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Determination of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) levels in packed and raw cow milk from Thrissur, Kerala

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ABSTRACT

The everincreasing incidences of contamination of food from different sources form an area of concern and dioxins are one of the major contaminants of dairy products. The present study was conducted to determine the presence of TCDD in cow milk used for consumption in Thrissur District of Kerala. The milk samples were subjected to liquid: liquid extraction, silica gel clean up and injected into a gas chromatograph mass spectrometer at EI mode. Single reaction monitoring programme was done. Two brands of packaged milk were found to have the presence of TCDD at 3.11 and 24.49 ng/g of fat which exceed the international standards of residue levels.

Keywords: Milk, Dioxin, Gas chromatography mass spectrometry.

INTRODUCTION

Dairy products form an important part of food of people in Kerala. Being a small state with limited animal population, people often depend on packed pasteurised milk for their use which come from local produce as well as from neighbouring states. There are various reports stating that the raw as well as pasteurised packed milk form a source of dioxins to human beings. According to World health Organisation, dioxins especially 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is one of the most potent carcinogens known. It is an endocrine disrupting chemical with immunosuppressing, teratogenic and mutagenic properties. It is also linked with various life style diseases including cardiovascular, diabetic and other complications. No authentic work has been carried out in southern part of India for the detection of dioxins in the food of animal origin. Hence this work was undertaken to screen the cattle milk available in Thrissur district of Kerala for the presence of TCDD.

MATERIAL AND METHOD

2.1 Sample collection

Fresh milk samples were collected from cattle Farm, College of Veterinary & Animal Sciences Mannuthy in clean glass bottles and Standard of 2,3,7,8 tetrachloro dibenzo-p-dioxin (TCDD) containing 10µg/mL in toluene from M/ s SIGMA ALDRICH was spiked with 100, 50, 25, 10 and 1 ppb of TCDD as described earlier. They were vortexed for 10 minutes and allowed to stand overnight and then processed as described and injected into GCMS to find out the recovery percentages.

2.2 Extraction and clean up

Extraction of TCDD from milk samples was done as per the method adopted by Kawashiro *et al.* 2008 and Rezaei, *et al.* 2015 with minor modifications. In short 100 mL of the collected milk sample was taken in a 250 mL separating funnel and added 4 mL of 25 per cent calcium oxalate. Then 100 mL of ethyl alcohol, 50mL of diethyl ether and 60 mL of n-hexane were added to the milk successively, mixed well, shaken vigorously many times and kept 3 hours for complete extraction into the solvent phase. The organic layer was collected in an amber collected glass stoppered bottle and additional 60mL of hexane was added and shaken vigorously for a minute and the process was repeated thrice. The organic layer was then washed with 25mL each of 5,10 and 20 per cent sodium chloride solution and was dehydrated by passing through sodium sulphate. Then it was evaporated in a rotary vacuum evaporator and the weight of lipids was calculated. Immediately afterwards, it was dissolved in 4 mL of n-hexane and used for clean up protocols. Each sample was extracted in triplicate. Two connected chromatographic columns were used for the clean up[2] The upper column was filled with 2g anhydrous sodium sulphate, 1 g silver nitrate silica gel, 0.5 g silica gel, 0.5 g 44 per cent sulphuric acid silica gel, 0.5 g 0.22 percent silica gel, 1 g anhydrous silica gel and lower column with 1g anhydrous sodium sulphate, 0.5 g activated carbon silica gel and 1g anhydrous sodium sulphate. The concentrated extract which was dissolved in four milli litres of hexane was percolated with 50 mL hexane in the columns and were collected by passing 50mL dichloromethane- hexane (3:1) and 130 mL toluene respectively through lower column, then concentrated in a rotary vacuum evaporator and eluted in one milli litres of toluene.

2.3 Calibration curve

TCDD standard (1µg/mL) in toluene purchased from sigma was diluted to 250, 200,150,100 and 50ppb and injected into the gas chromatograph mass spectrometer as detailed below. A standard graph was plotted by using the software and regression equation was fit in.

Gas chromatography

TCDD was analysed in a gas chromatography Mass spectrometers attached to RXi5 30mx0.25m column having an internal diameter of 0.25µm. Helium was used as the carrier gas with a flow rate of 1 mL /min. The column oven temperature was maintained at 120°C for three minutes. Then, it was programmed to 210°C at a ramp rate of 19°C/minute, then to 275°C at a ramp rate of 3°C/minute and the final temperature 300°C at a ramp rate of 25°C/minute. Injector temperature and detector temperature were optimized at 250 and 280°C, respectively. The MS operating parameters were as follows: ionization energy, 70 eV; ion source temperature, 270°C; solvent delay, 1.0 minutes. Total run time was 30.4 minutes. Selected reaction monitoring (SRM) was done for precursor mass of 321.89m/z and product mass of 258.93 m/z. Additional scanning was done for precursor mass of 319.89m/z and product mass of 256.93m/z to confirm the presence or absence in the samples. The collision energy was 20.00eV. The SRM time was 0.300 seconds.

RESULT AND DISCUSSION

3.1 Analytical method standardisation

Gas chromatography mass spectrometry (GC MS) was used for the estimation of TCDD and Single Ion monitoring was done for detection and analysis. Initially 500 ppb of the standard was injected using toluene as solvent and the same was detected and retention time (Rt) was measured. The procedure was repeated and then the Rt value was confirmed. Under the standard operating conditions of GC-MS, the retention time of TCDD was 19.64. Linearity of the isomers were confirmed at different concentrations of the standard (50, 100, 150 and 250 ppb) and the results are shown in Fig 1. The limit of detection was found to be 0.1ppb where as the limit of quantification was found to be 2 ppb.

3.2 Recovery study

The procedures for extraction and clean up of field milk samples were tested for recovery percentage by

fortifying fresh milk samples from University livestock farm, Mannuthy using 100, 50, 25, 10 and 5 ppb of standard of TCDD in toluene. Recovery studies revealed that the percent of recovery ranged from 78 to 90 per cent (Table 3.1 and Fig 1.).

3.3 Sample collection

The samples were collected during a 6 month period from March 2018 to September 2018 and were processed immediately on the day of sampling. A total of fourteen different brands of packaged milk were available for consumers in Mannuthy. Atleast three brands provided their milk in different ranges of fat percentage ranging from 3.5 per cent to 7 per cent. A total of twelve different packets were collected from twelve different brands. Samples were collected consecutively for twelve days from two brands and processed simultaneously and the complete processing of samples of packaged milk were done as six batches.

Table 3.1 Recovery percentage of different concentrations of standards of TCDD (per cent)

Sr.No	Concentration added (ppb)	Concentration detected (ppb)	Percentage of recovery (%)
1	100	79.79	79.79
2	50	42.24	84.48
3	25	22.285	89.14
4	10	7.843	78.43
5	5	4.503	90.06

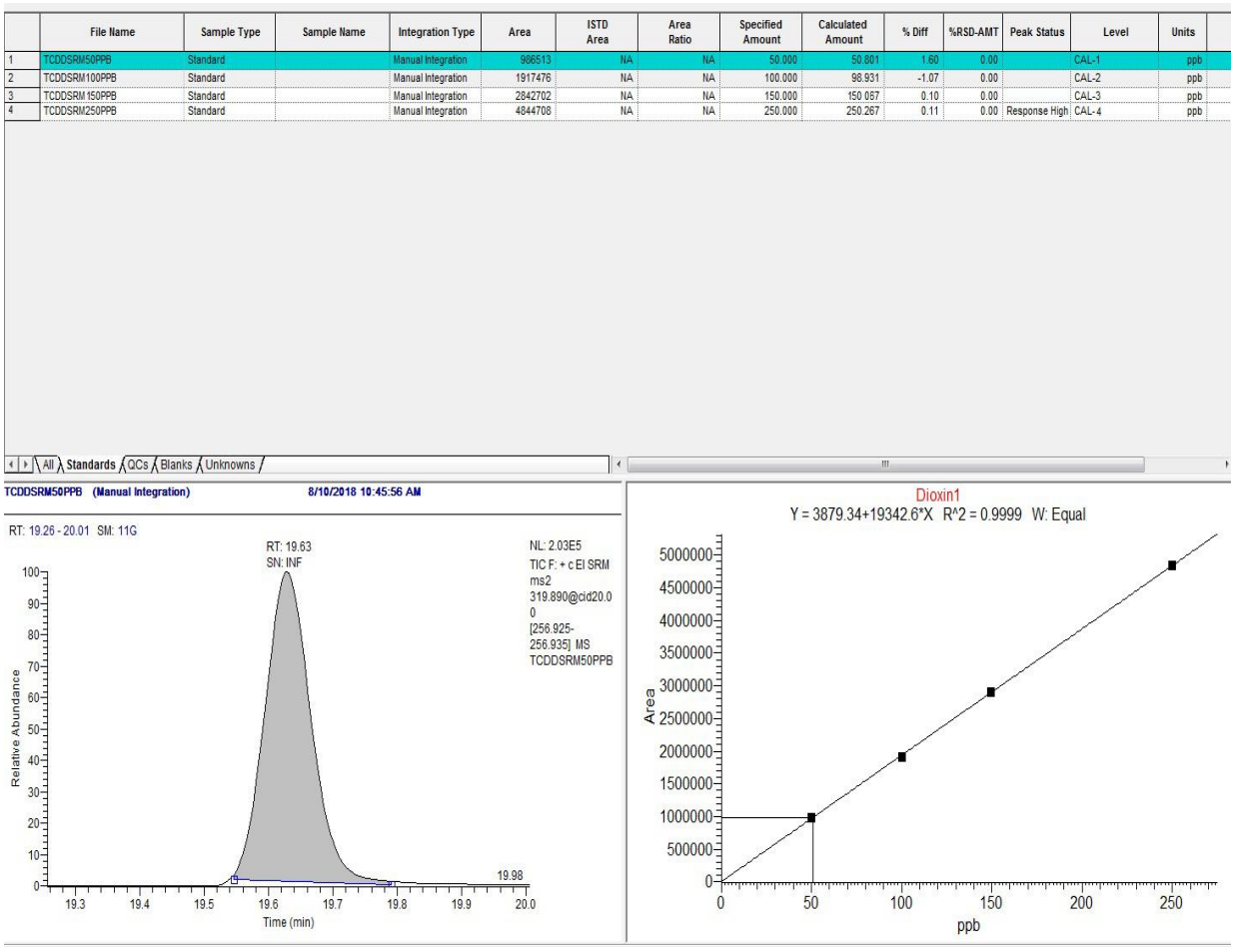
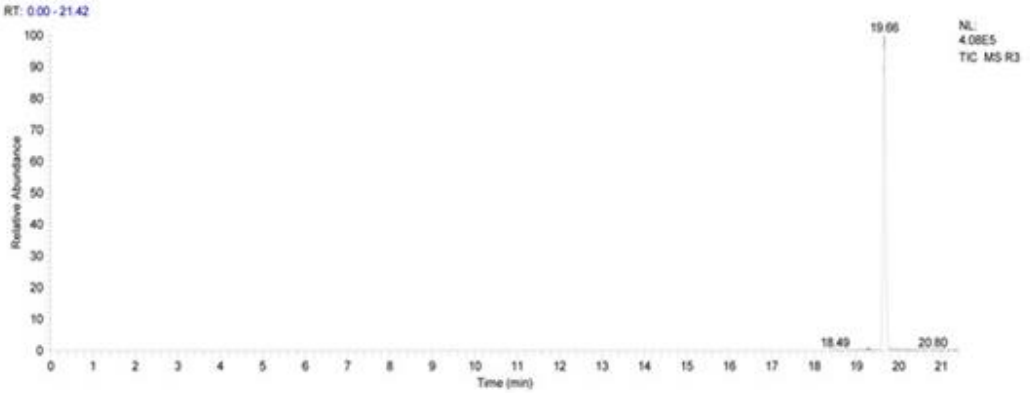
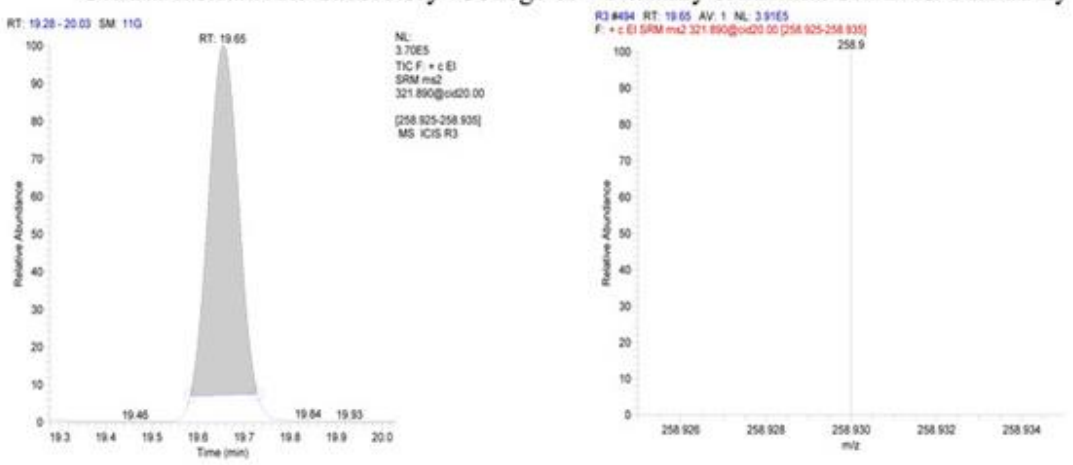


Fig 1. Linearity of standards of TCDD injected



Component Name	RT	Calculated Amount	Units
Dioxin2	19.65	79.788	ppb
Dioxin1	19.66	76.683	ppb

Central Instruments Laboratory- College of Veterinary & Animal Sciences- Mannuthy



Component Name:	Dioxin2	Actual RT(min):	19.65
Area(cts-sec):	1481173.76	Signal To Noise:	4270.98
Calculated Amount:	79.79		

Fig 2. Recovery per centage after spiking with 100ppb TCDD

The sample processing took two days of clean up for each batch. Those packets from which milk were collected on a day were resealed and refrigerated and the same was used on the third day for heating experiments. One brand formed a single pool and was injected as single unit into GC-MS. Fresh milk samples were collected from small holders and milk societies of different areas in Thrissur district. Samples were collected from morning milking and transported in glass containers and were used for processing without any storage. A total of 240 samples were collected from twelve different destinations. Each destination was considered as a lot for injection into GCMS. All the extracted samples from a single lot was pooled together and mixed thoroughly and 1 mL from the same was used for injection into the GCMS.

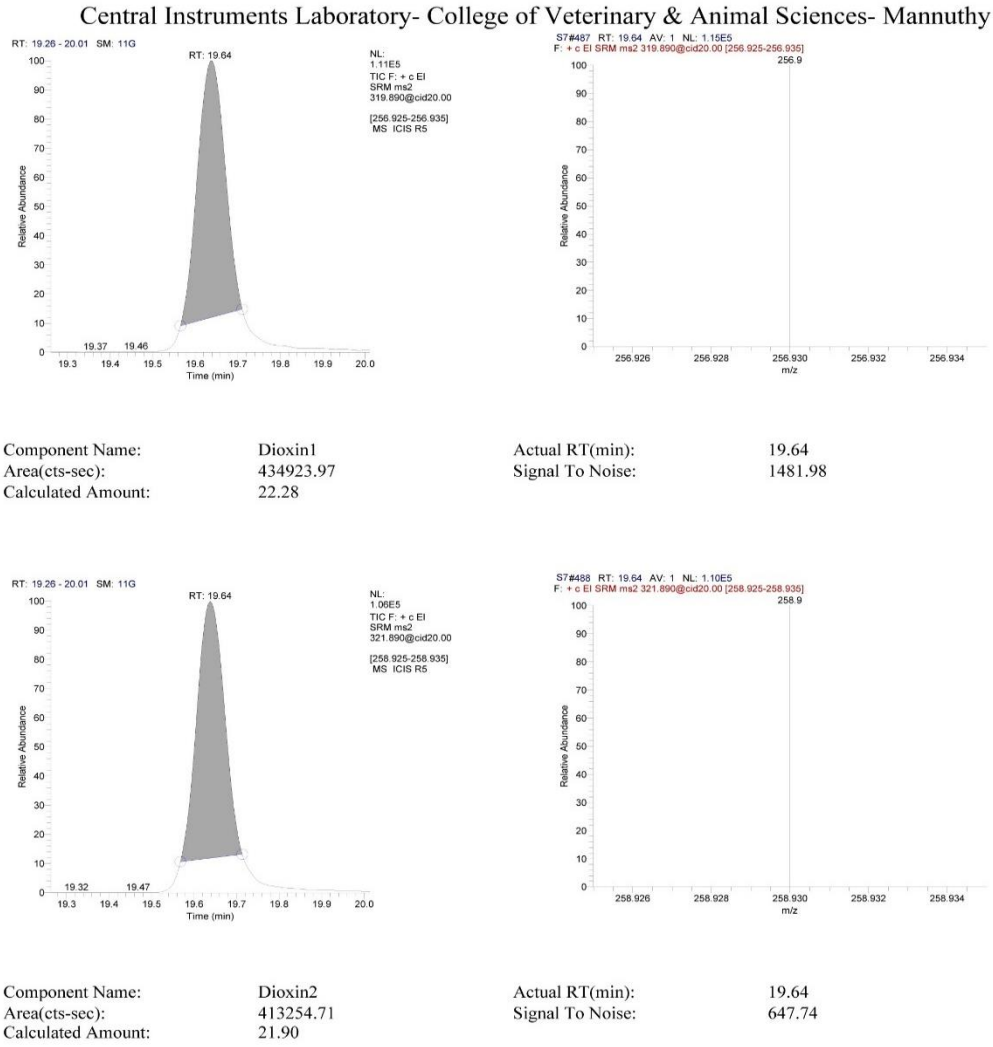


Fig 3. Chromatogram of sample tested positive for TCDD

3.4 Detection of dioxin from samples

Of the twelve brands of packaged milk tested for TCDD, two brands were tested positive where first brand contained 3.11 ng/g where as other contained 24.49 ng/g of fat (Fig 2 and 3). TCDD could not be detected from any of the raw milk samples collected from twelve different destinations of Thrissur district.

DISCUSSION

There are several reports of the presence of TCDD in packaged and raw milk samples. Maximum levels of TCDD was seen in milk of cattle near the chemical plant in Sevoso Italy which suffered accidental leakage of the toxicant to atmosphere and upto 7 ppb was found in human beings and the levels were attributable to the levels in dairy products (Fanelli *et al* 1980). There was presence of PCDD, PCDF in milk from animals near industrialised areas, vicinity of metal reclamation plants and cardboard packed milk and about 20 per cent of TCDD was carried over from paper container to milk (Beck *et al.* 1990). Baars *et al.* 2004, reported the presence of dioxins, dioxin like PCB's and non-dioxin like PCB's in food stuffs of Netherlands and found that the dioxin level was less than 3pg/g of fat in the case of dairy products. The maximum limits of dioxins in meat and milk of bovines stipulated by European Union were 3.0 pg TEQ/g fat where as the levels for poultry meat and eggs were 2.0 and 3.0 pg TEQ/g fat respectively.

Dioxins that are released from combustion of chlorine containing materials get deposited in plants and water forming a source of entry into food chain. Animals that feed on contaminated roughage or soil accumulate dioxins in their fat and human beings get exposure from consumption of such animal produce (Fries, 1995). Douben *et al.* 1997 explained that the presence of dioxins in the surface of leaves were mainly due to air borne or uptake except for cucrbits and this forms with negligible soil uptake and this forms the major source of poisoning in cattle and human beings. Transfer of dioxins from atmosphere to grass was proportional to the fallout from atmosphere where in about 35 per cent is retained in grass. Even though there was variations in deposition in plants and transfer rates, the mean concentration of dioxins in milk during were not affected (Schuler *et al.* 1997).

The results of the present study along with various other reports cited earlier shows that TCDD at the concentrations detected act as an endocrine disrupting agent. Hence, a general awareness should be created among public on the ways of reducing the levels of dioxins that are released into environment by unscientific waste management.

CONCLUSION:

From the study, it could be concluded that there is presence of TCDD in the packaged milk samples with concentrations ranging from 3.11 to 24.49 ng/g of fat. Eventhough the incidence is only less, screening for presence of TCDD should be made routine so as to reduce the chances of ill effects on human beings. Further studies need to be carried out to find out the source of contamination and incidence of other dioxins or polychlorinated biphenyls in milk.

Conflicts of Interest: The authors declare no conflict of interest.

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