

# Supercritical Fluid Application Notes

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## Determination of Steroids in Animal Tissues by Supercritical Fluid Extraction and Inline Trapping



### Introduction

The use of anabolic steroids in food producing animals is illegal among countries in the European Union. In order to enforce regulations, laboratories responsible for testing animal tissues have traditionally relied on extraction methods such

as liquid to liquid extraction, GC-MS, and HPLC. Unfortunately, these methods are time consuming, require large volumes of organic solvent, and must be optimized for every new steroid-matrix combination.

SFE is an alternative technique using supercritical carbon dioxide to extract analytes from a variety of matrices. SFE significantly reduces the use, exposure to, and disposal of hazardous solvents, while providing comparable extraction results to standard methods in less time.



There have been some problems identified with using typical SFE

methods to isolate drug residue from tissue matrices. One of the main difficulties is that when trace levels of residues are isolated from fat tissue by SFE using CO<sub>2</sub>, fat is co-extracted. If a modifier is used with CO<sub>2</sub>, the resultant extract becomes more complex and the desired analyte is more difficult to recover from the mixture.

A solution to these problems is to use an SFE instrument and method that simplifies the separation and recovery of trace level drug residues from an analyte/fat matrix. This application describes a procedure for coupling SFE technology with an inline trapping technique to quickly and easily extract residue levels of steroids from animal tissue samples without co-extracting fat.

### Equipment

- ✓ Applied Separations' *Spe-ed*<sup>TM</sup>SFE-4 or Helix Supercritical Extraction System
- ✓ Analytical Balance
- ✓ Evaporation Apparatus
- ✓ GC-MS
- ✓ Teflon Inline Cartridge Holder- Cat. #7923

### Materials

- ✓ Steroids
- ✓ Bovine muscle tissue
- ✓ SPE cartridge -3mL, 2.0 g.
  - Aluminium oxide N, Applied Separations
- ✓ *Spe-ed* Matrix (Cat. #7950)
- ✓ *Spe-ed* Polypropylene Wool (Cat. #7952)
  - #7952)
- ✓ Carbon dioxide -SFC/SFE grade without helium head pressure
- ✓ *tert*-butyl methyl ether (TBME)

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- ✓ heptafluorobutyric anhydride (HFBA)
- ✓ hydrochloric acid
- ✓ acetate buffer
- ✓ potassium hydroxide
- ✓ Water
- ✓ Acetone
- ✓ Ethanol
- ✓ Methanol
- ✓ *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA)
- ✓ ammonium iodide
- ✓ dithioerythritol
- ✓ isooctane

## Method

Homogenize 5.0 g of tissue and freeze dry. When ready for extraction, blend freeze dried tissue with 3.5 g of *Spe-ed* Matrix. Add internal standard and 1 mL of water. Remove the end flanges of a 3 ml SPE alumina cartridge and insert into an appropriately sized teflon holder. Close one end of an extraction vessel, and place the cartridge/holder into the vessel. Set the cartridge luer into the outlet of the vessel. Next, place a plug of *Spe-ed* Wool on top of cartridge/holder and compress with a stainless steel tamping rod. Pour the sample mixture of tissue and *Spe-ed* Matrix into the extraction vessel and tamp down with rod. Fill the remaining void in the extraction vessel with another plug of *Spe-ed* Wool, and then use a stainless steel tamping rod to tightly pack the vessel. Close vessel with an end-cap and perform extraction to the following conditions:

## Extraction Conditions

Extraction vessel: 24 mL  
Sample: 5.0 g  
Pressure: 450 BAR

Temperature: 50°C  
Valve temperature: 110°C  
CO<sub>2</sub> Flow Rate: 2 L/min (gas)  
Collection: 60 mL pre-weighed vial  
Dynamic time: 10 minutes

## Analyte Recovery

When the extraction sequence is complete, remove the SPE column and elute with 6 ml of methanol-water (65+35; v/v).

## Post-SFE Analysis

Divide the eluate into two equal portions.

### *Derivatisation Method I:*

Evaporate the solvent of the first portion and dissolve the residue in 0.2 ml of alkaline hydrolysis solution. (Prepare alkaline solution by dissolving 5.6 g of potassium hydroxide in 100 ml of methanol).

Incubate this mixture for 30 minutes at 37 °C. Add 1.0 ml of acidic buffer. (The acidic buffer is prepared by mixing 1.7 ml of hydrochloric acid (37%) with 98.3 ml of acetate buffer (2 mol l<sup>-1</sup>). Extract the mixture twice with 6 ml of TBME and evaporate the combined extract to dryness under a gentle stream of nitrogen. Transfer the residue to a derivatisation vial filled with 0.5 ml of ethanol. Evaporate the ethanol and add 0.05 ml of HFBA-acetone (1/4; v/v). Vortex mix the vial and incubate at 60 °C for 1 hr. Once incubation is complete, evaporate the reaction mixture to dryness under a gentle stream of nitrogen at 50 °C. Next, dissolve the derivatised residue in 0.025 ml of isooctane and transfer into a GC injection vial with a micro-insert. Derivatisation method I can be used for the following analytes:  
Medroxy-progesterone Medroxy-progesterone-*d*<sub>3</sub>

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Megesterol  
Megesterol-*d*<sub>3</sub>  
Melengesterol  
Melengesterol-*d*<sub>3</sub>  
Chloromadinone  
[<sup>37</sup>C]Chloromadinone  
Delmadinone  
β-Estradiol  
β-Estradiol-*d*<sub>3</sub>  
Ethinylestradiol  
Ethinylestradiol-*d*<sub>4</sub>  
Testosterone  
Testosterone-*d*<sub>2</sub>

## Derivatisation Method II:

Evaporate the second portion of the eluate under a gentle stream of nitrogen in a water-bath at 50 °C. Add 2 mLs of water and mix in vortex for 30 s. Next, extract mixture twice with 6 ml of TBME. Evaporate TBME and transfer residue into a derivatisation vial with 0.5 ml of ethanol. Evaporate ethanol and add 25 µl of MSTFA-ammonium iodide-dithioerythritol (1000+2+4, v/w/w). Mix vial for 30 s and incubate for 1 hour at 60 °C.

When incubation is complete, evaporate reaction mixture to dryness under a gentle stream of nitrogen at 50 °C. Dissolve derivatised residue in 0.025 ml of isooctane. Derivatisation method two can be used for the following analytes:

Nortestosterone  
Nortestosterone-*d*<sub>3</sub>  
Methylboldenone  
Methylboldenone-*d*<sub>3</sub>  
Norethandrolone  
Norgestrel  
Chlorotestosterone acetate  
Chlorotestosterone acetate-*d*<sub>3</sub>

## Analysis

GC-MS

## Results

### Validation results of SFE-GC-MS of Steroids from Fortified Bovine Muscle\*

Analyte	Repeatability, RSD (%) (n=9)	Within-laboratory Reproducibility RSD (%) (n=3)
Methylboldenone	12	23
B-Nortestosterone	4	2
Ethinylestradiol	7	9
Chlorotestosterone acetate	5 (n=6)	4
Norethandrolone	21	37
Norgestrel	15	31

\*(MSTFA Derivative)

## Conclusion

The supercritical carbon dioxide extraction of steroids from animal tissue samples offers a viable alternative to solvent-based procedures. The accuracy and precision of the results were comparable to the standard method while extraction times were reduced. In addition, levels of detection were 2 µg/k for melengestrol acetate.

## References

Stolker, A.; Zoontjes, P.; and van Ginkel, L. "The use of Supercritical fluid extraction for the determination of steroids in animal tissues." *The Analyst*. 1998, **123**, 2671-2676.

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