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**ROHM AND HAAS COMPANY**

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STUDY TITLE

Bound RH-9090 Residue Analytical Method for Milk

DATA REQUIREMENT

Guideline 171-4

AUTHORS

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S. S. Stavinski

STUDY COMPLETED ON

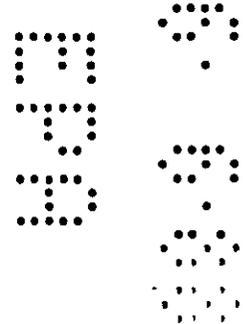
June 1988

PERFORMING LABORATORIES

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LABORATORY PROJECT ID

Technical Report No. 34S-88-15



STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA § 10(d)(1)(A), (B), or (C).

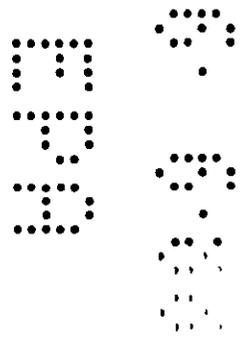
COMPANY: Rohm and Haas Company

COMPANY AGENT: Michael A. Morelli

Product Registration Manager  
TITLE

DATE: 6/2/88

Michael A. Morelli  
SIGNATURE



GOOD LABORATORY PRACTICE STATEMENT

No GLP statement is required for this type of study under 40CFR160.

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SIGNATURE

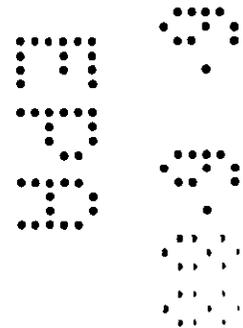
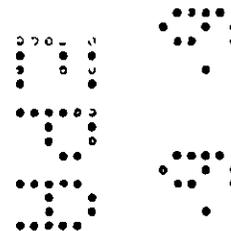


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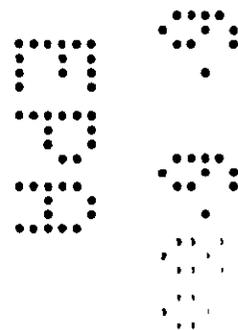
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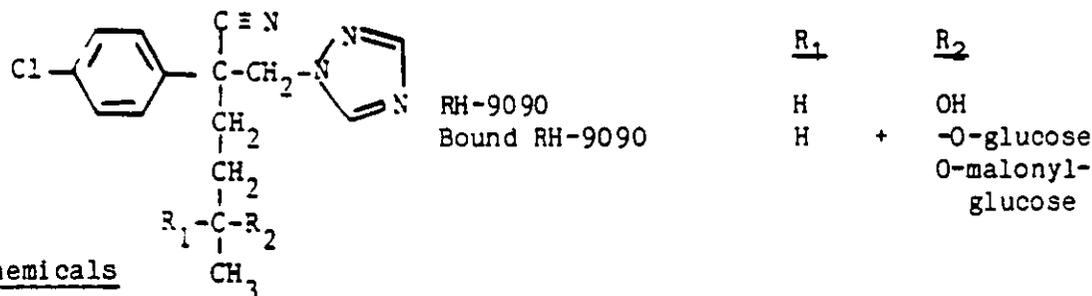


SUBJECT: Bound RH-9090 Residue Analytical Method for Milk

1. Introduction

This report details the residue analytical method for residues of Bound RH-9090 in milk. This method is a modification for milk of the Systhane Total Residue Analytical Method for Apple and Grape (TR 310-84-27, Addendum 31S-87-46), which has passed EPA validation trials. Residues in milk are refluxed overnight with 0.5 N HCl/MeOH. A 6 hour reflux may be used in lieu of an overnight reflux if this is more convenient to the analyst. The reflux converts Bound RH-9090 residues to RH-9090. The extract is purified by a petroleum ether partition, two methylene chloride partitions, Chelex 100-Fe<sup>+++</sup> affinity chromatography, and Bio-Sil A column chromatography. RH-9090 quantitation is performed by GLC on a Supelco Sup-Herb Megabore column with an ECD detector.

2. Experimental Compounds



3. Chemicals

<u>Item</u>	<u>Grade</u>	<u>Source</u>
1. Bio-Sil A	Reagent	Bio-Rad
2. Boiling stones	Reagent	Hengar Co.
3. Chelex 100, 50-100 mesh	Reagent	Bio-Rad
4. Ferric chloride	Reagent	Mallinckrodt
5. Hydrochloric acid	Reagent	Mallinckrodt
6. Hydrogen	HP	Air Products
7. Litmus paper	---	Micro Essential Lab
8. Methanol	Pesticide	Burdick & Jackson
9. Methylene chloride	Pesticide	Burdick & Jackson
10. P-10 gas	HP	Air Products
11. Petroleum ether	Pesticide	Burdick & Jackson
12. RH-9090	Standard	Rohm and Haas
13. Bound RH-9090	Standard	Rohm and Haas
14. Sodium borohydride	Reagent	Baker
15. Sodium chloride	Reagent	Fisher
16. Sodium hydroxide, 50% W/W	Reagent	Fisher
17. Sodium sulfate, anhydrous granular	Reagent	Mallinckrodt
18. Toluene	Pesticide	Burdick & Jackson
19. Water	HPLC	Milli-Q Purification System

4. Apparatus

Columns -

Glass 250 mm length, 10.5 mm I.D.,  
Teflon stopcock, 200 ml reservoir,  
Kontes

-

Glass 250 mm length, 25 mm I.D.,  
Teflon needle delivery valve, 500 ml  
reservoir, Fischer-Porter

Flasks -

Erlenmeyer, 500 ml, 2000 ml, Arthur H.  
Thomas Co.  
Round bottom, 500 ml, 300 ml, Arthur  
H. Thomas Co.  
Volumetric, 100 ml, 50 ml, Arthur H.  
Thomas Co.

Funnels -

Powder, 10 cm  
Separatory, 500 ml, Arthur H. Thomas Co.

Gas Chromatographs -

Hewlett-Packard 5890, <sup>63</sup>Ni electron  
capture detector

Graduated Cylinders -

1000 ml, 250 ml, 100 ml, Arthur H.  
Thomas Co.

Pipets -

10 ml, 5 ml, 1 ml, glass, disposable,  
Arthur H. Thomas Co.

Rotary Evaporator -

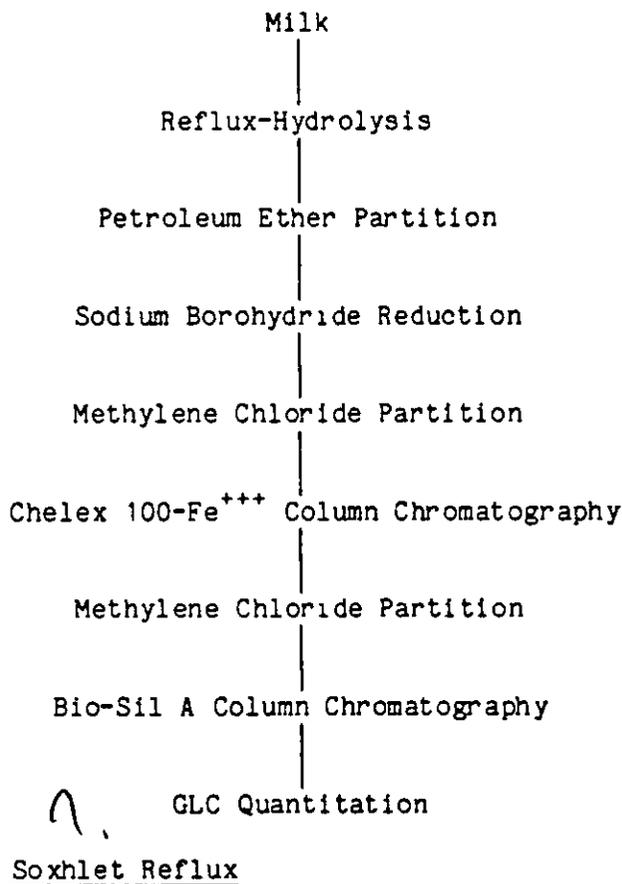
Buchi Rotovapor R, Brinkmann

Soxhlet Extractor -

Model K-586000, size 23, Kontes.

5. Method

5.1 Flow Chart



Weigh a representative 10. g sample of milk into a 24/40 500 ml round bottom flask. Add several boiling stones and 300 ml of 0.5 N HCl/MeOH into the 500 ml round bottom flask; attach to the soxhlet extractor, and reflux for 16 hours overnight. A 6 hour soxhlet reflux may be used in lieu of a 16 hour overnight reflux, if this is more convenient to the analyst. After cooling, measure final volume. Bound RH-9090 will be hydrolyzed during reflux to RH-9090.

5.3 Petroleum Ether Partition

Add a 10% aqueous sodium chloride solution to give a 1:5 water:methanol ratio. Transfer to a 500 ml separatory funnel containing 100 ml of petroleum ether. Gently shake the separatory funnel for 30 sec. After phase separation, draw off the lower water-methanol layer into a 500 ml separatory funnel.

5.4 Sodium Borohydride Reduction

Make the water-methanol layer from section 5.3 basic (litmus paper) by addition of 6-7 ml of 50% (w/w) sodium hydroxide. Add 200 mg sodium borohydride, swirl, and let stand at room temperature for 20 minutes. This reduction step results in a significantly cleaner final

sample.

#### 5.5 Methylene Chloride Partition No. 1

Add 10% aqueous sodium chloride to the separatory funnel to give a final 1:1 water-methanol ratio. Add 150 ml of methylene chloride. Shake vigorously for 1 min. After phase separation, collect the lower methylene chloride layer. Repeat the partitioning step exactly as described with a second 150 ml portion of methylene chloride. Combine both methylene chloride fractions in a 500 ml 24/40 3 round bottom flask. Evaporate to dryness on a rotary evaporator at 50°C under diminished pressure.

#### 5.6 Chelex 100-Fe<sup>+++</sup> Column Chromatography

Place 600 g of Bio-Rad Chelex-100 in a 2 liter Erlenmeyer Flask. Add 1 liter of methanol, swirl, let settle and decant. Repeat for a total of four methanol washes. Repeat process with four 1 liter Milli-Q water washes. This pre-wash process reduces artifacts on GLC chromatography due to Chelex 100.

Prepare the Chelex 100-Fe<sup>+++</sup> resin fresh daily by adding 100 ml of a 0.5% FeCl<sub>3</sub> solution (0.5 g FeCl<sub>3</sub>/100 mL water) to 400 mL of hydrated Chelex 100. Wash twice with 1000 mL of 1:4 (v/v) methanol-water and prepare column as described.

Slurry pack a 25 cm x 2.5 cm I.D. glass column to a height of approximately 8 cm with the iron-activated Chelex 100. Equilibrate the column with 100 ml of 1:4 (v/v) methanol-water at a flow rate of 10 ml/min.

Dissolve the residue from the methylene chloride partitioning step, section 5.5, in 15 ml 1:4 (v/v) methanol-water. Apply this solution to the Chelex 100-Fe<sup>+++</sup> column and elute to the top of the resin bed at a flow rate of 4 ml/min. Wash the 500 ml round bottom flask with a second 15 ml aliquot. Apply this aliquot to the column and elute exactly as described above.

Rinse flask with 25 ml of 1:4 (v/v) methanol-water and then add to the column and again elute to the top of the resin bed. Discard all fractions collected.

Wash the 500 ml round bottom flask with 200 ml of 1:1 (v/v) methanol-water. Apply to the Chelex 100-Fe<sup>+++</sup> column and elute at a flow rate of 10 ml/min, collecting the eluant, until the column is dry. Add 1 g sodium chloride to the eluant.

#### 5.7 Methylene Chloride Partition No. 2

Partition the Chelex 100-Fe<sup>+++</sup> eluant with two 150 ml aliquots of methylene chloride in a 500 ml separatory funnel, shaking vigorously for 30 seconds each time. Pass the lower methylene chloride phase through anhydrous sodium sulfate contained in a powder funnel with cotton plug into a 500 ml round bottom flask. Combine both extracts in the 500 ml

round bottom flask. Evaporate the methylene chloride to dryness at 50°C under reduced pressure by rotary evaporator.

5.8 Bio-Sil A Column Chromatography

Bio-Sil A, 100-200 mesh, is purchased from Bio-Rad Laboratories. Activate the Bio-Sil A by heating at 200°C for 48 hours. Remove and store dessicated in 8 oz. tightly capped glass vials.

Pack a 25 cm x 10.5 mm I.D. glass column with 13 ml (packed) of activated Bio-Sil A. Top the column with 1 g of anhydrous granular sodium sulfate.

Redissolve the residue from the methylene chloride partitions, Section 5.7, in 25 ml of 2% (v/v) acetone/toluene. Quantitatively transfer this solution to the column and elute to the top of the sodium sulfate layer. Wash the 500 ml round bottom flask with 10 ml of 2% (v/v) acetone/toluene, add the wash to the column, and elute to the top of the sodium sulfate layer. Wash the column with 125 ml of 20% (v/v) acetone/toluene. Add 40 ml of 35% (v/v) acetone/toluene to the column and continue elution until the liquid has reached the top of the sodium sulfate layer. Discard all washes. Elute the RH-9090 with 75 ml of 50% (v/v) acetone/toluene and collect the eluant in a 250 ml 24/40 round bottom flask. Evaporate to dryness by rotary evaporator at 50°C under diminished pressure. Add 10 ml of 23% isopropanol/isooctane to dissolve the residue. The sample is now ready for GLC analysis of RH-9090.

Standardize each new batch of Bio-Sil A to ascertain there are no batch to batch differences.

5.9 GLC Quantitation

5.9.1 Instrument and Conditions

RH-9090

GLC:	Hewlett Packard 5890
Detector:	Hewlett Packard <sup>63</sup> Ni ECD
Column:	Supelco Sup-Herb Megabore, 15 meter, 0.53 mm I.D.
Carrier Gas:	P-10 (10% methane/Argon)
Inlet Pressure:	80 PSI
Column Flow Rate:	8.5 ml/min
Detector Purge Flow Rate:	60 ml/min
Injector Temperature:	200°C
Detector Temperature:	280°C
Initial Temperature:	200°C
Initial Hold Time:	5 min.
Program Rate:	10°C/min.
Final Temperature:	250°C
Final Hold Time:	10 min.
Total Run Time:	20 min.
Injection Volume:	3 µl

5.9.2 Preparation of Standard Curves

Standard solutions of RH-9090 in 23% isopropanol/isooctane (v/v) were prepared by serial dilution in the concentration range of 0.2 µg/ml - 0.01 µg/ml. Three µl of each standard are injected and the resulting peak heights are measured. A standard curve of the peak heights measured vs. concentration (µg/ml) is constructed. The standard curve is linear within the concentration range. Standard curves are prepared for each analysis day.

5.9.3 Quantitation

Three microliters of the RH-9090 sample is injected into the GLC. If necessary, the sample is diluted to an appropriate volume to give a response within the standard curve range. The peak height is computed and the concentration is determined as follows:

$$\frac{\text{Total Volume (ml)} \times \text{Concentration From Standard Curve (µg/ml)} \times 100}{\text{Average Recovery (\%)}} = \text{Total µg}$$

$$\frac{\text{Total µg}}{\text{Sample Weight (g)}} = \text{ppm}$$

Sample chromatographs of standards, controls and fortifications of milk are illustrated in Figures 1-9.

5.9.4 Fortification Recovery

For samples fortified with known amounts of standards prior to extraction, measure the peak height, determine the concentration from the standard curve, and calculate % Recovery as follows:

$$\% \text{ Recovery} = \frac{(\text{µg/ml Found}) \times \text{Final Sample Volume} \times 100}{\text{Fortification (µg)}}$$

5.10 Confirmatory Analysis

Confirmatory analysis is performed on a Varian 3500 with direct on-column injection using a Nitrogen/Phosphorus (N/P) detector. The instrument settings are detailed below.

<u>Instrument and Conditions</u>	<u>Confirmatory RH-9090</u>
GLC:	Varian 3500
Detector:	Varian N/P
Column:	Supelco SPB-608, 15 m, 0.53 mm ID
Carrier Gas:	P-10 (10% methane/Argon)
Inlet Pressure:	80 PSI
Column Flow Rate:	21 ml/min
Column Temperature:	210°C
Injector Temperature:	260°C
Detector:	300°C

Illustrative chromatographs are shown in Figures 10-13. Table 1 shows a comparison of results for the primary analysis vs. the confirmatory analysis.

5.11 Recoveries

Table 2 details recovery data. The average recovery was  $82\% \pm 10\%$ .

5.12 Sensitivity

Sensitivity of the method for Bound RH-9090 residues was 0.010 ppm, determined by actual fortifications at this level.

Table 1  
Comparison of Primary Analysis vs. Confirmatory Analysis

<u>Sample</u>	<u>Analysis</u>	<u>µg/ml</u>	<u>µg Total</u>	<u>µg Cor<sup>1</sup></u>	<u>% Rec.</u>
Milk Fortification 0.125 ppm	Primary (Figure 9) ECD Detector	0.114	1.14	1.14	91
	Confirmatory (Figure 13) N/P Detector	0.118	1.18	1.12	90

<sup>1</sup> Corrected for apparent control residues

Table 2

Detailed Recovery Data of Milk Fortifications

<u>ppm Added</u>	<u>% Recovery</u>
0.010	79
0.010	69
0.010	79
0.010*	78
0.010*	85
0.010*	67
0.010*	80
0.010*	93
0.010*	106
0.020	95
0.020	70
0.020*	74
0.030*	76
0.030*	90
0.030*	83
0.040*	75
0.040	90
0.080	80
0.100	77
0.125	91
0.135	95
0.135	78

Average recovery = 82% ± 10%

\* Fortified with Bound RH-9090

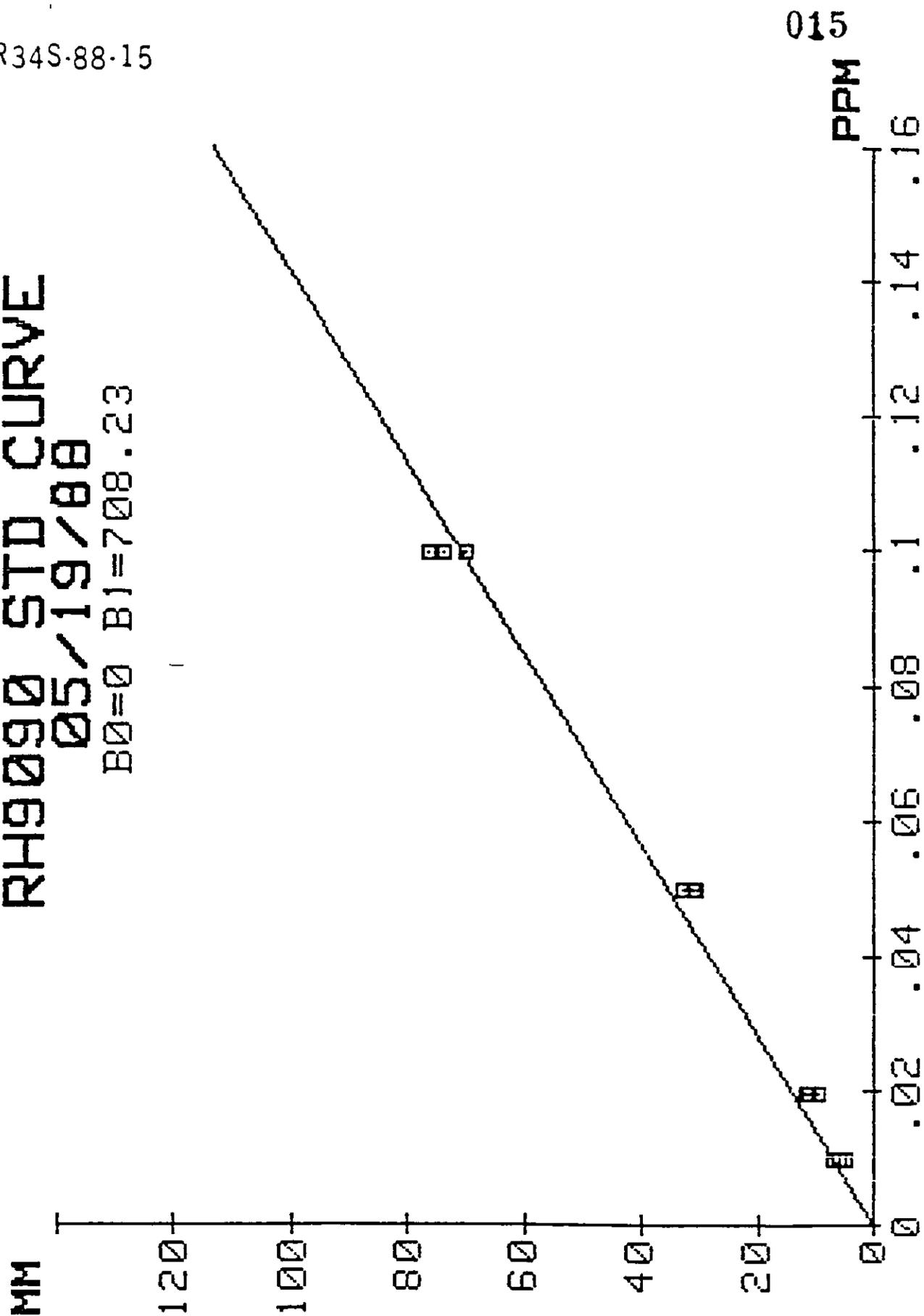
FIGURE 1

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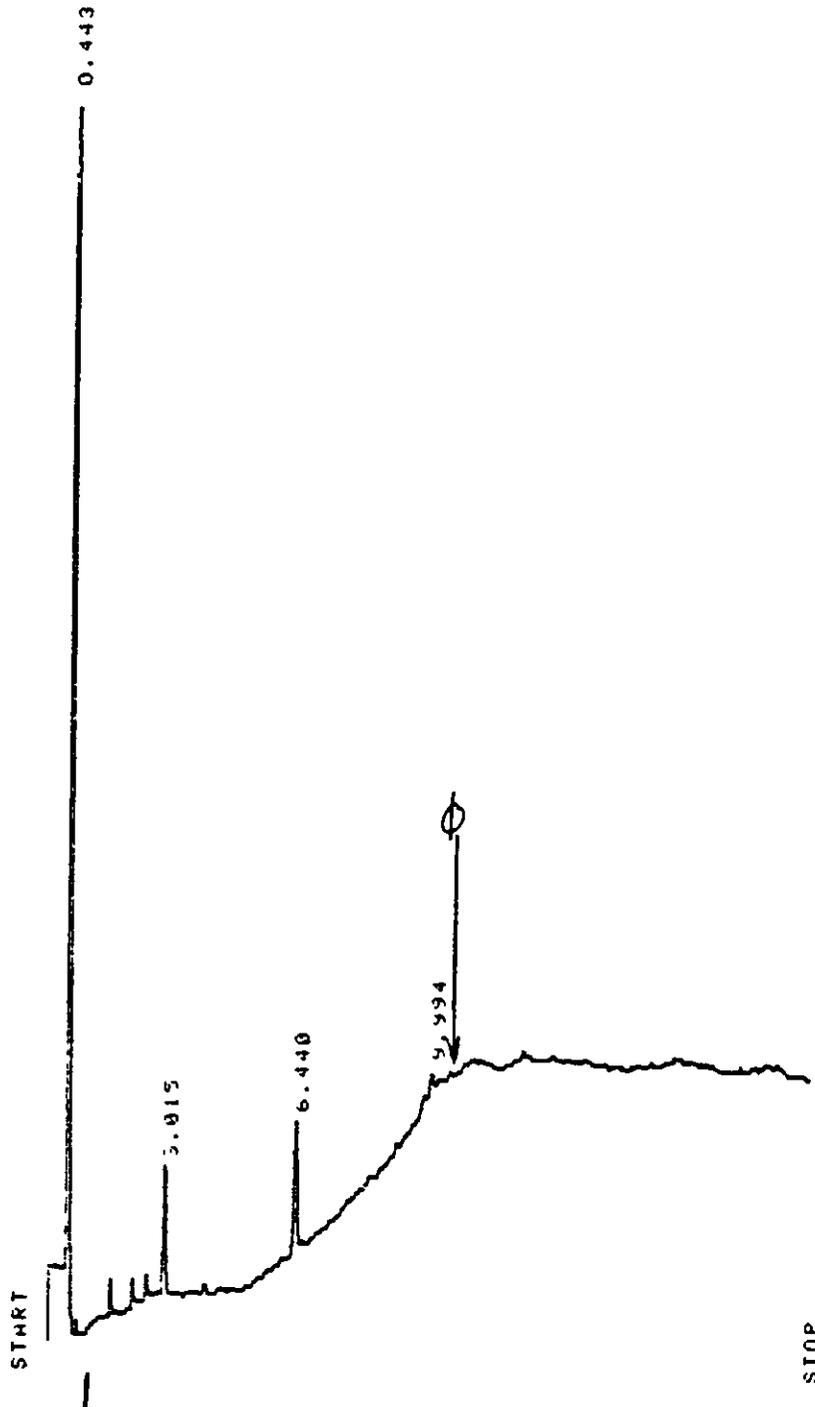
# RH9090 STD CURVE

05/19/88

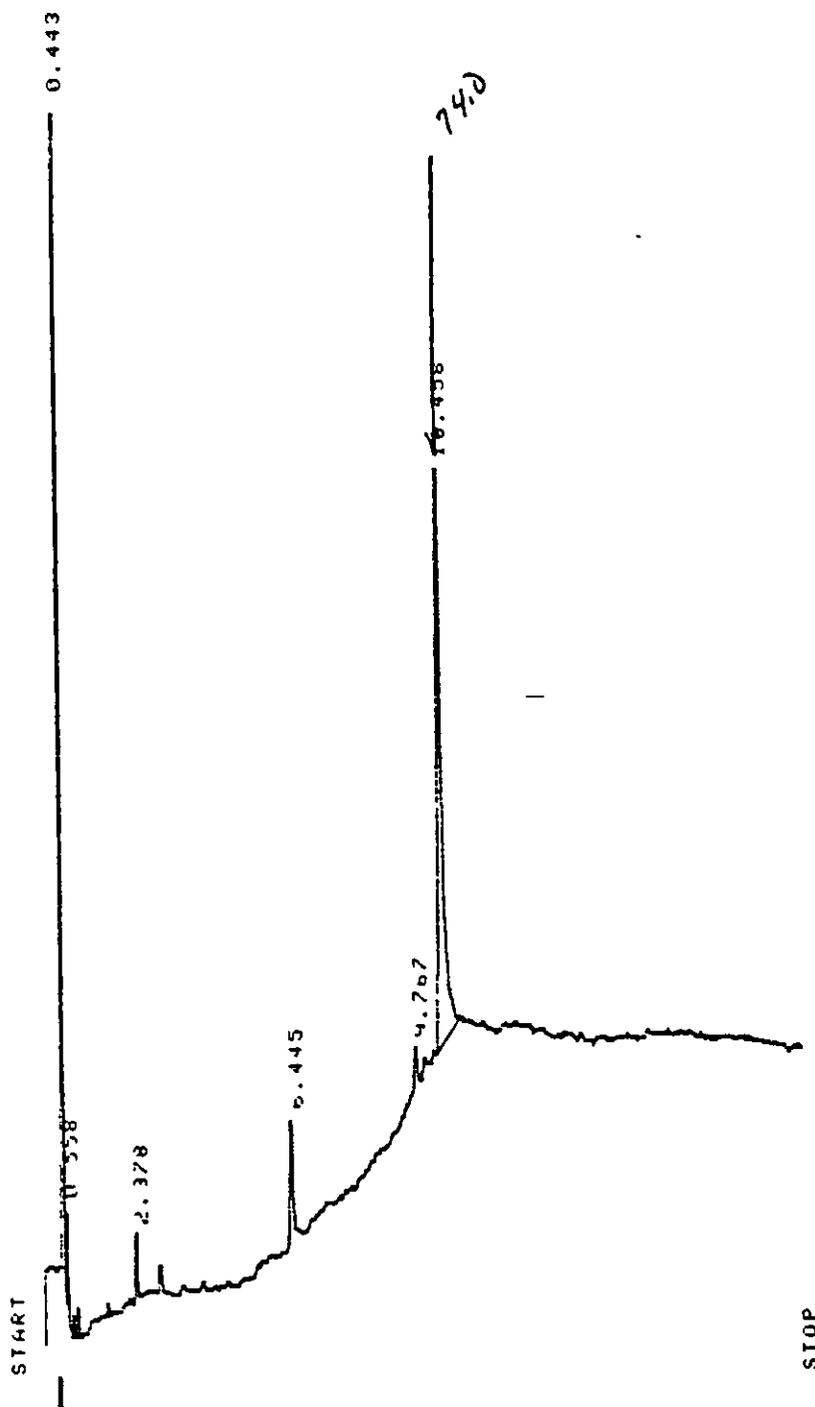
B0=0 B1=708.23



015



Solvent Injection  
STO CURVE 05/19/88

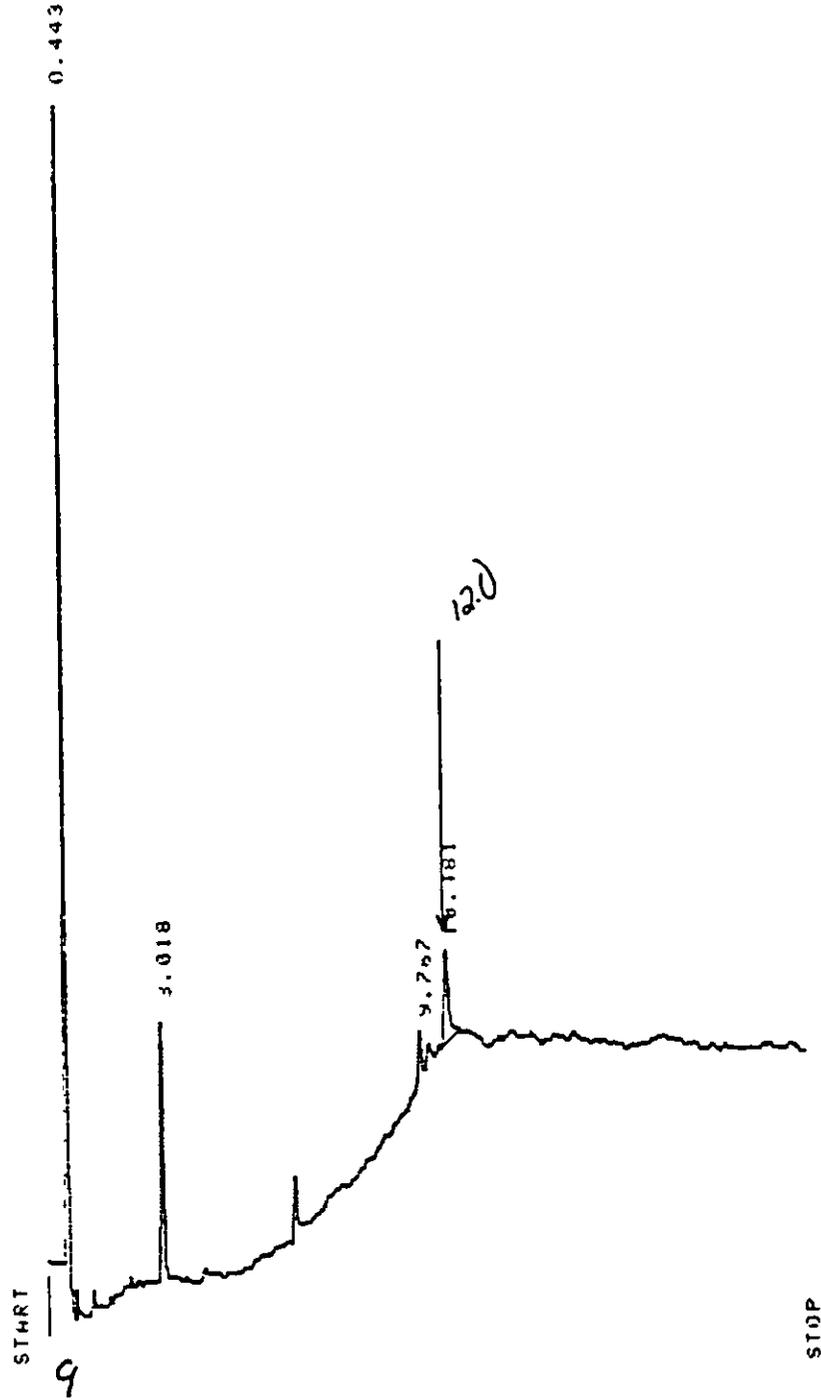


STD - 0.100 ppm

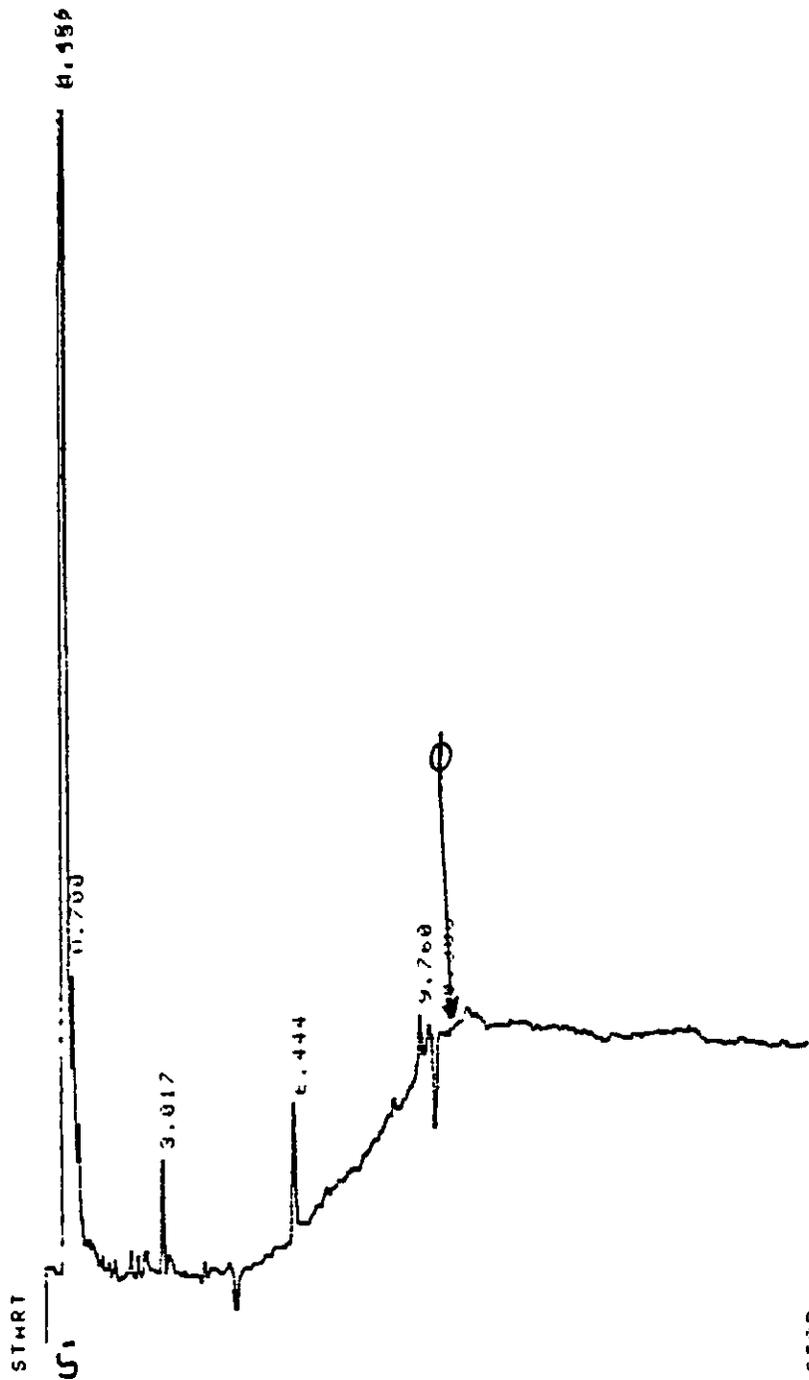
STD Curve 05/19/88

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018

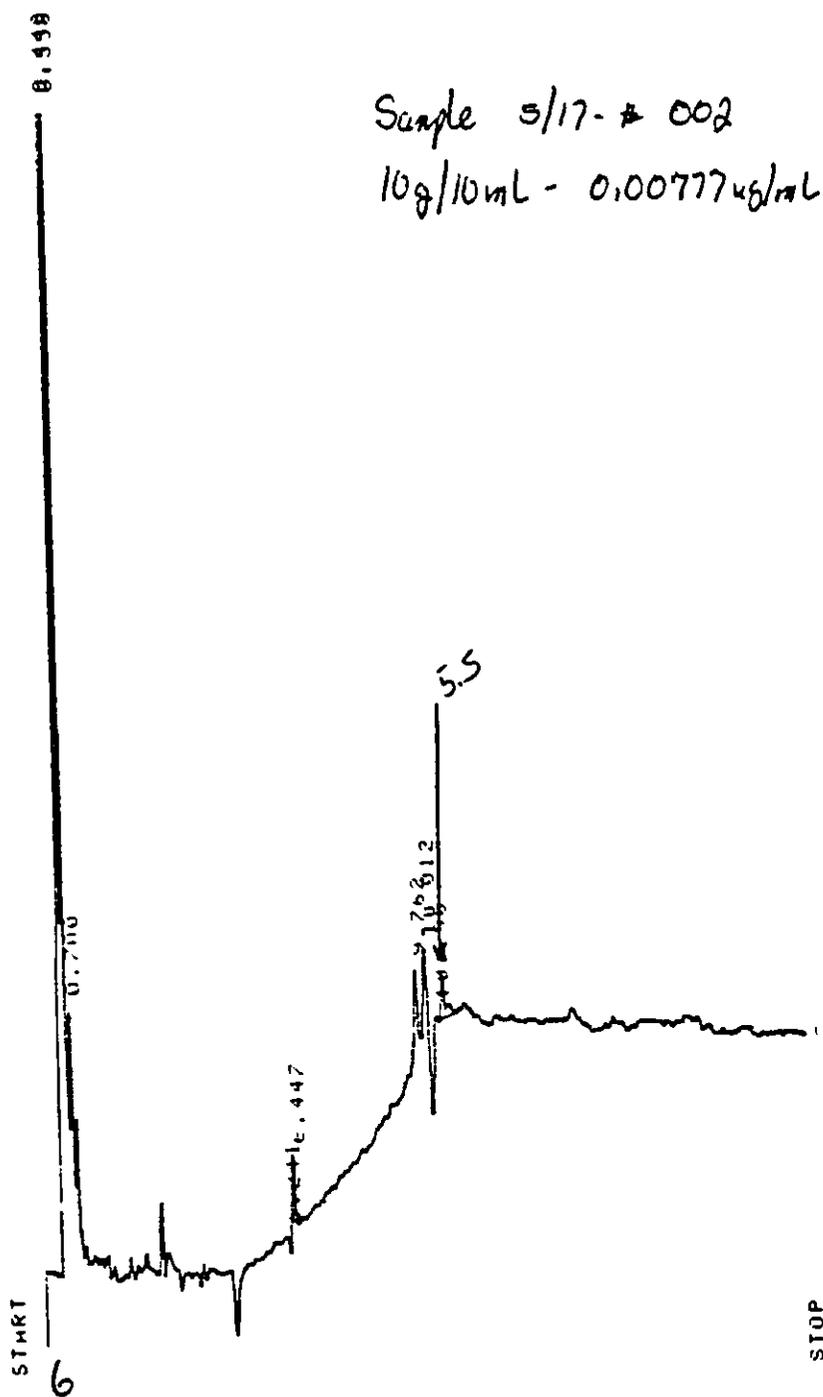


STD 0.020 ppm  
STD CURVE 05/19/88



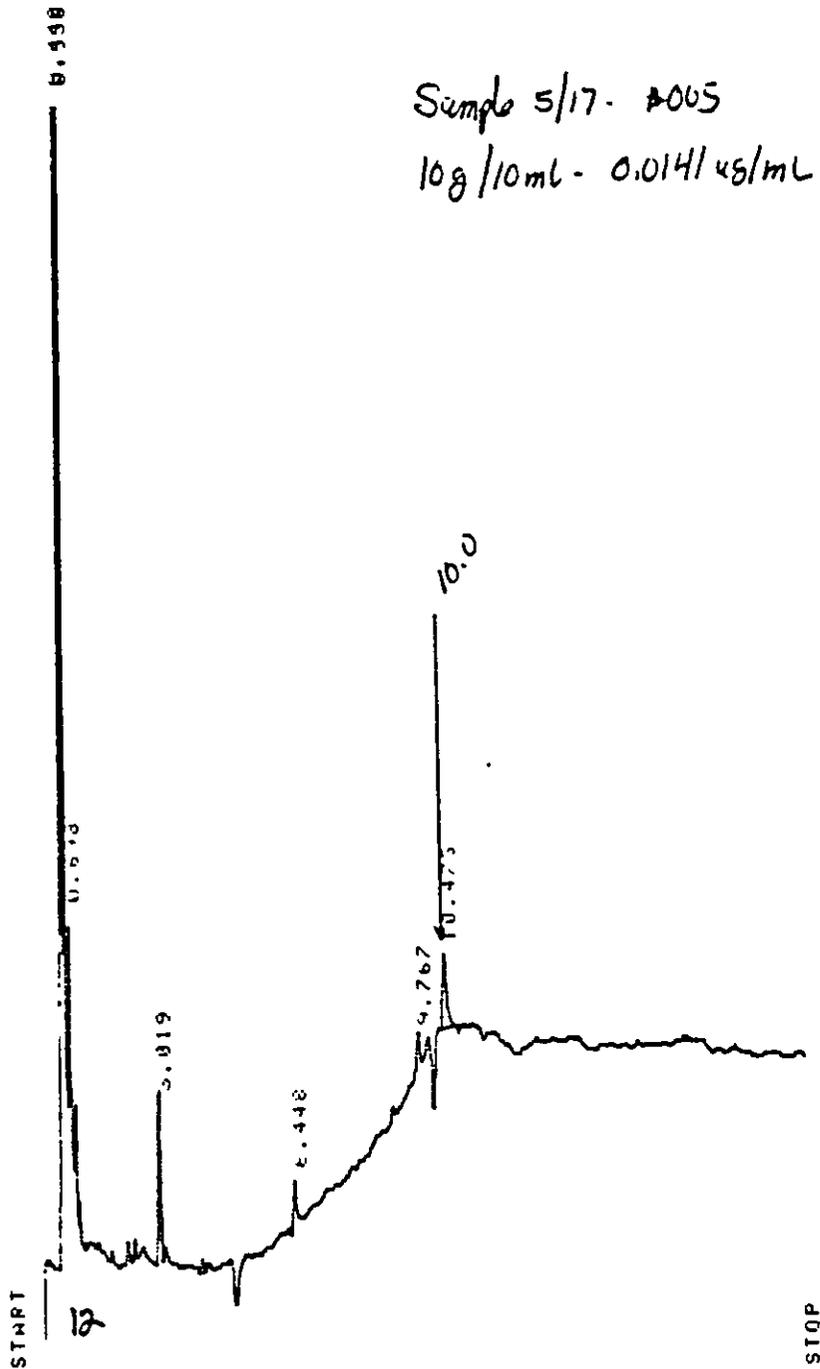
Milk CONTROL  
STD CURVE 05/19/88

TR34S-88-15



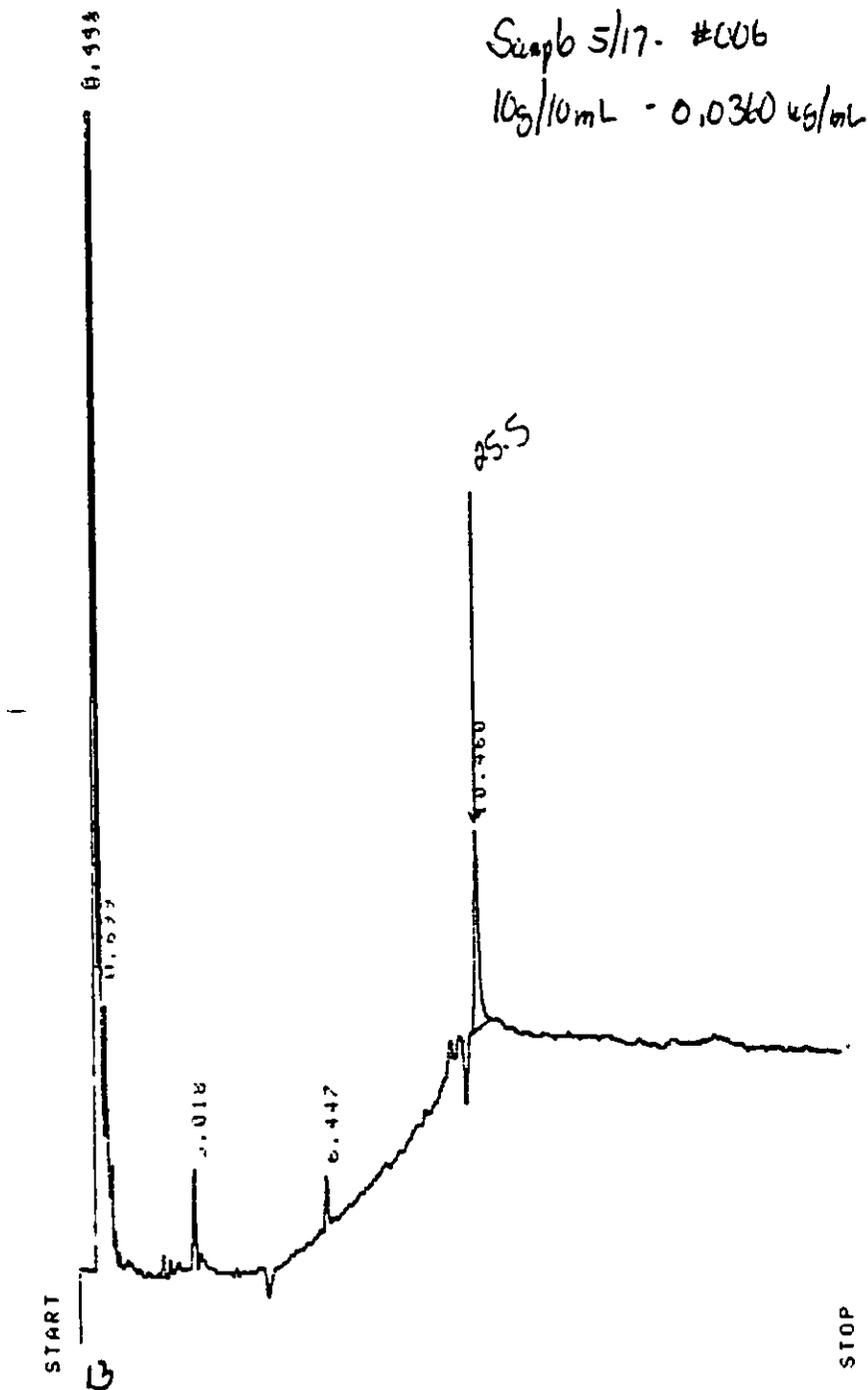
Milk Fortification - 0.010 ppm

STD CURVE 05/19/88



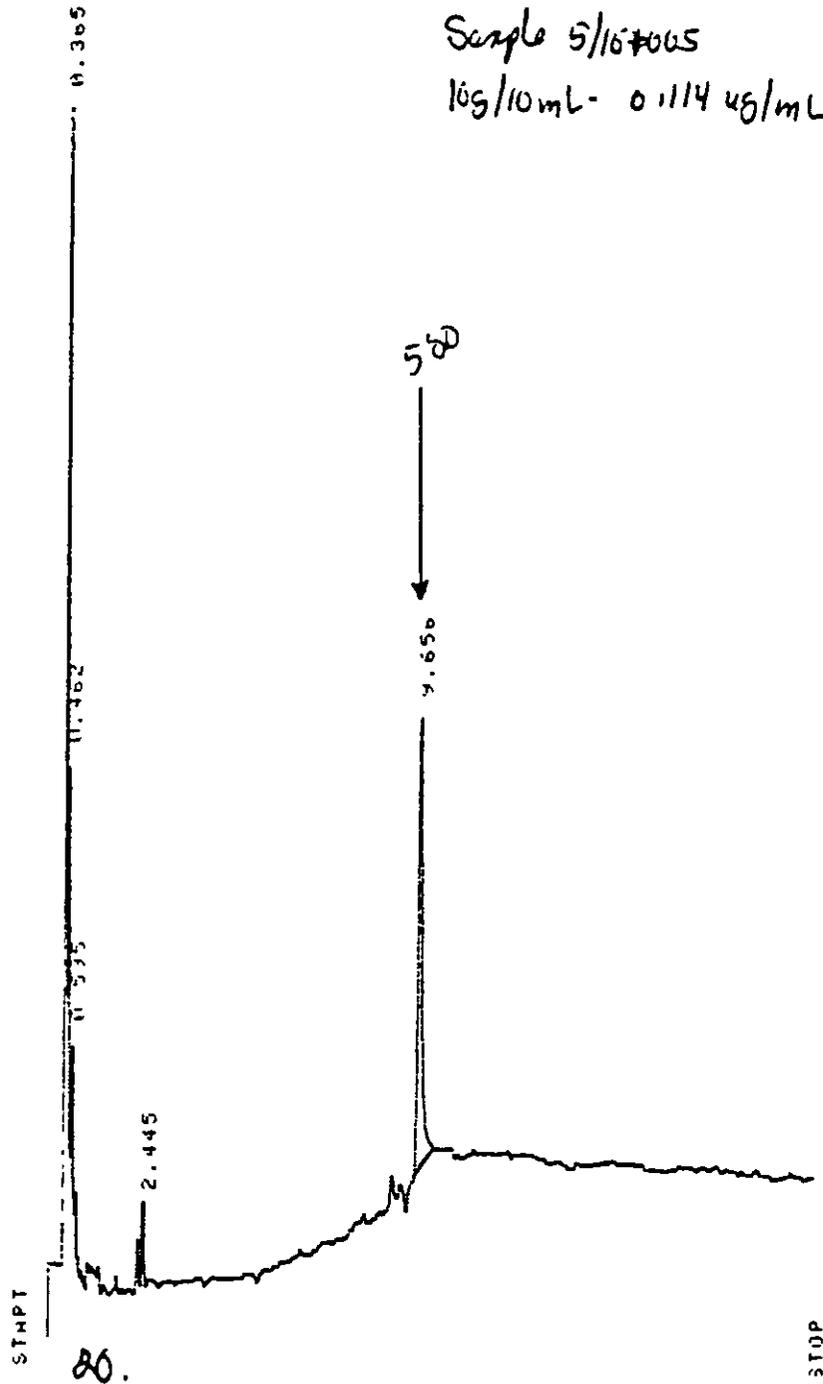
Milk FORTIFICATION 0.020 ppm

STD CURVE 05/19/88



Milk FORTIFICATION 0.040 ppm

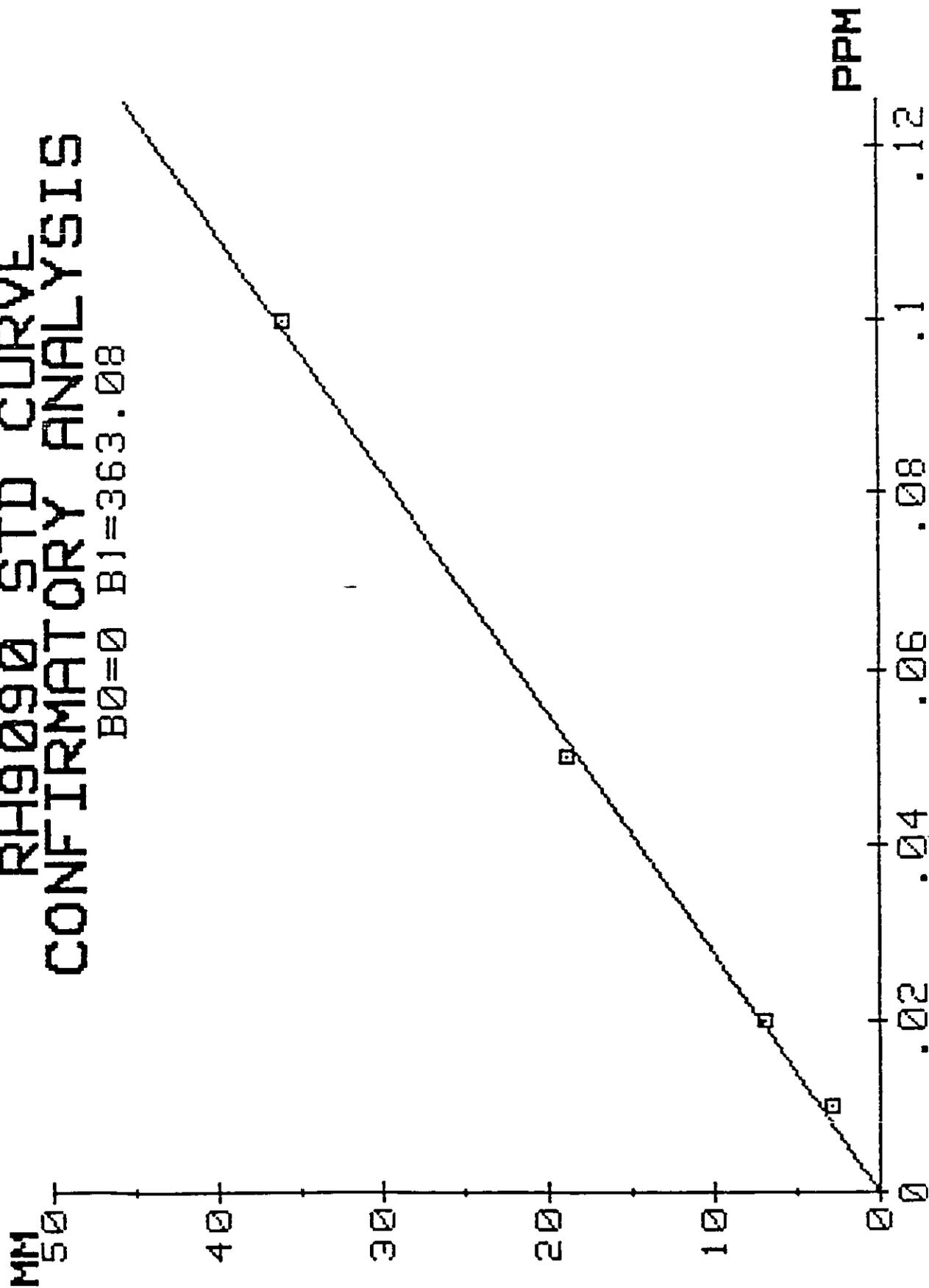
STU CURVE 05/19/88



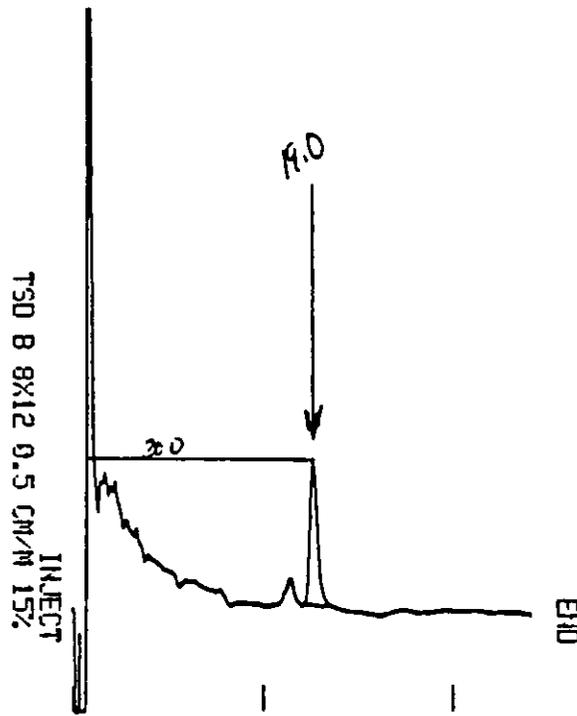
Milk Fortification 0.125 ppm  
STU CURVE 05/11/88

# RH9090 STD CURVE CONFIRMATORY ANALYSIS

$B0=0$   $B1=363.08$



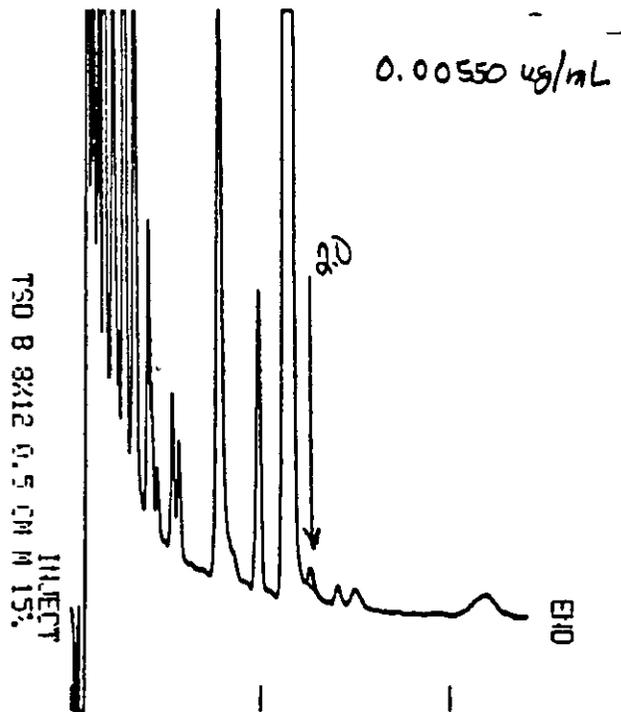
TR34S-88-15



STD 0.050 ppm

CONFIRMATORY ANALYSIS

TR34S-88-15



MILK CONTROL 10g/10mL

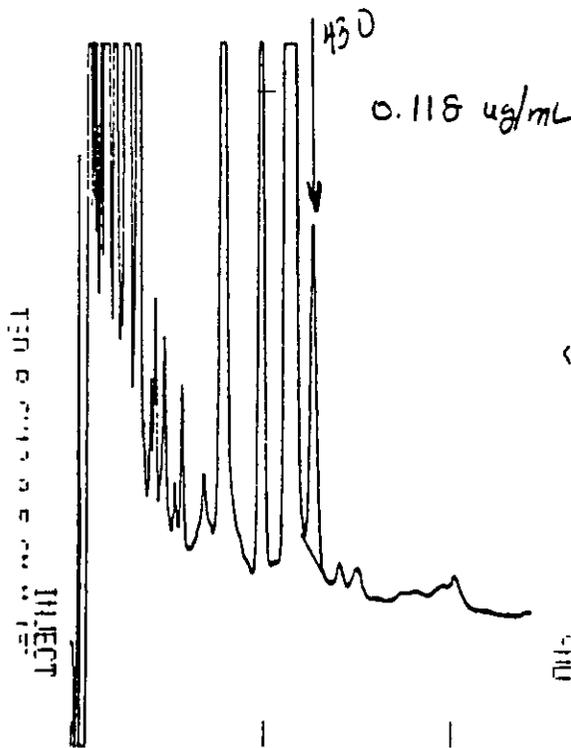
Sample 5/10 - # 001

CONFIRMATORY ANALYSIS

FIGURE 13

027

TR34S-88-15



MILK FORTIFICATION 0.125 ppm

Sample S/10- #005

CONFIRMATORY ANALYSIS