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Correlation Between Human Serum and Adipose Tissue Concentrations of 2,3,7,8-Tetrachlorodibenzo-p-dioxin

Center for Environmental Health
Centers for Disease Control
Atlanta, Georgia 30333

September 2, 1986

Correlation Between Human Serum and Adipose Tissue Concentrations of 2,3,7,8-Tetrachlorodibenzo-p-dioxin

INTRODUCTION

Non-volatile halogenated compounds tend to accumulate in the lipid stores of the body. Consequently, adipose tissue has been the matrix of choice for measuring these compounds at trace levels in the human body. We have used adipose tissue for determining the concentration of 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) in exposed and control populations in Missouri (1). In other studies (2,3,4), we have determined polychlorinated biphenyls (PCBs), DDT and its metabolites, and polybrominated biphenyls (PBBs) in serum. The primary advantage of serum in such studies is that it is collected by a much less invasive procedure. However, an important question about the interpretation of serum measurements is whether they reflect the adipose concentrations of these compounds. Some studies (5,6), in which organochlorine pesticides were measured on a whole weight basis in matched adipose and serum samples, have yielded inconclusive results as to how well adipose and serum measurements correlated. Other studies have found high correlations between adipose and serum (or plasma) levels for PCBs (7) and PBBs (8) on a whole weight basis. Eyster et. al, (8) have suggested that to accurately assess the partitioning between adipose and serum of chronically retained lipophilic xenobiotics, each measurement should be performed on a lipid weight basis rather than whole weight basis. Brown and Lawton (9), in their study of capacitor workers occupationally exposed to PCBs, also recommended calculating adipose to serum partitioning coefficients on a lipid weight basis for the various Aroclors they measured.

The objective of the current study was to determine how well adipose and serum levels of 2,3,7,8-TCDD correlated on either a whole weight or lipid weight basis.

MATERIALS AND METHODS

Study Population and Sample Acquisition

The population in this study consisted of a total of 50 persons in Missouri; persons with and without a history of exposure to 2,3,7,8-TCDD. The methods used for acquiring adipose are described elsewhere (1). Serum samples were not collected under fasting conditions. Serum samples of approximately 15 milliliters (ml) were collected during the same hospital stay that adipose tissue was collected. For some participants, serum samples of approximately 200 ml were collected in Missouri by the Red Cross at a time interval of 2 to 14 months after the collection of the individual's adipose tissue. These samples were sent to CDC in the original Travenol serum bags. All samples were coded so that the analytical laboratory could not link the adipose tissue with its corresponding serum sample.

Analytical methods

The analytical methods are discussed briefly here and presented in detail in the Analytical Methods Appendix. Adipose tissue and serum were analyzed for 2,3,7,8-TCDD by our methods (10,11) based on high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS) and a semi-automated sample preparation procedure (12). The adipose tissues were analyzed for 2,3,7,8-TCDD in analytical runs consisting of four unknown samples and a quality control pool sample. Every fourth analytical run consisted of two unknown samples, a method blank, a duplicate selected at random from an earlier run, and a different quality control pool sample.

The serum samples were analyzed for 2,3,7,8-TCDD in analytical runs consisting of a method blank, three unknown samples and a quality control pool sample. The composition and characterization of these quality control pools are described in more detail elsewhere (10,11). We determined percent lipid in adipose tissue by our gravimetric procedure (method B) (13). On some samples duplicate lipid determinations were made, in which case the mean is reported. The lipid determinations and 2,3,7,8-TCDD measurements were performed on different subsamples of the adipose tissue and at different times. The adipose samples were stored during the interim at -60 degrees Centigrade. Due to inadequate sample volume, seven of the adipose samples were not analyzed for percent lipid.

Total cholesterol, triglycerides, and high density lipoprotein cholesterol (HDL) were determined in duplicate on the serum samples by standard methods on the DuPont Automatic Chemical Analyzer. Total phospholipids were determined in duplicate by a modification (14) of the Folch procedure. Free cholesterol was determined in duplicate using an enzymatic method (15). For each analyte, the results of the duplicate analyses were averaged, and the mean reported. One serum sample was not available for serum lipid analysis. These results were used to calculate the concentrations of total lipids (16), low-density lipoprotein cholesterol (LDL) and very low-density lipoprotein cholesterol (VLDL) (17). All analytical runs for the 2,3,7,8-TCDD and lipid measurements were in control (10,11).

Statistical Analysis

Statistical analysis of the data was performed using the Statistical Analysis System (SAS) Version 5 (Sas Institute, Cary, NC 27511). Correlation coefficients are Pearson product-moment correlations.

RESULTS AND DISCUSSION

A description of the distributions of 2,3,7,8-TCDD levels in the adipose and serum of the 50 participants, reported on a whole weight basis, is given in Table 1. The adipose and serum TCDD levels ranged over approximately 2.5 orders of magnitude. Both distributions were markedly skewed to the right, so geometric means and standard deviations were also calculated. The results of the various serum lipid components and the adipose percent lipid measurements are summarized in Table 2. Although some individual values are outside of reference ranges, the results are overall what would be generally expected for lipid measurements.

The correlation analysis was performed for adipose 2,3,7,8-TCDD on both a whole weight and lipid weight basis with serum 2,3,7,8-TCDD on a whole weight basis and a lipid (or lipid fraction) weight basis. A lipid (or lipid fraction) weight basis means that the 2,3,7,8-TCDD level is expressed as grams of 2,3,7,8-TCDD per gram of total lipids (or lipid fraction). Since the distributions of adipose and serum 2,3,7,8-TCDD levels covered about 2.5 orders of magnitude (i.e., adipose 2 - 745 ppt, serum .013 - 8.33 ppt) and were markedly right skewed, log-transformed 2,3,7,8-TCDD levels were used in the correlation analysis. Results of the correlation analysis are given in Table 3. Figure 1 is a plot of adipose and serum 2,3,7,8-TCDD, both on a whole weight basis. Figure 2 is a plot of adipose and serum 2,3,7,8-TCDD levels, both on a lipid weight basis. The best correlation ($r = 0.976$) was found for adipose 2,3,7,8-TCDD on a lipid weight basis with serum 2,3,7,8-TCDD on a total lipid weight basis. This correlation was only marginally better than adipose 2,3,7,8-TCDD on a whole weight basis with serum 2,3,7,8-TCDD on a whole weight basis ($r = 0.967$). Adjusting for various serum lipid fractions offered no improvement over adjusting for serum total lipids.

The partitioning ratio of adipose 2,3,7,8-TCDD to serum 2,3,7,8-TCDD was also calculated on a whole weight and lipid weight basis. On a whole weight basis for both adipose and serum 2,3,7,8-TCDD levels, the mean of the partitioning ratios was 158 to 1 (std. dev. = 75.1, std. error = 10.6). On a lipid weight basis for both adipose and serum 2,3,7,8-TCDD levels, the mean of the partitioning ratios was 1.09 (std. dev. = 0.385, std. error = 0.060). The 95% confidence interval about the mean was 0.97 to 1.21, which includes 1.0. Thus on the basis of these data, a one to one partitioning ratio of 2,3,7,8-TCDD between adipose lipid and serum lipid cannot be excluded.

Adipose 2,3,7,8-TCDD measurements have generally been accepted as representing the body burden concentration of 2,3,7,8-TCDD. The high correlation between serum 2,3,7,8-TCDD levels and adipose 2,3,7,8-TCDD levels in this study indicates that serum 2,3,7,8-TCDD is a valid measurement of 2,3,7,8-TCDD body burden concentrations. The practical advantage of collecting serum rather than adipose samples should facilitate future epidemiologic studies which require estimates of 2,3,7,8-TCDD body burden.

Table 1

Adipose and serum 2,3,7,8-TCDD Levels
(Whole weight basis)

	Adipose 2,3,7,8-TCDD (parts per trillion)	Serum 2,3,7,8-TCDD (parts per trillion)
Mean	54.5	0.519
Standard deviation	125.8	1.314
Geometric mean	15.7	0.110
Geometric Std. deviation	4.25	0.053
10th percentile	3.2	0.018
50th percentile (median)	9.0	0.057
90th percentile	130	1.55
Range	2-745	0.013 - 8.30
N	50	50

Table 2
Lipid Measurements

	Total Lipids (mg/dl)	Total Cholesterol (mg/dl)	Total Triglyceride (mg/dl)	Total Phospholipids (mg/dl)	Free Cholesterol (mg/dl)	HDL Cholesterol (mg/dl)	LDL Cholesterol (mg/dl)	VLDL Cholesterol (mg/dl)	% Lipid in adipose (%)
Mean	608	189	124	206	59.0	46.0	118	24.8	79.4
Std. deviation	140	43.0	68.1	39.0	13.5	16.4	36.9	13.6	9.90
10th percentile	402	135	42	161	41	31	67.4	8.4	63.8
50th percentile (median)	612	196	116	209	61	42	113	23.2	82.5
90th percentile	803	246	215	254	77	64	170	43	89.7
Range	235-840	81-275	22-320	93-307	24-87	18-122	57-197	4.4-64	48.1-92.9
N	49	49	49	49	49	49	49	49	43

Table 3

Correlations of Adipose and Serum 2,3,7,8-TCDD*+

Serum 2,3,7,8-TCDD divided by the sample's	Correlation with Adipose 2,3,7,8-TCDD divided by sample's whole weight	Correlation with Adipose 2,3,7,8-TCDD divided by sample's total lipid weight
Whole weight	.967 (50)	.969 (43)
Total lipid weight	.969 (49)	.976 (42)
Total cholesterol weight	.966 (49)	.972 (42)
Total triglyceride weight	.939 (49)	.951 (42)
HDL weight	.951 (49)	.956 (42)
VLDL weight	.939 (49)	.951 (42)
LDL weight	.958 (49)	.964 (42)
LDL + VLDL weight	.966 (49)	.973 (42)

*The logarithm of each variable was used in the calculation of the correlation coefficient (see text).
+ P <0.0001 for all correlation coefficients. N is in parentheses.

ADIPOSE AND SERUM 2,3,7,8-TCDD LEVELS

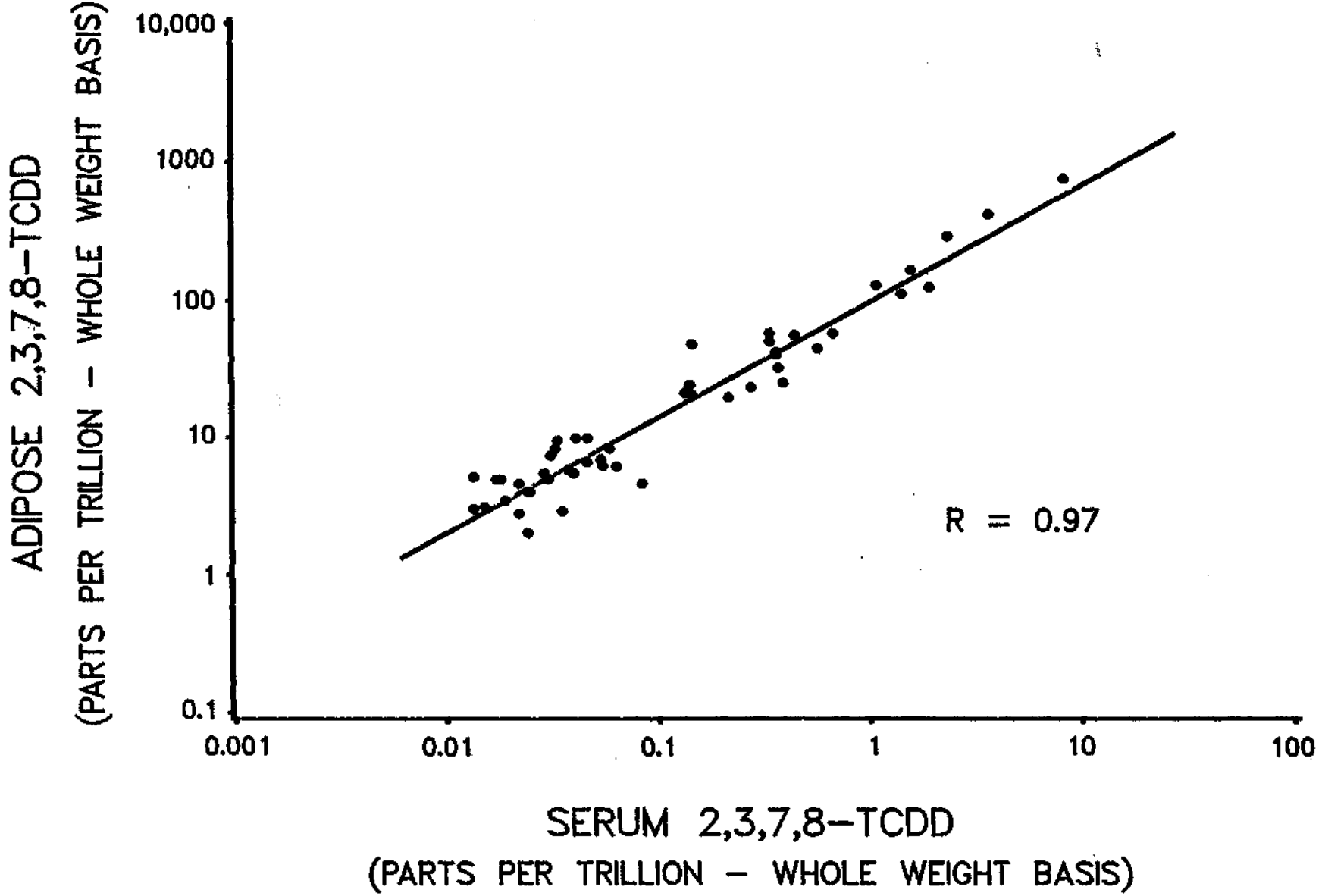

FIGURE 1

ADIPOSE AND SERUM 2,3,7,8-TCDD LEVELS

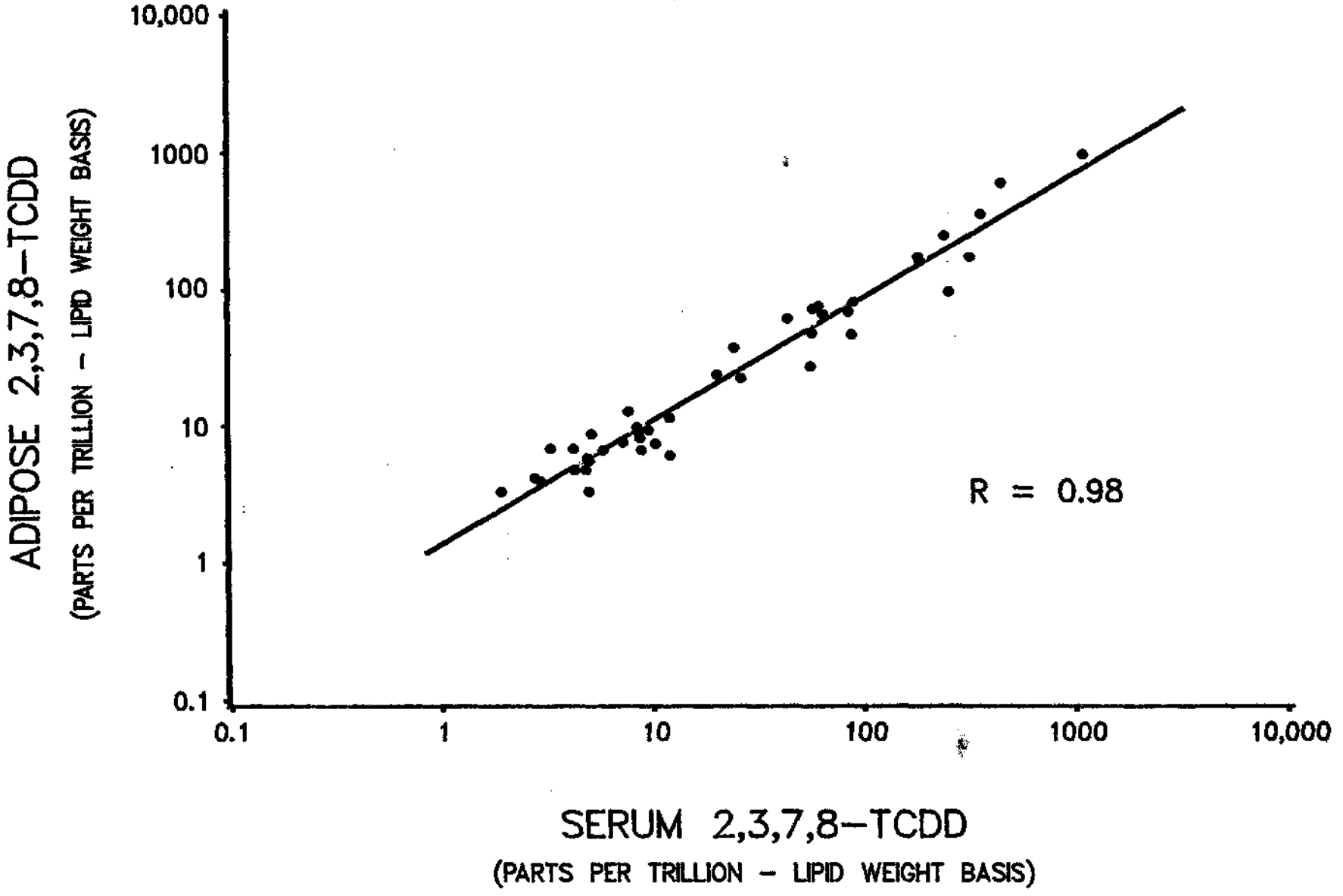

FIGURE 2

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ANALYTICAL METHODS

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High-Resolution Gas Chromatographic/High-Resolution Mass Spectrometric Analysis of Human Adipose Tissue for 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

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An analytical method is described for measuring 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and other TCDD isomers in human tissue samples using a highly specific cleanup procedure and high-resolution gas chromatography/high-resolution mass spectrometry. The 2,3,7,8-TCDD is quantified by the isotope dilution technique with [$^{13}C_{12}$]2,3,7,8-TCDD as the internal standard. Other TCDD isomers are estimated by use of relative response factors. The 1 part-per-trillion (pptr) limit of detection is adequate for determining 2,3,7,8-TCDD concentrations present in human adipose tissue specimens. Analytical accuracy is demonstrated by the results obtained in analyzing several spiked samples. The method is shown to be unaffected by a number of potentially interfering compounds. A series of quality control material is used to verify system performance. For samples containing 1.6 pptr 2,3,7,8-TCDD, a coefficient of variation (CV) of 19% is observed. For samples at the 8.4 pptr level, a CV of 11% is observed.

In recent reviews (*1–3*), the results of quantification of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in biological samples by using various approaches to gas chromatography/mass spectrometry (GC/MS) instrumentation have been summarized. Fish are the most studied group of animals, followed by cattle. A variety of other animals are the subject of single studies (*4–9*). As might be expected, animals inhabiting areas of high environmental contamination exhibit elevated body burdens.

The results of the study of 2,3,7,8-TCDD in human tissue samples have been reported in one article (*10*) and in oral presentations at three major conferences (*11–13*). Most human tissue samples analyzed so far have been reported to contain 2,3,7,8-TCDD at concentrations greater than 3 pptr. In most analytical studies, low-resolution mass spectrometry has been used, probably because this instrumentation is more accessible than the more definitive high-resolution mass spectrometry. In a review of the analytical problems of determining 2,3,7,8-TCDD in human tissue samples (*14*), the author recommended the use of high-resolution mass spectrometry because of the small sample extract for analysis and the highly political nature of the dioxin problem. Later a group of experts (*15*) recognized the acceptability of low-resolution mass spectrometry if sample cleanup is adequate.

We report an analytical method for measuring 2,3,7,8-TCDD in human tissue samples using the highly specific cleanup procedures developed by Smith et al. (*16*) and high-resolution gas chromatography/high-resolution mass spectrometry. This method provides a definitive quantitative determination for the 2,3,7,8-TCDD isomer and an estimate of any other tetrachlorodioxins present in the sample. The method with the instrumentation described has sufficient sensitivity for all human tissue samples analyzed so far. validation studies demonstrate the lack of interferences by various chlorinated organic compounds in the quantitative determination. Spiked recovery studies suggest that the method has sufficient accuracy for the proposed application to support a nationwide survey of TCDD levels in adipose tissue that would result in a reference "standard" for assessing exposure to and body burden of 2,3,7,8-TCDD. This analytical approach provides a sample extract, remaining after TCDD analysis, that may be used in the analysis for other chlorinated dioxins or chlorinated furans.

EXPERIMENTAL SECTION

Safety. Chemists undertaking this work with 2,3,7,8-TCDD and other such toxic compounds must understand the potential health effects of such compounds (*17*) and the prudent laboratory practices for handling toxic chemicals (*18*). Specific precautions related to TCDD analysis are described with analytical methods (*19, 20*). More recent Environmental Protection Agency (EPA) draft methods have emphasized specific aspects of protective equipment, training, personal hygiene, isolation of the work area, disposal of waste, laboratory cleanup, laundry, wipe testing, problems in inhalation, and accidents. Each laboratory involved in this work should have formal plans stating policy in each of these areas and mechanisms for ensuring compliance.

Enrichment Procedure. Tissue samples (spiked with isotopic marker compounds) are processed in a two-part procedure. In part I, the tissue/sodium sulfate mixture is subjected to solvent extraction and the extract is, in the same process, passed through a series of silica-based adsorbents and then through a carbon/glass fiber adsorbent. The extract passes through the adsorbents in the following order: potassium silicate, silica gel, and, finally, the

carbon adsorbent. The residues of interest [polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and non-ortho-substituted polychlorinated biphenyls (PCBs), as well as other chemical classes such as polychlorinated naphthalenes (PCNs), polychlorinated biphenylenes, and certain polyaromatic hydrocarbons] are retained on the carbon adsorbent and subsequently recovered by reverse elution with toluene.

In part II of the procedure, after a change of solvent to hexane, the sample is applied to a second series of adsorbents contained in two tandem columns. The first column contains small amounts of cesium silicate and sulfuric acid impregnated silica gel. The effluent from this column flows directly onto the second column, which contains activated alumina. After sample volume is reduced, analyses are carried out by high-resolution gas chromatography/high-resolution mass spectrometry.

The components of the apparatus used in part I of the enrichment procedure are as follows: column 1 (50 mm i.d. × 600 mm, Michel-Miller chromatography column 5795-54) is connected to column 2 (22 mm i.d. × 100 mm, Michel-Miller precolumn 5796-34) and to column 3 (11 mm i.d. × 50 mm, Michel-Miller custom column 5795-823, all from Ace Glass, Vineland, NJ) by means of Ace Glass No. 5801 Teflon end-fitting adapters and standard $^1/_8$ in. o.d. Teflon tubing and tube end fittings (available from most chromatographic supply companies). The washing solvent reservoir is also an Ace Glass chromatography column (25 mm i.d. × 450 mm, 5795-40).

The solvent-flow switching valves are Hamilton miniature inert valves (Hamilton Co., Reno, NV): selector valve (no. 86781) and bypass and reverse flow valves (no. 86779). The valving arrangement is designed to permit the following operations to be performed: venting of the solvent line from column 1, venting of the solvent reservoir, bypass of column 2, delivery of the effluent from column 1 to columns 2 and 3 sequentially, delivery of solvent from the reservoir sequentially to columns 2 and 3 or to column 3 only, reversal of solvent flow in columns 2 and 3, and stoppage of solvent flow in all lines. The solvent reservoir is routinely pressurized with 1–10 psi nitrogen during column washings. Column 1 is packed in the following sequence: one or two disks cut from glass microfiber filters (GF/F, 4.7-cm diameter, Whatman Inc., Clifton, NJ), a 2-cm depth of anhydrous sodium sulfate, 30 g of silica gel, 30 g of potassium silicate, another disk of glass microfiber filter (GF/D), 50 g of a 1 to 4 (w/w) mixture of the sample and anhydrous sodium sulfate, and, last, a 2-cm depth of anhydrous sodium sulfate. Column 2 is packed with equal volumes, 15 mL each, of potassium silicate and silica gel bracketed by plugs of glass wool or, preferably, disks of glass fiber filters (3 μm retention GF/D, Whatman, Inc., Clifton, NJ). Column 3 is packed with a mixture of activated carbon (Amoco PX-21, Amoco Research Corp., Chicago, IL) and glass fibers. The apparatus for part II of the enrichment procedure consists of two columns arranged in tandem. Column 4 is prepared from a disposable Pasteur pipet and is packed first with a plug of glass wool, then with 2 cm (0.50 g) of sulfuric acid impregnated silica gel, then with 3 cm (0.54 g) of cesium silicate, and, finally, with 0.5 cm of anhydrous sodium sulfate. Column 5 is constructed from a 225–mm length of 5 mm i.d. heavy-walled glass tubing fitted with a 50-mL reservoir and a 24/40 ground glass joint. Column 5 is packed with a plug of glass wool, followed by 3.50 mL (3.65 g) of activated alumina, and then 0.5 cm of anhydrous sodium sulfate. The alumina is packed firmly by sharply tapping the supporting clamp.

Materials. All solvents are glass-distilled grades (Burdick & Jackson, Muskegon, MI). Silica Gel 60, 70–230 mesh (EM Reagent, MC/B, Cincinnati, OH), acid alumina (AG4, Bio Rad Labs, Richmond, CA), and sodium sulfate (Mallinckrodt AR) were used. All glassware is washed with acetone, toluene, and, finally, with 50/50 hexane/methylene chloride (in that order) before use. Alumina is washed with methanol and then methylene chloride and oven activated at 190 °C for a minimum of 2 days (stored at 190 °C). Silica gel is washed in the same manner and activated at 130 °C for a minimum of 2 days (stored at 130 °C). Sodium sulfate is washed with hexane and then methylene chloride and oven activated at 190 °C for 2 days (stored at 190 °C). Two hundred milliliters of each solvent wash is used for every 100 g of adsorbent, and a slow flow of nitrogen is used to dry each adsorbent before activation. Potassium and cesium silicates are prepared from the reaction of the corresponding alkali metal hydroxides with silica gel in methanol at 55 °C for 90 min. The reaction is carried out in a 1- or 2-L round-bottom flask that is rotated and heated with a rotary evaporation apparatus. Sixty grams of cesium hydroxide (CsOH) (99+%, Aldrich Chemical Co., Milwaukee, WI) is dissolved in 200 mL of methanol and separated from insoluble material by decantation. An additional 200 mL of methanol is added, followed by 100 g of silica gel. For potassium silicate, 168 g of potassium hydroxide (KOH) (J. T. Baker Chemical Co., Phillipsburg, NJ), 300 g of silica gel, and ~700 mL of methanol is used; decantation is not necessary for KOH. After the reaction, the mixture is poured into a large glass column containing a plug of glass wool. Special care must be exercised to avoid contact, especially eye contact, with the extremely caustic solution. The adsorbent is washed into the column with methanol, and then 200 mL of methanol for every 100 g of silica gel is added to the column. The methanol can be pushed through the column under slight gas pressure, and as the level of the liquid reaches the bed of adsorbent, 200 mL of methylene chloride for every 100 g of silica gel is applied. The liquid is pushed through the column, and the silicate is partially or completely dried under a slow flow of nitrogen. Cesium silicate is dried completely under a stream of nitrogen and is not heat activated; potassium silicate is activated at 130 °C, and both are stored in a desiccator.

Sulfuric acid impregnated silica gel (40% w/w) is prepared by adding two parts of concentrated sulfuric acid to three parts of 130 °C activated silica gel, by weight, in a screw-capped flask and shaking until the mixture is completely free of lumps (about 15 min).

Nitrogen gas used for drying adsorbents and evaporating solvents is passed through a molecular sieve and then through activated carbon (coconut charcoal, Fisher Scientific Co., Pittsburgh, PA), bracketed by glass wool and glass microfiber filters. After the carbon trap, a high-purity filter (In-line filter no. 6185, Matheson Gas Products, East Rutherford, NJ) is inserted in the line in an attempt to prevent carbon particles from moving through the nitrogen line.

The carbon-on-glass fiber is prepared by cutting ~700 mg of glass fiber filter (Whatman GF/D) into small pieces and shredding the pieces briefly with a Janke and Kunkel "Tissumizer" with ~70 mL of methylene chloride as the suspending solvent. Amoco carbon (70 mg PX-21) is added to the slurry of glass fibers and mixed thoroughly. The solvent is decanted to remove some of the excess, unbound carbon particles and replaced with another 70 mL of methylene chloride. The slurry is packed into column 3 between layers of glass fiber filter (Whatman GF/D) and then flushed with clean nitrogen at >200 mL/min for at least an hour. Replace the fiber filters at the ends of the column before use.

Sample Preparation. Ten grams of adipose tissue is minced and combined with 40 g of sodium sulfate and about 25 to 30 mL of hexane/methylene chloride 50/50 (solvent A). The mixture is ground thoroughly with the Tissumizer, and an internal standard solution consisting of 240 pg of ^{13}C-labeled 2,3,7,8-TCDD is added to the sample. The standard (maintained at room temperature) is accurately measured with a Rainin Instrument Co., electronic digital pipet. The sample is then loaded into column 1 by using washings of solvent A.

Part I. The selector valve is positioned so that column 1 is connected to the vent line and air is allowed to escape. As the solvent level approaches the surface of the sample and adsorbent, 400 mL of solvent A is carefully applied to column 1, and the movement of the solvent front is observed. As the solvent front enters the transfer line (3–4 ft in length), air bubbles are removed by stopping the flow and tapping the line. When the solvent front reaches the selector valve, the latter is repositioned to direct the extract through columns 2 and 3. The effluent is collected in a 1-L flask positioned above columns 2 and 3 to maintain a positive back pressure on these columns. The height of column 1 above the collection flask is adjusted to produce a solvent flow of not more than 5 mL/min, but sufficient to complete the process overnight. Occasionally, the solvent flow will slow or stop during the first stage and will require that 1 or 2 psi of gas pressure be applied to the system at column 1. Rarely, the glass fiber filters on the inlet end of column 3 become clogged during the processing of decomposed or very oily samples. These complications can be reduced by placing a removable column (1.0 cm i.d. × 2 or 3

Table I. Estimated Concentration (Parts per Trillion) from Mass Spectrometry Calibration

95% control limits						
lower	upper	calibrator concn	obsd mean concn	bias, %	std dev	coeff of variation, %
		0.75	0.751	0.1	0.386	51
0.37	1.66	1.00	1.018	1.8	0.336	32
3.86	6.14	5.00	5.004	0.1	0.588	12
8.37	11.55	10.00	9.964	0.3	0.812	8
24.35	25.67	25.00	25.014	0.06	0.337	1

cm) containing four or five disks of glass microfibers in line at the exit end of column 2. This filter column, if clogged, can be replaced during the process.

After the first extraction/adsorption operation is completed, column 3 is washed (bypassing column 1 and 2) in the forward direction with 75 mL of solvent A and then with 50 mL of methylene chloride/methanol/benzene (75/20/5) at a flow of ~5 mL/min. These washings are collected in the flask with the initial eluate. The reservoir is then charged with 45 mL of toluene, which is passed through the carbon (column 3) in the reverse flow direction at ~2 mL/min and collected in a 125-mL round-bottom (24/40) flask. At this point, part I of the procedure is complete.

Column 2 is usually packed with fresh adsorbents for each sample, but this column can be used for more than one sample if the amounts of extracted materials, such as lipids, are small. The carbon adsorbent in column 3 is routinely reused after washings (under 3–8 psi nitrogen) between samples with the following solvent sequence: 100 mL each, in reverse flow, of toluene, methanol, toluene, and solvent A. Column 2 is bypassed during the washings with the first of these three solvents. During the final washing with solvent A, the solvent is directed through column 2 in the reverse direction to remove residual air and possible contaminants. Care must be taken to avoid passing methanol through column 2. Another 100 mL of solvent A is routinely passed through columns 2 and 3 in the forward direction as the final solvent washing. Complete displacement of toluene from column 3 is essential.

The sample in toluene is subjected to rotary evaporation at 55 °C under ~0.1 to 0.2 atm vacuum. The rotary evaporation system must be maintained in an uncontaminated condition by periodic washings with organic solvents. No lubricating greases can be used. The integrity of the sample is protected during rotary evaporation by the use of a vapor trap situated between the sample flask and the evaporation apparatus; the vapor trap is thoroughly washed with toluene between samples. The toluene solution (sample) is carefully taken just to dryness and removed immediately. At this point, the sample is ready for part II of the procedure.

Part II. Columns 4 and 5 are thoroughly washed before use, the former with 10 mL of hexane, the latter with 50 mL of hexane under ~5 psi nitrogen pressure to remove entrapped air. After the columns are washed, column 4 is partially inserted into column 5 so that the effluent from column 4 flows directly onto the adsorbent bed of column 5. The sample is transferred to the top of column 4 by using four separate 2-mL hexane washings of the flask, allowing each washing to pass through column 4 and onto column 5 before the next washing is applied.

Column 4 is now discarded and an additional 4.0 mL of hexane is applied to column 5, followed by 4.0 mL of 2% methylene chloride in hexane. Next, a 15-mL conical centrifuge tube is placed under column 5, and the compounds of interest are eluted with 14.0 mL of solvent A. This fraction is reduced in volume to ~0.5 mL under a stream of nitrogen in a 40–45 °C water bath. The sample is transferred to a conical minivial with four 0.5-mL washings with methylene chloride, each washing being reduced to a smaller volume under a stream of nitrogen before the next is added. The remaining solvent is then completely evaporated, and then the appropriate volume of desired solvent (usually 5–10 μL of toluene) is added just before the analysis. If the analysis is to be performed later, the sample can be kept in the dry state and stored in a freezer. Before the sample is injected, the solution is drawn up into the microliter syringe and applied repeatedly to the wall of the conical portion of the vial to bring the entire sample into solution. Gas chromatographic/mass spectrometric analyses are carried out on a 2-μL sample, which is injected by the direct injection technique.

Table II. Linear Regression Parameter Estimates[a]

	intercept	slope	coeff of determination
mean	0.0038	0.0252	0.9968
std dev	0.0170	0.0042	0.0047
CV, %	447	16.6	0.5

[a] n = 13 runs.

Instrument Analysis. Our analytical instrument system consists of a Vg ZAB-2F high-resolution mass spectrometer with a Vg 2250 data system and a Hewlett-Packard 5840 gas chromatograph. Other mass spectrometers capable of 10000 resolution, equipped with state-of-the-art data systems and capillary column capabilities could be used just as well. Our analyses are conducted in an isomer-specific mode, with a 60M SP2330 capillary column. The injection is splitless with a 30-s purge, then running in split mode with 100 mL/min split and 10 mL/min septum sweep. The mass spectrometer is operated in the high-resolution (static RP = 10000 at 10% valley) selected ion recording mode, with perfluorotributylamine used to provide a lock mass at m/z 314. Peak top jumping is accomplished by stepping the accelerating voltage after any necessary correction during the scan of the lock mass. Ions m/z 320 and 322 provide a measure of the native TCDD, and ions m/z 332 and 334 are monitored for the elution of the labeled internal standard. Five analytical standards that correspond to 0.75–25 pptr of 2,3,7,8-TCDD in the tissue samples have been used to establish a linear calibration curve. Data were obtained during an 18-month period and are tabulated in Tables I and II. The internal standard is the [$^{13}C_{12}$]-2,3,7,8-TCDD at a concentration corresponding to 24 pptr in the original samples. Other tetra isomers are estimated by assuming a relative response factor of 1.0. The best precision in quantitation is obtained by using chromatographic peak areas and by using the sum of the two ions for the native TCDD and two ions for the internal standard.

On a regular basis, isomer-specific chromatography is demonstrated by the analysis of a standard containing the 22 TCDDs. The calibration curve is verified by the analysis of an analytical standard during an 8-h shift. Instrument resolution at 10000 RP, tuning, and other parameters are checked on a regular basis to ensure optimum sensitivity and specificity. Criteria for a positive TCDD determination are as follows: (1) signal/noise greater than 3/1 for both signals on ions 320 and 322; (2) signal/noise greater than 10/1 for both signals on ions 332 and 334 from the internal standard; (3) observed retention times within ±1 scan of each other on ions 320 and 322 and the relative retention time (RRT) (to [$^{13}C_{12}$]-2,3,7,8-TCDD) must be within 2 ppt of the RRT of the analytical standard; (4) the ratio of the intensities of the ion 320 to 322 is within 10% of the theoretical value of 0.77 for a Cl_4 pattern.

Adipose-like quality control (QC) material has been prepared and is undergoing characterization. Incorporation of these materials in the analytical run will be used to set control limits and to provide a means for demonstrating that the analytical system is in control. Recovery data are calculated on the basis of the absolute area counts for m/z 332 + 334 in the samples vs. the standards.

QUALITY ASSURANCE PROGRAM

QC Materials. The main feature of the QC program is the use of matrix-based materials that are well characterized for

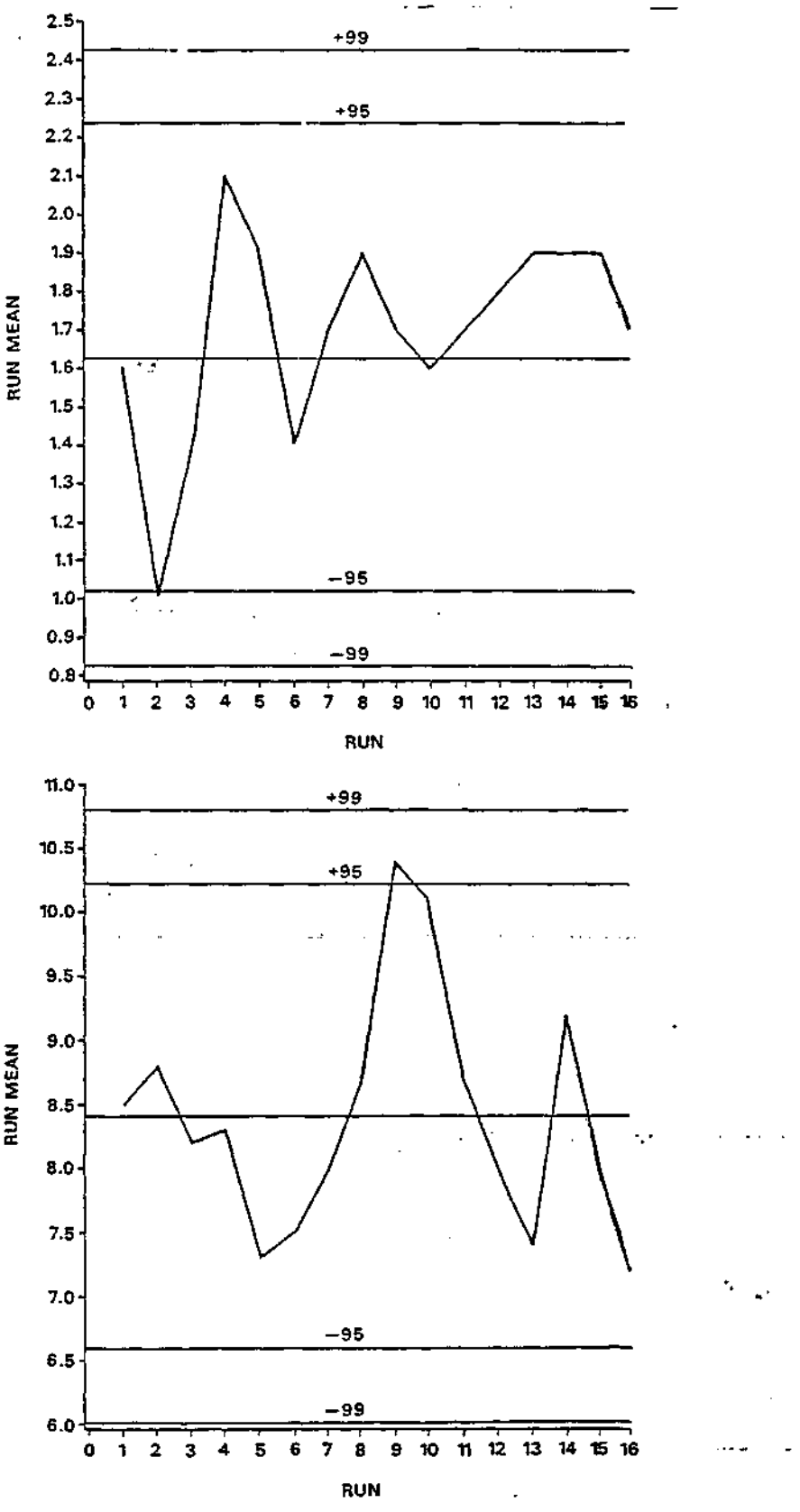

Figure 1. Quality control charts for pork fat pools C and E.

TCDD concentration to ensure that the analytical system is in control. Pork lard material has been spiked with various levels of 2,3,7,8-TCDD and dispensed in 11-g aliquots to simulate human adipose tissue samples. Five pools of material at different concentrations are available for insertion into the analytical run. Each of these materials contains the following dioxins and furans: 2,3,7,8-TCDD; 1,2,3,4-TCDD; 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (PnCDD); 1,2,4,7,8-PnCDD; 1,2,4,6,7,9-hexachlorodibenzo-*p*-dioxin (HxCDD); 1,2,3,6,7,9-HxCDD; 1,2,3,6,7,8-HxCDD; 1,2,3,7,8,9-HxCDD; 1,2,3,4,7,8-HxCDD; 1,2,3,4,6,7,9-HpCDD; 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin (HpCDD); octachlorodibenzo-*p*-dioxin (OCDD); 2,3,7,8-tetrachlorodibenzofuran (TCDF); and octachlorodibenzofuran (OCDF).

QC Charts. QC charts graphically document the analytical performance of the system. Figure 1 shows the QC charts for

Table III. Statistical Data for the Pork Fat QC Pools

	pool						
	A	B	C	D	E	F	G
concn of 2,3,7,8-TCDD, pptr	ND	1.0	1.63	5.85	8.39	15.35	43.2
N	9	1	16	4	16	4	12
std dev (σ)			0.32	0.23	0.92	0.55	2.36
CV			19.2	3.9	11.0	3.6	5.5
99% control limits, upper			2.43		10.78		
99% control limits, lower			0.82		6.00		

two levels of 2,3,7,8-TCDD that have been run over a period of 13 months; the statistical data are shown in Table III. QC charts similar to Figure 1 are also maintained for the 1,2,3,4-TCDD isomer in the QC material.

Details of the Analytical Run. The status of the specimens being analyzed is unknown to the laboratory analysts. Samples are received and arranged in analytical runs of five (four adipose tissue samples and one quality control sample from pool C). In every fourth analytical run a QC sample from pool E is substituted for a tissue sample and a blank is substituted for the pool C QC sample. In addition, a human adipose sample selected at random from one of the four analytical runs will be analyzed in duplicate. This duplicate analysis will be from a separate 10-g sample from the same individual. The samples are then submitted for cleanup by a manual method or an automated procedure (*21*) and then submitted to the mass spectrometry (MS) laboratory for analysis. The MS laboratory personnel are also unaware of the nature of the extract, and the samples are randomized before being analyzed.

To minimize the possibility for carryover or cross contamination of samples and analytical standards, analysts use separate syringes for samples and for each analytical standard. The sample syringe is periodically discarded or when an adipose sample is analyzed that contains more than 20 pptr of 2,3,7,8-TCDD. Between injections of a standard or a sample, the syringe is inserted through a septum, coated with Teflon, into a 15-mL vial containing 12 mL of toluene, and the barrel is filled and emptied 10 times. This process is repeated twice more with different 15-mL vials containing toluene. A final wash of the syringe is done by filling and expelling it 10 times from a fourth toluene wash solution. These wash solutions are discarded at the end of each working week. The final 5 μL of toluene for reconstituting of samples is then taken from a fifth toluene source.

The step-by-step procedure leading to an analytical result follows: (1) Inject a standard that contains the 22 TCDDs and begin a clearing run by programming the SP 2330, 60M capillary column, after an initial 2 min at 100 °C, to 180 °C at 20 °C/min, followed by an increase to 220 °C at 3 °C/min—the column temperature is then held at 220 °C for an additional 40 min for a total run time of 60 min. (2) Introduce FC-43 through the septum inlet system and tune the instrument to 10 000 RP at 10% overlap using m/z 313.9839. (3) Call up the selected ion recording (SIR) setup program and verify electrostatic analyzer (ESA) offset and instrument zero using masses 314 and 325. (4) Set the mass window to 125 ppm and load down the method file. (5) Inject 2 μL of a randomly selected analytical standard and verify that the signal-to-noise (S/N) ratio for the [$^{13}C_{12}$]TCDD is greater than 10 to 1. Calculate the concentration for this standard using the linear regression parameters from Table II. If this concentration is within the 95% confidence limits (Table I), the first sample of the analytical run may be analyzed. The data for this standard are saved to periodically check the calibration curve. (6) Reconstitute (with 5 μL of toluene) and analyze the first sample (samples are reconstituted at least 30 min before analysis to ensure that the sample is completely dissolved).

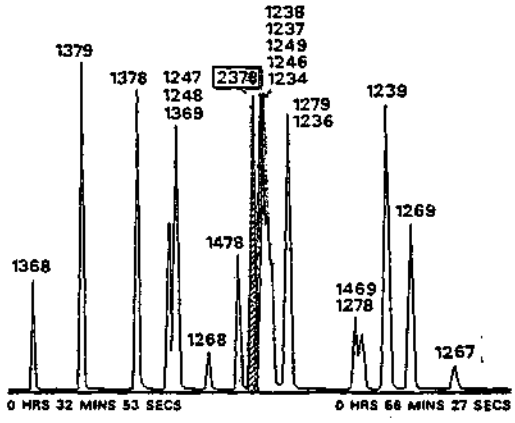

Figure 2. Reconstructed ion chromatogram for the 22 TCDDs.

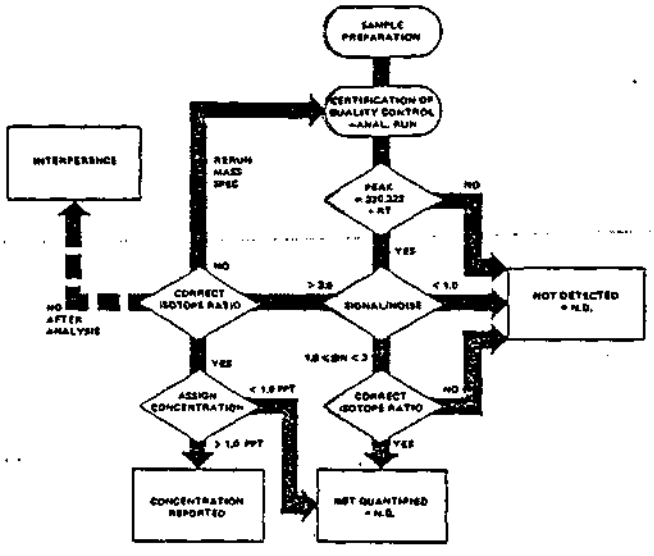

Figure 3. Criteria for reporting results as ND, NQ, or Q.

(7) Analyze the second, third, and fourth samples. (8) Analyze the fifth sample, which is a QC sample from pool C. This QC sample will be replaced with a blank sample every fourth analytical run. (9) Calculate the concentration of 2,3,7,8-TCDD in the QC sample and verify that it is within the 99% control limits from Table III. (10) Calculate the concentration of each sample using the calibration curve. The relative peak areas are used for quantitation by manually setting the base line. (11) Calculate the degree of isomer specificity from the analytical standard containing the 22 TCDDs (Figure 2) by determining the percent valley relative to 2,3,7,8-TCDD. A separation of at least 20% (valley definition) has been suggested (*22*) as a minimum for 2,3,7,8-TCDD quantification. (12) The results for each analytical run will contain at a minimum the following documentation: (a) the concentration of 2,3,7,8-TCDD reported in parts per trillion (picograms of 2,3,7,8-TCDD per gram of wet weight adipose tissue) and also the concentration of any other TCDD detected, based on response factors for those isomers; (b) the original wet weight

Table IV. Analytical Conditions and Criteria for TCDD Identification

analytical condition	requirement
GC column	SP 2330, 60M, capillary μ = 23 cm/s helium
GC conditions	2 min at 100 °C; to 180 °C at 20 °C/min; to 220 °C at 3 °C/min; hold 40 min at 220 °C
mass spec resolving power	10000 at 10% Valley
ions monitored	quantitation (FC-43), confirmation (PFK) 313.9839 (lock mass), 254.9854 (lock mass) 319.8964, 319.8964 321.8933, 321.8933 333.9335, 284.9274 331.9364, 286.9244 256.9324 258.9296 333.9335
retention time of monitored ions	each group of ions must maximize within one scan of each other. The relative retention time (to $[^{13}C_{12}]$TCDD) must be within 2 ppt of the RRT of the analytical std
ratio of isotopic ions monitored	for TCDDs, 0.77 ± 0.10 (m/z 320/322)
ratio of signal to noise	greater than or equal to 3.0 for quantitation
recovery of ^{13}C int std	S/N > 10/1 observed on all standards and samples

of the sample analyzed; (c) the percent lipid content of the sample; (d) if no TCDDs are quantifiable, then the appropriate result will be reported as indicated in Figure 3 [i.e., ND (calculated detection limit); NQ]; (e) the response ratios of m/z 320/322 and 332/334 for each detected TCDD; (f) the results of all blanks, split duplicates, and QC samples in each analytical run; (g) the current updated QC charts applicable to the analytical run; (h) the chromatograms for all samples and standards, including the raw peak response data; (i) the current calibration curve used for quantitation of 2,3,7,8-TCDD; (j) the current response factors used to semiquantitate TCDDs; and (k) the mass chromatograms for the determination of the degree of isomer specificity.

EVALUATION AND VALIDATION STUDIES

Interferences. The analytical characteristics that must be satisfied before an observed signal is assigned to a TCDD are listed in Table IV. The fulfillment of all of these requirements along with the extensive sample enrichment provides a high degree of specificity for the analysis. However, in the analysis for tetrachloro-*p*-dioxins, possible interferences are numerous. In a detailed analysis of many possible interferences in the analysis for PCDDs and PCDFs, Smith and Johnson (*23*), used the enrichment procedure outlined in the recent paper by Smith et al. (*16*). These authors have provided us with a sample of this same interference mixture, and the following compounds were spiked into blank pork fat at the 10-ppb level and carried through the cleanup procedure: Halowax 1014; 3,3′,4,4′-PCB; 3,3′,4,4′,5-PCB; 3,3′,4,4′,5,5′-PCB; 2′,3′,4′,5′,3-PCB-2-OCH_3; 2,4,3′,4′-PCB-3-OCH_3; 3,5,3′,4′-PCB-4-OCH_3; 3,3′,4,4′-DPE; 2,2′,4,4′,5-DPE; 2,3′4,4′,5,5′-DPE; 2,2′,3,3′,4,4′,5-DPE; 2,2′,3,3′,4,4′,5,6-DPE; decachloro-DPE; 3,4,5,2′,4′-DPE-2-hydroxy; nonachloro-DPE-2-hydroxy; nonachloro-DPE-3-hydroxy; 3,4,5,2′,4′-DPE-2-methoxy; nonachloro-DPE-2-methoxy; nonachloro-DPE-3-methoxy; 2,3,2′4′5′-PCB-4-hydroxy; and 2,2′,3′,4′,5′-PCB-2-hydroxy. The mass chromatograms for m/z 320 and 322 for the extract of this mixture are shown in Figure 4.

In addition, we formulated a mixture of the following compounds, which were spiked into blank pork fat at the 10-ppm level and carried through the cleanup procedure: Aroclor 1254; Aroclor 1260; hexachlorobenzene; γ-HCCH; *p,p′*-DDT; *o,p′*-DDT; *p,p′*-DDE; *o,p′*-DDE; *t*-Nonachlor; Oxychlordane; Heptachlor Epoxide; β-HCCH; HEOD; and Mirex. The mass chromatograms for m/z 320 and 322 for the extract of this mixture showed no interfering peaks.

The results of these analyses indicate that these potentially interfering compounds present in these mixtures at 10^3- to 10^6-fold excess are effectively removed during the multicolumn cleanup of the sample.

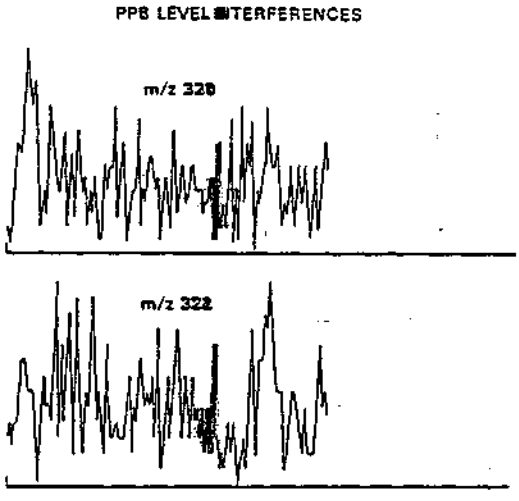

Figure 4. Mass chromatograms for m/z 320 and 322 from the extract of pork fat containing possible interferences at the 10-ppb level.

Validation of 2,3,7,8-TCDD Analytical Standard. A series of standards were prepared by diluting analytical standards of 2,3,7,8-TCDD received from various chemical suppliers and laboratories. The results of the analysis of these standards varied over a rather wide range from the stated concentrations. The stock standard solution used for our quantitative measurements was validated against 2,3,7,8-TCDD material that was synthesized in our laboratory (the other stocks varied from −65% to +35% of the stated concentration). The National Bureau of Standards plans to issue a Certified Reference Material for 2,3,7,8-TCDD in the near future that should help eliminate the wide variability in 2,3,7,8-TCDD standards.

Recovery of 2,3,7,8-TCDD. Blank pork fat was spiked at the 50- and 24-pptr levels with $[^{13}C_{12}]$-2,3,7,8-TCDD, processed through the entire five-column cleanup, and analyzed by using $[^{37}Cl_4]$-2,3,7,8-TCDD as the external standard to give average recoveries of 97% and 88%, respectively (Table V).

Recovery of the 22 TCDDs. Standards containing the 22 TCDDs (at levels corresponding to 20 pptr) were spiked at the 24-pptr level with $[^{13}C_{12}]$-2,3,7,8-TCDD. A standard that had been processed through the entire cleanup procedure was compared with a standard analyzed directly by GC/MS. The data in Table VI indicate that the recovery of the 22 TCDDs through the cleanup procedure is adequate for the quantitation of the 22 TCDDs.

Method Performance. All adipose tissue samples examined thus far have produced sufficiently strong signals (signal to noise ratio > 3/1) to permit quantification. The smallest

Table V. Recoveries of Internal Standard and Native 2,3,7,8-TCDD "Spiked" Human Adipose and Blank QC Pork Fat

pork fat			[$^{13}C_{12}$]-2,3,7,8-TCDD (% recovery)		amt of 2,3,7,8-TCDD, pptr	
10-g samples of pool A spiked with 50 pptr [$^{13}C_{12}$]TCDD			109, 97, 95, 91, 96			
10-g samples of pool A spiked with 24 pptr [$^{13}C_{12}$]TCDD			88, 89, 88			
10-g samples of pool A spiked with 1.07 pptr 2,3,7,8-TCDD					1.2, 0.8, 1.0	
10-g samples of pool A spiked with 0.54 pptr 2,3,7,8-TCDD					0.8, 1.2, 0.5	
			1 pptr spike		5 pptr spike	
human adipose	base (duplicate)		target	found	target	found
male (16 yr)	6.4	5.8	7.1	7.7 8.3	11.1	11.6
female (35 yr)	7.6	8.1	8.8	9.8 9.0	12.8	11.9 13.2
male (40 yr)	4.8	5.0	5.8	6.9 5.7	9.8	10.1 10.1

concentrations of 2,3,7,8-TCDD measured to date have been in the 3- to 5-pptr range. Absolute instrument sensitivity varies by a factor of 3 to 5 during the operation as the condition of the instrument source degrades. With regular routine maintenance, a limit of quantification of 1 pptr may be readily achieved for routine samples from epidemiological studies. For those samples with signals less than 3/1 but meeting all other criteria, we propose the designation of not quantifiable, NQ. Generally samples with a concentration of 0.5–1 pptr are characterized in this way. Samples without observable peaks at the retention time for 2,3,7,8-TCDD are reported as not detected, ND.

We have studied the signal-to-noise characteristics of the analytical standards to estimate the number of samples that would not be quantifiable by this approach. For samples at the 5-pptr level, we estimate that less than 1% of the samples will result in a signal to noise less than 3/1 and be characterized as NQ. For samples at the 1-pptr level, we estimate that 40% would be characterized as NQ. For samples at the 5-pptr level, none should exhibit a signal to noise less than 1 to 1 and be classified as ND. For samples at the 1 pptr level, less than 2% can be expected to be classified as ND because of a signal-to-noise level less than 1 to 1. These preliminary estimates are based on eight to ten samples at each part-per-trillion level. A Gaussian distribution of replicated measurements of the log S/N ratios is assumed.

The accuracy of the method is in part demonstrated by the spiked recovery experiments (Table V). In these tests, 1 or 5 pptr of 2,3,7,8-TCDD was spiked into a sample of human adipose that had been previously analyzed by this method in our laboratory. The values obtained upon analysis are in good agreement with the expected value. A number of human adipose tissue samples have been analyzed in duplicate, and agreement between duplicate samples is usually within 10%. This reproducibility is particularly remarkable because it includes any biological variability in the samples. Another measure of system performance is the precision associated with characterizing the quality control materials (see Table III). For samples at 1.6 pptr, a coefficient of variation (CV) of 19% was observed. For samples at the 8.4-pptr level, a CV of 11% was observed and at the 43-pptr level, a CV of 6% was observed.

Stability of 2,3,7,8-TCDD in Human Adipose. Large pieces of human adipose samples taken at autopsy were stored at −40 °C and analyzed at various times during the method development phase of this study. Samples of ~10 g were analyzed from a number of individuals over a period of 13 months. The data in Table VII indicate that the concentration of 2,3,7,8-TCDD does not appear to have changed over this period and that storage at −40 °C is probably adequate for this analysis. The large pieces of adipose were not homogenized nor manipulated in any way before storage, thereby mimicking the storage conditions of samples in actual studies. The replicate data in Table VII include any variability in the 2,3,7,8-TCDD distribution within the sample as well as any variability in percent lipid content within the large sample from which the 10-g aliquots were taken.

Table VI. Analytical Data for the 22-TCDDs

TCDD[a]	RT[b]	RRT[c]	% recovery through cleanup procedure
1,3,6,8	34:05	0.773	114.5
1,3,7,9	36:20	0.825	112
1,3,7,8	38:52	0.882	124
1,2,4,7 / 1,2,4,8 / 1,3,6,9	40:20	0.915	116
	40:37	0.922	114
1,2,6,8	42:11	0.957	122
1,4,7,8	43:31	0.988	115
[$^{13}C_{12}$]-2,3,7,8	44:04	1.000	
2,3,7,8	44:10	1.002	107
1,2,3,7 / 1,2,3,8 / 1,2,4,6 / 1,2,4,9 / 1,2,3,4	44:41	1.014	104
1,2,7,9 / 1,2,3,6	45:54	1.042	106
1,4,6,9 / 1,2,7,8	48:56	1.110	101
	49:14	1.117	94
1,2,3,9	50:20	1.142	92
1,2,6,9	51:23	1.166	94
1,2,6,7	53:22	1.211	105
1,2,8,9	60:00	1.362	*d*

[a] TCDD assignments based on "Supelco Reporter", Vol 1 (4), 4th ed. (1984, Figure A-1). [b] GC program: 100 °C for 2 min—20 °C/min to 180 °C—3 °C/min to 220 °C—hold for 40 min. [c] RRT = (RT of 322)/(RT of 334). [d] Separate run.

Formation of TCDDs from Pre-Dioxins during Cleanup. The interference mixture provided by Smith and Johnson (23) contained, among other compounds, 2',3,4,4',5-pentachloro-2-hydroxydiphenyl ether. The cyclization of this

Table VII. Concentration[a] of 2,3,7,8-TCDD in Human Adipose Samples

area	sex	race	age	concentration[b] (months stored at −40 °C)	type sample[c]
Atlanta	M	W	16	6.4 (0); 5.8 (0); 5.2 (13)	adipose
Atlanta	F	W	35	7.6 (0); 8.1 (0); 7.7 (13)	adipose
Atlanta	M	W	45	4.8 (0); 5.0 (0); 4.4 (13)	adipose
Atlanta	M	W	69	6.0 (0); 6.5 (0); 6.3 (9)	adipose
Atlanta	F	W	57	9.6 (0); 9.1 (9); 9.5 (9)	adipose
Atlanta	M	W	48	3.8 (0); 3.2 (0); 1.9 (10)	adipose
Atlanta	M	W	71	8.6 (0); 8.6 (6); 7.4 (6)	adipose
Atlanta	M	W	71	15.2 (0); 17.0 (4); 12.9 (6); 18.6 (6); 15.3 (6); 15.8 (9)	adipose
Atlanta	M	B	28	6.7 (5); 6.4 (6)	adipose
Atlanta	M	B	19	7.6 (5); 7.2 (6)	adipose
Atlanta	M	B	69	5.7 (5)	adipose
Atlanta	F	B	39	3.3 (5)	adipose
Atlanta	M	B	78	8.9 (5)	adipose
Atlanta	F	W	46	8.6 (6)	adipose
Atlanta	M	B	67	6.5 (6)	adipose
Atlanta	F	B	56	8.2 (6)	adipose
Atlanta	F	B	68	19.0 (6); 19.6 (7); 19.2 (7)	adipose
Atlanta	F	B	78	12.2 (7)	adipose
Atlanta	M	B	53	9.0 (7)	adipose
Atlanta	F	W	63	6.8 (7)	adipose
Atlanta	M	B	85	7.4 (6)	adipose
Atlanta	F	W	82	11.8 (6)	adipose
Atlanta	F	B	65	12.8 (6); 10.9 (6)	adipose
Atlanta	F	B	37	5.0	adipose
Atlanta	F	B	67	10.6 (6)	adipose
Atlanta	M	B	48	6.4 (6)	adipose
Atlanta	F	B	75	10.7 (6)	adipose
Atlanta	F			6.1	breast fat
Atlanta	F			7.2	breast fat
Atlanta	F			8.9	breast fat
Atlanta	F	W	27	ND (<0.4)	breast milk
Utah	M	W	57	7.1 (6)	adipose
Utah	M	W	51	3.2 (6); 3.8 (9)	adipose
Utah	M	W	59	6.7 (6)	adipose
Utah	M	W	52	5.6 (6)	adipose
Utah	M	W	64	2.7 (6)	adipose
Utah	F	W	76	11.3 (6); 12.9 (8)	adipose
Utah	F	W	41	7.2 (6)	adipose
Utah	F	W	18	3.8 (6)	adipose

[a] Concentrations are given as parts per trillion. [b] Two or more results are for separate 10-g samples from the same large piece of adipose tissue. [c] All samples are ~10 g wet weight (weighed to the nearest tenth of a gram).

o-hydroxydiphenyl ether anywhere in the analytical system would produce 1,2,3,8-TCDD and 1,2,3,7-TCDD ("Smiles rearrangement"). This material was carried through the multicolumn cleanup procedure at the 10-ppb level, and neither of these TCDDs were detected (see Figure 4). In addition, two different samples of human adipose were processed through the cleanup procedure after all acidic or basic chromatographic materials were replaced with neutral material, and the concentrations were the same (within the error of the method) as those obtained by using the normal acidic and basic chromatographic supports.

RESULTS AND DISCUSSION

Criteria for TCDD Identification and Quantification. The quantitative scheme in this method requires that a number of criteria be met if successful quantitative results for 2,3,7,8-TCDD and other TCDDs are to be obtained. The analytical system must be shown to be in control by obtaining satisfactory results with the quality control materials incorporated within the analytical runs. Other requirements for successful quantification are given in Table IV. Our experience suggests that TCDDs will meet the analytical criteria in Table IV for relative retention time and ion maxima even at these low levels. Because of the large uncertainty associated with quantitative results from very small signals, only those samples that result in characteristic peaks and a signal to noise greater than 3 to 1 will be reported quantitatively. The requirement of 10 to 1 signal to noise on the internal standard ensures adequate recovery in chromatographic cleanup and instrument sensitivity. We have mainly used the SP 2330 (Supelco) capillary column to obtain isomer-specific results. As fused silica capillary column technology evolves, other column phases may be more suitable for this work. We are evaluating the specificity and durability of the CP Sil 88 column system (Chrompack).

Samples containing greater than 20 pptr of 2,3,7,8-TCDD are reanalyzed in the confirmation mode. At these higher levels, the additional ions noted (Table IV) may be observed and may provide additional confidence about the presence of TCDD.

Human Adipose Results. During the method development phase of this study, we have analyzed various human adipose tissue samples collected from local and regional centers for 2,3,7,8-TCDD. These results are summarized in Table VII along with the only information that we have about these samples (age, sex, race, location). Only very limited data are available on the levels of 2,3,7,8-TCDD that may be found in human adipose tissue. The critical data needed to define expected levels of 2,3,7,8-TCDD in the general population, or in selected cohorts, do not exist. Many biological, environmental, and other factors that may influence the observed 2,3,7,8-TCDD levels must be carefully described and defined before these data can be validly interpreted. At this time, we can only state that the observed 2,3,7,8-TCDD levels in these

samples appear to be consistent with level reported by Gross et al. (*10*).

Future Method Development. The final phase of method development requires that results obtained from this analytical method be compared with those obtained in other laboratories. We are scheduled to participate in a pending Environmental Protection Agency/Veteran Administration (EPA/VA) interlaboratory study of human adipose tissue. If necessary, the Centers for Disease Control will sponsor a second interlaboratory study to complete validation of this method.

A key to understanding human body burden is knowing how TCDD is distributed throughout the various tissues types. Unless such information becomes available, additional research will be needed to relate adipose tissue samples to total body burden and to verify that this material is the best specimen for TCDD analysis.

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Automated Apparatus for the Extraction and Enrichment of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in Human Adipose Tissue

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An apparatus is described for the automation of the first part of a proven method for part-per-trillion dioxin analysis. A microprocessor controls the valves and solvent flow for the sequential extraction and enrichment of five tissue samples over a 20-h period. Sample and solvent selection for the multicolumn system and reversed flow elution, fraction collection, and column regeneration are all accomplished without operator attention. The time required by the analyst per sample has been reduced 50% compared with the totally manual method, resulting in greater throughput and lower personnel costs. The apparatus can be easily assembled with commercially available components.

In recent years interest in the analysis of tissue samples for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has rapidly increased (*1*), but an accurate determination at the part-per-trillion level presents many problems to the public health toxicologist. The toxicity of the analyte (*2*) and the required internal standard, the extraction of polychlorinated dibenzo-*p*-dioxins (PCDDs) and related compounds from the biological matrix, the removal of interfering polychlorinated biphenyls (PCBs) and pesticide residues (often present at parts-per-million levels) (*3*), and the separation, detection, and identification of specific isomers of interest must all be addressed before reliable results can be reported. Our effects to overcome these difficulties have led to the development of a reliable method (*4*); but it is long, involved, and expensive, and over 8–12 h, it requires many manipulations per sample. To increase sample throughput and reduce costs and the tedious attention required of the analyst (*5*, *6*), we have automated part of the procedure. The method adopted by the Centers for Disease Control (CDC) and modified for analysis

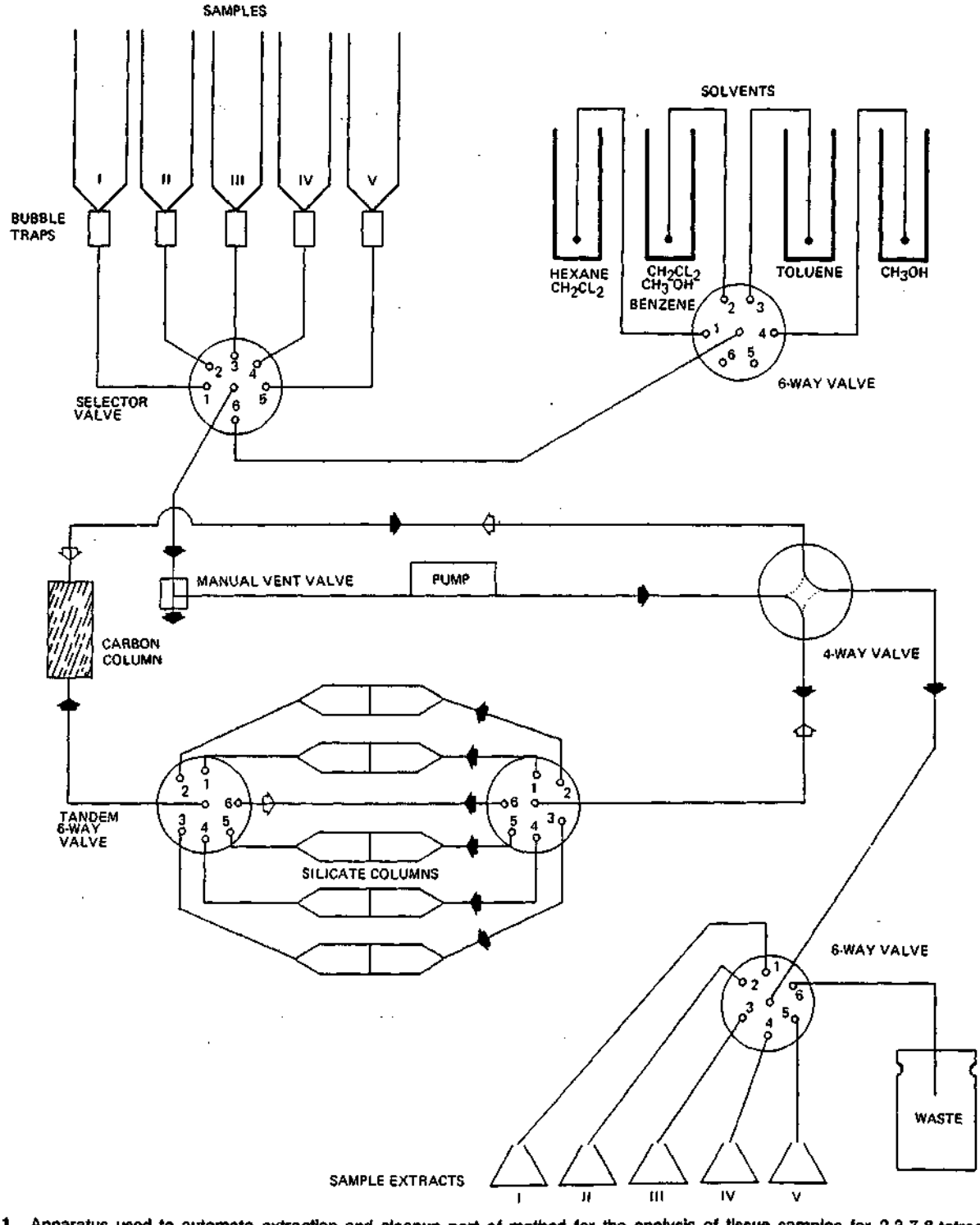

Figure 1. Apparatus used to automate extraction and cleanup part of method for the analysis of tissue samples for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

of human adipose was based on the method developed by Smith et al. (*7*) as a modular approach to sample cleanup that could be easily automated (*8*, *9*). This method is carried out in three stages: extraction and cleanup, fractionation of residues, and analysis by high-resolution gas chromatography/mass spectrometry (GC/MS). The apparatus used to automate the first part of the method is described here.

EXPERIMENTAL SECTION

Apparatus. The entire system is constructed of commercially available components and requires minimal mechanical skills to set up and operate (see Figure 1). The samples and their extracts contact only glass and Teflon, thereby minimizing the possibility of contamination. The carbon/glass fiber and potassium silicate/silica gel columns are reusable and are regenerated through solvent washings during the automated run.

All valves, containers, and columns are interconnected with flanged tubing made of Teflon and threaded polypropylene bushings and couplings (Universal Scientific, Atlanta, GA). Solvents (Burdick and Jackson) are pumped directly from the 4-L bottles in which they are shipped by the Fluid Metering, Inc., Model RP-SY-ICSC pump, fitted with a micrometer low-flow adjustment kit. High-pressure liquid chromatography, low-pressure solvent filters may be used, and they also serve as weight to maintain the pickup of solvent from the bottom of the container. Because solvent volumes are controlled by the flow set at the pump (5.0 mL/min), air bubbles must be kept out of the system and especially out of the pump head where they could become trapped, allowing little, if any, solvent to pass. This can be prevented by (1) locating the pump below the level of the solvent reservoirs, (2) purging the system of air before starting the pump, and (3)

Table I. Comparison of Manual and Automated System

	manual				automated			
sample	mean, pptr[a]	SD[b]	*N*	RSD[c]	mean, pptr	SD	*N*	RSD
QC								
low	1.6	0.32	16	20.0	1.8	0.17	14	9.5
high	8.6	0.95	11	11.0	8.0	0.78	5	9.7
human adipose								
A	16.1		2		15.3	2.0	13	13.3
B	9.6		1		9.3		2	
C	6.2		2		6.3		1	
D	7.8		2		7.7		1	
E	4.9		2		4.4		1	

[a] Parts per trillion. [b] Standard deviation. [c] Relative standard deviation.

including bubble traps. The bubble traps are the same columns as those used for the carbon column and are attached to the outlet of the sample column with an 11-mm coupling (5805-15).

The three glass ace-thred columns are described as follows: sample extraction column (600 × 50 mm 5795-54); silicate column (5796-34 precolumn); carbon column and bubble traps (11 mm custom 5795-823); and threaded, end fitting adapters made of Teflon (5801-14) are supplied by Ace Glass, Inc., Vineland, NJ.

Rheodyne low-pressure valves made of Teflon are used to control flow directions and to select samples and solvents for each step of the procedure. Three configurations of the valves are required: six-position rotary (three each), four-way rotary, and a tandem six-position rotary. All are pneumatically actuated and controlled by 120-V ac solenoid valves.

The multisample, multistep enrichment process (*4*) is automatically controlled through the use of an Eldex Laboratories (Menlo Park, CA) Chromat-A-Trol programmable microprocessor. This unit is easily programmed with up to 250 instructions (1.25K RAM with battery protection) and allows files to be looped or stacked, programs to be altered in progress, and any function to be performed manually at any time. Current channel status and elapsed run time are displayed at all times on the control panel. The unit is programmed to turn the pump on and off and to supply voltage to each valve's controlling solenoid through the accessory ac power module. The Chromat-A-Trol also provides for selection of each sample, through the Eldex selector valve, without pneumatic interfacing.

Operation. The Chromat-A-Trol is preprogrammed to control the switching of valves and pumping of selected solvents for up to five samples (1/file). At time 0, power is applied to the pump, and the selector valve is then set to receive sample extract from the first sample column. As the program progresses (time being determined by the amount of solvent pumped at 5 mL/min), the silicate column is removed from the flow, the carbon column is washed and then eluted in the reverse direction, and finally, the carbon and silicate columns are regenerated with multiple solvent washings. The pump is then turned off, or if more samples are to be extracted, the selector valve is switched to the next position and the cycle is repeated—all under the control of the Chromat-A-Trol (a sample program can be obtained upon request from the authors).

Preparation for a run of five samples is as follows. The four solvent containers are filled with appropriate amounts of solvents, and any air in the lines is bled by pulling the solvent through the vent valve with a gas-tight syringe. Next, the silicate columns are packed (or judged reusable), and the 50/50 hexane/methylene chloride solvent mix is pumped through the system to remove any air. The sample columns are then packed with adsorbents, and the samples are prepared for extraction (see ref 4). The first sample may then be loaded into the extraction column. With the selector valve set for the appropriate column and the vent valve open, air is allowed to escape the system until the first of the extraction solvent approaches the sample valve. The valve is then advanced to the next column, and its sample is loaded and the lines are bled as before. Finally, 500 mL of the 50/50 solvent mix is added to each extraction column. Before the program is begun, the valves must be in their starting positions, the solvent reservoirs full, and the collection vessels in place. With all samples loaded and all air removed from the system, the program is ready to take over and complete the extraction of the five samples without further attention. In its present configuration, the system can handle five adipose samples from initial homogenization through enrichment of planar trace-level environmental contaminants over a period of 20 h.

At this point the enriched extract, in 50 mL of toluene, must be handled manually (*4*) to exchange the solvent for hexane and be carried through the final chromatography steps before being analyzed by high-resolution GC/MS. Further development may allow for the removal of solvent to be automated, or the procedure may be changed so that the final separation can be included without solvent exchange.

RESULTS AND DISCUSSION

Performance Evaluation. The system has been tested for false positives, carryover from sample to sample, and precision, and it has been found to perform as well as its manual counterpart. A system blank consisting of all adsorbents, solvents, and internal standard but no tissue has been included in each set of five samples processed. None of these blanks analyzed would have met our criteria (*4*) for being reported as positive for TCDD. Possible carryover was evaluated by placing 1200 pg of ^{13}C-labeled 2,3,7,8-TCDD (our internal standard) in sample positions 1, 2, and 4 and processing positions 3 and 5 as blanks. Analysis of the two blanks showed no detectable 2,3,7,8-TCDD, indicating no anticipated carryover from a 10-g adipose sample containing up to 120 pptr (parts per trillion) 2,3,7,8-TCDD.

Precision of the method was determined by comparing results of dioxin analysis obtained following either manual or automated cleanup (Table I). Samples were either human adipose or spiked animal fat from our quality control (QC) pools.

The automated system has been accepted for routine use at CDC and has been used for processing samples from several cases of possible dioxin exposure. Our routine analytical run consists of five samples, including one sample from our QC pool (high, medium, or low), and four unknowns.

Our plans for future development of the system include modification or replacement of the control module so that the pneumatic valves can be replaced with electronic ones like the selector valves. In addition, we may be able to modify the procedure to eliminate the need for a solvent exchange, thereby allowing the alumina chromatography step to be included in the automation. Because the system is modular and very flexible, the apparatus could perhaps be adapted to other applications that require multicolumn chromatography cleanup or multisolvent elution, or both.

The advantages of automating this or any labor-intensive procedure are many. Tedious, repetitive manipulations are performed mechanically, reducing the analyst's boredom and exposure to hazardous substances while increasing the procedure's precision and reliability. Complete runs can be set up to process sequentially QC and analytical samples. Previously, only two samples per 8-h day were processed (on two

separate apparatuses), but now five samples can be processed automatically overnight, resulting in more than a 50% reduction in the time required of the analyst per sample.

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DETERMINATION OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN (TCDD) IN HUMAN ADIPOSE TISSUE ON WHOLE-WEIGHT AND LIPID BASES

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Abstract

Our method for determining 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) in human adipose tissue on a whole-weight basis has been modified for the same determination on a lipid basis. The method is to be used to assess TCDD in both exposed and unexposed human populations.

Introduction. The objective of this work is to develop a definitive analytical method for determining 2,3,7,8-tetrachlorodibenzodioxin (2,3,7,8-TCDD) and other TCDD congeners in human tissue samples, both on a whole-weight and a lipid basis. This method is to be used in our laboratory in several proposed studies. The data are needed to develop more accurate risk assessment policies related to human exposure to point sources.

Sample Preparation: Whole-Weight Basis. A 10-gram adipose tissue sample from the anterior, abdominal wall is mixed with 40 g sodium sulfate and 25-30 mL hexane/methylene chloride (1:1), and then homogenized. The homogenate is spiked with a 10-µl solution of $^{13}C_{12}$-2,3,7,8-TCDD in isooctane. This mixture is subjected to a semiautomated modification (1) of the cleanup procedure developed by Smith, Stalling, and Johnson (2).

Sample Preparation: Lipid Basis - Procedure A. The approximately 10-g adipose tissue sample is inspected, any obvious nonfatty portions on the exterior are removed, and five 100-mg slices of the tissue are taken and independently analyzed for lipids by our method (3).

Procedure B. The remaining portion (after Procedure A) of the 10-g adipose tissue sample is homogenized and spiked with internal standard as in the whole-weight procedure (4). However, instead of being subjected to the automated system, the homogenate in hexane/methylene chloride is filtered through a 25-x-450-mm glass column containing 2- to 3-μ glass fiber filters, a small plug of glass wool, and 2 cm anhydrous sodium sulfate. The eluate is collected in a 100-mL volumetric flask until that volume is reached. Two 1-mL aliquots of this eluate are removed with a pipet, placed into a tared 20-ml beaker, and allowed to evaporate to dryness at ambient temperatures, and weighed.

Instrumental Analysis. Our analytical instrument system consists of a Vg ZAB-2F high-resolution mass spectrometer with a Vg 2250 data system and a Hewlett Packard 5840 gas chromatograph. We analyze samples in an isomer-specific mode, using a 60M CPSIL-88 capillary column. The mass spectrometer is operated in the high-resolution (static RP = 10,000 at 10% valley), selected ion recording mode, with perfluorotributylamine used to provide a lock mass at m/z = 314. Peak top jumping is accomplished by stepping the accelerating voltage after any necessary correction during the scan of the lock mass. Ions m/z = 320 and 322 provide a measure of the native TCDD, and ions m/z = 332 and 334 are monitored for the elution of the labeled internal standard. Each observed chromatographic peak is examined for a retention time corresponding to 2,3,7,8-TCDD or another TCDD isomer and to see if the isotope ratio is consistent with a tetrachloro compound. Five analytical standards that correspond to 0.75 to 25 ppt of 2,3,7,8-TCDD in the tissue samples are used to establish a linear calibration curve (coefficient of determination = 0.9949). The other 21 tetra isomers are calculated on the basis of quantitative response factors (5) generated from pure analytical standards of the TCDDs, which were synthesized in-house. The best precision in quantitation is obtained by using chromatographic peak areas and by using the sum of the two ions for the native TCDD and two ions for the internal standard.

Accuracy and Validation. The standard stock solutions used for quantitation are validated against 2,3,7,8-TCDD material synthesized in our laboratory. Recoveries of the internal standard, which is spiked at a concentration equivalent to 24 ppt, are generally greater than 85%. Recoveries for the other 21 isomers range from 92% to 124%. A number of duplicate human tissue samples have been analyzed, and agreement between duplicate samples is usually within 10%. Additionally, several tissue samples were analyzed, and then duplicates of these samples were spiked with 10 pg and 50 pg per 10-gram sample and analyzed. The average agreement between observed and expected values was 9.3% and 3.3%, respectively. Our quality control program is described in another presentation (6) from the 5th International Symposium on Dioxins and Related Compounds.

Results: The overall means for the percent lipids by Procedure A and Procedure B are 79.9% and 80.5%, which are not statistically different, but the precision associated with Procedure B is better than that from Procedure A. Procedure A, however, can be performed independently of the TCDD determination, whereas in Procedure B, the entire 10-g sample is used; therefore, the lipids and dioxin determinations must be done on the same sample. This undoubtedly leads to more accurate percent lipid values, but we have been unable to incorporate that step into our automated procedure. We have compensated for the less precise and accurate numbers resulting from Procedure A by analyzing multiple slices of each 10-g sample. On the basis of a comparison of procedure A and B, the overall mean bias of Procedure A compared with Procedure B was less than 1% and does not differ significantly from zero. We determined that the mean lipid value from the analysis of three slices would maintain the mean bias to within ± 5% at a 95% statistical confidence level.

For the Atlanta/Utah Autopsy Study (6) we determined the percent lipids by Procedure A, using three slices. However, for analyzing tissues that contain less adipose and for which the fatty portions are not so evenly distributed (e.g., liver), a procedure such as Procedure B must be used because the fatty portion contains most of the dioxins and related compounds.

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HIGH-RESOLUTION GAS CHROMATOGRAPHIC/HIGH-RESOLUTION MASS SPECTROMETRIC ANALYSIS OF HUMAN SERUM ON A WHOLE-WEIGHT AND LIPID BASIS FOR 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN

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Abstract

An analytical method is described for measuring 2,3,7,8-tetrachloridibenzo-p-dioxin (TCDD) and other TCDD isomers in human serum using a highly specific cleanup procedure and high-resolution gas chromatography/high-resolution mass spectrometry. The 2,3,7,8-TCDD is quantified by the isotope dilution technique with [$^{13}C_{12}$]-2,3,7,8-TCDD as the internal standard. Other TCDD isomers are estimated by use of published relative response factors. The 1.25 part-per-quadrillion (ppq) limit of detection for 200g samples is more than adequate for determining 2,3,7,8-TCDD concentrations present in human serum specimens. Modifications to the method are presented for the analysis of 50g and 10g serum samples. Analytical accuracy is demonstrated by the results obtained in analyzing spiked samples. The method is shown to be unaffected by a number of potentially interfering compounds. A series of quality control material is used to verify system performance. For 200g samples containing 25.1 ppq 2,3,7,8-TCDD, a coefficient of variation (CV) of 11.5% is observed.

Introduction

Intense public health interest continues to focus on the polychlorinated dibenzo-p-dioxins and related compounds. Humans are exposed to many of these compounds from such sources as municipal incinerators (1) and automobile exhausts (2); other sources, such as the manufacture and use of compounds for which 2,4,5-trichlorophenol is a synthetic intermediate, give rise to primarily 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD). The 2,3,7,8-TCDD congener, one of 22 tetra isomers, is reportedly the most toxic of these compounds. Its toxicological properties, such as acute oral LD50 (3), tetratogenicity (4), carcinogenicity (5), and fetotoxicity (6) in selected animal species, have been documented. However, human toxicity associated with exposure to 2,3,7,8-TCDD has not been as documented. Findings of a recent study of the residents of a mobile home park in Missouri suggested that long-term exposure to 2,3,7,8-TCDD is associated with depressed cell-mediated immunity, although the effects did not result in an excess of clinical illness (7). However, in this health effect study and another (8), exposure was derived from self reported histories and not by body burden measurements. Such measurements are needed in these health related studies.

Polychlorinated dibenzo-p-dioxins are very lipophilic and are thus found in stores in the body that are high in lipid content. Body burden measurements for 2,3,7,8-TCDD in humans have been determined in human milk (8-10) and adipose tissue (11-13). The primary disadvantage of the former matrix in health-related studies is that the cohort is limited to females within a limited age range. The primary disadvantage of the latter matrix is that surgical procedure is required for taking the sample. Therefore, a biological specimen, such as blood or its components, that is obtainable with a less invasive procedure than adipose

and that is available from all participants, is highly desirable.

Other lipophilic xenobiotics, such as the chlorinated hydrocarbon pesticides and polychlorinated biphenyls, have been determined in both human adipose tissue and serum for years. Because the fat content of serum is much less than that in adipose tissue, these compounds are in higher concentrations in the adipose tissue. Consequently, a large volume of serum is normally used when determining these compounds in humans. Because 2,3,7,8-TCDD is present at the picogram per gram or parts per trillion (ppt) level in adipose tissue, any method for determining it in whole blood, plasma or serum would have to be particularly sensitive as well as selective. These criteria necessitate the need for methods based on gas chromatography/mass spectrometry (14). We report herein a high resolution gas chromatographic/high-resolution mass spectrometric method for determining 2,3,7,8-TCDD in human serum; this method is an adaptation of our method for determining this xenobiotic in human adipose tissue (15,16).

Experimental Section

Safety. Chemists undertaking this work with 2,3,7,8-TCDD and other such toxic compounds must understand the potential health effects of such compounds (17) and prudent laboratory practices for handling toxic chemicals (18). Specific precautions related to TCDD analysis are described with analytical methods (19,20). More recent Environmental Protection Agency (EPA) draft methods have emphasized specific aspects of protective equipment, training, personal hygiene, isolation of the work area, disposal of waste, laboratory cleanup, laundry, wipe testing, problems in inhalation, and accidents. We have

described safety precautions in the operation of our Chemical Toxicant Laboratory (21) and other laboratories have also described their procedures (22).

Materials. In addition to the materials already described (15,16); ethanol (anhydrous reagent, J.T. Baker, Phillipsburg, NJ), ammonium sulfate (certified primary standard, Fisher Scientific, Fair Lawn, NJ) and sulfuric acid (Reagent ACS, Fisher Scientific Co., Fair Lawn, NJ) were used.

Sample Preparation. Two hundred grams of serum is weighed into a 500 mL Teflon bottle (Nalge Company, Rochester, NY). An internal standard solution consisting of 240 pg of ^{13}C-labeled 2,3,7,8-TCDD is added to the sample. The standard (maintained at room temperature) is accurately measured, using an EDP electronic digital pipette (Rainin Instrument Company Inc., Woburn, MA) with disposable microliter pipette tips. The disposable pipette tip is primed by dipping the tip 2 to 3 mm below the surface of the standard and operating the pipette for pickup and dispensing back into the standard. A repeat operation is done for pickup and dispensing onto the side of the bottle containing the 200 g of serum. The sample is capped tightly and shaken vigorously using a wrist action shaker Model 75 (Bunnell corp, Pittsburgh, PA) for 30 min. To the sample is then added 100 mL of aqueous saturated ammonium sulfate solution (50mL to 10-50g sample), 100 mL of absolute ethanol (50 mL to 10-50g sample) and 100 mL of hexane (50mL to 10-50g sample). The flask is then capped tightly and shaken vigorously for 30 min using the wrist action shaker. The emulsion that is formed after shaking is centrifuged for 10 min at 1500 rpm. The top hexane layer is transferred to a 500 mL Teflon

separatory funnel. To the bottom aqueous layer is added another 100 mL of hexane, followed by vigorous shaking, centrifuging and combining of the two hexane extracts. The combined hexane extracts (200 mL) are then extracted with concentrated sulfuric acid using a 500 ml separatory funnel and a total of 200 mL concentrated sulfuric acid (70 mL for 10-50g samples) in 20 mL aliquots. The first three extractions are not shaken vigorously in order to prevent the formation of an emulsion. The acid washed hexane is then extracted with a total of 75 mL of deionized water in 25 mL aliquots. The hexane is then transferred to a 250 mL Erlenmeyer flask followed by the addition of 10 g of sodium sulfate and evaporation under a nitrogen stream to ~75 mL. The sample is then loaded onto the first chromatography column for part I of a two part sample cleanup procedure (15,16) which is capable of processing five samples unattended overnight.

The sample from part I in toluene is then subjected to rotary evaporation at 55°C under ~0.1 to 0.2 atm vacuum after the addition of 50 µL of dodecane (99%, Aldrich Chemical Co., Inc., Milwaukee, WI). The toluene solution is carefully taken to about 1 mL and then blown to dryness under a gentle stream of nitrogen. After reconstitution of the sample in hexane, it is ready for part II of the procedure (15). Prior to evaporation of the final extract to dryness, one microliter of dodecane is added to the conical sample vial. This sample extract is reconstituted to 5 µL with toluene just prior to analysis by high-resolution gas chromatography high-resolution mass spectrometry (15).

Instrument Analysis. Our analytical instrument system consists of a Vg ZAB-2F high-resolution mass spectrometer with a Vg 2250 data system and a

Hewlett-Packard 5840 gas chromatograph. Our analyses are conducted in an isomer-specific mode, with a 60 M SP2330 capillary column. The injection is splitless with a 30-s purge. The injection temperature is 250°C. The initial column temperature of 100°C is held for 2 min, programmed to 250°C at 15°C/min, and held for 14 min. The average linear velocity of helium is 23 cm/sec. The mass spectrometer is operated in the high-resolution (static RP=10000 at 10% valley) selected in recording mode, with perfluorotributylamine used to provide a lock mass at m/z 314. Peak top jumping is accomplished by stepping the accelerating voltage after any necessary correction during the scan of the lock mass. Ions m/z 320 and 322 provide a measure of the native TCDD, and ions m/z 332 and 334 are monitored for the eluation of the labeled internal standard. Five analytical standards that correspond to 1.25-25 ppq of 2,3,7,8-TCDD in a 200 g serum sample have been used to establish a linear calibration curve. The data for the standard curve are tabulated in Tables I and II and shown graphically in Figure 1. The internal standard is the [$^{13}C_{12}$]-2,3,7,8-TCDD at a concentration corresponding to 1.2 ppq in the original samples. The other TCDD isomers can be quantitated by using our published (23) response factors relative to 2,3,7,8-TCDD. The best precision in quantitation is obtained by using chromatographic peak areas and by using the sum of the two ions for the native TCDD and two ions for the internal standard.

On a regular basis, isomer-specific chromatography is demonstrated by the analysis of a standard containing the 22-TCDDs. Our serum QC pools L and H contain among other dioxins and furans, the 1,2,3,4-TCDD isomer which can be used to establish isomer specificity for 2,3,7,8-TCDD in analytical runs containing a sample from these QC pools (see Figure 2). The calibration curve is verified by the analysis of an analytical standard during an 8-h shift.

Instrument resolution at 10000 RP, tuning, and other parameters are checked on a regular basis to ensure optimum sensitivity and specificity. Criteria for a positive TCDD determination are as follows: (1) signal/noise greater than 3/1 for both signals on ions 320 and 322; (2) signal/noise greater than 10/1 for both signals on ions 332 and 334 from the internal standard; (3) observed retention times within ± 1 scan of each other on ions 320 and 322 and the relative retention time (RRT) (to [$^{13}C_{12}$]-2,3,7,8-TCDD) must be within 2 ppt of the RRT of the analytical standard; (4) the ratio of the intensities of the ion 320 to 322 and 332 to 334 is within the 95% confidence intervals established for these ratios (see Table III).

Spiked and non-spiked human serum quality control (QC) material has been prepared and characterized. Incorporation of these materials in the analytical run is used to set control limits and to provide a means for demonstrating that the analytical system is in control. Recovery data are calculated on the basis of the absolute area counts for m/z 332 + 334 in the sample vs. the standards.

Quality Assurance Program

QC Materials. The main feature of the QC program is the use of matrix-based materials that are well characterized for TCDD concentration to ensure that the analytical system is in control. Human serum has been dispensed into various sized aliquots. Some of the pools have been spiked with various levels of dioxins and furans while other pools have not been spiked. Six pools of material are available for insertion into the analytical run. The spiked pools contain the following dioxins and furans: 2,3,7,8-TCDD; 1,2,3,4-TCDD, 1,2,3,7,8-pentachlorodibenzo-p-dioxin (pnCDD); 1,2,4,7,8-PnCDD;

1,2,4,6,7,9-hexachlorodibenzo-p-dioxin (HxCDD); 1,2,3,6,7,9-HxCDD; 1,2,3,6,7,8-HxCDD; 1,2,3,7,8,9-HxCDD; 1,2,3,4,7,8-HxCDD; 1,2,3,4,6,7,9-heptachlorodibenzo-p-dioxin (HpCDD); 1,2,3,4,6,7,8-HpCDD; octachlorodibenzo-p-dioxin (OCDD); 2,3,7,8-tetrachlorodibenzofuran (TCDF); and octachlorodibenzofuran (OCDF).

QC Charts. QC charts graphically document the analytical performance of the system. Figure 3 shows the QC charts for pools L, H, and I which were established during the development of this method; the statistical data are shown in Table III.

Details of the Analytical Run. The status of the specimens being analyzed is unknown to the laboratory analysts. Samples are received and arranged in analytical runs of five (four serum samples and one QC sample from pool I or L). In every fourth analytical run a QC sample from a different pool is substituted for a serum sample and a blank is substituted for the pool I or L QC sample. In addition, a serum sample selected at random from one of the four analytical runs will be analyzed in duplicate providing that there is sufficient serum available. The samples are then submitted for cleanup by a manual method (15) or an automated procedure (16) and then submitted to the mass spectrometry (MS) laboratory for analysis. The MS personnel are also unaware of the nature of the extract.

To minimize the possibility for carryover or cross contamination of samples and analytical standards, analysts use separate syringes for samples and for each analytical standard. The sample syringe is periodically discarded or when a serum sample is analyzed that contains more than 75 ppq of 2,3,7,8-TCDD. Between injections of a standard or a sample, the syringe is

inserted through a septum, coated with Teflon, into a 15-mL vial containing 12 mL of toluene, and the barrel is filled and emptied 10 times. This process is repeated twice more with different 15-mL vials containing toluene. A final wash of the syringe is done by filling and expelling it 10 times from a fourth toluene wash solution. These wash solutions are discarded at the end of each working week. The final 5 µl of toluene for reconstituting of samples is then taken from a fifth toluene source. The step-by-step procedure leading to an analytical result; the accompanying documentation; the criteria for TCDD identification and for reporting results have been reported previously (15).

Evaluation and Validation Studies

Interferences. We have previously described (15) our studies to verify the elimination by the cleanup process of compounds which could interfere in the analysis for TCDDs. The results of these analyses indicated that these potentially interfering compounds present at 10^3 to 10^6 fold excess are effectively removed during the multicolumn cleanup of the sample.

Validation of 2,3,7,8-TCDD Analytical Standards. We previously analyzed a series of 2,3,7,8-TCDD standards received from various laboratories and chemical suppliers and found that the stocks varied from -65% to +35% of the stated concentration (15). Because of these findings, we validated our stock solution against 2,3,7,8-TCDD which we had synthesized and characterized in our laboratory. At the time of the previous report, the National Bureau of Standards (NBS) had plans to issue a Certified Reference Material for 2,3,7,8-TCDD, which is now available. We have, therefore, validated our stock

standard solution used for our quantitative measurements against CDC synthetic material, EPA and NBS analytical standards. The results of the measurements are given in Table IV. The agreement among these standards is very good and well within the stated uncertainty.

Recovery of 2,3,7,8-TCDD. Human serum samples (200g, 50g, 10g) were spiked with 240 pg of [$^{13}C_{12}$]-2,3,7,8-TCDD, processed through the entire cleanup procedure described above as well as the entire five-column cleanup described previously (15,16). These samples were analyzed by using [$^{37}Cl_4$]2,3,7,8-TCDD as the external standard to give average recoveries of 69%, 54%, and 68%, respectively (Table V).

Recovery of the 22 TCDDs. A standard that contained the 22-TCDDs that had been processed through the five column cleanup procedure was compared with a standard analyzed directly by GC/MS. The results which ranged from 95% to 124% recovery (15) were adequate for the quantitation of the 22 TCDDs.

Within-Vial Variability. Periodically we need to rerun the mass spectral analysis of a sample due to a number of possible hardware or software problems (for example: electral failure in the laboratory; poor signal-to-noise; incorrect isotope ratios; high voltage shut down; data system crash; etc). In addition, high samples may sometimes saturate the detector and require dilution prior to a second analysis. We have examined the variability involved in a reanalysis from a sample vial (2µl of 3µl) which was analyzed (2µl of 5 µl) the same day, one day earlier, and 27 days earlier (Table VI). The data in Table VI indicate that the samples may be reanalyzed

up to a month later with less than a 10% bias introduced.

Method Performance. All 200g serum samples examined thus far have produced sufficiently strong signals (signal to noise ratio > 3/1) to permit quantification. With regular routine maintenance, a limit of quantification of 5 ppq for a 200g sample may be readily achieved for routine samples from epidemiological studies. We have previously defined our criteria (15) for reporting samples as Q (quantifiable), NQ (non-quantifiable), and ND (non detect).

The accuracy of the method is in part demonstrated by the spiked recovery experiments (Table V). In these tests three different calibrated aliquots of 2,3,7,8-TCDD were spiked into separate 200g samples of Pool I. This experiment was performed twice by each of two different analytes. The values obtained upon analysis are in good agreement with the expected values. We have also conducted a series of experiments in which we combined vials of QC pool L to provide ~ 20, 30, and 40 g samples which were spiked with 240 pg of $^{13}C_{12}$-2378-TCDD and carried through the analytical procedure. The expected and observed values (Table VII) are in good agreement Another measure of system performance is the precision associated with characterizing the quality control materials (see Table III). For 10g samples at 1.9 ppt, a coefficient of variation (CV) of 15% was observed. For 200g samples at the 25.1 ppq level, a CV of 12% was observed.

Stability of 2,3,7,8-TCDD in Human Serum. Samples of QC Pools I and L were stored at -40°C and analyzed at various times during the method development phase of this study.

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Table I. Estimated Concentration (Parts per Quadrillion in a 200g Sample) from Mass Spectrometry Calibration

95% Control limits Lower	Upper	Calibrator Concn	Obsd Mean Concn	Bias, %	Std Dev	Coeff of Variation, %
0.0139	0.0346	0.025	0.0243	- 3	0.0053	22
0.0384	0.0595	0.050	0.0490	- 2	0.0053	11
0.0793	0.1012	0.100	0.0900	-10	0.0054	6
0.2438	0.2935	0.250	0.2687	7	0.0127	5
0.4676	0.5148	0.500	0.4912	- 2	0.0121	2

Table II. Linear Regression Parameter Estimates

	intercept	slope	coeff of determinations
mean	0.000816705	0.0241131	0.9941
Std Dev.			
CV, %			

Table III. Statistical Data for the Human Serum QC Pools

	POOL			
	B(pptr)[a]	L(pptr)[b]	H(pptr)[b]	I(ppq)[c]
concn of 2,3,7,8-TCDD		1.93		25.1
N (sample size, g)		22(10)		18(200)
std dev (σ)		0.30		2.9
CV		15.5		11.5
99% control limits, upper		2.70		32.5
99% control limits, lower		1.16		17.7
95% control limits, upper		2.51		30.8
95% control limits, lower		1.35		19.5
m/z 320/322 RATIO				78.5
N(sample size, g)				18(200)
std dev (σ)				8.9
CV				11.4
99% control limits, upper				101.4
99% control limits, lower				55.6
95% control limits, upper				96.0
95% control limits, lower				61.0
m/z 332/334 RATIO				77.7
N(sample size, g)				18(200)
std dev (σ)				4.9
CV				6.4
99% control limits, upper				90.4
99% control limits, lower				65.0
95% control limits, upper				87.4
95% control limits, lower				68.0

a This pool unspiked composite serum from

b These pools spiked with dioxins and furans described in text.

c This pool unspiked composite serum from 67 individuals.

Table IV. Validation of CDC Quantitation Stock Solution Against EPA and NBS Material for 2,3,7,8-TCDD

	Reported	Found[b]				
	Concentration[a]	Mean[a]	SD	CV	N	% Bias
NBS[c]	67.8 ± 2.3	69.4	3.0	4.3	4	+2.4
SRM 1614						
EPA[d]	78.7 ± 7.9	79.6	2.7	3.4	4	+1.1
7.87± .78µg/mL						
CAS:1746-01-6						
CDC-A[e]	3.77	3.34	0.22	6.8	4	-11
CDC-B[e]	5.02	4.46	0.23	5.2	4	-11
CDC-C[d]	25.1	24.9	1.51	6.1	4	-0.8
CDC-D[d]	50.2	49.4	4.23	8.6	4	-1.6
CDC-E[d]	125.6	123.4	7.47	6.1	2	-1.7

[a]Concentration in pg/µL. [b]Each standard was made in duplicate and each vial was analyzed on two different days. 2400 pg of $^{13}C_{12}$-2,3,7,8-TCDD was added to each vial as internal standard. After evaporation to dryness, the vials were reconstituted to 50 µL with toluene prior to mass spectral analysis of 2 µL aliquots. [c]25 µL of this standard was added to each vial. [d]These standards were diluted 1:100 prior to taking 25 µL aliquots.

[e]These standards were prepared by an additional 1:10 dilution of the stock solution used to make CDC - C, D, and E. The increased bias for these two standards may reflect the extra 1:10 dilution of the stock solution required in order to prepare these two standards.

TABLE V. Recoveries of Internal Standard and Native 2,3,7,8-TCDD "Spiked" Human Serum

Aliquot Added	TARGET[a]	FOUND (200g SAMPLES) 1	2	3	4	Mean Observed	% BIAS
A	49.1±4.8	42.0	39.0	32.0	64.2	44.3 ± 12.0	-9.8
B	71.5±3.0	78.5[b]	65.5	74.0	80.9	74.7 ± 5.9	+4.5
C	94.8±6.7	75.5	73.5	90.0	112.5	87.9 ± 15.6	-7.3

	$[^{13}C_{12}]$-2,3,7,8-TCDD (% recovery)
200-g samples of human serum spiked with 240 pg $[^{13}C_{12}]$ TCDD	56,50,84,93,48,63,81,80,66,59,76
50-g samples of human Serum spiked with 240 pg $[^{13}C_{12}]$ TCDD	58,58,63,51,64,32
10-g samples of human Serum spiked with 240 pg $[^{13}C_{12}]$ TCDD	65,68,61,54,50,100,77

[a] The target value was calculated by adding calibrated aliquots to the pool I (x = 25.1 ± 2.9 ppq). Aliquot A = 24.0 ± 3.8 ppq (N = 5); B = 46.4 ± 0.6 ppq (N = 3); C = 69.7± 6.0 ppq (N = 5).
[b] Undetermined amount of sample spilled. The internal standard ion counts were low.

TABLE VI. Within-Vial Variability

	Day			
	1	1	Maximum	Average
Sample	pg (ppt)	pg (ppt)	% Bias	% Bias
1	19.7(1.87)	18.9(1.79)	4.2	
2	19.8(1.87)	21.0(1.98)	6.1	
3	14.7(73.5)[a]	13.5(67.5)[a]	8.9	5.4
4	4.3(21.5)[a]	4.4(22.0)[a]	2.3	
	1	2		
5	60.7(5.77)	69.7(6.62)	14.7	
6	57.3(5.49)	56.4(5.40)	1.6	5.7
7	71.9(3.45)	75.6(3.62)	5.1	
8	48.0(1.53)	47.5(1.51)	1.1	
9	78.0(1.85)	82.5(1.96)	5.8	
	1	27		
10	18.6(1.75)	18.7(1.76)	0.6	
11	18.6(1.77)	13.7(1.30)	36	9.9
12	38.9(1.85)	41.8(1.98)	7.0	
13	53.9(1.71)	51.5(1.64)	4.3	
14	75.3(1.79)	76.7(1.82)	1.7	
	1	18		
15	12.8(64.2)[a]	11.7(58.7)[a]	9.4	

[a] parts-per-quadrillion.

Table VII. Quantitation of Combined Aliquots of QC Pool L

Pool	Experiment	Level (g)	Expected[a] X ± SD pg (ppt)	Observed pg (ppt)	% Bias
L	1	10.6	20.5 (1.93±0.29)	18.6(1.75)	-9.1
		10.53	20.3 (1.93±0.29)	18.6(1.77)	-8.5
		21.06	40.6(1.93)	38.9 (1.85)	-4.3
		31.45	60.7(1.93)	53.9(1.71)	-11.2
		42.14	81.3(1.93)	75.3(1.79)	-7.4
	2	21.115	40.8(1.93)	44.4(2.1)	+8.9
		31.795	61.4(1.93)	59.5(1.87)	-3.0
		42.369	81.8(1.93)	105.2(2.48)	+28.6
	3	10.525	20.3(1.93±0.29)	21.1(2.0)	+3.9
		20.984	40.5(1.93)	39.1(1.86)	-3.4
		31.587	61.0(1.93)	69.1(2.19)	+13.3
		42.152	81.4(1.93)	88.5(2.1)	+8.8

[a] See Table III for QC Pool L data.

Figure Legends

Figure 1. Standard Curve for 2,3,7,8-TCDD in Human Serum

Figure 2. RIC of 22-TCDDs Analyzed on a SP2330 Capillary Column

Figure 3. Quality Control Chart for Unspiked Human Serum Pool I (200g Samples)

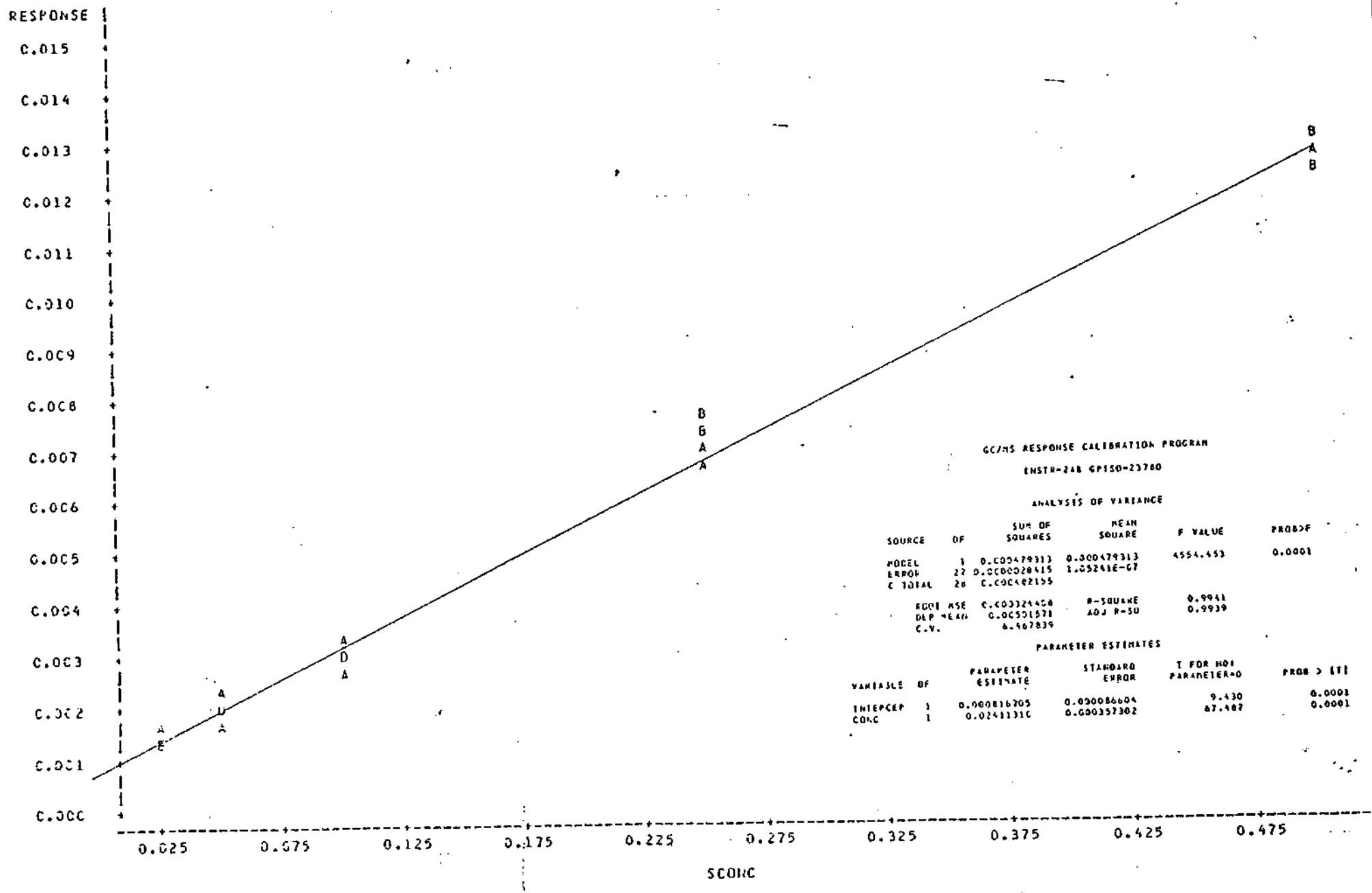
Figure 4. Quality Control Chart for Spiked Human Serum Pool L (10g Samples)

Figure 1

GC/MS RESPONSE CALIBRATION PROGRAM 12:12 FRIDAY, MAY 30, 1986 12

INSTR=ZAB GPISO=23780

PLOT OF RESPONSE*SCONC LEGEND: A = 1 OBS, B = 2 OBS, ETC.



GC/MS RESPONSE CALIBRATION PROGRAM

INSTR=ZAB GPISO=23780

ANALYSIS OF VARIANCE

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PROB>F
MODEL	1	0.000479313	0.000479313	4554.453	0.0001
ERROR	27	0.000002841	1.052418-07		
C TOTAL	28	0.000482155			

ROOT MSE	0.000324408	R-SQUARE	0.9941
DEP MEAN	0.005501571	ADJ R-SQ	0.9939
C.V.	5.897839		

PARAMETER ESTIMATES

VARIABLE	DF	PARAMETER ESTIMATE	STANDARD ERROR	T FOR HO: PARAMETER=0	PROB > |T|
INTERCEP	1	0.000816705	0.000086604	9.430	0.0001
CONC	1	0.024811310	0.000367302	67.487	0.0001

Fig 2

E 0.00001 058669.SD RUN - 1 HUMAN SERUM DEVEL SP2330 60M .25MM .
A314L B320S B322S D332S E334S

RETN.TIME	HEIGHT	AREA	UNCALIBRATED.
0:24:01	13.23	75.43	

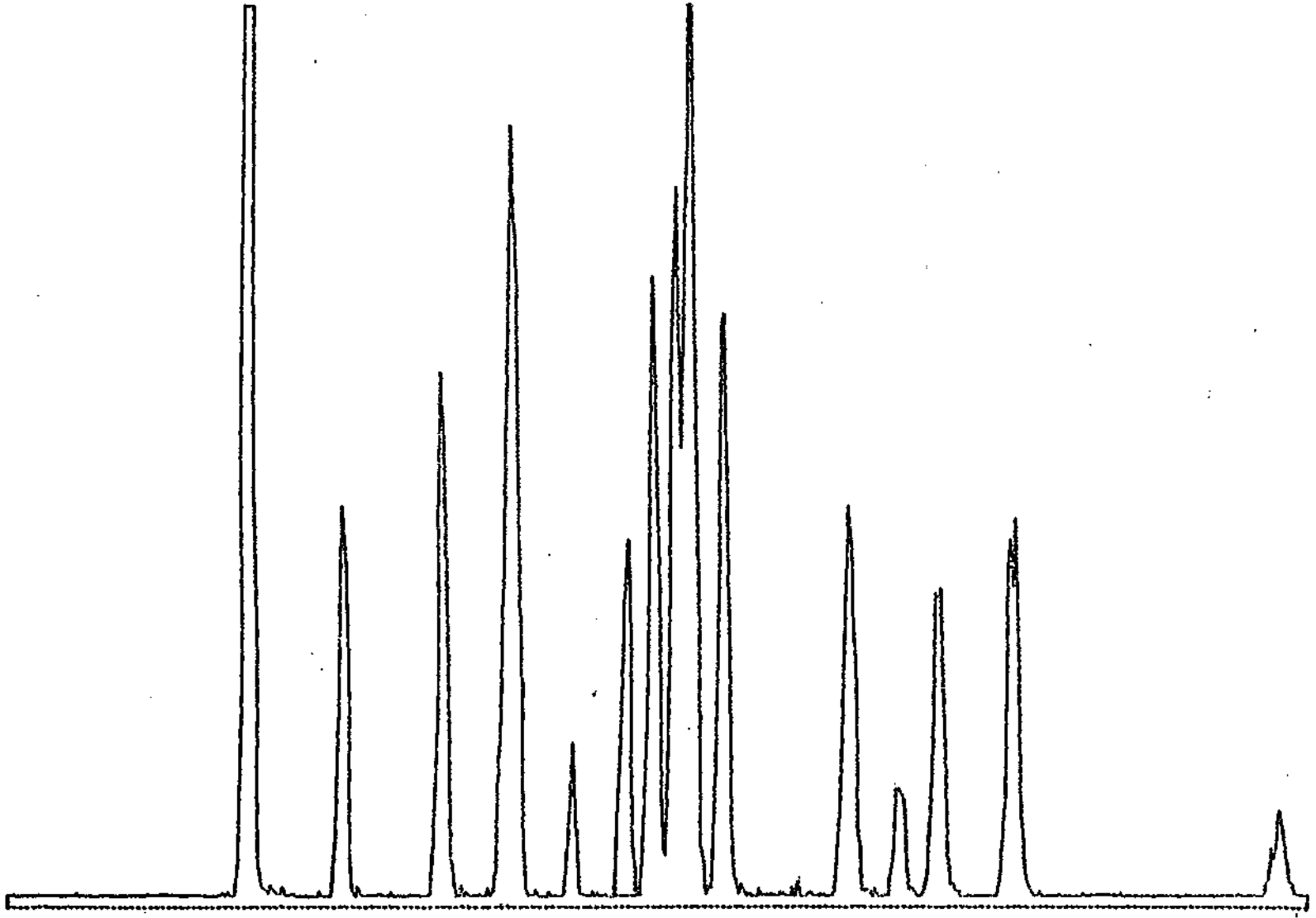
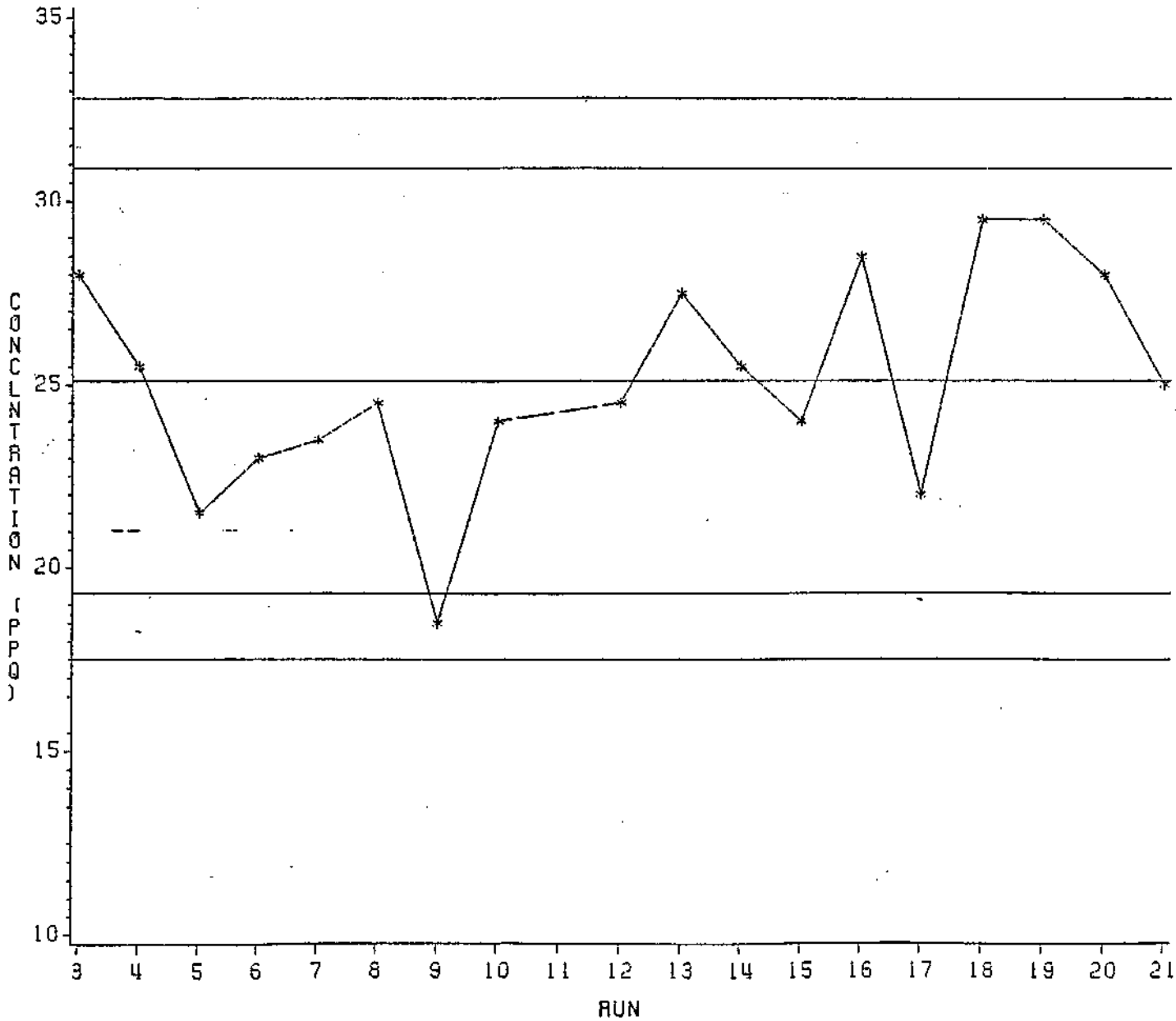


Figure 3

SERUM DIOXIN QUALITY CONTROL

PARTS PER QUADRILLION
POOL I (200 GRAMS)
CENTERS FOR DISEASE CONTROL
CENTERS FOR ENVIRONMENTAL HEALTH
DIVISION OF LABORATORY SCIENCES



PROGRAM NAME IS DSERQC
18 RUNS AS OF 7/17/1986

REVIEW OF MEASURMENT PROCESS BY EXPERTS

REVIEW OF THE MEASUREMENT PROCESS FOR 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN IN HUMAN ADIPOSE AND SERUM

Center for Environmental Health
Centers for Disease Control
Atlanta, Georgia 30333

August 19 and 20, 1986

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Centers for Disease Control
Atlanta GA 30333

August 19, 1986

INTRODUCTION

Thank you for accepting our invitation to serve as an expert in the review of our measurement process for 2,3,7,8-TCDD in human adipose and serum during your 2-day stay in the Center for Environmental Health (CEH) of the Centers for Disease Control.

Currently, the CEH laboratory is determining 2,3,7,8-TCDD in serum samples from Missouri residents whose adipose tissue samples we previously analyzed. From these data, we hope to define a partitioning ratio between adipose tissue and serum for 2,3,7,8-TCDD. If such a correlation exists, then future studies can be performed using serum, the advantages of which are numerous.

Your mission is to evaluate the CEH laboratory's methods, equipment, and personnel for the determination of 2,3,7,8-TCDD in human adipose and serum and the CEH laboratory having been "blinded" as to the matching of the adipose with its corresponding serum sample.

Enclosed is an organizational chart of those within the Center for Environmental Health who have worked on this project. During your two days here, you will be working most closely with members of the Toxicology Branch of the Division of Environmental Health Laboratory Sciences (EHLS). However, the other groups shown on this chart have also been involved in this project and you have complete access and freedom to contact any of these individuals. We are looking for an objective evaluation of the laboratory's methods, equipment and personnel and the fact that the laboratory is blinded in conducting these analyses. Also enclosed are copies of pre-prints and reprints that we believe will aid you in your evaluation.

I ask that before you leave on Wednesday, you will have submitted your final signed remarks. Again, I thank you and welcome you to the Center for Environmental Health, Centers for Disease Control.

Sincerely yours,

Vernon N. Houk, M.D.
Assistant Surgeon General
Director
Center for Environmental Health

REVIEW OF THE MEASUREMENT PROCESS FOR 2,3,7,8-TCDD
IN HUMAN ADIPOSE AND SERUM
August 19-20, 1986

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August 20, 1986

TO: Vernon N. Houk, M.D.
Assistant Surgeon General
Director, Center for Environmental Health
Centers for Disease Control

FROM: David L. Stalling
Research Scientist
Columbia National Contaminant Research Center
Convener of Experts to Review Measurement of 2,3,7,8-TCDD at CDC

SUBJECT: Review of TCDD Analyses

I am pleased to provide you with this summary stating a positive review by the experts invited to CDC, August 19-20, 1986, for the purpose of assessing the data integrity of samples of serum and adipose analyzed for 2,3,7,8-TCDD. An observer from the National Academy of Science also attended the meetings.

The experts have responded to the four questions that you set forth in the materials provided. The questions were:

> Are the analytical methods, equipment, and personnel of the CEH laboratory appropriate for determining 2,3,7,8-TCDD in human serum and adipose tissue?
>
> Are appropriate Quality Assurance/Quality Control procedures being used in determining 2,3,7,8-TCDD in human serum and adipose tissue?
>
> Is the laboratory sufficiently blinded in its ability to link the serum sample with the respective adipose tissue sample?
>
> Is the proposed method of data analysis appropriate for determining whether or not serum and adipose levels are correlated?

For the record, I asked the experts if additional questions should be developed or were required to fully evaluate the data analysis and quality. The experts did not propose or adopt additional questions. Each of the four questions was then reviewed and discussed to ensure that we agreed upon their meaning and scope.

The CDC technical and management staff are to be commended for their technical and program presentations. They provided clear, well-defined, accurate and scientifically sound information to the panel. The technical review panel toured the laboratory operations and after the CDC presentations, we discussed details of the analyses in subsequent meetings with those individuals responsible for the sample processing, analysis, data reporting, quality control, and other aspects of this project.

Each expert has provided you with his evaluation of each question and separately stated recommendations for your staff to consider. These recommendations are primarily aimed at increasing the information content of the analyses or enhancing the quality assurance of the data and in no way do these recommendations reflect adversely on existing data.

Some attention should be directed toward specifying Standard Operating Procedures (SOPs). The analytical SOPs presently are not referenced in the study protocol and a minimum effort should remedy this aspect of Quality Assurance. Some additional development of SOPs should also occur for some laboratory operation.

David L. Stalling, Ph.D.

Concurrence:

Jackson Lay, Ph.D.

John S. Stanley, Ph.D.

Fred L. DeRoos, Ph.D.

Ronald K. Mitchum, Ph.D.

Patrick W. O'Keefe, Ph.D.

Question 1
Are the analytical methods, equipment, and personnel of the CEH laboratory appropriate for determining 2,3,7,8-TCDD in human serum and adipose tissue?

A. Methods

The CEH laboratory of CDC is staffed by trained scientists who are competent to carry out ultratrace analyses for determining levels of 2,3,7,8-TCDD in human serum and adipose tissue. They have developed well documented and validated analytical methods that achieve detection limits that are appropriate for these matrices. Their mass spectrometry equipment is state-of-the-art and is maintained and operated by well trained staff.

The sample workup and mass spectrometry facility staff are familiar with analytical methods that have been developed by other researchers. They have visited several of these peer laboratories and have developed CEH methods in a timely and efficient manner by incorporating validated techniques. They have designed modifications to many of these techniques in order to improve sensitivity, accuracy, and reliability.

Their detection and quantification technique is based on capillary column gas chromatography/high resolution mass spectrometry using multiple ion detection. This is the most sensitive and selective analytical technique available for ultratrace analyses of this type. The CEH methods use 2,3,7,8-TCDD-$^{13}C_{12}$ as an internal standard to correct for analyte losses during sample cleanup and for quantification of the native 2,3,7,8-TCDD levels. This is the most accurate method of quantification since it corrects each sample for variations in the cleanup efficiency as well as sensitivity changes of the mass spectrometer.

Signature Fred L DeRoos Date 8-20-86

Fred. L. DeRoos, Ph.D.

6707L

Question 2

Are appropriate Quality Assurance/Quality Control procedures being used in determining 2,3,7,8-TCDD in human serum and adipose tissue?

The QA/QC procedures are adequate to assure that high quality data are being produced. Approximately 40% of the sample analyses are for QA/QC purposes. QA/QC procedures are followed in both the sample work up and mass spectrometry facilities.

The QA/QC samples consist of sample method blanks, pooled adipose/serum samples and duplicate samples. The sample method blanks are processed using the same procedures as used for the actual samples and will indicate if contamination of the samples has occurred. The pooled samples serve as reference samples that can be used to evaluate the long term precision of the analytical methods. The duplicate samples provide an additional check on the precision of the analytical methods.

Additional QA/QC procedures carried out in the mass spectrometry facility include checks of the mass resolution and the absolute sensitivity of the mass spectrometer. In addition, the raw mass spectral data are evaluated manually in the mass spectrometry facility as the data are acquired in order to provide rapid indication of any problems. This evaluation includes the chlorine isotope ratios, TCDD isomer resolution, accuracy of standard quantification, absence of contamination in method blanks, and reproducibility of pooled samples analyses.

Signature Fred L DeRoos Date 8-20-86

Fred. L. DeRoos, Ph.D.

6707L

Question 3
Is the laboratory sufficiently blinded in its ability to link the serum sample with the respective adipose tissue sample?

The sample identification numbers are assigned such that it would be very difficult to link serum samples to their respective dipose tissue samples. The adipose tissue samples can be related to the subject, however, the 200g serum samples delivered to CEH have only a Red Cross identification number. Data necessary to link the 10 g serum samples and their respective adipose tissue samples are available within CEH, however, these data cannot be easily retrieved and compared. The sample numbering system employed by CEH also provides additional blinding of the sample identification. The sample markup facility assigns a new identification number to each sample during workup. This number is then used as the identification number by the mass spectrometry facility.

Signature Fred L DeRoos Date 8-20-86
Fred. L. DeRoos, Ph.D.

6707L

QUESTION 4

Is the proposed method of data analysis appropriate for determining whether or not serum and adipose levels are correlated?

The reviewer is not qualified to fully respond to this question. It is reasonable, however, to expect 2,3,7,8-TCDD levels in serum to be a function of the proposed variables. Other correlation studies involving PCBs have shown one or more of these relationships. If a correlation between the 2,3,7,8-TCDD levels in human serum and adipose tissue exists, it should be revealed by the proposed data reduction method.

Signature Fred L DeRoos Date 8-20-86

Fred. L. DeRoos, Ph.D.

6707L

Recommendations:

Question 1

The following recommendations would enhance laboratory measurements in future studies, but are not meant to imply any deficiency in the quality of current results.

Additional confidence could be added to the analyses if a third native TCDD ion mass, such as m/z 324, was observed during the analyses and profile data were acquired for selected samples. The additional ion mass peak would further define the responses as being from 2,3,7,8-TCDD, while the profile data would reveal interferences that are only partially mass resolved.

It would be beneficial to include the limit of detection for analyses in which 2,3,7,8-TCDD is not detected.

It would be advisable to use a second standard, such as 1,2,3,4-TCDD-$^{13}C_{12}$, to calculate the recovery of the internal standard. Although the absolute recovery of the internal standard should not effect the accuracy of the data, it is useful to know whether high detection limits are the result of poor internal standard recovery or chemical interferences.

A surrogate standard could also be spiked into the sample before extraction. Its concentration should be at, or slightly above, the desired limit of detection. It would serve to demonstrate that the limit of detection was actually achieved for each analysis.

It would also add to the validity of the study if selected samples and/or sample extracts were analyzed by another laboratory.

Signature Fred L DeRoos Date 8-20-86

Fred. L. DeRoos, Ph.D.

6707L

Question 2

The following recommendations would enhance laboratory measurements in future studies, but are not meant to imply any deficiency in the quality of current results.

It is recommended that CEH prepare Standard Operating Procedures (SOP) for all analytical procedures, including sample workup, analyses, and data reduction. It is also recommended that a full sample identification number be used on all bottles/vials that contain samples or sample extracts. Sufficient cross reference data should be available to allow samples to be rapidly tracked through the entire analytical procedure.

Signature Fred L DeRoos Date 8-20-86

Fred. L. DeRoos, Ph.D.

6707L

QUESTION 1

Are the analytical methods, equipment, and personnel of the CEH laboratory appropriate for determining 2,3,7,8-TCDD in human serum and adipose tissue?

A. Methods

The extraction of samples and the cleanup and gas chromatography/mass spectrometry methods are state-of-the-art and, as demonstrated by literature reports from the laboratory, are capable of producing results with the accuracy and precision required for analyses in the ppt and ppq ranges.

B. Equipment

The mass spectrometry and sample cleanup laboratories are well-equipped and the instruments appear to be maintained at a high level of efficiency. The use of automated procedures for sample cleanup is commendable and the laboratory should be encouraged to make further progress in this area if large numbers of samples are to be processed. Reliance on capillary gas chromatography/high resolution mass spectrometry provides for a high degree of confidence in the analytical data. Three instruments are available with each instrument having the capability of detecting femtogram quantities of 2,3,7,8-TCDD.

C. Personnel

The professional personnel demonstrated by their presentations that they were very knowledgeable in their respective areas of dioxin analysis. Statisticians and computer personnel are also providing important support to the programme. Laboratory technicians appear to be well trained and are qualified at the B.S. level.

Signature Patrick W. O'Keefe

Patrick W. O'Keefe, Ph.D.

August 20, 1986

6706L

QUESTION 2

Are appropriate Quality Assurance/Quality Control procedures being used in determining 2,3,7,8-TCDD in human serum and adipose tissue?

(1) Several sample identification systems are used from the point at which samples arrive in the laboratory to the point where analytical reports are generated. The LIMBS (Laboratory Information System Number) given to the samples initially is translated into a laboratory notebook number on arrival of the sample in the sample cleanup laboratory. This is acceptable as it helps to keep the GC/MS laboratory "blind" with regard to duplicates. However, the cleanup analyst uses one digit numbers to track samples as they move through the cleanup. With several analysts working on sample groups at the same time and each one using the same set of numbers, the possibility for loss of sample identity cannot be ruled out.

(2) Other aspects of the Quality Control program, such as inclusion of blanks, spikes and duplicates, provide for a high level of confidence in the data.

Signature Patrick W. O'Keefe

Patrick W. O'Keefe, Ph.D.

August 20, 1986

6706L

QUESTION 3

Is the laboratory sufficiently blinded in its ability to link the serum sample with the respective adipose tissue sample?

While the laboratory does not attempt to make the internal quality control samples blind, coding procedures insure that nobody outside the Red Cross field sampling unit will be aware of the relationship between serum and adipose tissue samples.

Signature Patrick W. O'Keefe, Ph.D. August 20, 1986

6706L

QUESTION 4

Is the proposed method of data analysis appropriate for determining whether or not serum and adipose levels are correlated?

I have insufficient background in statistics to address this question.

Signature Patrick W. O'Keefe, Ph.D August 20, 1986

6706L

RECOMMENDATIONS

The following recommendations would enhance laboratory measurements in future studies, but are not meant to imply any deficiency in the quality of current results.

(1) The laboratory found that pork fat did not contain detectable concentrations of 2,3,7,8-TCDD and was able to use this material for Q.C. fortification with 2,3,7,8-TCDD. Consideration should be given to using a pork serum pool for the serum analyses. This would be important if a number of serum samples were found to have less than 25 ppq 2,3,7,8-TCDD, the background concentration in the human serum pool for QC.

(2) Calibration curves are constructed using all available data. Consideration might be given to constructing new curves when new standards are prepared to see if the new data deviate in any consistent way from previous data.

Signature Patrick W. O'Keefe

Patrick W. O'Keefe, Ph.D.

August 20, 1986

6706L

Question 1

Are the analytical methods, equipment, and personnel of the CEH laboratory appropriate for determining 2,3,7,8-TCDD in human serum and adipose tissue?

The analytical methods, equipment, and standards are appropriate and sample preparation and mass spectrometry personnel are appropriate for determination of 2,3,7,8-TCDD in human adipose at the ppt level and serum at the ppq level.

A. Analytical methods

The semi-automatic sample preparation (i.e., extraction and cleanup) is recognized as an effective procedure for achieving sample extracts of the necessary cleanliness and quality for achieving the required limits of detection. This procedure was adapted from work that has been previously presented by other laboratories for low level work in environmental analyses (fish, water, etc.) and has been used by at least two other laboratories in a previous interlaboratory study (Anal. Chem. by Albro, et al.). Other laboratories have also incorporated components of this procedure into their own sample preparation practices.

B. Equipment

The semi-automated preparation procedures result in reproducible handling of serum and adipose samples. Evidence for this reproducibility is presented through repeated analyses of both spiked and unspiked control samples (adipose and serum). The high resolution mass spectrometry equipment is state-of-the-art. The levels of detection demonstrated in the data provided could not have been achieved without this level of sophistication.

Signature  Date 8/20/86

John S. Stanley, Ph.D.

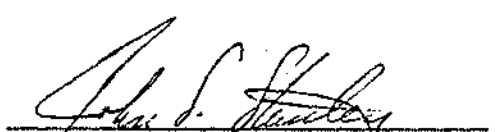
Doc. 6705L

C. Personnel

All persons involved with this program are obviously devoted to achieving the best level of performance as observed through laboratory practices, approach to the analysis, and quality of the resulting products. Specific responsibilities in the areas of sample control (blinding), sample preparation, instrumental analysis, synthesis of standards, data analysis, and overall project management have obviously contributed to this apparent rapid development of ultratrace capability at CDC.

D. Standards

The analytical standards (specifically 2,3,7,8-TCDD) synthesized by CDC have been demonstrated to be equivalent in quality to certified materials available through EPA's Reference Materials Branch and the NBS Standard Reference Materials.

Signature  Date 8/20/86

John S. Stanley, Ph.D.

Doc. 6705L

Question 2

Are appropriate Quality Assurance/Quality Control procedures being used in determining 2,3,7,8-TCDD in human serum and adipose tissue?

The Quality Control procedures implemented and practiced throughout the analytical programs reviewed are extensive and have been consistently applied.

Quality Control aspects include:

. Blind codes assigned to actual specimens

. Sample batches include:
  - 4 actual samples
  - 1 method blank
  - 1 QC (control) sample is substituted every 4th sample batch
  - This results in 4 method blanks (system blanks) and 1 control sample which are indicators of data quality for every 15 blind samples.

. Control samples – Documentation was provided to demonstrate that CDC has encountered at least one instance where a control samples did not meet the QC criteria and corrective action was implemented. This required a reanalysis of a sample set. Data for the reanalysis of this set was remarkably close to the original analysis for the unknowns.

. Instrumental control parameters or criteria are established and consistently practiced. Documentation of this activity is accomplished through both the mass spectrometry facility and a statistical analysis group.

Signature  Date 8/20/86

John S. Stanley, Ph.D.

Doc. 6705L

- Data interpretation – qualitative criteria and quantitative procedures are established and consistently followed.
  All data require a minimum of three levels of review.
  Once quantitative data are reviewed, they are documented and incorporated in the study file.

The QC program practiced is a strength in the CDC analytical approach and provides relevant data necessary to assess the credibility of the values reported for individual samples.

In terms of overall quality assurance, specific SOPs are presented for field collection of samples and analytical methods descriptions exist in general laboratory guidelines and are detailed in the scientific literature.

All personnel are well trained and qualified in their respective disciplines. Procedures for sample handling, preparation, analysis, and data interpretation are obviously routinely followed. The data generated to date are credible and supported by QC controls.

Signature John S. Stanley

John S. Stanley, Ph.D.

Date 8/20/86

Doc. 6705L

Question 3

Is the laboratory sufficiently blinded in its ability to link the serum sample with the respective adipose tissue sample?

Laboratory staff are blind to the exact nature of a specific sample through the unique sample coding operation and physical separation of the sample retrieval group and the sample analysis group. Sample information can be tracked from the Mass Spectrometry facility back to the sample preparation facility. However, decoding of the sample identity requires access to a data base which requires a password assigned to specific personnel.

Once data have been fully reviewed, a hard copy of the data is entered into the file. This hard copy requires a sign-off by two individuals. Hence, any change in the data base requires some formal documentation (tracking).

Signature  Date 6/20/96

John S. Stanley, Ph.D.

Doc. 6705L

Question 4

Is the proposed method of data analysis appropriate for determining whether or not serum and adipose levels are correlated?

This reviewer is not qualified to comment on the statistical analysis of data.

Signature John S. Stanley

John S. Stanley, Ph.D.

Date 8/20/84

Doc. 6705L

RECOMMENDATIONS

The following recommendations would enhance laboratory measurements in future studies, but are not meant to imply any deficiency in the quality of current results.

Question 1

A procedure should be implemented to address recovery of the internal standard for each sample. This will provide a means for tracking differences resulting from the sample preparation step. These recoveries may provide an indication of differences in laboratory staff, reagents, spiking accurary (internal standard), etc. Also some form of interlaboratory study should be completed before proceeding with additional studies using serum as the sample media.

Question 2

In lieu of future programs in this area, it is recommended that a formal SOP is established to describe in detail the exact responsibilities and procedures for each team member. This will be crucial to a transfer of technology in each discipline in the event that a large cohort study requires involvement of additional staff.

Signature John S. Stanley Date 8/30/66

John S. Stanley, Ph.D.

Doc. 6705L

QUESTION 1

Are the analytical methods, equipment, and personnel of the CEH laboratory appropriate for determining 2,3,7,8-TCDD in human serum and adipose tissue?

A. Methods

Yes, to all three aspects, with one recommendation to better document QC (See question #2)

Isomer specialty and cleanup or isolation of TCDD—the data being generated have high integrity and the level of sensitivity achieved is "state-of-the-art".

The QC compliance criteria and method performance demonstrate that these TCDD measurements have validity and adequate work to determine the analytical variability has been done to permit an evaluation of the study objectives.

Recommendation For present samples in which no TCDD is detected, the data report should include the limit of detection (LOD) and perhaps the recovery of ^{13}C-TCDD internal standard. The data for these calculations are available from present measurements.

B. Equipment

The equipment is properly maintained and its sensitivity/performance are among the best I have seen in the U.S. and on a comparable par with that of Dr. Rappe in Sweden. This reflects well the deducation and diligence of the MS staff. The automation of the cleanup has certainly been an important contribution to achieving the goals of the study. This ensures that precision and freedom from chemical interfereences are maintained.

C. Personnel:

Having visited this facility about two years ago, I am especially impressed with the scientific growth the laboratory personnel have made. The dedication and effort to develop the analytical resources are commendable. The staff have adequate training and the experience and work quality are reflected in the data quality evaluated in this review.

Signature David L. Stalling

David L. Stalling, Ph.D.

Date: August 20, 1986

Doc. 6703L

RECOMMENDATIONS

Question 1

The following recommendations would enhance laboratory measurements in future studies, but are not meant to imply any deficiency in the quality of current results.

For future studies in which serum or tissue sample may contain a larger percentage of non-detected levels of TCDD, consideration should be given to establishing a "Method performance spike", e.g. 1,2,3,4-^{13}C-TCDD that would be added in addition to 2,3,7,8-^{13}TCDD at a level that is 2-3 times the limit of detection for the method. This would enhance statistical evaluation of data.

Signature David L. Stalling

David L. Stalling, Ph.D.

Date: August 20, 1986

Doc. 6703L

QUESTION 2

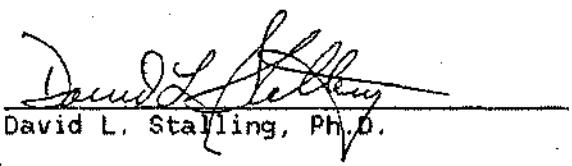
Are appropriate Quality Assurance/Quality Control procedures being used in determining 2,3,7,8-TCDD in human serum and adipose tissue?

Yes--to Quality Control. Laboratory protocols and experimental procedures document the sample processing, cleanup, and analysis steps. The data quality is of the highest caliber that I have personally evaluated.

It appears, though, that the laboratory Quality Assurance documentation needs to be formalized and included in the Study Protocol. Specifically, the sample flow, workup, analysis, and data reporting steps should point to QC or Standard Operating Procedures documents to be used as well as stating what training or instructional efforts are to be carried out by laboratory supervisors if new personnel are added.

This material should be included as an Attachment 10. to the Protocol Study document or as a memo from the appropriate management to formally charge other managers responsible for adherence to QA.

This recommendation IN NO WAY reflects adversely on any laboratory procedures or data quality! But it is made to assist you in meeting aspects of Good Laboratory Practices.

Signature 
David L. Stalling, Ph.D.

Date: August 20, 1986

Doc. 6703L

QUESTION 3

Is the laboratory sufficiently blinded in its ability to link the serum sample with the respective adipose tissue sample?

The procedures reviewed appear to maintain an adequate level of "blindness" and the analytical reports are transmitted by formal memo to provide a hard copy of the results to validate the laboratory data base in the future. Field collection and number assignment steps are well documented in the protocol for the study. It appears that the present system is perhaps overdone with respect to blindness. I would recommend that the numbering system be reviewed with regard to increasing the ruggedness of the numbering. The use of single letters in the workup may be revised. In larger studies, confusion could easily develop. There is no problem with the present system. It is well documented.

Signature David L. Stalling, Ph.D. Date: August 20, 1986

Doc. 6703L

QUESTION 4

Is the proposed method of data analysis appropriate for determining whether or not serum and adipose levels are correlated?

The proposed approach seems quite adequate. The lipid measurements in the present study should be used to evaluate and/or classify individuals (using a multivariate approach—principal components or partial least squares in latent variables (PLS-2)* classifications to determine which individuals have similar lipid biochemistry. Partitioning values may be more similar in these individuals. In addition to TCDD, DDE values should be measured, especially in future studies to enhance the evaluation of partitioning of TCDD.

*Reference: Sjostrom, M., S. Wold, and B. Soderstrom. 1986. "PLS Discriminant Plots" in Pattern Recognition in Practice II, Eds. Gelsema, E.S. and L.N. Kanal. Elsevier Science Publishers B.V. (North-Holland), pp. 461-70.

Signature _____ Date: August 20, 1986

David L. Stalling, Ph.D.

6703L

RECOMMENDATIONS

Question 4

The following recommendations would enhance laboratory measurements in future studies, but are not meant to imply any deficiency in the quality of current results.

Provisions for measuring a persistent contaminant associated with dietary intake (e.g., DDE) should be made to provide an alternate means of evaluating serum/fat TCDD ratios. The rationale for this recommendation is to provide a means of evaluating the partitioning of TCDD between serum/fat if there appears to be a peer correlation of TCDD partitioning. Considerable information is available regarding DDE levels and its half-life, etc. By ratioing TCDD/DDE levels in serum and in fat, it should be possible to better evaluate variations in partitioning by referencing DDE serum/fat ratios. Material for performing these analyses in remaining plasma samples should be available if the cleanup effluent from the initial carbon columns were collected. Also, the cost for this measurement is low and the amount of sample required for fat is quite small as well. If different type of lipid biochemistry is represented in individuals, grouping these cases should be done prior to regression modeling.

Signature David L. Stalling

David L. Stalling, Ph.D

Date: August 20, 1986

6703L

QUESTION 1

Are the analytical methods, equipment, and personnel of the CEH laboratory appropriate for determining 2,3,7,8-TCDD in human serum and adipose tissue?

A. Methods

The methods are clearly appropriate for analysis of adipose tissue. ALthough only a few "pooled" samples of serum have been assayed, the modifications made to the cleanup procedure used for adipose as well as the larger sample sizes (200 g) should allow accurate measurement of 2,3,7,8-TCDD in serum samples.

B. Equipment

The GC/MS methodology is clearly the analytical method of choice. Further, the 100-fg sensitivity (on column) of the ZAB should be more than sufficient for the analysis of the adipose and serum samples, provided that the proposed serum samples are of the large sizes (200 g) proposed in the methodology.

C. Personnel

The reviewer is favorably impressed with the personnel involved in the study. They have demonstrated in peer-reviewed publications that they can reproducably assay adipose samples for 2,3,7,8-TCDD. Because the same staff, with the possible exception of one individual no longer employed in the laboratory, is to be utilized for serum assays, the reviewer feels that the laboratory has adequately trained staff for the analysis of the serum as well.

Signature Jack Lay, Ph.D. Date: August 20, 1986

Doc. 6700L

QUESTION 2

Are appropriate Quality Assurance/Quality Control procedures being used in determining 2,3,7,8-TCDD in human serum and adipose tissue?

The QC procedures insure that the precision of the measurements will fall within a well defined set of confidence limits. Further, 'out-of-control' samples are identified readily and are reanalyzed according to a standard set of criteria.

The accuracy of the method cannot be evaluated in the absence of a standard reference material. The reviewer strongly recommends that serum samples which are "not detected" be checked for the percent recovery and that the measured detection limit be reported along with the fact that the sample was "not detected."

The reviewer is not well versed in the legalistic aspects of QA (i.e., GLP compliance). However, the absence of a clear and simple summary of procedures in the laboratory seems to be a deficiency which could become a serious problem if staff members were removed from or left the project. This is the only significant deficiency noted by this reviewer.

Signature Jonathan O. Lay Jr. Date 8/20/86
Jack Lay, Ph.D.
Doc. 6700L

QUESTION 3

Is the laboratory sufficiently blinded in its ability to link the serum sample with the respective adipose tissue sample?

The laboratory has an excellent blinding system. The use of the Red Cross "ID" number allows the entire laboratory to be blinded. Further, the use of the Red Cross numbers, until the assays are completed, makes the integrity of that portion of the serum blinding unquestionable. Furthermore, the reanalysis of randomly selected samples (for adipose) provides additional blinding. The reviewer suggests that perhaps some serum extracts could be recoded and reassayed blindly. (This is recommended because the entire serum sample is used in the assay.)

Signature Jackson O Lay Jr Date: August 20, 1986
Jack Lay, Ph.D.
Doc. 6700L

QUESTION 4

Is the proposed method of data analysis appropriate for determining whether or not serum and adipose levels are correlated?

The reviewer feels that he is not qualified as an "expert" in statistics. However, it appears to the reviewer that display of the adipose values and blood values on axes of two-dimensional plots, as proposed, should provide an indication of any potential relationship.

Signature Jackson O. Lay Jr.

Date: August 20, 1986

Jack Lay, Ph.D.
oc. 6700L

Question 1

Are the analytical methods, equipment, and personnel of the CEH laboratory appropriate for determining 2,3,7,8-TCDD in human serum and adipose tissue?

The requirements for an operational laboratory performing analytical chemistry associated with the ppt and ppq determination of TCDD in human adipose and serum must meet the highest standards. The methods must be validated by intensive laboratory study; the equipment must be state of the art in order to achieve consistent ppt/ppq operational levels; the personnel must be professionals and have the technical ability and scientific status to plan, develop, and interpret a complex study. The laboratory has published a number of scientific papers detailing the methods and method performance for adipose and serum assays. The assays are based upon sound scientific principles which have been proven for highly lipophilic matrices. The use of labelled internal standards achieves adequate precision for both adipose and serum. Recoveries based upon matrix spikes were within expected values for in-control analytical methodologies. The modification of the adipose method to accommodate the increased sera complex triglycerides has been shown to provide adequate detection limits for the 200 ml serum samples. The laboratory equipment consists of four state-of-the-art mass spectrometers which represent specific sensitive analytical quantitative tools for dioxin analysis. The use of high resolution mass spectrometry provides an additional level of specificity for accurate mass and isomer assignment. The laboratory personnel are made up of Ph.D.-trained chemists, trained medical technicians, trained laboratory technicians, and trained electronic technicians. Although the dioxin program at the CEH has existed only a short time, the laboratory personnel have received outside training and have over the past two years gained valuable experience in dioxin analysis.

Signature  Date: August 20, 1986

Ronald K. Mitchum, Ph.D.

Doc. 6701L

In summary the analytical methods have been single-laboratory, validated by the CEH. A limited number of collaborative study results have also been obtained. Since the laboratory will perform the analysis in-house and since a common link exists between standards associated with the study and those certified by NBS and EPA, no data bias is expected. The laboratory has state-of-the-art equipment well suited to the analytical needs of the project. The personnel are highly qualified professionals who, by the nature of the priority of the project, work solely on dioxin methodology and the analytical chemistry associated with the quantitation of the samples. Such a focused program results in quality procedures and a consistency of the analytical chemistry. The levels of dioxin to be determined in human sera are at the limits of existing technology and that must be considered along with the expectations of the study. The analytical results at hand at the time of this review represent excellent data obtained at the cutting edge of technology. The variability in the data is what can be expected and little improvement would be expected or should be required. This should be factored into the data analysis for determining if a correlation exists.

Signature 

Ronald K. Mitchum, Ph.D.

Date: August 20, 1986

Doc. 6701L

QUESTION 2

Are appropriate Quality Assurance/Quality Control procedures being used in determining 2,3,7,8-TCDD in human serum and adipose tissue?

There are two aspects to be considered when determining if the data generated from this study is of known quality. Quality assurance (QA) is a management tool which addresses the procedures necessary to assure the data is of known quality. This includes problem definition, SOPs for the performance of the experiment, controls in place to insure SOPs are followed, and birth to death project management. Quality control (QC) is a laboratory tool to ensure QA procedures are met. An assessment of the QA procedures in place in the laboratory which includes adequate management controls, problem deifnition, data quality objectives, program managmenert indicates that many QA procedures are in place and none which are not in place would impact the data assessment. QA procedures associated with critical parameters such as data traceability and independent statistical analysis of QC runs, are in place. In critical areas such as QA checking for transcription errors and calibration criteria for analytical balances were not in place but would not be considered critical. SOPs for some of the general laboratory operation were not in place however, due to the small laboratory groups (i.e., supervisor to employee ratio 3:1 ensures the general method and analysis validity). The QA/QC criteria in place in the laboratory is adequate to assess the quality of the data. The EHLS should be commended for actively incorporating QC materials and control into the experiment, therefore ensuring the quality of the data.

Signature 

Date: August 20, 1986

Ronald K. Mitchum, Ph.D.

Doc. 6701L

QUESTION 3

Is the laboratory sufficiently blinded in its ability to link the serum sample with the respective adipose tissue sample?

The blinding protocol as presented and as found to be in place was appropriate for the study. In addition to the defined blinding process, there also exists internal binding within the laboratory due to record-keeping practices in place. An example would be the analytical mass spectrometry laboratory being blind to the LIMS ID number. The procedures are controlled. Protocols for blinding as well as for sample collection were in place. Unblinding either inadvertently or on purpose would be difficult, if not impossible, with the strict controls in place.

Signature 

Ronald K. Mitchum, Ph.D.

Date: August 20, 1986

Doc. 6701L

QUESTION 4

Is the proposed method of data analysis appropriate for determining whether or not serum and adipose levels are correlated?

A protocol for the correlation was provided and details the procedure for determining correlation between adipose and serum values. Sample size and power estimates were determined. Data treatment was proposed and based upon sound statistical principles. A multivariate approach is suggested to correlate lipid type LDH, HDL, LLDL with the adipose serum correlation.

The appropriateness of the correlation method should be reviewed by professional statisticians with the laboratory data variability being a critical factor. If there is not a strong correlation, the data variability taken from the laboratory duplicates and QC samples will prove useful.

Signature _____ Date: August 20, 1986

Ronald K. Mitchum, Ph.D.

Doc. 6701L

Recommendations:

The following recommendations would enhance laboratory measurements in future studies, but are not meant to imply any deficiency in the quality of current results.

a) Laboratory data intercorrelation tables should be constructed. That is, sample ID #, such as LIM #, wet lab notebook #, MS file # should be correlated with each other to allow the ready tracking of samples.

b) SOPs should be in place for the overall laboratory operation. This should include balance calibration SOPs, extraction, data analysis, data storage and retrieval. These are examples and others at that detail level should also be incorporated.

c) Data transcription error checking should be implemented.

d) The laboratory should consider a limited multilab test if contracting out becomes necessary.

Signature 

Ronald K. Mitchum, Ph.D.

Date: August 20, 1986

Doc. 6701L

MISSOURI ADIPOSE STUDY

Levels of 2,3,7,8-Tetrachlorodibenzo-p-dioxin in Adipose Tissue of Exposed and Control Persons in Missouri

-An Interim Report-

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CONFIDENTIAL

The compound 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), commonly known as dioxin, has been measured in the adipose tissue of 39 persons with a history of residential, recreational, or occupational exposure in Missouri and in 57 persons in a control group. All participants had detectable levels of TCDD in their adipose tissue, but the exposed group had significantly higher levels ($p < 0.001$). Levels of six of the exposed were greater than 5 times higher than the level of the highest control. Measuring TCDD in adipose tissue provides a much improved index of exposure, an important advance for research studies evaluating the possible health effects of this compound.

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD, commonly known as dioxin) is created as an unwanted byproduct during the synthesis of 2,4,5-trichlorophenol, a precursor of a number of commercial formulations[1], which include 2,4,5-trichlorophenoxyacetic acid and hexachlorophene. In 1971, industrial sludge waste from a hexachlorophene production facility in Verona, Missouri, was mixed with waste oil and sprayed for dust control on residential, commercial, and recreational areas of eastern Missouri near St. Louis. As of April 1986, 40 sites that were related either to the contaminated oil or to other waste products generated at the Verona chemical facility have been found to be contaminated at levels $\geq$ one part TCDD per billion parts soil[2].

Since TCDD is extremely toxic to certain animal species, epidemiologic studies were undertaken to determine whether exposure to TCDD in Missouri has resulted in adverse health effects to humans. An important component of these studies is the estimate of exposure to TCDD. Individuals are generally classified as exposed to TCDD if they were physically located at or near a contaminated site. More refined estimates of exposure require information on the length of time the individual was at a contaminated site, the concentration of TCDD at the site, the type of activity at the site, and the amount of TCDD entering humans through ingestion, inhalation, and dermal routes. These refined estimates require information that is either difficult or impossible to obtain on an individual basis.

Consequently, a potential major advance in estimating exposure would be the direct measurement of TCDD in the body. In the last few years, methods based on gas chromatography-mass spectrometry have been developed to measure

TCDD in human adipose tissue[3]. Although a few such measurements have been made on individuals with a history of exposure to TCDD, most measurements reported are from samples of adipose tissue obtained at autopsy from individuals with no known TCDD exposure.

The purposes of this study were to determine (1) whether concentrations of TCDD in adipose tissue differ in exposed and control groups and (2) whether TCDD levels are associated with specific demographic and exposure characteristics. We report here the results for 39 persons with a history of exposure to TCDD and 57 persons with no known exposure. This is an interim report, because the investigation is still in progress.

METHODS

Study Design

The study was a cross-sectional comparison of TCDD levels in an exposed group and a control group from Missouri. From January 1983 to November 1985, the Missouri Department of Health compiled a central listing of individuals who believed they had been exposed to TCDD. The listing consisted of persons who volunteered to complete a questionnaire concerning their medical history, health habits, work history, and potential exposure to TCDD. Based on questionnaire responses, the eligible exposed group for this study consisted of approximately 400 persons who were (1) exposed to areas with TCDD levels in soil between 20 and 100 parts TCDD per billion parts soil (ppb) for two or more years or (2) exposed to TCDD levels greater than 100 ppb for at least six months. These soil levels were determined by the Environmental Protection Agency (EPA) and reported to the State of Missouri in 1983[2].Persons who met these criteria were classified as having one of three types of exposure: (1) residential, i.e., either living in close proximity to areas with TCDD

contaminated soil or having evidence of contamination inside the home; (2) recreational, i.e., riding or caring for horses in TCDD-contaminated stable arenas at least one time per week ; or (3) occupational, i.e., working either in a hexachlorophene production facility or at truck terminals where the grounds had been sprayed with TCDD contaminated waste oil.

Each exposed participant donated approximately 20 grams of subcutaneous adipose from the anterior abdominal wall. A plastic surgeon administered local anesthesia, removed the tissue, and provided follow-up surgical care. Every eligible person who volunteered was included in the study, except one individual who was taking corticosteroids and was advised by his physician not to participate.

The control group was selected from persons undergoing elective abdominal surgery in one of three hospitals located in St. Louis, Kansas City, and Springfield. Each participant in the control group donated approximately 20 grams of subcutaneous adipose tissue from the anterior abdominal wall during an elective surgical procedure. When the study is completed, it is expected that the control group will be composed of persons from a broad age range, from both sexes, from white and other than white races, and from both urban and rural residence locations.

Based on responses from a Questionnaire, individuals were excluded from the control group who had (1) more than one month of potential direct soil contact at a confirmed TCDD-contaminated site in Missouri[2], (2) a history of military duty in Southeast Asia at any time from 1962 through 1970, or (3) occupational or commercial contact with trichloro-phenol or its derivatives. Also excluded were persons who (1) had abnormal fat metabolism or were in a catabolic state (e.g., had diabetes, lipodystrophies, excessive weight loss, or cancer); (2) were at increased risk of complications from the biopsy (e.g., had a bleeding disorder or immunosuppression); (3) were known to have AIDS,

hepatitis, or active tuberculosis, thus imposing unacceptable risks to laboratory personnel processing the specimens; and (4) were part of a high-risk special study population (e.g., children, pregnant females, and prisoners).

All adipose tissue samples were collected from July 1985 through November 1985. All participants were white. Informed consent was obtained from all participants after the nature of the procedure had been fully explained.

The data were analyzed by using standard programs from the Statistical Analysis System (SAS) Version 5 program package. The TCDD levels were approximately log-normally distributed. Regression analyses were performed with the natural log of TCDD as the dependent variable and age, sex, and exposure status as independent variables. Nonlinear and interaction terms were also tested in the multiple regression models but these terms were not statistically significant ($p > 0.05$).

Laboratory Analysis

The adipose specimens were collected, frozen under dry ice, and shipped to the Centers for Disease Control. Levels of TCDD in 10 grams of adipose tissue were measured by high-resolution gas chromatography, high-resolution mass spectrometry and are reported on a whole-weight basis[4,5]. The detection level of TCDD in 10 grams of adipose tissue was one part TCDD per trillion parts tissue (ppt). The analyst was blind to whether the sample was from an exposed or a control individual. The analyst used two quality control pools that had been previously characterized by this method and had been found to contain 1.6 and 8.4 ppt of TCDD. A sample of the 1.6-ppt quality control pool and four adipose samples from Missouri made up an analytical run. Every fourth run, however, consisted of a sample of the 8.4-ppt quality control pool, a duplicate fat sample selected from a preceding run, two more adipose samples from Missouri, and a reagent blank. An analytical run was considered

out of control and was rerun if any one of the following conditions occurred: (1) the analytical standard was outside its 95% control limits, (2) the quality control pool sample was outside its 99% control limits, or 3) two consecutive results from the quality control pool were outside the 95% control limits.

RESULTS AND DISCUSSION

All persons in both the exposed and control groups had detectable levels of TCDD in their adipose tissue, as shown in the Figure and described in Table 1. Nineteen of the 39 (49%) exposed persons had measurements higher than the highest TCDD level (20.2 ppt) of the 57 controls. Six (15%) of the 39 exposed persons had TCDD levels greater than 100 ppt, which was five times higher than the level of the highest control. On the other hand, there were persons from all three exposure subgroups (residential, recreational, occupational) with TCDD levels within the range of the control group. Levels in the occupational group were, in general, higher than those in the residential group.

The control group was analyzed to determine how age and sex influenced the TCDD levels. Regression analysis showed age to be a significant predictor of TCDD level ($p < 0.001$), with TCDD increasing approximately 1.3 ppt per decade. After adjustment for age differences, females had slightly higher TCDD levels that were of borderline statistical significance ($p = 0.047$). In Table 2, TCDD levels in the control group of this study are compared with levels for persons with no known exposure reported from four other studies. The mean values and ranges of values among these studies are quite similar.

The exposed and control groups differed in mean age and percent of males (Table 1). After controlling for these differences by using multiple regression, exposure status was a significant predictor of TCDD levels

($p < 0.001$). Even after persons with TCDD levels greater than 100 ppt were excluded, TCDD levels in the exposed group were significantly higher than those in the controls ($p < 0.001$). The geometric mean for the exposed group was more than twice that of the controls.

As shown in the Figure, five of the six values greater than 100 ppt were from persons exposed to TCDD during the production of hexachlorophene. The other high value (577 ppt) was found in a man exposed to TCDD while horseback riding in a contaminated arena. Prior to this report, the two highest levels of TCDD in adipose tissue were (1) 1,840 ppt, in a specimen obtained at autopsy from a 55-year-old woman who had been exposed to TCDD in Seveso, Italy,[6] and (2) 130 ppt, in a specimen obtained at autopsy in Canada.[7]

Information on the length of time since last exposure is important for determining the half-life of TCDD in humans. The half-life of TCDD in primates has been estimated to be about one year.[8] Recently, an investigator in Switzerland voluntarily ingested radiolabeled TCDD, and from subsequent measurements on urine and feces, he estimated the half-life to be 4.95 years.[9] Rappe measured TCDD in the adipose tissue of a man 31 years after he was exposed in an industrial accident and found a TCDD level of 100 ppt[10]. Assuming first-order kinetics and a one year half-life, the original level of TCDD in this man would have been 200,000,000,000 ppt, or 20% of his fat mass--clearly impossible. However, if the half-life were 8 years, the original level of TCDD in Rappe's subject would have been 1,600 ppt--a more plausible number based on our findings. In the present study, the individuals with TCDD levels of 122 ppt, 166 ppt, and 745 ppt gave a history of last exposure to TCDD from 12 to 14 years before their biopsy. Again, a one-year half-life leads to unacceptably high estimates of original TCDD levels in these men, supporting the contention that the half-life of TCDD in human

adipose tissue is longer than one year. These data are consistent with 5 to 8 years.

The exposed participants were probably not a representative sample of the total exposed population. Because of the selection biases of volunteering, first for the central listing and second for excisional adipose tissue biopsy, true population means and ranges of persons exposed to TCDD in Missouri cannot be inferred from these data. We cannot predict the direction and magnitude of these biases. Likewise, the control group is drawn from hospitalized, surgical patients and is a nonrandom sample. As a result, the values for the control group must be viewed as a reference range, not as the normal range for the general population of Missouri.

We conclude that the measurement of TCDD in adipose tissue is a much improved exposure index for studies evaluating the possible health effects of TCDD. Unfortunately, the specimen must be obtained by a surgical procedure, albeit minor, thus precluding its widespread use. This problem could be overcome either by the development of a method for measuring TCDD levels in a more easily obtained specimen, such as blood, or by mathematical modeling of predictors of adipose TCDD levels that would permit construction of an accurate, noninvasive exposure index. Completion of this study will provide additional information on the epidemiologic correlates of exposure and on the range of TCDD values in a broad sample of the Missouri population.

Table 1. Comparison of the TCDD Levels (ppt) in the adipose of the exposed and control groups

	CONTROLS	EXPOSED		
		Total	Recreational	Residential
N	57	39	8	16
Arithmetic mean	7.4	79.7	90.8	21.1
Median	6.4	17.0	23.5	14.5
Range	1.4-20.2	2.8 - 750	5.0 - 577	2.8 - 59.1
Geometric mean	6.4	21.8	24.8	15.3
Mean age (SD)	52.6 (15.7)	44.3 (13.7)	42.1 (14.7)	39.7 (14.9)
Percent male	35.1	61.5	37.5	43.8

Table 2. Comparison of the adipose tissue concentration of TCDD in populations that have had no known TCDD exposure

Source of Specimens	N	Mean (ppt)	Range (ppt)
Present study: Elective surgical patients in Missouri	57	6.4[a]	1.4-20.2
Autopsy specimens from Ohio[11]	6	-	5-12
Autopsy specimens from St. Louis[12] and Canada[7], and adipose from veterans[15]	61[b]	7.5	1-15
Autopsy specimens from sudden deaths in St. Louis[13]	35	7.2[a]	-
Autopsy specimens from Georgia and Utah[14]	35	7.1[a]	2.7 - 19

[a] - Geometric mean
[b] - Combination of results from references 7,12, and 15. Three results from persons known to have had exposure to 2,4,5-trichlorophenol were excluded.

Legend for figure

Figure. Levels of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), commonly known as dioxin, in exposed and control persons in Missouri.

Acknowledgments

Chester R. Lapeza and William T. Belser performed the adipose sample cleanup. Louis R. Alexander, Ph.D.; Ralph C. O'Connor, Ph.D.; and Vincent Maggio performed the high-resolution mass spectral analysis. S. Jay Smith, M.S., was the quality control officer. The following individuals provided technical assistance and consultation for the study: John Liddle, Ph.D.; James Holler, Ph.D; Brenda Lewis; Francis Paletta, Sr., M.D.; Francis Paletta, Jr., M.D.; Paul Koontz, M.D.; Wayne Schramm, M.S.; Cindy Kempker; Tina Luebhering; Donald Kaminski, M.D.; Miriam JoAnn Wollard, R.N.; Rebecca Snow; Beverly Heinzelman, R.N.; Jeff Staake, M.P.A.; David Forney; Florence Neider, R.N.; and Mary Frey, R.N.

This study was partially funded by the Agency for Toxic Substances and Disease Registry, Public Health Service, U.S. Department of Health and Human Services, Atlanta, Georgia 30333

The project described and the data reported in this publication were developed under a Cooperative Agreement between the Missouri Department of Health and the Centers for Disease Control, U.S. Department of Health and Human Services. Additional information on the project may be obtained by writing:

Missouri Dioxin Study
Missouri Department of Health
P.O. Box 570
Jefferson City, Missouri 65102.

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DRAFT
CONTROLS
EXPOSED
RESIDENTIAL
OCCUPATIONAL
RECREATIONAL
NUMBER OF PERSONS
DIOXIN ADIPOSE LEVEL (parts per trillion)
0
2
4
6
8
10
12
14
16
18
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
100
200
300
400
500
600
700
800

REVIEW OF FINAL REPORT
BY CDC

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Centers for Disease Control

Memorandum

Date September 2, 1986

From Assistant Director for Science

Subject Report of the Ad Hoc Committee for Review of the Agent Orange Study Protocol

To Director, CDC

In response to your August 21, 1986, request, an Ad Hoc Committee for Review of the Agent Orange Study Protocol was convened to review a document from the Center for Environmental Health (CEH), entitled "Correlation Between Human Serum and Adipose Tissue Concentration of 2,3,7,8-Tetrachlorodibenzo-p-dioxin." On September 2, 1986, the Committee, composed of Drs. David Bayse, Carol Hogue, Peter Layde, James Melius, Gladys Reynolds, and Gary Noble (chair), received and reviewed the report and interviewed senior CEH staff working on the study.

We understand the responsibility of the Ad Hoc Committee was to review the following questions:

1. Are the analytical procedures, as presented in this study, appropriate for the measurement of 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) concentration in serum and adipose tissue?

2. Is there is a sufficiently high correlation between the concentration of 2,3,7,8-TCDD in human serum and adipose tissue to allow the use of serum measurement of 2,3,7,8-TCDD as an alternative for adipose assays?

The Ad Hoc Committee responses follow:

1. Are the analytical procedures, as presented in this study, appropriate for the measurement of 2,3,7,8-TCDD concentration in serum and adipose tissue?

   Based on the review done by a committee of outside experts on August 19-20, 1986, the analytical procedures as presented appear adequate for the measurement of 2,3,7,8-TCDD in human serum and adipose. Moreover, the scientific expertise exhibited by this documentation would identify the CEH laboratory as one of the foremost laboratories in the area of trace organic compound analysis. The consideration of various lipid components in the analysis adds further scientific validity and rigor.

2. Is there a sufficiently high correlation between the concentration of 2,3,7,8-TCDD in human serum and adipose tissue to allow the use of serum measurement of 2,3,7,8-TCDD as an alternative for adipose assays?

The Committee found that the high correlation between adipose tissue and serum measurements reported in this study should allow the use of serum measurements as an alternative to adipose tissue measurement of 2,3,7,8-TCDD for estimating dioxin exposure. The measurement of 2,3,7,8-TCDD in both adipose tissue and serum on a whole-weight and lipid-weight basis provides valuable information. We suggest that consideration be given to continuing similar analyses in the next phase of this study.

We anticipate that the next phase of this study will address whether the precision of this analytical procedure is sufficient to determine with adequate certainty the correlation between serum dioxin concentrations and military Agent Orange exposure records, in a cohort of Vietnam veterans.

We would like to commend the staff of CEH for a very well-performed study and well-written report. Despite the constraints of time, the quality of the product is outstanding.

Gary R. Noble, M.D.

APPENDIX A

Correlation Between Human Serum and Adipose Tissue Concentration of 2,3,7,8-Tetrachlorobibenzo-p-dioxin

(with reports of independent reviews of laboratory procedures)

Correlation Between Human Serum and Adipose Tissue Concentrations of 2,3,7,8-Tetrachlorodibenzo-p-dioxin

Center for Environmental Health
Centers for Disease Control
Atlanta, Georgia 30333

September 2, 1986

Correlation Between Human Serum and Adipose Tissue Concentrations of 2,3,7,8-Tetrachlorodibenzo-p-dioxin Levels

INTRODUCTION

Non-volatile halogenated compounds tend to accumulate in the lipid stores of the body. Consequently, adipose tissue has been the matrix of choice for measuring these compounds at trace levels in the human body. We have used adipose tissue for determining the concentration of 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) in exposed and control populations in Missouri (1). In other studies (2,3,4), we have determined polychlorinated biphenyls (PCBs), DDT and its metabolites, and polybrominated biphenyls (PBBs) in serum. The primary advantage of serum in such studies is that it is collected by a much less invasive procedure. However, an important question about the interpretation of serum measurements is whether they reflect the adipose concentrations of these compounds. Some studies (5,6), in which organochlorine pesticides were measured on a whole weight basis in matched adipose and serum samples, have yielded inconclusive results as to how well adipose and serum measurements correlated. Other studies have found high correlations between adipose and serum (or plasma) levels for PCBs (7) and PBBs (8) on a whole weight basis. Eyster et. al, (8) have suggested that to accurately assess the partitioning between adipose and serum of chronically retained lipophilic xenobiotics, each measurement should be performed on a lipid weight basis rather than whole weight basis. Brown and Lawton (9), in their study of capacitor workers occupationally exposed to PCBs, also recommended calculating adipose to serum partitioning coefficients on a lipid weight basis for the various Aroclors they measured.

The objective of the current study was to determine how well adipose and serum levels of 2,3,7,8-TCDD correlated on either a whole weight or lipid weight basis.

MATERIALS AND METHODS

Study Population and Sample Acquisition

The population in this study consisted of a total of 50 persons in Missouri; persons with and without a history of exposure to 2,3,7,8-TCDD. The methods used for acquiring adipose are described elsewhere (1). Serum samples were not collected under fasting conditions. Serum samples of approximately 15 milliliters (ml) were collected during the same hospital stay that adipose tissue was collected. For some participants, serum samples of approximately 200 ml were collected in Missouri by the Red Cross at a time interval of 2 to 14 months after the collection of the individual's adipose tissue. These samples were sent to CDC in the original Travenol serum bags. All samples were coded so that the analytical laboratory could not link the adipose tissue with its corresponding serum sample.

Analytical methods

The analytical methods are discussed briefly here and presented in detail in the Analytical Methods Appendix. Adipose tissue and serum were analyzed for 2,3,7,8-TCDD by our methods (10,11) based on high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS) and a semi-automated sample preparation procedure (12). The adipose tissues were analyzed for 2,3,7,8-TCDD in analytical runs consisting of four unknown samples and a quality control pool sample. Every fourth analytical run consisted of two unknown samples, a method blank, a duplicate selected at random from an earlier run, and a different quality control pool sample.

The serum samples were analyzed for 2,3,7,8-TCDD in analytical runs consisting of a method blank, three unknown samples and a quality control pool sample. The composition and characterization of these quality control pools are described in more detail elsewhere (10,11). We determined percent lipid in adipose tissue by our gravimetric procedure (method B) (13). On some samples duplicate lipid determinations were made, in which case the mean is reported. The lipid determinations and 2,3,7,8-TCDD measurements were performed on different subsamples of the adipose tissue and at different times. The adipose samples were stored during the interim at -60 degrees Centigrade. Due to inadequate sample volume, seven of the adipose samples were not analyzed for percent lipid.

Total cholesterol, triglycerides, and high density lipoprotein cholesterol (HDL) were determined in duplicate on the serum samples by standard methods on the DuPont Automatic Chemical Analyzer. Total phospholipids were determined in duplicate by a modification (14) of the Folch procedure. Free cholesterol was determined in duplicate using an enzymatic method (15). For each analyte, the results of the duplicate analyses were averaged, and the mean reported. One serum sample was not available for serum lipid analysis. These results were used to calculate the concentrations of total lipids (16), low-density lipoprotein cholesterol (LDL) and very low-density lipoprotein cholesterol (VLDL) (17). All analytical runs for the 2,3,7,8-TCDD and lipid measurements were in control (10,11).

Statistical Analysis

Statistical analysis of the data was performed using the Statistical Analysis System (SAS) Version 5 (Sas Institute, Cary, NC 27511). Correlation coefficients are Pearson product-moment correlations.

RESULTS AND DISCUSSION

A description of the distributions of 2,3,7,8-TCDD levels in the adipose and serum of the 50 participants, reported on a whole weight basis, is given in Table 1. The adipose and serum TCDD levels ranged over approximately 2.5 orders of magnitude. Both distributions were markedly skewed to the right, so geometric means and standard deviations were also calculated. The results of the various serum lipid components and the adipose percent lipid measurements are summarized in Table 2. Although some individual values are outside of reference ranges, the results are overall what would be generally expected for lipid measurements.

The correlation analysis was performed for adipose 2,3,7,8-TCDD on both a whole weight and lipid weight basis with serum 2,3,7,8-TCDD on a whole weight basis and a lipid (or lipid fraction) weight basis. A lipid (or lipid fraction) weight basis means that the 2,3,7,8-TCDD level is expressed as grams of 2,3,7,8-TCDD per gram of total lipids (or lipid fraction). Since the distributions of adipose and serum 2,3,7,8-TCDD levels covered about 2.5 orders of magnitude (i.e., adipose 2 - 745 ppt, serum .013 - 8.33 ppt) and were markedly right skewed, log-transformed 2,3,7,8-TCDD levels were used in the correlation analysis. Results of the correlation analysis are given in Table 3. Figure 1 is a plot of adipose and serum 2,3,7,8-TCDD, both on a whole weight basis. Figure 2 is a plot of adipose and serum 2,3,7,8-TCDD levels, both on a lipid weight basis. The best correlation ($r = 0.976$) was found for adipose 2,3,7,8-TCDD on a lipid weight basis with serum 2,3,7,8-TCDD on a total lipid weight basis. This correlation was only marginally better than adipose 2,3,7,8-TCDD on a whole weight basis with serum 2,3,7,8-TCDD on a whole weight basis ($r = 0.967$). Adjusting for various serum lipid fractions offered no improvement over adjusting for serum total lipids.

The partitioning ratio of adipose 2,3,7,8-TCDD to serum 2,3,7,8-TCDD was also calculated on a whole weight and lipid weight basis. On a whole weight basis for both adipose and serum 2,3,7,8-TCDD levels, the mean of the partitioning ratios was 158 to 1 (std. dev. = 75.1, std. error = 10.6). On a lipid weight basis for both adipose and serum 2,3,7,8-TCDD levels, the mean of the partitioning ratios was 1.09 (std. dev. = 0.385, std. error = 0.060). The 95% confidence interval about the mean was 0.97 to 1.21, which includes 1.0. Thus on the basis of these data, a one to one partitioning ratio of 2,3,7,8-TCDD between adipose lipid and serum lipid cannot be excluded. This finding suggests that 2,3,7,8-TCDD is stored to approximately the same extent in lipid whether the lipid is in adipose or serum.

Adipose 2,3,7,8-TCDD measurements have generally been accepted as representing the body burden concentration of 2,3,7,8-TCDD. The high correlation between serum 2,3,7,8-TCDD levels and adipose 2,3,7,8-TCDD levels in this study indicates that serum 2,3,7,8-TCDD is a valid measurement of 2,3,7,8-TCDD body burden concentrations. The practical advantage of collecting serum rather than adipose samples should facilitate future epidemiologic studies which require estimates of 2,3,7,8-TCDD body burden.

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Table 1

Adipose and serum 2,3,7,8-TCDD Levels
(Whole weight basis)

	Adipose 2,3,7,8-TCDD (parts per trillion)	Serum 2,3,7,8-TCDD (parts per trillion)
Mean	54.5	0.519
Standard deviation	125.8	1.314
Geometric mean	15.7	0.110
Geometric Std. deviation	4.25	0.053
10th percentile	3.2	0.018
50th percentile (median)	9.0	0.057
90th percentile	130	1.55
Range	2-745	0.013 - 8.30
N	50	50

Table 2
Lipid Measurements

	Total Lipids (mg/dl)	Total Cholesterol (mg/dl)	Total Triglyceride (mg/dl)	Total Phospholipids (mg/dl)	Free Cholesterol (mg/dl)	HDL Cholesterol (mg/dl)	LDL Cholesterol (mg/dl)	VLDL Cholesterol (mg/dl)	% Lipid in adipose (%)
Mean	608	189	124	206	59.0	46.0	118	24.8	79.4
Std. deviation	140	43.0	68.1	39.0	13.5	16.4	36.9	13.6	9.90
10th percentile	402	135	42	161	41	31	67.4	8.4	63.8
50th percentile (median)	612	196	116	209	61	42	113	23.2	82.5
90th percentile	803	246	215	254	77	64	170	43	89.7
Range	235-840	81-275	22-320	93-307	24-87	18-122	57-197	4.4-64	48.1-92.9
N	49	49	49	49	49	49	49	49	43

Table 3

Correlations of Adipose and Serum 2,3,7,8-TCDD*+

Serum 2,3,7,8-TCDD divided by the sample's	Correlation with Adipose 2,3,7,8-TCDD divided by sample's whole weight	Correlation with Adipose 2,3,7,8-TCDD divided by sample's total lipid weight
Whole weight	.967 (50)	.969 (43)
Total lipid weight	.969 (49)	.976 (42)
Total cholesterol weight	.966 (49)	.972 (42)
Total triglyceride weight	.939 (49)	.951 (42)
HDL weight	.951 (49)	.956 (42)
VLDL weight	.939 (49)	.951 (42)
LDL weight	.958 (49)	.964 (42)
LDL + VLDL weight	.966 (49)	.973 (42)

*The logarithm of each variable was used in the calculation of the correlation coefficient (see text).
+ P <0.0001 for all correlation coefficients. N is in parentheses.

ADIPOSE AND SERUM 2,3,7,8-TCDD LEVELS

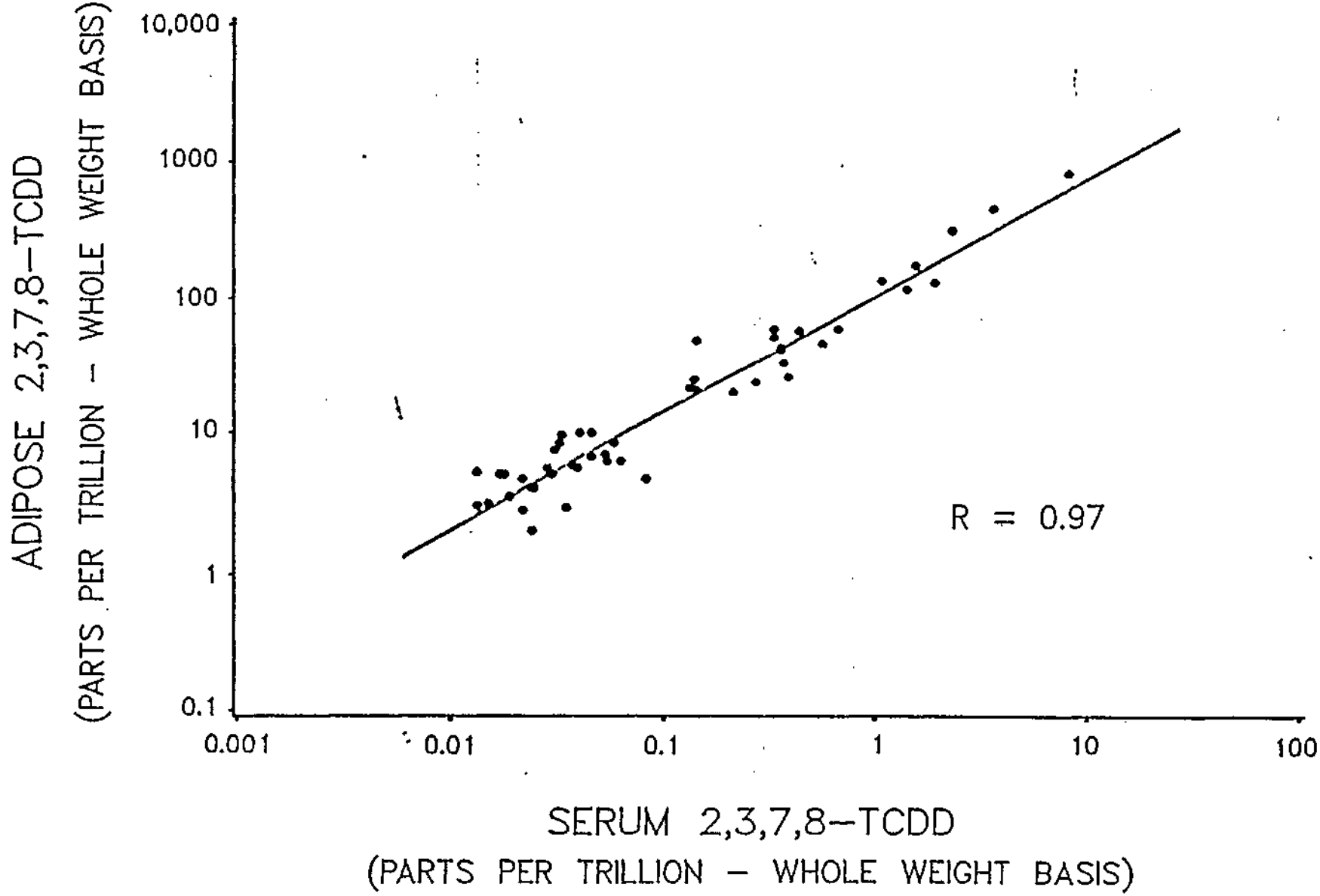

FIGURE 1

ADIPOSE AND SERUM 2,3,7,8-TCDD LEVELS

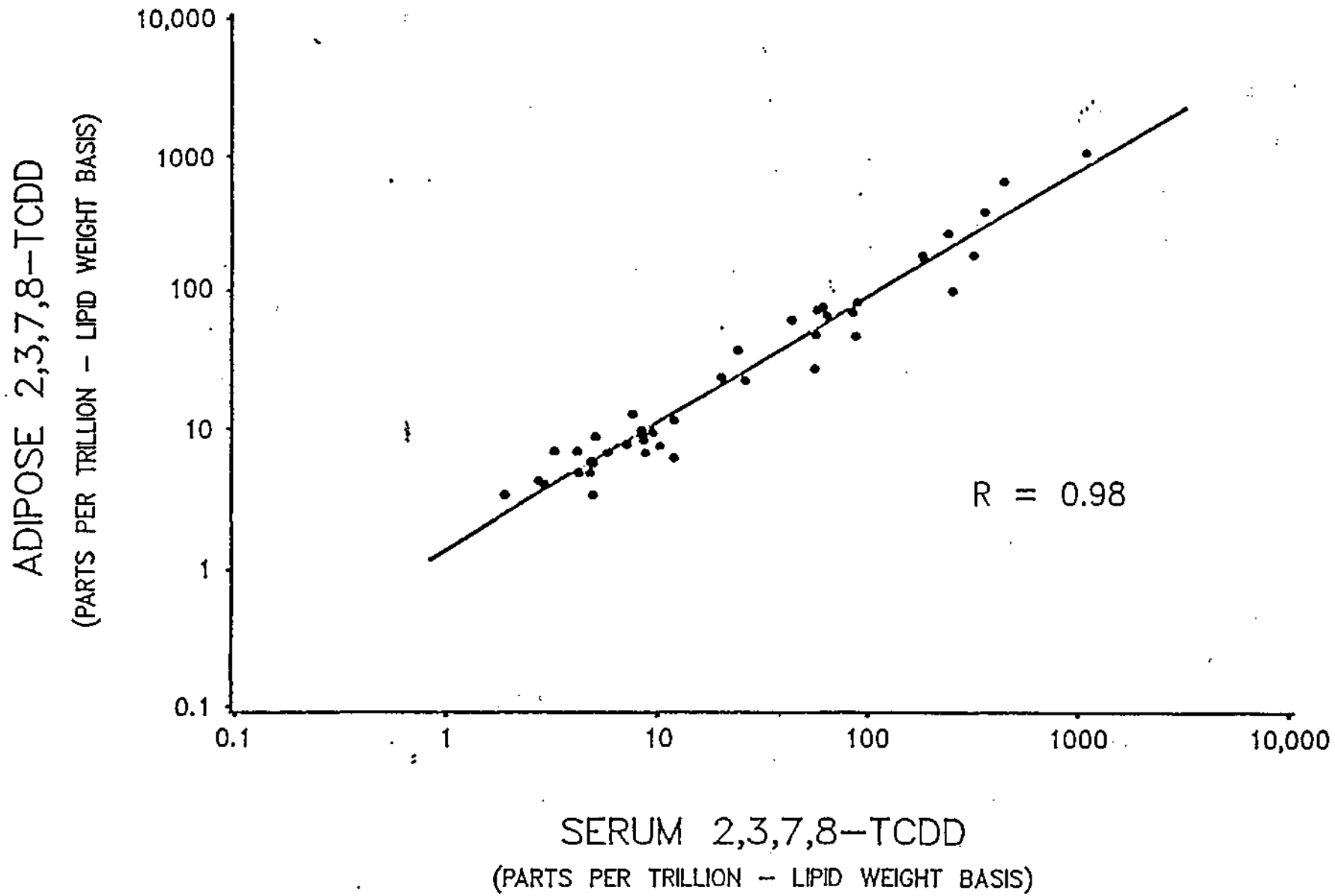

FIGURE 2

REVIEW OF THE MEASUREMENT PROCESS FOR 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN IN HUMAN ADIPOSE AND SERUM

Center for Environmental Health
Centers for Disease Control
Atlanta, Georgia 30333

August 19 and 20, 1986

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Centers for Disease Control
Atlanta GA 30333

August 19, 1986

INTRODUCTION

Thank you for accepting our invitation to serve as an expert in the review of our measurement process for 2,3,7,8-TCDD in human adipose and serum during your 2-day stay in the Center for Environmental Health (CEH) of the Centers for Disease Control.

Currently, the CEH laboratory is determining 2,3,7,8-TCDD in serum samples from Missouri residents whose adipose tissue samples we previously analyzed. From these data, we hope to define a partitioning ratio between adipose tissue and serum for 2,3,7,8-TCDD. If such a correlation exists, then future studies can be performed using serum, the advantages of which are numerous.

Your mission is to evaluate the CEH laboratory's methods, equipment, and personnel for the determination of 2,3,7,8-TCDD in human adipose and serum and the CEH laboratory having been "blinded" as to the matching of the adipose with its corresponding serum sample.

Enclosed is an organizational chart of those within the Center for Environmental Health who have worked on this project. During your two days here, you will be working most closely with members of the Toxicology Branch of the Division of Environmental Health Laboratory Sciences (EHLS). However, the other groups shown on this chart have also been involved in this project and you have complete access and freedom to contact any of these individuals. We are looking for an objective evaluation of the laboratory's methods, equipment and personnel and the fact that the laboratory is blinded in conducting these analyses. Also enclosed are copies of pre-prints and reprints that we believe will aid you in your evaluation.

I ask that before you leave on Wednesday, you will have submitted your final signed remarks. Again, I thank you and welcome you to the Center for Environmental Health, Centers for Disease Control.

Sincerely yours,

Vernon N. Houk, M.D.
Assistant Surgeon General
Director
Center for Environmental Health

REVIEW OF THE MEASUREMENT PROCESS FOR 2,3,7,8-TCDD
IN HUMAN ADIPOSE AND SERUM
August 19-20, 1986

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August 20, 1986

TO: Vernon N. Houk, M.D.
Assistant Surgeon General
Director, Center for Environmental Health
Centers for Disease Control

FROM: David L. Stalling
Research Scientist
Columbia National Contaminant Research Center
Convener of Experts to Review Measurement of 2,3,7,8-TCDD at CDC

SUBJECT: Review of TCDD Analyses

I am pleased to provide you with this summary stating a positive review by the experts invited to CDC, August 19-20, 1986, for the purpose of assessing the data integrity of samples of serum and adipose analyzed for 2,3,7,8-TCDD. An observer from the National Academy of Science also attended the meetings.

The experts have responded to the four questions that you set forth in the materials provided. The questions were:

> Are the analytical methods, equipment, and personnel of the CEH laboratory appropriate for determining 2,3,7,8-TCDD in human serum and adipose tissue?
>
> Are appropriate Quality Assurance/Quality Control procedures being used in determining 2,3,7,8-TCDD in human serum and adipose tissue?
>
> Is the laboratory sufficiently blinded in its ability to link the serum sample with the respective adipose tissue sample?
>
> Is the proposed method of data analysis appropriate for determining whether or not serum and adipose levels are correlated?

For the record, I asked the experts if additional questions should be developed or were required to fully evaluate the data analysis and quality. The experts did not propose or adopt additional questions. Each of the four questions was then reviewed and discussed to ensure that we agreed upon their meaning and scope.

The CDC technical and management staff are to be commended for their technical and program presentations. They provided clear, well-defined, accurate and scientifically sound information to the panel. The technical review panel toured the laboratory operations and after the CDC presentations, we discussed details of the analyses in subsequent meetings with those individuals responsible for the sample processing, analysis, data reporting, quality control, and other aspects of this project.

Each expert has provided you with his evaluation of each question and separately stated recommendations for your staff to consider. These recommendations are primarily aimed at increasing the information content of the analyses or enhancing the quality assurance of the data and in no way do these recommendations reflect adversely on existing data.

Some attention should be directed toward specifying Standard Operating Procedures (SOPs). The analytical SOPs presently are not referenced in the study protocol and a minimum effort should remedy this aspect of Quality Assurance. Some additional development of SOPs should also occur for some laboratory operation.

David L. Stalling, Ph.D.

Concurrence:

Jackson Lay, Ph.D.

John S. Stanley, Ph.D.

Fred L. DeRoos, Ph.D.

Ronald K. Mitchum, Ph.D.

Patrick W. O'Keefe, Ph.D.

Question 1
Are the analytical methods, equipment, and personnel of the CEH laboratory appropriate for determining 2,3,7,8-TCDD in human serum and adipose tissue?

A. Methods

The CEH laboratory of CDC is staffed by trained scientists who are competent to carry out ultratrace analyses for determining levels of 2,3,7,8-TCDD in human serum and adipose tissue. They have developed well documented and validated analytical methods that achieve detection limits that are appropriate for these matrices. Their mass spectrometry equipment is state-of-the-art and is maintained and operated by well trained staff.

The sample workup and mass spectrometry facility staff are familiar with analytical methods that have been developed by other researchers. They have visited several of these peer laboratories and have developed CEH methods in a timely and efficient manner by incorporating validated techniques. They have designed modifications to many of these techniques in order to improve sensitivity, accuracy, and reliability.

Their detection and quantification technique is based on capillary column gas chromatography/high resolution mass spectrometry using multiple ion detection. This is the most sensitive and selective analytical technique available for ultratrace analyses of this type. The CEH methods use 2,3,7,8-TCDD-$^{13}C_{12}$ as an internal standard to correct for analyte losses during sample cleanup and for quantification of the native 2,3,7,8-TCDD levels. This is the most accurate method of quantification since it corrects each sample for variations in the cleanup efficiency as well as sensitivity changes of the mass spectrometer.

Signature Fred L DeRoos Date 8-20-86

Fred. L. DeRoos, Ph.D.

6707L

Question 2
Are appropriate Quality Assurance/Quality Control procedures being used in determining 2,3,7,8-TCDD in human serum and adipose tissue?

The QA/QC procedures are adequate to assure that high quality data are being produced. Approximately 40% of the sample analyses are for QA/QC purposes. QA/QC procedures are followed in both the sample work up and mass spectrometry facilities.

The QA/QC samples consist of sample method blanks, pooled adipose/serum samples and duplicate samples. The sample method blanks are processed using the same procedures as used for the actual samples and will indicate if contamination of the samples has occurred. The pooled samples serve as reference samples that can be used to evaluate the long term precision of the analytical methods. The duplicate samples provide an additional check on the precision of the analytical methods.

Additional QA/QC procedures carried out in the mass spectrometry facility include checks of the mass resolution and the absolute sensitivity of the mass spectrometer. In addition, the raw mass spectral data are evaluated manually in the mass spectrometry facility as the data are acquired in order to provide rapid indication of any problems. This evaluation includes the chlorine isotope ratios, TCDD isomer resolution, accuracy of standard quantification, absence of contamination in method blanks, and reproducibility of pooled samples analyses.

Signature Fred L DeRoos Date 8-20-86
Fred. L. DeRoos, Ph.D.

6707L

Question 3
Is the laboratory sufficiently blinded in its ability to link the serum sample with the respective adipose tissue sample?

The sample identification numbers are assigned such that it would be very difficult to link serum samples to their respective adipose tissue samples. The adipose tissue samples can be related to the subject, however, the 200g serum samples delivered to CEH have only a Red Cross identification number. Data necessary to link the 10 g serum samples and their respective adipose tissue samples are available within CEH, however, these data cannot be easily retrieved and compared. The sample numbering system employed by CEH also provides additional blinding of the sample identification. The sample markup facility assigns a new identification number to each sample during workup. This number is then used as the identification number by the mass spectrometry facility.

Signature Fred L DeRoos Date 8-20-86
Fred. L. DeRoos, Ph.D.

6707L

QUESTION 4

Is the proposed method of data analysis appropriate for determining whether or not serum and adipose levels are correlated?

The reviewer is not qualified to fully respond to this question. It is reasonable, however, to expect 2,3,7,8-TCDD levels in serum to be a function of the proposed variables. Other correlation studies involving PCBs have shown one or more of these relationships. If a correlation between the 2,3,7,8-TCDD levels in human serum and adipose tissue exists, it should be revealed by the proposed data reduction method.

Signature Fred L DeRoos Date 8-20-86

Fred. L. DeRoos, Ph.D.

6707L

Recommendations:

Question 1

The following recommendations would enhance laboratory measurements in future studies, but are not meant to imply any deficiency in the quality of current results.

Additional confidence could be added to the analyses if a third native TCDD ion mass, such as m/z 324, was observed during the analyses and profile data were acquired for selected samples. The additional ion mass peak would further define the responses as being from 2,3,7,8-TCDD, while the profile data would reveal interferences that are only partially mass resolved.

It would be beneficial to include the limit of detection for analyses in which 2,3,7,8-TCDD is not detected.

It would be advisable to use a second standard, such as 1,2,3,4-TCDD-$^{13}C_{12}$, to calculate the recovery of the internal standard. Although the absolute recovery of the internal standard should not effect the accuracy of the data, it is useful to know whether high detection limits are the result of poor internal standard recovery or chemical interferences.

A surrogate standard could also be spiked into the sample before extraction. Its concentration should be at, or slightly above, the desired limit of detection. It would serve to demonstrate that the limit of detection was actually achieved for each analysis.

It would also add to the validity of the study if selected samples and/or sample extracts were analyzed by another laboratory.

Signature Fred L DeRoos Date 8-20-86

Fred. L. DeRoos, Ph.D.

6707L

Question 2

The following recommendations would enhance laboratory measurements in future studies, but are not meant to imply any deficiency in the quality of current results.

It is recommended that CEH prepare Standard Operating Procedures (SOP) for all analytical procedures, including sample workup, analyses, and data reduction. It is also recommended that a full sample identification number be used on all bottles/vials that contain samples or sample extracts. Sufficient cross reference data should be available to allow samples to be rapidly tracked through the entire analytical procedure.

Signature Fred L DeRoos Date 8-20-86

Fred. L. DeRoos, Ph.D.

6707L

QUESTION 1

Are the analytical methods, equipment, and personnel of the CEH laboratory appropriate for determining 2,3,7,8-TCDD in human serum and adipose tissue?

A. Methods

The extraction of samples and the cleanup and gas chromatography/mass spectrometry methods are state-of-the-art and, as demonstrated by literature reports from the laboratory, are capable of producing results with the accuracy and precision required for analyses in the ppt and ppq ranges.

B. Equipment

The mass spectrometry and sample cleanup laboratories are well-equipped and the instruments appear to be maintained at a high level of efficiency. The use of automated procedures for sample cleanup is commendable and the laboratory should be encouraged to make further progress in this area if large numbers of samples are to be processed. Reliance on capillary gas chromatography/high resolution mass spectrometry provides for a high degree of confidence in the analytical data. Three instruments are available with each instrument having the capability of detecting femtogram quantities of 2,3,7,8-TCDD.

C. Personnel

The professional personnel demonstrated by their presentations that they were very knowledgeable in their respective areas of dioxin analysis. Statisticians and computer personnel are also providing important support to the programme. Laboratory technicians appear to be well trained and are qualified at the B.S. level.

Signature Patrick W. O'Keefe

Patrick W. O'Keefe, Ph.D.

August 20, 1986

6706L

QUESTION 2

Are appropriate Quality Assurance/Quality Control procedures being used in determining 2,3,7,8-TCDD in human serum and adipose tissue?

(1) Several sample identification systems are used from the point at which samples arrive in the laboratory to the point where analytical reports are generated. The LIMBS (Laboratory Information System Number) given to the samples initially is translated into a laboratory notebook number on arrival of the sample in the sample cleanup laboratory. This is acceptable as it helps to keep the GC/MS laboratory "blind" with regard to duplicates. However, the cleanup analyst uses one digit numbers to track samples as they move through the cleanup. With several analysts working on sample groups at the same time and each one using the same set of numbers, the possibility for loss of sample identity cannot be ruled out.

(2) Other aspects of the Quality Control program, such as inclusion of blanks, spikes and duplicates, provide for a high level of confidence in the data.

Signature Patrick W. O'Keefe, Ph.D. August 20, 1986

6706L

QUESTION 3

Is the laboratory sufficiently blinded in its ability to link the serum sample with the respective adipose tissue sample?

While the laboratory does not attempt to make the internal quality control samples blind, coding procedures insure that nobody outside the Red Cross field sampling unit will be aware of the relationship between serum and adipose tissue samples.

Signature Patrick W. O'Keefe

Patrick W. O'Keefe, Ph.D.

August 20, 1986

6706L

QUESTION 4

Is the proposed method of data analysis appropriate for determining whether or not serum and adipose levels are correlated?

I have insufficient background in statistics to address this question.

Signature Patrick W. O'Keefe

Patrick W. O'Keefe, Ph.D

August 20, 1986

6706L

RECOMMENDATIONS

The following recommendations would enhance laboratory measurements in future studies, but are not meant to imply any deficiency in the quality of current results.

(1) The laboratory found that pork fat did not contain detectable concentrations of 2,3,7,8-TCDD and was able to use this material for Q.C. fortification with 2,3,7,8-TCDD. Consideration should be given to using a pork serum pool for the serum analyses. This would be important if a number of serum samples were found to have less than 25 ppq 2,3,7,8-TCDD, the background concentration in the human serum pool for QC.

(2) Calibration curves are constructed using all available data. Consideration might be given to constructing new curves when new standards are prepared to see if the new data deviate in any consistent way from previous data.

Signature Patrick W. O'Keefe

Patrick W. O'Keefe, Ph.D.

August 20, 1986

6706L

Question 1

Are the analytical methods, equipment, and personnel of the CEH laboratory appropriate for determining 2,3,7,8-TCDD in human serum and adipose tissue?

The analytical methods, equipment, and standards are appropriate and sample preparation and mass spectrometry personnel are appropriate for determination of 2,3,7,8-TCDD in human adipose at the ppt level and serum at the ppq level.

A. Analytical methods

The semi-automatic sample preparation (i.e., extraction and cleanup) is recognized as an effective procedure for achieving sample extracts of the necessary cleanliness and quality for achieving the required limits of detection. This procedure was adapted from work that has been previously presented by other laboratories for low level work in environmental analyses (fish, water, etc.) and has been used by at least two other laboratories in a previous interlaboratory study (Anal. Chem. by Albro, et al.). Other laboratories have also incorporated components of this procedure into their own sample preparation practices.

B. Equipment

The semi-automated preparation procedures result in reproducible handling of serum and adipose samples. Evidence for this reproducibility is presented through repeated analyses of both spiked and unspiked control samples (adipose and serum). The high resolution mass spectrometry equipment is state-of-the-art. The levels of detection demonstrated in the data provided could not have been achieved without this level of sophistication.

Signature 

John S. Stanley, Ph.D.

Date 8/20/86

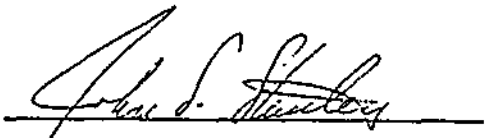
Doc. 6705L

C. Personnel

All persons involved with this program are obviously devoted to achieving the best level of performance as observed through laboratory practices, approach to the analysis, and quality of the resulting products. Specific responsibilities in the areas of sample control (blinding), sample preparation, instrumental analysis, synthesis of standards, data analysis, and overall project management have obviously contributed to this apparent rapid development of ultratrace capability at CDC.

D. Standards

The analytical standards (specifically 2,3,7,8-TCDD) synthesized by CDC have been demonstrated to be equivalent in quality to certified materials available through EPA's Reference Materials Branch and the NBS Standard Reference Materials.

Signature  Date 6/24/86

John S. Stanley, Ph.D.

Doc. 6705L

Question 2

Are appropriate Quality Assurance/Quality Control procedures being used in determining 2,3,7,8-TCDD in human serum and adipose tissue?

The Quality Control procedures implemented and practiced throughout the analytical programs reviewed are extensive and have been consistently applied.

Quality Control aspects include:

- Blind codes assigned to actual specimens

- Sample batches include:
  - 4 actual samples
  - 1 method blank
  - 1 QC (control) sample is substituted every 4th sample batch
  - This results in 4 method blanks (system blanks) and 1 control sample which are indicators of data quality for every 15 blind samples.

- Control samples - Documentation was provided to demonstrate that CDC has encountered at least one instance where a control samples did not meet the QC criteria and corrective action was implemented. This required a reanalysis of a sample set. Data for the reanalysis of this set was remarkably close to the original analysis for the unknowns.

- Instrumental control parameters or criteria are established and consistently practiced. Documentation of this activity is accomplished through both the mass spectrometry facility and a statistical analysis group.

Signature  Date 8/20/86

John S. Stanley, Ph.D.

Doc. 6705L

Data interpretation – qualitative criteria and quantitative procedures are established and consistently followed.
All data require a minimum of three levels of review.
Once quantitative data are reviewed, they are documented and incorporated in the study file.

The QC program practiced is a strength in the CDC analytical approach and provides relevant data necessary to assess the credibility of the values reported for individual samples.

In terms of overall quality assurance, specific SOPs are presented for field collection of samples and analytical methods descriptions exist in general laboratory guidelines and are detailed in the scientific literature.

All personnel are well trained and qualified in their respective disciplines. Procedures for sample handling, preparation, analysis, and data interpretation are obviously routinely followed. The data generated to date are credible and supported by QC controls.

Signature _____ Date 8/20/86

John S. Stanley, Ph.D.

Doc. 6705L

Question 3

Is the laboratory sufficiently blinded in its ability to link the serum sample with the respective adipose tissue sample?

Laboratory staff are blind to the exact nature of a specific sample through the unique sample coding operation and physical separation of the sample retrieval group and the sample analysis group. Sample information can be tracked from the Mass Spectrometry facility back to the sample preparation facility. However, decoding of the sample identity requires access to a data base which requires a password assigned to specific personnel.

Once data have been fully reviewed, a hard copy of the data is entered into the file. This hard copy requires a sign-off by two individuals. Hence, any change in the data base requires some formal documentation (tracking).

Signature  Date 6/20/96

John S. Stanley, Ph.D.

Doc. 6705L

Question 4

Is the proposed method of data analysis appropriate for determining whether or not serum and adipose levels are correlated?

This reviewer is not qualified to comment on the statistical analysis of data.

Signature John S. Stanley Date 8/20/84

John S. Stanley, Ph.D.

Doc. 6705L

RECOMMENDATIONS

The following recommendations would enhance laboratory measurements in future studies, but are not meant to imply any deficiency in the quality of current results.

Question 1

A procedure should be implemented to address recovery of the internal standard for each sample. This will provide a means for tracking differences resulting from the sample preparation step. These recoveries may provide an indication of differences in laboratory staff, reagents, spiking accurary (internal standard), etc. Also some form of interlaboratory study should be completed before proceeding with additional studies using serum as the sample media.

Question 2

In lieu of future programs in this area, it is recommended that a formal SOP is established to describe in detail the exact responsibilities and procedures for each team member. This will be crucial to a transfer of technology in each discipline in the event that a large cohort study requires involvement of additional staff.

Signature _____ Date 6/30/66

John S. Stanley, Ph.D.

Doc. 6705L

QUESTION 1

Are the analytical methods, equipment, and personnel of the CEH laboratory appropriate for determining 2,3,7,8-TCDD in human serum and adipose tissue?

A. Methods

Yes, to all three aspects, with one recommendation to better document QC (See question #2)

Isomer specialty and cleanup or isolation of TCDD—the data being generated have high integrity and the level of sensitivity achieved is "state-of-the-art".

The QC compliance criteria and method performance demonstrate that these TCDD measurements have validity and adequate work to determine the analytical variability has been done to permit an evaluation of the study objectives.

Recommendation For present samples in which no TCDD is detected, the data report should include the limit of detection (LOD) and perhaps the recovery of ^{13}C-TCDD internal standard. The data for these calculations are available from present measurements.

B. Equipment

The equipment is properly maintained and its sensitivity/performance are among the best I have seen in the U.S. and on a comparable par with that of Dr. Rappe in Sweden. This reflects well the deducation and diligence of the MS staff. The automation of the cleanup has certainly been an important contribution to achieving the goals of the study. This ensures that precision and freedom from chemical interfereences are maintained.

C. Personnel:

Having visited this facility about two years ago, I am especially impressed with the scientific growth the laboratory personnel have made. The dedication and effort to develop the analytical resources are commendable. The staff have adequate training and the experience and work quality are reflected in the data quality evaluated in this review.

Signature David L. Stalling

David L. Stalling, Ph.D.

Date: August 20, 1986

Doc. 6703L

RECOMMENDATIONS

Question 1

The following recommendations would enhance laboratory measurements in future studies, but are not meant to imply any deficiency in the quality of current results.

For future studies in which serum or tissue sample may contain a larger percentage of non-detected levels of TCDD, consideration should be given to establishing a "Method performance spike", e.g. 1,2,3,4-^{13}C-TCDD that would be added in addition to 2,3,7,8-^{13}TCDD at a level that is 2-3 times the limit of detection for the method. This would enhance statistical evaluation of data.

Signature David L. Stalling

David L. Stalling, Ph.D.

Date: August 20, 1986

Doc. 6703L

QUESTION 2

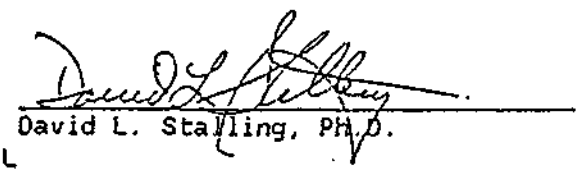
Are appropriate Quality Assurance/Quality Control procedures being used in determining 2,3,7,8-TCDD in human serum and adipose tissue?

Yes—to Quality Control. Laboratory protocols and experimental procedures document the sample processing, cleanup, and analysis steps. The data quality is of the highest caliber that I have personally evaluated.

It appears, though, that the laboratory Quality Assurance documentation needs to be formalized and included in the Study Protocol. Specifically, the sample flow, workup, analysis, and data reporting steps should point to QC or Standard Operating Procedures documents to be used as well as stating what training or instructional efforts are to be carried out by laboratory supervisors if new personnel are added.

This material should be included as an Attachment 10. to the Protocol Study document or as a memo from the appropriate management to formally charge other managers responsible for adherence to QA.

This recommendation IN NO WAY reflects adversely on any laboratory procedures or data quality! But it is made to assist you in meeting aspects of Good Laboratory Practices.

Signature  Date: August 20, 1986

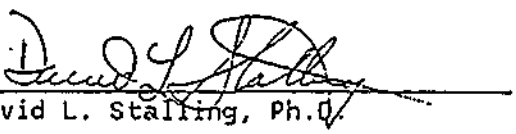
David L. Stalling, Ph.D.

Doc. 6703L

QUESTION 3

Is the laboratory sufficiently blinded in its ability to link the serum sample with the respective adipose tissue sample?

The procedures reviewed appear to maintain an adequate level of "blindness" and the analytical reports are transmitted by formal memo to provide a hard copy of the results to validate the laboratory data base in the future. Field collection and number assignment steps are well documented in the protocol for the study. It appears that the present system is perhaps overdone with respect to blindness. I would recommend that the numbering system be reviewed with regard to increasing the ruggedness of the numbering. The use of single letters in the workup may be revised. In larger studies, confusion could easily develop. There is no problem with the present system. It is well documented.

Signature  Date: August 20, 1986

David L. Stalling, Ph.D.

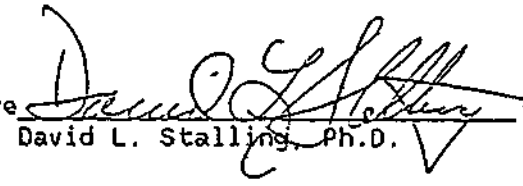
Doc. 6703L

QUESTION 4

Is the proposed method of data analysis appropriate for determining whether or not serum and adipose levels are correlated?

The proposed approach seems quite adequate. The lipid measurements in the present study should be used to evaluate and/or classify individuals (using a multivariate approach—principal components or partial least squares in latent variables (PLS-2)* classifications to determine which individuals have similar lipid biochemistry. Partitioning values may be more similar in these individuals. In addition to TCDD, DDE values should be measured, especially in future studies to enhance the evaluation of partitioning of TCDD.

*Reference: Sjostrom, M., S. Wold, and B. Soderstrom. 1986. "PLS Discriminant Plots" in Pattern Recognition in Practice II, Eds. Gelsema, E.S. and L.N. Kanal. Elsevier Science Publishers B.V. (North-Holland), pp. 461-70.

Signature  Date: August 20, 1986

David L. Stalling, Ph.D.

6703L

RECOMMENDATIONS

Question 4

The following recommendations would enhance laboratory measurements in future studies, but are not meant to imply any deficiency in the quality of current results.

Provisions for measuring a persistent contaminant associated with dietary intake (e.g., DDE) should be made to provide an alternate means of evaluating serum/fat TCDD ratios. The rationale for this recommendation is to provide a means of evaluating the partitioning of TCDD between serum/fat if there appears to be a peer correlation of TCDD partitioning. Considerable information is available regarding DDE levels and its half-life, etc. By ratioing TCDD/DDE levels in serum and in fat, it should be possible to better evaluate variations in partitioning by referencing DDE serum/fat ratios. Material for performing these analyses in remaining plasma samples should be available if the cleanup effluent from the initial carbon columns were collected. Also, the cost for this measurement is low and the amount of sample required for fat is quite small as well. If different type of lipid biochemistry is represented in individuals, grouping these cases should be done prior to regression modeling.

Signature David L. Stalling

David L. Stalling, Ph.D

Date: August 20, 1986

6703L

QUESTION 1

Are the analytical methods, equipment, and personnel of the CEH laboratory appropriate for determining 2,3,7,8-TCDD in human serum and adipose tissue?

A. Methods

The methods are clearly appropriate for analysis of adipose tissue. ALthough only a few "pooled" samples of serum have been assayed, the modifications made to the cleanup procedure used for adipose as well as the larger sample sizes (200 g) should allow accurate measurement of 2,3,7,8-TCDD in serum samples.

B. Equipment

The GC/MS methodology is clearly the analytical method of choice. Further, the 100-fg sensitivity (on column) of the ZAB should be more than sufficient for the analysis of the adipose and serum samples, provided that the proposed serum samples are of the large sizes (200 g) proposed in the methodology.

C. Personnel

The reviewer is favorably impressed with the personnel involved in the study. They have demonstrated in peer-reviewed publications that they can reproducably assay adipose samples for 2,3,7,8-TCDD. Because the same staff, with the possible exception of one individual no longer employed in the laboratory, is to be utilized for serum assays, the reviewer feels that the laboratory has adequately trained staff for the analysis of the serum as well.

Signature Jack O. Lay Jr. Date: August 20, 1986
Jack Lay, Ph.D.
Doc. 6700L

QUESTION 2

Are appropriate Quality Assurance/Quality Control procedures being used in determining 2,3,7,8-TCDD in human serum and adipose tissue?

The QC procedures insure that the precision of the measurements will fall within a well defined set of confidence limits. Further, 'out-of-control' samples are identified readily and are reanalyzed according to a standard set of criteria.

The accuracy of the method cannot be evaluated in the absence of a standard reference material. The reviewer strongly recommends that serum samples which are "not detected" be checked for the percent recovery and that the measured detection limit be reported along with the fact that the sample was "not detected."

The reviewer is not well versed in the legalistic aspects of QA (i.e., GLP compliance). However, the absence of a clear and simple summary of procedures in the laboratory seems to be a deficiency which could become a serious problem if staff members were removed from or left the project. This is the only significant deficiency noted by this reviewer.

Signature Jack O. Lay Jr. Date 8/20/86
Jack Lay, Ph.D.
Doc. 6700L

QUESTION 3

Is the laboratory sufficiently blinded in its ability to link the serum sample with the respective adipose tissue sample?

The laboratory has an excellent blinding system. The use of the Red Cross "ID" number allows the entire laboratory to be blinded. Further, the use of the Red Cross numbers, until the assays are completed, makes the integrity of that portion of the serum blinding unquestionable. Furthermore, the reanalysis of randomly selected samples (for adipose) provides additional blinding. The reviewer suggests that perhaps some serum extracts could be recoded and reassayed blindly. (This is recommended because the entire serum sample is used in the assay.)

Signature John O Lay Jr

Date: August 20, 1986

Jack Lay, Ph.D.
Doc. 6700L

QUESTION 4

Is the proposed method of data analysis appropriate for determining whether or not serum and adipose levels are correlated?

The reviewer feels that he is not qualified as an "expert" in statistics. However, it appears to the reviewer that display of the adipose values and blood values on axes of two-dimensional plots, as proposed, should provide an indication of any potential relationship.

Signature Jackson O. Lay Jr. Date: August 20, 1986
Jack Lay, Ph.D.
Doc. 6700L

Question 1

Are the analytical methods, equipment, and personnel of the CEH laboratory appropriate for determining 2,3,7,8-TCDD in human serum and adipose tissue?

The requirements for an operational laboratory performing analytical chemistry associated with the ppt and ppq determination of TCDD in human adipose and serum must meet the highest standards. The methods must be validated by intensive laboratory study; the equipment must be state of the art in order to achieve consistent ppt/ppq operational levels; the personnel must be professionals and have the technical ability and scientific status to plan, develop, and interpret a complex study. The laboratory has published a number of scientific papers detailing the methods and method performance for adipose and serum assays. The assays are based upon sound scientific principles which have been proven for highly lipophilic matrices. The use of labelled internal standards achieves adequate precision for both adipose and serum. Recoveries based upon matrix spikes were within expected values for in-control analytical methodologies. The modification of the adipose method to accommodate the increased sera complex triglycerides has been shown to provide adequate detection limits for the 200 ml serum samples. The laboratory equipment consists of four state-of-the-art mass spectrometers which represent specific sensitive analytical quantitative tools for dioxin analysis. The use of high resolution mass spectrometry provides an additional level of specificity for accurate mass and isomer assignment. The laboratory personnel are made up of Ph.D.-trained chemists, trained medical technicians, trained laboratory technicians, and trained electronic technicians. Although the dioxin program at the CEH has existed only a short time, the laboratory personnel have received outside training and have over the past two years gained valuable experience in dioxin analysis.

Signature 

Date: August 20, 1986

Ronald K. Mitchum, Ph.D.

Doc. 6701L

In summary the analytical methods have been single-laboratory, validated by the CEH. A limited number of collaborative study results have also been obtained. Since the laboratory will perform the analysis in-house and since a common link exists between standards associated with the study and those certified by NBS and EPA, no data bias is expected. The laboratory has state-of-the-art equipment well suited to the analytical needs of the project. The personnel are highly qualified professionals who, by the nature of the priority of the project, work solely on dioxin methodology and the analytical chemistry associated with the quantitation of the samples. Such a focused program results in quality procedures and a consistency of the analytical chemistry. The levels of dioxin to be determined in human sera are at the limits of existing technology and that must be considered along with the expectations of the study. The analytical results at hand at the time of this review represent excellent data obtained at the cutting edge of technology. The variability in the data is what can be expected and little improvement would be expected or should be required. This should be factored into the data analysis for determining if a correlation exists.

Signature 

Ronald K. Mitchum, Ph.D.

Date: August 20, 1986

Doc. 6701L

QUESTION 2

Are appropriate Quality Assurance/Quality Control procedures being used in determining 2,3,7,8-TCDD in human serum and adipose tissue?

There are two aspects to be considered when determining if the data generated from this study is of known quality. Quality assurance (QA) is a management tool which addresses the procedures necessary to assure the data is of known quality. This includes problem definition, SOPs for the performance of the experiment, controls in place to insure SOPs are followed, and birth to death project management. Quality control (QC) is a laboratory tool to ensure QA procedures are met. An assessment of the QA procedures in place in the laboratory which includes adequate management controls, problem deifnition, data quality objectives, program managmenert indicates that many QA procedures are in place and none which are not in place would impact the data assessment. QA procedures associated with critical parameters such as data traceability and independent statistical analysis of QC runs, are in place. In critical areas such as QA checking for transcription errors and calibration criteria for analytical balances were not in place but would not be considered critical. SOPs for some of the general laboratory operation were not in place however, due to the small laboratory groups (i.e., supervisor to employee ratio 3:1 ensures the general method and analysis validity). The QA/QC criteria in place in the laboratory is adequate to assess the quality of the data. The EHLS should be commended for actively incorporating QC materials and control into the experiment, therefore ensuring the quality of the data.

Signature 

Date: August 20, 1986

Ronald K. Mitchum, Ph.D.

Doc. 6701L

QUESTION 3

Is the laboratory sufficiently blinded in its ability to link the serum sample with the respective adipose tissue sample?

The blinding protocol as presented and as found to be in place was appropriate for the study. In addition to the defined blinding process, there also exists internal binding within the laboratory due to record-keeping practices in place. An example would be the analytical mass spectrometry laboratory being blind to the LIMS ID number. The procedures are controlled. Protocols for blinding as well as for sample collection were in place. Unblinding either inadvertently or on purpose would be difficult, if not impossible, with the strict controls in place.

Signature  Date: August 20, 1986

Ronald K. Mitchum, Ph.D.

Doc. 6701L

QUESTION 4

Is the proposed method of data analysis appropriate for determining whether or not serum and adipose levels are correlated?

A protocol for the correlation was provided and details the procedure for determining correlation between adipose and serum values. Sample size and power estimates were determined. Data treatment was proposed and based upon sound statistical principles. A multivariate approach is suggested to correlate lipid type LDH, HDL, LLDL with the adipose serum correlation.

The appropriateness of the correlation method should be reviewed by professional statisticians with the laboratory data variability being a critical factor. If there is not a strong correlation, the data variability taken from the laboratory duplicates and QC samples will prove useful.

Signature 

Ronald K. Mitchum, Ph.D.

Date: August 20, 1986

Doc. 6701L

Recommendations:

The following recommendations would enhance laboratory measurements in future studies, but are not meant to imply any deficiency in the quality of current results.

a) Laboratory data intercorrelation tables should be constructed. That is, sample ID #, such as LIM #, wet lab notebook #, MS file # should be correlated with each other to allow the ready tracking of samples.

b) SOPs should be in place for the overall laboratory operation. This should include balance calibration SOPs, extraction, data analysis, data storage and retrieval. These are examples and others at that detail level should also be incorporated.

c) Data transcription error checking should be implemented.

d) The laboratory should consider a limited multilab test if contracting out becomes necessary.

Signature 

Date: August 20, 1986

Ronald K. Mitchum, Ph.D.

Doc. 6701L

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Centers for Disease Control
Atlanta GA 30333

9/8/86
The following are additional comments from the CDC Ad Hoc Committee.

Mark

September 3, 1986

NOTE TO: Vern Houk

SUBJECT: Additional comments on "Correlation Between Human Serum and Adipose Tissue Concentration of 2,3,7,8-Tetrachlorobenzo-p-dioxin."

The Ad Hoc Committee again wishes to commend the CEH staff for the outstanding report (titled above) and wishes to provide a few additional comments that may be useful in preparation of the next phase of the Agent Orange Study. It could be useful to perform serum 2,3,7,8-TCDD measurements on Vietnam veterans in all studies focused on health effects of Agent Orange, to determine the validity of the epidemiologic exposure indices. This could include measurements of serum 2,3,7,8-TCDD in a sample of the Ranch Hand Study population. If you propose the use of a cut-off value for serum or adipose 2,3,7,8-TCDD concentration to classify individuals as exposed or non-exposed, rather than a continuous measure of exposure permitting dose-response assessment, it would be important to estimate the degree to which an individual would be classified differently using the various available methods of exposure assessment, i.e., serum and adipose 2,3,7,8-TCDD measurements and an epidemiologic exposure model using military records.

Further consideration might also be given to assessing the importance of the variability observed among individuals in their ratios of serum and adipose 2,3,7,8-TCDD concentrations.

Gary R. Noble, M.D.

cc:
Dr. Mason
Ad Hoc Committee Members

APPENDIX B

Agent Orange Working Group Science Subpanel

July, 1986, report on

Exposure Assessment for the Agent Orange Study

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
National Institutes of Health

Memorandum

Date July 10, 1986

From Carl A. Keller, Ph.D. CAK
Chairman, Science Panel, AOWG

Subject Review of Exposure Assessment Subpanel Report

To Donald M. Newman
Chairman, Agent Orange Working Group

At our meeting of June 17, 1986, the Science Panel discussed the Exposure Assessment Subpanel Report. I am attaching the minutes from that meeting as the Science Panel's report to the Agent Orange Working Group regarding exposure assessment for the Agent Orange Study. As noted in the minutes, our conclusions are based on the subpanel report.

Attachment

cc:
Members of the Science Panel, AOWG

MINUTES OF THE MEETING ON JUNE 17, 1986
SCIENCE PANEL OF THE AGENT ORANGE WORKING GROUP

The Science Panel met from 10:00 AM to 1:30 PM in room 729G of the Humphrey Building in Washington, D.C. Members present at the meeting are listed on the attached sign-in sheet.

The purpose of the meeting was to review the report of the exposure assessment subpanel which had been commissioned by the acting chair of the Agent Orange Working Group. The Science Panel based its conclusions on material in the report and on scientific issues alone, and did not bring in independent evidence or experts. Although it does not agree with all of the conclusions and statements in the subpanel report, the Science Panel considered the information and recommendations contained in the report, and concluded as follows:

* Pertinent military records have been used appropriately to locate all known herbicide spraying operations and military units and to identify individuals who may have had opportunities for exposure to Agent Orange. Limitations on the assessment of exposure opportunities are due to limitations in the records themselves. It seems inevitable that veterans' opportunities for exposure to Ranch Hand defoliation missions will be over-ascertained due to troop dispersion while those with opportunities for exposure to herbicide used to clear base perimeters will be under-ascertained due to unrecorded spraying operations.

* There is unanimous agreement that an epidemiological study of ground troops' possible exposures to Agent Orange disseminated by Operation Ranch Hand fixed-wing aerial spraying, based solely on military records, does not appear to be scientifically feasible.

* Pilot study results indicate that veterans had more opportunities for exposure via helicopter operations and ground spraying of base perimeters than from Operation Ranch Hand defoliation missions.

* With the exception of one member, all Science Panel members agree that the potential for misclassification of the exposure status of ground troops (from Ranch Hand spraying or otherwise) will preclude scientifically valid results from any epidemiological study based on military records alone.

* During the meeting, the Centers for Disease Control provided the Science Panel with its concept for the "Use of Biological Samples as a Surrogate for Exposure to Agent Orange" (attached), which might be developed into a verification study. The Science Panel recognizes that the accurate measurement of current adipose tissue levels for 2,3,7,8-TCDD, as proposed by the Centers for Disease Control, may provide the only viable basis for assessing past exposure to Agent Orange. However, it is not clear how this information will be used to validate individuals' exposures, nor how military records can be used to generate exposed and unexposed cohorts for a large scale epidemiology study. The Science Panel does not object to further development of a detailed protocol, although members were divided on whether to encourage such an effort. Some feel that an attempt at verification may be useful in determining whether the Agent Orange study can go forward or not. Other opinions ranged from those who feel that the proposed validation method is most unlikely to be useful, to those who feel that it must be attempted despite uncertainty about its usefulness.

* There is no agreement at this time whether a feasible and accurate method for validation of individual exposure status can be devised, nor on the elements of a verification study. Until a detailed protocol for a verification study is available, it will not be possible to evaluate the feasibility of any proposal for validating individual exposures.

* As indicated previously, the results of a verification study should have a decisive influence on conducting the Agent Orange study. Therefore, the Science Panel recommends that both the Agent Orange Working Group and the Congressional Office of Technology Assessment review any proposal which may be developed for its scientific suitability, before the sponsoring agency proceeds with such a study.

* There is unanimous agreement that if a well-designed exposure verification study fails to validate individuals' exposures as determined from military records, the Agent Orange Epidemiological Study should be discontinued.

Carl A. Keller

Carl A. Keller, Chairman
Science Panel, AOWG

Attachment

APPENDIX C

Area Approach System Methodology

for Determination of Agent Orange Exposure Status

AREA APPROACH SYSTEM METHODOLOGY

FOR DETERMINATION OF AGENT ORANGE EXPOSURE STATUS

OF U.S. ARMY PERSONNEL

IN THE REPUBLIC OF VIETNAM

Prepared by Shelby L. Stanton, J.D.

January 20, 1986

For Agent Orange Projects

Division of Chronic Disease Control

Center For Environmental Health

Centers for Disease Control

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AREA APPROACH SYSTEM METHODOLOGY

FOR DETERMINATION OF AGENT ORGANGE EXPOSURE STATUS

OF U.S. ARMY PERSONNEL IN THE REPUBLIC OF VIETNAM

By Shelby L. Stanton

1. Scope and Purpose of This Report:

The use of herbicides in Vietnam was authorized by President Kennedy as early as 1961. During the period 1965 to 1970, chemical herbicides were used as a form of combat support to defoliate vegetated areas which were used by the Viet Cong as base areas or which provided cover for VC attacks against U.S. and allied forces. They were also used to destroy enemy crops.[1]

The Area Approach System Methodology for Determination of Agent Orange Exposure Status is designed to render reasonably accurate conclusions as to the likelihood of exposure to Agent Orange by service members operating in the III Corps Tactical Zone of Vietnam during the Second Indochina War. This system compensates for the lack of precise information in both daily individual ground and/or unit placement data, as well as for the lack of specific aircraft chemical delivery records (especially in regard to rotary-wing [helicopter] missions), by proposing a rational formula based on an individual soldier's presence in a given geographical area.

This system is necessitated by the present incomplete state of the military records with respect to daily unit locations as well

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1. Engineer Strategic Studies Group, Office, Chief of Engineer, Department of the Army, Herbicides and Military Operations, Volume II, dtd February 1972, hereafter cited as Herbicides and Military Operations.

as helicopter spray missions. The need for this system is explained by discussing first the magnitude of delivery by army rotary-wing aircraft and the reasons for a lack of permanent records therefrom, and secondly, by discussing the extent of locally authorized herbicide applications. This groundwork is then fused to show that as a matter of operational methodology Agent Orange application was largely confined to combat areas requiring deprivation of enemy use of cover and concealment or regions requiring crop destruction. The operational areas of United States troops within III Corps Tactical Zone were affected by the Rules of Engagement, which for practical reasons limited significant herbicide application to certain designated areas.

2. Delivery by Rotary-Wing Aircraft (Helicopters):

One of the essential factors that must be ascertained in any complete herbicide spray study is the degree of spray missions performed in addition to the Air Force RANCH HAND program. While the Air Force maintained rather complete documentation on its fixed-wing herbicide mission under Operation RANCH HAND, these spray tapes only partially disclose the total area sprayed with Agent Orange. In addition to Air Force C-123 aircraft sprayings, Agent Orange was dispersed by a variety of other means to include boat, truck, manpack, and helicopter. Of these other methods, only aerial helicopter (rotary-wing) application of the chemical reached such significant levels that any Agent Orange study would

be adversely affected without either exact documentation or system development properly accounting for helicopter utilization.

The first helicopter crop destruction missions were flown in Phuoc Long Province using H-34 helicopters equipped with hand sprayers as early as November 21 - 23, 1962. These helicopter-delivered applications of Agent Orange ruined three hundred hectares of crops, including the estimated destruction of one thousand tons of rice.[2] Crop destruction missions were carried out mainly by the H-34, since each helicopter had a capability similar to the C-123 aircraft in hours of flying time and could provide more accuracy in ground coverage. For example, helicopter crop destruction missions over Binh Thuan Province during July and August of 1964 resulted in 80% destruction of suspected Viet Cong crops.[3] The fact that these helicopters lacked the area coverage of fixed-wing aircraft and could carry less chemical was compensated by their ready availability and more frequent use. Each H-34 could fly forty-five hours a month and perform from twenty to twenty-five spray missions.[4]

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2. Dept. of Defense, Herbicide Operations in the Republic of Vietnam, undated.

3. Dept. of the Air Force Report, Evaluation of Crop Destruction in RVN, dtd 1 July 1966, in 12th ACS Files.

4. Department of Defense, Herbicide Program in the Republic of Vietnam, dtd 18 December 1964.

The great, indeed "pin-point", chemical delivery provided by helicopter defoliation spraying was quickly realized by the military. Flying at less than one-fourth their capacity (23%), H-34 helicopters mated with HIDAC delivery systems flew 128 herbicide sorties and destroyed over 2,605 hectares of crops from May through October of 1964. It is important to remember that these Agent Orange sprayings were conducted outside of the RANCH HAND program. To offer a comparison of the helicopter expenditure effort, consider that a total of 6,086 hectares (or 15,215 acres) were sprayed by both Air Force RANCH HAND and H-34 helicopters during the entire year. Thus, computing the available figures for helicopter sprayings - which only cover the six months of 1964 from May through October - against the known total, almost half of all spray missions were conducted by rotary-wing means.[5]

Army Major John Dalton Howard, who graduated from the United States Military Academy in 1964, later discussed the pervasive use of army helicopter spray missions during the Vietnam War in his postwar thesis, Herbicides in Support of Counterinsurgency Operations: A Cost-Effectiveness Study. Major Howard stated, "In certain areas, ground commanders are authorized to conduct local herbicide operations. When UC-123 aircraft are not available to

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5. Dept. of Defense, Herbicide Program in the Republic of Vietnm, dtd 18 December 1964.

the job or the target is too small to merit fixed-wing sorties, the UH-1 helicopter (commonly known as the "Huey") can be equipped with an internal tank and spray booms. In initial operations in Vietnam, some U.S. Army units used a field expedient which employed a 55-gallon drum fitted with rubber hoses and sprayers mounted on the helicopter skids. The second generation system used in the UH-1 is the AGAVENCO sprayer, developed by a Las Vegas firm for use in agricultural work. This system can be mounted in the aircraft in less than one-half hour and consists of a 200-gallon tank, pump, and pressurized nozzles [Department of the Army (DA) Training Circular (TC) 3-16, 1969]. The UH-1 fitted with the AGAVANECO provides the same dissemination rates as the UC-123 but its capacity is considerably less.

"The use of the helicopter in the Republic of Vietnam for delivery of herbicides has been far less standard than the operations of the (Air Force) 12th Special Operations Squadron. Since division commanders were the controlling authorities for these missions in each AO (Area of Operations) the methods used varied considerably throughout the theater. Ideally, several "Hueys" should be employed for efficiency's sake. However, since no helicopters were set aside specifically for herbicide missions, they were normally diverted on a one-by-one basis from other combat sorties. The security escorts, the AH-1G ("Huey Cobra") helicopters, faced the same problem, and while a

defoliation helicopter should be supported by two Cobras, on many occasions none were available. However, this lack of security did not curtail the missions."[6]

The UH-1 "Huey" helicopter had a 90% coverage efficiency for its 100-gallon load (while helicopter tank capacity was 200 gallons, weight limitations under combat conditions curtailed this maximum load). Helicopters were employed under the same operational and climatic conditions as the C-123 RANCH HAND missions, with the exception that helicopters were more accurate and more cost effective. Due to these factors, it is estimated that from 40% - 60% of herbicide application around army fire support bases and base camps was accomplished by helicopter, and the remainder largely rendered by vehicular, manpack, or boat employment. Since U.S. soldiers normally conducted combat operations from within or in close proximity to fire support bases as a matter of standard military practice, the significance of such helicopter sprayings of Agent Orange reaches critical proportions in any exposure study. In such cases, RANCH HAND is a supplemental factor.[7]

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6. Major John Dalton Howard, Herbicides in Support of Counterinsurgency Operations: A Cost-Effectiveness Study, Naval Postgraduate School, Monterey, California, March 1972, Pages 16-17. Howard's thesis formed the basis for his Master of Science in Operations Research. Underlining added by the author.

7. Military Assistance Command, Herbicide Operations Report, August 1969.

3. Extent of Locally Authorized Herbicide Spray Missions:

By 1966, the commander of Military Assistance Command Vietnam (General William C. Westmoreland) and the Ambassador, who had the authority to approve United States herbicide missions, had delegated this authority to senior U.S. advisors at corps and division level.[8] Thus, these field officers could approve defoliation requests within certain guidelines. This guidance paralleled the same criteria allowing ground commanders to engage suspected enemy targets by fire, and varied with the complex set of rules governing conduct of air and ground battles in Vietnam commonly known as the "Rules of Engagement". These rules restricting allied ability to combat the enemy were incorporated into MACV Directive 525-13, and subordinate units issued the instructions as regulations.

In general, the Rules of Engagement severely restricted U.S. and allied initiation of combat around populated regions, and gave much greater freedom of action in less populated areas and certain designated war and special zones. Defoliation guidelines for ground commanders, which closely followed specific instructions on the use of firepower, likewise allowed much greater latitude for helicopter-delivered spray missions in war

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8. Headquarters, PACAF Directorate, Tactical Evaluation of Herbicide Operations in Southeast Asia, July 1961 - June 1967, dtd 11 October 1967, P. 35.

zones, special zones, and selected free-fire zones than across the general countryside. The effect of these rules were incorporated into specific policy areas contained in MACV Directive 525-1, Military Operations - Herbicide Operations, which stated in Paragraph 3(b), "In consonance with the desires of the Government of the Republic of Vietnam, herbicide operations will be limited to areas of low population". Further, Paragraph 3(f) confirmed the approval authority for use of U.S. assets "for defoliation by helicopter in support of local base defense and on known small enemy ambush sites along lines of communication, or the maintenance of deforested areas"as delegated to senior advisors.[9]

As previously cited, in fact the authority to conduct helicopter spray missions was delegated to division commanders, two-star generals who entrusted their staff chemical officers with determination of defoliation request validity and the allocation of local division resources to meet these requests. In addition to the employment of hand-spray and ground-based power spray apparatus, division resources included organic and attached helicopter assets which could be utilized on a mission-ready basis. These were all non-RANCH HAND techniques, and the recordation of their employment was usually

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9. MACV Directive 525-1, Military Operations - Herbicide Operations, dtd 12 August 1969. Earlier versions of this regulation also existed.

non-permanent. In other words, the requests followed standard operating procedures which were not maintained on a permanent basis as a matter of army recordskeeping. To buttress this contention, the author cites Page 5 of the Department of the Army Office of the Chief of Engineers' Volume I of Herbicides and Military Operations, "Use of herbicides, for example around friendly base perimeters, were at the discretion of area commanders. Such use seemed so obvious and so uncontroversial at the time that little thought was given to any detailed or permanent record of the uses or the results."

A parallel in demonstrating the current lack of records pertaining to helicopter herbicide employment is provided by the manner in which ammunition, a much higher priority item of greater significance in the Vietnam war, was regulated and expended. While extensive bookkeeping was maintained by the army to fix chain of responsibility and trace allocation, the distribution of ammunition and its ultimate expenditure at firing unit level was a low-echelon level concern only requiring temporary supply records. Distribution and dispensation of ammunition to individual units or machines, whether tanks or helicopters, was not maintained as part of the permanent army records system during the Vietnam conflict. Utilization of ammunition and herbicide on an aerial mission basis within the army supply system framework was a local supply matter, recorded in such documents as the helicopter crew chief logs. Expenditure

of both ammunition and herbicide in any given area or by any specific aircraft would be impossible to reconstruct due to the nature of the records themselves. Individual army helicopter sortie logs do not now exist and the more extensive recordation typified by helicopter flight records was never permanently maintained.

The data that now exists on helicopter-delivered herbicide applications, especially with regard to missions conducted in response to intradivisional requests, is very incomplete. What information does exist comes merely from chance entries in unit daily journals and command messages responding to specific inquiries from higher headquarters. No concerted effort was made by the army to track either its specific ammunition or herbicide expenditures. In this regard, the deliberate Air Force effort to permanently record RANCH HAND operations (just as the Air Force permanently records delivery of ammunition in bombing sorties) represents a much greater wealth of data than what currently exists in army records.

While the RANCH HAND tapes give us the bulk of aerial spraying records that now exist, they do not necessarily represent the bulk of actual aerial sprayings conducted in Vietnam. In fact, they may represent far less than what was actually delivered by aerial means, since army helicopters often sprayed the areas closest to troops due to their greater versatility, response, and

accuracy. The tapes being produced on army rotary-wing sprayings are not based on any complete set of records, but rather on secondary information sources which by their very composition are incomplete. Thus, the actual extent of Agent Orange chemical dispensation is unknown. Through knowledge of divisional chemical employment and judicious application of the Rules of Engagement, it is possible to reconstruct the areas likely to be sprayed. However, any attempt to reconstruct the exact areas which were sprayed - without the actual records of army chemical dispersion either by aircraft or ground mode - is impossible. Attempts to reconstruct such application from incomplete data will render incomplete results, and can be successfully challenged in any specific instance not covered by a currently existing official report.

One option available to circumvent the absence of these specific records is to reach a solution premised on a determination of the extent of herbicide spraying based on the Area Approach System methodology developed by this author and outlined below.

4. Area Approach System Methodology for Determination of Agent Orange Exposure Status:

The Area Approach System compensates for the lack of complete aircraft chemical dispersion records and the lack of precise daily troop locations basis. A determination of Agent Orange

exposure is based on a geographical breakdown of III Corps Tactical Zone into sectors based on the application of herbicides in conformity with the Rules of Engagements as practised in the Vietnam War.

Herbicides were used in Vietnam to deny the enemy those military advantages which dense foliage lends to the following military activities: ambush and harassment along transportation routes (land and water), infiltration, enemy base areas, and surprise attack on friendly bases. Herbicides were also used to destroy crops grown in enemy-held areas. [10]

This system is based on three principles which apply to the use of herbicides in conjunction with army ground operations. (1) Agent Orange was a defoliant principally used to deny the enemy cover and concealment in this context, and missions expressly flown to deprive the Viet Cong of crop utilization were utilized in areas not under friendly control. (2) Division commanders (who were authorized to spray Agent Orange as explained in Section 2) employed this chemical to assist components under their command or control, and authorized helicopter missions to accomplish the pinpoint sprayings required around firebase kill zones and other likely enemy approach lanes. There was a great need for sizeable perimeters clear of all vegetation high enough

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10. Herbicides and Military Operations, Volume II, P. B-4-2.

to conceal the movements of crawling men. Under cover of darkness, the Viet Cong hid in the tall grass or brush even when flares were released. Without herbicides, control of grass and weeds in the barbed wire barriers was very difficult.[11] (3) The Rules of Engagement as applied to firepower also generally governed the army utilization of chemical spraying, and thus spraying was more likely in "free-fire" zones rather than populated areas, where clearances and approval would be more difficult.

Five well-defined and charted sectors of III Corps Tactical Zone were considered contested territory during the Vietnam war, and utilization of Agent Orange within them meet all of the above requirements. Units normally operated in these five sectors on a transient basis, and were garrisoned in major base camps either on their periphery or some distance away. Perhaps, most significantly, these were areas of low population density specifically designated by MACV as satisfying the most lenient Rules of Engagement in terms of firepower and/or herbicide application. The areas which meet the area approach system guidelines, along with their selection basis, are given on the next three pages:

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11. Herbicides and Military Operations, P. B-4-18.

1. War Zone C:

Herbicides within War Zone C were employed intensively to clear landing zones and fire support base areas. Mention of this technique is repeated throughout the Operational Reports of units operating in this region northwest of Saigon. Units operating in this area from 1967-1968 normally consisted of 25th Infantry Division elements (to include the 3d Brigade of the 4th Infantry Division until it was merged with the 25th), but the 11th Armored Cavalry Regiment, 196th Infantry Brigade, 173d Airborne Brigade, and 1st Infantry Division also entered this zone for specific, large-scale operations. Special Forces occupied several campsites in War Zone C on a long-term basis.

2. War Zone D:

War Zone D was defoliated primarily because the area was being used as an enemy base camp.[12] Troops operating in this area during the 1967-1968 time frame were mostly from the 1st Infantry Division, 11th Armored Cavalry Regiment, some elements of the 199th Infantry Brigade, and Special

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12. Herbicides and Military Operations, P. B-4-15.

Forces.

3. The Iron Triangle:

The Iron Triangle, for a time a favorite Viet Cong staging base near Saigon, was intensively sprayed with Agent Orange on a repeated basis throughout the war.[13] Units operating in this area included the 173d Airborne Brigade, 1st Infantry Division, 11th Armored Cavalry Regiment, 25th Infantry Division, and 196th Infantry Brigade.

4. The Rung Sat Special Zone (RSSZ):

The RSSZ was the main shipping route to Saigon for oceangoing vessels and a vital supply link, where dense canopy formed by the mangrove forests provided a unique VC hideout. The water there is affected by the tides and much of the land surface is inundated at high tide. Although traveling on foot in this area is difficult because of mud and the intricate system of streams and canals, these channels provide good transportation for the VC using small

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13. Heavy herbicide dosage within the Iron Triangle was commonplace. For example, see Department of the Air Force Project CHECO Report, Herbicide Operations in Southeast Asia, July 1961 to June 1967, dtd 11 October 1967, P. 29 - 32.

boats. Defoliation of this area began in the middle 1960's, and most of the mangrove forests adjacent to shipping lanes were defoliated by the late 1960s.[14] Troops operating in the RSSZ consisted mostly of Australians, Marines, and infantrymen from the 1st Infantry Division.

5. Canal Network and Plain of Reeds west of Saigon:

These targets were selected and variously mentioned in local army reports, and were sprayed mostly due to their line of communication and ambush value to the Viet Cong. Units operating in this area included Special Forces, a brigade of the 25th Infantry Division, and for a limited period, elements of the 199th Infantry Brigade.

These five areas represented much of the combat region within III Corps Tactical Zone. Under the Area Approach System, units entering these areas would be considered to have contact with Agent Orange, primarily from helicopter-applied sprayings in the absence of specific RANCH HAND mission tapes. These were the areas in which fire support bases required clear kill zones and freedom from infiltration/sapper lanes, and in which the Rules of Engagement allowed the full spectrum of either herbicide or

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14. Herbicides and Military Operations, Volume II, P. B-4-6.

firepower utilization to protect American lives.

Troops entering such areas under this system approach would be credited with Agent Orange Exposure for the number of days spent in such a zone. While precise grid coordinates of units may be impossible to ascertain exactly on a daily basis, studious review of all available records (Operational Reports - Lessons Learned, Combat After Action Reports, Daily Journals, Daily Situation Reports, etc.) can disclose whether any given unit was inside or outside of one of these areas on any given day. Troop assignment to any organization can be determined with a high degree of certainty based on individual military service records, and the number of days credited accordingly.

Example:

Battery B of the 1st Battalion, 8th Artillery (25th Infantry Division) is demonstrated to have entered War Zone C during two separate occasions in one month for ten days on each occasion; July 1 - 10 and July 15 - 24, 1967. Battery B is automatically assigned a high degree of risk in exposure to Agent Orange for the twenty days cited in July. This risk accompanies the high likelihood of helicopter spray missions in applying herbicide coverage around fire support bases within this region, even in the absence of specific information to this effect (since most of the flight information records have been destroyed as non-permanent documents as explained in Section 3). Finally,

Private Joe Tentpeg is shown to have joined Battery B on July 17 from a unit not operating in one of the five identified regions, based on his military service record. In this example, Joe Tentpeg is credited with eight days of likely exposure during July.

5. Conclusion:

The Area Approach System for Determination of Agent Orange Exposure Status is proposed as a logical solution in reaching viable conclusions as to individual soldier exposure, in the absence of complete military unit locational and spray mission data. Since units operated in and out of the five cited regions, and these regions harmonize with actual firepower and herbicide utilization in Vietnam under the Rules of Engagement, and personnel transferred in and out of such units, it is possible to assign fixed percentiles of exposure likelihood to individual service members. The Area Approach System is a valid means of determining Agent Orange exposure which takes cognizance of military operational reality, the present incomplete state of the army's Vietnam war records, and compensates for loss of data in the interest of both the service and the public in rendering a proper degree of flexiblity in meeting individual claim criteria.

COMPARATIVE SUMMARY

Current AOP Approach Method

1. Determination of exact area sprayed by herbicides. Strength: complete objectivity. Weakness: insufficient data due to inherent incompleteness of herbicide spray mission records.

2. Determination of exact daily location of unit. Strength: complete objectivity. Weakness: absence of data for certain periods.

3. Individual service member is identified with specific unit through military service record and exposure premised on his unit being in area which was sprayed. Strength: complete objectivity. Weakness: Questionable determination due to insufficient spray data and possible absence of data on true unit locations.

Proposed Area Approach Method

1. Determination that certain areas are Agent Orange-contaminated based on rules of engagement, actual spraying methodology, and military operating procedures in Vietnam. These areas are War Zones C and D, the Iron Triangle, the Rung Sat Special Zone, and certain canal network and Plain of Reeds locations. Strength: Valid assessment of areas in III CTZ with high Agent Orange risk. Weakness: Subjective, with possible overextension of Agent Orange risk area.

2. Determination that units are either inside or outside the above cited areas. Strength: Reliable gauge of general unit location in relation to Agent Orange-contaminated areas. Weakness: More general approach.

3. Individual service member is identified with specific unit through military service record and exposure premised on his unit being in a cited area. Strength: A simple test whereby an individual is determined to have a high potential of exposure to Agent Orange within certain zones, and determined to have a low potential of exposure to Agent Orange outside of cited areas, open to individual proof that he was affected on a case-by-case basis even outside such zones. Weakness: More liberalized, general approach.

Summation

The current AOP Approach, while objective in theory, fails in reality due to the incompleteness of the data assembled and current state of the army's Vietnam records. Thus, resort must be made to a quasi-objective approach in order to accurately determine the true extent of Agent Orange exposure. This status can be reasonably calculated by recourse to the area approach system outlined in this proposal.

APPENDIX D

Distribution of E3 and Area Scores

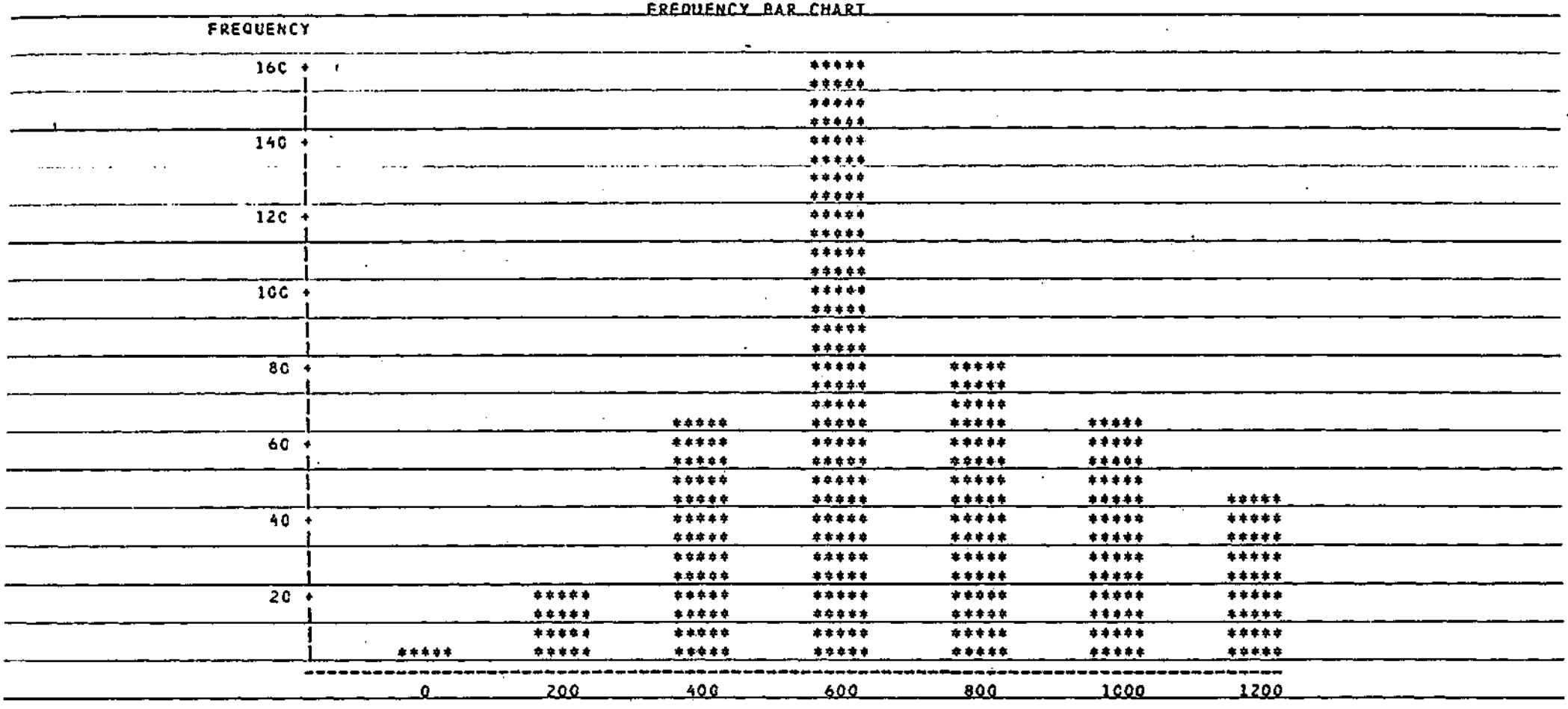
APPENDIX D

The distributions of E3 scores in men selected for the "high hits" group and for the "low hits" group are illustrated in Figures 1 and 2, respectively. As the figures illustrate, the range of E3 scores among the men selected as candidates for each group is wide, so that we should be able to investigate the association between TCDD levels and the E3 scores using linear regression techniques such as inclusion of a term of group (e.g. 1 for high hits, 0 for low hits), we should be able to study the association in both groups together. Approximate transformations will be used as necessary to accomplish these linear regression analyses.

The distributions of the areas scores are shown in Figures 3 and 4, again illustrating a wide range of scores among the men selected for the study. This wide range should permit appropriate application of linear regression techniques, allowing us to investigate the association of the area scores with TCDD levels.

3 SCORE

E3 Scores, High Hits Group

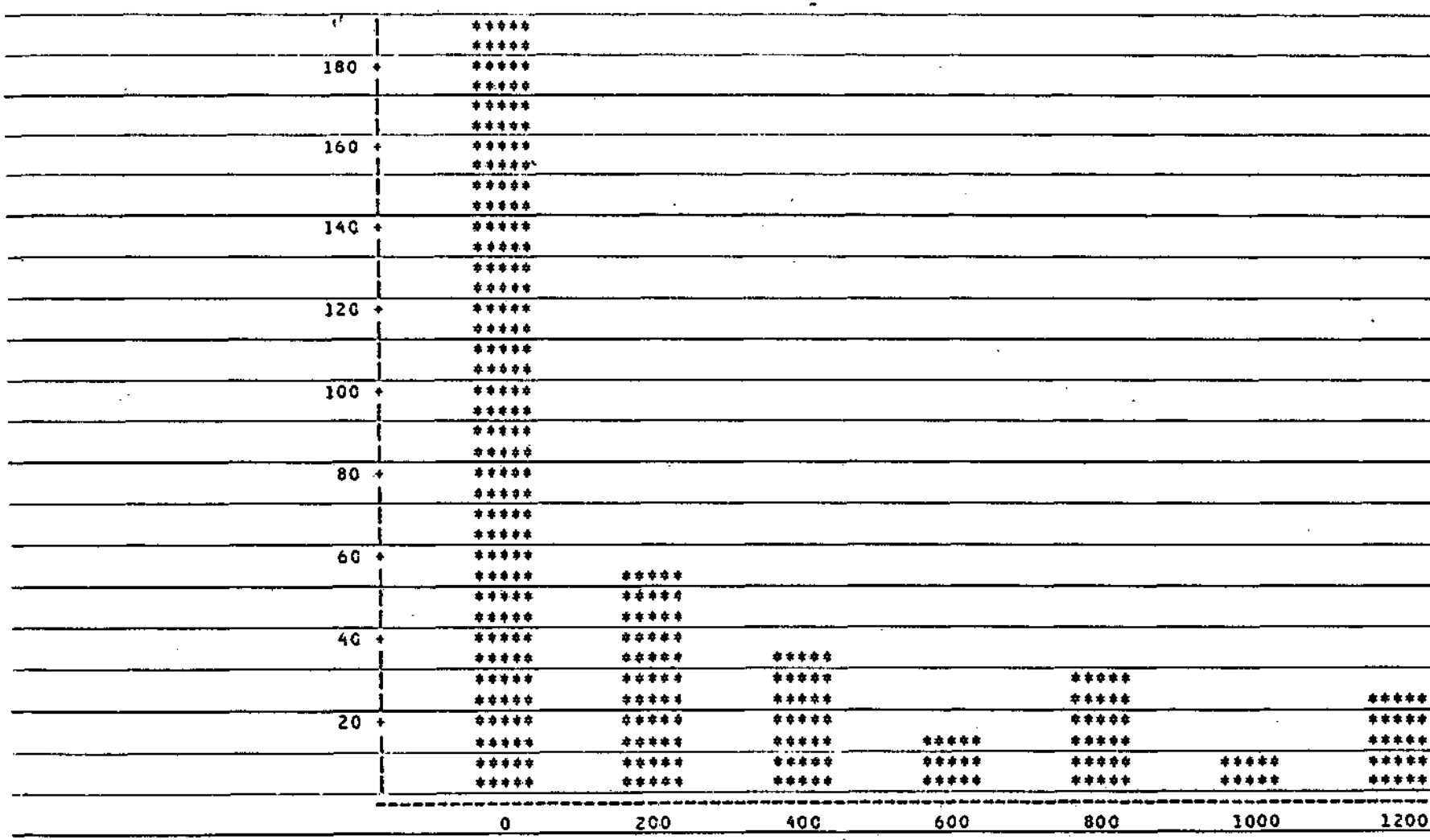


E3_SCORE

FIGURE 2

E3 Scores, Low Hits Group

FREQUENCY BAR CHART

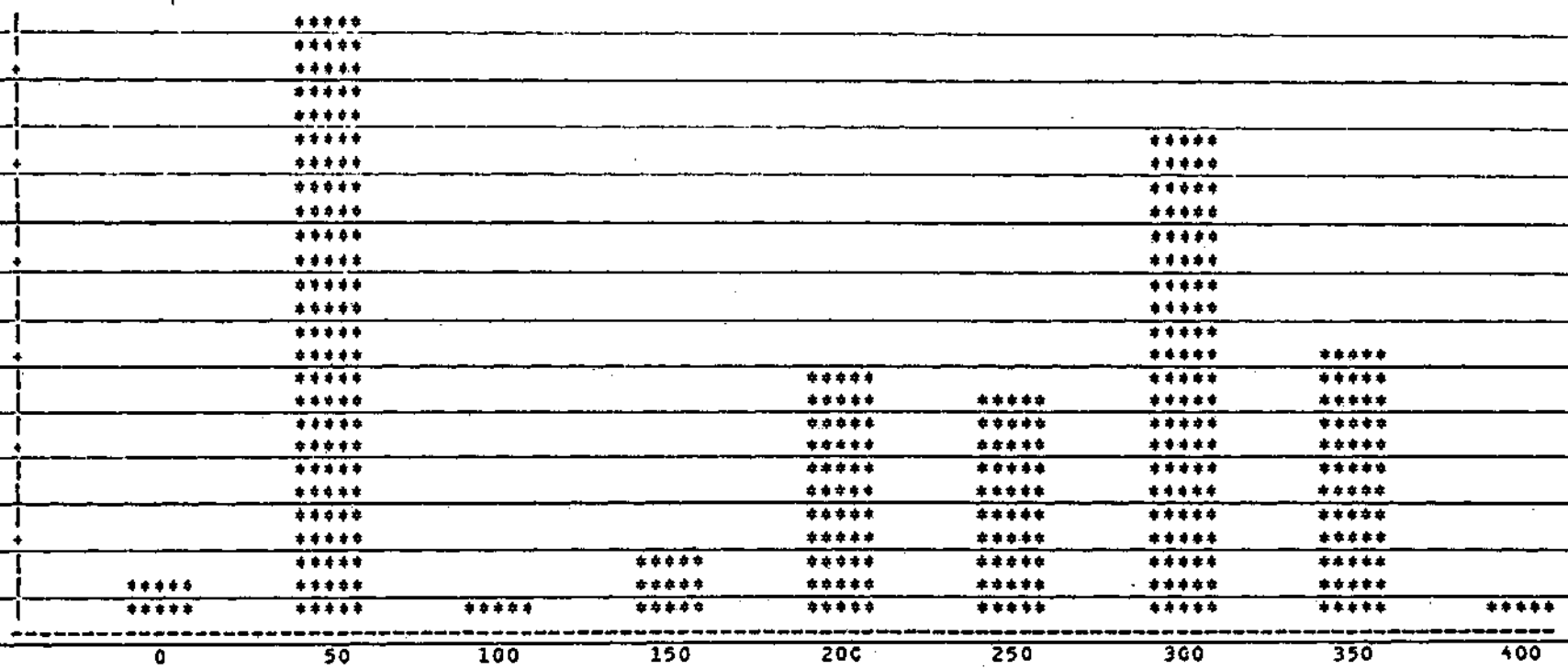


AREA SCORE

FIGURE 3

AREA SCORE, HIGH HITS GROUP

FREQUENCY BAR CHART

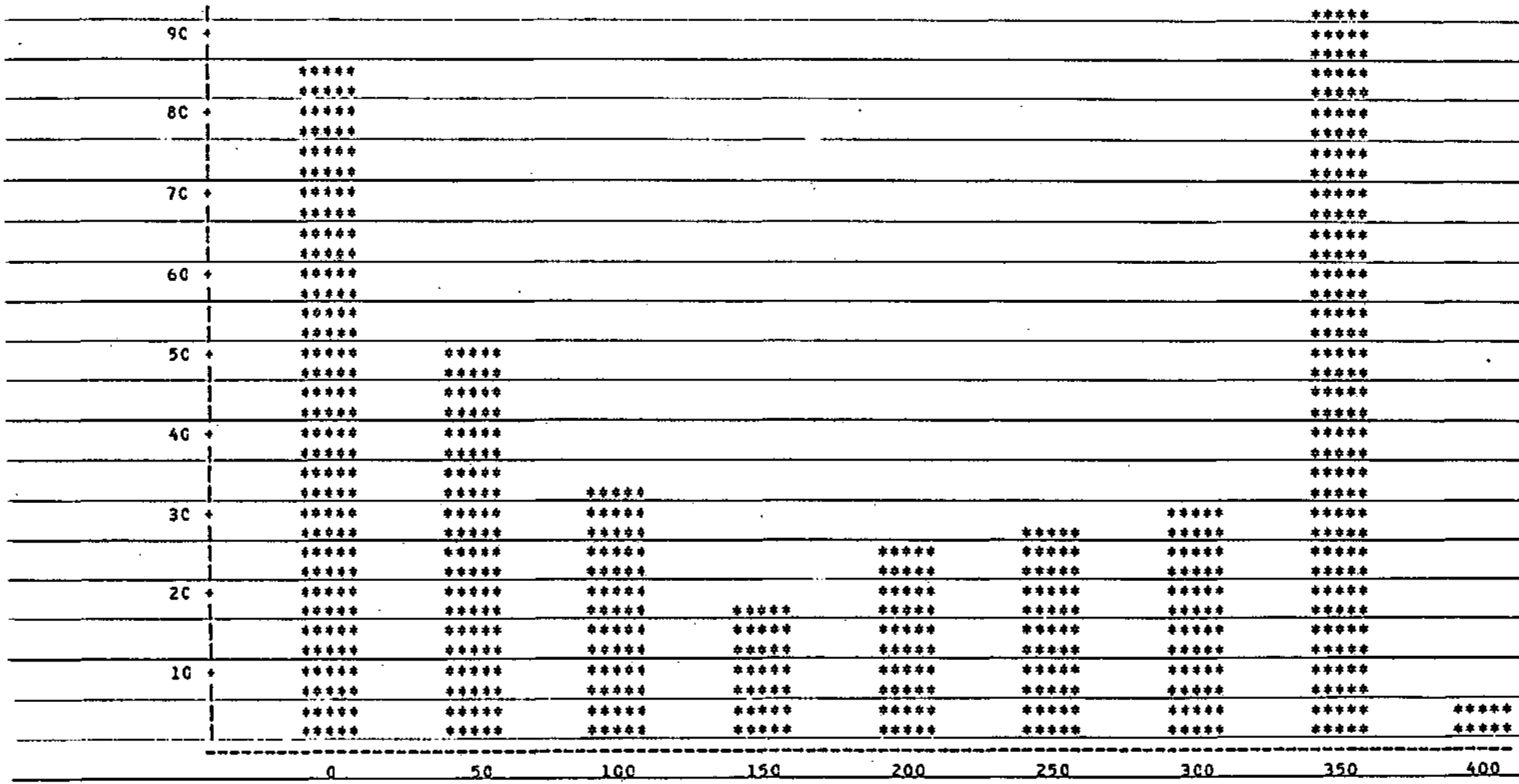


AREA SCORE

FIGURE 4

AREA SCORES, LOW HITS GROUP

FREQUENCY BAR CHART



APPENDIX E

Exposure Questionnaire

(To be added to current Medical History Questionnaire)

EXPOSURE QUESTIONNAIRE

SECTION A

A–1. Exam Code __ __ __ __

A–2. Participant ID __ __ __ __ __ __ __

[Participant's last name _____]

A–3. Exam date Month __ __
Day __ __
Year __ __

A–4. Interviewer ID __ __ __ __

A–5. Completion status __
1=Complete
2=Partial
3=Refused

INTRODUCTION

Hello Mr. _____. My name is (interviewer's name) and I would like to explain the special questionnaire we have for you at this station. We want to ask you a series of questions about your Army service and about various jobs you may have had in civilian life. Many of the questions concern your possible contact with chemicals, especially herbicides that are used to kill weeds and destroy other forms of vegetation. CDC needs this information to classify your examination and test results correctly with those of other veterans of similar background and experiences. Some of the questions may sound like ones you were asked in your telephone interview but we have to ask them again here.

SECTION B

MILITARY EXPERIENCE

The first part of this questionnaire is about your tour of duty in Vietnam. There are a number of questions about the units you were assigned to and the locations where you served. Please take time to recall as much as you can about where you were and what your assignments were. We need to get the most accurate information we can.

B-1. How many different tours of duty did you serve in Vietnam?

Tours __ __

DK.....98

ASK B-2 through B-5 FOR EACH TOUR OF DUTY	a. FIRST TOUR	b. SECOND TOUR	c. THIRD TOUR
B-2. What was the name of the unit in which you served the longest during your (first, second, etc.) tour in Vietnam? Please give me as much information as you can, in other words as many organizational levels as you remember. (RECORD VERBATIM)			
B-3. What kind of unit was that? Was it an infantry, artillery, engineer, intelligence unit, or something else? (RECORD VERBATIM)			
B-4. Where in Vietnam were you located with this unit? In what military region or corps were you located? And within that region, what were the names of the places or areas near where you served? (RECORD VERBATIM)			
B-5. What were your primary duties? (RECORD VERBATIM)			

B-6. I would now like to ask if you remember coming into contact with chemicals in various ways while you were in Vietnam. Were you ever present when others were spraying chemicals?

Yes.....1 (B-7)

No.....2 (B-10)

DK.....8 (B-10)

B-7. Were any of the chemicals used to kill weeds, destroy vegetation or make leaves fall off trees?

Yes.....1 (B-8)

No.....2 (B-10)

DK.....8 (B-10)

B-8. On how many occasions were you present when others were spraying <u>those</u> <u>kind</u> of chemicals?

Number __ __ __

DK.....998

B-9. What were the names of those chemicals? (RECORD VERBATIM)

_____

_____

DK.....998

B-10. Did you ever get chemicals on your skin or clothing?

Yes.....1 (B-11)

No.....2 (B-14)

DK.....8 (B-14)

B-11. Were any of the chemicals used to kill weeds, destroy vegetation, or make leaves fall off trees?

Yes.....1 (B-12)

No.....2 (B-14)

DK.....8 (B-14)

B-12. On how many occasions did you get those kind of chemicals on your skin or clothing?

Number __ __ __

DK.....998

B-13. What were the names of those chemicals? (RECORD VERBATIM)

_____

_____

DK.....998

B-14. Did you ever handle equipment or containers that had been used with chemicals?

Yes.....1 (B-15)

No.....2 (B-18)

DK.....8 (B-18)

B-15. Were any of the chemicals used to kill weeds, destroy vegetation, or make leaves fall off trees?

Yes.....1 (B-16)

No.....2 (B-18)

DK.....8 (B-18)

B-16. On how many occasions did you handle equipment or containers that were used with those kind of chemicals?

Number __ __ __

DK.....998

B-17. What were the names of those chemicals? (RECORD VERBATIM)

_____

_____

DK.....998

B-18. Did you ever spray chemicals yourself?

Yes.....1 (B-19)

No.....2 (B-22)

DK.....8 (B-22)

B-19. Were any of the chemicals used to kill weeds, destroy vegetation, or make leaves fall off trees?

Yes.....1 (B-20)

No.....2 (B-22)

DK.....8 (B-22)

B-20. On how many occasions did you spray those kind of chemicals?

Number __ __ __

DK.....998

B-21. What were the names of those chemicals? (RECORD VERBATIM)

_____

_____

DK.....998

B-22. Do you recall ever passing through an area where food crops or vegetation looked like it had been treated with chemicals to kill it?

Yes.....1 (B-23)

No.....2 (B-24)

DK.....8 (B-24)

B-23. On how many occasions did you pass through these kinds of areas?

Number __ __ __

DK.....998

B-24. While in Vietnam did you ever clear vegetation around base camps, fire support bases, roads, or waterways after they had been sprayed with chemicals?

Yes.....1 (B-25)

No.....2 (SECT C)

DK.....8 (SECT C)

B-25. On how many occasions did you do this?

Number __ __ __

DK.....998

SECTION C

CIVILIAN OCCUPATIONAL EXPOSURES

The next set of questions are about different kinds of jobs you may have had in civilian life. Please tell me if you ever had any of these jobs, even if it was for a short time or happened before you went in the Army.

C-1. Did you ever live or work on a farm or ranch of any kind?

Yes.....1 (C-2)

No.....2 (C-14)

C-2. Did you live there, work there, or both?

Lived.....1

Worked.....2

Both.....3

C-3. In what year did you first live or work on a farm or ranch?

19__ __

DK.....98

C-4. In what year did you last live or work on a farm or ranch?

19__ __

DK.....98

C-5. For how many years in total did you live or work on a farm or ranch?

Number of years __ __

Less than one year....95

Continuous.....96

DK.....98

C-6. Were chemicals ever used on any of the farms to kill weeds or other vegetation?

Yes.....1 (C-7a)

No.....2 (C-14)

DK.....8 (C-14)

C-7a. Did you ever work in, or near, an area where these chemicals were used?

Yes.....1 (C-7b)

No.....2 (C-14)

DK.....8 (C-14)

C-7b. What was the average number of days per year you worked in, or near, an area where these chemicals were used?

1 to 5.....1

6 to 10.....2

11 to 20.....3

21 to 40.....4

More than 40.....5

DK.....8

C-8a. Did you ever apply or spray these chemicals?

Yes.....1 (C-8b)

No.....2 (C-9a)

DK.....8 (C-9a)

C-8b. What was the average number of days per year you applied or sprayed these chemicals?

1 to 5.....1

6 to 10.....2

11 to 20.....3

21 to 40.....4

More than 40.....5

DK.....8

C-9a. Did you ever mix or prepare these chemicals?

Yes.....1 (C-9b)

No.....2 (Box A)

DK.....8 (Box A)

C-9b. What was the average number of days per year you mixed or prepared these chemicals?

1 to 5.....1

6 to 10.....2

11 to 20.....3

21 to 40.....4

More than 40.....5

DK.....8

BOX A
Did R answer YES to C-8a or C-9a?
Yes (C-10)
No (C-14)

C-10. What were the names of the chemicals that were used? (RECORD VERBATIM)

_____

_____

DK.....998

C-11. In what year did you first come into contact with these chemicals?

19__ __

DK.....98

C-12. In what year did you last come into contact with these chemicals?

19__ __

DK.....98

C-13. For how many years in total did you come into contact with these chemicals?

Number of years __ __

Less than one year....95

Continuous.....96

DK.....98

C-14. Now, I'd like to ask about some other jobs you might have had. Did you ever do right-of-way maintenance along highways, railroads, or power lines?

Yes.....1 (C-15)

No.....2 (C-26)

DK.....3 (C-26)

C-15. In what year did you first do this type of work?

19__ __

DK.....98

C-16. In what year did you last do this type of work?

19__ __

DK.....98

C-17. For how many years altogether did you do this type of work?

Number of years __ __

Less than one year....95

Continuous.....96

DK.....98

C-18. Were herbicides or weed killers ever used in this work?

Yes.....1 (C-19a)

No.....2 (C-26)

DK.....8 (C-26)

C-19a. Did you ever work in, or near, an area where herbicides or weed killers were used?

Yes.....1 (C-19b)

No.....2 (C-26)

DK.....8 (C-26)

C-19b. What was the average number of days per year you worked in, or near, an area where herbicides or weed killers were used?

1 to 5.....1

6 to 10.....2

11 to 20.....3

21 to 40.....4

More than 40.....5

DK.....8

C-20a. Did you ever apply or spray them?

Yes.....1 (C-20b)

No.....2 (C-21a)

DK.....8 (C-21a)

C-20b. What was the average number of days per year you applied or sprayed them?

1 to 5.....1

6 to 10.....2

11 to 20.....3

21 to 40.....4

More than 40.....5

DK.....8

C-21a. Did you ever mix or prepare them?

Yes.....1 (C-21b)

No.....2 (Box B)

DK.....8 (Box B)

C-21b. What was the average number of days per year you mixed or prepared them?

1 to 5.....1

6 to 10.....2

11 to 20.....3

21 to 40.....4

More than 40.....5

DK.....8

BOX B
Did R answer YES to C-20a or C-21a?
Yes (C-22)
No (C-26)

C-22. What were the names of the herbicides? (RECORD VERBATIM)

_____

_____

DK.....998

C-23. In what year did you first come into contact with herbicides in this type of work?

19__ __

DK.....98

C-24. In what year did you last come into contact with herbicides in this this type of work?

19__ __

DK.....98

C-25. For how many years in total did you come into contact with herbicides in this type of work?

Number of years __ __

Less than one year....95

Continuous.....96

DK.....98

C-26. Did you ever have a job in professional lawn care, landscaping or grounds maintenance?

Yes.....1 (C-27)

No.....2 (C-38)

DK.....8 (C-38)

C-27. In what year did you first do this type of work?

19__ __

DK.....98

C-28. In what year did you last do this type of work?

19__ __

DK.....98

C-29. For how many years altogether did you do this type of work?

Number of years __ __

Less than one year....95

Continuous.....96

DK.....98

C-30. Were herbicides or weed killers ever used in this work?

Yes.....1 (C-31a)

No.....2 (C-38)

DK.....8 (C-38)

C-31a. Did you ever work in, or near, an area where herbicides or weed killers were used?

Yes.....1 (C-31b)

No.....2 (C-38)

DK.....8 (C-38)

C-31b. What was the average number of days per year you worked in, or near, an area where herbicides or weed killers were used?

1 to 5.....1

6 to 10.....2

11 to 20.....3

21 to 40.....4

More than 40.....5

DK.....8

C-32a. Did you ever apply or spray them?

Yes.....1 (C-32b)

No.....2 (C-33a)

DK.....8 (C-33a)

C-32b. What was the average number of days per year you applied or sprayed them?

1 to 5.....1

6 to 10.....2

11 to 20.....3

21 to 40.....4

More than 40.....5

DK.....8

C-33a. Did you ever mix or prepare them?

Yes.....1 (C-33b)

No.....2 (Box C)

DK.....8 (Box C)

C-33b. What was the average number of days per year you mixed or prepared them?

1 to 5.....1

6 to 10.....2

11 to 20.....3

21 to 40.....4

More than 40.....5

DK.....8

BOX C
Did R answer YES to C-32a or C-33a?
Yes (C-34)
No (C-38)

C-34. What were the names of the herbicides? (RECORD VERBATIM)

_____

_____

DK.....998

C-35. In what year did you first come into contact with herbicides on this job?

19__ __

DK.....98

C-36. In what year did you last come into contact with herbicides on this job?

19__ __

DK.....98

C-37. For how many years in total did you come into contact with herbicides on this job?

Number of years __ __

Less than one year....95

Continuous.....96

DK.....98

C-38. Did you ever have a job in logging or forestry?

Yes.....1 (C-39)

No.....2 (C-50)

DK.....8 (C-50)

C-39. In what year did you first do this type of work?

19__ __

DK.....98

C-40. In what year did you last do this type of work?

19__ __

DK.....98

C-41. For how many years altogether did you do this type of work?

Number of years __ __

Less than one year....95

Continuous.....96

DK.....98

C-42. Were herbicides or weed killers ever used in this work?

Yes.....1 (C-43a)

No.....2 (C-50)

DK.....8 (C-50)

C-43a. Did you ever work in, or near, an area where herbicides or weed killers were used?

Yes.....1 (C-43b)

No.....2 (C-50)

DK.....8 (C-50)

C-43b. What was the average number of days per year you worked in, or near, an area where herbicides or weed killers were used?

1 to 5.....1

6 to 10.....2

11 to 20.....3

21 to 40.....4

More than 40.....5

DK.....8

C-44a. Did you ever apply or spray them?

Yes.....1 (C-44b)

No.....2 (C-45a)

DK.....8 (C-45a)

C-44b. What was the average number of days per year you applied or sprayed them?

1 to 5.....1

6 to 10.....2

11 to 20.....3

21 to 40.....4

More than 40.....5

DK.....8

C-45a. Did you ever mix or prepare them?

Yes.....1 (C-45b)

No.....2 (Box D)

DK.....8 (Box D)

C-45b. What was the average number of days per year you mixed or prepared them?

1 to 5.....1

6 to 10.....2

11 to 20.....3

21 to 40.....4

More than 40.....5

DK.....8

BOX D
Did R answer YES to C-44a or C-45a?
Yes (C-46)
No (C-50)

C-46. What were the names of the herbicides? (RECORD VERBATIM)

_____

_____

DK.....998

C-47. In what year did you first come into contact with herbicides on this job?

19__ __

DK.....98

C-48. In what year did you last come into contact with herbicides on this job?

19__ __

DK.....98

C-49. For how many years in total did you come into contact with herbicides on this job?

Number of years __ __

Less than one year....95

Continuous.....96

DK.....98

C-50. Did you ever have any other job in which you came into contact with herbicides or weed killers?

Yes.....1 (C-51)

No.....2 (C-61)

DK.....8 (C-61)

C-51. What kind of job was that? (RECORD VERBATIM)

_____

_____

DK.....998

C-52. In what year did you first do this type of work?

19__ __

DK.....98

C-53. In what year did you last do this type of work?

19__ __

DK.....98

C-54. For how many years altogether did you do this type of work?

Number of years __ __

Less than one year....95

Continuous.....96

DK.....98

C-55. How did you come into contact with herbicides or weed killers in this work?

Applied or sprayed them.....1

Mixed or prepared them.....2

Handled items that had been treated with them.....3

Walked through area that had been treated with them...4

Present when others were applying or spraying them.....5

Other.....6

Multiple ways.....7

DK.....8

C-56. What was the average number of days per year you came into contact with them?

1 to 5.....1

6 to 10.....2

11 to 20.....3

21 to 40.....4

More than 40.....5

DK.....8

C-57. What were the names of the herbicides? (RECORD VERBATIM)

_____

_____

DK.....998

C-58. In what year did you first come into contact with herbicides on this job?

19__ __

DK.....98

C-59. In what year did you last come into contact with herbicides on this job?

19__ __

DK.....98

C-60. For how many years in total did you come into contact with herbicides on this job?

Number of years __ __

Less than one year....95

Continuous.....96

DK.....98

C-61. Did you ever work in a lumber mill or wood-treating plant?

Yes.....1 (C-62)

No.....2 (C-72)

DK.....8 (C-72)

C-62. In what year did you <u>first</u> do this type of work?

19 __ __

DK.....98

C-63. In what year did you <u>last</u> do this type of work?

19 __ __

DK.....98

C-64. For how many years <u>altogether</u> did you do this type of work?

Number of years __ __

Less than one year....95

Continuous.....96

DK.....98

C-65. Were wood preservatives ever used in this work?

Yes.....1 (C-66a)

No.....2 (C-72)

DK.....8 (C-72)

C-66a. Did you ever use them?

Yes.....1 (C-66b)

No.....2 (C-72)

DK.....8 (C-72)

C-66b. What was the average number of days per year you used them?

1 to 5.....1

6 to 10.....2

11 to 20.....3

21 to 40.....4

More than 40.....5

DK.....8

C-67a. Did you ever mix or prepare them?

Yes.....1 (C-67b)

No.....2 (Box E)

DK.....8 (Box E)

C-67b. What was the average number of days per year you mixed or prepared them?

1 to 5.....1

6 to 10.....2

11 to 20.....3

21 to 40.....4

More than 40.....5

DK.....8

BOX E
Did R answer YES to C-66a or C-67a?
Yes (C-68)
No (C-72)

C-68. What were the names of the wood preservatives? (RECORD VERBATIM)

_____

_____

DK.....998

C-69. In what year did you <u>first</u> come into contact with wood preservatives in this work?

19 __ __

DK.....98

C-70. In what year did you last come into contact with wood preservatives in this work?

19 __ __

DK.....98

C-71. For how many years altogether did you come into contact with wood preservatives in this work?

Number of years __ __

Less than one year....95

Continuous.....96

DK.....98

C-72. Did you ever work at an incinerator or industrial power plant?

Yes.....1 (C-73)

No.....2 (C-76)

DK.....8 (C-76)

C-73. In what year did you first work at an incinerator or industrial power plant?

19 __ __

DK.....98

C-74. In what year did you last work at an incinerator or industrial power plant?

19 __ __

DK.....98

C-75. How many years altogether did you work at an incinerator or an industrial power plant?

Number of years __ __

Less than one year....95

Continuous.....96

DK.....98

C-76. Did you ever have a job in metalworking where cutting oils were used?

Yes.....1 (C-77)

No.....2 (C-80)

DK.....8 (C-80)

C-77. In what year did you first do this type of work?

19 __ __

DK.....98

C-78. In what year did you last do this type of work?

19 __ __

DK.....98

C-79. How many years altogether did you do this type of work?

Number of years __ __

Less than one year....95

Continuous.....96

DK.....98

C-80. Did you ever have a job in which you handled animal hides, skins or pelts?

Yes.....1 (C-81)

No.....2 (C-84)

DK.....8 (C-84)

C-81. In what year did you first do this type of work?

19 __ __

DK.....98

C-82. In what year did you last do this type of work?

19 __ __

DK.....98

C-83. How many years altogether did you do this type of work?

Number of years __ __

Less than one year....95

Continuous.....96

DK.....98

C-84. Did you ever work in a paper or pulp mill?

Yes.....1 (C-85)

No.....2 (C-88)

DK.....8 (C-88)

C-85. In what year did you first do this type of work?

19 __ __

DK.....98

C-86. In what year did you last do this type of work?

19 __ __

DK.....98

C-87. How many years altogether did you do this type of work?

Number of years __ __

Less than one year....95

Continuous.....96

DK.....98

C-88. Did you ever have a job that involved chemical waste disposal?

Yes.....1 (C-89)

No.....2 (C-92)

DK.....8 (C-92)

C-89. In what year did you first do this type of work?

19 __ __

DK.....98

C-90. In what year did you last do this type of work?

19 __ __

DK.....98

C-91. How many years altogether did you do this type of work?

Number of years __ __

Less than one year....95

Continuous.....96

DK.....98

C-92. Did you ever have a job where you came into contact with the chemical hexachlorophene?

Yes.....1 (C-93)

No.....2 (SECT D)

DK.....8 (SECT D)

C-93. In what year did you first do this type of work?

19 __ __

DK.....98

C-94. In what year did you last do this type of work?

19 __ __

DK.....98

C-95. How many years altogether did you do this type of work?

Number of years __ __

Less than one year....95

Continuous.....96

DK.....98

C-96. How often did you come into contact with hexachlorophene in that job?

Every day.....1

Some days, every week.2

Several days a month..3

Less than one day a month.....4

DK.....8

SECTION D

OTHER ACTIVITIES

D-1. Now I have some questions about activities you may have engaged in outside of your jobs. In maintaining your lawn or garden, did you ever use weed killers for more than one year?

Yes.....1 (D-2)

No.....2 (END)

DK.....8 (END)

D-2. In what year did you first use weed killers or herbicides?

19__ __

DK.....98

D-3. In what year did you last use weed killers or herbicides?

19__ __

DK.....98

D-4. For how many years altogether did you use them?

Number of years __ __

Continuous.....96

DK.....98

D-5. When you were using them, about how many times a year, on the average, did you use them?

Number of times __ __

DK.....98

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