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Title: Determination of Carbadox Metabolite by GC/ECD		
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A. INTRODUCTION

1. Theory

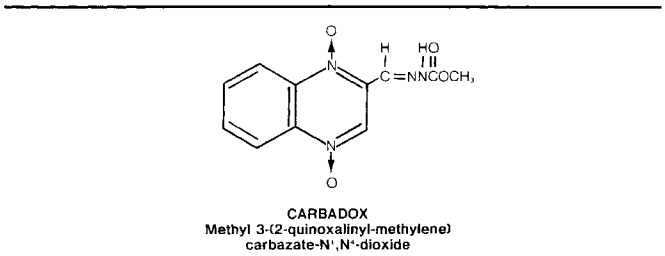
Carbadox is approved for use in swine weighing less than 75 Ibs to prevent or treat enteritis and for increased feed efficiency and weight gain. Since the parent compound is a liver carcinogen, carbadox is monitored in domestic hogs, boars, and sows.

Carbadox metabolic residues are determined as quinoxaline-2-carboxylic acid (QCA), which is isolated from the tissue after alkaline hydrolysis, sequential extraction into ethyl acetate and pH 6 buffer, and ion exclusion chromatography. The column eluate is extracted with chloroform and derivatized with methanolic sulfuric acid. The methyl ester derivative, methyl quinoxaline-2-carboxylate (QME) is then quantitated by GC-ECD.

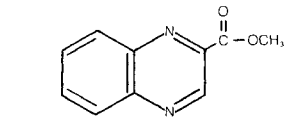
2. Applicability

This method is applicable for analysis of Carbadox in swine liver at levels ≥ 15 ppb.

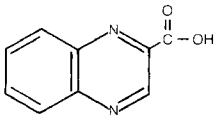
3. Structure



CARBADOX
Methyl 3-(2-quinoxalinyl-methylene) carbazate-N¹,N⁴-dioxide



Methyl Quinoxaline-2-carboxylate
"QME" or CP-25,536



Quinoxaline-2-carboxylic Acid
"QCA" or CP-16,505

B. EQUIPMENT

1. Apparatus

   Note: An equivalent can be substituted for any apparatus listed below.

   a. Centrifuge - With rotor(s) able to accept 50 mL and 15 mL tubes and maintain 1500 rpm.

   b. Pipet, disposable - Pasteur: 5 ¾ inch, Cat. No. 53283-910, VWR.

   c. Dispensers - bottle-top, 5 mL, 50 mL and 100 mL. Labmax, Cat. No. 40000-062, -066, -070, VWR.

   d. Pipet - volumetric class A, 1.0 mL, Cat. No. 37007-1 and 10.0 mL, Cat. No. 37007-10, Kimble.

   e. Pipet - precision, mL, 100µL - 1000µL Eppendorf.

   f. Centrifuge tube - 15 mL glass heavy duty, with screw cap, Cat. No. 73785-15, Kimble.

   g. Centrifuge tube – 50 mL glass, heavy duty, with screw cap, 25 x 150 mm and 29 x 122 mm. Cat. No. 9826-25 and 8422-50, Corning.

   h. Volumetric flask - class A, glass stoppered 100 mL, 200 mL, and 1000 mL capacity, Cat. No. KT850100-0013,-0016,-0022, VMR.

   i. Chromatography columns - 250 mm x 11 mm i.d. with Teflon stopcock and 200 mL reservoir Kontes, Cat. No. KT420280-0213, VWR.

   j. Oil bath - Fisher HiTemp: 4 L, Cat. No. 11-481, Fisher.

   k. Round-bottom flask - single neck, 250 mL capacity, Cat. No. 4320-250. Pyrex.

   l. Test tube rack - for 15 mL and 50 mL centrifuge tubes.

   m. Test tube mixer - Vortex, Cat. No. K-550-G, VWR.

   n. Separatory funnels - 60 and 250 mL capacity with Teflon stopcocks and glass stoppers, Cat. No. 29048F-60 and 29048F-250, Kimble.

   o. Rotary evaporator with water bath - Buchi RE 111, Bath, Buchi 461.

   p. pH meter - with electrode, Corning model 430, Cat. No. 475301, VWR.

   q. Thermometer - glass or digital, 0 - 150 °C range, accurate to 1 °C.

   r. Top-load balance - sensitive to 0.01 g, Mettler model PM 360.

   s. N-Evap - with water bath, using nitrogen for purging, Organomation Associates model 112.

t. Graduated cylinder - class A: 50 mL, 100 mL Kimble #20026 -50 -100.

u. Ring Support - cork for round bottom flask, 110 mm o.d. x 60 mm i.d. Cat. No. 56250-046, VWR.

v. GC auto-sampler vial kit - 2 mL vial with Teflon/silicone/Teflon septa, Cat. No. C4000-86W, National Scientific.

w. Digital timer/stopwatch - Cat. No. 62344-588, VWR.

2. Instrumentation

Note: An equivalent can be substituted for any instrumentation listed below.

a. Gas chromatograph - Hewlett Packard 6890 GC Equipped with an electron capture detector and appropriate analytical software and hardware to support the analysis.

b. GC column - 30 m x 450 µm x 0.7 µm film thickness, Cat. No. DB608, Agilent.

C. REAGENTS AND SOLUTIONS

1. Reagents

Note: An equivalent reagent or solution may be substituted.

a. Chloroform - amylene stabilized, ACS grade, Cat. No. AH049-4, Burdick and Jackson.

b. Methanol - Cat. No. GC 230-4, GC grade, Burdick and Jackson.

c. Toluene - Cat. No. AH 347-4, Burdick and Jackson.

d. Ethyl acetate - Cat. No. AH 100-4, ACS grade, min. 99.5%, Burdick and Jackson.

e. Citric acid - Monohydrate, granular, reagent grade, Cat. No. CX1725-1, EM Science.

f. Sodium Hydroxide – 3 N, Cat. No. VW3472-1, VWR.

g. Sodium Hydroxide – 5 N, Cat. No. VW3225-1, VWR.

h. Sodium sulfate - Anhydrous granular, Cat. No. 6639-1, EM Science.

i. Silicone fluid - #510 to operate at 100 °C or higher, Cat No. 13-874-60B, Fisher.

j. Hydrochloric acid - Conc. reagent grade, Cat. No.HX0603P-5, EM Science.

k. Sulfuric acid - Conc. reagent grade, Cat. No. SX1244-11, EM Science.

l. Chromatography resin - AGMP-50, 100 - 200 mesh, Cat. No. 1430841, Bio-Rad Laboratories.

2. Solutions

   a. Hydrochloric acid, 1 M:

      Dilute 83.3 mL of concentrated HCI to 1000 mL with distilled water.

   b. 10/90 Methanol:Water (v/v):

      Dilute 10.0 mL of reagent grade methanol to 100 mL with distilled water.

   c. 3% Sulfuric Acid:Methanol (v/v):

      Dilute 0.15 mL of concentrated H_2SO_4 to 5 mL with methanol that has been dried over anhydrous Na_2SO_4. Use an ice bath to cool the methanol before adding the acid. Prepare daily.

   d. Citric acid, 1 M:

      Dissolve 210.0 g of citric acid monohydrate in distilled water and dilute to 1 L.

   e. Citric acid buffer, 0.5 M:

      Adjust the pH of 100 mL of 1 M citric acid to pH 6.0 with 5 M sodium hydroxide (ca. 55 mL), using a pH meter. Cool the buffer to room temperature. Adjust the final volume to 200 mL with distilled water.

D. STANDARDS

1. Source

   a. Quinoxaline-2-carboxylic acid (QCA), stock standard A (15.0 µg/mL), part # 91819, Absolute Standards, Inc.

   b. Methyl quinoxaline-2-carboxylate (QME), stock solution, (15.0 µg/mL), part # 91820 Absolute Standards, Inc.

2. Preparation

   a. QCA working standard solution B (0.150 µg/mL):

      Pipet 1 .0 mL of stock standard A (D.1.a) (15.0 µg/mL) into a 100 mL volumetric flask and dilute to volume with distilled water.

   b. QCA working standard solution C (0.150 µg/mL):

      Pipet 1.0 mL of stock standard A (D.1.a) (15.0 µg/mL) into 100 mL volumetric flask and dilute to volume with methanol.

c. Processed GC injection standard (0.015 µg/mL):

Pipet 1.0 mL of working standard solution C (D.2.b) into a 15 mL centrifuge tube and follow the extraction procedure starting at F.2.d.vi.

d. QME injection standard (0.015 µg/mL):

Pipet 0.10 mL of QME stock solution (D.1.b) into a 100 mL volumetric flask and dilute to volume with toluene

3. Storage Conditions

Standard solutions can be stored in tightly closed glass bottles at room temperature.

4. Shelf Life Stability

a. Stock standard – According to the manufacturer expiration date.

b. QME injection standard - 6 months.

c. Working standards - 1 month.

E. SAMPLE PREPARATION

Homogenize liver samples using a blender or food processor and freeze prior to extraction.

F. ANALYTICAL PROCEDURE

1. Column Preparation

a. Slurry 7.0 g of AGMP-50 resin in 1 N HCl and transfer to a 10.5 mm i.d. glass column containing a small glass wool plug. Allow the resin to settle for at least 10 minutes, then drain a small volume of the HCl to complete the settling and cap the resin bed with a glass wool plug. Maintain the liquid level above the resin.

2. Extraction Procedure

a. Weigh 5.0 ± 0.1 g of blank tissue to a 25 x 150 mm centrifuge tube and fortify with 1.0 mL of QCA working standard solution B. This will yield a 30 ppb fortified control.

b. Dissolution and hydrolysis

i. Weigh 5.0 ± 0.1 g of freshly sliced frozen tissue in a 25 x150 mm centrifuge tube.

ii. Pipet 10 mL of 3 M sodium hydroxide into the tube, cap lightly, and place it in a preheated 95 -100 °C silicone oil bath for 30 minutes. The liquid level of silicone oil in the bath should exceed that of sample in the tube.

iii. Cool the alkaline hydrolysate in an ice bath and acidify to pH ≤1 with 4 mL of concentrated HCI. Cap and vortex the sample (pH can be measured with pH paper). Transfer to 29 x 122 mm centrifuge tube.

iv. Add 15 mL of ethyl acetate to the acidified hydrolysate, cap tightly, and extract by shaking for at least 40 seconds.

v. Centrifuge the mixture at 1500 rpm for 5 minutes to clarify the ethyl acetate phase. Transfer the extract to a 60 mL separatory funnel.

vi. Re-extract the hydrolysate with two additional 15 mL portions of ethyl acetate and combine the organic extracts. Do not contaminate the ethyl acetate phase with interfacial material during these extractions.

vii. Add 5 mL of 0.5 M citric acid buffer to the ethyl acetate extract, shake, and allow the lower phase to clarify (at least 10 minutes).

viii. Collect the aqueous phase in a 15 mL centrifuge tube.

ix. Re-extract the ethyl acetate phase with an additional 5 mL 0.5 M citric acid buffer. Allow the aqueous phase to clarify. Combine the aqueous extracts in the centrifuge tube.

x. Add 2 mL of concentrated HCI and mix

c. Ion exclusion chromatography-sample elution.

i. Transfer the sample to the ion exclusion column prepared in F.1.a. above. Drain the extract to the top of the resin bed. Wash the tube and resin with 20 mL of 1 N HCI. Drain through the column. Rewash the column with an additional 20 mL of 1 N HCI. Discard this and previous effluents from the column.

ii. Place a 150 mL beaker under the column and elute the column with 75 mL of methanol: water (10:90). The column may be allowed to run dry in this step. The flow rate of the effluent should be <1.2 mL/min.

Stopping point: The samples may be covered and stored in the hood overnight.

Note: The resin may be discarded after each assay or it may be regenerated by washing in sequence with methanol, water, and 1 N HCI.

d. Concentration of the quinoxaline-2-carboxylic acid eluate.

i. Transfer contents of beaker to a 250 mL separatory funnel using a small amount of 10:90 methanol: water to rinse beaker.

ii. Add 1 mL of concentrated HCI.

iii. Extract with three 50 mL portions of chloroform. Collect the extract in a 250 mL round-bottom flask.

iv. Evaporate extract to dryness on a rotary evaporator at 45 - 50 °C.

v. Transfer the residue to a 15 mL centrifuge tube by washing the flask with three small portions, approximately 1 mL each, of methanol. Use a disposable Pasteur pipet to transfer the methanolic solvents. Prepare a processed GC injection standard (D.2.c).

vi. Place the tube in N-Evap bath maintained at 50 - 55 °C and evaporate the solvent to dryness under a stream of nitrogen.

Stopping point: The samples may be stored in a refrigerator overnight.

e. Esterification of quinoxaline-2-carboxylic acid.

i. Reconstitute the residue with 0.2 mL of freshly prepared 3% sulfuric acid:methanol

ii. Cap tightly and heat at 50 - 55 °C in a water bath for 30 minutes.

iii. Remove the tube from the water bath, add 1.0 mL toluene to the tepid esterification solution, and mix thoroughly in a test tube mixer.

iv. Add 1 mL water and mix thoroughly. Centrifuge to clarify.

v. In a separate vial dilute 100 µL of the toluene extract to 1.0 mL with toluene, cap and mix. The solution is ready for GC-ECD analysis.

3. Instrument Conditions

Note: Other settings may be necessary to optimize results, depending on the equipment used.

Detector temperature	325 °C
Injector temperature	250 °C
Oven temperature: ramp settings:	
Initial temperature	100 °C
Hold time	2.00 min

Rate	15 °C/min to 160 °C
Hold time	10.0 min
Rate	5 °C/min to 200 °C
Hold time	1 min
Post Temperature	250 °C
Post time	5.00 min
Make-up gas	Nitrogen 99.9%
Carrier gas	Helium (UHP grade)
Flow rate	10 mL/min
Injection volume	1.0 µL
Attenuation	8
Approximate retention time	19.4 min

Note: Initially prepare calibration table. Inject reagent blank followed by working standard 2 into the gas chromatograph to determine retention and evaluate the response of the EC detector. Prepare a single level calibration table using the average of at least three injections.

4. Order of injection
   a. QME injection standard (D.2.d)
   b. Triplicate injections of processed GC injection standard (D.2.c)
   c. Samples
   d. QME injection standard (D.2.d)

G. CALCULATIONS

Calculate the results as below:

$$\text{ppb QCA found} = \frac{\text{Peak area of sample} \times 30 \text{ ppb}}{\text{Peak area of processed GC injection standard}}$$

$$\text{Percent conversion of QCA to QME} = \frac{\text{Peak area of processed GC injection standard}}{\text{Peak area of QME x 1.08}} \times 100$$

30 = concentration of processed GC injection standard (ppb)

Note: Peak height may be used instead of peak area if chromatographic conditions permit.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment - Safety Glasses, Nitrile gloves, Lab coat and Fume Hood.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Chloroform	Listed as a carcinogen by EPA; Volatile. May be fatal if swallowed, inhaled or absorbed through skin. Causes irritation to skin, eyes and respiratory tract. May affect central nervous system, cardiovascular system, liver and kidneys.	Use under well-ventilated hood. Avoid contact with skin, eyes.
Toluene Methanol Ethyl acetate	Flammable, poisonous; inhalation will cause headache, fatigue, nausea.	Same as above
Sodium Hydroxide	Very corrosive to skin and eyes. Ingestion will cause severe chemical burns to mouth, gastrointestinal tract.	Same as above Use eye protection.

Sulfuric acid, Hydrochloric acid	Very corrosive to skin and eyes. Ingestion will cause severe chemical burns to mouth, gastrointestinal tract.	Use under well-ventilated hood. Avoid contact with skin, eyes. Use eye protection.
Standards		
QCA QME	Limited toxicological data from Pfizer.	Same as above

3. Disposal Procedures

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Organic solvents	See above	Collect waste and store in a tightly sealed container. Store away from non-compatibles in a cool, well ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, State, and Federal regulations.
All acid/base solutions	See above	Collect waste and store in a tightly sealed container. Store away from non-compatibles in a cool, well ventilated, acid liquid storage area/cabinet for disposal in accordance with local, state, and Federal regulations.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

Analyte	*Analytical Range*	*Acceptable Recovery*	*Acceptable Repeatability (CV)*
Carbadox	15 – 60 ppb	50 – 100%	≤ 20

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	Sample weight	5.0 ± 0.1 g
b.	Silicone oil bath temperature	95 -100 °C

c.	pH adjustment of the alkalinehydrolysate	≤ 1
d.	Resin weight	7.0 ± 0.1 g
e.	Column effluent flow rate	≤ 1.2 mL/min
f.	Rotary evaporator temperature	45 – 50 °C
g.	N-Evap temperature	50 – 55 °C
h.	Methanol-sulfuric acid	0.2 mL
i.	Extract dilution	1.0 mL w/toluene

3. Readiness To Perform

   a. Familiarization

      i. Phase I: Standards - Standard curve on each of 3 different days, which will include the following:

         (a) 0

         (b) 15 ppb

         (c) 30 ppb

         (d) 60 ppb

      ii. Phase II: Fortified samples-0, 15 ppb, 30 ppb, and 60 ppb in duplicate over a period of 3 different days.

         NOTE: Phase I and Phase II may be performed concurrently.

      iii. Phase III: Check samples for analyst accreditation.

         (a) 8 unknown samples fortified at levels between 1 - 4 times MPL using concentrations unknown to the analyst. Set must include 1 blank and fortified samples.

         (b) Report analytical findings to the Quality Assurance Manager (QAM) and Supervisor.

         (c) Authorization from QAM and Supervisor is required to commence official analysis.

   b. Acceptability criteria.

      Refer to I. 1.

4. Intralaboratory Check Samples

   a. System, minimum contents.

i. Frequency: One sample per week per analyst when samples are analyzed.

ii. Records are to be maintained.

b. Acceptability criteria.

Refer to I. 1.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst with this method.

ii. Take corrective action.

5. Sample Acceptability and Stability

a. Matrix: Swine liver

b. Sample receipt size: minimum 30 g

c. Condition upon receipt: Cold < 10 °C

d. Sample storage:

i. Time: 6 months

e. Condition: Value < -20°C

6. Sample Set

Note: Each sample set must include:

a. Blank tissue

b. Blank tissue fortified with 30 ppb.

c. Samples

7. Analyst Capability

a. Minimum proficiency level (MPL): 15 ppb

J. WORKSHEET

Following worksheet is an example.

Carbadox Determination by GC/ECD

Analyst:
Start Date:
End Date:
Peer Review/Date:
Supervisor Review/Date:

Weighing Information		
Weighing Notebook		
Page Number (QC Tissue / Samples) (4.9 - 5.1 g)	/	
Resin Page Number and ID (6.9 - 7.1 g)		

Dissolution, hydrolosis, and elution	ID	Pipet/Dispenser/Info
Fortification Std (1000 uL)		
3M Sodium hydroxide (NaOH) (10mL)		
Oil Bath (30 min)		**N/A**
Timer		**N/A**
Thermometer & Temperature of Oil Bath		(95-100 ºC)
Hydrochloric acid, conc. (HCl) (4 mL)		
pH (≤1)		**yes / no**
Ethyl acetate ($C_4H_8O_2$) (3 x 15 mL)		
Centrifuge (1500 rpm/5min)		**N/A**
0.5 M citric acid buffer (2 x 5 mL)		
Hydrochloric acid, conc. (HCl) (2 mL)		
Ion exclusion column	**(New / Remade)**	(date)
1 N hydrochloric acid (2 x 20 mL)		
10:90 methanol: water (75 mL)		
Flow rate (<1.2 mL/min)	**yes / no**	**N/A**
Stopping point one used? How long? (F.2.c.ii)	**yes / no**	

Concentration	ID	Pipet/Dispenser/Info
Hydrochloric acid, conc. (HCl) (1 mL)		
Chloroform ($CHCl_3$) (3 x 50 mL)		
Rotary Evaporator		**N/A**
Roto-Vap Temperature		(45-50 ºC)
Methanol (3 x ~1 mL)		**N/A**
Process Standard (1 mL)		
N-Evap		**N/A**
N-Evap Thermometer and Temperature		(50-55 ºC)
Stopping point two used? How long? (F.2.d.vi)	**yes / no**	

Carbadox Determination by GC/ECD

Esterification	ID	Pipet/Dispenser/Info
3% sulfuric acid in methanol (200 uL)		
Water bath		N/A
Timer and Time for water bath		
Thermometer and Temperature for water bath		(50-55 ºC)
Toluene (1 mL)		
Water (1 mL)	N/A	
Centrifuge		N/A
Extraction (100 uL)	N/A	
Toluene (900 uL)		
QME		N/A

Instrument	ID
GC	
Method	
Sample List or Data Folder	

QC	Spiking Level	Recovery (ppb) / % Recovery
Recovery sample	30 ppb	/
Intra (if run with set)		/
% recovery = (amount recovered / spiking level)*100 (must meet 45-95% rec)		

Sample ID	Amt Found (ppb)	Comment
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		

Comments:

K. APPENDIX

1. Column preparation and resin regeneration

   a. Column Preparation:

      i. Plug the bottom of a chromatography column – measuring 25 cm long x 10.5 mm id, with a 200 mL reservoir – with a small wad of fine glass wool.

      ii. Weigh 7.0 ± 0.1 g of resin – AGMP-50, 100 - 200 mesh – in a 50 mL screw cap tube. Add 20 mL of 1 N HCl and shake to slurry.

      iii. Using a small-tube funnel, quickly pour the slurry into the column. Place upright in a clamp on a ringstand, open the stopcock and drain the HCl through the resin into a beaker. Use the eluate to rinse the reservoir and sides of the column.

      iv. Check the resin bed to insure that there are no breaks in the bed. Do not reuse a packed resin bed if any breaks in the bed are observed. Allow the resin to settle for at least 10 minutes, then drain a small volume of the HCl to complete the settling.

      v. Drain; neutralize the rinse and discard.

      vi. Place a small plug of glass wool on top of the resin bed. Add 10 -15 mL of 1 N HCl and allow 2 - 3 mL to flow out. Close the stopcock; maintain acid above the resin bed until ready to use.

      vii. Neutralize the acid and discard.

   b. Column Regeneration:

      i. After use the resin may be regenerated by sequential washing with methanol, water and 1 N HCl. Add 80 mL methanol to the column reservoir and drain through the resin completely to wash away organic residues. Discard eluate in a flammable waste container.

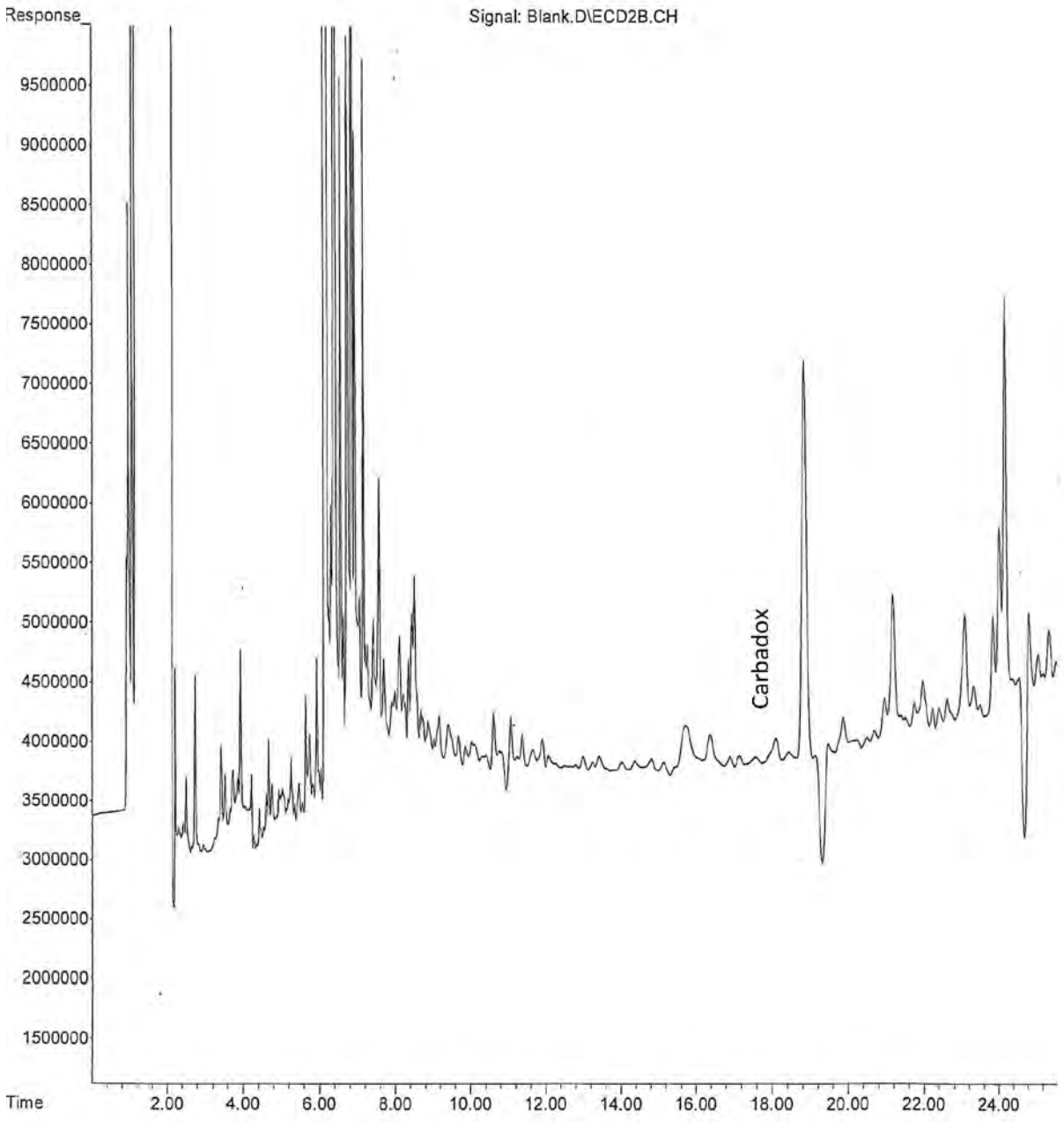
      ii. Follow with 200 mL distilled water. Drain completely to wash all of the methanol from the resin. Discard the eluted water.

      iii. Pour 20 mL 1 N HCl onto the column. Drain off 5 -10 mL to reactivate the resin and close the stopcock. Maintain acid above the resin bed until ready to use. Neutralize and discard the eluted HCl.

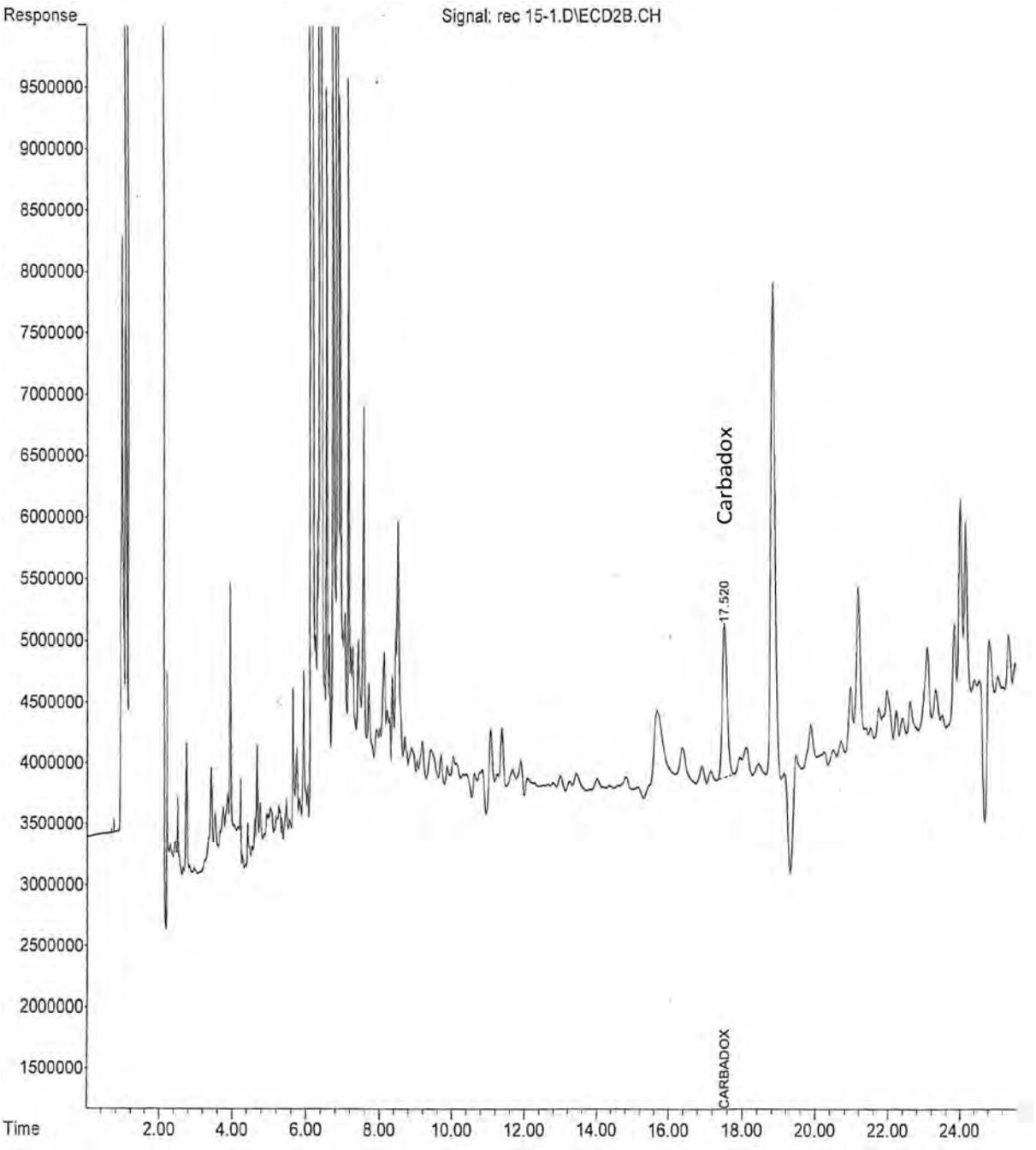
      Note: The resin bed may be used up to four times (three regenerations).

2. Chromatograms:

Blank swine liver



15 ppb swine liver



L. APPROVALS AND AUTHORITIES

1. Approvals on file.

2. Issuing Authority: Director, Laboratory Quality Assurance Division.