



**U.S. FOOD & DRUG  
ADMINISTRATION**

## **FDA Foods Program Compendium of Analytical Laboratory Methods: Chemical Analytical Manual (CAM)**

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**METHOD TITLE:** Determination of Chloramphenicol and Nitrofurantoin Metabolites in Cobia, Croaker, and Shrimp Using Microwave-Assisted Derivatization, Automated SPE, and LC-MS/MS

**VALIDATION STATUS:** Level 3 multi-laboratory validation (MLV) using Foods Program Guidelines for Validation of Chemical Methods, 2<sup>nd</sup>. Edition

**AUTHOR(S):** Brian Veach

**METHOD SUMMARY/SCOPE:**

**Analyte(s):** Chloramphenicol and Nitrofurantoin metabolites

**Matrices:** Cobia, Croaker and Shrimp (MLV); Crawfish, Catfish, Frog, Crab and Barramundi (Single-laboratory validation)

**REVISION HISTORY:**

**OTHER NOTES:**

# Determination of Chloramphenicol and Nitrofuran Metabolites in Cobia, Croaker, and Shrimp Using Microwave-Assisted Derivatization, Automated SPE, and LC-MS/MS

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**2019.1 METHOD TITLE: Determination of Chloramphenicol and Nitrofuran Metabolites in Cobia, Croaker, and Shrimp Using Microwave-Assisted Derivatization, Automated SPE, and LC-MS/MS**

**2019.2 SCOPE OF APPLICATION**

The method describes a high-throughput and automated procedure for the quantitation of chloramphenicol and nitrofuran metabolites in various species or aquaculture. The method has been validated in the following food matrices:

Matrices	Validation	Date	Analyst
Shrimp, Crawfish, Catfish, Frog, and Crab	Single lab validation <sup>1</sup>	2014	Brian Veach
Cobia, Croaker, and Barramundi	Single lab validation <sup>1</sup>	2017	Brian Veach
Shrimp, Cobia, and Croaker	Multi-lab validation <sup>2</sup>	2019	POC: Brian Veach 3 laboratories participated

This method should be used by analysts experienced in the use of LC-MS/MS, including but not limited to operation of the instrumentation and software, data analysis and reporting results. Analysts should also be able to identify chromatographic and mass spectrometric interferences during sample analysis and take necessary actions following validated procedures for their correction to achieve reliable identification and quantitation. The method should be used only by personnel thoroughly trained in automated solid-phase extractions and microwave operation.

The use of trade names in this method constitutes neither endorsement nor recommendation by the U. S. Food and Drug Administration (FDA). Equivalent performance may be achievable using apparatus and materials other than those cited here.

**2019.3 PRINCIPLE**

Microwave-assisted derivatization has been shown to be an effective alternative to the extended acid hydrolysis and derivatization process. Additionally, the labor-intensive liquid-liquid extraction

procedure can be eliminated by utilizing the use of an automated SPE cartridge (ASPEC) system. The combination of these two approaches allows for the simultaneous extraction of nitrofurantoin metabolites and CAP. Analysis can then be performed using high-pressure liquid chromatography (HPLC) coupled with triple-quadrupole MS (MS/MS). Positive atmospheric pressure chemical ionization (APCI) was used for the nitrofurantoin metabolites and negative electrospray ionization (ESI) was used for chloramphenicol (CAP) analysis <sup>1</sup>.

#### 2019.4 REAGENTS

- (1) Acetonitrile —LC-MS grade
- (2) Methanol —LC-MS grade (*mobile phase*)
- (3) Methanol —HPLC grade (*extractions and reagents*)
- (4) Water —LC-MS grade (*mobile phase and reagents*)
- (5) Water —HPLC grade (*extractions*)
- (6) Ammonium acetate —LC-MS grade
- (7) 0.1% Formic acid in water —LC-MS grade
- (8) Ethyl acetate —HPLC grade
- (9) 0.125 M hydrochloric (HCl) acid —Reagent grade
- (10) Dipotassium hydrogen phosphate —Reagent grade
- (11) 2-Nitrobenzaldehyde — Reagent grade

#### 2019.5 STANDARDS

- (1) Semicarbazide hydrochloride as semicarbazide reference standard. —Sigma-Aldrich (St. Louis, MO) Part No 33656.
- (2) AMOZ reference standard. —Sigma-Aldrich Part No 33349.
- (3) AOZ reference standard. —Sigma-Aldrich Part No 33347
- (4) 1-Aminohydantoin hydrochloride as 1-aminohydantoin (AHD) reference standard—Sigma-Aldrich Part No 33655.
- (5) Chloramphenicol reference standard—Sigma-Aldrich. Part No. C0378.
- (6) Chloramphenicol-d<sub>5</sub> internal standard—Cambridge Isotopes (Andover, MA) Part No. DLM-1190-0.
- (7) SCA-<sup>13</sup>C-<sup>15</sup>N<sub>2</sub> internal standard. —Sigma-Aldrich (St. Louis, MO) Part No. 33882.
- (8) AMOZ-d<sub>5</sub> internal standard. —Sigma-Aldrich (St. Louis, MO). Part No. 33881

(9) AHD-<sup>13</sup>C<sub>3</sub> internal standard—Santa Cruz Biotechnology (Santa Cruz, CA) Part No. 208596.34

## 2019.6 PREPARATION OF STANDARDS, REAGENTS AND SAMPLES

The following are suggested preparation instructions. They may be modified as needed, if the ratios of the components are equivalent.

**2019.6.1** Derivatization solution, 100 mM 2-nitrobenzaldehyde (2-NBA), is prepared by dissolving 152 mg of 2-NBA in 10.0 mL of HPLC grade methanol (MeOH). This solution should be prepared fresh daily.

**2019.6.2** Hydrochloric acid (0.125 M): The 0.125 M solution can be purchased commercially or prepared by adding 10.3 mL of 37% HCl to 1-liter HPLC grade water. The prepared solution has a shelf life of three months; the commercially purchased solution's shelf life shall be determined by the reagent manufacturer.

**2019.6.3** Dipotassium hydrogen phosphate (1.0 M) can be purchased commercially or prepared by dissolving 174.2 g in 1 liter of HPLC grade water. The prepared solution should be sonicated for about 5 minutes to ensure the dipotassium hydrogen phosphate has dissolved. It may be stored at room temperature for one month. Commercially purchased solutions storage and shelf life will be determined by the manufacturer.

**2019.6.4** Nitrofurans metabolite mobile phase A: Ammonium acetate mobile phase (8.5 mM ammonium acetate buffer) is prepared by dissolving 0.655 g of ammonium acetate (LCMS grade) in 1000 mL of 0.1 % formic acid in water (LCMS grade) and mixing until completely dissolved and sonicated for about 5 minutes. This solution was prepared fresh monthly.

**2019.6.5** Reconstitution solution is equivalent to mixed mobile phase (40% methanol and 60% 8.5 mM ammonium acetate in 0.1% Formic Acid). This solution has a shelf life of one month.

### 2019.6.6 Standard Preparation

Nitrofurans analytical and internal standard stock solutions (100 µg/mL) were prepared in an 80:20 methanol:water (v/v) solution. These solutions are stable for up to 6 months when stored at ≤ 5°C. Chloramphenicol stock and internal standard solutions (20.0 µg/mL) were prepared in methanol. These solutions are stable for up to 1 year when stored at ≤ 5°C. A mixed intermediate analytical standard was then prepared to a concentration of 25.0 ng/g for each of the nitrofurans and 5.00 ng/g for chloramphenicol in an 80:20 methanol:water (v/v) solution. Additionally, a mixed internal standard solution was prepared in an 80:20 methanol:water (v/v) solution at a concentration of 80.0 ng/g for each deuterated or isotopically labeled nitrofurans standard, and 24.0 ng/g for the deuterated chloramphenicol standard. The mixed analytical standard and the mixed internal standard are stable for up to 6 months when stored at ≤ 5°C.

### **2019.6.6** Sample Preparation

Control cobia, croaker and shrimp products were obtained from commercially available sources for the demonstration of negative controls and spike recoveries. The edible portion of tissue was homogenized with dry ice and a blender until powdery consistencies were obtained. Samples should sublime for a minimum of 12 hours in a refrigerator prior to use<sup>1-3</sup>.

### **2019.7 APPARATUS/INSTRUMENTATION**

- (1) LC-MS/MS – AB Sciex (Framingham, MA) QTRAP 5500 coupled to an Agilent (Santa Clara, CA) 1260 HPLC
- (2) Chromatographic column – Agilent Technologies Zorbax Eclipse SDB-C18 1.8  $\mu$ M, 4.6 x 50 mm.
- (3) Centrifuge – Capable of 3700 g.
- (4) Microwave reaction system – CEM (Mathews, NC) MARS 6
- (5) Microwave carousel – DV-50
- (6) Blender – Robotcoupe (Ridgeland, MS)
- (7) SPE cartridges – Waters (Milford, MA) Oasis HLB 3 cc X 60 mg.
- (8) Automated solid-phase extraction system – Gilson (Middleton, WI) GX-274

### **2019.8 METHOD**

#### **2019.8.1** Sample Extraction

A volume of 50.0  $\mu$ L of mixed internal standard solution was added to 2.00 grams ( $\pm$  .03) of negative control matrix in a 50 mL centrifuge tube. Approximately 10 mL of 0.125 M HCl was added to each tube. Additionally, 200  $\mu$ L of 100 mM 2-nitrobenzaldehyde (2-NBA) prepared fresh daily in methanol was added to each vessel. Each vessel was then vortexed (~20 seconds) and placed in the microwave for 6 minutes (5-minute ramp from ambient temperature to 95°C and hold 1 minute).

Upon microwave completion, 5 mL of 1.0 M dipotassium hydrogen phosphate was added to each vessel to adjust the pH to 7.3 ( $\pm$  0.3). If the desired pH is not achieved, the extract can be further adjusted by the addition of dilute sodium hydroxide or hydrochloric acid. Contents were then centrifuged for 10 minutes at 3700g, and the supernatant was subsequently transferred into a 0.45 $\mu$ m PVDF 50 mL filtration tube where it was centrifuged for 5 minutes at 2500g. Utilizing the ASPEC system, the HLB (3 cc X 60 mg), solid-phase extraction cartridges were conditioned with 3 mL of ethyl acetate, 3 mL of methanol, and 3 mL of water. The ASPEC system loaded the samples onto the conditioned solid phase extraction (SPE) cartridges and washed the cartridges with 2 mL of water and 2 mL of 30% methanol in water. Subsequently the ASPEC system dried the SPE cartridges and eluted them with 3 mL of ethyl acetate. The eluent was evaporated to dryness and reconstituted with 250  $\mu$ L of reconstitution solution

(40% methanol and 60% 8.5 mM ammonium acetate in 0.1% Formic Acid). Reconstituted samples were vortexed (~10 seconds), sonicated (~5 minutes) and filtered through a 0.45 µm PVDF syringe filter) into a 2 mL autosampler vial with insert.

## 2019.8.2 LC-MS/MS

The prepared extracts were analyzed under two separate LC-MS/MS conditions. Nitrofurantoin metabolites extracts have been shown to be stable for approximately 3 days when stored at 5 °C. However, chloramphenicol extracts have proven to be stable for up to 6 months when stored at 5 °C. Considering the stability of the extracts, it was believed the sample vials should first be analyzed for nitrofurantoin, and then subsequently assayed for chloramphenicol.

Nitrofurantoin metabolites were assayed using APCI. Samples were injected (10 µL) onto a 50 mm x 4.6 mm x 1.8 µm XDB-C-18 reverse phase column and eluted under isocratic conditions with an 8.5 mM ammonium acetate in 0.1% Formic Acid (mobile phase A) and methanol (mobile phase B) followed by column cleaning and regeneration. Mobile phase A was held at 60% (600 µL/min) for 2.3 minutes. At 2.4 minutes mobile phase B (600 µL/min) was ramped up to 80% for 1 minute. At 3.5 minutes mobile phase B was ramped down to 40% (750 µL/min) and held for 1.5 minutes. Data from the mass spectrometer was acquired utilizing SRM (selected reaction monitoring) in positive APCI mode (precursor ions and MS/MS fragment ions are listed in Table 1).

**Table 1:** MS/MS acquisition parameters for nitrofurantoin metabolite using APCI.

nitrofurantoin metabolite	precursor ion (m/z)	product ions (m/z)	CE (volts)	retention time (min)
NBA-AHD	249.0	<b>134.0</b> , 104.0, 178.1	17, 30, 22	2.1
NBA-AOZ	236.0	<b>134.0</b> , 104.0, 149.0	18, 29, 20	2.2
NBA-AMTZ	335.1	<b>291.2</b> 262.2, 128.0	16, 23, 30	1.7
NBA-SC	209.1	<b>166.1</b> , 134.0, 192.1	14, 15, 15	2.3
NBA-AHD <sup>13</sup> C <sub>3</sub>	252.0	134.0	16	2.1
NBA-AMTZ d <sub>5</sub>	340.1	296.2	16	1.7
NBA-SC <sup>13</sup> C <sup>15</sup> N <sub>2</sub>	212.1	168.1	14	2.3

Chloramphenicol was analyzed using ESI. Samples were injected (5 µL) onto a 50 mm x 4.6 mm x 1.8 µm XDB-C-18 reverse phase column. Chloramphenicol was eluted from the column with a gradient flow (750 µL/min) of water (mobile phase A) and acetonitrile (mobile phase B). Mobile phase A was held an

initial time of 0.5 minutes at 75%, then the proportion of mobile phase B was increased linearly to 50% in the following 3 minutes. At 3.6 minutes the proportion of mobile phase B was increased to 90%, followed by a hold of 1 minute at 90%. At 4.7 minutes mobile phase B was returned to 25% and re-equilibrated for 1.3 minutes prior to subsequent injection. The mass spectrometer data were collected in negative ESI mode using SRMs (precursor and MS/MS fragment ions obtained are listed in Table 2).

**Table 2:** MS/MS acquisition parameter for chloramphenicol analysis using ESI.

analyte	precursor ion (m/z)	product ions (m/z)	CE (volts)	retention time (min)
CAP	321.1	151.8, 193.8, 257.2	-23, -16, -16	3.1
CAP d <sub>5</sub>	326.1	262.2	-16	3.1

## 2019.9 DATA ANALYSIS

Quantitation was performed for each analyte of interest by calculating the ratio of the chromatographic area of the quantitation ion with respect to the chromatographic area of the internal standard (AMOZ/AMOZ d<sub>5</sub>, SC/SC <sup>13</sup>C <sup>15</sup>N<sub>2</sub>, AOZ/ SC <sup>13</sup>C <sup>15</sup>N<sub>2</sub>, AHD/AHD <sup>13</sup>C<sub>3</sub>, and CAP/CAP d<sub>5</sub>). Each representative ratio was plotted against the concentration of the corresponding matrix extracted calibration standard. The linear calibration curve fit obtained should yield a regression (R<sup>2</sup>) of ≥ 0.995. For positive confirmation all product ions must be detected, the associated chromatographic peak must exhibit a retention time within 5% of the average retention time of the calibration standards, and the product ion ratios must be within 20% (absolute value) of the product ion ratios obtained from calibration standards<sup>4-5</sup>.

## 2019.10 VALIDATION INFORMATION/STATUS

This method has undergone multi-laboratory validation (MLV) for shrimp, cobia, and croaker using the FDA Foods Program Guidelines for the Validation of Chemical Methods, 2<sup>nd</sup> Edition. The validation will be published and referenced in a future update to this SOP. Until that time, the method author may be contacted to obtain validation information. Single-laboratory validation information may be found in reference 1 below.



## 2019.11 REFERENCES

1. Veach, B. T.; Baker, C. A.; Kibbey, J. H.; Fong, A.; Broadaway, B. J.; Drake, C. P., Quantitation of Chloramphenicol and Nitrofurantoin Metabolites in Aquaculture Products Using Microwave-Assisted Derivatization, Automated SPE, and LC-MS/MS. *J. AOAC Int.* 2015, 98, 588-594.
2. Veach, B.; Hawk, H.; Baker, C.; Linder, S., Determination of Chloramphenicol in Shrimp, Crawfish, Crab, and Frog Legs Using High Throughput LC-MS/MS. *FDA/ORA/DFS Lab. Inf. Bull.* 2011, 27, 4473, 1-15.
3. An, H.; Cain, T.; Henry, M.; Tran, B.; Paek, H. C.; Farley, D., *FDA/ORA/DFS Lab. Inf. Bull.* 2011, 27, 4482, 1-35.
4. U.S. Food and Drug Administration (2015), *Guidelines for the Validation of Chemical Methods for the FDA Foods Program*. 2nd edition ed.; US FDA: Washington DC.
5. *Guidance for Industry — Mass Spectrometry for Confirmation of the Identity of Animal Drug Residues*. *Fed. Regist.*, 2003; Vol. 66, pp 31938–31939.