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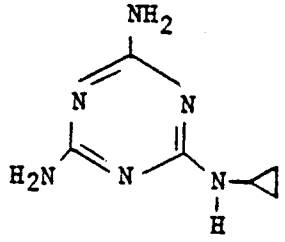
1 of 13	AG-376	DETERMINATION OF CGA-72662 IN FEED
EDITION 2/18/82		
SUBMITTED BY: K. Balasubramanian, L. E. Williams and R. K. Williams		
		APPROVED BY:

1.0 SCOPE

This method is used for the extraction, cleanup and final determination of CGA-72662 residues in chicken feed samples. The limit of detection for the method is 1.0 ppm.

CGA-72662:



N-cyclopropyl-1,3,5-triazine-2,4,6-triamine.

2.0 PRINCIPLE

Feed samples are extracted by refluxing with glacial acetic acid for one hour. An aliquot of the extract is evaporated to dryness and cleaned up by silica gel column chromatography prior to gas chromatographic analysis. This method is outlined in Figure 1.

3.0 APPARATUS

3.1 Air manifold, N-Evap. by Organomation or equivalent.

3.2 Column, chromatographic, 19-mm i.d., with Teflon stopcock.

3.3 Flask, round bottom, 250-ml, 500-ml.

3.4 Pipette, 10-ml.

3.5 Rotary evaporator, Büchi or equivalent.

3.6 Sample vial, 20-ml.

3.7 Variable transformer, Powerstat.

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3.8 Ultrasonic bath.

3.9 Vortex mixer.

3.10 Reflux condenser.

4.0 REAGENTS

4.1 Acetic acid, glacial, reagent grade.

4.2 Acetone, distilled in glass, Burdick and Jackson.

4.3 Methanol, reagent grade.

4.4 CGA-72662, analytical standard.

4.5 Silica gel (Woelm 02747) 100-200 mesh, ICN Catalogue #402747.

5.0 PROCEDURE

5.1 Preparation of Sample

Thoroughly mix the feed sample.

5.2 Extraction

5.2.1 Weigh a 10-g representative subsample into a 500-ml round bottomed flask. Add 100-ml of glacial acetic acid, fit with a reflux condenser and reflux for one hour.

5.2.2 Allow the mixture to cool to room temperature.

5.2.3 Pipette a 10-ml aliquot of the supernatant liquid (1-g aliquot) into a 250-ml round bottomed flask.

5.2.4 Evaporate the sample to dryness using a rotary evaporator (bath temperature 40-50°C). Add methanol and reevaporate if necessary to completely remove all solvent.

5.3 Silica Gel Cleanup

5.3.1 Fill a chromatographic column (19-mm i.d.) containing glass wool at the bottom with 2% methanol in acetone. Measure 25 ml of silica gel (Woelm) 100-200 mesh using a 50-ml graduated cylinder and add to the column. Tap gently to remove any trapped air bubbles. (The column is approximately 3 inches in height.)

5.3.2 Transfer the sample to the column using two 10-ml portions of 2% methanol/acetone using an ultrasonic bath to dissolve the residue. Discard the eluate.

5.3.3 Add 2 ml of methanol to the sample flask and use an ultrasonic bath to dissolve the residue. Add 98 ml of acetone to the flask and swirl. Transfer the contents of the flask to the column by filtering through a funnel containing glass wool. Discard the eluate.

5.3.4 Add 150 ml of 15% methanol in acetone to the sample flask. Transfer this solution to the column, again filtering through the glass wool, and collect the eluate in a 500-ml round bottom flask.

5.3.5 Evaporate the sample to dryness using a rotary evaporator (bath temperature 40-50°C).

5.3.6 Quantitatively transfer the contents of the flask to a 20-ml sample vial using 10 ml of methanol. Evaporate the sample to dryness using a gentle stream of air in an N-Evap.

6.0 GAS CHROMATOGRAPHIC ANALYSIS

Sample residues (Section 5.3.6) are dissolved in methanol prior to gas chromatographic analysis. CGA-72662 residues are detected by gas chromatography using an alkali flame ionization detector in the nitrogen-specific mode. Gas chromatographic conditions are given in Table I.

6.1 Standardization

6.1.1 Prepare a stock solution containing 20 mg of CGA-72662 in 100 ml of methanol (200 ng/µl). Make serial dilutions with methanol until working solutions containing 1 ng/µl and 10 ng/µl are achieved.

6.1.2 Standardize the gas chromatograph, operating under the conditions specified in Table I, by injecting 2- to 8-µl aliquots of the 1 ng/µl or 10 ng/µl solution. This represents a working range of 2 to 80 nanograms.

6.1.3 Determine the peak height or area for the injected standards. Typical chromatograms of standards are shown in Figure 2. Typical standardization data are given in Table III.

6.1.4 Construct a standard curve, plotting detector response (peak height or area) versus nanograms injected. A typical standard curve is presented in Figure 3. Alternatively, enter the standardization data into an appropriate electronic calculator (e.g. Texas Instruments Model TI55) to calculate a least squares standard curve.

6.2 Fortification Experiments

This method is validated for each set of samples analyzed by including an untreated control sample and one or more control samples fortified prior to extraction with 1 ppm or more of CGA-72662.

6.2.1 Add 10 µg of standard CGA-72662 in 2 ml or less of methanol to 10 g of control sample of feed in the extraction step (Step 5.2.1) for a 1-ppm fortification. Use correspondingly greater concentrations of standard for higher fortifications. Analyze the control and fortified samples by the procedures of the method.

6.2.2 Calculate the final ppm value for the control and fortified samples according to the equation in 6.3.3 using R=1.

6.2.3 Correct the residue value (ppm) by subtracting the residue value, real or apparent, found in the control sample. Calculate the recovery factor in percent, by the following equation:

$$R\ (\%) = \frac{\text{ppm found (corrected)}}{\text{ppm added}} \times 100$$

6.3 Detection of Sample Residues

6.3.1 Dissolve the residue from Section 5.3.6 in an appropriate volume of methanol.

6.3.2 Inject an aliquot into the gas chromatograph (e.g. dissolving the residue in 2.0 ml of methanol and injecting 4 µl gives 2 mg of sample equivalent injected:4 µl/2000 µl X 1000 mg = 2 mg). Compare peak heights or areas of unknown samples with the standard curve to determine the CGA-72662 amounts in the aliquot injected. Typical chromatograms for feed are shown in Figure 4.

6.3.3 Calculate residue results in ppm by the following equation:

$$\text{ppm} = \frac{\text{CGA-72662 found (ng)}}{\text{mg sample injected}} \div R$$

The recovery factor (R) is determined in Step 6.2.3 and is expressed as a decimal (100% = 1.00, etc.).

7.0 ALTERNATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) DETECTION

The HPLC conditions given in Table III have also been used to determine CGA-72662 in feed samples.

8.0 DISCUSSION

1. Typical recoveries of CGA-72662 from feed samples fortified at 1.0 to 50 ppm are 92 $\pm$ 11 (N=5) for GC/AFID detection.

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TABLE I: GAS CHROMATOGRAPHIC CONDITIONS

Instrument	Tracor 560 equipped with an Alkali Flame Ionization Detector (Perkin Elmer)
Column Packing	Ultrabond II. Supelco Chromatography Supplies, Inc.
Column	Pyrex 2' X 2 mm i.d.
Temperatures	
Column	175°C
Injector	250°C
Detector	250°C
Gas Flows	
He carrier	40 ml/min.
H_2 reaction gas	3.0 ml/min. (regulated)
Compressed air	125 ml/min.
Attenuation	10 X 8
Bead Current Setting	690
Minimum Detection Limit	2 nanograms
Volumes Injected	2-8 µl
Chart Speed	1 cm/minute
Retention Time	~ 2.5 minutes

TABLE II. TYPICAL STANDARD CURVE FOR GAS CHROMATOGRAPHIC DETERMINATION OF CGA-72662

Amount CGA-72662 Injected (ng)	Peak Height (cm)
2	0.6
8	1.9
20	5.4
40	10.2
80	17.4

See Figure 3 for plot.

Standardization Data:

n = 5
Correlation Coefficient: 0.9958
Slope: 1.2170
Intercept: 0.5474

TABLE III: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY CONDITIONS

Equipment:

Pump :	Waters Model 6000A
Injector:	Waters WISP 710B Automatic Injector
Detector:	Waters Model 441 UV Detector, 214 nm
Column:	Whatman Partisil PXS 10/25 PAC
Solvent:	68% Isooctane, 8% 2-Propanol, 24% Methanol
Flow Rate:	1.1 ml/min
Injection Volume:	10-20 µl
Minimum Detection Limit	5 nanograms
Retention Time	8.5 minutes

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Figure 1: Flow Diagram of the Analytical Procedure for the Determination of CGA-72662 Residues in Chicken Feed

Sample (10g)
↓
Reflux in 100 ml glacial acetic acid 1 hour
↓
Let cool to room temperature
↓
Aliquot 10 ml (1g) of supernatant
↓
Evaporate to dryness
↓
Silica gel Column
3" X 19 mm i.d.
↓
20 ml 2% Methanol/Acetone
100 ml 2% Methanol/Acetone
150 ml 15% Methanol/Acetone
↓
Evaporate
↓
Analyze by GC-AFID

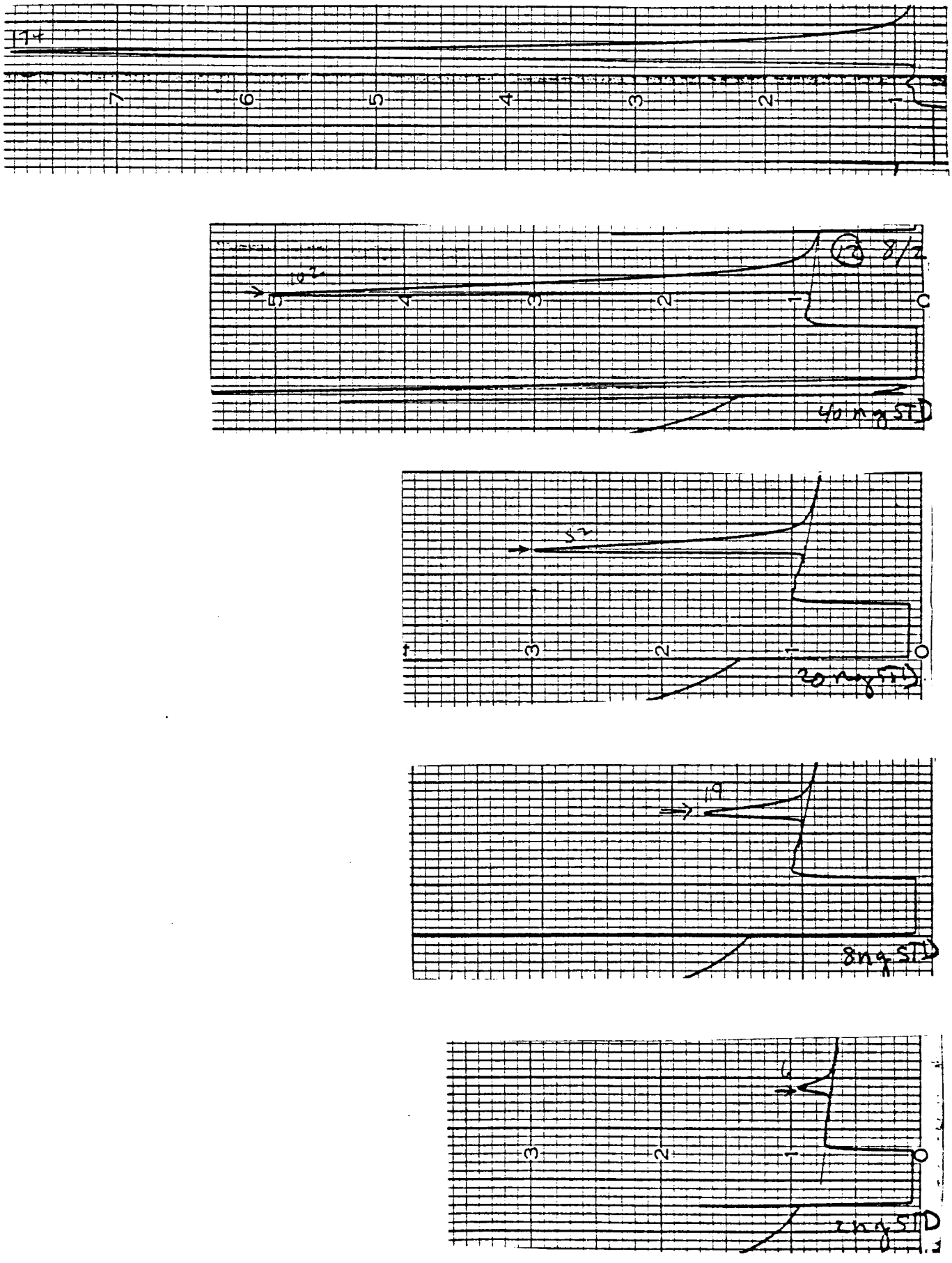

Figure 2: Typical Gas Chromatograms of CGA-72662 Standards

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Figure 3. Typical Standard Curve for Gas Chromatographic Determination of CGA-72662. (See Table III for Data)

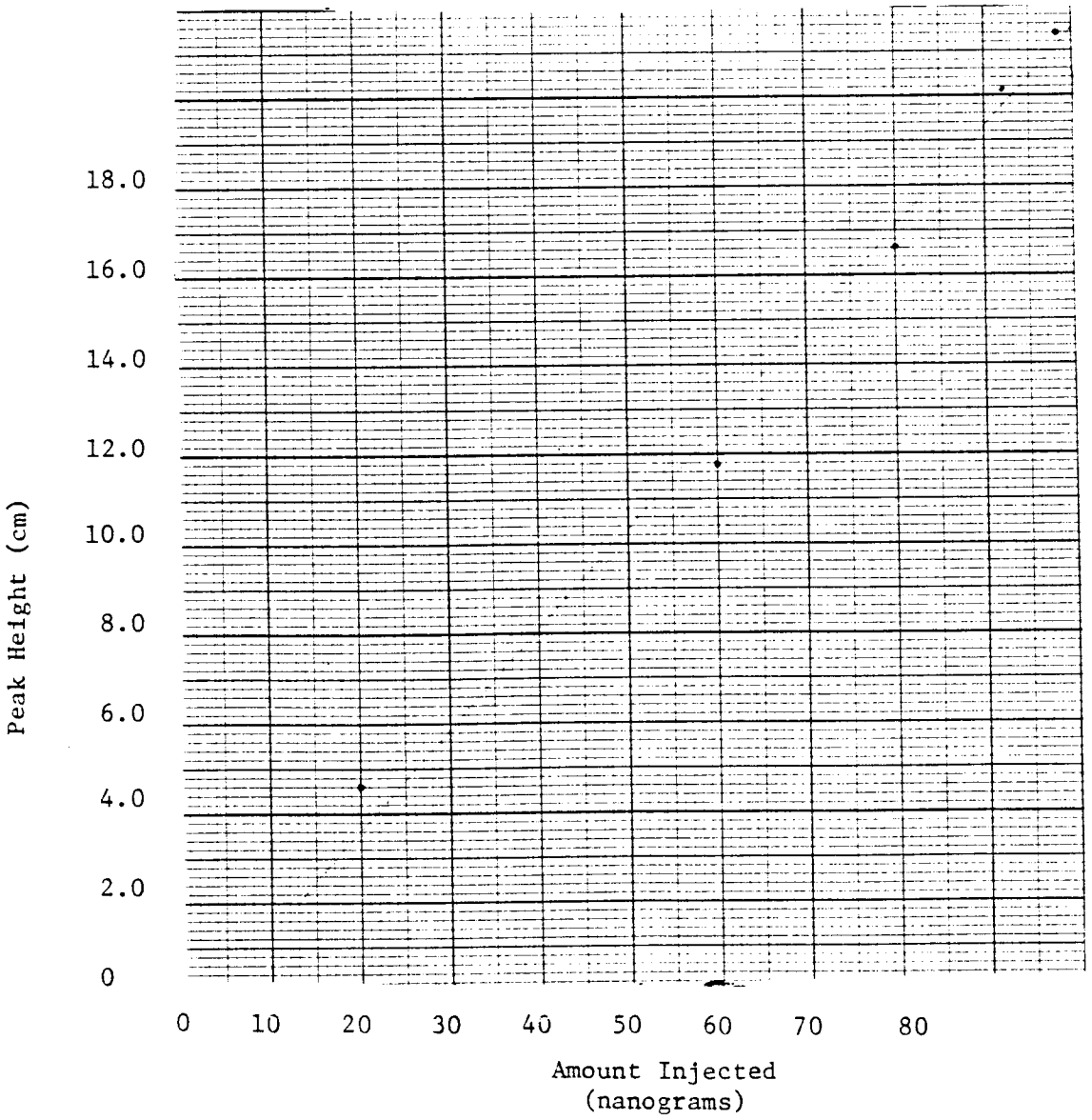

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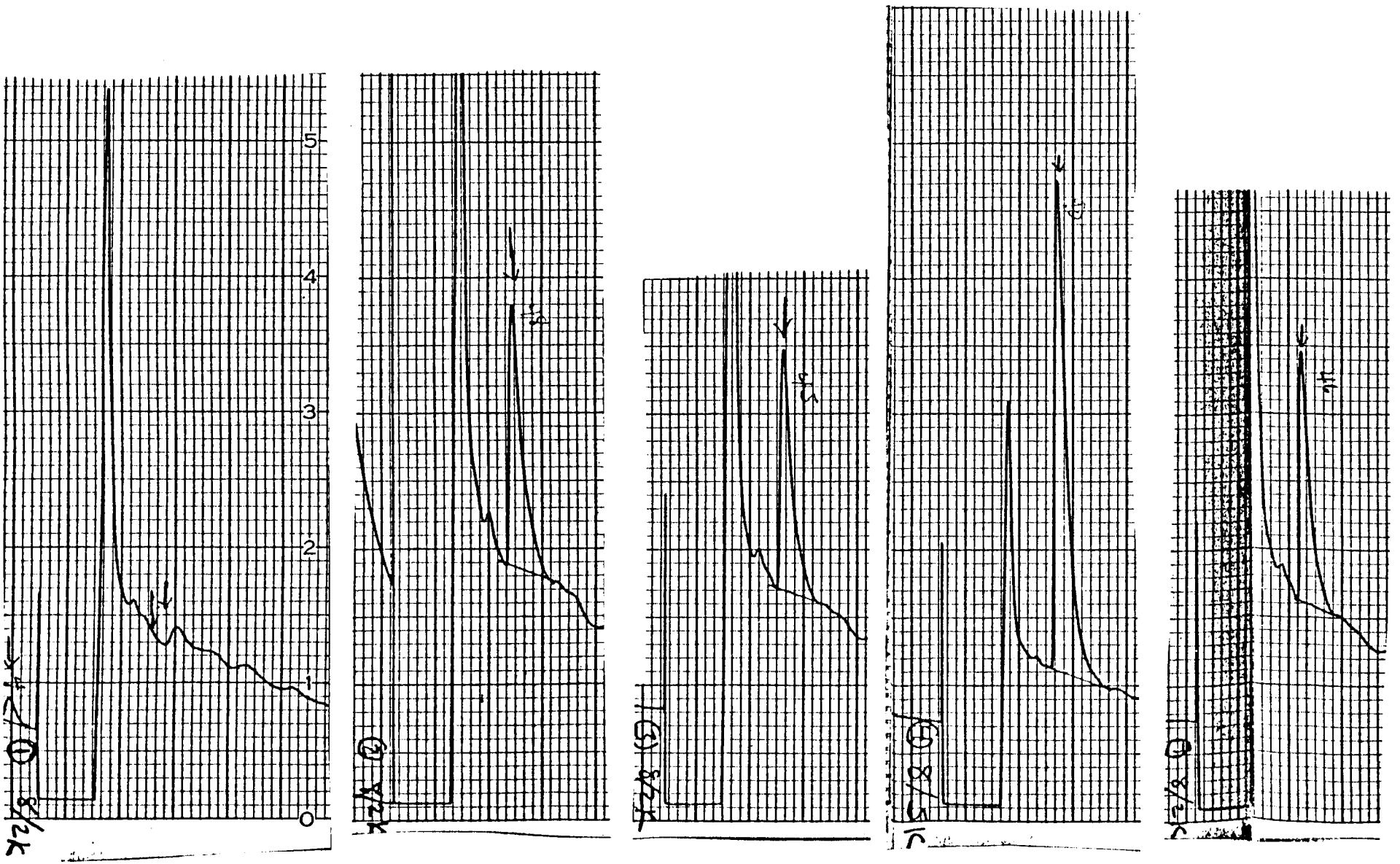
Figure 4: Typical Gas Chromatograms of CGA-72662 Determinations In Feed Sample (AG-A 6510)



A

Check
4.0 mg injected
Found: <1.0 ng
<1.0 ppm

B

Check + 5.0 ppm
4.0 mg injected
Found: 20 ng
4.9 ppm
98% recovery

C

0-Day, 5 ppm Treated
4.0 mg injected
Found: 18 ng
4.6 ppm

D

0-Day, 25 ppm Treated
1.6 mg injected
Found: 37 ng
23 ppm

E

7-Day, 5 ppm Treated
4.0 mg injected
Found: 19 ng
4.7 ppm