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Title: Determination and Confirmation of Chloramphenicol		
Revision: .07	Replaces: CLG-CAM.06	Effective: 04/13/2018

Contents

PART I: Determinative Method

A. INTRODUCTION

1. Background

   Chloramphenicol (CAM) has a broad spectrum of activity against gram-positive and gram-negative bacteria and rickettsia. It is banned in the U.S. for use in animals used as food.

2. Summary of Procedure

   Meta-chloramphenicol is added to the sample as a recovery index. The sample is then incubated with β-glucuronidase to convert any chloramphenicol monoglucuronide to free chloramphenicol. CAM is extracted from muscle with ethyl acetate and the ethyl acetate is concentrated to about 1 mL. A 4% sodium chloride solution is added and the remaining ethyl acetate is purged with nitrogen. The salt solution is applied to the top of a C_{18} SPE column, the cartridge is washed with methanol: water (20:80) and the CAM is eluted with acetonitrile. The eluate is evaporated to dryness and silanized. CAM is quantitated by GC/ECD using a DB-1 capillary column. Confirmation is accomplished by GC/MS, using an OV-1 capillary column and negative ion chemical ionization.

3. Applicability

   This method is suitable for the determination and confirmation of chloramphenicol in bovine muscle at levels ≥ 0.25 ppb, and poultry and fish of the order Siluriformes (catfish) muscle at levels ≥ 0.3 ppb and swine and equine muscle at concentrations ≥ 0.5 ppb.

   Note: Refer to 21CFR for tolerance values set by FDA and 40CFR for tolerance values set by EPA.

B. EQUIPMENT

Note: Equivalent equipment may be substituted.

1. Apparatus

   a. Vacuum manifold device - for aid in washing and elution of C18 cartridges. Optional, but allows for multiple C18 elutions. J.T. Baker or Analytichem.

   b. N-Evap - Organomation Associates.

   c. Pipettes - disposable, glass serological (10 mL) and Eppendorf (50-200 µL).

   d. Test tube racks.

   e. 50 mL polypropylene conical tubes - Falcon BlueMax.

f. Microliter syringes - 10 μL, for injection. Hamilton #701 and #1 701.

g. Centrifuge - Damon IEC Division Model #PR-7000 with IEC #253 rotor, cup size IEC catalog #320.

h. Vortex mixer - Labline Supermixer Model #1290.

i. Homogenizer - Ultra Turrax, Tekmar Model SDT, with microshaft.

j. Incubator - low temperature, Precision Scientific Freas Model 825.

k. Heating module - Reacti-therm, Pierce Model #18780.

l. Conical 1 mL autosampler vials - available from Chemical Research Supplies, combo pack with polyethylene P8-6.

m. Culture tubes - 5 mL borosilicate, dimensions 13 x 10 mm, Corning #99445.

n. Pasteur pipettes - Kimble #72050.

o. Baker 10 SPE C18 (octadecyl) columns - 3 mL capacity, Catalog No. 7020-3.

p. Borosilicate glass disposable centrifuge tube - 15 mL screwthread, Kimble #73785-15 and phenolic cap (PTFE faced, rubber liner), Kimble #73802-15415.

q. Shaker - Eberbach, variable speed.

r. Robot Coupe processor - Robot Coupe U.S.A., Inc.

2. Instrumentation

a. Gas chromatograph - Hewlett-Packard 6890 with capillary inlet (splitless injection) fitted with electron capture Ni-63 detector.

b. Gas chromatographic column - Agilent Technologies-DB-1, 30 meter length, 0.250 mm i.d., having a film thickness of 0.25 µm-Part #: 122-0132.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents / solutions may be substituted. The maximum length of time that a working reagent shall be used is 1 year unless the laboratory has produced extension data.

1. Reagents

a. Methanol - High-purity solvent product 230-4, Burdick and Jackson (B&J).

b. Ethyl acetate - Omni Solv product EX0241 -1.

c. Hexane - UV, B&J high-purity solvent product 216.

d. High-purity water - 18 megaohm/cm specific resistance.

e. Acetonitrile - UV, B&J high-purity solvent product 015.

f. Cyclohexane - pesticide grade, Fisher C-553.

g. Potassium Phosphate, Monobasic - ACS grade, Sigma, P-0662.

h. Sodium Phosphate, Dibasic - Sigma, S-5136.

i. β-glucuronidase - Sigma, G-7396.

j. Sylon HTP - Supelco, 3-3038.

k. Sodium Chloride - ACS grade, Mallinckrodt, 7581.

2. Solutions

a. Type IX-a β-glucuronidase:

Dilute with buffer (refer to item b below) to a concentration of 4,000 units/mL. Use the units/gram listed on the bottle to calculate the amount of dry reagent needed. Prepare fresh daily. Store dry β-glucuronidase below 0 ºC.

b. Buffer solution:

Mix approximately 41 g of KH_2PO_4 and 43 g of Na_2HPO_4 (ACS reagent grade) in 3 L of water aqueous, pH 6.8 ± 0.1. Adjust pH to 6.8 with the appropriate dry reagent.

c. 4 % Sodium chloride:

Prepare 4% aqueous solution with distilled water, by mixing 20.8 g of sodium chloride with 500 mL of water. Store at room temperature.

d. Cyclohexane/hexane (60:40):

Mix 60 mL cyclohexane and 40 mL hexane in a graduated cylinder.

e. Methanol/Water (20:80):

Mix 200 mL methanol and 800 mL deionized water in a 1 L volumetric flask.

D. STANDARD(S)

Note: Equivalent standards / solutions may be substituted. Purity and counterions are to be taken into account when calculating standard concentrations. In-house prepared standards shall be assigned an expiration date that is no later than the stability stated in the method. The maximum length of time that an in-house prepared standard shall be used is 1 year unless the laboratory has produced extension data.

1. Standard Information

a. Chloramphenicol
Sigma Chemical Co.

b. Metachloramphenicol (Internal Standard)

Sigma Chemical Co.

2. Preparation of Standard Solution(s)

   a. Stock Solution (500 µg/mL):

      Prepare individual 500 µg/mL stock solutions of chloramphenicol and metachloramphenicol by transferring 50 mg standard to a 100 mL volumetric flask and diluting to volume with methanol. This standard is stable for 1 year when stored in amber glassware at < -10ºC unless the note under the Section D heading applies.

   b. Intermediate Solution (50 µg/mL):

      Prepare individual intermediate solutions at 50 µg/mL by transferring 10 mL of stock into a 100 mL volumetric flask and diluting to volume with methanol. This standard is stable for 6 months when stored in amber glassware at < -10ºC unless the note under the Section D heading applies.

   c. Working Solutions (100 ng/mL):

      Prepare individual working solutions at 100 ng/mL by transferring 200 µL of the intermediate solution into a 100 mL volumetric flask and diluting to volume with methanol. This standard is stable for 3 months when stored in amber glassware at < -10ºC unless the note under the Section D heading applies.

E. SAMPLE PREPARATION

Prepare muscle tissue as follows:

1. Cut lean tissue from different parts of muscle sample. Avoid the fat and connective tissue as much as possible.

2. Cut just enough of the muscle to make the sample (approximately ¾ to 1 pound).

3. Cut the muscle tissue into small ½ to 1 inch cubes before processing. Cut the muscle tissue into cubes smaller than ½ to 1 inch if the sample tissue is tough.

4. Process the muscle tissue using Robot Coupe or grinder until the sample is homogeneous.

F. ANALYTICAL PROCEDURE

1. Preparation of Controls and Samples

   a. Weigh 10 ± 0.1 g of blank tissue into a 50 mL centrifuge tube.

   b. Prepare 1 blank muscle and 4 fortified blank muscle samples to be analyzed with each sample set as follows:

      i. Add 100 µL metachloramphenicol internal standard (100 ng/mL in

methanol, 1 ppb) to the control tissues.

ii. Fortify the recovery (1ppb) by adding 100 µL of working standard.

iii. Fortify the recovery curve samples:

(a) 0.5 ppb (50 µL of working standard),

(b) 1.0 ppb (100 µL of working standard),

(c) 2.0 ppb (200 µL of working standard).

Data generated from fortified samples will be used for calculations.

Note: To quantitate positives below 0.5 ppb prepare a fortified curve at 0.25 ppb, 0.5 ppb, and 1.0 ppb.

2. Extraction Procedure

a. Weigh 10 ± 0.1 g muscle tissue into a 50 mL centrifuge tube.

b. To each sample add 100 µL metachloramphenicol internal standard (100 ng/mL in methanol, 1 ppb).

c. Add 15 mL phosphate buffer (pH 6.8 ± 0.1) and 200 µL ß-glucuronidase (800 units) solution to all blanks, fortified controls, and sample tubes.

d. Blend in a tissuemizer for 30-60 sec at room temperature or shake on a mechanical shaker at high speed for at least 5 minutes.

e. Incubate all tubes at least 90 min at 37 ± 2 °C. After incubation, samples may be left in refrigerator overnight.

f. Bring tubes to room temperature.

g. Add 15 mL ethyl acetate to each tube.

h. Mix tubes on vortex mixer for 30 sec or shake on a mechanical shaker for one minute to extract chloramphenicol.

i. Centrifuge at approximately 2,000 rpm for at least 2 min to separate phases.

j. Transfer ethyl acetate (upper phase) with a disposable pipette to a clean 15 mL tube. Evaporate extract to approximately 1 mL at 65 ± 5 °C.

k. Repeat extraction of sample (steps g-j) and combine extracts.

l. Reduce ethyl acetate volume to 1 mL on an N-Evap, or equivalent, under a gentle stream of nitrogen, using a sand bath or water bath temperature of approximately 65 ± 5 °C.

m. Add 4 mL aqueous 4% NaCl solution to all tubes and vortex for approximately 5 - 10 sec.

n. Continue evaporation of ethyl acetate on N-Evap until ethyl acetate layer is

absent, leaving an oily residue over the NaCl solution.

o. Add 5 mL hexane to the 4 mL 4% aqueous NaCl layer. Vortex for 10 sec. Centrifuge at 1000 rpm for 1 min. Remove top layer and discard.

p. Repeat step o.

Note: Steps r through u should be performed immediately, one after the other. Do not allow the sorbent to dry.

q. Precondition a C18 column for each sample, blank, and fortified control by passing 10 mL methanol, and 10 mL distilled water sequentially through the column. Discard all washes.

r. Transfer the entire aqueous extract onto the C18 column. Discard the eluate.

s. Rinse the sample tube by vortexing twice with 1 mL distilled water and adding the rinses onto the C18 column. Discard eluate.

t. Wash each C18 column with 1 mL water followed by 2 mL methanol-water (20:80). Allow the last wash to elute completely through the column. Discard washes.

u. Elute the chloramphenicol from the C18 column with acetonitrile, 3 mL, collecting the eluate in a clean 5 mL culture tube.

Note: Samples may be stored in a freezer at this point.

v. Evaporate the acetonitrile eluate to dryness using a sand bath or water bath at a temperature of 65 ± 5 °C and a gentle stream of nitrogen.

CAUTION: Avoid moisture from this point forward.

w. To the dried residue, add 200 µL Sylon HTP.

x. Stopper and vortex 5 sec. React at 65 ± 5 °C in a sand bath or water bath for 15 min.

y. Evaporate excess reagents on heating module or water bath at 65 ± 5 °C with gentle stream of nitrogen to approximately 10 µL.

CAUTION: Excessive drying time at this step may result in loss of analyte.

z. Reconstitute residue in 200 µL cyclohexane/hexane (60:40).Vortex 5 sec.

aa. Inject suitable microliter volume of derivatized material into GC for quantitative determination.

2. Instrumental Settings

Note: The instrument parameters may be optimized to ensure system suitability.

The following conditions are for the 6890 Hewlett-Packard GC as described in section B.2 and should be considered an example only. The analyst should optimize these parameters for the instrument being used.

a.	Carrier gas:	Helium, linear velocity 15 cm/sec
b.	Make-up gas:	Argon/methane, 95/5, flow rate 50 mL/min
c.	Initial column temperature:	100 °C, hold for 1 min
d.	Temperature programming:	Program at 20 °C/min to 290 °C; hold for 15 min or until the Meta isomer and chloramphenicol have eluted. Then program at 20 °C/min to 310 °C; hold for 2 min to make sure all the sample has eluted.
e.	Injector temperature:	260 °C
f.	Detector temperature:	330 °C
g.	Sensitivity setting:	2/8 attenuation
h.	Expected retention time:	Chloramphenicol 11 to 16 min metachloramphenicol 11 to 16 min
i.	Expected response:	50 % full-scale deflection for 0.20 ng chloramphenicol

3. Injection sequence /Sample Set

   Each sample set must contain one QA sample/20 samples

   a. Tissue blank

   b. Recovery Curve at 0.25 ppb (if needed), 0.5 ppb, 1.0 ppb, and 2.0 ppb

   c. Recovery

   d. Check (if necessary)

   e. Samples

   f. Recovery (safeguard)

G. CALCULATIONS / IDENTIFICATION

1. Procedure

   a. Metachloramphenicol is used as an internal standard for calculating chloramphenicol concentration. Proceed in the following manner to calculate linear regression calibration curves and chloramphenicol concentration. By an acceptable means, measure the peak height or peak area for each component in the fortified samples that have been processed through the procedure. Calculate the peak height or peak area ratios for chloramphenicol by dividing its peak height or peak area by that of the metachloramphenicol peak height or peak area.

b. Using the ratios and associated ppb values, calculate a linear regression calibration curve by least squares computation.

$y = mx + b$, where

m = slope

b = y intercept

$y = \frac{\text{chloramphenicol peak height or area}}{\text{metachloramphenicol peak height or area}}$

x = chloramphenicol concentration in ppb

This calibration curve is then used to calculate values for additional samples from the sample set.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment - Safety glasses, polyvinyl or latex gloves, and lab coat.

2. Hazards

CAUTION: Do not swallow, inhale, or absorb through skin any chemical, as complete toxicological properties for most chemicals are unknown

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Enzyme: β-glucuronidase, type IX-a	May be harmful by inhalation, ingestion/skin absorption. May cause irritation/allergic reactions in certain sensitive individuals.	The analysis should be done under an efficient fume hood.
Ethyl acetate, Hexane, Methanol, Cyclohexane	Flammable. Vapors are corrosive to the skin, eyes, and respiratory system.	Avoid contact or prolonged exposure to vapors. Work in a fume hood. Keep away from flame or heat.
Analyte: Chloramphenicol Metachloramphenicol	Chloramphenicol has a wide range of possible adverse effects ranging from dermatitis, embryo and fetal death, to death of adults. Chloramphenicol has been associated with inducing aplastic anemia. Those who survive	Because the unique toxicity to humans has been well documented, the utmost care in handling chloramphenicol is recommended. See the material safety data sheet for information on how to handle any spills.

aplastic anemia have a high incidence of developing acute leukemia. It is considered a carcinogen. The Meta isomer is assumed to have similar toxicity.

3. Disposal Procedures

Follow local, state and federal guidelines for disposal.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

Analyte	Analytical Range (ppb)	Acceptable Recovery %	Acceptable Repeatability (CV)
Chloramphenicol	0.25 - .49 0.5 - 2.0	77 -131 85 -115	< 20 at 0.25 ppb < 20 at 1.0 ppb

Standard curve correlation coefficient ≥ 0.9945.

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	(ß-glucuronidase) solution	Prepare fresh daily.
b.	Incubation time	Minimum incubation time is 90 minutes.
c.	Incubation temperature	37 ± 2 °C
d.	Sample size	10 ± 0.1 g
e.	Working standard solution volume	Depends on fortification level.
f.	Sylon HTP volume	200 µL ± 20 µL
g.	Reaction time	15 min ± 1 min
h.	Reaction temperature	65 ± 5 °C
i.	Evaporation of excess Sylon HTP	See section F.2. y.

3. Intralaboratory Check Samples

a. System, minimum contents.

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

ii. Records are to be maintained.

b. Acceptability criteria.

Refer to I. 1.

If unacceptable values are obtained, then:

i. Investigate following established procedures.

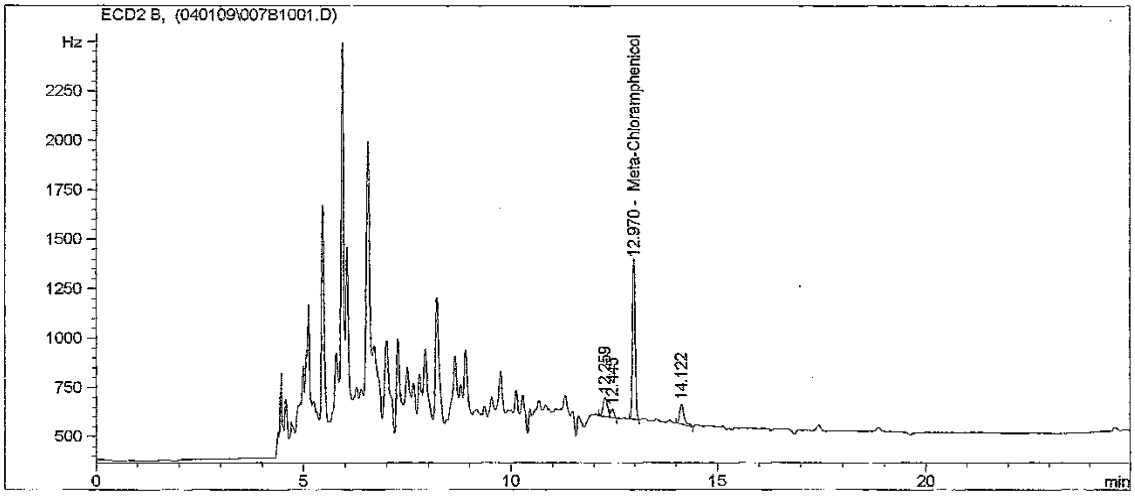
ii. Take corrective action as warranted.

4. Condition upon receipt: Cold

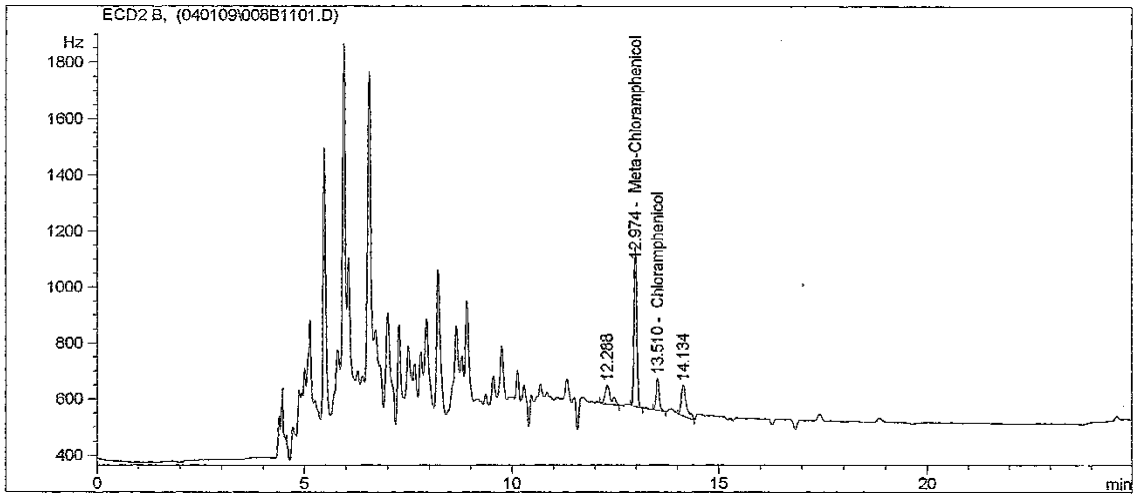
J. APPENDIX

1. Chromatograms/spectra

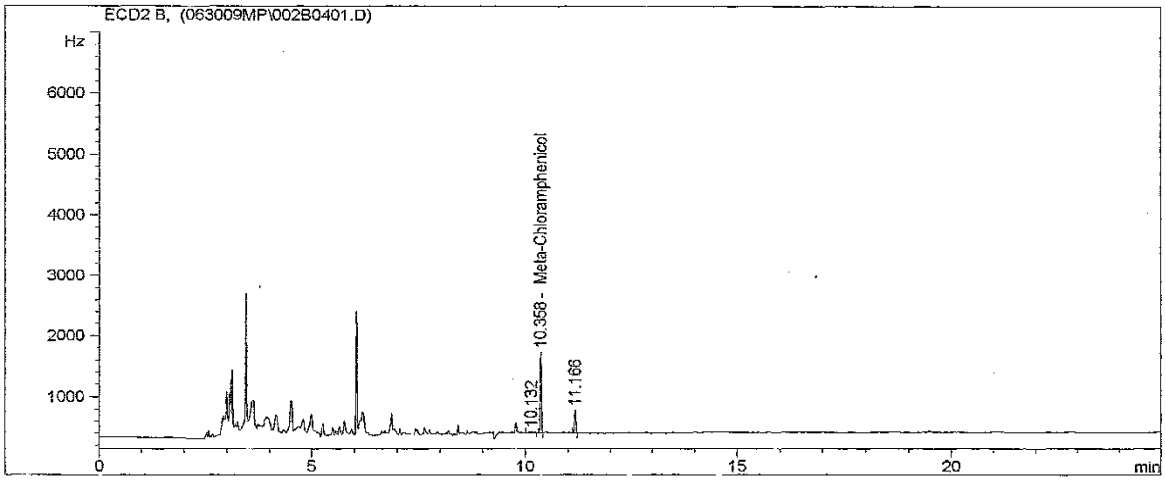
a. Blank bovine muscle



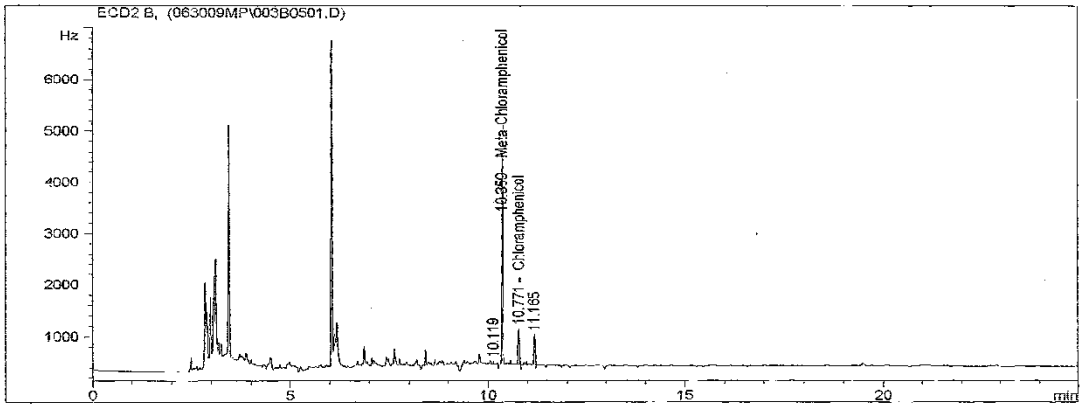
b. Bovine muscle fortified with 0.25 ppb chloramphenicol



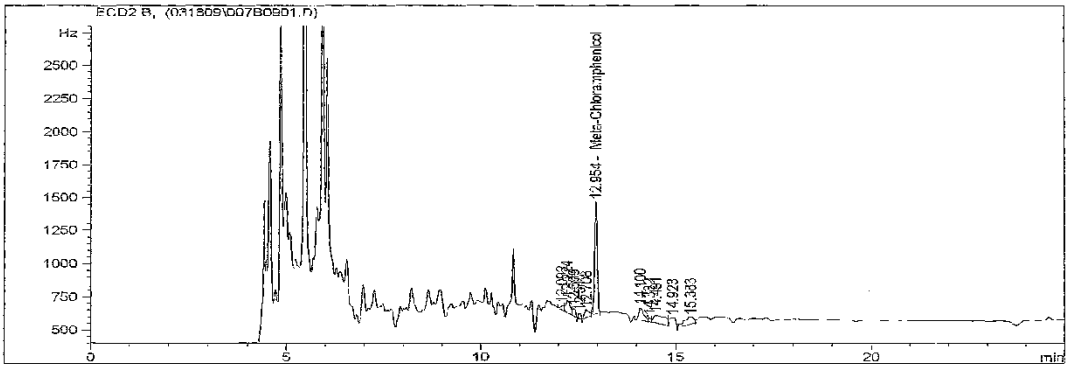
c. Blank turkey muscle



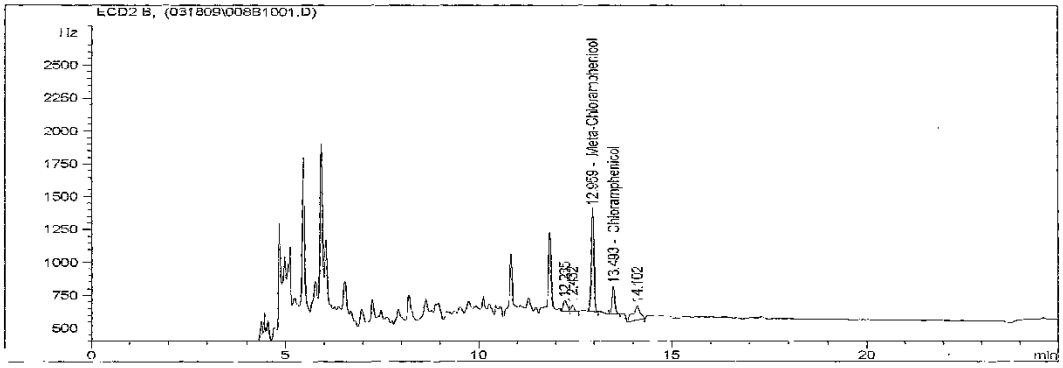
d. Turkey muscle fortified with 0.3 ppb chloramphenicol



e. Blank catfish muscle



f. Catfish muscle fortified with 0.3 ppb chloramphenicol



PART II: Confirmatory Method

A. INTRODUCTION

Refer to Part I: Determinative Method, section A.

B. EQUIPMENT

Note: Equivalent equipment may be substituted.

1. Apparatus
   a. Refer to Part I: Determinative Method, section B.1
2. Instrumentation
   a. GC/MS - HP 6890/5973N Mass Selective Detector, quadrupole with capillary inlet, splitless injection fitted crosslink methyl silicone column, film thickness 0.33 µm, length, 25 meters, diameter 0.20 mm, column part number 19091S-602.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents / solutions may be substituted. The maximum length of time that a working reagent shall be used is 1 year unless the laboratory has produced extension data.

1. Reagents
   a. Perfluorotributylamine (PFTBA)
   b. Refer to Part I: Determinative Method, section C.1.
2. Solutions
   a. Refer to Part I: Determinative Method, section C.2.

D. STANDARD(S)

Note: Equivalent standards / solutions may be substituted. Purity and counterions are to be taken into account when calculating standard concentrations. In-house prepared standards shall be assigned an expiration date that is no later than the stability stated in the method. The maximum length of time that an in-house prepared standard shall be used is 1 year unless the laboratory has produced extension data.

1. Standard Information

a. See Part I: Determinative Method, section D.1.

2. Preparation of Standard Solution(s)

a. The mass spectrometer operator will use the same sample set that is used in the Determinative Method for confirmation. Only the 1.0 ppb standard is usually analyzed.

b. See Part I: Determinative Method, section D.2.

E. SAMPLE PREPARATION

Refer to Part I: Determinative Method, section E

Note: Samples with excessive connective tissue may produce unacceptable confirmatory results.

F. ANALYTICAL PROCEDURE

1. Preparation of Controls and Samples

Refer to Part I: Determinative Method, Section F.1.

2. Extraction Procedure

a. Refer to Part I: Determinative Method, Section F.2.

b. Inject a suitable microliter volume of derivatized extract generated from step F.2.z. of Part I: Determinative Method into the GC/MS for confirmation.

3. Instrumental Settings

Note: The instrument parameters may be optimized to ensure system suitability.

Note: The following conditions are for the instrument described in section B.2 and are given as an example only. The analyst should optimize the parameters for the instrument being used

a. GC conditions and parameters

i.	Column:	Crosslinked Methyl Siloxane, Capillary, 25 meters
ii.	Injector:	230 °C
iii.	Temperature programming:	150 °C with no initial hold, program to 300 °C at 20 °C/min. Final hold 10 min.
iv.	Transfer line temperature:	300 °C
v.	Helium flow rate:	29 cm/sec, splitless injection

b. Mass spectrometer conditions

i.	Detection:	Negative ion chemical ionization (NICI)
ii.	Ionization gas:	Methane
iii.	Source temperature:	260 °C
iv.	Mode of operation:	SIM-NICl
v.	Dwell time:	100 millisec
vi.	Calibration standard:	PFTBA: Tune acceptance is based on manufacturer's instructions

c. Required Samples for GC/MS Analysis

Set up instrument as described in section 1 above to monitor chloramphenicol and the internal standard metachloramphenicol. Inject 2 to 5 µL of the external standard and analyze results to verify that the system is functioning properly. If not, make any necessary adjustments in operating parameters or standard concentration, then re-inject to verify performance. Inject 2 to 5 µL each of the confirmation sample, the recovery, and the tissue blank. Confirmatory analysis is required in all samples found positive by the determinative method

G. CALCULATIONS / IDENTIFICATION

1. Criteria for Confirmation

a. Retention time specification. Compare the retention time of the sample with the retention time of the fortified tissue. The times should match ± 2.0%.

The following is an example only as other retention times are acceptable.

Compound	*Expected Retention Time (min)*
Metachloramphenicol	7.7 ± 0.15
Chloramphenicol	7.9 ± 0.16

b. Ions 468, 466, 322, and 304 must be present. In samples with high levels of chloramphenicol, ions m/z 358 and 360 can also be monitored.

c. Compute at least the following three ratios for the standard, fortified control, and samples: 466/468, 322/466, and 304/466.

For successful confirmation between levels 0.25 ppb and 2.0 ppb, the 466/468 and either the 322/466 or the 304/466 ion for the sample must agree within ± 20% relative of the ratio for fortified tissue.

In samples with high levels of chloramphenicol, ion ratios for 358/466 and 358/360 may also be used to confirm.

The ion ratios of the fortified standard must match the ion ratios of the sample by ± 20 % relative. The following are expected ion ratios for the conditions cited.

466/468 = 1.43

322/466 = 0.49

304/466 = 0.57

Note: The ion ratios are dependent upon the source temperature.

Refer to the Appendix section J.3. for postulated structured of confirming ions.

H. SAFETY INFORMATION AND PRECAUTIONS

Refer to Part I: Determinative Method, section H.

I. QUALITY ASSURANCE PLAN

1. Performance Standard
   a. No false positives at 0 ppb.
   b. No false negatives at 0.25 ppb.
2. Critical Control Points and Specifications

   See Part I: Determinative Method, section I.2.
3. Intralaboratory Check Samples
   a. System, minimum contents.
      i. Frequency: One per week per analyst when samples analyzed.
      ii. Records are to be maintained.
   b. Acceptability criteria.

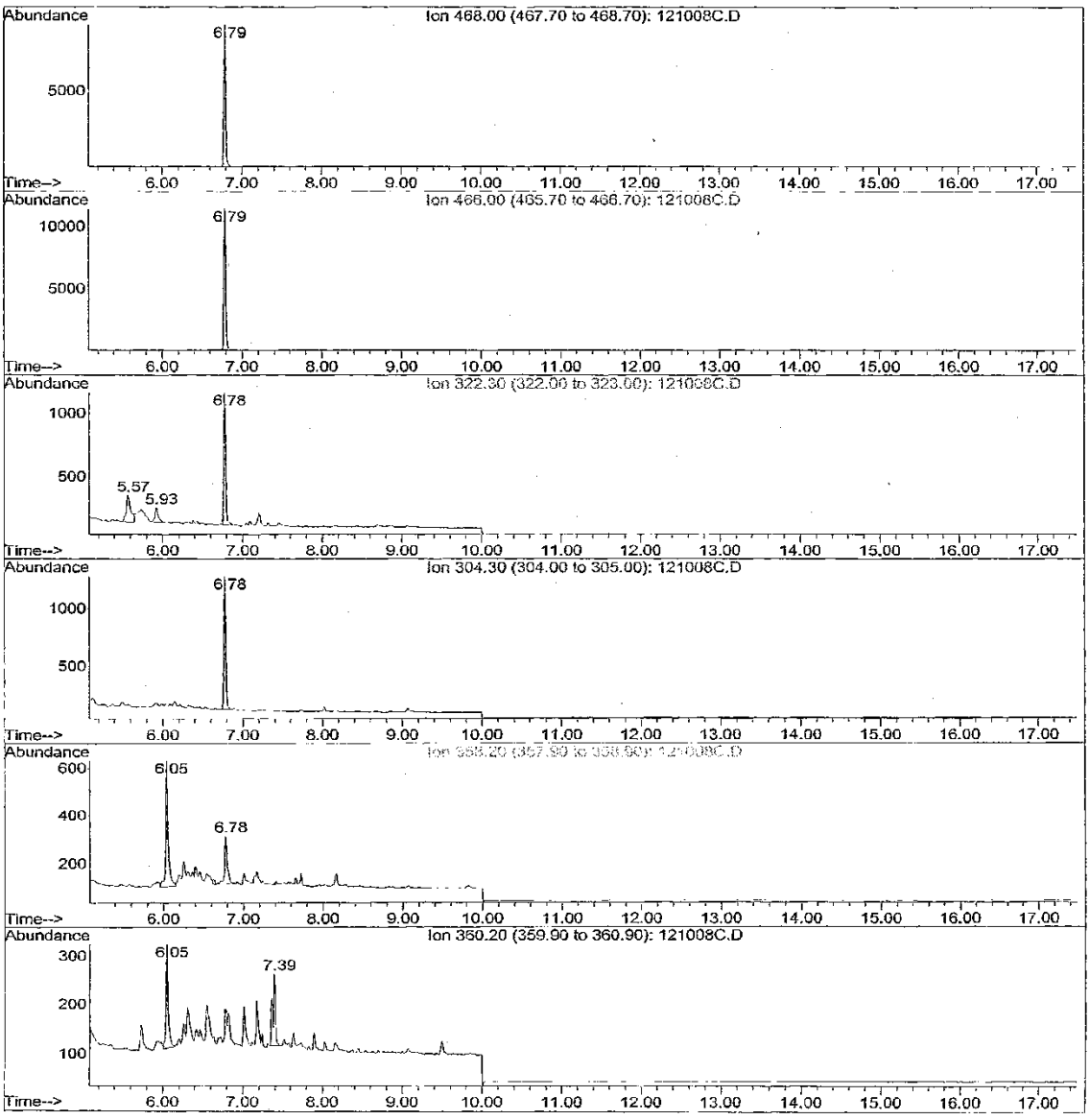
      Refer to I. 1.

      If unacceptable values are obtained, then:
      i. Investigate following established procedures.
      ii. Take corrective action as warranted.
4. Condition upon receipt: Cold.

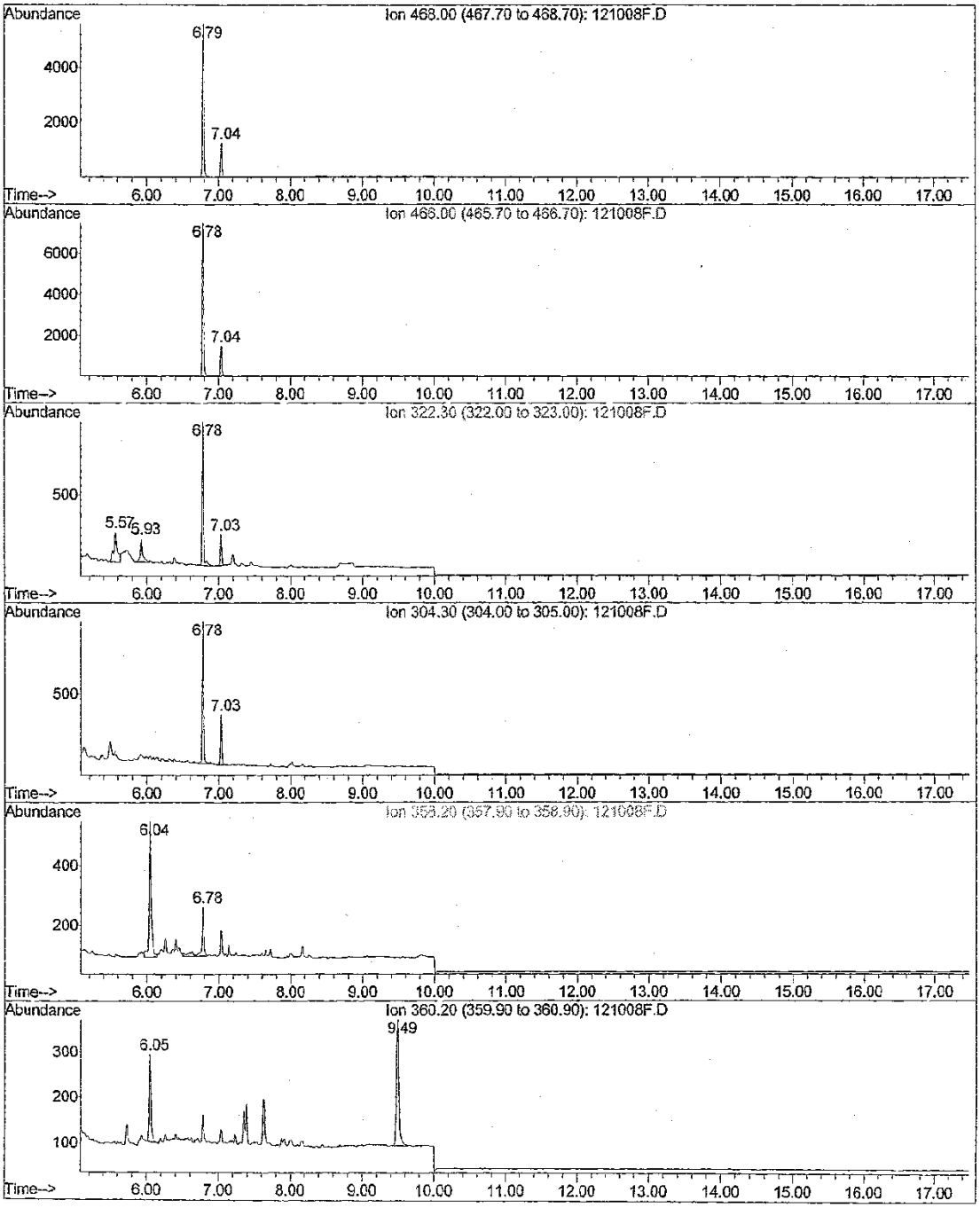
J. APPENDIX

1. Chromatograms/spectra

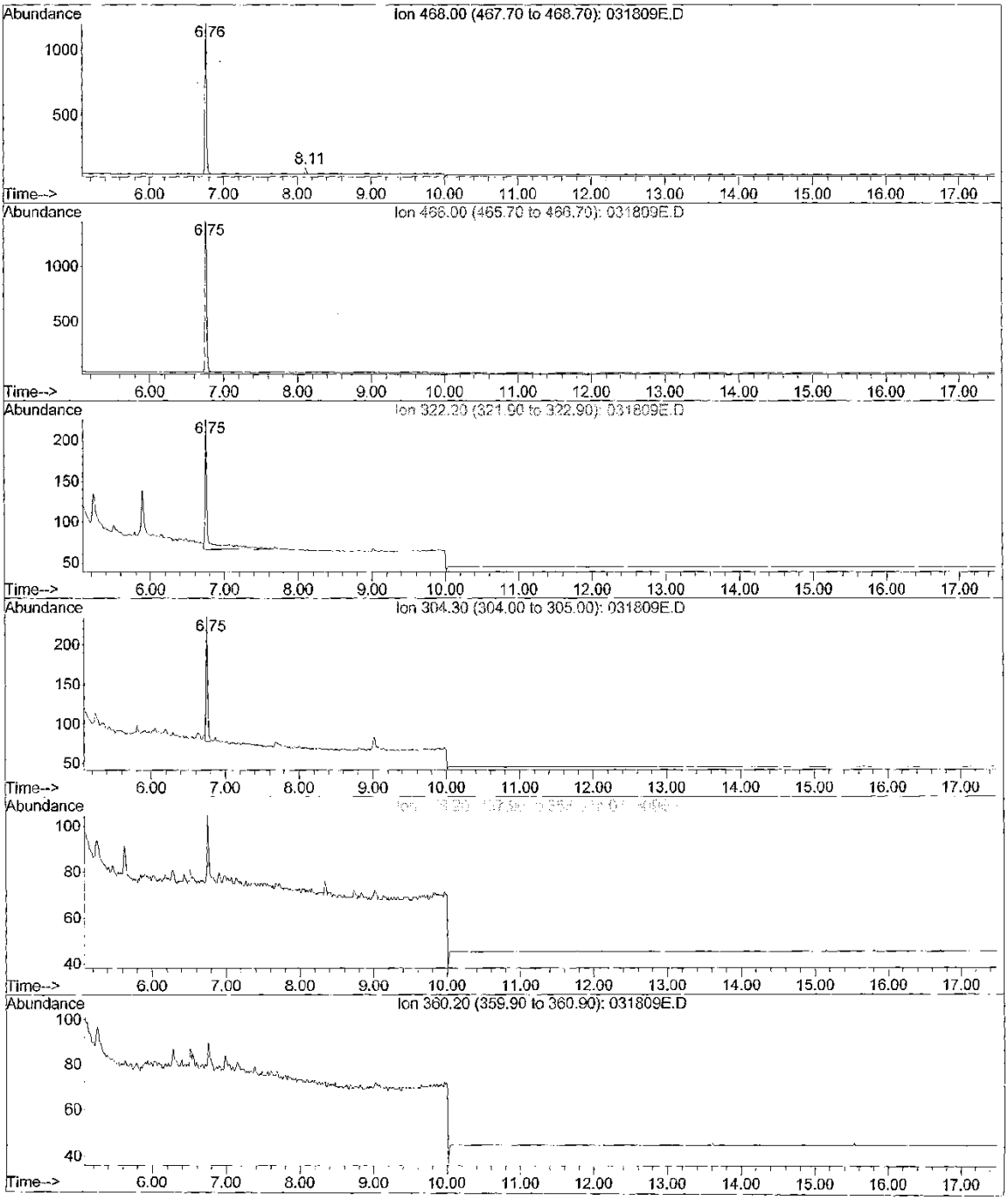
a. Blank turkey muscle



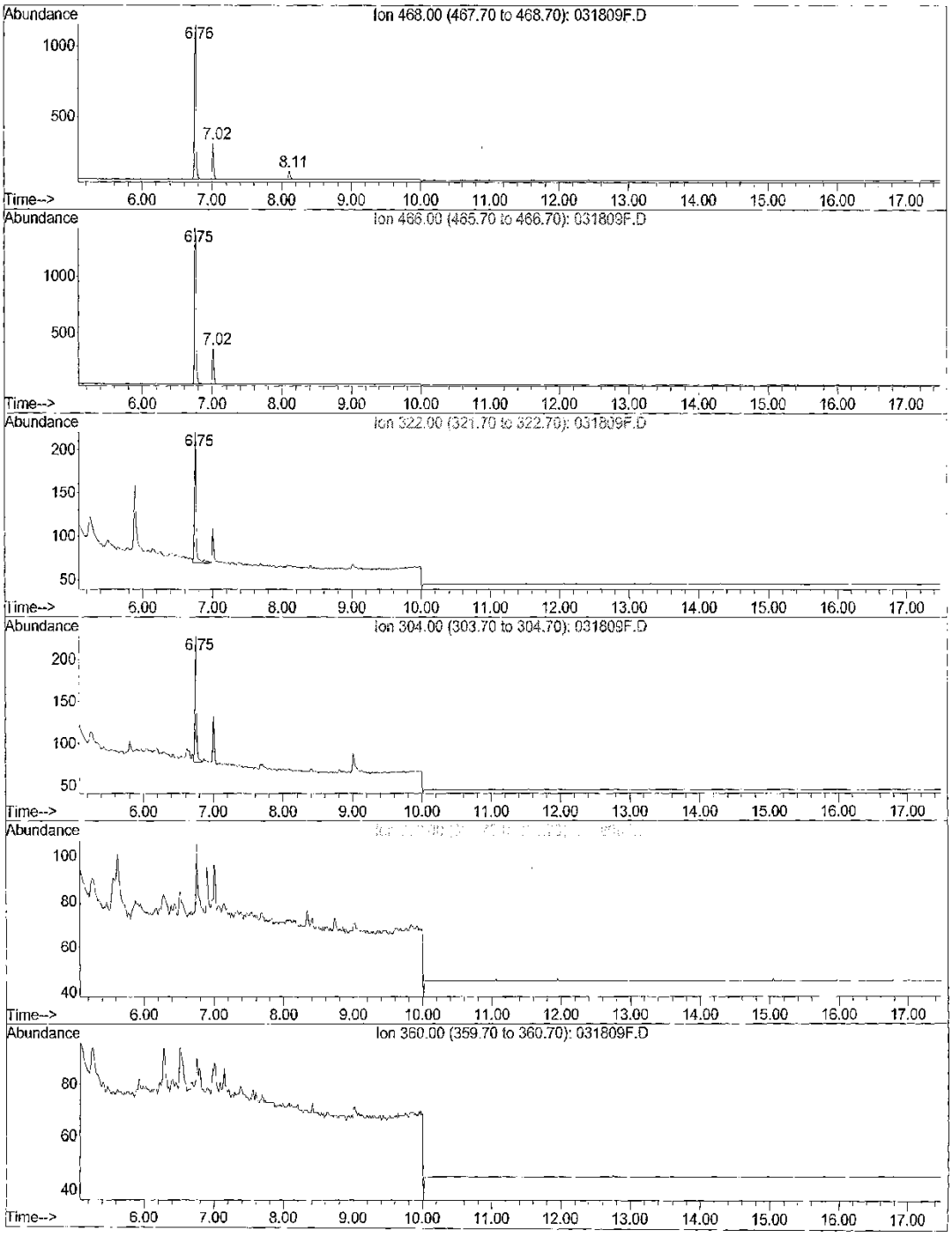
b. Turkey muscle fortified with 0.3 ppb chloramphenicol



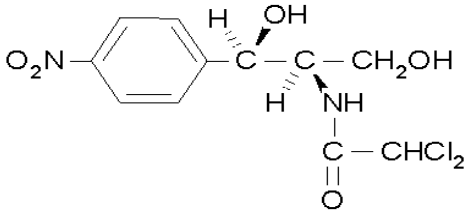
c. Blank catfish muscle



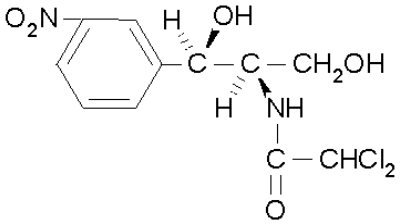
d. Catfish muscle fortified with 0.3 ppb chloramphenicol



2. Structures



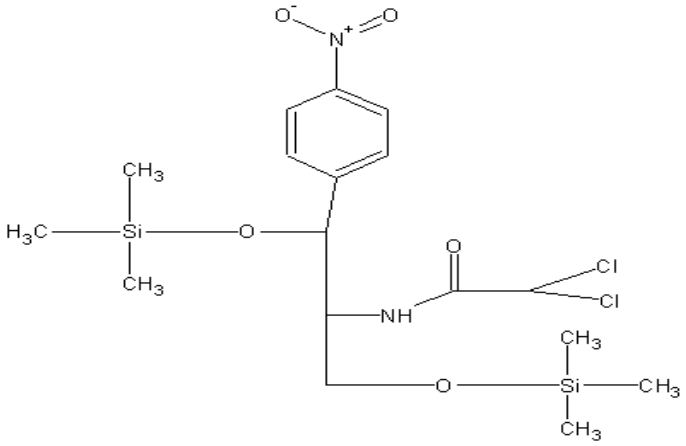
Chloramphenicol



Metachloramphenicol

3. Postulated Structure of some confirming Ions

MASS
468 AND 466
Due to Cl isotopes



K. APPROVALS AND AUTHORITIES

1. Approvals on file.

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