Simultaneous Analysis of Free Steroids and Sulfate Conjugates by Solid-Phase Extraction and LC-MS/MS

UCT Part Numbers

SSHLB066
Styre Screen® HLB
6 mL, 60 mg sorbent

SCS27-DA1021
SelectraCore® DA UHPLC Column
100 X 2.1 mm, 2.7 µm

SCS27-DAGDC21
SelectraCore® DA Guard Column
5 X 2.1 mm, 2.7 µm

SLGRDHLDR-HPOPT
UHPLC Direct Connect Guard Cartridge Holder

Summary:

Steroids are common analytes tested by forensic, clinical, and anti-doping laboratories. Analysis of free steroids generally requires a hydrolysis step, most commonly performed using an enzyme to cleave glucuronide groups. However, recently, there is a growing interest in the direct analysis of steroid sulfate conjugates rather than targeting free steroids originating from glucuronide conjugates after hydrolysis. This is because the ratio between glucuronide and sulfate metabolites is different from person to person and sulfate conjugates may even exceed the glucuronide-bound steroids in some cases, potentially leaving a large degree of analytes unanalyzed.[1] Also, sulfate metabolites are excreted at a slower rate, meaning their abundance is dependent on the time and route of administration.[2] Current research suggests that steroid sulfate markers can increase the detection window for the identification of analytes that are potentially being abused by athletes. Sulfate metabolites increase with time after use and remain present in the body longer than glucuronide metabolites.[2]

This application note outlines a highly efficient method for the simultaneous analysis of free and sulfated steroids from urine, plasma, and blood utilizing solid-phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Styre Screen® HLB extraction cartridges consist of a highly retentive hydrophilic and lipophilic sorbent which can effectively retain these challenging analytes leading to high recoveries.
The SelectraCore® DA UHPLC column provided exceptional retention and peak shape for the wide range of steroids included in the method.

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Sample Pretreatment:

Urine
- In a test tube, add 0.5 mL sample, internal standards, 200 µL of methanol, and 1.3 mL of DI water
- Vortex

Blood and Plasma
- In a test tube, add 0.25 mL of sample, internal standards, and 0.75 mL acetonitrile
- Vortex and centrifuge
- Decant supernatant in 5 mL of DI water
- Vortex

SPE Procedure:

1. Condition Column

a) 1 x 3 mL methanol
b) 1 x 3 mL DI water

2. Load Sample

a) Load the samples at 1 to 2 mL/min

3. Wash Column

a) 1 x 3 mL 60 mM HCl in DI water
b) 1 x 3 mL 30% methanol in DI water

4. Dry

a) Dry columns for 10 mins at full vacuum or pressure

5. Elute Analytes

a) 1 x 3 mL 50:50 methanol:acetonitrile

6. Evaporate and Reconstitute

a) Evaporate to full dryness at 10 psi, 40°C
b) Reconstitute in 1 mL 80:20 water:acetonitrile or other appropriate volume and solvent

Notes:

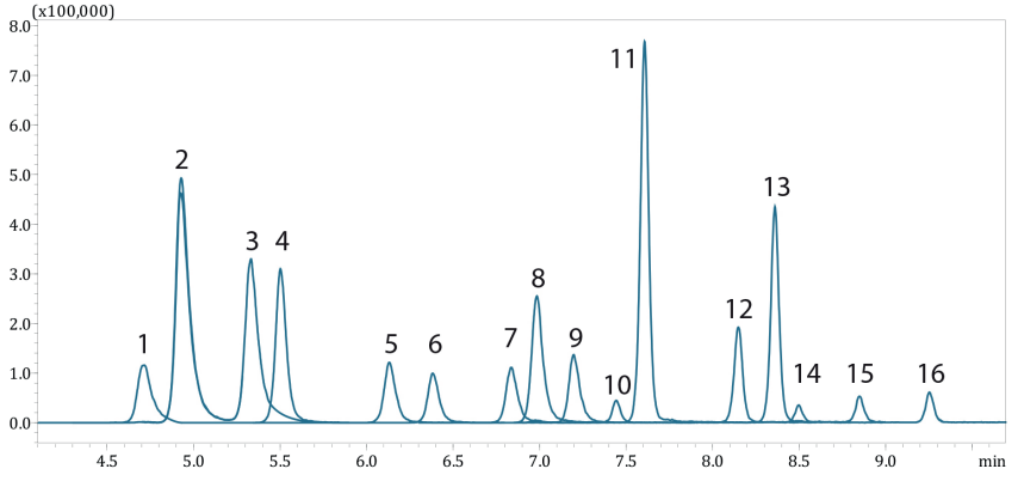
Steroids are endogenous substances; this was accounted for in the following ways:

- Synthetic urine was used as a surrogate matrix for urine
- Surrogate matrices were not used for blood and plasma, results were obtained by using a background subtraction method (See **Equations 1, 2**).

LC-MS/MS Parameters	
System	Shimadzu Nexera LC-30AD w/ MS-8050
UHPLC Column	SelectraCore® DA 100 x 2.1 mm, 2.7 µm (**P/N: SCS27-DA1021**)
Guard Column	SelectraCore® DA 5 x 2.1 mm, 2.7 µm (**P/N: SCS27-DAGDC21**)
Column Temperature	40°C
Column Flow Rate	0.4 mL/min
Injection volume	5 µL

Gradient Program		
Time (min)	**Mobile Phase A (%)** 0.1% Formic Acid in Water	**Mobile Phase B (%)** Acetonitrile
0	80	20
5	70	30
8.5	55	45
10.5	0	100
11.5	0	100
11.6	80	20
15.6	80	20

Chromatogram:



1. 17β-estradiol-17-sulfate
2. 17β-estradiol-3-sulfate
3. 17α-estradiol Sulfate
4. Boldenone Sulfate
5. Nandrolone Sulfate
6. Testosterone Sulfate
7. Estrone-3-sulfate
8. DHEA sulfate
9. Androsterone Sulfate
10. Estradiol
11. Boldenone
12. Nandrolone
13. Testosterone
14. DHEA
15. Estrone
16. Androsterone

Figure 1: Standards mix at 25 ng/mL in 80:20 water:acetonitrile

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MRM Table:

MRM						
Analyte	**Parent Ion**	**Product Ion 1**	**CE**	**Product Ion 2**	**CE**	**RT (mins)**
17β-estradiol-17-sulfate	351.1	97.0	39	80.0	55	4.70
17β-estradiol-3-sulfate	351.1	271.2	35	145.1	55	4.92
17*α*-estradiol Sulfate	351.1	271.2	35	145.1	54	5.33
Boldenone Sulfate	365.1	350.2	30	96.9	49	5.50
Nandrolone Sulfate	353.1	97.0	40	79.9	54	6.12
Testosterone Sulfate	367.2	97.1	42	351.2	44	6.37
Estrone-3-sulfate	349.1	269.3	34	145.0	53	6.83
DHEA Sulfate	367.1	97.0	33	-	-	6.97
Androsterone Sulfate	369.2	97.0	39	-	-	7.19
Estradiol	255.0	159.0	21	95.5	30	7.44
Boldenone	287.2	121.1	25	135.1	15	7.60
Nandrolone	275.1	109.1	27	257.3	17	8.14
Testosterone	289.2	97.1	27	97.1	23	8.36
DHEA	270.8	253.0	15	97.1	40	8.50
Estrone	270.8	253.0	14	79.1	47	8.84
Androsterone	291.2	273.3	10	255.3	15	9.25

*CE=collision energy, RT=retention time

Note: Free steroid compounds were analyzed in positive mode while the sulfate steroid conjugates were analyzed in negative mode

Representative Calibration Curves:

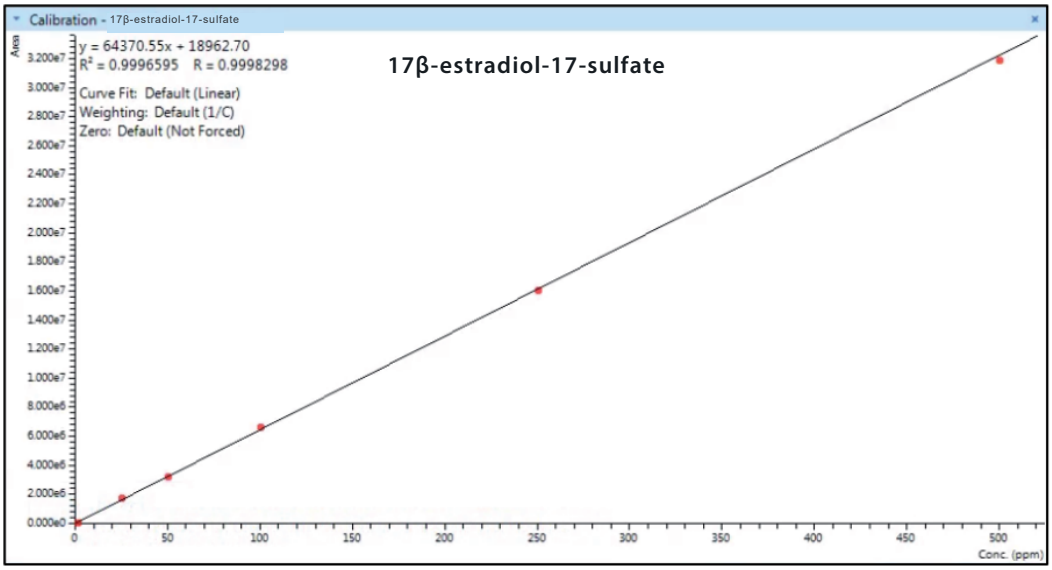

Figure 2a: Example of a 6-point solvent calibration curve for 17β-estradiol-17-sulfate with linear equation & R^2 value (1, 25, 50, 100, 250 & 500 ng/mL)

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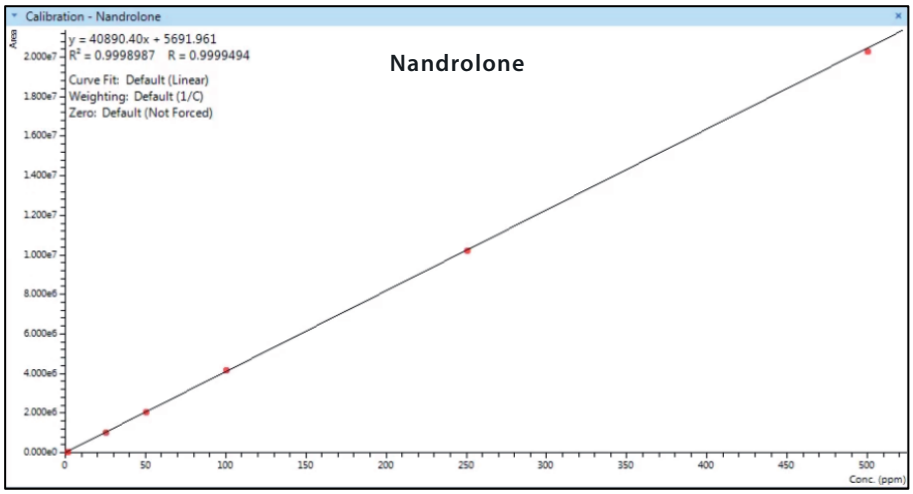

Figure 2b: Example of a 6-point solvent calibration curve for Nandrolone with linear equation & R^2 value (1, 25, 50, 100, 250 & 500 ng/mL)

Results:

Urine Extraction									
	5 ng/mL (n=5)			50 ng/mL (n=5)			250 ng/mL (n=5)		
Analyte	Recovery (%)	RSD (%)	Matrix Effects (%)	Recovery (%)	RSD (%)	Matrix Effects (%)	Recovery (%)	RSD (%)	Matrix Effects (%)
17β-estradiol-17-sulfate	85	3	-5	89	7	-12	86	18	-13
17β-estradiol-3-sulfate	89	3	-1	96	3	-7	93	7	-8
17α-estradiol Sulfate	91	1	0	100	3	-12	96	3	-13
Boldenone Sulfate	92	2	0	98	2	-6	98	3	-9
Nandrolone Sulfate	90	2	1	97	2	-5	98	5	-9
Testosterone Sulfate	91	4	0	97	3	-10	96	14	-12
Estrone-3-sulfate	85	7	9	85	3	13	103	9	-8
DHEA Sulfate	90	2	14	86	5	6	100	12	-9
Androsterone Sulfate	88	4	2	92	5	-7	92	1	-13
Estradiol	87	1	9	97	1	1	99	1	2
Boldenone	92	1	3	97	1	0	100	0	-2
Nandrolone	92	1	13	96	1	6	103	1	-1
Testosterone	91	2	12	96	1	7	102	1	2
DHEA	87	6	11	94	1	9	98	2	2
Estrone	93	4	2	95	2	2	99	1	1
Androsterone	98	4	0	95	1	1	98	7	-1

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Plasma Extraction									
	5 ng/mL (n=5)			50 ng/mL (n=5)			250 ng/mL (n=5)		
Analyte	Recovery (%)	RSD (%)	Matrix Effects (%)	Recovery (%)	RSD (%)	Matrix Effects (%)	Recovery (%)	RSD (%)	Matrix Effects (%)
17β-estradiol-17-sulfate	82	2	-5	83	3	-3	80	4	-5
17β-estradiol-3-sulfate	80	2	-4	82	2	-2	81	2	-4
17α-estradiol Sulfate	85	3	-5	88	2	-4	85	2	-5
Boldenone Sulfate	86	2	-4	88	2	-2	89	1	-4
Nandrolone Sulfate	88	2	-5	88	2	-3	89	2	-4
Testosterone Sulfate	85	2	-4	88	2	2	88	2	-3
Estrone-3-sulfate	84	3	-3	84	2	-3	81	2	-5
DHEA Sulfate	59*	2	-77*	90	2	-12	86	1	-5
Androsterone Sulfate	76*	1	-41*	86	2	-9	85	4	-6
Estradiol	87	3	-8	92	3	-8	89	3	-9
Boldenone	89	1	0	89	1	2	92	1	1
Nandrolone	89	1	-1	89	1	2	91	1	-1
Testosterone	87	2	-5	88	1	-1	91	2	-5
DHEA	84	3	3	90	2	-3	90	3	-8
Estrone	86	6	-3	93	2	-8	89	5	-9
Androsterone	84	3	-13	92	3	-17	92	6	-23

*LLOQ must be higher than 5 ng/mL for reproducibility of DHEA sulfate and androsterone due to endogenous background concentrations (See Conclusion/Discussion section).

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Results:

Blood Extraction									
	5 ng/mL (n=5)			50 ng/mL (n=5)			250 ng/mL (n=5)		
Analyte	Recovery (%)	RSD (%)	Matrix Effects (%)	Recovery (%)	RSD (%)	Matrix Effects (%)	Recovery (%)	RSD (%)	Matrix Effects (%)
17β-estradiol-17-sulfate	76	2	3	73	2	1	77	6	0
17β-estradiol-3-sulfate	77	1	-1	77	1	2	78	3	0
17α-estradiol Sulfate	78	1	2	80	2	1	82	3	0
Boldenone Sulfate	85	1	1	82	1	2	86	2	0
Nandrolone Sulfate	84	1	0	81	1	0	86	3	-1
Testosterone Sulfate	84	10	24	81	2	2	87	3	2
Estrone-3-sulfate	77	3	3	77	2	0	79	4	-1
DHEA Sulfate	113*	2	27*	79	1	6	86	4	3
Androsterone Sulfate	101*	3	10*	76	1	3	83	4	1
Estradiol	81	2	-2	77	3	-1	84	3	-3
Boldenone	82	1	-2	83	2	0	86	3	0
Nandrolone	85	1	10	83	2	1	86	2	0
Testosterone	85	2	4	83	1	-1	87	2	-1
DHEA	89	1	1	84	2	-6	85	2	-4
Estrone	83	2	-3	78	3	-8	84	1	-6
Androsterone	80	4	5	82	2	-4	86	1	-3

*LLOQ must be higher than 5 ng/mL for reproducibility of DHEA sulfate and androsterone due to endogenous background concentrations (See Conclusion/Discussion section).

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Conclusion/Discussion:

A method was developed for the simultaneous extraction of both free and sulfated steroids in urine, plasma, and blood. Steroids are inherently difficult to work with because they are endogenous, especially DHEA sulfate, androsterone, and testosterone, making accurate analysis challenging. As a solution to this, synthetic urine was used as a surrogate matrix for urine. For blood and plasma, a suitable surrogate matrix is not available, so background subtraction was employed to determine recoveries. This was done by extracting a set of blank matrices simultaneously with the samples and subtracting the steroids present in the blanks from the samples. This was particularly necessary for DHEA sulfate, androsterone sulfate, and testosterone since they are present in the blank matrix in the highest amounts. Recoveries were calculated using **Equation 1** and matrix effects were determined using **Equation 2**.[3] However, for a background subtraction method to be reproducible, the lower limit of quantitation (LLOQ) for endogenous analytes should be at least 15-20% of the background peak concentrations.[4] For this reason, the results for DHEA sulfate and androsterone spiked at 5 ng/mL are irreproducible and insignificant in practice. A higher LLOQ would be required for these two analytes specifically because they are limited by the endogenous background concentrations rather than the analytical sensitivity of the method.

$$\%\ recovery = \frac{average\ area\ pre\ spiked\ samples - average\ area\ blank\ matrix}{average\ area\ post\ spiked\ samples - average\ area\ blank\ matrix}$$

Equation 1: Determination of percent recovery of endogenous substances

$$\%\ matrix\ effects = \left(\frac{average\ area\ post\ spiked\ samples - average\ area\ blank\ matrix}{average\ area\ solvent\ calibrator}\right) - 1$$

Equation 2: Determination of percent matrix effects of endogenous substances

Working with both free and sulfated steroids was further challenging because the sulfate conjugates are always negatively charged while the free steroids are always neutral, making reverse phase SPE an evident strategy for these analytes. Additionally, during LC-MS/MS analysis, sulfated steroids were ionized in negative mode, while the free steroids were ionized in positive mode. Meaning, the LC-MS/MS method required polarity switching for the simultaneous detection of target ions in both positive and negative ionization modes.

The steroid sulfates are also different from free steroids because the sulfate conjugates are highly protein bound, making the protein precipitation step necessary in order to analytically observe these substances. This part of the method also helps to clean up the sample and remove potential matrix interferences before it is even introduced to the SPE cartridge, which is preferable for blood matrices extracted on a polymeric cartridge, like the Styre Screen® HLB cartridge utilized in this method.

The full SPE method including the protein crash, wash solvents, and elution solvents were optimized to achieve the highest recoveries with the lowest matrix effects. Recovery for analytes at low, medium, and high concentrations in all three matrices ranged from 73-103% with low relative standard deviations (RSD) and matrix effects. Negative and positive matrix effects represent ion suppression and ion enhancement respectively. A robust analysis method for steroids was developed that can readily be implemented by clinical, forensic, and anti-doping labs.

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[2] Esquivel, A., Alechaga, É., Monfort, N., & Ventura, R. (2018). Sulfate metabolites improve retrospectivity after oral testosterone administration. Drug Testing and Analysis. doi:10.1002/dta.2529

[3] Hess, C., Sydow, K., Kueting, T., Kraemer, M., & Maas, A. (2018). Considerations regarding the validation of chromatographic mass spectrometric methods for the quantification of endogenous substances in forensics. Forensic science international, 283, 150-155.

[4] Thakare, R., Chhonker, Y. S., Gautam, N., Alamoudi, J. A., & Alnouti, Y. (2016). Quantitative analysis of endogenous compounds. Journal of pharmaceutical and biomedical analysis, 128, 426-437.

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