COMPREHENSIVE QUANTITATIVE ANALYSIS OF MULTICLASS STEROIDS IN SERUM USING THE STEROID PANEL LC-MS KIT AND AGILENT 6495 TRIPLE QUADRUPOLE LC-MS.

Semi-Automation-Sample Preparation with 96-Well Plate Format.

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ABSTRACT.

This application note details a robust workflow for quantifying a broad panel of steroids using the Tecan® Steroid Panel LC-MS kit targeted for Agilent 6495 Triple Quadrupole LC-MS. The method from the RUO kit (Cat no: 30220266*) was optimized for chromatographic separation and mass spectrometric detection, enabling confident quantification of multiple analytes, even those with similar structures, with an Agilent LC-MS set-up. Precision was demonstrated with CVs below 10% for most analytes and below 3% for testosterone, estradiol, androstenedione, and 11-deoxycorticosterone. Linearity was excellent (R^2 >0.99), and trueness at QC levels was within ±15% for most analytes. All QC ranges specified by the manufacturer were met. The method, adapted on the Agilent 6495 LC-MS/MS, provides a reliable and accurate solution for comprehensive steroid profiling in clinical research.

EXPERIMENTAL.

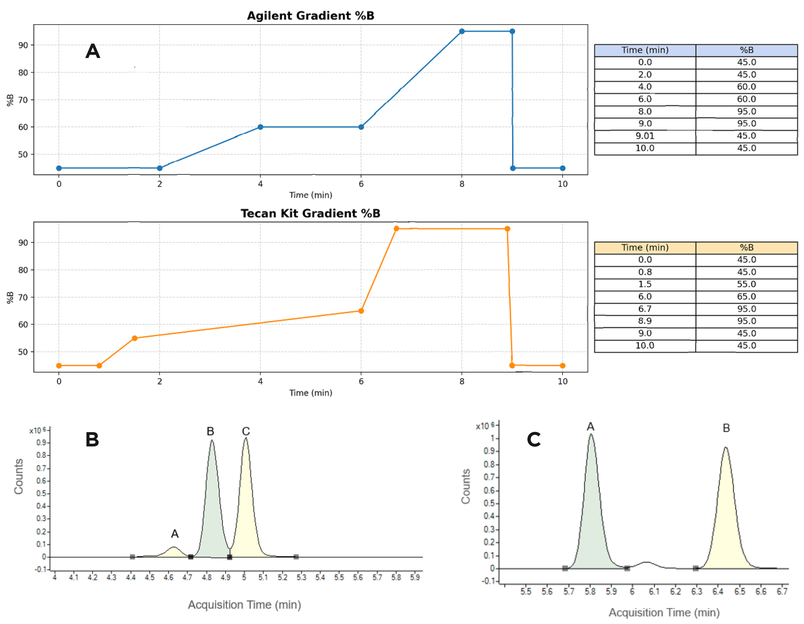
Steroid analysis, was performed using an Agilent 1290 Infinity III LC system coupled to an Agilent 6495 triple quadrupole mass, spectrometer with C8 column distrubuted by Tecan. Sample preparation involved semi-automated SPE using the Tecan Resolvex® A200 in a 96-well plate format. Mobile phases contained water or methanol with ammonium fluoride. Data were acquired in positive/negative ESI mode using Agilent MassHunter software, and quantification was based on calibration curves from kit standards (see Table 1 and Table 2).

Table 1: Overview of LC-MS/MS Method Parameters

Parameter	Details
LC-System	Agilent 1290 Infinity III LC
MS System	Agilent 6495 Triple Quadrupole Mass Spectrometer
Column	C8 column, Tecan (REF: 30215928)
LC-Modules	Binary pump (G7120A), multisampler (G7167B), multicolumn thermostat (G7116B)
Inline Filter	0.3 µm (part no. 5067-6189)
Mobile Phases	A: Water + ammonium fluoride (<5 mM); B: Methanol + ammonium fluoride (<5 mM)
Gradient	0–2 min: 45% B; 4 min: 60% B; 8–9 min: 95% B; 10 min: 45% B; flow rate 0.35 mL/min
Injection Volume	20 µL
Temperatures	Autosampler: 10°C; Column oven: 40°C
Ionization	Positive/Negative ESI (AJS)
Acquisition	dMRM, cycle time 0.5 s, stop time 10 min
Sample Prep	Semi-automated SPE (Tecan Resolvex A200, 96-well plate format)
Chemicals	LC/MS-grade MeOH, water, ammonium fluoride
Software	Agilent MassHunter (v12.2)
Calibration	Kit standards and internal standards; quantification by calibration curves

METHOD ADAPTION.

Chromatographic separation was improved by C8 column distributed by Tecan and optimizing both the gradient and MRM transitions for the LC/TQ system. These adjustments enabled robust resolution of all 18 analytes, including challenging pairs, within a shortened 10-minute run time (see Figure 1 and 2). The method supports simultaneous analysis in both positive and negative ionization modes, ensuring comprehensive analyte coverage and reliable quantification.

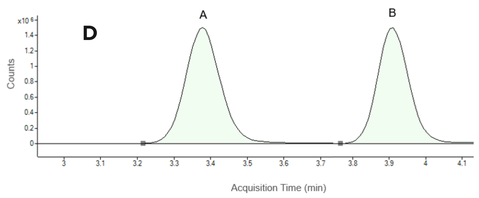


Time (min)	%B
0.0	45.0
2.0	45.0
4.0	60.0
6.0	60.0
8.0	95.0
9.0	95.0
9.01	45.0
10.0	45.0

Time (min)	%B
0.0	45.0
0.8	45.0
1.5	55.0
6.0	65.0
6.7	95.0
8.9	95.0
9.0	45.0
10.0	45.0

Disclaimer: The combined use of the reagents, process script and instrument has to be validated individually on site by each laboratory.
*For research use only, not for use in diagnostic procedures.

Figure 1: Gradient Optimization and Chromatographic Performance: Comparison of Agilent and Tecan kit gradients (Figure A) shows that adapting the Agilent gradient to the Tecan protocol improves analyte separation and reduces run time to 10 minutes. Chromatograms demonstrate clear separation of challenging steroid pairs: 21-deoxycortisol, corticosterone, and 11-deoxycortisol (Figure B) and 11-deoxycorticosterone and 17-hydroxyprogesterone (Figure C) and cortisone and cortisol (Figure D).



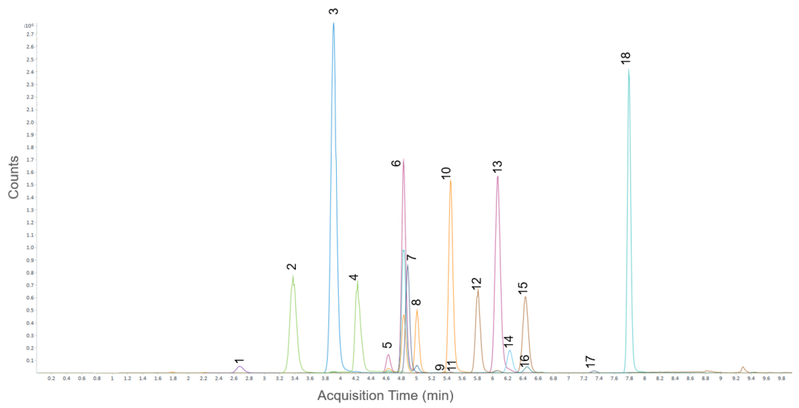


Figure 2: MRM trace overlay of 18 analytes detailing the elution profile. The data shown were acquired using calibrator C. Numbers are assigned to analytes which are explained in table below.

Table 2. Overview of Target Analytes, Retention Times, and MRM Transition

No	Target	RT (min)	Transition (m/z)
1	aldosterone	2.65	361.2 → 315.1
2	cortisone	3.37	361.2 → 163.0
3	cortisol	3.9	363.2 → 121.0
4	dehydroepiandrosterone sulfate (DHEAS)	4.2	271.2 → 253.1
5	21-deoxycortisol	4.59	347.2 → 121.0
6	corticosterone	4.82	347.2 → 329.1
7	dexamethasone	4.84	393.2 → 373.1
8	11-deoxycortisol	4.97	347.2 → 97.0
9	*estrone	5.38	269.2 → 145.0
10	androstenedione	5.45	287.2 → 97.0
11	*estradiol	5.45	271.2 → 183.0
12	11-deoxycorticosterone	5.81	331.2 → 97.0
13	testosterone	5.91	289.2 → 97.0
14	dehydroepiandrosterone (DHEA)	6.19	289.2 → 271.0
15	17-hydroxyprogesterone (17OHP4)	6.37	331.2 → 97.0
16	*17-hydroxypregnenolone (17OHP5)	6.46	331.2 → 303.2
17	dihydrotestosterone (DHT)	7.31	291.2 → 255.2
18	progesterone	7.75	315.2 → 97.0

* Indicates analytes measured in negative ionization mode.

LINEARITY.

Linearity was evaluated for each analyte using calibration standards from the Tecan kit. All analytes showed good linearity (R^2 >0.99), confirming a strong correlation between concentration and response. Most fit a linear model, while a few required a quadratic fit. For example, the calibration curve for cortisol (see Figure 3) demonstrates the method's robust and reliable quantification for comprehensive steroid profiling.

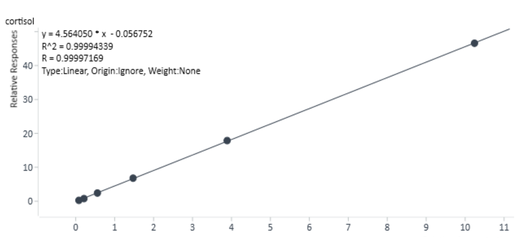


Figure 3: Linearity of cortisol quantification across six calibration levels (x-axis: Relative Concentration; y-axis: Relative Response)

On our homepage you will find further information on the Steroid Panel LC-MS, including three interesting videos.

TRUENESS AND PRECISION.

Trueness was assessed by analyzing QC samples with metrological traceability at both high and low concentrations. For each analyte, measured concentrations were compared to assigned QC values, and percent deviation calculated. Most analytes demonstrated excellent trueness, with deviations well within ±20% at both QC levels. Key analytes such as progesterone, DHT, DHEA, testosterone, and estradiol showed deviations below 15%. While a few analytes exhibited minor positive or negative bias, all results remained within accepted limits, confirming the method's accuracy (see Table 3).

Precision was determined as CV% across six concentration levels (four replicates each). Most analytes, including testosterone, estradiol, androstenedione, and 11-deoxycorticosterone, had CVs below 10%. Even at low and high levels, most CVs remained under 20%, confirming high repeatability and reproducibility of the adapted method (see Table 4).

Table 3. Trueness of the adapted method: Percent deviation from assigned values at high and low QC levels for each analyte.

Analyte	Trueness	
	QC high	QC low
progesterone	9.30%	2.80%
dihydrotestosterone	5.20%	2.10%
17-hydroxypregnenolone	0.10%	-5.90%
17-hydroxyprogesterone	-12.50%	-2.20%
dehydroepiandrosterone	4.40%	3.80%
testosterone	11.90%	10.90%
11-deoxycorticosterone	3.60%	11.00%
estradiol	1.00%	3.00%
androstenedione	15.60%	1.30%
estron	0.33%	-0.18%
11-deoxycortisol	-2.00%	-2.60%
dexamethasone	-7.30%	16.20%
corticosterone	-0.90%	8.00%
21-deoxycortisol	-12.30%	-11.50%
dehydroepiandrosterone sulfate	7.70%	10.40%
cortisol	8.80%	-2.20%
cortisone	3.70%	9.80%
aldosterone	18.70%	13.40%

Table 4. Precision (CV%) of adapted method at Six QC Levels: Coefficient of variation (CV%) for each analyte across six different QC levels (S1–S6, four replicates each).

Analyte	CV% Precision					
	S1	S2	S3	S4	S5	S6
progesterone	2.00%	3.40%	11.70%	2.60%	10.60%	10.70%
dihydrotestosterone	2.90%	5.80%	4.10%	3.20%	5.00%	12.60%
17-hydroxypregnenolone	0.90%	7.40%	5.80%	5.80%	4.20%	4.30%
17-hydroxyprogesterone	3.70%	12.30%	16.20%	8.20%	6.60%	5.90%
dehydroepiandrosterone	2.00%	0.90%	1.80%	8.70%	8.10%	10.80%
testosterone	1.20%	2.10%	1.00%	1.90%	0.90%	2.10%
11-deoxycorticosterone	2.50%	2.00%	1.80%	1.90%	1.50%	2.20%
estradiol	2.00%	3.10%	2.20%	3.20%	2.70%	11.60%
androstenedione	1.30%	2.00%	2.40%	1.70%	1.70%	4.10%
estron	0.40%	5.10%	1.90%	3.10%	1.00%	3.30%
11-deoxycortisol	0.70%	1.60%	4.70%	3.50%	5.10%	3.60%
dexamethasone	3.20%	4.10%	9.90%	4.30%	2.10%	6.10%
corticosterone	1.90%	2.20%	4.10%	2.30%	6.10%	3.80%
21-deoxycortisol	4.60%	7.60%	12.20%	0.90%	3.30%	2.40%
dehydroepiandrosterone sulfate	5.00%	0.70%	12.70%	8.90%	8.60%	8.20%
cortisol	0.50%	4.10%	11.60%	3.20%	7.30%	12.30%
cortisone	1.50%	1.60%	5.20%	1.10%	3.80%	4.70%
aldosterone	2.10%	7.00%	12.10%	1.70%	5.00%	12.20%

CONCLUSION.

This study demonstrates that targeted optimizations enable comprehensive analyte quantification in a single 10-minute run without compromising performance. Results show high precision (CVs <10%), good linearity, and reliable trueness across a broad range. Compatibility with the Agilent platform ensures robust and reproducible outcomes. Semi-automated sample preparation streamlines workflow and enhances laboratory efficiency. Overall, the Steroid Panel LC-MS kit tailored for an Agilent LC-MS set-up provides a reliable and efficient solution for advanced steroid profiling.