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**PROTOCOL FOR VETERANS ADMINISTRATION/ENVIRONMENTAL PROTECTION AGENCY
RETROSPECTIVE STUDY OF DIOXIN AND FURANS IN HUMAN ADIPOSE TISSUE**

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Preface

This protocol is prepared as a part of the on-going effort by the Veterans Administration (VA), Agent Orange Projects Office (AOPO), and the Environmental Protection Agency (EPA), Office of Toxic Substances (OTS) to assess exposure of military and civilian populations to potentially toxic residues from phenoxy herbicides and other sources of dioxins and furans.

The proposal describes the approach of the VA and EPA to the comparison of dioxin levels in the fat of Vietnam veterans potentially exposed to Agent Orange with levels from men with no history of Vietnam service or Agent Orange exposure.

This proposal was prepared by Co-Principal Investigators Michele Flicker, M.D., Ph.D., and Han K. Kang, Dr.P.H., in collaboration with Joseph S. Carra (EPA).

Facilities and resources to conduct Phase I of the program have been drawn from the Veterans Administration and the Environmental Protection Agency. Resources for phases II and III are to come from the same sources.

Questions concerning the technical aspects of this proposal should be directed to Dr. Flicker or Dr. Kang. Dr. Flicker's address is Veterans Administration Medical Center, 4801 Linwood Boulevard, Kansas City, Missouri 64128, Mail Code 11C, (816) 861-4700, ext. 620; FTS 8-754-1620. Dr. Kang's address is Research Section, Agent Orange Projects Office (10A7B), Veterans Administration Central Office, 810 Vermont Avenue, N.W., Washington, D.C. 20420, (202) 389-5534; FTS 8-389-5534. Mr. Carra's address is Office of Toxic Substances, Environmental Protection Agency (TS-798), 401 M Street, S.W., Washington, D.C. 20460, (202) 382-3886.

Executive Summary

An interagency VA/EPA study is herein described. It is a retrospective analysis of dioxins, furans, and related compounds found in one of the world's few collections of archived adipose tissue of some 36 or more Vietnam veterans and several hundred Vietnam-era controls. The adipose tissue was collected within 13 years of military service. The study's purpose is to determine whether men who served in Vietnam had higher adipose tissue levels of 2,3,7,8-tetrachlorodibenzodioxin (2,3,7,8-TCDD)*, a contaminant of the phenoxy herbicide Agent Orange, than their counterparts who never served in Vietnam. Such a determination will add to the on-going Federal efforts to answer the question, "were Vietnam veterans who served in Vietnam more heavily exposed to 2,3,7,8-TCDD than their counterparts?" The degree to which this study answers the preceding question will depend upon future studies clarifying the relationship, if any, of 2,3,7,8-TCDD exposure with adipose tissue levels -- a link which to date remains unproven and which this study is not designed to answer.

Study Objectives

Study objectives include: 1) Development of reliable assay methods for 2,3,7,8-TCDD and related compounds at the parts per trillion level. 2) Determination of baseline levels of these compounds in the archived fat of the American adult male population. 3) Analysis of those specimens from individuals who had military service in Vietnam and comparison of their levels with the baseline. 4) Pattern recognition analysis of the data from all specimens for selected dioxins, furans, and chemical relatives as exposure markers for non-phenoxy herbicide sources of 2,3,7,8-TCDD.

Study Plan

The study plan is outlined in Figures 1-3 on the following three pages. Of note in the plan is the reference fat, 30 kilograms of adipose tissue which will be homogenized and "spiked" with 2,3,7,8-TCDD, polychlorinated dibenzodioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) and sent to as many as eleven coordinating laboratories for the "interlaboratory study." The interlaboratory study will yield data concerning the precision and accuracy of the analytical methods as well as the capabilities of the participating laboratories. These data will be

RETROSPECTIVE STUDY SCHEME

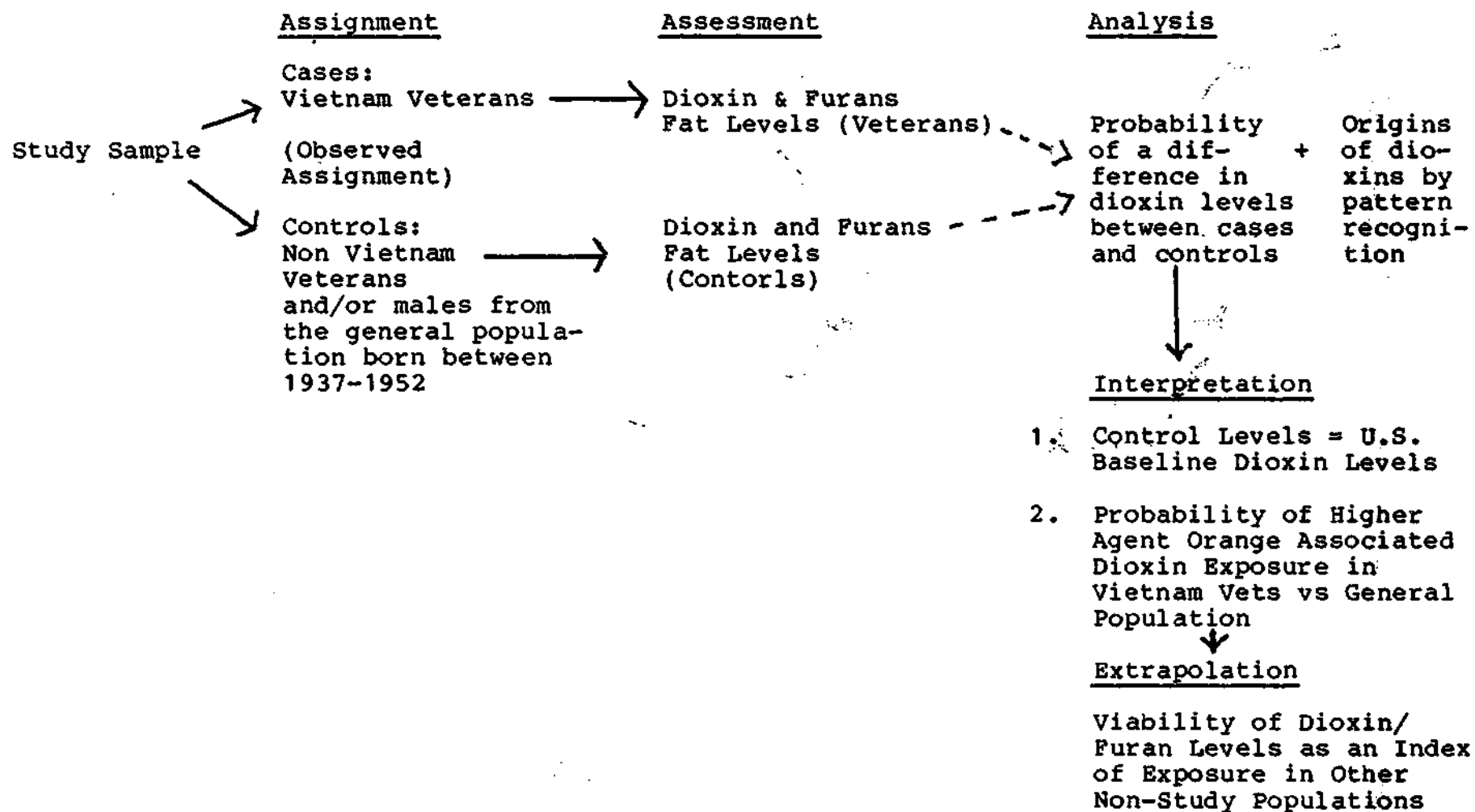
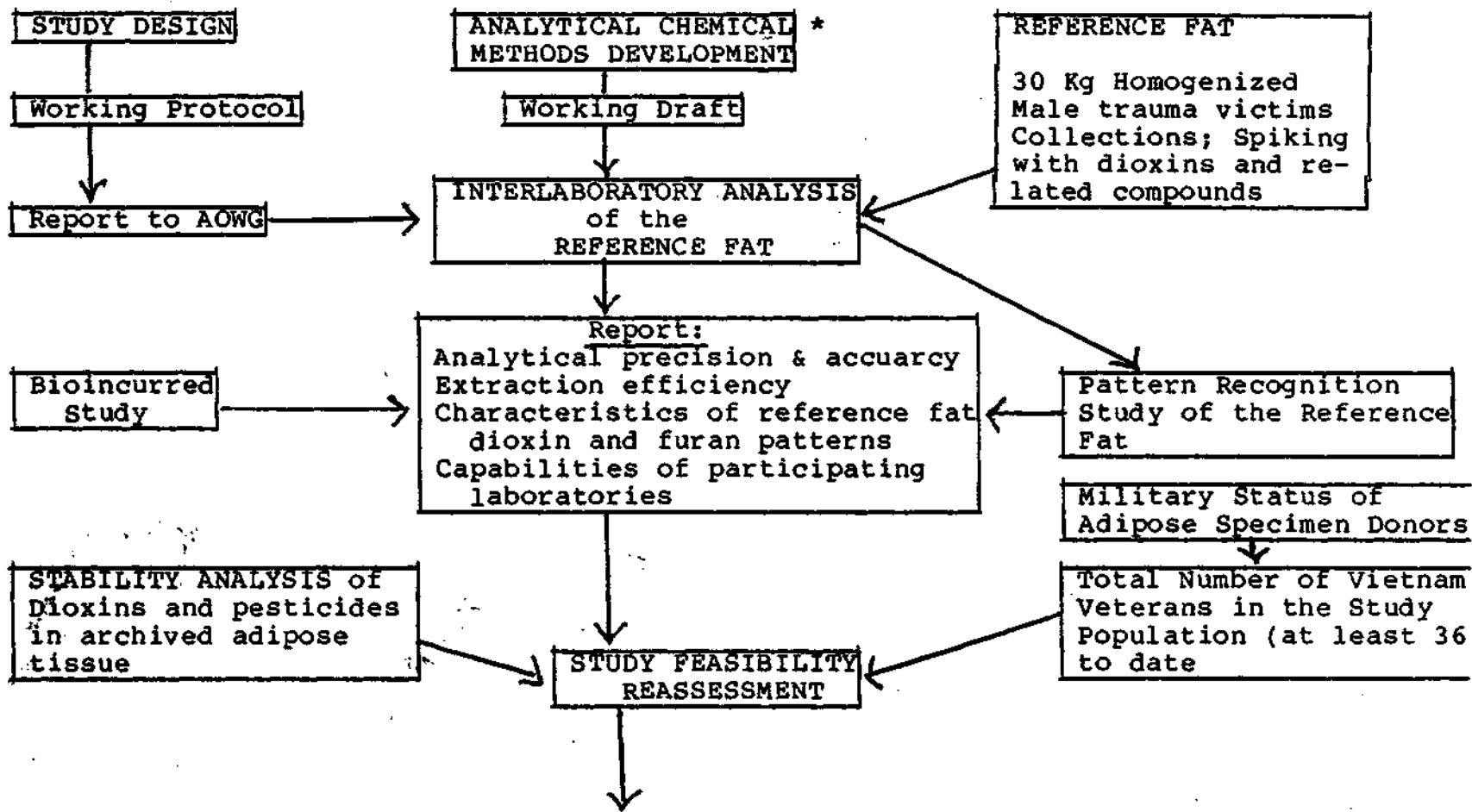


Figure 1.

1. Study Hypothesis: There is a difference between the dioxin levels in the adipose tissue of Vietnam veterans and that of non-Vietnam veterans or of the age-matched general male population.
2. Null Hypothesis: There is no difference between the dioxin levels in the adipose tissue of Vietnam veterans compared to that of non-Vietnam veterans or that of the age-matched general male population.



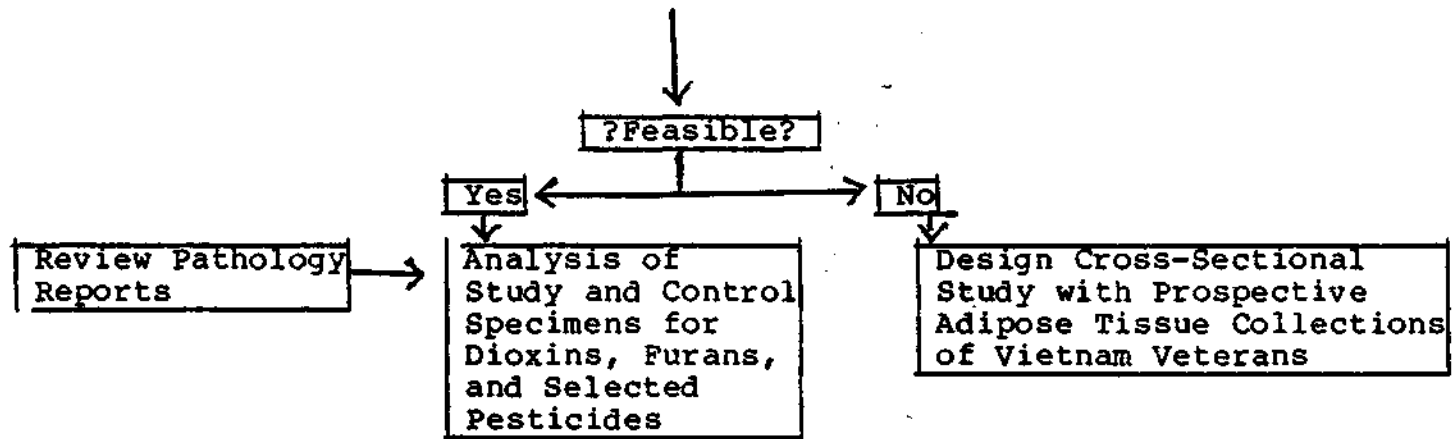


Figure: Algorithm for VA/EPA Retrospective Study of Dioxins and Furans in Human Adipose Tissue

*See detail, Figure 2, next page

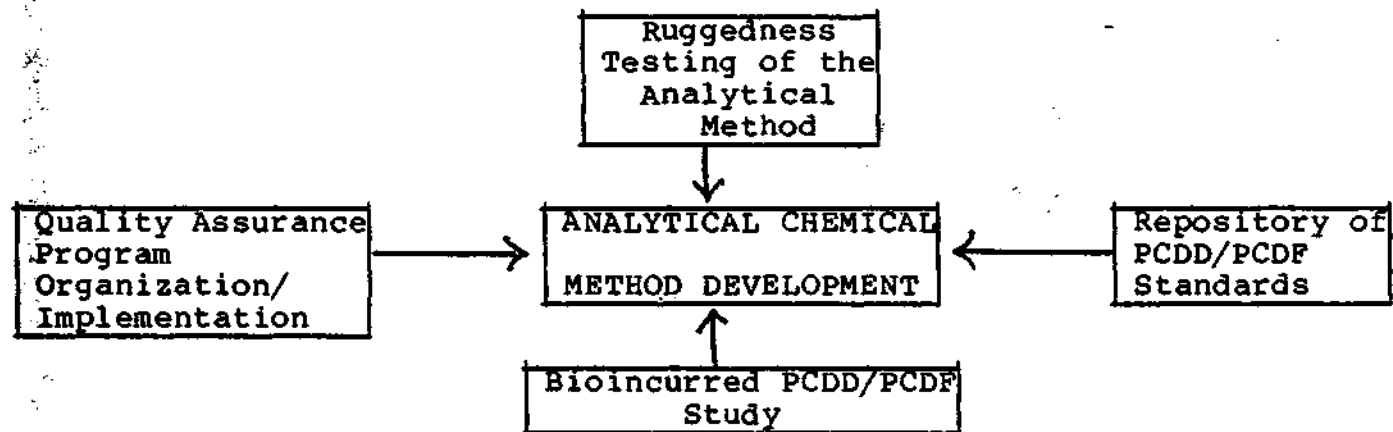


Figure 3: Necessary steps to preliminary analytical chemicals methods development

sp.
combined with the results of ancillary studies to reassess the feasibility of detecting differences in TCDD between veterans and controls. In other words, the actual analysis of the precious adipose tissues from the veterans will not be attempted without confirmation of the study's statistical power. This statistical power will be assessed from the results of the ancillary study of the stability of dioxins and surrogates in adipose tissue, from the extraction efficiencies determined from the "bioincurred" feeding study (radiolabelled PCDDs fed to animals), and from the final tally of the number of Vietnam veteran samples available in the archives.

Study Limitations

Inherent limitations of the proposed retrospective study include relatively small sample size (between 36 to 50 veterans), variability of possible PCDD exposure while in Vietnam, and variability in the time interval between donor exposure to TCDD in Vietnam and specimen procurement.

Small Sample Size: Preliminary power calculations suggest that even with moderate to high overall variability (i.e., analytical error, random variability) differences of veterans' level of 2-3 times controls could be detected at the 95% confidence level. Pattern recognition profiling for pesticides and other PCDDs and PCDFs may offer a means of screening for confounding exposures and variables.

Variability of possible PCDD exposure while in Vietnam: Such variability is a recognized feature of the data set. An analogous variability, it may be argued, obtains with respect to non-phenoxy herbicide sources of TCDD exposure for the comparison groups as well.

Exposure variability may be reflected in data point spread the magnitude of which cannot be predicted in advance. The possibility of such a spread does not invalidate the need to obtain the data; awareness of this factor is necessary to the careful interpretation of and extrapolation from the resultant data but does not invalidate the usefulness of the study.

Variability in the time interval between possible donor exposure to TCDD in Vietnam and specimen collection: This time delay is a known variable for the veterans and will be determined for each donor from the military service record and the pathologist's report. The time delay between non-herbicide TCDD exposure and sample collection is an unknown variable for both the veterans and the non-veteran population.

Study Advantages

Advantages of proceeding with this study through to the reassessment of feasibility stage include the opportunity to refine and validate the chemical methodology for the analysis of dioxins and furans in adipose tissue and, by means of the interlaboratory analysis of the reference fat, an objective assessment and comparison of the capabilities of many laboratories currently performing dioxin and furan analysis.

Should it be deemed appropriate to proceed with the analysis of the archived veterans' and non-veterans' tissues, additional advantages include 1) the possibility of assessing U.S. national baseline levels of selected dioxins and furans in human adipose tissue, 2) rapid generation of study results by virtue of the retrospective nature of the study design, possibly within a year of commencement of actual specimen analysis 3) the possibility of detecting additional sources of dioxin and furan contamination (e.g., fly ash, fish, hexachlorophene, and pentachlorophenol) in both veterans and non-veterans by pattern recognition techniques, and 4) the generation of data important in the regulation of hazards. The specimen bank available for this study represents the world's only known collection of adipose tissue samples from a large number of males of the Vietnam veteran age group. For the actual in-country Vietnam veterans represented in the donor population, many of the specimens will have been obtained within a relatively short time following herbicide (and possible dioxin) exposure. The analysis of these specimens would be of great interest to all concerned with the Agent Orange issue.

Results of Analysis of TCDD in Human Adipose Tissue^a

VA code number	Concentration (ppt) ^b	Detection limit (ppt)	Percent recovery	Ratio ^c
<u>"Heavily Exposed Veterans"</u>				
10	23	4	65	.85
10	35	9	100	.75
19	ND ^b	3	20	--
26	99	10	90	.77
26	63	6	45	--
<u>"Lightly Exposed Veterans"</u>				
1	ND	5	50	--
13	ND	2	80	--
28	7	4	50	.88
28	8	6	40	.78
34	5	3	100	.85
<u>"Possibly Exposed Veterans"</u>				
6	5	3	65	.90
8	5	3	50	.90
9	ND ^a	3	40	--
11	3	2	55	.77
12	9	3	60	.88
14	4	3	65	.74

Results of Analysis of TCDD in Human Adipose Tissue^a
Continued

VA code number	Concentration (ppt) ^b	Detection limit (ppt)	Percent recovery	Ratio ^c
<u>"Possibly Exposed Veterans"</u>				
16	ND	4	60	--
24	5	3	80	.71
24	5	4	45	--
25	12	4	45	--
25	10	3	100+	.78
27	ND	6	100	--
29	13	5	60	.88
30	ND	3	95	--
<u>"Controls"</u>				
5	4	4	65	1.02
7	3	2	60	.92
17	4,3	3	75	.84
18	ND	4	30	--
20	5	4	50	.86
21	6	3	35	1.07
23	8	2	100	.78
23	6	3	55	--
31	7	4	50	.98
32	4	4	60	.74
33	14	7	100	.94

Results of Analysis of TCDD in Human Adipose Tissue^a
Continued

VA code number	Concentration (ppt) ^b	Detection limit (ppt)	Percent recovery	Ratio ^c
<u>"USAF Scientists"</u>				
2	5	2	50	.77
3	4	1	85	.94
4	6	2	50	.76

Sources: Hobson, L., 1983
Gross, M. L., 1984

a sample sizes ranged from 2.2 to 11.6g for each extraction. Internal standard amounts used varied from 2.0 - 2.6 ng/extraction.

b ND= not detected

c Ratio of intensities of m/z 320 and m/z 322. Acceptable values are not 0.78 ± 0.10 .

I. Introduction and Background

A. Scope of the Study

In the midst of the political turmoil surrounding the Vietnam conflict, veterans have sought an answer to the questions "how heavily were Vietnam veterans exposed to 2,3,7,8-TCDD during Vietnam service and are there any adverse health effects from exposure of that magnitude?"

Agent Orange is the code name for the principal defoliating herbicide used in Vietnam between 1965-1971. It was a 50-50 mixture of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) contaminated with 1-50 parts per million of 2,3,7,8-TCDD. Because this dioxin is believed to be responsible for most of the herbicide's toxicity, it has become the focus of much concern in recent years.

The means by which to measure the magnitude of a veteran's exposure to 2,3,7,8-TCDD has remained controversial and frustratingly elusive. As early as 1980, congressional hearings explored the possibility of using 2,3,7,8-TCDD in veterans' adipose tissue to substantiate exposure to Agent Orange, (Cleland, 1980). Preliminary data which supported this possibility included measurements of 2,3,7,8-TCDD in the adipose tissue of a woman living near the Seveso, Italy, shortly before her death (Facchetti, 1981). In addition, the Agent Orange Working Group, established by the White House Cabinet Council on Human Resources to coordinate and oversee all federal studies related to Agent Orange, saw data in 1983 that suggested a link between 2,3,7,8-TCDD in veterans' adipose tissue and service in Vietnam (Table 1).

No known correlation exists between the exposure levels probable in Vietnam and adverse health effects.¹

Because exposure to TCDD has not yet been proven to correlate with adipose tissue levels, this study seeks to answer a more limited question, "Do Vietnam veterans have higher levels of 2,3,7,8-TCDD in their adipose tissue than their veteran counterparts who were not stationed in Vietnam or than non-veterans?"

Should future work prove an association between TCDD concentrations in adipose tissue and exposure and an association between low level exposure and health effects interpretation of a greater scope may then be possible.

¹ The FDA (Wipf, H.K., 1983) has calculated the "no observable effect level" (NOEL) of 2,3,7,8-TCDD for humans to be 70 ng/man/day. It is possible to perform orders of magnitude calculations for all three exposure routes: dermal absorption, inhalation, and ingestion. The magnitudes of these exposures may then be compared with the NOEL.

Absorption: The dermal absorption of dioxin for hairless rats from a soil-water suspension of 500 ppb is 0.1% (Table 2). Given the soil concentration of dioxin in Vietnam as less than or equal to 0.016 ppb (Young, 1975), the average man covered with a kilogram of dirt would absorb less than 16×10^{-3} ng per day ($16 \text{ ppt} \times 1 \text{ Kg dirt} \times 10^{-3}$ absorption factor). The NOEL of 70ng/man/day is thus over 4,000 times this absorption level.

Inhalation: Inhalation is even less likely to have contributed significantly to exposure. Using the method of diDomenico (1980) the daily amount inhaled from dust containing 16 ppt 2,3,7,8-TCDD would be 0.02 picograms/day or three million times less than the NOEL. Even soil with 1 ppb dioxin would give a daily amount inhaled of 1.4 picograms or 5,000 times less than the NOEL.

Ingestion: Ingestion of contaminated food and water is the final possible exposure modality for veterans. The poor solubility, rapid photodegradability, tendency of 2,3,7,8-TCDD to sink to the subsurface silt of rivers, and minimal uptake by plants negate most food and water sources as significant risks. However, 2,3,7,8-TCDD was determined in South Vietnamese fish at levels ranging from 18 to 810 parts per trillion by non-isomer specific techniques (Baugham and Meselson, 1973). At the average dioxin concentration of 300 parts per trillion in the fish, one could ingest half a pound of fish per day and still be at the NOEL.

Dermal Absorption of Dioxin

<u>Vehicle</u>	<u>Percentage</u>
Methanol	40
Polyethylene glycol	25
Vaseline	4
Soil-water	
26 ppm	6
0.5 ppm	0.1
Activated carbon	
26 ppm	<0.1
0.5 ppm	<0.1

From data in Poiger H, Schlatter C:
Fd Cosmet Toxicol 18: 477-481, 1980.
(Soil used in this experiment was from
Seveso, Italy)

Table 2

B. The Agencies Involved

Human exposure to chemicals regarded as environmental pollutants and the health risks they impose have received much public attention. In response to this concern, private industry, academia, and public agencies have committed vast human and financial resources to research the sources of contamination, toxicology, and environmental fate of these chemicals. Much of this research has been focused on the phenoxy herbicides and their 2,3,7,8-TCDD contaminant.

The federal agencies participating in such research include the Veterans Administration (VA), the Environmental Protection Agency (EPA), and the Centers for Disease Control (CDC). The VA, involved for many years with the question of the exposure of U.S. military personnel to Agent Orange, has been conducting and supporting numerous projects. These include on-going health surveillance of veterans (the Agent Orange Registry, Young, et al., 1985), basic research on the toxicology of dioxins, and epidemiological studies of Vietnam veterans such as the Soft Tissue Sarcoma Study and the Vietnam Veterans Mortality Study (Kang, 1984). The VA has supported the CDC's Vietnam Veterans Ground Troop Epidemiologic Study. The EPA, in addition to performing studies of herbicide usage and its impact upon human reproduction (Johnson, 1979 and Nelson, et al., 1979), has been monitoring the concentration of various pesticides in the adipose tissue of the American population for more than a decade via its National Human Monitoring Program. CDC has completed a study of birth defects and their relationship to military service in Vietnam (Erickson, et al., 1984), utilizing the birth defects registry it has maintained in the Atlanta area for many years.

The possibility of performing the present study was investigated recently by Hobson for the VA (Hobson, et al., 1983). He set out to determine whether 2,3,7,8-TCDD was detectable in human adipose tissue. The study was carried out on adipose tissue from three groups of adult males:

1. Twenty veterans who claimed health problems related to Agent Orange exposure;
2. Three U.S. Air Force officers with known heavy and relatively recent exposure in connection with herbicide disposal operations but who did not serve in Vietnam; and
3. Ten veterans with no service in Vietnam and no known exposure to herbicides.

The study results, as shown in Table 1, revealed that very low levels of TCDD, believed to be 2,3,7,8-TCDD, could be detected in human adipose tissue in the range of 3 to 99 parts per trillion (ppt). Further interpretation of the levels, however, could not be made without additional data on background levels of TCDD in the general U.S. population and a larger "exposed" sample size.

The VA recognized in the EPA National Human Monitoring Program's adipose tissue collection, which includes at least 494 Vietnam-era males, the opportunity to continue to explore the relative levels of 2,3,7,8-TCDD in veterans and non-veterans. The EPA and the CDC have acknowledged parallel interests in the development of the analytical methodology to determine 2,3,7,8-TCDD in human adipose tissue for use in future epidemiological studies. The present study, then, represents a cooperative effort by all three agencies.

C. The Compounds of Interest

Polychlorinated dibenzo-p-dioxins and furans are aromatic compounds formed as inevitable contaminants during the manufacture of a wide variety of organochlorine commercial products originating from chlorophenols (e.g., phenoxy herbicides, wood preservatives containing pentachlorophenol, and hexachlorophene). They are also formed during low temperature combustion of wastes containing chlorinated precursors. The number of chlorine atoms in the dioxins and furans can vary from 1 to 8 producing up to 75 PCDD and 135 PCDF positional isomers. Toxicities vary widely with the structures. The 2,3,7,8-substituted dioxin and furan are the most toxic. Agent Orange was contaminated with only this 2,3,7,8 dioxin and no other dioxins or furans.

The chemical structures of these compounds are given in Figure 4.

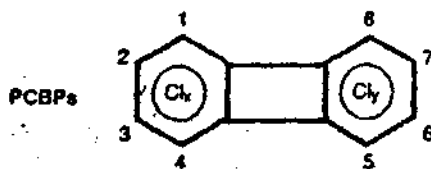
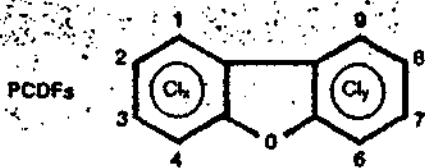
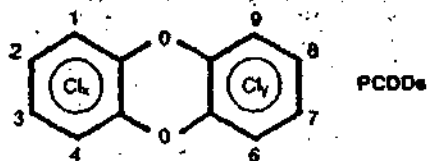
Dioxin and Furan Formation: Agent Orange is contaminated with dioxin from 2,4,5-trichlorophenol, the precursor to 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and its parent compound, tetrachlorobenzene through the intermediate 2,4,5-trichlorophenate (Figure 6). Mass action considerations favor the formation of the furan early in the production of trichlorophenol when the concentration of the phenate is low, (Figure 7), while dioxin formation is favored later in the reaction when there is a higher concentration of trichlorophenate, thereby increasing the probability of dimerization between two phenate molecules (Figure 8).

Methodological modifications in the manufacturing process have decreased the amount of 2,3,7,8-TCDD to less than 0.1 ppm (generally 0.01 ppm) in the 2,4,5-trichlorophenol being produced today.

Toxicology: 2,3,7,8-TCDD displays wide species variability in toxicity (Table 3). While touted as one of the most toxic chemicals known to man (Sun, 1983), it is interesting that the LD₅₀ of 2,3,7,8-TCDD is of the same order of magnitude as the standard cardiac medication digitoxin (Table 4).

Knowledge of sublethal human dioxin toxicity comes from studies of survivors of the more than twenty industrial accidents worldwide and of the property owners and children present during the contamination of a Missouri Horse Arena by dioxin-containing waste oil in 1971. Principal target organs injured by 2,3,7,8-TCDD include skin (chloracne), liver (elevated liver function tests), and nervous system (peripheral neuropathy). Human systemic toxicity is unusual in the absence of

FIGURE A
Structures of PCDDs, PCDFs, and PCBPs



$x + y = 1-8$

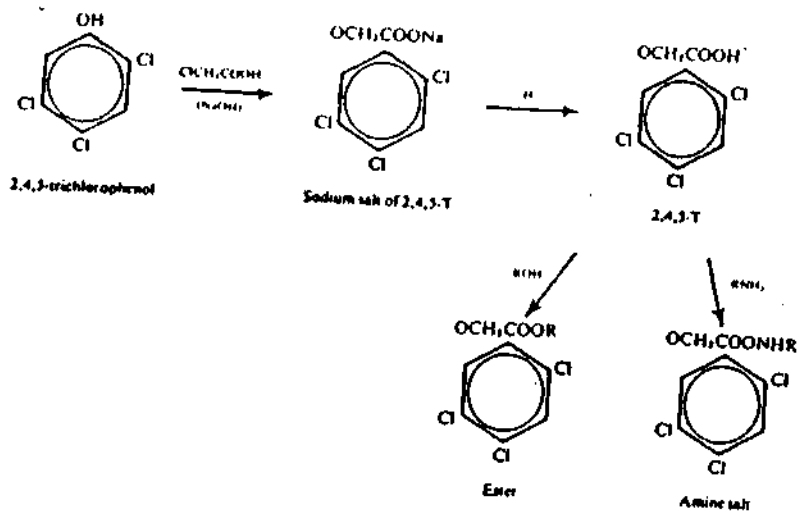
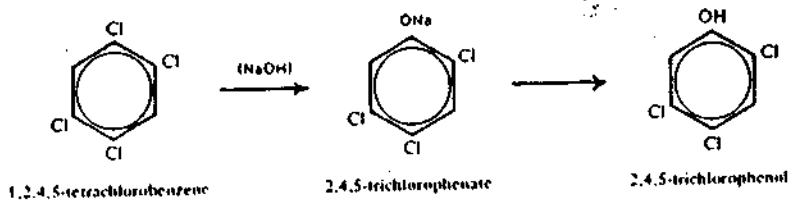


Fig. 5

Formation of 2,4,5-T and its ester and amine salt



Hydrolysis of tetrachlorobenzene to 2,4,5-trichlorophenol.

Fig. 6

chloracne, which itself has come to be regarded as a form of systemic toxicity. While reproductive effects such as birth defects, abortions, and carcinogenesis have been demonstrated in animals after chronic exposure, they have never been proven in humans.

There is recent evidence (Birnbaum et al., in press; Rizzardini et al., 1983, and Weber et al., 1984) that, at least in animals, dioxin toxicity is modified by the contaminant presence of polychlorinated biphenyls (PCB's) and furans.

Partitioning and Metabolic Fate: Data from the Seveso, Italy, industrial accident (Table 5) and from animal work (Gasiewicz, 1983) indicate that adipose and liver tissue are the major storage sites for 2,3,7,8-TCDD and other dioxins and furans. Adipose to plasma 2,3,7,8-TCDD concentration ratios approximate 100-1,000 to 1 in humans.

Knowledge of human metabolism is incomplete, but studies of patterns of dioxins and furans present in the blood and liver of exposed people (Rappe, 1983, and 1979, Masuda, 1982) suggest that the rate of excretion of 2,3,7,8 isomers is slow (incomplete eleven years after exposure) compared to less toxic isomers with two adjacent unsubstituted carbons and compared to animals such as rodents, in whom 2,3,7,8-TCDD is reported to have a half life of only a few weeks (Rose, et al., 1976).

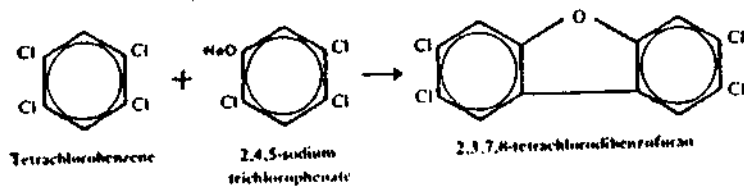


FIG. 7 Condensation reaction involving tetrachlorobenzene and sodium trichlorophenate to form 2,3,7,8-tetrachlorodibenzofuran.

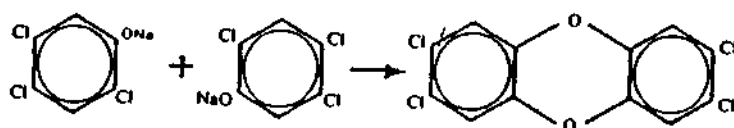


FIG. 8 Condensation of 2 molecules of sodium trichlorophenate to form 2,3,7,8-tetrachlorodibenzo-p-dioxin.

Single Dose LD₅₀ Values for TCDD

<u>Species</u>	<u>µg/kg body weight</u>
Guinea pig	2
Rat, female	25
Rat, male	60
Monkey	50
Rabbit	115
Mouse	
C57B1/6J	132
DBA/2J	620
B6D2F ₁ /J ^a	300
Hamster	>3,000

From Neal RA, et al: Drug Metab Rev, 13: 355-385, 1982.

Table 3

Relative Toxicity of Several Chemicals

Agent	Lethal Dose (<u>µg of chemical</u>) (kg of body wt)
<hr/>	
	*
Dioxin	50
Hydrogen cyanide	1,000
Cyanide salts	2,000
Cantharidin	900
Nicotine	600
Colchicine	100
Digitoxin	50

From Dunagin, W.G.: J. Am. Acad. Derm. 10: 688-700, 1984

Table 4

TCDD in TISSUES of

a SEVESO WOMAN

<u>Organ</u>	<u>PPI</u>
Fat	1840
Pancreas	1040
Liver	150
Thyroid	85
Brain	60
Lung	60
Kidney	40
Blood	6

From Facchetti et.al., 1981.

Table 5

(Rappe, 1983, and 1979, Masuda, 1982) suggest that the rate of excretion of 2,3,7,8 isomers is slow (incomplete eleven years after exposure) compared to less toxic isomers with two adjacent unsubstituted carbons and compared to animals such as rodents, in whom 2,3,7,8-TCDD is reported to have a half life of only a few weeks (Rose, et al., 1976).

D. Study Population

The EPA has been conducting the National Human Adipose Tissue Survey (NHATS) since 1970, collecting over 21,000 adipose specimens from a representative sample of the U.S. population. These specimens were collected by the Field Studies Branch (FSB) of EPA's Office of Toxic Substances (OTS) through the National Human Monitoring Program (NHMP) for the purpose of assessing human exposure to organic chemicals including pesticides. Unused portions of 8,000 of the original 21,000 specimens remain and are presently archived in Kansas City.

The VA has identified in these archived tissues the opportunity to perform dioxin analyses on a large number of human adipose tissue specimens, including a subset of 494 men born in the Vietnam-era years of 1937-1952. At least 120 of these specimen donors have been identified as U.S. veterans, and at least 36 of these veterans have served in Vietnam. It is probable that specimens from approximately 50 Vietnam veterans will be available for this study. The identification process is currently in the final stages.

The total number of Vietnam veterans will be an important determinant of the statistical power of the study (see Section IV., pp. 45 ff).

E. Analytical Techniques

Analytical chemical techniques now exist capable of analyzing dioxins and their relatives, the furans, at the parts per trillion level. These techniques are refinements of the combination of gas chromatography and mass spectrometry originally developed by Baughan and Meselson (1973).

Because laboratories working in this field differ somewhat in their methodological approaches to dioxin and furan analysis and because no single consensus method exists, the VA requested EPA assistance in developing and validating a narrower spectrum of methods for the reliable, precise, and sensitive determination of 2,3,7,8-TCDD in human adipose tissue.

Under EPA direction, the Midwest Research Institute (MRI) in Kansas City completed an extensive literature review and coordinated several peer reviews of the existing analytical chemical detection methodology for 2,3,7,8-TCDD and related compounds at the parts per trillion level. Based upon this research, MRI has outlined a summary of analytical methods incorporating the basic techniques used by the relatively few laboratories capable of performing isomer-specific analyses of dioxins, furans, and other polyhalogenated aromatic compounds in adipose tissue and other biological tissue (see page 59). The MRI summary is meant to serve as an operational framework for the laboratories participating in this study, imparting some homogeneity in methodology while not imposing strict uniformity of detail. Some methodologic variations among laboratories are thus to be expected during the course of this study.

Comparability of the data generated by the different laboratories will be assessed by means of the "interlaboratory study" (Section IV, page 67). In this study the 30 kilograms of reference fat will be completely homogenized, spiked with known dioxins and furans, and sent to all participating laboratories for analysis. Pattern recognition techniques will then be used to analyze results, thereby assessing the precision and accuracy of the analytical methods and the relative capabilities of the involved laboratories.

II. Program Objectives

A. Assay Development

The initial objective of this multi-agency research program is the further development and validation of a reliable, precise, and very sensitive (parts per trillion) method for the determination of 2,3,7,8-tetrachlorodibenzo-p-dioxins and possibly other polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzo-p-furans (PCDFs) that may occur in human adipose tissue.

B. National Baseline Levels of TCDD

A second objective is to determine whether these compounds can be detected in the fat of the American adult male population; levels detected will serve as a baseline estimate of background levels which may exist in the general population.

C. Comparative TCDD Levels in Vietnam Veterans

A third objective is to discover whether individuals who were in military service in Vietnam have significantly higher levels of 2,3,7,8-TCDD than other American men.

D. Identification of Non-Phenoxy Herbicide Sources of TCDD

A fourth objective is to identify residues of non-phenoxy herbicide sources of TCDD by means of computerized pattern recognition analysis for selected dioxins, furans, and other halogenated aromatic compounds which characterize these sources. (2,3,7,8-TCDD may be found in non-herbicide sources such as pentachlorophenol, fly ash, hexachloropene and fish. Thus, exposure to such sources may contribute to the total body burden of TCDD.)

III. Statement of Work

Achievement of the first three program objectives (assay development, national baseline TCDD levels in adipose tissue, comparative TCDD levels in Vietnam Veterans) will involve three phases: 1) sample characterization and study design, 2) analytical method of development and ancillary study design, and 3) actual dioxin and furan analysis; completion of ancillary studies.

Phase I: Sample Characterization and Study Design

<u>Goal</u>	<u>Responsible Agency/ (Investigator)</u>	<u>Status</u>
A. Inventory of remaining number of adipose specimens in NHATS as of 1982 (= 8,000 remaining from original 21,000)	EPA-MRI	Completed
B. Relocation of the 8,000 adipose specimens to Kansas City from the National Space Technology Laboratory at Bay St. Louis, Mississippi	EPA-MRI (Hosenfeld)	Completed
C. Choice of selection criteria for adipose specimens: male, dates of birth 1937-1952, 3-5 grams of tissue.	VA	Partially completed (specimen weight determination in progress)
D. Inventory of 8,000 specimens for specimens meeting selection criteria (1c above) - (- #528) by merging Master and Inventory files.	EPA	Completed

Phase I: Sample Characterization and Study Design

<u>Goal</u>	<u>Responsible Agency/ (Investigator)</u>	<u>Status</u>
E. Information Retrieval: Identification of specimens from Vietnam veterans donors		
1) Contact hospital pathologists for social security numbers, and/ or names, dates of birth of donors (#494 specimens)	EPA (Carra, Remmers Frankenberry)	Completed
2) Determination of Vietnam Service status of the 494 specimens.	VA (Kang)	In progress
F. Specimen characterization		
1) Identification of 6,783 archived adipose specimens collected 1972 - 1983 with previously detected levels of ortho- para DDE, hexachlorbenzene (HCB), and chlordane component trans- nonachlor for reanalysis of these compounds (Purpose: assessment of their stability in archived adipose tissue- see Section III. L, Statement of Work)	EPA (Colorado State Tessari)	Completed
2) Assessment of existing pesticide and PCB data (ortho- para DDE, HCB, Trans non, PCB's) for the 528 study specimens so that reanalysis of these compounds during Phase III will yield stability informa- tion.	EPA (Hosenfeld) VA (Flicker)	Proposed
G. Statistical design and sample size-power calculations	EPA (Carra) VA (Kang)	Completed
H. Drafting of preliminary study proposal for presentation to AOWG (This document)	VA (Flicker, Kang) EPA (Carra)	Completed

Phase II: Analytical Method Development and Ancillary Studies Design

<u>Goal</u>	<u>Responsible Agency/ (Investigator)</u>	<u>Status</u>
A. Scientific meetings of nationally recognized experts to define requirements of an acceptable analytical method and to present state-of-the-art data.	EPA/VA/MRI	Held April 1983 (MRI) Held September 1983, VACO
B. Review of the scientific literature and statement of a proposed analytical method	EPA/MRI (Stanley)	Completed
C. Intralaboratory trials of extraction and analytical methods for "ruggedness," i.e., precision, practicality, tolerable range of methodological variation which yields reliable results.	EPA/MRI (Stanley)	In Progress
D. Collection of 30 kg reference adipose tissue to generate a homogenous sample matrix for interlaboratory methods validation and quality assurance program.	EPA (Strassman-Sundy) MRI (Stanley, Hosenfeld)	Completed
E. Homogenization and spiking of the reference material with known analytes (PCDD's, PCDF's, etc).	EPA/MRI (Stanely) VA (Flicker) FDA (Firestone)	Proposed
F. Provide on-site VA physician at MRI as Co-Investigator.	VA (Flicker)	Completed
G. Presentation of Protocol to AOWG	VA (Shepard, Kang, Flicker)	Proposed

Phase II: Analytical Method Development and Ancillary Studies Design

<u>Goal</u>	<u>Responsible Agency/ (Investigator)</u>	<u>Status</u>
H. Establish a repository of PCDD/PCDF standards of known quality: 1. Inventory existing research teams (MRI, NBS) 2. Investigate commercial sources, (Pathfinder labs, Cambridge Isotope Labs, Stohl/Kor Isotopes)	EPA/MRI (Stanley) VA (Flicker)	Proposed
I. Develop a quality assurance program	EPA/MRI (Stanley) VA (Flicker)	Partially
J. Interlaboratory Study 1. Compilation of names, address, telephone numbers of participating laboratories	VA (Flicker)	In Progress
2. Compilation of laboratory instrumentation capabilities of participating labs (e.g. negative chemical ionization)	VA (Flicker)	In Progress
3. Full characterization of reference material (II D, II E) and bioincurred samples (II M) for PCDD's, PCDF's and interfering substances by interlaboratory validation	EPA/MRI (Stanley) EPA (Harless) USDI/FWS (Stalling) FDA (Firestone) CDC (Bayes) U. of Nebraska (Gross) U. of Umea, Sweden (Rappe) Wright State U., Ohio (Tiernan, Taylor) Health Protection Branch, Canada (Ryan, Tosine) Monsanto (Hileman) Dow (Shadoff)	Proposed

Phase II: Analytical Method Development and Ancillary Studies Design

<u>Goal</u>	<u>Responsible Agency/ (Investigator)</u>	<u>Status</u>
K. Pattern recognition study of dioxin, furan data from the interlaboratory analysis of the 30 kg. homogenized reference tissue (Purpose: choice of best computer program for this study; quality control; possible dioxin source information)	USDI/FWS (Stalling) U. of Illinois (Dunn)	Proposed
L. Stability Study: Design a study of the effects of long-term cold storage on TCDD levels in adipose, including freeze-thaw conditions	VA (Flicker) Monsanto (Hileman)	In Progress
Design a similar study of ortho-para DDE, HCB, Trans-non, and PCB's as TCDD surrogates	EPA	Completed
Stability Study: analysis TCDD	Monsanto (Hileman) Health Protection Branch, Canada (Ryan) EPA (Harless)	In Progress (1986 Completion Goal)
Surrogates	Colorado State (Tessari) for EPA	In Progress (1986 Goal)
M. Design and initiate study of "biologically incurred" TCDD - i.e. radioactivity labeled TCDD fed to animals. Adipose from these animals would be analyzed as part of the quality control program to calculate true TCDD extraction efficiencies.	NIEHS, Triangle Park, NC (McKinney) VA (Flicker)	Proposal simultaneous ancillary study

Phase III: Actual Analysis

A. Analysis of archived fat samples for dioxins and furans (and related "marker" chemicals)	EPA/MRI (Stanley)	Proposed
B. Pattern recognition analysis or archived fat data	EPA/MRI (Stanley) U of IL (Dunn) USDI/FWS (Stalling)	Proposed
C. Statistical interpretation of the analytical and pattern recognition data	EPA (Carra) VA (Kang)	Proposed
D. Preparation and coordination of final report.	VA (Flicker)	Proposed

IV. Study Plan

This section contains expansions of selected portions of the statement of work.

Background of the Adipose Tissue Specimen Collection (Phase I: A-D)

The National Human Adipose Tissue Survey (NHATS) is a national, statistically designed survey to monitor human adipose tissue for selected chemicals and pesticides. Up to 1,000 adipose tissue specimens are collected annually by pathologists and medical examiners across the country, analyzed by participating laboratories, and sent to the central facility for data archiving and storage.

After geographical stratification of the forty-eight co-terminous states into areas coinciding with the nine Census Divisions, the Primary Sampling Units (PSU's) are selected, two to seven Standard Metropolitan Statistical Areas (SMSA's) from each Census Division, for a total of forty. One or more hospitals or medical examiners are selected from each SMSA to comprise the Secondary Sampling Units (SSU's). At the final stage, the specimens themselves are selected at each facility according to age, sex and race quotas, and with a preference for sudden traumatic death (for specimens from the medical examiners) so as to preclude any disease condition that may be a potential confound to the chemical level detected.

The sampling scheme provides a representative sample of SMSA's, selected with probabilities proportional to their populations. The number of SMSA's selected within a Census Division is determined by its relative population with respect to the general U.S. population. Within each SMSA the hospitals, medical examiners' offices, and specimens are selected in a nonprobabilistic manner, based on the EPA guidelines and judgment of the cooperating professionals. Quotas are specified, however, for age, sex and race of the individuals, so that the sums of the weights assigned for these categories equal the appropriate population counts for each Census Region. Statistical analyses of the data from these samples have consequently assumed that the specimens within the SMSA's are representative of the target population of non-institutionalized persons living in the corresponding Census Division. Given the demographic quotas specified and the weighting scheme based on "approximate" probabilities of selection, this has not been an unreasonable assumption. Furthermore, unpublished data have shown no evidence of differences between specimens from the sample of cadavers and those from living surgical patients.

Figure 9 depicts the multistage sampling process. Specimens are collected, frozen, shipped to the laboratory, and analyzed for over twenty organochlorines and pesticides. Only one chemical analysis is performed on each specimens, and any tissue remaining after the analysis is archived and stored for the possibility of use in future studies such as the present one.

In order to ensure uniformity of sampling technique, EPA prepared a detailed protocol which was distributed to all participating pathologists (Appendix). Personal site visits were made by EPA-FSB officials to each pathologist to review and clarify the procedure.

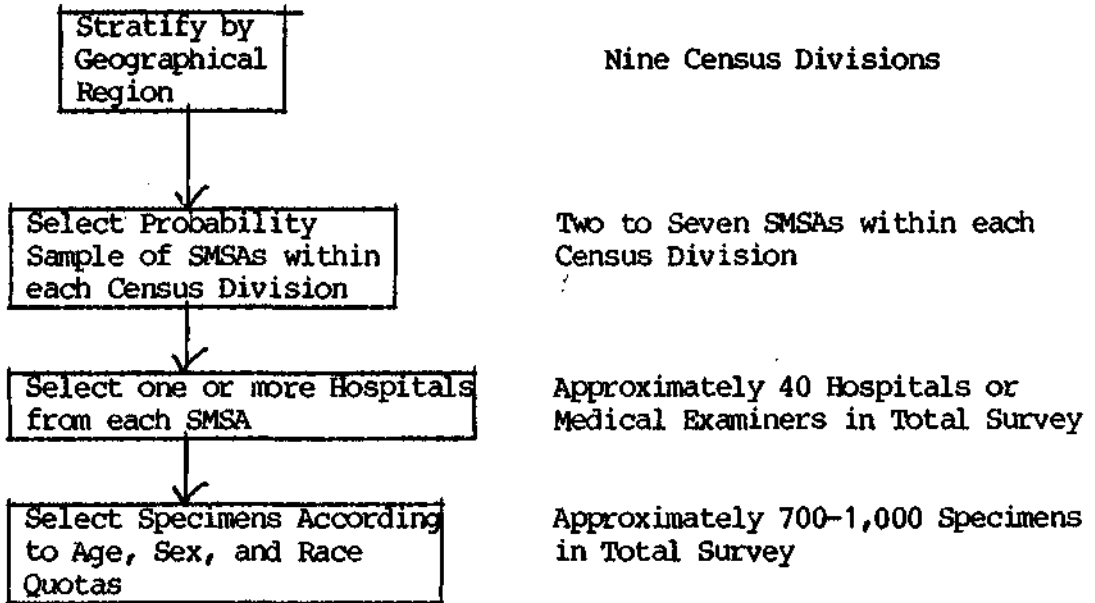


Figure 9. Overview of the Sample Selection Process

Information Retrieval (Phase I E)

In order to select and define a subgroup of Vietnam veterans from among the archived tissues, identifying information leading to the determination of military status of the individual contributors was necessary. Since the initial operation of NHATS in 1970, EPA has never collected any individual identifying information on the NHATS specimens. The NHATS Master File of information on 21,000 specimens has contained the age, race, and sex of the donor, donor initials, hospital or autopsy identification number and date of collection of the specimen. No personal identifying information, such as name or Social Security Number (SSN) has ever been requested, and all chemical results have always been reported in the aggregate for sex, age, race, year or region of the country.

The initial task, therefore, was to inventory the specimen and data banks and determine first, of the 21,000 specimens ever collected, how many had adequate tissue remaining for the analysis; and secondly, of the remaining tissue samples, how many met the criteria of age and sex of the donor to have been contributed by Vietnam veterans.

To this end an extensive inventory of the sample bank was carried out in 1982 to record how many specimens from each year of collection and analysis remained, the weights of the remaining specimens, location codes, and hospital/facility/patient identification numbers for each specimen in the bank. A new data file was created for the 8,000 inventoried specimens that were found to remain. The first limiting factor for the study, therefore, was the number of specimens remaining in the bank, a total of just under 8,000.

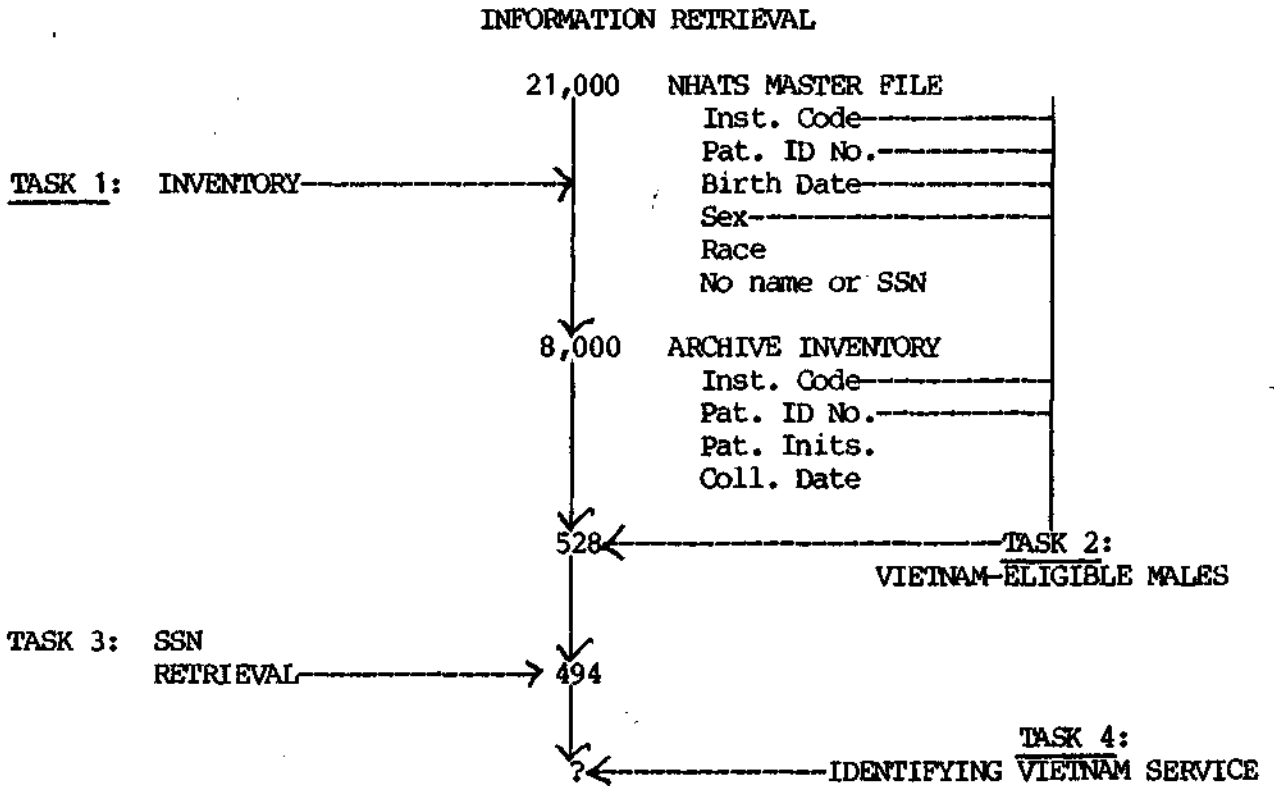
Next, criteria of eligibility for service in Vietnam were set by the VA to be all males born between the years of 1937 and 1952, inclusive. A second task was then to merge Master File with Inventory File to determine which of the specimens remaining in the inventory were donated by males born between 1937 and 1952. This effort yielded a total of 528 specimens eligible for analysis, the second limiting factor for the study.

The third and most sizeable task has been the effort to reconstruct the hospital or medical examiner originally collecting each of the 528 specimens to obtain enough identifying information on the donor of the specimen to determine his military status. A list of the relevant specimens of interest was supplied to each facility by patient/autopsy number and year of collection. Initially, the Social Security Number (SSN) and the autopsy/pathology report for each of the donors was

requested, with name still removed. When it became apparent that roughly only about 60% of hospitals use or record SSN's from their patients, names were requested from the facility to accompany the date of birth and/or death information in identifying each individual. The collection effort has yielded this information for 494 of the 528 specimens, or close to 94%.

The final limiting factor for the study will now be the number of Vietnam veterans identified from among the 494 Vietnam-era males whose names/SSN's have been supplied by EPA and the VA to the National Personnel Records Center for determination of Vietnam service. These Vietnam veterans, if sufficient number exist, will then be matched with either one or multiple comparison or "control" cohorts for the analysis of dioxin in the tissues. Figure 10 depicts these steps in the identification and retrieval effort.

Figure 10



- Define potential Vietnam Veteran—male, born 1937-1952.
- Match archive inventory with master file to obtain potential Vietnam Veterans
- Contact institutions to obtain social security numbers.
- Check against VA and DoD records to determine Vietnam service.

Study Design (Phase I G)

Before beginning the chemical analysis of the specimens, it will be necessary to determine if an adequate number of Vietnam veterans exist in the sample to detect significant differences from the "controls," if any exist. A simple design of one-to-one matching was employed for the purpose of doing preliminary sample size-power calculations.

Theoretically matching by age, race, and geographic location of each Vietnam veterans one similar Vietnam-era male from the pool of the 494, calculations were made of the ratio of dioxin levels that could be detected between the two groups, given likely sample sizes and likely variability from a number of sources. The factors that contribute to the overall variability in the analysis are:

1. Specimen age;
2. A combined variability due to the analytical measurement process random variation in dioxin levels from year to year.

Figure 11 shows the assumptions made about the specimen age differences in calculating the differences between the groups:

- for sample sizes of 30, 60, and 120 Vietnam veterans;
- for statistical false positive and false negative rates of 5% and 5% or 10% and 10%;
- for an overall combined variability of low, low-to-moderate, moderate-to-high, and high.

The model for these calculations and for the study design assumes that deterioration of the specimens over the years, if any, is at a constant rate, that any change in average dioxin level in the population from year to year is at a constant rate, and that the Vietnam veterans and controls are a random sample of the specimen-age distribution of the eligible specimens. While there is no reason to expect any deterioration of the samples from year to year, first assumption will be checked, at least for the organochlorine data, in an independent study.

STATISTICAL POWER CALCULATIONS

- Sample Sizes:
 - 30, 60, 120
- False Positive (alpha) and False Negative Rates (beta):
 - alpha = 5% and beta = 5%
 - alpha = 10% and beta = 10%
- Specimen Age:
 - Any deterioration of specimens is at a constant rate
 - Any change in average dioxin level from year to year is at a constant rate
 - Veterans and controls are a random sample of the specimen-age distribution of the eligible specimens
- Variability:
 - Analytical measurement variability: 10% coefficient of variation (C.O.V.)
 - Random variability: Low: 40% C.O.V.; High: 100% C.O.V.

Combine to give a measure of overall variability

Figure 11

In additional, the model does not take into account the varying lengths of time between exposure to TCDD in Vietnam for each individual and the date of his specimen collection. Estimation of the importance of this omission awaits further advances in the understanding of human dioxin metabolism.

Table 6 gives the ratios of dioxin levels of Vietnam veterans to "controls" that would be detectable for sample sizes of 30, 60, and 120 Vietnam veterans, allowing for a false positive and false negative rate of 5% each. Detectable ratios range from 1.3-to-1 for the large sample size of 120 (Vietnam veterans) with overall low variability, to 7.0-to-1 for the smallest sample size of 30, given high overall variability. With this one-to-one matching, at the 5% level of allowable error, the expected sample size of 30 to 60 with an overall variability of moderate-to-high would yield detectable differences for levels in the Vietnam group that were two to three times higher than those in the "controls".

Table 7 shows the same calculations, given a slightly relaxed level of allowable error, a 10% chance of false positive and false negative results. Here the range of detectable ratios between the two groups runs from 1.2-to-1 in the best case to 4.5-to-1 for the smallest sample size with highest variability. The detectable ratio of levels between the two groups, given expected variability and sample size of 30 to 60 is approximately 2 to 1.

The sample pool of 494 specimens, however, affords the possibility for multiple matching of "controls" to Vietnam veterans and a resulting increasing in statistical power. Independent calculations predict an even better detectable ratio of dioxin levels between the groups, down to 1.5-to-1, Vietnam veterans to controls, with a sample size of 60 and low-to-moderate overall variability. This low ratio of detectable differences can become even more sensitive as precision increases in the laboratory.

Ratio of Dioxin Levels in Vietnam Veterans, to those of Controls, Detectable with Different Sample Sizes (False Positive Rate = 5%, False Negative Rate = 5%).

Sample Size	<u>Overall Variability</u>			
	High	Moderate to High	Moderate to Low	Low
30	7.0	3.1	2.2	1.7
60	3.9	2.2	1.8	1.4
120	2.2	1.8	1.5	1.3

Ratio of Dioxin Levels in Vietnam Veterans, to those of Controls, Detectable with Different Sample Sizes (False Positive Rate = 10%, False Negative Rate = 10%).

Sample Size	<u>Overall Variability</u>			
	High	Moderate to High	Moderate to Low	Low
30	4.5	2.4	1.9	1.5
60	2.9	1.9	1.6	1.3
120	2.1	1.5	1.4	1.2

The Analytical Method (Phase II A,B)

I. General Considerations

(Results of Scientific Meetings and Literature Review)

A. Method Capability

Analytical methods selected for the study should, given sufficient sample size, be capable of:

1. Isomer specific determination of 2,3,7,8-TCDD
 2. Determination of higher-chlorinated 2,3,7,8-substituted PCDD's and PCDF's
 3. Measurement of total PCDD's and PCDF's by homolog
 4. Detection of 1,3,4,6,7,9 (or 1,2,4,6,8,9) hexachloroxanthene
- (See section on Pattern Recognition, pages 70-77, for the rationale for points 2-4 above).

B. Instrumentation Modalities and Sample Preparation

1. Analytical determination of TCDD:

The scientific meetings held at MRI in April 1983, resulted in the following assessment of the instrumentation necessary to detect TCDD in human adipose tissue at the parts per trillion level (Stanley, 1984):

a. Instrumentation

"It was a consensus that mass spectrometry is necessary for the identification and quantitation of PCDDs and PCDFs. The criteria for qualitative identification of PCDDs and PCDFs are similar regardless of whether low resolution or high resolution mass spectrometry is used for analysis. These criteria include (1) coincident response of at least two ions characteristic of the molecular ion cluster of a specific homolog, (2) the proper ion response ratio, and (3) the correct retention times. In addition, response of a fragment ion characteristic of the loss of COCI is necessary to confirm the presence of a PCDD congener.

Electron impact ionization mass spectrometry was presented as the most useful for analysis of PCDDs and PCDFs. It was pointed out, however, that other mass spectrometry methods, negative ion chemical ionization in particular, are applicable to the analysis of specific PCDD or PCDF congeners. The alternate mass spectrometry methods also provide additional sensitive confirmatory information."

RELATIVE EFFICIENCY OF VARIOUS METHODS USED
AT EACH STAGE OF ANALYSIS

Method ^a	Description
Stage I: sample preparation	
L	Chemical treatment and/or extraction without chromatograph
M	L + column chromatography
H	M + HPLC
Stage II: sample introduction	
L	No gas chromatography (direct probe)
M	Packed column GC
H	Capillary column GC
Stage III: mass spectrometry	
L	Low resolution (300-2,000)
M	Medium resolution (>2,000-9,000)
H	High resolution (>9,000)

Source: Mahle, N. H., and L. A. Shadoff, "The Mass Spectrometry of Chlorinated Dibenzo-p-dioxins," Biomedical Mass Spectrometry, 9:45-60 (1982).

^a L = Low, M = medium, H = high.

Table 8

Two years earlier, Mahle and Shadoff arrived at similar conclusions, rating the combination of gas chromatography/mass spectrometry the most highly efficient modalities in the analysis of the PCDD's (Table 8).

b. Detection Limits

Method detection limits for analysis of 2,3,7,8-TCDD were estimated at 1 to 5 parts per trillion (ppt), providing that the original sample size is at least 1 to 3 g. It was recognized by the meeting participants that this small sample size may not be sufficient to allow analysis for other PCDDs and PCDFs. The only means of extending a small sample for the analysis of all PCDDs and PCDFs is to isolate the different chlorinated homologs using liquid chromatography techniques. Estimates for method detection limits of octachlorodibenzo-p-dioxins and octachlorodibenzofurans ranged from 20 to 100 ppt.

It was generally recognized that the use of high resolution rather than low resolution is based on the extent that potential interferences are removed from the sample extract. If sufficient extract cleanup is achieved, low resolution mass spectrometry is acceptable for the analysis of PCDDs and PCDFs at low parts per trillion."

c. Chemical Interferences

"Compounds that are known to interfere with the analysis of 2,3,7,8-TCDD were presented in the literature review. The potential interferences to the analysis of higher chlorinated PCDDs and PCDFs have not been identified. It was speculated that compounds similar to the interferences for 2,3,7,8-TCDD analysis, but with greater chlorine substitution, may interfere with the analysis of other PCDDs and PCDFs. These compounds include the polychlorinated biphenyls, benzoquinones, benzylphenyl ethers, and diphenyl ethers."

2. Extraction

a. Methods

Three methods for extraction of PCDD's from biological matrices have been reported: Neutral extraction with anhydrous sodium sulfate (Ryan, 1980; Albro and Corbett, 1977; Hass et. al. 1978; O'Keefe, 1978, and Shadoff, 1980); alcoholic potassium hydroxide extraction (Baughman and Meselson, 1973); and acidic digestion (Langhorst and Shadoff, 1980).

While there has been no study directly comparing the advantages of one procedure over another, Firestone (1977) and Lamparski et. al. (1978) have noted that the alcoholic potassium hydroxide process may lead to the destruction of higher chlorinated homologs of the PCDD's (hepta's and octa's). The method described below thus employs the neutral extraction process which has had the most extensive literature verification.

b. Extraction Efficiency

To date extraction efficiencies have been estimated by recovery of carbon-13 or chlorine-37 labelled surrogate PCDD's which had been spiked into the sample prior to extraction.

Such post mortem spiking techniques, however, may not accurately reflect the true extractability of PCDD's and PCDF's bound to the biological matrix into which they were incorporated from exposure during life. Thus the rationale for the "bioincurred" study in this proposal in which carbon-14 radiolabelled PCDD's and PCDF's are fed to (or otherwise introduced into) live experimental animals whose tissues are harvested and analyzed by both radiodetection and GC/MS techniques. In this way the true extraction efficiencies of PCDD's and PCDF's from adipose tissue be determined (See section IV, p. 79).

3. Cleanup

Cleanup procedures, the separation of PCDD's and PCDF's from interferences (PCB's; 2,4-D; ,2,4,5-T, etc.) must be considered with respect to the final instrumental technique chosen. Two such schemes are presented in Figures 12 and 13. When high resolution mass spectroscopy is available, less stringent cleanup may be performed (Figure 12). Pathways to both high and low resolution MS involve bulk matrix cleanup with a modified silica gel to remove excess lipid and oxidizable compounds followed by removal of chemical interferences (PCB's, chlorinated diphenyl ethers) using an alumina macrocolumn. Should separation of PCDD's and PCDF's by homolog be desired, an HPLC step may be necessary (not shown).

With low resolution MS as the final instrumentation, an additional step involving an activated charcoal (carbon) adsorption column is suggested.

II. Proposed Analytical Method for PCDDs and PCDFs in Human Adipose Tissues

The proposed method has been developed from these meetings to provide for the determination of PCDD's and PCDF's, particularly 2,3,7,8-TCDD in human adipose. It utilizes High Resolution Capillary Gas Chromatography/Mass Spectrometry (HRGC/MS) with selected ion monitoring techniques, and is flexible to allow for both Low Resolution Mass Spec (LRMS) and High Resolution Mass Spec (HRMS), depending on the instrumentation of the particular laboratory. The method as proposed is sensitive in the range of 1 to 10 parts per trillion for the analysis of one to ten grams of adipose tissue. It was presented at the September 1983 meeting of the American Chemical Society, with the recommendation for evaluation by intralaboratory ruggedness testing and interlaboratory validation. Preliminary discussions have been held about the possibility of soliciting the assistance of the CDC as referee or participating laboratory for this evaluation. Method detection limits for higher chlorinated PCDDs and PCDFs will vary depending on the degree of chlorination and specific isomer. The analyst should demonstrate that instrumental sensitivity is sufficient to provide identification of the PCDDs and PCDFs of interest (e.g., 1 to 5 pg of 2,3,7,8-TCDD) injected on-column before proceeding with the analysis of actual human adipose samples. This method must be fully evaluated through intralaboratory ruggedness testing and interlaboratory validation before is applied to any study dealing with population exposure to PCDDs and PCDFs.

Summary of the Method

Figure 12 and 13 are schematics of the analytical procedures for final analyses by HRGC/HRMS and HRGC/LRMS respectively. Both procedures require accurate mass measurement of the original adipose tissue specimen. One to 10 g of the sample are spiked with stable isotope labelled PCDDs, typically 1 to 5 ng for each sample. The PCDD and PCDF analytes are isolated along with lipid materials through neutral extraction or alcoholic saponification¹. Excess lipid material in the sample matrix is removed using acid and base modified silica gels. Chemical interference such as polychlorinated biphenyls (PCBs), chlorinated diphenylethers, etc., are separated from the PCDDs and PCDFs using chromatographic separation on an aluminum macro column. The final extract is analyzed at this point by HRGC/HRMS at a minimum resolution (m/m) of 10,000. If HRMS instrumentation is not available the sample extract should be taken through an additional cleanup step using a carbon adsorbent to remove potential interfering compounds. Analysis by HRGC/LRMS may now be performed.

¹ Alcoholic saponification is not recommended should the study compounds of interest include the hepta- and octachlorinated dioxins (see page 56).

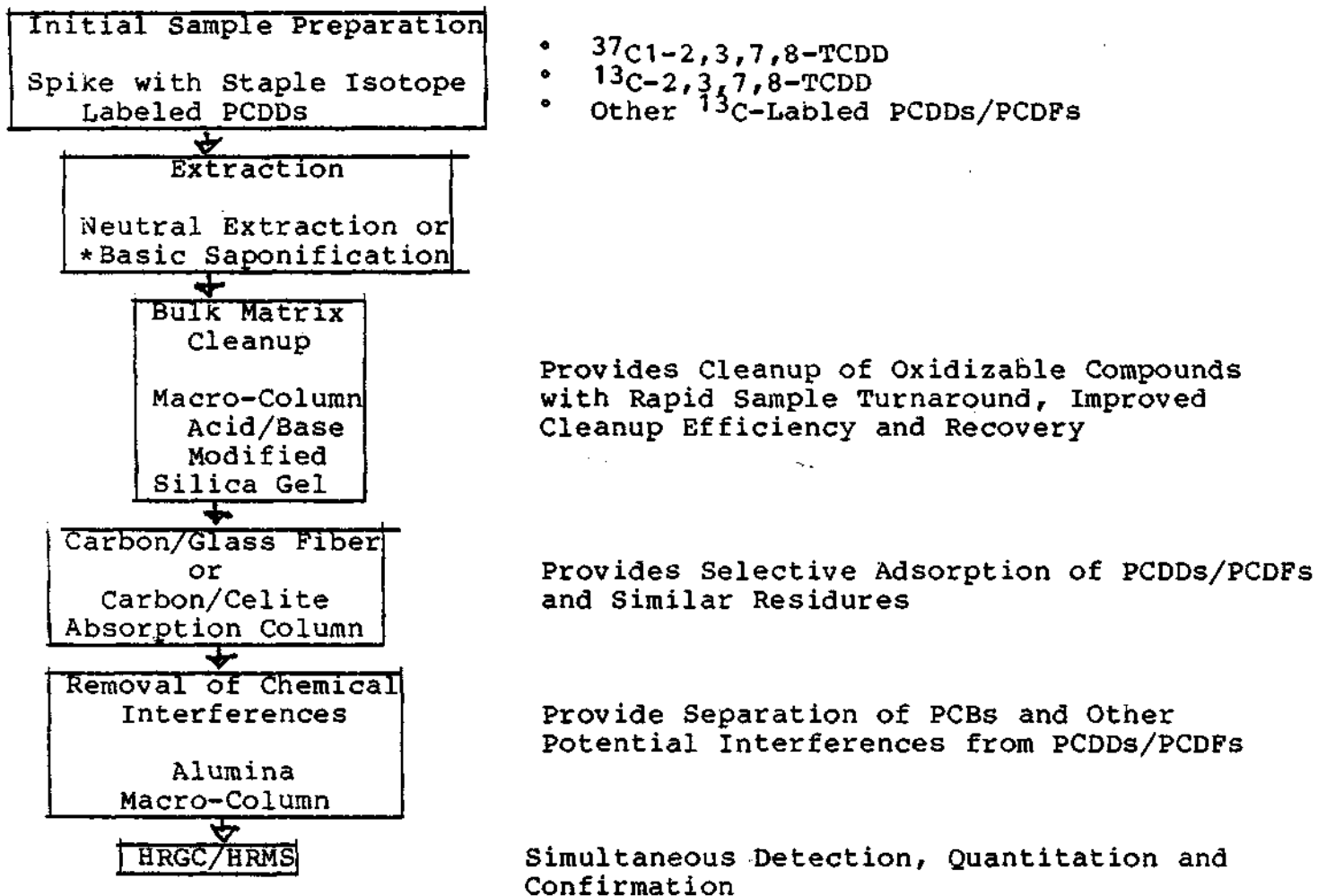


Figure 12. Schematic of proposed analytical method using low resolution mass spectrometry (LRMS).

* See footnote proceeding page

Initial Sample Preparation
Spike with Stable Isotope
Labeled PCDDs

- $^{37}\text{C}1-2,3,7,8\text{-TCDD}$
- $^{13}\text{C}-2,3,7,8\text{-TCDD}$
- Other ^{13}C -Labeled PCDDs/PCDFs

Extraction
Neutral Extraction or
*Basic Saponification

Bulk Matrix
Cleanup
Macro-Column
Acid/Base
Modified
Silica Gel

Provides Cleanup of Oxidizable Compounds
with Rapid Sample Turnaround, Improved
Cleanup Efficiency and Recovery

Removal of Chemical
Interferences
Alumina Macro-Column

Provide Separation of PCBs and Other
Potential Interferences from PCDDs

HRGC/HRMS

Simultaneous Detection, Quantitation and
Confirmation

Figure 12. Schematic of proposed analytical method using high resolution mass spectrometry (HRMS).

* See note on page

A stringent quality assurance/quality control program should be followed to ensure the validity of data. The quality control samples should include blanks, duplicate samples if enough specimen is available, control samples spiked with PCDDs and PCDFs near the desired detection limit, and frequent analysis of blind adipose specimens spiked by an independent quality control laboratory. The HRGC/MS instrumentation must be calibrated daily and consistency of response factors of quantitation standards must be established through correct ion ratio responses at appropriate retention time windows. If isomer specific measurements are necessary, specific HRGC columns will be required to separate isomers of the same homolog group.

Intralaboratory Testing and Method Development (Phase II.C.)

In order to define the limitations and critical variable of the method, "ruggedness testing" will be performed. This involves varying sample sizes, quantities of adsorbent, volumes of solvent, and other methodological parameters. Individual steps of the method will be characterized using adipose tissue spiked with PCDD's and PCDF's; radiolabelled if available to help define critical variables more rapidly than can be achieved with HRGC/MS.

Finally, the total method including HRGC/MS will be challenged with potential interference spiked into the sample matrix.

Tissue Program: Collection of the Reference Fat (Phase II D and E)

A large pool of homogeneous adipose tissue is needed to prepare quality control (Q.C.) samples for the overall QA program and interlaboratory validation studies. It is estimated that the 30 kg of adipose tissue collected to date will be sufficient to prepare a number of control samples at known spiked concentration levels with and without the addition of potential interferences. The samples have been collected

through the National Human Monitoring Program and are now being stored in Kansas City. They will be pooled and possibly rendered to provide a homogeneous matrix that will be subdivided for spiking procedures.

The adipose tissues were collected from male trauma victims within 24 hours after death and frozen until composited for homogenization with other specimens.

A background analysis of the homogenized tissue is necessary to provide information on the levels of PCDD's, PCDF's, and potential interferences. It is recognized that the assistance of the laboratories detailed in the Statement of Work, Phase II, Section J, with experience in the analysis of PCDD's and PCDF's will be of benefit in obtaining this information in the most expedient manner. These background analyses must be completed before proceeding with subdividing the homogenous tissue for spiking purposes in the manner to be described under the QA program.

Standards Program (Phase II. H.)

A repository of standard radiolabelled and unlabelled PCDD and PCDF compounds as well as of potential interferences of known quality is essential to achievement of consistent results from the intralaboratory methods development, interlaboratory study, and final analysis program. These compounds will be used as surrogates -- that is, internal standards, -- for the study. A survey of participating laboratories' inventories is currently underway. In this manner the need for procurement or synthesis of specific congeners will be identified.

Commercially available compounds include PCDD's as carbon-13, chlorine-37, and carbon-14 labelled TCDD. Chlorine-13 labelled 1,2,3,4,6,7,8 heptachlorodibenzo-p-dioxin and octachlorodibenzo-p-dioxins, and carbon-13 labelled 2,3,7,8-tetrachlorodibenzofurans are also available, as are the interference compounds polychlorinated biphenyls and DDE.

Presently unavailable compounds include radiolabelled penta-, hexa- and heptachloro-PCDD's or any of the PCDF's. Less available interferences include the chlorinated diphenyl ethers, chlorinated benzylphenyl ethers, and chlorinated benzoquinones.

Once the purity of all standards and interference is documented and the repository established, distribution of compounds to collaborators may occur, probably by supplying solutions of accurately determined concentrations.

Quality Assurance Program (Phase II. I.)

The quality assurance program will be designed to operate throughout all phases of the study: intralaboratory method development, interlaboratory characterization of the reference fat and bioincurred fat, and actual analysis of the adipose tissue study samples.

The quality assurance (QA) program will:

- a. Provide details for preparation of fortified tissue samples containing PCDDs and PCDFs with at least one isomer from each PCDD and PCDF homolog.
- b. Provide details for tissue samples spiked with potential interferences (compounds known to interfere with the analysis of PCDDs and PCDFs). This type of performance evaluation sample will provide information on the potential for false positive results.
- c. Specify procedures for sample handling, coding and distribution.
- d. Determine frequency of the spiked quality control samples.
- e. Specify data handling and decoding procedures.

Interlaboratory Study (Phase II. J.)

Before proceeding with the analysis of the archived adipose specimens from veterans and controls, interlaboratory study of the homogenized reference fat is proposed. Such a study, in which all participants will receive samples from the homogenized tissue and analyze them by their own individualized methods within the general guidelines of Figures 12 and 13, will permit objective comparison of each laboratory's capabilities as well as define the precision and accuracy of their respective analytical methods.

It will be this data regarding method precision and accuracy, combined with the final total sample size of the study population, which will determine the feasibility of proceeding with Phase II, the actual analysis of veteran and control tissues.

The interlaboratory study will also generate data to be used in the initial pattern recognition study (Phase II. K., pages) to select the computer program most useful for this study.

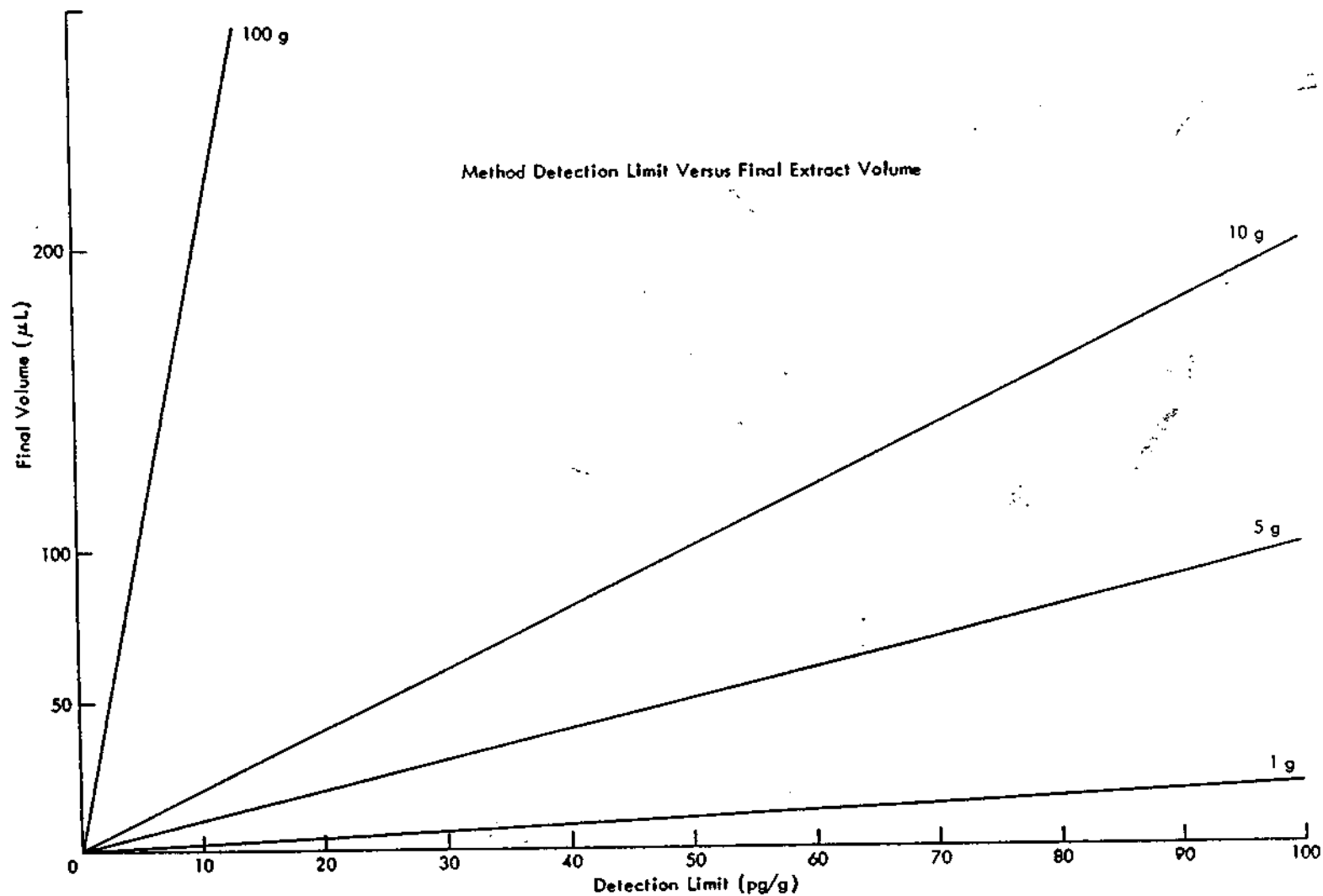


Figure 14. Method detection limit versus final extract volume and initial sample size assuming a GC/MS instrumental detection limit of 5 pg/ μ l on-column. (Stanley, 1984)

Should time and resources permit, a preliminary study may be conducted with samples of known concentration to identify potential difficulties and to modify the guidelines of the analytical methodology suggested on pages .

The full scale interlaboratory study will include:

- a. Samples of the unadulterated homogenized reference fat;
- b. Reference fat spiked with known amounts of PCDD's near the limits of analytical detection;
- c. Reference fat with both PCDD's and interferences and possibly;
- d. Actual adipose tissue specimens from the EPA/VA selected control population as representative of the general population and Vietnam veterans.

Specimens from B and C would be prepared as "Youden¹" pairs (paired samples which differ in the analyte of interest by approximately ten percent). Sample sizes would be varied to explore the relationship of detection limit as a function of sample size (Figure 14) for each laboratory.

¹ See Glossary

A schematic of such a study appears in Figure 15. "TAC" numbers are meant to represent actual archived specimen I.D. numbers.

All samples will be prepared under the quality assurance program guidelines and will be distributed to participants under blind codes.

<u>Sample Method Validation</u>	<u>Laboratory</u>							
	A	B	C	D	E	F	G	H
Fat	X	X	X	X	X	X	X	X
Fat	X	X	X	X	X	X	X	X
Fat + Interf.	X	X	X	X	X	X	X	X
Fat + Interf.	X	X	X	X	X	X	X	X
Fat + PCDD	X	X	X	X	X	X	X	X
Fat + PCDD	X	X	X	X	X	X	X	X
Fat + PCDD + Interf.	X	X	X	X	X	X	X	X
Fat + PCDD + Interf.	X	X	X	X	X	X	X	X
<u>TAC</u>								
352	X				X			
353		X				X		
354			X				X	
355				X				X
356	X				X			
357		X				X		
358			X				X	
359				X				X

Figure 15. Example of possible interlaboratory organization.

Pattern Recognition (Phase II. K.)

A growing body of data worldwide supports the desirability of broadening the scope of the adipose tissue analysis to encompass dioxins and furans not originally present in Agent Orange because background levels suggest a baseline pattern not originating with the phenoxy herbicides (Ryan, et al., 1984; Rappe, et al., 1984b; Graham, et al., 1984; Schechter, et al., 1984; and Stalling, et al., 1984 and in press).

Such an expanded analysis is necessary for quality control, co-toxin classification, and dioxin source identification. The data set generated by this analysis may be large and difficult to interpret because there are 75PCDD's, 135 PCDF's, and 209 PCB's.

Pattern recognition or "chemometrics" may facilitate the interpretation of such data sets by taking advantage of the computer's ability to analyze multivariable systems. As noted by Stalling et al., 1983, the power of principal components modeling (Wold, et al., 1983) in describing multivariate data associated with residue analysis for dioxins, furans, and polychlorinated biphenyls lies in the ability of these computer programs to present these data in both graphical and statistical terms. Data are presented as two or three-dimensional projections from higher dimensional space while preserving most of the existing relationships among samples and variables (Wold and Sjorstrom, 1977). This feature is especially helpful because data of more than three dimensions cannot be readily visualized. In addition, it is possible to determine whether an unknown sample is a member of a given class of samples (e.g., a "typical" background PCDD-PCDF pattern) by comparing it with the class model of other samples that are related to the problem under study.

The advantages of pattern recognition analysis for this study are discussed below.

I. Rationale for Pattern Recognition of the Homogenized Reference Fat

A. Quality Control

Pattern recognition analysis of data generated in the interlaboratory study of the homogenized reference fat will be a more powerful quality control tool by virtue of its multivariate analysis than mere comparison of the results of TCDD concentration determinations alone. In addition, pattern recognition can be used as a determinant of intralaboratory

quality by measuring the pattern-space of the standards and control samples analyzed with the samples. Outliers, i.e., control samples results outside the pattern-space of results for the laboratory, can be identified and thus the validity of the data set can be determined.

B. Choice of Computer Program for Best Data Classification

Several pattern recognition programs exist, permitting different levels of data classification (Dunn et. al., 1984; Stalling et. al., in press). At the first or lowest level are the class discrimination methods such as the linear learning machine (LLM) and linear discriminant analysis (LDA) which permits classification of a sample as belonging to one of a set of predefined classes. A second level of analysis, the distance based methods such as K-nearest neighbor (KNN), permit classification of an unknown sample into one of a number of defined classes with the additional possibility that it is not a member of any of them. The highest level of classification-- and the one most relevant to the study objectives, permits a sample to be assigned a class probability and then to be further analyzed, for example, to ascertain whether it may be a mixture of several classes. Soft Independent Modeling of Class Analogy (SIMCA), for example, is a set of programs useful for such classification. Data from the reference tissue may be used to select the programs most suited to the adipose study, as well as to determine whether the reference tissue, itself, appears to fit the general population patterns already known from preliminary work in Sweden (Rappe, Banbury Report, in press), and Canada (Ryan and Williams, 1983, and Ryan, 1984).

II. Rationale for pattern recognition analysis of the adipose samples from veterans and controls

A. Quality Control

In the case of the study tissue samples, pattern recognition analysis would provide not only quality control or participating laboratories but also of the pathologists' collection techniques by recognizing patterns suggestive of contamination or mishandling.

B. Co-Toxins

Data now exist which suggest that toxicity of TCDD may be altered by the presence of other chemicals. Birnbaum and McKinney et. al., (in press) have demonstrated potentiation of TCDD teratogenicity for cleft palate in mice by one of the polychlorinated biphenyls (2,3,4,5,3',4' hexachlorobiphenyl (HCB)). This effect takes place at concentration of HCB which, in the absence of TCDD, are not teratogenic. 2,3,4,5,3',4' HCB has been shown to be present in human tissue (Masuda et. al., 1982).

Dioxin-furan interaction, in general competitive (Rizzardini et. al., 1983) or additive depending upon their relative concentrations (Vecchi et al., 1984). Thus, interpretation of potential toxicity of TCDD concentration levels is incomplete without simultaneous assessment of concentration patterns of associated co-toxin PCB's and furans.

C. Identification of Non-Agent Orange Dioxin Sources

The phenoxy herbicides are by no means the only sources of exposure to 2,3,7,8-TCDD for both veterans and civilians. In addition to the high risk occupations (crop dusting, sawmill, leather, textile) the average citizen may be exposed to numerous pentachlorophenol-containing products, use hexachlorophene, inhale municipal fly ash dust, and eat fish.

The chemical composition profiles of these alternative 2,3,7,8-TCDD sources differ from one another not only in the identity of their component dioxins, furans, and related compounds but also in the concentration distributions of the chemicals they share in common. In addition, evidence exists that these "marker chemicals" and concentration patterns persist in the environment and in living organisms despite the potential of the latter for idiosyncratic metabolic modifications of the patterns.

For example, Rappe (1983c) describes the relationship between the chlorinated phenolic compounds to which textile and tannery workers were exposed and the dioxins and furans in their blood. The blood reflected the composition of the parent compound, and concentrations correlated with amount and duration of exposure. Rappe (1985, in press) later described the presence of a pentachlorinated dioxin, 1,2,3,7,8-pentachlorodioxin, never detected in any commercial product but present in incinerator fly ash, which he has found in Baltic animals (fish-eating herring gulls, fish, and seal fat), human adipose tissue from North Sweden, and mother's milk from North Sweden and Germany.

The 1,2,3,7,8-pentachlorodioxin is present in higher concentration than the 2,3,7,8-tetrachlorodioxin.

Another example of a "marker" chemical was found during the analysis of soil from the hexachlorophene-contaminated Shenandoah stables in Missouri (Buser, 1978; Buser and Rappe, 1980). This soil analysis revealed 2,3,7,8-TCDD and 1,2,3,7,8-TCDD with the major component 1,2,4,7,8-hexachloroanthene, a compound which can serve as a marker for this type of contamination. The xanthene is a normal by-product of hexachlorophene production and has never been associated with the production of 2,4,5-trichlorophenol or 2,4,5-T derivatives.

Alternative Dioxin Sources and Their Marker Chemicals

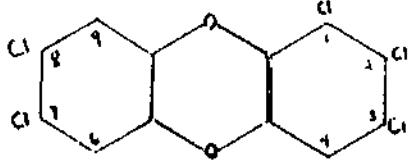
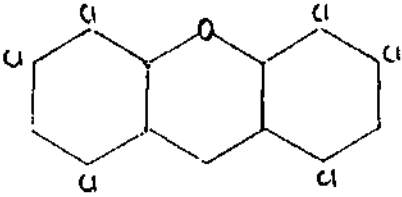
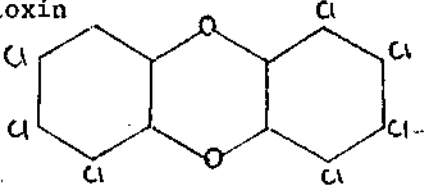
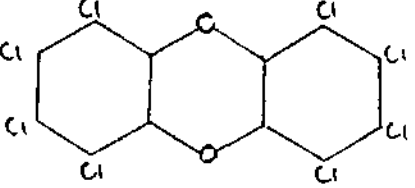
<u>Source</u>	<u>Marker</u>	<u>Structure</u>
Fish	1,2,3,7,8 Pentachlorodioxin	
Fly Ash	" "	
Hexachlorophene	1,2,4,6,8,9 Hexachloroxanthene	
Pentachlorophenol	1,2,3,4,6,7,8 Heptachlorodioxin	
	Octachlorodioxin	

Table 9

Possible Adipose Composition

	<u>2,3,7,8-TCDD</u>	<u>"Marker"</u>
Agent Orange	+	-
Agent Orange + Other TCDD Source (Marker Retained)	+	+
Agent Orange + Other TCDD Source (Marker Excreted)	+	-
Other TCDD Source	+	+

Table 10: Possible combination of TCDD and Marker chemicals resulting from different exposures and metabolic handling.

The alternative sources of 2,3,7,8-TCDD and the structures of their associated "marker" chemicals are summarized below in Figure 16.

Note that none of the "marker" chemicals are found in Agent Orange, yet all of the above sources have 2,3,7,8-TCDD as a contaminant. Thus, the presence of the "marker" chemicals in adipose tissue would imply exposure to a non-Agent Orange source of 2,3,7,8-TCDD, while the absence of the marker chemical would not exclude exposure to non-Agent Orange sources of 2,3,7,8-TCDD. Such thinking is summarized in Table 10.

It is noteworthy that the relative contribution of Agent Orange versus the alternate TCDD sources is not yet quantifiable given the present lack of knowledge of the degree to which a source's "pattern" is metabolically altered in human beings. Should such information ever become available, however, the data could be analyzed by pattern recognition techniques which have successfully performed Arochlor source quantitation for PCB's in toxic oils (Dunn et. al., 1984; Stalling et. al., in press).

Stability Study (Phase II. L.)

Because the study population of adipose tissue will have been stored at -20° C for varying lengths of time and, in some cases, subjected to accidental freeze-thaw cycles, the question of the stability of 2,3,7,8-TCDD in adipose tissue under such storage conditions arises. The EPA is also interested in the stability of related aromatic halogenated hydrocarbons such as ortho-para DDE, hexachlorobenzene, trans-nonachlor, and polychlorinated biphenyls.

A stability study has already been initiated by the EPA for the latter compounds at Colorado State. The structural similarities of these compounds may allow extrapolation of their results to TCDD as "surrogates".

In addition, Dr. Fred Hileman of Monsanto has specimens of human adipose tissue archived at -20° C since June 1983, with data points for 2,3,7,8-TCDD concentrations for June and November 1983, and July 1984. Current data points are being collected.

We are presently designing a formal stability study of this tissue, probably involving the subsectioning of subsections to freeze-thaw cycles and temperatures above -20° C to hasten any temperature and time related deterioration of the samples. Results of such a study should be available by 1986.

Other investigators, such as J. Ryan in Canada, have indicated their willingness to contribute data points from their archived adipose specimens.

Bioincurred Program (Phase II. M.)

The need exists to investigate the extraction efficiency of PCDD's and PCDF's from adipose tissue. The extractability from adipose tissue of TCDD acquired during the life of the organism ("biologically incurred" or "bioincurred") may differ from that of extrinsically introduced or "spiked" TCDD in vitro because of matrix binding considerations. Such differences could result in a loss of accuracy in the final TCDD concentration determinations. Thus, a feasible approach to study this extraction efficiency is through the use of tissue with bioincurred compounds.

Carbon-14 radiolabelled TCDD could be fed¹ to or intravenously introduced into experimental animals (miniature pigs have been suggested) and the fat from such animals removed for further study.

The fat would then be "spiked" with stable isotope labelled TCDD or with native TCDD and analyzed both by radioactive counting techniques and by standard GC/MS (Figure 17).

¹ The possibility of radioactive contamination of the pig pens by scattered radioactive food has led to the suggestion of intravenous introduction of the radiolabelled TCDD (Keith, 1984).

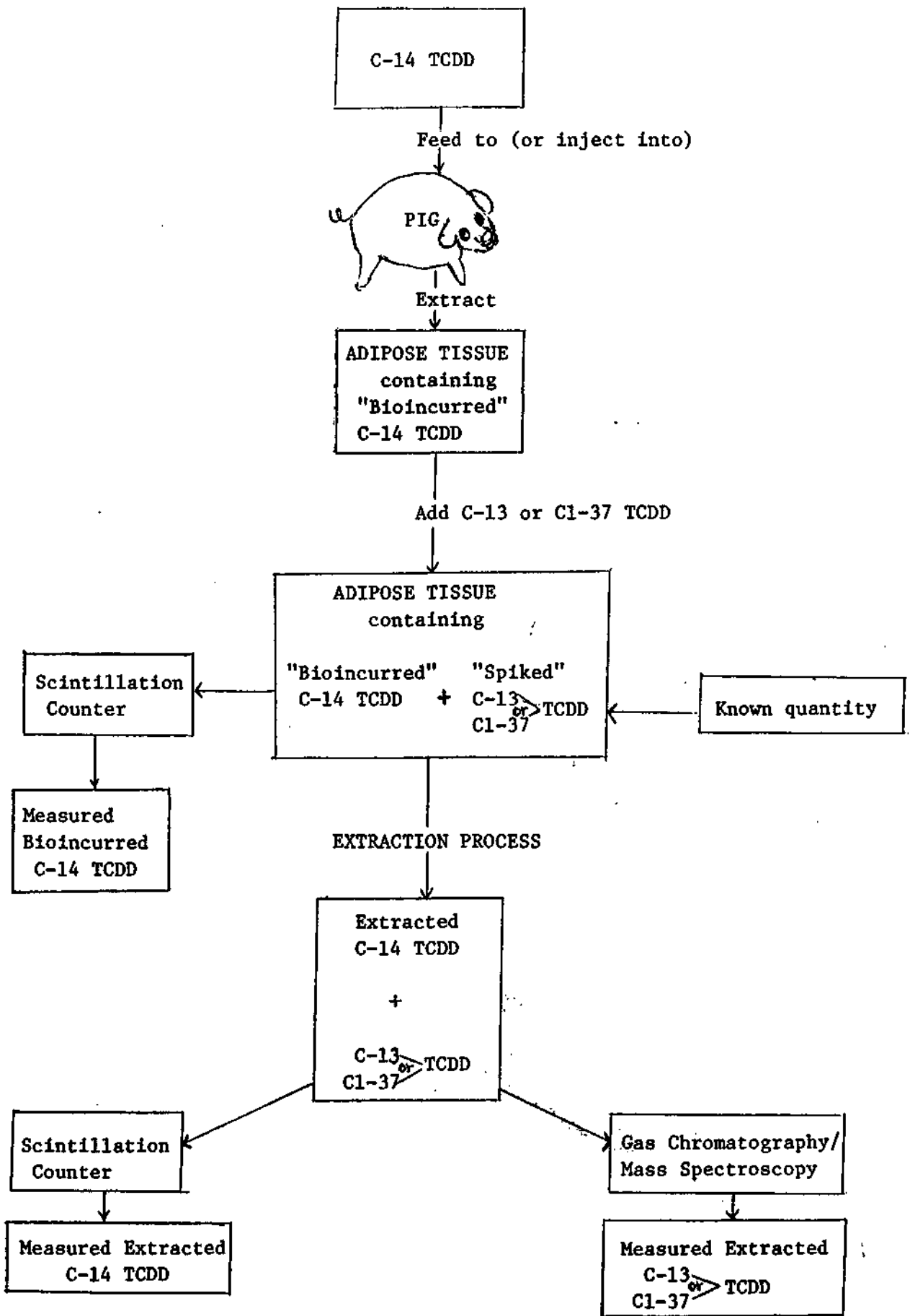


Figure 17.

The ratio of the quantity of extracted bioincurred C-14 labelled TCDD with its pre-extraction value gives the absolute extraction efficiency:

$$\text{Extraction Efficiency (true)} = \frac{\text{Measured Extracted C-14 TCDD}}{\text{Measured Bioincurred C-14 TCDD}}$$

Similarly, the ratio of the extracted stable-labelled TCDD with its pre-extraction value gives the extraction for the "spiked" compounds:

$$\text{Extraction Efficiency (spiked)} = \frac{\text{Measured Extracted C1-37 or C-13 TCDD}}{\text{Known Amount of Spiked C-13 or C1-37 TCDD}}$$

Comparison of the absolute extraction efficiency with the "spiked" extraction efficiency serves as an indication of the adequacy of the sample spiking procedures.

Epilogue

Work on the retrospective portion of this collaborative study is proceeding as final sample identification and selection is being made and the groundwork is laid for the interlaboratory testing and validation of the analytical method. Both the VA and EPA welcome the participation of other agencies in this effort, many of whom collaborated in the methodological review and symposiums held last year. It is our belief that the National Human Adipose Tissue Survey (NHATS) Archive can be a rich source of information for similar studies such as the present, and that the on-going NHATS may provide a definitive, statistically-based sampling frame for possible prospective studies in the future.

APPENDIX I

SPECIAL PROJECT - CHLORODIOXINS HUMAN ADIPOSE TISSUE SAMPLING

General Information and Guidelines Regarding the Collection of Human Adipose

The Survey and Analysis Division of the U.S. Environmental Protection Agency is conducting a special study to examine human adipose tissue for the presence of chlorodioxins. Chlorodioxins have been found as contaminants of several pesticides - including silvex and 2,4,5,-T. These chlorodioxins, particularly 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), are highly toxic and teratogenic. As a mechanism for investigating general population exposure to these contaminants, human adipose tissue samples will be chemically analyzed. Data from these analyses will prove invaluable in evaluating various factors and conditions pertaining to human health and pesticide regulation.

The analysis of these chlorodioxins in adipose tissue collected from the general population is an extremely difficult technical procedure. However, we do have indications that they may be present in human tissues. The U.S. Environmental Protection Agency is conducting this and similar projects to insure that the people of the United States are protected from such hazards through effective chemical regulation.

The human tissues for this study will be secured through the cooperation of participating pathologists who obtain the material during postmortem examinations. Following analysis, reports of our findings will be returned to each participating physician for the tissues submitted. Summaries comparing results from other locations also will be returned to each participant as they are developed.

Collection of Adipose Tissue During Postmortem Examination

If present, we expect to detect chlorodioxins in the low parts per trillion range. This necessitates that all adipose tissue be collected in strict accord with the guidelines detailed below. Failure to comply with this protocol will severely compromise the analytical integrity of our findings.

- * Adipose tissue samples must be collected from unembalmed cadaver over the age of 15 years. The interval between death and tissue collection should be as short as possible, must not exceed 24 hours (assuming adequate refrigeration).

- The individuals sampled should have no known medical history or evidence of barbiturate therapy or usage.
- Samples of adipose tissue should fill the 4-oz bottle provided. Tissue should be removed from the cadaver and placed directly into the specially prepared container provided. Do not substitute any other container.
- Submit only high lipid-containing tissue such as perirenal, subcutaneous, or mesenteric fat. Do not submit omentum as it contains too much connective tissue for satisfactory analysis.
- Adipose tissue should be taken dry, and should not be rinsed before placing it in the provided container. Many water supplies contain materials which may interfere with chemical analyses.
- A completed label should be affixed to each specimen container. Wrap each jar in gauze to prevent breakage.
- Specimens should be stored at -20°C (-4°F) without any fixatives or preservatives until shipped.
- During all phases of this study avoid all contact with:
 - disinfectants such as hexachlorophene
 - formalin or other preservatives
 - parafin
 - plastics
 - any substance containing phenolic compounds
- Instruments should be well rinsed in distilled water and soaked in undenatured alcohol or acetone for approximately 20-30 minutes. Again thoroughly rinse the instruments in distilled water and place them within a folded sterile towel to dry before use.

Completion of the Patient Summary Report

A Patient Summary Report should be completed for each patient from whom a sample was taken. Special attention should be given to the completeness of the data. All medical information submitted is protected from disclosure or release by U.S.C. 552, (b)(6); 45 CFR Part 5 and the Privacy Act of 1974.

First and last initials, in that order, should be used instead of the complete name to insure that confidentiality is maintained. The initials

along with the date of birth, sex, and race, are used in this office to compose the AMA identification number. The patient's identification number and/or the pathology department's accession number are for your information in referring back to the individual patient when you receive the results of the pesticide analysis.

Confirmed diagnosis should be detailed in the spaces provided. Only the major ones should be supplied.

Other required information should be completed as accurately as possible. The completed forms should be held and sent under the inner lid of the insulated container when shipment is made.

Packing and Shipping

Tighten all lids on the specimen bottles carefully. This is important since we are required to use special caps which make tightening a little difficult. Be certain that a completed bottle label is firmly attached to each specimen bottle. Wrap each bottle in gauze or paper to prevent breakage during shipment and to keep the label on the container. Place the specimen bottles in the insulated mailer and fill it with dry ice. If you have difficulty obtaining dry ice, please call us so that we may arrange alternative methods of refrigeration for you.

New mail transport regulation require that any materials shipped in dry ice be specially labeled and accompany by a Shippers Certification for Restricted Articles. Since frozen medical and/or diagnostic specimens are exempt from some of these requirements, be sure that the contents of the shipping containers are clearly indicated by ORM-A: FROZEN MEDICAL SPECIMENS. We have provided a special label for this purpose. If, for some reason, you do not have this labeling, please attach a card or piece of wide adhesive tape with ORM-A: FROZEN MEDICAL SPECIMENS indicated in indelible ink.

The Shipper's Certification forms have been filled out for you in triplicate as required. Please sign where indicated, fold, and place all three copies into the provided Packing List Enclosed envelope. This envelope should be attached to the outside of the metal shipping container.

When you have completed your collections, please contact Ms. Madeline Dean by calling (collect) 202/755-8060. We will arrange to have an air courier service pick up the samples and directly transport them to our laboratory.

Legal Consideration

The National Human Monitoring Program is both interested and deeply concerned about the legal ramification of this human research project. Since this study is operative in six States, it is not feasible for us to handle the variety of local or state interpretations from our location in Washington. Therefore, as a matter of policy, legal requirements such as informed consent, confidentiality, etc. are matters for your consideration and resolution. Collections for this project must be made in conformance with the applicable HEW guidelines on the protection of human subjects of biomedical and behavioral research. We will, however, be pleased to assist you in any way possible.

We have completed several studies on these matters and do not believe that they present major obstacles to your participation. In most documents authorizing postmortem examinations, there is a clause granting the examining physician permission to remove tissues for research purposes. We consider this project as being included in that category.

As you will notice in our discussion of data needed for each patient sampled, we do have several mechanisms to assure confidentiality. In fact, the disclosure or release of certain data is protected by several federal statutes. The fees paid to you by our program are solely intended to remunerate you or your designee for professional services rendered. They are not intended to pay for tissues received.

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LIST OF TERMS, ABBREVIATIONS, AND SYMBOLS

Accuracy	Closeness of analytical result to "true" value.
AOAC	Association of Official Analytical Chemists.
Congener	One of 75 PCDDs or 135 PCDFs, not necessarily the same homolog.
DDE	1,1,-Dichloro-2,2-bis(p-chlorophenyl)-ethylene.
DDT	1,1,1-Trichloro-2,2,-bis(p-chlorophenyl)-ethane.
2,4-D	2,4-Dichlorophenoxyacetic acid.
ECD	Electron capture detector.
EI	Electron impact ionization (mass spectrometry).
EIMS	Electron impact mass spectrometry.
FID	Flame ionization detector.
GC	Gas liquid chromatography (column type unspecified).
GC/MS	Gas liquid chromatography/mass spectrometry (ionization mode unspecified).
HCDD	Hexachlorodibenzo-p-dioxin.
HpCDD	Heptachlorodibenzo-p-dioxin.
Homolog	One of the eight degrees of chlorination of PCDDs and PCDFs.
HPLC	High performance liquid chromatography.
HRGC silica.	High resolution gas chromatography, glass or fused silica.

HRMS	High resolution electron impact mass spectrometry.
Internal standard	Standards used expressly for quantitation added to sample extract immediately prior to the analytical determination. Internal standards are used for PCDD and PCDF analyses to accurately measure recoveries of spiked surrogate compounds.
Isomer	One of up to 22 PCDDs or 38 PCDFs possessing the same degree of chlorination (1,2,3,4-TCDD and 2,3,7,8-TCDD are different isomers).
KOH	Potassium hydroxide.
LOD	Lower limit of detection (see also MDL). Lowest concentration at which an analyte can be identified as present in a sample at a stated statistical confidence level.
LOQ	Lower limit of quantitation. Lowest concentration to which a value can be assigned at a stated statistical confidence level.
LRMS	Low resolution mass spectrometry.
MDL	Method detection limit.
Mean	Arithmetic mean.
MRI	Midwest Research Institute
MS	Mass spectrometry.
m/z	Mass-to-charge ratio.
NHATS	National Human Adipose Tissue Survey
NRCC	National Research Council of Canada.
OCDD	Octachlorodibenzo-p-dioxin.
PCB	Polychlorinated biphenyl.
PCDD	Polychlorinated dibenzo-p-dioxin (including monochlorodibenzo-p-dioxins).
PCDF	Polychlorinated dibenzofuran (including monochlorodibenzofuran).
PGC	Packed column gas liquid chromatography.
ppb	Parts per billion (1×10^{-9} g/g, ng/g).
ppm	Parts per million (1×10^{-6} g/g, µg/g).

ppt	Parts per trillion (1×10^{-12} g/g, pg/g).
Precision	Reproducibility of an analysis, measured by standard deviation (SD) of replicates.
QA	Quality assurance. An organization's program for assuring the integrity of data it produces or uses.
QC	Quality control. The specific activities and procedures designed and implemented to measure and control the quality of data being produced.
RP-HPLC	Reverse phase high performance liquid chromatography.
RSD	Percent relative standard deviation (SD/mean x 100).
SD	Standard deviation.
Sensitivity	The slope of instrument response with respect to the amount of analyte. Also used colloquially to refer to lowest detectable amount of analyte.
SIM	Selected ion monitoring (also mid or mass fragmentography).
Surrogate	Standard compounds added to the sample prior to any analytical manipulations for the express purpose of measuring recovery through extraction, cleanup, etc., and to provide true internal standard quantitation.
TCDD	Tetrachlorodibenzo-p-dioxin.
$^{13}\text{C}_{12}$ -TCDD	Carbon-13 stable isotope labeled TCDD.
$^{37}\text{Cl}_4$ -TCDD	Chlorine-37 stable isotope labeled TCDD.
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid.