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Determination of Some Congeners of Polychlorinated Dibenzo-p-Dioxins (PCDDs) in Milk Using Gas Chromatography

Iffat Abdul Tawab Khan · Zahida Tasneem Maqsood

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Abstract A method for determination of four congeners of polychlorinated dibenzo-p-dioxins (PCDDs) namely, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 1,2,3,7,8-pentachlorodibenzo-p-dioxin (PeCDD), 1,2,3,6,7,8-hexachlorodibenzo-p-dioxin (HxCDD) and 1,2,3,4,6,7,8-heptachlorodibenzo-p-dioxin (HpCDD) in milk is presented. Limit of detection (LOD) was found to be 0.002 ng for PeCDD while for other three it was found to be 0.005 ng. Recoveries for PeCDD were checked by spiking the milk at 0.020, 0.050 and 0.10 ng g^{-1} levels and recovered in the range of 81.03%–120.17%. TCDD, HxCDD and HpCDD were checked at 0.05, 0.10 and 0.5 ng g^{-1} spiked levels and recovered in the range of 80.47%–133.30%, 88.40%–128.02% and 76.97%–132.55% respectively. Limit of quantification (LOQ) was found to be 0.1 ng g^{-1} for PeCDD whereas 0.5 ng g^{-1} for others. %RSD was in the range of 4.30–15.79.

Keywords PeCDD · TCDD · HxCDD · HpCDD · LOD · LOQ

Milk occupies an important place particularly in the diet of infants and children and plays a pivotal role in their growth and development. The presence of PCDDs in milk is, therefore, toxic and hazardous. Because of their chemical stability, refractivity to biotransformation and lipophilic nature, they tend to be biomagnified and accumulate in the food chain and can persist in fat and mammary glands of animals and pass out in milk (Vieth et al. 2000). Over 98% of human intake of dioxins is via food, especially from beef meat and milk. The dioxins deposit on hay and feed crops are consumed by cows, which concentrate the dioxins in their fat tissues. Thus the general public is exposed to dioxins primarily through consumption of fish, meat and dairy products (Taioli et al. 2005). It is therefore, essential to obtain information on residue levels of them in milk in order to assess their health significance. PCDDs have been detected in cow and human's milk samples in various monitoring program conducted in several countries like Japan (Saito et al. 2005), in Taiwan (Hsu et al. 2007), in Italy (Taioli et al. 2005), in Spain (Eljarrat et al. 2002; Ramos et al. 1997) and in Germany (Vieth et al. 2000). Rodas–Ortiz et al. (2008) and Raab et al. (2008) also investigated the organochlorinated compounds in human milk. Thus there was a need to develop multiple detections system for all congeners of all groups of dioxins in Pakistan. The objective of the present study was to develop a systematic method for analyzing four selected congeners of PCDDs in milk. The next step will be monitoring but at present it is very difficult to conduct because of unavailability of more sensitive and modern techniques for clean up and quantification.

The four congeners of polychlorinated dibenzo-p-dioxins (PCDDs) were selected for this study, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 1,2,3,7,8-pentachlorodibenzo-p-dioxin (PeCDD), 1,2,3,6,7,8-hexachlorodibenzo-p-dioxin (HxCDD) and 1,2,3,4,6,7,8-heptachlorodibenzo-p-dioxin (HpCDD). No technical data in this regard is available yet in Pakistan. In order to achieve the goal for proper recommendations, knowledge of these aspects is very essential. In the present study samples of milk were screened for a mixture of four selected congeners of PCDDs gas chromatography with electron capture detector.

I. A. T. Khan (✉) · Z. T. Maqsood
Department of Chemistry, University of Karachi, Karachi 75270, Pakistan
e-mail: iffku@yahoo.com

Materials and Methods

Petroleum ether, dichloromethane, concentrated sulphuric acid; sodium chloride and anhydrous sodium sulphate were of analytical reagent grade and were purchased from Merck (Darmstadt, Germany). Aluminium oxide pH 4.5 ± 0.5, activity 1 was of analytical reagent grade purchased from Faluka Laboratory Supplies (Switzerland). Anhydrous sodium sulphate was dried at 120°C while aluminium oxide activated at 450°C for 3 h. Cotton wool washed with dichloromethane prior to analysis. PCDDs congeners were purchased from Wellington laboratories (www.well-labs.com) (Canada) with concentration of 50 ± 2.5 μg mL^{-1}. Stock standard solutions of the congeners were prepared 10 μg mL^{-1} in n-hexane. Stock and working standard solutions were kept at 4°C protected from light (as recommended). Then mixture of working standard solutions was prepared as per requirement from the pesticide stock solution in hexane or acetone. These solutions were all stable for at least 1 month if stored in the dark at 4°C.

A gas chromatograph (Agilent technologies 6890 N, USA), equipped with an auto injector (7683 series) and Ni^{63} electron capture detector, for data collection a software enhanced data analysis was present in the system. The laser-jet printer (Hewlet Packard 2300) and capillary column of 30 m × 0.25 mm id (HP-5MS) with film thickness 0.25 μm was used. Operating parameters were set as 280°C for injector, for column oven 250°C with nitrogen (99.9995% pure) flow rate 0.5 mL min^{-1} and for detector 300°C. Makeup flow for nitrogen was set as 60 mL min^{-1} and split less injection mode was used.

Milk was drawn from local supplier and treated as control. Then 10 g milk was taken in triplicate for each experiment and known amount of mixture of standard congeners was added to milk and shaken for five minutes. The fortified sample was allowed to stand for half an hour at room temperature so that the congeners were thoroughly absorbed. These samples along with a blank (only solvent passed through the all steps) as well as control sample (the sample free from any pesticides) were then passed through the following procedure and finally analyzed for percent recovery by GC.

10 g of milk was transferred into a 100 mL separatory funnel. 5 mL concentrated sulphuric acid was slowly added drop wise through the side of a separatory funnel, which was then fitted with a glass stopper. If acid is added to fast or allowed to drop directly into the milk, there may be a violent reaction, which may decrease extraction efficiency. Acid forms lower layer. Acid and milk were shaken with a gentle rotary movement until a homogenous mixture was formed and curd particles no longer visible. During mixing, the separatory funnel was kept dipped in iced cold water in order to keep its temperature under control. After mixing, the solution was allowed to cool at room temperature and the contents transferred to a 100 mL conical flask. 20 mL petroleum ether was added to the mixture and the flask was shaken for 5 min on an electric shaker. The contents were again transferred to the separatory funnel and waited until two layers clearly separated. In some cases an emulsion was formed which did not break of its own. In such cases, 5 mL saturated solution of sodium chloride was slowly added from the sides, the contents shaken gently and the layers allowed to separate. Petroleum ether layer was collected while lower aqueous layer was drawn in a conical flask, process repeated twice with 15 and 10 mL petroleum ether. Aqueous layer was finally discarded in each case and different portions of organic layer were combined. Since the organic layer so obtained was colored, 5 mL of concentrated sulphuric acid was added to the organic layer for decolorisation, shaken thoroughly and layers separated. Acid layer was discarded in each case. Total volume of extract in each case was noted and dried by passing through a polypropylene mini column (1.2 cm id × 10.5 cm long) containing about 4 gm of anhydrous sodium sulphate. For quantitative extraction, sodium sulphate in the column was washed with a little petroleum ether, which was then combined with the original extract. It was then concentrated down to about 1 mL in a rotary vacuum evaporator at 40°C. The method was further little bit modified with addition of propylene mini column containing acidic aluminum oxide to remove the remaining fat and co-extractives in the extract because insufficient cleanup of sample causes rapid deterioration of GC system especially ECD thereby precluding reliable results.

For column clean-up a polypropylene mini column (1.2 cm id × 10.5 cm long) was plugged with cotton, a small quantity of anhydrous sodium sulphate was poured into it and then about 4 g mixture of activated aluminium oxide was added to it with continuous tapping. Little amount of anhydrous sodium sulphate was again added to the column so as to form a bed on the top of the column. Two same columns were used for single analysis (doublet). These were arranged in an iron stand using two clamps in up and down position after washing individually. The columns have been pre-washed with 10 mL dichloromethane and the 1 mL concentrated extract was then transferred on to a polypropylene mini column. 15 mL eluate was collected with flow rate adjusted to 1 mL min^{-1} and concentrated to dryness. The residues were dissolved in 1 mL hexane or acetone. Then injected 1 μL of dissolved residues to gas chromatograph through auto injector and peaks areas were compared with those obtained from similar injections of mixture of standards.

Results and Discussion

Method of Zahida and Zafar (1988), which applied for organochlorine pesticides was followed with slight modification. The cleanup step was little bit modified with addition of propylene mini column to remove the remaining fat and coextractives in the extract because insufficient cleanup of sample causes rapid deterioration of GC system especially ECD thereby precluding reliable results. Blank as well as control sample also treated in the same manner which did not show any contamination peaks except of two peaks which appeared just after solvent peak but those could not be attributed to the studied congeners. For the quantitative determination, electron capture detector was used. The detection limits were found to be 0.005 ng for three congeners; TCDD, HxCDD and HpCDD whereas 0.002 ng for PeCDD based on signal-to-noise ratio (S/N) of $\geq$3. Linear calibration curves were obtained for all of the four congeners constructed by plotting the analyte concentration against peak areas under the proposed chromatographic conditions. Statistical parameters (linear ranges, correlation equations with correlation coefficients) derived were presented in Table 1. Which show that a good linearity was achieved from five point calibration curves for selected calibration range. Quantitative analysis was performed by the external standardization method. Accuracy depends mainly on the ability to inject exact amounts of the sample with a syringe. In present study auto sampler was used and it was previously calibrated. Three sample injections were carried out on each extract to check re-producibility of results.

Percent recoveries were checked for TCDD, HxCDD and HpCDD applied fortification levels on milk 0.05, 0.1 and 0.5 ng g^{-1} whereas 0.02, 0.05 and 0.1 ng g^{-1} levels applied to PeCDD. Therefore, LOQ of the method was found to be 0.5 ng g^{-1} for TCDD, HxCDD and HpCDD and 0.1 ng g^{-1} for PeCDD based on signal to noise ratio $\geq$10. Recoveries and precision obtained for selected fortification levels for milk were listed in Table 2, which show that the average percent recoveries of the studied congeners at LOQ, determined by the method fell within satisfactory range. PeCDD was recovered in the range of 81.03%–120.17% with %RSD range 4.87%–10.25%. TCDD, HxCDD and HpCDD were recovered in the range of 80.47%–133.30%, 88.40%–128.02% and 76.97%–132.55% respectively and %RSD values were in the range of 4.30–11.85, 9.96–15.79 and 4.97–11.35 respectively.

These results show the method has relatively good reproducibility. The above data show that recovery of pesticides is quite satisfactory. It is interesting to note in all cases at lower fortification levels the percentage recovery decreases. It is due to the fact that at lower residue

Table 1 Statistical parameters for calibration of studied congeners

Congeners	Linear range (ng)	Correlation equation	Correlation coefficient
TCDD	0.0050–0.50	$y = 808.2 + 16265950x$	0.9991
PeCDD	0.0020–0.10	$y = 733.3 + 58882431x$	0.9989
HxCDD	0.0050–0.50	$y = 8765.9 + 23045998x$	0.9988
HpCDD	0.0050–0.50	$y = 457.7 + 14346032x$	0.9976

Table 2 Fortification levels, percent recoveries and percent RSD values for studied congeners in milk

Congeners	Fortification levels (ng g^{-1})	Recoveries (%)	Mean (%)	RSD (%)
TCDD	0.05	111.18, 147.08, 141.62	133.30	11.85
	0.1	121.54, 118.76, 131.19	123.83	4.30
	0.5	79.44, 88.03, 73.95	80.47	7.20
PeCDD	0.02	125.74, 131.88, 103.21	120.17	10.25
	0.05	129.64, 105.21, 122.26	119.04	8.60
	0.1	76.45, 86.07, 80.57	81.03	4.87
HxCDD	0.05	126.33, 104.15, 153.59	128.02	15.79
	0.1	140.87, 104.03, 128.05	124.32	12.28
	0.5	100.03, 86.46, 78.71	88.40	9.96
HpCDD	0.05	139.40, 137.72, 120.52	132.55	6.43
	0.1	131.16, 128.55, 116.81	125.51	4.97
	0.5	82.14, 84.10, 64.67	76.97	11.35

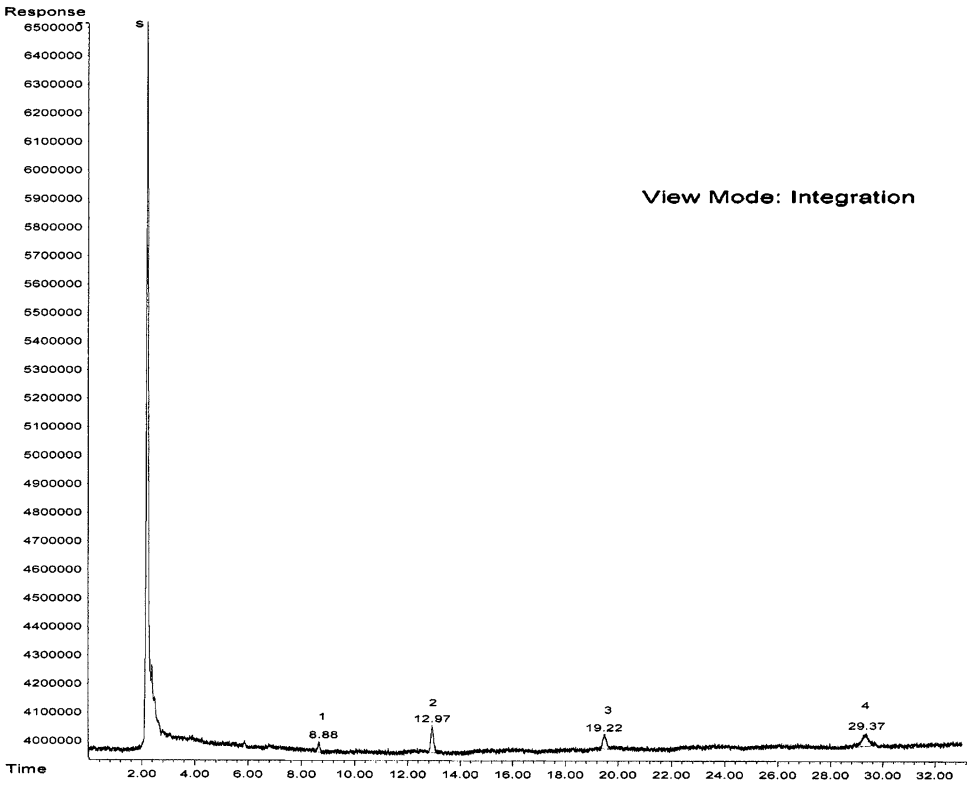

Fig. 1 GC-ECD chromatogram obtained for studied congeners of PCDDs (0.002 ng each)

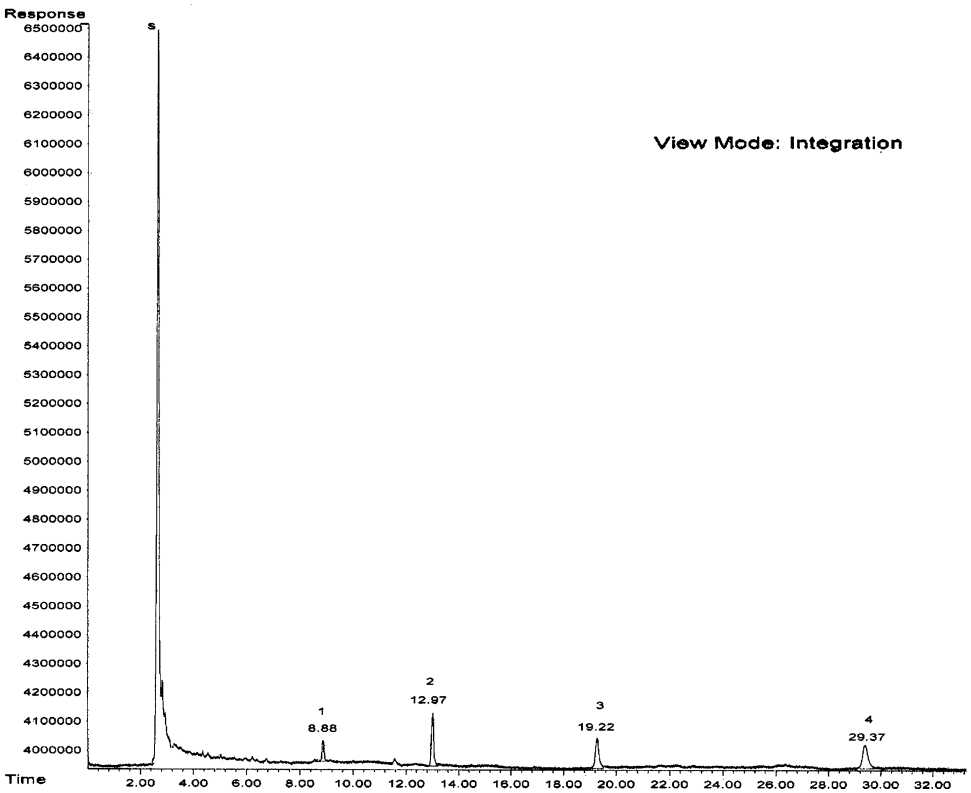

Fig. 2 GC-ECD chromatogram obtained for studied congeners of PCDDs (0.005 ng each)

concentrations chances of error are usually enhanced but the reported results are well within the range of permissible error. Gas chromatogram of a mixture of studied congeners 0.002 ng and 0.005 ng of each standard are presented in Figs. 1 and 2 respectively. Gas chromatogram of a fortified milk sample is presented in Fig. 3.

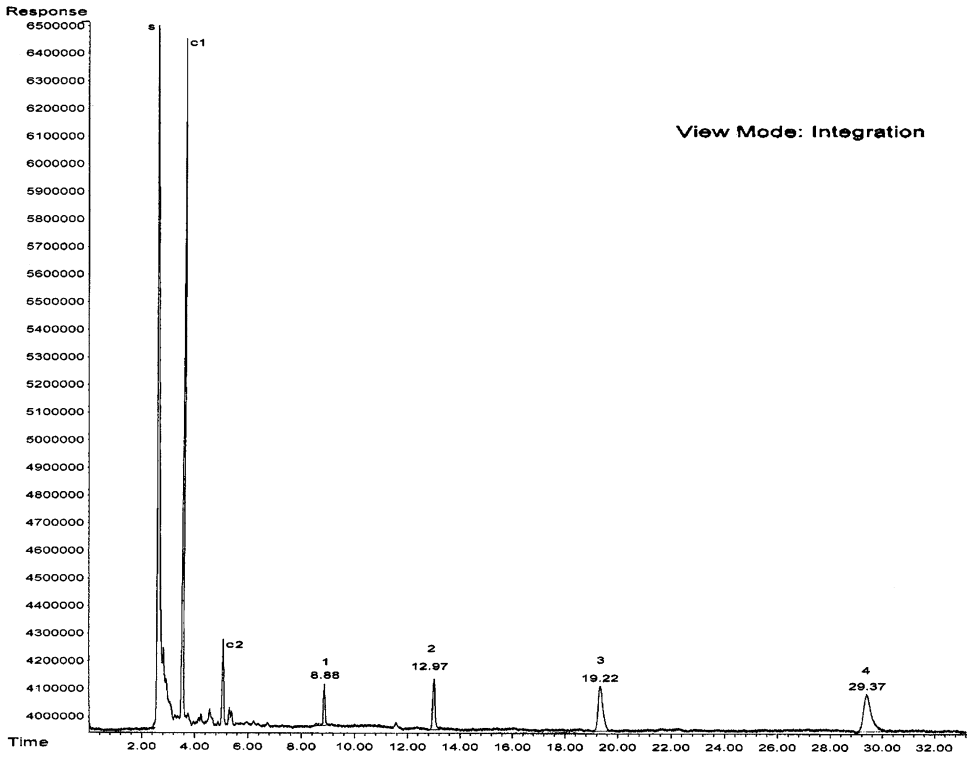

Fig. 3 GC-ECD chromatogram obtained for fortified milk sample, Fortification level: TCDD, HxCDD, HpCDD = 0.50 ng g^{-1}; PeCDD = 0.10 ng g^{-1}; *1* TCDD, *2* PeCDD, *3* HxCDD, *4* HpCDD; *s* peak of solvent; *c1* and *c2* contamination peaks

In the present study, an evaluation of several parameters involved has been undertaken with the goal to achieve good recoveries and at the same time, making maximum lipid removal possible. We have developed an easy and inexpensive method for determination of some congeners of PCDDs in milk first time in Pakistan. This study focused on easy operation. Recommended value for allowable/tolerable daily intake (ADI/TDI) is 3 pg according to European commission regulations (2001) that is too far from our quantification limit but this target also can be achieved by applying more sensitive and automated techniques for clean up and quantification.

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