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Title: Determination for Ceftiofur-Related (DCA) Residues by HPLC		
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Contents

A. INTRODUCTION

1. Background

Ceftiofur (NAXCEL®/EXCENEL®) is used in several countries for the treatment of bacterial respiratory diseases in cattle and swine. Ceftiofur is rapidly metabolized by loss of the furoic acid moiety to produce the central active residue, desfuroylceftiofur (DFC). [Beconi-Barker, 1996] This metabolite has been shown to rapidly conjugate with cysteine and glutathione, to dimerize to a disulfide dimer, and to bind to proteins. Since parent ceftiofur has such a short half-life once administered, DFC becomes the “metabolite” of interest from a residue monitoring perspective. Muscle and kidney are target tissues for a regulatory assay of ceftiofur and related metabolites in bovine and swine. The development and validation of a determinative method is, therefore, required to monitor for violative residues above MRL. This report describes a method that was developed and validated for the determination of ceftiofur and related metabolites in bovine and swine muscle and kidney.

2. Summary of Procedure

In this procedure, tissue is homogenized and incubated with a solution of dithioerythritol (DTE) which cleaves ceftiofur and desfuroylceftiofur-related metabolites from proteins or other sulfur-containing compounds, yielding a common desfuroylceftiofur (DFC) moiety. The DFC is reacted with iodoacetamide to yield the stable desfuroylceftiofur acetamide derivative (DCA). This derivatization does not distinguish between parent or metabolites and all are converted to the DCA derivative. The derivative is first extracted onto a C-18 solid phase extraction column. Further purification is performed on a strong anion exchange (SAX) extraction column, followed by clean-up using a strong cation exchange (SCX) column. The derivatized residues are then analyzed via gradient UHPLC using a C-18 column with UV absorbance detection.

3. Applicability

This method is suitable for the determination of ceftiofur and related metabolites in bovine and swine muscle and kidney at levels ≥ 0.1 µg/g (ppm).

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. Water bath, shaking, Lab Line Co.

b. Visiprep Solid Phase Extraction Vacuum Manifold, Supelco, equipped with PTFE or plastic tips.

c. Mechanical shaker - flatbed, 2 speed, Eberbach.

d. Beckman Zeromatic Model SS-3 pH meter, Beckman Instruments.

e. Sorvall Model RC5C centrifuge equipped with SS-34 rotor, Sorvall Instruments.

f. Rainin Pipetman Model P-200 and P-1000 Digital Pipettes, Rainin Instruments.

g. Repeater Pipet: Eppendorf, with Combitips (12.5, 25 mL), Brinkman Instruments.

h. Tubes, centrifuge, 50 mL round bottom, polypropylene, Nalgene.

i. Multipurpose container, 4.5 oz, (a.k.a. Baxter Cups), Baxter Healthcare. (NOTE: The cork borer can be used to bore a hole in the plastic lid for easy access by the homogenizer probe.)

j. Vial, scintillation, 20 mL, borosilicate glass, disposable. Kimble.

k. Mega Bond Elut C-18 SPE cartridges, 1 g 6 mL, Agilent Sample Preparation Products; Part number 1225-6001.

l. Bond Elut LRC SAX SPE cartridges, 500 mg 10 mL, Agilent Sample Preparation Products; Part number 1211-3043.

m. Bond Elut LRC SCX SPE cartridges, 100 mg 10 mL, Agilent Sample Preparation Products; Part number 1211-3013; or equivalent.

Note: SPE (Solid-Phase Extraction) cartridges are an important part of this procedure. Products from other vendors should be evaluated before use.

2. Instrumentation

a. LC system: (HPLC, or UHPLC, etc.): Agilent1290 Infinity UHPLC Model G422A Binary pump and Model G4226A Autosampler.

b. UV Detector: Model G1314E variable wavelength detector with 266nm-capable UV wavelength detector, and appropriate data collection/integration software.

c. Column: Phenomenex Kinetex 1.7µm, 2.1 x 50mm (Cat. No. OOB-4475-AN).

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents / solutions may be substituted. The stability time frame of the solution is dependant on the expiration date of the components used or the listed expiration date, whichever is soonest.

1. Reagents

a. Acetonitrile, high purity, Baxter Healthcare Corp., Burdick and Jackson.

b. Calcium chloride ($CaCl_2$) anhydrous, Mallinckrodt.

c. Dithioerythritol, (DTE), 99%, Aldrich Chemical Co.

d. Glacial acetic acid, AR grade, Mallinckrodt.

e. Iodoacetamide, 97%, Aldrich Chemical Co.

f. Phosphoric acid, 85%, AR grade, Mallinckrodt.

g. Potassium chloride (KCl), AR grade, Mallinckrodt.

h. Potassium hydroxide (KOH), 45% w/v, AR grade, Mallinckrodt.

i. Potassium phosphate monobasic (KH_2PO_4), AR grade, Mallinckrodt.

j. Methanol, high purity, Baxter Healthcare Corp., Burdick and Jackson.

k. Sodium borate ($Na_2B_4O_7 \bullet 10H_2O$), AR grade, Mallinckrodt.

l. Sodium chloride (NaCl), AR grade, Mallinckrodt.

m. Sodium hydroxide (NaOH), 50% w/v, AR grade, Mallinckrodt.

n. Trifluoroacetic acid (TFA), 99%, Aldrich Chemical Co.

o. Water (high purity), Baxter Healthcare Corp, Burdick & Jackson.

2. Solutions

a. Borate buffer, 0.05M, pH~9: Weigh 19g of sodium borate and 3.7g of KCl, and q.s. to 1000 mL with water.

b. Phosphate buffer, 0.025M, pH=7: Weigh 3.4g of KH_2PO_4 and q.s. to 1000 mL with water. Adjust pH with KOH.

c. Extracting Solution (0.4% DTE w/v): Dissolve 1g of DTE in 250 mL borate buffer. The Extracting Solution (DTE) has a limited shelf life and MUST be prepared daily.

d. Iodoacetamide Solution (14% w/v): Dissolve 7g in 50 mL of phosphate buffer. The Iodoacetamide Solution has a limited shelf life and MUST be prepared daily.

e. 0.01N Sodium hydroxide: Place in volumetric flask 0.52 mL NaOH q.s to 1000 mL with water.

f. 0.1M Sodium chloride: Weigh 5.9g of NaCl and q.s. to 1000 mL with water.

g. 0.1M Calcium chloride: Weigh 11.1g of $CaCl_2$ and q.s. to 1000 mL with water.

h. 25 % Phosphoric acid: Place in volumetric flask 25 mL phosphoric acid q.s. to 100 mL with water.

i. 5 % Acetic acid: Place in volumetric flask 5 mL acetic acid q.s. to 100 mL with water.

j. C-18 Elution Solution: Mix 15:85 acetonitrile: water.

k. SAX Prewash Solution: Mix 25:75 methanol: 0.1M NaCl.

l. SAX Elution Solution: Mix 5:95 acetonitrile: 5% acetic acid in water.

m. SCX Prewash Solution: Mix 25:75 methanol: 0.1M $CaCl_2$.

n. SCX Elution Solution: Mix 5:95 acetonitrile: 0.1M NaCl.

o. HPLC Mobile phases:

i. Mobile Phase A (0.1 % TFA in water): 1 mL trifluoroacetic acid q.s. to 1000 mL with water.

ii. Mobile Phase B (0.1 % TFA in acetonitrile): 1 mL trifluoroacetic acid q.s. to 1000 mL with acetonitrile.

D. STANDARD(S)

Note: Equivalent standards / solutions may be substituted. Purity and counterions are to be taken into account when calculating standard concentrations. The stability time frame of the solution is dependant on the expiration date of the components used or the listed expiration date, whichever ends sooner.

1. Standard Information

a. Name: Ceftiofur hydrochloride

b. Molecular Weight: 559.02 ($C_{19}H_{16}N_5O_7S_3$ ●HCl)

c. Supplier: The Pharmacia & Upjohn Co.

d. Purity: 893 µg/mg potency as ceftiofur free acid or as labeled.

e. Storage: Store frozen at -10°C with desiccant.

2. Preparation of Standard Solution(s)

a. Ceftiofur Reference Solution 1 (100 µg Ceftiofur Free Acid Equivalents) (CFAE)/mL):

Dissolve an accurately weighed amount of ceftiofur hydrochloride, approximately 11.2 mg of ceftiofur hydrochloride into a 100 mL volumetric flask and bring it to volume with phosphate buffer to give approximately 100 µg of ceftiofur free acid/mL stock solution. This weight is based on potency of 893 µg/mg, which may change with different lots. Transfer this solution into 3-mL freezer-safe vials and store at < -10°C for up to 6 months.

b. Ceftiofur Reference Solution 2 (10.0 µg CFAE/mL): Prepare fresh daily

Dispense 0.500 mL of the Ceftiofur Reference Solution 1 into a 5-mL volumetric flask and bring it to volume with phosphate buffer.

c. Ceftiofur Reference Solution 3 (1.0 µg CFAE/mL): Prepare fresh daily

Dispense 0.500 mL of the Ceftiofur Reference Solution 2 into a 5-mL volumetric flask and bring it to volume with phosphate buffer.

3. Preparation of Calibration Curve

Prepare the Calibration Standards by adding aliquots (see Table 1) of Ceftiofur Reference Solution 1, 2 or 3 to separate 50 mL tubes, each containing 15 mL of the extracting solution. Derivatize and process the standards according to the procedures below, beginning at the incubation step in Section F.2.d

Table 1 Preparation of Calibration Standards

Calibration Curve Standard Concentration (µg CFEA/g tissue)	Ceftiofur Reference Solution 1 (µL)	Ceftiofur Reference Solution 2 (µL)	Ceftiofur Reference Solution 3 (µL)
0.075			75.0
0.25			250.
0.50		50.0	
2.5		250.	
7.5	75.0		
12.5	125		

E. SAMPLE PREPARATION

Samples collected fresh must be kept cold before and during shipping to the laboratory. Once received at the laboratory, samples must be frozen (< -10 °C) prior to grinding if they cannot be prepared on the day of receipt. Once frozen, the sample should be allowed to thaw, while keeping it as cold as possible. Dissect away fat and connective tissue. Grind tissue in blender or vertical cutter-mixer until homogeneous. Store samples frozen (< -10 °C) prior to analysis.

F. ANALYTICAL PROCEDURE

1. Preparation of Controls and Samples

a. Weigh 2 portions of 10 g of a known blank tissue into separate Baxter cups. Note(s): Weigh an additional 10 g portion for check sample, if needed. Weigh appropriate controls for each tissue combination for samples to be analyzed.

b. Fortify the recovery cup with appropriate Ceftiofur Reference Solution according to Table 2 to attain the necessary tissue equivalent concentration.

Table 2 Fortification of Tissues

Concentration (µg CFAE/ g tissue)	Aliquot of Ceftiofur Reference Solution 1	Aliquot of Ceftiofur Reference Solution 2	Aliquot of Ceftiofur Reference Solution 3
0.0	-	-	-
0.10	-	-	1.00mL
0.25		0.250 mL	
0.40		0.400 mL	
1.0	-	1.00 mL	-
2.0	0.200 mL		
10	1.00 mL	-	-

Note: Fortify bovine kidney at 0.40 µg/g, bovine muscle at 1.0 µg/g, swine kidney at 0.25 µg/g, and swine muscle at 2.0 µg/g per established tolerances (see A.3). Changes to these recoveries must be in accordance with changes in established tolerances and be within the established range of the method.

2. Extraction Procedure

a. Place 10.0 ± 0.1 g of tissue into a Baxter cup and add 140 mL of the fresh Extracting Solution to the controls and samples and shake on high on a flat-bed shaker for 10 minutes.

Note: Tissue may require centrifugation before proceeding (e.g. 40 mL in a 50 mL tube at 4000RPM for 5 minutes).

b. Transfer 15.0 mL (1 g tissue equivalent) of the above final homogenate to a 50 mL centrifuge tube.

STOPPING POINT

c. Incubate the centrifuge tubes in a shaking water bath at 50 ± 1°C for 15 minutes at 20 - 30 RPM.

d. To derivatize add 3 mL of the iodoacetamide solution to each tube.

Note: The iodoacetamide solution has a limited shelf life and must be prepared daily.

e. Mix the tubes well and let them sit at room temperature for 30 minutes.

STOPPING POINT

f. Centrifuge the tissue homogenate samples at approximately 48,000 x g for 20 minutes at 4°C.

STOPPING POINT

g. Precondition the C-18 SPE cartridges with 4 mL of methanol followed by 5 mL of phosphate buffer.

h. Charge the supernatant onto the cartridges using gravity feed. (See Notes in Section J.3.a)

i. Wash the cartridges with 5 mL of phosphate buffer, followed by 3 mL of 0.01N sodium hydroxide.

j. Insert the scintillation vial to collect the eluate, and put the cartridges back on the manifold.

k. Add 3 mL of the C-18 Elution Solution and collect the eluate, allowing it to drain by gravity feed.

l. Use increased vacuum to recover the remaining solution in the cartridge.

m. Remove the collection vials

n. Dilute with 15 mL of water to give a total volume of 18 mL.

STOPPING POINT - If stopping, store sample solutions in a **refrigerator** overnight. Bring solutions to room temperature before continuing analysis.

o. Mix the contents well.

p. Precondition the SAX cartridges with 2 mL of methanol followed by 2 mL of SAX Prewash Solution and two times with 1 mL of water.

q. Transfer the samples (diluted extract from C-18 SPE) to the SAX cartridges, allow to drain by gravity feed and wash with 1 mL water. (See Notes in Section J.3.a)

r. Place new collection vials within the manifold and place the cartridges back on it.

s. Elute the cartridge contents with 2.5 mL of SAX Elution Solution by gravity feed then emptied with increased vacuum.

STOPPING POINT

t. Add 10 mL of water to each of the collection vials for a total of 12.5 mL.

u. Mix well.

v. Precondition the SCX cartridges with 1 mL of methanol followed by 2 mL of SCX Prewash Solution and twice with 2 mL of water.

w. Transfer the diluted extract from the SAX cleanup to the SCX cartridges and allow to drain by gravity feed. Wash with 1 mL water.

x. Elution of the Desfuroylceftiofur Acetamide (DCA) Derivative: Prior to elution, the SCX columns should be dried with high vacuum for up to 30 seconds to remove most of the solvent. Dry the tips within the manifold using a Kim- Wipe if necessary.

y. Place the new collection tubes into the manifold.

z. Elute the cartridge contents with 2.5 mL of SCX Elution Solution using gravity

feed, then recover completely with increased vacuum.

aa. Mix well and transfer the extract to the appropriate autosampler vial.

bb. Analyze this extract by HPLC.

Note: If stopping, store samples in a freezer. Thaw solutions before continuing analysis.

3. Instrumental Settings

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

a. Mobile phase:

Mobile Phase A – 0.1 % TFA in water
Mobile Phase B – 0.1 % TFA in acetonitrile

Allow column to equilibrate at initial conditions.

b. UHPLC gradient program: (Table 3)

Table 3 LC gradient

Time (min)	**%A**	**%B**
0.00	98	2
10.00	90	10
10.01	50	50
10.50	50	50
10.90	98	2
12.50	98	2

c. Autosampler program

i. Flow rate: 1.2 mL/min

ii. Column temperature: 35^{o}C

iii. Injection volume: 20 µL

iv. Run time: 12.5 min

v. Detector settings: 266 nm

4. Injection sequence / Sample Set

   Example Injection sequence

   a. External curve at 0.0750, 0.250,0.500,2.50,7.50,12.5 µg/g
   b. Recovery
   c. Blank
   d. Check sample (if necessary)
   e. Up to 19 samples
   f. External curve standard or recovery

G. CALCULATIONS / IDENTIFICATION

1. Calculations

   A standard curve is generated from the DCA peak area vs. the ceftiofur concentration (µg/mL) of the standards using least squares regression. The accuracy of the regression (observed X / backcalculated X) must be checked and recorded. Weighted regression ($1/conc^2$, where conc = concentration) generally provides the best fit. (See Notes in Section J.3)

   Sample DCA concentrations are calculated by interpolating the sample DCA peak area into the standard curve. The sample concentration in the tissue is calculated as follows.

$$\text{Sample Concentration (µg/g)} = \frac{(\text{DCA Area - intercept (a)}) \text{ X Dilution Factor}}{\text{Slope (b) x 1g (tissue equivalent)}}$$

   Calculations are usually performed by the computer data system and allow for complete documentation of the calibration curve. For a sample which exceeds the uppermost point on the curve, the final extract shall be diluted and reinjected for the response to be within the range of the curve. Add the dilution factor into the calculation above as needed.

2. Criteria

   If the recovery of the fortified sample(s) or the curve correlation coefficient is unacceptable, the samples may be re-injected and analyzed. If the re-analysis still produces an unacceptable recovery, the sample set shall be rejected and the extraction repeated.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment — Protective clothing, eyewear, and gloves, where applicable.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Acetonitrile	Flammable, toxic, may be fatal if inhaled or absorbed.	Use only in a fume hood. Keep away from flame or heat.
Methanol	Flammable, harmful if swallowed.	Use only in a fume hood. Keep away from flame or heat.
Glacial Acetic Acid	Harmful if inhaled. Causes skin and eye burns.	Use only in fume hood. Wear protective clothing, gloves and safety glasses.
Phosphoric Acid	Corrosive. Causes skin burns and eye burns.	Use only in a fume hood. Wear suitable protective clothing, gloves and safety glasses.
Trifluoroacetic acid	Harmful if inhaled and causes skin burns and eye burns.	Use only in a fume hood. Wear suitable protective clothing, gloves and safety glasses.
Sodium Hydroxide	Corrosive. Contact with liquids can result in burns and severe skin, eye and respiratory irritation.	Use only in a fume hood. Wear gloves and safety glasses.
Potassium Hydroxide	Corrosive. Contact with skin and eyes causes burns. Very harmful if inhaled.	Use only in fume hood. Wear protective clothing, gloves and safety glasses.
Iodoacetamide	Harmful if inhaled and causes skin and eye irritation	Use only in fume hood. Wear protective clothing, gloves and safety glasses.
Dithioerythritol	Irritating to eyes, respiratory system and skin	Use only in fume hood. Wear protective clothing, gloves and safety glasses.
Ceftiofur Std	Harmful if inhaled. Causes skin and eye irritation.	Use only in a fume hood. Wear protective clothing, gloves and safety glasses.

3. Disposal Procedures

   Follow local, state and federal guidelines for disposal.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

Tissue	*Analytical Range*	*Acceptable Recovery*
Kidney	0.1 µg/g – < 0.4 µg/g	53 - 139%
	0.4 µg/g – 10 µg/g	67 - 118%
Muscle	0.1 µg/g – < 0.4 µg/g	64 - 134%
	0.4 µg/g – 10 µg/g	75 - 119%

$R^2 \geq 0.995$, where R^2 = curve correlation coefficient

2. Critical Control Points and Specifications

   None

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. Sample Condition upon Receipt - Cool or Frozen

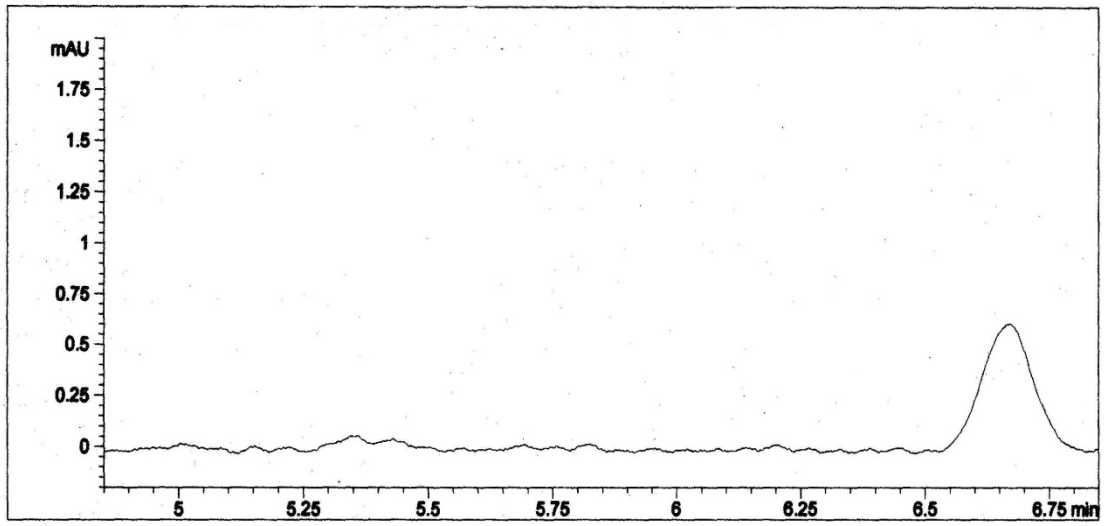
J. APPENDIX

1. References

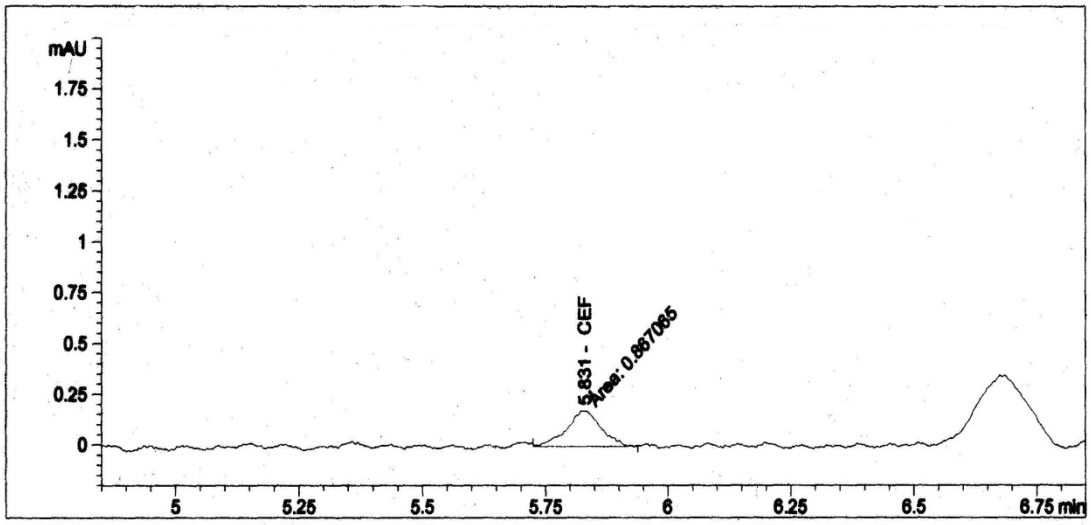
Hornish, Rex E., and Boof, Ryan D., Laboratory Procedure for Determinative Method for Ceftiofur-Related in Bovine and Swine Muscle and Kidney(HPLC-DCA),Pharmacia Animal Health a0067665, version 2.0, pp1-45,(2002).

2. Chromatograms

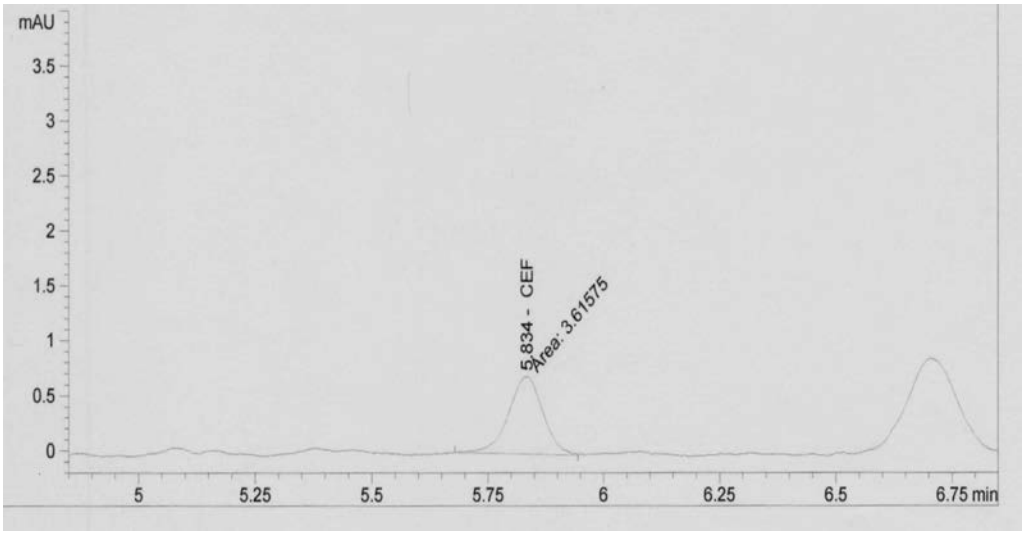
a. Blank



b. 0.1 µg/g Standard



c. 0. 4 µg/g Recovery



3. Notes

a. Solid-phase extraction is a routine laboratory technique, though differences in style may affect success. The use of light positive pressure and/or gentle vacuum is acceptable to start columns dripping or to maintain flow, but the flow should not exceed 1 mL/min.

b. The standard curve calibration using weighted regression may be corrected if necessary by dropping outlying standards which don't meet the ±10% backcalculated criteria. Care should be used while adjusting the weighted regression since the $1/conc^2$ weight can cause significant shifts to the curve depending upon the levels of the standards and the concentration of a point which is dropped. Especially at the lowest end of the calibration curve, dropping the lowest point reduces the total weight significantly and improve fit, while dropping the second-lowest point can sometimes improve fit while preserving the range of the standard curve.

c. If weighted regression is not readily available, the calibration data (concentrations and peak heights) can be transformed and a conventional nonweighted least squares regression can be used to calculate the slope and intercept variables from the same data as if ($1/conc^2$) weighting were used. The data is transformed and new variables will be calculated from the Standard Concentration (X) and DCA Peak Response (Y) data from calibration standards. The Y/X ratio is used as the surrogate dependant variable (Y'), and the 1/X ratio

is used as the surrogate independent variable (X'). Common LS regression using these terms (do not include the 0,0 origin) will calculate the slope and intercept terms, which now must be swapped so that the calculated Intercept is equal to the SLOPE from the ($1/conc^2$) weighted regression, while the calculated Slope is equal to the INTERCEPT from the weighted regression. Calculate the concentrations of samples as detailed above. Multiply by a dilution factor if necessary.

d. Tissue homogenates have been shown to be viable for at least 48 hours when stored at < -10°C. If a sample needs to be re-assayed in order to provide for dilution or other lab failure, a frozen homogenate may be used as long as the QA samples are also included in the repeat assay to control for storage.

K. APPROVALS AND AUTHORITIES

1. Approvals on file.

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