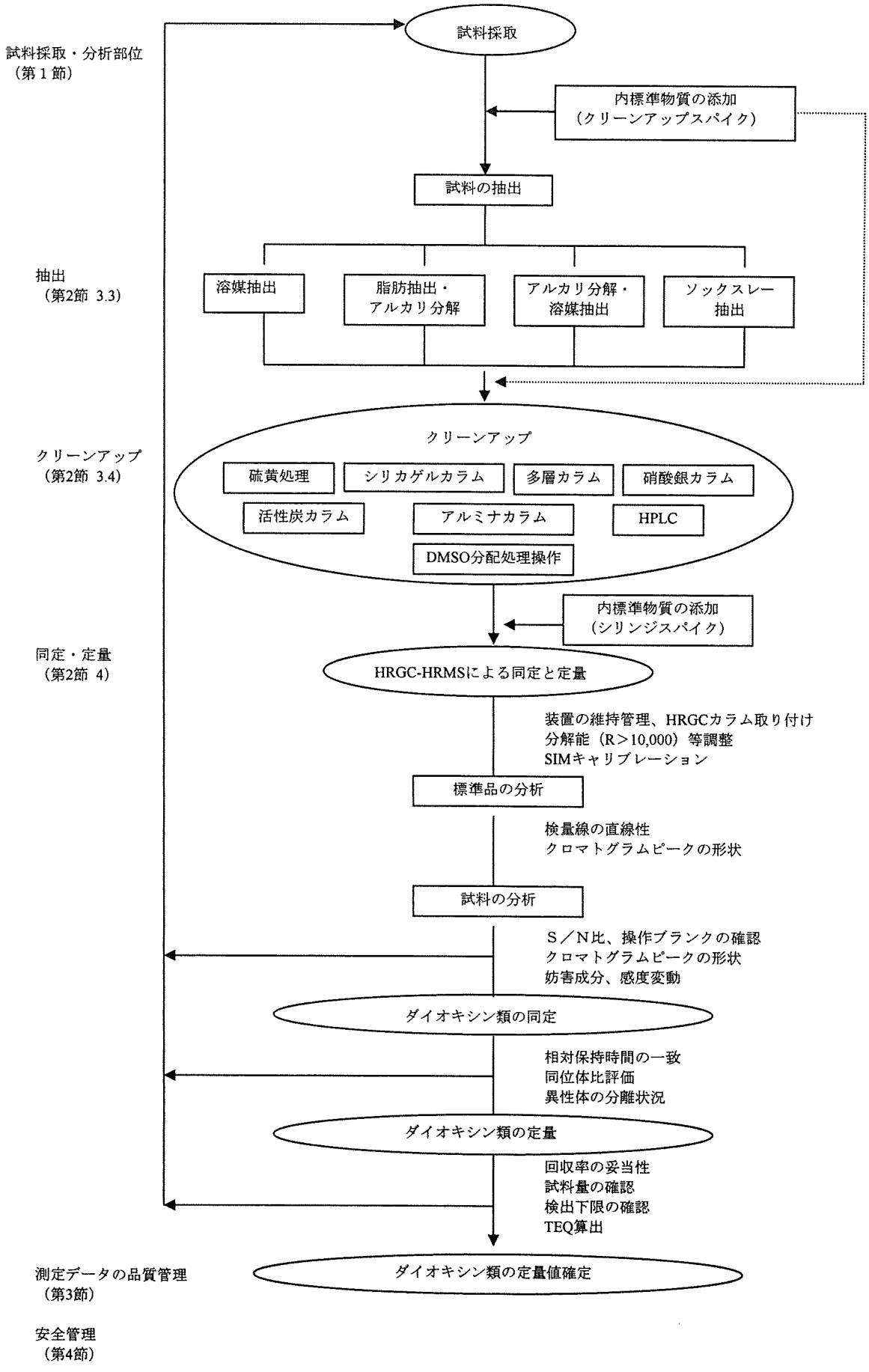

図1-1　分析フローと本ガイドラインでの記述箇所