United States Department of Agriculture
Food Safety and Inspection Service, Office of Public Health and Science

SOP No: CLG–CBX1.01		Page 1 of 22
Title: Determination of Carbadox Metabolite by GC/ECD		
Revision: .01	Replaces: .00	Effective: 11-10-03

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A. INTRODUCTION

1. Theory

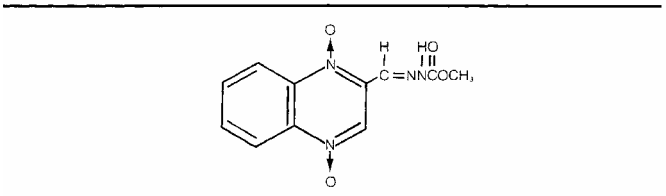
   Carbadox is approved for use in swine weighing less than 75 Ibs to prevent or treat enteritis and for increased feed efficiency and weight gain. Since the parent compound is a liver carcinogen, carbadox is monitored in domestic hogs, boars, and sows.

   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.

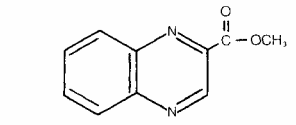
2. Applicability

   This method is suitable for analysis of Carbadox in swine liver.

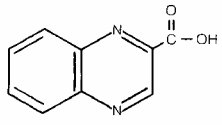
3. Structure



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 of 50 mL and 15 mL tubes, and maintaining 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, 0.1 mL, 0.2 mL, and 1.00 mL, series 2000, 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 150mm and 29 x 122mm. 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. 5640-100, -200, Kimble.

   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: 4L, 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 - 2 place Mettler sensitive to 0.01 g, 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, J&W Scientific.

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 - 3N, Cat. No. VW3472-1, VWR.

g. Sodium Hydroxide - 5N, 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 3.0 mL of concentrated H_2SO_4, to 100 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, 0.5M:

      Adjust the pH of 100 mL of 1 M citric acid to pH 6.0 with 5M 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

   Quinoxaline-2-carboxylic acid (QCA) and Methyl quinoxaline-2-carboxylate (QME) standards can be purchased, as 15.0 µg/ml solutions, from:

   Absolute Standards, Inc.
   44 Rossoto Drive
   Hamden, CT 06514

2. Reference Standard Preparation

   a. Quinoxaline-2-carboxylic acid (QCA) solutions

      i. Stock solution A (15.0 µg/mL):

         Quinoxaline-2-carboxylic acid in methanol as purchased.

      ii. Working standard solution B (0.150 µg/mL):

         Pipet 1 .0 mL of stock solution A into 100 mL volumetric flask and dilute to volume with distilled water.

iii. Working standard solution C (0.150 µg/mL):

Pipet 1.0 mL of stock solution A into 100 mL volumetric flask and dilute to volume with methanol.

iv. GC process standard (0.015 µg/mL):

Pipet 1.0 mL of working standard solution C into a 15 mL centrifuge tube and evaporate to dryness under a stream of nitrogen at 50 - 55 °C.

Add 0.2 mL of sulfuric acid:methanol, stopper and heat in a water bath, 50 - 55 °C for 30 minutes. Extract and dilute 1:10 with toluene. Esterify standards concurrently with samples as directed in section F.2.e. below.

b. Methyl quinoxaline-2-carboxylate (QME) solutions.

i. Stock solution 1, (15.0 µg/mL):

Methyl quinoxaline 2 carboxylate in methanol, as purchased..

ii. GC working standard 2, (0.015 µg/mL):

Pipet 0.10 mL of QME stock solution 1 into 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 - three years in sealed ampules.

b. GC 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 3M 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 0.5M 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 0.5M 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 <1.2mL/min.

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 process standard, at this point, by pipetting 1.0 mL of working standard solution C into a 15 mL centrifuge tube.

vi. Place the tube in N-Evap bath maintained at 50 - 55 °C and evaporate the solvent to dryness under a stream of nitrogen. The residue may be stored refrigerated 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	Argon-Methane (95:5)
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. Inject working standard 2 in duplicate followed by duplicate GC process standard, samples and by another injection of working standard 2.

G. CALCULATIONS

Calculate the results as below:

$$\text{ppb QCA found} = \frac{\text{Peak area of sample x 30 ppb}}{\text{Peak area of process standard}}$$

$$\text{Percent conversion of QCA to QME} = \frac{\text{Peak area of process standard x 100}}{\text{Peak area of QME x 1.08}}$$

30 = concentration of process standard (ppb)

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

H. HAZARD ANALYSIS

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	See Above.	Same as above Use eye protection
<u>Standards</u>		
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 (ppb)*	*Acceptable Recovery %*	*Acceptable Repeatability (CV)*
Carbadox	15 - 60	45 - 95	≤ 20

2. Critical Control Points and Specifications

Record	*Acceptable Control*
Sample weight	5.0 ± 0.1 g
Silicone oil bath temperature	95 -100 °C
pH adjustment of the alkaline hydrolysate	≤ 1
Resin weight	7.0 ± 0.1 g
Column effluent flow rate	≤ 1.2 mL/min
Rotary evaporator temperature	45 – 50 °C
N-Evap temperature	50 – 55 °C
Methanol-sulfuric acid	0.2 mL
Extract dilution	1.0 mL w/toluene

3. Readiness To Perform (FSIS Training Plan)

   a. Familiarization

      i. Phase I: Standards –Duplicate standard curve at each of the following concentration levels: 0, 15 ppb, 30 ppb, and 60 ppb on three different days.

      ii. Phase II: Fortified samples - Analyses of fortified samples at 0, 15, 30, and 60 ppb in duplicate on 3 working days. Phase II may be run along with Phase I.

      iii. Phase III: 14 unknown samples fortified at levels between 1 - 4 times MPL using concentrations unknown to the analyst. Set must include 1 blank and fortified samples.

         (a) Samples submitted by the supervisor or designee.

         (b) Report data to QAM.

(c) Letter from QAM is required to commence official analysis.

b. Acceptability criteria.

Refer to section I.1. above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: One sample per week per analyst as samples analyzed.

ii. Records are to be maintained for review.

b. Acceptability criteria:

If unacceptable values are obtained, then:

i. Stop all official carbadox analyses by that analyst.

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

ii. Condition: Frozen < -20 °C.

6. Sample Set

Note: Each sample set must include:

a. Blank tissue

b. Blank tissue fortified with 30 ppb.

c. Samples

7. Sensitivity

a. Minimum proficiency level (MPL): 15 ppb

J. WORKSHEET

The worksheet, on the following page, is only an example and can be removed for photocopying.

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CARBADOX ANALYSIS

ANALYST: _____
SET NO.: _____
START DATE: _____

SAMPLE NO	SAMPLE WT	RESULTS
CONTROL		
RECOVERY 1		
1		
2		
3		
4		
5		
6		
7		
8		
9		
RECOVERY 2		
RECOVERY 3		

ANALYTICAL PARAMETERS

CLEAN-UP

BALANCE #: _____
STD B: _____
PROC. STD.: _____
3M NaOH: _____
CONC. HCl: _____
ETHYL ACETATE: _____
pH BUFFER: _____
RESIN:_____PREP'D:_____
1N HCl: _____
METHANOL/H_2O: _____
CHLOROFORM: _____
METHANOL: _____
METHANOL/H_2SO_4: _____
TOLUENE: _____

CONDITIONS

BATH TEMP: _____
SAMPLE pH: _____
RESIN WT: _____
COL FLOW TEMP: _____
ROTO VAP TEMP: _____
N-EVAP TEMP: _____
CENTRIFUGE 1: _____
CENTRIFUGE 2: _____

ANALYSIS

CG #: _____
GC STD (30 ppb): _____
GC METHOD: _____

CALCULATIONS:

PROC. STD., % _____
RECOVERY 1, % _____
RECOVERY 2, % _____
AVG (last 10) _____

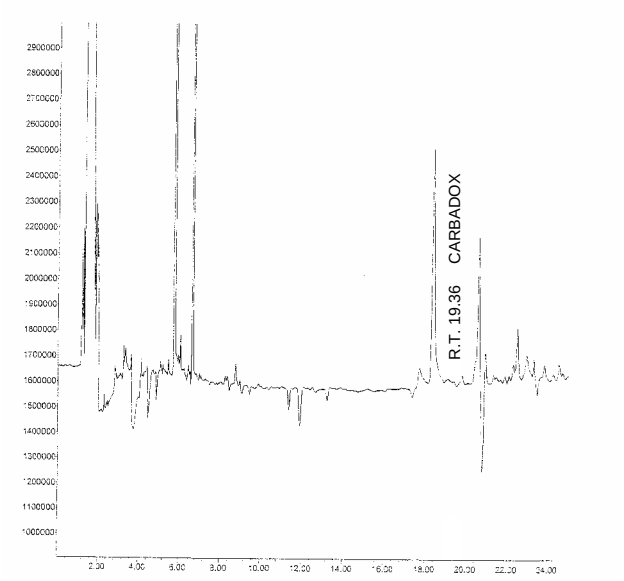
REPORT DATE: _____
INITIALS: _____
REVIEWED BY: _____
SUPERVISOR: _____

K. APPENDIX

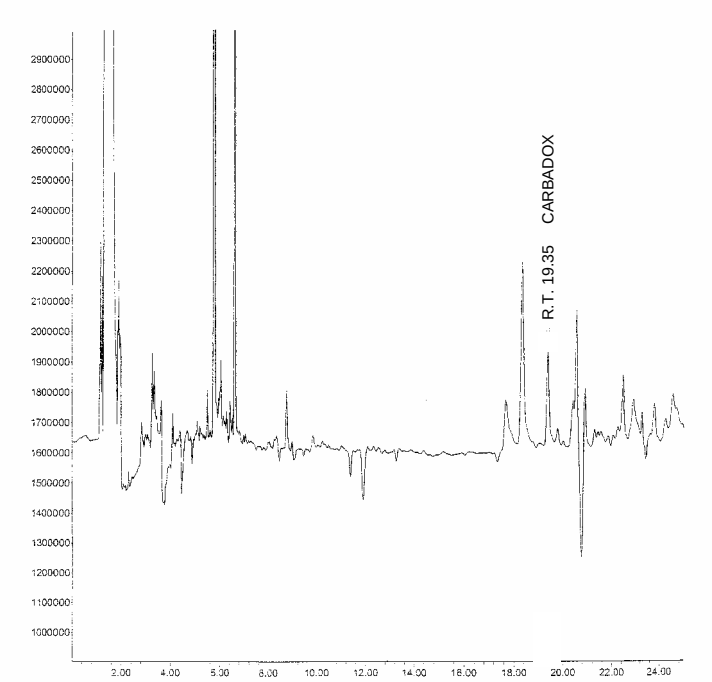
1. Column preparation and resin regeneration
   a. Column Preparation:
      i. Plug the bottom of a chromatography column – measuring 25 cm long x 10.5 mm id, with a 200 mL reservoir – with a small wad of fine glass wool.
      ii. Weigh 7.0 ± 0.1 g of resin – AGMP-50, 100 - 200 mesh – in a 50 mL screw 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).

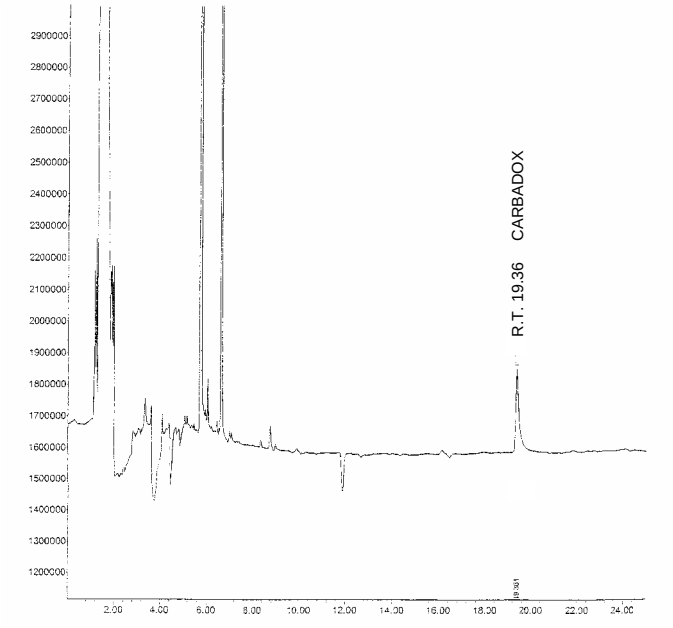
2. Chromatograms:



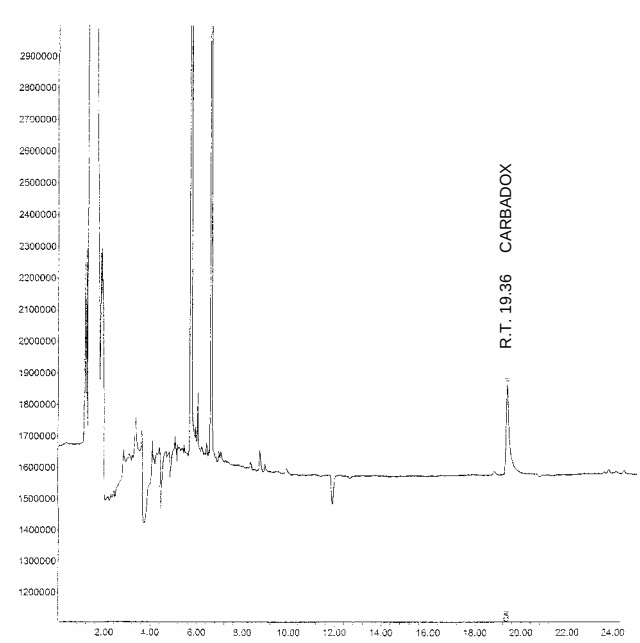
Blank Tissue



30 ppb Recovery in Tissue



30 ppb Standard



30 ppb Process Standard

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Approved By:	Date Approved:
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Leon Ilnicki	Oct. 15, 2003
Stephen Powell	Oct. 08, 2003
Jess Rajan	Oct. 14, 2003
Charles Pixley	Oct. 08, 2003
Phyllis Sparling	Oct. 09, 2003

Approvals on file.