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Item ID Number	01649
Author	Gross, Michael L.
Corporate Author	United States Environmental Protection Agency
Report/Article Title	Direct Testimony of Dr. Michael L. Gross, in re: The Dow Chemical Company, et al.
Journal/Book Title	
Year	0000
Month/Day	
Color	☐
Number of Images	45
Description Notes	FIFRA Docket Nos. 415, et al., EPA Exhibit No. 223 Purpose of Gross' testimony was to provide information on the detectin and measurement of TCDD in some human and environmental samples.

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

BEFORE THE ADMINISTRATOR

In re:	
The Dow Chemical Company, et al.	FIFRA Docket Nos. 415, et al.

DIRECT TESTIMONY OF DR. MICHAEL L. GROSS*/

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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

BEFORE THE ADMINISTRATOR

In re:	
The Dow Chemical Company, et al.	FIFRA Docket Nos. 415, et al.

DIRECT TESTIMONY OF DR. MICHAEL L. GROSS*/

My name is Michael Lawrence Gross. I am professor of Chemistry and Director of the Midwest Center of Mass Spectrometry at the University of Nebraska, Lincoln, Nebraska. The mass spectrometry center is one of six regional instrumentation facilities that were formed in 1978 by the National Science Foundation. I received my Ph.D. in Organic Chemistry with minor in Physical Analytical Chemistry in 1966 at the University of Minnesota in Minneapolis, Minnesota. After one year of postdoctoral research at the University of Pennsylvania and one year at Purdue University, I was appointed assistant professor of chemistry at the University of Nebraska in 1968. In 1972, I was promoted to associate professor (with tenure), and in 1978, to professor. A curriculum vitae is attached.

My research interests are divided among three areas, all related to gas-phase ion chemistry and mass spectrometry: (1) fundamental properties of organic gas-phase ions, (2) environmental chemistry, and (3) instrumental developments in mass spectrometry. In the environmental area, our laboratory has performed numerous analyses

*/ EPA Exhibit No. 223.

at trace levels for pesticide residues and toxic substances such as polychlorinated dibenzo-p-dioxins.

The purpose of my testimony is to provide information on the detection and measurement of TCDD in some human and environmental samples. This information is based on analyses in my laboratory which show that measurable amounts of TCDD have been found in some samples of soil, fish and other aquatic biologicals, and in deer and human fat tissue.

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I. BACKGROUND

Since 1977, our laboratory has provided analytical support for several different projects involving the determination of TCDD levels in human and environmental samples. These projects have been sponsored by various private and governmental agencies, such as the Environmental Protection Agency, the U.S. Air Force, the U.S. Department of Agriculture and the National Science Foundation. A brief overview of these several projects is presented below.

Work in our laboratory with tetrachlorodibenzo-p-dioxin (TCDD)*/ began in 1977 and was supported by a contract with the Environmental Protection Agency. The focus of this research was the analysis of TCDD in a variety of environmental and biological samples including soil, blood, adipose, liver and fish tissue. Also during 1977, the analytical methodology currently employed by the EPA and collaborating laboratories was validated over a range of 2,3,7,8-TCDD concentrations from 9-81 parts-per-trillion.

During 1978, our work in this area continued with support from the U.S. Air Force (Major A.L. Young was the project officer). During that year we analyzed 100 biological and soil samples taken from sites contaminated with TCDD. The purpose of the work was to evaluate the environmental fate of TCDD and TCDD uptake into animal populations. This work was continued in 1979 under a

*/ TCDD can exist as 22 different isomers or as various mixtures of these isomers. Throughout this document, the term "TCDD" is used to indicate a mixture of TCDD isomers. When the term "2,3,7,8-TCDD" is used, it refers to that specific isomer of TCDD.

second contract directed by Major Young. A new issue was addressed in 1979: the movement of TCDD in biological samples in the vicinity of the Herbicide Orange storage area at Gulfport, Mississippi. The results of the second study have been published (Ref. 1).

In addition to the Air Force sponsored research, we did the lead analytical work under a Cooperative Agreement with the EPA directed at the analysis of TCDD in human milk from mothers in the western forest areas of the United States. Prior to beginning this work, we participated in a validation study of the human milk methodology at TCDD levels of 0-20 parts-per-trillion. Sediment and water from Oregon were also analyzed during 1979. This cooperative research program has been extended into 1980.

Another study conducted during 1979 was aimed at evaluating the possible accumulation of TCDD in deer tissue in the Blodgett National Forest in California. This work was supported by a grant from the U.S. Forest Service, U.S. Department of Agriculture.

A number of other TCDD monitoring studies were conducted in 1979 principally under the auspices of the National Science Foundation Regional Instrumentation Facility Grant. Three of these studies deserve mention here. In the first study, we assisted scientists from the New York State Department of Health with a preliminary survey of fish from the .iagara River and from Lake Ontario. The results of this investigation were announced publicly by Dr. David Axelrod, state health commissioner, on April 24, 1979 (Ref. 2).

The second study was a collaborative effort with Dr. Brenda Kimble of the Laboratory for Energy Related Health Research of the University of California-Davis, and was directed at evaluating TCDD production in a coal-fired power house. This work has been published (Ref. 3).

The third project was with the U.S. Veterans Administration. Its purpose was to examine whether Vietnam veteran exposure to Herbicide Orange, a 1:1 mixture of 2,4,5-T and 2,4-D which contained high concentrations of TCDD,*/ could be documented by assaying adipose tissue taken ten years after the alleged contact with Herbicide Orange.

In the remainder of this document, I will discuss the analytical methodology used in our laboratory, and the results of some of the above projects which are based on our analytical studies.

II. Analytical Methods

The complete analysis of a sample for TCDD consists of two operations: sample extraction and clean-up, followed by the actual analysis. The sample extraction method for tissue employed in our laboratory is called the acid/base procedure and was originally introduced by Baughman and Meselson (Ref. 4) and then perfected by scientists at Dow Chemical Company (Ref. 5). It is essentially the same method as that employed by Dr. Aubry Dupuy and his co-workers at Bay St. Louis, Mississippi.

*/ To date, there is no firm evidence indicating that 2,4-D contains TCDD. Therefore, it is assumed that the TCDD in Herbicide Orange comes from the 2,4,5-T.

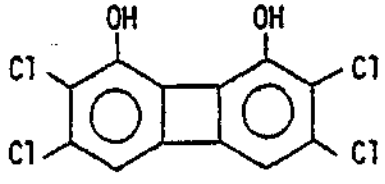
Although the methods vary somewhat with sample type, there are some steps which are common to all. A tissue sample is first dissolved in an alkaline solution which serves to solubilize in water all the fat and protein material. Nonpolar materials, such as TCDD, are then extracted into hexane, and most of the original sample material is left behind in the water layer. (When analyzing a soil sample the first step is the extraction of nonpolar components into hexane.) Once the sample is in hexane, the hexane layer is cleaned up by washing with concentrated sulfuric acid and performing one or more liquid chromatography steps. These procedures remove low concentrations of potential interfering compounds so that TCDD can be freed and analyzed at the parts-per-trillion level without interference from other materials. The extract is then stored until analysis. Details of these procedures are contained in Appendix 1.

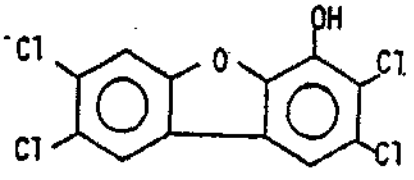
We employ packed column gas chromatography/high resolution mass spectrometry (GC/MS) for the analysis of the extract. The mass spectrometer (a Kratos MS-50) possesses the highest resolving power available in a commercial instrument. We have demonstrated an ultimate resolution of 180,000 (10% valley) which means we could distinguish mass 180.000 from 180.001. This resolving power is not employed for the analysis of TCDD because the sensitivity would be very poor; rather the resolution is chosen to be 10,000 which is adequate to separate TCDD from other interferences, notably polychlorinated biphenyls and DDE (a metabolite of DDT), which could lead to false positives. A

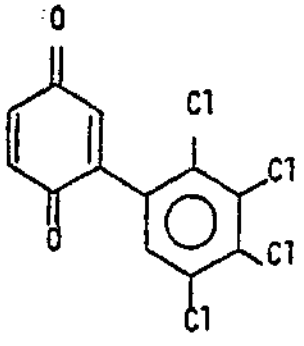
description of the GC/MS analysis and a typical output showing the resolution of potential interferences are presented in Appendix 2.

Some considerations of our analytical procedure should be now pointed out. First of all, the method allows us to monitor each sample at the exact mass of TCDD: 321:8936 ± 0.0015. This is accomplished through the use of an internal standard (TCDD with all chlorine atoms as Cl-37) which serves as a mass standard. As a result, the analysis is highly specific for any substance which has the formula $C_{12}H_4O_2{}^{35}Cl_3{}^{37}Cl$. All of the tetrachloro-dibenzo-p-dioxins have this formula. */

*/ There are also other compounds which have this combination of elements. The examples below are illustrative of substances having the same mass as TCDD. However, having the same mass does not automatically mean that a compound will behave as TCDD in our analytical procedure. None of these subatances has been identified in our analyses, and we postulate that they would be removed in the sample clean-up or by the gas chromatography.







Second, the possibility of assigning a positive detection for TCDD to the side of a peak from a very intense interference signal is minimal because entire peak profiles are gathered for each analysis. Consequently, only a complete peak corresponding to TCDD is designated as TCDD. Therefore, the method is reasonably unsusceptible to "false positives." Third, if a detection is made, the analysis is repeated and both $C_{12}H_4O_2{}^{35}Cl_3{}^{37}Cl$ (m/z = 321.8936) and $C_{12}H_4O_2{}^{35}Cl_4$ (m/z = 319.8967)*/ are monitored. These are both expected signals from the TCDD molecule and should be in the ratio of 1.00:0.78 (we expect to reproduce this ratio to within $\pm$ 10-15%). This procedure adds additional assurance to a detection. Fourth, the measurement of the amount of TCDD, if detected, is made by considering the intensity of the signal relative to the internal standard. This method (called the internal standard method) is recognized as the best way to obtain accurate quantitative results.

As is well-known, TCDD can exist as 22 different isomers. Probably the most toxic isomer, that produced in the manufacture of 2,4,5-trichlorophenol, 2,4,5-T and silvex, is 2,3,7,8-TCDD. The mass spectrometer, as we operate it, is not capable of distinguishing among isomers; only gas chromatography can do this. Therefore, a gas chromatograph is used in series with a mass spectrometer in our and most laboratories doing TCDD analyses. Our gas chromatography column, developed and evaluated by Dow

*/ m/z = mass to charge ratio. This ratio is also sometimes designated as m/e.

scientists (Ref. 6), permits us to conclude that, if a detection is made, it may be 2,3,7,8-TCDD or one of eight of the remaining 21 isomers. Thus, our methodology has some, but not total, specificity for 2,3,7,8-TCDD. However, we often collaborate with the EPA laboratory of Mr. Robert Harless, who employs even higher resolving power gas chromatography that can distinguish all of the isomers, to verify positive detections.

III. Validation Studies

It is well appreciated among analytical chemistry scientists that analysis at parts-per-trillion levels is extremely difficult. This is primarily because of the low levels involved. To gain an appreciation for this, consider that if one were measuring time instead of chemical concentrations, one part-per-trillion corresponds to about one second in just over 30,000 years. Compounding the problem is the fact that most samples contain a multitude of interferences which can complicate analyses at these levels.

Because of these difficulties, it is important to validate the analytical procedures used at these low levels. Therefore, it has become customary for the EPA and its collaborating scientists to periodically engage in validation studies, principally when a new method or sample medium is used. Our laboratory has participated in two validation studies which were designed to test the analytical capabilities of the method we employ. Both studies were conducted blindly; that is, extracts containing unknown amounts of TCDD were prepared by other scientists (Dr. Aubry Dupuy and his co-workers at the EPA Toxicant Analysis Center in

Bay St. Louis, Mississippi) and then sent to us in such a way that we could not know the amounts involved. After analysis, the results were then compared with the true values.

In the 1977 validation study, standard solutions and extracts from "spiked" beef fat were studied. See Figures 1 and 2 for a graphical picture or our results. The original data reported by us to EPA are given in Table 1V of Exhibit 224.

As can be seen from our results plotted in Figures 1 and 2, the agreement between the reported value and the true value is quite good. Furthermore, no "false positives" or "false negatives" */ were obtained. Successful detections of TCDD in these 5 gram samples were in the range of 9-81 parts-per-trillion. Samples containing 1 and 4 parts-per-trillion TCDD were not identified because they were lower than our detection limit (ca. 5 ppt). **/ This study shows that TCDD can be reliably extracted, detected, and quantitated at the low parts-per-trillion level.

Before beginning the human milk monitoring project in 1979, it was necessary to validate the extraction and analysis of TCDD in this medium. This was done in early 1979 in the same manner as the 1977 validation of standard solutions and beef fat. The results (submitted to EPA in letter reports --- see Exhibit 225)

*/ "False positives" are analyses in which TCDD appears to be detected even though none is present in the sample. "False negatives" refer to analyses in which TCDD is present but is not detected.

**/ Detection limit is the minimum concentration of sample that can be detected in any given analysis.

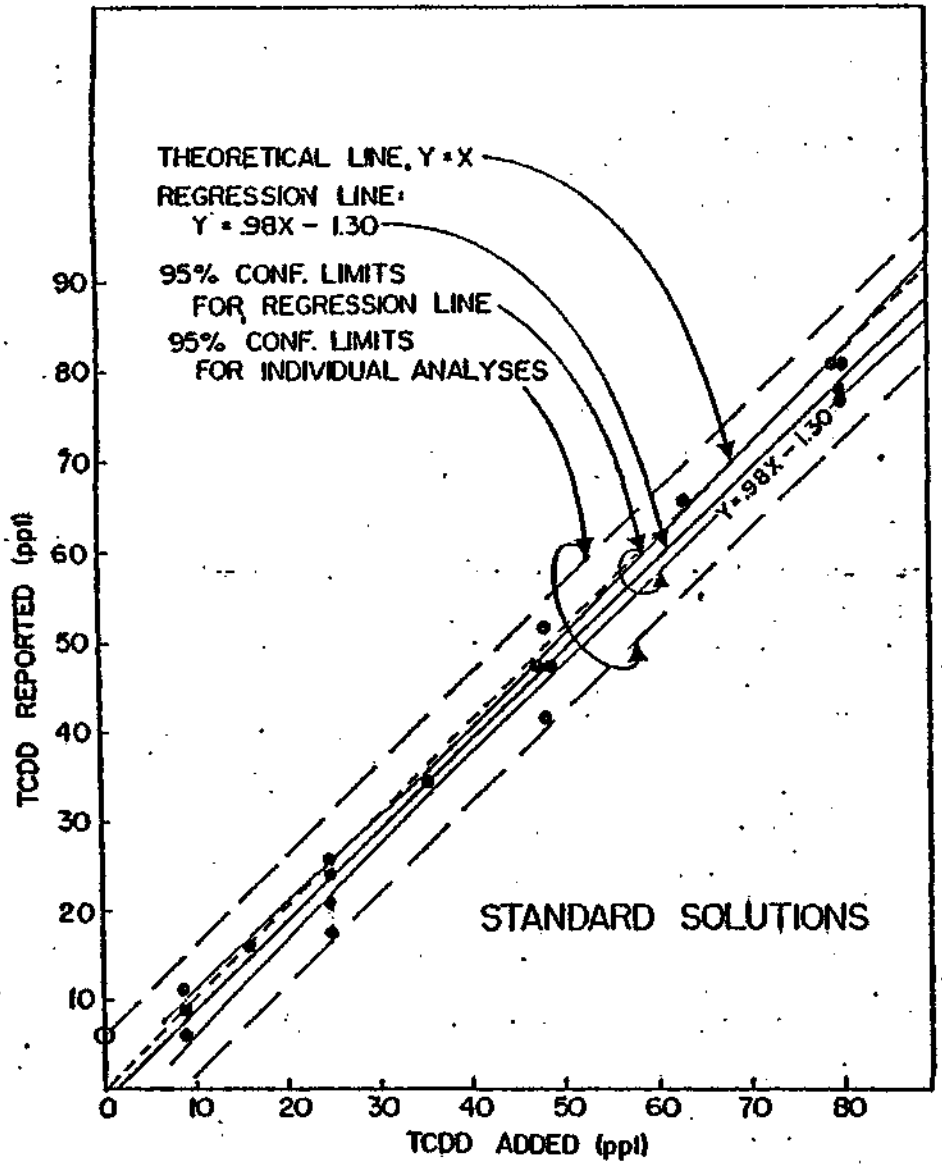


Figure 1

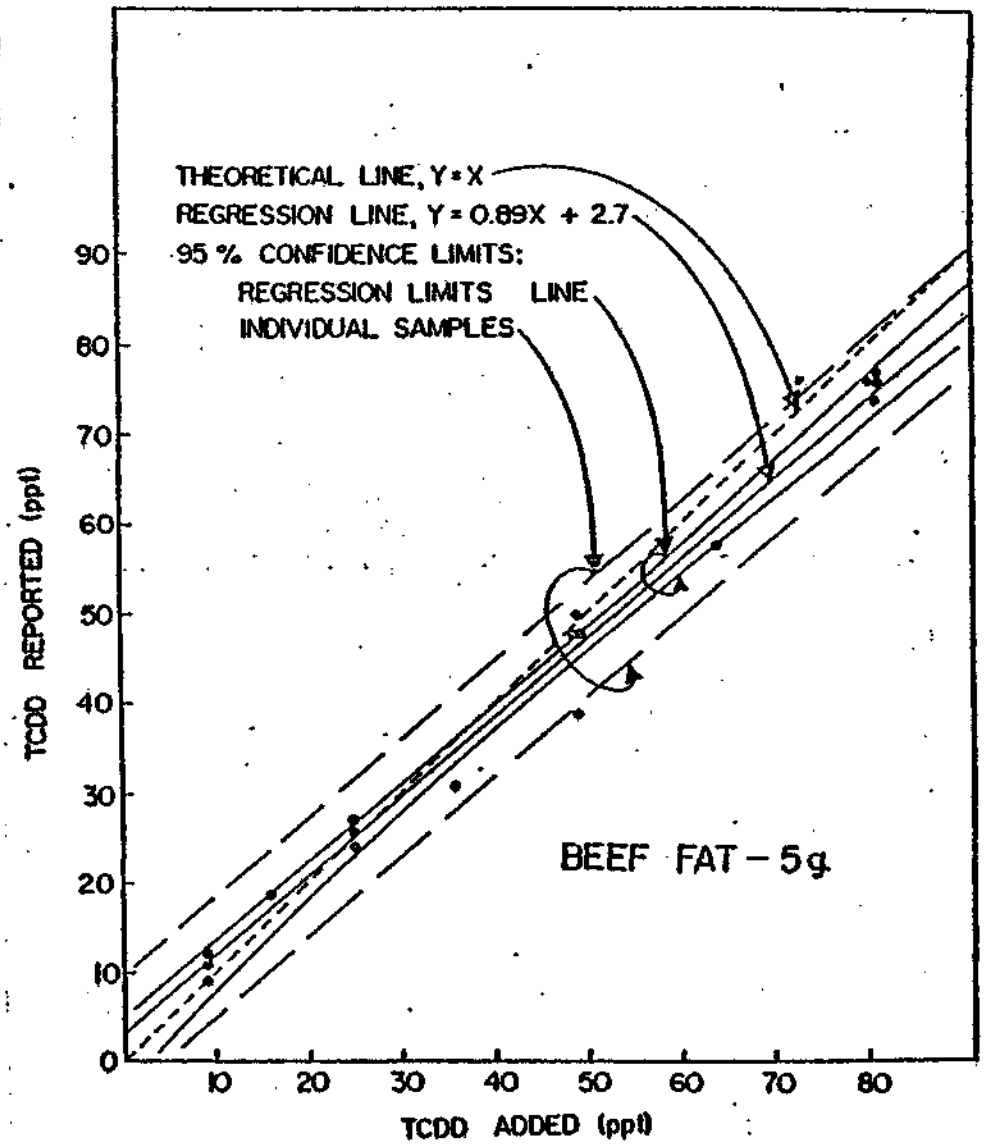


Figure 2

are shown graphically in Figures 3 and 4. The correlation of our results is less good, principally because the samples contain lower concentrations of TCDD (1-20 parts-per-trillion). Nevertheless, the results show that TCDD can be reliably extracted, detected, and quantitated (albeit less accurately than at higher levels) in human milk. Furthermore, no "false positives" and only one "false negative" was found in the validation study and in the quality control samples */ which were submitted along with the extracts of the actual human milk samples.

To minimize the possibility of false positives and to add certainty to analyses in which detectable levels of TCDD were observed, a method validation approach was instituted in the monitoring program as a standard procedure in 1979. According to this procedure, a sample showing detectable levels of TCDD in one laboratory is reextracted and reanalyzed in another laboratory for confirmation. Most of our collaborative work has been conducted with the EPA extractions laboratory (Dr. Aubry Dupuy) at the Toxicant Analysis Center, Bay St. Louis, Mississippi, and the EPA analysis laboratory (Mr. Robert Harless) at Research Triangle Park, North Carolina. **/

*/ Quality control samples are extracts containing known amounts of TCDD which are analyzed along with the true unknowns.

**/ The analysis procedure of Mr. Harless is complementary to our own. He employs capillary column gas chromatography, high resolution mass spectrometry, which, as previously mentioned, is capable of distinguishing the TCDD isomers. However, unlike our laboratory, he does not record signal profiles and works at somewhat lower mass resolutions.

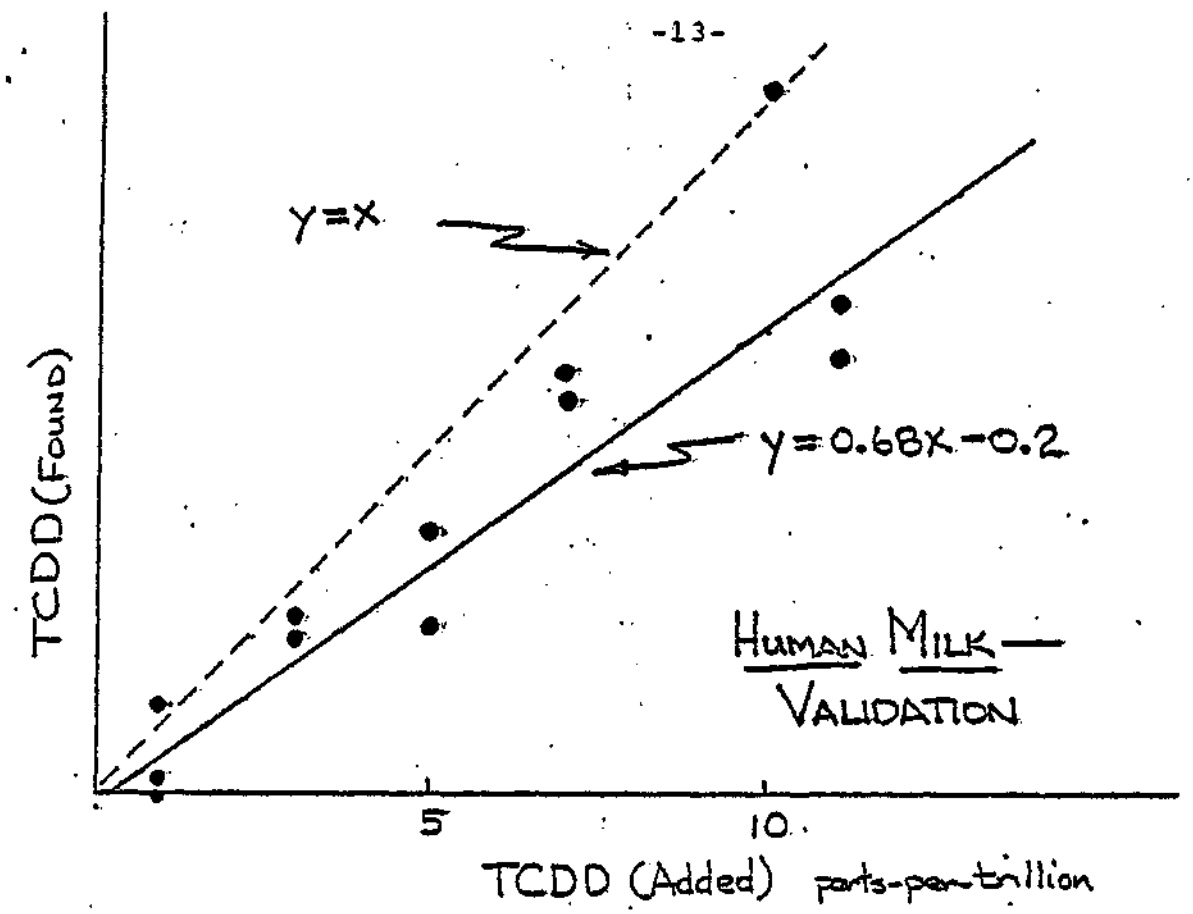


Figure 3

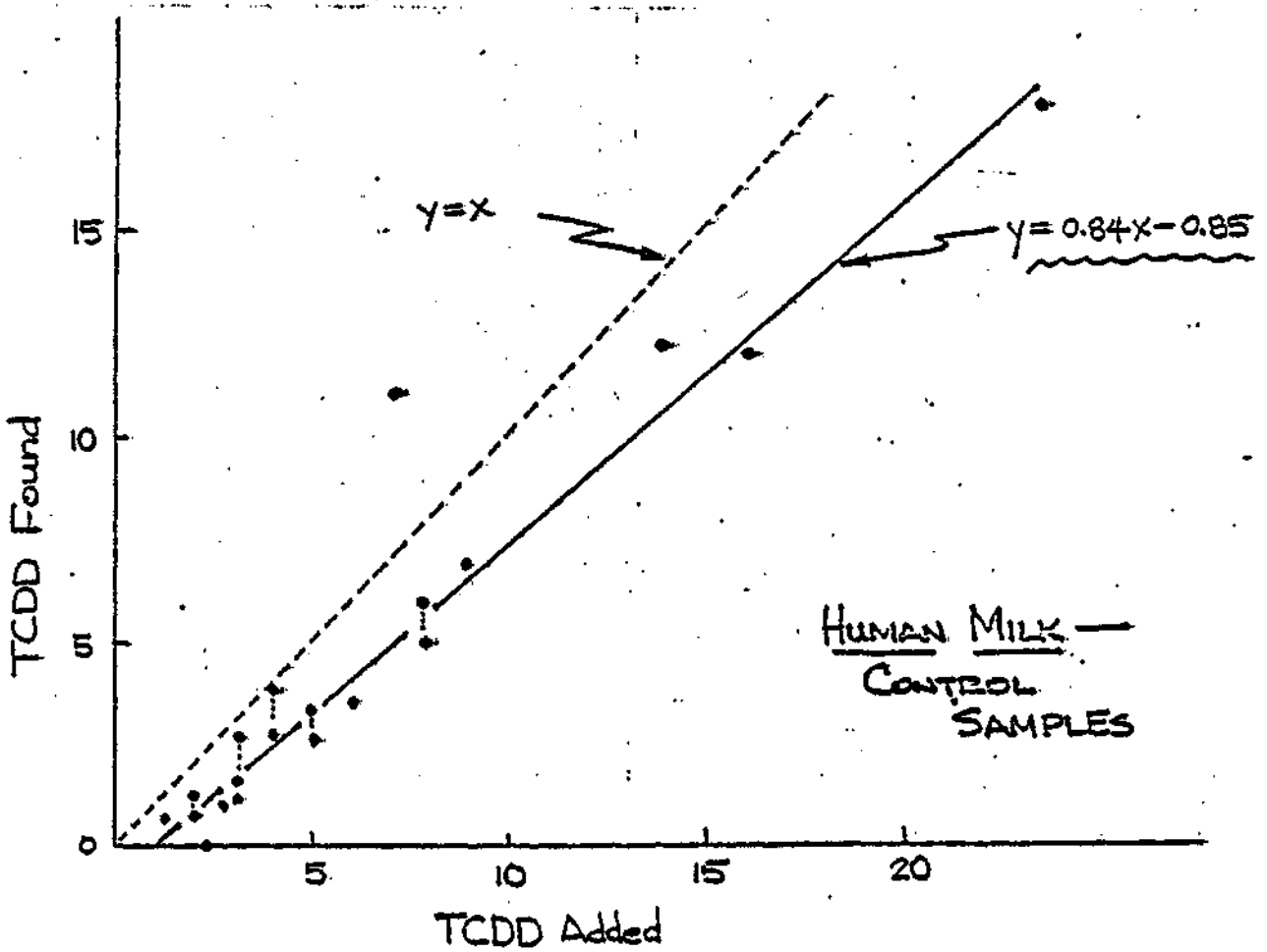


Figure 4

Our criteria for assigning a positive detection of TCDD are as follows:

1. The gas chromatography retention time must be the same as authentic TCDD.
2. The suspected samples must give a signal at 321.8936 $\pm$ 0.0015 as determined by real-time peak matching using the internal standard TCDD-^{37}Cl as the reference mass.
3. The signal intensity ratio of m/z 320:322 must be 1.00:0.78 ($\pm$ 0.10).
4. The exact mass of m/z 320 must be 319.8965 $\pm$ 0.0015 using m/z 321.8936 as a mass standard.
5. The detected signal at m/z 321.8936 must be at least 2.5 times the noise level (refered to hereafter as a 2.5 S/N criterion).

Concluding this section, we note that TCDD can be reliably analyzed in fat and milk at levels extending down to one part-per-trillion in milk. These observations are important for they lend credence to all trace analyses of TCDD done in this laboratory. Furthermore, as a result of application of the stringent requirements cited above and employment of a "method validation approach", we postulate that TCDD can be reliably detected at levels down to 1 part-per-trillion in similar types of samples.

IV. Analyses of Environmental Samples

Our laboratory has participated in several studies which have involved the analysis of environmental samples for TCDD.

Environmental media which have been analyzed in these studies include soil, water, sediment, fish and other aquatic biological, and fat tissue from deer. Four of these studies are discussed below.

A. EPA Soil Study

During 1977, 16 soil samples from the forest area of Grays Harbor County in the State of Washington were extracted and analyzed in our laboratory. These soil samples were collected three hours after a normal spray of 2,4,5-T and then 15 days, 37 days, and 72 days after spraying. They were composites of the first 1" or the first 2" of topsoil. No analyses were conducted on the 15-day samples.

Twelve samples were found to contain detectable amounts of TCDD varying from 8 to 40 parts-per-trillion (Ex. 224), with an average detection limit of 6 parts-per-trillion and a range of 2 to 9 parts-per-trillion. No trend could be distinguished in terms of sampling time. That is, 1 of 2 samples taken on the day of spraying contained 20 parts-per-trillion TCDD; 5 of 6 samples taken after 37 days showed TCDD at levels ranging from 8-31 parts-per-trillion, and 6 of 8 samples after 72 days contained TCDD at levels of 12 to 40 parts-per-trillion.

In addition, ten animal tissue specimens were extracted and analyzed, but no TCDD was detected at an average detection limit of 4 parts-per-trillion (range of 1 to 7 parts-per-trillion).

B. Sediment and Water Study

In 1979, various samples of sediment and water taken from the Alsea, Oregon area were extracted by EPA and analyzed in our laboratory (Ex. 225). The water samples were surface water taken from fourteen sites in the Alsea area. The samples were sub-divided, extracted, and portions were analyzed by both Mr. Robert Harless and by us. No TCDD was detected by either laboratory; i.e., the detection limit varied between 0.008 and 0.020 parts-per-trillion in Mr. Harless' laboratory, and 0.004 and 0.080 parts-per-trillion in our laboratory. The average detection limit using the lower value reported for each sample was 0.011 parts-per-trillion (11 parts-per quadrillion) with a range of 0.004 to 0.020 parts-per-trillion. We can conclude with high certainty that no TCDD exists in these particular surface water supplies at levels of 0.100 parts-per-trillion or higher.

Sediment samples were taken below the water at ten of the fourteen sites investigated above. The samples were extracted by Dr. Dupuy and coworkers using both a neutral extraction and an acid/base procedure. In the neutral procedure, any TCDD in the sediment was removed by extraction into hexane/acetone followed by the usual clean-up. In the acid/base procedure, the sample was heated with an alkaline solution (as if it were a tissue sample) and the resulting mixture was extracted to remove any TCDD. The samples were analyzed by Mr. Robert Harless and by us.

No TCDD was detected at seven of the ten sites investigated at a detection limit of less than one part-per-trillion. We

believe we can say with high certainty that no more than one part-per-trillion of TCDD exists at these seven sites. However, at two sites, the results were more ambiguous. For example, at site #1, one analysis of the five conducted gave a positive result at 2 parts-per-trillion; all the other analyses showed no TCDD detected at detection limits ranging from 0.6 to 4 parts-per-trillion. At site #4, two analyses were positive at 1.0 and 1.5 parts-per-trillion, but the results could not be duplicated by additional extractions and analyses. We can therefore conclude with certainty that no TCDD exists at these two sites at levels exceeding 3 parts-per-trillion; however, lower concentrations may be present.

Finally, the sediment from one of the sites (#9) was shown to contain significant amounts of TCDD. The results for the several analyses of this particular sample are as follows:

Lab	Analysis Identification	Level TCDD		Detection Limit	
RTP-1 */	Neutral Extraction of 50 g.	28	ppt	3	ppt
RTP-2	Acid/Base Extraction	10	ppt	1	ppt
UNL-2	Split of RTP-2	2.3	ppt	0.5	ppt
RTP-3	Acid/Base Extraction	17	ppt	3	ppt
UNL-3	Split of RTP-3	4	ppt	1	ppt

*/ RTP refers to Research Triangle Park EPA Lab of Mr. Robert Harless. UNL refers to the University of Nebraska-Lincoln.

The quality control samples done in our laboratory at the time of our analyses of these sediment extracts tended to be low, and, therefore, we think that our results are low. Accordingly, we postulate that TCDD does exist in these samples at a level between 10 and 20 parts-per-trillion. Although the quantitative results show some variability, we suggest that the important observation is that all analyses of this sediment showed detectable levels of TCDD in the parts-per-trillion range.

C. Gulfport Study

Also during 1979, we collaborated with Major A.L. Young of the United States Air Force to monitor biological samples for TCDD in the area of Gulfport, Mississippi (Ex. 226). Specifically, the samples were taken near the Naval Construction Battalion Center (NCBC), the site where over 15,000 drums (more than 80,000 gallons) of Herbicide Orange had been stored from mid 1968 until mid-1977 (Ref. 1).*/ This area was unfortunately contaminated with Herbicide Orange, including its TCDD component, because of leaks in the drum containers.

The concentration of TCDD in the soil is highly variable at this 1-2 acre site depending on whether or not spills had occurred. The mean concentration of TCDD at spill sites in July, 1977 was 240 parts-per-billion.**/ In 1978, additional monitoring was done (Ref. 1). Sites where no obvious spill had taken place contained

*/ In the summer of 1977, the Herbicide Orange was removed from Gulfport and destroyed.

**/ Parts-per-billion = 1000 x parts-per-trillion.

less than 0.2 parts-per-billion TCDD, whereas "old" spill (light stain, mild odor) and "new" spill (heavy stain, strong odor) sites were contaminated at levels of about 40 parts-per-billion and 200 parts-per-billion, respectively.

Clearly, the area contains TCDD at levels much higher than soil exposed tc normal spray application of 2,4,5-T. However, the high levels allowed Major Young to develop an analytical program to address the question of movement of TCDD in the environment. Our role in the program was to analyze biological samples.

As has been reported by Young, Thalken, and Cairney (Ex. 226), we found detectable levels of TCDD in a variety of biological samples (crayfish, mosquitofish, frog, etc). */ The concentration of TCDD decreased smoothly from levels of 100 to 7200 parts-per-trillion on site to levels of 45 parts-per-trillion at a distance of 7000 feet from the site and 20 parts-per-trillion at 9000 feet.

The same trend was observed for samples collected later, in June, 1979. For example, for composite crayfish/mosquitofish samples, levels of 175, 88, 31, 20, and 6 parts-per-trillion were

*/ We have also participated in monitoring of TCDD from a more normal environment. One study was conducted on fish in the Niagara River and in Lake Ontario in collaboration with scientists at the New York Department of Health. TCDD was detected at levels of 4.6 parts-per-trillion in a small mouth bass and 6.5 parts-per-trillion in a brown trout, both from Lake Ontario (Ref. 2). The results are consistent with the observations from Gulfport, but the origin of the TCDD and the extent of the contamination is not clear from this preliminary study. It is our understanding that more extensive investigations are underway in the New York Department of Health.

found on site and at 3000', 7000', 9000', and 12,000' from the storage area. The complete data and a map of the area are shown in Tables 1 and 2, and Figure 5.

These observations appear to be at odds with the commonly held view that TCDD in soil is immobile (Ref. 7,8). We must point out, however, that for the usual environmental situation, movement studies of this nature would not be feasible because the site of origin of the TCDD (a spray site, for example) would be contaminated at levels lower than the at parts-per-billion levels found at NCFBC in Gulfport. Clearly Major Young and his coworkers have shown farsightedness in taking advantage of an inadvertant contamination incident to generate useful environmental data.

Young and coworkers (Ref. 9) have also coordinated studies on other biologicals at Eglin Air Force Base, Florida, a one square mile site that was heavily sprayed with 2,4,5-T (161,000 pounds between 1962 and 1970). The area contained rather high levels of TCDD in 1973 (10-710 parts-per-trillion). In 1978, TCDD was still present, but no comparison can be made because of sampling differences. Based on results from many biological analyses, it was shown that TCDD accumulates in animal tissues (detectable levels were observed in nine species of animals). For example, the liver of beach mice was shown to have up to 1500 parts-per-trillion TCDD, several times the amount in its surrounding habitat. This work is ongoing, and we are collaborating by providing analytical support.

Table 1. TCDD Residues in Biologicals from Gulfport (1976 Results)

Aquatic Sampling Site	Distance from Storage Area (feet)	TCDD Residues in Biologicals (ppt) a/
I	Immediate Area	140 - 3500 1600 - 7200 b/
II	3,000	200 - 2200
III	7,000	45
IV	9,000	20
V	12,000	ND

ND= Not detected at detection limit of 8 ppt.
a/ Detection limit ranged from 50 to 5 ppt.
b/ Sample collected in 1979

Table 2. TCDD Residues in Biologicals from Gulfport (1979 Results)

Aquatic Sampling Site	Distance from Storage Area (feet)	Nature of Sample	TCDD Residues (ppt) b/
II	3,000	Composite a/	175
III	7,000	Composite	88
		Turtle (fat)	ND
IV	9,000	Composite	31
V	12,000	Composite	20
		Frog (whole body)	6

ND= Not detected at detection limit of 35 ppt.
a/ Composite samples consisted of mosquitofish and small crayfish.
b/ Detection limit ranged from 35 to 5 ppt.

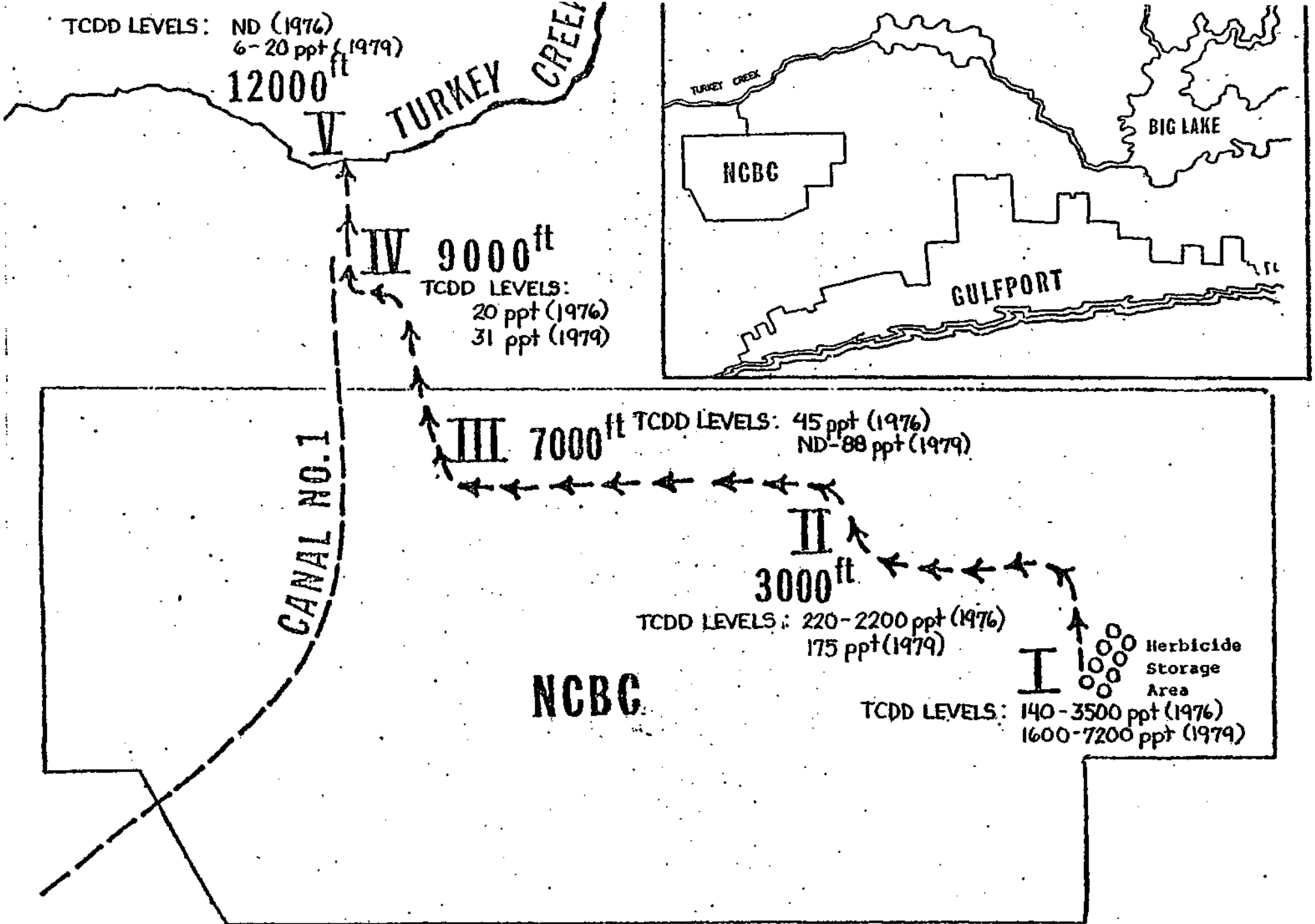


Figure 5. Locations of the aquatic sampling sites in relation to the herbicide storage area. Relationship of the Naval Construction Battalion Center (NCBC) to the major popu- center and associated aquatic system (Insert).

D. Blodgett Forest Study

Finally, a carefully designed study on the impact of 2,4,5-T on Blodgett Forest located in central California has been undertaken by the U.S. Forest Service, and is nearing completion. We participated in a part of this study testing whether TCDD would accumulate in deer tissue. Deer were penned in an 11 acre area in the Blodgett Forest. The herbicide 2,4,5-T was sprayed at an application dose of three pounds per acre. Prior to the spraying, fat biopsies were taken from all deer to be used as controls. After the spraying, three deer each were sacrificed at intervals of 2, 14, and 28 days. Again samples of tissue (fat, liver, muscle, and bone marrow) were taken. Two additional deer were collected from areas not known to have been sprayed with 2,4,5-T, and tissues from these deer were used as controls (Ref. 10).

The tissue samples were extracted and cleaned up by Dr. Aubry Dupuy at the Toxicant Analysis Center in Bay St. Louis, Mississippi using the "acid/base" procedure. The extracts were submitted to us (along with a suitable number of control samples) for analysis using our standard method of gas chromatography-high resolution mass spectrometry. The results of our analysis as originally reported to the Forest Service are presented in Table 3.

TCDD was detected in 18 of the samples of deer tissue that were submitted to us for analysis (Ex. 227). We have recently learned that nine of these samples were quality control samples that had been spiked with known amounts of TCDD (Ex. 228). There appears to

Table 3. Summary of Positives in Deer from Blodgett Forest

Sample Number	Sample Type	TCDD Residue (ppt)	Detection Limit (ppt)
FS-3	Deer Muscle	3.3	1
FS-4	" "	1.6	1
FS-7	" "	7.4	0.8
FS-8	" "	3.4	1
FS-11	" "	4.6	0.7
FS-18	Deer Adipose	12	2
FS-19	" "	2	2
FS-20	" "	2.9	1
FS-23	" "	4.9	1
FS-24	" "	2.2	1
FS-27	" "	3.6	0.7
FS-29	Deer Liver	14	0.8
FS-31	" "	2.5	1
FS-34	" "	2.1	1
FS-36	" "	4.5	2
FS-40	" "	7.8	2
FS-42	" "	3	3
FS-45	Bone Marrow	3.9	3

be a systematic error in the quantitation of these quality control samples. Specifically, our results are a factor of about 3-3.5 times lower than the "true" values. Because our determined values are always low, we surmise that the error must be systematic and occurred either in the spiking and extraction laboratory or in the analysis laboratory. Studies are underway to determine the source of the error. We believe these errors do not significantly reflect on the validity of the study. In our view, the key point is whether TCDD can be detected in these samples; the exact levels at the low parts-per-trillion range are of secondary concern.

Knowing that nine of the eighteen deer samples were spiked as quality control samples, we concluded that the remaining nine samples of deer tissue contain TCDD at an apparent level of 1-10 parts-per-trillion. However, if these determinations are also systematically low because of an error made in our analysis, then the levels would have to be raised by about a factor of three, i.e., the real levels would range from 3 to 30 parts-per-trillion.

We do not have knowledge of the sample code at this writing. */ For example, we are unsure whether positive detections were found only in exposed deer or only in control animals (not exposed), or both. Therefore, we cannot comment on any trends in the data and we cannot conclude that spraying of 2,4,5-T leads to accumulation of TCDD in deer tissue. However, the final results from this study should permit us to refute or substantiate

*/ Because analysis of some samples is still in progress, the Department of Agriculture has not released the code which identifies particular samples as coming from exposed or from control deer.

the hypothesis that TCDD accumulation does occur in animal tissue as a result of normal usage of 2,4,5-T. This is particularly true if these results are consistent (as they appear to be) with the results from an ongoing EPA study on TCDD residues in deer and elk from 2,4,5-T treated forests. */ Clearly, this present study will be important for understanding the fate of TCDD which enters the environment by a "normal" spray application route, and further details should be sought.

V. Analysis of Human Samples

Our laboratory has provided the analytical support for three studies designated to determine whether or not TCDD accumulates in human tissue. These studies involve (a) human liver and adipose from autopsy cases in the United States rice growing area, (b) human milk from women in the western forest areas of the United States, and (c) human adipose from veterans of the Vietnam conflict. They will be discussed in turn.

A. Human Adipose and Liver Study

During 1977, 44 samples of human adipose and liver were extracted and analyzed for TCDD in our laboratory. The samples originated from autopsy cases in four southern hospitals in the U.S.: Greenwood Leflore Hospital, Greenwood, Mississippi; Veterans Administration Hospital, Memphis, Tennessee; University Medical Center, Memphis, Tennessee; and Veterans Administration Hospital, Little Rock, Arkansas. These hospitals were chosen because they handle patients residing in or about the principal rice-growing

*/ Preliminary results from the EPA study indicate that measurable amounts of TCDD can be found in animals that have gazed in 2,4,5-T treated forests (Ref. 12). Other witnesses in this hearing will testify on this study.

areas of this country. Herbicides, including 2,4,5-T and silvex, are used annually for this application. No TCDD was found in any of the samples at a detection limit of 5 parts-per-trillion on the average (the range of the detection limit for 41 samples was 1 to 12 parts-per-trillion). Three samples had somewhat higher detection limits: 17, 19, and 160 parts-per-trillion because of small sample sizes for the first two and a poor percent recovery for the third (ca. 1%).

B. Human Milk Study

In 1979, we analyzed 103 human milk samples under the auspices of a Cooperative Agreement with the EPA. The 72 study cases were mothers residing in northern California, Oregon, and Washington state. Control samples were taken from mothers in Los Angeles and in Alaska, two areas where 2,4,5-T or Silvex exposure is not likely. The mothers were all volunteers.

Each sample was divided into two equal volumes, and one volume was extracted and cleaned-up by Dr. Aubry Dupuy and his coworkers. (The second volume was reserved in the event that reextration and reanalysis became necessary in order to resolve questionable results from the first extraction.) The extracts along with controls were then submitted to us for analysis by GC/High Resolution Mass Spectrometry. As was discussed previously, the sample extraction, clean-up, and analysis was first validated using control samples (see Ex. 225).

No TCDD was detected in any of the samples at detection limits averaging about 1 part-per-trillion. The average detection

limits for each sample region are as follows:

State	Average Detection Limit	Range
Washington	0.9 ppt	0.2-2.0 ppt
Oregon	1.9 ppt	0.5-3.0 ppt
Northern California	2.7 ppt	0.4-6.0 ppt
Controls	1.8 ppt	0.1-3.0 ppt

Clearly, no human exposure, documentable by milk analyses, is evident at the detection limits shown above.

In the course of this study, an important observation was made with reference to the analytical science of parts-per-trillion detections. Nine samples appeared to be positive at levels of 0.7 to 11 parts-per-trillion during the first round of analyses in our laboratory. These samples were then reextracted using the second milk portion and submitted to us and to Mr. Robert Harless at the EPA Research Triangle Park laboratory. The sample identity was not known to us. In all cases, the reanalyais gave a "not detected" result in both laboratories at detection limits lower than for the first analyses.

This approach of checks and replicate analyses, carefully designed by EPA scientists, permitted the analytical workers to avoid reporting out "false positives". Moreover, it is evidence of the nonprejudicial manner in which government science has sometimes been undertaken. We can speculate that the first round of apparent positives was due to handling errors either in the

extraction or the analysis laboratories. Furthermore, the positive detections, later corrected, illustrate the need for caution in the interpretation of analytical results at these low levels.

C. Vietnam Veterans Study

The third study on potential human exposure to TCDD in which we participated was conducted by the Veteran Administration, and involved the analysis of human adipose taken from veterans of the Vietnam war and a control group. Fat samples were taken from 33 men, including a control group of 10 who had not served in Vietnam. As of December, 1979, 22 of the 33 samples had been analyzed, and dioxin was found in 10 of them (Ref. 11). The Veterams Administration is currently reviewing the results of the analyses, and their possible implications. */

VI. Conclusion

There now exist reliable methods for the extraction, detection, and quantitation of TCDD at low parts-per-trillion levels in soils, sediment, and tissue. The validity of the methods is insured by the validation studies, the stringent criteria employed

*/ Our laboratory reported the results of its analyses to the Veterans Administration on February 13, 1980. In addition, the results from some of these samples have been qualitatively confirmed by Mr. Harless at the EPA analysis laboratory. Because of the possible public reaction to the results and implications of this study, the Veterans Administration has asked that the data from our laboratory not be presented in an open forum before the entire study has been reviewed by independent scientists. However, the Veterans Administration is amenable to their use in an in camera session, and negotiations are currently underway to provide protective arrangements for the use of our data in this hearing. Once these protective arrangements are in place, or the review is complete, I agree to be recalled in this hearing in order to present data from this study.

in the collaborating laboratories, and the confirmation or method validation procedure involving checks in a second laboratory.

Application of these analytical methods to real samples permits us to conclude that measurable amounts of TCDD can be found in some human and environmental samples. In many instances, the presence of the TCDD can be associated with exposure to 2,4,5-T.

Michael L. Gross by par

Michael L. Gross

APPENDIX 1

Sample Extraction Procedure for Tissue

A 1-10 g sample was accurately weighed and spiked with a known amount (2.0 - 2.5 ng) of ^{37}Cl-TCDD. It was then saponified in 15 ml of ethanol*/ and 30 ml of 40% aqueous KOH in a reflux apparatus for 60 minutes with stirring. The sample should be completely hydrolyzed before terminating the saponification.

The solution was transferred to a 250 ml separatory funnel and diluted with 20 ml of ethanol and 40 ml of water and extracted four times with nanograde hexane. The first extraction was done with 25 ml of hexane, shaking vigorously for one minute. The lower aqueous layer was removed to a clean beaker, and the upper hexane layer was decanted to a 125 ml separatory funnel. The aqueous layer was then extracted three times more with 15 ml portions of hexane, each time adding the hexane to the 125 ml separatory funnel. The combined hexane extracts were washed with 10 ml water to remove excess base.

The combined hexane extracts were washed 4 times with 10 ml concentrated H_2SO_4, or until both layers were clear. As many as 8 extractions may be necessary, depending upon the sample. Again the hexane was washed with 10 ml water. The hexane layer was decanted to a 2 ounce jar and concentrated under a stream of dry nitrogen to approximately one ml.

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*/ All solvents are of the highest grade and suitable for residue analysis.

Three chromatography steps were done, the first being a silica gel column. No activation of silica was necessary. A 5 cm column was prepared using a disposable pipet plugged with glass wool. The silica was capped with 1/4 cm anhydrous sodium sulfate to remove water, and then wetted with hexane. The sample, dissolved in 1 ml of hexane, was transferred to the column. A second ml of hexane was used to rinse the jar and was subsequently added to the column. Dioxin was eluted with 3 ml of 20% (V/V) benzene in hexane. All the eluate was collected in another 2 ounce jar and concentrated to a volume of 1 ml.

Alumina was washed by saturating with methylene chloride, removing excess solvent, then activating at 225°C for 24 hours. A column was prepared in the same manner as the silica column above. The column was cooled to room temperature in a dessicator before use.

Hexane was used to wet the column before transferring the sample. The jar was again rinsed with one ml of hexane which was transferred to the column. The alumina was eluted with two 3 ml portions of pesticide grade CCl_4, then with 4 ml of CH_2Cl_2. These solvents were used to rinse the jar before being transferred to the column. The methylene chloride fraction was collected in a clean 2 ounce jar and concentrated under nitrogen while replacing the volatile CH_2CL_2 with hexane. All other fractions can be discarded.

The final step was florisil chromatography. The florisil was saturated with methylene chloride and activated in a oven at 165°C for 24 hours. The packing was allowed to cool in a vacuum dessicator. A five cm column was prepared in a disposable pipet plugged with glass wool. The column was packed with 10 ml of hexane under light nitrogen pressure, in an attempt to remove all air pockets.

The sample, dissolved in one ml of hexane, was added to the florisil column. The container was rinsed with one ml of 8% (by volume) methylene chloride in hexane. The column was eluted with nine ml of 8% CH_2Cl_2 in hexane (which removed 80-85% of the PCB's) and then with eight ml of CH_2Cl_2. The dichloromethane fraction, which contained the TCDD, was collected in a centrifuge tube, and the solvent was evaporated to a small volume under a stream of dry nitrogen. The sides of the centrifuge tube were rinsed down with one ml of hexane and again the volume was reduced. The tube was rinsed a final time with one ml of hexane and the solvent evaporated until the volume was less than 100ul. The centrifuge tube was capped with a teflon-lined screw cap and stored in a freezer at about -20°C until analysis.

List of Materials Used in Tissue Extractions

Acetone, OmniSolv*/, MCB

Benzene, OmniSolv, MCB

Carbon tetrachloride, OmniSolv, MCB

Ethyl alcohol, OmniSolv, MCB

Hexane, Omnisolv, MCB, non UV

Methylene chloride, OmniSolv, MCB

Sulfuric acid, concentrated, analytical reagent, Mallinckrodt

Water, distilled in glass

Potassium hydroxide, analytical grade, Mallinckrodt

Sodium sulfate (anhydrous), analytical grade, Fisher

Aluminum oxide, neutral, activity grade I, Woelm Pharma

Florisil, 60-100 mesh, Fisher

Silica gel, 60-200 mesh, reagent grade, Baker Chemical Co.

Dry nitrogen (boil-off from liquid N_2)

*/ All OmniSolv line solvents are distilled in glass, suitable for chromatography and residue analysis.

Sample Extraction Procedure for Soil

A 5-10g sample was accurately weighed in a 125 ml Erlenmeyer flask and spiked with a known amount of ^{37}Cl- TCDD (2.0 - 2.5 ng). The spike was allowed to dry on the soil before proceeding. Four ml of 0.2 M ammonium chloride solution (10.7 g/liter) were added to saturate the soil. The soil was allowed to stand for several minutes.

Fifty ml of 1:1 (by volume) hexane/acetone solution was then added; the solution was stirred for 15 minutes using a magnetic stirrer. The solvent was carefully decanted into a 250 ml separatory funnel, filtering suspended particles through glass wool. Another 40 ml of hexane/acetone was added and the soil was allowed to stir for another 30 minutes. Again, solvent was decanted and filtered.

The hexane/acetone solution was extracted twice with 25 ml of 1N KOH, followed by one extraction with 25 ml of distilled water. (Any emulsions formed were broken up by addition of a few drops of concentrated H_2SO_4.) Several washings with concentrated H_2SO_4 were done, approximately 10-15 ml each, until hexane and acid layers were clear. Four or five extractions were generally necessary.

The hexane layer was washed with 100 ml of distilled water and excess acid was neutralized by addition of amounts of solid Na_2CO_3 to the water/hexane mixture until the neutralization reaction subsided. The water layer was then removed.

Sodium carbonate columns were prepared by adding anhydrous Na_2CO_3 to a height of 8 cm in 25 ml burettes. The burettes were plugged with a generous amount of glass wool to prevent Na_2CO_3 leakage (Na_2CO_3 was not packed tightly). The column was wetted with hexane before transferring the hexane layer from the separatory funnel, followed by a rinse of 25 ml of hexane. All eluate was collected in 4 ounce jars, then concentrated under a stream of dry nitrogen to approximately 1 ml.

Alumina was washed by saturating with methylene chloride, removing excess solvent, then activating at 225°C for at least 24 hours. The column was cooled to room temperature in a dessicator before use. A 5 cm column was prepared using a disposable pipet plugged with a small amount of glass wool. The alumina was capped with 1/4 cm anhydrous sodium sulfate to remove water.

Hexane was used to wet the column before transferring the sample, dissolved in 1 ml hexane, to the column. A second 1 ml of hexane was used to rinse the jar and was subsequently added to the column. The alumina was eluted with two 3 ml portions of CCl_4, then with 4 ml of CH_2Cl_2. These solvents were used to rinse the jars before being transferred to the column. The methylene chloride fraction was collected in a 12 ml conical centrifuge tube and again concentrated under nitrogen while replacing the volatile CH_2Cl_2 with isooctane. All other fractions can be discarded. The isooctane was concentrated to a volume of less than 100 ul, capped with a teflon-lined screw cap, and stored in a freezer at about -20°C until analysis.

List of Materials Used in Soil Extractions

Acetone - Omnisolv*/, MCB

Carbon tetrachloride - Omnisolv, MCB

Hexane - Omnisolv, MCB, non UV

Methylene chloride - Omnisolv, MCB

Sulfuric Acid, concentrated - analytical reagent, Mallinckrodt.

Water - distilled in glass

2,2,4-trimethylpentane (isooctane) - Omnisolv, MCB

Aluminum oxide-neutral, Activity Grade I, Woelm Pharma

Ammonium chloride - analytical reagent, Fisher

Potassium hydroxide - analytical reagent, Mallinckrodt

Sodium carbonate (anhydrous) - analytical reagent, Fisher

Sodium sulfate (anhydrous) - analytical reagent, Fisher

Dry nitrogen (boil-off from liquid N_2)

*/ All Omnisolv line solvents are distilled in glass, suitable for chromatography and residue analysis.

APPENDIX 2

Gas Chromatography/High Resolution Mass Spectrometry (GC/HRMS) Analysis

At the time of analysis, the side of the centrifuge tubes was washed thoroughly with aproximately 100 ul of hexane or isooctane using a graduated syringe. During the washing, the solvent was allowed to evaporate until a volume of~50 ul remained. This remaining volume was accurately measured; usually three-fourths was replaced in the centrifuge tube, and the fourth remaining in the syringe was used for the gas chromatography/ mass spectrometry analysis.

Mass Spectrometer

A Kratos MS-5076 ultra high resolution mass spectrometer was used for this analysis (ultimate resolution = 180,000). The mass spectrometer was interfaced via a direct coupling to a Perkin Elmer Sigma II gas liquid chromatograph. Data acquisition was accomplished with a Nicolet Model 1170 signal averaging computer.

Gas Chromatography

The column was a 6' x 1/4" O.D. glass containing a Dow mixed phase packing. Typical operating conditions were: Helium flow rate of 15 cc/min; injector 270°: column temperature program 1.5 min at 250° and then ramped at 10° c/min to 300°C and held there until the dioxin had eluted. The GC/MS interface was a simple glass lined stainless steel capillary and was held at an average temperature of 250°C. Typical retention time was 3.4 minutes (peak width at 10% height approximately 40 seconds).

Mass Spectrometer Conditions

The electron impact source was used at 70 eV ionizing energy and an accelerating voltage of 8 KV. The source was set at 260°C. The instrument was tuned to a resolving power of 10,000 (10% valley definition).

Data were acquired using the standard ion switching feature provided with the MS-50 (dual ion monitoring). The first analysis was made monitoring one channel m/z 321.8936 (the most abundant molecular ion of TCDD having natural isotopic elemental abundances) and m/z 327.8848 ($^{37}Cl_4$-TCDD, the internal standard) on the second channel. The complete peak profiles were acquired at a bandwidth of 3000 Hz by scanning of a frequency of about 2 Hz, corresponding in each case to a mass range of 300 ppm (0.096 amu). The output of the mass spectrometer was accumulated over about 75 sweeps per channel using a Nicolet Model 1170 signal averager. The resulting signals were submitted to a three-point smoothing routine prior to print-out on an X-Y recorder.

Calculation of Results

Quantitation was achieved by employing the internal standard "ratio method." Throughout the analysis period, standard samples containing TCDD and internal standard were analyzed. From these results, a calibration curve can be prepared by plotting ratio of the weights of TCDD and internal standard versus the ratio of signal intensities (intensity at m/z 321.8936; intensity at m/z 327.8848). Residues of TCDD in actual samples were obtained by

measuring the ratio of the signal intensities at m/e 322 and 328 (internal standard) and reading the concentration of TCDD from the calibration plot. The detection limit in the actual samples was obtained by multiplying the noise level by 2.5 which was considered the maximum amount of TCDD which could be present in the sample.

The percent recovery was measured using the absolute signal intensity for the internal standard and mass spectrometer response factors measured by analyzing standard solutions of internal standard.

Validation

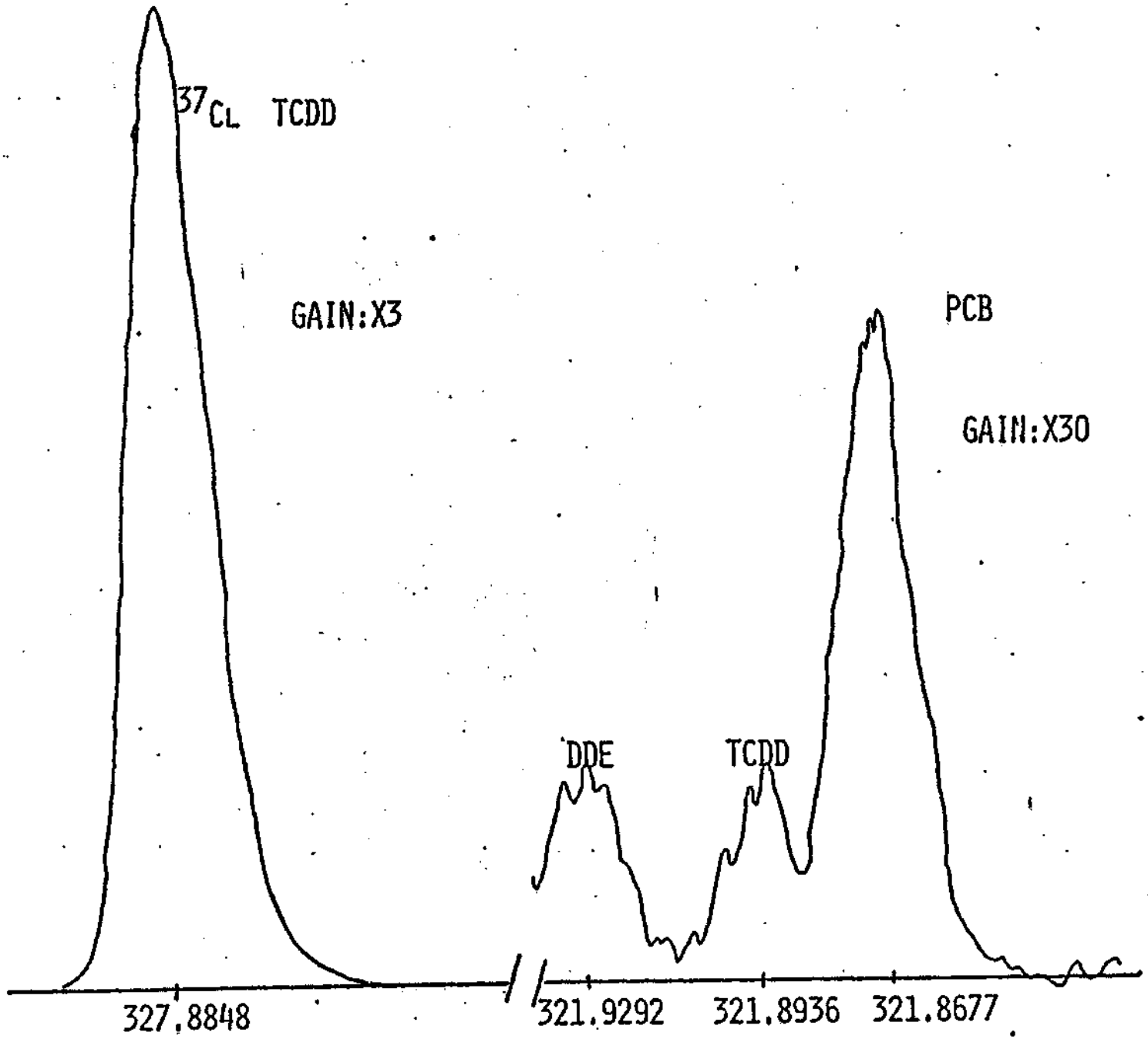
Samples which showed detectable concentrations of TCDD or which were questionable were reanalyzed by removing a second aliquot and reinjecting onto the GC/HRMS. For this validation, the high mass channel is centered at 321.8936 and the low mass channel at 319.8965, the second most abundant molecular ion of TCDD. All other conditions were as reported above. The theoretical ratio of intensities is 0.78 (m/z 319.8965: m/z 321.8936).

The analysis permits us to calculate a concentration of TCDD based on the absolute signal intensity observed at m/z 321.8936 using response factors determined for the mass spectrometer from analysis of standard solutions of TCDD. Based on the percent recovery measured above, the quantitation is adjusted to 100% discovery.

Validation of TCDD is considered acceptable if the observed ratio of signals is 0.78 $\pm$ 0.10.

Figure 1 TYPICAL COMPUTER OUTPUT FOR SOIL ANALYSIS. CONCENTRATION TCDD =

16 PARTS-PER-TRILLION



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