Application Note

Small Molecule Pharma

Comprehensive Quantitative Analysis of Multiclass Steroids in Serum

Using a 96-well format with the Tecan Steroid Panel LC-MS kit and Agilent 6495 triple quadrupole LC/MS

Authors

Ayham Al Ahmad
Tecan, IBL International GmbH, Flughafenstraße 52a, 22335 Hamburg, Germany

Christian Hegmanns
Agilent Technologies Deutschland GmbH, Hewlett-Packard-Straße 8, 76337 Waldbronn, Germany

Abstract

This application note describes a robust and reliable workflow for the quantitative analysis of a broad panel of steroids using the Tecan Steroid Panel LC-MS kit.

The method from the Steroid Panel LC-MS kit (Cat no: 30220266, Research use only (RUO)) was optimized for chromatographic separation and mass spectrometric detection, enabling confident quantification of multiple analytes, even those with closely related chemical structures and mass transitions. The analytical performance was evaluated to demonstrate the reliability of this method. The precision of using samples (spiked human serum) with six different concentration levels was evaluated, demonstrating coefficients of variation (CV%) less than 10% for most analytes, with particularly low CVs (< 3%) observed for testosterone, estradiol, androstenedione, and 11-deoxycorticosterone. While a few analytes, such as 17-hydroxyprogesterone (17-OHP4), 21-deoxycortisol, and aldosterone, exhibited higher CVs at certain levels, All results were within the acceptable limit of 20%.

Linearity was established for all analytes, with R^2 values up to 0.999 and most exceeding 0.990, indicating excellent linearity across the concentration range for all 17 steroids and dexamethasone. Most compounds followed a linear calibration model, though a quadratic fit was applied where necessary to increase reliability.

Trueness, assessed at both high and low QC levels, was within ± 15% for most analytes, confirming the method's accuracy and reliability. Additionally, the QC ranges provided by the manufacturer were met for all analytes.

Overall, the results confirm that the Steroid Panel LC-MS kit adapted on an Agilent 6495 triple quadrupole LC/MS provides a robust, precise, and accurate solution for comprehensive steroid profiling, supporting its application in clinical research environments where high analytical performance is required.

Introduction

Steroids are critical endogenous metabolites that regulate various physiological processes, making their accurate quantification essential for clinical research applications. Conventional immunoassays, while widely used, are often limited by cross-reactivity and insufficient analytical specificity, particularly when measuring structurally similar steroid compounds. Liquid chromatography coupled with mass spectrometry (LC/MS) has, therefore, become the gold standard for steroid analysis, offering superior specificity, sensitivity, and the ability to detect analytes at trace levels.[1]

A persistent challenge in steroid analysis is achieving high precision and accuracy across a broad panel of analytes and concentration ranges.[2] The method described here, using the Steroid Panel LC-MS kit from Tecan, was meticulously developed to address these demands, delivering robust quantification with excellent repeatability and linearity. Chromatographic conditions were carefully optimized to resolve analytes with closely related structures and overlapping mass transitions, thereby ensuring accurate identification and quantification even in complex sample matrices.

This application note details the integration of the Steroid Panel LC-MS kit with the 6495 LC/TQ, providing a comprehensive and efficient workflow for the simultaneous quantification of 17 steroids and dexamethasone. The streamlined protocol minimizes manual intervention, enhances sample throughput, and ensures high analytical performance, establishing a reliable solution for advanced steroid profiling in research settings.

Experimental

Instrumentation and workflow

The Steroid Panel LC-MS workflow was established using the following instrumentation and components:

Chromatographic separation was performed on an Agilent 1290 Infinity III liquid chromatography system coupled to a 6495 triple quadrupole mass spectrometer. Separation was achieved using a C8 column distributed by Tecan (Tecan p/n 30215928). The 1290 Infinity III LC system was configured with the following modules:

– Agilent 1290 Infinity III binary pump (G7120A)

– Agilent 1290 Infinity III multisampler (G7167B)

– Agilent 1290 Infinity III multicolumn thermostat (G7116B)

A 0.3 µm inline filter (Agilent p/n 5067-6189) was installed between the autosampler injection valve and the multicolumn compartment to protect the column from particulates. The detailed LC conditions are provided in Table 1. Steroid hormone detection and quantification were performed using a 6495 LC/TQ. The instrument operated in both positive and negative electrospray ionization (ESI) modes, with multiple reaction monitoring (MRM) transitions individually optimized for each analyte. Data acquisition and processing were performed using Agilent MassHunter software, version 12.2. The 6495 LC/TQ parameters are shown in Table 2.

Table 1. Agilent 1290 Infinity III LC parameters.

Parameters	Value
Multiwash	Step / Time (sec) / Solvent 1 / 5 / ACN* 2 / 5 / MeOH* 3 / 5 / Water:MeOH 55:45 (v:v)* *Seat backflush and needle wash
Analytical Separation	C8 column distributed by Tecan (Tecan p/n 30215928)
Autosampler Temperature	10 °C
Multicolumn Thermostat Temperature	40 °C
Injection Volume	20 µL
Mobile Phase A	Water with ammonium fluoride (< 5 mM)
Mobile Phase B	Methanol with ammonium fluoride (< 5 mM)
Gradient	Time / %B / Flow rate (mL/min) 0.00 / 45 / 0.35 2.00 / 45 / 0.35 4.00 / 60 / 0.35 6.00 / 60 / 0.35 8.00 / 95 / 0.35 9.00 / 95 / 0.35 9.01 / 45 / 0.35 10.0 / 45 / 0.35

Table 2. Agilent 6495 triple quadrupole LC/MS configuration and parameters.

Parameter	Value
Ionization Mode	Positive/negative ESI with Agilent Jet Stream technology ion source (AJS)
Acquisition Type	dMRM
Cycle Time	0.5 sec
Stop Time	10.00 min
Gas Temperature	120 °C
Gas Flow	16 L/min
Nebulizer	30 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	3,500 V (+)/2,500 (–)
Nozzle Voltage	500 V (+)/500 (–)

MRM optimization

Target-specific MRM transitions and collision energies were optimized using MassHunter software, version 12.2. MS/MS optimization was conducted without chromatographic separation by injecting 2 µL of neat solutions of individual analytes at approximately 1,000 µg/L. For instrument optimization and tuning, Tecan Steroid Tuning Mix 1 – 4 (Tecan p/n 30227628, 30227629, 30227630, and 30227631) were used according to the manufacturer's instructions. The optimized LC parameters, along with the 6495 LC/TQ collision energy values, were incorporated into the final dMRM method for data acquisition. The complete set of optimized MRM settings used for the analysis is presented in Table 3.

Data acquisition and analysis

Data acquisition and processing were conducted using MassHunter software, version 12.2. Quantification was based on calibration curves generated from kit standards and internal standards; for fitting details, see Table 4.

Sample preparation

Sample extraction was performed using a semi-automated positive pressure manifold (Tecan Resolvex® A200) for solid phase extraction (SPE). Extraction columns and deep well collection plates were supplied as part of the kit. The extraction procedure was carried out exactly as described in the Instructions for Use (IFU).

Chemicals and reagents

Agilent InfinityLab LC/MS-grade methanol (MeOH), water, and ammonium fluoride were used for the study. Chemicals were purchased from VWR International GmbH (Darmstadt, Germany).

Table 3. Quantifier transitions, retention times, and collision energies for LC/MS/MS steroid analysis (continued on the next page).

Compound	Precursor Ion (*m/z*)	Product Ion (*m/z*)	RT (min)	Time Window (min)	CE (V)	Polarity
11-Deoxycorticosterone	331.2	109.0	5.81	10.0	30	Positive
11-Deoxycorticosterone	331.2	97.0	5.81	10.0	26	Positive
11-Deoxycorticosterone-$^{13}C_3$	334.2	112.1	5.81	10.0	28	Positive
11-Deoxycorticosterone-$^{13}C_3$	334.2	100.1	5.81	10.0	26	Positive
11-Deoxycortisol	347.2	109.0	5.03	10.0	32	Positive
11-Deoxycortisol	347.2	97.0	5.03	10.0	30	Positive
11-Deoxycortisol-D_5	352.2	113.1	4.98	10.0	32	Positive
11-Deoxycortisol-D_5	352.2	100.1	4.98	10.0	28	Positive
17-Hydroxypregnenolone	331.2	303.2	6.46	20.0	19	Negative
17-Hydroxypregnenolone	331.2	287.2	6.46	20.0	21	Negative
17-Hydroxypregnenolone-$^{13}C_2$-D_2	335.3	316.2	6.42	20.0	19	Negative
17-Hydroxypregnenolone-$^{13}C_2$-D_2	335.3	289.2	6.42	20.0	19	Negative
17-Hydroxyprogesterone	331.2	109.0	5.82	10.0	28	Positive
17-Hydroxyprogesterone	331.2	97.0	5.82	10.0	26	Positive
17-Hydroxyprogesterone-D_8	339.3	113.1	6.38	10.0	32	Positive
17-Hydroxyprogesterone-D_8	339.3	100.0	6.38	10.0	30	Positive
21-Deoxycortisol	347.2	311.1	4.84	1.0	15	Positive
21-Deoxycortisol	347.2	121.0	4.84	1.0	30	Positive
21-Deoxycortisol-D_8	355.3	319.2	4.60	1.0	15	Positive
21-Deoxycortisol-D_8	355.3	125.1	4.60	1.0	32	Positive
Aldosterone	361.2	343.1	2.70	1.0	15	Positive
Aldosterone	361.2	315.1	2.70	1.0	19	Positive
Aldosterone-D_4	365.2	319.1	2.70	1.0	19	Positive
Aldosterone-D_4	365.2	97.0	2.70	1.0	41	Positive
Androstenedione	287.2	109.1	5.46	1.0	26	Positive
Androstenedione	287.2	97.0	5.46	1.0	24	Positive

Table 3. Quantifier transitions, retention times, and collision energies for LC/MS/MS steroid analysis (continued from the previous page).

Compound	Precursor Ion (*m/z*)	Product Ion (*m/z*)	RT (min)	Time Window (min)	CE (V)	Polarity
Androstenedione-$^{13}C_3$	290.2	112.1	5.46	1.0	28	Positive
Androstenedione-$^{13}C_3$	290.2	100.0	5.46	1.0	24	Positive
Corticosterone	347.2	329.1	4.84	1.0	14	Positive
Corticosterone	347.2	311.1	4.84	1.0	14	Positive
Corticosterone-$^{13}C_3$	350.2	332.2	4.84	1.0	14	Positive
Corticosterone-$^{13}C_3$	350.2	124.0	4.84	1.0	24	Positive
Cortisol	363.2	121.0	3.93	1.0	28	Positive
Cortisol	363.2	91.0	3.93	1.0	36	Positive
Cortisol-D_4	367.2	331.2	3.92	1.0	14	Positive
Cortisol-D_4	367.2	121.0	3.92	1.0	26	Positive
Cortisone	361.2	163.0	3.42	1.0	24	Positive
Cortisone	361.2	91.0	3.42	1.0	60	Positive
Cortisone-$^{13}C_3$	364.2	166.1	3.40	1.0	24	Positive
Cortisone-$^{13}C_3$	364.2	105.0	3.40	1.0	40	Positive
Dehydroepiandrosterone	289.2	271.0	6.07	1.0	6	Positive
Dehydroepiandrosterone	289.2	253.0	6.07	1.0	8	Positive
Dehydroepiandrosterone sulfate	271.2	253.1	4.34	1.0	8	Positive
Dehydroepiandrosterone sulfate	367.2	96.9	4.34	1.0	38	Negative
Dehydroepiandrosterone sulfate-D_5	276.2	258.2	4.33	1.0	10	Positive
Dehydroepiandrosterone sulfate-D_5	372.2	97.9	4.33	1.0	40	Negative
Dehydroepiandrosterone-D_5	294.2	258.0	6.20	1.0	12	Positive
Dehydroepiandrosterone-D_5	294.2	218.0	6.20	1.0	19	Positive
Dexamethasone	393.2	373.1	4.89	1.0	4	Positive
Dexamethasone	393.2	355.1	4.89	1.0	8	Positive
Dexamethasone-D_5	398.2	378.2	4.86	1.0	4	Positive
Dexamethasone-D_5	398.2	360.2	4.86	1.0	10	Positive
Dihydrotestosterone	291.2	255.2	7.34	1.0	13	Positive
Dihydrotestosterone	291.2	159.0	7.34	1.0	22	Positive
Dihydrotestosterone	291.2	43.0	7.34	1.0	60	Positive
Dihydrotestosterone-D_3	294.2	258.2	7.31	1.0	15	Positive
Dihydrotestosterone-D_3	294.2	43.0	7.31	1.0	54	Positive
Estradiol	271.2	183.0	5.46	1.0	51	Negative
Estradiol	271.2	145.0	5.46	1.0	45	Negative
Estradiol-$^{13}C_3$	274.2	186.0	5.46	1.0	45	Negative
Estradiol-$^{13}C_3$	274.2	148.0	5.46	1.0	45	Negative
Estrone	269.2	145.0	5.38	1.0	45	Negative
Estrone	269.2	143.0	5.38	1.0	69	Negative
Estrone-$^{13}C_3$	272.2	148.0	5.38	1.0	45	Negative
Estrone-$^{13}C_3$	272.2	146.0	5.38	1.0	60	Negative
Progesterone	315.2	109.0	7.80	1.0	30	Positive
Progesterone	315.2	97.0	7.80	1.0	26	Positive
Progesterone-D_9	324.3	113.1	7.76	1.0	30	Positive
Progesterone-D_9	324.3	100.1	7.76	1.0	26	Positive
Testosterone	289.2	109.0	6.08	1.0	26	Positive
Testosterone	289.2	97.0	6.08	1.0	24	Positive
Testosterone-$^{13}C_3$	292.2	112.1	6.08	1.0	26	Positive
Testosterone-$^{13}C_3$	292.2	100.0	6.08	1.0	22	Positive

Results and discussion

SPE sample cleanup and elution profile

The Steroid Panel LC-MS workflow utilizes Narrow Bore Extraction™ plates manufactured by Tecan for the extraction of target analytes and the removal of matrix components such as salts and phospholipids. This 96-well plate-based SPE protocol was performed using the Tecan Resolvex A200 positive pressure processor, which ensures uniform and automated sample processing, representing a significant improvement over traditional liquid-liquid extraction (LLE) methods. The protocol is fully compatible with automation, allowing for increased throughput and reduced manual handling.

A particular advantage of this workflow is the integrated "Sample Dry" function of the Resolvex A200, which enables efficient drying of samples directly on the SPE plate. For this purpose, an evaporation plate (Tecan p/n 30138271) is used, which focuses the nitrogen flow onto the wells to facilitate evaporation.

This evaporation plate minimizes the risk of contamination compared to conventional evaporators and eliminates the need to heat samples up to 40 °C, as recommended in many protocols, thereby reducing the risk of analyte degradation.

Stable pressure profiles are maintained throughout the process, further enhancing reproducibility and reliability. Additionally, a reusable evaporation plate, distributed by Tecan, is employed, which can be used for multiple sample preparation cycles, supporting both consistent evaporation and cost efficiency.

Chromatographic separation was performed using a C8 column, distributed by Tecan, providing robust resolution of all analytes within a short analysis time. Critical analyte pairs with similar MRM transitions, such as corticosterone, 11-deoxycortisol, and 21-deoxycortisol (Figure 1), were effectively separated using the optimized LC conditions. Compared to the LC conditions provided with the Steroid Panel LC-MS kit, both the gradient and several transitions were modified to tailor the method to the LC/TQ system in use. The adjustment of transitions was necessary due to the use of a different MS instrument and ion source geometry. Additionally, the gradient was further optimized to achieve improved separation, as demonstrated in Figure 2. Screenshots are included to illustrate these improvements. It should also be noted that a different LC system was used compared to the original protocol.

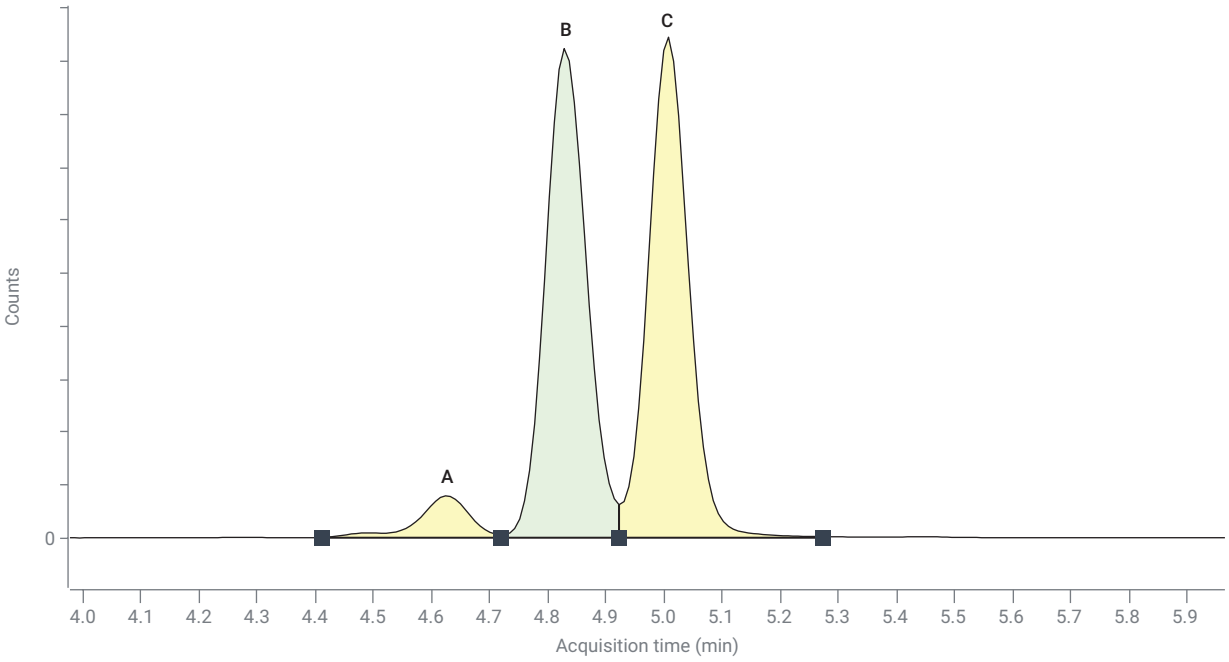
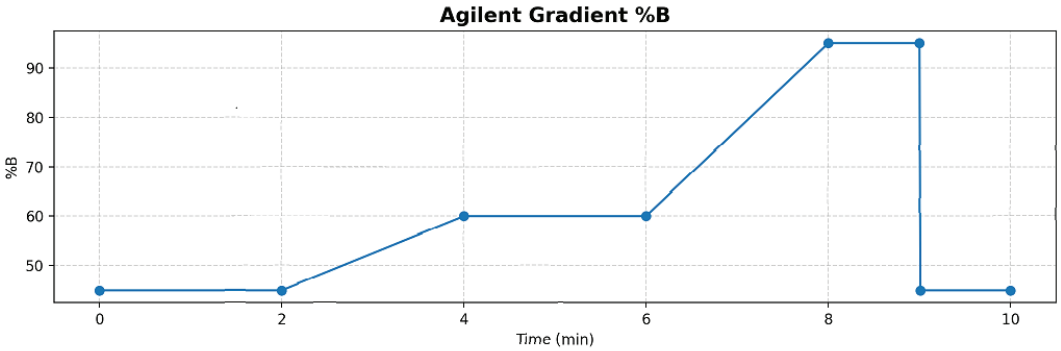
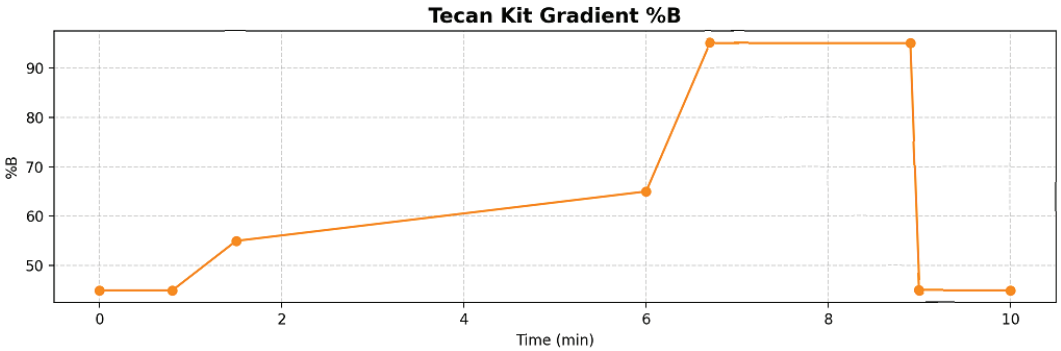


Figure 1. Chromatographic separation of 21-deoxycortisol (A), corticosterone (B), and 11-deoxycortisol (C) in Cal E. Retention times were 4.58 minutes for 21-deoxycortisol, 4.83 minutes for corticosterone, and 4.97 minutes for 11-deoxycortisol. The concentrations in calibrator E, according to the Tecan Steroid Panel LC-MS kit IFU, were as follows: 21-deoxycortisol: 4.86 ng/mL, corticosterone: 14.6 ng/mL, and 11-deoxycortisol: 4.86 ng/mL.



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

Figure 2. Comparison of Agilent and Tecan Steroid Panel LC-MS kit gradient profiles (%B over time). The Agilent gradient was modified to match the Tecan gradient as provided in the kit. Plots show the %B composition as a function of time for both methods, with corresponding data tables displayed on the right.

Furthermore, the method achieved baseline separation of challenging pairs such as cortisone and cortisol (Figure 3) and 11-deoxycorticosterone and 17α-hydroxyprogesterone (Figure 4), supporting confident MRM-based quantitation.

To provide an overview of the method's chromatographic performance and analyte coverage, Figure 5 and Table 4 are presented. Figure 5 shows the MRM trace overlay of all 18 target analytes, illustrating their elution profile and separation under the described LC/MS conditions. Each peak is labeled with the corresponding analyte number, as detailed in Table 4. Table 4 summarizes the analytes included in the panel, their retention times, and the MRM transitions used for quantification.

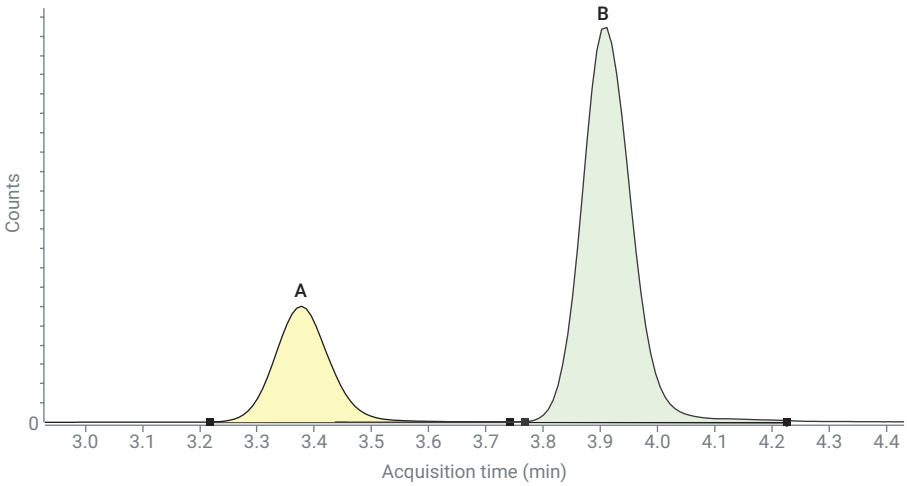


Figure 3. Chromatographic separation of cortisone (A) and cortisol (B) in Cal E. Retention times were 3.3 minutes for cortisone and 3.9 minutes for cortisol. The concentrations in calibrator E, according to the Tecan Steroid Panel LC-MS kit IFU, were as follows: cortisone: 24.3 ng/mL and cortisol: 97.2 ng/mL.

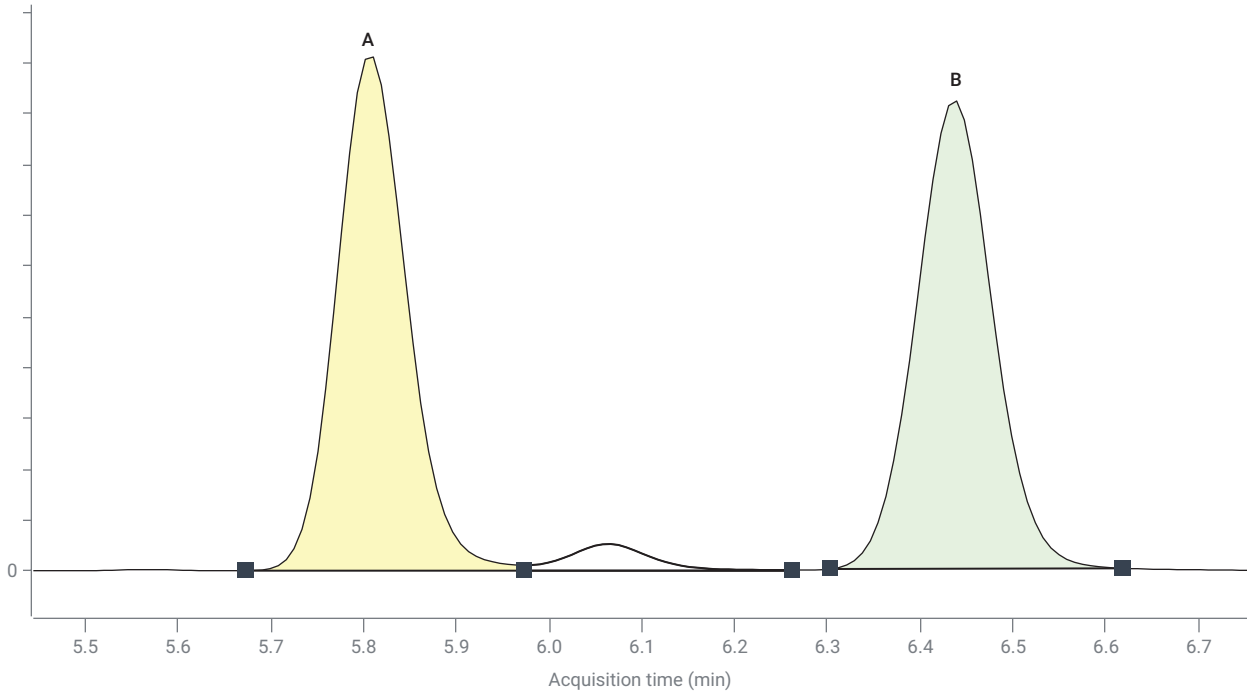


Figure 4. Chromatographic separation of 11-deoxycorticosterone (A) and 17-hydroxyprogesterone (B) in Cal E. Retention times were 5.8 minutes for 11-deoxycorticosterone and 6.4 minutes for 17-hydroxyprogesterone. The concentrations in calibrator E, according to the Tecan Steroid Panel LC-MS kit IFU, were as follows: 11-deoxycorticosterone: 1.94 ng/mL and 17-hydroxyprogesterone: 4.86 ng/mL.

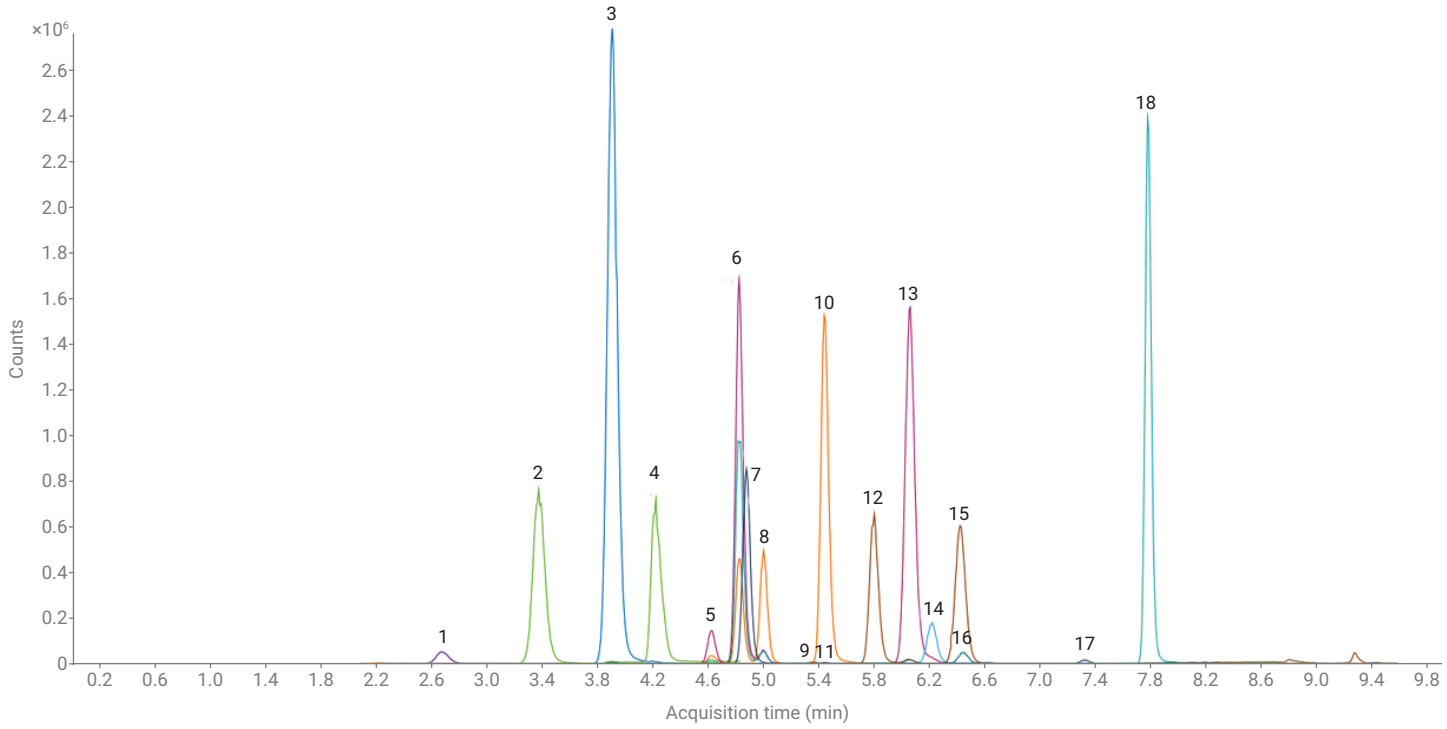


Figure 5. MRM trace overlay of 18 analytes detailing the elution profile. The data shown were acquired using calibrator C. Numbers are assigned to analytes, as explained in Table 5.

Table 4. Overview of target analytes, retention times, and MRM transitions.

Number	Target	Retention Time (min)	Transition (*m/z*)
1	Aldosterone	2.65	361.2 → 315.1
2	Cortisone	3.37	361.2 → 163.0
3	Cortisol	3.90	363.2 → 121.0
4	Dehydroepiandrosterone sulfate (DHEAS)	4.20	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.

Precision

Precision, expressed as coefficient of variation (CV%), was assessed with six different concentration levels for each analyte, with four re-extraction replicates per level (see Table 5). Most analytes, including testosterone, estradiol, androstenedione, and 11-deoxycorticosterone, showed repeatability, with CVs typically less than 10%. Even at the more challenging low and high sample levels, most analytes-maintained CVs within the acceptance criterion of 20%. Some analytes, such as 17-OHP4 and aldosterone, exhibited higher CVs at certain sample levels (up to 16.2% and 13.4%, respectively), but these values remained within the generally accepted limit of 20% for quantitative assays. These results indicate that the Steroid Panel LC-MS method is suitable for precise and reproducible quantification of steroids.

Table 5. Precision (CV%) of Tecan Steroid Panel LC-MS kit at six different QC levels (four replicates each).

Analyte	Precision (CV%)					
	S1	S2	S3	S4	S5	S6
Progesterone	2.0	3.4	11.7	2.6	10.6	10.7
Dihydrotestosterone	2.9	5.8	4.1	3.2	5.0	12.6
17-Hydroxypregnenolone	0.9	7.4	5.8	5.8	4.2	4.3
17-Hydroxyprogesterone	3.7	12.3	16.2	8.2	6.6	5.9
Dehydroepiandrosterone	2.0	0.9	1.8	8.7	8.1	10.8
Testosterone	1.2	2.1	1.0	1.9	0.9	2.1
11-Deoxycorticosterone	2.5	2.0	1.8	1.9	1.5	2.2
Estradiol	2.0	3.1	2.2	3.2	2.7	11.6
Androstenedione	1.3	2.0	2.4	1.7	1.7	4.1
Estrone	0.4	5.1	1.9	3.1	1.0	3.3
11-Deoxycortisol	0.7	1.6	4.7	3.5	5.1	3.6
Dexamethasone	3.2	4.1	9.9	4.3	2.1	6.1
Corticosterone	1.9	2.2	4.1	2.3	6.1	3.8
21-Deoxycortisol	4.6	7.6	12.2	0.9	3.3	2.4
Dehydroepiandrosterone Sulfate	5.0	0.7	12.7	8.9	8.6	8.2
Cortisol	0.5	4.1	11.6	3.2	7.3	12.3
Cortisone	1.5	1.6	5.2	1.1	3.8	4.7
Aldosterone	2.1	7.0	12.1	1.7	5.0	12.2

Linearity

Linearity was systematically evaluated for each analyte by constructing calibration curves using calibrators from the Tecan kit across the relevant concentration ranges. Each calibration curve consisted of six points and a zero calibrator. All analytes demonstrated outstanding linearity, as indicated by high coefficients of determination (R^2), reflecting a strong and consistent correlation between analyte concentration and instrument response (see Table 6). Most analytes were best described by a linear regression model; however, a subset including progesterone, 17-OHP4, 21-deoxycortisol, and aldosterone required a quadratic model to achieve optimal accuracy. The application of appropriate weighting factors, such as $1/x$ or $1/x^2$, further enhanced the fit of the calibration curves for certain analytes, ensuring precise quantification across the calibration range (see Table 6).

Table 6. Calibration curve characteristics: model fit, weighting, and coefficient of determination (R^2) for each analyte.

Analyte	Fit	Weight	R^2
Progesterone	Quadratic	None	0.998
Dihydrotestosterone	Linear	None	0.999
17-Hydroxypregnenolone	Quadratic	None	0.999
17-Hydroxyprogesterone	Quadratic	1/x	0.999
Dehydroepiandrosterone	Quadratic	None	0.999
Testosterone	Linear	$1/x^2$	0.989
11-Deoxycorticosterone	Linear	None	0.999
Estradiol	Linear	None	0.999
Androstenedione	Linear	$1/x^2$	0.995
Estrone	Linear	None	0.999
11-Deoxycortisol	Linear	1/x	0.996
Dexamethasone	Linear	None	0.998
Corticosterone	Linear	None	0.999
21-Deoxycortisol	Quadratic	None	0.999
Dehydroepiandrosterone sulfate	Linear	1/x	0.991
Cortisol	Linear	None	0.999
Cortisone	Linear	None	0.999
Aldosterone	Quadratic	None	0.999

As demonstrated in Figure 6, the calibration curve for cortisol across six concentration levels highlights the excellent linearity achieved with this method. These results confirm the robustness and reliability of the method for quantitative analysis, supporting its suitability for comprehensive steroid profiling.

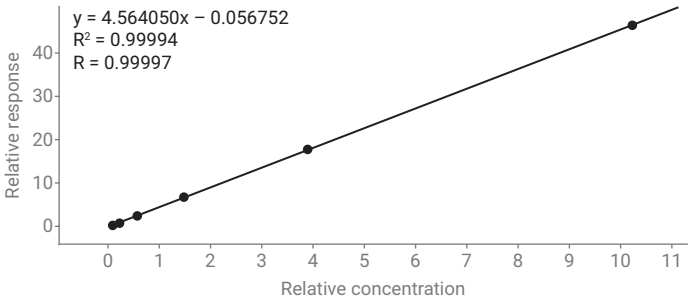


Figure 6. Linearity of cortisol quantification across six calibration levels (X-axis: relative concentration; Y-axis: relative response).

Trueness

The trueness of the adapted method was evaluated by analyzing QC samples with demonstrated metrological traceability according to ISO 17511:2020 at both high and low concentration levels. For each analyte, multiple re-extraction replicates of the QC samples were processed using the Tecan 96-well plate-based SPE protocol and the Resolvex A200 positive pressure processor, followed by LC/TQ analysis. The measured concentrations were compared to the assigned QC values given in the QC certificate, and the age deviation was calculated to assess trueness.

The results demonstrated excellent trueness for most analytes, with values well within ± 20 % at both QC high and QC low levels, fully meeting established acceptance criteria for quantitative LC/TQ assays. In particular, analytes such as progesterone, DHT, DHEA, testosterone, and estradiol exhibited deviations well below 15%, underscoring the high trueness and robustness of the adapted method. While a few analytes, including 17-OHP4 and 21-deoxycortisol, showed a slight negative bias at both QC levels, and others, such as androstenedione and aldosterone, displayed a higher positive bias at the high QC level, all results remained within acceptable limits. Overall, these findings highlight the reliability and trueness of the Tecan sample preparation workflow in combination with the adapted LC/TQ method for accurate quantification across a comprehensive panel of steroids. It is recommended that trueness be comprehensively evaluated using NIST standards, where available, to further ensure the comparability of results. A summary of the trueness results for each analyte at both QC high and QC low levels is provided in Table 7.

Table 7. Trueness of Tecan Steroid Panel LC-MS kit: percent deviation at high and low QC levels for each analyte (n = 19 replicates).

Analyte	Trueness (%)	
	QC High	QC Low
Progesterone	9.3	2.8
DHT	5.2	2.1
17-OHP5	0.1	–5.9
17-OHP4	–12.5	–2.2
DHEA	4.4	3.8
Testosterone	11.9	10.9
11-Deoxycorticosterone	3.6	11.0
Estradiol	1.0	3.0
Androstenedione	15.6	1.3
Estrone	0.33	–0.18
11-Deoxycortisol	–2.0	–2.6
Dexamethasone	–7.3	16.2
Corticosterone	–0.9	8.0
21-Deoxycortisol	–12.3	–11.5
DHEAS	7.7	10.4
Cortisol	8.8	–2.2
Cortisone	3.7	9.8
Aldosterone	18.7	13.4

Conclusion

To address specific analytical requirements and improve overall efficiency, the Tecan Steroid Panel LC-MS kit has undergone targeted optimization for use on the Agilent 6495 triple quadrupole LC/MS. These modifications, including adjustments to instrument parameters, have made it possible to combine two separate runs into a single run completed within 10 minutes. Notably, these enhancements have been achieved without compromising analytical performance; the kit continues to deliver robust and reliable results, as demonstrated in this evaluation. Importantly, the Steroid Panel LC-MS method has been shown to perform exceptionally well on the 6495 LC/TQ, confirming its compatibility and effectiveness with this widely used instrument.

The method demonstrates high precision and reproducibility across a wide concentration range, with most analytes exhibiting CVs far less than 10%. Linearity was excellent for all analytes, as shown by high R^2 values and appropriate curve fitting. Trueness was also satisfactory for most analytes, supporting the method's accuracy. While a few analytes exhibited slightly higher variability or bias at certain QC levels, these deviations were limited and did not significantly impact the overall reliability of the assay. The use of concentrated solvents and robust sample preparation further enhances the practicality and efficiency of the method.

Although the preliminary work for method development has been completed, end users are required to perform validation within their own laboratory environments. The semi-automated workflow, particularly when using the Resolvex A200, offers significant advantages by increasing throughput, reducing manual handling errors, and ensuring consistent sample preparation, thereby enhancing overall laboratory efficiency.

In summary, the Steroid Panel LC-MS kit is a reliable and robust tool for comprehensive steroid profiling on different mass spectrometry systems, making it suitable for more specialized applications.

Disclaimer

This product and the described workflow are for Research Use Only (RUO) and are not intended for diagnostic or clinical use. The optimized method and instrument-specific modifications described here are not included in the Instructions for Use (IFU) and have not been validated by the manufacturer for clinical or diagnostic applications. End users are responsible for validating the method in their own laboratories and ensuring its suitability for their specific research purposes.

References

1. Vogeser, M.; Parhofer, K. G. Liquid Chromatography Tandem-Mass Spectrometry (LC-MS/MS)–Technique and Applications in Endocrinology. *Ann. Lab. Med.* **2007**, *27(5)*, 401–410. https://doi.org/10.1055/s-2007-981458
2. Keevil, B. G. Novel Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS) Methods for Measuring Steroids. *Best Pract. Res. Clin. Endocrinol. Metab.* **2016**, *27(5)*, 663–674. https://doi.org/10.1016/j.beem.2013.05.015

www.agilent.com

RA250918.498

Printed in the USA, October 23, 2025
5994-8729EN

Agilent
Trusted Answers