図1 低濃度のスクリーニングの CaM の測定におけるヒストグラムと正規確率プロット
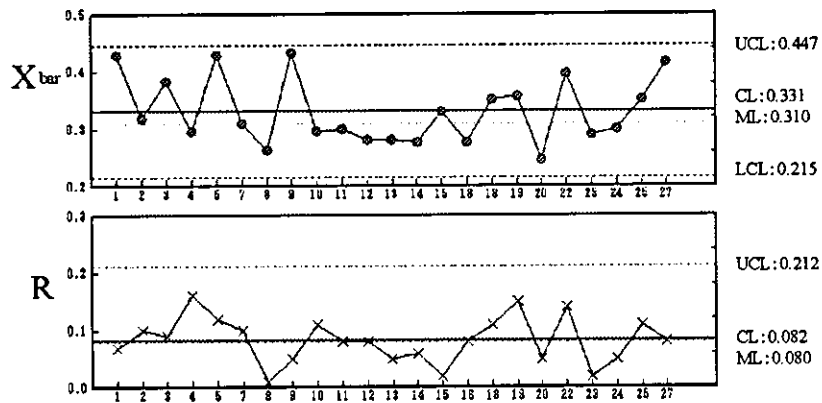


図2 低濃度のスクリーニングの CaM の測定における X－R 管理図
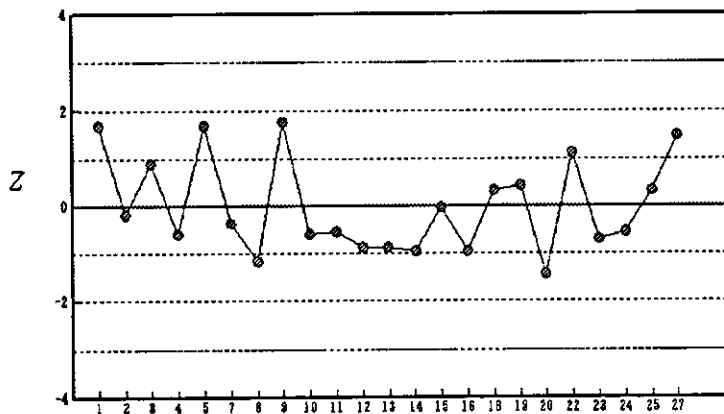


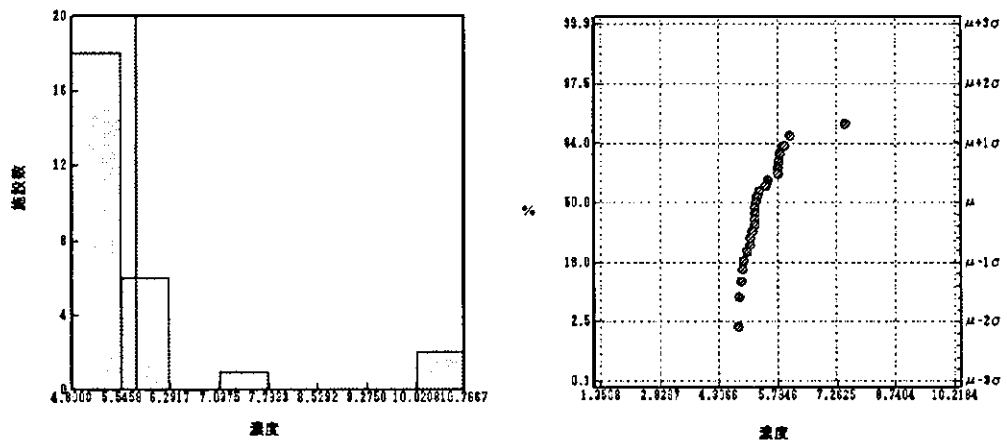図3 低濃度のスクリーニングの CaM の測定における z－スコア

図4 高濃度のスクリーニングの CaM の測定におけるヒストグラムと正規確率プロット



図5 高濃度のスクリーニングの CaM の測定における X－R 管理図
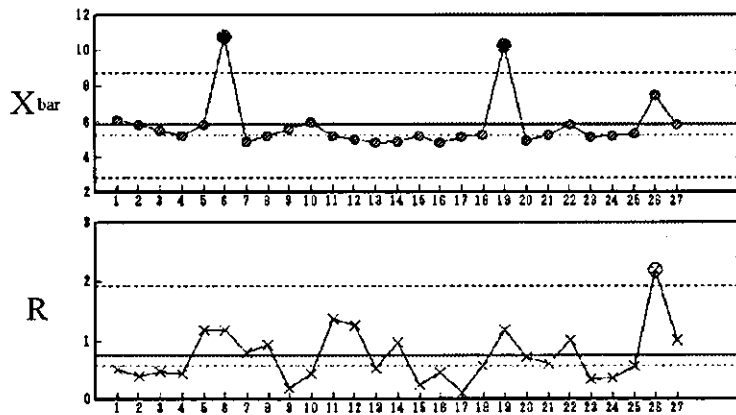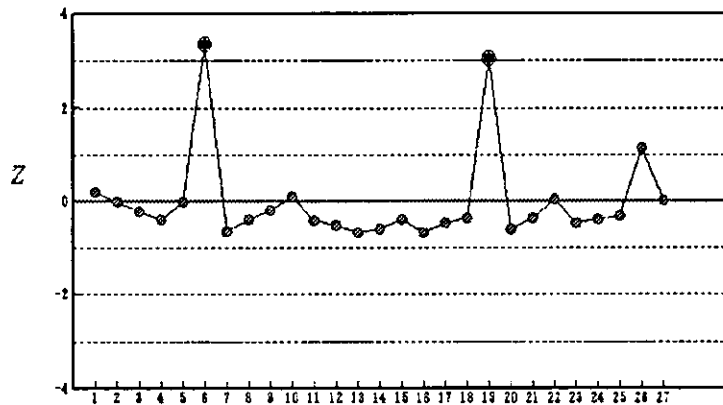


図6 高濃度のスクリーニングの CaM の測定における z－スコア
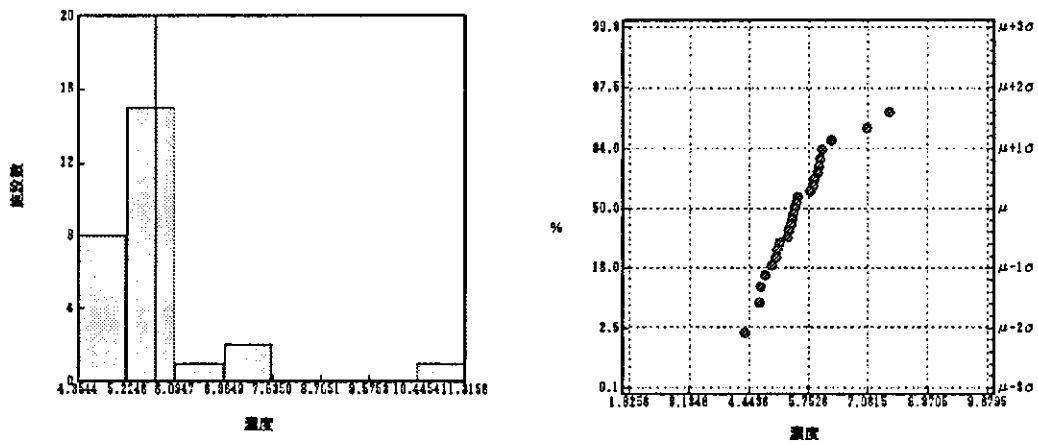
図7 系統特異的定量の Mon810 の測定におけるヒストグラムと正規確率プロット
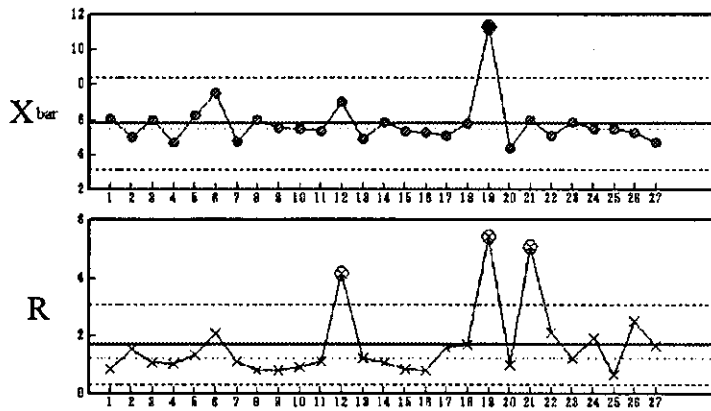


図8 系統特異的定量の Mon810 の測定における X−R 管理図
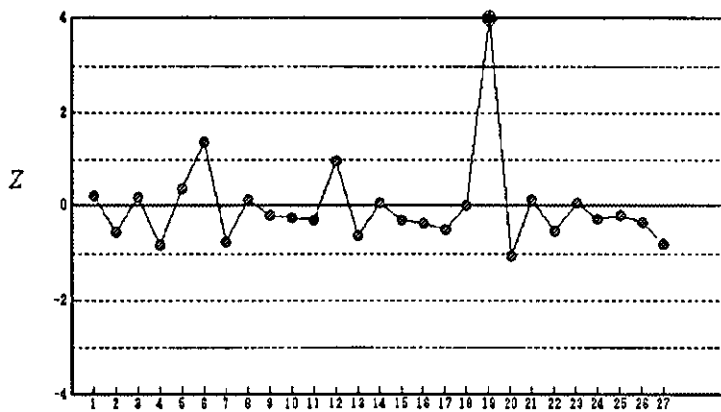


図9 系統特異的定量の Mon810 の測定における z−スコア
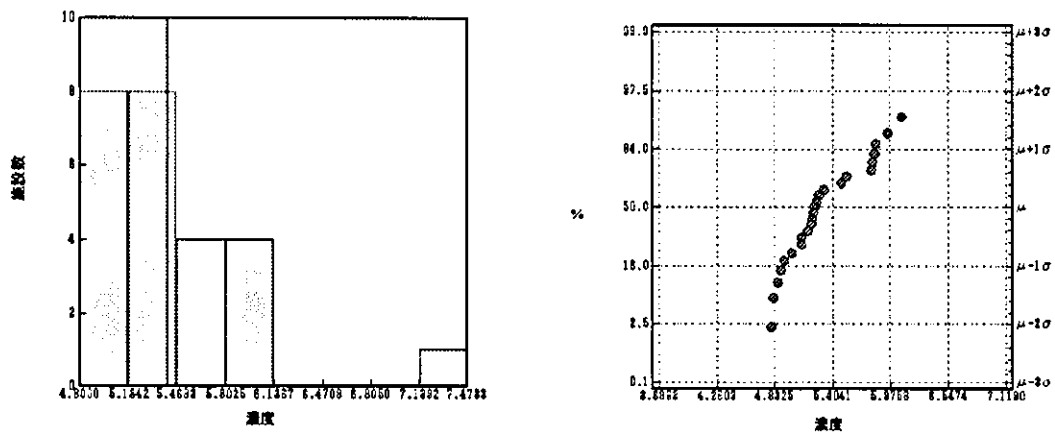
図10 高濃度のスクリーニングの CaM の測定におけるヒストグラムと正規確率プロット
（定量 PCR 装置に機種 F を用いた機関の測定値を除外した場合）
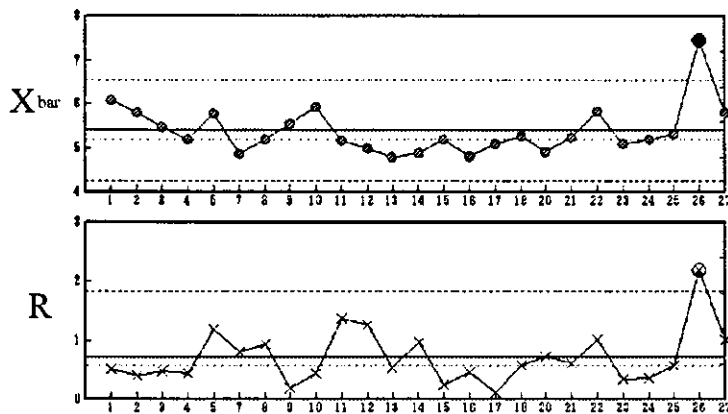


図11 高濃度のスクリーニングの CaM の測定における X－R 管理図
（定量 PCR 装置に機種 F を用いた機関の測定値を除外した場合）
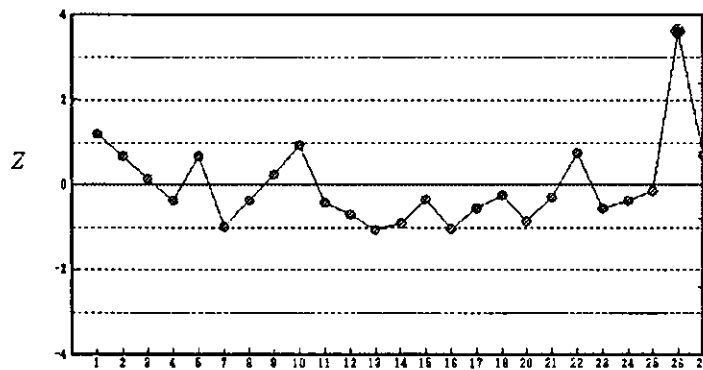


図12 高濃度のスクリーニングの CaM の測定における z－スコア
（定量 PCR 装置に機種 F を用いた機関の測定値を除外した場合）
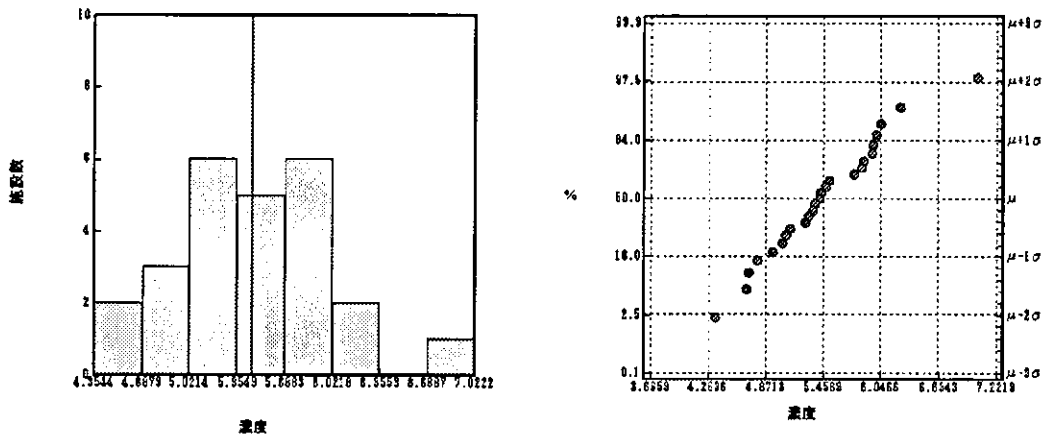
図 13　系統特異的定量の Mon810 の測定におけるヒストグラムと正規確率プロット
（定量 PCR 装置に機種 F を用いた機関の測定値を除外した場合）



図 14　系統特異的定量の Mon810 の測定における X－R 管理図
（定量 PCR 装置に機種 F を用いた機関の測定値を除外した場合）
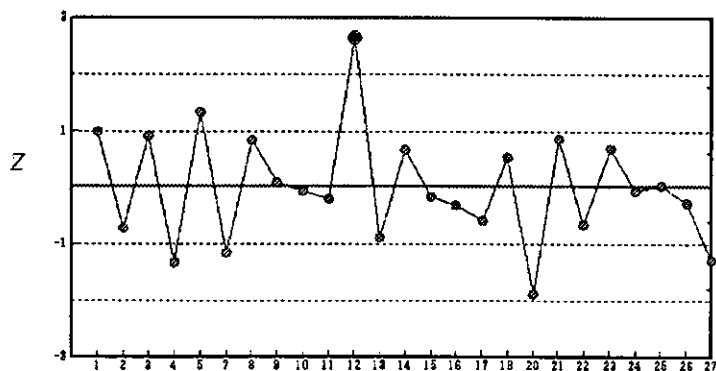


図 15　系統特異的定量の Mon810 の測定における z－スコア
（定量 PCR 装置に機種Fを用いた機関の測定値を除外した場合）

厚生労働科学研究費補助金（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」


（平成16年度）
研究成果に関する刊行物一覧表

# 研究成果の刊行に関する一覧表

書籍

| 著者氏名 | 論文タイトル名 | 書籍全体の編集者名 | 書籍名 | 出版社名 | 出版地 | 出版年 | ページ |
|---|---|---|---|---|---|---|---|
| なし | | | | | | | |

雑誌

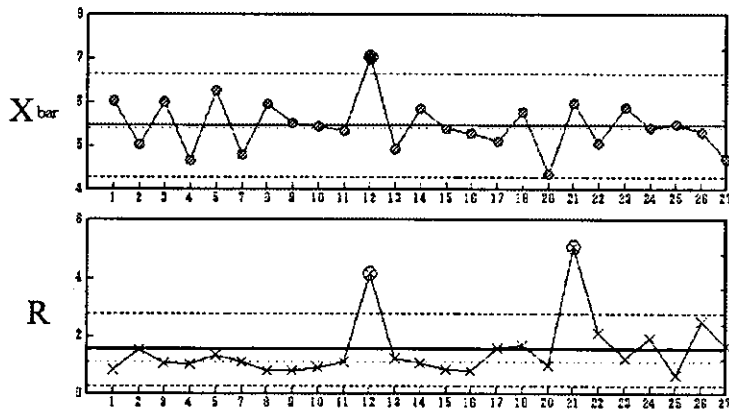| 発表者氏名 | 論文タイトル名 | 発表誌名 | 巻号 | ページ | 出版年 |
|---|---|---|---|---|---|
| 中澤班<br>(1) Koihci Saito, Masakazu Ogawa, Mikiko Takekuma, Atsuko Ohmura, Migaku Kawaguhci, Rie Ito, Koichi Inoue, Yasuhiko Matsuki, and Hioryuki Nakazawa | Systematic analysis and the overall toxicity evaluation of dioxins and hexachlorobenzene in human milk. | Organohalogen Compounds | 66 | 38-41 | 2004 |
| (2) Koichi Saito, Andreas Sjodin, Courtney D. Sandau, Mark D, Davis, Hiroyuki Nakazawa, Yasuhiko Matsuki, and Donald G Patterson, Jr. | Development of a Accelerated Solvent Extraction and Gel Permeation Chromatography Analytical Method for Measuring Persistent Organohalogen Compounds in Adipose and Organ Tissue Analysis. | Chemosphere, | 57 | 373-381 | 2004 |
| (3) Koihci Saito, Masahiro Ishizuka, Yukio Sugawara, Hioryuki Nakazawa and Yasuhiko Matsuki | Cleanup Method Using Disposable Tandem Cartridge System for the Determination of Dioxins in Human Milk by Enzyme-Linked Immunosorbent Assay. | Bull Environ Contam Toxicol. | 73 | 17-23 | 2004 |
| 織田班<br>(4) 熊谷信二、織田肇、田淵武夫、赤阪進、小坂 博、吉田仁、甲田茂樹、毛利一平 | 自治体焼却施設における堆積粉塵中ダイオキシン類濃度と労働者の血清中ダイオキシン類濃度との関係 | 産業衛生学雑誌 | 46 | 1-9 | 2004 |
| 米谷班<br>(5)R. Matsuda, T.Tsutsumi, M. Toyoda, T. Maitani | The Proficiency Testing of Determination of Dioxins in Food | Organohalogen Compounds | 66 | 576-581 | 2004 |
| 松木班<br>(6)Mitsunobu Okuyama, Norihiro Kobayashi, Wakako Takeda, Takako Anjo, Yasuhiko Matsuki, Junichi Goto, Akira Kambegawa, Sinjiro Hori | Enzyme-Linked Immunosorbent Assay for Monitoring Toxic Dioxin Congeners in Milk Based on a Newly Generated Monoclonal Anti-Dioxin Antibody. | Analytical Chemistry | 76 (7) | 1948-1956 | 2004 |
| | | | | | |

| (7) 亀山浩, 渡邉敬浩, 笠間菊子, 松木容彦, 米谷民雄 | 遺伝子組換えトウモロコシ（CBH351）および遺伝子組換えジャガイモ(NewLeaf Plus and NewLeaf Y)の検知用試料の作製と調査成績 | 食品衛生研究 | 54(4) | 25-35 | 2004 |
|---|---|---|---|---|---|
| (8)<br>Takahiro Watanabe, Hideo Kuribara, Takashi Mishima, Hiroyuki Kikuchi, Misao Kubo, Takashi Kodama, Satoshi Futo, Kikuko Kasama, Akie Toyota, Masanori Nouno, Ayako Saita, Kunihiko Takahashi, Akihiro Hino, Hiroshi Akiyama, Tamio Maitani | New Qualitative Detection Methods of Genetically Modified Potatoes | Biol Pharm Bull | 27(9) | 1333-1339 | 2004 |

学会発表

| 発表者氏名 | タイトル名 | 発表学会名 | 出版年 |
|---|---|---|---|
| 斉藤貢一, 小川政彦, 竹熊美貴子, 大村厚子, 中澤裕之, 松木容彦, | 母乳中ダイオキシンとヘキサクロルベンゼンの系統分析及び総合的毒性評価 | 日本薬学会第124年会（大阪） | 2004年 |
| 斉藤貢一, 大村厚子, 竹熊美貴子, 伊藤里恵, 井之上浩一, 松木容彦, 中澤裕之 | ベビーフード中ダイオキシン類の分析および摂取量評価 | 環境ホルモン学会（名古屋） | 2004年 |
| R. Matsuda, T.Tsutsumi, M. Toyoda, T. Maitani | The Proficiency Testing of Determination of Dioxins in Food | Daioxin（Berlin） | 2004年 |
| 渡邉敬浩, 亀山浩, 米谷民雄, 笠間菊子, 松木容彦, 児玉貴志, 栗原秀夫, 日野明寛 | 遺伝子組換え大豆定量検査法の外部精度管理について | 全国衛生化学協議会第40回年会(和歌山) | 2003年 |
| ○川崎 勝 大島赴夫, 松木容彦, 鈴木敏之, 山本茂貴, 伊藤嘉典, 町井研士 | 精度管理用貝毒検査試料中の遊離脂肪酸の測定 | （社）日本食品衛生学会第88回学術講演会（広島） | 2004年11月 |

厚生労働科学研究費補助金（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」


（平成 16 年度）
研究成果に関する刊行物

論文

(1)

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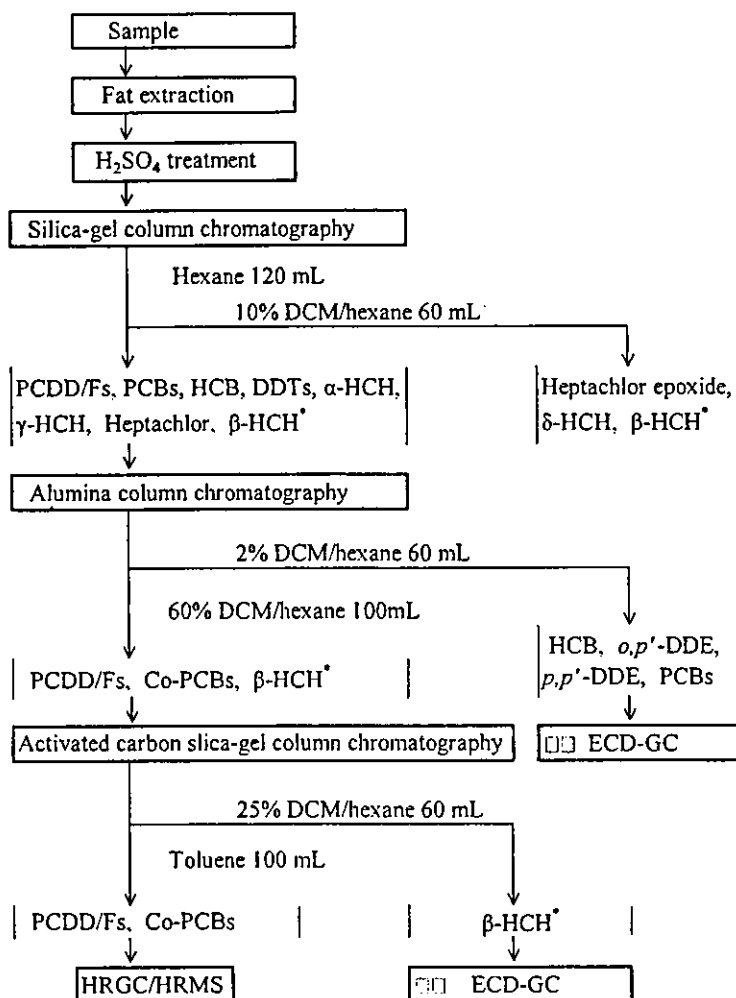
Fig. 1. Flowchart of systematic analysis of dioxins and hexachlorobenzene in human milk

Table 1. Residual concentration of HCB, heptachlor epoxide and $\beta$-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 |
| $\beta$-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).

**References**
1. Sakai S., Hirai Y., Takatsuki H. (2001) Waste Management Research 12, 349.
2. Hahn M.E., Goldstein J.A., Linko P., Gasiewicz T.A. (1989) Arch. Biochem. Biophys. 270, 344.
3. Van Bilgelen A.P.J.M. (1998) Environ. Health Perspect. 106, 683.

4.  Polder, A., Becher, G., Savinova, T.N., Skaare, J.U., 1998. Dioxins, PCBs and some chlorinated pesticides in human milk from the Kola Peninsula, Russia. Chemosphere 37, 1795-1806.

# 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*

## 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.
© 2004 Elsevier Ltd. All rights reserved.

## 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).

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, $\alpha$-HCH, $\beta$-HCH, $\gamma$-HCH, $\delta$-HCH, heptachlor, heptachlor epoxide, $\alpha$-chlordane, $\gamma$-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 pre-washed 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
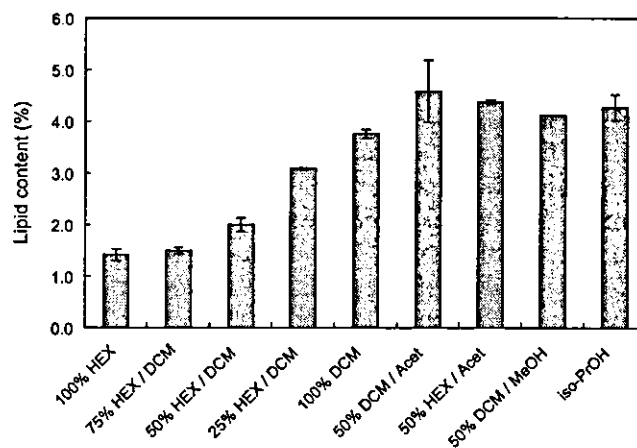


Fig. 1. Extractable lipid content determined by ASE with various solvent combinations from swine liver tissue.

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.

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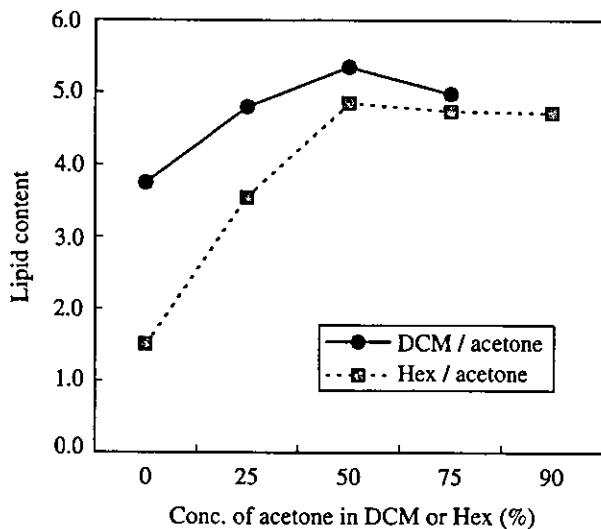
Fig. 2. Comparison of extractable lipid content by dichloromethane/acetone and hexane/acetone from swine liver tissue.
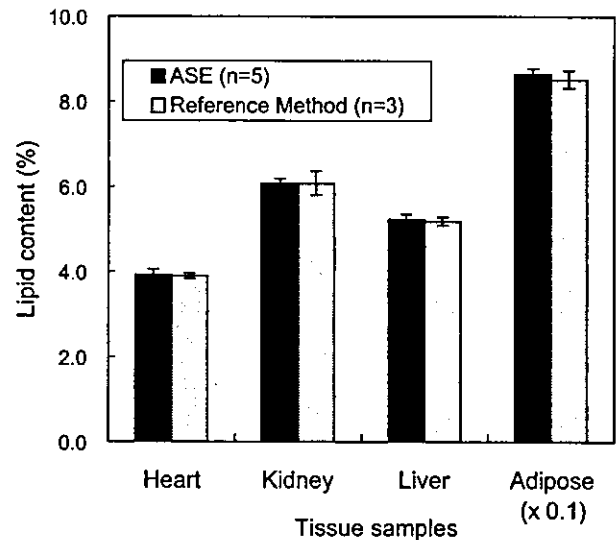


Fig. 3. Comparison of extractable lipid content by the ASE and reference method for swine heart, kidney and liver tissue and cattle adipose tissue.

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% |

Tissue sample (1 - 5 g)
↓
Homogenize (mix with Hydro-Matrix)
↓
ASE (DCM/acetone(1:1), 100    1500 psi) —— [Automated]
↓
Partirioning (Hex/MTBE(9:1) - 0.1M $H_3PO_4$ with 1% KCl)
↓
(Lipid)
↓
GPC (Bio Beads S-X3; DCM/Hex(1:1)) —— [Automated]
↓
SPE (silica-gel) —— [Automated]

Fraction A               Fraction B
non-polar compounds      polar compounds
   (PBDEs)                   (HO-PCBs)
   (PCBs)                    (MeSO₂-PCBs)
   (PCNs)                    (TBBPA)
   (OCPs)                    (PCP)
                            (δ-HCH)
                            (Endosulfan II)
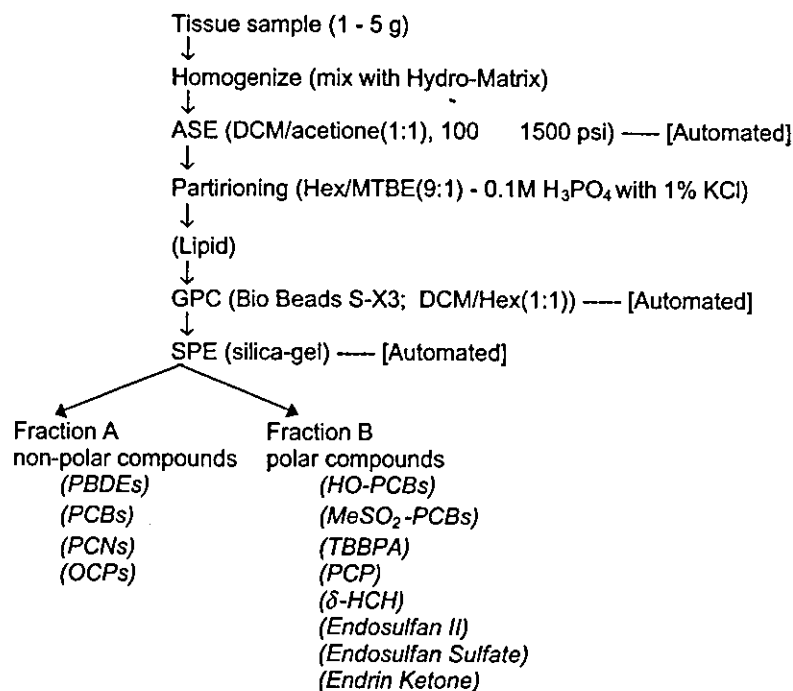                            (Endosulfan Sulfate)
                            (Endrin Ketone)

Fig. 4. Proposed analytical method scheme.