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(食品の安全性高度化推進研究事業)

# ダイオキシン類等の化学物質の食品及び生体試料検査における信頼性確保と生体曝露モニタリング法の確立に関する研究

研究成果に関する刊行物

(平成14～16年度)

## 論文

(1)

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Environmental Contamination and Toxicology

# Enzyme-Linked Immunosorbent Assay Toxicity Evaluation Method for Dioxins in Human Milk

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The contamination of food and the ecosystem by dioxins and its resultant effects on our health have been drawing much attention from the public. Thus, the investigation of human exposure to dioxins is an urgent and important task for the government. Since the Law Concerning Special Measures against Dioxins (Environmental Agency of Japan, 1999) became effective in Japan in 1999, the number of substances to be sampled and measured are expected to increase.

Conventionally, high resolution gas chromatography/mass spectrometry (HR-GC-MS) has been used for the measurement of dioxins. However, a GC-MS method has a drawback in that it requires a complicated cleanup procedure for measurement of all types of samples as well as it is very time-consuming and extremely expensive to perform. Thus, the development of a method for measuring dioxins, which is inexpensive, easy to perform, and highly sensitive, has been highly demanded by the public and the government. One of the methods that may satisfy these requirements is an enzyme-linked immunosorbent assay (ELISA), and there have been some reports on the measurement of dioxins using ELISA. However, most of the reports dealt with standard substances (Stanker et al. 1987; Sugawara et al. 1998; Carlson et al. 1998), fly ash (Harrison et al. 1997; Zennegg et al. 1998; Li et al. 1999), soil (Harrison et al. 1997; Harrison et al. 1998; Harrison et al. 1999), sediment (Li et al. 1999), and chimney soot (Zennegg et al. 1999), which contained dioxins in high concentrations. There has been no report on a practical assay that can deal with biological samples containing dioxins in extremely low concentrations such as human milk and blood. The conventional ELISA has been considered as a simple screening method, but less reliable compared with the GC-MS method.

In this report, we constructed a basic strategy for the development of a toxicity evaluation method for dioxins in human milk by ELISA. Also, we selected an optimal isomer to be detected, which is the key factor in the development of this ELISA method. Furthermore, to make the proposed ELISA method compatible with the conventional GC-MS method, it was tried to use the same preprocessing operation, and it was also examined if an extremely complicated conventional preprocessing operation could be simplified. For these objectives, we made a

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prototype of a three-layer $H_2SO_4$/silica-gel cartridge that took the place of a multi-layer silica-gel column, and also evaluated its applicability for practical use.

## METHODS AND MATERIALS

All of the dioxin standards were from Wellington Laboratories and were diluted with decane to the appropriate concentrations. Most of the organic solvents such as hexane, dichloromethane, toluene and diethylether were of dioxin analysis quality from Kanto Kagaku (Tokyo, Japan) or Wako Pure Chemicals (Osaka, Japan). Silica-gel impregnated activated carbon, and 44 % $H_2SO_4$/silica-gel were from Wako Pure Chemicals. The prototype of the three-layer $H_2SO_4$/silica-gel column packed in a disposable cartridge tube (inside diameter 15 mm x length 75 mm; made of polypropylene) was made by a special request to Supelco (USA). The cartridge was washed with 40 ml of hexane prior to use.
Three-layer $H_2SO_4$/silica-gel column: one g silica-gel, 2 g 44% $H_2SO_4$/silica-gel, 0.5 g silica-gel, and 1 g $Na_2SO_4$ were sequentially layered from the lower layer into a glass column having an inside diameter of 1cm by slurry packing with hexane. The column was then washed with 40 ml of hexane prior to use.
Human milk samples, at about 30 days after birth, were collected from 100 Japanese primiparae whose mean age was 28.5 years old.

As for the analysis of dioxin in human milk by the GC-MS method, ten kinds of stable isotopes of the 2,3,7,8-substituted congeners of PCDDs and PCDFs, and $^{13}C_{12}$-1,2,3,4-TCDF were added as surrogate after the fat was extracted from the human milk according to the Official Methods of Analysis of the AOAC International (Association of Official Analytical Chemists, 1995). The fat was then subjected to concentrated sulfuric acid washing and to various chromatographies (silica-gel, alumina, and activated carbon silica-gel) as the cleanup operation, followed by the GC-MS measurement. The PCDD/Fs were analyzed by HR-MS using a JEOL JMS-700 MStation mass spectrometer coupled to a HP-6890 HR-GC with a capillary column of DB-17HT (30 m x 0.25mm i.d., film thickness 0.15 um; J & W Scientific) for the isomer specific separation. The GC program was as follows: 150°C for 1 min, increased 20°C /min to 220°C and subsequently 4°C /min to 280°C, then maintained for 11.5 min. Helium was used as the carrier gas. The injector temperature was 280°C and the GC-MS interface temperature was held at 280°C. The MS was operated in the selected ion monitoring mode with a mass resolution of 10000, and the electron impact ionization energy was 38 eV, with an ion source temperature of 260°C. Quantification was done by the isotopic dilution method, i.e. the PCDD/Fs congeners were quantified by comparison with their respective reference $^{13}C_{12}$-labeled standards in the following two ways; in one way, one kind of stable isotope in each congener of the PCDD/Fs was used as the internal standard, and in the other method, $^{13}C_{12}$-1,2,3,4-TCDF was for the tetra to hexa-CDD/Fs, and $^{13}C_{12}$-OCDD was for the hepta to octa-CDD/Fs, and then the PCDD/Fs concentrations were calculated on the fat basis. The toxicity equivalent quantity

(TEQ) was calculated using WHO-TEF(1998).

As for ELISA, the fat was extracted from human milk, and surrogates were added to the fat, and then 40 ml of 1N-KOH/ethanol was added and stirred. The solution was then allowed to stand at room temperature overnight. The alkaline solution was diluted with 40 ml of water, followed by liquid-liquid extraction with 40 ml of hexane (twice). After the hexane layer was dehydrated and concentrated, the extracted material was processed with the three-layer $H_2SO_4$/silica-gel column using 40 ml of hexane as the eluent. The eluate was split equally into two aliquots; one was for ELISA and the other was for the GC-MS analysis. The eluate for ELISA was dried by a nitrogen stream, and the residue was re-dissolved into 60 μl of MeOH-DMSO (1:1) with 100 ppm Triton X-100. The ELISA was done according to the method previously reported by Sugawara et al. (Sugawara et al. 1998). The antiserum used was the same one that Sugawara et al. (Sugawara et al. 1998) had already developed and reported.

## RESULTS AND DISCUSSION

We consider that an ELISA suitable for the measurement of dioxins in biological samples should satisfy the following requirements:

1) The ELISA should be able to evaluate the toxicity instead of providing only a simple screening method,

2) The ELISA should share a common pre-treatment procedure with the conventional GC-MS method so that valuable biological samples are effectively used, and also be compatible with the GC-MS. Moreover, the ELISA should not require a complicated pre-treatment procedure.

3) The data obtained from actual samples (human milk) by the ELISA should be highly correlated with those obtained by the GC-MS method.

In this report, we will focus on 1) and 2), and 3) will be reported in another paper.

In this study, we constructed a basic strategy for the development of a highly sensitive and simple method for the measurement of dioxins in human milk by ELISA and for the evaluation of the method as a toxicity evaluation method. We presented ELISA as a method for directly evaluating toxicity and not as a simple screening method. This is a new approach to ELISA. That is, after the fat is extracted from human milk, a simple pre-treatment is performed on the fat, and then half of the pre-treated fat as a testing solution is immediately evaluated for its toxicity by ELISA. If the concentration of each isomer needs to be measured or the TEQ needs to be confirmed, the results obtained by ELISA are feed-back, and the remaining half of the sample is subjected to cleanup, followed by measurement using the GC-MS method. This method is considered to increase the additional value of the data obtained by ELISA and enable ELISA to be compatible with the GC-MS method.

We determined the isomer to be targeted for the construction of a toxicity evaluation method. Since ELISA has the property that it can perform a specific measurement only on a specific chemical substance, it cannot separate or

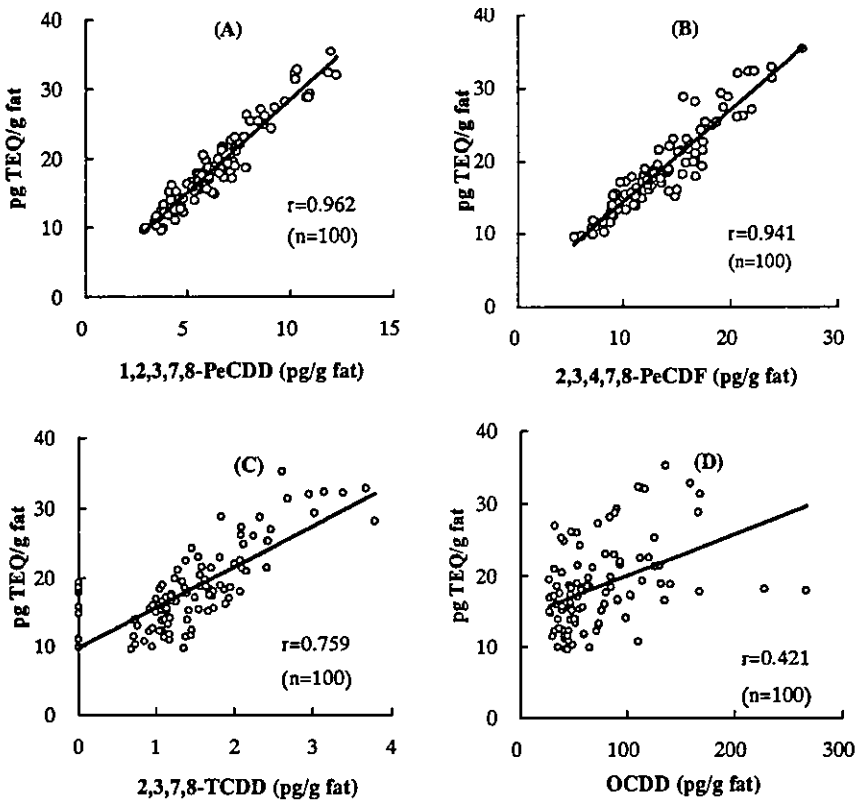


**Figure 1.** Correlation between TEQ of PCDD/Fs and each isomer ;
(A) 1,2,3,7,8-PeCDD, (B) 2,3,4,7,8-PeCDF, (C) 2,3,7,8-TCDD, and (D) OCDD.

determine different isomers unlike the GC-MS method. However, by using an antibody that is highly responsive to an isomer having a high toxicity and poorly responsive to an isomer having a low toxicity, the results by ELISA provide not only the detection values but also the degrees of toxicity. In order to develop a toxicity evaluation method by ELISA based on this concept, it was necessary to examine the relationship between the various isomers detected from biological samples and the TEQs. Accordingly, 100 samples of human milk were analyzed by the GC-MS method to find isomers that are highly correlated with the TEQ and detected in large amounts. As shown in Figure 1, either isomer of 1,2,3,7,8-PeCDD or 2,3,4,7,8-PeCDF was found to highly correlate with the TEQ (r=0.962, r=0.941, respectively). The most likely reason for this is that both have high toxicity equivalent factors (TEF) and are detected in relatively large amounts. In contrast, a typical dioxin isomer, 2,3,7,8-TCDD, was found not to correlate as highly with the TEQ (r=0.759) as the above two isomers. This is probably due to the smaller amount of detected 2,3,7,8-TCDD. OCDD had the highest concentration among the isomers detected from human milk, but showed a low correlation with the TEQ (r=0.421) because of its extremely low TEF of 0.0001.

Based on these results, the optimal isomers to be targeted for measurement by ELISA were determined to be 1,2,3,7,8-PeCDD and 2,3,4,7,8-PeCDF.
The ELISA used in this investigation showed that 1,2,3,7,8-PeCDD has almost the same cross-reactivity to 2,3,7,8-TCDD (Sugawara et al., 1998). However, the other isomers had a low reactivity in the ELISA. Therefore, it is considered that this ELISA detected the total amount of 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD. These results show that the ELISA worked advantageously for the toxicity evaluation based on the TEQ, since the TEF of both 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD is 1 according to the latest WHO-TEF (1998).

The conventional GC-MS method for dioxin analysis needs appropriate surrogates spiked prior to sample cleanup. However, as the surrogates usually used are 2,3,7,8-substituted $^{13}C_{12}$-congeners, which react with an antibody used for ELISA, the test solution prepared for the GC-MS method cannot be applied to ELISA. Thus, in this study, we decided to examine a surrogate that does not cause a cross reaction with ELISA, and that also enables a GC-MS method to measure the isomers with good accuracy. In order to achieve this objective, the following requirements should be satisfied.

1) The cross reactivity in ELISA should be extremely small.

2) As the conventional GC-MS method separately measures two groups of congeners; one consists of 4-6CDD/Fs and the other consists of 7-8CDD/Fs, the surrogate should be made to enter by one kind in each group.

3) Quantification values measured by the selected surrogate should agree with those measured by a conventional method.

The antibody used in this study showed a tendency to strongly react with congeners of high TEFs (Sugawara et al., 1998). Therefore, we selected $^{13}C_{12}$-1,2,3,4-TCDF for the 4-6CDD/Fs measurement and $^{13}C_{12}$-OCDD for the 7,8CDD/Fs measurement as surrogates that meet these requirements. The TEFs of these two congeners are 0 and 0.0001, respectively. The two congeners also showed less cross-reactivity in the ELISA we used. In general, we know that 4CDD/Fs and 8CDD/F are eluted from the column chromatographies, such as silica-gel, alumina, and activated carbon silica-gel, in the order first and last, respectively, or possibly in the opposite order if the chromatography packing material is different, when the chromatography is carried out as a conventional sample cleanup operation. Therefore, if good recoveries of the surrogates of 4CDD/Fs and 8CDD/F are confirmed, it is expected that the 5-7CDD/Fs are also collected in sufficient amounts.
As for requirement No.3, we made a comparative study between the use of ten kinds of surrogates and the two surrogates selected above. For that, we added the ten kinds of surrogates (one kind for each congener) and the two surrogates selected above to 19 samples of human milk at the same time and did a series of analyses. As shown in Table 1, the resulting correspondence between the values for each congener was excellent; furthermore, excellent correlation ($r=0.956$) was also obtained between both for the TEQ (Figure2). Based on these results, using $^{13}C_{12}$-1, 2, 3, 4- TCDF and $^{13}C_{12}$-OCDD as surrogates makes a common

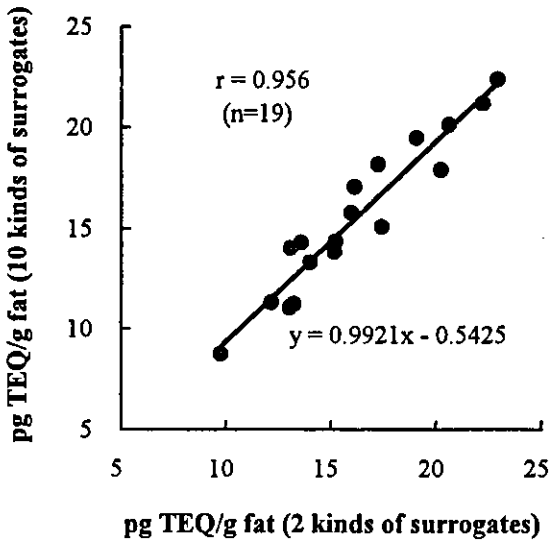


**Figure 2.** Correlation between TEQ data calculated with 2 kinds of surrogates and 10 kinds of surrogates.

**Table 1.** Correlation between PCDD/Fs data calculated by 2 kinds of surrogates and by 10 kinds of surrogates

| | 2 kinds | 10 kinds | Correlation Coefficient |
|---|---|---|---|
| Dioxin isomers | Mean (pg/g fat) | | (n=19) |
| 2,3,7,8-TCDD | 1.6 | 1.5 | 0.982 |
| 1,2,3,7,8-PeCDD | 10.0 | 7.3 | 0.958 |
| 1,2,3,4,7,8-HxCDD | 2.4 | 2.3 | 0.977 |
| 1,2,3,6,7,8-HxCDD | 19.2 | 18.5 | 0.872 |
| 1,2,3,7,8,9-HxCDD | 3.9 | 3.9 | 0.985 |
| 1,2,3,4,6,7,8-HpCDD | 11.3 | 9.4 | 0.936 |
| OCDD | 73.2 | 75.0 | 1.000 |
| 2,3,7,8-TCDF | 0.4 | 0.7 | 0.991 |
| 1,2,3,7,8-PeCDF | 0.6 | 0.8 | 0.996 |
| 2,3,4,7,8-PeCDF | 10.4 | 13.6 | 0.955 |
| 1,2,3,4,7,8-HxCDF | 4.5 | 4.6 | 0.978 |
| 1,2,3,6,7,8-HxCDF | 5.3 | 5.5 | 0.960 |
| 2,3,4,6,7,8-HxCDF | 3.4 | 3.6 | 0.969 |
| 1,2,3,7,8,9-HxCDF | 0.3 | 0.2 | 0.997 |
| 1,2,3,4,6,7,8-HpCDF | 3.3 | 3.0 | 0.932 |
| 1,2,3,4,7,8,9-HpCDF | ND | ND | — |
| OCDF | 0.1 | 0.1 | 1.000 |

preprocessing operation for the GC-MS and ELISA methods possible.
Simplifying the preprocessing operation was studied, because conventional preprocessing operations such as sulfuric acid washing and various chromatographies for dioxin analysis of human milk require a complicated cleanup which are very time consuming. Particularly, the sulfuric acid washing has a difficulty in handling concentrated sulfuric acid and also has a problem in treating the waste fluid. Therefore, the alkali decomposition and the multi-layer silica-gel column processing method were employed as a preprocessing operation for ELISA. In this study, we examined a technique by which a multi-layer silica-gel column, which is important in a cleanup operation, can be simplified.

A conventional multi-layer silica-gel column is prepared by accumulating 7 layers with 5 kinds of packing materials, and also requires relatively large amounts of materials. In addition, the packing operation of the column is extremely complex and requires skill to accomplish. We then examined a way of omitting some of the packing materials used in the multi-layer column and reducing the amounts of the packing materials. It was considered that impurities were significantly excluded before the multi-layer silica-gel column treatment because the fat extracted from human milk was processed by decomposition in alkali. Therefore, it has been understood that a total amount of 1/5 or less of the packing materials is sufficient. In addition, we sequentially removed several of the accumulated packing materials, and examined the influence on the GC-MS chromatograms and on the recoveries of the surrogates. As a result, it has been shown that neither the silica-gel impregnated silver nitrate nor the silica-gel impregnated potassium hydroxide was necessary. Moreover, either 22% or 44% $H_2SO_4$/silica-gel was sufficient. On the other hand, impurities such as pigment and relatively polar substances from the sample still existed in the hexane extract after the alkaline treatment. Therefore, carbonization occurred in the upper part of the column as the impurities reacted with the sulfuric acid when this extract came in contact with the $H_2SO_4$/silica-gel. The impurities with a relatively large polarity of these sample origins were then adsorbed by the silica-gel by accumulating a small amount of silica-gel in the upper part of the $H_2SO_4$/silica-gel. This accumulation was able to considerably reduce the load of the $H_2SO_4$/silica-gel. On the other hand, the coexisting material newly generated by the $H_2SO_4$/silica-gel processing was occasionally eluted from the column and was mixed in the dioxin fraction. When another silica-gel layer was accumulated in the lower part of the $H_2SO_4$/silica-gel, the above-mentioned coexisting material was removed. On the basis of these results, it has been shown that the column having three layers with two kinds of packing materials is sufficient for cleanup. To remove moisture in the sample, a small amount of sodium sulfate is usually used, but it is not always necessary because moisture in the sample is dehydrated before the column chromatography.
In this study, the performance of the prototype, which was filled with the three-layer $H_2SO_4$/silica-gel in the above-mentioned disposable cartridge for saving labor during the packing operation, was also evaluated. As a result, the

elution behavior of dioxins, the recoveries of the surrogates, and the chromatograms of real samples were similar to those when the above-mentioned three-layer $H_2SO_4$/silica-gel column was used. In addition, it was confirmed that there was no problem during practical use. Accordingly, it has been shown that using the simplified cartridge column developed in this research makes a prompt analysis possible.

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# Extraction and cleanup methods of dioxins in house dust from two cities in Japan using accelerated solvent extraction and a disposable multi-layer silica-gel cartridge

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**Abstract**

A simple and rapid method for the extraction and cleanup of dioxins from house dust was developed using an accelerated solvent extraction (ASE) method and a multi-layer silica-gel cartridge. It was found that the WHO-TEQ levels of dioxins extracted from the house dust obtained by both a conventional soxhlet extraction and the ASE were almost equal, when the data obtained by both methods were compared. As for the cleanup method, a multi-layer silica-gel cartridge yielded higher dioxin recoveries than the alkaline digestion method. The average values of the dioxins in house dust from Kumagaya city and Sendai city in Japan (Sendai city is bigger than Kumagaya city with respect to the population and industry), were 15.6 pg TEQ/g (8.6–26.0 pg TEQ/g, $n = 5$, Kumagaya city) and 16.0 pg TEQ/g (5.9–30.5 pg TEQ/g, $n = 5$, Sendai city), respectively.
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*Keywords:* PCDDs; PCDFs; House dust; GC/MS; Multi-layer silica-gel cartridge

## 1. Introduction

The dioxins unintentionally generated by waste incineration are polluting the ecosystem and food supply. This has lead to the inexposure to humans in the food chain; in addition, there is a concern about the transmission of this pollutant from mothers to the next generation through human milk. Based on current research, it has been clarified that the intake of dioxins from food accounts for 90% or more as the exposure route of dioxins to humans, and that the proportion of exposure to dioxin from the environment such as the atmosphere and the soil is low. However, the exposure due to indoor pollution has yet to be completely examined. In general, young generations such as newborn babies and infants mainly spend their life indoors. Moreover, there is a pressing need to investigate the inexposure influence from house dust because they live in the space near the floor. However, there have been few reports concerning house dust polluted by dioxins (Wittsiepe et al., 1997), PCB (Seidal et al., 1996; Chuang et al., 1998; Vorhees et al., 1999), pesticides (Reighard and Olesik, 1997; Berger-Preiss et al., 1997; Colt et al., 1998) and other

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persistent organic pollutants such as polycyclic aromatic hydrocarbons (Chuang et al., 1995; Monserrate and Olesik, 1997). We then did a comparative study using soxhlet extraction and accelerated solvent extraction (ASE) as the extraction methods of dioxins from house dust. In addition, we also made a comparative study of the alkaline digestion method and the multi-layer silica-gel cartridge method as cleanup operations. Moreover, the pollution levels of dioxins in house dust from two districts ($n = 10$) in Japan were clarified.

## 2. Materials and methods

### 2.1. Materials and chemicals

All of the dioxin standards were from Wellington Laboratories (USA) and were diluted with decane to the appropriate concentrations. The surrogates we used for the PCDD/F analyses were $^{13}$C-2,3,7,8-TCDD, $^{13}$C-1,2,3,7,8-PeCDD, $^{13}$C-1,2,3,6,7,8-HxCDD, $^{13}$C-1,2,3,4,6,7,8-HpCDD, $^{13}$C-OCDD, $^{13}$C-2,3,7,8-TCDF, $^{13}$C-1,2,3,7,8-PeCDF, $^{13}$C-1,2,3,4,7,8-HxCDF, $^{13}$C-1,2,3,4,6,7,8-HpCDF and $^{13}$C-OCDF. The surrogates for the non-ortho PCB analyses were $^{13}$C-TeCB (#77), $^{13}$C-PeCB (#126) and $^{13}$C-HxCB (#156), and those for the mono-ortho PCB analyses were $^{13}$C-PeCB (#123), $^{13}$C-PeCB (#118), $^{13}$C-PeCB (#114), $^{13}$C-PeCB (#105), $^{13}$C-HxCB (#167), $^{13}$C-HxCB (#156), $^{13}$C-HxCB (#157) and $^{13}$C-HpCB (#189). The internal standards for the syringe spike for the PCDD/F recovery check were $^{13}$C-1,2,3,4-TCDD and $^{13}$C-1,2,3,4,7,8,9-HpCDF, and that for the non-ortho PCBs was $^{13}$C-TeCB (#81), while those for the mono-ortho PCBs were $^{13}$C-PeCB (#101), $^{13}$C-HxCB (#138) and $^{13}$C-HpCB (#178). Most of the organic solvents such as hexane, acetone, dichloromethane, toluene, diethylether and ethanol were of dioxin analysis quality from Kanto Kagaku (Tokyo, Japan) or Wako Pure Chemicals (Osaka, Japan). Decane was of special quality grade and was redistilled prior to use. Silica-gel impregnated activated carbon was from Wako Pure Chemicals. An activated carbon column was prepared as follows: 0.5 g of sodium sulfate and 0.5 g of the carbon silica-gel were sequentially accumulated in a glass column (20 cm × 0.8 cm i.d.), and a slight amount of sodium sulfate (ca. 0.2 g) was gently placed on the top of the carbon silica-gel.

A three-layer $H_2SO_4$/silica-gel column containing 1 g silica-gel, 2 g 44% $H_2SO_4$/silica-gel and 0.5 g silica-gel were sequentially layered from the bottom into a disposable polypropylene cartridge tube (75 mm × 15 mm i.d.). It was made after a special request to Supelco (USA).

The multi-layer $H_2SO_4$/silica-gel column packed in a disposable cartridge tube was from GL Sciences Inc. (Tokyo, Japan). It is made of 0.9 g silica-gel, 3 g 2% KOH/silica-gel, 0.9 g silica-gel, 4.5 g 44% $H_2SO_4$/silica-gel, 22% $H_2SO_4$/silica-gel, 0.9 g silica-gel, 10% $AgNO_3$/silica-gel and 6 g sodium sulfate. The cartridge was washed with 100 ml of hexane prior to use. The water was deionized and distilled, and also washed with hexane. All the other chemicals were of PCB analysis quality grade or special quality grade, and used without further purification.

1 M KOH/ethanol: 56 g of KOH was dissolved in 100 ml of water, followed by diluting with ethanol to 1 l.

### 2.2. Extraction (soxhlet extraction and ASE)

The house dust was collected from five residential homes in each of the two districts (Kumagaya city and Sendai city) in Japan. All the dust samples were collected from the dust bags of vacuum cleaners, and were passed through two kinds of sieves (mesh size: 1 mm and 75 μm). The comparatively big solids and minute powders were removed. The dust samples having a particle size of 75 μm–1 mm were used for the analysis. Each sample ($n = 5$) from Sendai city was divided into two portions for the soxhlet extraction and ASE. Five gram samples were used for the soxhlet extraction and ASE. The soxhlet extraction was carried out for 16 h with toluene as the extraction solvent. On the other hand, the ASE extraction was carried out using Dionex ASE 200 with toluene as the extraction solvent at a temperature of 150 °C and pressure of 2000 psi, with three extracting cycles per sample. Each extracted material was concentrated with a rotary evaporator in order to remove the toluene, and the resulting fatty residue was obtained. The obtained residue was considered the amount of lipid in the house dust.

### 2.3. Cleanup procedure

#### 2.3.1. Alkaline digestion

According to the conventional method, the stable isotopes of the dioxins ($^{13}C_{12}$-PCDD/Fs: 100 pg, non-ortho PCBs: 500 pg, mono-ortho PCBs: 2000 pg) were added as dioxin surrogates after the lipid was extracted from the samples. Forty milliliters of 1 M KOH/ethanol was then added. It was stirred, and the solution was then allowed to stand at room temperature overnight. The alkaline solution was diluted with 40 ml of water, followed by two liquid–liquid extractions with 40 ml of hexane. After the hexane layer was dehydrated and concentrated, the extracted material was processed using a three-layer $H_2SO_4$/silica-gel cartridge, which we developed in a previous study (Saito et al., 2003), followed by activated carbon silica-gel column chromatography. The mono-ortho PCBs fraction was obtained by eluting with 40 ml of 25% dichloromethane/hexane, and the

fraction of the PCDD/Fs and non-ortho PCBs was eluted using 150 ml of toluene.

*2.3.2. Multi-layer silica-gel cartridge*

The extracted lipid was dissolved in a small amount of hexane, the surrogates were added to the lipid, and then charged into a pre-packed multi-layer silica-gel cartridge previously well-washed with hexane. After 150 ml of hexane was added for elution, the eluate was concentrated, followed by activated carbon silica-gel column chromatography.

*2.4. GC/MS measurement*

The PCDD/Fs were analyzed by HR-GC/MS using a JEOL JMS-700 mass spectrometer equipped with a capillary column of CP-SIL88 (30 m × 0.25 mm i.d., 0.1 μm film thickness) for the 4–6CDD/Fs, DB-17HT (30 m × 0.25 mm i.d., 0.15 μm film thickness) for the 7–8CDD/Fs and non-ortho Co-PCBs, and DB-5MS (30 m × 0.25 mm i.d., 0.25 m film thickness) for the mono-ortho PCBs, with helium as the carrier gas at a linear velocity of 35 cm/s 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, for the DB-17HT and the DB-5MS, and 120 °C (1 min) to 180 °C (0 min) at 20 °C/min and subsequently at 2 °C/min to 250 °C, then maintained for 20 min at 250 °C, for the CP-SIL88. The injector temperature was 280 °C and the GC/MS interface temperature was held 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 PCDD/Fs, non-ortho PCBs and mono-ortho PCBs were quantified using a molecular ion (M), M + 2 ion or M + 4 ion. The detection limits (pg/g) for the respective analytes were as follows: 0.2 for 4–5CDD/Fs, 0.5 for 6–7CDD/Fs, 1 for OCDD/F, non-ortho PCBs and mono-ortho PCBs. The toxic equivalent quantity (TEQ) was calculated using WHO-TEF, 1998 (Van den Berg et al., 1998).

## 3. Results and discussion

*3.1. Extraction procedure*

House dust is composed of complicated matrices, which are of outdoor origin and of indoor origin. Examples of the former include atmospheric dust particles, soil, etc., which have been carried by the wind or clothes. The latter are fiber rubbish from indoor carpets or clothes, dead skin, dirt, hair, etc., which are of animal or human origin. We made a comparative study of the extraction efficiency of the lipid and the dioxin isomer concentration as indices for the soxhlet extraction method and the ASE method.

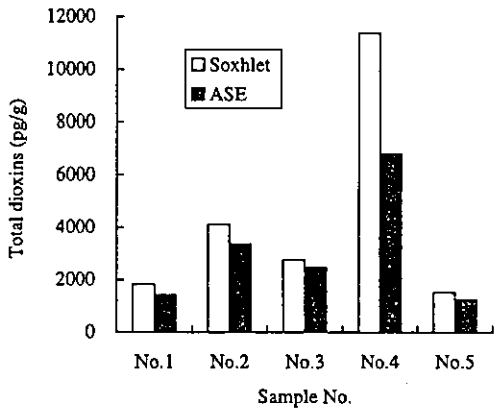


Fig. 1. Comparison of total dioxin contents obtained from the soxhlet extraction and ASE methods.

Five samples were divided half, and each sample was extracted using the soxhlet extraction and ASE methods. Both methods yielded approximately the same lipid content, i.e., about 6% for each sample. These values are very similar to the values reported by Johanna and van Bronswijk (1981) for the lipid content in house dust. It is an interesting fact that the lipid content rate in house dust of residential house origins that are not mutually related indicated almost the same value. Fig. 1 shows the total dioxin content (net value) that is composed of the 29 kinds of dioxin isomers having TEF, and Fig. 2 shows their WHO-TEQ. Although the dioxin level (net value), which had been obtained by the ASE method indicated slightly lower values, compared with those obtained by the soxhlet method, the WHO-TEQ levels obtained by the ASE method was almost equal to those

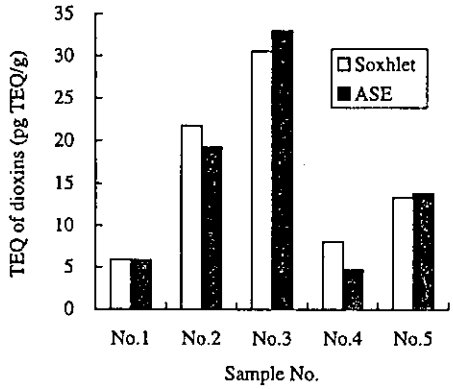


Fig. 2. Comparison of dioxin WHO-TEQ obtained from the soxhlet extraction and ASE methods.

obtained by the soxhlet extraction. When the content of each dioxin isomer was examined in detail, the low chlorinated isomers that significantly contributed to the WHO-TEQ value showed approximately the same level. The OCDD content (net value) was the highest among the dioxin isomers. The soxhlet extraction yielded a higher OCDD content than the ASE method. It appears that this difference is reflected in Figs. 1 and 2. As for the pattern of each isomer, the house dust showed the patterns of no biological origins such as human milk and blood, etc., but environmental origins such as the atmosphere or soil. These results suggested that the dioxins in house dust significantly depended on environmental influences such as the atmosphere or soil, which had entered the residential house from the outdoors. Based on these results, it was understood that the soxhlet extraction method was better than the ASE method in order to completely extract the OCDD. However, the dioxin level for human risk assessment is usually discussed in terms of its WHO-TEQ values. Accordingly, the ASE method is preferable as a practical method for the dioxin extraction from house dust, because the ASE extraction is more rapid than the soxhlet extraction, and it also yields almost the same WHO-TEQ values as the soxhlet method.

In sample no. 4, a big difference was observed between the soxhlet extraction method and the ASE method (Figs. 1 and 2). It was postulated that the lipid extraction was insufficient during the ASE. When ASE was carried out using sample no. 4, the total extract was not obtained after the second cycle extraction. The percentage of lipid in sample no. 4 extracted by ASE was 4.4%, whereas that by the soxhlet was 6.0%. A conceivable reason for this difference is as follows: since the moisture in the sample evaporated in the cell, the pressure exceeded 2000 psi, and consequently the toluene was not sufficiently replenished.

### 3.2. Simple cleanup using a multi-layer silica-gel cartridge

A cleanup method for the analysis of human milk, in which an alkaline digestion step and a three-layer $H_2SO_4$/silica-gel cartridge were used, was reported in a previous paper (Saito et al., 2003). Although an excellent cleanup effect can be obtained by employing this method, it requires an overnight alkaline digestion treatment (about 12 h) and a liquid–liquid distribution extraction using hexane. When the lipid extracted from house dust was directly applied to the "three-layer $H_2SO_4$/silica-gel cartridge", the purification resulted in insufficient purification due to the overload of impurities. However, the three-layer $H_2SO_4$/silica-gel cartridge was applicable if a sample was applied after the alkaline digestion step. On the other hand, a conventional multi-layer column seemed to have the ability to clean up the lipid without the alkaline digestion step. In this study, we tried to develop a simpler cleanup method that allows the extracted lipid to be directly treated using a multi-layer silica-gel column without subjecting it to the alkaline digestion step. There used to be a drawback to this in that the preparation of a multi-layer silica-gel column required complicated procedures, however, a pre-packed multi-layer silica-gel in a disposable cartridge has now become available.

On the other hand, we processed other house dust samples using the above-mentioned alkaline digestion method in order to compare them with the multi-layer silica-gel cartridge method. We studied the recoveries of the dioxin surrogates and the influence of coexisting impurities on the GC/MS chromatograms. As a result, although the recoveries of the dioxin surrogates (PCDD/Fs, non-ortho Co-PCBs, mono-ortho PCBs) were indicated as sufficient in both methods, the multi-layer silica-gel cartridge method showed higher recoveries than the alkaline digestion method (Table 1). Moreover, as for the GC/MS chromatograms, the influence of the coexisting impurities was not observed in either cleanup method.

On the basis of these results, it was determined that the combination of the ASE extraction, the multi-layer silica-gel cartridge treatment, and the activated carbon silica-gel column chromatography was suitable for the rapid extraction and simple cleanup operation required for house dust analysis.

### 3.3. Dioxins in house dust

The samples that had been measured at this time were collected from five residential homes in each of the two districts in Japan. These two districts (Kumagaya city and Sendai city) were located in the center (Kumagaya) of Japan and the northern section (Sendai), and

Table 1
Comparison of recovery rate of dioxin surrogates obtained from alkaline digestion and multi-layer silica-gel cartridge methods

| | Alkaline digestion recovery (%) | | Multi-layer silica-gel cartridge recovery (%) | |
|---|---|---|---|---|
| | Mean ($n = 5$) | Range | Mean ($n = 5$) | Range |
| PCDD/Fs | 74.0 | 66.0–78.3 | 89.6 | 75.6–90.8 |
| Co-PCBs | 78.1 | 53.3–90.1 | 94.4 | 88.2–99.7 |
| PCDD/Fs and Co-PCBs | 76.2 | 64.5–82.3 | 92.2 | 86.7–98.8 |

are well separated from each other. The population in Sendai city is over one million, and it is about 10 times bigger than Kumagaya city. In addition, the number of industries in Sendai city is about four times greater than that in Kumagaya city. For the measurement results, the average value of the dioxins from Kumagaya city was 15.6 pg TEQ/g (8.6–26.0 pg TEQ/g, $n = 5$), and that from Sendai city was 16.0 pg TEQ/g (5.9–30.5 pg TEQ/g, $n = 5$). Furthermore, the data of each dioxin congener detected in both cities were similar to each other (Table 2). These data suggested that there was no significant regional difference between the two cities, and that the dioxin level in the house dust samples was near to the level usually found in the soil of Japan (Osaki et al., 1992; Hashimoto et al., 1999).

Wittsiepe et al. also reported the dioxin concentration in house dust samples in Germany (Wittsiepe et al., 1997). In this report, the average level of PCDD/Fs in normal house dust was 101 ng I-TEQ/kg (range: 7.83–332 ng I-TEQ/kg), which was relatively higher than ours. This difference might be derived from various factors such as soil, atmosphere, combustion soot, insecticides, PCB, etc. that have some possibility of polluting the indoor environment.

As for the risk assessment, it is generally thought to be necessary to estimate the risk of dioxin intake from house dust for small children, because small children, especially a newborn baby and infant spend their life (i.e., breathing and meals) in the space near the floor. However, it is difficult for us to estimate the risk in

Table 2
Each dioxin isomer content, WHO-TEQs and lipid content of house dust from the two districts in Japan

| Dioxin isomer | Kumagaya city | | Sendai city | |
|---|---|---|---|---|
| | Mean ($n = 5$) (pg/g) | Range (pg/g) | Mean ($n = 5$) (pg/g) | Range (pg/g) |
| 2,3,7,8-TCDD | 1.0 | 0.4–2.0 | 0.3 | <0.2–1.3 |
| 1,2,3,7,8-PeCDD | 2.2 | 1.1–3.5 | 3.0 | <0.2–8.0 |
| 1,2,3,4,7,8-HxCDD | 1.8 | 0.9–3.5 | 0.8 | <0.5–1.8 |
| 1,2,3,6,7,8-HxCDD | 4.7 | 2.6–7.7 | 4.1 | 0.7–7.6 |
| 1,2,3,7,8,9-HxCDD | 3.1 | 1.9–4.8 | 1.6 | <0.5–3.3 |
| 1,2,3,4,6,7,8-HpCDD | 68.4 | 41.5–96.1 | 76.1 | 43.8–171 |
| OCDD | 698 | 422–881 | 884 | 522–1500 |
| ∑PCDDs (WHO-TEQ) | 4.9 | 2.5–8.1 | 4.8 | 0.9–10.6 |
| 2,3,7,8-TCDF | 3.6 | 1.9–6.8 | 8.0 | 2.0–29.3 |
| 1,2,3,7,8-PeCDF | 8.3 | 2.6–18.2 | 10.8 | 2.5–32.9 |
| 2,3,4,7,8-PeCDF | 8.2 | 5.6–11.5 | 8.6 | 2.0–23.8 |
| 1,2,3,4,7,8-HxCDF | 10.5 | 5.4–19.4 | 7.5 | 3.1–10.9 |
| 1,2,3,6,7,8-HxCDF | 10.8 | 5.4–20.3 | 7.2 | 2.6–12.5 |
| 2,3,4,6,7,8-HxCDF | 15.7 | 8.1–32.2 | 8.2 | 3.2–12.7 |
| 1,2,3,7,8,9-HxCDF | 0.5 | <0.5–2.4 | <0.5 | <0.5–<0.5 |
| 1,2,3,4,6,7,8-HpCDF | 41.6 | 18.4–69.5 | 32.1 | 15.4–47.5 |
| 1,2,3,4,7,8,9-HpCDF | 3.9 | 1.5–6.1 | 2.6 | 1.3–3.8 |
| OCDF | 52.5 | 23.4–100 | 31.4 | 17.4–57.2 |
| ∑PCDFs (WHO-TEQ) | 9.1 | 5.2–15.3 | 8.3 | 2.4–20.4 |
| ∑PCDDs + PCDFs (WHO-TEQ) | 14.0 | 7.7–23.4 | 13.1 | 4.3–25.8 |
| 3,4,4′,5-TeCB (#81) | 4.3 | 2.6–7.1 | 9.8 | 3.7–28.9 |
| 3,3′,4,4′-TeCB (#77) | 139 | 55.4–247 | 136 | 58.9–351 |
| 3,3′,4,4′,5-PeCB (#126) | 13.8 | 7.3–22.7 | 23.5 | 8.8–44.4 |
| 3,3′,4,4′,5,5′-HxCB (#169) | 3.1 | <1.0–6.2 | 3.9 | 1.7–5.2 |
| 2′,3,4,4′,5-PeCB (#123) | 76.8 | 51.8–114 | 238 | 55.4–697 |
| 2,3′,4,4′,5-PeCB (#118) | 531 | 388–860 | 1510 | 352–5220 |
| 2,3,4,4′,5-PeCB (#114) | 37.3 | 23.0–66.4 | 99.8 | 31.7–271.5 |
| 2,3,3′,4,4′-PeCB (#105) | 225 | 160–392 | 832 | 172–2970 |
| 2,3′,4,4′,5,5′-HxCB (#167) | 26.6 | 19.2–42.2 | 95.2 | 25.8–286 |
| 2,3,3′,4,4′,5-HxCB (#156) | 54.3 | 35.2–83.7 | 216 | 40.6–721 |
| 2,3,3′,4,4′,5′-HxCB (#157) | 17.5 | 11.3–28.4 | 56.3 | 12.2–180 |
| 2,3,3′,4,4′,5,5′-HpCB (#189) | 9.0 | 6.5–13.8 | 13.9 | 6.0–25.9 |
| ∑Co-PCB (WHO-TEQ) | 1.6 | 0.8–2.6 | 2.9 | 1.0–4.7 |
| WHO-TEQ (PCDD/Fs + PCBs) | 15.6 | 8.6–26.0 | 16.0 | 5.9–30.5 |
| Lipid (%) | 6.1% | 5.4%–7.2% | 5.4% | 2.8%–6.8% |

comparison to the TDI of 1–4 pg WHO-TEQ/kg bw/day, because we cannot calculate the intake amount of house dust by small children. Anyway, the data in this report suggested that measuring dioxin in house dust would be useful for examining the indoor pollution by dioxin.

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# Levels of PCDDs, PCDFs and Co-PCBs in human milk in Saitama, Japan, and epidemiological research

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**Abstract**

From 1998 to 2000, the dioxin levels were measured in the milk of 299 mothers who lived in Saitama prefecture, Japan. Factors that influenced the dioxin levels were investigated based on a questionnaire given to the milk donors at that time. It was found that the dioxin levels in the milk of the mothers who smoked were lower than those of non-smokers, and the dioxin levels were generally low in the mothers who were heavy smokers. The average dioxin levels in the milk of mothers who had been breast-fed were higher than those given formula, and there was a significant difference in the dioxin congeners. It was also found that the dioxin levels in milk of the women who regularly consumed fish and shellfish were generally higher. There was a strong correlation between $\sum$TEQ and PCB126. The data suggested that PCB126 could be a useful indicator for the simplified analysis of dioxin in human milk.
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*Keywords:* PCDDs; PCDFs; Co-PCBs; Human milk; Japanese; Dietary habits; Lactation; Smoking habits; A simplified analysis

## 1. Introduction

Recently, dioxin pollution has been acknowledged as a widespread social problem in Japan. This is especially true in Saitama, where industrial waste incinerators have proliferated, and residents' concern has risen since high-level dioxin pollution was reported in the surrounding soil. Our group was asked by the Saitama Prefectual Health Promotion Division to investigate whether the dioxin levels in human milk might be higher in this area than in other regions of Saitama prefecture. Therefore, we measured the dioxin levels in the milk of mothers who resided in various parts of Saitama.

Moreover, we investigated the specific factors influencing the dioxin levels in human milk referring to Fürst et al. (1992) and Pluim et al. (1993). A questionnaire given to the milk donors contained the following: age, BMI (body mass index; body weight/height$^2$), days after childbirth when human milk was collected, residence region (whether industrial waste incinerators were present or not), residence years, distance to the nearest waste incinerator from the dwelling, smoking habits (including level of tobacco consumption), lactation history (breast-feeding or formula or mixed feeding), and dietary habits (meat and eggs, fish and shellfish, milk and dairy products).

## 2. Materials and methods

### 2.1. Collection samples

Human milk samples were collected from the primiparas who volunteered for the investigation in 1998–2000 ($n = 299$): The planned time to collect the

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Table 1
Dioxin levels in human milk ($n = 299$)[a,b]

| Compounds | Mean | SD | Min | Max |
|---|---|---|---|---|
| 2,3,7,8-TCDD | 1.54 | 0.66 | ND | 3.79 |
| 1,2,3,7,8-PeCDD | 6.30 | 2.00 | 2.56 | 12.96 |
| 1,2,3,4,7,8-HxCDD | 2.04 | 1.13 | ND | 5.41 |
| 1,2,3,6,7,8-HxCDD | 19.00 | 6.57 | 6.04 | 52.61 |
| 1,2,3,7,8,9-HxCDD | 3.62 | 1.85 | ND | 11.51 |
| 1,2,3,4,6,7,8-HpCDD | 10.86 | 5.58 | ND | 39.84 |
| OCDD | 74.01 | 48.58 | 20.00 | 397.72 |
| 2,3,7,8-TCDF | 0.76 | 0.48 | ND | 3.02 |
| 1,2,3,7,8-PeCDF | 0.51 | 0.43 | ND | 2.78 |
| 2,3,4,7,8-PeCDF | 13.51 | 4.11 | 3.75 | 31.94 |
| 1,2,3,4,7,8-HxCDF | 4.63 | 2.01 | ND | 25.39 |
| 1,2,3,6,7,8-HxCDF | 5.78 | 2.73 | ND | 38.02 |
| 2,3,4,6,7,8-HxCDF | 3.35 | 1.70 | ND | 9.46 |
| 1,2,3,4,6,7,8-HpCDF | 2.33 | 1.39 | ND | 9.18 |
| PCB77 | 5.93 | 3.29 | ND | 25.68 |
| PCB126 | 60.90 | 26.29 | 20.41 | 196.15 |
| PCB169 | 35.38 | 12.79 | ND | 93.91 |
| ∑PCDDs | 117.40 | 59.01 | 36.10 | 469.77 |
| ∑PCDFs | 30.88 | 9.80 | 8.88 | 84.98 |
| ∑PCDDs + PCDFs | 148.22 | 64.47 | 52.01 | 507.83 |
| ∑Co-PCBs | 102.20 | 36.63 | 35.80 | 274.12 |
| ∑PCDD/Fs + Co-PCBs | 250.42 | 86.08 | 116.94 | 634.05 |
| ∑PCDDs (TEQ) | 7.34 | 2.30 | 2.78 | 16.44 |
| ∑PCDFs (TEQ) | 8.25 | 2.47 | 2.37 | 18.76 |
| ∑PCDDs + PCDFs (TEQ) | 15.59 | 4.54 | 5.14 | 30.62 |
| ∑Co-PCBs (TEQ) | 6.45 | 2.71 | 2.18 | 20.30 |
| ∑PCDD/Fs + Co-PCBs (TEQ) | 22.03 | 6.62 | 7.32 | 49.68 |

[a] Results are given in pg/g; SD = standard deviation; ND = not detected.
[b] 1,2,3,7,8,9-HxCDF, 1,2,3,4,7,8,9-HpCDF and OCDF were below detection limit.

was assumed that the levels of each compound were influenced not by one factor but by various factors, and thus, when the standardization partial regression coefficients according to the factor for each compound were compared, three factors (the fat content in human milk, the presence of smoking and age) were remarkable. Table 2 shows the result of a main multivariate linear regression analysis (coefficient of non-standard, standard error, and significant probability). There were many compounds, especially OCDD, whose levels significantly increased with age. In contrast, when the fat content in human milk was high, the levels significantly decreased. Overweight persons showed the levels of compounds significantly increasing or decreasing. Three items are omitted in the table because of space limitations: when the milk and dairy products indices were high, the levels of OCDD (RC = −2.707, SE = 1.371, $P = 0.049$) and ∑PCDDs (RC = −3.340, SE = 1.677, $P = 0.047$) significantly decreased; when the distance from the waste incinerator was far, the levels of 2,3,7,8-TCDF (RC = 0.063, SE = 0.024, $P = 0.010$) and 1,2,3,7,8-PeCDF (RC = 0.058, SE = 0.022, $P = 0.010$) significantly decreased; and for residents who lived in industrial waste incinerator regions, the level of OCDD (RC = −12.239, SE = 5.597, $P = 0.030$) was significantly low compared with residents who lived in another region. In general, the principal ingredient of the PCDD/Fs of combustion origin is an eight-chlorinated structure, which is probably one of the causes for OCDD producing a regional difference of dioxin in the atmosphere. However, in the investigation region of anxiety in Saitama, the level of OCDD assumed to be of combustion origin was significantly low compared with the other regions.

### 3.1. Smoking habits

The mean level of the total dioxin in the milk of 95 mothers who smoked was 227.3 pg/g fat (19.8 pg TEQ/g fat), while the mean level of 204 mothers who did not smoke was 261.2 pg/g fat (23.1 pg TEQ/g fat). Most congener levels of the mothers who smoked were lower than the mothers who did not smoke, and the covariance analysis showed that significant differences were found