Modifications to LIB 4597 for the analysis of nitrofuran metabolites and chloramphenicol in aquaculture products using LC-MS/MS

Brian T. Veach, Victoria L. Owens, Bryanna J. Broadaway, Nilantha Bandara, John H. Kibbey, and Chris A. Baker

Office of Regulatory Affairs, Arkansas Laboratory, U.S. Food and Drug Administration, 3900 NCTR Road, Jefferson, Arkansas 72079, United States

ABSTRACT

Laboratory Information Bulletin 4597 is a harmonized method that has undergone a level 3 validation. Although the method has proven to be effective for regulatory use, substantial modifications were performed to add 3,5-dinitrosaliscylic acid hydrazide, an additional nitrofuran metabolite to the assay. These modifications were all implemented in the method as described herein and did not affect quantitation, robustness, or confirmation abilities. A modification was made to the solid-phase extraction elution solvent from the previous method to improve the absolute recoveries of the 3,5-dinitrosalisylic acid hydrazide. Validation of the method demonstrated acceptable recoveries and detection limits.

Correspondence addressed to Brian T. Veach, Tel (870)-543-4085; fax (870)-543-4041; email brian.veach@fda.hhs.gov

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INTRODUCTION

The potential use of nitrofurans and chloramphenicol in aquaculture products continues to be of great concern for the U.S. Food and Drug Administration (FDA) due to their potential for negative health effects, which include but is not limited to aplastic anemia and carcinogenicity [1-3] . One of the primary methods that has been used for this analysis is FDA Laboratory Information Bullet (LIB) # 4597, which was developed at the FDA's Arkansas Laboratory. This method utilizes novel techniques to improve analytical results and drastically reduce analysis times. These analytical techniques include microwave assisted derivatization and the use of an automated solid-phase extraction system. LIB 4597 assays chloramphenicol and four nitrofuran metabolites, which are 3-amino-2-oxazolidinone (AOZ), semicarbazide (SEM), 1-aminohydantoin (AHD), and 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ). The method was harmonized among the FDA aquaculture laboratories and went through a multi-laboratory validation in 2020 [4-6].

Recently, new concerns have grown from the European Union (EU) and the FDA about the potential use of an additional nitrofuran drug in aquaculture products. That drug is nifursol. Nifursol is often administered through livestock feed, where it exhibits both anti-microbial and anti-parasitic activities. Nifursol, like other nitrofurans is a prodrug and is rapidly metabolized. The primary nifursol metabolite that is observed is 3,5-dinitrosalicylic acid hydrazide (DSH) [7-9].

The Arkansas Laboratory initially attempted to do a simple analyte extension to LIB 4597 to include DSH. Unfortunately, the analyte has some chemical properties that prevented it from being recovered in sample matrices. In order to include DSH, along with the other nitrofuran metabolites and chloramphenicol, significant method modifications were needed. This method captures all modifications of LIB 4597 so that chloramphenicol and five nitrofuran metabolites can be quantitated and confirmed by LC-MS/MS.

EXPERIMENTAL

Equipment:

(a) *LC-MS/MS* **–** Sciex (Framingham, MA) QTRAP 6500^{+}, Analyst® and OS® software

(b) *HPLC* – Sciex Exion AC series

(c) *Chromatographic column* – Agilent Poroshell 120 EC-C18 (3.0 mm X 150 mm X 2.7µm)

(d) *Centrifuge* – Fisher Scientific (Houston, TX), Capable of holding 50 mL tubes and maximum speed of ≥ 4800g

(e) *PTFE Syringe filter* – Fisher Scientific, (0.45 µm X 13 mm)

(f) *Centrifuge* – Fisher Scientific, Capable of holding 50 mL tubes and max speed of ≥ 20,000g and 4ºC

(g) *Microwave reaction system* – CEM (Matthews, NC) MARS 6 microwave system with a DV-50 High Throughput Accessory Set (CEM P.N. 430420) and appropriate temperature probe

(h) *Blender* – RobotCoupe (Ridgeland, MS)

(i) *Automated solvent evaporation system* – Biotage (Charlotte, NC) TurboVap LV

(j) *Automated solid-phase extraction system (ASPEC)* – Gilson (Middleton, WI) GX-274

(k) *Solid-phase extraction cartridges* – Gilson HLB 3 cc X 60 mg

(l) *2mL Autosampler vials with inserts* – Agilent Technologies (Santa Clara, CA)

(m) *pH-indicator paper* – Sigma Aldrich, (St. Louis, MO) pH 6.4–8.0 Special Indicator

(n) *Autosampler vial caps* – Agilent Technologies

STANDARDS AND REAGENTS

(a) *Acetonitrile* – Fisher Scientific, LCMS grade

(b) *Methanol* – Fisher Scientific, HPLC grade

(c) *Water* – Fisher Scientific, LCMS grade

(d) *Ammonium acetate* – Fisher Scientific, LCMS grade

(e) *0.1% Formic acid in water* – Fisher Scientific, LCMS grade

(f) *Ethyl acetate* – Honeywell – Burdick & Jackson (Muskegon, MI), HPLC grade

(g) *0.125 N Hydrochloric acid* – Fisher Scientific

(h) *1 M Potassium phosphate dibasic solution* – Sigma Aldrich

(i) *1 N Sodium Hydroxide* – Fisher Scientific

(j) *1 N Hydrochloric acid* – Fisher Scientific

(k) *2-Nitrobenzaldehyde (2-NBA)*– Sigma Aldrich

(l) *Semicarbazide hydrochloride (SEM)* – Sigma-Aldrich, CAS # 563-41-7

(m) 3-amino-2-oxazolidinone *(AOZ)* – Sigma-Aldrich, CAS # 80-65-9

(n) 3-amino-5-morpholinomethyl-2-oxazolidinone *(AMOZ)* – Sigma-Aldrich, CAS # 43056-63-9

(o) *1-Aminohydantoin HCl* (AOZ) – Sigma-Aldrich, CAS # 2827-56-7

(p) 3,5-dinitrosalicylic acid hydrazide *(DSH)* – Santa Cruz Biotechnology (Santa Cruz, CA) CAS # 955-07-7

(q) *Chloramphenicol* – Sigma-Aldrich, CAS # 56-75-7

(r) *Chloramphenicol-d_5 internal standard* – Cambridge Isotopes (Andover, MA) CAS # 202480-68-0

(s) *Semicarbazide-^{13}C-$^{15}N_2$ HCl internal standard* – Sigma-Aldrich, CAS # 1173020-16-0

(t) *AMOZ-d_5 internal standard* – Sigma-Aldrich, CAS # 1017793-94-0

(u) *AHD-$^{13}C_3$ internal standard* – Santa Cruz Biotechnology (Santa Cruz, CA) CAS # 957509-31-8

(v) *AOZ-d_4 internal standard* – Sigma-Aldrich, CAS # 1188331-23-8

(w) *3,5-Dinitrosalicylic acid- $^{13}C_6$ hydrazide (DSH $^{13}C_6$)* – WITEGA (Berlin, Germany)

METHOD

Reagents and Standard Preparation.

a. 2-NBA (100 mM) solution in methanol: This solution should be prepared fresh daily.

b. Mobile phase A: 2 mM ammonium acetate mobile phase solution in LCMS grade water.

c. Reconstitution solution: 60:40 (v/v) 2 mM ammonium acetate in 0.1% formic acid: acetonitrile.

d. Solid-phase extraction (SPE) elution solvent: 90:10 (v/v) ethyl acetate: acetonitrile.

e. Stock Internal Standard Solutions (ISTD): Separate internal standard stock solutions were prepared as follows:

   i. 100 µg/mL for AOZ d_4, AMOZ d_5, AHD $^{13}C_3$, and SEM ^{13}C-$^{15}N_2$ prepared in 80:20 methanol:water (v/v) solution.

   ii. 40.0 µg/mL for DSH $^{13}C_6$ prepared in methanol.

   iii. 20.0 µg/mL for CAP d_5 prepared in methanol.

f. Stock Standard Solutions for Calibration Standards:

   i. 80.0 µg/mL for AOZ, AMOZ, AHD, and SEM prepared in 80:20 methanol:water (v/v) solution.

   ii. 40.0 µg/mL for DSH prepared in methanol.

   iii. 20.0 µg/mL for CAP prepared in methanol.

g. Mixed Intermediate Internal Standard Solution: Prepare an intermediate ISTD solution in 80:20 methanol:water (v/v) as described in Table 1.

Table 1: Preparation of Intermediate ISTD

Internal Standard	Conc. Of Stock Solution	Volume Used	Final Volume	Final Conc.
AOZ d_4	100 µg/mL	80.0 µL	100 mL	80.0 ng/mL
AMOZ d_5	100 µg/mL	80.0 µL	100 mL	80.0 ng/mL
AHD $^{13}C_3$	100 µg/mL	80.0 µL	100 mL	80.0 ng/mL
Semicarbazide ^{13}C-$^{15}N_2$	100 µg/mL	80.0 µL	100 mL	80.0 ng/mL
DSH $^{13}C_6$	40.0 µg/mL	200 µL	100 mL	80.0 ng/mL
CAP d5	20.0 µg/mL	100 µL	100 mL	20.0 ng/mL

h. Intermediate Analytical Calibration Standard: Prepare the standard solution in 80:20 methanol:water (v/v) as described in Table 2.

Table 2: Preparation of Intermediate Calibration Standard Solution

Analytical Standard	Conc. Of Stock Solution	Volume Used	Final Volume	Final Conc.
AOZ	80.0 µg/mL	25.0 µL	100 mL	20.0 ng/mL
AMOZ	80.0 µg/mL	25.0 µL	100 mL	20.0 ng/mL
AHD	80.0 µg/mL	25.0 µL	100 mL	20.0 ng/mL
SEM	80.0 µg/mL	25.0 µL	100 mL	20.0 ng/mL
DSH	40.0 µg/mL	50.0 µL	100 mL	20.0 ng/mL
CAP	20.0 µg/mL	25.0 µL	100 mL	5.00 ng/mL

i. Intermediate analytical ICV standard solution can be prepared as shown in Table 2.

Sample Preparation and Extraction:

1. An appropriate amount of edible tissue (i.e., ≥100 grams) should be placed in a Robot-Coupe® food processor with an adequate amount of dry ice. The contents should be homogenized into a powder like form with no visible clumps of product present. The homogenized product should be stored in a freezer or refrigerator for a minimum of 12 hours to allow the dry ice to sublime.

2. Measure 2.00 grams (± 0.03) of the homogenized tissue into 50 mL centrifuge tubes. Blank matrix matched tissue, without compounds of interest, is used for quality control and calibration standards.

3. All samples, calibration standards and quality control samples are fortified with 50.0 µL of the mixed intermediate internal standard solution. This correlates to a concentration of 0.500 ng/g for chloramphenicol and 2.00 ng/g for the nitrofuran metabolites.

4. Calibration standards and the ICV are fortified at the levels listed in Table 3 below.

Table 3: Calibration Standards and ICV

Extracted Calibration Curve	Volume of Mixed Intermediate Standard	Nitrofurans Con. (ng/g)	Chloramphenicol Conc. (ng/g)
Calibration Standard 1	20.0 µL	0.200	0.0500
Calibration Standard 2	40.0 µL	0.400	0.100
Calibration Standard 3	80.0 µL	0.800	0.200

Extracted Calibration Curve	Volume of Mixed Intermediate Standard	Nitrofurans Con. (ng/g)	Chloramphenicol Conc. (ng/g)
Calibration Standard 4	160 µL	1.60	0.400
Calibration Standard 5	400 µL	4.00	1.00
ICV	80.0 µL	0.800	0.200

5. Add 10 mL of 0.125 N HCl and 200 µL of 100 mM 2-NBA to each centrifuge tube.

6. The tubes are vortexed/shaken for ~ 10 seconds. Some matrices (i.e., crab) may require up to 10 minutes of agitation to achieve complete homogenization.

7. Microwave the vessels with a 5-minute ramp to temperature at 95ºC, with a 1-minute hold at 95ºC, and the wattage set to 800 watts if 24 or fewer vessels with tissue are present. If 25 or more vessels with tissue are used, then the wattage should be adjusted to 1080 watts. Temperatures should be adjusted to elevation so that the maximum temperature is below the boiling point of the solution. *If the temperature fails to reach 95ºC, as long as the wattage referenced above is being applied then the temperature read back is insignificant. Different matrix types may yield different temperatures (i.e., frog). The temperature is only a guide to prevent too much wattage from being applied and causing degradation or over pressuring the centrifuge tube.*

8. Upon completion of the microwave digestion, add ~ 5 mL of 1 M K_2HPO_4 solution to each centrifuge tube. The tubes are capped and vortexed/shaken for ~10 seconds *(if crab matrix is being analyzed, it is recommended to vortex the samples for 10 minutes to achieve complete neutralization)*. If the pH of the sample matrix is not 7.3 (±0.3), then the pH can be adjusted with 1N NaOH or 1N HCl.

9. Centrifuge the 50 mL tubes at 4700 x g for 10 minutes. Decant the supernatant into a clean 50 mL tube. Additional sample cleanup is needed prior to performing the solid-phase extraction (SPE) in order to help prevent plugging the SPE cartridges. This can be done by any of the following processes:
   i. Ultra-centrifuge (20,000 x g for 15 minutes at 4ºC)
   ii. 50 mL filtration tubes (2,500 x g for 5 minutes)[4]
   iii. 20 µm frits into 20 mL reservoir tubes. Decant the supernatant into the filtration module and apply slight positive pressure with nitrogen to force flow through the reservoir into a 50 mL centrifuge tube [4].

10. Condition the HLB (3cc X 60 mg) SPE cartridges with 3 mL of SPE elution solvent, 3 mL of methanol, and 3 mL of water.

11. Load the sample onto the SPE columns, then wash the SPE cartridges with 2 mL of water, followed by 2 mL of 30% methanol in water (v/v).

12. Dry the columns and then elute with 3 mL of SPE elution solvent. *Note: Ethyl acetate can degrade into ethanol and acetic acid. This can change the pH of the extracted sample and cause chromatography issues.*

13. Evaporate the samples to dryness at 45ºC (± 4ºC) with nitrogen pressure up to 20 psi.

14. Reconstitute the samples with 250 µL of the reconstitution solution, followed by sonication (~5 minutes) and vortexing (~20 seconds).

15. Filter the sample through a PTFE (0.45 µm X 13 mm) syringe filter into an autosampler vial with insert for analysis.

Chromatography:

Table 4: HPLC Gradient

Time (min)	Flow (µL/min)	% Mobile Phase A	% Mobile Phase B
0.0	400	70	30
2.2	400	70	30
4.5	400	40	60
4.6	400	10	90
8.0	400	10	90

*A 3.5-minute post run was used to re-equilibrate the column.

Mobile phase A: 2 mM ammonium acetate in LCMS grade water
Mobile phase B: LCMS acetonitrile
Column: Agilent Poroshell 120 EC-C18 (3.0 mm X 150 mm X 2.7 µm)
Column Temperature: 40ºC
Injection volume: 5.0 µL
Autosampler Temperature: 5ºC

Mass Spectrometry using Electrospray Ionization:

The mass spectrometer was tuned and calibrated in positive and negative ionization modes according to the manufacturer's instructions. Compound

optimization was performed by flow injection analysis, (50:50) 2 mM ammonium acetate in LCMS grade water and acetonitrile at a flow rate of 400 µL/min, to optimize electronic voltages and gas flows.

The mass spectrometer utilized polarity switching. The suggested voltages and settings are shown below:

- Curtain Gas: 30 psi
- Electrospray voltage: ±4500 V
- Source Heater: 600ºC
- Ion Source Gas 1: 70 psi
- Ion Source Gas 2: 65 psi
- Entrance Potential: ±10 eV
- Collision Cell Exit Potential: ±16 eV

Table 5: Mass Spectrometer Scheduled MRM Settings[4, 7]:

Q1	Q3	Time (min)	Identification	Declustering Potential (V)	Collision Energy (V)	Polarity
209.1	**166.1**	**3.1**	**SEM 1**	**+80**	**+14**	**Positive**
209.1	134.0	3.1	SEM 2	+80	+15	
209.1	192.1	3.1	SEM 3	+80	+15	
236.0	**134.0**	**4.7**	**AOZ 1**	**+70**	**+10**	**Positive**
236.0	104.0	4.7	AOZ 2	+70	+17	
236.0	149.0	4.7	AOZ 3	+70	+20	
249.0	**134.0**	**3.3**	**AHD 1**	**+70**	**+17**	**Positive**
249.0	104.0	3.3	AHD 2	+70	+30	
249.0	178.1	3.3	AHD 3	+70	+17	
335.1	**291.2**	**5.1**	**AMOZ 1**	**+70**	**+16**	**Positive**
335.1	262.2	5.1	AMOZ 2	+70	+23	
335.1	128.0	5.1	AMOZ 3	+70	+30	
374.0	**183.0**	**6.7**	**DSH 1**	**-70**	**-35**	**Negative**
374.0	226.0	6.7	DSH 2	-70	-30	

Q1	Q3	Time (min)	Identification	Declustering Potential (V)	Collision Energy (V)	Polarity
374.0	182.0	6.7	DSH 3	-70	-30	
321.1	**151.8**	**4.1**	**CAP 1**	**-70**	**-23**	**Negative**
321.1	193.8	4.1	CAP 2	-70	-16	
321.1	257.2	4.1	CAP 3	-70	-16	
340.1	296.2	5.1	AMOZ d_5	+70	+16	Positive
212.1	168.1	3.1	SEM ^{13}C $^{15}N_2$	+80	+14	Positive
252.0	134.0	3.3	AHD $^{13}C_3$	+70	+16	Positive
240.0	134.0	4.7	AOZ d_4	+70	+10	Positive
326.1	262.2	4.1	CAP d_5	-70	-16	Negative
380.0	188	6.7	DSH $^{13}C_6$	-70	-30	Negative

- Ions listed in bold are quantitation ions.

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_5, SEM/SEM ^{13}C $^{15}N_2$, AOZ/ AOZ d_4, AHD/AHD $^{13}C_3$, DSH/ DSH $^{13}C_6$ and CAP/CAP d_5). Each representative ratio was plotted against the concentration of the corresponding matrix extracted calibration standard. The linear calibration curve fit yielded a regression (R^2) of ≥ 0.995.

For positive confirmation, all product ions must be detected, and the associated chromatographic peak must exhibit a retention time within 5% of the average retention time of the calibration standard(s). If a precursor ion selected by the MS^n is completely dissociated and only two structurally specific product ions are monitored in MS^{n+1}, the relative abundance ratio should match the comparison standard(s) within ± 10%. If three or more structurally specific ions are monitored, the relative abundance ratios should match the comparison standard(s) within ± 20%. The acceptability range is calculated by addition and subtraction. For example, at 50% relative abundance, the acceptability range would be 40–60%, not 45–55%.[10].

Analysis of Reference Materials and Commercial Products

Reference materials were obtained from commercially available sources and were prepared as described in the sample preparation section. Samples were quantitated using matrix-matched extracted standards that were previously screened and determined to be free of chloramphenicol and nitrofuran metabolites of interest using external methods.

Limits of Detection and Quantitation Studies

The method detection limits (MDL) and limits of quantitation (LOQ) for each analyte were determined on the basis of replicate analysis. The MDL of each analyte was calculated by the multiplication of the standard deviation by the student's t-value at the 99% confidence level, and the LOQ by multiplying the standard deviation by ten [11].

RESULTS AND DISCUSSION

Method Optimization

Original efforts were focused on simply adding DSH to LIB 4597 and avoiding any method modification. The fortified solvent blanks that were analyzed during the instrument optimization portion demonstrated an acceptable amount of response for DSH. However, once the matrix was introduced the response diminished to a point that DSH could no longer be detected at or near a target testing level. Because of this, it was apparent that major method modifications were needed to analyze DSH, along with the other compounds assayed in LIB 4597.

Because DSH was recovered in fortified solvent blanks but not in fortified matrix blanks, it was believed to be a solubility issue and a different reconstitution solution would be needed. Since DSH is a more non-polar compound than the other nitrofuran metabolites, the decision was made to change from a 40% methanol solution to a 40% acetonitrile solution. This would enhance solubility for DSH and not negatively impact the other compounds of interest. The addition of acetonitrile immediately improved recoveries for DSH in matrix. Henceforth, it was promptly implemented in all method development.

One of the drawbacks of LIB 4597 has always been that it required two separate injections. The original LIB 4597 nitrofurans were analyzed using positive ionization mode with atmospheric pressure chemical ionization (APCI), and chloramphenicol was analyzed in negative ionization mode with electrospray ionization (ESI). Although it was not optimal for efficiency, it did provide the most sensitivity for the extremely low levels of residues that are required for

this analysis. During the development of LIB 4597, ESI and APCI both were evaluated to determine if a single injection could acquire all the compounds. With the instrumentation and the mobile phases used, it was felt as though none of these approaches provided the sensitivity needed. However, while working on the development of this method, it was noticed that one publication assayed multiple nitrofuran metabolites with ESI by using a lower concentrated ammonium acetate mobile phase than what is used in LIB 4597 [7]. By using this mobile phase and polarity switching, it was believed that the targeted nitrofurans and chloramphenicol might be acquired in a single acquisition.

The laboratory began using these types of mobile phase compositions found in other publications [7-9]. Although the different mobile phases and gradients referenced did work well for the nitrofurans, it was less than optimal for chloramphenicol. It was suspected that substituting acetonitrile for methanol could boost chloramphenicol response and that matching the same composition to our reconstitution solution would provide better long-term results. Therefore, acetonitrile was substituted for methanol. With the use of acetonitrile, the gradient had to be drastically changed. At this time, the decision was also made to switch to a longer column to improve chromatographic resolution.

During the method development process, it was discovered that the original PVDF syringe filters that were used for LIB 4597 were not acceptable for use per the manufacturer. This was because the reconstitution solution had been changed from 40% methanol to 40% acetonitrile. The switch was then made from PVDF to nylon. The nylon filters were acceptable for use with acetonitrile per the manufacturer, and they were readily available for use. Unfortunately, after analyzing multiple fortified matrix blanks, it was discovered that DSH has a strong binding to the nylon phase. This prompted a study to be conducted on various filter phases to determine which would be the best before starting the validation of the method. The study analyzed PVDF, PTFE, and nylon. The results were as expected with PTFE and PVDF being relatively equal for all compounds and the nylon again showing a strong affinity for the DSH. PTFE was chosen to be used in the validation efforts since it is recommended for use with acetonitrile, whereas PVDF is not recommended for use with acetonitrile.

As validation efforts began, it was noticed in the first two sets that the reagent blank and some laboratory fortified matrix blanks would have retention time shifts for AHD but no other compounds of interest. With it being only one compound, it was suspected the issue might be pH related. It was apparent that this problem would need to be addressed before continuing with validation. Therefore, a small amount of acidified mobile phase was added to the samples in question. As a result, the retention time for AHD shifted back to the expected time. With the discovery that a small amount of acid to adjust the pH would eliminate retention time issues for AHD, 0.1% formic acid was added to the

ammonium acetate mobile phase. Although no other retention time shifts for AHD were ever observed again, the drop in sensitivity for chloramphenicol was too substantial and made the acidified mobile phase not a viable option. This left acidifying the reconstitution solution as the only option.

A reconstitution solution consisting of a 2 mM ammonium formate in 0.1% formic acid and an acetonitrile solution (60:40 v/v) was evaluated with multiple sample sets. The reconstitution solution did not show any adverse effects on the chloramphenicol sensitivity, and no further significant AHD retention time shifts were observed. Unfortunately, with this additional method modification the previous validation work could not be used.

After the analysis of hundreds of sub-samples, it became apparent that the DSH absolute recoveries fluctuated a great deal more than the other analytes. Depending upon the matrix, the signal could be extremely low at the limit of quantitation. Further research into the issue revealed that DSH recovery is largely dependent upon the choice of solvents eluting the analytes from the SPE columns. Numerous solvents and different mixtures of solvents were extensively tested to provide the best results for not only DSH, but all compounds assayed in the method. Conclusive results showed that a combination of ethyl acetate and acetonitrile (90:10 v/v) provided the best overall results for all compounds.

Method Validation

The validation efforts focused on 10 different types of shrimp, one frog, one crab, and one crawfish matrix. These validation recovery studies were conducted according to the "U.S. Food and Drug Administration Guidance for Industry for Mass Spectrometry Confirmation and Identification of Animal Drug residues, and The Guidelines for Validation of Chemical Methods for the FDA Foods Program 3rd edition" [10, 11]. Each individual matrix was assayed on separate days.

During the course of validation, it was noted that the crawfish matrix analyzed had numerous coeluting peaks. This caused poor quantitation and led to the exclusion of this matrix from the validation study. For shrimp, frog, and crab, the validation consisted of a total of 159 different laboratory fortified matrix blanks and 45 matrix blanks. Method accuracies and precision using matrix matched extracted calibration standards were acceptable for the fortified matrices. All 159 assayed laboratory fortified matrix blanks met the required confirmation criteria for all residues of interest, and no matrix blanks or reagent blanks met the confirmation criteria for any residue. The validation results are outlined in Tables 6 – 8. Chromatography examples from low-level laboratory fortified blanks are illustrated in Figures 1 – 6.

CONCLUSION

A new quantitative and confirmatory method for chloramphenicol and five different nitrofuran metabolites was validated at the Arkansas Laboratory. This method uses LIB 4597 as the foundation of the extraction procedure; however, it incorporates some significant changes on the extraction to accommodate an additional analyte and reduce the overall cost of the method. The new instrument acquisition method incorporates a new mobile phase, gradient, and column so that all compounds can now be analyzed in a single acquisition. This reduces the amount of instrument run time, simplifies data processing, and reduces the number of required instruments for sample analysis. The substantial benefits from the cost saving measures, enhanced efficiency, and the addition of a nitrofuran drug make this method a viable option for regulatory laboratories to use for nitrofuran and chloramphenicol analysis in aquaculture.

REFERENCES

1. McCalla, D.R., *Mutagenicity of nitrofuran derivatives: Review.* Environmental Mutagenesis, 1983. **5**(5): p. 745-765.
2. Schilling, C.L., T; and Uden, D, *Chloramphenicol-Associated Aplastic Anemia.* Journal of Pharmacy Technology, 1988. **4**(2).
3. Cohen, S.E., E; Von Esch, M; Crovetti, J; and Bryan, G, *Carcinogenicity of 5-Nitrofurans and Related Compounds With Amino-Heterocyclinc Subsitutents.* Journal of the National Cancer Institute, 1975. **54**(4).
4. Veach, B.T.B., C.A.; Kibbey, J.H.; Fong, A.; Broadaway, B.J.; and Drake, C.P, *Quantitation of Chloramphenicol and Nitrofuran Metabolites in Aquaculture Products Using Microwave-Assisted Derivatization, Automated Solid-Phase Extraction and LC-MS/MS.* Lab. Inf. Bull. of US FDA, 2015(4597).
5. Veach, B.T.B., C.A.; Kibbey, J.H.; Fong, A.; Broadaway, B.J.; and Drake, C.P, *Quantition of Chloramphenicol and Nitrofuran Metabolites in Aquaculture Products Using Microwave-Assisted Derivatization, Automated SPE, and LC-MS/MS.* AOAC International, 2015. **98**(3).
6. Veach, B., T, *Determination of Chloramphenicol and Nitrofuran Metabolites in Cobia, Croaker, and Shrimp Using Microwave-Assisted Derivatization, Automated SPE, and LC-MS/MS-Results from a U.S. Food and Drug Administration Level Three Inter-Laboratory Study.* Journal of AOAC International, 2020. **103**: p. 1043-4051.
7. Guichard, P.L., M.; Hurtaud-Pessel, D.; and Verdon, E, *Confirmation of five nitrofuran metabolites including nifursol metabolite in meat and aquaculture products by liquid chromatogrphy-tandem mass spectrometry: Validatio according to European Union Decision 2002/657/EC.* Food Chemistry, 2021. **342**.
8. Barbosa, J.F., A; Mourao, J; NOronha da Silveira, M and Ramos, F, *Determination of Furaltadone and Nifursol Residues in Poultry Eggs by Liquid Chromatography-Electrospray Ionizatin Tandem Mass Spectrometry.* J. Agric. Food Chem., 2012. **60**: p. 4227-4234.
9. Wang, C.Q., L; Liu, X; Zhao, C; Zhao, F, Huang, F; Zhu, Z; and Han, C, *Determination of a metabolite of nifursol in foodstuffs of animal origin by liquid-liquid extraction and liquid chromatography with tandem mass spectrometry.* Journal of Separation Science, 2016. **40**(3): p. 671-676.
10. *Guidance for Industry — Mass Spectrometry for Confirmation of the Identity of Animal Drug Residues*. 2003: Fed. Regist. p. 31938–31939.
11. *Guidelines for the Validation of Chemical Methods for the FDA Foods Program*. 2015; 3 rd edition:[Available from: https://s27415.pcdn.co/wp-content/uploads/2020/01/64ER20-7/Validation_Methods/b-Chemical-Methods-Validation-Guidelines_3rd-ed_RSSC-508_final_12_06_19.pdf.

Table 6. Quantitative Data for Shrimp:
n = number of replicates.

Compound	Level of Interest ng/g	Cal Std 3 level (X) ng/g	% Recovery (% RSD)			MDL ng/g n = 7	LOQ ng/g n = 7
			¼ X n=70	X n=30	2X n=30		
SEM	0.5	0.800	89.9 (21)	97.0	97.4	0.0171	0.0543
AOZ	0.5	0.800	104 (10)	102	100	0.0168	0.0535
AHD	0.5	0.800	106 (8)	102	100	0.0251	0.0799
AMOZ	0.5	0.800	91.4 (15)	90.7	89.8	0.0204	0.0648
DSH	0.5	0.800	107 (9)	102	100	0.0234	0.0746
CAP	0.15	0.200	105 (21)	100	98.0	0.0141	0.0450

Table 7. Quantitative Data for Frog Legs:

Compound	Level of Interest ng/g	Cal Std 3 level (X) ng/g	% Recovery (% RSD)			MDL ng/g n = 7	LOQ ng/g n = 7
			¼ X n=7	X n=3	2X n=3		
SEM	0.5	0.800	105 (4)	94.5	100	0.0260	0.0828
AOZ	0.5	0.800	90.9 (7)	93.2	104	0.0395	0.126
AHD	0.5	0.800	103 (3)	97.6	103	0.0198	0.0631
AMOZ	0.5	0.800	96.1 (2)	101	102	0.0147	0.0467
DSH	0.5	0.800	95.5 (6)	94.1	101	0.0373	0.119
CAP	0.15	0.200	93.1 (4)	94.5	104	0.00657	0.0209

Table 8. Quantitative Data for Crab:

Compound	Level of Interest ng/g	Cal Std 3 level (X) ng/g	% Recovery (% RSD)			MDL ng/g n = 7	LOQ ng/g n = 7
			¼ X n=7	X n=3	2X n=3		
SEM	0.5	0.800	108 (2)	97.0	98.8	0.0174	0.0552
AOZ	0.5	0.800	102 (4)	102	100	0.0263	0.0838
AHD	0.5	0.800	105 (2)	98.6	98.8	0.0162	0.0516
AMOZ	0.5	0.800	104 (4)	99.4	101	0.0248	0.0789
DSH	0.5	0.800	96.8 (5)	92.6	94.4	0.0320	0.102
CAP	0.15	0.200	108 (4)	98.5	95.2	0.00610	0.0194

Figure 1. SEM Chromatogram of Fortified Blank at 0.200 ng/g.

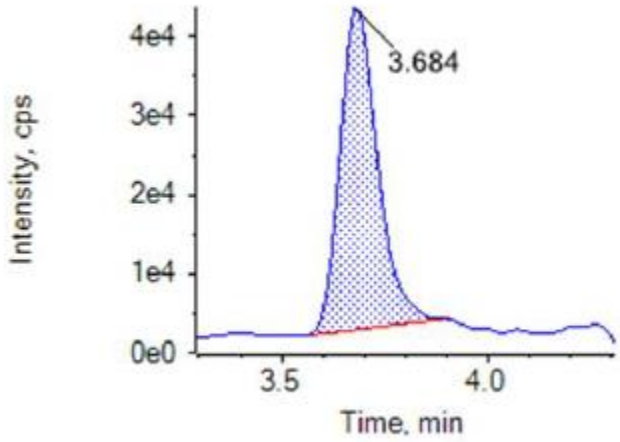

Figure 2. AOZ Chromatogram of Fortified Blank at 0.200 ng/g.

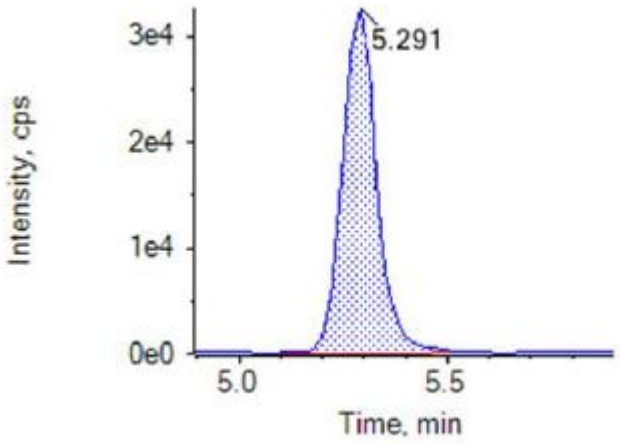

Figure 3. AHD Chromatogram of Fortified Blank at 0.202 ng/g.

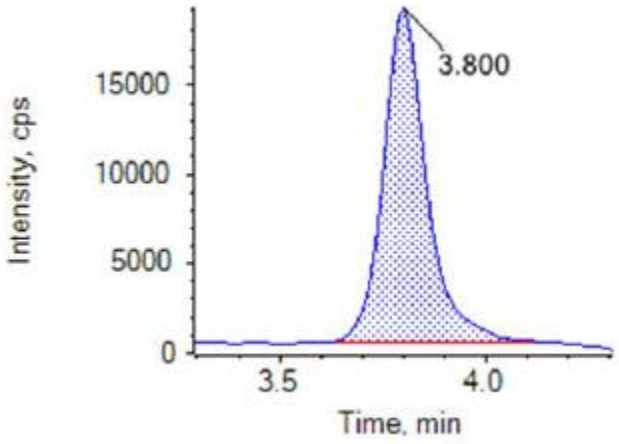

Figure 4. AMOZ Chromatogram of Fortified Blank at 0.200 ng/g.

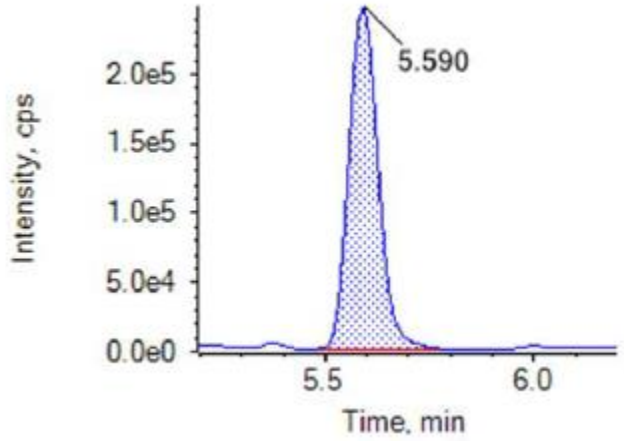

Figure 5. DSH Chromatogram of Fortified Blank at 0.200 ng/g.

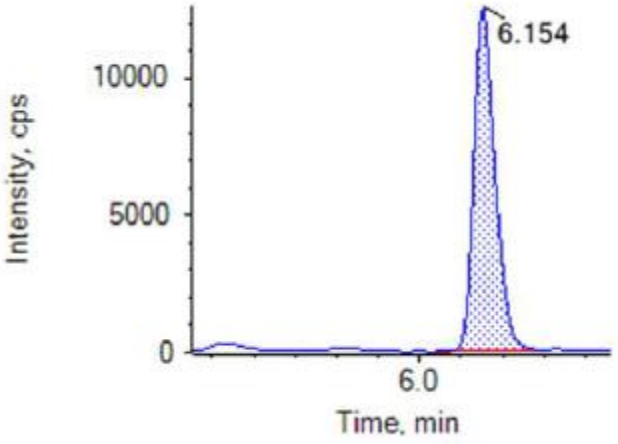

Figure 6. CAP Chromatogram of Fortified Blank at 0.0500 ng/g.