Systematic analysis and the overall toxicity evaluation of dioxins and hexachlorobenzene in human milk

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Introduction

The hexachlorobenzene (HCB), a type of organochlorine pesticide (OCP), was used as a fungicide for seed, and as a wood preservative. Also, HCB exists in the by-products found in the manufacturing process of chlorinated organic chemicals, and is generated by garbage incineration[1]. The HCB is a so-called, unintended toxic pollutant as well as dioxins, and HCB is then specified for Persistent Organic Pollutants (POPs). According to a recent study, it was pointed out that HCB binds to the aryl hydrocarbon (Ah) receptor[2,3], resulting in dioxin-like effects and bioaccumulates. Therefore, the overall toxicity evaluation of dioxins and HCB in human body, especially in human milk, should be examined, because HCB is universally detected in human milk. Until now, many studies regarding the dioxins or OCPs polluted in human milk have been reported. However, there are only a few reports that analyze both dioxins and HCB in the same sample[4], because repeated sampling and large amounts of samples of human milk were generally difficult to acquire. Moreover, few studies are available for the overall toxicity evaluation of dioxins and HCB in human milk.

The aim of the present study was to develop the systematic analysis method of dioxins and HCB, and to obtain additional information about the overall toxicity evaluation of dioxins and HCB in human milk. The correlation between the HCB residue level and each dioxin isomer in the human milk was also considered.

Materials and Methods

Chemicals: All of the dioxin standards were from Wellington Laboratories. The OCPs were HCB, α-hexachlorocyclohexane (HCH), β-HCH, γ-HCH, δ-HCH, *o,p'*- DDT, *p,p'*-DDT, *o,p'*- DDD, *p,p'*-DDD, *o,p'*- DDE, *p,p'*-DDE, heptachlor and heptachlor epoxide, all of which were from Wako Pure Chemical Industries (Osaka, Japan). Most of the organic solvents such as hexane, acetone, dichloromethane (DCM), toluene, diethylether and ethanol were of dioxin analysis quality from Kanto Kagaku (Tokyo, Japan) or Wako Pure Chemical Industries. All the other chemicals were from PCB analysis quality grade or special quality grade.

Analysis of dioxins and HCB: Human milk samples were collected from 100 Japanese primiparae. Approximately 50 g milk samples were used for the analysis. The sample pretreatment for dioxin analysis was carried out in accordance with the manual compiled by the Ministry of Health, Labour and Welfare, Japan. Briefly, a stable isotope of each congener of the PCDD/Fs and Co-PCBs were added as a surrogate after the fat was extracted from the human milk. The fat was subjected to a concentrated sulfuric acid washing and then to chromatographies such as silica-gel column (1.5g of silica-gel; eluted with 120 mL hexane, followed by 60 mL of 10% DCM/hexane), alumina column (6.5 g of basic alumina; eluted with 60mL of 2% DCM/hexane, followed by 100 mL of 60% DCM/hexane) and activated carbon silica-gel column (0.5 g of activated carbon silica-gel; eluted with 60 mL of 25% DCM/hexane, followed by 100 mL of toluene) as the cleanup operation, followed by the GC/MS measurement for dioxins.

As for the analysis of HCB, the fraction of 2% DCM/hexane eluate from alumina column was evaporated near dryness in vacuo, and the residue was dissolved with 1mL of hexane, followed by the GC-ECD (electron capture detection) measurement. For other OCPs such as heptachlor epoxide and a part of β-HCH, the fraction of the 10% DCM/hexane eluate from the silica-gel column was used in a similar manner. For the remaining β-HCH, the fraction of 25% DCM/hexane eluate from activated carbon silica-gel column was also used in a similar manner.

GC/MS measurement: The PCDD/Fs were analyzed by HR-GC/MS using a JEOL JMS-700 mass spectrometer equipped with a capillary DB-17HT column (30 m x 0.25 mm i.d., film thickness 0.15 μm) in the splitless injection mode (1 μl). The GC program was as follows: 150 °C (1 min) to 220 °C (0 min) at 20 °C/min and subsequently at 4 °C /min to 280 °C, then maintained for 16.5 min at 280 °C. The MS was operated in the selected ion monitoring mode with a mass resolution of 10,000, and the electron impact ionization energy was 38 eV with an ion source temperature of 260°C. The toxic equivalent quantity (TEQ) was calculated using the international toxic equivalency factors (I-TEF, 1988), WHO/IPCS-TEF (1993) or WHO-TEF (1998).

GC-ECD measurement: The HCB and other OCPs were analyzed by GC-ECD using a HP5890 SERIES II (Agilent) equipped with a capillary DB-5.625 column (30 m x 0.25 mm i.d., film thickness 0.25 μm) in the splitless injection mode (1 μl). The GC program was as follows: 70 °C (1 min) to 150 °C (0 min) at 20 °C/min and subsequently at 3 °C /min to 270 °C, then maintained for 10 min at 270 °C. The injector temperature was 200°C and the detector temperature was held at 300°C. The quantification of the OCPs was carried out using the absolute standard curve method.

Results and Discussion

Behavior of HCB and other OCPs in preprocessing process of dioxin analysis: The behavior of the HCB and other OCPs in each column chromatography was examined. In the silica-gel column, the HCB and most of OCPs were eluted in the first fraction (hexane 120mL) except heptachlor epoxide, δ-HCH and a part of β-HCH. The heptachlor epoxide and δ-HCH besides β-HCH of the remainder were eluted in the second fraction (10% DCM/hexane 60mL). In the alumina column, HCB, *o,p'*-DDE and *p,p'*-DDE were eluted in the first fraction (2%DCM/hexane, 60mL). However, the other OCPs excluding β-HCH were eluted neither in the first fraction nor in the second fraction (60% DCM/hexane 100mL). On the other hand, in the activated carbon silica-gel column, all the

OCPs were eluted in 25%DCM/hexane 60mL. From the above-mentioned results, the fractionation of HCB and other OCPs was shown in the flowchart of Fig.1. The HCB and some pesticides such as *o,p'*-DDE, *p,p'*-DDE, heptachlor epoxide, β-HCH and δ-HCH were found to have the possibility to construct a systematic analysis with dioxins using the preprocessing of dioxin analysis. In the present study, HCB, heptachlor epoxide and β-HCH were determined.

Recovery study: The bovine milk samples fortified at a level of 10 ng/g each of HCB, heptachlor epoxide and β-HCH were used for the recovery study. The overall mean recoveries were 60.3 – 70.5 % and the standard deviations (SD) were less than 9%. The β-HCH was calculated by the summation of the recovery from a silica-gel column and an activated carbon silica-gel column as shown in Fig.1.

Investigation of HCB pollution level in human milk: In Table 1, there are summarized levels of OCPs determined in our study concerned with the examination of the set of 100 human milk samples. The residual level of HCB was 4.1 – 91.8 ng/g fat (mean; 33.9 ng/g fat). The heptachlor epoxide and β-HCH were also found in all of the samples. These data suggested that the human milk had been polluted by these persistent organochlorine contaminants.

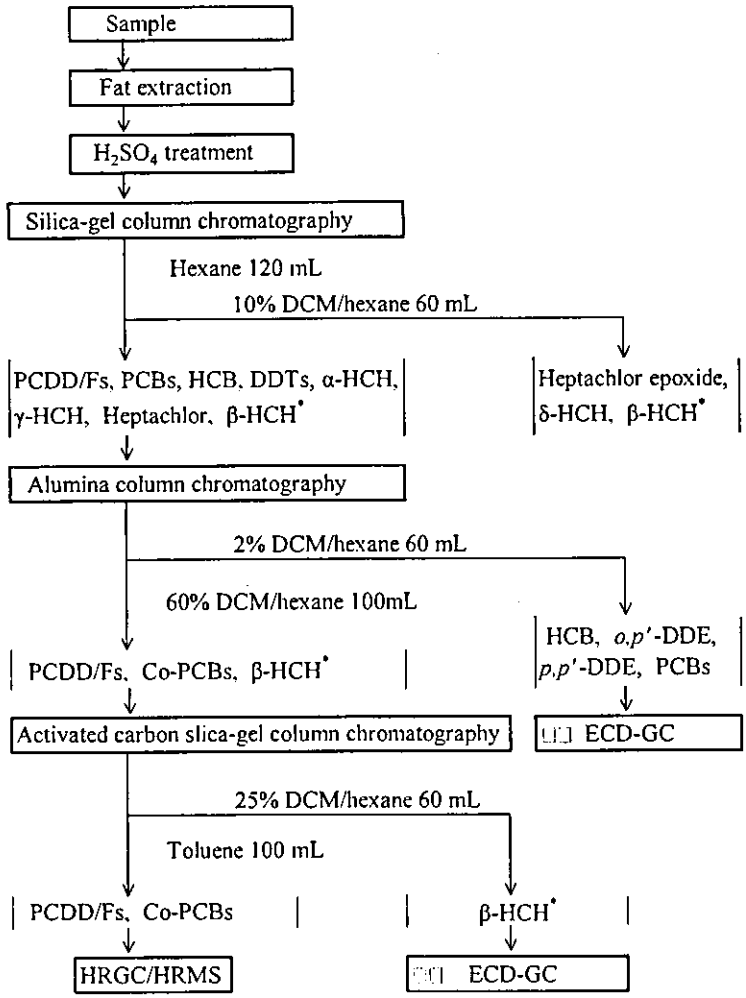

Fig. 1. Flowchart of systematic analysis of dioxins and hexachlorobenzene in human milk

Table 1. Residual concentration of HCB, heptachlor epoxide and β-HCH

Peticide	Mean	Min	Max	SD
	(ng/g fat; n=100)			
HCB	33.9	4.1	91.8	16.2
Heptachlor epoxide	7.4	1.4	22.1	4.0
β-HCH	62.7	8.1	610.3	80.8

Correlation analysis: The Pearson's correlation coefficients among the residue levels of OCPs and each isomer of the dioxin in human milk were examined with the data of 100 samples. The HCB showed a significant positive correlation ($p < 0.01$) with most of the dioxin isomers. On the other hand, heptachlor epoxide and β-HCH showed a poor correlation with the dioxin isomers. In addition, more significant positive correlations were found for the HCB and the dioxin isomers with a high TEF such as 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD and 2,3,4,7,8-PeCDF. These results suggest that the behavior of the exposure root and the cumulative exposure to the human body throughout the lifetime by HCB was assumed to be similar to those of the dioxins.

Overall toxicity evaluation of dioxins and HCB: According to a recent study[3] the binding activity to the Ah receptor of HCB, it is said that the toxicity equivalency factor (TEF) of HCB corresponds to 0.0001. This value is as low as OCDD and OCDF. However, so far it is reported that the contaminated level of HCB is higher than that of the dioxins in human milk. When the HCB toxicity was calculated using the TEF (0.0001), the TEQ of HCB in human milk yielded 0.41-9.2pg TEQ/g fat (Mean value:3.4 pg TEQ/g fat, n=100) (Table 2). It yielded an increase of about 16% (average value) when these results were summed with the TEQ (calculated using I-TEF) of dioxins. Because the mono-ortho-PCBs were not determined in the dioxin analysis at this time, the total TEQ in the WHO-TEF(1998) was calculated using the guessing value (it was assumed that the TEQ mono-ortho-PCBs accounted for about 13% of total TEQ by WHO-TEF). As a result, the increase in TEQ by HCB became about 12%.These data were almost in the same range as those reported in the literature[3], which described that HCB could add 10 - 60 % total TEQ in human milk samples. On the basis of these results, it was understood that evaluating the overall toxicity by adding HCB was necessary for the dioxin toxicity evaluation in human milk.

Table 2. Increase of dioxin toxicity (TEQ) in human milk when including HCB toxicity

	Toxicity (pg TEQ/g fat)*		Increasing rate
HCB	3.4		
Dioxins (I-TEF)	21.8		
Dioxins (WHO-TEF 1998)**		28.5	
HCB + Dioxins (I-TEF)	25.2		116%
HCB + Dioxins (WHO-TEF 1998)		31.9	112%

* □□Average of 100 human milk samples

**□The data of mono-ortho PCBs was calculated using presumed value (13% of Total TEQ).

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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
, ŋ b 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
, ŋ b 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

生体試料中ダイオキシン ELISA の構築および試料精製法の開発

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【目的】 内分泌撹乱化学物質による環境や食物の汚染とそれによるヒトの健康影響に対する社会的関心が急速に高まってきている。特にダイオキシン類はその毒性が極微量で発現することから、ヒトや生態系に及ぼす影響が懸念されている。しかし、その毒性発現機序や感受性の動物種差などについては未だ不明な点も少なくない。ヒトの曝露状況や環境・食物の汚染実態を経年的推移を含めて把握することは、こうした問題を解決する上で極めて重要である。

従来、ダイオキシンの測定には高分解能ガスクロマトグラフィー／マススペクトロメトリー（HRGC/HRMS）が用いられているが、いずれの試料についても長時間にわたる多段階で煩雑なクリーンアップ操作が必要で、その測定費用は著しく高価なものとなっている。今後、測定対象試料の種類と検体数は益々増加することが予測されるため、安価で簡便・迅速かつ高感度なダイオキシン測定法の開発が強く求められている。以上の観点から我々は、母乳を含む生体試料中のダイオキシン簡易測定系の確立を目標として、毒性の強いダイオキシン異性体に親和性の高いモノクローナル抗体を新たに作製して ELISA を構築した。今回、目的とするダイオキシン類を脂質から簡便、迅速に精製する方法を開発し、ELISA の前処理法としての有用性に検討を加えた。

【方法】 前処理法：試料に水酸化カリウム（最終濃度：1 mol/L）およびエタノールを加え、室温で 2 時間撹拌して脂質を加水分解した後、*n*-ヘキサンでダイオキシン類を抽出し、濃硫酸でヘキサン相を洗浄した。ヘキサンを乾固した後、残渣に濃硫酸を添加して脂質を完全に分解し、再びヘキサンで抽出した。 5% 炭酸水素ナトリウムおよび水で洗浄したヘキサン相をダイオキシン吸着カラム（和光純薬工業試作品）に負荷した。ヘキサンでカラムを洗浄後、ベンゼン／ヘキサン（1:3）混液で溶出し、溶出液に Triton X-100（Sigma）を加えて有機溶媒を乾固した後、残渣を緩衝液に溶解して ELISA 用試料とした。

ELISA：第二抗体をコーティングした 96 穴 ELISA プレートのウェルにペルオキシダーゼ標識ダイオキシン 50μL およびダイオキシン（標準品または試料）とモノクローナル抗ダイオキシン抗体混液 50μL を添加して 4℃で一晩反応させた。ウェルを PBS で洗浄し、基質溶液（0.05% *o*-フェニレンジアミンおよび 0.01% H_2O_2 を含有する 50

mmol/L 酢酸ナトリウム-クエン酸緩衝液 (pH5.0)) 100μL を加えて室温で 30 分間反応後、3 mol/L 硫酸 50μL を添加して酵素反応を停止し、波長 490nm における各ウェルの吸光度をマイクロプレートリーダー (Bio-Tek Instrument Inc. BL312e) により測定した。

HRGC/HRMS：上記の前処理法により精製した試料に ^{13}C 標識内部標準物質を添加し、以下の条件でガスクロマトグラフ (Hewlett Packard 5890-II) ／マススペクトロメーター (JEOL JMS-700,Mstation) を用い、SIM 法により測定した。

GC 条件

カラム：Supelco 2331　60m x 0.25mm i.d.　膜厚 0.25μm

昇温プログラム：130℃ (2 min)、130-200℃ (15℃ /min)、200-260℃ (3℃ /min)、260℃ (30 min)

キャリヤーガス：He　　カラムヘッドプレッシャー：168 Kpa

注入口温度：270℃　　注入量：2 μL

MS 条件

検出モード：EI　　イオン源温度：270℃

イオン化電流：600 μA　　イオン化エネルギー：38 eV

加速電圧：10 KV

【結果および考察】　本 ELISA の測定範囲は 1～100 pg/assay であり、母乳など生体試料中のダイオキシン類を測定できる感度を有していた。

脂肪を水酸化カリウムおよび濃硫酸で完全に分解した後に、ダイオキシン吸着カラムで精製することにより、ELISA に適用可能な試料を調製しうることが判明した。本法で精製したダイオキシン添加バターまたは牛乳を ELISA により測定した結果、その測定値はほぼ満足できるものであり、HRGC/HRMS による測定値とも良好な相関性がみられた。また、母乳中の毒性等価量 (TEQ) の大部分を占める 3 種のダイオキシン類、2,3,7,8-TCDD、1,2,3,7,8-PeCDD および 2,3,4,7,8-PeCDF の回収率 (HRGC/HRMS による) はそれぞれ 64%、75%および 85%であった。

ダイオキシン類のように、水に難溶性で試験器具に吸着しやすく、かつ超微量 (ppt レベル) を測定するには、界面活性剤や吸着防止剤の使用が必須で、その種類や濃度を適切に設定することが重要となる。本研究では、Triton X-100 の添加が有効で、上記のように満足のいく回収率を得ることに成功した。

今回開発した精製法を組み合わせた ELISA は、安価で簡便・迅速なダイオキシン類のモニタリングおよびスクリーニング法となることが期待される。

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Development of Simple and Rapid Purification Methods for Bioanalytical Detection of Dioxins

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Introduction

Polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) are organochlorinated pollutants found in various environmental matrices. These compounds are known to be bioaccumulative exhibiting a variety of toxic effects including tumor promotion, immunotoxicity, reproductive toxicity and endocrine disruption. In consequence PCDD/Fs are recognized as a potential threat to human health and to be a serious ecological issue.

High-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) has conventionally been used for detection of PCDD/Fs because of the advantages of separating the numerous congeners and simultaneous quantification of each component with reasonable accuracy and precision. However, the HRGC/HRMS procedures require time-consuming and expensive pretreatment method to extract a trace amount of the target compounds.

Thus, several rapid and high-throughput bioanalytical methods, involving enzyme-linked immunosorbent assay (ELISA)[1,2] and AhR-dependent assay[3-5], have recently been developed. These procedures could be much more feasible, cheaper and suitable for routine monitoring of potential toxicity due to PCDD/Fs in various samples with a reasonably low detection limit. However, a proper sample pretreatment is still necessary to avoid interference particularly due to

lipophilic substance: that masks the target compounds and prohibit to be bound by antibody or receptor.

In this study, we established simple, rapid and inexpensive purification procedures for an ELISA of PCDD/Fs using a solid phase extraction (SPE) cartridge, Wakogel P-29. Moreover, we attempted to develop an immunoaffinity extraction method that could allow even simpler and faster pretreatment for bioanalyses of PCDD/Fs. To achieve this aim, previously generated monoclonal antibodies, whose cross-reactivity to dioxin congeners was corresponding to the toxic equivalence factors (TEF), were employed for preparing immunosorbent.

Materials and Methods

1. Chemicals

PCDD/Fs congeners were purchased from Wellington Laboratories. SPE cartridges, Wakogel P-29 and abselut NEXUS, were purchased from Wako Pure Chemicals and Varian, respectively. Cyanogen bromide (CNBr)-activated Sepharose 4FF was obtained from Amersham Pharmacia Biotech, and Affi-Gel 10 was from Bio-Lad.

2. Clean-up Procedure for ELISA

Fat was saponified with 1 mol/L KOH and extracted with *n*-hexane. The organic layer was washed with H_2SO_4 until the sulfuric acid layer had become to be clear. The extract was loaded to Wakogel P-29 cartridges, which were washed with *n*-hexane and then eluted with hexane/benzene (3:1). After addition of Triton X-100 (0.1% in MeOH; 25 μL) to the effluent, the solvent was evaporated and the residue was dissolved in PBS, then the following ELISA performed.

3. ELISA

The ELISA was carried out using monoclonal antibody D9-36 as described previously[2]. Briefly, horseradish peroxidase-labeled hapten, the monoclonal antibody and standard dioxin or the sample prepared as above were added to the second antibody-coated wells in a 96-well microtiter plate. After overnight incubation at 4°C, the wells were washed with PBS, and the bound enzyme activity was measured using H_2O_2 and *o*-phenylenediamine as a substrate.

4. Preparation of Immunosorbent and Affinity Column

A solution of the IgG fraction (monoclonal antibody D2-37, D9-36 or D35-42)[2] was added to the CNBr-activated Sepharose 4FF or Affi-Gel 10. After gentle stirring of the mixture overnight at 4°C, remaining reactive groups of the gel were blocked by addition of 0.1 mol/L Tris-HCl buffer (pH 8.0). Then the gel was washed serially with 0.1 mol/L acetate buffer (pH 4.0), 95% MeOH, water and PBS, and a portion of the resulting gel (1 mL) was packed into a disposable column.

5. Immunoaffinity Extraction-Based Pretreatment

Fat specimen was treated as described above, and the residue was dissolved in PBS and applied to the immunoaffinity column. After washing serially with PBS, water and 10% MeOH, PCDD/Fs were eluted with 95% MeOH. This fraction was diluted with water to reduce the MeOH concentration to be less than 60%, added to the NEXUS cartridge and washed with 90% MeOH. Then PCDD/Fs fraction was eluted with acetone, and submitted to the ELISA.

6. GC/MS Measurement

After purification by Wakogel P-29, PCDD/Fs in the purified extract were determined by HRGC/HRMS on a Hewlett Packard 5890-II gas chromatograph-JEOL JMS-700 (Mstation) mass spectrometer at a resolution of R=10000 in the selected ion monitoring mode (SIM).
The HRGC/HRMS conditions were as follows: column, Supelco 2331 60 m x 0.25 mm i.d., 0.25 μm thickness; column temperatureprogram, 130°C (2 min), 130-200°C at 15°C/min, 200-260°C at 3°C/min, 260°C (30 min); carrier gas, He; column head pressure, 168 Kpa; injection temperature, 270°C; injection volume, 2 μL (splitless). The MS conditions: detection mode, EI; ion source temperature, 270°C; ionizing current, 600 μA; ionizing energy, 38 eV; accelerating voltage, 10 KV. The results were corrected for the recovery of $^{13}C_{12}$-labeled internal standards.

Results and Discussion

Saponification of the fat with KOH and washing with H_2SO_4 were essential for developing clean-up procedures of dioxins. After the purification of the extract using Wakogel P-29 cartridge, we could obtain reasonable assay values for PCDD/Fs using the ELISA, which were in good correlation with the values obtained with the GC/MS procedure (fig.1). Recoveries of 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD and 2,3,4,7,8-PeCDF through the clean-up procedure (determined by the GC/MS) were 64%, 75% and 85%, respectively. In the case of the immunoaffinity extraction-based pretreatment, the recovery of 2,3,7,8-TCDD from fat sample (measured by the ELISA) was about 70%.

Conclusion

It has been shown that the present clean-up procedure using Wakogel P-29 is useful for developing an ELISA system which is available as a simple, rapid and inexpensive method for screening and monitoring of PCDD/Fs. The immunoaffinity extraction using monoclonal antibodies equipping high affinity to toxic dioxin congeners is expected to be a novel pretreatment procedure that is available not only for ELISAs but also for the AhR-dependent assays.

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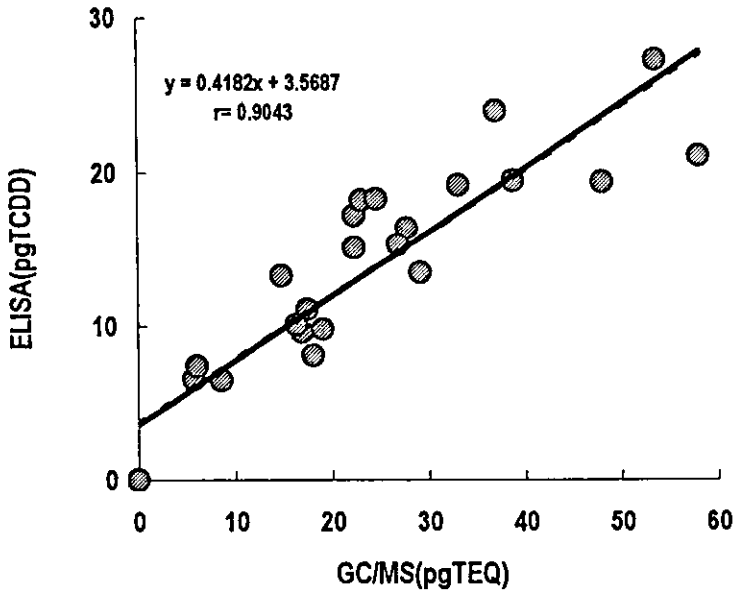

Fig.1 Correlation between GC/MS and ELISA for Fat Samples

遺伝子組替え大豆定量検査法の外部精度管理について

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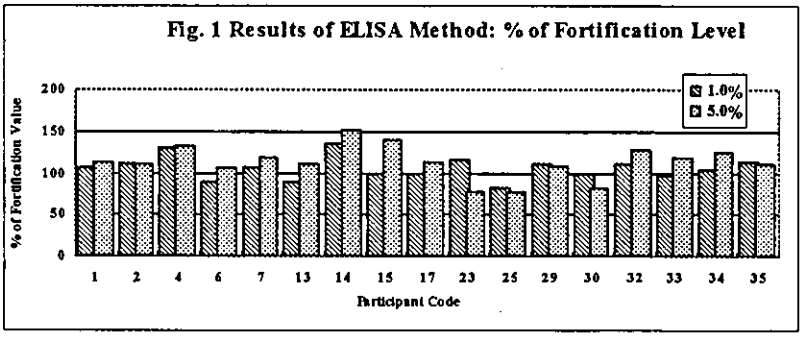
【緒言】厚生労働省では平成 13 年 4 月以降,公衆衛生の観点から遺伝子組換え(GM)食品の安全性審査を義務付けるものとし，併せて表示制度を施行している．またこれに伴い，流通ならびに表示の科学的検証を目的とした検査方法を記した「組換え DNA 技術応用食品の検査方法について」を医薬局食品保健部長通知し，GM 食品の検査方法を定めた．当該検査方法は平成 13 年 3 月 27 日に通知されて以降，科学的検証法の改良や対象 GM 食品の変遷等に合わせて順次改訂を重ね，平成 15 年 6 月 18 日に通知された食発第 0618001 号においては，分析適応可能機種の拡充に伴い，定量 PCR 法の項を中心とした大幅な改訂を行ったところである．

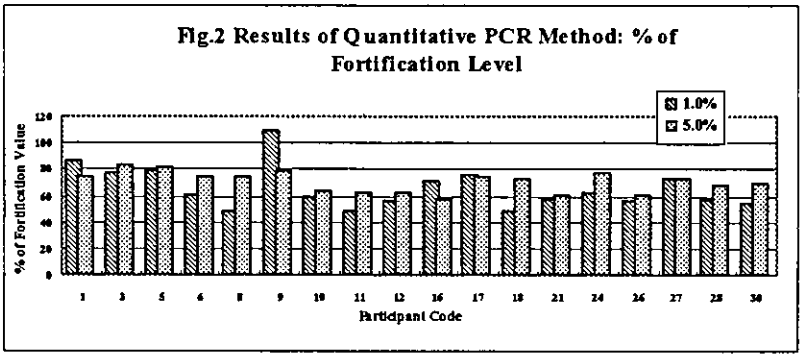
GM 食品のうち，安全性審査を終了した GM 食品に関しては食品衛生法に規格基準が設けられており，意図せざる場合，5%の混入率を目安に分別流通管理が正しく行われているか否かが判断される．行政上,この数値をもって判断がなされるため，当検査方法を用いて得られる測定結果の信頼性を確保することが大変重要である．この測定結果の正当性を保証するためには，精度管理が不可欠である．すなわち，諸検査機関が厚生労働省通知法に従い配布検査試料を同一時期に分析し，その分析結果をもとに，当該分析法における検査機関間のデータのばらつきの程度ならびに検査水準を把握すること，さらには，検査担当者が自己の技術を客観的に認識し，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．しかしながら GM 食品定量検査方法に採用されている ELISA 法,ならびに定量 PCR 法を対象とした外部精度管理方法については国際的にもほとんど検討されておらず，既存の外部精度管理方法が適用できるのかについての情報は少ない．本研究では，昨年度本会において報告した GM トウモロコシおよび GM ジャガイモの定性検査法に引き続き，安全性審査の終了した GM 大豆の定量検査方法を対象とした外部精度管理方法を検討することを目的とし，35 機関(定量 PCR 法のみ：18 機関，ELISA 法のみ：13 機関，両法：4 機関)による試験を実施し，集計された共通未知試料の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．

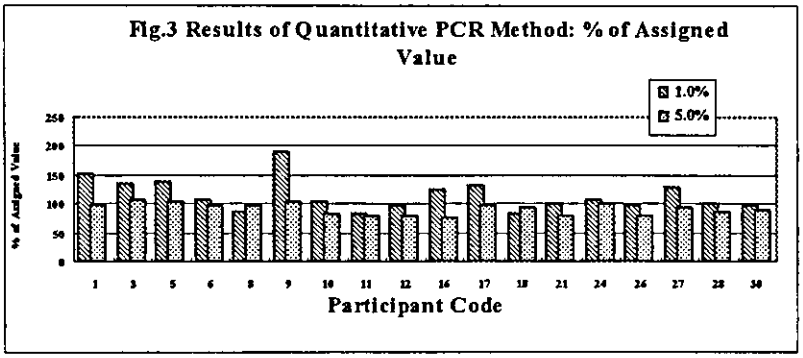
【方法】GM 大豆試料は厚生労働省医薬局食品保健部監視安全課を通じ，また 0%試料ならびに疑似混入試料調製時のマトリクスとして使用した非遺伝子組換え(non-GM)大豆試料(アメリカ産大豆)は（独）食品総合研究所を通じ入手した．入手した全ての大豆試料を粒径が均一に 200 μm となるよう粉砕し，その後，被験物質の混入率が重量換算で 1.0%, 5.0%となるよう混合した．混合は以下の通り行った．まず，均一に粉砕した試料を再度凍結乾燥処理した．その後，上記重量比となるよう 0%試料と被験物質を正確に秤量し，全量を 500 g としてプラスチック製の袋に量り採った．袋中で充分な混合を行った後，篩にかけ，再び袋中で混合を繰り返した．この混合操作は合計で 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，疑似混入試料とした．疑似混入試料調製後, 0%試料ならびに疑似混入試料を 6.5 g (定量 PCR 法) または 2.0 g (ELISA 法)となるよう，それぞれ 50 mL，あるいは 15 mL 容遠沈管 50 本に秤量分注し小分け試料とした． 小分け試料のうち 6 点を無作為選出し，定量 PCR 法を用いた均一性試験を食品総合研究所で，ELISA 法を用いた均一性試験を国立衛研で実施した．試験の結果は統計的に処理し，十分な均一性を確認した．安定性試験は小分け試料 4 点を無作為選出し，試料配布日を 0 日目とし，-20°Cの条件で 30 日間保存した後に，試験終了時としての測定を実

施した. 測定試験はPCR法, ELISA法の両法を用いて行い, 外部精度管理実施期間中における保存安定性について検討した. 均一性確認後, 各小分け試料は0%試料を「低濃度」, 1.0%試料を「中濃度」, 5.0%試料を「高濃度」検体と表記し参加機関に送付した. またこれに合わせELISA法, 定量PCR法ともに, 厚生労働省通知法食発第0618001号に従い試験するものとし, 当該通知法に従ったマニュアルを作成し, 同送した. 報告された測定データの統計処理は, ISO/IEC GUIDE 43-1等の国際基準を参考に実施した.

【結果及び考察】ELISA法を実施した各機関において得られた定量値を, 送付検体のGM大豆混入率に対する相対値に換算した結果をFig.1に示す. 5%検体を測定した場合, 機関14ならびに15においてそれぞれ152.4%(定量値：7.62), 140.8%(定量値：7.04)と高めの値が報告された. また一方で, 機関23ならびに25からはそれぞれ76%(定量値：3.82)と若干低めの値が報告されている. これら機関間にみられる測定値のばらつきの原因については現在解析しているところであるが, 全体としては1.0%検体を測定した平均値が1.06%(相対値：105.8%), 5%検体を測定した場合の平均値が5.66%(相対値：113.2%)と報告されており, 良好な試験が行われたものと判断している. 次に定量PCR法の結果を示す. 定量PCR法を実施した22機関中18の機関がシリカゲル膜タイプキット法(QIAGEN DNeasy Plant mini kit)を用いてDNA抽出を行っていた. Fig.2には, それら機関より報告された定量値を送付検体のGM大豆混入率に対する相対値に換算した結果を示す. シリカゲル膜タイプキット法を用いた機関から報告された定量値は, 1.0%検体の平均値が0.66%(相対値：65.7%), 5%検体の平均値が3.53%(相対値：70.7%)と混入率に比較して低めであった. しかし, Fig.3に示すように, 国立衛研において同法を用いて得られた定量値に対し相対値を算出した場合には, 機関番号1ならびに6の機関から報告された1.0%検体に対する結果を除き, 良好な試験が行われたことが示唆されている(1.0%検体相対値：115.3%, 5%検体相対値:91.3%). また, 他のDNA抽出法を採用した機関からは混入率に比較し良好と判断される結果が報告されている(1.0%検体平均値:0.87%, 5%検体平均値：4.78%). これらの結果から, 大豆検体からシリカゲル膜タイプキット法(QIAGEN Dneasy Plant mini kit)を用いて抽出されたDNAを対象にPCR法を実施した場合, 何らかの要因により測定値が低くなる可能性が示唆され, 通知の改正が必要と考えられた.







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