Articles

Analytical Method for Dioxin and Organo-Chlorinated Compounds: (I) Pretreatment of Milk Samples for Dioxin Analysis

Jeong Soo Yang, Jin Young Kim†, Yong Wook Choi†, and Dai Woon Lee*

Department of Chemistry, Yonsei University, Seoul 120-749, Korea
†Korea Basic Science Institute, Taejon 305-333, Korea
‡Department of Chemistry, Jeonju University, Jeonju 560-759, Korea
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A simple and reproducible pretreatment method was developed for the determination of dioxins in milk sample. Liquid-liquid extraction (LLE) was used for the initial extraction of the analyte from milk. For the elimination of interferences coextracted from milk, acid treatment followed by multilayer silica gel, and then alumina column clean-up were performed. The clean extract could be obtained without carbon column or high performance liquid chromatographic (HPLC) clean-up procedure. Polychlorinated biphenyles (PCBs) and dioxins were separated on neutral alumina activated at 180 °C for 12 hours. The final extract was analyzed by HPLC and high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS). The recovery of dioxins spiked in milk at 75-300 ppt level was 83.3-98.9% and their relative standard deviation was 4.1-14%.

Introduction

Polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are the most toxic organic pollutants known to mankind.[1] The determination of these compounds in environmental and biological samples has been the subject of intense research.[2,3] Dioxins are characterized by a relatively high environmental persistence and toxicity.[4] Because of their highly lipophilic nature and slow elimination kinetics, these compounds have strong tendencies to bioaccumulate in lipid-rich compartments of organisms. Because the general public is exposed to dioxins mainly through contaminated food (fish, meat, dairy products, etc.), the investigations of dioxins in food are crucial.[5-7]

The low concentration level of these compounds in biological samples, typically in the low pg/g range, requires a highly sensitive and specific method of analysis. A combination of several different clean-up techniques was used to remove potential interferences and to obtain a clear sample extract.[8,9] Commonly used extraction and clean-up techniques include LLE,[10] solid phase extraction(SPE),[11-13] HPLC clean-up,[14] and column chromatography using a variety of adsorbents such as acid and base modified silica gel,[15,16] alumina,[17] florisil[18] and charcoal.[19] Super critical fluid extraction (SFE)[20,21] and gel permeation chromatography[13,22] have been recently applied to dioxin analysis.

Utilization of multiple clean-up techniques often allows attainment of clean sample extract, but the working procedure is very laborious and tedious. These complex techniques are also less applicable to routine analysis of large number of samples.[22] A goal of this study is to develop a cost effective, rapid and less hazardous pretreatment generating results with acceptable precision and accuracy for dioxin analysis at a few of ng level.

In this study, the clean-up methods based on multilayer silica gel, alumina, carbon and HPLC column were described for the determination of dioxins in milk sample. The activation of alumina, which is one of the most selective adsorbents, was studied for the separation of PCBs and dioxins. The performance of each pretreatment with regard to elimination of interfering compounds and recovery was examined by analyzing a milk sample spiked with dioxins.

Experimental

Reagents. Acetonitrile, dichloromethane, hexane, and toluene were of HPLC grade (Burdick & Jackson). Ethanol (Hayman LTD) and diethylether (Duksan Pharmaceutical) were of HPLC grade. Silica gel and alumina activity I were obtained from Merck. Prior to use, they were washed with methanol and dichloromethane twice, followed by being activated overnight at 180 °C. Carbon(100 mesh) was obtained from Darco. All other reagents(sulfuric acid, sodium sulfate and sodium oxalate) were of analytical reagent grade.

Standards. 2,2'3,3'6,6'-hexaCB (HxCB) and 1,2,3,4-tetrachlorinated dibezodioxin (TCDD), 1,2,3,4,7-pentaCDD (PeCDD), 1,2,3,4,7,8-hexaCDD (HxCDD), 1,2,3,4,6,7,8-heptaCDD (HpCDD) and 1,2,3,4,6,7,8,9-octaCDD (OCDD) were obtained from Ultra Scientific.

Extraction of certified sample. 100 mL of a commercial milk was fortified with a standard mixture containing each 7.5 ng of 1,2,3,4-TCDD, 1,2,3,4,7-PeCDD, 1,2,3,4,7,8-HxCDD, 1,2,3,4,6,7,8-HpCDD and 300 ng of OCDD at lower ppb level. After homogenization, the extraction of lipids from milk was performed according to the method described by Rappe et al.[23] Sodium oxalate and ethanol were added to the milk sample and the fat fraction containing the dioxins was isolated by LLE using diethylether and hexane. The

*To whom correspondence should be addressed.

combined ether and hexane fractions were dried.

Acid clean-up. The dried fat was redissolved in 25 ml of hexane. The hexane solution was treated with 10-20 mL of concentrated sulphuric acid repeatedly until organic phase became colorless and was washed twice with 100 mL of water. The organic portion was dried with anhydrous sodium sulfate and concentrated to 1mL at low temperature in the rotary evaporator under vacuum.

Silica gel column clean-up. Silica gel was packed in the glass column (2×20 cm) in the following order; silica gel (1 g), silica gel impregnated with sodium hydroxide (2 g),[24] silica gel (1 g), silica gel impregnated with concentrated sulphuric acid (4 g),[15,24] silica gel (2 g) and anhydrous sodium sulfate (2 g), and was prewashed with 50 mL of hexane. After loading of the acid-treated sample extract, the column was eluted with 90 mL of hexane at a flow rate of 1 mL/min. The eluate was concentrated up to 1 mL.

Alumina column clean-up. The concentrated extract was cleaned using a glass column (1×20 cm) packed with 5 g of neutral alumina which had been previously activated by heating for 12 hours at 180 °C. Fractionation was started by eluting with 50 mL hexane and then 40 mL of hexane/dichloromethane (9/1, v/v). Dioxins were eluted in the second fraction. The eluate was concentrated and dried under a stream of nitrogen.

Carbon column clean-up. After alumina column clean-up, the concentrated extract was loaded into a glass column (0.8×10 cm) filled with the mixture of carbon (0.02 g) and sodium sulfate (0.2 g) and eluted in the order of 20 mL of hexane, 10 mL of hexane/dichloromethane (6/4), 20 mL of dichloromethane, 10 mL of dichloromethane/toluene (7/3), and 80 mL of toluene. Dioxins were eluted in 10 mL of dichloromethane/toluene and 80 mL of toluene fraction. The eluate was collected, concentrated and dried under a stream of nitrogen.

HPLC column clean-up. HPLC used in this work was Shimadzu Liquid Chromatograph equipped with an SPD-10A UV-visible detector and C-R6A integrator. The monitoring wavelength was 240 nm (A.U.F.S.=0.005). A 5 μm Shodex C18-5B column(250×4.6 mm i.d.; Shoko, Kyoto, Japan) was used with a 100 μL sample loop. The mobile phase consists of 7% water in acetonitrile. The flow rate was 1.0 mL/min. From the retention times measured (1,2,3,4-TCDD: 10 min, 1,2,3,4,7-PeCDD: 12.6 min, 1,2,3,4,7,8-HxCDD: 16.0 min, 1,2,3,4,6,7,8-HpCDD: 18.5 min and OCDD: 22 min) the fraction of 9.5-22.5 min was taken and dried through sodium sulfate column. The eluate was concentrated and dried under a stream of nitrogen.

Determination of PCDDs. The appropriate volume of solvent was added to the pretreated sample extracts and then they were analyzed by HPLC and HRGC/HRMS. The column and mobile phase were the same as those used for HPLC clean-up. Each sample extract was dissolved in 500 μL of acetonitrile for HPLC analysis. 20 μL aliquots of them was injected onto the column.

JMS-SX 102A mass spectrometer (JEOL, Japan) with DS 6200 GC (Donam, Korea) was used for confirmation of dioxins in SIM mode of the positive electron impact ionization (EI) at resolution of 10,000. DB-5 (30 m×0.25 μm, J&W, USA) fused silica column was used. The temperature program for the GC was 80 °C isothermal for 2 min then 80-200 °C (20 °C/min), followed by 200-280 °C (4 °C/min). The temperature was then kept at 280 °C for 25 min. The samples were dissolved in 20 μL of nonane and a 1 μL aliquot was injected.

Recovery data for the dioxins spiked in milk were calculated from the area ratio of peak in comparison to reference samples obtained by HPLC.

HRGC/HRMS confirmation. Because some interferences occurring from complex sample matrix could be considered as dioxins in HPLC chromatogram, the confirmation by HRGC/HRMS was necessary.[24] The main ions used for confirmation purposes in SIM (selected ion monitoring) mode were $[M]^+$ and $[M+2]^+$ or $[M+2]^+$ and $[M+4]^+$(Table 1).

Results and Discussion

LLE was performed to extract dioxin-containig lipid from milk. The removal of potential interferences in the extract was carried out by the various steps, such as acid and base modified silica gel, alumina, carbon, and HPLC column clean-up (Figure 1).

Liquid-Liquid Extraction. LLE is a well established technique for the extraction of organic micro-pollutants from biological samples.[10] LLE is simple and does not require time-consuming steps such as preparation of column packing. Though emulsion formation was a commonly encountered problem in LLE,[12] small addition of ethanol to emulsion layer could break it in this work. The amount of extract from the 100 mL milk sample was about 8 g. But, this procedure isolated the fat matrix and the fat soluble residues together. An extensive clean up and fractionation should be involved in further steps.

Acid clean-up. For the dioxin analysis in the biological sample, the initial step of clean-up is the removal of lipids and other lipophilic biogenic interferences from the extract. Saponification,[10] concentrated sulfuric acid treatment,[18] gel permeation chromatography[13,25] and column chromato-

Table 1. Exact masses and molecular data (m/z, relative abundances) of HxCB and dioxins

Compounds	Retention time (min)	Molecular ion (m/z)			Relative abundance		
		exact M	M+2	M+4	M	M+2	M+4
HexaCB ($^{35}Cl_6$)	15.7	357.8515			1		
TetraCDDs	18.3	319.8965	321.8936		77	100	
PentaCDDs	21.6		355.8546	357.8518		100	65
HexaCDDs	25.1		389.8158	391.8128		100	81
HpetaCDDs	32.1		423.7769	425.7739		100	97
OctaCDD	32.1		457.7380	459.7350		89	100

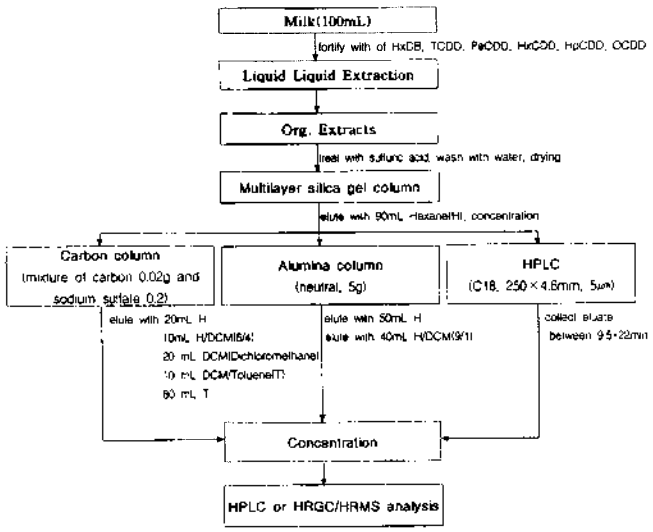

Figure 1. Flow chart of analytical method for determination of dioxins in milk. Carbon or HPLC clean-up can be an alternative to alumina clean-up.

graphy using adsorbent[10,15] are generally used for elimination of lipids. Saponification has a limitation for destroying some higher chlorinated dioxins, particularly OCDD,[11,26,27] and chromatographic methods require preparation of large amount of adsorbents. On the other hand, acid treatment is extremely effective for destroying and removing lipids and polar compounds such as amines, aldehydes and some pesticides which are vulnerable to acid.[18] Another advantage is that a relatively large amount of fat can be treated. Recovery studies of dioxins and dioxin-like compounds on acid treatments were already reported in the literatures and showed good results.[10,18] In this work, the importance of acid treatment was confirmed as we could get a cleaner sample extract through repeated acid treatment until organic phase had no color.

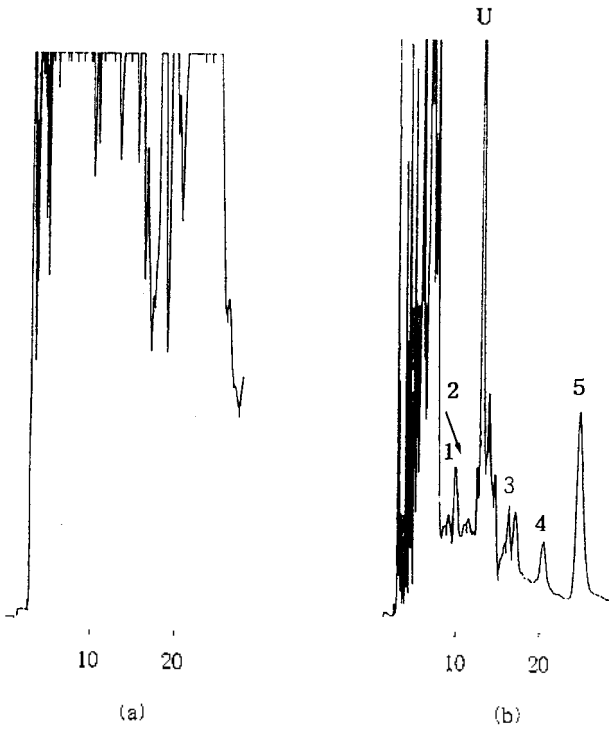

Figure 2. HPLC chromatogram before and after multilayer silica gel column clean-up of milk extract. (a) before, (b) after, Peak I.D., 1: 1,2,3,4-TCDD (M+2, m/z=321.8936), 2: 1,2,3,4,7-PeCDD (M+2, m/z=355.8546), 3: 1,2,3,4,7,8-HxCDD (M+2, m/z=389.8158), 4: 1,2,3,4,6,7,8-HpCDD (M+2, m/z=423.7769), 5: OCDD (M+4, m/z=459.7350), U: unknown.

Silica gel column clean-up. Silica gel column has been most widely used for purification of organic com-

Table 2. Recoveries of HxCB and dioxins in the successive fractions depending on activation condition of alumina

Activation condition temperature	time (hr)	Experiment	Fraction	Recoveries (%)[1] HxCB[2]	TCDD	PeCDD	HxCDD	HpCDD	OCDD
room temp.	3	A	I[3]	121.4	94.0	86.6	94.1	90.5	93.5
			II[4]	-	-	-	-	-	-
	12	B	I	129.9	95.0	91.1	96.2	93.9	94.5
			II	-	-	-	-	-	-
100 °C	3	C	I	133.4	96.4	91.7	92.8	91.5	92.0
			II	-	-	-	-	-	-
	12	D	I	138.3	100.5	95.5	96.3	93.4	97.2
			II	-	-	-	-	-	-
150 °C	3	E	I	132.4	97.7	92.5	95	91.3	92
			II	-	-	-	-	-	-
	12	F	I	145.0	98.8	90.6	91.0	88.3	88.5
			II	-	-	-	-	-	-
180 °C	3	G	I	89.6	97.4	98.1	96.5	91.3	75.4
			II	-	-	-	-	-	-
	12	H	I	122.8	-	-	-	-	-
			II	-	103.3	96.0	87.8	99.1	96.7

[1]Each value was the average of three measurements. [2]HxCB: 2,2'3,3'6,6'-HxCB. [3]50 mL hexane (P'=0). [4]40 mL hexane/dichloromethane (9/1, P'=0.34).

pounds.[28] Remaining lipid residues and other polar organic compounds such as phenols and OH-PCBs after acid treatment were removed in multilayer silica gel column. Two chromatograms obtained before and after silica gel column clean-up are shown in Figure 2(a) and 2(b). It was confirmed that a large amount of interference was removed by multilayer silica gel column clean-up. But after silica gel clean-up, some compounds which made quantification of dioxins difficult still remained.

Alumina column clean-up. Alumina is one of the most suitable and selective adsorbents for separation of dioxins from other organochlorine compounds.[29] The retention of the compounds on the surface of alumina depends on the planarity of the molecule, the localization of π-electron cloud, and the surface area of the compound molecule. The planar and symmetrical compounds like 2,3,7,8-isomers of dioxins can interact with alumina surface more strongly.[30] For the optimization of separation, volume, selectivity and polarity of solvent and surface activity of adsorbent should be considered.[30] In this study, the effect of activation of alumina by heating was also examined. Table 2 shows the elution patterns of HxCB and dioxins from neutral alumina activated under different conditions. Fraction I and II were eluted with 50 mL of hexane(solvent polarity parameter, P'= 0), and 40 mL of the mixture of hexane/dichloromethane (9/1,v/v, P'=0.34), respectively.

If the activation by heating is insufficient (experiment A-G in Table 2), electrons of dioxins and dioxin-like compounds could not be localized onto hydroxyl acidic sites of alumina. HxCB and dioxins were eluted early by even weak solvent (fraction I, P'=0) without separation. Therefore, alumina should be activated at 180 °C over 12 hours for good separation of dioxins from PCBs and other organochlorine compounds (experiment H).

Solvent also plays a major role in the separation of diox-

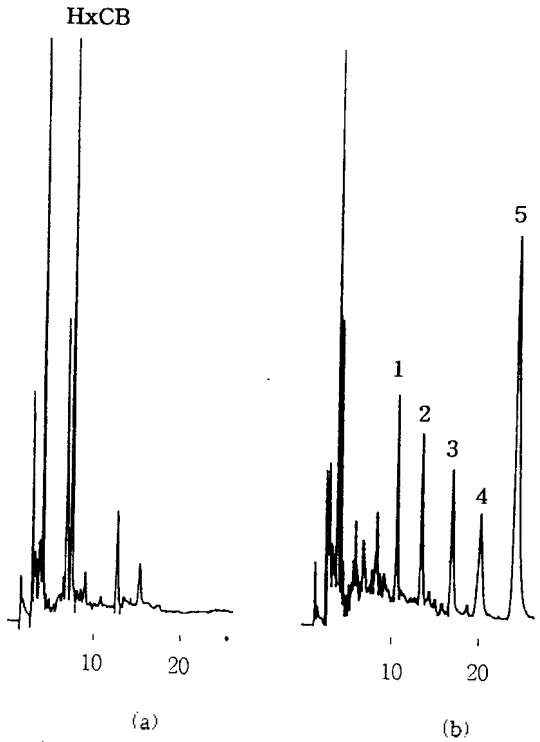

Figure 3. HPLC chromatogram of extract after alumina clean-up of milk extract. (a) fraction (I) of 50 mL hexane, (b) fraction (II) of 40 mL mixture of hexane/dichloromethane (9/1), Peak numbers refer to Figure 2.

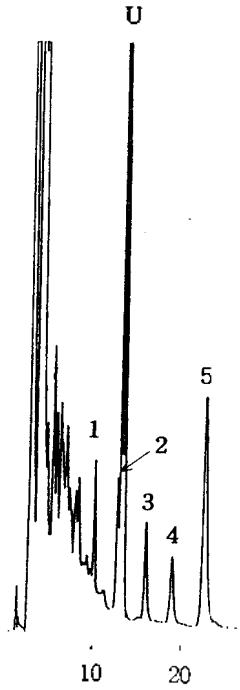

Figure 4. HPLC chromatogram of fraction of mixture of 10 mL dichloromethane/toluene (7/3) and 80 mL toluene in carbon column clean-up procedure. Peak numbers refer to Figure 2.

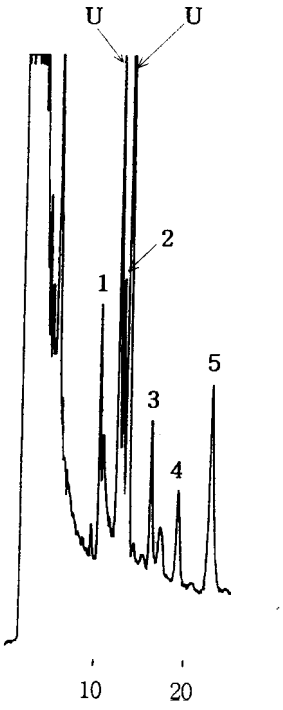

Figure 5. HPLC chromatogram of fraction between retention time 9.5 and 22.5 min in HPLC clean-up procedure. Peak numbers refer to Figure 2.

ins and PCBs.[29] It has been suggested that the oxygens of dioxin play a role on adsorption.[16] They act as weak Lewis bases which give the dioxin as additional set of sites for localization onto alumina surface. On the other hand, PCBs which lack oxygens are eluted earlier than dioxins.

Dichloromethane was chosen as the modifier to hexane for increasing solvent strength in this study. HxCB and dioxins were eluted in fraction I using 50 mL of hexane and fraction II using 40 mL of the mixture of hexane/dichloromethane (9/1,v/v), respectively (Table 2). Chromatograms obtained by this method are shown in Figure 3(a) and 3(b). Dioxins were separated from HxCB without interference.

Carbon column clean-up. After multilayer silica gel clean-up, the carbon clean-up instead of alumina clean-up was followed for the specific fractionation of planar aromatic molecules such as dioxins. The retention of molecules is based on the coplanarity of closely situated aromatic systems between carbon and molecules and is increased by

Table 3. Recoveries of dioxins spiked to milk (N=3)

PCDDs	levels of dioxins spiked to milk (ppt)	Recovery (%) ±RSD (%)
1,2,3,4-TCDD	75	88.2±14
1,2,3,4,7-PeCDD	75	83.3±4.1
1,2,3,4,7,8-HxCDD	75	92.7±5.0
1,2,3,4,6,7,8-HpCDD	75	98.7±14
OCDD	300	98.9±9.9

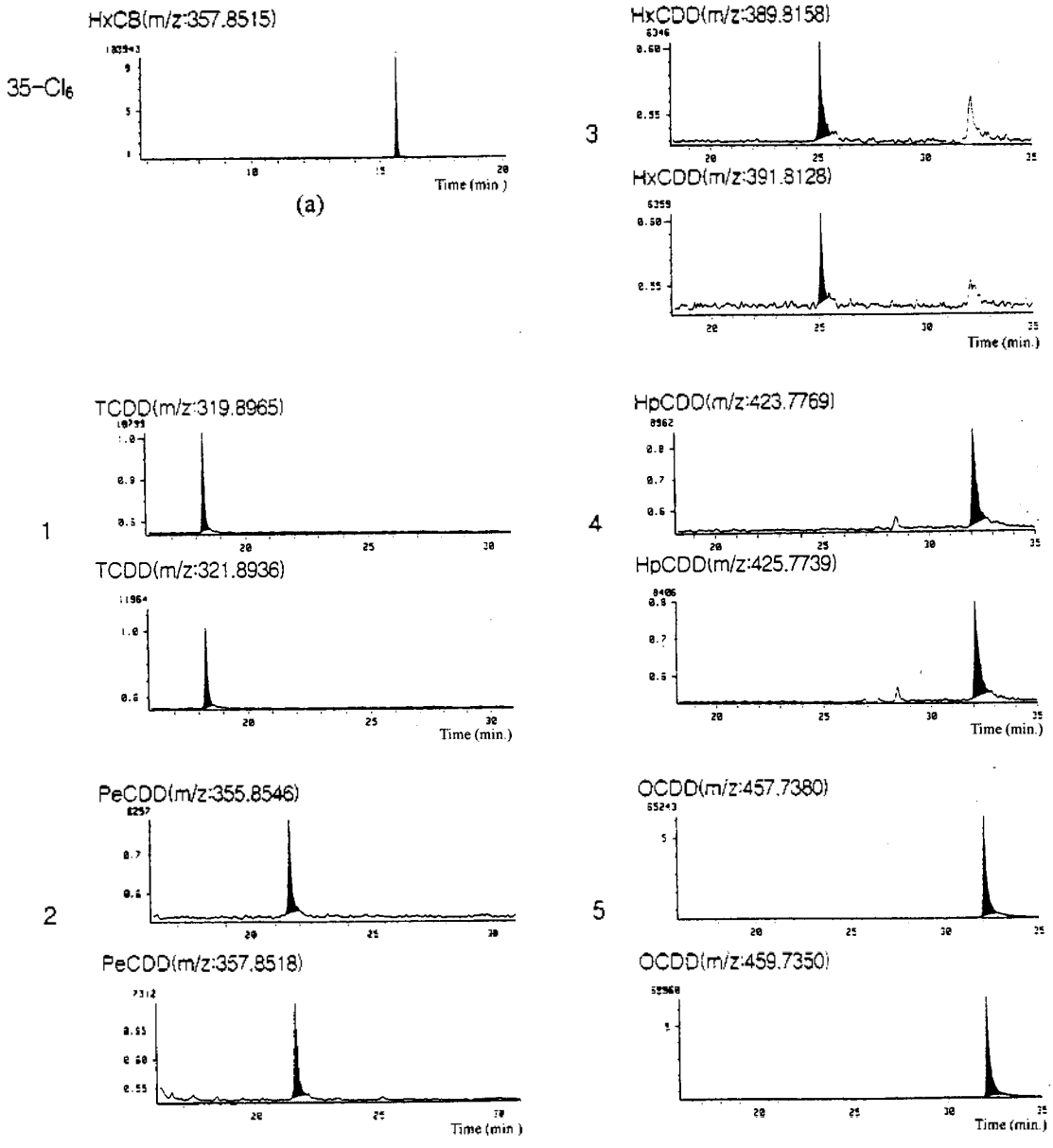

Figure 6. Selected ion HRGC/HRMS chromatogram of HxCB and dioxins of cleaned extract from milk. (a) fraction (I) of 50 mL hexane, HxCB (M=357.8515) (b) fraction (II) of 40 mL mixture of hexane/dichloromethane (9/1), Peak numbers refer to Figure 2.

electronegativity of chlorine, bromine and nitro substituents of molecules on the aromatic systems.[19,28] But the results allowed us to check the efficiency of the carbon clean-up in the process. Chromatogram of Figure 4 showed the similar result to that obtained after alumina clean-up (Figure 3-b). It was rather worse to present unknown peak by concentration of large volume of solvent.

Carbon column clean-up resulted in a lengthy procedure using several eluents of large volume. It is due to the high affinity of coplanar compounds to the carbon and the inhomogeneity of the active sites of the carbon. This is the reason why the optimization of the experimental conditions for carbon clean-up was difficult.

HPLC column clean-up. After multilayer silica gel clean-up, the HPLC clean-up instead of alumina clean-up was followed. HPLC clean-up has a high separation potential and its process can be monitored directly by UV detection.[10] Figure 5 shows the chromatogram of eluate after HPLC clean-up. The result was very similar to that of carbon clean-up. Though HPLC clean-up was relatively simple, the additional drying procedure of the eluate by sodium sulfate was needed because of water contained in mobile phase.

Application to milk sample. The clean-up procedure of acid treatment, multilayer silica gel and alumina column were applied to the determination of dioxins in milk sample. The recoveries of dioxins spiked to milk at lower ppb level were 83.3-98,9%. The results are given in Table 3. In order to confirm dioxins, HRGC/HRMS analysis of eluate after alumina clean-up was performed. HRGC/HRMS chromatogram is shown in Figure 6.

Conclusions

In this study, the efficiency of clean-up methods for the analysis of dioxins in milk sample was examined. LLE method was used to extract lipid phase containig dioxins from milk. Acid treatment and multilayer silica gel column clean-up were followed for elimination of lipids and polar interferences from extract. Alumina column clean-up was required to separate the remaining chlorinated organics and dioxins. The surface activation of alumina and strength of eluting solvent were considered for optimization of separation in alumina clean-up. Alumina activated at 180 °C over 12 hours and 50 mL of hexane followed by 40 mL of hexane/dichloromethane (9/1, v/v) have been required for the separation of PCB and dioxins at 7.5-30 ng level. Though carbon column and HPLC column clean-up showed good separation of PCBs and dioxins, these methods were lengthy procedures and needed the consumption of toxic solvent. The clean-up methods of acid treatment, multilayer silica gel and alumina column were applied to milk containing dioxins spiked at 7.5-30 ng level. The recovery and relative standard deviation of dioxins from milk sample were 83.3-98.9% and 4.1-14%, respectively.

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