

A Look at Column Reproducibility: Analysis of Endogenous Steroid Hormones in Serum Using Three Historical Batches of 1.8 μ m HSS T3 Stationary Phase

Kenneth D. Berthelette, Jennifer M. Nguyen, Chelsea Plummer, Kim Haynes, Jonathan E. Turner, Jonathan P. Danaceau

Waters Corporation

Abstract

Batch to batch testing of stationary phases for a given assay is a common method robustness experiment.¹⁻³ Having reproducible columns is critical for the success of these tests, as well as ensuring lifelong performance for the assay. To demonstrate the reproducibility of Waters column chemistries, the analysis of endogenous steroid hormones was performed on three batches of ACQUITY UPLC HSS T3 manufactured over a five-year span. Low %RSD values for retention time, peak area, peak width, and peak symmetry demonstrate that the HSS T3 stationary phase is highly reproducible, and little to no chromatographic differences were seen between the oldest and newest batches.

Benefits

- Comparable batch to batch performance for three historical batches of HSS T3 material
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- Improved signal and detection using Solid Phase Extraction over Protein Precipitation with ACQUITY UPLC HSS T3 columns

Introduction

Having high quality products, particularly liquid chromatography (LC) stationary phases and columns, ensures that the data generated for an assay is accurate and reproducible over time. The quality of LC stationary phases can be measured by batch-to-batch reproducibility testing for a given assay to ensure that each manufactured lot of material performs similarly. Most batch-to-batch tests are performed using three batches of material made within a certain time period, such as those within a method validation kit. While these materials can work well, their use may only provide a snapshot of the overall quality of the product. To get the full picture, historical batches of material should be tested, as this provides a better scope of overall product quality.

The ACQUITY HSS T3 stationary phase is a commonly used C₁₈ bonded phase using fully porous silica particles. This phase is designed to retain polar analytes better than a traditional C₁₈ without compromising the separation of hydrophobic compounds. This phase has been used in many applications and publications for separations ranging from small molecule pharmaceuticals in dosage form to bioanalytical applications.⁴⁻⁶ To demonstrate the quality of the HSS T3 material, three columns were packed with different batches of material. These materials were made five years apart ranging from 2016 to 2021. All three columns were then used to separate endogenous steroid hormones in human serum to assess the reproducibility of the data across all columns.

Experimental

Sample Description

Human serum was prepared by solid phase extraction (SPE) and protein precipitation (PPT). PPT using acetonitrile (3:1 acetonitrile:serum) was performed. Samples were vortexed, centrifuged, and the supernatant removed for analysis. SPE was performed using Oasis HLB 1 cc 30 mg cartridges (p/n: [WAT094225](https://www.waters.com/nextgen/global/shop/sample-preparation--filtration/wat094225-oasis-hlb-1-cc-vac-) < <https://www.waters.com/nextgen/global/shop/sample-preparation--filtration/wat094225-oasis-hlb-1-cc-vac->

[cartridge-30-mg-sorbent-per-cartridge-30--m-1.html](#)>). Sorbent conditioned with methanol and equilibrated with water as outlined in the literature.⁷ Generic SPE conditions were used, and elution performed using 100% acetonitrile. The eluate was dried under nitrogen at 60 °C and reconstituted in 100 µL Milli-Q water for analysis.

LC Conditions

LC systems:	ACQUITY UPLC I-Class PLUS with Binary Solvent Manager (BSM), Sample Manager Flow Through Needle (SM-FTN), and PDA Detector
Detection:	MRMs of Serum Hormones outlined in Table 1
Column(s):	ACQUITY UPLC HSS T3 1.8 µm, 2.1 x 50 mm (p/n: 186009467) Three (3) batches of material: 189 – Manufactured Oct-2016 233 – Manufactured Nov-2019 270 – Manufactured Nov-2021
Column temp.:	30 °C
Sample temp.:	10 °C
Injection volume:	5.0 µL
Flow rate:	0.6 mL/min
Mobile phase A:	0.1% Formic acid in Milli-Q Water
Mobile phase B:	0.1% Formic acid in acetonitrile
Gradient profile:	5% to 95% B in 5.0 minutes, hold at 95% B for 0.5

minutes.

MS Conditions

MS system:	Xevo TQ-S micro
Ionization mode:	ESI+
Acquisition range:	MRMs outlined in Table 1
Capillary voltage:	3.0 kV
Cone voltage:	Analyte dependent (Table 1)
Desolvation temp.:	500 °C
Desolvation flow:	500 L/hr
Cone gas flow:	10 L/hr

Compound	ESI mode	Precursor mass (m/z)	Cone voltage	Product ions (m/z)	Collision energy (V)
Androstenedione	+	287.3	70	97.1 / 109.1	24 / 24
Testosterone	+	289.3	70	97.1 / 109.1	22 / 22
Progesterone	+	315.3	70	97.1 / 109.1	24 / 24
Cortisol	+	363.3	40	91.1 / 121.2	24 / 24

Table 1. MRM conditions for four steroid hormones detected in serum.⁸

Data Management

Results and Discussion

Prior to column testing two different sample preparation techniques were assessed to optimize the assay. PPT and SPE were performed on a sample of human serum. The SPE protocol outlined in the experimental section using Oasis HLB 1 cc 30 mg cartridges was not optimized for this experiment. Generic SPE conditions were used to demonstrate effectiveness of SPE compared to PPT. Further improvements in signal could be achieved by optimization of SPE protocol. Both sample preparation techniques were analyzed using a single batch of ACQUITY UPLC HSS T3 1.8 μm , 2.1 x 50 mm to evaluate mass spectrometry (MS) signal intensity of expected analytes. Figure 1 shows the comparison of SPE vs PPT results for the steroid hormones detected in serum.

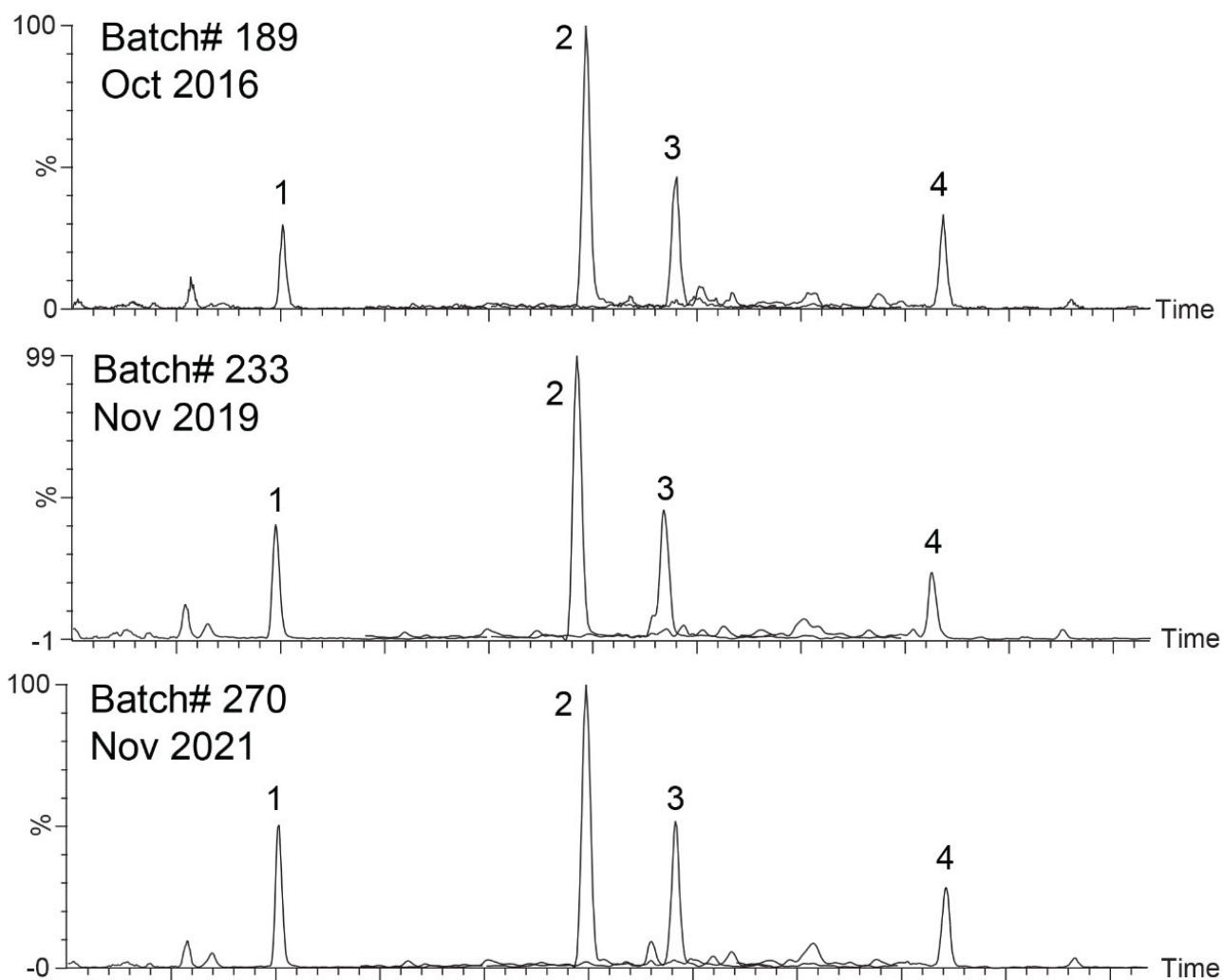


Figure 1. Comparison of SPE vs PPT as sample preparation of human serum for the detection of endogenous steroid hormones. Signal intensity differences circled.

A panel of potential steroids in serum were examined.⁸ Four steroid hormones were detected with signal $>1e5$ after SPE under these conditions. The remaining steroids were not detected. Peak identification was performed using a standard mixture of the steroids and the test conditions indicated. The results after PPT show only two steroid hormones, testosterone, and cortisol, with considerably lower signals compared to SPE. Androstenedione and progesterone were not detected after PPT. SPE produced a 5x increase in signal for cortisol and about a 30x increase in signal for testosterone. This provides not only a more easily detectable peak, but also reduces the

LOD and LOQ of the assay and can give a wider dynamic range for SPE recovery studies. SPE also creates a cleaner sample by removing phospholipids and other problematic compounds.^{9,10} Cleaner samples reduce the rate of instrument and column fouling while also reducing matrix effects such as ion suppression or enhancement. Moving forward to column comparisons SPE will be used for sample preparation.

Fresh samples were prepared by SPE to evaluate three historical batches of ACQUITY UPLC HSS T3 1.8 μm material. The batches were created over a five-year span, with the oldest batch being manufactured and tested in October 2016. The remaining batches were manufactured in November 2019 and November 2021. Figure 2 shows the chromatograms of the four endogenous steroids on the three columns. Replicate injections ($n=3$) of serum were performed on each column, with two blanks before and after to ensure there was no system carryover.

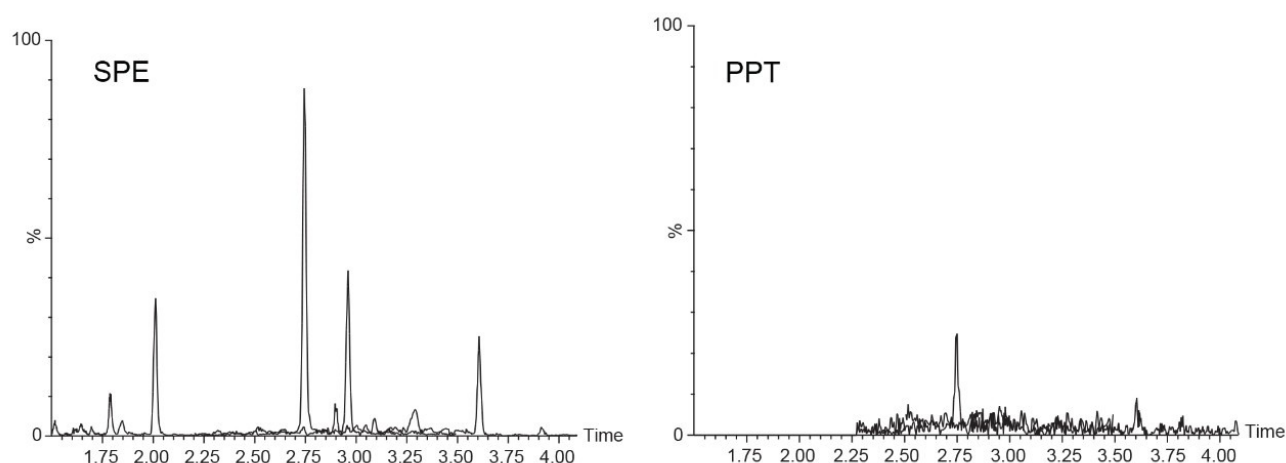


Figure 2. Batch to batch column comparison of endogenous steroid hormones in serum. Batch numbers and manufacturing date shown.

1) Cortisol, 2) Testosterone, 3) Androstenedione, and 4) Progesterone.

All three columns achieve comparable results for the analysis of endogenous steroids. Peak retention times, peak areas, peak widths, and peak symmetry were monitored and assessed for all three columns, as well as pooled data samples, Table 2. %RSDs for retention time were <1% for all probes on each column ($n=3$) as well as the pooled data set ($n=9$). Peak area reproducibility was good with a %RSD <5% for each column and pooled data samples. Similarly, low %RSDs were obtained for peak width (half height) and peak symmetry.

Cortisol								
Retention time (min)		Peak area		Peak width (HH) (sec)		Peak symm.		
Batch #	Avg	%RSD	Avg	%RSD	Avg	%RSD	Avg	%RSD
189	2.004	0.12	4991	1.23	1.27	1.49	1.14	3.00
233	1.988	0.00	5039	0.37	1.32	4.54	1.18	1.20
270	2.008	0.00	4937	4.76	1.37	4.56	1.17	2.21
Pooled data	2.000	0.53	4989	1.02	1.32	3.86	1.17	1.84
Progesterone								
Retention time (min)		Peak area		Peak width (HH) (sec)		Peak symm.		
Batch #	Avg	%RSD	Avg	%RSD	Avg	%RSD	Avg	%RSD
189	3.589	0.07	3987	3.27	1.25	3.40	1.13	1.88
233	3.565	0.00	4096	0.86	1.23	0.78	1.12	1.31
270	3.606	0.00	4015	4.56	1.24	1.62	1.18	2.00
Pooled data	3.587	0.57	4033	1.40	1.24	0.97	1.14	3.03
Testosterone								
Retention time (min)		Peak area		Peak width (HH) (sec)		Peak symm.		
Batch #	Avg	%RSD	Avg	%RSD	Avg	%RSD	Avg	%RSD
189	2.733	0.00	14683	1.99	1.93	2.54	0.99	0.96
233	2.711	0.00	14884	0.73	1.97	1.65	1.05	3.67
270	2.744	0.00	14365	2.90	1.86	1.88	1.01	2.87
Pooled data	2.729	0.62	14644	1.79	1.92	2.70	1.01	2.94
Androstenedione								
Retention time (min)		Peak area		Peak width (HH) (sec)		Peak symm.		
Batch #	Avg	%RSD	Avg	%RSD	Avg	%RSD	Avg	%RSD
189	2.947	0.07	7156	3.29	1.50	4.94	1.03	3.66
233	2.922	0.06	7268	3.65	1.61	1.61	1.02	4.20
270	2.958	0.00	6897	4.10	1.47	1.46	1.06	2.89
Pooled data	2.942	0.63	7107	2.68	1.53	4.88	1.04	2.12

Table 2. Tabular data for batch-to-batch testing of HSS T3. Samples injected in replicate (n=3) on each column. Pooled data (n=9) used for calculations of overall reproducibility.

Good batch-to-batch reproducibility for this assay is achieved even with materials that were manufactured over a five-year span. Using materials with this level of reproducibility ensures that an assay will perform the same over its lifetime which can be several years. Waters columns provide this level of reproducibility through stringent quality control and manufacturing processes, to ensure the data generated on those columns is of the highest possible quality.

Conclusion

To demonstrate the quality of Waters particle and column manufacturing processes, three batches of ACQUITY HSS T3 stationary phase, manufactured over a five-year span, were used to separate endogenous steroid hormones in human serum. SPE was used for all sample preparation for reproducibility testing.

The three batches showed reproducible chromatographic performance. %RSD values for retention time were <1% for each column as well as the pooled data set (n=9). Additionally, %RSDs for peak area, peak width, and peak symmetry were <5% for each detected analyte. These results indicate that even though the materials were made five years apart, the same quality of data can be generated. This level of assurance, that similar data can be achieved over years, is critical when developing new methods. Knowing that the selected stationary phase will be the same lot-to-lot and year-to-year allows for a longer assay lifetime without the need for additional rework.

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