ATTACHMENT 4

FMC AGRICULTURAL CHEMICAL GROUP
Princeton

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12/10/84

Title: ANALYTICAL PROCEDURE FOR THE DETERMINATION OF FMC 54800 IN MILK AND TISSUES

Project No. and Title: G182 - FMC 54800

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Study Number: S 182 84 07

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Period Covered: June - November, 1984

ABSTRACT

A method for the routine analysis of parent FMC 54800 residues in milk and tissues has been developed. The analytical procedure involved an acetone (for milk) or acetone/hexane (for tissues) blend followed by concentration into hexane, a hexane/water partition, and then by gel-permeation and Florisil^{R} column clean-up. The FMC 54800 was quantified by gas chromatography employing a ^{63}Ni electron capture detector.

Method sensistivities were established at 0.02 ppm for milk, 0.10 ppm for fat, and 0.05 ppm for all other tissues. Method detectabilities were 0.005 and 0.01 ppm for milk and tissues, respectively. Average method recoveries were 89% for milk, 78% for fat, and 106% for all other tissues.

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TABLE OF CONTENTS

I. INTRODUCTION

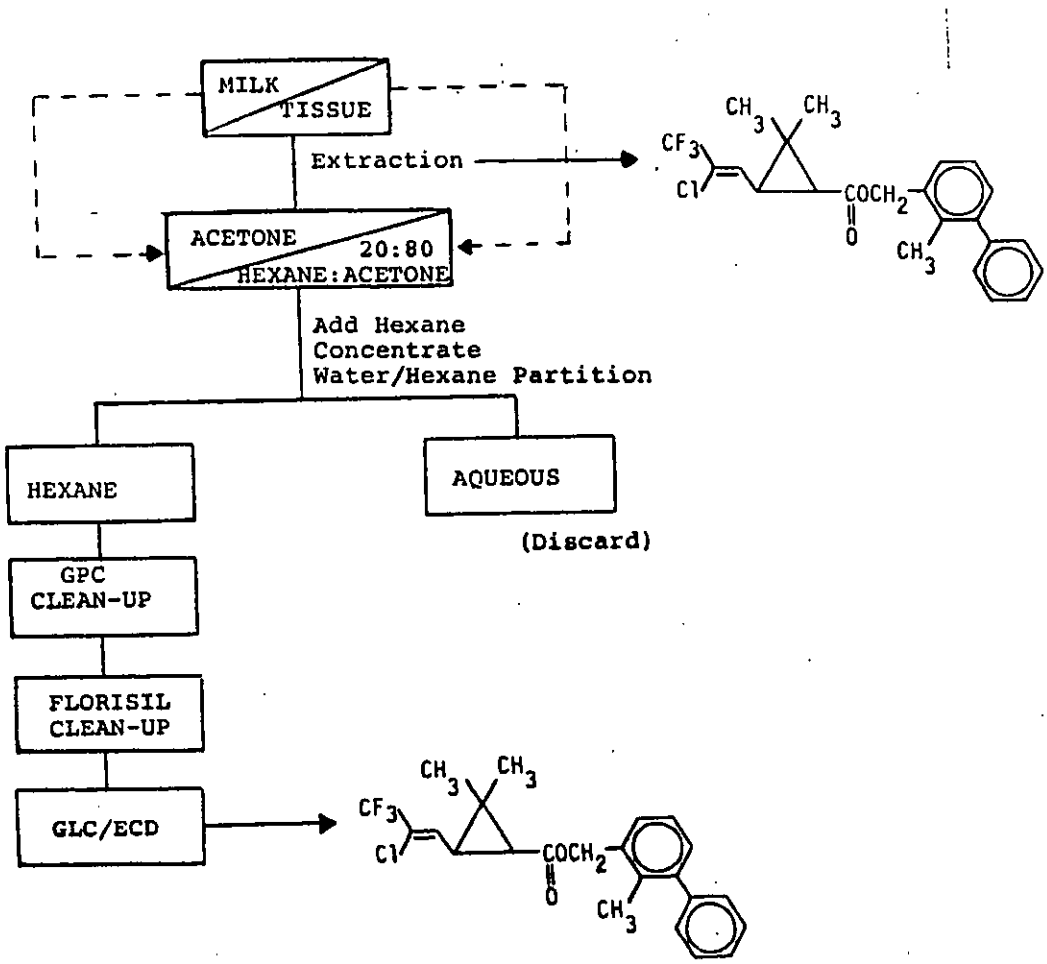
An analytical method for the routine analysis of parent FMC 54800 residues in milk and tissues has been developed. A method originally developed for analysis of this compound in apples (A1) was adapted for use on the animal (milk and tissues) study.

II. ANALYTICAL METHOD

Figure 1 is a schematic representation of the analytical procedure employed in the determination of parent FMC 54800 residues in cow milk and tissues.

FIGURE 1

METHOD OF ANALYSIS OF FMC 54800 IN MILK AND TISSUES



A. Apparatus

Autoprep 1001[R] GPC, ABC Laboratories
Bio-Beads[R] S-X3, Bio-Rad Laboratories
Chromatographic column, 15 mm O.D. X 240 mm, 250 ml reservoir
Cylinder, graduated, 10, 50, 100, 500 ml
Flask, Erlenmeyer, 250 and 500 ml
Flask, round-bottom, 100 ml wit 24/40 ℨ neck opening
Funnel, glass 4" diameter
Gas chromatograph, Hewlett-Packard 5880A fitted with an Auto sampler and Level 4 terminal
Hobart[R] food processor
Kuderna-Danish evaporative concentrator, 500 ml with a 24/40 ℨ top joint and a 24/25 ℨ bottom joint
N-EVAP[R] evaporator
Snyder distilling column, 3 ball, 250 mm
Steam bath
Syringe, Hamilton, 10, 25, 50, 100 ul
Turbomatic Jr. glassware washer, series 7000, Better-Built Machinery Corp.
Ultrasonic extractor-Tissumizer[R], Tekmar Co.

B. Reagents

Acetone, Distilled in Glass[R], Burdick and Jackson
Analytical standards, FMC Corporation, ACG, Princeton, NJ
Cyclohexane, Distilled in Glass[R], Burdick and Jackson
Ethyl acetate, Resi-Analyzed, Baker
Filter paper, fluted, grade 515, size 18.5 cm, Eaton-Dikeman
Florisil[R], 100/200 mesh, Floridin
Glass wool, filtering fiber, Corning Glass Works
Hexane, UV-Analyzed, Burdick and Jackson
Methylene chloride, Distilled in Glass[R], Burdick and Jackson
Sodium chloride, reagent grade, Fisher
Sodium sulfate, anhydrous, Fisher
Water, high purity, Burdick and Jackson

C. Sample Preparation

Milk samples were thawed overnight at ambient hood temperature and AM and PM samples for each test day were combined and thoroughly mixed by hand-shaking. The duplicate AM and PM milk samples were handled in the same manner and maintained in separate containers. Frozen tissue samples were chopped into small pieces with a machete and processed further with a Hobart[R] food chopper, adding liquid nitrogen, as necessary, to prevent thawing.

D. Procedure

All glassware were thoroughly washed in a Turbomatic washer using the cycle water rinse, non-phosphorous detergent wash, water rinse, and a distilled water rinse. A final acetone rinse was employed prior to use.

1. Extraction

Twenty-gram aliquots of pooled whole milk were blended with 200 ml of acetone for two minutes with an ultrasonic extractor. A similar sample size (20 g) was used for tissues, but with an 80:20 acetone:hexane (v/v) extraction solvent. The mixture (milk/tissue) was filtered through acetone-washed (100 ml) fluted filter paper (fat samples required suction filtration). The blending flask and filter cake were washed with 50-ml of acetone and the combined acetone filtrate (~250 ml) was adjusted to 280 ml with acetone. A 70-ml aliquot (5 g milk or tissue equivalent) of each extract was transferred to a 500 ml Kuderna-Danish (K-D) concentrator equipped with a 100 ml round-bottom flask. One hundred ml of hexane was added followed by concentration of the mixture to ~20 ml on a steam bath. The concentrated solution was transferred into a 500 ml separatory funnel containing ~5 g NaCl in solution in 50 ml of high purity (H-P) water. The K-D flask was washed with 100 ml of hexane followed by 50 ml of H-P water with both washes transferred to the separatory funnel. The mixture was shaken vigorously and left to stand for phase separation. The lower aqueous phase was drained into a 250 ml Erlenmeyer flask, while the hexane extract (upper phase) was filtered through anhydrous Na_2SO_4 and glass wool into another 500 ml K-D concentrator. The aqueous phase was re-extracted twice with 100 ml of hexane and then discarded. The combined hexane fractions (~300 ml) were first concentrated to ~10 ml on a steam bath and then to ~1 ml using a nitrogen evaporator (N-EVAP). The concentrated extract was made up to 10 ml with cyclohexane/methylene chloride (85/15, v/v) for clean-up by gel permeation chromatography (GPC).

2. Gel Permeation Column Clean-Up - Autoprep 1001 GPC

Each sample was loaded into a sample loop of 5.0 ml capacity and the Autoprep 1001 GPC system was programmed (based on previous calibration) to elute the samples sequentially through the Bio-Bead^R column. Appropriate fractions were then discarded or collected from the column eluate. Operating conditions for the system are as follows:

Column:	25 mm I.D. x 300 mm glass, packed with ~50 g Bio-Beads S-X3 (200/400 mesh) compressed to a bed length of 220 mm with a plunger assembly

Solvent System:	Cyclohexane/methylene chloride (85/15, v/v)
Flow Rate:	4.04 ml/min
Dump Time:	18 min
Collect Time:	9 min
Wash Time:	15 min

After preparing the instrument, each loop was washed with ~8 ml of solvent and the 10 ml solution extracts were injected into numbered loops via a sample introduction valve. The sample loop system retained 5.0 ml of each extract and automatically discarded the rest. After loading the samples the Auto start button was engaged to start the elution cycle. The FMC 54800 fractions were collected in 50 ml centrifuge tubes and transferred to K-D concentrators with 100 ml of hexane. They were then concentrated on a steam bath to about 10 ml for Florisil clean-up.

3. Florisil Column Clean-Up

The glass chromatographic column was plugged with glass wool and filled with 100 ml hexane. Ten grams of deactivitated Florisil (3% H_2O) was slowly added. The Florisil was allowed to settle and capped with a 0.5 inch layer of anhydrous Na_2SO_4. The solvent was drained to about 2 mm above the sodium sulfate. The concentrated extract in hexane was quantitatively transferred to the Florisil column. The concentration tube was washed with about 2 x 2 ml of hexane with washings added to the column. Next, 100 ml of hexane was added to the column and allowed to elute, with the eluant discarded. The FMC 54800 was then eluted from the column with 100 ml of 5% ethyl acetate in hexane (v/v). The eluant was collected in a K-D evaporator and concentrated to about 10 ml. This solution was then transferred quantitatively to a 13 ml centrifuge tube and further concentrated with a N-EVAP to the appropriate volume for GC analysis.

4. Gas Liquid Chromatography (GLC)

The FMC 54800 was analyzed with a Hewlett-Packard (HP) 5880A gas chromatography equipped with a ^{63}Ni electron capture detector and a HP 7672A automatic liquid sampler. Operating parameters are as follows:

Column:	2 mm I.D. x 122 cm glass containing 4% SE-30/6% OV-210 on 100/200 Chromosorb W HP (Supelco)

Injector Temperature:	250°C
Column Oven Temperature:	220°C (Liver - 210°C)
Detector Temperature:	350°C
Carrier Gas and Flow:	5% methane 95% argon at 30 ml/min
Attenuation:	2^{11} (for residues >0.05 ppm)
	2^{10} (<0.05 ppm in milk)

III. QUANTITATION

Quantitation of FMC 54800 was automatically performed by a Hewlett-Packard 5880A Level 4 Terminal using peak area integration and comparison to external standard calibrations. Results of all analyses were reported in parts per million (ppm) by using appropriate dilution factors for samples injected. External standard calibrations were updated at the start of analysis of a series of samples. In practice a standard was injected following every two samples to obtain updated calibration and chromatographic information for subsequent sample analysis. The following standard (in hexane) were used for quantitation and fortification: 1 ug/ul, 0.1 ug/ul, and 0.5 ng/ul.

IV. ANALYTICAL LIMITS

Quantitatively reliable measurement of response from actual sample matrices (method sensitivity) was established as 0.02 ppm for milk, 0.10 ppm for fat, and 0.05 ppm for all other tissues. These limits were based on satisfactory recovery of FMC 54800 from fortified check samples. Visual recognition of detector response (method detectability) was set at 0.005 ppm for milk and 0.01 ppm for tissues. Any apparent response below these limits of detectability was considered non-detectable (ND). Residue values between method detectability and sensistivity were reported as estimated values, as their quantitative reliability was not proven by actual fortifications of check sammples, below the sensitivities quoted. Data from analyses of untreated milk and tissue samples (prefix coded VC for "Vehicular Control") are summarized in Tables 1 and 2.

V. FORTIFICATION RECOVERIES

Untreated cow milk and tissue samples (prefix coded VC for "Vehicular Control") were fortified prior to the addition of any extraction solvent. Fortification levels ranged from 0.02 to 1.00 ppm for milk and 0.05 to 2.00 ppm for tissues. A check sample was analyzed along with each batch of fortified samples. Individual recovery data for milk and tissue samples are listed in Tables 3 and 4.

VI. LITERATURE CITED

A. FMC Literature

1. Witkonton, S. "Analytical Method for the Residue Analysis of FMC 54800 in Apples", FMC Agricultural Chemical Group, Princeton, NJ, Report P-0757, November 14, 1983.

VII. SIGNATURES

We the undersigned hereby declare that this study was performed under our supervision according to the procedures herein described, and that this report provides a true and accurate record of the results obtained.

AUTHOR: F.H. Akkari DATE: 12/17/84

TITLE: Research Chemist

SUPERVISOR: R F Cook DATE: 17 DEC 84

TITLE: Manager, Residue Chemistry

VIII. TABLES

TABLE 1

FMC 54800 BACKGROUND IN UNTREATED MILK SAMPLES (VC-VEHICULAR CONTROL)

Sample Identification	FMC 54800 Level, ppm
VC-1, Day 12	0.01
VC-1, Day 16	0.01
VC-1, Day 28	0.01
VC-3, Day 0	ND[1/]
VC-3, Day 3	0.01
VC-3, Day 8	ND
VC-3, Day 24	ND
VC-2, Day 0	ND
VC-2, Day 1	ND
VC-2, Day 5	0.01
VC-2, Day 28	ND
	Mean ± S.D. 0.005 ± 0.005

[1/] ND means none detected (<0.005 ppm)

TABLE 2

FMC 54800 BACKGROUND IN UNTREATED
TISSUE SAMPLES (VC-VEHICULAR CONTROL)

Tissue Type	Sample Identification	FMC 54800 Level, ppm
Muscle (Adductor)	VC-2, Day 28	ND[1/] 0.02
Muscle (Pectoral)	VC-2, Day 28	0.02 0.01
Muscle (Cardial)	VC-2, Day 28	ND 0.01
Liver	VC-2, Day 28	ND ND
Kidney	VC-2, Day 28	0.02 0.02
		Mean ± S.D 0.01 ± 0.01
Fat, subcutaneous	VC-2, Day 28	0.04 0.03
Fat, peritoneal	VC-2, Day 28	0.03 ND
		Mean ± S.D. 0.025 ± 0.017

[1/] ND means none detected (<0.01 ppm)

TABLE 3

RECOVERY OF FMC 54800 FROM FORTIFIED
UNTREATED COW MILK SAMPLES

Sample Identification	Fortification Level, ppm	FMC 54800 in Sample (ppm[1/])	Recovery Level (%)
VC-3, Day 3	0.02	0.022	110
VC-3, Day 3	0.03	0.029	97
VC-3, Day 3	0.04	0.040	100
VC-3, Day 3	0.05	0.046	92
VC-3, Day 8	0.05	0.055	110
VC-2, Day 5	0.10	0.093	93
VC-2, Day 28	0.10	0.106	106
VC-1, Day 1	0.10	0.086	86
VC-1, Day 5	0.10	0.071	71
VC-1, Day 28	0.50	0.376	75
VC-2, Day 0	0.50	0.373	75
VC-1, Day 20	0.50	0.425	85
VC-1, Day 16	1.00	0.730	73
VC-1, Day 31	1.00	0.753	75
		Mean ± S.D.	89 ± 14

[1/] Values corrected for background in corresponding checks.

TABLE 4

RECOVERY OF FMC 54800 FROM FORTIFIED
UNTREATED COW TISSUE SAMPLES

Sample Identification	Fortification Level, ppm	FMC 54800 in Sample (ppm[1/])	Recovery Level (%)
Muscle, adductor	0.05	0.055	110
VC-2, Day 28	0.10	0.072	72
Muscle, pectoral	0.05	0.067	134
VC-2, Day 28	0.10	0.080	80
Muscle, cardial	0.05	0.056	112
VC-2, Day 28	0.10	0.121	121
Liver	0.05	0.059	118
VC-2, Day 28	0.10	0.099	99
Kidney	0.05	0.065	130
VC-2, Day 28	0.10	0.084	84
		Mean ± S.D.	106 ± 22
Fat, subcutaneous	0.50	0.358	72
VC-2, Day 28	1.00	0.934	90
Fat, peritoneal	1.00	0.806	81
VC-2, Day 28	2.00	1.417	70
		Mean ± S.D.	78 ± 9

[1/] Values corrected for background in corresponding checks.

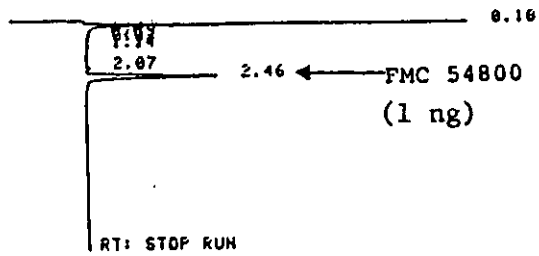
IX. CHROMATOGRAMS

Typical chromatograms from milk and tissue analyses are presented in Figures 2 through 16. Those shown are representative of fortification levels used for milk and tissues, and only one muscle (pectoral) and fat (subcutaneous) type because of similarities in chromatogram characteristics.

INDEX TO CHROMATOGRAMS

Figure	Description
2	FMC 54800 standard
3	Milk, check
4	Milk, check + 0.1 ppm FMC 54800
5	FMC 54800 standard
6	Muscle, pectoral, check
7	Muscle, pectoral, check + 0.1 ppm FMC 54800
8	FMC 54800 standard
9	Liver, check
10	Liver, check + 0.1 ppm FMC 54800
11	FMC 54800 standard
12	Kidney, check
13	Kidney, check + 0.1 ppm FMC 54800
14	FMC 54800 standard
15	Fat, subcutaneous, check
16	Fat, subcutaneous, check + 0.5 ppm FMC 54800

FIGURE 2
FMC 54800 Standard
1 ng Injected

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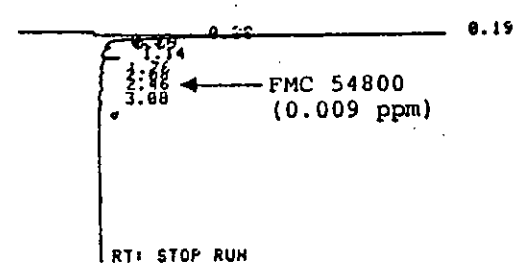
OFF
ESTD

   RT            AREA   TYPE CAL    AMOUNT  NAME

  2.46        53853.50    PB    1    0.997  54800

MULTIPLIER = 1
```

FIGURE 3
Milk, Check
5 mg Injected

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OFF
ESTD

   RT            AREA   TYPE CAL    AMOUNT  NAME

  2.46         2340.50    VP    1 8.669E-03  54800

MULTIPLIER = 0.2
```
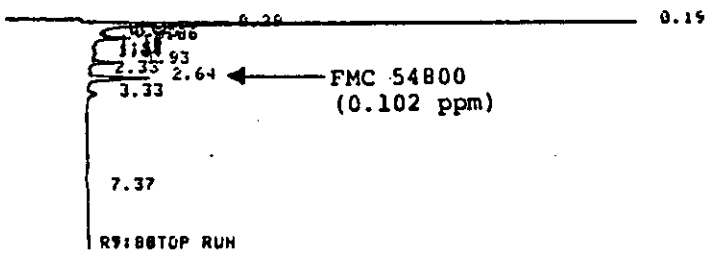

FIGURE 4
Milk, Check
Fortified at 0.1 ppm
5 mg Injected

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0
ESTD

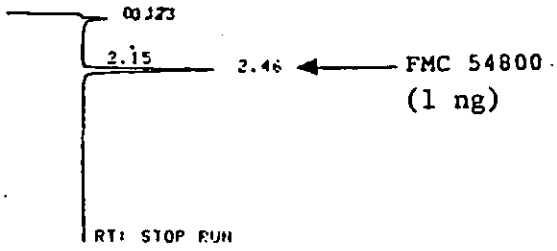
   RT            AREA   TYPE CAL    AMOUNT  NAME

  2.64        25913.50    VP    1     0.102  54800

MULTIPLIER = 0.2
```

FIGURE 5

FMC 54800 Standard
1 ng Injected

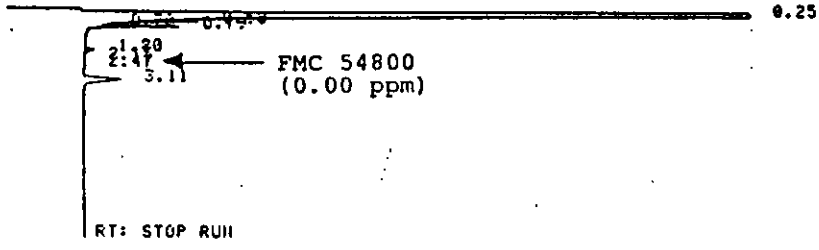


EHP3 5880A SAMPLER INJECTION @ 16:16 OCT 4, 1984
SAMPLE # : ID CODE :
2 54800-1.0
OFF
ESTD

RT	AREA	TYPE	CAL	AMOUNT	NAME
2.46	52131.10	VB	1	0.973	54800

FIGURE 6

Muscle, Pectoral, Check
5 mg Injected



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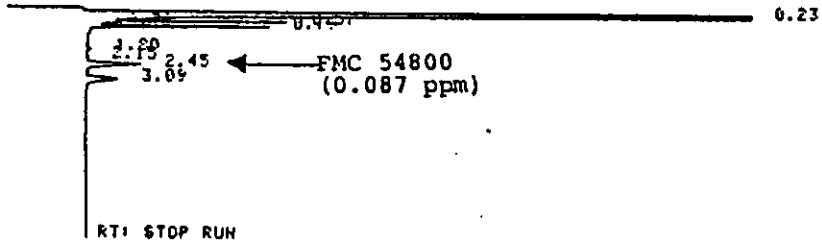
4 1.2.1000
OFF
ESTD

RT	AREA	TYPE	CAL	AMOUNT	NAME
2.47	1751.93	VV	1	6.721E-03	54800

MULTIPLIER = 8.2

FIGURE 7

Muscle, Pectoral, Check
Fortified at 0.1 ppm
5 mg Injected



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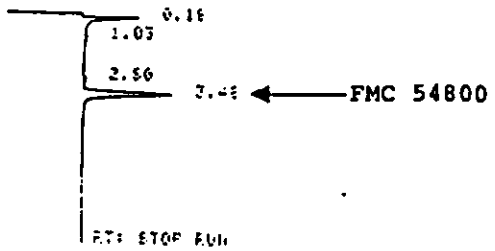
6 2.2.1000
OFF
ESTD

RT	AREA	TYPE	CAL	AMOUNT	NAME
2.45	22692.48	VV	1	8.706E-02	54800

FIGURE 8

FMC 54800 Standard
1 ng Injected

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OFF
ESTD

   RT              AREA  TYPE CAL     AMOUNT  NAME

 3.48         54356.20    PB    1      1.023  54800

MULTIPLIER = 1
```
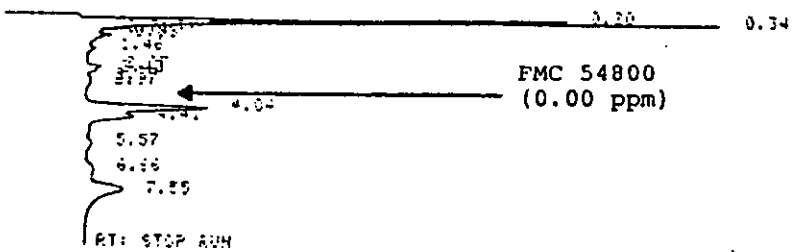

FIGURE 9

Liver, Check
5 mg Injected

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  SAMPLE # :  ID CODE   :
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METHOD ABORTED
AREA %
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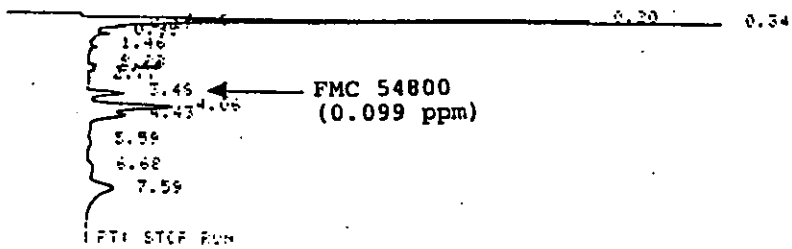
FIGURE 10

Liver, Check
Fortified at 0.1 ppm
5 mg Injected

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OFF
ESTD

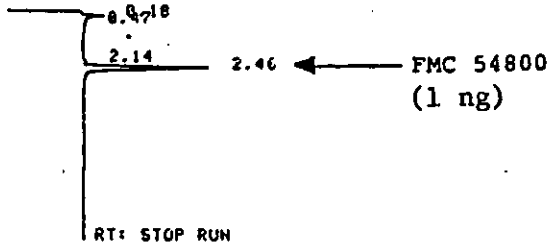
   RT              AREA  TYPE CAL     AMOUNT  NAME

 3.48         26336.40    VV    1 9.510E-02  54800

MULTIPLIER = 9.2
```

FIGURE 11

FMC 54800 Standard
1 ng Injected

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OFF
ESTD

   RT              AREA   TYPE CAL    AMOUNT  NAME

  2.46         52367.80    VB    1     1.045  54800

MULTIPLIER = 1
```
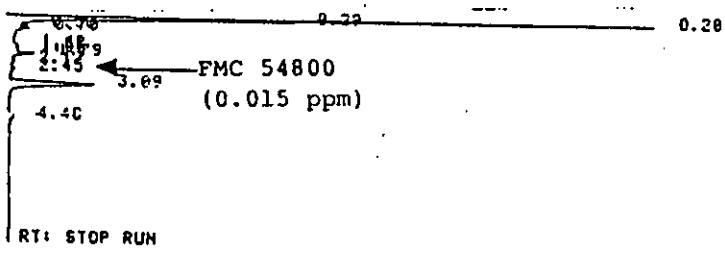

FIGURE 12

Kidney, Check
5 mg Injected

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OFF
ESTD

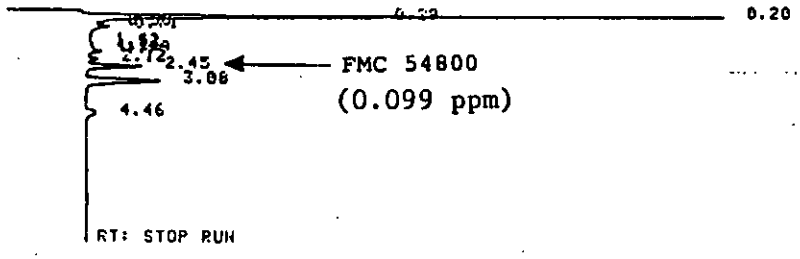
   RT              AREA   TYPE CAL    AMOUNT  NAME

  2.45          3703.02    VV    1 1.478E-02  54800

MULTIPLIER = 0.2
```

FIGURE 13

Kidney, Check
Fortified at 0.1 ppm
5 mg Injected

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OFF
ESTD

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  2.45         24780.00    VV    1 9.891E-02  54800

MULTIPLIER = 0.2
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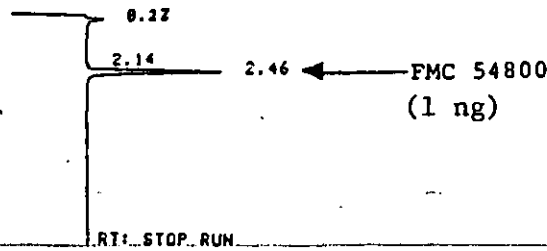

FIGURE 14
FMC 54800 Standard
1 ng Injected

```
[HP] 5880A  SAMPLER INJECTION @ 14:26  OCT  5, 1984
  SAMPLE # :  ID CODE   :
          2    54800-1.0
OFF
ESTD

   RT              AREA   TYPE CAL    AMOUNT  NAME

 2.46         55487.70    VB    1     0.996  54800

MULTIPLIER = 1
```

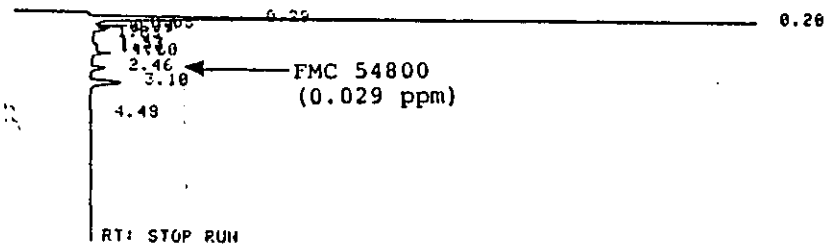

FIGURE 15
Fat, Subcutaneous, Check
5 mg Injected

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[HP] 5880A  SAMPLER INJECTION @ 14:50  OCT  5, 1984
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OFF
ESTD

   RT              AREA   TYPE CAL    AMOUNT  NAME

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MULTIPLIER = 0.2
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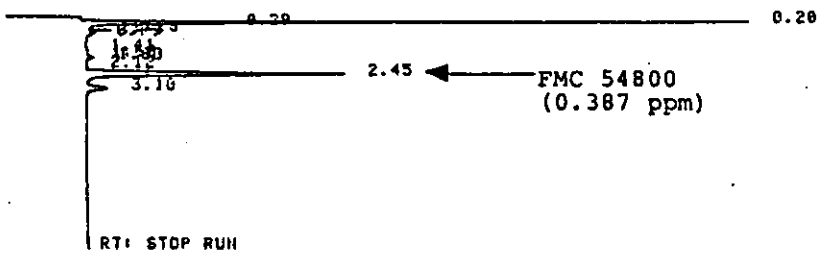

FIGURE 16
Fat, Subcutaneous, Check
Fortified at 0.5 ppm
5 mg Injected

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[HP] 5880A  SAMPLER INJECTION @ 15:14  OCT  5, 1984
  SAMPLE # :  ID CODE   :
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OFF
ESTD

   RT              AREA   TYPE CAL    AMOUNT  NAME

 2.45        107852.00    VV    1     0.387  54800

MULTIPLIER = 0.2
```