Temporal Changes in Purity and Specific Activity Associated with Use of Tritiated 2,3,7,8-Tetrachlorodibenzo-p-dioxin for Rapid Analysis in Toxicology Studies.

Philip M. Cook, Joseph D. Fernandez, Brian C. Butterworth, and Steven P. Bradbury, U.S. Environmental Protection Agency, NHEERL, Mid Continent Ecology Division, 6201 Congdon Blvd., Duluth Minnesota 55804.

Abstract Changes in the specific activity (***S***) and radiopurity (***R***) of tritiated 2,3,7,8-tetrachlordibenzo-p-dioxin, [^{3}H]TCDD, were measured by gas chromatography/mass spectrometry in order to accurately characterize TCDD doses received by invertebrates, fish and fish embryos in several toxicology studies conducted over a three year period. The [^{3}H]TCDD sample was found to consist of six TCDD analogs involving hydrogen, deuterium and tritium substitution of the 1,6 positions and a complex mixture of impurities (with and without tritium labels). Carbon column chromatography resulted in incomplete purification of the [^{3}H]TCDD solutions due to the presence of planar aromatic impurities which were identified as tolyl-TCDD adducts. These adducts resulted from the decay of ^{3}H radiolabels to produce TCDD carbonium ions which reacted with the solvent, in this case, toluene. Similar tolyl adducts of trichlorodibenzo-p-dioxin (TriCDD) were also identified. Radiolysis during storage of solutions caused loss of a chlorine from both TCDD and tolyl-TCDDs which resulted in formation of TriCDD and tolyl-TriCDDs through free radical reactions. Radiolysis, however, did not change [^{3}H]TCDD analog ratios, and thus did not contribute to changes in ***S***. Autoradiolysis did not appear to be a significant route for transformation of TCDD. Hydroxylated TCDD is the expected decay reaction product in tissues and may be misidentified as a metabolite. Changes in ***S*** over time were accurately modeled as a function of the conversion of each [^{3}H]TCDD analog to a solvent-TCDD analog at the rate of loss of ^{3}H from decay. Storage, purification and use of tritiated chemicals for environmental toxicology studies requires consideration of the decay-related phenomena. Measurements of dose and toxicokinetic parameters should account for changes in ***S*** and ***R*** due to reactions which occur over the duration of an experiment.

Introduction Tritium (^{3}H) - labeled compounds are often used in toxicology studies because of analytical advantages that include rapid sample throughput associated with liquid scintillation counting (LSC), the ability to analyze a large number of samples at low cost, and low levels of detection due to highspecific activity (***S***). In aquatic toxicology studies, [^{3}H]TCDD has been especially useful for determination of bioconcentration factors and dose delivered to tissues in toxicity studies employing small fish and/or early life stages that require analysis of tissue samples too small for use of radiocarbon-(^{14}C) labeled TCDD due to lower ***S***. [^{3}H]TCDD used in these studies typically is reported to be [1,6-$^3H_2$]TCDD but actually is a complex mixture of double and single tritiated and unlabeled TCDD molecules[1]. While the use of ^{3}H-labeled chemicals, including [^{3}H]TCDD, offers practical advantages, characteristics of the labeling need

to be accurately determined to ensure proper interpretation of qualitative and quantitative analytical results. Of particular concern is the determination of the labeling site(s), the radiochemical purity, and the amount of label (***S***) associated with the chemical[2]. The 12.3 year half-life ($t_{1/2}$) of ^3H, associated with the natural radioactive decay of $^3_1H \rightarrow {}^3_2He^- + \beta^-$, has clear implications for the need to re-confirm ***S*** and radiochemical purity on a regular basis. In preparations with high ***S*** and/or in situations where labeled molecules are very concentrated, ^3H can cause extensive extramolecular decomposition of the labeled chemical through radiolysis.

Mass spectrometry has been used in quality control of radiolabeled compounds[3,4] From measurements of the amount of label associated with the chemical of interest and knowledge of the decay constant (λ, where $\lambda = \ln 2/t_{1/2}$) of the radionuclide(s) used as the label, the ***S*** of the chemical can be calculated. A comparison of the calculated or measured radioactivity associated with the chemical of interest in a unit of volume or mass of sample to the measured radioactivity of the sample determines the degree of radiopurity (***R***). Recently in our laboratory, several studies[5,6,7,8,9] with aquatic organisms utilized a sample of [^{3}H]TCDD to determine concentrations of TCDD in tissues and exposure media. In all of these studies dose-response relationships were based on whole-body or tissue-specific accumulation of [^{3}H]TCDD as determined by tissue combustion or digestion followed by LSC. In support of these studies, GC/MS was used to: 1) establish ***R*** and ***S*** of [^{3}H]TCDD and 2) confirm the LSC-measured concentrations of TCDD in tissues from the aquatic organisms exposed to [^{3}H]TCDD[10]. We summarize here factors which were found to impact the measurement and interpretation of ***R*** and ***S*** of [^{3}H]TCDD preparations. These discoveries were incorporated into a mass balance model which was used to account for changes in ^3H, TCDD, and impurities over time, both in solutions and in test organisms[10].

Experimental Methods [^{3}H]TCDD, as acquired from Cambridge Isotope Laboratories (Lot No. AWN-729-87) in toluene, was reported to have been synthesized and purified in October 1987 with a resulting concentration of 8 μg/mL, ***S*** = 40Ci/mMole, and ***R*** = 97%. In June, 1993 in preparation for toxicity studies, the solution was analyzed by GC/MS to determine ***R*** and ***S***. Because the mass spectra indicated only 60% of total ^3H activity measured by LSC was attributable to [^{3}H]TCDD, the existence of additional ^3H activity associated with a large amount of unidentified contaminants was suspected.

LSC analyses were conducted on a Model 2500TR Liquid Scintillation Counter (Packard Instrument Co. Meriden CT). Automatic quench control was based on quench standard sets traceable to National Institute of Standards and Technology (NIST). Samples were counted for a maximum of one hour or until a 2 sigma error level of 2% was attained, counting precision ranged from 7% to 2%. GC/MS analyses were performed with a Finnigan-MAT Model 95S double focusing high resolution GC/MS system. A 30-meter DB5 capillary column (J&W Scientific, Folsum CA) was used in the Varian 3400 gas chromatograph. Using a Finnigan A200S autosampler, injections were made on-column via a Varian Septum-equipped Programmable Injector (SPI). Preliminary analyses of the [^{3}H]TCDD exposure solution were performed with the mass spectrometer operated in the electron impact ionization (70 eV) and low mass-resolution scanning modes. Measurements of [^{3}H]TCDD and impurities were made with multiple ion detection (MID). MID analyses were complicated by the presence of analogs of each chemical with two, one or no ^3H labels. Since each analog produces a cluster of

molecular ions which result from the presence of the naturally occurring isotopes of chlorine and carbon, the analytical challenge arises from the similarity of the difference in mass units between ^{35}Cl and ^{37}Cl (1.9970 amu) and the difference in mass units between ^{1}H and ^{3}H (2.0082 amu); only 0.0112 amu separate the two differences. Total TCDD concentration was determined from the combined response of all ions in the range of m/z 320 to m/z 330. Unlabeled TCDD could be measured directly since the m^{-1} molecular ion (nominal m/z 320) is exclusive to that analog. The concentration of the tritiated analogs was calculated from the difference between the theoretical relative abundances and the measured responses at each of the other masses[1].

Tissues were prepared for analyses of TCDD and impurities with techniques similar to those described by Marquis et al (1994)[11]. Homogenized tissue samples (1 to 10 grams) were mixed with sodium sulfate, spiked with $^{13}C_{12}$ 2,3,7,8-TCDD (Cambridge Isotope Laboratories), and soxhlet extracted with hexane/methylene chloride. The extracts were purified with glass columns containing, in sequence, plugs of sodium sulfate, sulfuric acid on celite, potassium silicate and silica gel which were eluted with a mixture of hexane and methylene chloride. To isolate TCDD, the concentrated eluants were placed on columns containing activated carbon on silica gel and were eluted first with methylene chloride and then with toluene in the reverse direction. The toluene eluants were concentrated, and spiked with $^{13}C_{12}$ 1,2,3,4-TCDD (Cambridge Isotope Laboratories) prior to GC/MS analysis.

Results and Discussion Detection in the mass spectra of [^{3}H]TCDD of extraordinary amounts of deuterium (^{2}H) associated with the ^{3}H radiolabel resulted in modification of the TCDD quantitation method to account for the effect on the mass spectra of three additional TCDD analogs. The RIC (Fig 1) of the [^{3}H]TCDD solution indicated that TCDD comprised less than 25% of the chemicals present. Large amounts of dimethylbiphenyls (m/z of 182), tolyl benzylethers (m/z of 198), and methylphenylesters of benzoic acid (m/z of 212) without tritium labels indicated that radiolysis of toluene created tolyl and benzyl free radicals that reacted with toluene or oxygen and toluene to form the impurities. Three major impurities associated with [^{3}H]TCDD had greater gas chromatography retention times and parent ions in the mass spectra with a m/z of 410. All three impurities displayed similar mass spectral fragmentation patterns with loss of chlorine atoms and a tolyl substituent. The large amount of tolyl-TCDDs with either one or no ^{3}H was consistent with formation of adducts of the solvent, toluene, with [^{3}H]TCDD accompanied by loss of ^{3}H from TCDD and ^{1}H from toluene. Such reactions probably occur as the result of a rate determining step involving the radioactive decay of ^{3}H from the $^{1}H,^{3}H$-TCDD, $^{2}H,^{3}H$-TCDD and $^{3}H_{2}$-TCDD analogs present in solution (Fig 2). After a decay event, the resulting helium (^{3}He) is released to solution and the TCDD molecule becomes electron deficient as a carbonium ion which rapidly reacts with the solvent. This reaction mechanism allows the formation of six ditolyl-TCDDs from the $^{3}H_{2}$-TCDD analog as indicated by the mass spectrum (Fig 1).

The gradual increase of tritiated trichlorodibenzo-p-dioxin (TriCDD) and tritiated tolyl-TriCDD in solutions following purification by carbon column chromatography suggested dechlorination of [^{3}H]TCDD as a result of radiolysis followed free radical reactions with toluene. As predicted, mass spectra of tolyl-triCDDs, ditolyl-triCDDs, and tritolyl-triCDDs were obtained from the original solution (Fig. 1). The addition of tolyl as result of radiolysis occurs at the 2,3,7,8

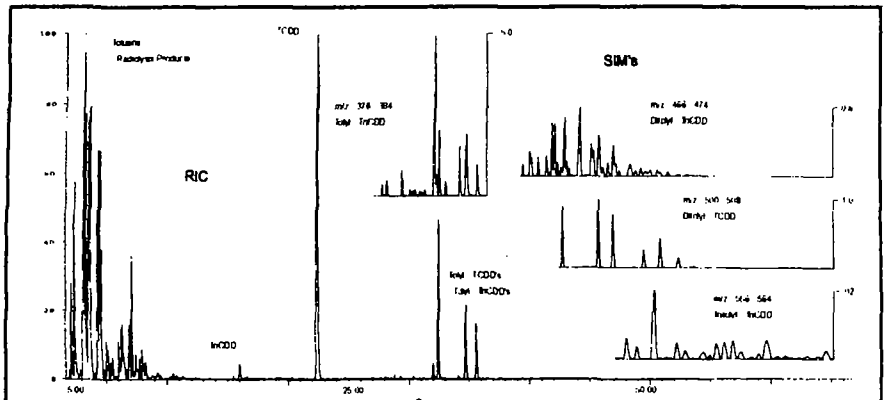


Figure 1. GC/MS analyses of an eight year old solution of [^{3}H] TCDD in toluene. TCDD and major contaminants are represented in the reconstituted ion chromatogram (RIC). Other decay and radiolysis products are presented in selected ion monitoring (SIM)mass chromatograms.

Figure 2. Tritium Decay-Intiated Reactions of ^{3}H-TCDD with Solvent

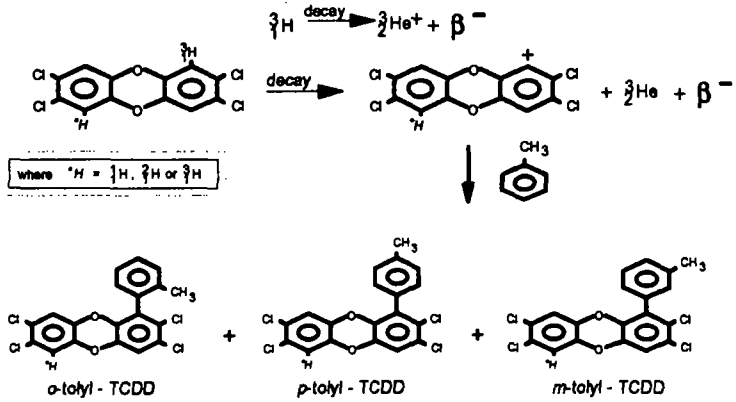
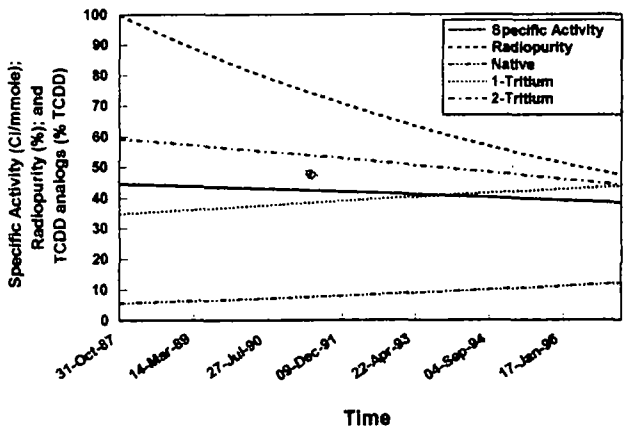
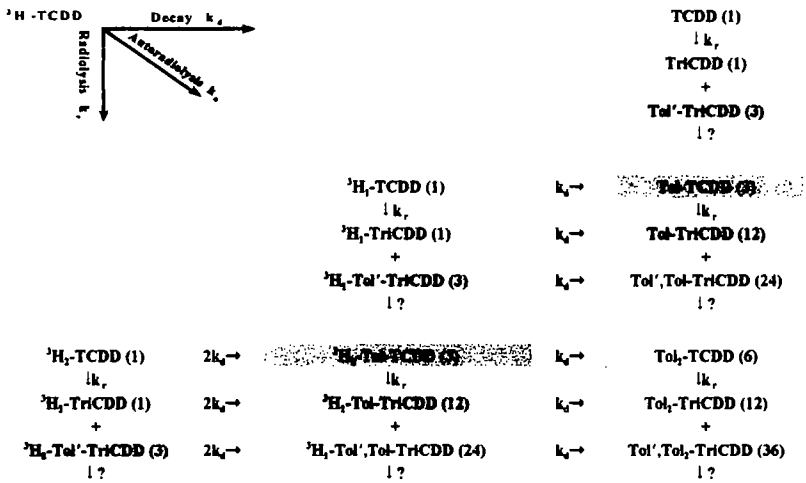


Figure 4. Changes in Radiolabeled TCDD over a 9-Year Period. Radiopurity is estimated from measurements of tritium distribution in solution.



dibenzo-p-dioxin positions, whereas addition of tolyl as a result of the decay reaction $^3H_2$-TCDD occurs at the 1,6 positions (Fig. 2). Figure 3 summarizes the reaction pathways and products (98 structures and 147 masses due to ^3H substitution) that were identified by GC/MS. Because there was no evidence for significant formation of tolyl-triCDDs through autoradiolysis, change in ***S*** with time could be modeled as a function of the loss of TCDD and ^3H through the decay reaction (Fig. 2). Figure 4 illustrates the model's predictions of changes in [^{3}H]TCDD analog composition, ***S*** and ***R*** during storage of the toluene solution. The results are in excellent agreement with GC/MS measurements of the solution over the period of 1993-1997. The model was used to adjust values of ***S*** used with LSC measurements to quantify concentrations of TCDD in organisms during long term toxicity tests.

Figure 3. Reactions of [^{3}H]TCDD in Toluene



The number in parentheses = number of structures possible.
Tol = tolyl substituted at 1,6 positions on dibenzo-p-dioxin.
Tol' = tolyl substituted at 2,3,7,8 positions on dibenzo-p-dioxin.

Because carbon column chromatography did not remove the more planar tolyl-CDDs from [^{3}H]TCDD solutions used in toxicology studies, GC/MS analysis of tissue samples was necessary in order to confirm ***S*** and determine if retention of radioimpurities by the organsms contributed to total radioactivity measured by LSC. Dietary exposure of adult brook trout resulted in accumulation of [^{3}H]TCDD in tissues and eggs with greatly reduced or nondetectable concentrations of the tolyl-TCDD and tolyl-TriCDD impurities that remained in the exposure solution. Less than 1% of the LSC counts could be attributed to the impurities. The absence of the impurities in brook trout tissues was probably due to metabolism, rather than lack of assimilation from food. In exposures of fish eggs to [^{3}H]TCDD via water with 0.5% acetone the contribution of impurities to total LSC counts, measured in embryos early in development, ranged from <1 to 7%, depending on species.

This study has implications for many other investigations which have used or may use ^3H or other β^- emitting radioisotopes with short half lives for LSC determination of concentrations of radiolabeled chemicals. Experiments in which the ^{3}H-labeled chemical is traced for long periods

of time are subject to the types of reactions found for [^{3}H]TCDD and consequent changes in ***S*** and ***R***. Changes in ***S*** can not be predicted accurately without knowledge of the distribution of the ^{3}H atoms among molecules of the chemical (analog ratio) at some point in time. Only mass spectroscopy can determine the analog ratio needed to predict how the sample will change in storage or during the course of experiments. Shorter term experiments may also be affected if the chemical is not purified effectively just before use. [^{3}H]TCDD solutions can most effectively be purified with HPLC techniques[12]. The decay related reactions of ^{3}H-labeled chemicals in tissues need to be considered when analytical data are used for identification of metabolites or measurement of rates of metabolism. Finally, it is our impression that many experiments are insufficiently documented in the literature to allow understanding of the actual degree of radiopurity present

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