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# Characterization of 2,3,7,8-Substituted Chlorodibenzo-*p*-dioxins using LRGC-MS/MS

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Exquisitely sensitive and specific GC-MS capable of resolving low levels of dioxins and potential toxic congeners in environmental matrices is must. The work aims to resolve the most toxic 2,3,7,8-substituted dibenzo-*p*-dioxins in soil sample by low resolution gas chromatography coupled to ion-trap mass spectrometry (LRGC-MS/MS). In particular, the study mainly focuses on simple and efficient method for analyzing 2,3,7,8-tetrachlorodibenzo-*p*-dioxins (2,3,7,8-TCDD) using different extraction methods. This paper illustrates optimization steps required for 2,3,7,8-TCDD analysis using LRGC-MS/MS. Finally, quality control measures include the calibration exercises. Successful results were achieved using LRGC-MS/MS in the analysis of simulated soil samples. In general, LRGC-MS/MS in conjunction with rotatory shaker extraction method constitutes an interesting and simple alternative for routine analysis of such matrices.

**Key Words: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxins, GC-MS/MS, Soxhlet extraction, Rotatory shaker extraction, Standard deviation.**

## INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxins (2,3,7,8-TCDD) has been recognized as an extremely toxic[1], teratogenic, mutagenic[2] and possibly carcinogenic[3] compound stable in biological systems and pose a threat to life and environment. WHO has set a tolerable daily intake (TDI) of 1-4 pg/kg bw/d. Each of the seventeen highly dioxins/furans are assigned as Toxic Equivalency Factor (TEF) based on a particular chemical's toxicity relative to 2,3,7,8-TCDD, with the toxicity of TCDD being equal to 1.0. Because of the extreme toxicity of 2,3,7,8-chlorine substituted dibenzo-*p*-dioxins, sensitive and specific analytical technique for measurement is required.

The analysis at parts per trillion levels of environmental samples is complicated due to multitude of interfering compounds ranging from natural products to industrial pollutants. The paper describes an approach on the sample processing, separation techniques followed by GC-MS for the analysis of 2,3,7,8-TCDD in environmental samples. The careful statistical examination of the data has been made in the selection of efficient and specific sample preparation procedure.

## EXPERIMENTAL

A gas chromatograph/mass spectrometer (GC-MS) with data system, Varian Saturn 3800 GC was used in 2,3,7,8-TCDD analysis. A fused silica column (25 m × 0.32 mm ID) coated with BP-5, 1.0 μm film thickness has been used to isolate 2,3,7,8-TCDD.

**Gas chromatograph-mass spectrometer interface:** The gas chromatograph coupled directly with a Saturn 2200 ion-trap mass spectrometer source has been used.

**Mass spectrometer:** The static resolution of the instrument has been maintained at a minimum of 1 amu. The mass spectrometer has been operated in a full scan Electron Impact (EI) mode.

**Data system:** A dedicated computer data system has been employed to control the rapid multiple ion monitoring process and to acquire the data. Quantification of data (peak areas) and EI traces (displays of intensities of each m/z being monitored as a function of time) has been acquired during the analyses. Quantifications have been reported based upon the computer-generated peak areas.

**Reference compounds:** Reference standard of 2,3,7,8-TCDD was obtained from Wellington laboratories (Ontario, Canada).

### Sample preparation

The simulated soil samples of 2,3,7,8-TCDD were prepared by adding known amount (25-100 ng) of reference standard in nonane. The samples were thoroughly mixed by preparing the slurry with acetone and allowed to dry and equilibrate at room temperature.

### Optimization of GC-MS conditions

An ion trap GC-MS equipped with electron impact (EI) mode and MS capability was used for 2,3,7,8-TCDD analysis. The various conditions set for the GC-MS and ion-trap for the GC resolution and mass spectra of 2,3,7,8-TCDD are given in Tables 1-3. The ion trap was held at 200°C. The manifold temperature was 80°C. The ion trap was connected by a heated (300°C) transfer line to GC. BP-5 minibore capillary column has been used and connected to on-column injector directly into ion source of ionpole mass spectrometer. The EI mode was operated at electron energy of 70eV. The gas flow of 1.5 mL/min has been optimized. A 59 min temperature programme has been used to separate 2,3,7,8-TCDD (Table-3). The samples (1-2 μL) were injected and the column temperature has been programmed as follows 70°C isothermal for 1.5 min, with rise of

10°C/min to 235°C for 10 min, 5°C/min to 275°C for 3 min, 5°C/min to 325°C for 10 min. The filament of the ion source was switched off during elution of the solvent for mass specific detection (mass fragmentography) of 2,3,7,8-TCDD.

TABLE-1

GC-MS COLUMN OVEN PROGRAMME FOR 2,3,7,8-TCDD

| Temperature (ºC) | Rate (ºC/min) | Hold (min) | Total (min) |
|---|---|---|---|
| 70 | 0.0 | 1.50 | 1.50 |
| 235 | 10.0 | 10.00 | 28.00 |
| 275 | 5.0 | 3.00 | 39.00 |
| 325 | 5.0 | 10.00 | 59.00 |

TABLE-2

GC-MS CONDITIONS OPTIMIZED FOR 2,3,7,8-TCDD

| Parameters | Conditions |
|---|---|
| Make | Varian |
| Model | CP-3800 gas chromatograph-Saturn 2200 GC/MS/MS |
| Carrier gas | Helium |
| Flow rate (mL/min) | 1.5 |
| Split ratio | 1 : 20 |
| Trap temperature (ºC) | 200 |
| Manifold temperature (ºC) | 80 |
| Transfer line temperature (ºC) | 310 |
| Column | BP-5 capillary column<br>Length : 25 m<br>Diameter : 0.32 mm<br>Film thickness : 1.0 μm |

TABLE-3

GC-MS ION TRAP CONDITIONS OPTIMIZED FOR 2,3,7,8-TCDD

| Parameters | Conditions | Parameters | Conditions |
|---|---|---|---|
| Temperature (ºC) | 200.00 | Threshold (count) | 2 |
| Emission current (μA) | 90.00 | Background mass (amu) | 99 |
| RF storage | 48.00 | Mass range (amu) | 90-500 |
| Scan rate (s/scan) | 0.75 | PFTBA* tuning | Target |
| Filament delay (min) | 10.00 | | |

*Perfluorotributylamine

Initial calibration of the GC-MS system is required before any samples are analysed for 2,3,7,8-TCDD. Solutions of 10, 50, 100, 200, 300 and 500 pg μL$^{-1}$ of 2,3,7,8-TCDD were prepared for the initial calibration. The instrument was tuned with a reference compound perfluorotributylamine (PFTBA). A six point standard calibration curve was determined using six different concentrations of 2,3,7,8-TCDD solution. The relative standard deviation was found to be 3.09 %.

The mass spectrum of 2,3,7,8-TCDD was monitored for the following characteristics ions:

| m/z | Compound |
|---|---|
| 322 | Unlabeled TCDD |
| 320 | Unlabeled TCDD |
| 257/259 | TCDD-COCl |
| 194/196 | TCDD-2COCl |
| 285/287 | TCDD-Cl |
| 250/252 | TCDD-$Cl_2$ |
| 222/224 | TCDD-COCl-Cl |

The ratio of integrated ion current for m/z 320 to 322 for 2,3,7,8-TCDD was between 0.67 and 0.87 (± 13 %). The ion current response for m/z 259, 320 and 322 reached their maxima simultaneously and all the ion current intensities were $\geq$ 2.5 times noise level for the positive identification of 2,3,7,8-TCDD.

The EI mass spectra of 2,3,7,8-TCDD show strong molecular ions ($M^+$). Fragmentation occurs through the loss of CO and Cl radicals. Majors ions are at $M^+$-63 ($M^+$-COCl) and $M^+$-126 ($M^+$-2COCl), both characteristic and diagnostically important for 2,3,7,8-TCDD. Minor fragmentation ions are $M^+$-35, $M^+$-70 and $M^+$-98 (loss of Cl, $Cl_2$ and COCl + Cl, respectively). Based on molecular ions and fragmentation mode, 2,3,7,8-TCDD has easily been distinguished from other chlorinated pollutants, such as polychlorinated diphenyl ether, polychlorinated biphenyls, polychlorinated naphthalene, polychlorinated dibenzofurans and chlorine containing pesticides.

The gas chromatogram and mass spectrum of 2,3,7,8-TCDD reference standard show the molecular ion and its isotope peaks at m/z 320, 322 and 324. Major ions are at m/z 257, 259 ($M^+$-COCl), 194, 196 ($M^+$-2COCl).

## Procedure

The specific set of soil samples in duplicate were extracted with three different organic solvents phases *viz.* dichloromethane, acetone-hexane (1:1) mixture and toluene for a fixed period using two different extraction methods *viz.*, rotatory shaker and soxhlet extraction assembly. 2,3,7,8-TCDD was extracted from homogenized soil samples by continuous rotatory shaker and soxhlet extraction assembly using bulk of the solvent was removed by evaporation in especially fabricated K-D equipment. Interfering materials were then partially removed from the extracts by successive clean-up using chromatographic columns of silica gel, alumina and carbopack-C/celite columns. The eluates of the columns were concentrated to 10-50 and 1-2 μL aliquot was used for GC-MS analysis.

**Sample clean-up:** Sample clean-up processing included clean-up of the concentrated extract in succession with three different chromatographic columns of silica gel, alumina (acidic) and carbopack-C/celite 545.

**Column preparation:** The columns of No.1-silica gel, 2-alumina and 3-carbopack-celite were prepared as per the following procedure: A chromatographic column of dimension 1 × 20 cm is packed with a glass wool plug, followed by 1 g of silica gel, 2 g of KOH impregnated silica gel, 1 g of silica gel, 4 g of 40 % (w/w) sulfuric acid impregnated silica gel and 2 g of silica gel, respectively. Tap the column gently after each addition. Pack a second chromatographic column (1 × 30 cm) with a glass wool plug and 6 g of acidic alumina and top it with a 1 cm layer of sodium sulfate. Add hexane to the columns until they are free of channels and air bubbles. A small positive pressure of clean nitrogen can be used if needed.

Quantitatively transfer the sample extract (1 mL) from the concentrator tube to the top of the silica gel column No.1. Rinse the concentrator tube with 0.5 mL portions of hexane. Transfer the rinses to the top of the silica gel column.

Elute the extract from the silica gel column with 90 mL of hexane directly into a K-D evaporative flask. Concentrate the eluate to 0.5 mL, using nitrogen blowdown, as necessary.

Transfer the concentrate (0.5 mL) to the top of the alumina column No.2. Rinse the K-D assembly with two 0.5 mL portions of *n*-hexane and transfer the rinses to the top of the alumina column. Elute the alumina column with 20 mL hexane until the hexane level is just below the top of the sodium sulfate. Discard the eluate. Do not let the columns reach dryness (*i.e.*, maintain a solvent 'head').

Add 30 mL of 20% (v/v) dichloromethane in hexane on top of the alumina column and elute the 2,3,7,8-TCDD from the column. Collect this fraction in a 50 mL flask.

Activated carbon selectively retains the planar molecules such as 2,3,7,8-TCDD and which are then removed from the column by elution with toluene. The column No. 3 is packed with 18% carbopack-C/celite 545 mixture thoroughly by mixing 3.6 g carbopack C (80/100 mesh) and 16.4 g celite 545 in a 40 mL vial. Activate the mixture at 130ºC for 6 h and store it in a dessicator. A glass column of 7 mm ID and 30 cm length is taken and a plug of glass wool is inserted inside at one end. 340 mg of the activated carbopack-C/celite is added into the column with continuous tapping and closed with another glass wool plug at other end. Using two glass rods, both glass wool plugs are pushed simultaneously toward the carbopack-C/celite plug to a length of 2.0 to 2.5 cm. Pre-elute the column with 2 mL of toluene followed by 1 mL of 75:20:5 dichloromethane/methanol/benzene, 1 mL of 1:1 cyclohexane in dichloromethane and 2 mL of hexane. The flow rate should be less than 0.5 mL per min. While the column is still wet with hexane, add the entire elute (30 mL) from the alumina column to the top of the column. Rinse the Erlenmeyer flask that contained the extract twice with 1 mL of hexane and add the rinsates to the top of the column. Elute the column sequentially with two 1 mL aliquots of hexane, 1 mL of 1:1 cyclohexane in dichloromethane and 1 mL of 75:20:5 dichloromethane/methanol/benzene. Turn the column upside down and elute the 2,3,7,8-TCDD fraction into a concentrator tube with 15 mL of toluene. Warm the concentrator tube to approximately 60ºC and reduce the toluene volume to approximately 0.2-0.3 mL using a stream of nitrogen. Store the sample in refrigerator until GC-MS analysis is performed.

## RESULTS AND DISCUSSION

The careful statistical analysis has been made for the selection of most efficient and specific sample preparation procedure using the data of laboratory exercises for the estimation of 2,3,7,8-TCDD in soil samples. The findings of 2,3,7,8-TCDD measurements in simulated samples are given in Tables 4-6.

TABLE-4
2,3,7,8-TCDD ANALYSIS IN SOIL USING DICHLOROMETHANE

| Sample | Initial conc. (ng) | Concentration (ng) | | | |
|---|---|---|---|---|---|
| | | Soxhlet | | Rotatory | |
| | | Observed | % Recovery | Observed | % Recovery |
| Blank | – | – | – | – | – |
| S-1 | 50 | 8.600 | 17.2 | 12.669 | 25.34 |
| S-2 | 50 | 6.169 | 12.3 | 19.246 | 38.50 |
| S-3 | 50 | 17.746 | 35.5 | 10.025 | 20.05 |
| S-4 | 50 | 10.800 | 21.6 | 9.382 | 18.76 |
| S-5 | 50 | 10.100 | 20.2 | 11.900 | 23.80 |
| S-6 | 50 | 13.090 | 26.2 | 17.755 | 35.50 |

TABLE-5
2,3,7,8-TCDD ANALYSIS IN SOIL USING HEXANE-ACETONE

| Sample | Initial conc. (ng) | Concentration (ng) | | | |
|---|---|---|---|---|---|
| | | Soxhlet | | Rotatory | |
| | | Observed | % Recovery | Observed | % Recovery |
| Blank | – | – | – | – | – |
| S-1 | 50 | 12.720 | 25.4 | 8.000 | 16.00 |
| S-2 | 50 | 5.100 | 10.2 | 8.540 | 17.10 |
| S-3 | 50 | 8.700 | 17.4 | 5.160 | 10.32 |
| S-4 | 50 | 5.700 | 11.4 | 9.990 | 19.98 |
| S-5 | 50 | 7.750 | 15.5 | 5.220 | 10.44 |
| S-6 | 50 | 6.450 | 12.5 | 5.150 | 10.30 |

TABLE-6
2,3,7,8-TCDD ANALYSIS IN SOIL USING TOLUENE

| Sample | Initial conc. (ng) | Concentration (ng) | | | |
|---|---|---|---|---|---|
| | | Soxhlet | | Rotatory | |
| | | Observed | % Recovery | Observed | % Recovery |
| Blank | – | – | – | – | – |
| S-1 | 50 | 50.00 | 100.00 | 31.90 | 63.80 |
| S-2 | 50 | 25.36 | 50.72 | 30.30 | 60.60 |
| S-3 | 50 | 49.15 | 98.30 | 30.11 | 60.22 |
| S-4 | 50 | 37.00 | 74.00 | 40.04 | 80.08 |
| S-5 | 50 | 36.40 | 72.80 | 43.90 | 87.80 |
| S-6 | 25 | 17.00 | 68.00 | 20.52 | 82.00 |
| S-7 | 25 | 19.25 | 77.00 | 20.43 | 81.72 |
| S-8 | 100 | 83.20 | 83.20 | 79.90 | 79.90 |
| S-9 | 100 | 72.80 | 72.80 | 70.56 | 70.56 |
| S-10 | 100 | 68.00 | 68.00 | 70.99 | 70.99 |
| S-11 | 100 | – | – | 68.78 | 68.78 |

Nearly 60 simulated samples in the concentration range of 25-100 ng were subjected to extraction procedures and clean-up using dichloromethane, hexane-acetone and toluene (Tables 4-6).

**Statistical evaluation for efficient procedure:** The results of response factor as % recovery of 2,3,7,8-TCDD using two different methods have been analysed using student's 't' test for comparison of the mean recovery for each of the solvents, used in extraction separately. The null hypothesis ($H_O$) has been formulated that there is no significant difference between the mean results of % recovery of 2,3,7,8-TCDD by the methods using a particular solvent. The hypothesis has been tested against the alternative hypothesis ($H_1$) that the recovery results by two methods differ significantly. The 't' statistic has been calculated using the standard formula[4]. The calculated value of 't' has then been compared with the critical value from the Table-7 for (m + n - 2) degrees of freedom where m and n are the number of observation in the rotatory shaker and soxhlet extraction assembly, respectively.

TABLE-7
STUDENT'S 't' TEST FOR COMPARISON OF ROTATORY SHAKER AND SOXHLET EXTRACTION METHODS

| Solvent | Number of observations | Calculated value of 't' | Degree of freedom (m + n - 2) | Critical value of 't' |
|---|---|---|---|---|
| Dichloromethane | m = 6, n = 6 | 0.128 | 10 | 2.228 |
| Hexane-acetone (1 : 1) | m = 6, n = 6 | 0.483 | 10 | 2.228 |
| Toluene | m = 12, n = 10 | 0.600 | 20 | 2.086 |

At 5 % level of significance shows that the calculated value of 't' is less than the critical value in each of the three solvents. It has been inferred from the data evaluation that for all the solvents the recovery % age obtained by using rotatory shaker and soxhlet extraction assembly is almost equal. Both the methods are equally good in % recovery of 2,3,7,8-TCDD and are almost comparable. Though both the methods have been found to be compatible and yield almost the same % recovery, rotatory shaker method has been preferred because of the less solvent consumption, less time consuming and ease in carrying out the extraction.

The mean % recovery, standard deviation and coefficient of variation of 2,3,7,8-TCDD for each of the three extracting solvents separately using the rotatory shaker method has been calculated (Table-8).

Dichloromethane and hexane-acetone solvents have almost same coefficient of variation and average recovery (Table-8). In case of toluene, the average recovery is very high (73.43%) and the corresponding coefficient of variation is very low (12.35%). Thus toluene has been found as very efficient solvent for recovery of 2,3,7,8-TCDD and precise while using rotatory shaker method, when compared with the other two solvents results.

TABLE-8
STATISTICAL EVALUATION OF SOLVENTS EXTRACTION EFFICIENCY FOR 2,3,7,8–TCDD IN SOIL

| Solvent | Mean recovery (%) | Standard deviation | Coefficient of variation (%) |
|---|---|---|---|
| Dichloromethane | 27.00 | 8.15 | 30.20 |
| Hexane-acetone (1:1 v/v) | 14.02 | 4.24 | 30.26 |
| Toluene | 73.43 | 9.07 | 12.35 |

To assure the validity and reliability of analytical procedure in estimating 2,3,7,8-TCDD in soil, extensive internal quality assurance programme has been carried out. A number of laboratory exercises in the estimation of 2,3,7,8-TCDD in soil have been made for the selection of most efficient and specific solvent. The findings of statistical analysis show that both the methods are equally good in % recovery and are almost comparable. The mean % recovery, standard deviation and coefficient of variation of 2,3,7,8-TCDD among three extracting solvents dichloromethane, hexane-acetone and toluene have been calculated using rotatory and soxhlet extraction assembly. The most efficient average recovery of 73.43% and the corresponding coefficient of variation (12.35%) have been found in case of toluene.

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