COMPARISON OF ACCELERATED SOLVENT EXTRACTION AND ALKALINE DIGESTION-HEXANE SHAKING EXTRACTION FOR DETERMINATION OF DIOXINS IN ANIMAL-ORIGIN FOOD SAMPLE

Hori T[1], Yasutake D[1], Tobiishi K[1], Ashizuka Y[1], Kajiwara J[1], Nakagawa R[1], Iida T[2], Tsutsumi T[3], Sasaki K[3]
[1]Fukuoka Institute of Health and Environmental Sciences, 39 Mukaizano, Dazaifu-shi, Fukuoka 818-0135, Japan; [2]Kitakyushu Life Science Center, 1-4 Nakabarushinmachi, Kitakyushu-shi, Fukuoka 804-0003, Japan; [3]National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Abstract
We studied the progressive analytical method for dioxins in animal-origin food samples such as fish, meat and dairy products. This study aimed to establish a highly sensitive and rapid analytical method using HRGC/HRMS equipped with a solvent cut large volume (SCLV) injection system and accelerated solvent extraction (ASE). When ASE was applied to extract fat from dried milk powder, high fat amounts were obtained in the case where the temperature was set to 150 °C and acetone/n-hexane (1:1, v/v) was used as the extraction solvent. A high extraction efficiency in these conditions was also found in quantitative results for 29 kinds of dioxin congeners on the identical sample. Using these conditions, a freeze-dried *tuna* homogenate was extracted by ASE and we performed a standard alkaline digestion followed by a n-hexane shaking extraction on the identical sample. The concentrations of each dioxin congener were very similar in both extraction methods. Our analysis of 20 g of various animal-origin food items according to the present method, including the ASE and SCLV injection technique, showed recovery rates for labeled congeners within the range recommended by the Japanese analytical guideline of dioxins in food (40%-120%).

Introduction
We previously developed a highly sensitive method for determining dioxin content in food using a solvent cut large volume (SCLV) injection system coupled to a cyanopropyl phase capillary column[1]. The SCLV injection system coupled to a 40m-length Rtx-2330 column showed sufficient separation of 2,3,7,8-chlorine-substituted isomers and had at least five-times higher sensitivity than the conventional injection technique[2]. In the conventional method, a large volume of sample (generally 100g) must be treated collectively in order to attain the desirable limit of detection (LODs) at low ppt levels, namely, 0.01pg/g for 2,3,7,8-tetraCDD/F. The SCLV injection technique method allows the reduction of a sample volume from 100g to 20g when such usual LODs are demanded and is expected to improve the efficiency of laboratory performance, especially when it is coupled to an automated extraction method such as accelerated solvent extraction (ASE). In order to examine the applicability of ASE for the determination of dioxins in food samples, it is important to verify the extraction efficiency of this method against that of the conventional technique.
We reported the applicability of an ASE for the determination of dioxins in plant food samples and compared the method's performance with that of the standard conventional shaking extraction (separatory funnel extraction) regarding recovery rates and quantitative determination[3]. The results showed that ASE could extract dioxins at high efficiency using a low-volume solvent and could provide a high level of performance for various plant matrices, especially regarding those, such as seaweed powder, from which dioxins are difficult to extract using conventional shaking extraction.
In the present study, the applicability of the combined SCLV injection and ASE methodology is evaluated for use regarding animal-origin fatty food samples. It is considered that homogeneous tissue, such as dried milk powder, is suitable for the method's quantitative validation.

Materials and Methods
Dried milk powder on the market was used for the examination of extraction conditions. For the comparison of quantitative determinations, about 300 g of the edible parts of *tuna* were purchased at a market in Japan. They were homogenized using a food processor, freeze dried and homogenized again. For the examination of the recovery rate, extracts were prepared from homogenates of animal-origin food samples (cow's milk, cheese, yogurt, and so on). The recovery rates for 17 kinds of ^{13}C-labeled 2,3,7,8-substituted PCDD/Fs and 12 kinds of ^{13}C-labeled dioxin-like PCBs were evaluated.

The analytical procedures used in this study are summarized in Table 1. In Method 1, the conventional standard method, the sample was treated with 100 ml of 1 N potassium hydroxide/ethanol for two hours with stirring at room temperature. The alkaline hydrolyzate was extracted twice with 100 ml of n-hexane using a separatory funnel for one hour each time, and then the concentrated extract was treated with 15 ml of concentrated sulfuric acid. By contrast, in Method 2, automated extraction was performed using an ASE-300 (Dionex, CA) under conditions of 1500 psi. Four individual experiments and four simultaneous blank tests were performed for each extraction method.

Dioxins were analyzed using a model 6890 gas chromatograph (Agilent Technologies, CA) coupled to a model Autospec-Ultima mass spectrometer (Micromass, UK). We employed an Rtx-2330 (0.18mm x 40m) capillary column (Restek, PA) on an SCLV injection system (SGE, Australia) in order to determine tetra- and pentaCDD/Fs, and hexaCDFs. The details of the operating conditions for the SCLV injection system are described in another paper[2]. The LOD for each congener was determined according to the provisional guidelines for analysis of dioxins in foods issued by the Ministry of Health and Welfare of Japan in 1999 ("*Guideline*"): An absolute quantity corresponding to S/N = 3 was evaluated on HRGC/HRMS chromatograms using verification standards.

Table 1 Analytical procedures for determination of dioxins in food.

		Method 1	Method 2
Extraction		Alkaline digestion (KOH/ethanol) followed by shaking extraction* Sample size: 20g Time: 60 min x 2 (120 min) Solvent: n-hexane 200 ml (100ml x 2)	Accelerated solvent extraction (ASE) Sample size: 20 g Time: 25 min Solvent: acetone/n-hexane (1:1, v/v) 120 ml
Cleanup		Sulfuric acid treatment ↓ Multi-layer silica gel column ↓ Active carbon-dispersed silica gel column	
HRGC/ HRMS analysis	PCDD/DFs and non-*ortho* PCBs	SCLV injection Injection volume: 4 µL / 20µL Pre-column:BPX-5 (0.25mm x 5m) Analytical columns: a) Rtx-2330 (0.18mm x 40m) b) BPX-5 (0.15mm x 30m)	
	Mono-*ortho* PCBs	Splitless injection Injection volume: 1µL/20 µL Analytical column: HT8-PCB (0.25mm x 60m)	

* Method recommended for fat, fish and shellfish, meats, eggs, milk and dairy products in "*Guideline*".

Results and Discussion

Twenty grams of milk powder were extracted by ASE. After the extracts were evaporated and dried, fat contents were measured gravimetrically. Three individual experiments were performed for each extraction condition shown in Table 2. As a result, the largest fat content was obtained under the condition of 150 ^{o}C, acetone/n-hexane (1:1, v/v). This highest value agreed with that obtained from the standard fat extraction method by shaking its reconstituted aqueous solution with diethyl ether/petroleum ether (1:1, v/v). Data regarding the quantification of dioxin congeners in milk powder (pg/g whole weight basis) are shown in Table 3. Trace data showing the concentrations of the congeners under detection limits were re-evaluated and are shown in parentheses to compare concentrations between the methods. Generally, high concentrations and a large number of detected congeners were found under the condition of 150 ^{o}C, acetone/n-hexane (1:1, v/v), compared to other conditions. By contrast, there were no obvious differences among the computed data showing 29 kinds of labeled compound recoveries in each extraction condition (data not shown), all of which were adapted to the range recommended in the "*Guideline*" (40%-120%). The above results suggested that differences in quantification values between extraction conditions were due to differences in the extraction efficiency of dioxin molecules from the tissue. Hence, validation tests comparing ASE to the conventional method were carried out using the condition of "150 ^{o}C, acetone/n-hexane," which demonstrated the high extraction efficiency of the compounds and the fat content's similarity to the standard fat extraction method.

Table 2 Fat contents (%) of milk powder under various extraction conditions

	Temperature (°C)	Solvent	Trial	Milk powder weighed (g)	Fat obtained (g)	Fat contents (%)
ASE	100	n-hexane	1st	20.01	0.20	1.0
			2nd	20.34	0.31	1.5
			3rd	20.00	0.30	1.5
		acetone/n-hexane (1:1)	1st	20.21	0.69	3.4
			2nd	20.08	0.93	4.7
			3rd	20.01	1.12	5.6
	150	n-hexane	1st	20.16	3.02	15.0
			2nd	20.33	3.22	15.8
			3rd	20.27	3.13	15.5
		acetone/n-hexane (1:1)	1st	20.24	5.30	26.2
			2nd	20.27	5.24	25.8
			3rd	20.26	5.30	26.1
Shaking extraction		diethyl ether/petroleum ether (1:1)	1st	5.06	1.24	24.4
			2nd	4.96	1.19	23.9
			3rd	4.96	1.16	23.3

Table 3 Concentrations of dioxins (pg/g whole weight basis) in dried milk powder; comparison of temparature and solvent used.

Temperature (℃)		100						150					
Solvent		n-hexane			acetone/n-hexane			n-hexane			acetone/n-hexane		
Congener	LOD (pg/g)	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
2,3,7,8-TeCDD	0.01	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
1,2,3,7,8-PeCDD	0.01	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
1,2,3,4,7,8-HxCDD	0.02	nd	nd	nd	nd	nd	nd	nd	nd	0.021	nd	0.020	(0.011)
1,2,3,6,7,8-HxCDD	0.02	nd	nd	nd	nd	nd	nd	0.026	0.034	0.035	0.046	0.035	0.026
1,2,3,7,8,9-HxCDD	0.02	nd	nd	nd	nd	nd	nd	nd	nd	0.013	(0.018)	(0.018)	0.020
1,2,3,4,6,7,8-HpCDD	0.02	nd	nd	nd	(0.064)	(0.061)	0.13	0.25	0.19	0.21	0.34	0.38	0.31
OCDD	0.05	0.19	0.25	0.22	0.51	0.62	0.99	2.1	2.2	2.2	3.3	3.6	2.9
2,3,7,8-TeCDF	0.01	nd	nd	nd	0.019	0.016	0.017	0.055	0.055	0.049	0.082	0.080	0.067
1,2,3,7,8-PeCDF	0.01	nd	nd	nd	nd	nd	nd	0.037	0.021	0.030	nd	nd	nd
2,3,4,7,8-PeCDF	0.01	nd	nd	nd	nd	nd	nd	0.043	0.035	0.036	nd	nd	0.024
1,2,3,4,7,8-HxCDF	0.02	nd	nd	nd	nd	(0.0091)	(0.019)	0.031	0.026	0.029	0.051	0.064	0.036
1,2,3,6,7,8-HxCDF	0.02	nd	nd	nd	nd	nd	(0.012)	0.021	nd	(0.012)	0.029	0.030	0.027
1,2,3,7,8,9-HxCDF	0.02	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.014
2,3,4,6,7,8-HxCDF	0.02	nd	nd	nd	nd	nd	(0.0080)	0.022	(0.013)	(0.016)	0.028	0.032	0.020
1,2,3,4,6,7,8-HpCDF	0.02	nd	nd	nd	0.024	0.020	(0.019)	0.056	0.054	0.085	0.10	0.10	0.11
1,2,3,4,7,8,9-HpCDF	0.02	nd	nd	nd	nd	nd	(0.010)	nd	nd	nd	nd	nd	nd
OCDF	0.05	nd	nd	nd	(0.024)	(0.014)	0.056	0.14	0.15	0.16	0.25	0.19	0.20
3,3',4,4'-TeCB(#77)	0.1	(0.075)	(0.096)	(0.087)	(0.099)	(0.064)	0.10	0.13	0.11	0.15	0.14	0.15	0.22
3,4,4',5-TeCB(#81)	0.1	nd	nd	nd	(0.015)	nd	nd	(0.012)	nd	(0.011)	(0.013)	nd	(0.019)
3,3',4,4',5-PeCB(#126)	0.1	nd	nd	nd	(0.031)	(0.022)	nd	(0.082)	(0.099)	0.11	0.13	0.11	0.11
3,3',4,4',5,5'-HxCB(#169)	0.1	nd	nd	nd	nd	(0.0083)	nd	(0.057)	(0.048)	(0.052)	(0.061)	(0.074)	(0.063)
2,3,3',4,4'-PeCB(#105)	1	(0.23)	(0.31)	(0.32)	(0.53)	(0.55)	(0.88)	1.7	1.6	1.8	2.1	2.1	2.2
2,3,4,4',5-PeCB(#114)	1	(0.028)	(0.029)	(0.031)	(0.046)	(0.044)	(0.075)	(0.18)	(0.18)	(0.18)	(0.22)	(0.26)	(0.23)
2,3',4,4',5-PeCB(#118)	1	(0.81)	1.1	1.2	1.8	2.0	3.1	6.9	6.5	7.1	8.9	9.9	9.3
2',3,4,4',5-PeCB(#123)	1	(0.019)	(0.032)	(0.025)	(0.042)	(0.039)	(0.066)	(0.12)	(0.087)	(0.092)	(0.13)	(0.13)	(0.17)
2,3,3',4,4',5-HxCB(#156)	1	(0.12)	(0.11)	(0.16)	(0.33)	(0.38)	(0.56)	1.4	1.4	1.4	2.0	2.2	1.9
2,3,3',4,4',5'-HxCB(#157)	1	(0.033)	(0.045)	(0.036)	(0.095)	(0.11)	(0.16)	(0.39)	(0.36)	(0.40)	(0.50)	(0.51)	(0.47)
2,3',4,4',5,5'-HxCB(#167)	1	(0.054)	(0.070)	(0.064)	(0.12)	(0.14)	(0.21)	(0.50)	(0.48)	(0.47)	(0.72)	(0.75)	(0.73)
2,3,3',4,4',5,5'-HpCB(#189)	1	(0.022)	(0.030)	(0.027)	(0.050)	(0.056)	(0.11)	(0.22)	(0.20)	(0.20)	(0.26)	(0.37)	(0.28)

Table 4 shows the dioxin concentrations and RSD values obtained from the two extraction methods using freeze-dried *tuna* homogenates. RSD values in ASE ranged from 4% to 19%, similar to the results in alkaline digestion (1% to 27%). The concentrations of 29 kinds of dioxin congeners were close for both extraction methods other than OCDD; the ratios of estimated concentrations from ASE compared to those from the alkaline digestion-hexane shaking extraction method ranged from 0.96 to 1.4, except 2.0 for OCDD. It is considered that this result was due to ASE's high extraction efficiency compared with the shaking extraction; a tendency like this was observed in our previous examination using dried seaweed powder, in which the extraction efficiency of ASE was found to be superior to that of conventional separatory funnel extraction [3].

A recovery test in the present method, including the ASE and SCLV injection technique, was performed using 18 food items, mainly dairy products. The results showed that recovery rates for 29 kinds of labeled congeners ranged from 41% to 108 %, within the range recommended by the Japanese analytical guideline for dioxins in food (40%-120%). Our results suggest that the present method is available for rapid and sensitive determination

of dioxins in animal-origin fatty food samples of low sample size and requiring only a small volume of extraction solvent compared to the conventional extraction method. The ASE condition suited for dioxins in the animal-origin sample presented here is identical to that proposed for plant food samples[3]. Therefore, independent extraction conditions could be available for both animal- and plant-origin food samples. Moreover, fat content values obtained from the present extraction method of dioxins could be directly applied to the calculation of fat weight-based concentrations. The applicability of the combined SCLV injection and ASE methodology has been continuously verified for use regarding food mixture samples, e.g., total diet study samples.

Table 4 Concentrations of dioxins (pg/g whole weight basis) in dried *tuna* homogenates; conparison between ASE and alkaline digestion followed by hexane shaking.

Congener	ASE (n=4)			Alkaline digestion-hexane shaking (n=4)			a / b
	Range	Mean[a]	RSD(%)	Range	Mean[b]	RSD(%)	
2,3,7,8-TeCDD	0.61 - 0.67	0.64	4	0.60 - 0.72	0.67	7	0.96
1,2,3,7,8-PeCDD	0.75 - 0.83	0.80	5	0.76 - 0.80	0.77	3	1.0
1,2,3,4,7,8-HxCDD	0.023 - 0.035	0.028	19	0.020 - 0.030	0.024	16	1.2
1,2,3,6,7,8-HxCDD	0.20 - 0.22	0.21	5	0.20 - 0.22	0.21	4	0.99
1,2,3,7,8,9-HxCDD	0.026 - 0.037	0.032	17	0.022 - 0.028	0.025	12	1.2
1,2,3,4,6,7,8-HpCDD	0.058 - 0.067	0.065	7	0.055 - 0.058	0.057	2	1.1
OCDD	0.15 - 0.17	0.16	6	0.070 - 0.094	0.081	13	2.0
2,3,7,8-TeCDF	4.4 - 5.5	5.0	9	4.8 - 5.3	5.1	5	0.98
1,2,3,7,8-PeCDF	0.92 - 1.1	0.99	6	0.94 - 1.0	0.96	3	1.0
2,3,4,7,8-PeCDF	2.7 - 3.1	2.9	5	2.7 - 2.8	2.7	2	1.1
1,2,3,4,7,8-HxCDF	0.15 - 0.21	0.19	14	0.15 - 0.25	0.18	26	1.1
1,2,3,6,7,8-HxCDF	0.14 - 0.21	0.18	19	0.17 - 0.21	0.18	11	1.0
1,2,3,7,8,9-HxCDF	0.11 - 0.15	0.13	14	0.12 - 0.14	0.13	7	1.0
2,3,4,6,7,8-HxCDF	nd nd	-	-	nd nd	-	-	-
1,2,3,4,6,7,8-HpCDF	0.067 - 0.079	0.072	7	0.050 - 0.063	0.055	11	1.3
1,2,3,4,7,8,9-HpCDF	nd nd	-	-	nd nd	-	-	-
OCDF	nd nd	-	-	nd nd	-	-	-
3,3',4,4'-TeCB(#77)	220 - 270	240	9	260 - 280	270	3	0.91
3,4,4',5-TeCB(#81)	17 - 21	19	9	20 - 21	20	1	0.94
3,3',4,4',5-PenCB(#126)	210 - 250	230	6	230 - 240	230	1	0.99
33'44'55'-HxCB(#169)	26 - 29	27	5	28 - 28	28	1	0.97
233'44'-PeCB(#105)	60000 - 72000	64000	9	50000 - 68000	61000	14	1.1
2344'5-PeCB(#114)	2700 - 3400	3100	10	2700 - 2900	2800	4	1.1
23'44'5-PeCB(#118)	100000 - 120000	110000	6	110000 - 130000	120000	8	0.96
2'344'5-PeCB(#123)	2600 - 4100	3600	19	1900 - 3700	2700	27	1.4
233'44'5-HxCB(#156)	31000 - 37000	36000	9	32000 - 42000	39000	12	0.92
233'44'5'-HxCB(#157)	8500 - 10000	9400	8	8000 - 11000	9700	13	0.97
23'44'55'-HxCB(#167)	20000 - 23000	22000	7	20000 - 25000	23000	11	0.96
233'44'55'-HpCB(#189)	4900 - 5900	5600	8	4400 - 5300	5000	9	1.1

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