

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

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Title: Quantitation and Confirmation of Desfuoylceftiofur Cysteine Disulfide by LC-MS-MS		
Revision: 02	Replaces: CLG-CEF2.01	Effective: 12/07/20

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A. INTRODUCTION

1. Background / Summary of Procedure

Ceftiofur is a cephalosporin β -lactam antibiotic widely used for treating certain bacterial respiratory infections in swine and cattle. This is a method for the determination and confirmation of a ceftiofur metabolite, desfuroylceftiofur cysteine disulfide (DCCD). Tissue sample (~ 0.4 g) is extracted with 1% phosphate buffer. The crude extract is cleaned up by reversed-phase SPE. The analyte in the samples is quantitated and confirmed by LC-MS/MS in positive ESI mode.

2. Applicability

This method is suitable for the quantification and confirmation of DCCD in tissues as shown below:

	Quantitation ($\mu\text{g/g}$)	Confirmation ($\mu\text{g/g}$)
Bovine Muscle	0.5 - 2	≥ 0.5
Bovine Kidney	> 0.2	> 0.2
Porcine Muscle	1 - 4	≥ 1
Porcine Kidney	0.125 - 0.5	≥ 0.125

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. Analytical Balance – Mettler - Model X-205 Dualrange.
- b. Glassware – Class A
- c. Adjustable pipettes - Eppendorf 100 μL , 1000 μL and 5 mL or 10 mL - Brinkman Instruments Inc.
- d. Centrifuge – Thermo IEC GP-R8 –R - Centra
- e. Reciprocating Shaker - Eberbach Cat. No. 6010
- f. Spatulas - stainless steel or disposable
- g. Tubes - 15 mL Corning Cat. No. 05-538-51 - Fisher Scientific Co

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- h. Centrifuge tubes – polypropylene (PP), 15 mL, Falcon - VWR
 - i. SPE columns - Strata X, 33 µm, 60 mg, 3 mL – Phenomenex
 - j. Solvent evaporator - TurboVap LV with a 15 mL test tube rack – Zymark
 - k. PVDF syringe filter disk –0.2 µm Mfr. No. 9474051 – Xpertext
 - l. Plastic disposable – 3 mL syringe Cat. No. 03-377-21 - Fisher Scientific
 - m. Vortex mixer - Genie 2 mixer - Scientific Industries
 - n. Ultrasonic cleaner - Model 175D – Crest Ultrasonics Corp
 - o. Re-pipet dispensers - capable of dispensing variable volumes between 1 to 20 mL
 - p. Zymark® RapidTrace™ SPE Workstation - 3-mL syringe barrel columns – Biotage
 - q. Solid phase extraction vacuum manifolds - 12 and 24-port - Supelco/Sigma-Aldrich
 - r. Multi-tube vortexer - VX-2500 analog vortexer – VWR
 - s. Autosampler vials - 2 mL amber glass vial with pre-slit PTFE/silicone septa – Waters
 - t. Freezer capable of ≤ -10°C
 - u. Freezer capable of ~ -80°C
 - v. Nalgen cryogenic vials - Cat. No. 500-0020 - Thermo Scientific
 - w. Syringless filter device - PVDF 0.2 µm Cat. No. US203NPEAQU - Whatman
2. Instrumentation
- a. Thermo Scientific Ultimate 3000 RS Pump and RS Autosampler
 - b. Thermo Scientific Ultimate 3000 Liquid Chromatograph
 - c. Thermo Scientific TSQ Quantum Access Max with ESI source using TraceFinder version 3.2.512.0
 - d. Waters Acquity BEH C18 (1.7 µm 50 x 2.1 mm) (Cat. No. 186002350) with Waters Acquity BEH C18 Guard (1.7 µm 5 x 2.1 mm) (Cat. No. 186003975)

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents / solutions may be substituted.

1. Reagents

- a. Methanol (MeOH) – LC grade, Cat. No. AH230-4 - Burdick and Jackson

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- b. Acetonitrile (MeCN) – LC grade, Cat. No. AH015-4 - Burdick and Jackson
- c. Potassium phosphate monobasic - Fisher Scientific
- d. Potassium phosphate dibasic - Fisher Scientific
- e. Formic Acid (FA) - Reagent grade, Cat. No. F0507 - Sigma Aldrich

2. Solutions

- a. Mobile Phase A - 0.1% aqueous formic acid
Add 1.0 mL of formic acid to 1000 mL Milli-Q water and mix. This solution may be used for one month at HPLC conditions.
- b. Mobile Phase B - 0.1% formic acid in acetonitrile
Add 1.0 mL of formic acid to 1000 mL MeCN and mix. This solution may be used for one month at HPLC conditions.
- c. 1% Phosphate buffer (pH ~ 6.2)
Dissolve 8.0 g of KH₂PO₄ and 2 g of K₂HPO₄ in a 1 L volumetric flask using Milli-Q water as solvent. This solution may be stored for 2 months at room temperature.

D. STANDARD(S)

Note: Equivalent standards / solutions may be substituted. Purity and counter ions 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.

1. Standards Information

- a. DesfuroylCeftiofur Cysteine Disulfide
 - i. Molecular weight: 548.64
 - ii. Supplier: Toronto Research Chemicals - Cat No. - D289905
 - iii. Purity: Contains 5% Ceftiofur
 - iv. Storage: Store in freezer ≤ -10° C
- b. DesfuroylCeftiofur Cysteine Disulfide-d₃
 - i. Molecular weight: 551.66
 - ii. Supplier: Toronto Research Chemicals - Cat No. - D289907
 - iii. Purity: ~ 90%

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iv. Storage: Store in freezer $\leq -10^{\circ}\text{C}$

2. Preparation of Standard Solution(s)

a. DCCD stock solution - ~ 1 mg/mL

Weigh appropriate amount of DCCD in a weighing boat. Transfer it to a volumetric flask using Milli-Q water and mix well. Acidified water with a small amount of acetonitrile aids in the solubility of the stock as will gently heating the solution. Correct the concentration for purity. For example, weigh ~ 5.2 mg of DCCD (96% purity) and transfer it to a 5-mL volumetric flask using Milli-Q water and mix well to make a 1.0 mg/mL stock solution. Subdivide in aliquots of ~ 1 mL using cryogenic vials and store in an ultra-low (~ -80°C) freezer. The stability of this solution is up to 12 months in a ~-80°C freezer.

b. Internal Standard (IS: d3-methoxy-DCCD) stock solution - 0.37 mg/mL

Weigh appropriate amount of IS in a weighing boat. Transfer it to a volumetric flask using Milli-Q water for dissolution and mix well. Acidified water with a small amount of acetonitrile aids in the solubility of the stock as will gently heating the solution. Correct the concentration for purity. For example, weigh ~ 1.9 mg of DCCD (96% purity) and transfer it to a 5-mL volumetric flask using Milli-Q water and mix well to make a 0.37 mg/mL stock solution. Subdivide in aliquots of ~ 1 mL into cryogenic vials and store in a ~-80°C freezer. The stability of this solution is up to 12 months in a ~-80°C freezer.

c. DCCD intermediate solutions - 20 µg/mL

The DCCD intermediate solutions are prepared from a fresh stock solution or a freshly thawed vial of stock solution (see D.2.a, ~ 1 mg/mL). Calculate the volume of the stock solution that is required to prepare 25 mL of DCCD solution at a concentration of 20 µg/mL. Accurately measure and transfer the calculated volume of the concentrated stock solution (~ 1 mg/mL) into a 25 mL volumetric flask using an adjustable micropipette. Bring to volume with Milli-Q water and mix. Prepare aqueous DCCD working solutions from the corresponding Intermediate Stock Solution according to Table I. Use 10-mL volumetric flasks, adjustable micropipettes. For example, to prepare working standard at level 6 (8.0 µg/mL) working solution, transfer 4.0 mL of Intermediate Stock Solution to a 10-mL volumetric flask and dilute to the mark with Milli-Q water and mix well. Subdivide in aliquots of ~ 1 mL using cryogenic vials and store in a ~-80°C freezer. The stability of DCCD intermediate solution is up to 12 months in a ~-80°C freezer and 1 week in a 2-8 °C refrigerator.

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Table 1 - Preparation of intermediate solutions

Solution Level	(mL to aliquot)	Volume of Flask (mL)	Concentration ($\mu\text{g/mL}$)	Equivalent Conc. in Tissue* (ng/g)
L1	0.2	10	0.4	100
L2	0.3	10	0.6	150
L3	0.5	10	1.0	250
L4	1.0	10	2.0	500
L5	2.0	10	4.0	1000
L6	4.0	10	8.0	2000
L7	8.0	10	16.0	4000

* Fortification of 0.4 g of tissue with 100 μL of each intermediate solution.

d. IS working solution - 3 $\mu\text{g/mL}$

Transfer appropriate amount of IS stock solution (e.g., 810 μL of 0.37 mg/mL) to a 100 mL volumetric flask and add Milli-Q water to the mark. Mix well. Subdivide in aliquots of ~ 5 mL and store in a $\sim -80^{\circ}\text{C}$ freezer. The stability of this solution is up to 12 months when stored in a $\sim -80^{\circ}\text{C}$ freezer.

3. Preparation of External Calibration Curve

- a. Bring DCCD intermediate solutions (L1 – L7) and IS working solution to room temperature and shake.
- b. To prepare 1 mL of the individual solvent-based working standards, transfer 100 μL of the IS working solution into 7 amber autosampler vials. Add 100 μL of each of the DCCD intermediate standards (L1 – L7) to the autosampler vials followed by 800 μL of Milli-Q water and vortex-mix. The working standard at L3 can be injected as the system suitability sample for the batch.

E. SAMPLE RECEIPT AND PREPARATION

Homogenize tissue samples using a blender or food processor.

F. ANALYTICAL PROCEDURE

1. Preparation of Controls and Samples

- a. Weigh 0.4 g (± 0.008 g) test and control samples (recovery, blank, and check sample, if needed) while still partially frozen into 15mL Falcon polypropylene centrifuge tubes. Record the weight to three decimal places.

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- b. Add 100 μ L of the IS working solution to all samples and controls.
- c. Prepare a fortified analyst recovery. Add 80 μ L of the L4 intermediate standard solution to the appropriate tube for bovine kidney, 100 μ L of the L5 intermediate for bovine muscle, 100 μ L of the L3 intermediate for porcine kidney, or 100 μ L of the L6 intermediate for porcine muscle. Fortify analyst check sample, if needed.

2. Extraction Procedure

- a. Add 3.5 mL 1% phosphate buffer to all sample tubes.
- b. Shake the samples for 10 min on a shaker.
- c. Centrifuge the tubes at ~4500 rpm for 20 min at room temperature.
- d. The supernatant is cleaned up by SPE as following:
 - i. Condition Strata-X 60 mg, 3 mL SPE cartridges with 2 mL MeOH, followed by 2 x 2 mL Milli-Q water.
 - ii. Load the supernatant onto the SPE cartridge at ~ 1 mL/min. Do not apply vacuum.
 - iii. Wash SPE column with 2 mL Milli-Q water.
 - iv. Elute analytes from the SPE columns with 2 mL of 50% MeCN/H₂O (v/v) into clean 15 mL polypropylene centrifuge tubes at ~ 1 mL/min.
- e. Evaporate MeCN under nitrogen at about 15 psi and ~40°C to \leq 1 mL.
- f. Bring the volume in all tubes to the 1-mL mark with Milli-Q water and vortex. Centrifuge the sample at ~4500 rpm at room temperature for 10 min. Filter through a 0.2 μ m PVDF filter into pre-labeled LC-MS/MS vials. Inject 10 μ L into the LC-MS/MS.

Note: The working standards and extracts may be stored at 2-8°C for 2 days or at -20°C or lower for 1 month.

3. Instrumental Settings

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

UHPLC conditions:

Column: Waters Acquity BEH C18 1.7 μ m, 50 x 2.1 mm

Waters Acquity BEH C18 1.7 μ m 5 x 2.1 mm

Column temperature: 30°C

Autosampler temperature: 10°C

Injection volume (μ L): 10

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Run time (min): 7

Flow (mL/min): 0.300

Mobile phase: Binary gradient Mobile Phase A: Mobile Phase B

Strong Needle Wash: 50%MeCN/water (v/v)

Weak Needle Wash: 10%MeCN/water (v/v)

Mobile Phase A: 0.1% aqueous formic acid

Mobile Phase B: 0.1% formic acid in MeCN

Table 2 - UPLC mobile phase gradient composition

Time (minutes)	% Mobile Phase A	% Mobile Phase B
0	100	0
1.0	100	0
4.0	65	35
4.1	5	95
5.1	5	95
6.2	100	0
7.0	100	0

Table 3 - Mass Spectrometer Parameters

Parent	Product	Width	Time	CE	Q1 PW	Q3 PW	Tube Lens
549.053	183.041	0.200	0.100	26	0.70	0.70	91
549.053	240.954	0.200	0.100	20	0.70	0.70	91
552.031	243.943	0.200	0.100	20	0.70	0.70	85

Instrument Method Settings TSQ Quantum

MS Run Time: 7.00 min

Divert Valve: in use

0.00 to 0.49 inject to waste

0.49 to 5.47 Load to detector

5.47 to 7.00 to waste

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Tune Parameters TSQ Quantum:

Spray Voltage: 3600

Vaporizer Temperature: 325

Sheath Gas Pressure: 30

Ion Sweep Gas Pressure: 0

Aux Gas Pressure: 15

Capillary Temperature: 350

Tube Lens Offset: 60

Skimmer Offset: 0

4. Sample Set

- a. Solvent Blank (if applicable)
- b. System Suitability Standard (L3 standard)
- c. Standards
- d. Solvent Blank
- e. Blank
- f. Recovery
- g. Intra-laboratory check sample (if needed)
- h. Up to 22 Samples
- i. Other Solvent Blanks (if needed)
- j. Reinjection of L3 standard

G. DECISION CRITERIA / CALCULATIONS

1. Calculations for determinative analysis

- a. Use the instrument software to smooth and integrate the product-ion chromatogram of the most intense peak of each analyte and to calculate the signal-to-noise ratio. Inspect the automatic peak integrations and manually correct if base-to-base integration was not achieved. Manual integrate peaks in the blanks, if necessary.
- b. Use the internal standard to prepare a linear calibration curve. Use 1/X weighting and plot the response of the analyte in each standard versus its nominal concentration.

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$$\text{Response} = \frac{\text{Analyte peak area} \times \text{IS concentration}}{\text{IS peak area}}$$

- c. Calculate the linear regression parameters for the calibration curve and interpolate the concentrations of the samples from the regression parameters. Do not use zero as a regression data point.
- d. Export the processed data into Microsoft Excel or equivalent spreadsheet program for further calculations. The experimental analyte concentrations (except for standards, controls and fortified samples) are corrected for the mass difference of the individual samples to the nominal mass of sample used to calculate the concentration of the analytes in the calibration standards. Use the equation:

$$\text{Mass corrected concentration (ng/g)} = \frac{\text{experimental conc.} \times \text{nominal mass}}{\text{Mass of sample (g)}}$$

- e. The accuracy of fortified samples is calculated using the following equation:

$$\text{Accuracy (\%)} = \frac{\text{Mass corrected concentration} \times 100}{\text{Nominal concentration}}$$

- f. If an interference is observed in the negative control at the retention time of the analyte, the amount of the interference must be < 5% of the analyst recovery amount.

$$\text{Percent interference} = \frac{\text{Amount of interference in negative control} \times 100}{\text{Amount of analyst recovery}}$$

- g. If carryover is observed from the highest calibration standard to the next blank injection, calculate the percent carryover as follows:

$$\text{Percent carryover} = \frac{\text{Area of blank injection} \times 100}{\text{Area of Level 7 standard}}$$

- h. Report values rounded to one decimal place.

2. Calculations for the confirmatory analysis

- a. Integrate the two product-ion chromatograms for each analyte. Inspect the automatic peak integrations and manually correct if base-to-base integration was not achieved.
- b. Calculate the percent interference as described in G.1.f and the percent carryover as described in G.1.g

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- c. Calculate the ion-abundance ratio from the two monitored ions using the most intense ion as the denominator.

$$\text{Ion-abundance ratio} = \frac{\text{Area of less intense product ion}}{\text{Area of most intense product ion}}$$

- d. Compare the ion-abundance ratios of the unknowns and fortified samples to those of the standards. Confirmation of the presence of the analyte in a sample requires the relative abundance of the product ions in the sample to be within $\pm 10\%$ (absolute) their mean abundance in the standards. For example, if the average ion-abundance ratio of the standards is 0.74, the acceptance range for the samples is 0.64 – 0.84.

3. Acceptance criteria for quantitation

- a. The analyte quantitation peak must be present with a signal-to-noise > 10 and its retention time must agree within $\pm 2\%$ (relative) its mean retention time in the standards.
- b. If an interference is observed in the negative control at the retention time of the analyte, the amount of the interference must be $< 5\%$ of the analyst recovery amount.
- c. Carryover from the highest standard to the following blank injection is $< 1\%$.
- d. The coefficient of determination (r^2) for the calibration curve is > 0.99 .
- e. The deviations of calibration standards are within 15% of their nominal values.
- f. The last injection of SS L3 should be within $\pm 15\%$ of its nominal value.

4. Acceptance criteria for confirmation

- a. The two product ions associated with the analyte are present and have a signal-to-noise (S/N) ratio $> 10:1$. Visually inspect the chromatograms of control and low level standards to verify that the signal-to-noise ratio calculated by the processing software is acceptable.
- b. The retention time of each of the two product ions of the analyte in the samples must be within 2% the mean retention time of the analyte in the standards.
- c. Carryover from the highest level standard into the following blank injection is $< 1\%$.
- d. If interference is observed in the negative control at the retention time of the

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analyte, the amount of the interference must be < 5% of the analyst recovery amount.

- e. The abundance-ion-ratio of the analyte in the samples is arithmetically within 10% of the average ion-abundance-ratio of the standards. For example, if the average ion-abundance ratio of the standards is 0.74, the acceptance range for the ion-abundance-ratio of the samples is 0.64 – 0.84.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Personal Protective Equipment — Safety eyewear, protective gloves, and lab coat.
2. Hazards
Consult all Safety Data Sheets (SDS) associated with the method.
3. Disposal Procedures
Follow federal, state and local regulations.

I. QUALITY ASSURANCE PLAN

1. Performance Standard - Criteria must meet guidelines in section G.3 for quantitation and G.4 for confirmation

For quantitation:

<i>Analyte</i>	<i>Analytical Range (µg/g)</i>	<i>Acceptable Recovery (%)</i>
Bovine Muscle	0.5 - 2	81 - 126
Bovine Kidney	> 0.2	83 - 120
Porcine Muscle	1 - 4	83 - 122
Porcine Kidney	0.125 - 0.5	89 - 119

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2. Intra-laboratory Check Samples

a. Acceptability criteria.

Refer to I. 1.

If unacceptable values are obtained, then:

- i. Investigate following established procedures.
- ii. Take correction or corrective action as warranted.

J. APPENDIX

1. References

- a. The US Food and Drug Administration. Guidance for Industry No.118. Mass Spectrometry for Confirmation of the Identity of Animal Drug Residues, May 1, 2003.
<http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/ucm052658.pdf>
- b. Feng, S., Chattopadhaya. C., Kijak. P.J., Chiesa O.A., and Tall E.A. Determinative and Confirmatory Method for Ceftiofur Metabolite Desfuroylceftiofur Cysteine Disulfide in Bovine Kidney by LC-MS/MS. Journal of Chromatography B. 2012, 898: 62– 68.

2. Chromatograms/spectra

Reserved

K. APPROVALS AND AUTHORITIES

1. Approvals on file.
2. Issuing Authority: Director, Laboratory Quality Assurance Staff