milk was 30 days after childbirth. After collection, the samples were frozen and stored at −20 °C until analysis.

2.2. Dioxin analysis

Approximately 50 g milk samples were used for the analysis. The sample pretreatment was carried out in accordance with the manual compiled by the Ministry of Health, Labour and Welfare, Japan. Briefly, the fat was extracted from the milk, then, $|^{13}C_{12}|$-PCDDs, $|^{13}C_{12}|$-PCDFs, $|^{13}C_{12}|$-Co-PCBs as internal standards were added. The fat was cleaned by concentrated sulfuric acid decomposition or alkaline digestion, followed by silica gel column, alumina column and activated carbon column chromatographies.

The PCDD/Fs and non-*ortho* Co-PCBs were analyzed by HR-GC/MS using a JEOL JMS-700 mass spectrometer equipped with a capillary DB-17HT column (30 m×0.25 mm i.d., film thickness 0.15 μm). The MS was operated in the selected ion monitoring mode with a mass resolution of 10 000.

The detection limit values were TCDD/F, PeCDD/F: 0.02 pg/g; HxCDD/F, HpCDD/F: 0.05 pg/g; OCDD/F: 0.1 pg/g; Co-PCB: 0.1 pg/g for the whole basis. Levels under the detection limits were treated as 0. However, the dioxin levels in this report are described on a fat basis. As for the toxic equivalency factors (TEFs), the International-TEFs (I-TEF: 1988) were applied for PCDD/F, and the WHO/IPCS-TEFs (1993) for Co-PCBs.

2.3. Dietary habits index

To objectively compare the dietary habits, we first set standard servings of various foods, and we then calculated the dietary habits index from a comparison between the frequency and the amount of the food eaten. The standard serving of steak eaten at one time, for example, was set at 150 g, and the index for a person who ate this amount once a day was denoted "1". The number was "0.5" for a person who ate half or less of the standard serving, "1.5" for a person who ate 1.5 times or more than the standard, or who ate it more frequently. "0.5" also represents the consumption of a dietary item 3–4 times a week, or "2.5" for consumption several times daily. Food items are those used in the large-scale cohort research of the Ministry of Health, Labour and Welfare: We investigated fish and shellfish, about 19 items, meat and eggs, about 17 items, and dairy products, three (milk, cheese and yogurt). We divided the dietary items into three categories and summed the food indices to arrive at the fish/shellfish index, the meat/eggs index and the dairy products index.

2.4. Statistical analysis

A multivariate linear regression analysis, partial correlation analysis, Pearson correlation analysis and covariance analysis were used. The statistical test for a significant difference was set at 5%.

3. Results and discussion

The mothers were born in 1959–1979, and their average age was 29.3 years (range: 21–40 years). Their average BMI was 20.9 (range: 15–30). The average collection time was 30.6 days after childbirth (range: 12–61 days). The number of mothers who lived in industrial waste incinerator regions was 185, and the number of those who lived in other regions was 114. The average number of residence years was 20.6 years (range: 1–37 years). The distance to the nearest waste facility from the dwelling was 1.8 km on average (range: 0.1–13.5 km). The number of mothers who smoked was 95, and the number of mothers who did not smoke was 204. The amount of smoking is shown by the smoking index (multiplying smoking years by number of cigarettes per day). The smoking index of smoking mothers was 116.6 on average (range: 8–440). The number of mothers who had had only breast-feeding in infancy was 108, and the number of those who had had only formula in infancy was 53, and the number of those who had had mixed feeding in infancy was 130. There were eight uncertain cases. The meat and eggs index was 1.7 on average (range: 0.0–9.1). The milk and dairy products index was 2.2 on average (range: 0–17.7). The fish and shellfish index was 1.3 on average (range: 0–5.5).

The fat content in human milk was 4.0% on average (range: 0.7–10.5%). The total dioxin level in human milk was 250.4 pg/g fat on average (range: 116.9–634.1 pg/g fat) for PCDDs + PCDFs + Co-PCBs, and their toxic equivalent was 22.0 pg TEQ/g fat on average (range: 7.3–49.7 pg TEQ/g fat) (Table 1). The OCDD level was the highest, accounting for 29.6%. Next came PCB126, 24.3%, then PCB169, 14.1%. As for the contribution to TEF, 2,3,4,7,8-PeCDF accounted for 30.7%, PCB126 accounted for 27.6% and 3,3′,4,4′,5-PeCDD accounted for 14.3%.

To exclude the influences among the 13 factors considered, the levels of each compound in the human milk and these compounds were analyzed by individual multivariate linear regressions. The results of the analyses showed nine items to be significant for dioxin: age, fat content, BMI, the presence of smoking, breast-feeding, fish and shellfish index, milk and dairy products index, the distance to the nearest waste facility from the dwelling, and residence region. Four items were not significant, i.e., days after childbirth, residence years, meat and eggs index, and formula during lactation. It

Table 2
Considering influence of various factors on dioxin levels in human milk, this table is a result of multivariate linear regression analysis ($n = 299$)[a]

Compounds	Age			Fat content			BMI		
	RC	SE	P	RC	SE	P	RC	SE	P
2,3,7,8-TCDD	0.020	0.013	0.123	Δ 0.033	0.027	0.229	Δ 0.020	0.015	0.209
1,2,3,7,8-PeCDD	0.073	0.039	0.060	Δ 0.250	0.083	0.003	Δ 0.093	0.047	0.047
1,2,3,4,7,8-HxCDD	0.031	0.022	0.158	0.056	0.047	0.236	Δ 0.017	0.026	0.522
1,2,3,6,7,8-HxCDD	0.299	0.128	0.020	Δ 0.977	0.273	0.000	0.111	0.154	0.470
1,2,3,7,8,9-HxCDD	Δ 0.023	0.036	0.521	Δ 0.208	0.078	0.008	0.118	0.044	0.008
1,2,3,4,6,7,8-HpCDD	0.186	0.102	0.070	Δ 0.574	0.219	0.009	0.410	0.124	0.001
OCDD	4.505	0.902	0.000	Δ 2.696	1.932	0.164	3.836	1.089	0.000
2,3,7,8-TCDF	0.019	0.009	0.033	0.016	0.019	0.419	Δ 0.017	0.011	0.120
1,2,3,7,8-PeCDF	Δ 0.007	0.008	0.377	Δ 0.000	0.018	0.983	0.006	0.010	0.547
2,3,4,7,8-PeCDF	0.089	0.078	0.253	Δ 0.490	0.166	0.003	Δ 0.224	0.094	0.018
1,2,3,4,7,8-HxCDF	Δ 0.027	0.040	0.498	Δ 0.193	0.086	0.025	Δ 0.039	0.048	0.415
1,2,3,6,7,8-HxCDF	Δ 0.021	0.054	0.701	Δ 0.315	0.116	0.007	Δ 0.049	0.065	0.452
2,3,4,6,7,8-HxCDF	Δ 0.014	0.031	0.651	Δ 0.197	0.067	0.003	0.020	0.038	0.594
1,2,3,4,6,7,8-HpCDF	Δ 0.057	0.028	0.041	Δ 0.060	0.059	0.309	0.033	0.033	0.317
PCB77	0.148	0.063	0.019	Δ 0.499	0.135	0.000	Δ 0.098	0.076	0.196
PCB126	0.174	0.499	0.728	Δ 1.476	1.069	0.169	Δ 0.202	0.603	0.738
PCB169	0.697	0.241	0.004	Δ 1.536	0.516	0.003	Δ 1.078	0.291	0.000
∑PCDDs	5.090	1.104	0.000	Δ 4.683	2.363	0.049	4.346	1.332	0.001
∑PCDFs	Δ 0.018	0.189	0.926	Δ 1.240	0.404	0.002	Δ 0.270	0.228	0.238
∑PCDDs + PCDFs	5.072	1.216	0.000	Δ 5.923	2.604	0.024	4.076	1.468	0.006
∑Co-PCBs	1.019	0.691	0.142	Δ 3.511	1.480	0.018	Δ 1.378	0.834	0.100
∑PCDD/Fs + Co-PCBs	6.091	1.620	0.000	Δ 9.434	3.469	0.007	2.698	1.956	0.169
∑PCDDs (TEQ)	0.093	0.044	0.037	Δ 0.279	0.095	0.004	Δ 0.037	0.054	0.492
∑PCDFs (TEQ)	0.039	0.047	0.402	Δ 0.315	0.100	0.002	Δ 0.120	0.056	0.035
∑PCDDs + PCDFs (TEQ)	0.133	0.087	0.127	Δ 0.594	0.185	0.002	Δ 0.157	0.105	0.135
∑Co-PCBs (TEQ)	0.024	0.051	0.635	Δ 0.163	0.110	0.139	Δ 0.031	0.062	0.618
∑PCDD/Fs + Co-PCBs (TEQ)	0.157	0.125	0.212	Δ 0.757	0.268	0.005	Δ 0.188	0.151	0.216

	Smoking habit			Breast-feeding			Fish and shellfish index		
	RC	SE	P	RC	SE	P	RC	SE	P
2,3,7,8-TCDD	Δ 0.135	0.082	0.102	0.166	0.074	0.026	0.133	0.055	0.016
1,2,3,7,8-PeCDD	Δ 0.435	0.249	0.081	0.375	0.224	0.095	0.315	0.167	0.059
1,2,3,4,7,8-HxCDD	Δ 0.641	0.141	0.000	0.145	0.127	0.254	0.089	0.094	0.346
1,2,3,6,7,8-HxCDD	Δ 0.903	0.822	0.273	0.558	0.739	0.451	0.276	0.550	0.617
1,2,3,7,8,9-HxCDD	Δ 0.510	0.234	0.030	0.026	0.210	0.901	0.110	0.157	0.482
1,2,3,4,6,7,8-HpCDD	Δ 3.277	0.660	0.000	0.712	0.593	0.231	0.098	0.442	0.824
OCDD	Δ 0.018	5.811	0.998	9.646	5.222	0.066	Δ 3.099	3.890	0.426
2,3,7,8-TCDF	Δ 0.191	0.058	0.001	0.063	0.052	0.226	0.110	0.039	0.005
1,2,3,7,8-PeCDF	Δ 0.142	0.054	0.008	0.057	0.048	0.237	0.067	0.036	0.064
2,3,4,7,8-PeCDF	Δ 1.067	0.500	0.034	1.210	0.449	0.008	0.705	0.335	0.036
1,2,3,4,7,8-HxCDF	Δ 0.186	0.258	0.471	0.268	0.232	0.248	0.264	0.173	0.127
1,2,3,6,7,8-HxCDF	0.038	0.349	0.914	0.249	0.314	0.429	0.392	0.234	0.095
2,3,4,6,7,8-HxCDF	Δ 1.276	0.201	0.000	0.220	0.181	0.223	0.290	0.135	0.032
1,2,3,4,6,7,8-HpCDF	Δ 0.240	0.178	0.178	Δ 0.111	0.160	0.486	Δ 0.052	0.119	0.660
PCB77	0.072	0.405	0.860	0.109	0.364	0.765	0.793	0.271	0.004
PCB126	Δ 14.732	3.216	0.000	3.853	2.890	0.183	6.337	2.153	0.004
PCB169	Δ 3.031	1.552	0.052	2.064	1.395	0.140	1.851	1.039	0.076
∑PCDDs	Δ 5.919	7.108	0.406	11.628	6.388	0.070	Δ 2.078	4.759	0.663
∑PCDFs	Δ 3.064	1.217	0.012	1.956	1.093	0.075	1.776	0.814	0.030
∑PCDDs + PCDFs	Δ 8.983	7.832	0.252	13.584	7.038	0.055	Δ 0.302	5.243	0.954

Table 2 (*continued*)

	Smoking habit			Breast-feeding			Fish and shellfish index		
	RC	SE	*P*	RC	SE	*P*	RC	SE	*P*
∑Co-PCBs	Δ 17.692	4.452	0.000	6.026	4.001	0.133	8.981	2.980	0.003
∑PCDD/Fs + Co-PCBs	Δ 26.675	10.435	0.011	19.610	9.377	0.037	8.679	6.986	0.215
∑PCDDs (TEQ)	Δ 0.591	0.286	0.040	0.443	0.257	0.086	0.336	0.192	0.080
∑PCDFs (TEQ)	Δ 0.704	0.301	0.020	0.687	0.271	0.012	0.461	0.202	0.023
∑PCDDs + PCDFs (TEQ)	Δ 1.296	0.558	0.021	1.129	0.501	0.025	0.797	0.373	0.034
∑Co-PCBs (TEQ)	Δ 1.504	0.331	0.000	0.406	0.297	0.173	0.653	0.222	0.003
∑PCDD/Fs + Co-PCBs (TEQ)	Δ 2.799	0.807	0.001	1.535	0.726	0.035	1.450	0.540	0.008

[a] RC = regression coefficient; SE = standard error; Δ = negative.

with most congeners (Table 3). Next, the amount of smoking was examined, with correction by a multivariate linear regression analysis and Pearson correlation analysis, and these results are shown in Table 4. Each congener showed a negative correlation as the smoking index was raised, and the correlation of 2,3,4,6,7,8-HxCDF was especially strong (Fig. 1). The amount of smoking showed the dioxin levels decreasing with increasing tobacco consumption, supporting Fürst et al. (1992), who reported that mothers who are active, or even passive, smokers contain, on average significantly, lower PCDD/F levels than non-smoking women. While one report (Pluim et al., 1993) found that smoking habits were not related to dioxin levels, results here

Table 3
Mean levels of dioxin in human milk associated with smoking habits, and the result of covariance analysis[a]

Compounds	Active smoker		*P*
	Yes ($n = 95$)	No ($n = 204$)	
2,3,7,8-TCDD	1.41	1.59	0.105
1,2,3,7,8-PeCDD	5.91	6.49	0.071
1,2,3,4,7,8-HxCDD	1.61	2.24	0.000
1,2,3,6,7,8-HxCDD	18.19	19.37	0.322
1,2,3,7,8,9-HxCDD	3.24	3.79	0.023
1,2,3,4,6,7,8-HpCDD	8.46	11.97	0.000
OCDD	71.62	75.13	0.948
2,3,7,8-TCDF	0.60	0.83	0.001
1,2,3,7,8-PeCDF	0.40	0.56	0.009
2,3,4,7,8-PeCDF	12.58	13.94	0.035
1,2,3,4,7,8-HxCDF	4.43	4.71	0.492
1,2,3,6,7,8-HxCDF	5.70	5.81	0.919
2,3,4,6,7,8-HxCDF	2.43	3.78	0.000
1,2,3,4,6,7,8-HpCDF	2.16	2.41	0.166
PCB77	5.79	5.99	0.917
PCB126	49.96	65.99	0.000
PCB169	32.83	36.57	0.056
∑PCDDs	110.44	120.58	0.382
∑PCDFs	28.31	32.04	0.012
∑PCDDs + PCDFs	138.75	152.63	0.235
∑Co-PCBs	88.58	108.54	0.000
∑PCDD/Fs + Co-PCBs	227.34	261.17	0.010
∑PCDDs (TEQ)	6.83	7.57	0.040
∑PCDFs (TEQ)	7.65	8.53	0.020
∑PCDDs + PCDFs (TEQ)	14.48	16.10	0.021
∑Co-PCBs (TEQ)	5.33	6.97	0.000
∑PCDD/Fs + Co-PCBs (TEQ)	19.81	23.07	0.001

[a] Results are given in pg/g; n = number of samples.

Table 4
Correlation between the dioxin levels in human milk and the smoking index ($n = 299$)[a]

Compounds	Partial	P	Pearson	P
2,3,7,8-TCDD	Δ 0.064	0.275	Δ 0.064	0.267
1,2,3,7,8-PeCDD	Δ 0.094	0.108	Δ 0.090	0.120
1,2,3,4,7,8-HxCDD	Δ 0.226	0.000	Δ 0.219	0.000
1,2,3,6,7,8-HxCDD	Δ 0.082	0.163	Δ 0.062	0.283
1,2,3,7,8,9-HxCDD	Δ 0.132	0.025	Δ 0.125	0.031
1,2,3,4,6,7,8-HpCDD	Δ 0.298	0.000	Δ 0.266	0.000
OCDD	Δ 0.030	0.615	0.005	0.928
2,3,7,8-TCDF	Δ 0.113	0.055	Δ 0.104	0.074
1,2,3,7,8-PeCDF	Δ 0.145	0.013	Δ 0.154	0.008
2,3,4,7,8-PeCDF	Δ 0.087	0.138	Δ 0.093	0.110
1,2,3,4,7,8-HxCDF	Δ 0.025	0.666	Δ 0.039	0.498
1,2,3,6,7,8-HxCDF	0.023	0.700	0.011	0.846
2,3,4,6,7,8-HxCDF	Δ 0.366	0.000	Δ 0.357	0.000
1,2,3,4,6,7,8-HpCDF	Δ 0.062	0.289	Δ 0.076	0.190
PCB77	Δ 0.015	0.795	Δ 0.049	0.933
PCB126	Δ 0.248	0.000	Δ 0.244	0.000
PCB169	Δ 0.063	0.282	Δ 0.057	0.325
∑PCDDs	Δ 0.076	0.198	Δ 0.040	0.494
∑PCDFs	Δ 0.119	0.042	Δ 0.128	0.027
∑PCDDs + PCDFs	Δ 0.087	0.137	Δ 0.056	0.336
∑Co-PCBs	Δ 0.204	0.000	Δ 0.196	0.001
∑PCDD/Fs + Co-PCBs	Δ 0.153	0.009	Δ 0.125	0.031
∑PCDDs (TEQ)	Δ 0.113	0.055	Δ 0.103	0.076
∑PCDFs (TEQ)	Δ 0.101	0.084	Δ 0.107	0.064
∑PCDDs + PCDFs (TEQ)	Δ 0.113	0.055	Δ 0.110	0.057
∑Co-PCBs (TEQ)	Δ 0.244	0.000	Δ 0.240	0.000
∑PCDD/Fs + Co-PCBs (TEQ)	Δ 0.178	0.002	Δ 0.174	0.003

[a] Partial correlation coefficient and Pearson correlation coefficient; Δ = negative.

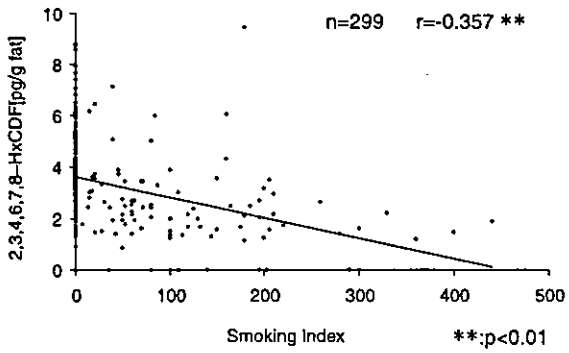

Fig. 1. 2,3,4,6,7,8-HxCDF levels in human milk in relation to the smoking index.

showed that the dioxin levels in milk of mothers who smoked were lower than for mothers who did not smoke, and the dioxin levels were generally lower for mothers who were heavy smokers. It is thought that smoking somehow influences the accumulation of dioxin in the body.

3.2. Lactation of mother in infancy

Mothers who were given only breast-feeding in infancy (108) showed a mean level of total dioxins in their human milk of 263.7 pg/g fat (23.1 pg TEQ/g fat). Mothers who had mixed feeding (130) had a mean level of 246.2 pg/g fat (21.8 pg TEQ/g fat), and mothers who had only formula (53), a mean level of 235.6 pg/g fat (20.7 pg TEQ/g fat). There were eight uncertain cases. The three methods were then compared with dioxin congeners. A multivariate linear regression analysis showed that the levels of 2,3,7,8-TCDD, 2,3,4,7,8-PeCDF, ∑PCDD/Fs + Co-PCBs, ∑PCDFs (TEQ), ∑PCDDs + PCDFs (TEQ) and ∑PCDD/Fs + Co-PCBs (TEQ) in human milk of those who had only breast-feeding were significantly high. After this, the three lactation methods were compared with each congener. Most congener levels showed a ranking in the order of breast-feeding, mixed feeding and formula. Next, a factor found to be significant in the multivariate linear regression analysis was assumed to be the amount of the covariant, and a covariance analysis was then

Table 5
Mean levels of dioxin in human milk associated with lactation of mother in infancy, and the result of covariance analysis[a]

Compounds	Breast-feeding $n = 108$	Mixed feeding $n = 130$	Formula $n = 53$	P
2,3,7,8-TCDD	1.65	1.51	1.39	0.056
1,2,3,7,8-PeCDD	6.52	6.26	6.11	0.398
1,2,3,4,7,8-HxCDD	2.17	2.05	1.82	0.301
1,2,3,6,7,8-HxCDD	19.19	19.07	18.63	0.888
1,2,3,7,8,9-HxCDD	3.62	3.60	3.63	0.992
1,2,3,4,6,7,8-HpCDD	11.67	10.65	9.49	0.111
OCDD	80.09	70.85	69.65	0.294
2,3,7,8-TCDF	0.80	0.74	0.73	0.653
1,2,3,7,8-PeCDF	0.54	0.50	0.52	0.706
2,3,4,7,8-PeCDF	14.32	13.31	12.60	0.017
1,2,3,4,7,8-HxCDF	4.78	4.69	4.19	0.153
1,2,3,6,7,8-HxCDF	5.83	5.98	5.27	0.274
2,3,4,6,7,8-HxCDF	3.55	3.32	3.04	0.201
1,2,3,4,6,7,8-HpCDF	2.20	2.38	2.52	0.419
PCB77	5.83	6.31	5.31	0.322
PCB126	64.14	60.25	56.09	0.236
PCB169	36.83	34.74	34.63	0.347
∑PCDDs	124.91	114.00	110.72	0.324
∑PCDFs	32.01	30.91	28.87	0.114
∑PCDDs + PCDFs	156.92	144.91	139.59	0.274
∑Co-PCBs	106.79	101.30	96.02	0.218
∑PCDD/Fs + Co-PCBs	263.71	246.21	235.61	0.165
∑PCDDs (TEQ)	7.60	7.29	7.02	0.305
∑PCDFs (TEQ)	8.70	8.18	7.67	0.021
∑PCDDs + PCDFs (TEQ)	16.31	15.46	14.69	0.071
∑Co-PCBs (TEQ)	6.78	6.38	5.96	0.231
∑PCDD/Fs + Co-PCBs (TEQ)	23.09	21.84	20.65	0.073

[a] Results are given in pg/g; n = number of samples.

done. As a result, significant differences were found for 2,3,4,7,8-PeCDF, and ∑PCDFs (TEQ) ($p < 0.05$) (Table 5). Because of the long half-lives of the PCDDs/PCDFs, usually years or tens of years (Ryan et al., 1993), body burdens can be estimated from samples collected much later than first exposure and the dose extrapolated to estimate the original exposure (Ott et al., 1993). Ryan et al. (1994) reported that there was a good correlation between weeks of breast-feeding and body burden at one year for 2,3,4,7,8-PeCDF and 1,2,3,4,7,8-HxCDF, which is similar to our results. Moreover, the following is thought to explain the higher dioxin levels in milk of those who grew up with only breast-feeding. Our 299 mothers were born in the period 1959–1979, when environmental pollution by dioxin was first noticed but no restrictions had been placed on it. There is a report that shows the data. It is the data of dioxin levels in human milk taken from mothers living in Osaka for 1973–1996 (Hori et al., 1999): the level of total dioxin in human milk in 1973 was 1920 pg/g fat (57.1 pg TEQ/g fat) for PCDDs + PCDFs + Co-PCBs, while the level of total dioxin in human milk in 1996 was 279.9 pg/g fat (24.1 pg TEQ/g fat) for PCDDs + PCDFs + Co-PCBs. Thus, dioxin levels showed a decreasing tendency from 1973, and it has been reported that the level decreased 85% (toxic equivalent: 58%) during the period of 1973–1996.

3.3. Dietary habits

By controlling other factors using a multivariate linear regression and Pearson analysis, the consumption of fish/shellfish had the greatest influence on dioxin in the body, among the dietary habits. These results are shown in Table 6. Numerous congeners were associated with the fish and shellfish index. A scattering diagram of PCB77 is shown in Fig. 2. The dioxin levels of fish or shellfish are higher than for the other food groups (Beck et al., 1989; Fürst et al., 1990), and generally, the Japanese tend to consume large amounts of fish and shellfish compared with Westerners. In the report of Toyoda et al. (1999), the dietary daily intake of PCDDs, PCDFs, and Co-PCBs as TEQs from fish and shellfish in Japan accounted for 62.4% of the total intake. It is probable

Table 6
Correlation between the dioxin levels in human milk and the fish and shellfish index ($n = 299$)[a]

Compounds	Partial	P	Pearson	P
2,3,7,8-TCDD	0.169	0.004	0.159	0.006
1,2,3,7,8-PeCDD	0.127	0.030	0.123	0.034
1,2,3,4,7,8-HxCDD	0.094	0.111	0.083	0.154
1,2,3,6,7,8-HxCDD	0.061	0.298	0.044	0.447
1,2,3,7,8,9-HxCDD	0.053	0.366	0.012	0.837
1,2,3,4,6,7,8-HpCDD	0.036	0.537	Δ 0.021	0.714
OCDD	Δ 0.051	0.387	Δ 0.082	0.159
2,3,7,8-TCDF	0.149	0.011	0.131	0.023
1,2,3,7,8-PeCDF	0.143	0.015	0.109	0.060
2,3,4,7,8-PeCDF	0.153	0.009	0.147	0.011
1,2,3,4,7,8-HxCDF	0.108	0.065	0.093	0.109
1,2,3,6,7,8-HxCDF	0.112	0.055	0.099	0.089
2,3,4,6,7,8-HxCDF	0.137	0.019	0.093	0.107
1,2,3,4,6,7,8-HpCDF	Δ 0.008	0.889	Δ 0.037	0.523
PCB77	0.180	0.002	0.164	0.004
PCB126	0.181	0.002	0.142	0.014
PCB169	0.121	0.039	0.132	0.023
∑PCDDs	Δ 0.021	0.720	Δ 0.056	0.330
∑PCDFs	0.152	0.009	0.130	0.024
∑PCDDs + PCDFs	0.005	0.934	Δ 0.032	0.583
∑Co-PCBs	0.189	0.001	0.163	0.005
∑PCDD/Fs + Co-PCBs	0.085	0.147	0.045	0.434
∑PCDDs (TEQ)	0.131	0.026	0.114	0.048
∑PCDFs (TEQ)	0.162	0.006	0.151	0.009
∑PCDDs + PCDFs (TEQ)	0.154	0.008	0.140	0.016
∑Co-PCBs (TEQ)	0.181	0.002	0.145	0.012
∑PCDD/Fs + Co-PCBs (TEQ)	0.181	0.002	0.155	0.007

[a] Partial correlation coefficient and Pearson correlation coefficient; Δ = negative.

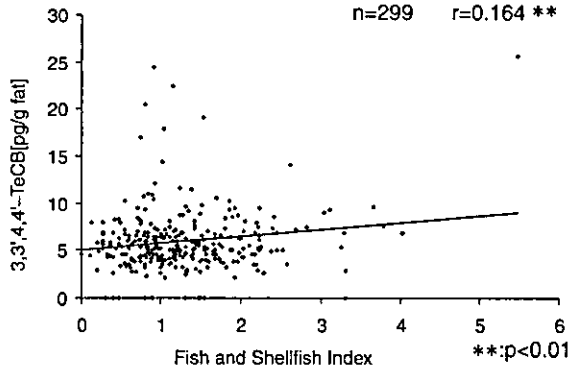

Fig. 2. 3,3',4,4'-TeCB (PCB77) levels in human milk in relation to the fish and shellfish index.

that the high intake of fish and shellfish is deeply involved in the accumulation of dioxin among the Japanese.

3.4. ∑TEQ and Co-PCBs

In our survey, the level of PCB126 was the highest after OCDD, accounting for 24.3%. Moreover, even when judging by toxicity, PCB126 was the highest after 2,3,4,7,8-PeCDF, accounting for 27.6%. Accordingly, the correlation for each congener and ∑TEQ was examined. Again controlling the factor influences by means of a multivariate analysis and Pearson analysis, the results are shown in Table 7. A strong correlation between ∑TEQ and PCB126 was found (Fig. 3). Whether PCB126 is a reliable marker to evaluate the toxicity of dioxin exposure, even considering the evidence of the scatter diagram in Fig. 3, can only be estimated. The analysis of dioxin is generally tedious and time consuming. Compared with dioxin, the level of PCB126 in human milk is high (20.4–196.2 pg/g fat; in this investigation) and measurement is easy. It is thought that the toxic evaluations of dioxin in human milk could be more efficiently done if this congener was assumed to be an indicator.

Table 7
Correlation between the dioxin levels in human milk and ∑PCDD/Fs + Co-PCBs (TEQ) ($n = 299$)[a]

Congeners	Partial	P	Pearson	P
2,3,7,8-TCDD	0.754	0.000	0.770	0.000
1,2,3,7,8-PeCDD	0.852	0.000	0.860	0.000
1,2,3,4,7,8-HxCDD	0.640	0.000	0.649	0.000
1,2,3,6,7,8-HxCDD	0.735	0.000	0.730	0.000
1,2,3,7,8,9-HxCDD	0.589	0.000	0.580	0.000
1,2,3,4,6,7,8-HpCDD	0.536	0.000	0.553	0.000
OCDD	0.376	0.000	0.353	0.000
2,3,7,8-TCDF	0.412	0.000	0.453	0.000
1,2,3,7,8-PeCDF	0.431	0.000	0.449	0.000
2,3,4,7,8-PeCDF	0.883	0.000	0.887	0.000
1,2,3,4,7,8-HxCDF	0.585	0.000	0.597	0.000
1,2,3,6,7,8-HxCDF	0.541	0.000	0.549	0.000
2,3,4,6,7,8-HxCDF	0.624	0.000	0.660	0.000
1,2,3,4,6,7,8-HpCDF	0.339	0.000	0.317	0.000
PCB77	0.275	0.000	0.333	0.000
PCB126	0.830	0.000	0.841	0.000
PCB169	0.736	0.000	0.750	0.000

[a] Partial correlation coefficient and Pearson correlation coefficient.

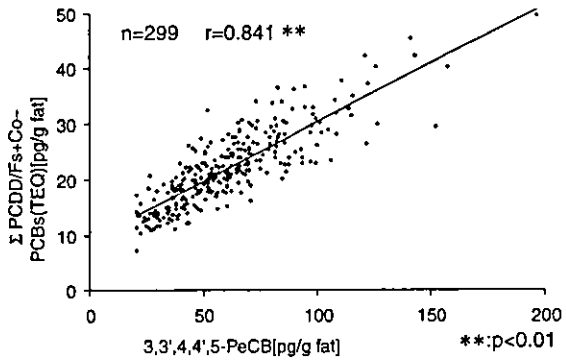

Fig. 3. 3,3′,4,4′,5-PeCB (PCB126) levels in human milk in relation to ∑PCDDs + PCDFs + Co-PCBs (TEQ).

4. Conclusions

It seems clear that it is necessary to consider age, smoking history, lactation in infancy and dietary habits, when dioxin levels are compared among individuals. It is particularly clear that the influence of smoking is significant. In our study, life-style factors—such as smoking—and the mother's body factors—such as age—influenced the dioxin levels in human milk more than environmental factors—such as area of residence.

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

Koichi Saito[1], Masahiko Ogawa[1], Mikiko Takekuma[1], Atsuko Ohmura[1], Migaku Kawaguchi[2], Rie Ito[2], Yasuhiko Matsuki[3], Hiroyuki Nakazawa[2]

[1]Dioxin Research Group, Saitama Institute of Public Health, Saitama
[2]Department of Analytical Chemistry, Hoshi University, Tokyo
[3]Institute of Food Hygiene, Japan Food Hygiene Association, Tokyo

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.

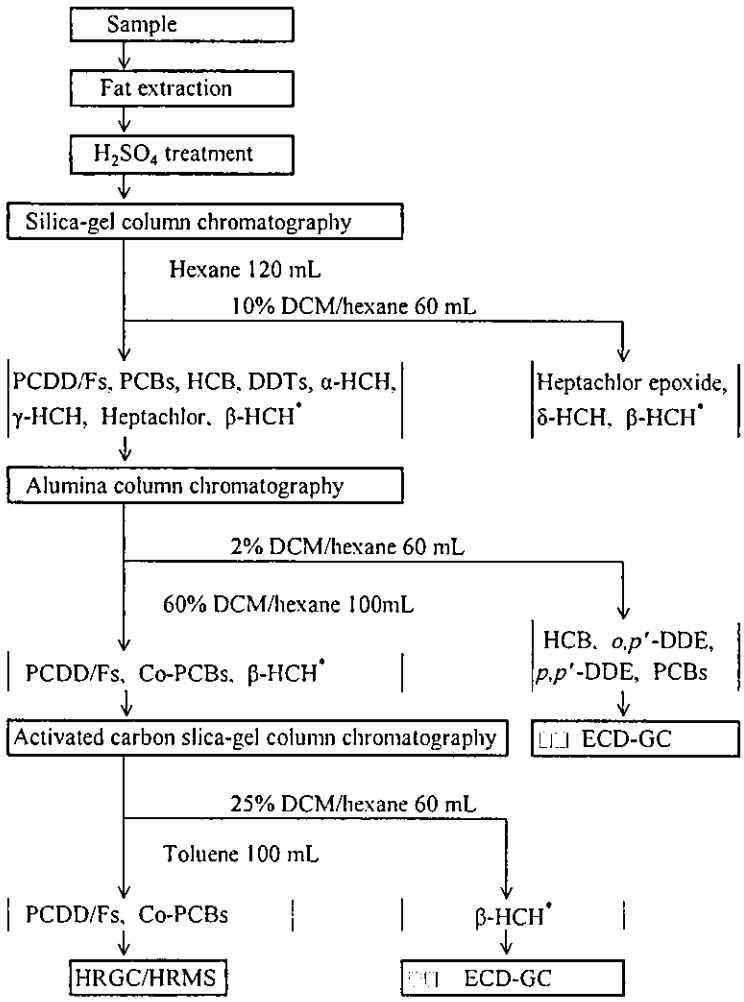

Fig. 1. Flowchart of systematic analysis of dioxins and hexachlorobenzene in human milk

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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Development of a accelerated solvent extraction and gel permeation chromatography analytical method for measuring persistent organohalogen compounds in adipose and organ tissue analysis

Koichi Saito [a], Andreas Sjödin [b,*], Courtney D. Sandau [b], Mark D. Davis [b], Hiroyuki Nakazawa [c], Yasuhiko Matsuki [d], Donald G. Patterson Jr. [b]

[a] *Dioxin Research Group, Saitama Institute of Public Health, 639-1, Kamiokubo, Saitama 338-0824, Japan*
[b] *Centers for Disease Control and Prevention, 4770 Buford Hwy, NE, Mailstop F17 Atlanta, GA 30341-3724, USA*
[c] *Department of Analytical Chemistry, Hoshi University, 2-4-41, Ebara, Shinagawa-ku, Tokyo 142-8501, Japan*
[d] *Food and Drug Safety Center, Hatano Research Institute, 729-5, Ochiai, Hadano, Kanagawa 257-8523, Japan*

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Abstract

A new analytical method has been developed for the quantification of 59 different persistent organohalogen compounds, such as polybrominated diphenyl ethers (PBDEs), polychlorinated naphthalenes (PCNs), polychlorinated biphenyls (PCBs), PCB metabolites, organochlorine pesticides (OCPs) in biological organ tissues. The optimum extraction and cleanup procedures were examined using accelerated solvent extraction (ASE), automated gel permeation chromatography (GPC) on Biobeads S-X3 and automated solid phase extraction (SPE) on silica-gel. The target compounds were divided into two fractions, non-polar compounds and more polar compounds, which in the latter fraction was subsequently methylated using diazomethane. Detection can be achieved by GC/MS in negative chemical ionization (NCI) mode. The average recoveries of the compounds spiked in swine liver, heart, kidney, and cattle adipose tissues were considered satisfactory, and it was confirmed that the method could be used in routine analysis.

Keywords: Persistent organic pollutants; Brominated flame retardants; Accelerated solvent extraction; Gel permeation chromatography; Negative chemical ionization

1. Introduction

Persistent organohalogen compounds such as dioxins, PCBs, OCPs, and brominated flame retardants (BFRs) are known environmental contaminants that are present in both the indoor and outdoor environments (Sjödin et al., 2001; Strandberg et al., 2001). Most of the organohalogen compounds were commercially produced for use in agricultural, industrial, and/or household applications, while others such as dioxins were formed unintentionally during municipal waste incineration, in other combustion and thermal processes or as by-products in the chemical industry. These organohalogen compounds are generally known to biomagnify in the fatty tissues of wildlife and humans (Vallack et al., 1998), and may influence humans through environmental background, occupational or accidental

* Corresponding author. Tel.: +1-770-488-4207; fax: +1-770-488-4546.
E-mail address: zrq4@cdc.gov (A. Sjödin).

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exposure (Sjödin et al., 1999). According to the United Nations Environmental Program, 12 chlorinated compounds: PCBs, dioxins, furans, DDT, aldrin, dieldrin, endrin, chlordane, heptachlor, hexachlorobenzene, mirex and toxaphene, have recently been defined as persistent organic pollutants (POPs), and are restricted in their manufacture and use since the agreement was adopted at the international conference held in Stockholm in 2001. PCB metabolites such as hydroxylated PCBs (HO-PCBs) and methylsulphonyl PCBs ($MeSO_2$-PCBs) have been found in blood (Bergman et al., 1994), liver and lung tissue (Brandt and Bergman, 1987). The HO-PCBs have also been reported to be potential endocrine-disrupting compounds (Kramer et al., 1997). Due to their potential health effects in humans (dermal toxicity, immunotoxicity, reproductive effects, teratogenicity, carcinogenicity and endocrine disruption), monitoring for these metabolites in humans is of significant concern (WHO, 1998).

Some PBDEs are indicated as dioxin-like chemicals that interfere with the aryl hydrocarbon receptor (Meerts et al., 1998; Chen et al., 2001). Even though the acute toxicity of most PBDEs seems to be fairy low, some have shown similar toxic effects as PCBs and dioxins. Thus, PBDEs are a potential hazard to the environment and their levels in the environment must be monitored.

Recently, considerable emphasis has been placed on the study and monitoring of selected POPs or BFRs in biological samples such as breast milk (Noren and Meironyte, 2000; Thomsen et al., 2002a), serum (Sjödin et al., 1999; Jakobsson et al., 2002; Thomsen et al., 2002b), plasma (Sjödin et al., 2000; Sandau et al., 2003), hair (Covaci and Schepens, 2001), adipose (Meneses et al., 1999; Vetter, 2001; Covaci et al., 2002), and other biological tissues (Sellstrom et al., 1993; Ikonomou et al., 2002) as well as in environmental samples (Sjödin et al., 1999) and food stuffs (Christensen, 2002). However, there have been few reports concerning the comprehensive and simultaneous determination of both POPs and BFRs in biological organ tissues.

In this research, the extraction and cleanup procedures using ASE, automated GPC and automated SPE were examined in order to develop a simple, rapid and reliable method to measure 59 organohalogen compounds such as PBDEs, tetrabromobisphenol A (TBBP-A), PCNs, PCBs, PCB metabolites, and OCPs in biological organ tissues.

2. Materials and methods

2.1. Samples

Cattle fat and swine internal organ tissues (heart, kidney, and liver), which were bought in supermarkets in the USA, were used as the biological tissue samples for method development.

2.2. Reagents

Standards for the BFRs included were: triBDE-17, triBDE-28, tetraBDE-47, tetraBDE-66, pentaBDE-100, pentaBDE-99, pentaBDE-85, hexaBDE-154, hexaBDE-153, hexaBDE-138, heptaBDE-183, and TBBP-A; the PCNs were: pentaCN-52, hexaCN-66, heptaCN-73, and octaCN-75; the PCBs were: pentaCB-92, pentaCB-101, pentaCB-119, pentaCB-118, pentaCB-105, hexaCB-151, hexaCB-144, hexaCB-134, hexaCB-158, hexaCB-128, hexaCB-157, heptaCB-191, heptaCB-190, nonaCB-208, nonaCB-207 and decaCB-209; the HO-PCBs were 4-OH-pentaCB-107, 4-OH-hexaCB-130, and 4-OH-heptaCB187; the $MeSO_2$-PCBs were 4-$MeSO_2$-pentaCB-87, 4-$MeSO_2$-hexaCB-132, and 4-$MeSO_2$-heptaCB-174; the OCPs were pentachlorobenzene, hexachlorobenzene, α-HCH, β-HCH, γ-HCH, δ-HCH, heptachlor, heptachlor epoxide, α-chlordane, γ-chlordane, 4,4′-DDE, aldrin, dieldrin, endrin, endrin aldehyde, endrin ketone, endosulfan I, endosulfan II, endosulfan sulfate, octachlorostyrene, and pentachlorophenol. These standards were purchased from Wellington Laboratories (Ont., Canada) or Cambridge Isotope Laboratories (MA, USA).

Acetone, dichloromethane (DCM), *n*-Hexane and methanol were of pesticide analysis quality and obtained from TEDIA (Fairfield, OH). Isopropanol, pesticide grade was obtained from Mallinckrodt (Paris, KY) Methyl *t*-butyl ether (MTBE) and isooctane was obtained from Aldrich (Milwaukee, WI). The water was deionized and distilled.

Hydromatrix an inert diatomaceous earth, was purchased from Varian Inc. (CA, USA), and was washed using ASE with DCM, followed by drying in an oven at 150 °C prior to use. The Hydromatrix support aids in dispersing the sample and absorbing water from different sample matrices.

Silica-gel (high-purity grade, 70–230 mesh, 60 Å) used for the SPE fractionation was from Sigma-Aldrich Inc. (USA), and was heated at 180 °C for at least 3 h.

Silca-gel columns were manually prepared by weighing 0.95 g of activated silica-gel into a 3 ml polypropylene SPE cartridge (Varian Inc., USA). Frits were pre-cleaned by sonication them for 20 min in hexane and drying prior to use. The SPE procedures such as prewashing, sample application, and elution were carried out using a RapidTrace® (Zymark, USA), an automatic preprocessing device.

Diazomethane used for the methylation of the phenolic compounds, was prepared from *N*-nitroso-*N*-methyl urea (Sigma-Aldrich, USA), according to the procedure of Blatt, 1943.

2.3. Lipid extraction (ASE method)

To 5 g of each homogenized tissue sample (1 g in the case of adipose tissue), 6–7 g of Hydromatrix (about 1.2–1.4 times heavier amount than the sample weight) was added in order to dehydrate and disperse the sample. Two pieces of cellulose filter paper, which had been previously placed on the bottom of the ASE cell (33 ml volume), and 1 g of intact Hydromatrix and the mixed sample described above were sequentially layered from the bottom of the ASE cell. In addition, the appropriate amount of Hydromatrix was packed on the top of the sample to fill the gap in the ASE cell. The ASE extraction was carried out using a Dionex ASE 200 with DCM/acetone (1:1) as the extraction solvent at a temperature of 100 °C and pressure of 1500 psi, with two extractions per sample. The extracting solvent was reduced in volume using a RapidVap® (Labconco, Missouri, USA). To the residue, 10 ml of hexane/MTBE (9:1) and 10 ml of 0.1 M H_3PO_4/1% KCl aqueous solution were added, and the mixed solution was then rocked back and forth 30 times to thoroughly mix. After centrifugation of the solution at 1500 rpm for 5 min, the organic phase (upper layer) was transferred to another test tube. This procedure was repeated again after the addition of 10 ml of hexane/MTBE(9:1) to the lower layer. After the two organic phases (upper layer) were combined, the solution was evaporated to dryness using the RapidVap®. The residue was weighed and used to calculate the percent lipid in the sample.

2.4. Lipid extraction (conventional reference method)

A standard method (Jensen et al., 1969) were used with modifications for comparison to the developed ASE based method here given in brief. To a chopped tissue sample placed in a 50 ml glass pot, 15 ml of acetone/hexane (7:2) was added, and subsequently they were homogenized using Polytron® (Kinematica GmbH, Luzern, Switzerland), a high speed homogenizer. The homogenate was transferred to a centrifugation tube, and it was then centrifuged at 3000 rpm for 10 min. After the supernatant solution was decanted into another centrifugation tube, 10 ml of hexane/MTBE (9:1) was added to the residue. The contents were stirred and mixed for 1 min using a vortex mixer. After centrifugation (3000 rpm, 10 min), the supernatant solution was combined with the solution previously separated. After the two organic phase solutions (upper layer) were combined, 10 ml of 0.1 M H_3PO_4/1% KCl was added, and the mixed solution was then rocked back and forth 30 times. After centrifugation (2000 rpm, 5 min), the supernatant solution was decanted into a 60 ml test tube (an extraction tube for ASE). To the lower layer, 10 ml of hexane/MTBE (9:1) was added, and then the rocking and centrifugation were repeated. The supernatant solution was combined with the solution previously separated, and this solution was evaporated to dryness using the RapidVap®. The residue was weighed to determine the lipid percent.

2.5. Cleanup by GPC and SPE

The lipid was dissolved in 5 ml of DCM/hexane (1:1), and then purified using gel permeation chromatography (GPC) with a Biobeads® S-X3 column (35 g dry material was packed in 55 cm×27 m i.d. glass column) with DCM/hexane as the eluting solvent at a flow rate of 5 ml/min. This GPC system was operated using an AutoPrep 2000® (OI Analytical, USA), an automated GPC system. The first 90 ml fraction of the eluant, containing the lipids, was discarded. The next 70 ml fraction was collected, and then evaporated to near dryness leaving a small amount of an oily residue. To remove the remaining trace amount of the lipid, the residue was dissolved with 0.5 ml of hexane, followed by loading onto a silica-gel SPE cartridge, which was prewashed with 6 ml of 10% methanol/DCM and then 8 ml of 5% DCM/hexane. The SPE operation was carried out by a RapidTrace®, an automatic SPE system. Two fractions were collected from the SPE cartridge, the first eluted with 8 ml of 5% DCM/hexane (Fraction-A) and the second eluted with 8 ml of 10% methanol/DCM (Fraction-B). Fraction-A contained PCBs, PBDEs, PCNs, and most of the non-polar chlorinated pesticides, and Fraction-B contained PCP, HO-PCBs, $MeSO_2$-PCBs, TBBP-A, and some more polar chlorinated pesticides. To each fraction 100 μl of iso-octane was added as a keeper. After the solvent of Fraction-A was evaporated to 100 μl, 20 μl of a syringe spike solution (^{13}C-PCB153, 516 pg/ml) was added, and the total volume was then made up to 200 μl with hexane. The compounds in fraction-A were measured by NCI-GC/MS in the selected ion monitoring (SIM) mode. Fraction-B was concentrated to about 0.5 ml, and then 3 ml of diazomethane/hexane solution was added and allowed to stand for 3 h at ambient temperature to derivatize phenolic compounds into their corresponding methyl ethers. After the excess derivatizing reagent was removed using the RapidVap to evaporate the sample to a volume of 100 μl, 20 μl of the syringe spike solution (^{13}C-PCB153, 516 pg/ml) was added, and the total volume was then made up to 200 μl with hexane. The compounds in fraction-B were measured by NCI-GC/MS in the SIM mode.

2.6. GC/MS measurement

The POPs were analyzed by GC/MS using a HP5973MSD (Agilent, USA) mass spectrometer coupled to a HP6890 gas chromatograph with a capillary

column of DB-5MS (30 m×0.25 mm i.d., 0.25 μm film thickness; J & W Scientific, USA), with helium as the carrier gas at a linear velocity of 35 cm/s. Volumes of 2 μl were injected in the splitless mode at an injector temperature of 280 °C. The GC oven temperature was programmed to run at 80 °C (held for 1 min), increased at 15 °C/min to 275 °C, and increased at 10 °C/min to 320 °C, then maintained for 5 min. The transfer line temperature of the GC/MS interface and the ion source temperature were held at 280 and 260 °C, respectively. The MS was operated in the NCI mode with methane as the reagent gas. The most abundant ions within the molecular ion clusters of each of the target compounds were recorded using the SIM mode. Quantification of the compounds was based on signals in the mass chromatograms and on comparison with the $^{13}C_{12}$-PCB153 used as a syringe spike. Other information about the quantification by GC/MS was set based on the previous paper (Sandau et al., 2003).

3. Results and discussion

3.1. Dehydration of tissue samples with hydromatrix

It is necessary to dehydrate the sample beforehand in order to extract the lipid from the biological tissues using ASE. Anhydrous sodium sulfate or Hydromatrix are usually used as a dehydrating agent for ASE extraction of environmental samples such as soil. It was difficult to dehydrate the biological tissue samples using anhydrous sodium sulfate because the samples usually contain a large amount of water. On the other hand, the Hydromatrix was able to sufficiently dehydrate the tissue samples. Even in the case of a liver sample that usually has more moisture compared to the other tissues, an amount of Hydromatrix that was 1.2–1.4 the weight of the tissue was sufficient to dry the sample. Furthermore, the mixture of the sample and the Hydromatrix was easy to pack into the ASE cell, because it consisted of free-flowing granules.

3.2. Operating conditions of ASE for lipid extraction

Since many organohalogen compounds accumulate in the lipid portion of tissues, the levels are often normalized to the lipid content in the tissues. The method related differences in the lipid yield might cause discrepancies in the results, even when the same amounts of analyte is extracted. Because lipids with a high polarity such as phospholipid are not likely to be easily extracted into organic solvent, it is necessary to determine the efficiency of the lipid extraction from biological samples (Ryan and Mills, 1997).

ASE has been reported to be a good method for the extraction of POPs and other halogenated compounds from environmental matrices, such as soil (Bjorklund et al., 2000; Hubert et al., 2000), plant (Hubert et al., 1998), and house dust (Saito et al., 2003). However, there are few reports, which examine the lipid extraction efficiency from biological tissues using ASE.

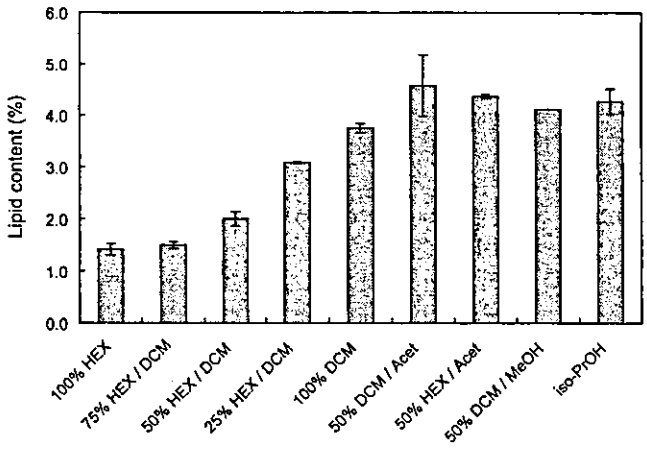

Fig. 1. Extractable lipid content determined by ASE with various solvent combinations from swine liver tissue.

The optimum extraction solvent for ASE was examined using swine liver tissue. This tissue contains much more phospholipid than the other organ tissues. At first, the mixture ratio of hexane/DCM was varied in order to examine the lipid extraction efficiency. The extraction efficiency of the lipid was increased with an increase in the DCM content of the solvent mixture, as shown in Fig. 1. When other solvent systems such as DCM/acetone, hexane/acetone, DCM/methanol, and 100% isopropanol were examined, it was found that the DCM/acetone and hexane/acetone systems produced better lipid extraction efficiencies than all the others. The optimum extraction efficiency for both extraction solvent systems was examined by changing the percent of acetone in both solvent systems. Fig. 2 shows that DCM/acetone (1:1) was the best combination for lipid extraction efficiency. Two static extraction cycles were necessary for the optimum lipid extraction efficiencies from the swine liver samples shown in Fig. 2.

3.3. Dehydration processing of ASE extract

A small amount of moisture and powder were eluted from the sample and/or the Hydromatrix during the ASE lipid extraction step. In order to remove these impurities, two different methods were evaluated. In the first method, the extract was passed through a column packed with sodium sulfate. This method gave no recovery for phenolic compounds such as HO-PCBs and PCP from the Na_2SO_4 column. The second method consisted of rocking the extract against a solution of 0.1 M H_3PO_4 in 1% NaCl (X ml). After phase separation by centrifugation the water layer was reextracted with a

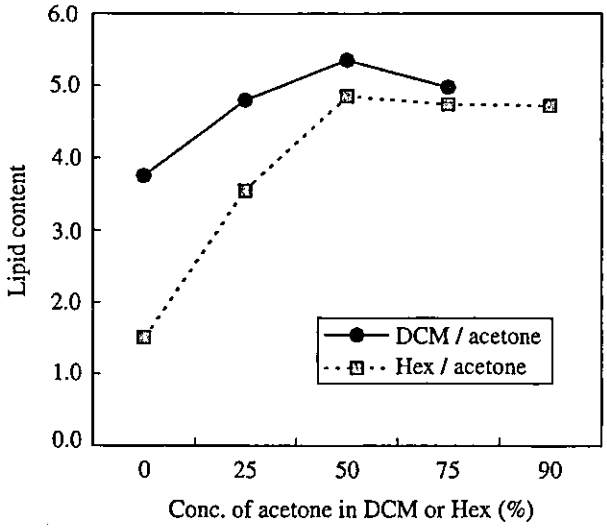

Fig. 2. Comparison of extractable lipid content by dichloromethane/acetone and hexane/acetone from swine liver tissue.

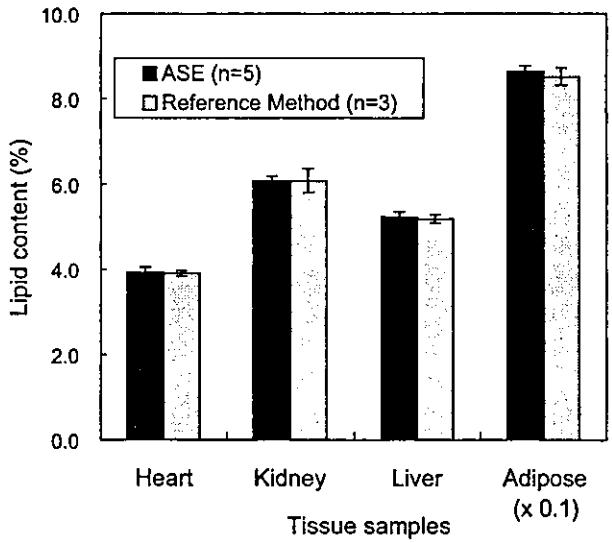

Fig. 3. Comparison of extractable lipid content by the ASE and reference method for swine heart, kidney and liver tissue and cattle adipose tissue.

solution of hexane/MTBE(9:1). The combined organic phases were evaporated to dryness for lipid weight determination. This second method gave much better recoveries of the target compounds.

3.4. Comparison of the ASE method with the reference method

A comparison of the lipid extraction efficiency of the ASE and the conventional reference method was

Table 1
Lipid removal efficiency by GPC cleanup

Sample ($n = 5$)	Lipid (g)	After GPC (g)	Removal rate (%)
Adipose (beef)	0.9022	0.0007	99.9%
Heart (pork)	0.1975	0.0011	99.4%
Kidney (pork)	0.3047	0.0017	99.4%
Liver (pork)	0.2677	0.0014	99.5%

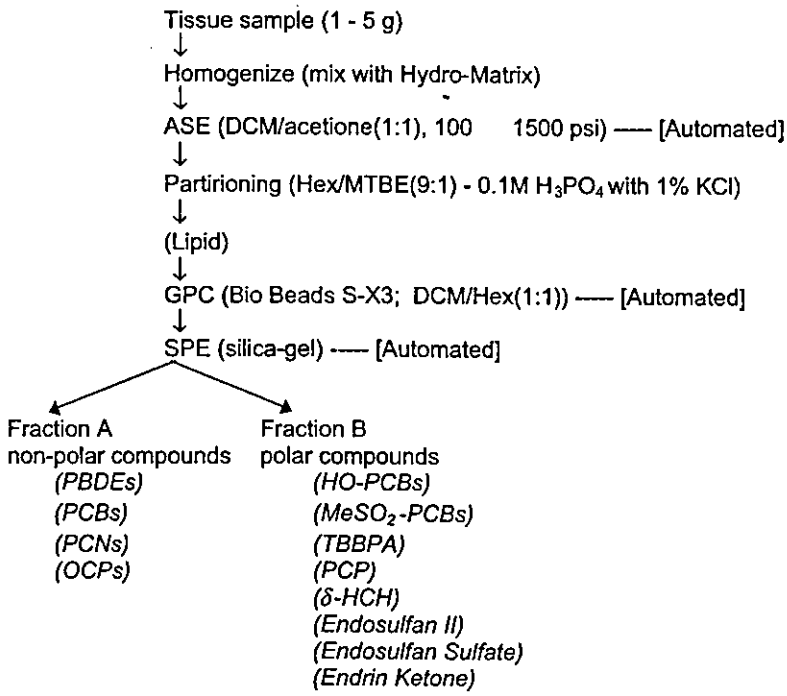

Fig. 4. Proposed analytical method scheme.

Table 2
Recoveries of representative BFRs, PCNs, PCBs, and PCB metabolites from tissue samples

Classes and individual compounds		Adipose (cattle)		Heart (swine)		Kidney (swine)		Liver (swine)	
	Spiked	5 ng/g		1 ng/g		1 ng/g		1 ng/g	
	Fraction	Mean (%)	(Std) ($n = 3$)	Mean (%)	(Std) ($n = 3$)	Mean (%)	(Std) ($n = 3$)	Mean (%)	(Std) ($n = 3$)
Lipid content (%)		86.5	(1.4)	3.88	(0.16)	6.05	(0.14)	5.23	(0.06)
PBDEs (tri-to heptaBDE)									
triBDE-17	A	88.0	(2.6)	83.5	(11)	70.9	(4.6)	74.5	(10)
triBDE-28	A	86.3	(2.3)	80.1	(11)	68.2	(5.7)	70.1	(9.6)
tetraBDE-47	A	99.8	(9.3)	93.7	(11)	75.8	(1.0)	87.9	(11)
tetraBDE-66	A	88.5	(2.0)	80.1	(9.1)	70.3	(4.9)	72.8	(8.0)
pentaBDE-100	A	94.9	(4.1)	85.8	(11)	74.1	(5.3)	77.6	(6.3)
pentaBDE-99	A	93.5	(5.4)	86.6	(7.6)	74.2	(6.0)	77.5	(6.4)
pentaBDE-85	A	103	(5.8)	91.8	(13)	80.5	(5.2)	84.0	(8.0)
hexaBDE-154	A	112	(3.8)	104	(14)	89.4	(8.3)	90.7	(7.8)
hexaBDE-153	A	115	(3.6)	106	(13)	88.2	(5.8)	92.4	(9.0)
hexaBDE-138	A	109	(5.2)	101	(13)	85.4	(7.0)	81.6	(5.9)
heptaBDE-183	A	119	(5.2)	113	(12)	95.3	(7.5)	86.9	(4.5)
TBBP-A	B	41.2	(14)	21.6	(7.0)	78.6	(15)	34.8	(14)
Polychlorinted naphtalenes (PCNs)									
pentaCN-52	A	98.3	(5.5)	91.3	(13)	77.1	(8.8)	77.8	(11)
hexaCN-66	A	69.0	(2.3)	87.3	(11)	75.3	(6.8)	76.9	(11)
heptaCN-73	A	96.1	(0.1)	92.3	(12)	77.7	(6.2)	81.2	(9.5)
octaCN-75	A	118	(4.6)	102	(13)	87.7	(9.3)	94.9	(12)
PCBs (penta-to decaCB)									
pentaCB-92	A	84.3	(2.1)	80.6	(11)	67.0	(6.1)	70.3	(9.2)
pentaCB-101	A	84.6	(3.7)	84.0	(11)	69.9	(6.1)	72.3	(9.0)
pentaCB-119	A	98.5	(14)	86.7	(21)	70.7	(5.6)	79.8	(17)
pentaCB-118	A	83.4	(1.8)	75.4	(9.5)	63.7	(4.6)	66.4	(10)
pentaCB-105	A	81.6	(1.2)	80.9	(18)	62.5	(4.3)	83.9	(11)
hexaCB-151	A	82.3	(1.4)	76.8	(10)	64.5	(5.1)	67.8	(9.3)
hexaCB-144	A	82.5	(2.3)	77.3	(10)	64.9	(5.9)	67.7	(9.9)
hexaCB-134	A	81.0	(1.6)	74.7	(9.5)	62.0	(5.2)	65.5	(11)
hexaCB-158	A	82.6	(1.0)	76.3	(10)	64.2	(4.8)	67.2	(9.7)
hexaCB-128	A	80.9	(1.4)	74.3	(10)	63.1	(4.6)	66.2	(9.3)
hexaCB-157	A	81.0	(1.8)	74.1	(9.1)	62.4	(4.9)	66.4	(8.5)
hepaCB-191	A	83.6	(1.3)	76.6	(11)	63.9	(4.4)	68.1	(7.8)
hepaCB-190	A	81.8	(1.8)	74.5	(10)	62.5	(4.7)	66.7	(8.7)
nonaCB-208	A	83.6	(1.0)	76.8	(9.8)	64.0	(4.4)	68.1	(8.8)
nonaCB-207	A	83.0	(1.8)	76.4	(10)	63.5	(4.6)	67.4	(8.7)
decaCB-209	A	81.8	(2.4)	74.9	(9.8)	62.8	(4.6)	66.3	(7.9)
Hydroxylated PCBs (HO-PCBs)									
4-HO-pentaCB107	B	64.6	(4.9)	47.4	(9.1)	68.8	(7.9)	40.4	(6.7)
4′-HO-hexaCB130	B	63.1	(5.5)	57.3	(8.2)	68.2	(3.7)	43.7	(8.7)
4-HO-heptaCB187	B	58.8	(5.7)	56.3	(9.8)	57.5	(6.2)	36.2	(5.1)
Methylsulfonated PCBs ($MeSO_2$-PCBs)									
4-$MeSO_2$-pentaCB87	B	64.2	(7.5)	106	(14)	98.8	(16)	82.9	(20)
4-$MeSO_2$-hexaCB132	B	69.4	(6.7)	87.0	(11)	87.6	(14)	73.7	(17)
4-$MeSO_2$-heptaCB174	B	75.8	(7.1)	119	(16)	73.5 ($n = 1$)		76.1	(16)

conducted using tissue samples of swine heart, kidney, liver, and cattle adipose tissue. The reference method is widely used to extract the lipids from marine animals (Jensen et al., 1969). The lipid content obtained by both

the ASE method and the reference method were quite consistent with each other as shown in Fig. 3.

3.5. GPC cleanup

A sulfuric acid decomposition method or an alkaline digestion method have been the two major methods used for the cleanup of environmental pollutants such as dioxins, PCBs, OCPs and BFRs in biological and environmental samples. However, it is well known that certain kinds of organochlorine pesticides such as dieldrin, endrin and DDTs are decomposed by these methods. We have, therefore, tried GPC as a non-destructive cleanup method. The elution profile of the 59 target compounds and the efficiency of the lipid removal were examined using a GPC column of Bio-Beads S-X3 with DCM/hexane (1:1) as the mobile phase. All of the target compounds were eluted in the fraction from 90 to 160 ml (18 to 32 min retention time). When the elution profile of the lipid extracted from swine liver was examined, the majority of lipids was eluted in the first 90 ml of the eluate (0–18 min retention time). The GPC cleanup gave an excellent efficiency for the lipid removal as shown in Table 1.

3.6. Fractionation by SPE and methylation of phenolic compounds

A silica-gel SPE procedure was used to examine the removal of a small amount of coextracted impurities that still remained in the fraction eluted from the GPC column. This procedure fractionated the non-polar compounds such as the PCBs, PBDEs, PCNs, and most of the OCPs from the more polar compounds such as the PCB metabolites (HO-PCBs, $MeSO_2$-PCBs), TBBP-A, PCP, and certain kinds of OCPs. The reasons for the fractionation step were that some phenolic compounds such as HO-PCBs and PCP required derivatization prior to the GC/MS analyses, and that the levels of the metabolites were low compared with the parent compounds.

When the elution behavior of the above mentioned target compounds during silica-gel SPE chromatography was examined, the PBDEs, PCBs, PCNs and most of the OCPs were found in the 5% DCM/hexane 8 ml Fraction-A. On the other hand, the PCB metabolites (HO-PCBs, $MeSO_2$-PCBs), TBBP-A, PCP, δ-HCH, endosulfan II, endosulfan sulfate and endrin ketone were eluted in the 10% methanol/DCM 8 ml Fraction-B. In addition, the coextracted substances that interfered

Table 3
Recoveries of representative organochlorine pesticides from tissue samples

Classes and individual compounds		Adipose (cattle)		Heart (swine)		Kidney (swine)		Liver (swine)	
	Spiked	5 ng/g		1 ng/g		1 ng/g		1 ng/g	
	Fraction	Mean (%)	(Std) ($n = 3$)	Mean (%)	(Std) ($n = 3$)	Mean (%)	(Std) ($n = 3$)	Mean (%)	(Std) ($n = 3$)
Organochlorine pesticides									
Pentachlorobenzene	A	40.9	(0.8)	50.8	(12)	48.8	(9.5)	24.3	(5.2)
Hexachlorobenzene	A	79.2	(3.0)	64.5	(16)	56.3	(14)	46.4	(8.3)
α-HCH	A	68.0	(2.2)	66.5	(13)	54.7	(9.4)	50.7	(9.9)
β-HCH	A	80.1	(4.9)	75.9	(14)	68.5	(3.3)	66.8	(17)
γ-HCH	A	88.7	(16)	88.4	(16)	72.6	(11)	63.4	(14)
δ-HCH	B	73.8	(8.0)	71.4	(18)	59.4	(5.6)	60.9	(8.4)
Heptachlor	A	79.4	(16.1)	74.2	(15)	60.2	(9.6)	46.4	(11)
Heptachlor epoxide	A	80.7	(6.2)	66.4	(9.6)	53.6	(4.8)	53.8	(8.9)
α-Chlordane	A	82.8	(2.6)	77.5	(10)	64.9	(6.3)	65.7	(10)
γ-Chlordane	A	81.7	(2.8)	81.3	(11)	68.6	(6.4)	65.4	(9.6)
4,4′-DDE	A	85.5	(2.1)	68.1	(9.5)	58.5	(4.9)	63.0	(10)
Aldrin	A	111	(20)	110	(20)	90.0	(11)	84.3	(13)
Dieldrin	A	64.6	(5.9)	43.7	(4.8)	37.9	(2.6)	36.3	(5.1)
Endrin	A	52.5	(2.6)	45.4	(5.9)	37.6	(3.5)	37.6	(6.3)
Endrin aldehyde	A	47.9	(2.3)	45.2	(6.1)	39.1	(3.3)	37.1	(5.5)
Endrin ketone	B	72.7	(6.4)	122	(38)	77.3 ($n = 1$)		88.5	(14)
Endosulfan I	A	82.7	(3.6)	78.0	(10)	65.0	(7.1)	65.0	(10)
Endosulfan II	B	79.5	(2.9)	88.5	(14)	73.3	(6.6)	79.2	(8.4)
Endosulfan sulfate	B	70.1	(6.6)	83.3	(13)	67.8	(5.5)	65.8	(11)
Octachlorostyrene	A	94.5	(7.0)	90.4	(14)	75.3	(9.2)	76.5	(11)
Pentachlorophenol	B	94.0	(11)	92.6	(23)	82.2	(12)	106	(11)