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Title: Confirmation of Carbadox Metabolite in Swine Liver by GC/EI Ion-trap MS		
Revision .00	Replaces: CBX part II, 1991	Effective: 8/22/05

Contents

A. INTRODUCTION

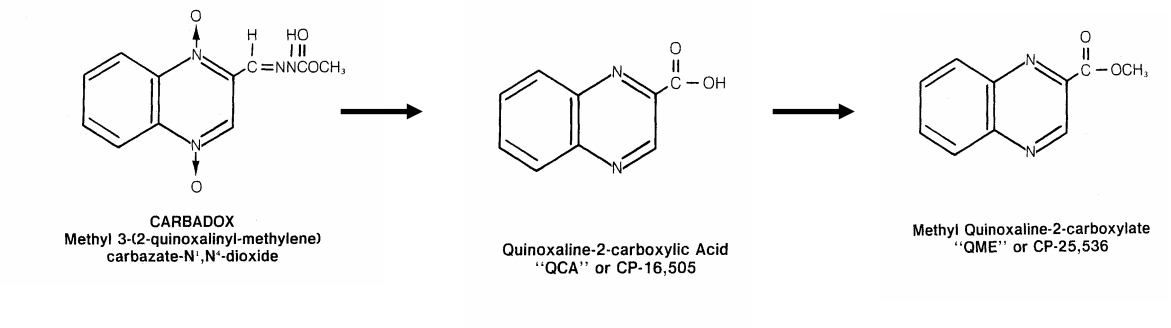
1. Theory

   Carbadox metabolite derivative methyl quinoxaline-2-carboxylate (QME) is confirmed in extracts from the determinative method CLG-CBX1 by Gas Chromatography/Electron Ionization Ion-Trap MS (GC/EI IT-MS) in Selective Ion Monitoring (SIM) mode. Confirmation is based on comparison of sample GC retention time and relative ion abundance ratios against those obtained for a reference standard.

2. Applicability

   This method is applicable to quinoxaline-2-carboxylate in swine liver at ≥ 30 ppb.

3. Structure



B. EQUIPMENT

1. Apparatus

   Note: Equivalent apparatus may be substituted for the following:

   a. Eppendorf pipettors - Variable volume pipettes: 2 - 20 µL, Cat No. 05-402-46, 10 - 100 µL, Cat No. 05-402-48, 50 - 200 µL, Cat. No. 05-402-49, 100 -1000 µL, Cat. No. 05-402-50 and 500 - 2500 µL, Cat. No. 05-402-51, Fisher Scientific.

   b. Teflon® membrane Syringeless Filter Device, 0.2 µm Pore Size, Cat. No. UN203NPENYL, Whatman.

2. Instrumentation

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

   a. Mass spectrometer - Varian Saturn 2000.

   b. Gas Chromatograph - Varian Chrompack CP3800 GC equipped with Varian

CP8200 AutoSampler.

c. Analytical column - DB-5MS, 30 m x 0.25 mm id, 0.25 µm film thickness, Part No. 122-5532, JW Scientific.

C. REAGENTS AND SOLUTIONS

1. Reagents

Note: Equivalent reagents or solutions may be substituted for the following.

a. Iso octane - Cat. No. 362-4, Burdick & Jackson.

b. Toluene - Cat. No. AH 347-4, Burdick & Jackson.

D. STANDARDS

GC/MS External Standard – 0.15 µg/mL methyl quinoxaline-2-carboxylate (QME):

Pipet 10 µL Stock Solution 1 from CLG-CBX1 D.2.b.i into GC vial. Add 990 µL Toluene and mix.

E. SAMPLE PREPARATION

See CLG-CBX1 section E.

F. ANALYTICAL PROCEDURE

1. Weigh, extract, and clean up the sample as described in Determinative Method CLG-CBX1, sections F.1 and F.2.

Note: Confirmatory sample sets require a tissue blank and a fortified recovery spiked at a concentration necessary to yield an extract of approximately the same concentration as the sample to be confirmed.

2. Filter the undiluted toluene extract, from step F.2.e.iv of the determinative method, through a Teflon® membrane. The extract is ready for GC-MS analysis using the Ion Trap MS.

3. Set Instrument Operating Conditions:

Note: The instrument parameters listed here may be optimized, if necessary, to accommodate differences between individual instruments.

a. Gas Chromatograph Parameters:

Carrier Gas	Helium
Column Flow Rate	1.0 mL/min

Injector Temperature	220 °C
Injection Volume	2 µL
Injection Mode	Pulse injection, splitless at 30 psi for 1 min.
Temperature Program:	
Initial temp:	100 °C
Initial hold time	1 min
Program rate up to 180 °C	20 °C/min
Hold time at 180 °C	2 min
Program rate up to 270 ºC	25 ºC/min
Program rate up to 270 °C	5 min
Run time	15.6 min

b. Mass Spectrometer Parameters:

EM voltage	Autotune to autotune +300 as needed.
Electron energy	70 eV
Emission current	25 µA
Detector temperature	150 °C
Manifold temperature	80 °C
Transferline temperature	220 °C

c. Data Acquisition Selected Ion monitoring mode:

Ion 76, scan width 5d, ionization time factor 100 %

Ion 102, scan width 5d, ionization time factor 100 %

Ion 130, scan width 5d, ionization time factor 50 %

Ion 158, scan width 5d, ionization time factor 100 %

4. Optimize MS

a. Tune the instrument on the day of the analyses.

b. Before analyzing a set of samples, verify system suitability by injecting a 0.15 µg/mL QME external standard.

5. Analyze sample set

Recommended injection sequence for analysis set)

a. Standard

b. Solvent blank (if necessary)

c. Blank (Negative tissue control)

d. Sample

e. Solvent blank (if necessary)

f. Recovery

g. Solvent blank (if necessary)

h. Standard

Note: Carry-over may be observed when too high a concentration of QME sample is injected. It is always prudent to inject the solvent blank after a high concentration of QME sample is analyzed. Multiple sample and/ or reference injections may be used to provide averaged response values.

G. CONFIRMATION

1. For each chromatogram showing a positive analyte response, record the retention time and ion abundance for each ion peak detected. Calculate ion abundance ratios, relative to the ion showing the highest abundance in the external standard.

2. Compare response for each sample against that obtained for the nearest reference. Normally the external standard should be used for this purpose. However, if sample response shows evidence of matrix effects, the recovery may be substituted. Compute a % difference for the retention times and for each ion abundance ratio, where % Difference = 100 X (Sample value – Reference value)/(Reference Value).

3. Confirmation of QME presence in a sample extract requires that the following criteria be met:

   a. The retention time of the QME peak in the sample chromatogram is within ± 2 % of that determined for the reference.

   b. All monitored ions (76, 102, 130, and 158) are present in the sample and reference.

   c. At least 2 fragment ion ratios calculated for the sample match those of the reference within a relative difference of ± 20 % for ratios between 20 - 100 %, and within ± 50 % for ratios <20 %.

   d. The negative control extract does not contain QME.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment - Safety glasses, disposable gloves, lab coats.

2. Hazards

Reagents / Solutions	Hazard	Recommended Safe Procedure
Iso octane, Toluene	Flammable	Wear gloves and work in the hood. Use protective eyewear.

3. Disposal Procedures

Reagents / solutions	Hazard	Recommended Safe Procedure
Iso octane, Toluene	See above	Store waste in a tightly sealed container away from non-compatibles in a cool, well ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, state, and federal regulations.

I. QUALITY ASSURANCE PLAN

1. Performance Standards
   a. No false positives from blank tissues.
   b. No false negatives at ≥ 30 ppb.

2. Readiness To Perform (FSIS Training Plan)
   a. Familiarization
      i. Phase I, Standard(s):

      Analyze a 0.15 µg/mL QME injection standard solution for fragment ions, 76, 102, 130 and 158 using GC/ EI-IT-MS in SIM mode. Repeat this analysis on three different days to verify parameters.

      ii. Phase II, Fortified samples:

      Analyze 3 replicates at 0 and 30 ppb 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) Analyze a minimum of 8 check samples provided by the Supervisor/Quality Assurance Manager (QAM). At least one check sample should be negative. At least 4 samples should be fortified at ≥30 ppb level.

(b) Report analytical findings to Supervisor/QAM.

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

3. Intralaboratory check samples

a. System, minimum contents.

i. Frequency: A minimum of four per year.

ii. Records are maintained.

b. Acceptability criteria: Refer to sections I.1 above.

If unacceptable results are obtained, then:

i. Stop the sample analysis.

ii. Take corrective action.

4. Sample set must include

a. Positive control (recovery).

b. Tissue blank.

c. Sample extract(s).

5. Sensitivity

a. Minimum Proficiency Level (MPL): 30 ppb.

J. WORKSHEET

QME CONFIRMATION WORKSHEET

Name of analyst	**Internal Lab No.**
Date Started	**Form No.**
Date Completed	**Tissue Code**
Instrument	**Dilution or Concentration Factor**
Injection Volume	**Result Confirmed (yes or no)**

CONFIRMATION

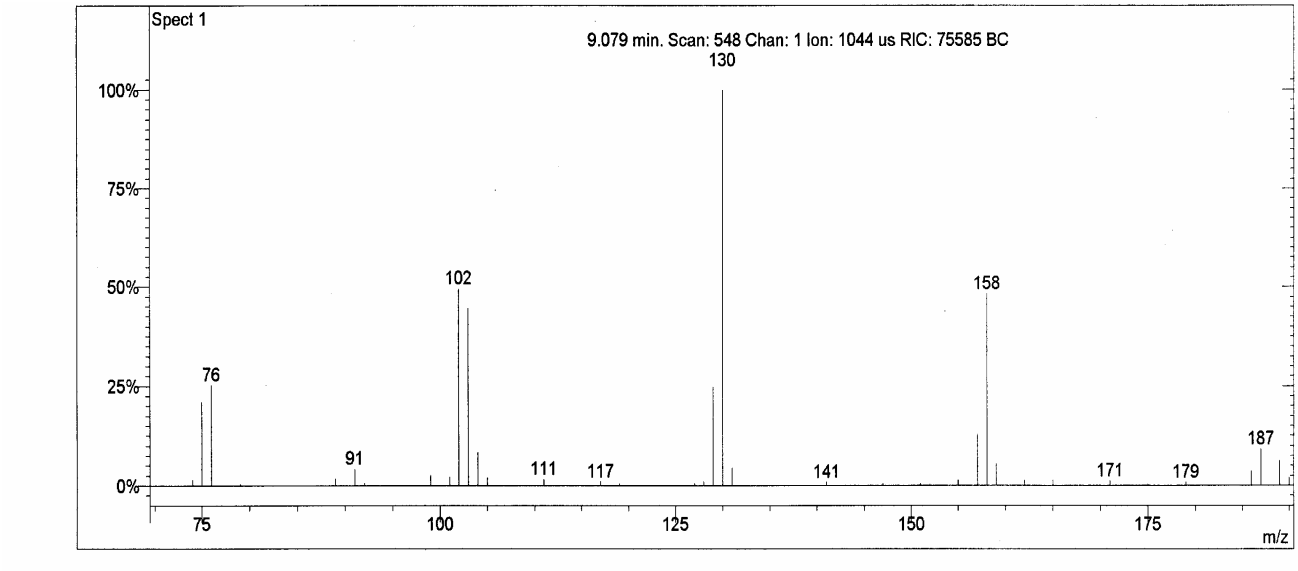
Sample Name:							
		Sample	**Std**	**%Diff Rel. Ion**	**Sample**	**Rec**	**%Diff Rel. Ion**
	m/z	**Rel. Ion %**	**Rel. Ion %**	**Rec - Sample**	**Rel. Ion %**	**Rel. Ion%**	**Std - Sample**
	76						
	102						
	130						
	158						
				%Diff RT			**%Diff RT**
Retention time (min):							

Comments

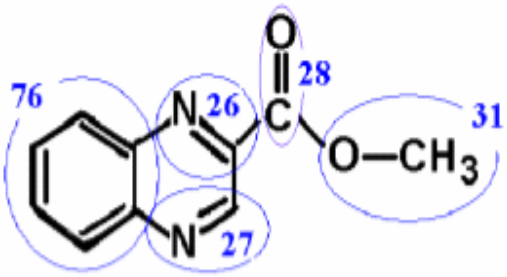
K. APPENDIX

1. Chromatograms and Spectra

Figure 1. GC/EI IT mass spectrum of QME



QME Structure and Ions



Molecular weight of QME: 188 g/mol
Quasi molecular ion: m/z [MH]+ 189

Structurally significant fragment ions:
- m/z [MH – OCH_3]+ 158
- m/z [MH – $COOCH_3$]+ 130
- m/z [MH – H – $COOCH_3$]+ 129
- m/z [MH – N=$CCOOCH_3$]+ 103
- m/z [MH – H – N=$CCOOCH_3$]+ 102
- m/z [MH – N=C(C=N)$COOCH_3$]+ 76
- m/z [MH – H – N=C(C=N)$COOCH_3$]+ 75

Figure 2. TIC of External Standard Equivalent to 30 ppb QME

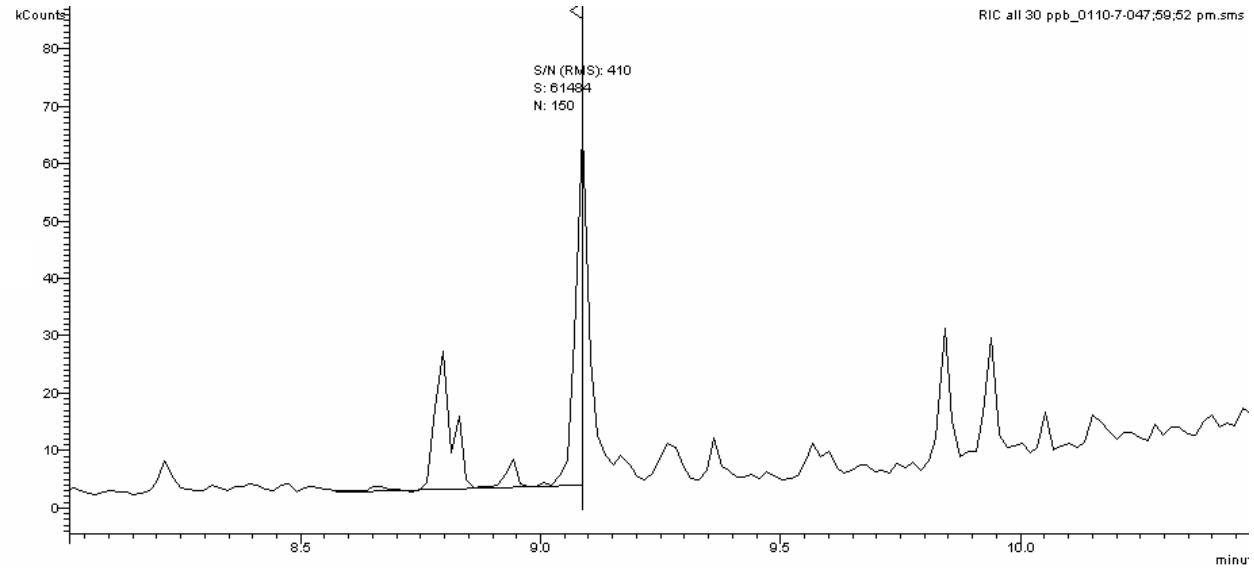


Figure 3. TIC of Tissue Blank

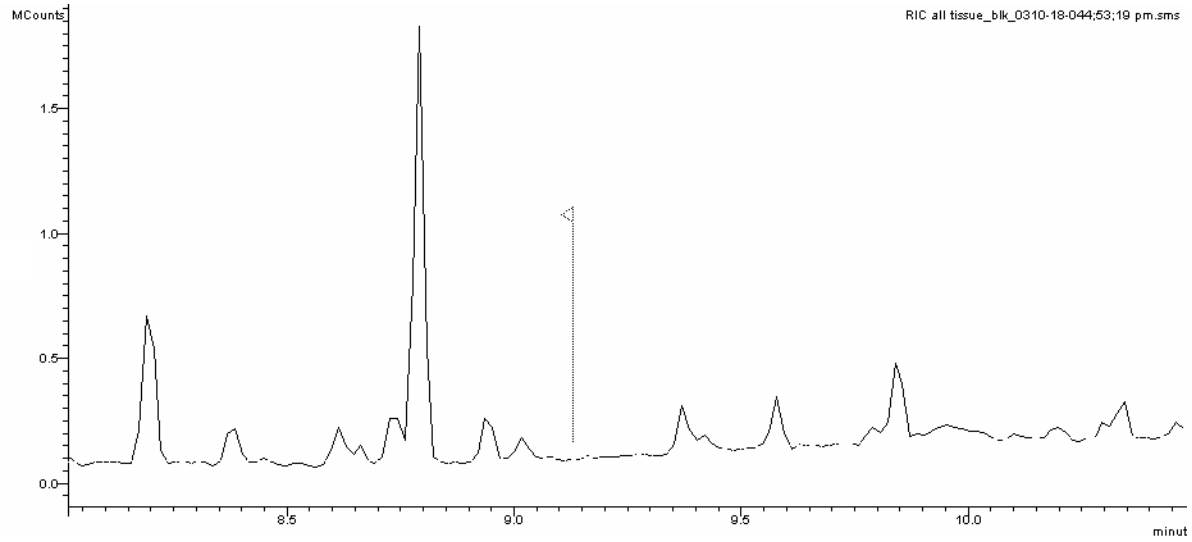


Figure 4. TIC of 30 ppb Recovery

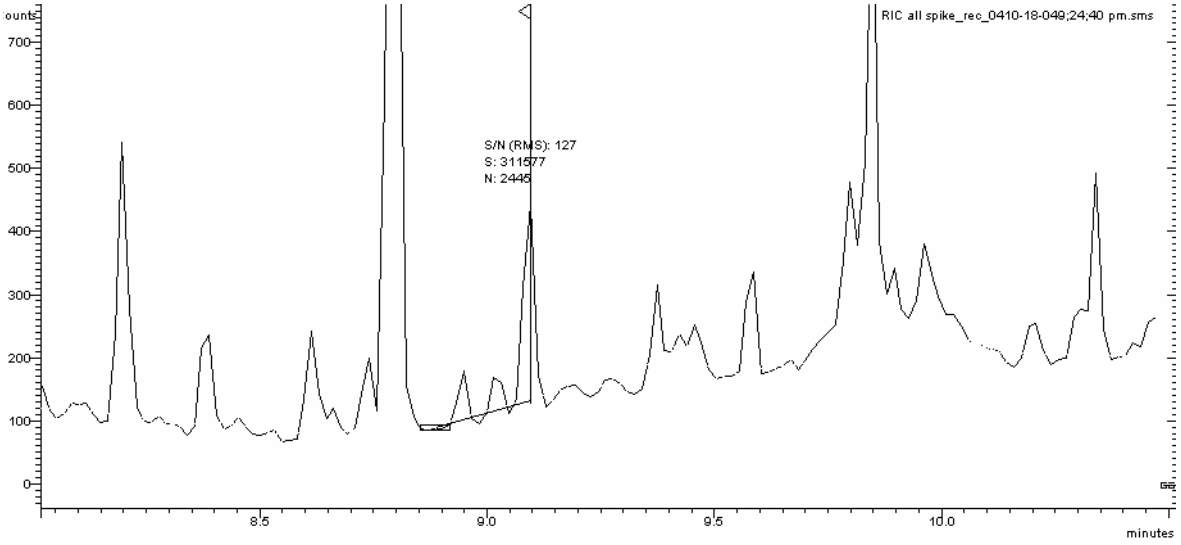
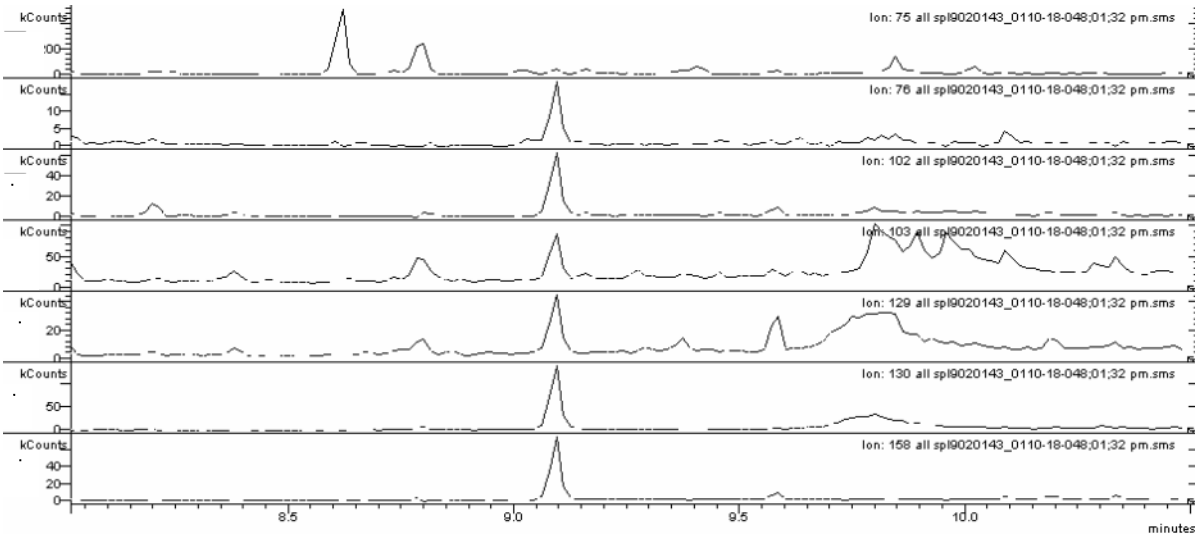


Figure 5. Ion Chromatograms for 30 ppb Recovery



2. Reference:

Carbadox metabolite in swine tissue, II. Confirmatory method, FSIS Chemistry Laboratory Guidebook, July, 1991.

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Approval records are on file.