C, Farlow

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

JAN 30 1991

OFFICE OF
PESTICIDES AND TOXIC
SUBSTANCES

MEMORANDUM

SUBJECT: Fermenta ASC Corporation: Response to the Chlorothalonil Reregistration Standard: Metabolism Studies in Goats (MRID #'s 41576001 and 41576002, DEB # 7112.)

FROM: R. B. Perfetti, Ph.D., Chemist
Reregistration Section
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RB Perfetti

THRU: W. J. Boodee, Section Head
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Chemistry Branch II: Reregistration Support
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TO: Reto Engler, Ph.D., Chief
Science Analysis and Coordination Branch
Health Effects Division (H7509C)

and

L. Rossi, Chief
Reregistration Branch
Special Review and Reregistration Division (H7508C)

Attached is a review of two goat metabolism studies submitted by Fermenta ASC Corporation in response to the chlorothalonil Reregistration Standard. These studies were reviewed by Acurex Corporation under supervision of CBRS, HED.

This information has undergone secondary review in CBRS and has been revised to reflect the Branch policies.

Please see our conclusions in the attachment regarding the adequacy of the information provided by the Registrant.

If you need additional input please advise.

Attachment 1 : Review of Chlorothalonil Goat Metabolism Studies.

cc: With Attachment 1: R. B. Perfetti, J. Burrell (PIB/FOD), Chlorothalonil Reregistration Standard File, Chlorothalonil Subject File, C. Furlow (PIB/FOD), Acurex, Circ. (7).

cc: Without Attachment: P. Fenner-Crisp (HED), R. Schmitt and RF.

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CHLOROTHALONIL
(DEB No. 7112)

TASK 3

Registrant's Response to Residue Chemistry Data Requirements

January 14, 1991

Contract No. 68-DO-0142

Submitted for:

U.S. Environmental Protection Agency
Arlington, VA 22202

Submitted by:

Acurex Corporation
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CHLOROTHALONIL (DEB No.7112)

REGISTRANT'S RESPONSE TO RESIDUE CHEMISTRY DATA REQUIREMENTS

Task-3

BACKGROUND

The Chlorothalonil Guidance Document dated 9/88 concludes that the qualitative nature of the residue in animals is not adequately understood, and requires data concerning the nature of the terminal residues in poultry and ruminants. The 9/88 requirement specifies dosing animals with [^{14}C]chlorothalonil only. The previously issued Guidance Document dated 9/84 includes a requirement for dosing with the metabolite [^{14}C]4-hydroxy chlorothalonil as well as the parent compound. In response to these requirements, Fermenta ASC Corporation (DEB No. 7112) has submitted two volumes of data pertaining to metabolism of chlorothalonil (1990; MRID 41576001) and 4-hydroxy chlorothalonil (1990; MRID 41576002) in goats. These data are reviewed here for their adequacy in fulfilling outstanding residue chemistry data requirements.

In addition, MRID 41576002 contains data pertaining to the recovery of ^{14}C-residues using an unspecified residue analytical method for the 4-hydroxy metabolite. These data are discussed below following the details of the metabolism studies.

CONCLUSIONS

1a. The qualitative nature of the residue in animals dosed with chlorothalonil in the diet is not adequately understood because. Only 45.4, 6, and 3% of the total residue in milk, liver, kidney, respectively were conclusively identified. The 4-hydroxy metabolite of chlorothalonil was the only component identified. However, for liver and kidney, only a range of levels of the 4-hydroxy metabolite were reported and exact amounts in specific samples could not be determined. Insoluble residues in liver and kidney accounting for up to 33% of the residue in liver and 43% in kidney were not characterized. Radioactivity in the aqueous fraction of milk, accounting for up to 56% of the ^{14}C-activity was insufficiently characterized. Evidence was presented to support a proteinaceous nature of the milk aqueous residue, but the existence of conjugated residues of concern in this fraction could not be ruled out. Putative protein fractions from liver and kidney, containing up to 30 and 17% of the total tissue radioactivity, respectively, were not adequately characterized. In addition, the up to 24% of the total liver residue in the saline wash of the organosoluble fraction and the 17% of the kidney residue that appear to be low molecular weight conjugates warrant further characterization. ^{14}C-Residues in muscle and fat from the [^{14}C]chlorothalonil study were not characterized. The levels of total radioactivity in these tissues warrant investigation of the solvent extraction properties of these residues.

1b. The registrant needs to report storage information for samples collected in the [^{14}C]chlorothalonil study. If the duration of storage was greater than 6 months, a storage stability study must be conducted.

2. The results of the study in which goats were dosed with the 4-hydroxy metabolite indicate that little metabolism of this compound occurs in animals. The unchanged test substance accounted for 88-99% of the total radioactivity in milk and edible tissues.

3. The registrant has obtained data pertaining to the ability of an unspecified residue analytical method to recover ^{14}C-residues from tissues collected in the study with the 4-hydroxy metabolite. The data indicate that recoveries were 106% from milk, 100% from liver, 61-68% from muscle, and 140% from kidney.

RECOMMENDATIONS

The registrant should be informed that additional data are needed in order to resolve questions raised regarding the data presented on [^{14}C]chlorothalonil metabolism. Residues in a several fractions were not adequately characterized. The insoluble fractions from extraction of residues from liver and kidney should be characterized further, as should the putative protein-bound residues from the aqueous fractions of these tissues. Also, the aqueous-soluble residues from milk, the low-molecular-weight, non-proteinaceous conjugates from kidney, and the residues in the saline wash of the organic fraction of liver need to be better characterized. Enzymatic and other hydrolysis procedures could release identifiable residues from these fractions and better separation and additional chromatography systems could be employed. In conducting extraction and characterization analyses, the registrant should be advised that extractable and non-extractable, identified and unidentified activity, and any losses of ^{14}C-activity are to be reported in terms of the total radioactive residue (TRR) for a given sample expressed in ppm chlorothalonil equivalents. Extraction and characterization should be attempted on the same samples for which the TRR has been determined.

We also recommend that the registrant conduct solvent extractions of ^{14}C-residues in muscle and fat from the high-dose goat. If residue levels in the resulting organic fractions permit, chromatographic characterization of these residues should be attempted.

Finally, the registrant should provide information regarding the intervals and conditions of sample storage, and, if for samples stored longer than 6 months, provide data depicting residue decline in samples stored for corresponding intervals.

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DETAILED CONSIDERATIONS

Qualitative Nature of the Residue in Animals

Study 1. Goats administered [^{14}C]chlorothalonil.

Fermenta ASC Corporation (1990; MRID 41576001) submitted data pertaining to the metabolism of chlorothalonil in goats. The test substance, uniformly ring labeled [^{14}C]chlorothalonil (specific activity 78.74 mCi/mmole radiochemical purity 99.2%) was diluted with non-labeled chlorothalonil to a final specific activity of 3.09 uCi/mmole. Lactating goats (breed not specified, two animals per treatment group) were dosed orally for 8 consecutive days with gelatin capsules containing [^{14}C]chlorothalonil at levels equivalent to 3 and 30 ppm in the diet (the actual dose levels were 3.1 - 3.2, and 30 - 31 ppm for low and high doses, respectively). A fifth goat was administered a control gelatin capsule consisting of the suspension medium only. Capsules were administered via balling gun.

According to the registrant, the dosing levels represent ca. 1x and 10x the maximum theoretical dietary burden of 3 ppm for goats. A more accurate estimate is 2.1 ppm, based on tolerances and feeding rates for a combination of the following feed items: dried tomato pomace (5 ppm, 25%), spent mint hay (2 ppm, 40%), and soybean seed (0.2 ppm, 35%). The low and high administered doses for goats used in the study were approximately 1.5 and 15 x the theoretical dietary burden of 2.1 ppm.

Milk from untreated and treated goats was collected twice daily. Feces and urine were collected daily. The animals were sacrificed following the end of the 8-day dosing period. Fat, kidneys, liver, gall bladder contents, heart, and muscle were collected. Blood was sampled prior to the last dose and just prior to termination. Samples were frozen prior to shipment to Fermenta ASC Corp for further analysis.

Total Radioactive Residues (TRR)

Radioactivity in milk, urine, feces and tissues was quantified at Analytical Bio Chemistry Laboratories Inc., following the in-life portion of the study. Radioactivity in urine and milk was quantified by liquid scintillation spectroscopy (LSS) and feces samples were combusted to quantify radioactivity. Subsamples of non-fatty tissues were combusted for quantification of ^{14}C-activity. Radioactivity in fatty tissues was quantified using LSS; solubilizing tissue in scintillant at room temperature for 72 hours and then heating to 50 C. The efficiency of this technique was not reported. The limit of detection was 0.001 ppm for milk and blood, 0.002 ppm for urine and feces, and 0.001 to 0.002 ppm for tissues. The registrant indicated that the radio-analyses conducted at ABC Labs were the basis of the TRR values considered in the data calculations. At Fermenta ASC Corp., milk and tissue samples were also radioassayed, prior to characterization. Radioactivity in milk samples and tissue homogenates suspended in

water were quantified by LSS. It was reported that the radioassay results from both facilities were within an average ± 10 percent. The registrant did not submit data in support of the contention that quantification of radioactivity in tissue homogenate suspensions in water at Fermenta ASC Corp is equivalent to combustion analysis conducted previously at ABC Labs. The storage duration and conditions prior to analysis were not described.

The recovery of total dose and distribution of total radioactive residue (TRR) are given below in Table 1. ^{14}C-Activity was detectable in all tissues and milk. The TRRs in goats administered [^{14}C]chlorothalonil at 3 ppm in the diet were: liver 0.085, kidney 0.24, muscle and fat 0.004, heart 0.011, and milk 0.015 ppm (day 6). Excreta accounted for 67.1 and 72.5% of the total low and high dose, respectively. Feces and urine comprised most of the total recovered dose (greater than 98%). The recovery of radioactivity was 68.0 and 73.5% for low and high doses, respectively. The authors speculate that the unaccounted radioactivity remained in the gastrointestinal tract, but no evidence was presented for this contention.

Table 1. Total radioactive residues (TRR) and distribution of total dose in milk and tissues of a lactating goat administered [^{14}C]chlorothalonil at 3 ppm (low dose) and 30 ppm (high dose).[a]

Matrix	Low dose		High dose	
	Recovery (%)	TRR (ppm)	Recovery (%)	TRR (ppm)
Urine	7.1		7.5	
Feces	60.0		65.0	
Blood[b]	0.2		0.2	
Liver	0.2	0.085	0.1	0.68
Kidney	0.1	0.24	0.1	2.10
Muscle	0.1	0.004	0.1	0.032
Fat	0.1	0.004	0.1	0.038
Heart	0[c]	0.011	0[c]	0.110
Milk	0.2	0.015[d]	0.4	0.146[d]
Total	68.0		73.5	

[a] Results from combustion analysis performed at ABC labs prior to shipping samples to Fermenta ASC Corp. The cage wash was less than 0.05 ppm of the total dose for both samples.

b Afternoon sample.

[c] Residues in heart samples were < 0.05 ppm of the total dose.

[d] Day 6 afternoon milk.

Extraction

Milk samples (80 g) were diluted with 95% ethanol (75 mL) and then acidified with concentrated sulfuric acid (4 mL). The samples were then partitioned five times with 60 mL diethyl ether:hexane (1x 75:25, 2x 80:20, 2x 100:0). Organic phases were pooled, radioassayed and partitioned with 20 mL of aqueous saturated sodium chloride (10%). The organic phase was evaporated and residues then dissolved in hexane. The hexane-soluble residue was then partitioned with acetonitrile. The hexane and acetonitrile fractions were evaporated and the acetonitrile fraction was solubilized in methanol prior to reverse phase high-performance liquid chromatography (HPLC) analysis. The hexane fraction was cleaned up on a Florisil column and the resulting peaks were analyzed through normal phase HPLC. Greater than 97% of the radioactivity in the aqueous fraction was precipitated by aqueous sodium chloride. This precipitate (aqueous precipitate) was solubilized in ammonium carbonate and subjected to size exclusion chromatography.

The distribution of radioactivity in extracts of milk is summarized in Table 2. The total radioactive residue in day 6 afternoon milk samples was 0.015 ppm. The residue in each fraction from the low dose was less than 0.01 ppm. The recovery of ^{14}C-activity was 97.7 and 95.8% for low and high dose goat milk, respectively.

Table 2. Distribution of total recovered radioactivity in extracts of milk from a lactating goat administered [^{14}C]chlorothalonil[a].

Fraction	Low dose		High dose	
	percent	ppm	percent	ppm
Aqueous precipitate	39.0	0.006	28.2	0.042
Hexane	12.5	0.002	22.8	0.034
Acetonitrile	(46.2)		(44.8)	
4-Hydroxy	45.5	0.007	28.8	0.043
Other	0.7	0.0001	16.0	0.024
TOTAL	97.7	0.0151	95.8	0.143

[a] Day 6, pm milk samples (low dose = 3 ppm, goat 10; high dose = 30 ppm, goat 3)

Homogenized liver and kidney samples (10 g) were diluted with 30 mL water and then extracted with concentrated sulfuric acid:acetone (1:40). The mixture was centrifuged and the

solids re-extracted with sulfuric acid:acetone. The supernatants were pooled, radioactivity was quantified, and the acetone removed. The remaining non-extractable solids were suspended in water and radioactivity quantified by LSS. The supernatant was diluted with water and partitioned four times with 40 mL diethyl ether:hexane (70:30 two times, 80:20, 100:0). Radioactivity in the aqueous phase was quantified by LSS. The organic phases were pooled, radioassayed, and residues partitioned with a sodium chloride solution. The organic phase was evaporated and the residue partitioned with acetonitrile:hexane (5:2). The pooled acetonitrile fractions were washed with hexane and radioactivity quantified in all fractions. The acetonitrile fraction was concentrated prior to HPLC analysis.

The distribution of radioactivity in extracts of liver and kidney is summarized in Tables 3 and 4, respectively. The recovery of ^{14}C-activity in liver was 99.7 and 85.5% for low and high dose goat milk, respectively. The recovery of ^{14}C-activity in kidney for low dose goat was 84.5 and for high dose goat was 102.5%. The non-extractable fraction of liver was 36.1% of the TRR and 43.2% for kidney of low dose animals. The non-extractable residues in liver and kidney extracts were not characterized further.

Table 3. Distribution of total recovered radioactivity in extracts of liver from a lactating goat administered [^{14}C]chlorothalonil[a].

Fraction	Low dose		High dose	
	percent	ppm[b]	percent	ppm[b]
Non-extractable	36.1	0.031	29.7	0.202
Aqueous	20.8	0.018	25.4	0.173
Saline	24.2	0.021	12.3	0.084
Organic	(30.8)[c]		(20.6)	
Hexane	4.6	0.003	4.2	0.029
Hexane wash	0.6	0.001	0.02	0.001
Acetonitrile	13.4	0.011	13.9	0.094
TOTAL	99.7	0.085	85.52	0.583

[a] Low dose equals 3.0 ppm from goat 10; high dose equals 30 ppm from goat 3.
[b] Data calculated by the reviewer using TRR and percent values provided by the registrant. The TRR used in calculations was 0.095 for low and 0.68 ppm for high dose.
[c] Values in parentheses represent the percent of the TRR in the initial organic phase.

Table 4. Distribution of total recovered radioactivity in extracts of kidney from a lactating goat administered [^{14}C]chlorothalonil[a].

Fraction	Low dose		High dose	
	percent	ppm[b]	percent	ppm[b]
Non-extractable	43.2	0.104	38.2	0.802
Aqueous	19.3	0.046	47.9	1.007
Saline	8.9	0.021	3.8	0.080
Organic	(11.6)[c]		(12.2)	
Hexane	4.4	0.011	4.1	0.085
Hexane wash	0.3	0.001	0.2	0.004
Acetonitrile	8.4	0.020	8.3	0.174
TOTAL	84.5	0.203	102.5	2.152

[a]Low dose was 3 ppm (goat 10) and high dose was 30 ppm (goat 3).
[b]Data calculated by the reviewer from TRR and percent values provided by the registrant. The TRR used in calculations was 0.24 for low and 2.10 ppm for high dose.
[c] Values in parentheses represent the percent of the TRR in the initial organic phase.

A second procedure consisting of a neutral buffer extraction with saline solution was conducted with high dose kidney samples to characterize suspected protein conjugates. Kidney homogenates were diluted with 20 mL 0.05 M potassium phosphate in 0.9% sodium chloride at pH 7. The resulting mixture was centrifuged and the supernatant decanted and re-centrifuged to produce a second supernatant and precipitate. The original precipitate was re-suspended in saline solution and centrifuged to produce a third supernatant and precipitate. The second extraction procedure resulted in three supernatants and three non-extractable fractions. Radioactivity in the soluble fractions were quantified and some of the fractions were characterized through size exclusion chromatography and reverse phase HPLC. The recovery of radioactivity was 91.9% by this procedure. The precipitated (non-extractable) activity consisted of 30.9% of the total recovered activity and was not characterized further.

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Hydrolysis of Residues

The aqueous fraction of liver was acid hydrolyzed using dilute hydrochloric acid (HCl) in n-butanol. Aqueous liver extracts were flash evaporated and an equal amount of butanol added and the mixture again evaporated. Butanol (30 mL) and 0.4 mL 6N HCl were then added and the mixture refluxed. The sample was flash evaporated, diluted with water and partitioned three times with 30 mL ethyl acetate (saturated with 6N HCl). The n-butanolic acid hydrolysis resulted in 95 to 98% of the ^{14}C-activity extracted into ethyl acetate.

Characterization of Residues

The hexane and acetonitrile fractions of milk, liver and kidney samples were analyzed by high performance liquid chromatography (HPLC). Standards were co-injected with test standards. The standards used for the particular HPLC system are listed in Table 5. Peaks were identified through ultraviolet absorbance at 254 nm for the buffered and non-buffered reverse phase and 265 nm for the non-aqueous phase HPLC. HPLC fractions were collected and radioactivity quantified using liquid scintillation spectroscopy.

The acetonitrile fractions of milk samples were analyzed by non-buffered reverse phase HPLC. The solvent system employed (HPLC system 1) was a linear gradient of 100% water to a 100% methanol (20 minutes), 2 mL per minute flow rate.

The acetonitrile fraction of liver and kidney samples were analyzed by buffered reverse phase HPLC. The following is the solvent system employed:

HPLC System 2

A: Potassium phosphate (0.05M, ph 7);
B: 100% Methanol, 1.5 mL per minute

Time (min.)	Solvents		Gradient
0	A:B	(85:15)	isocratic
0 to 20	to A:B	(30:70)	linear
20 to 30	to A:B	(0:100)	linear
> 30	A:B	(0:100)	isocratic

The hexane fraction of milk, liver, and kidney samples, and the acetonitrile fraction of methylated samples were analyzed by nonaqueous-phase HPLC using the following solvent system:

HPLC System 3
A:Hexane;
B:Ethyl acetate, 2 mL per minute flow rate

Time (min.)	Solvents		Gradient
0	A:B	(93:7)	isocratic
1.1 - 8	to A:B	(89:11)	linear
8.1 - 10	to A:B	(84:16)	linear
10.1 - 20	to A:B	(10:90)	linear
20.1 - 25	to A:B	(0:100)	linear
25 to > 30	A:B	(0:100)	

The registrant indicated that a majority of the milk acetonitrile fraction (0.007 ppm, 45.5% of the TRR) consisted of the 4-hydroxy metabolite. The major radioactive peak in the acetonitrile fraction of milk samples co-chromatographed with the 4-hydroxy standard. Acetonitrile extracts were subjected to HPLC separation and samples corresponding to the 4-hydroxy metabolite were brought to dryness and solubilized in ether. A portion of diazomethane reagent and a catalyst (HCl:methanol, 2:1) was then added (twice) to methylate the samples. The methylated samples were evaporated and residues solubilized in methanol prior to analysis with Gas Chromatography/Mass Spectrometry (GCMS). Methylated [^{14}C]4-hydroxy chlorothalonil (SDS 4824) was used as a standard to confirm the identity of the ^{14}C-activity.

An unknown radioactive component in the acetonitrile fraction comprised up to 25.8% of the TRR in day 1 milk for a 0.0024 ppm residue level of low dose goats. It was reported that this unknown in high dose goats would have been 0.018 ppm for day 1 and 0.007 ppm for day 6. The registrant was unsuccessful in characterizing this unknown through GCMS. A metabolite tentatively identified as a dimethoxy derivative (2,5,-dichloro-4,6-Dimethoxyisophthalonitrile, SDS 5080) in the hexane fraction, comprised 9.2% of the TRR on day 1 and 4.5% on day 6 in afternoon milk of a goat receiving high dose treatments. This metabolite would account for less than 0.01 ppm at the low dose level.

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Table 5. Chlorothalonil and metabolite standards used in the HPLC systems employed for characterization of residues in goat tissue.

Chemical Name	Common Name	System 1	2	3	4	5
2,4,5,6-Tetrachloroisophthalonitrile	Chlorothalonil	X	X	X		
4-Hydroxy-2,5,6-trichloroisophthalonitrile	SDS-3701	X	X		X	X
2,4,5,6-Tetrachloro-3-cyanobenzamide	SDS-19221	X	X		X	
2,5,6-Trichloro-4-methoxyisophthalo-nitrile	SDS-4824			X	X	X
2,5-Dichloro-4,6-dimethoxyisophthalonitrile	SDS-5080			X	X	X
2,5,6-Trichloro-3-cyano-benzamide	SDS-47524	X	X		X	
2,4,5-Trichloro-6-hydroxy-3-cyano-benzamide	SDS-47525	X	X		X	
2,4,5-Trichloroisophthalamic acid	SDS-46851	X	X		X	
S-[2,4-Dicyano-3,5,6-trichlorophenyl] N-acetyl cysteine	SDS-66430		X			
5,5'-[2,4-Dicyano-3,6-dichlorophenyl] diglutathione	SDS-66432		X			
2,5,6-Trichloro-4-methylthioisophthalonitrile	SDS-22913			X	X	
2,5-Dichloro-4,6-bis (methylthio) isophthalonitrile	SDS-65960		X	X		
5-Chloro-2,4,6-tris (methylthio) isophdthalonitrile	SDS-66272			X		
2,4,5,6-Tetramethylthioisophthalo-nitrile	SDS-3819			X		
S-[2,4-Dicyano-3,5,6-trichlorophenyl] glutathione	SDS-66382		X			
5,6-Dichloro-2,4-dihydroxyisophthalonitrile	SDS-66581			X	X	
2,5,6-Trichloro-4-mercaptoisophthalonitrile	SDS-13353		X		X	

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The registrant indicated that the milk aqueous precipitate fraction is probably proteinaceous in nature because greater than 97% of the ^{14}C-activity was found as a precipitate upon acid hydrolysis and a majority of this fraction solubilized in ammonium carbonate. The ammonium carbonate soluble fraction was subjected to size exclusion chromatography. The molecular weight of the majority of the ^{14}C-activity was greater than 700; raw data were not provided. The aqueous precipitate from the low dose milk (39% of the TRR) was not adequately characterized. A more rigorous acidic, or, enzymatic extraction may be useful in characterizing the aqueous precipitate fraction. Molecular size and solubility characteristics determinations do not rule out the presence of bound residues of concern in the putative protein fraction.

The 4-hydroxy metabolite was tentatively identified in the acetonitrile extracts from liver and kidney fractions of high dose goats through co-chromatography. The results for the 4-hydroxy metabolite were presented as a range of ppm and percent values, and data from individual samples were not reported. The registrant reported that the range of residues were 0.03 to 0.04 ppm (3-6% of the TRR) in liver and 0.05 to 0.07 ppm (2.4-3.2% of the TRR) in kidney. The value for the 4-hydroxy metabolite in a low dose estimated from a high dose was 0.004 and 0.007 ppm for liver and kidney samples, respectively. Other radiolabeled components of the acetonitrile fraction were not identified; an unknown component comprised 20 to 30% of the liver acetonitrile fraction. The registrant indicated that there was insufficient material to analyze using GC-MS. The major portion of the acetonitrile fraction of kidney and liver co-chromatographed with the 4-hydroxy metabolite. Raw data may be useful for estimating the levels of the 4-hydroxy metabolite for the low dose from high dose analysis and determining the levels of the unknown ^{14}C-activity in the acetonitrile fraction of liver and kidney.

The ethyl acetate-soluble residues from the hydrolyzed aqueous fraction of liver was analyzed by HPLC. Approximately 48% of the ^{14}C-activity was converted to non-polar compounds with retention times greater than the parent compound. None of the ^{14}C-activity co-chromatographed with any known metabolites. Clean-up attempts by thin-layer chromatography were unsuccessful. Size exclusion chromatography was also conducted on the liver aqueous fraction. The registrant reported that the retention volumes of a majority of the ^{14}C-activity corresponded to 700 to 1,300 molecular weight, with 10% in the 700 to 1,300 molecular weight range. The registrant suggested that the aqueous fraction consists of a mixture of conjugates over a range of molecular weights. Actual values and raw data were not presented for size exclusion chromatography.

The aqueous fraction (up to 48% of the TRR) from the acidic acetone extraction of kidney was also analyzed by size exclusion chromatography. The registrant reported that three peaks made up this fraction. The first peak comprised 20% of the ^{14}C-activity on the column and was of high molecular weight. The UV absorbance ratio suggested that this peak is proteinaceous in nature. The second peak comprised 32% of the ^{14}C-activity with a molecular weight range of 400 to 600 with a retention time similar to paranitrobenzyl-glutathione. The registrant suggested that several components make up the second peak. The

third peak, a broad band, amounted to 24% of the radioactivity applied to the column. These procedures are insufficient to rule out the existence of residues of concern in these aqueous fraction of liver and kidney samples. A clean-up procedure such as using a C-18 SepPak and separation/chromatographic procedures may be useful in characterizing radiolabeled components in the aqueous fraction.

The registrant conducted column chromatography on the hexane fraction of kidney. Several fractions that comprised 50 to 80% of the recovered ^{14}C-activity eluted with polar solvents. The registrant indicated that these fractions contained substantial interfering substances and they were unable to analyze these fractions further. The saline fractions of kidney and liver were not characterized. The insoluble residues liver and kidney were not characterized. A more rigorous acidic, or, enzymatic extraction may be useful in characterizing the non-extractable fraction of liver and kidney.

The supernatants of the neutral buffer extraction of kidney were analyzed by HPLC. For one supernatant containing 47.1% of the radioactivity, about half of this ^{14}C-activity was retained on the column. One peak that comprised 16.3% of the recovered radioactivity was preliminarily identified as the 4-hydroxy metabolite at a 0.08 ppm level (ca.3-4% of the TRR). Three other peaks, each equivalent to 2-3% of the TRR were not characterized further. Data for HPLC analysis of the second supernatant, comprising 13.9% of the TRR, were not provided. The precipitates, comprising 30.9% of the ^{14}C-activity, were not adequately characterized. Supernatants for the neutral buffer extraction were also characterized by size exclusion chromatography; recovery ranged from 88 to 109%. Two major peaks were present; the first peak comprised 17% of the TRR and the second peak 15-17%. The retention volumes of the first peak indicated molecular weights in the range of 45,000 to 54,000, and was designated protein-bound residue and the second peak, at molecular weights of 330 to 360, was reported to consist of low molecular weight conjugates. The ultraviolet absorbance properties of the material in the first peak are consistent with the theoretical proteinaceous character of this fraction; the second peak did not exhibit these characteristics. The registrant reported that the second peak contained multiple components when analyzed by HPLC, but none were identified.

The qualitative nature of the residue in animals dosed chlorothalonil in the diet is not adequately understood because residues were inadequately characterized. The 4-hydroxy metabolite of chlorothalonil accounted for 45.4% of the total residue in milk, 6% of that in liver, and 3% of that in kidney and was the only component identified; it should be noted that, for liver and kidney, only a range of levels of this compound were reported and the amount in any given sample could not be determined. Insoluble residues in liver and kidney accounting for up to 33% of the residue in liver and 43% in kidney were not characterized. Radioactivity in the aqueous fraction of milk, accounting for up to 56% of the ^{14}C-activity was insufficiently characterized; although evidence to support a proteinaceous nature of these residues was presented, the existence of conjugated residues of concern in this fraction could not be ruled out. Similarly, putative protein fractions from liver and kidney, containing up to 30 and 17%, respectively, were not adequately characterized. In addition, the up to 24% of

the total liver residue in the saline wash of the organosoluble fraction and the 17% of the kidney residue that appear to be low molecular weight conjugates warrant further characterization. ^{14}C-Residues in muscle and fat were not characterized and should be investigated with respect to the solvent extraction properties of these residues.
Finally, the registrant needs to report information pertaining to the conditions and intervals of sample storage, and, if the duration of storage was greater than 6 months, a storage stability study must be conducted.

Study 2. Goats administered [^{14}C]4-hydroxy chlorothalonil.

Fermenta ASC Corporation submitted data pertaining to the metabolism of the 4-hydroxy metabolite of chlorothalonil (4-hydroxy-2,5,6-trichloroisophthalonitrile, SDS-3701) in goats (1990; MRID 41576002). The average specific activity of the test substance, uniformly ring labeled [14C]4-hydroxy chlorothalonil, was 38,222 dpm per ug. Lactating goats (breed not specified, two animals per treatment group) were dosed orally for 9 consecutive days with gelatin capsules containing [^{14}C]4-hydroxy chlorothalonil at levels equivalent to 0.2 and 2 ppm in the diet. The actual dose levels based on the analysis of the dose capsules and feed were 0.24-0.29 and 2.5-2.4 ppm for low and high doses, respectively. A fifth goat was administered a control gelatin capsule consisting of the suspension medium only. Capsules were administered via balling gun.

The registrant indicated that the ration intake for the control goat was 2.0 kg/day, the maximum level. The ration intake for the treated animals was 1.7, 1.38, 1.61 and 1.66 kg/day. We note that the registrant indicated that the animals lost weight over the course of the study. Raw data concerning the pre-dosing ration intake, weight loss, animal health and final pathology report may be useful in evaluating the study. The registrant indicated that the optimum dosing levels represent ca. 1 and 10x the maximum dietary burden of 0.2 ppm for goat. The registrant did not indicate the basis for these estimates.

Total Radioactive Residues (TRR)

Milk from untreated and treated goats was collected twice daily. Feces and urine were collected daily. The goats were sacrificed within 8 hours of the last dose at the end of the 9-day dosing period. Fat, kidneys, liver, gall bladder contents, heart, and muscle samples were collected. Blood was sampled prior to the last dose and just prior to termination. Samples were frozen (-18 C) prior to shipment to Fermenta ASC Corp for further analysis.

Radioactivity in milk, urine, feces, and tissue samples were quantified at Analytical Bio Chemistry Laboratories Inc. following the in-life portion of the study. Aliquots of milk and urine were taken and the ^{14}C-activity quantified through liquid scintillation spectroscopy (LSS). Homogenized feces and tissue samples were combusted and radioassayed through LSS. The limit of detection of the radioassay was: milk 0.007 ppm, urine 0.009; feces

0.0054; and tissues 0.0011 to 0.0017 ppm. Milk, urine and tissue samples were also radioassayed at Fermenta ASC Corp. Aliquots of milk and urine samples were taken and radioassayed by LSS. Tissue aliquots were combusted and radio-assayed by LSS. The limit of detection of these latter radio-assays was not provided. The values for the TRR were based on the second radioassay. The registrant reported that values for both radio-assays were similar all tissues except for kidney. The duration and conditions of sample storage were not reported.

The recovery of total dose and distribution of total radioactive residue (TRR) are given below in Table 6. The TRR in day 7 afternoon milk was 0.134 and 0.922 ppm for low and high doses, respectively. The TRRs in kidney were 0.260 and 0.821 ppm for animals treated at the low and high dose, respectively. The TRRs in liver were 0.068 ppm for the low dose and 0.567 ppm for the high dose. The TRRs in muscle were 0.019 ppm for low and 0.108 ppm for high dose levels. The TRRs in fat were 0.017 ppm and 0.084 ppm, respectively, for low and high dose levels. Urine and feces accounted for 24.0 and 28.5% of the ^{14}C-activity in the total dose for low and high doses, respectively. Milk accounted for 18.0% of the total administered low dose and 22.6% of the high dose. Blood accounted for 12.5 and 11.9% of the total administered dose for low and high doses, respectively. The recovery of radioactivity was 67.8 for low dose and 71.0% for high dose. The authors speculate that the unaccounted radioactivity remained in the gastrointestinal tract, although no evidence for this was presented.

Table 6. Total radioactive residues (TRR) and recovery of ^{14}C-activity in milk and tissues of a lactating goat administered 4-hydroxy chlorothalonil.[a]

Matrix	TRR				Factor[b]
	Low dose		High dose		
	Recovery	TRR	Recovery	TRR	
	%	ppm	%	ppm	
Urine	6.5		9.8		
Feces	17.5		18.7		
Blood[c]	12.5	0.181	11.9	1.57	
Liver	2.2	0.068	1.6	0.567	8.34
Kidney	1.1	0.260	0.4	0.821	3.16
Muscle	6.0	0.019	3.7	0.108	5.68
Fat	3.5	0.017	1.9	0.084	4.94
Heart	0.4	0.052	0.3	0.390	7.50
Milk	18.0	0.134[d]	22.6	0.922[d]	6.88
Cage wash	0.1		0.1		
Total	67.8		71.0		

[a] Results from in-life study analysis (ABC Labs.), recovery expressed as a percent of total dose (low dose = 0.2 ppm, goat 3; high dose = 2.0 ppm, goat 2).
[b] Multiplication factor calculated by the reviewer to estimate residue for low dose based on high dose levels for characterization of residues (TRR high dose / TRR low dose).
[c] Average of three samples.
d Day 7 afternoon milk.

Extraction

The extraction efficiency was greater than 98% for all milk, urine and tissue samples. Milk samples were extracted three times with 100 mL acetonitrile. The precipitates remaining after acetonitrile extraction were dissolved in 20 mL water and combined with the aqueous fraction prior to partitioning with acetonitrile the second and third time. The acetonitrile fractions were pooled, washed with hexane (250 mL) and the organic phase removed until only an

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aqueous phase remained. The aqueous phase was acidified (1 mL hydrochloric acid (HCl)) and partitioned (three times) with methylene chloride (100 mL). The methylene chloride fractions (organic) were pooled, evaporated and the residue dissolved in methanol (1 mL). The ^{14}C-activity remaining in the aqueous and hexane fractions were radioassayed through LSS. Non-extractable precipitates were suspended in water and radioassayed by LSS. Milk samples were methylated using diazomethane for analysis by Gas Chromatography/Mass Spectrometry (GCMS). Samples were brought to dryness and a portion of the diazomethane reagent and a catalyst (HCl:methanol, 2:1) added to the samples twice. The methylated samples were evaporated and residues solubilized in methanol prior to purification through HPLC and analysis by GCMS.

Non-fatty tissue homogenates (10 g) were extracted with acetone (twice, 50 mL) containing 2% HCl and filtered. The acetone fraction was evaporated and water (25 mL) added. The aqueous solution was then partitioned with methylene chloride (three times, 50 mL). The radioactivity in the aqueous and methylene chloride fractions was quantified using LSS and the methylene chloride fraction was concentrated for HPLC analysis. The radioactivity in the non-extractable fraction was quantified through combustion and LSS.

Fat samples were extracted three times (100 mL) with acetonitrile containing 2% HCl. The acetonitrile fraction was then filtered and washed with hexane (three times, 100 mL). The acetonitrile fraction was reduced in volume, deionized water added (100 ml), and this solution partitioned (3 times) with methylene chloride (50 mL). The methylene chloride fractions were pooled, and along with the aqueous fraction, radioactivity quantified through LSS. The methylene chloride fraction was then concentrated for HPLC analysis. The non-extractable fractions were dried and radioactivity quantified through combustion and LSS.

Urine samples (50 mL) were acidified with 12N HCl to pH 2.0 and then extracted with equal volumes of ethyl acetate (three times). The radioactivity in the ethyl acetate phase was quantified through LSS and the phase concentrated, dried and dissolved in methanol prior to HPLC analysis. The radioactivity in the aqueous phase was quantified through LSS.

The TRR and distribution of ^{14}C-activity in the tissue fractions following extraction is summarized in Table 7. A majority of the TRR was in the organic fraction; greater than 96% for all matrices.

Radioactivity in the aqueous, hexane and non-extractable milk fractions was less than 2% of the TRR except for heart tissue. The heart aqueous fraction contained 3.2% of the residue and was equivalent to 0.014 ppm for the high dose, the level for the low dose rate would be less than 0.01 ppm. The residue level for the aqueous, non-extractable, and hexane fractions for tissues other than heart, was less than 0.01 ppm at the high dose.

Table 7. The TRR and distribution of total recovered activity in tissue, milk and urine fractions from lactating goat administered [^{14}C]4-hydroxy chlorothalonil at a high dose (2.0 ppm), and estimated residue of the 4-hydroxy metabolite for a low dose (0.2 ppm)[a].

Matrix	TRR	Aqueous	NE[b]	Organic[c]	4-hydroxy[d] high dose	4-hydroxy[e] low dose
	ppm	%	%	%	ppm	ppm
Kidney	0.88	0.1	0.8	99.1	0.89	0.28
Liver	0.56	1.5	0.9	97.6	0.51	0.06
Rear leg muscle	0.12	0	0.9	99.1	0.11	0.02
Loin muscle	0.13	0.1	0.7	99.2	0.12	0.02
Omental fat	0.08	0	1.8	98.2	0.08	0.02
Perirenal fat	0.09	1.7	1.7	96.6	0.08	0.02
Heart	0.44	3.2	<0.1	96.8	0.40	0.05
Milk[f]	0.94	<0.1	0.2	99.7	0.86	0.13
Urine[g]	0.26	1.6	0	98.4	0.23	

[a] The values presented have been adjusted by the reviewer to total 100%.
[b] NE equals non-extractable precipitate.
[c] The organic fraction was methylene chloride for milk and tissues and ethyl acetate for urine samples.
[d] A component of the organic fraction, identification of the 4-hydroxy metabolite was confirmed for milk samples by GC-MS.
[e] Estimated value from the high dose samples using the multiplication factor in Table 6.
[f] Day 7 afternoon sample, hexane fraction equals approximately 0.1% of the TRR.
[g] Day 6 samples, rep 1. The organic fraction consisted of four peaks that co-chromatographed with the 4-hydroxy metabolite (90.2%, 0.23 ppm), 2,5,6-trichloro-3-cyanobenzamide (3.2%, 0.008 ppm), 2,4,5-trichloro-6-hydroxy-3-cyanobenzamide (< 1%), and an unknown (4.6%, 0.011 ppm). The 2,4,5-trichloro-6-hydroxy-3-cyanobenzamide metabolite was present in rep 2 at 2.4% at a level of 0.007 ppm.

Characterization of Residues

The samples from high dose treated goats were characterized. The methylene chloride fraction of milk and tissue samples, and the ethyl acetate of urine were analyzed by high performance liquid chromatography (HPLC) using an analytical ODS column. Two solvent systems were employed using a linear gradient and flow rate of 1.5 mL per minute described below.

HPLC System 4

A:20mM potassium phosphate buffer (200ml 20mM KH_2PO_4 & 300 mL 20mM K_2HPO_4)
B: Acetonitrile (100%)

Time (min)	Solvents
10	A:B (100:0)
10	A:B (80:20)
5	A:B (75:25)
*	A:B (10:90)

HPLC System 5

A: potassium phosphate buffer (300ml 20mM KH_2PO_4 & 200 mL 200mM K_2HPO_4)
B: Acetonitrile (100%)

Time (min)	Solvents
5	A:B (100:0)
13	A:B (55:45)
*	A:B (25:75)

* time interval not given

A non-labeled standard of 4-hydroxy chlorothalonil and nine potential metabolites were co-injected with the test fractions (Table 5). The registrant did not indicate the means of detection (ie. absorbance wavelength) used to identify the metabolites. Fractions were collected at one minute intervals and ^{14}C-activity quantified using liquid scintillation spectroscopy (LSS).

In general, many of the HPLC scans are unreadable and the standards were not identified. The specific HPLC system used for a particular scan was not identified. For the test samples

other than milk, the identification of the 4-hydroxy metabolite was not confirmed by another procedure such as GCMS.

The distribution of TRR in milk and tissue fractions, and an estimation of the 4-hydroxy metabolite in low dose goats are presented in Table 7. The milk organic fraction was characterized using HPLC and it was reported that over 97% of the ^{14}C-activity co-chromatographed with the 4-hydroxy metabolite of chlorothalonil. GCMS using a [^{14}C]4-hydroxy standard confirmed the identity of the 4-hydroxy metabolite. It was reported that the TRR for day 7 high dose milk is 0.94 ppm and the level for the 4-hydroxy metabolite is 0.86 ppm. The estimated level of the 4-hydroxy metabolite for the low dose is 0.13 ppm.

Approximately 99% of the kidney organic fraction co-chromatographed with the 4-hydroxy metabolite of chlorothalonil. The registrant indicated that the 4-hydroxy metabolite equals 0.89 ppm for the high dose rate. An estimation of the 4-hydroxy residue in the low dose is 0.28 ppm.

The registrant reported that 92% of the liver organic fraction co-chromatographed with the 4-hydroxy metabolite and was equivalent to 0.51 ppm. An estimation of the 4-hydroxy metabolite for low dosed liver is 0.06 ppm. Results are presented in the submitted document for kidney and liver HPLC scans where the fractions that correspond to the 4-hydroxy metabolite elute off at different times. The HPLC system used for a particular scan is not identified.

The registrant reported that 94 and 92%, respectively, of the organic fraction of rear leg and loin muscle co-chromatographed with the 4-hydroxy metabolite. This equaled 0.11 ppm for rear leg and 0.12 ppm for loin muscle. The estimated level in rear leg and loin muscle for the low dose is 0.02 ppm for both tissues. The registrant reported that another component of the organic fraction of the rear leg samples with a retention time greater than the 4-hydroxy standard may also be this metabolite.

The registrant reported that 94.6 and 95.4% of the radioactivity in the organic fraction co-chromatographed with the 4-hydroxy metabolite for omental and perirenal fat, respectively. This is a residue level of 0.08 ppm for both omental and perirenal fat with an estimated value of 0.02 ppm for a low dose.

The registrant reported that a majority (90.2%) of the radioactivity in the urine organic fraction co-chromatographed with the 4-hydroxy metabolite. Two other metabolites were tentatively identified through retention times, 2,5,6-trichloro-3-cyanobenzamide (SDS 47524) and 2,4,5-trichloro-6-hydroxy-3-cyanobenzamide (SDS 47525) comprising 3.2 and < 1% of the TRR, respectively. The registrant reported an unknown metabolite ranged in value from 4 to 21% of the urine organic fraction over the course of the study. The registrant indicated that this unknown was only present in urine samples and was not characterized. It appears that this metabolite may also be present in liver tissue extracts at low levels, or may be an artifact of the extraction procedure as the unknown appears to be present in some of the spiked samples also.

Following the second extraction procedure, the registrant analyzed derivatized samples from milk and tissues by GC. The registrant attempted to compare values for the peak that co-chromatographed with the 4-hydroxy metabolite in HPLC studies with GC values of the diethyl ether fraction. GC results were not presented for the characterization of residues in the diethyl ether fraction. Raw data may be useful in characterization and quantification of residues by GC analysis.

The results of this study indicate that little metabolism of this compound occurs in animals. The unchanged test substance accounted for 88-99% of the total radioactivity in milk and edible tissues.

Recovery of ^{14}C-residues using the residue analytical method for 4-hydroxy chlorothalonil.

Samples from the goat administered the high dose of [^{14}C]4-hydroxy chlorothalonil were analyzed using GLC residue analytical methodology for the metabolite. Milk or tissue samples were macerated with 125 mL acidic acetone (20 mL 10 N sulfuric acid / 380 mL acetone) and the samples were filtered. The acetone in the filtrate was evaporated and the remaining aqueous phase was adjusted to pH 4.5 with sodium bicarbonate. The aqueous phase was then extracted with petroleum ether (twice, 50 mL). The ^{14}C-activity in the petroleum ether phase was quantified through LSS. The pH of the aqueous phase was adjusted to <2 and sodiun chloride added to obtain a 30% solution. The saline solution was partitioned twice with diethyl ether and the diethyl ether fractions combined. Radioactivity in the aqueous and diethyl ether fractions was quantified through LSS. The diethyl ether fraction and the [^{14}C]4-hydroxy metabolite standard were derivatized using diazomethane. A portion of a diazomethane reagent and catalyst (hydrochloric acid:methanol, 2:1) was added twice to methylate samples. Samples were evaporated and residues solubilized with methanol prior to analysis by GLC.

The results indicate that 80-95% of the TRR in milk and tissue samples was extracted into diethyl ether. GLC quantification of 4-hydroxy chlorothalonil resulted in residue values 106% of the radioassayed TRR in milk, 100% of that in liver, 61-68% of that in muscle, and 140% of that in kidney.