INTERLABORATORY METHOD VALIDATION STUDY FOR DIOXIN

AN INTERIM REPORT

by Robert G. Heath
Human Effects Monitoring Branch
OPP, OTS, EPA

January 5, 1979

Interlaboratory Method Validation Study for Dioxin

A. Introduction and Scope

B. Study Design

C. Results: Tables and Graphs

- Standards
- Beef Fat
- Human Milk
- Types and Frequencies of Errors

D. Statistical Analysis of Lab C Beef Fat Reports.

- The regression equation and confidence limits.
- Confidence limits for a predicted report value for a given spiking level.
- Spiking and extraction precision vs. quantitation (GC-MS) precision
- Detection limit characteristics

E. Estimation of a "true" TCDD level, with statistical confidence limits, from spiking study results (Lab C Beef Fat example)

F. Discussion

Acknowledgements page to be prepared.

A. Introduction and Scope

The Interlaboratory Method Validation Study for Dioxin was undertaken to measure the accuracy and precision with which 2,3,7,8-tetrachlorodiobenzo-p-dioxin (TCDD), when added to beef fat and human milk at low parts-per-trillion concentrations, can be extracted and quantified by methods of gas chromatography-mass spectrometry (GC-MS). Method validation also included quantitation of equivalent amounts of TCDD standards. In particular, the study was undertaken to develop regression statistics for converting reported TCDD concentrations to "best estimates" of actual (but unknown) concentrations and for expressing the reliability of such estimates in terms of statistical confidence limits. The study was also intended to determine the lowest concentration of TCDD that was identified with practicable consistency and the frequency of "false positive" and "false negative" reports.

All samples were prepared and extracted at the EPA Pesticide Monitoring Laboratory, Bay St. Louis, Mississippi. Analytical laboratories participating in all or part of the GC-MS quantitation were those of Dow Chemical Company, Harvard University, University of Nebraska, Wright State University and the EPA Health Effects Research Laboratory (HERL),

Research Triangle Park, North Carolina. Analytical laboratories are identified only as laboratory A,B,C,D, or E throughout the report; alphabetical order is independent of the above laboratory order. The number of samples, by type, quantified by each laboratory is shown in Table A-1.

TABLE A-1.

Number of Samples, by Type, Quantified by Participants

	Sample Description				
		Acid/base cleanup		Neutral Extraction	Lab
Laboratory	Standard	Beef fat	Human milk	Beef fat	Totals
A	26	26	26	0	78
B	25	26	26	0	77
C	26 + 3	26	0	16	71
D	0	6	0	16	22
E	1	0	11	0	12
Type totals	81	84	63	32	260

B. Study Design

Beef fat and human milk samples were "spiked" with ^{35}Cl TCDD at levels ranging from 0 to 81 ppt. Standards were prepared so as to contain equivalent amounts of the chemical. Samples were prepared from one of two pools of rendered beef fat (pools F and G) and one of two pools of human milk (pools M and N). Fat was from cattle without potential exposure to dioxin; the milk had been collected in regions where use of pesticides potentially contaminated with TCDD was incidental. Pools were constructed using equal amounts of fat or milk from each animal or donor, using a separate set of animals or donors for construction of each pool.

Eleven samples each were prepared from fat pool F and milk pool M--the major pools. The samples from each pool were spiked individually at 0, 0.5, 1, 4, 9, 16, 25, 36, 49, 64 or 81 ppt. The samples were then extracted by procedures developed/refined at PML, and the extract was divided into the required numbers of equal aliquots for shipment to the analytical laboratories. Spiking levels, excepting 0.5 ppt, were systematically incremented as the squares of the digits 0 through 9 to provide close spacing at low levels and a moderate, systematic increase in spacing with increasing levels.

To test the precision of the extraction methodology, five samples each were prepared from the minor pools (fat pool G and milk pool N). These samples were spiked individually at 0, 9, 25, 49, and 81 ppt, extracted by the same procedures used for samples from pools F and M, and divided into the required number of equal aliquots for shipment to the analytical laboratories. Thus, fat pools F and G and milk pools M and N provide replicate samples at the above levels of spiking for testing extraction precision.

To test the precision of GC-MS quantitation for comparison with that of extraction, laboratories were provided two aliquots of the G- and N-pool extracts, along with two aliquots from each of the matching extracts from pools F and N, so as to obtain duplicate analyses of the same extract. Labs also received four standards at each equivalent of 0, 9, 25, 49, and 81 ppt, as well as single standards at 0.5, 1, 4, 16, 36, and 64 ppt. Standards are denoted as S.

All samples--fat, milk and standards--were required to be prepared and shipped in random order, and laboratories were to analyze the samples in the order in which they were received. Samples were identified only by shipment number, so that laboratories knew neither the type of sample nor the TCDD level at the time of analysis.

A variation in the above procedure was developed for a set of beef fat samples analyzed at Lab D. The fat samples in that set were spiked at PML but were extracted at Lab D using a neutral extraction procedure rather than the acid/base procedure utilized throughout the study.

Accuracy (the degree of constant tendency to either under-report or over-report the true level) and precision (variation among repeated measurements of the same extract) have been measured by methods of regression analysis; comparisons of extraction vs quantitation precision are by analysis of variance based on those spiking levels for which there were duplicate analyses of replicate extractions. The analytical schedule is presented in Table B-1.

Table B-1.

Design Diagram for Phase II Dioxin Study

TCDD Level (ppt)	Beef Fat Measurements			Human Milk Measurements			Standard Measurements		
	Pool Code	Lab A	Lab B etc.	Pool Code	Lab A	Lab B etc.	Pool Code	Lab A	Lab B etc.
0	F	2	2	M	2	2	S	4	4
0	G	2	2	N	2	2			
1/2	F	1	1	M	1	1	S	1	1
1	F	1	1	M	1	1	S	1	1
4	F	1	1	M	1	1	S	1	1
9	F	2	2	M	2	2	S	4	4
9	G	2	2	N	2	2			
16	F	1	1	M	1	1	S	1	1
25	F	2	2	M	2	2	S	4	4
25	G	2	2	N	2	2			
36	F	1	1	M	1	1	S	1	1
49	F	2	2	M	2	2	S	4	4
49	G	2	2	N	2	2			
64	F	1	1	M	1	1	S	1	1
81	F	2	2	M	2	2	S	4	4
81	G	2	2	N	2	2			

C. General Results

Analytical results for the quantitation of standards are presented in Tables C-1 through C-3. Figures C-1 through C-3 (each figure follows its respective table) show the plotted results and the least squares regression lines and equations for reported values on spiked values. The theoretical line y=x, for perfect extraction and quantitation is also shown for comparison.

Equivalent results for beef fat samples are presented in Tables and Figures C-4 through C-9, and those for human milk appear in Tables and Figures C-10 through C-12.

An explanation of the types of reporting errors and an enumeration of those errors are presented in Tables C-13 through C-16. In this report, the reporting of a positive value in an unspiked sample is identifed as a "False Positive" (FP), and a positive report given when the detection limit exceeds the level of spiking is identified as a "false positive" (fp). A "false not detected" (fnd) is defined as a report of "nd" when, in fact, the level of detection is less than the level of spiking.

As might have been expected, the highest frequency of errors occurred at spiking levels below 9 ppt.

Table C-1.

Dioxin Phase II: Interlaboratory Quantitation Study

Type of Sample: Standard (5g equivalent)
Preparation Lab: PML
Quantitation Lab: Lab A WS

Sample ID		Ship-	Recov.	TCDD Levels (ppt)				Detection Limit	
					Reported				
Study	PML	ment	Cl^{37} (%)	Added	320	322	Avg.	320	322
$S\text{-}0_1$	ST-0	2	88	0		nd			0.3
$S\text{-}0_2$	ST-0	40	101	0		nd			6
$S\text{-}0_3$	ST-0	45	83	0		2			2
$S\text{-}0_4$	ST-0	49	94	0		nd			2
S-0.5	ST-.5	15	95	0.5		1			1
S-1	ST-1	19	141	1		nd			2
S-4	ST-4	10	77	4		2			2
$S\text{-}9_1$	ST-9	21	68	9		7			2
$S\text{-}9_2$	ST-9	28	93	9		4			2
$S\text{-}9_3$	ST-9	46	113	9		7			7
$S\text{-}9_4$	ST-9	47	69	9		10			3
S-16	ST-16	22	86	16		6			2
$S\text{-}25_1$	ST-25	16	93	25		nd			0.7
$S\text{-}25_2$	ST-25	42	90	25		19			3
$S\text{-}25_3$	ST-25	48	64	25		8			2
$S\text{-}25_4$	ST-25	52	131	25		18			2
S-36	ST-36	5	81	36		16			4
$S\text{-}49_1$	ST-49	13	67	49		23			2
$S\text{-}49_2$	ST-49	32	109	49		26			2
$S\text{-}49_3$	ST-49	44	98	49		18			1
$S\text{-}49_4$	ST-49	50	98	49		68			4
S-64	ST-64	17	107	64		nd			0.5
$S\text{-}81_1$	ST-81	12	46	81		44			2
S-8	ST-81	29	98	81		40			2
$S\text{-}81_3$	ST-81	43	102	81		44			2
$S\text{-}81_4$	ST-81	51	106	81		56			3

Figure C-1

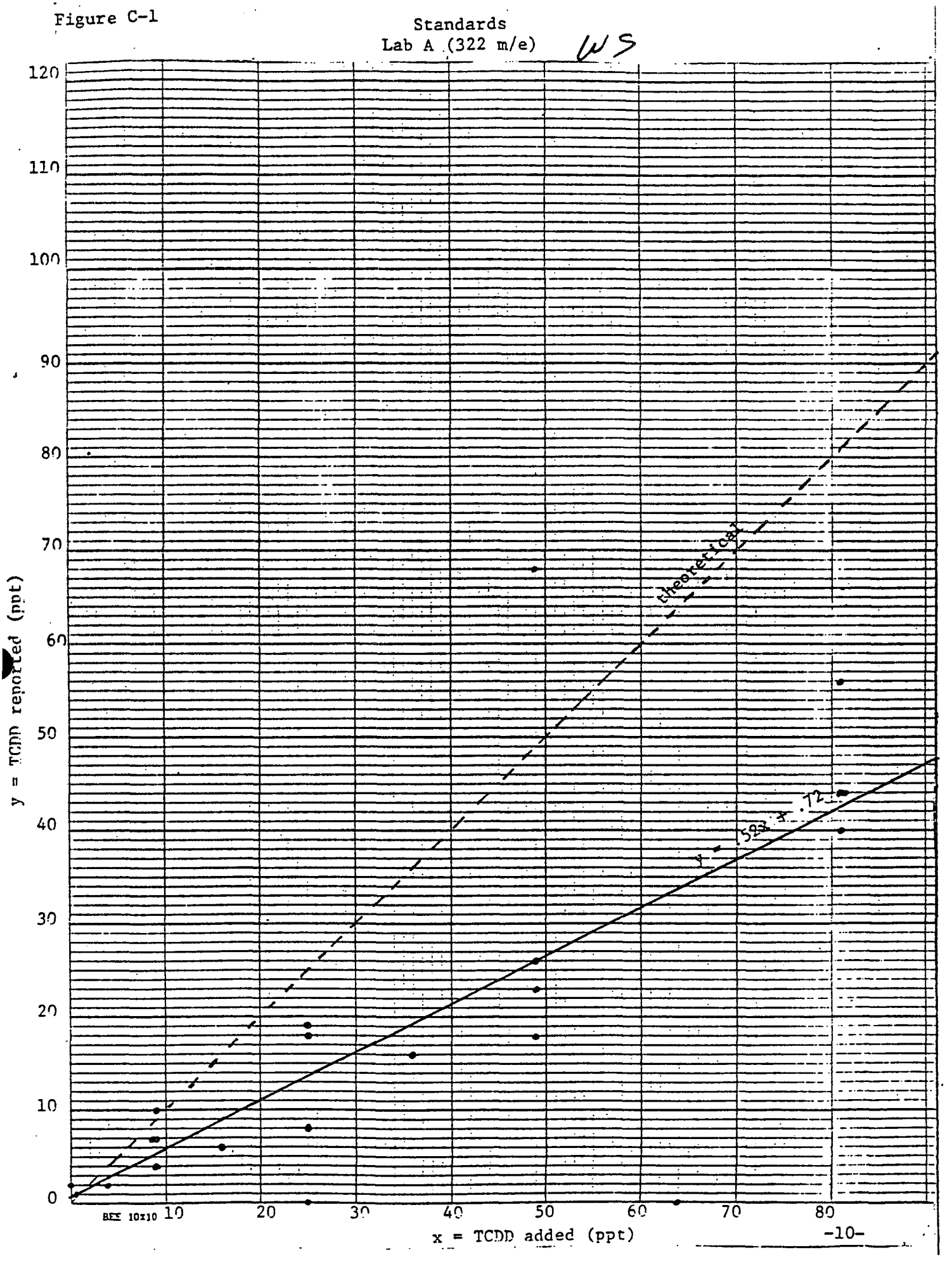

Standards
Lab A (322 m/e)
WS
120
110
100
90
80
70
60
50
40
30
20
10
0
y = TCDD reported (ppt)
theoretical
y = .583 x + .72
10
20
30
40
50
60
70
80
x = TCDD added (ppt)

Table C-2.

Dioxin Phase II: Interlaboratory Quantitation Study

Type of Sample: Standard (5g equivalent)
Preparation Lab: PML
Quantitation Lab: Lab B

Sample ID		Ship-	Recov.	TCDD Levels (ppt)				Detection Limit	
					Reported				
Study	PML	ment	Cl^{37}(%)	Added	320	322	Avg.	320	322
$S\text{-}0_1$	ST-0	2		0	nd	nd	nd	4	3
$S\text{-}0_2$	ST-0	40		0	nd	nd	nd	2	3
$S\text{-}0_3$	ST-0	45		0	3	nd	-	2	2
$S\text{-}0_4$	ST-0	49		0	nd	2	-	3	1
S-0.5	ST-.5	15		0.5	nd	nd	nd	3	4
S-1	ST-1	19		1	nd	nd	nd	4	5
S-4	ST-4	10		4	-	-	-	-	-
$S\text{-}9_1$	ST-9	21		9	4	5	4.5	3	2
$S\text{-}9_2$	ST-9	28		9	9	6	7.5	4	1
$S\text{-}9_3$	ST-9	46		9	4	6	5	2	2
$S\text{-}9_4$	ST-9	47		9	10	7	8.5	3	1
S-16	ST-16	22		16	10	10	10	2	1
$S\text{-}25_1$	ST-25	16		25	16	22	19	4	4
$S\text{-}25_2$	ST-25	42		25	17	13	15	2	1
$S\text{-}25_3$	ST-25	48		25	7	19	13	1	2
$S\text{-}25_4$	ST-25	52		25	29	21	25	3	1
S-36	ST-36	5		36	6	12	9	4	5
$S\text{-}49_1$	ST-49	13		49	13	22	17.5	5	3
$S\text{-}49_2$	ST-49	32		49	52	29	40.5	5	1
$S\text{-}49_3$	ST-49	44		49	40	18	29	1	1
$S\text{-}49_4$	ST-49	50		49	8	25	16.5	2	1
S-64	ST-64	17		64	28	33	30.5	3	2
$S\text{-}81_1$	ST-81	22		81	59	50	54.5	5	2
S-8	ST-81	29		81	21	23	22	1	1
$S\text{-}81_3$	ST-81	43		81	77	47	62	6	2
$S\text{-}81_4$	ST-81	51		81	83	43	63	3	2

Figure C-2a

Standards
Lab B (322 m/e)

Dow

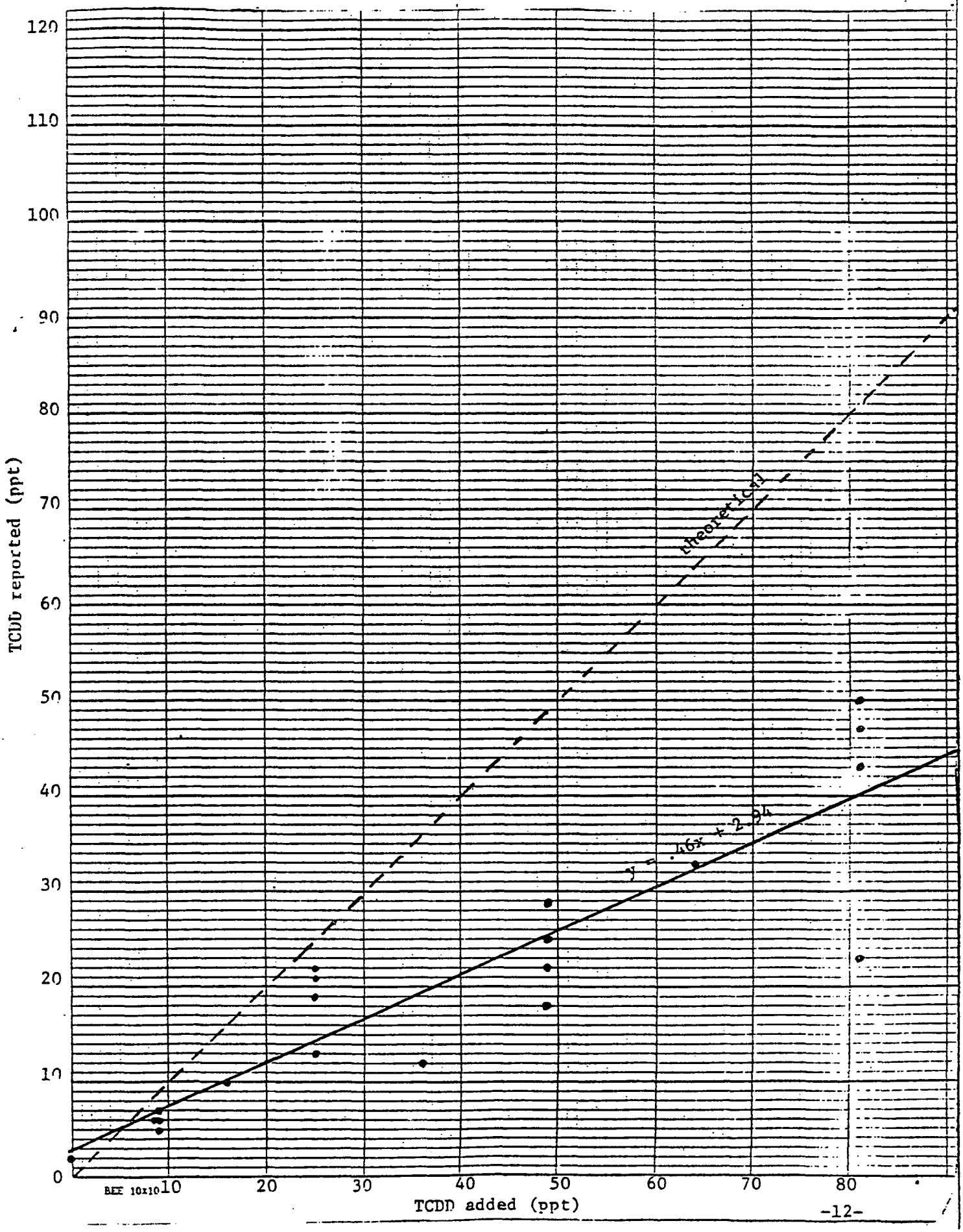


Figure C-2b

Standards
Lab B (320 m/e)

Dow

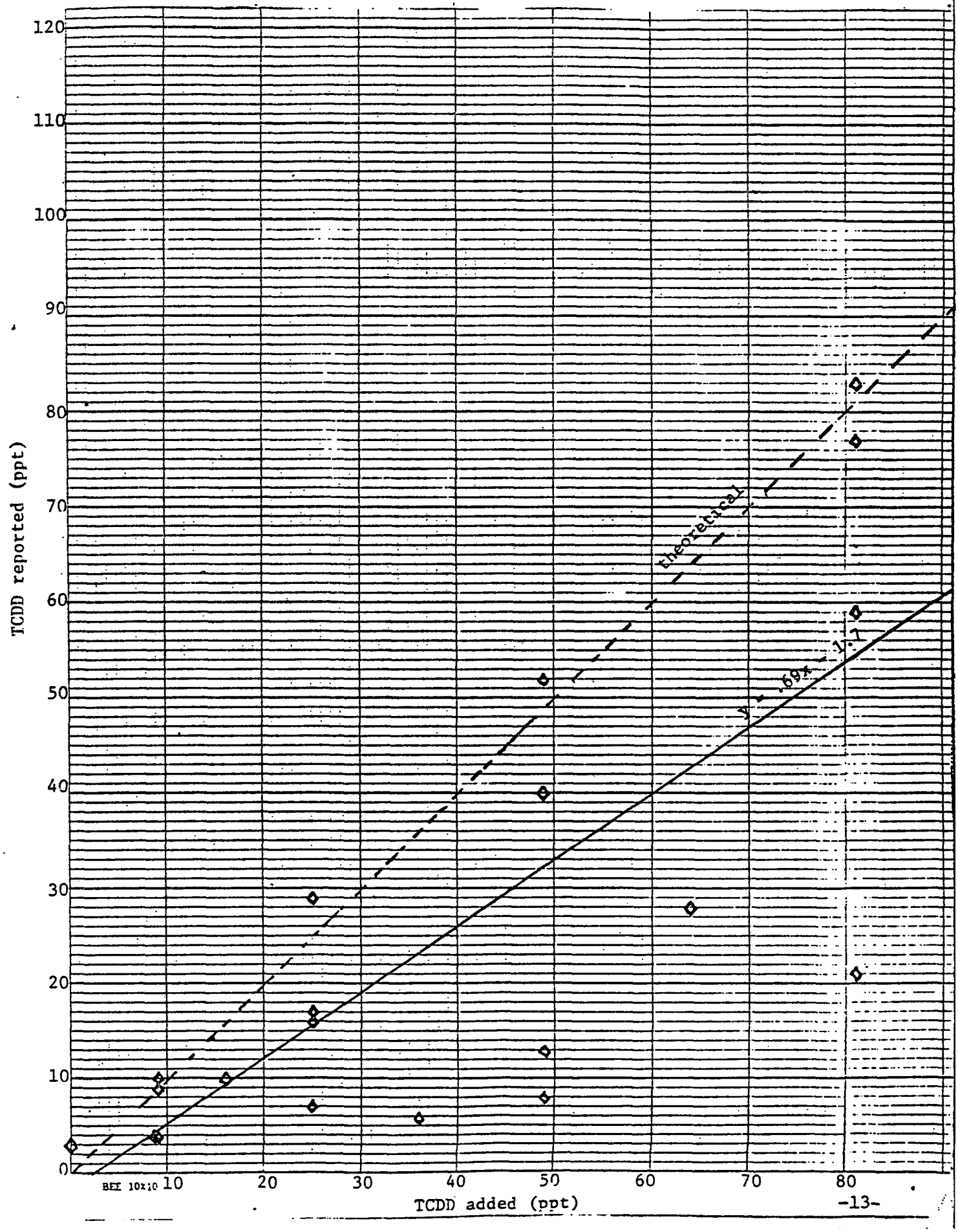


Table C-3.

Dioxin Phase II: Interlaboratory Quantitation Study

Type of Sample: Standard (5g equivalent)
Preparation Lab: PML
Quantitation Lab: Lab C Nebraska

Sample ID			Recov.	TCDD Levels (ppt)				Detection Limit	
					Reported				
Study	PML	Shipment	Cl^{37} (%)	Added	320	322	Avg.	320	322
$S\text{-}0_1$	ST-0	2		0		nd			10
$S\text{-}0_2$	ST-0	48		0		nd			4
$S\text{-}0_3$	ST-0	57		0		nd			4
$S\text{-}0_4$	ST-0	62		0		nd			4
S-0.5	ST-.5	15		0.5		nd			6
S-1	ST-1	19		1		nd			12
S-4	ST-4	10		4		nd			6
$S\text{-}9_1$	ST-9	21		9		nd			10
$S\text{-}9_2$	ST-9	56		9		11			5
$S\text{-}9_3$	ST-9	60		9		9			4
$S\text{-}9_4$	ST-9	64		9		6			4
S-16	ST-16	22		16		16			10
$S\text{-}25_1$	ST-25	16		25		21			4
$S\text{-}25_2$	ST-25	55		25		17			4
$S\text{-}25_3$	ST-25	63		25		25			4
$S\text{-}25_4$	ST-25	69		25		24			4
S-36	ST-36	5		36		34			16
$S\text{-}49_1$	ST-49	13		49		41			12
$S\text{-}49_2$	ST-49	51		49		51			3
$S\text{-}49_3$	ST-49	52		49		47			9
$S\text{-}49_4$	ST-49	70		49		47			5
S-64	ST-64	17		64		65			6
$S\text{-}81_1$	ST-81	12		81		76			6
S-8	ST-81	49		81		77			3
$S\text{-}81_3$	ST-81	59		81		80			3
$S\text{-}81_4$	ST-81	67		81		80			5

Figure C-3

Standards

Lab C (322 m/e) Nebraska

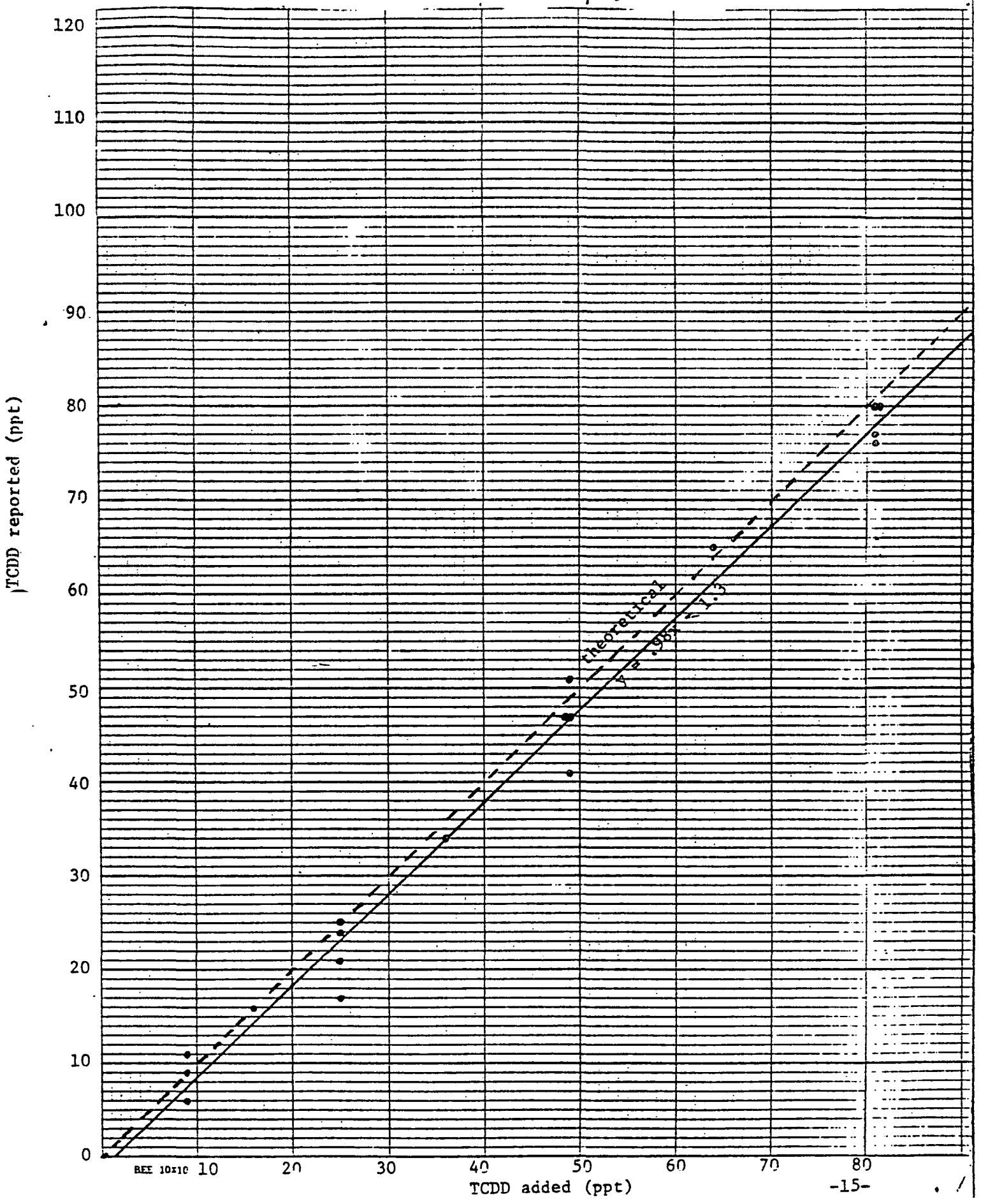


Table C-4.

Dioxin Phase II: Interlaboratory Quantitation Study

Type of Sample: Beef Fat (5g sample)
Extraction Lab: PML; Method: Acid/base
Quantitation Lab: Lab A WS

Sample ID			Recov.	TCDD Levels (ppt)				Detection Limit	
					Reported				
Study	PML	Shipment	Cl^{37} (%)	Added	320	322	Avg.	320	322
$F\text{-}0_1$	FE	11	71	0		103			7
$F\text{-}0_2$	FE	37	72	0		nd			5
$G\text{-}0_1$	GB	24	73	0		nd			4
$G\text{-}0_2$	GB	39	53	0		2			2
F-0.5	FG	1	69	0.5		12			0.4
F-1	FC	7	72	1		3			2
F-4	FI	6	70	4		28			5
$F\text{-}9_1$	FK	25	64	9		4			2
$F\text{-}9_2$	FK	41	75	9		9			1
$G\text{-}9_1$	GC	20	93	9		2			1
$G\text{-}9_2$	GC	30	68	9		nd			1
F-16	FL	26	72	16		8			4
$F\text{-}25_1$	FD	3	71	25		110			1
$F\text{-}25_2$	FD	34	77	25		22			6
$G\text{-}25_1$	GE	18	73	25		5			5
$G\text{-}25_2$	GE	38	68	25		8			1
F-36	FA	8	80	36		34			3
$F\text{-}49_1$	FH	27	73	49		18			2
$F\text{-}49_2$	FH	33	99	49		7			2
$G\text{-}49_1$	GA	14	87	49		6			0.8
$G\text{-}49_2$	GA	35	68	49		32			5
F-64	FB	9	101	64		86			3
$F\text{-}81_1$	FJ	23	53	81		25			2
$F\text{-}81_2$	FJ	31	89	81		22			4
$G\text{-}81_1$	GD	4	69	81		110			1
$G\text{-}81_2$	GD	36	71	81		40			2

Figure C-4

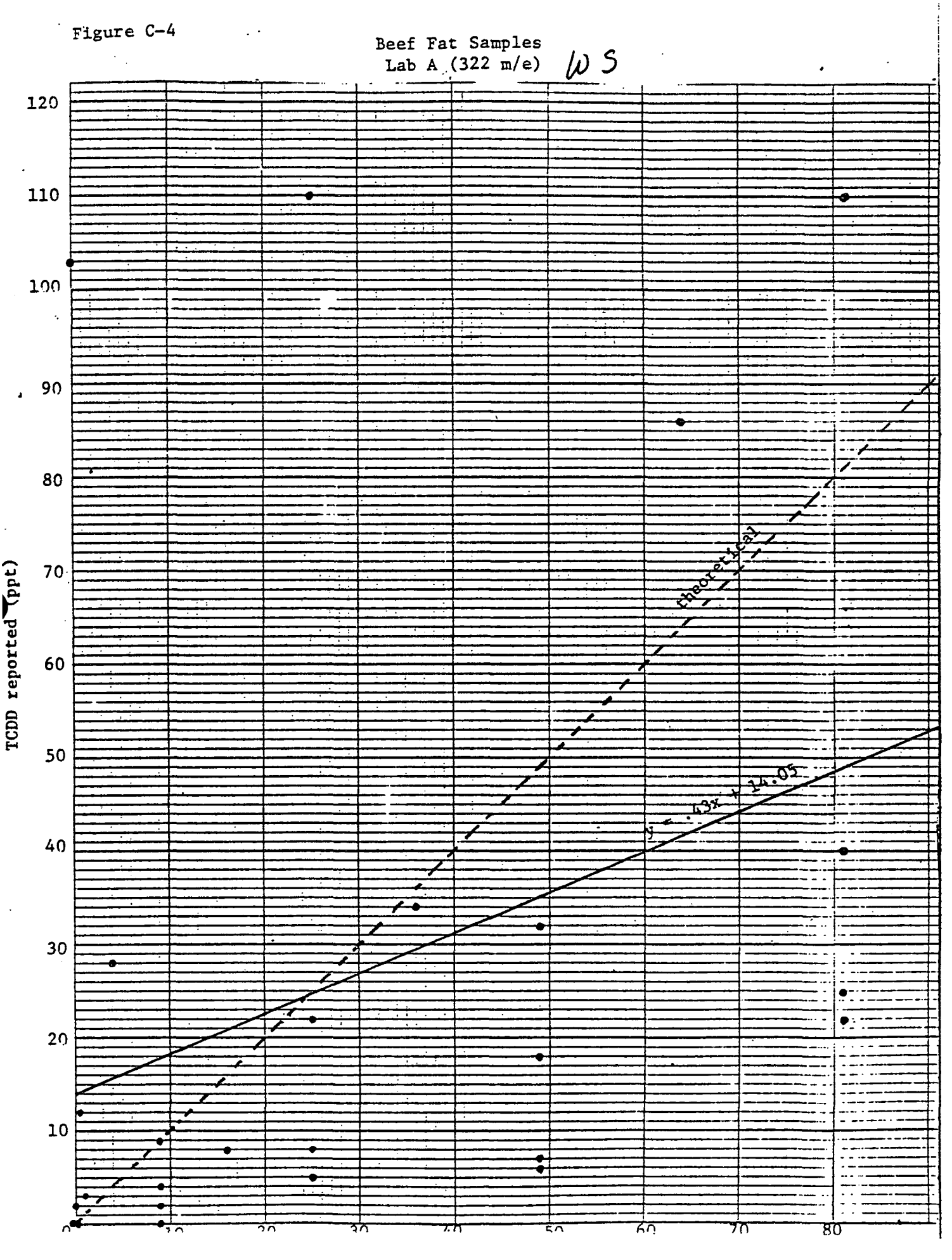

Beef Fat Samples
Lab A (322 m/e) WS
TCDD reported (ppt)
120
110
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
theoretical
y = .43x + 14.05

Table C-5.

Dioxin Phase II: Interlaboratory Quantitation Study

Type of Sample: Beef Fat (5g sample)
Extraction Lab: PML; Method: Acid/base
Quantitation Lab: Lab B Dow

Sample ID			Recov.	TCDD Levels (ppt)				Detection Limit	
					Reported				
Study	PML	Shipment	Cl^{37} (%)	Added	320	322	Avg.	320	322
$F\text{-}0_1$	FE	11		0	4	7	5.5	4	2
$F\text{-}0_2$	FE	37		0	13	6	9.5	2	3
$G\text{-}0_1$	GB	24		0	nd	10	-	4	4
$G\text{-}0_2$	GB	39		0	9	5	7	2	2
F-0.5	FG	1		0.5	7	12	9.5	4	3
F-1	FC	7		1	nd	nd	nd	4	4
F-4	FI	6		4	7	20	13.5	5	5
$F\text{-}9_1$	FK	25		9	7	13	10	5	3
$F\text{-}9_2$	FK	41		9	6	13	9.5	2	2
$G\text{-}9_1$	GC	20		9	8	21	14.5	7	5
$G\text{-}9_2$	GC	30		9	7	11	9	2	2
F-16	FL	26		16	13	18	15.5	7	2
$F\text{-}25_1$	FD	3		25	12	10	11	4	2
$F\text{-}25_2$	FD	34		25	12	17	14.5	2	3
$G\text{-}25_1$	GE	18		25	18	27	22.5	6	6
$G\text{-}25_2$	GE	38		25	12	8	10	3	2
F-36	FA	8		36	27	29	28	5	7
$F\text{-}49_1$	FH	27		49	21	22	21.5	3	2
$F\text{-}49_2$	FH	33		49	35	26	30.5	3	2
$G\text{-}49_1$	GA	14		49	38	43	40.5	7	8
$G\text{-}49_2$	GA	35		49	15	28	21.5	2	2
F-64	FB	9		64	54	25	39.5	8	2
$F\text{-}81_1$	FJ	23		81	42	34	38	5	1
$F\text{-}81_2$	FJ	31		81	27	32	29.5	2	3
$G\text{-}81_1$	GD	4		81	60	64	62	9	5
$G\text{-}81_2$	GD	36		81	39	32	35.5	2	2

Figure C-5a

Beef Fat Samples
Lab B (322 m/e) Dow

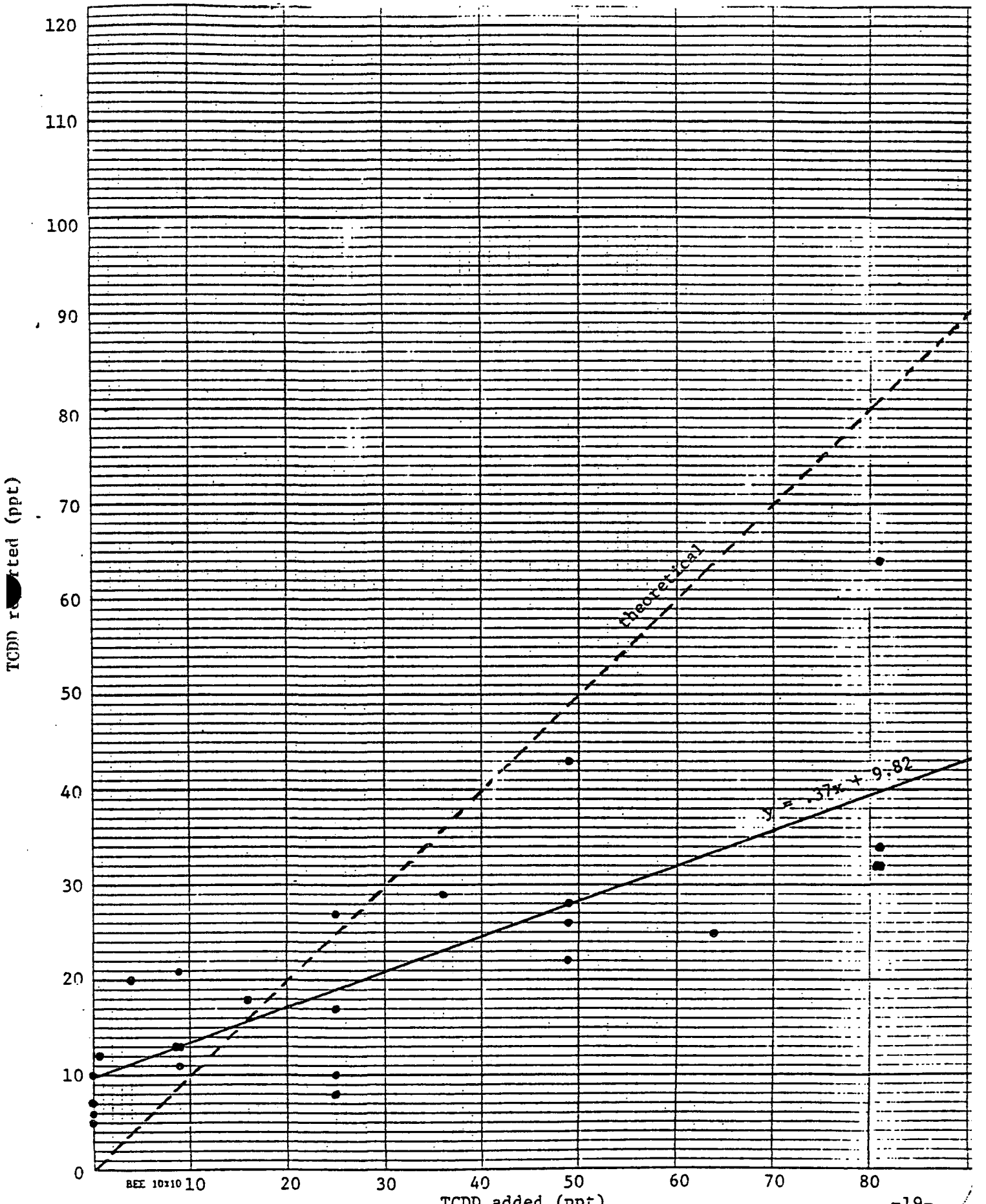


Figure C-5b

Beef Fat Samples

Lab B (320 m/e)

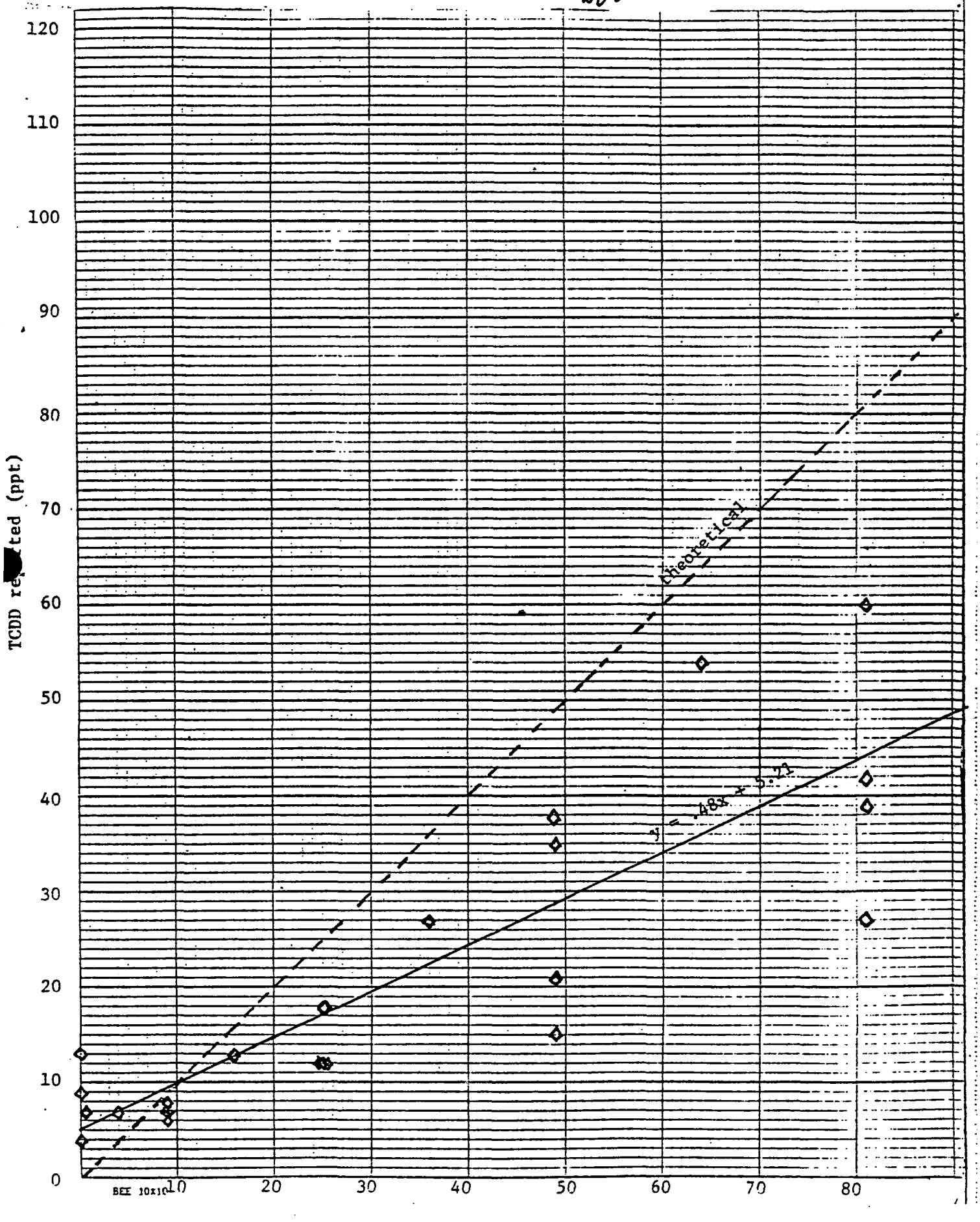


Table C-6.

Dioxin Phase II: Interlaboratory Quantitation Study

Type of Sample: Beef Fat (5g sample)
Extraction Lab: PML; Method: Acid/base
Quantitation Lab: Lab C

Sample ID			Recov.	TCDD Levels (ppt)				Detection Limit	
					Reported				
Study	PML	Shipment	Cl^{37} (%)	Added	320	322	Avg.	320	322
$F\text{-}0_1$	FE	11		0		nd			8
$F\text{-}0_2$	FE	54		0		nd			5
$G\text{-}0_1$	GB	24		0		nd			6
$G\text{-}0_2$	GB	65		0		nd			6
F-0.5	FG	1		0.5		nd			14
F-1	FC	7		1		nd			10
F-4	FI	6		4		nd			18
$F\text{-}9_1$	FK	25		9		9			4
$F\text{-}9_2$	FK	68		9		11			5
$G\text{-}9_1$	GC	20		9		nd			14
$G\text{-}9_2$	GC	50		9		12			5
F-16	FL	26		16		19			10
$F\text{-}25_1$	FD	3		25		(63)*			10
$F\text{-}25_2$	FD	47		25		24			2
$G\text{-}25_1$	GE	18		25		26			12
$G\text{-}25_2$	GE	71		25		27			4
F-36	FA	8		36		31			6
$F\text{-}49_1$	FH	27		49		50			6
$F\text{-}49_2$	FH	58		49		48			4
$G\text{-}49_1$	GA	14		49		39			10
$G\text{-}49_2$	GA	66		49		48			6
F-64	FB	9		64		58			4
$F\text{-}81_1$	FJ	23		81		76			6
$F\text{-}81_2$	FJ	61		81		74			4
$G\text{-}81_1$	GD	4		81		76			14
$G\text{-}81_2$	GD	53		81		77			3

*Aberrant value both included and excluded in calculations.

Figure C-6a

Lab C (322 m/e)
(n = 17)

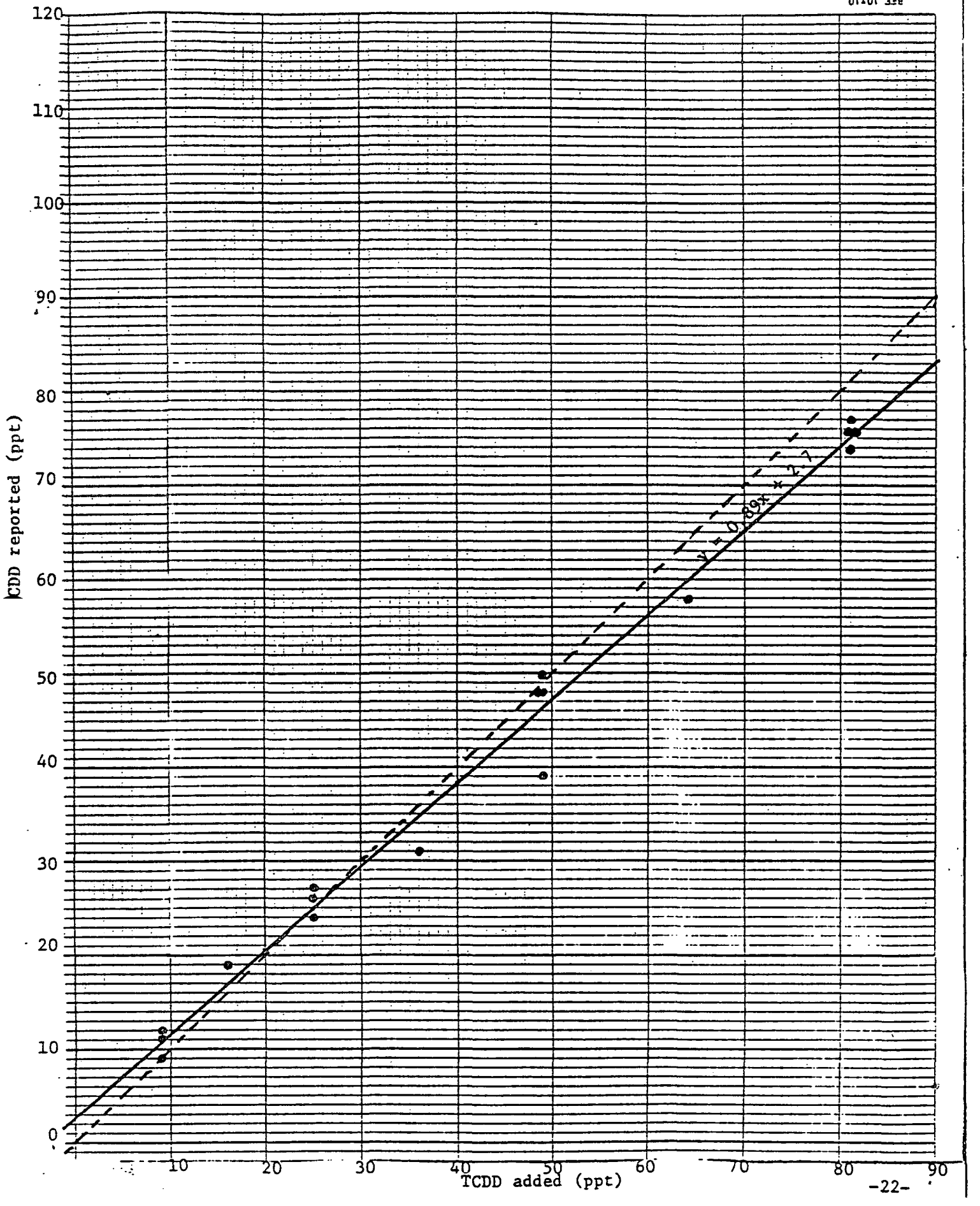


Figure C-6b

Beef fat (5 gm)
Lab C (322 m/e)
(n = 18)

Net

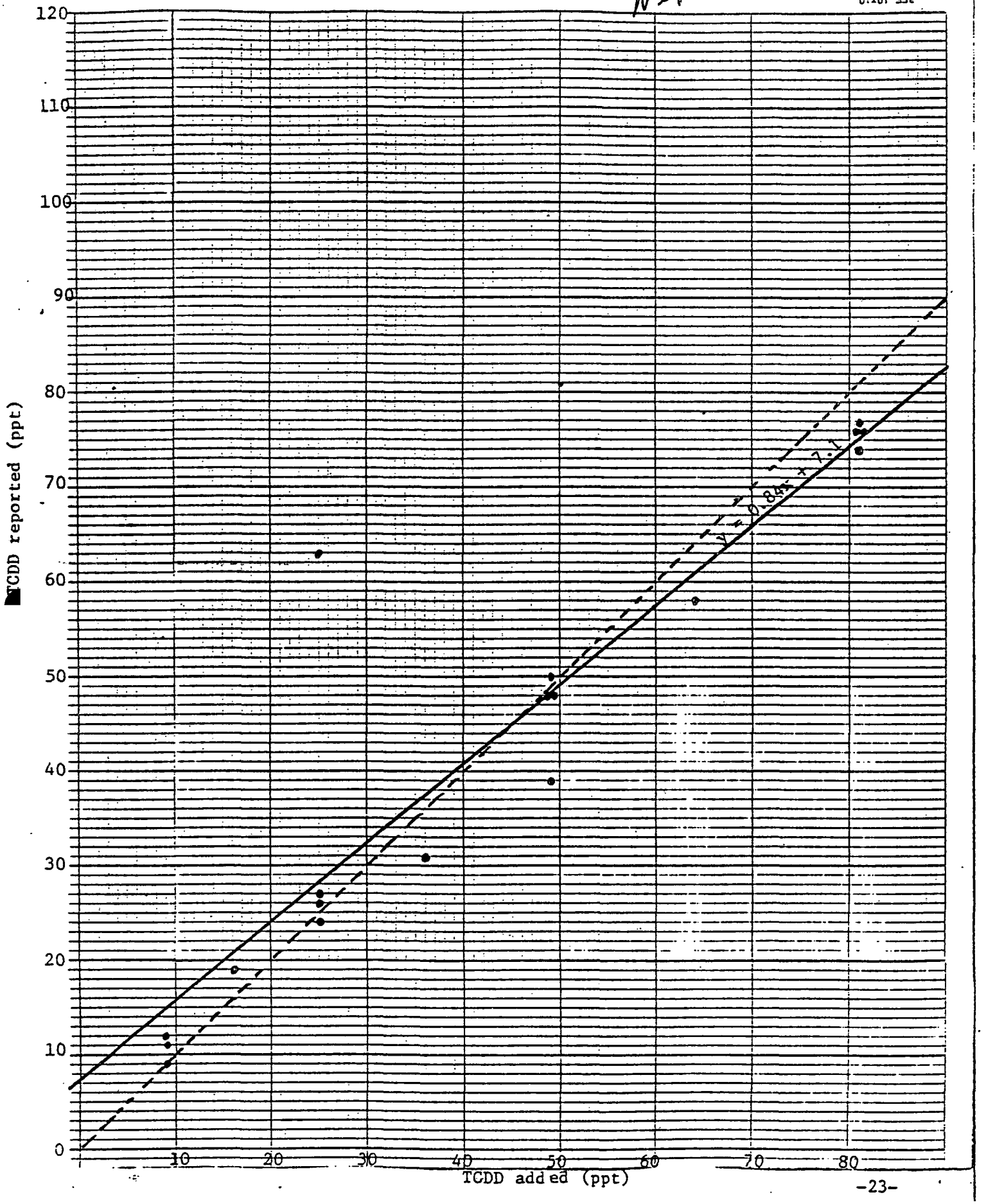


Table C-7.

Dioxin Phase II: Interlaboratory Quantitation Study

Type of Sample: Beef Fat (2.5g sample)
Extraction Lab: Lab D; Method: Neutral extraction
Quantitation Lab: Lab C

Sample ID			Recov.	TCDD Levels (ppt)				Detection Limit	
					Reported				
Study	PML	Ship-ment	Cl^{37} (%)	Added	320	322	Avg.	320	322
F'-0_1	S-12	41		0		nd			6
G'-0_1	S-1	28		0		nd			6
F'-0.5	S-4	31		0.5		nd			6
F'-1	S-9	37		1		nd			6
F'-4	S-2	29		4		17			6
F'-9_1	S-10	38		9		nd			8
G'-9_1	S-13	42		9		10			5
F'-16	S-3	30		16		12			6
F'-25_1	S-6	34		25		24			5
G'-25_1	S-11	40		25		25			10
F'-36	S-16	45		36		31			6
F'-49_1	S-15	44		49		45			9
G'-49_1	S-8	36		49		70			5
F'-64	S-14	43		64		52			5
F'-81_1	S-5	33		81		76			8
G'-81_1	S-7	35		81		70			3

Figure C-7

Beef Fat Samples
Lab C (322 m/e)
Neutral Extraction, 2.5 gm

Neb

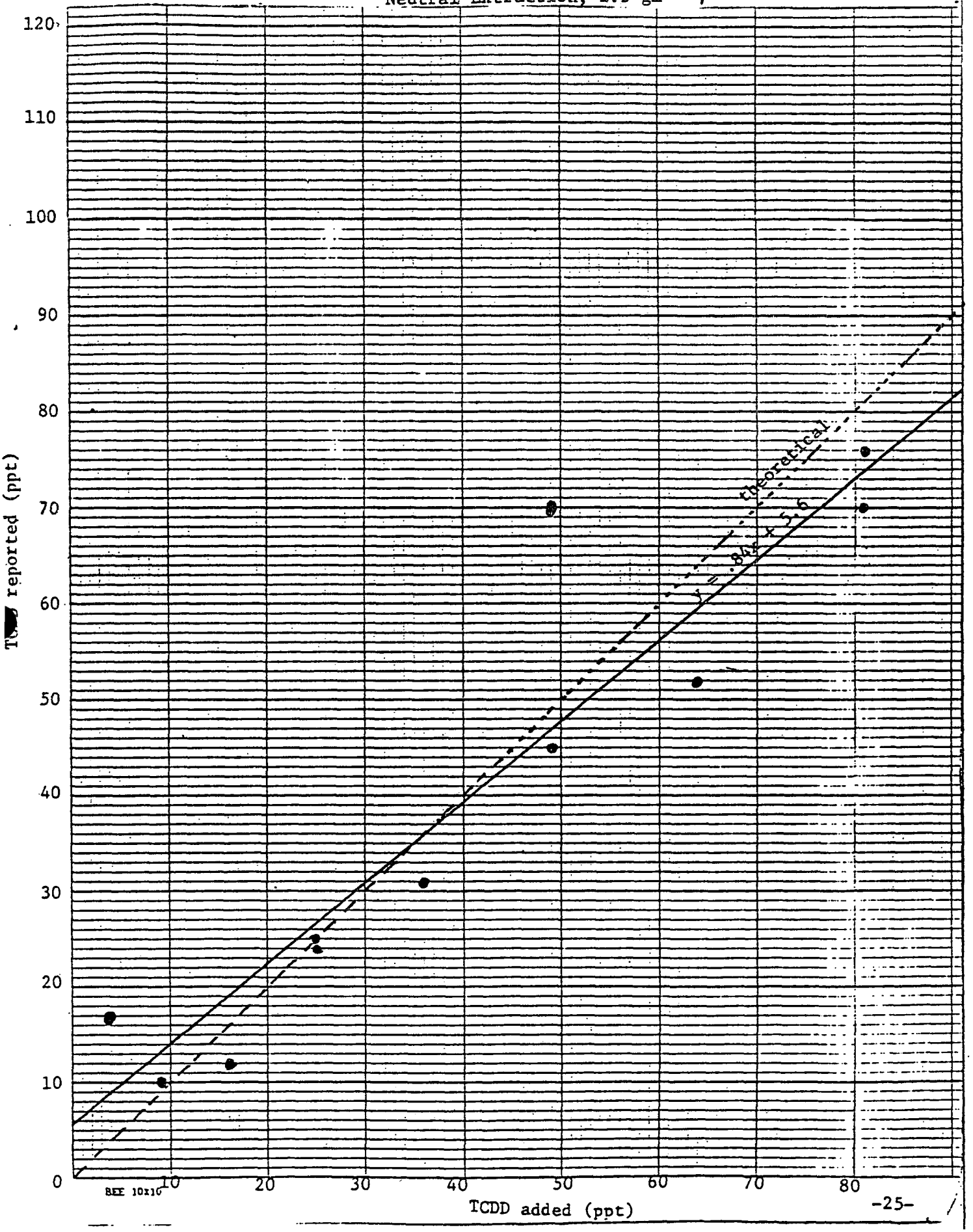


Table C-8.

Dioxin Phase II: Interlaboratory Quantitation Study

Type of Sample: Beef Fat
Extraction Lab: Lab D; Method: Neutral extraction
Quantitation Lab: Lab D

Harvard

Sample ID			Recov.	TCDD Levels (ppt)				Detection Limit	
					Reported				
Study	PML	Shipment	Cl^{37} (%)	Added	320	322	Avg.	320	322
F'-0_1	S-12	41	-	0	nd	nd	nd	23	7.7
G'-0_1	S-1	28	53	0	nd	23	(23)	35	6.9
F'-0.5	S-4	31-1	-	0.5	nd	nd	2.4	16	9.2
		31-2			2.5	2.3		0.8	0.5
F'-1	S-9	37-1		1	nd	-	nd	13	-
		37-2			0.9	nd		0.4	2.2
F'-4	S-2	29	57	4	42	45	44	16	15
F'-9_1	S-10	38	-	9	19	17	18	15	15
G'-9_1	S-13	42-1	81	9	nd	nd	11	16	46
		42-2		9	11	8.5			
F'-16	S-3	30	-	16	25	19	22	9.2	15
F'-25_1	S-6	34	38	25	29	26	28	12	12
G'-25_1	S-11	40	-	25	nd	25	(25)	62	10
F'-36	S-16	45	-	36	32	41	37	8.5	25
F'-49_1	S-15	44	-	49	62	73	68	22	33
G'-49_1	S-8	36	-	49	49	49	49	25	10
F'-64	S-14	43		64	50	54	52	34	8.5
F'-81_1	S-5	33	-	81	-	155	(155)	-	70
G'-81_1	S-7	35		81	89	86	88	24	15

Figure C-8

Beef Fat Samples
Lab D (Avg. of 320, 322 m/e)
Neutral Extraction

Harvard

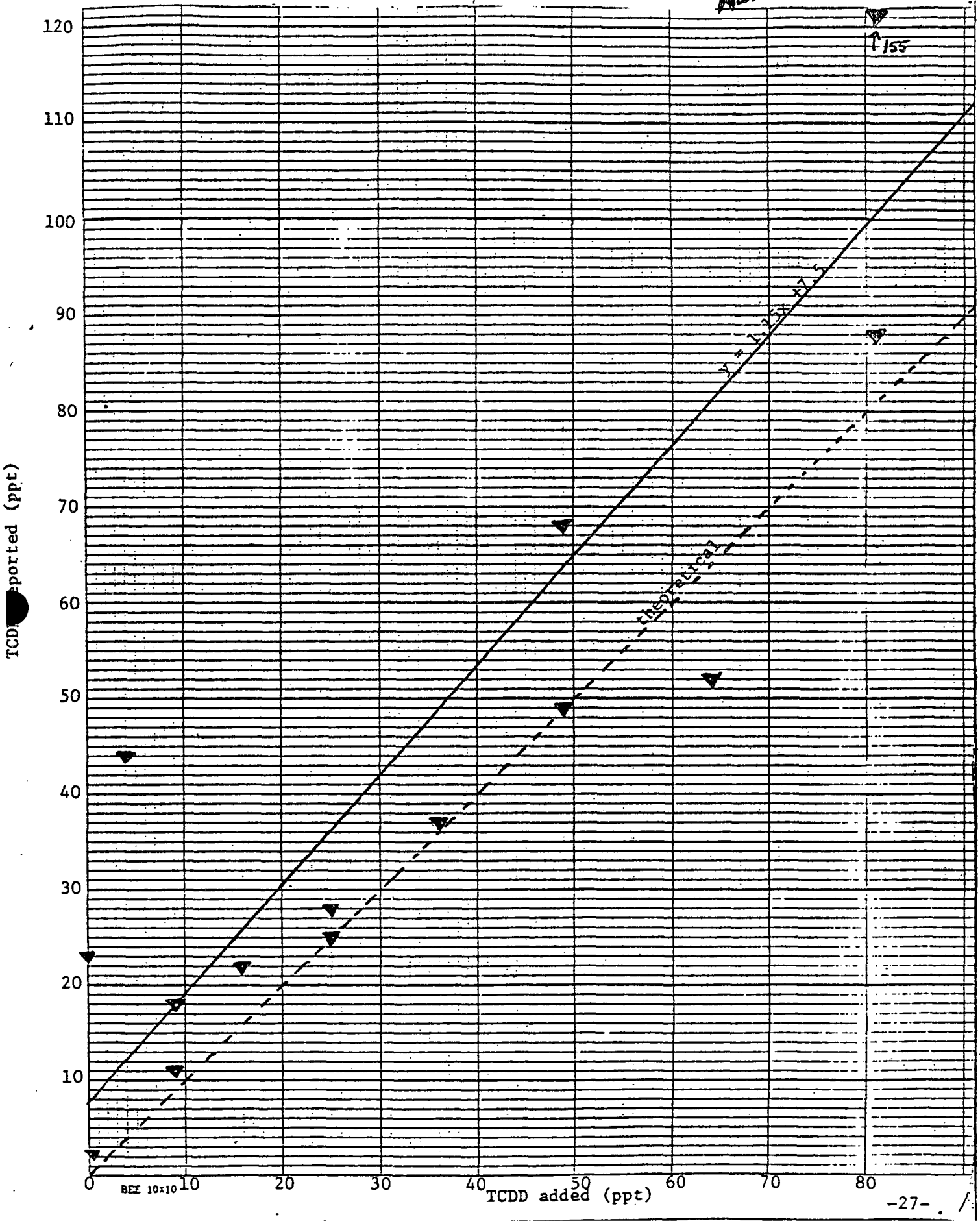


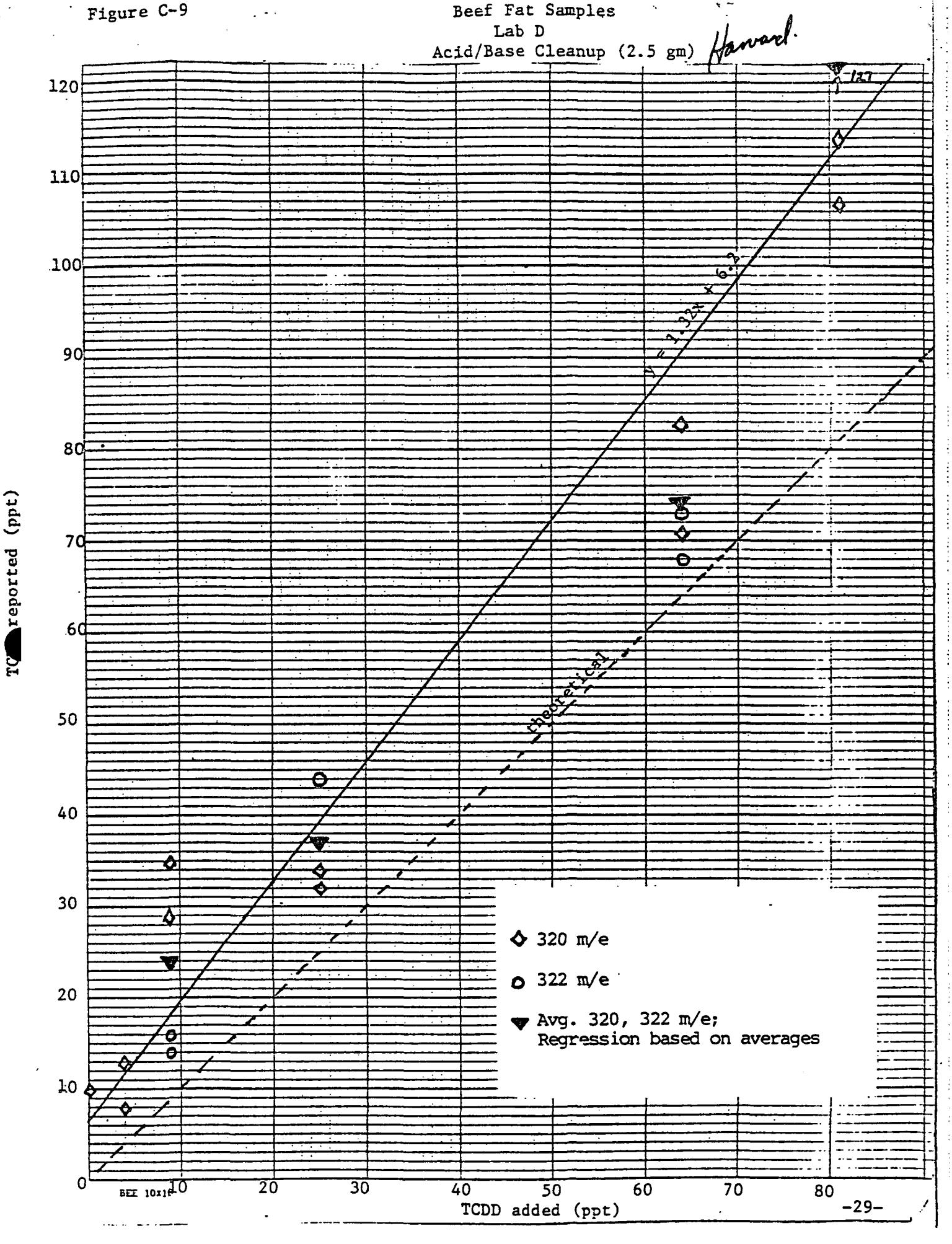
Table C-9.

Dioxin Phase II: Interlaboratory Quantitation Study

Type of Sample: Beef Fat
Extraction Lab: PML; Method: Acid/base
Quantitation Lab: Lab D Harvard

Sample ID			Recov.	TCDD Levels (ppt)				Detection Limit	
					Reported				
Study	PML	Shipment	Cl^{37} (%)	Added	320	322	Avg.	320	322
$F\text{-}0_1$	FE	S0-5	-	0	10	nd	nd	3	66
$F\text{-}0_2$	FE	-		0	(nd)	(nd)		(11)	(15)
$G\text{-}0_1$	GB	-		0		nd			6
$G\text{-}0_2$	GB	-		0		nd			6
F-0.5	FG	-		0.5		nd			14
F-1	FC	-		1		nd			10
F-4	FI	S0-4	-	4	13	nd,nd	nd	4	6.1 (3.1)
					(8.3)	nd,36		8.3	5.0 (7.0)
$F\text{-}9_1$	FK	S0-1	83	9	29	16	24	10	4.4
$F\text{-}9_2$	FK	-		9	35	(14)		9.8	(8)
$G\text{-}9_1$	GC	-		9					
$G\text{-}9_2$	GC	-		9					
F-16	FL	-		16					
$F\text{-}25_1$	FD	S0-3	-	25	32	44	37	3.0	5.0
$F\text{-}25_2$	FD	-		25	(34)	(37)		(8.0)	(7.0)
$G\text{-}25_1$	GE	-		25					12
$G\text{-}25_2$	GE	-		25					4
F-36	FA	-		36					6
$F\text{-}49_1$	FH	-		49					
$F\text{-}49_2$	FH	-		49					
$G\text{-}49_1$	GA	-		49					
$G\text{-}49_2$	GA	-		49					
F-64	FB	S0-6	-	64	71	68	74	10	10
					(83)	(73)		(14)	(13)
$F\text{-}81_1$	FJ	S0-2		81	114	143			6
$F\text{-}81_2$	FJ	-		81	(145)	(154)	127	(17)	
$G\text{-}81_1$	GD	-		81	107			10	
$G\text{-}81_2$	GD	-		81	(100)			(17)	

Figure C-9



BEE 10x10

Table C-10.

Dioxin Phase II: Interlaboratory Quantitation Study

Type of Sample: Human Milk (10 g sample)
Extraction Lab: PML; Method: Acid/base
Quantitation Lab: Lab A W.S.

Sample ID			Recov.	TCDD Levels (ppt)				Detection Limit	
					Reported				
Study	PML	Shipment	Cl^{37} (%)	Added	320	322	Avg.	320	322
$M\text{-}0_1$	AB	71	103	0		1			1
$M\text{-}0_2$	AB	77	92	0		4			2
$N\text{-}0_1$	BB	58	80	0		nd			2
$N\text{-}0_2$	BB	60	103	0		2			2
M-0.5	AD	54	99	0.5		nd			1
M-1	AE	59	91	1		nd			1
M-4	AF	63	92	4		1			1
$M\text{-}9_1$	AC	53	93	9		3			1
$M\text{-}9_2$	AC	55	89	9		5			1
$N\text{-}9_1$	BC	61	69	9		5			4
$N\text{-}9_2$	BC	64	107	9		4			1
M-16	AG	56	79	16		5			1
$M\text{-}25_1$	AH	70	99	25		15			2
$M\text{-}25_2$	AH	76	90	25		11			2
$N\text{-}25_1$	BD	66	81	25		15			1
$N\text{-}25_2$	BD	74	104	25		7			1
M-36	AI	69	96	36		28			1
M-49	AA	72	110	49		29			1
M-49	AA	78	119	49		29			3
N-49	BA	57	100	49		36			1
N-49	BA	62	98	49		39			1
M-64	AJ	68	85	64		36			2
M-81	AK	67	89	81		31			1
M-81	AK	75	104	81		21			2
N-81	BE	73	136	81		114			2
N-81	BE	79	121	81		24			1

Figure C-10

Human Milk Samples
Lab A (322 m/e) W.S.

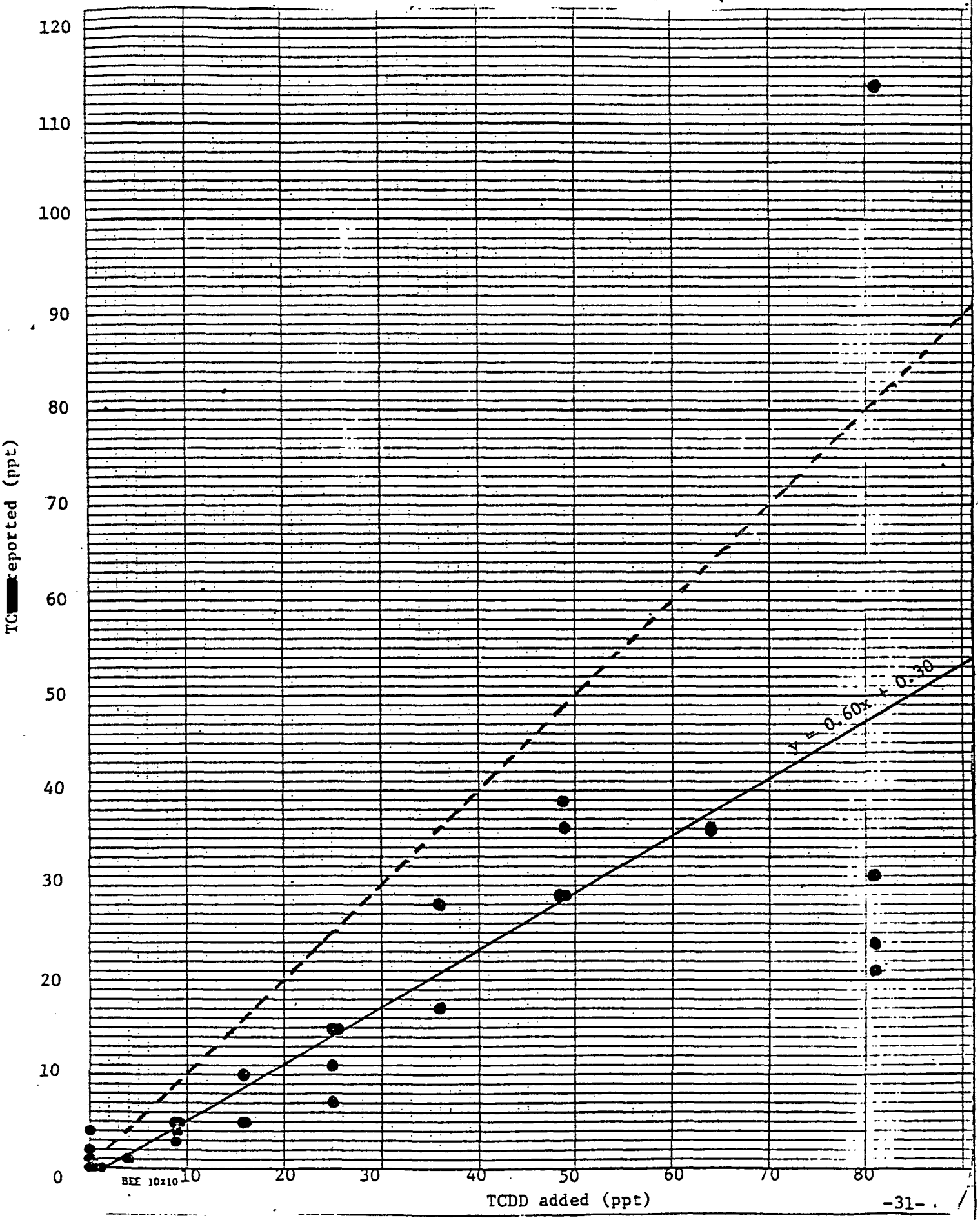


Table C-11.

Dioxin Phase II: Interlaboratory Quantitation Study

Type of Sample: Human Milk (10 g sample)
Extraction Lab: PML; Method: Acid/base
Quantitation Lab: Lab B Dow

Sample ID		Ship-	Recov.	TCDD Levels (ppt)				Detection Limit	
					Reported				
Study	PML	ment	Cl^{37}(%)	Added	320	322	Avg.	320	322
$M\text{-}0_1$	AB	71		0	-	10	(10)	-	2
$M\text{-}0_2$	AB	77		0	-	8	(8)	-	2
$N\text{-}0_1$	BB	58		0	2	17	9.5	2	3
$N\text{-}0_2$	BB	60		0	2	15	8.5	2	3
M-0.5	AD	54		0.5	-	3	(3)	-	1
M-1	AE	59		1	-	7	(7)	-	3
M-4	AF	63		4	nd	4	(4)	2	1
$M\text{-}9_1$	AC	53		9	3	7	5	2	2
$M\text{-}9_2$	AC	55		9	3	7	5	2	2
$N\text{-}9_1$	BC	61		9	2	10	6	2	2
$N\text{-}9_2$	BC	64		9	-	9	(9)	-	2
M-16	AG	56		16	7	9	8	2	2
$M\text{-}25_1$	AH	70		25	7	26	16.5	1	2
$M\text{-}25_2$	AH	76		25	24	35	29.5	4	2
$N\text{-}25_1$	BD	66		25	29	34	31.5	1	2
$N\text{-}25_2$	BD	74		25	-	32	(32)	-	2
M-36	AI	69		36	23	38	30.5	1	2
M-49	AA	72		49	45	69	57	3	2
M-49	AA	78		49	49	53	51	4	2
N-49	BA	57		49	17	21	19	1	2
N-49	BA	62		49	-	10	(10)	-	1
M-64	AJ	68		64	-	82	(82)	-	3
M-81	AK	67		81	-	110	(110)	-	3
M-81	AK	75		81	-	97	(97)	-	2
N-81	BE	73		81	-	110	(110)	-	2
N-81	BE	79		81	-	96	(96)	-	2

Figure C-11a

Human Milk Samples
Lab B (322 m/3) Dow.

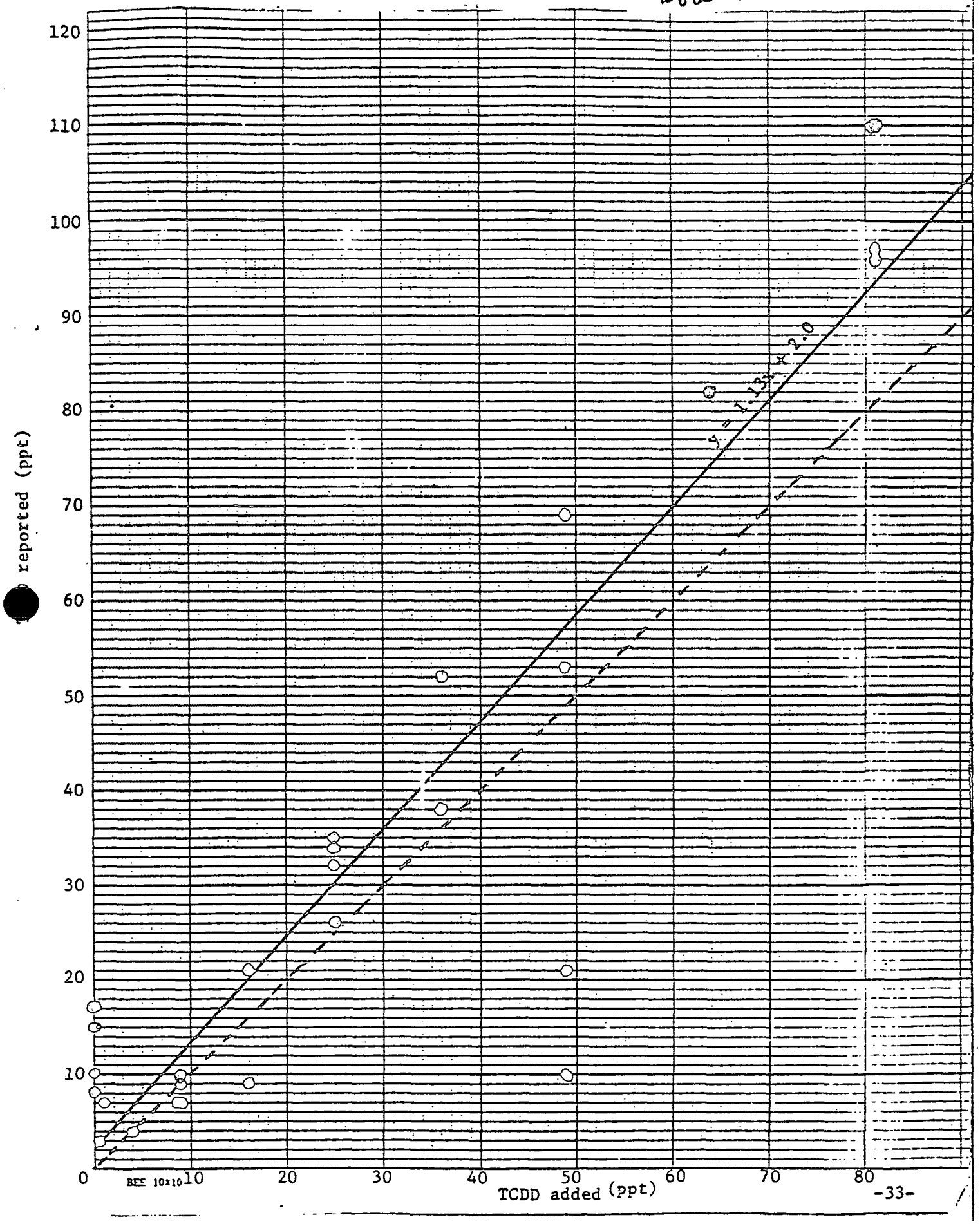


Figure C-11b

Human Milk Samples
Lab B (320 m/e) Dow

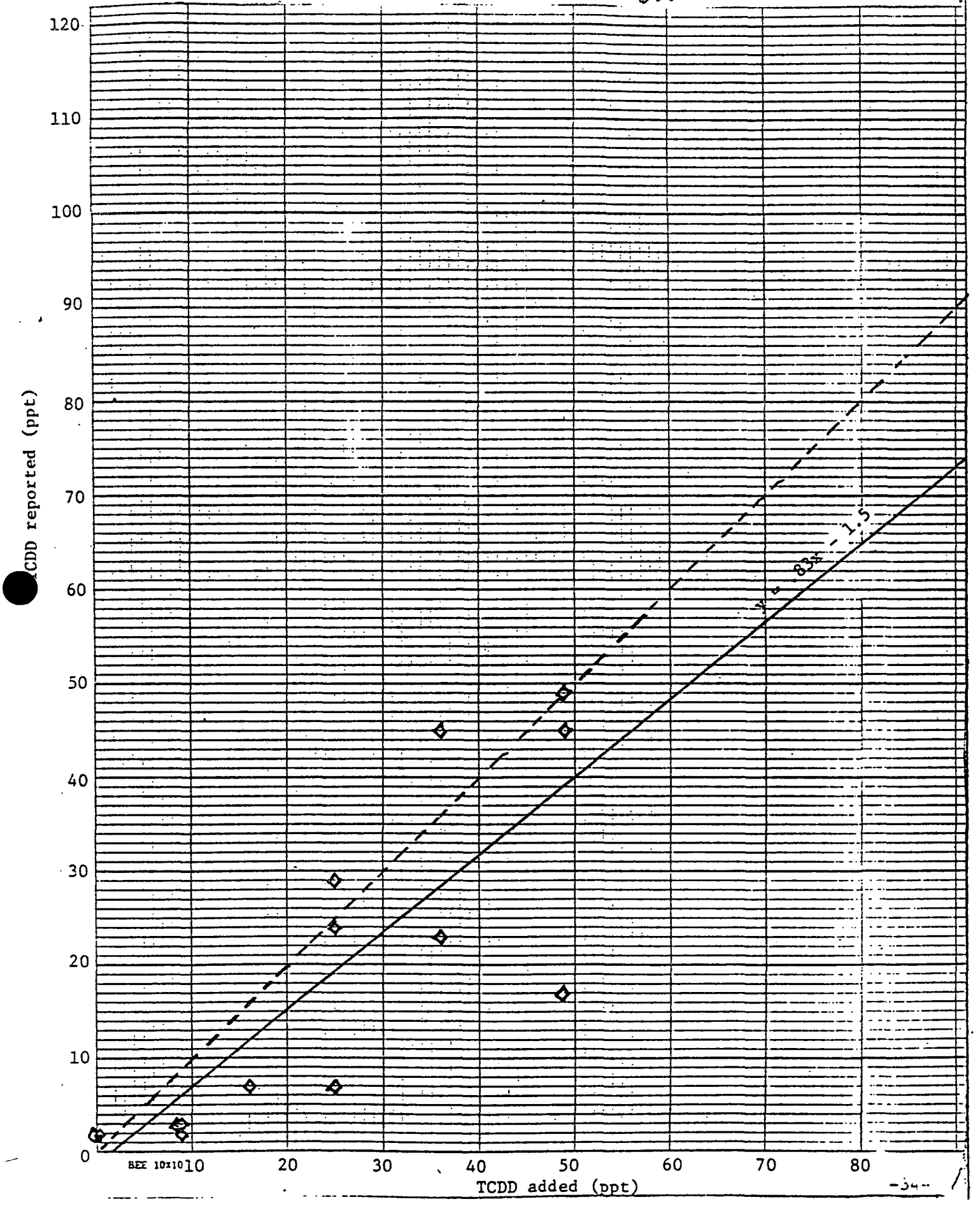


Table C-12.

Dioxin Phase II: Interlaboratory Quantitation Study

Type of Sample: Human Milk + 1 standard
Extraction Lab: PML; Method: Acid/base
Quantitation Lab: Lab E RTP

Sample ID			Recov.	TCDD Levels (ppt)				Detection Limit Avg.	
					Reported				
Study	PML	Shipment	Cl^{37}(%)	Added	320	322	Avg.	320	322
$M\text{-}0_1$	AB	HMT-3	100+	0			1.5		0.3
$M\text{-}0_2$	AB	-10	66	0			0.6		0.1
-	A2	-6	95	0.2			9		1
M-4	AD	-4	64	0.5			0		0.2
-	AI	-9	100	0.8			0.9		0.3
M-1	AE	-11	100	1			1.4		0.4
M-4	AF	-2	77	4			6		0.6
$M\text{-}9_1$	AC	-1	100+	9			14		3
$M\text{-}9_2$	AC	-7	100+	9			5.5		1
M-16	AG	-5	100+	16			29		2.1
M-25	AH	-8	100+	25			34		2
S-1	STD-1	-12	100	1			1.9		0.2

Figure C-12

Human Milk Samples

Lab E (Avg. of 320, 322 m/e) RTP

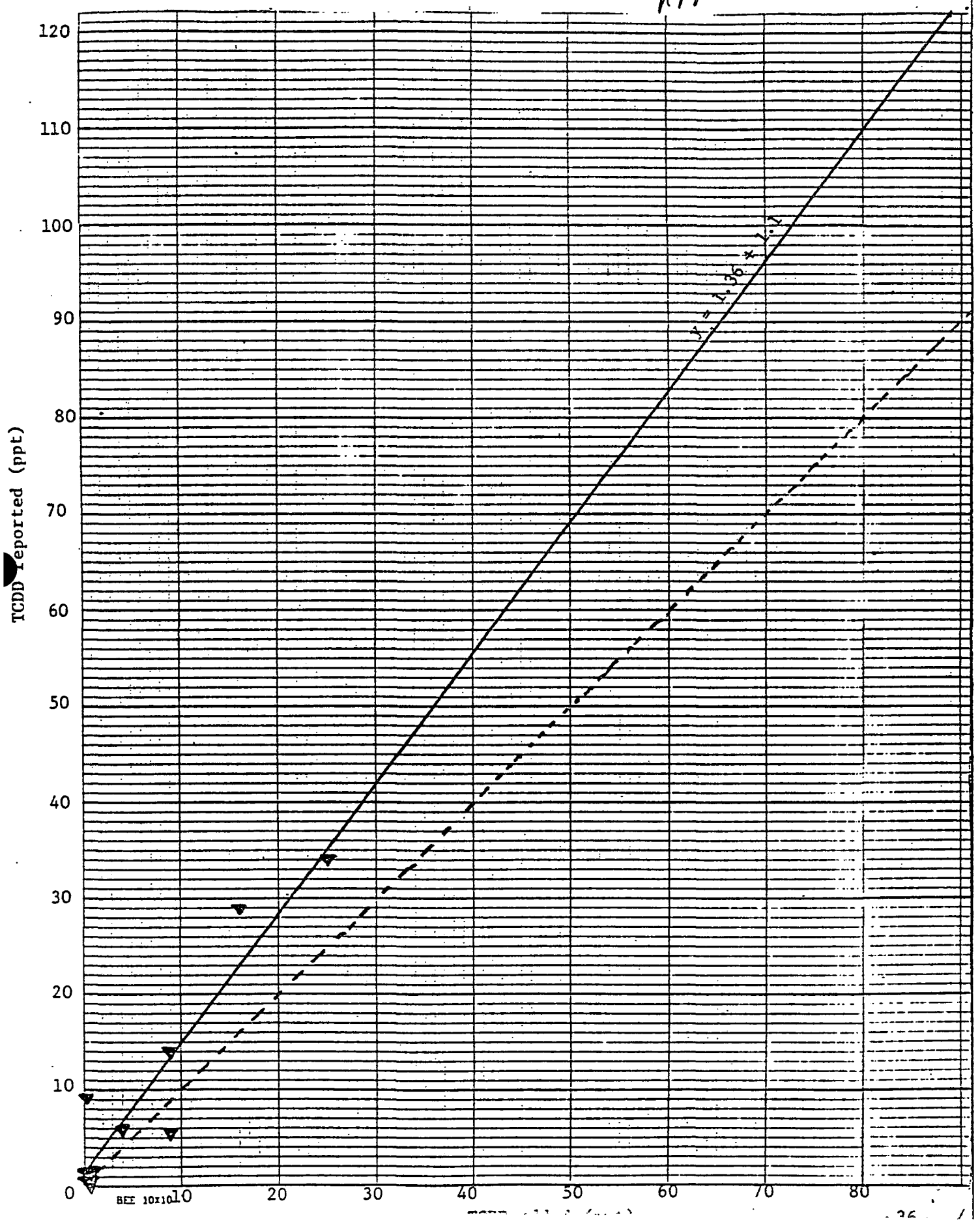


TABLE C-13.

Types of "Valid" and "Erroneous" Values

Reported for Spiked Samples

Laboratory Report	Spiking Category: Sample Not Spiked	Sample Spiked: S < DL 1/	Sample Spiked: S ≥ DL 2/
not detected (nd)	valid	valid	false nd (fnd)
positive value	False Positive (FP)	false positive (fp)	valid

1/ Spiking level less than the detection limit.
2/ Spiking level greater than or equal to the detection limit.

TABLE C-14.

Incidence of Reporting Errors[1/] for Standards

Number of Measurements (n) and Errors, by Types

Lab	m/e	Sample Not Spiked (n)	Sample Not Spiked FP	Sample Spiked: Spike < 9 ppt (n)	Spike < 9 ppt fp	Spike < 9 ppt fnd	Spike ≥ 9 ppt (n)	Spike ≥ 9 ppt fp	Spike ≥ 9 ppt fnd
A	322	(4)	1	(3)	0	0	(19)	0	2
B	320	(4)	1	(2)	0	0	(19)	0	0
B	322	(4)	1	(2)	0	0	(19)	0	0
C	322	(4)	0	(3)	0	0	(19)	0	0
D	-	(0)	-	(0)	-	-	(0)	-	-
	Avg:								
E	320, 322	(0)	-	(1)	0	0	(0)	-	-
Totals:		(16)	3	(11)	0	0	(76)	0	2

1/ See Table C-13 for error definitions

TABLE C-15.

Incidence of Reporting Errors[1] for Beef Fat Samples[2]

Number of Measurements (n) and Errors, by Type

Lab	m/e	Sample Not Spiked (n)	Sample Not Spiked FP	Sample Spiked: Spike < 9 ppt (n)	Spike < 9 ppt fp	Spike < 9 ppt fnd	Sample Spiked: Spike ≥ 9 ppt (n)	Spike ≥ 9 ppt fp	Spike ≥ 9 ppt fnd
A	322	(4)	2	(3)	2	0	(19)	0	1
B	320	(4)	3	(3)	2	0	(19)	0	0
	322	(4)	4	(3)	2	0	(19)	0	0
C	322	(4)	0	(3)	0	0	(19)	0	0
C	(NE)[2] 322	(2)	0	(3)	1	0	(11)	0	1
D	(NE) 320	(2)	1	(8)	2	0	(14)	1	0
D	(NE) 322	(2)	1	(7)	3	0	(13)	1	0
D	320	(2)	1	(2)	1	0	(10)	2	0
D	322	(2)	0	(4)	0	0	(8)	0	0
E	-	(0)	-	(0)	-	-	(0)	-	-
Totals:									
	Acid/base	(20)	10	(18)	7	0	(94)	2	1
	NE	(6)	1	(18)	6	0	(38)	2	1

1/ See Table C-13 for error definitions
2/ NE denotes neutral extraction; otherwise, acid/base cleanup utilized

TABLE C-16.

Incidence of Reporting Errors[1/] for Human Milk Samples[2/]

Number of Measurements (n) and Errors, by Types

Lab	m/e	Sample Not Spiked (n)	Sample Not Spiked FP	Sample Spiked: Spike < 9 ppt (n)	Spike < 9 ppt fp	Spike < 9 ppt fnd	Sample Spiked: Spike ≥ 9 ppt (n)	Spike ≥ 9 ppt fp	Spike ≥ 9 ppt fnd
A	322	(4)	3	(3)	0	1	(19)	0	1
B	320	(2)	2	(1)	0	1	(11)	0	0
	322	(4)	4	(3)	2		(19)	0	0
C	-	(0)	-	(0)	-	-	(0)	-	-
D	-	(0)	-	(0)	-	-	(0)	-	-
	Avg:								
E	320, 322	(2)	2	(5)	0	1	(4)	0	0
Totals:		(12)	11	(12)	2	3	(53)	0	0

1/ See Table C-13 for error definitions
2/ All extractions utilized acid/base cleanup

D. Statistical Analysis of Laboratory C Beef Fat and Standard Reports

For practicality, a detailed statistical analysis of analytical results is presented only for Laboratory C in order to determine the optimum known accuracy and precision that can currently be achieved in quantifying low ppt levels of TCDD in samples of the types analysed. (Complete statistical analyses of the results of other laboratories can be conducted if determined advisable.) Laboratory C quantified only standards and beef fat samples; therefore, the exact reliability of the analytical method for human milk is currently speculative.

Two types of upper and lower 95% confidence limits have been calculated for the regressions of reported values (Y) on spiking levels (X), as shown for standards in Figure D-1 and for beef fat in Figures D-2 and D-3. First are the 95% confidence limits for the line itself, as are graphed by the pairs of lines closest to the regression line in the above Figures. These limits are interpreted as follows: The true regression line (as would be determined if the experiment were repeated a countless number of times under the same conditions) lies within the confidence limits unless the test results are sufficiently unusual to be among those expected to occur less than 5% of the time.

The second set of confidence limits, depicted by the pair of lines furthest from the regression line, predict the 95% confidence limits for the result of a single analysis at a particular spiking level. Interpretation is as follows: The result (reported value) of a single analysis of a standard or beef fat sample spiked at a given level can be predicted to fall between the 95% confidence lines unless the analytical result (which includes extraction as well as GC-MS quantitation) is one sufficiently unusual to be expected to occur approximately 5% of the time.

In calculating the regression lines and confidence limits, values of "nd" have been excluded. For Lab C all spiking levels below 9 ppt were reported as nd, and therefore, the lower limit of quantitation in this study must be considered to fall somewhere between 5 and 9 ppt. Lab C gave no erroneous reports (i.e., no reports classified as FP, fp or fnd) for standards or for 5g beef fat samples when extraction utilized acid/base cleanup.

The calculated regression line for standards lies very close to the theoretical line, the slope of 0.983 being essentially equal to the theoretical slope of 1 and any point on the line being from 1 to 2 ppt less than the spiking level (Figure D-1). The 95% confidence limits for a predicted result of a single analysis fall only 6 to 7 ppt above and below the regression line. Thus, accuracy can be expressed as a negative

bias averaging about 2 ppt over the range of levels tested, and precision in terms of the confidence limits for the line and for predicted results of individual standards. There was no apparent tendency for increased variability among reported values at higher (or lower) spiking levels, i.e., variance about regression was apparently independent of the spiking level (See Table C-3 to compare values).

The results of the beef fat analyses were slightly more variable than those for standards, as might be expected. In particular, one value was an apparent outlier (reported value 63 ppt; spiking level 25 ppt) and has been both excluded (Figure D-2) and included (Figure D-3) in calculations. The rationale for excluding the value is based on a discussion with the principal investigator at Lab C; he was reasonably certain that on-the-spot calculation of separate measurements of the same GC-MS run would have revealed a discrepancy and the sample would have been rerun. Thus, exclusion of the value assumes a laboratory procedural modification to eliminate the possibility of reoccurance. The reported value of the sample's duplicate was 24 ppt. (A second sample--that spiked at 64 ppt--was originally reported as 32 ppt. Recalculation without knowledge of the spiking level revealed an arithmetic error, resulting in a revised value of 58 ppt, which has been used in calculations.)

Figure D-1

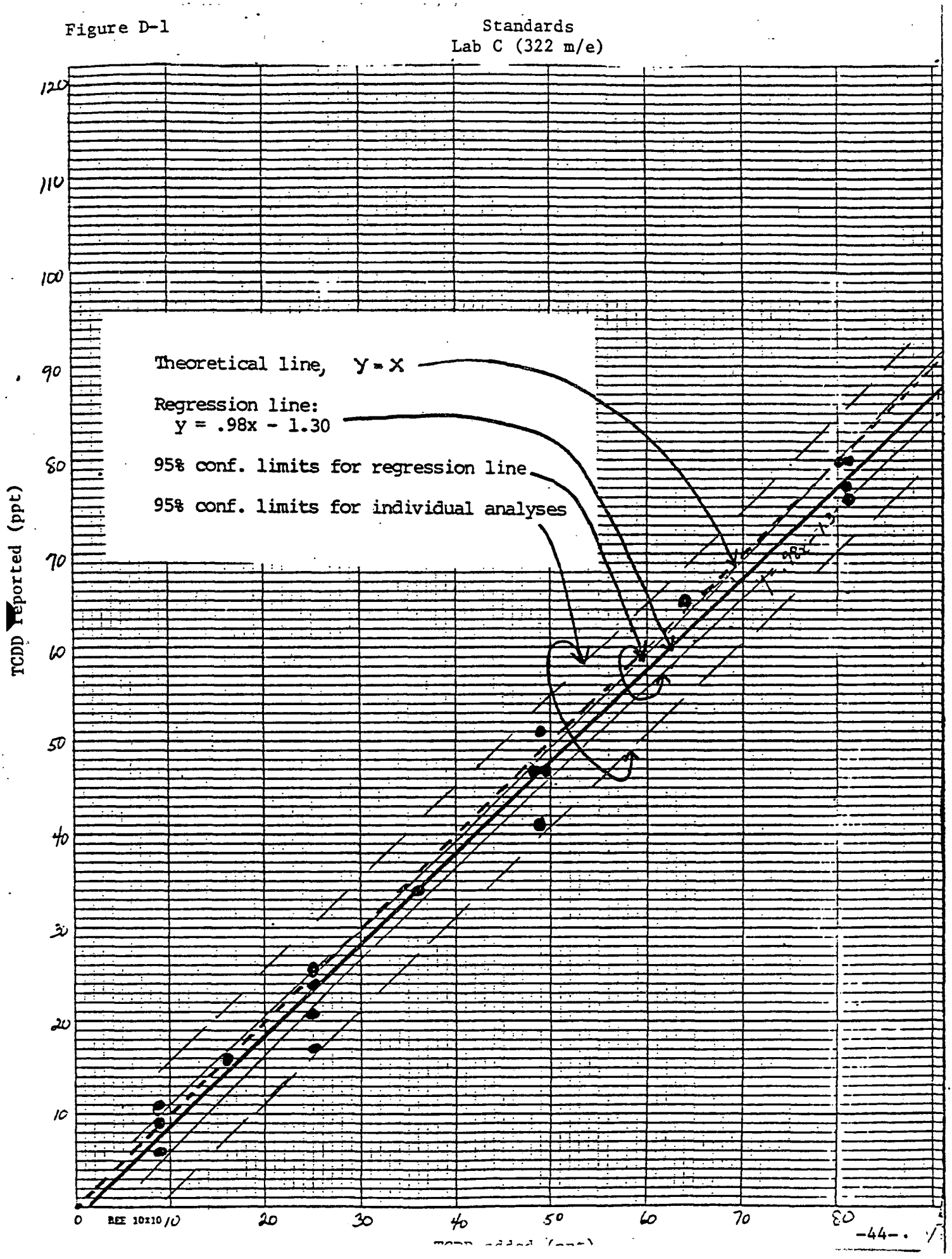

Standards
Lab C (322 m/e)
Theoretical line, Y = X
Regression line:
y = .98x - 1.30
95% conf. limits for regression line
95% conf. limits for individual analyses
TCDD Reported (ppt)
120
110
100
90
80
70
60
50
40
30
20
10
0
20
30
40
50
60
70
80

Figure D-2

Beef Fat Samples, Lab C (n = 17): 5g, acid/base, 322 m/e

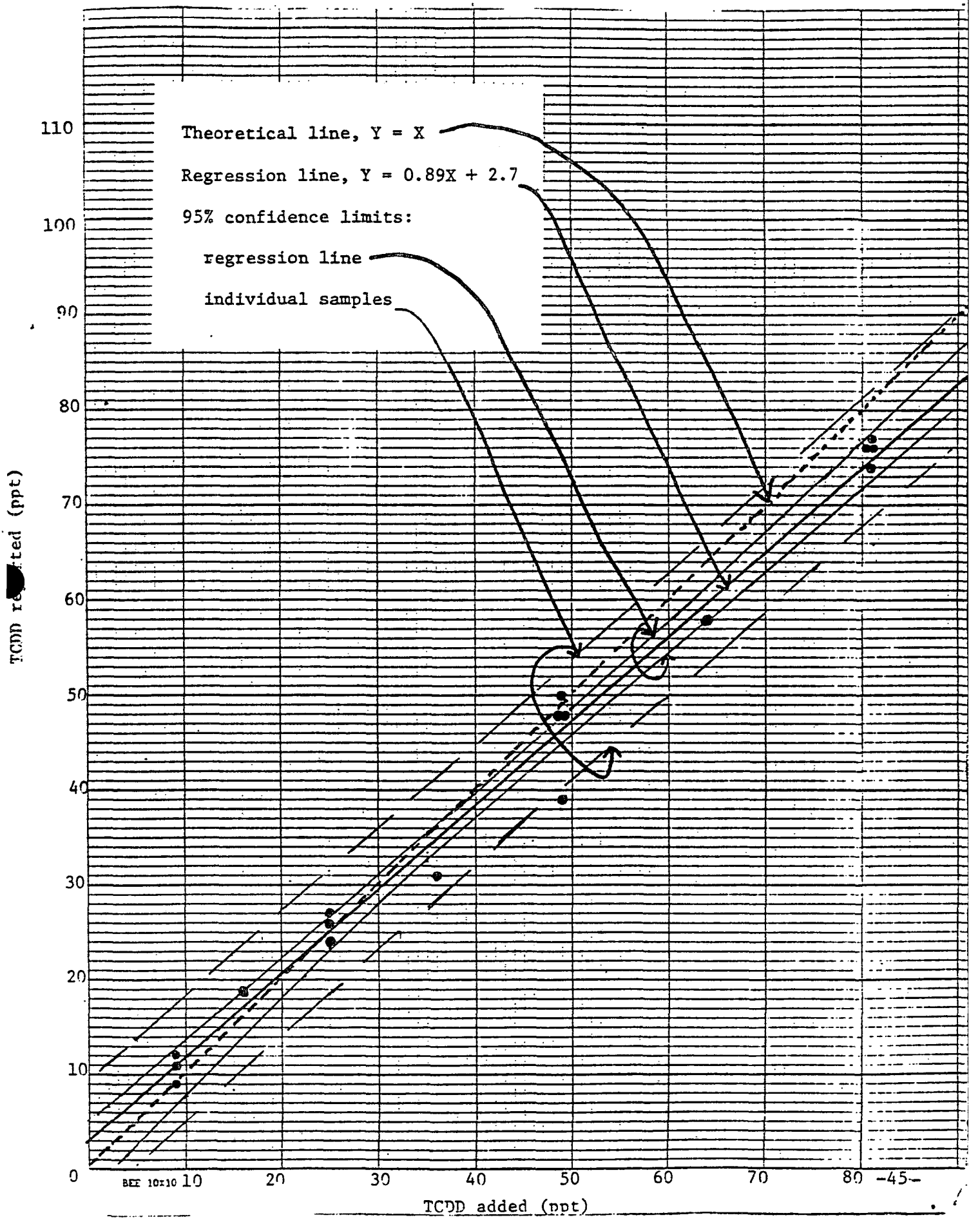


Figure D-3

Beef Fat Samples, Lab C (n=18): 5g, acid/base, 322 m/e

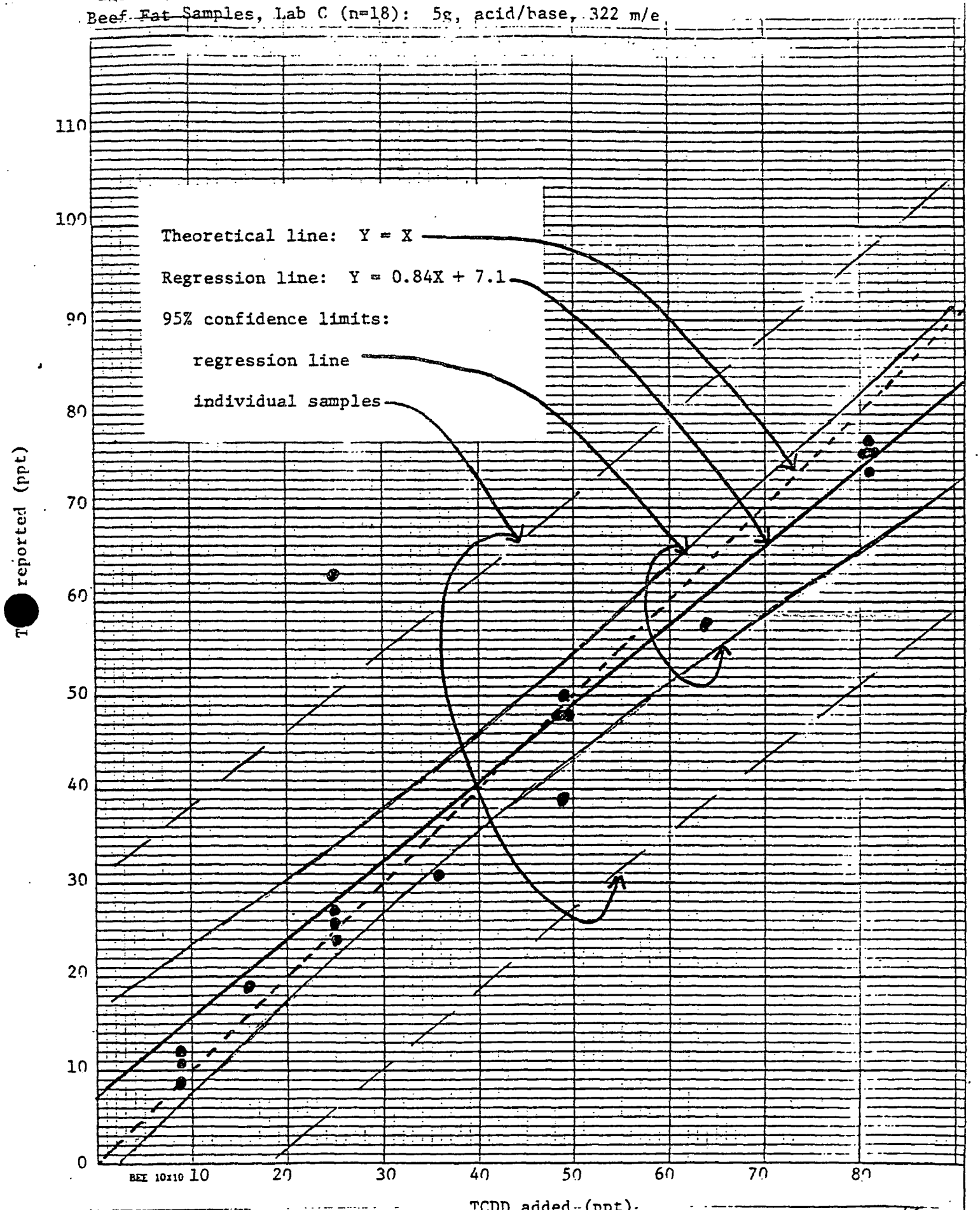


Figure E-2

Estimated True TCDD Level in Beef Fat: Lab C
(From regression analysis of 18 spiked samples)

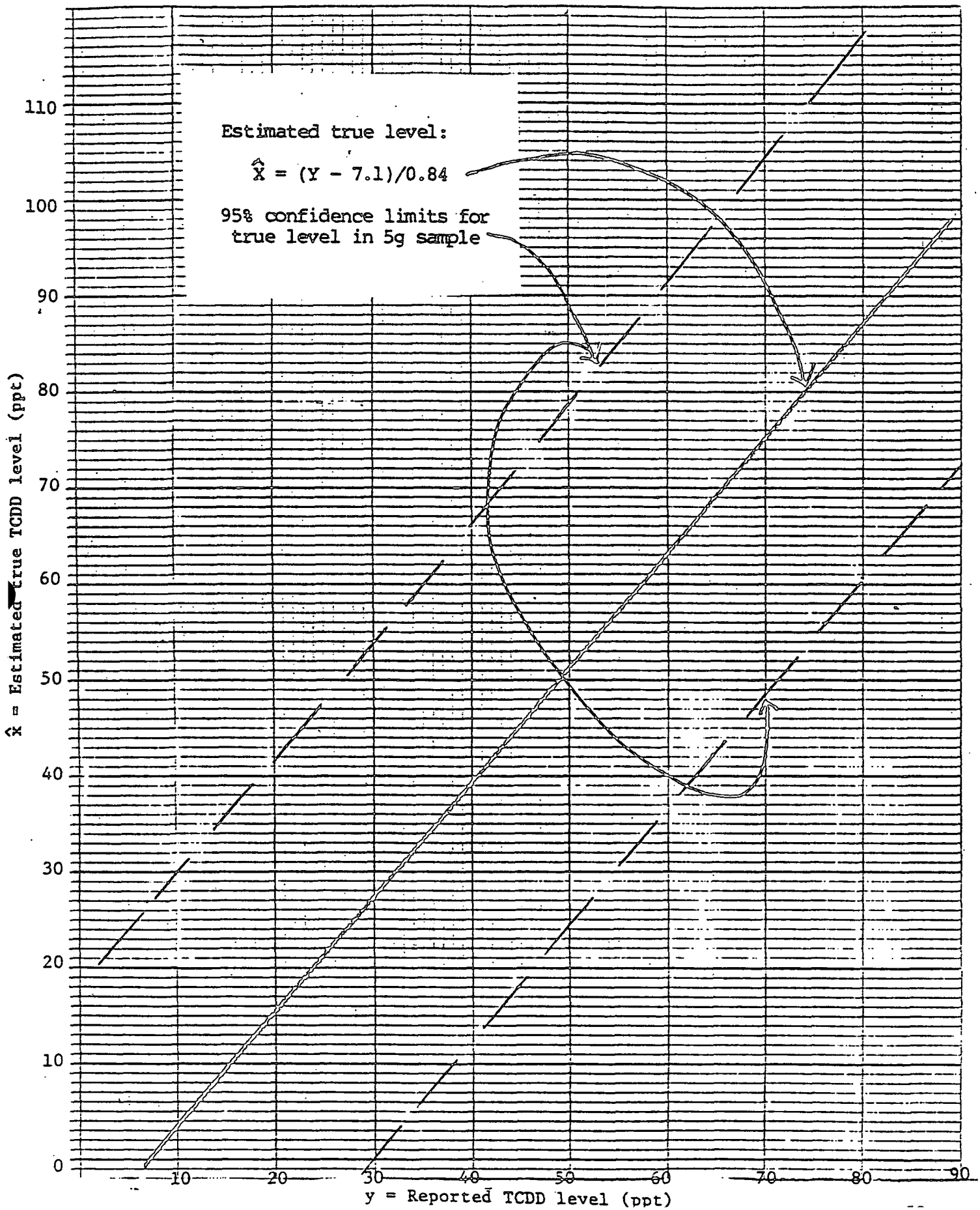
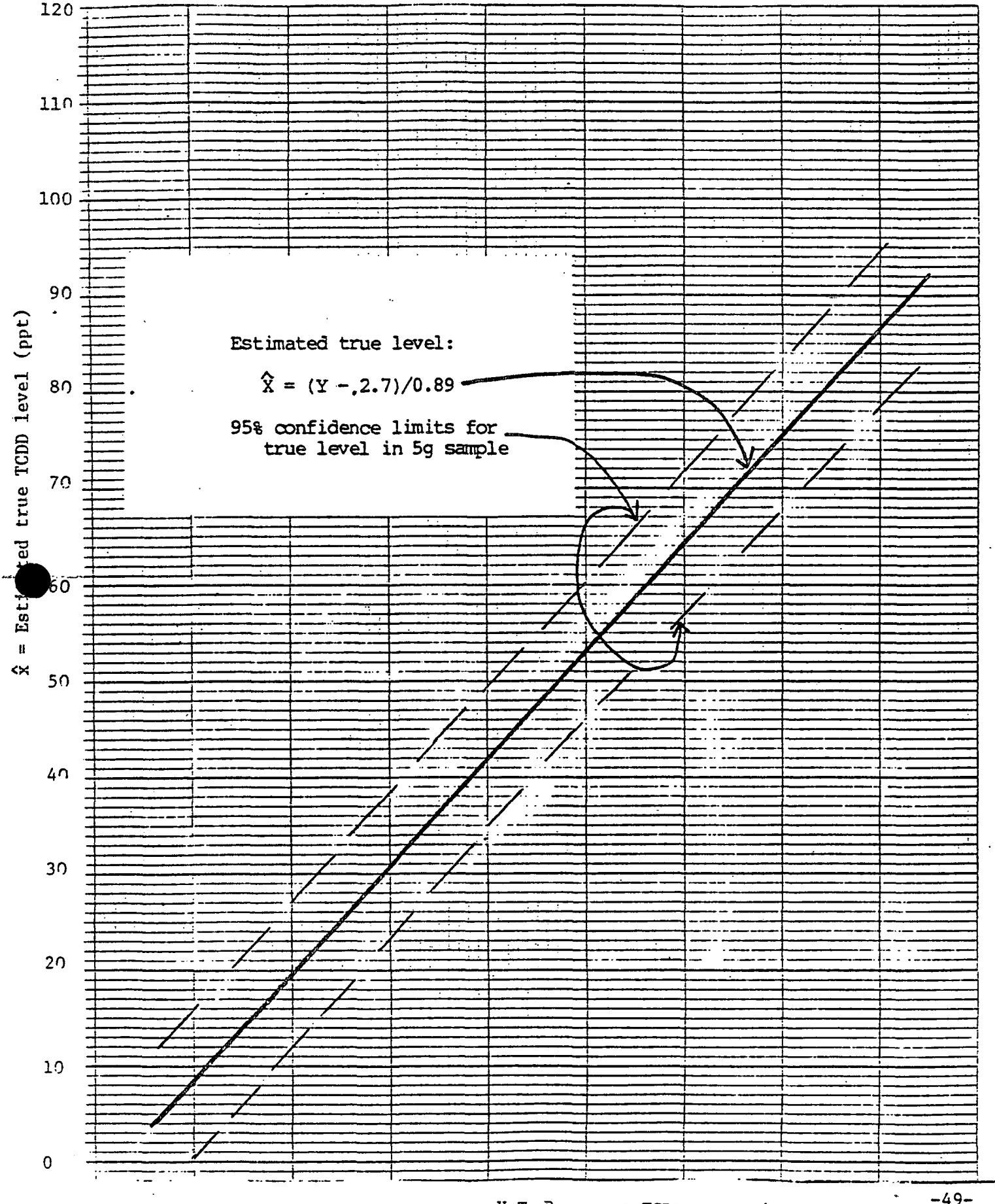


Figure E-1

Estimated True TCDD Level in Beef Fat: Lab C
(From regression analysis of 17 spiked samples)



calculations (Figure E-1) (see part D). When that value is included, the confidence limits are about four times as wide (Figure E-2). For example, the estimated actual value for a reported value of 40 ppt is 42 ppt with 95% confidence limits of 35 to 49 ppt when the aberrant 63 ppt has been excluded from regression calculations. When included, the estimated true level for the same reported value (40 ppt) is 39 ppt with confidence limits of 12 to 66 ppt. Again, the essentiality of developing procedures to detect and correct aberrant results at the onset is emphasized.

The confidence limits presented in the above graph are for a single extraction and GC-MS quantitation of a sample. Confidence limits can be narrowed if 2 or more independent extractions and quantitations of the cause sample are performed and the reported values averaged. This approach may be of value in applied TCDD residue evaluations.

E. Estimation of Actual TCDD Levels, with Confidence Limits, from Spiking Results: Lab C

Using regression statistics developed from spiking study data, a "best estimate" of the "true" level of TCDD in an unspiked sample can be derived from the reported level, as well as statistical confidence limits for that estimate. The procedure is that of estimating the value of the independent variable ($\hat{X}$) for a measurement of the dependent variable (Y), in this case the reported TCDD level in a sample. (The basic approach is used, for example, in estimating LD_{50}'s in dose-response studies.) Confidence limits for such estimates tend to be broad relative to these for the regression line per se.

Figures E-1 and E-2 present estimated "true" values of TCDD ($\hat{X}$) for reported values (Y) ranging from approximately 9 to 80 ppt. In both figures the reported values (Y) now appear on the horizontal axis and the estimated "true" values ($\hat{X}$) on the vertical axis. The slope of the new line is the reciprocal of the slope of the regression of Y on X. The new regression equations appear on the graphs.

The 95% confidence limits for estimates of actual values range from 6 to 7 ppt above and below the predicted value when the aberrant value of 63 ppt is eliminated from the

F. Discussion

This study has demonstrated that for standards and spiked samples of beef fat, the extraction and quantitation methodology exist to quantify TCDD at levels as low as 9 ppt with practicable accuracy and precision. This conclusion assumes that extraction methods are exactly those used at PML and quantitation utilizes procedures and instrumentation indentical or equivalent to that of Lab C. Otherwise, practicable precision has not been fully demonstrated.

The reliability of the above methodology for quantifying TCDD in human milk is yet to be determined. However, based on the fact that milk results are essentially as precise as those for beef fat among laboratories that performed both sets of analyses, a quantitation problem is not anticipated. None-the-less, the procedure needs to be verified with further testing.

Lab C has indicated that their instrumentation may be capable of quantifying TCDD levels below 9 ppt in samples of the type used in the study. Reports of False Positives (positive TCDD values reported for unspiked samples) by laboratories other than Lab C may present a basic problem when attempting to quantify in the range of 0 to possibly 8 ppt. Additional analyses of spiked samples are necessary to determine if a quantification level below 9 ppt can be achieved.

Reprinted from Analytical Chemistry, 1981, 53, 1902.

Method Validation for the Determination of Tetrachlorodibenzodioxin at the Low Parts-per-Trillion Level

M. L. Gross,* Tung Sun, P. A. Lyon, S. F. Wojinski, and D. R. Hilker

Department of Chemistry, University of Nebraska—Lincoln, Lincoln, Nebraska 68588

A. E. Dupuy, Jr.

USEPA, OPTS/OTS, EED, Field Studies Branch, Toxicant Analysis Center, Bay St. Louis, Mississippi 39529

R. G. Heath*

USEPA, OPP/OTS, HED, Health Effects Branch, 401 M St., S.W., Washington, D.C. 20460

This statistically designed study is directed at determining the precision and accuracy obtainable in the quantitation of 2,3,7,8-tetrachlorodibenzodioxin (TCDD) in standard solutions and in fortified beef adipose tissue. The TCDD was extracted after digestion of the adipose tissue in ethanolic potassium hydroxide, and the resulting solution was cleaned up using a concentrated sulfuric acid wash and short column liquid chromatography. The analysis was conducted with packed column gas chromatography interfaced to a high-resolution mass spectrometer operating at a mass resolution of 10 000–12 000 (10% peak width definition). Quantitation was achieved by employing an internal standard method which involved 2,3,7,8-TCDD labeled with ^{37}Cl. The results were submitted to comprehensive statistical analysis in order to determine "best estimates" of concentrations in actual samples and to express the reliability of such estimates in terms of statistical confidence limits.

Attention has been focused recently on the need for evaluation of data taken in environmental analysis (*1*). One aspect of evaluation is a quality assurance program which should include proficiency testing. This is a report of a blind study of the proficiency achievable for the analysis of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. The study also included comparison of replicate results with those obtained in other laboratories. Those results are available in another report (*2*).

This study was undertaken to measure the accuracy and precision with which 2,3,7,8-tetrachlorodibenzodioxin (TCDD), when added to beef fat at low parts-per-trillion concentrations, could be extracted and quantified using packed column gas chromatography–high-resolution mass spectrometry (GC-HRMS). Method validation also included quantitation of equivalent amounts of TCDD in standard solutions.

The extraction and analysis employed in this study stem from the pioneering work of Baughman and Meselson (*3*) and later O'Keefe, Meselson, and Baughman (*4*) who employed high-resolution dual ion monitoring with direct probe introduction of the sample. The disadvantage of this procedure has been overcome by scientists at Dow Chemical by developing packed column GC for sample introduction to the mass spectrometer (*5*). Although this approach is specific for the general class of TCDDs, it does not permit separation of all TCDD isomers. However, some can be distinguished (*6*); for example, 2,3,7,8-TCDD can be resolved from 1,3,6,8-TCDD using the gas chromatography employed in this study.

To meet the needs of environmental monitoring at the low parts-per-trillion range, particularly for samples relating to certain types of combustion (*7–11*), isomer specific methods have been developed. One approach is to use capillary column GC coupled with either low (*8, 12, 13*) or high-resolution (*14*) mass spectrometry. A single column can be used to separate 2,3,7,8-TCDD from all the other 22 isomers (*13*). A second approach involves a combination of high-pressure liquid chromatography and packed column GC/low-resolution MS

(6). This latter method is totally isomer specific.

Nevertheless, the methods evaluated here have advantages. The extraction and cleanup are relatively simple, and the GC/MS procedure is efficient. Therefore, the analysis is rapid and suitable for carefully controlled monitoring studies and for sample screening.

This extensive blind study is the first to be conducted for parts-per-trillion levels which has made use of rigorous statistical design and analysis of the data. Recently, analytical data for various chlorinated dioxins have been submitted to a similar statistical treatment (*15*). However, those results were not obtained in a blind study.

The specific goals of the investigation are to develop regression statistics for converting reported TCDD concentrations to "best estimates" of actual (but unknown) concentrations and to express the reliability of such estimates in terms of statistical confidence limits. In addition, we intend to determine the lowest concentration of TCDD detectable with practicable consistency and the frequency of "false positive" and "false negative" reports.

EXPERIMENTAL SECTION

Sample Extraction and Cleanup. The adipose tissue samples were extracted by methods described previously (*14*). Briefly, beef adipose was rendered to obtain a representative sample that was free of connective and other tissue. A 10-g sample was spiked directly with 20 ng of ^{37}Cl-TCDD and a known amount of nonenriched or "native" TCDD. After addition of 15 mL of distilled water, 20 mL of ethyl alcohol, and 40 mL of 45% potassium hydroxide, the mixture was refluxed with stirring for 2.5 h. After being cooled, the resulting homogenous solution was extracted 4 times with 25 mL of hexane each, and the hexane extracts were combined.

The combined hexane extracts were washed with base solution followed by four 50-mL portions of concentrated sulfuric acid. The hexane layer was then washed with distilled water, neutralized, and dried by passing it through a glass column packed with anhydrous sodium carbonate. The hexane was then concentrated in preparation for liquid chromatography.

The concentrate was transferred to a small alumina column (4.5 cm × 0.5 cm) prepared in a disposable Pasteur pipet. The column was eluted with 6 mL of carbon tetrachloride (discarded) followed by 4 mL of methylene chloride which was collected in a distillation receiver. The solvent was evaporated through a micro-Snyder column, and two separate 2-mL portions of hexane were added and also evaporated. The residue was taken up in 3 mL of hexane and rechromatographed on a second column as just described. After the methylene chloride was evaporated, 2 mL of benzene was added, the solution concentrated to 0.100 mL, transferred quantitatively to Chromaflex tube, and carefully concentrated to 0.060 mL using a stream of dry nitrogen. This extract was split into two equal portions and each was sealed in 3 mm i.d. × 7 cm glass tubing and stored in a freezer until the GC/MS analysis. Samples were shipped in dry ice cooled containers.

GC/MS Analysis. The sample extracts were analyzed at the University of Nebraska in two batches (see below) using a Pye Unicam Series 104 gas chromatograph which was coupled directly to a Kratos MS-50 high-resolution mass spectrometer. Data acquistion was accomplished by use of a 40K word Nicolet Model 1180 computer which was interfaced to the peak switching circuitry of the mass spectrometer.

An aliquot representing 20–30% of the sample was injected (injection at 250 °C) on a 183 cm × 0.64 cm o.d. GC column containing 3% OV-3 on Supelcoport (from Supelco, Inc., Bellefonte, PA). A helium flow rate of about 20 cm^3/m was used at a column temperature of 250 °C for 1 min and then the temperature increased linearly to 300 °C at a rate of 10 deg/min. The column was maintained at 300 °C for 7 m. The eluent from the column (principally solvent) was vented to the atmosphere for the first 1 min and then the entire gas flow was admitted directly to the mass spectrometer source. The interface consisted of 75 cm × 0.03 cm i.d. glass-lined stainless steel tubing connected to a 7.5 cm × 0.15 mm i.d. glass restrictor and an electrically nonconducting glass coil. The transfer line and the glass restrictor and coil were held at 250 and 220 °C, respectively. The retention time, determined by using standard solutions of 2,3,7,8-TCDD, was 4.4 and 5.3 min for the first and second batches, respectively, of sample analyses. The 2,3,7,8-TCDD and any coeluting isomers were quantitated by duel ion monitoring using real-time peak matching at a mass resolution of 10 000–12 000 (10% valley). One channel was centered at m/z 327.8848 (^{37}Cl-TCDD, the internal standard) and the other at m/z 321.8936 (the most abundant molecular ion of TCDD having natural isotopic abundances). The complete peak profiles were acquired by scanning at a frequency of 2 Hz over a masss range of 300 ppm (0.097 atomic mass unit). The output for about 120 sweeps was accumulated in the memory of the computer, submitted to a smoothing routine, and then plotted on an X-Y recorder (see Figure 1 for a typical output). The signal averaging was started at the first appearance of internal standard signal observed on the peak matching oscilloscope (*11*).

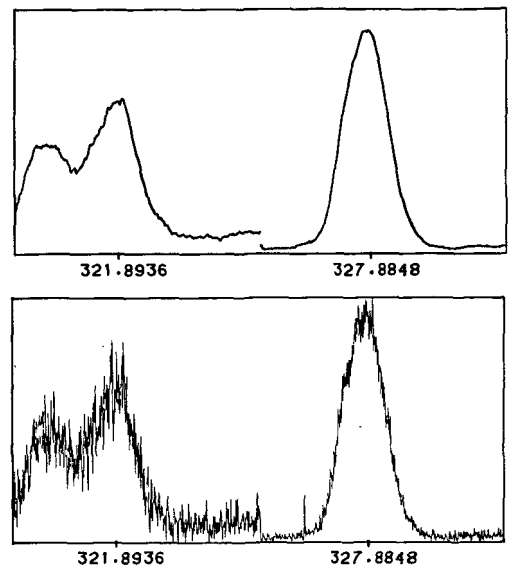


Figure 1. Actual signal output (lower trace) and smoothed output (51 point sliding window) for high-resolution dual ion monitoring of an adipose extract fortified with TCDD at 16 pptr. The signal on the left is for m/z 321.8936 (larger signal) and on the right is for m/z 327.8848, the internal standard. The reported concentration is 12 pptr.

Data Handling. The concentrations of TCDD were determined from a calibration plot using the ratio of the maximum peak heights at m/z 327.8848 and 321.8936. The calibration data were acquired by using solutions of the native TCDD and ^{37}Cl-TCDD which were analyzed at a rate of about one every two to three unkowns. The calibration was linear over a range of 5–150 pg of native TCDD. If no signal was observed at m/z 321.8936, the detection limit was calculated to be 2.5 times the noise amplitude (a 2.5:1 signal-to-noise criterion).

The percent recovery was calculated knowing the size aliquot removed from the original extact and measuring the absolute intensity for the isotopically labeled internal standard (m/z 327.8848). The response of the GC/MS to the internal standard was determined by injecting known amounts of the standard.

Further confidence for the assignment of the peak profile as TCDD can be obtained by injecting a second aliquot and monitoring m/z 320 and m/z 322. This was not done for these samples. The internal standard method (using m/z 322 and m/z 328 peak intensities) is preferred for quantitation.

The results were transmitted without knowledge of the code to the office of the coordinator of the Dioxin Monitoring Program, United States Environmental Protection Agency, Washington, DC. The data were then decoded and forwarded to one of the authors (R.G.H) for statistical analysis using standard methods (*16*).

RESULTS AND DISCUSSION

Study Design. Beef fat samples were "spiked" with native 2,3,7,8-TCDD at levels ranging from 0 to 81 pptr (parts per trillion). Standards were prepared so as to contain equivalent amounts of the chemical. Samples were developed from one

Table I. Sample Design for Validation Study

TCDD level[a]	beef fat pool code	beef fat no. of extracts	standards pool code	standards no. of solns
0	F	2	S	4
0	G	2		
1/2	F	1	S	1
1	F	1	S	1
4	F	1	S	1
9	F	2	S	4
9	G	2		
16	F	1	S	1
25	F	2	S	4
25	G	2		
36	F	1	S	1
49	F	2	S	4
49	G	2		
64	F	1	S	1
81	F	2	S	4
81	G	2		

[a] Parts per trillion.

of two pools of rendered beef fat (pools F and G) taken from cattle without potential exposure to TCDD. Pools were constructed by using equal amounts of fat from each animal. Tissue from a separate set of animals was used for construction of each pool (see Table I).

Eleven samples were prepared from fat pool F, the major pool. The samples were spiked individually with native 2,3,7,8-TCDD and the internal standard. They were then extracted as described in the Experimental Section. Spiking levels, excepting 0.5 pptr, were systematically incremented as the squares of the digits 0 through 9 to provide close spacing at low levels and a moderate, systematic increase in spacing with increasing levels.

To test the precision of the extraction methodology, we prepared five samples from fat pool G, the minor pool (see Table I). These samples were extracted by the same procedures used for samples from Pool F. Thus, fat pools F and G provided replicate samples at the above levels of spiking for testing extraction precision.

To test the precision of GC-HRMS quantitation for comparison with that of extraction, the analysis laboratory was provided two aliquots of the G-pool extracts, along with two aliquots from the matching extracts from pool F, so as to obtain duplicate analysis of the same extract. The laboratory also received four replicates of some of the standard solutions (see Table I).

All beef fat samples and standards were prepared or extracted and shipped in random order. In all, 52 samples were involved. They were transmitted to the University of Nebraska in four batches each containing about equal numbers of extracts. The first 27 samples were analyzed along with suitable standards and other samples over a 5-day period. The remaining 25 samples were analyzed over a 6-day period $4^1/_2$ months later. The samples were selected for analysis in random order. Samples were identified only by shipment number, so that neither the type of sample nor the TCDD level was known at the time of quantitation.

Accuracy and precision were measured by methods of regression analysis (*16*). The comparisons of extraction vs. quantitation precision have been made by analysis of variance based on those spiking levels for which there were duplicate analyses of replicate extractions.

Three possible types of analytical reporting errors are recognized in this study. The reporting of a positive value in an unspiked sample is identified as a "false positive" (FP), and a positive report given when the detection limit exceeds the level of spiking is identified as a "false positive" (fp). A

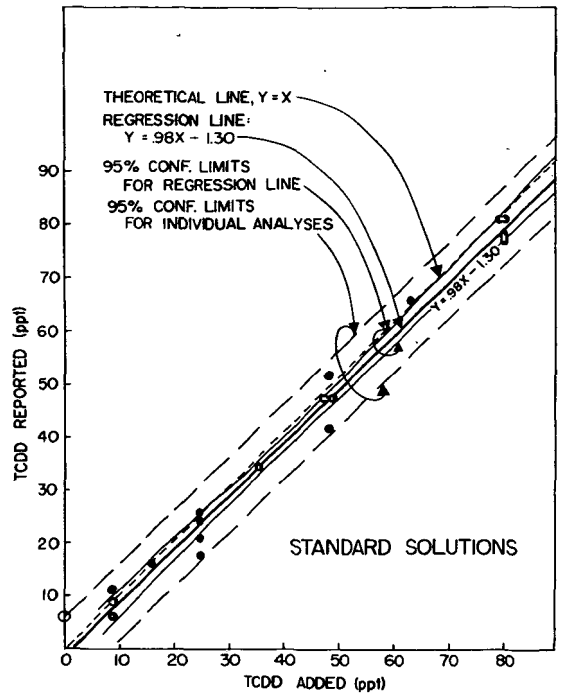


Figure 2. Reported concentration vs. concentration of TCDD actually added to standard solutions.

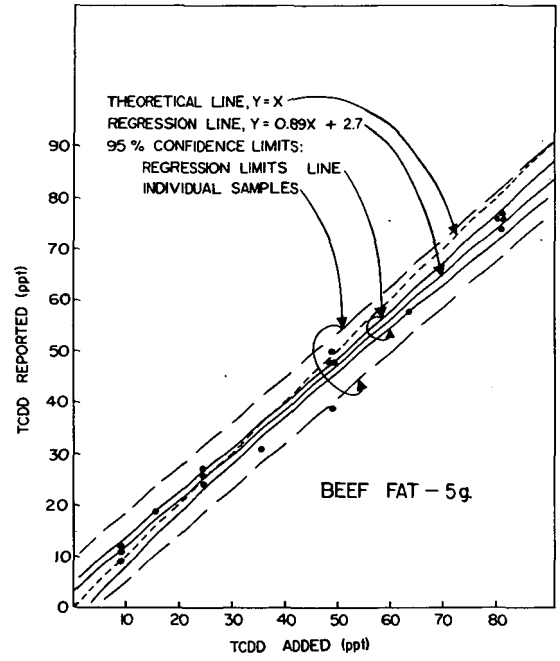


Figure 3. Reported concentrations vs. concentrations of TCDD actually added to beef adipose. The aberrant value (see text) has been included.

"false not detected" (fnd) is defined as a report of "nd" when, in fact, the level of detection is less than the level of spiking.

Statistical Analysis. Analytical results for the quantitation of standards and beef fat samples and the percent recoveries are presented in Tables II and III, respectively. The plotted results for standard solutions, the least-squares regression line, and equation for reported values are given in Figure 2. Figure 3 is a similar presentation for beef fat data but excludes an aberrant report of 63 pptr for a sample spiked at 25 pptr (see below). In each of the above figures, the theoretical line, $y = x$, representing perfect extraction and quantitation, is included for comparative purposes.

Two types of upper and lower 95% confidence limits or "bounds" have been calculated for the least-squares regressions

Table II. Results for Analysis of Standard Solutions (5 g Equivalent) for TCDD

study code[a]	analysis results: TCDD added[b]	TCDD reported[b]	detection limit[b]	% recovery[c]
$S\text{-}0_1$	0	nd	10	93
$S\text{-}0_2$	0	nd	4	85
$S\text{-}0_3$	0	nd	4	85
$S\text{-}0_4$	0	nd	4	85
S-0.5	0.5	nd	6	100
S-1	1	nd	12	94
S-4	4	nd	6	104
$S\text{-}9_1$	9	nd	10	76
$S\text{-}9_2$	9	11	5	80
$S\text{-}9_3$	9	9	4	95
$S\text{-}9_4$	9	6	4	100
S-16	16	16	10	94
$S\text{-}25_1$	25	21	4	99
$S\text{-}25_2$	25	17	4	85
$S\text{-}25_3$	25	25	4	95
$S\text{-}25_4$	25	24	4	75
S-36	36	34	16	110
$S\text{-}49_1$	49	41	12	130
$S\text{-}49_2$	49	51	3	90
$S\text{-}49_3$	49	47	9	90
$S\text{-}49_4$	49	47	5	80
S-64	64	65	6	90
$S\text{-}81_1$	81	76	6	104
$S\text{-}81_2$	81	77	3	100
$S\text{-}81_3$	81	80	3	75
$S\text{-}81_4$	81	80	5	70

[a] Code refers to sample numbers used for the entire study at TAC and at UN-L. [b] Parts per trillion. [c] Average recovery 92 ± 13% (standard deviation). The reported concentrations are corrected for recovery in the internal standard calculations method.

Table III. Results for Analysis of Extracts of 5 g of Beef Fat for TCDD

study code[a]	analysis results: TCDD added[b]	TCDD reported[b]	detection limit[b]	% recovery[c]
$F\text{-}0_1$	0	nd	8	67
$F\text{-}0_2$	0	nd	5	90
$G\text{-}0_1$	0	nd	6	75
$G\text{-}0_2$	0	nd	6	70
F-0.5	0.5	nd	14	93
F-1	1	nd	10	79
F-4	4	nd	18	92
$F\text{-}9_1$	9	9	4	66
$F\text{-}9_2$	9	11	5	75
$G\text{-}9_1$	9	nd	14	84
$G\text{-}9_2$	9	12	5	85
F-16	16	19	10	63
$F\text{-}25_1$	25	(63)[d]	10	67
$F\text{-}25_2$	25	24	2	100
$G\text{-}25_1$	25	26	12	75
$G\text{-}25_2$	25	27	4	75
F-36	36	31	6	68
$F\text{-}49_1$	49	50	6	72
$F\text{-}49_2$	49	48	4	85
$G\text{-}49_1$	49	39	10	120
$G\text{-}49_2$	49	48	6	75
F-64	64	58	4	79
$F\text{-}81_1$	81	76	6	61
$F\text{-}81_2$	81	74	4	85
$G\text{-}81_1$	81	76	14	57
$G\text{-}81_2$	81	77	3	75

[a] Code refers to sample numbers used for entire study. [b] Parts per trillion. [c] Average recovery 78 ± 14% (standard deviation). The reported concentrations are corrected for recovery in the internal standard calculations method. [d] Aberrant value (see text).

of reported values (y) on spiking levels (x), as shown for standard solutions (Figure 2) and for beef fat (Figure 3). First are the 95% confidence bounds for the regression line itself, as are graphed by the pair of lines closest to the regression line in each of the figures. These bounds are interpreted as follows: the true regression line (as would be determined if the study were repeated a countless number of times under the same conditions) lies within the confidence limits unless the analytical results are sufficiently unusual to be among those expected to occur less than 5% of the time.

The second set of confidence bounds, depicted by the outer pair of lines, constitute the 95% confidence limits for the result of a *single* analysis at a particular spiking level. The interpretation is as follows: the result (reported value) of a single analysis of a standard or beef fat sample spiked at a given level can be predicted to fall between the 95% confidence bounds unless the analytical result (which includes extraction as well as GC-HRMS quantitation) is among those sufficiently unusual to be expected less than 5% of the time.

In calculation of the regression lines and confidence limits, values of "nd" have been excluded. All spiking levels below 9 pptr were reported as "nd". Therefore, the lower limit of quantitation in this study must be considered to fall somewhere between 5 and 9 ppt.

The analysis laboratory gave no erroneous reports (i.e., no reports classified as FP, fp, or fnd) for standard solutions or for 5-g beef fat samples extracted utilizing acid/base cleanup.

The calculated regression line for standards solutions lies very close to the theoretical line, the slope of 0.981 being essentially equal to the theoretical slope of unity and any point on the line being from 1 to 2 pptr less than the spiking level. The 95% confidence limits for a predicted result of a single analysis fall only 7 pptr above and below the regression line. Thus, accuracy can be estimated as a negative bias averaging about 2 pptr over the range of levels tested, and precision can be expressed in terms of the confidence limits for the line and for predicted results of individual standards. There was no apparent tendency for increased variability among reported values at higher (or lower) spiking levels, i.e., variance about regression was apparently independent of the spiking level (sees Table II and III).

The rationale for excluding the aberrant value of 63 pptr for beef fat is as follows. The workers in the MS lab were suspicious of the first analysis and reanalyzed this sample obtaining a value of 24 pptr (true value, 25 pptr). However, calculations of TCDD concentrations were not made on-the-spot; therefore, the discrepancy was not realized until a later time when a third analysis was not possible. The 24 pptr result was not reported because the signal-to-noise ratio was worse than the first determination. We are reasonably certain that if calculations were made immediately, the discrepancy would have been realized and a third analysis conducted.

The calculated regression line for beef fat, based on 17 samples (aberrant value excluded), lies close to the theoretical line $y = x$ over the range of spiking levels tested (see Figure 3). Its slope of 0.89 (95% confidence limits 0.84–0.95) differs slightly from the theoretical slope of 1.0, the calculated line intersecting the theoretical line at approximately 25 pptr. Thus, bias, although small, is a function of the spiking level, ranging from approximately +2 pptr for samples spiked at 9 pptr to −6 pptr for those spiked at 81 pptr. Precision was comparable to that for standards: the mean squares for deviation from regression are 5.51 $pptr^2$ for standards and 5.15 $pptr^2$ for fat (5 df each).

Analysis of variance of the differences between reported and spiked TCDD levels for replicate fat samples and duplicate aliquots of the same extract shows that variation between replicates is not significantly greater than that for

duplicate aliquots (i.e., within replicates). The mean square for replicate samples was 10.68 pptr^2 and that for duplicate aliquots was 7.92 pptr^2 ($F_{4,6\,df}$ = 1.35; n.s.). This result demonstrates the reliability of the extraction methodology at low parts-per-trillion levels.

Estimation of True TCDD Levels in Beef Fat. By use of regression statistics developed from spiking study data, a "best estimate" of the true level of TCDD in an unspiked beef fat sample can be derived from the reported level, as well as statistical confidence limits for that estimate. The method is that of inverse prediction for estimating the value of the independent variable (the true TCDD level) for a given measurement of the dependent variable (the reported level).

The estimated true values of TCDD ($\hat{x}$) for the reported values (y) ranging from approximately 9 to 80 pptr has been obtained by replotting the reported values (y) on the horizontal axis and the estimated true values ($\hat{x}$) on the vertical axis. The slope of the new line is the reciprocal of the slope of the regression of y on x. Two new regression equations (eq 1 and 2) are obtained for the data excluding and including the aberrant value, respectively.

$$\hat{x} = (y - 2.7)/0.89 \quad (1)$$

$$\hat{x} = (y - 7.1)/0.84 \quad (2)$$

The 95% confidence limits for estimates of true values range from 6 to 7 pptr above and below the predicted value when the aberrant value of 63 pptr is eliminated from the calculations. When that value is included, the confidence limits are about 4 times as wide. For example, the estimated actual value for a reported value of 40 pptr is 42 pptr with 95% confidence limits of 35–49 pptr when the aberrant 63 pptr has been excluded from regression calculations. When included, the estimated true level for the same reported value (40 pptr) is 39 pptr with confidence limits of 12–66 pptr. It is essential that the procedure to detect and correct aberrant results be implemented at the time of analysis.

The confidence limits presented are for a single extraction and GC/MS quantitation of a sample. Confidence limits can be narrowed if two or more independent extractions and quantitations of the same sample are performed and the reproted values averaged. This approach may be of value in TCDD residue evaluations.

In conclusion, this study is a demonstration that for standards and spiked samples of beef fat, the extraction and quantitation methodology exist to quantify TCDD at levels as low as 9 pptr in 5-g samples with practicable accuracy and precision. This conclusion applies if extraction methods are exactly those used at TAC and for quantitation procedures and instrumentation equivalent to that of the University of Nebraska.

Improvements in the detection limit can be expected by making use of more thermally stable GC columns to reduce chemical noise and by turning to capillary column GC/MS to increase the instantaneous concentration of the TCDD in the ion source.

ACKNOWLEDGMENT

We thank Mary Frankenberry for help with the statistical analysis.

LITERATURE CITED

(1) ACS Committee on Environmental Improvement and Subcommittee on Environmental Analytical Chemistry *Anal. Chem.* **1980**, *52*, 2242.
(2) Heath, R. G. "Interlaboratory Method Validation Study for Dioxin, An Interim Report", United States EPA, Human Effects Monitoring Branch, Office of Toxic Substances, Washington, DC, 1979.
(3) Baughman, R. W.; Meselson, M. S. *EHP Environ. Health Persepct.* **1973**, *5*, 27.
(4) O'Keefe, P. W.; Meselson, M. S.; Baughman, R. W. *J. Assoc. Off. Anal. Chem.* **1978**, *61*, 621.
(5) Shadoff, L. a.; Hummel, R. A. *Biomed. Mass Spectrom.* **1978**, *5*, 1.
(6) Nestrick, T. J.; Lamparski, L. L.; Stehl, R H. *Anal. Chem.* **1979**, *51*, 2273.
(7) Olie, K.; Vermeulen, P. L.; Hutzinger, O. *Chemosphere* **1977**, *6*, 455.
(8) Buser, H. R.; Bosshardt, H. P.; Rappe, C. *Chemosphere* **1978**, *7*, 165.
(9) Eiceman, G. A.; Clement, R. E.; Karasek, F. W. *Anal. Chem.* **1979**, *51*, 2343.
(10) Bumb, R. R.; Crummett, W. B.; Cutie, S. S.; Gledhill, J. R.; Hummel, R. H.; Kagel, R. O.; Lamparski, L. L.; Luoma, E. V.; Miller, D. L.; Nestrick, T. J.; Shadoff, L. A.; Stehl, R. H.; Woods, J. S. *Science* **1980**, *210*, 385.
(11) Kimble, B. J.; Gross, M. L. *Science* **1980**, *207*, 59.
(12) Buser, H. R. *Anal. Chem.* **1977**, *49*, 918.
(13) Buser, H. R.; Rappe, C. *Anal. Chem.* **1980**, *52*, 2257.
(14) Harless, R. L.; Oswald, E. O.; Wilkinson, M. K.; Dupuy, A. E., Jr.; McDaniel, D. D.; Tai, Han *Anal. Chem.* **1980**, *52*, 1239.
(15) Langhorst, M. L.; Shadoff, L. A. *Anal. Chem.* **1980**, *52*, 2037.
(16) Snedecor, G.; Cochran, W. "Statistical Methods", 6th Ed., Iowa State University Press: Ames, IA, 1967.

RECEIVED for review February 19, 1981. Accepted July 22, 1981. This project has been funded at least in part with Federal Funds from the U.S. E.P.A. under Contract No. 68-01-4305. The content of this publication does not necessarily reflect the views of policies of the U.S. E.P.A., nor does mention of trade names, commerical products, or organizations imply endorsements by the U.S. Government.