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

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Title: Determination of Dipyrone-Related Residues by HPLC		
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Contents

A. INTRODUCTION

1. Theory

Dipyrone-related residues are extracted from the homogenized muscle tissue with buffer. The extract is filtered and further purified by passing through a C18 solid phase extraction column. The residues are eluted with methanol. The eluate is evaporated to near dryness, then re-dissolved in mobile phase, filtered, and analyzed by High Performance Liquid Chromatography (HPLC) with UV detection.

2. Applicability

This method is applicable to dipyrone-related residues (4-metylaminoantipyrine, 4-formylaminoantipyrine, and 4-aminoantipyrine) in bovine and porcine muscle at ≥ 0.2 ppm.

B. EQUIPMENT

Note: Equivalent equipment may be substituted for the following.

1. Apparatus

a. Centrifuge - With 50 mL tube carriers, model TJ-6, Beckman.

b. Culture tubes - 10 mL disposable glass, Cat. No. 14-961-29, Fisher, and rubber stoppers.

c. Centrifuge tubes - 50 mL, disposable polypropylene, Cat. No. 352098, Becton Dickinson.

d. Balance - Analytical, 0.01 g sensitivity, Mettler, PJ3600 Delta Range.

e. Sidearm flasks - Erlenmeyer, 125 mL.

f. Filter - 0.45 µm, nylon, acrodisc-13, Cat. No. 4551, Pall, Gelman Sciences, Inc.

g. Filter paper - Glass fiber, 5.5 cm, Cat. No. F2831-55, Whatman GF/B.

h. Funnel - Buchner, 5.5 cm, Cat. No. 30305-040, VWR.

i. Homogenizer - Polytron Model PT 10-35, Brinkmann.

j. Liquid dispenser - Adjustable, 5 - 25 mL, Brinkmann.

k. Micropipetters - Adjustable, 100 - 1000 µL and 10 - 100 µL, Eppendorf.

l. Nitrogen evaporator - N-Evap, Organomation Associates Inc.

m. Shaker - Horizontal flatbed, two speed, Cat. No. 511105, Eberbach.

n. Solid Phase Extraction (SPE) supplies - Bond Elut C18 columns, 6 mL, 500 mg, Cat. No. 12102052, adapter caps, Cat. No. 2131001; 60 mL reservoirs, Cat. No. 12131012, Varian.

o. Syringe - 3 mL, disposable, Cat. No. BD301077, Becton Dickinson.

p. Vacuum manifold - For solid phase extraction, Cat. No. 5-7030, Supelco.

q. Volumetric flasks - 1, 100, 500 and 1000 mL, Cat. No. 29620-029 (1 mL), VWR.

r. Vortex mixer - Variable speed, Cat. No. S8223-1, American Scientific Products.

2. Instrumentation

a. Liquid chromatograph - Agilent 1100 equipped with quaternary pump, vacuum degasser, ALS, heated column compartment, diode array detector, and Chem Station.

b. Analytical column - Inertsil ODS-3, 150 mm x 4.6 mm ID, 5 µm particle size, Cat. No. 0396-150x046, Metachem Technologies, Torrance, CA.

c. Guard column - SecurityGuard ODS, 4 mm x 3 mm (2 used in tandem), Cat. No. AJO-4287 (guard cartridges) KJO-4282 (cartridge holder kit), Phenominex, Torrance, CA.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents/solutions may be substituted for the following.

1. Reagents

a. Acetonitrile (ACN) - HPLC grade, Cat. No. AH015-4, Burdick & Jackson.

b. Water - Deionized water, HPLC Grade, Millipore Rx system.

c. Methanol (MeOH) - HPLC grade, Cat. No. AH230-4, Burdick & Jackson.

d. Sulfuric acid - Concentrated, reagent grade, Cat. No. 320501, Sigma-Aldrich.

e. Aluminum chloride hexahydrate ($AlCl_3$. $6H_2O$) - > 98%, reagent grade, Fisher Scientific.

f. Sodium sulfite - > 98%, reagent grade, Cat. No. 239321, Sigma-Aldrich.

g. Sodium thiosulfate - > 98%, reagent grade, Cat. No. 217263, Sigma-Aldrich.

2. Solutions

a. 0.2 M Aluminum chloride solution:

Dissolve 4.83 g of $AlCl_3 \bullet 6H_2O$ in 30 mL methanol in a 100 mL volumetric flask. Dilute to volume with water and mix. Prepare as needed.

b. Sulfuric acid (H_2SO_4), 25% (v/v):

Add 125 mL of concentrated sulfuric acid to approximately 250 mL of water in a 500 mL volumetric flask. Mix, cool to room temperature, and dilute to volume with water.

c. Extraction buffer (0.1 M Sodium Sulfite, pH 7.0 ± 0.1):

Dissolve 12.6 g of sodium sulfite in 900 mL water in a 1 L volumetric flask and adjust the pH to 7.0 with 25% H_2SO_4. Dilute to volume with water and mix.

d. Mobile phase buffer (0.05 M Sodium sulfite, pH 7.0 ± 0.1):

Dissolve 6.3 g of sodium sulfite and 1.1 g of sodium thiosulfate in 900 mL of water in a 1 L volumetric flask. Adjust pH to 7.0 with 25% H_2SO_4 and dilute to volume with water and mix. Filter through a 0.45 µm membrane filter.

D. STANDARDS

1. Source

Name	Chemical name	Source	Cat. No.
4-Aminoantipyrine (AA) (Ampyrone) $C_{11}H_{13}N_3O$, FW 203.2	4-amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one	Sigma-Aldrich	33528
4-Dimethylaminoantipyrine (DAA) (Aminopyrine) $C_{13}H_{17}N_3O$, FW 231.3	4-dimethylamino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one	Sigma-Aldrich	D8015
4-Formylaminoantipyrine (FAA) $C_{12}H_{13}N_3O_2$, FW 231.3	4-formylamino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one	Sigma-Aldrich	S349941
4-Methylaminoantipyrine (MAA) (4-methylaminophenazone hydrochloride) $C_{12}H_{15}N_3O$•HCl, FW 253.7	4-methylamino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one	LGC Promochem	MM 0052.10

2. Preparation of Standards

Note: If purity is less than 100%, make corrections based on the actual purity provided. Adjust amount of MAA if using the hydrochloride form.

a. Mixed Stock Standard Solution (1 mg/mL):

Weigh amount equivalent to 100 mg of each standard (AA, FAA, and MAA) recorded to nearest 0.1 mg, into a 100 mL volumetric flask. Dissolve in 60 mL

methanol and bring to volume with methanol. Stable for three months when stored at -20 ºC.

b. Working Standard Solution (100 µg/mL):

Add 100 µL of stock solution (a) to a 1 mL volumetric flask and dilute to volume with methanol. Prepare daily as needed.

c. Stock Internal Standard Solution:

Weigh amount equivalent to 100 mg of DAA standard recorded to nearest 0.1 mg, into a 100 mL volumetric flask. Dissolve in 60 mL methanol and bring to volume with methanol. Stable for three months when stored at -20 ºC.

d. Working Internal Standard (ISTD) Solution (100 µg/mL):

Dilute 100 µL of the DAA stock standard to 1 mL with methanol in a 1 mL volumetric flask. Prepare daily as needed.

E. SAMPLE PREPARATION

After removing excessive fat from muscle sample, cut it into smaller pieces and homogenize with a mechanical food processor. Transfer homogenized sample into plastic bags and store in a freezer at ≤ -20 ºC. Let the sample partially thaw prior to analysis.

F. ANALYTICAL PROCEDURE

1. Samples extraction and cleanup procedure

a. Weigh 5.0 ± 0.1 g of homogenized muscle sample into a 50 mL polypropylene centrifuge tube.

Note: Prepare blank and recoveries at this time using previously analyzed muscle tissue containing no dipyrone-related residues.

i. Tissue-based Calibration Curve Standards for Screening:

Add 20 µL of Working Standard Solution (D.2.b) (equivalent to 0.4 ppm) to a 5 g blank tissue.

ii. Tissue-based Calibration Curve Standards for quantitative analysis:

Add 10, 20, 40 and 80 µL of Working Standard Solution (D.2.b) (equivalent to 0.2, 0.4, 0.8 and 1.6 ppm, respectively) to 4 separate 5 g blank tissues.

iii. Use a blank and a 0.4 ppm recovery for both screening and quantitative analyses.

b. Add 20 µL of 100 µg/mL internal standard solution (D.2.d) to tube.

c. Add 10 mL of extraction buffer to tube.

d. Blend using a Polytron for 15 sec.

e. Rinse Polytron probe with 5 mL of water into tube.

f. Cap and gently shake on a horizontal shaker for 5 min.

g. Centrifuge at 1500 x g at room temperature for 12 min. Conditions may be adjusted so long as firm tissue packing is obtained.

h. Pour the supernatant into a second 50 mL centrifuge tube.

i. Add 15 mL of extraction buffer to the tissue plug and resuspend the tissue using a vortex mixer.

j. Cap and gently shake on a horizontal shaker for 5 min.

k. Centrifuge at 1500 x g at room temperature for 12 min. Conditions may be adjusted so long as firm tissue packing is obtained.

l. Combine the supernatants in the second centrifuge tube (step h).

m. Repeat steps i - k.

n. Place a GF/B filter paper in a Buchner funnel. Moisten the filter with buffer and apply vacuum. Pass the combined supernatants through the filter into a 125 mL sidearm flask.

o. Attach 60 mL reservoir onto C18 SPE column and place on the vacuum manifold. Condition the column by passing 10 mL of methanol followed by 10 mL of water and 10 mL of extraction buffer. Add the filtered supernatant to the reservoir and allow the sample to pass through at a flow rate of 1 - 2 mL/min.

   Note: Do not allow the column to run dry until the water rinse (step p) has passed through.

p. Rinse the sidearm flask with 5 mL of water and add to the reservoir when most of the sample has passed through the SPE. When the water rinse has passed through the column, dry the column by drawing air through it for 3 min with vacuum at maximum setting.

q. Dry the tip of the vacuum block with tissue paper and place labeled 10 mL culture tube in the receiving area of the block.

r. Elute the analytes from the SPE column with 5 mL of methanol.

s. Reduce methanol to near dryness on an N-Evap at 60 ± 5 ºC.

   Note: Analytes may be lost if solution is evaporated to dryness.

t. Reconstitute the residue with 1 mL of mobile phase.

u. Add 25 µL of 0.2 M aluminum chloride to the reconstituted residue.

v. Vortex to mix.

w. Filter samples through an Acro LC13 filter into an autosampler vial.

x. The sample is ready for HPLC analysis.

2. HPLC Analysis

Inject calibration standards (F.1.a.i or F.1.a.ii), recovery, and sample extracts onto the HPLC system.

Note: The HPLC conditions below are suggestions and may be adjusted to obtain acceptable chromatography.

a. HPLC parameters:

Column temperature: 25 ºC ± 2 ºC.

Injection volume: 30 µL.

Flow rate: 1 mL/min.

Detector wavelength: 265 nm.

Run Time 21.6 min (data is collected for only ~12 min).

b. Gradient settings:

Time	% Buffer	% MeOH	%ACN	% Water
0	69	29	2	0
12.6	0	85	2	13
15.6	69	29	2	0
21.6	69	29	2	0

c. Column conditioning:

At the end of each set, flush the system and column using the settings in the table below.

Time	% Buffer	% MeOH	%ACN	% Water
0	0	10	0	90
30	0	25	0	75

60	0	100	0	0
90	0	100	0	0

d. Approximate Retention Times:

FAA 4.4 min

DPN 5.6 min

AA 7.5 min

MAA 8.8 min

DAA 10.2 min

e. System suitability criteria for acceptability of chromatography:

The chromatographic conditions listed above should produce chromatograms with completely resolved peaks.

G. CALCULATIONS

1. Standard curve

a. Calculate a peak area ratio (analyte peak area / DAA peak area) for each analyte in all calibration standards included with the sample set.

b. Using linear regression analysis, construct a standard curve for each analyte by plotting area ratio vs. fortified concentration. Calculate the slope (m), intercept (b) and correlation coefficient (r) for each curve.

Construction of the standard curve can be automated through HPLC software.

2. Determination

Note: Quantitative analysis requires that the correlation coefficient calculated for the 5-point standard curve be ≥ 0.995.

Determine the analyte concentrations in each sample using the formula:

Analyte concentration, ppm = (y - b) / m, where

y = Analyte peak area ratio

m, b = slope and intercept, respectively, calculated from standard curve equation.

Samples found to contain any dipyrone-related residue in a screening run at levels ≥ 0.2 ppm, must be re-run using the quantitative option.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment - Safety glasses, disposable gloves, lab coats.

2. Hazards

Reagents / Solutions	Hazard	Recommended Safe Procedure
Methanol, acetonitrile	Flammable & poisonous	Wear gloves, work in fume hood.
LC Mobile phase containing a mixture of sodium sulfite and sodium thiosulfate of pH 7.0 (adjusted with sulfuric acid), methanol and acetonitrile	Irritation to skin, eyes, nose, mouth, throat and mucous membrane and may cause burns to skin	Wear gloves and work in hood. Use protective eyewear.

3. Disposal Procedures

Reagents / solutions	Hazard	Recommended Safe Procedure
Methanol and acetonitrile.	Flammable & poisonous	Collect in a tightly sealed container and store in the flammable liquid storage area for disposal in accordance with local, state, and Federal regulations.
LC Mobile phase containing a mixture of sodium sulfite and sodium thiosulfate of pH 7.0 (adjusted with sulfuric acid), methanol and acetonitrile.	Irritation to skin, eyes, skin, nose, mouth, throat and mucous membrane	Collect in a tightly sealed container and store in the flammable liquid storage area for disposal in accordance with local, state, and Federal regulations.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

Analyte	*Analytical Range (ppm)*	*Acceptable Recovery*	*Acceptable Repeatability (CV)*
FAA, AA, MAA	≥ 0.2	80 - 120*	≤ 20%

*Correlation coefficient must be ≥ 0.995 for tissue-based fortifications used for quantitation (0, 0.2, 0.4, 0.8 and 1.6 ppm).

2. Critical Control Points and Specifications

Record	*Acceptable Control*
C.2.c Extraction buffer pH	7.0 ± 0.1
C.2.d Mobile phase buffer pH	7.0 ± 0.1
F.1. n - p	Steps should be completed without interruption.

3. Readiness To Perform (FSIS Training Plan)

   a. Familiarization

   Phase I: External Calibration Curve: Generate a calibration curve using external standards at 0, 0.2, 0.4, 0.8, and 1.6 ppm, respectively (calculated on tissue-based fortifications). Verify instrument sensitivity. Use linear regression analysis to verify that the y-intercept of the regression line is approximately 0 and that the correlation coefficient ≥ 0.995.

   Phase II: Analyst fortified samples: Generate duplicate tissue based calibration curves by fortifying blank tissues at 0, 0.2, 0.4, 0.8 and 1.6 ppm fortification levels (see step F.1.a.ii). For each curve, verify correlation coefficients ≥ 0.995. Using standard curve constructed from first data set, quantitate all levels in second set and calculate recoveries. Repeat this analysis two more days using two different blank muscles.

   Note: Phases I and II can be performed concurrently.

Phase III: Check samples for analyst accreditation:

(a) A minimum of 8 blind fortified samples. At least one sample should be blank. Samples should be fortified at 0.2 – 1.6 ppm levels.

(b) Report analytical findings to Supervisor/Quality Assurance Manager (QAM).

(c) Notification from the QAM is required to commence official sample analysis.

4. Intralaboratory check samples

a. System, minimum contents.

i. Frequency: 1 per week as samples analyzed.

ii. Records are maintained.

b. Acceptability criteria: Refer to section I.1 above.

If unacceptable results are obtained, then:

i. Stop all sample analysis by the analyst.

ii. Take corrective action.

5. Sample acceptability and stability

a. Matrix: bovine and porcine muscle

b. Sample size: ≥ 50 g

c. Condition upon receipt: cold, not spoiled

d. Sample stability: 2 months at -20 ºC

6. Sample set must include:

a. For screening analysis:

i. 0.4 ppm recovery.

ii. Tissue blank.

iii. Samples.

b. For quantitative analysis:

i. 0.4 ppm recovery.

ii. Tissue blank.

iii. Samples.

7. Sensitivity

Minimum Proficiency Level (MPL): ≥ 0.2 ppm.

J. WORKSHEET

Start Date	
End Date	

Analyst	
Peer Review	
Supervisor Review	

Equipment	
freezer	
balance	
micropipettors	
dispensettes	
homogenizer	
shaker	
centrifuge	
vortexer	
HPLC	
N-evap	

HPLC	
data collection	12.0 min
total run time	21.6 min
injection vol.	30 μL
flow rate	1.0 mL/min
column temp.	25°C ± 2°C
detector λ	265 nm
column	Inertsil ODS-3, 150 mm x 4.6 mm I.D., 5mm, Metachem
guard	SecurityGuard ODS, 4 mm x 3 mm, (2 used in tandem), Phenomenex

Reagents	
0.2M aluminum chloride	
extraction buffer	
pH 7.0 ± 0.1	
mobile phase buffer	
pH 7.0 ± 0.1	
methanol	
acetonitrile	

Data	
folder	
files	

Standards	
working (AA, FAA, MAA)	
internal (DAA)	
mixed external	
DPN working	
DPN external	

Tissue-Based Calibration Curve				
DPN	AA	FAA	MAA	correlation
				r ≥ 0.995

External Standards				
DPN	AA	FAA	MAA	ppm based
				on calibration

Comments:

Sample	**weight**	**ppm measured**					**ppm**	**recovery**			
	5.0±0.1g	DPN	AA	FAA	MAA	DPN eq.	**spike**	DPN	AA	FAA	MAA

K. APPENDIX

1. Chromatograms

Chromatograms of blank and 0.4 ppm fortified muscle tissue, respectively.

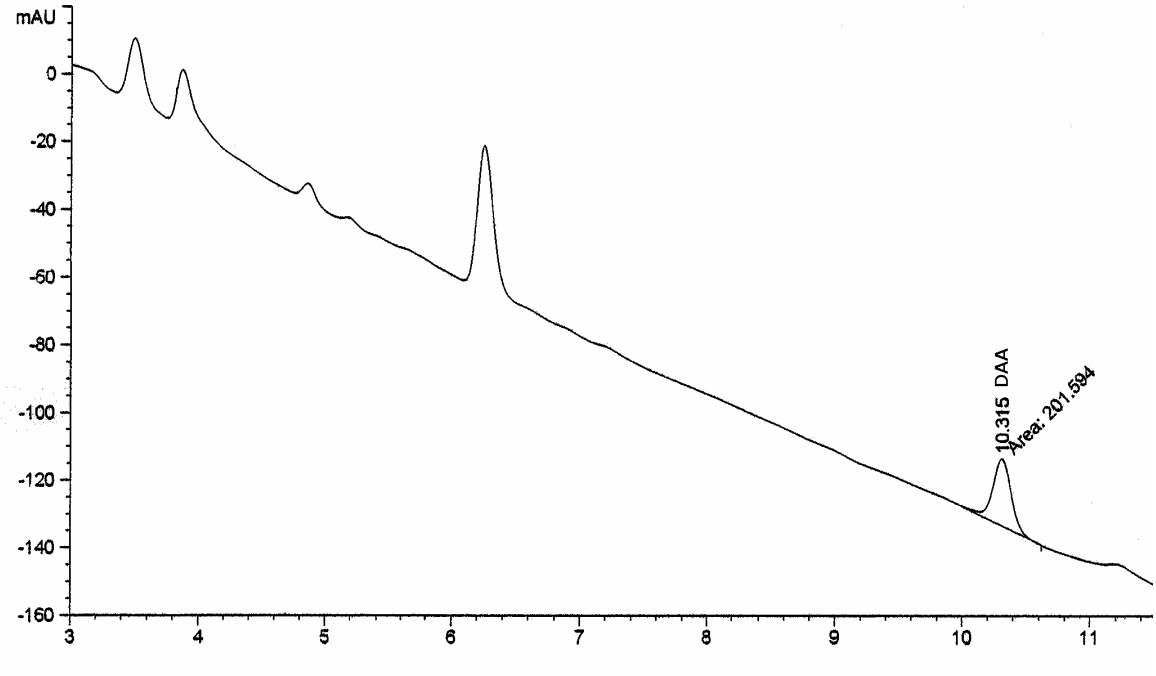

Figure 1. Blank Beef Muscle.

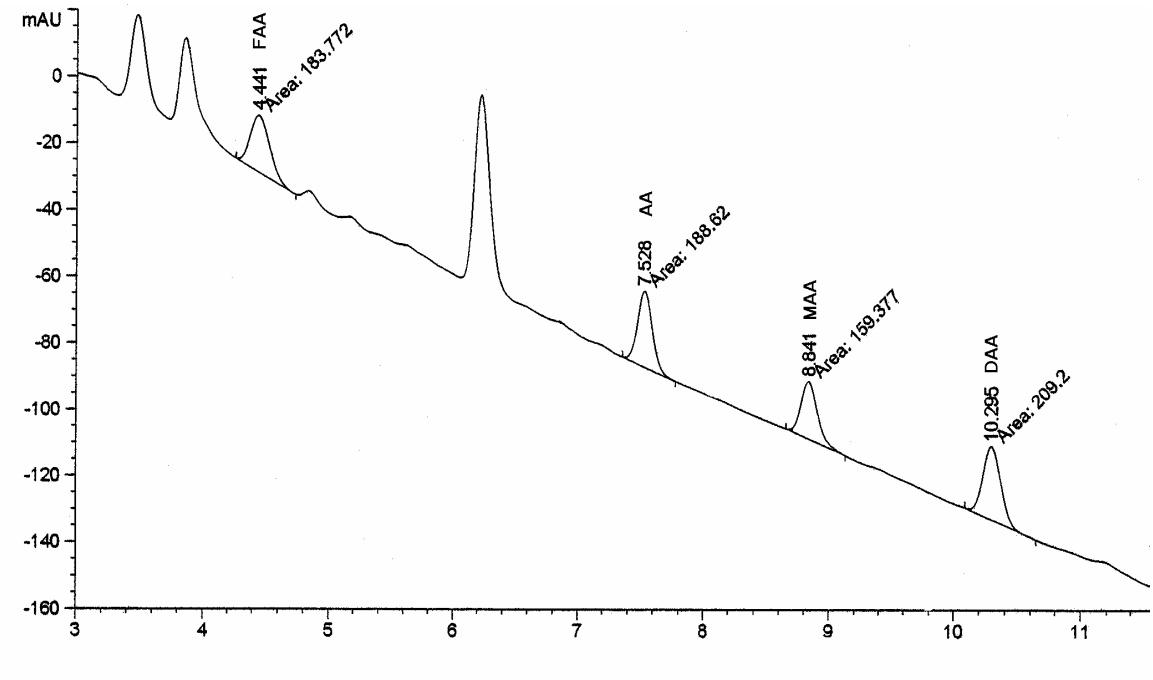

Figure 2. Mixed Recovery at 0.4 ppm in Muscle.

2. Reference

Screening and Determinative Method for Dipyrone-related Residues in Bovine and Porcine Muscle using Liquid Chromatography, CVDR, DPY-SP02, Canadian Food Inspection Agency, Saskatoon Laboratory and Centre for Veterinary Drug Residues, 116 Veterinary Road, Saskatoon, SK S7N 2R3.

Approved by:	Date Approved:
David Martin	10/14/04
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