Neutral Cleanup Procedure for 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Residues in Bovine Fat and Milk

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A neutral cleanup method for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in milk and animal tissue was developed involving solvent extraction and liquid adsorption chromatography on magnesia-Celite 545, alumina, and Florisil. Cleaned up extracts were subjected to dual-ion analysis in a direct probe high resolution mass spectrometer, interfaced to a multichannel analyzer for signal averaging. Calibration experiments were carried out with bovine milk and beef fat samples containing added TCDD. The ^{37}Cl isotopic isomer of TCDD was added as an internal standard. The response was linear for concentrations in the ppt range, with recoveries about 80%. Milk from a cow fed TCDD was cleaned up by the neutral procedure or, alternatively, a base-acid extraction procedure. The TCDD recoveries for both procedures were essentially the same. Recoveries of TCDD from liver samples of a rat given ^{14}C-TCDD intraperitoneally, subjected to neutral cleanup and radioactive counting, were about 70%.

The highly toxic compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is formed as a contaminant in the production of 2,4,5-trichlorophenol, an intermediate in the manufacture of the herbicide 2,4,5-T and certain other products (1). In order to investigate the possible bioaccumulation of TCDD under field conditions, Baughman and Meselson (2–5) developed a technique to determine TCDD residues at the picogram level in environmental samples. The cleanup procedure was a modification of one previously developed for the analysis of chlorodibenzo-*p*-dioxins in feed fats (6). In the initial stage of development the method involved base hydrolysis of a tissue or milk sample followed by extraction with sulfuric acid, chromatography on alumina, and collection of the TCDD fraction by preparative gas-liquid chromatography (GLC) before analysis by direct probe high resolution mass spectrometry (MS) (2, 3). This method was successfully used to identify TCDD at levels from 4 to several hundred parts per trillion (ppt) in samples of fish, crustaceans, and human milk collected in 1970 from areas in Vietnam treated with 2,4,5-T during the military defoliation program (3, 4). A similar base-acid cleanup method with the addition of a silicic acid column has recently been applied to environmental samples before analysis by coupled GLC-MS (7).

Recoveries of TCDD could be increased from approximately 30 to 60–80% when the preparative GLC step was replaced by a second alumina minicolumn (5). Comparison with the levels found in the 1970 Vietnamese samples and in spiked control samples showed that this simplified procedure gave essentially the same analytical results as did the procedure incorporating GLC. However, in the analysis of certain samples of fish and crustaceans from Vietnam collected in 1973 from areas exposed to 2,4,5-T, replacement of GLC by a second alumina minicolumn led to the appearance of a compound or compounds that seriously interfered with TCDD analysis. This material volatilized in the mass spectrometer later than authentic TCDD but gave a mass spectrum which included ions with the same m/e values as TCDD (4). On the possibility that treatment with strong base or acid might generate compounds which could form chlorinated dioxins in the mass spectrometer (8, 9), a neutral extraction procedure was then developed (4). Although this procedure eliminated the interferences encountered earlier, its limit of detection for TCDD in tissue samples was about 50 ppt. The present report describes the further development of the neutral extraction procedure, resulting in a method applicable to routine analysis of beef fat and milk at substantially lower TCDD levels.

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METHOD

Reagents and Apparatus

(a) *Standards.*—Dissolve ca 100 μg TCDD standard (supplied by D. Firestone, Food and Drug Administration, Washington, DC) in 1 ml benzene. For routine use, benzene solutions of TCDD, ^{37}Cl-TCDD (chlorination of dibenzo-*p*-dioxin (4)), and ^{14}C-TCDD (supplied by A. Poland, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI) are calibrated against standard solution by mass spectrometry. DDE, reagent grade (Analabs); polychlorinated biphenyl (PCB, Aroclor 1254), standard solution in benzene (Regis Chemical).

(b) *Solvents.* — Distilled-in-Glass (Burdick & Jackson Laboratories, Inc.) except ethanol, pesticide quality (Matheson, Coleman and Bell). (Caution: Benzene, carbon tetrachloride, and chloroform are possible carcinogens.)

(c) *Adsorptive magnesia.*—(Fisher Scientific). Activate magnesia-Celite 545 (2+1 by weight) 12 hr at 170°C and cool to room temperature in vacuum desiccator on day of use.

(d) *Sea sand.*—Washed and ignited (Fisher Scientific).

(e) *Alumina.* — Neutral activity, grade 1 (Woelm). Activate 12 hr at 400°C and cool to room temperature in vacuum desiccator on day of use.

(f) *Florisil.*—60–100 mesh (Fisher Scientific). Activate 12 hr at 170°C and cool to room temperature in vacuum desiccator on day of use.

(g) *Tissue solubilizer.*—Protosol (New England Nuclear).

(h) *Scintillation fluids.*—Aquasol and Omnifluor (New England Nuclear).

(i) *Liquid scintillation counter.*—Beckman LS-250 (Beckman Instruments).

(j) *Perfluorotributylamine (PFA).*—Mass spectral standard (PCR, Inc.).

(k) *Mass spectrometer.* — AEI Model MS-9 double focusing (AEI, Manchester, UK). Operating conditions: source 220°C, resolution 10,000 (10% valley between peaks), trap current 1.0 mamp (rhenium filament), electron multiplier 700, ionizing voltage 70 ev.

(l) *Signal-averaging computer.*—Varian Model TAC-1024 multi-channel analyzer operated at 4 scans/sec. Analyzer is interfaced to mass spectrometer essentially as described previously (2) but with additional modification for dual-ion monitoring (4), accomplished by introducing a relay into TAC-1024 circuit. Relay is activated by MS-9 peak switching relays, allowing signals at 2 different m/e values to be simultaneously averaged in separate banks of channels on TAC-1024.

Cleanup

Cut 30 g frozen beef fat into 1 cm cubes and place in beaker in 160°C oven. After 20 min, decant 10 g liquid fat into Erlenmeyer flask. When flask has cooled to 40°C, add 10 ml hexane and 1–10 ng ^{37}Cl-TCDD in 50 μl benzene.

Extract milk samples by procedure of Samuel (10). Mix 150 ml milk sample with 1–10 ng ^{37}Cl-TCDD, 2 g sodium oxalate, and 300 ml ethanol. Extract solution with ethyl ether-hexane (185+265) followed by 3 additional extractions with 265 ml portions of hexane. Combine extracts, wash with 200 ml water, and dry by passing through 200 × 20 mm column of Na_2CO_3. Concentrate organic phase to 20 ml in hot water bath with Snyder column attached to round-bottom boiling flask. Add 40 ml cyclohexane and reconcentrate to 12 ml.

Load hexane or cyclohexane solution on dry-packed magnesia-Celite 545 column (70 × 27.5 mm) with top layer of 40 mm Na_2SO_4. Elute with 250 ml ethyl ether-hexane (40+60) followed by 160 ml benzene, using nitrogen pressure of 15 psi. Concentrate (Snyder column) benzene fraction to 2 ml and add 10 ml cyclohexane. Reconcentrate (Vigreaux column) to 2 ml and repeat this step with 2 further 10 ml portions of cyclohexane. Pack alumina column (200 × 6 mm) under dry conditions followed by 1 cm layer of Na_2SO_4 and fill void spaces by forcing 20 ml CCl_4 through column under 15 psi nitrogen. Add sample extract in cyclohexane to column, followed by 2 ml hexane wash of sample flask. Elute column with following solvent systems: (*1*) 10 ml CCl_4, (*2*) 8 ml CH_2Cl_2-CCl_4 (10+90), and (*3*) 10 ml CH_2Cl_2. Save third fraction and replace CH_2Cl_2 with hexane, using procedures described above. Prepare alumina minicolumn by filling disposable Pasteur pipet (6 mm id), plugged with glass wool, to height of 40 mm with alumina. Add 5 mm layer of Na_2SO_4 and force 10 ml hexane through column under 15 psi pressure. Evaporate sample in hexane solution to ca 1 ml and load on alumina minicolumn, following this addition with 1 ml hexane wash from sample flask. Add 10 ml CCl_4 to column, followed by 5 ml CH_2Cl_2. Replace CH_2Cl_2 in second fraction with hexane as before. Chromatograph hexane residue on Florisil minicolumn. To prepare and prewash column, use procedure described above for alumina minicolumn. After adding sample, elute with 10 ml hexane followed by 5 ml CH_2Cl_2. Add 10 ml benzene to CH_2Cl_2 fraction and concentrate (Vigreaux column) to 1 ml. Transfer sample to microconcentration flask and concentrate to 60 μl under stream of nitrogen. Place 6 μl aliquots from cleaned up sample extract in capillary tubes sealed near one end. Constrict

tubes at point 10 mm from seal to limit rate of sample evaporation in mass spectrometer. After solvent removal under vacuum, seal constricted ends. Tubes may then be stored for extended periods of time before analysis.

Mass Spectrometry

Evaporate residue from sample tube into mass spectrometer over period of 45 sec to 1 min. Use peak-switching unit of the MS-9 to simultaneously monitor ions of selected mass from natural TCDD (322 m/e, 320 m/e, or fragment ion) and ions of another selected mass from ^{37}Cl-TCDD (328 m/e or 329 m/e, ^{13}C-isotope ion). All mass selections are relative to PFA as internal marker. Ions register in separate channels of multi-channel analyzer and time-averaged signals are recorded on XY recorder. For samples containing high levels of TCDD, time course of evaporation may be observed by recording signals on oscillographic recorder without signal averaging. Calculate level of natural TCDD in sample from ratio of peak height at 322 or 320 m/e to peak height at 328 or 329 m/e and from known amount (60–500 ppt) of ^{37}Cl added before cleanup. Calculate recovery from ratio of peak heights at 328 or 329 m/e to those at 322 or 320 m/e in separate analysis with natural TCDD added *after* cleanup. Amount of natural TCDD added is ca 1 ppb (lipid base) for environmental samples and ca 4 times greater than amount added prior to cleanup for spiked samples. In calculating TCDD levels, it is necessary to recognize differences in isotopic isomer distributions for molecular ions of TCDD and ^{37}Cl-TCDD. Since ^{37}Cl-TCDD internal standard is labeled with 95.5% ^{37}Cl, response for given amount of ^{37}Cl-TCDD at 328 m/e is approximately 2 times greater than for same amount of incurred TCDD at 322 m/e and 2.5 times greater than for same amount of incurred TCDD at 320 m/e. When mass spectrometer is operating under conditions of high sensitivity, signals from 328 m/e ions of ^{37}Cl-TCDD saturate amplification system of multi-channel analyzer. One may then monitor 329 (^{13}C-isotope) m/e ion which is 8.3 times less abundant than 328 m/e ion.

^{14}C-TCDD Levels in Rat Liver

Sacrifice male rat (Sprague-Dawley) 30 days after administration of intraperitoneal injection of 5 μg ^{14}C-TCDD (specific activity 98 mCi/mmole)/kg body weight. Dissect liver from other tissues and homogenize with 1 part water. Clean up 2 g aliquot from liver homogenate by neutral procedure described previously. Divide cleaned up residue in benzene solution into two 1 ml fractions; spike one with known amount of ^{14}C-TCDD for quantitation and dilute each fraction and solvent blank with 10 ml Omnifluor before counting by liquid scintillation. Digest 0.5 g aliquot from liver homogenate with 2 ml Protosol for 2 hr at 55°C in screw-cap vial. After cooling to room temperature, add 1 ml 30% hydrogen peroxide to decolorize. Divide digested material into 2 portions as described above, and dilute with 10 ml Aquasol. Before counting, neutralize excess base with dilute acid to minimize chemiluminescence.

Results and Discussion

The development in our laboratory of an instrumental modification for the alternate signal averaging of ion signals at 2 different m/e values by high resolution MS (4) offers the following advantages in comparison to single ion monitoring: (*1*) It is possible to directly determine the level of TCDD from a single analysis and in the absence of recovery data. Therefore, variations in instrument sensitivity do not affect quantitative results. (*2*) Ions from ^{37}Cl-TCDD serve as a useful means to reference the m/e values of ions from TCDD. There is, however, some loss in sensitivity since there is a reduction in the number of scans at each m/e value. Using the dual-ion mass spectrometry procedure for TCDD detection, the neutral method was calibrated for beef fat and bovine milk. TCDD was added to beef fat at levels of 0, 13, 25, 100, and 200 ppt and 4 replicates were analyzed at both 320 and 322 m/e at each concentration level. Similar experiments were carried out with bovine milk, using TCDD levels of 0, 0.7, 13, and 65 ppt. For the milk samples containing 0 and 0.7 ppt, analyses were carried out only at 322 m/e. The experimentally determined TCDD values and standard deviations for the calibration samples were, respectively, not detected (ND), 13±2, 20±1, 83±6, and 175±32 ppt for the beef fat; and ND, 0.6±0.1, 13±1, and 55±5 ppt for the bovine milk. The ^{37}Cl-TCDD recovery values ranged from 77±18 to 105±9% for the beef fat and from 71±12 to 87±21% for the bovine milk.

Thus, the analytical procedure provides linear results over the range of TCDD concentrations examined. TCDD signals from cleaned up samples of beef fat and bovine milk, containing the lowest levels of TCDD used in the calibration experiments (13 and 0.7 ppt, respectively), are shown in Figs 1 and 2. Corresponding mass spectra for controls without added TCDD are shown

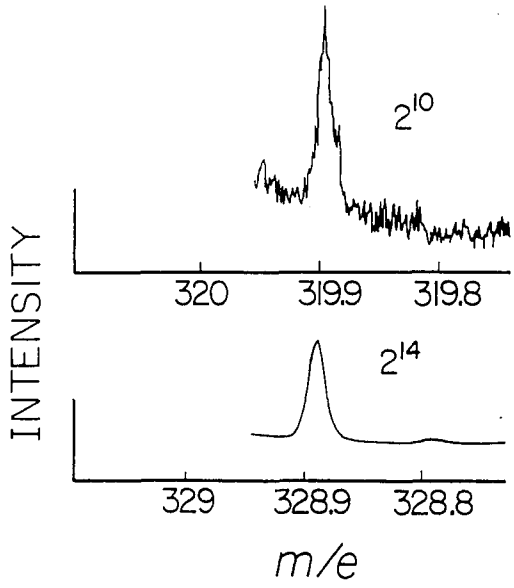

FIG. 1—Dual-ion MS (10,000 resolution) analysis of beef fat (10 g) containing added TCDD (13 ppt) and with ^{37}Cl-TCDD (390 ppt) as internal standard. Subsample equivalent to 10% of residue after neutral cleanup. Numbers to right of figure refer to signal attenuation. Similar notation is used in all subsequent figures.

in Figs 3 and 4. There are no peaks at the TCDD molecular ion m/e values.

Excess residue can depress the TCDD peak height in the mass spectrometer. Earlier work demonstrated that 5 μg squalene caused a 50% depression in the TCDD signal relative to a standard (2). The magnesia-Celite 545 column step is important in removing lipids. In an experiment with 8 g lipid from butterfish with added TCDD, 95% of the lipid was eluted in the ether-hexane fraction from the column while TCDD remained bound. The solvent system was selected to maximize the amount of lipid that can be handled by the cleanup procedure. Up to 10 g rendered beef fat from trimmings purchased at a local store could be successfully cleaned up after being dissolved in 10 ml hexane. When beef perirenal fat, collected as part of a TCDD field monitoring program, was handled in a similar manner, it solidified in the magnesia-Celite 545 column. However, an 8 g sample of this fat could be dissolved in 10 ml ether-hexane (1+25).

Two procedures, relying on partition chromatography rather than adsorption chromatography, have been reported in the literature for separating chlorinated pesticides from large quantities of lipids (11, 12). Their application to the cleanup of TCDD has not been investigated, although one of the methods (13) has been incorporated into a cleanup procedure for chlorodibenzofurans.

In addition to minimizing lipid components in the final extract, the extraction procedure must also minimize levels of certain chlorinated hydrocarbons that can form ions in the mass spectrometer at m/e values close to those of TCDD (319.897, 321.894) or its ^{37}Cl-isomer (327.885). The most likely sources of interference are DDE molecular ions at 319.933 and 321.930 m/e, DDT ions at 319.947 and 321.944, and PCB ions at 321.868 and 327.878 m/e. Although TCDD can be resolved from low concentrations at both 320 and 322 m/e when the mass spectrometer is operated at a resolution of 10,000, higher concentrations could present interference problems (2). To avoid this, Baughman and Meselson (3) modified the solvent system for elution of an alumina minicolumn previously developed for separating PCBs from chlorodibenzo-*p*-dioxins (14). When this column was incorporated into a base-acid cleanup procedure, microgram quan-

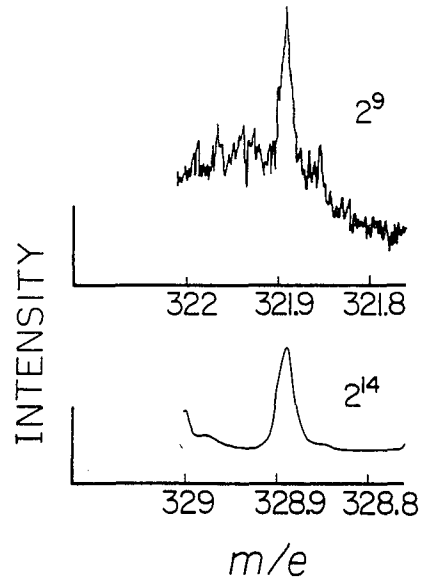

FIG. 2—Dual-ion MS (10,000 resolution) analysis of bovine milk (150 ml) containing added TCDD (0.7 ppt) and with ^{37}Cl-TCDD (66 ppt) as internal standard. Subsample equivalent to 10% of residue after neutral cleanup.

tities of PCB and DDE were reduced to the extent that it was possible to measure picogram quantities of TCDD. A Florisil minicolumn also provides an efficient means of separating DDE and PCB from TCDD (D. Firestone (1977) Food and Drug Administration, Washington, DC). The relative merits of the Florisil and alumina minicolumns in the last chromatography step in the neutral procedure were investigated with bovine milk samples spiked with 1 ppm DDE, 1 ppm PCB, and 66 ppt ^{37}Cl-TCDD. One sample was cleaned up by the present neutral procedure and another sample was cleaned up by the same procedure with the exception that a second alumina minicolumn was substituted for the Florisil minicolumn. After cleanup, sub-fractions were spiked with 60 pg TCDD. Although the use of a second alumina minicolumn succeeded in removing virtually all traces of DDE, there was still interference from PCB (Fig. 5). The use of a Florisil minicolumn reduced the PCB to an insignificant level (Fig. 6).

Although TCDD added to control samples was extracted efficiently, consideration must be given to the possibility that its distribution in environmental samples may render it less easily extractable. Three replicate samples of milk obtained from a cow fed TCDD were cleaned up by either the base-acid procedure or the neutral

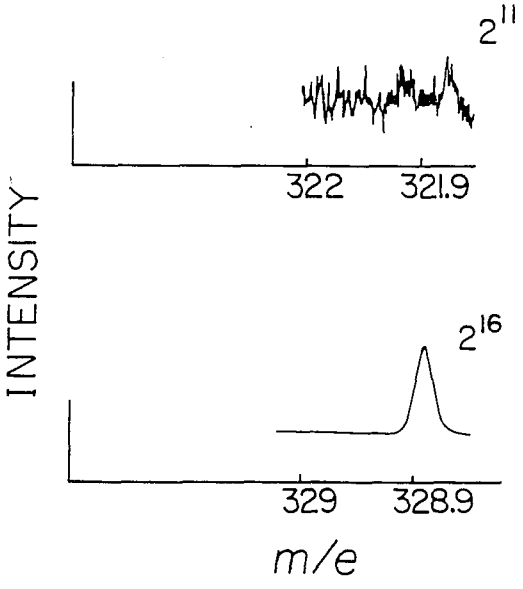

FIG. 4—Dual-ion MS (10,000 resolution) analysis of control bovine milk (150 ml) with no added TCDD but containing ^{37}Cl-TCDD (66 ppt) as internal standard. Sub-sample equivalent to 10% of residue after neutral cleanup.

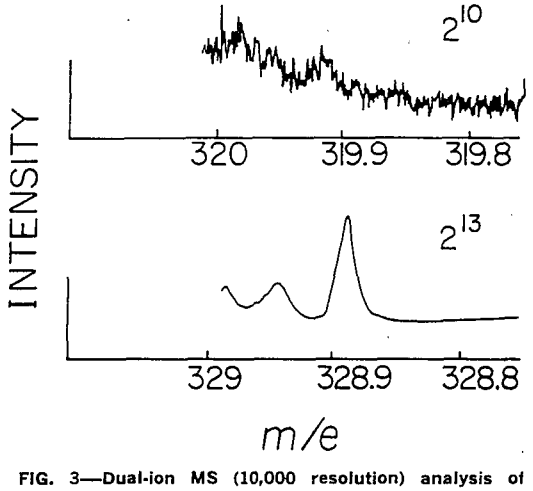

FIG. 3—Dual-ion MS (10,000 resolution) analysis of control beef fat (8 g) with no added TCDD but containing ^{37}Cl-TCDD (250 ppt) added as internal standard. Sub-sample equivalent to 10% residue after neutral cleanup. No TCDD signals have been observed in any of 7 independent analyses of beef fat obtained from local markets.

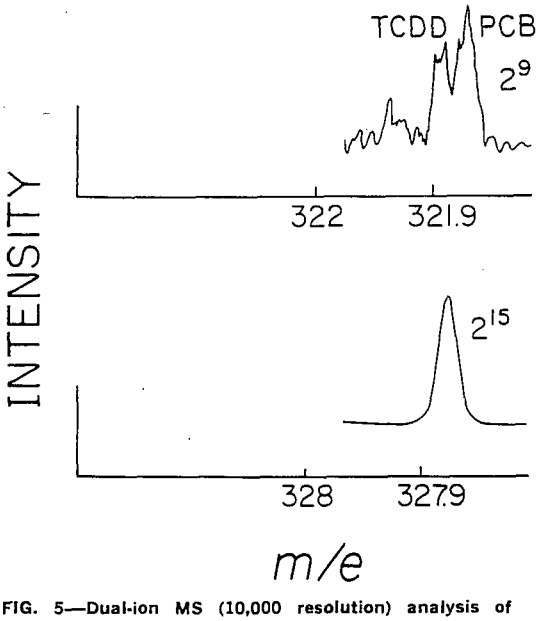

FIG. 5—Dual-ion MS (10,000 resolution) analysis of bovine milk after neutral cleanup without Florisil chromatography. Bovine milk (150 ml) was spiked with Aroclor 1254 (1 ppm), DDE (1 ppm), and ^{37}Cl-TCDD (66 ppt) before cleanup. Sub-sample equivalent to 10% of cleaned up residue was spiked with TCDD (60 pg) before analysis.

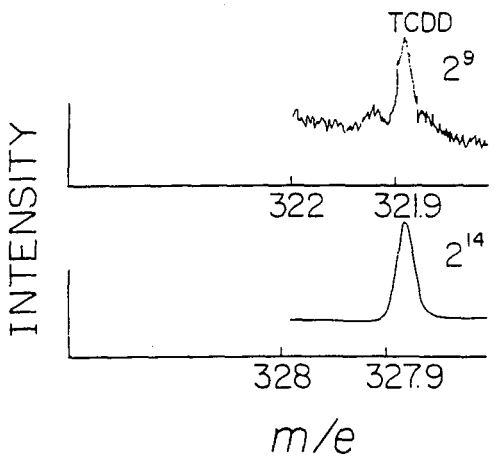

FIG. 6—Dual-ion MS (10,000 resolution) analysis of bovine milk after neutral cleanup with Florisil chromatography. Bovine milk (150 ml) was spiked with Aroclor 1254 (1 ppm), DDE (1 ppm), and ^{37}Cl-TCDD (66 ppt) before cleanup. Sub-sample equivalent to 10% of cleaned up residue was spiked with TCDD (60 pg) before analysis.

procedure and were found to have essentially the same levels of TCDD, 91±7 ppt for base-acid cleanup and 96±5 ppt for neutral cleanup. Liver homogenate was prepared from a rat killed 1 month after intraperitoneal injection of ^{14}C-TCDD; 72% of the radioactivity of the homogenate was recovered after neutral cleanup (3 replicates). These results show that the extraction of TCDD by the neutral procedure was nearly complete. The method described here has been applied to the analysis of selected samples of beef fat from cattle grazed on rangeland treated with 2,4,5-T. TCDD was found in several of the samples, the highest level being approximately 70 ppt. These results will be reported in detail elsewhere.

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