Extraction of Anabolic Steroids from Horse Urine Using ISOLUTE® SLE+ Prior to LC-MS/MS Analysis

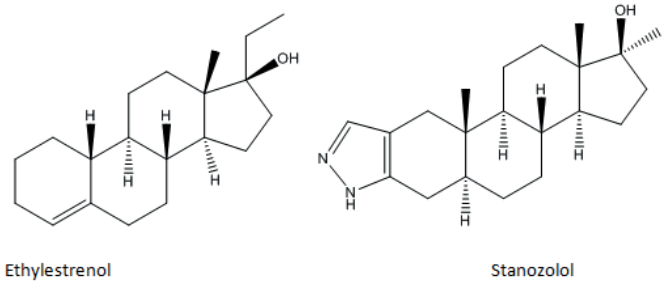

Figure 1. Structures of Ethylestrenol and Stanozolol

Introduction

Ethylestrenol and stanozolol are anabolic steroids which can be used to increase muscle mass and enhance performance. These drugs have been linked to instances of doping in race horses. This application note describes a Supported Liquid Extraction (SLE) protocol for the extraction of ethylestrenol and stanozolol from horse urine prior to LC-MS/MS analysis.

The method described in this application note achieves high reproducible analyte recoveries from both gelding and filly urine. Protocols for 48-well, 96-well plate and column formats are described.

ISOLUTE® SLE+ Supported Liquid Extraction plates and columns offer an efficient alternative to traditional liquid-liquid extraction (LLE) for bioanalytical sample preparation, providing high analyte recoveries, no emulsion formation, and significantly reduced sample preparation.

Analytes

4-Ethylestrenol and Stanozolol.

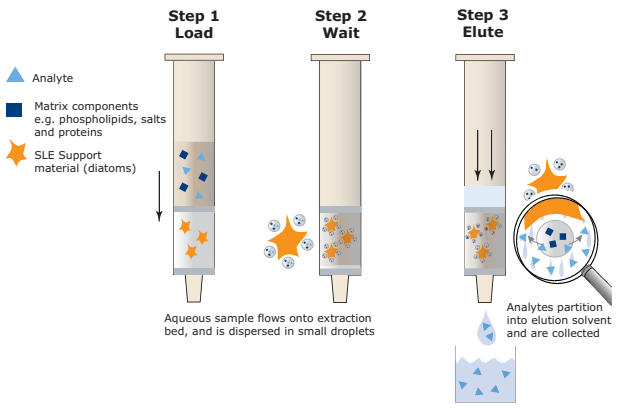

Figure 1. Supported liquid extraction mechanism.

Sample Preparation Procedure

Format: ISOLUTE® SLE+ 1 mL Supported Liquid Extraction plate, 48-well, part number 820-1000-Q01. Protocols for 96-well plates and column formats are also included, see **Table 1.**

Sample Pre-treatment

Take appropriate volume of urine and add same volume of H_2O. Mix.

Sample Loading

ISOLUTE SLE+ 48-well plate: Load the pre-treated sample (800 µL) to each well of the 48-well plate followed by a pulse of vacuum or positive pressure to initiate flow. Leave to absorb for 5 minutes.

Elution

Ensure a suitable collection vessel is in place.

Apply 1 mL of MTBE and allow to flow under gravity.

Apply a second 1 mL of MTBE and allow to flow under gravity.

Apply a third 1 mL of MTBE and allow to flow under gravity until the solvent reaches the top frit. Pull through the remaining solvent with vacuum or positive pressure for 10–20 seconds.

Note: DCM is a suitable alternative elution solvent

Post Elution

Evaporate to dryness at 40 °C in a stream of air or nitrogen.

Reconstitution*

Reconstitute using 500 µL of 20/80 H2O/ACN with 0.1% Formic acid. Mix gently.

*Recovery and reproducibility for ethyestrenol was found to be affected by non-specific binding and/or losses on evaporation and reconstitution. We recommend that collection vessels, evaporation vessels and reconstitution solvents are investigated during method development to minimize losses/variability.

Table 1. Loading and elution details for alternative ISOLUTE SLE+ formats

Format	Load Volume	Elution Protocol	Reconstitution Solvent
ISOLUTE SLE+ 400 µL capacity plate	300 µL of pre-treated urine	2 x 600 µL MTBE or DCM	200 µL 20/80 H2O/MeOH 0.1%Formic acid.
ISOLUTE SLE+ 400 µL capacity column	300 µL of pre-treated urine	2 x 600 µL MTBE or DCM	200 µL 20/80 H2O/MeOH 0.1% Formic acid.
ISOLUTE SLE+ 1 mL capacity column	800 µL of pre-treated urine	1 x 3 mL MTBE or DCM	200 µL 20/80 H2O/MeOH 0.1%Formic acid.

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UPLC Conditions

Instrument
Waters Acquity UPLC (Waters Assoc., Milford, MA, USA)

Column
ACE Excel 2 C18 column (50 x 2.1 mm id)

Mobile Phase
A: 2 mM ammonium acetate 0.1% Formic Acid (aq)
B: 2 mM ammonium acetate 0.1% Formic Acid (MeOH)

Flow Rate
0.4 mL/min

Table 2. UPLC Gradient Conditions.

Time	%A	%B	Curve
0	25	75	
0.25	25	75	6
1.50	10	90	6
1.51	0	100	6
3.50	0	100	6
3.51	25	75	1
4.00	25	75	1

Curve 6: Linear Gradient

Injection Volume
10 µL (partial loop with overfill)

Sample Temperature
20 °C

Column Temperature
40 °C

Mass Spectrometry Conditions

Instrument
Premier XE triple quadrupole mass spectrometer equipped with an electrospray interface for mass analysis.

Desolvation Temperature
450 °C

Ion Source Temperature
120 °C

Positive ions acquired in the multiple reaction monitoring (MRM) mode:

Table 3. Mass Spectrometry Conditions.

Compound	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
Stanozolol	329.0 > 94.8	50	40
Stanozolol	329.0 > 106.8	50	40
Ethylestrenol	271.1 > 120.8	35	15
Ethylestrenol	274.1 > 146.8	35	15

Results

Analyte Recovery and Reproducibility
Horse urine (gelding and filly) was spiked (n=7 for each matrix) with both analytes at a sample concentration of 40 ng/mL. Both dichloromethane and MTBE were evaluated as elution solvents. Typical recovery and reproducibility for each format are shown in **Table 4** below.

Table 4. Typical analyte recovery and reproducibility using various ISOLUTE® SLE+ formats

Elution Solvent	Format	Stanozolol (40 ng/ mL)		Ethylestrenol (40 ng/ mL)	
		Recovery	% RSD	Recovery	% RSD
DCM	ISOLUTE SLE+ 400 µL capacity plate	>85%	<3%	>84%	<7%
DCM	ISOLUTE SLE+ 400 µL capacity column	>87%	<7%	>82%	<12%
MTBE	ISOLUTE SLE+ 1 mL capacity plate (48-well)	>90%	<9%	>88%	<11%
DCM	ISOLUTE SLE+ 1 mL capacity column	>89%	<11%	>85%	<16%

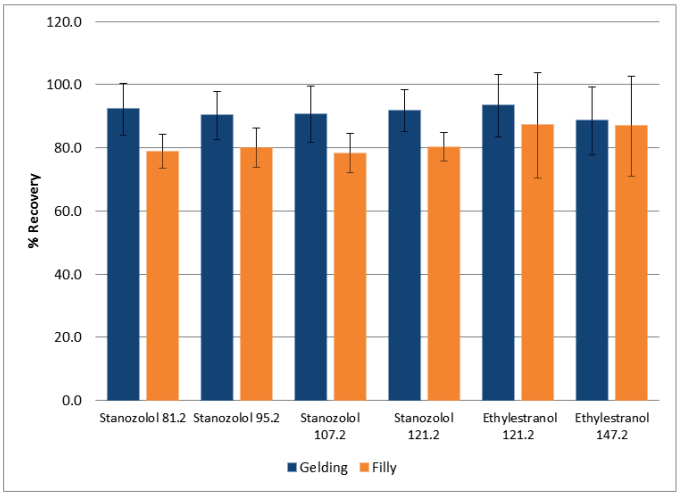

Figure 2. Recovery profile for anabolic steroids with MTBE elution, 20:80 H2O:ACN 0.1% Formic acid reconstitution solution using 48-well plate format.

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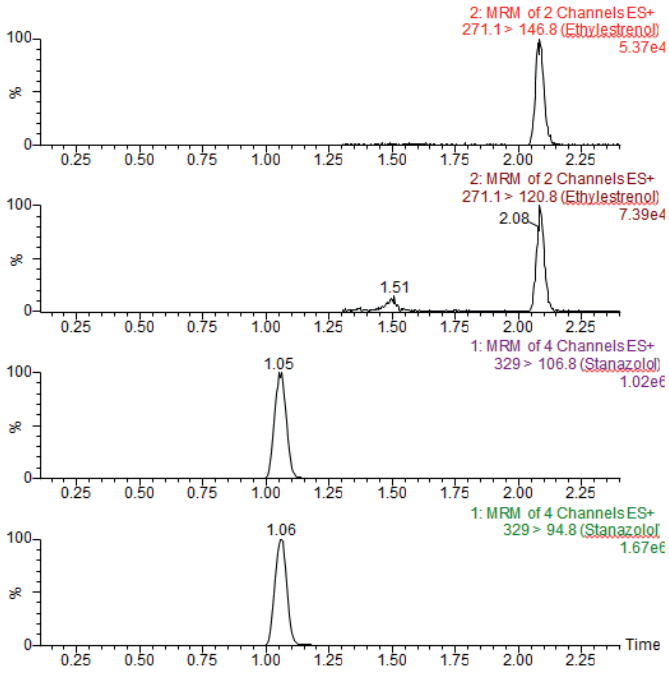

Figure 3. LC/MS chromatography

Ordering Information

Part Number	Description	Quantity
820-0400-P01	ISOLUTE® SLE+ 400 Supported Liquid Extraction Plate	1
820-1000-Q01	ISOLUTE® SLE+ 1 mL Supported Liquid Extraction Plate, 48-well	1
820-0055-B	ISOLUTE® SLE+ 400 µL Sample Volume Columns	50
820-0140-C	ISOLUTE® SLE+ 1 Sample Volume Columns	30
121-9600	Biotage® VacMaster™-96 Sample Processing Manifold	1
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
SD-9600-DHS-EU	Biotage® SPE Dry Sample Concentrator System 220/240 V	1
SD-9600-DHS-NA	Biotage® SPE Dry Sample Concentrator System 100/120 V	1
C103263	TurboVap®96, Evaporator 100/120V	1
C103264	TurboVap® 96, Evaporator 220/240V	1

Additional Notes

Buffer Preparation

1. 2 mM ammonium acetate aq: Weigh 0.15416 g and dissolve in H_2O. Dilute and make up to 1 L in H_2O then add 0.1 mL Formic acid.
2. 2 mM ammonium acetate in methanol: Weigh 0.15416 g and dissolve in methanol. Dilute and make up to 1 L in methanol then add 0.1 mL Formic acid.

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