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Improved Analytical Method for Residual Dioxins in Human Milk

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Polychlorinated dibenzo-*p*-dioxins(PCDDs), polychlorinated dibenzofurans(PCDFs) and coplanar polychlorinated biphenyls (Co-PCBs) are globally distributed toxic chemicals and were found in human milk (Tuinstra *et al.* 1994, Beck *et al.* 1994, González *et al.* 1996, Kiviranta *et al.* 1999, Iida *et al.* 1999 and Yang *et al.* 2002) and foods (Rhijn *et al.* 1993, Ramos *et al.* 1999, Malisch *et al.* 2000 and Thorpe *et al.* 2001). As the 2,3,7,8-substituted PCDD/PCDF congeners are highly toxic among these compounds, there is much concern about their potential entry into the food chain. These compounds are lipophilic and persistently accumulate in the human adipose tissue and then these compounds accumulate in the human adipose tissue. Their elimination and lipophilic metabolites from adipose tissue is very slow. As the fat content of human breast milk is relatively high, these levels in milk are strongly correlated to the fat content (Norén, 1988) and influenced by the concentration of adipose tissue. Numerous analytical methods have been published for the determination of trace levels of these compounds in human milk. Most methods start with addition of ^{13}C-labelled standard congeners and fat extraction, treatment with alkaline saponification and/or sulfuric acid and applied with silica gel, alumina and activated carbon chromatography. Isomer identification and quantification have been separated and resolved with polar and apolar columns using high resolution mass spectrometry. In this paper we described simultaneous determination of PCDDs, PCDFs and Co-PCBs congeners in human milk. And we improved several cleanup steps around column chromatography operations. Further, we performed a survey study of the PCDDs, PCDFs and Co- PCBs contents of human milk donated from various areas in Tokyo by this improved method.

MATERIALS AND METHODS

Native and $^{13}C_{12}$-PCDDs, PCDFs and Co-PCBs as authentic standards were purchased from Wellington Laboratories (Guelph, Ontario, Canada). Active carbon-impregnated silica gel was purchased from Wako Pure Chemicals Industries (Osaka, Japan) as dioxin analysis grade. Silica gel 60 was

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purchased from Merck (Darmstadt, Germany). All solvents were obtained analytical grade of dioxin (Wako) and all other chemicals analytical grade.
GC-MS was performed on an Autospec Ultima (VG Analytical, Manchester, UK) fitted with an HP 6890 gas chromatograph (Hewlett Packard, Palo Alto, CA) and an HP 7683 auto injector. The GC was fitted with a DB-5MS (J&W Scientific, Folsom, CA), fused silica capillary column (30m;0.25 mm i.d.; 0.25 μm film thickness) and utilized with helium as the carrier gas at a column head pressure of 175 KPa. . The column temperature was programmed from 100°C (hold 1 min) to 200°C at 20°C/min rate (hold 1 min), then to 270°C at 5°C/min rate. Final temperature was maintained for 30 min. Selected ion monitoring was employed using the two most intense ions from the molecular ion cluster for each homologue. Data processing was performed using standard VG OPUS soft ware for automatic peak area measurement and to calculate the mass of each compound present. Human milk samples were obtained from healthy native Japanese mothers living in the Tokyo region over five years. The first samples were collected from May to August in 2000 from 120 volunteer mothers from Tokyo after inquiring about their residence. They were all provided with pre-cleaned glass vials to collect approximately 50 mLs manually from the breast into the cooled container. The samples were kept frozen at -20°C until analysis. The mothers ages ranged from 25 to 34 in primiparas and multiparas.

The frozen milk sample was thawed and homogenized with ultrasonic bath. A 50 mL aliquot of the milk was spiked with a set of carbon-13 labeled PCDDs, PCDFs and Co-PCBs internal standards. Each 100μl of eight $^{13}C_{12}$- labeled PCDDs, ten PCDFs and twelve Co-PCBs congeners were 50 mL aliquots of milk at levels of 2, 2, and 10 ng/kg, respectively. Except OCDD and OCDF, which were spiked 4 ng/kg. They were extracted with a mixture of 10 mL of saturated sodium oxalate solution, 75 mL of ethanol, 50 mL of ethyl ether and 50 mL of hexane in a 300 mL of separatery funnel. After shaking, the upper layer was transferred to another funnel, the hexane extraction, repeated twice. The organic layers were collected and washed with 50 mL of 20% sodium chloride solution, then 10% and 5%, respectively. The organic layer was dried with sodium sulfate, evaporated to dryness and fat content determined by weighing. The residue was dissolved in 100 mL of hexane and treated with 25 mL of concentrated sulfuric acid, the operation, being repeated four times. The hexane layer was rinsed with 50 mL of water, several times, dried with sodium sulfate and evaporated. The residue was dissolved in 3 mL of hexane and applied to a silica gel (prewashd with hexane using Soxhlet extractor, 48 hrs and activated 130°C 18 hrs) connected to an activated carbon-impregnated column, eluted with 80 mL of hexane. The eluate was passed through the silica gel column to the carbon column. Then, the silica gel column was detached from the carbon column (the hexane fraction was discard). The carbon column eluted with 80 mL of 20%(v/v) dichloromethane/hexane (mono-*ortho*-PCBs Frs), eluted with 250 mL of toluene (non-*ortho*-PCBs, PCDDs and PCDFs Frs).

Both fractions were evaporated and reconstructed with 50 μL of ^{13}C-labelled 1,2,3,4-TCDD and 2,3',4',5-TCB as external standards. One μL aliquot of the external solution was injected to GC/MS. A minimum resolution of 10,000 was used when operating with the MS instrument. A blank sample was run every 5 human milk samples in order to check for any contamination throughout the analytical procedure.

RESULTS AND DISCUSSION

To evaluate the precision and accuracy of this cleanup method , the following

Table 1. Recoveries of 13C-PCDDs, PCDFs and Co-PCBs (%, n=120).

Congener	Min	Max	Mean	S .D.
13C-2,3,7,8-TeCDD	44	87	69.9	8.60
13C-1,2,3,7,8-PeCDD	59	105	85.8	7.20
13C-1,2,3,4,7,8-HxCDD	50	109	85.9	10.28
13C-1,2,3,6,7,8-HxCDD	57	116	98.6	9.74
13C-1,2,3,7,8,9-HxCDD	68	118	100.6	9.88
13C-1,2,3,4,6,7,8-HpCDD	54	108	86.9	12.62
13C-OCDD	43	98	64.3	9.69
13C-2,3,7,8-TeCDF	47	88	70.2	8.93
13C-1,2,3,7,8-PeCDF	59	97	81.4	6.60
13C-2,3,4,7,8-PeCDF	68	99	85.7	6.84
13C-1,2,3,4,7,8-HxCDF	54	105	84.8	9.80
13C-1,2,3,6,7,8-HxCDF	58	108	93.4	9.08
13C-1,2,3,7,8,9-HxCDF	57	106	89.4	10.37
13C-2,3,4,6,7,8-HxCDF	53	109	91.1	9.72
13C-1,2,3,4,6,7,8-HpCDF	56	111	89.8	11.44
13C-1,2,3,4,7,8,9-HpCDF	51	106	80.6	11.31
13C-OCDF	46	97	61.5	9.47
13C-1,3,6,8-TeCDD	48	88	70.2	8.43
13C-1,3,7,9-TeCDD	52	89	71.0	8.11
13C-3,3',4,4'-TeCB (#77)	44	99	78.0	13.16
13C-3,4,4',5-TeCB (#81)	46	98	80.7	12.15
13C-3,3',4,4',5-PeCB (#126)	61	113	95.7	11.63
13C-3,3',4,4',5,5'-HxCB (#169)	73	117	104.6	8.85
13C-2',3,4,4',5-PeCB (#123)	56	106	84.3	8.27
13C-2,3',4,4',5-PeCB (#118)	53	103	86.9	7.28
13C-2,3,4,4',5-PeCB (#105)	58	101	88.6	6.10
13C-2,3,3',4,4'-PeCB (#114)	59	104	89.9	6.16
13C-2,3',4,4',5,5'-HxCB (#167)	55	103	82.2	12.77
13C-2,3,3',4,4',5-HxCB (#156)	59	110	87.9	13.87
13C-2,3,3',4,4',5'-HxCB (#157)	58	109	87.4	14.30
13C-2,3,3',4,4',5,5'-HpCB (#189)	47	105	82.9	15.88

Table 2. PCDDs, PCDFs and Co-PCBs levels (pg/g, Fat) in human milk from Tokyo and fat concentrations (%).

Congener	Mean	S.D.	TEQ	TEF
2,3,7,8-TeCDD	1.3	0.63	1.3	1
1,2,3,7,8-PeCDD	5.3	2.4	5.3	1
1,2,3,4,7,8-HxCDD	2.2	0.96	0.22	0.1
1,2,3,6,7,8-HxCDD	16.2	7.1	1.6	0.1
1,2,3,7,8,9-HxCDD	3.1	1.3	0.31	0.1
1,2,3,4,6,7,8-HpCDD	8.6	4.2	0.086	0.01
OCDD	61.5	47.2	0.006	0.0001
Total PCDDs			8.9	
2,3,7,8-TeCDF	1.0	0.42	0.10	0.1
1,2,3,7,8-PeCDF	0.66	0.31	0.033	0.05
2,3,4,7,8-PeCDF	10.4	4.48	5.2	0.5
1,2,3,4,7,8-HxCDF	3.4	1.28	0.34	0.1
1,2,3,6,7,8-HxCDF	3.9	1.7	0.39	0.1
1,2,3,7,8,9-HxCDF	0.78	0.74	0.078	0.1
2,3,4,6,7,8-HxCDF	2.7	1.2	0.27	0.1
1,2,3,4,6,7,8-HpCDF	2.2	1.6	0.022	0.01
1,2,3,4,7,8,9-HpCDF	<1.25	—	0	0.01
OCDF	<2.5	—	0	0.0001
Total PCDFs			6.4	
Total (PCDDs + PCDFs)			15.3	
1,3,6,8-TeCDD	1.4	2.8	0	0
1,3,7,9-TeCDD	0.24	0.81	0	0
3,3',4,4'-TeCB (#77)	10.4	5.4	0.001	0.0001
3,4,4',5-TeCB (#81)	3.2	1.8	0.0003	0.0001
3,3',4,4',5-PeCB (#126)	53.9	27.5	5.4	0.1
3,3',4,4',5,5'-HxCB (#169)	30.8	16.2	0.31	0.01
2',3,4,4',5-PeCB (#123)	<0.75	—	0	0.0001
2,3',4,4',5-PeCB (#118)	10200	4860	1.0	0.0001
2,3,4,4',5-PeCB (#105)	2000	1090	0.20	0.0001
2,3,3',4,4'-PeCB (#114)	697	526	0.35	0.0005
2,3',4,4',5,5'-HxCB (#167)	1140	540	0.011	0.00001
2,3,3',4,4',5-HxCB (#156)	3060	1580	1.53	0.0005
2,3,3',4,4',5'-HxCB (#157)	732	364	0.37	0.0005
2,3,3',4,4',5,5'-HpCB (#189)	221	111	0.022	0.0001
Total Co-PCBs			9.2	
Total (PCDDs+PCDFs+Co-PCBs)			24.5	

	Min	Max	Mean	S.D.
Fat concentration (%)	1.00	8.70	4.00	1.398

quality control samples were analysed. Cow's milk sample obtained from the market, was used for this study. Thirty one $^{13}C_{12}$- labeled PCDDs, PCDFs and Co-PCBs congeners were added to 50 mL aliquots of milk at levels of 2-10 ng/kg. Triplet analyses were made at the spike level. The recoveries range from 66.8 to 98.5%, with the exceptions of OCDD and OCDF with recoveries 63.4 and 58.7%, respectively. We applied this cleanup method for the analysis of PCDDs, PCDFs and Co-PCBs in human milk. Table 1 summarizes the experimental data obtained. The average recoveries are in the range 64.3 -100.6% for PCDDs, 61.5-93.4% for PCDFs and 78.0-104.6% for Co-PCBs, which are in good agreement with the results obtained by other procedures (Chang *et al.* 1993, Soboleva *et al.* 1997). Mean recovery of OCDD and OCDF were lower than other congeners. The accuracy and precision of the clean-up procedure for human milk was demonstrated by the validation study. Therefore, the method was used for the analysis of PCDDs, PCDFs and Co-PCBs in the sub-ppt-range. We were able to shorten the clean-up time by 20% using this method compared with an alumina column procedure. Method inearity was determined by preparing 5 solutions of each native standard in triplicate, over the concentration range of 0.02-20ng/mL (OCDD and OCDF; 0.04-40 ng/mL, Co-PCBs;0.1-100ng/mL). The detection limit was 0.01 pg/g for tetra- to penta- dioxin and furan, 0.05 pg/g for hexa- to hepta-, 0.1 pg/g for octa- and 0.03 pg/g for Co-PCBs, respectively (whole basis).

The levels of PCDD and PCDF congeners for 120 samples are given in Table 2. The mean concentration of PCDDs in human milk (pg TEQ/g fat) was 8.9 (36.3%), PCDFs 6.4 (26.1%), Co-PCBs 9.2 (37.6%), PCDDs+PCDFs 15.3 (62.4%) and PCDDs+PCDFs+Co-PCBs 24.5, respectively. Penta- chlorinations were predominating, followed by hexa-, tetra-, hepta- and octa-, respectivelly (PCDDs, PCDFs). A similar pattern was found for non-*ortho* Co-PCBs, penta-chlorinations (#126) were predominant, followed by hexa- (#169). But in mono-*ortho* Co-PCBs, predominant was hexa- (#156) followed by penta- (#118) >hexa- (#157) ≧ penta-(#114)>penta-(#105), respectively. The non-*ortho* was 62% of total Co-PCBs(TEQ).

The concentrations of PCDDs, PCDFs and Co-PCBs in human milk samples of mothers living in Tokyo are similar or lower than those previously reported for many countries (Beck *et al.*1994, González *et al.*1996, Yang *et al.*2002).
Fat content in human milk was on average 4.0±1.40% (ranged from 1.0 to 8.7%) in Tokyo(Table 2).

We detected non 2,3,7,8-chlorine-substituted congeners in mother's milk, which were 1,3,6,8- and 1,3,7,9- TeCDD. The former congener was detected 102 positive samples and the latter 31 by all 120 samples (Table 2). But we couldn't detect 1,2,3,6,8-PeCDD in these samples (Masunaga *et al.*2001). The correlation between 1,3,6,8- and 1,3,7,9- concentrations among human milk samples was calculated. The linear regression correlation coefficient(R) for the breast milk

was 0.9545. Both 1,3,6,8- and 1,3,7,9- congeners didn't contribute to TEQ. The detection reason of these congeners in breast milk was thought to be the result of impurities of diphenyl ether herbicides, i.e., 2,4,6-trichlorohenyl-4'-nitrophenyl ether (chloronitrofen, CNP). CNP had been used as a paddy field herbicide in Japan from 1970's until 1995 (Sakurai *et al.* 1996, Masunaga *et al.*2001). So, the concentrations of 1,3,6,8- and 1,3,7,9- in human milk will be gradually decreasing.

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