Sensitivity improvements in the quantitation of steroids on the SCIEX 7500+ system

Aidan Harrison[1], Natalie ZM Homer[2], Ruth Andrew[2], Scott G Denham[2], Joanna P Simpson[2], Pierre Negri,[3] Michael Jarvis[4]
[1]SCIEX, UK; [2]University of Edinburgh, UK; [3]SCIEX, USA; [4]SCIEX, Canada.

This technical note demonstrates a method for the analysis of 18 steroids in solvent, achieving lower limits of quantitation (LLOQ) as low as 2.5 fg on column using the SCIEX 7500+ system. The advantage of this high sensitivity was explored by comparing the results with the previous generation of SCIEX mass spectrometer (MS), the QTRAP 6500+ system. The results showed an increase in sensitivity for the SCIEX 7500+ system (Figure 1), shown in significant gains for both peak area, and signal-to-noise (S/N), utilizing optimization of the Q0 dissociation (Q0D) parameter, a feature unique to the improved front-end technology of the SCIEX 7500+ system. This increase in sensitivity could allow for simplified sample preparation, reduced injection volume or a decrease in the sample volume required compared to those previously achieved in similar workflows.

Key features of the quantitation of steroids in solvent using the SCIEX 7500+ system

- **Enhanced sensitivity unlocked:** Improved front-end technology enabled average raw signal gains of 14-fold and S/N gains of 4.7-fold achieved when compared to the QTRAP 6500+ system
- **High level of sensitivity:** LLOQ levels as low as 2.5 fg on column achieved for a range of steroids in solvent
- **Improved analytical performance:** Almost 3-fold improvement in precision (%RSD) for the LLOQ, with good linearity, accuracy and precision with the new, lower LLOQ achieved for steroid compounds over the QTRAP 6500+ system

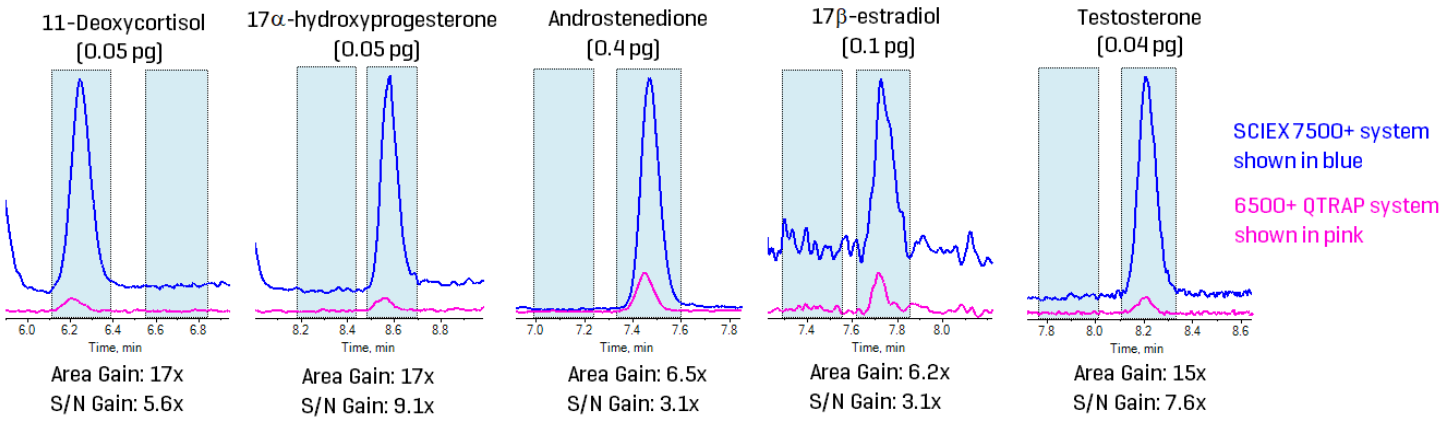


Figure 1: Improved sensitivity on the SCIEX 7500+ system for the detection of five steroids. Extracted ion chromatogram (XIC) comparisons between the SCIEX 7500+ system (blue) and QTRAP 6500+ system (pink) for five steroids shows significant increase in sensitivity, shown as increases in both peak area and signal-to-noise (S/N). Values are reported as pg on column.

Introduction

Steroid measurement is essential in both biochemical research and clinical diagnostics due to the significant role steroids play in regulating metabolism, immune responses, and reproductive functions. Accurate quantitation of steroid levels is vital for diagnosing and managing various health conditions.

Historically, steroids have been measured using immunoassays and GC-MS, but these methods have limitations. Immunoassays often suffer from cross-reactivity, leading to inaccurate results, while GC-MS requires extensive sample preparation including derivatisation and long analytical run times. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become a preferred method for steroid analysis, offering high sensitivity and specificity with minimal sample preparation. LC-MS/MS can simultaneously measure multiple steroids in a single sample, making it ideal for clinical research and diagnostics.

High sensitivity and wide dynamic range are crucial in steroid analysis because steroids are found from picomolar to micromolar ranges within biological samples. Enhanced sensitivity enables measurement in samples of smaller volumes and reduced injection volumes, which is beneficial when sample volumes are limited, particularly in paediatric clinical settings or rare specimen research. Therefore, advancements in LC-MS technology that improve peak height, area, and S/N ratios for analytes are the preferred approach to boosting sensitivity.

Methods

Samples and reagents: All reagents used were of analytical grade or better. Cortisol (F), cortisone (E), corticosterone, (B), 11-deoxycortisol (S), 21-deoxycortisol (21DF), 11-deoxycorticosterone (11DOC), testosterone (T), androstenedione (A4), 5α-dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), progesterone (P4), pregnenolone (P5), 17-hydroxypregnenolone, (17OHP5), 17α -hydroxyprogesterone (17OHP4), aldosterone (Aldo), estrone (E1), 17β -estradiol (E2), estriol (E3) were all purchased from Sigma-Aldrich and Cerilliant. 11-dehydrocorticosterone (A) was purchased from Steraloids, UK.

Sample preparation: An intermediate stock solution was prepared in methanol containing steroids at concentrations ranging from 0.5-10.00 µg/mL. Subsequently, a calibration curve was generated over 5 orders of magnitude at physiologically relevant concentrations for human plasma for each steroid in a 30:70 (v/v) methanol/water mixture through serial dilutions of the stock solution.

Chromatography: Chromatographic separation used a previously established method[1] and was performed using a SCIEX ExionAE LC system at a flow rate of 0.3 mL/min on a Phenomenex Kinetex C18 (2.1 x 150 mm, 2.6 µm, 100 Å) column. A 16-minute gradient (Table 1) was run to separate steroids using 0.05mM ammonium fluoride in water as mobile phase A and 0.05mM ammonium fluoride in methanol as mobile phase B. An injection volume of 10 µL was used for analysis. A mixture of 1:1:1 (v/v/v) acetonitrile/methanol/water was used as a needle wash solvent

Table 1: Chromatographic gradient for steroid analysis.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.0	50	50
4.0	50	50
9.0	25	75
10.0	0	100
12.0	0	100
12.1	50	50
16.0	50	50

Mass spectrometry: Samples were analyzed using the SCIEX 7500+ system equipped with an OptiFlow Pro ion source with E lens and electrospray ionization (ESI) analytical probe. Samples were also analyzed using a QTRAP 6500+ system equipped with an IonDrive Turbo V source with ESI probe. The same multiple-reaction-monitoring (MRM) transitions were analyzed on each MS system, with previously optimized compound parameters used for the QTRAP 6500+ system,[1] while all parameters for steroids were reoptimized on the SCIEX 7500+ system, including those for Q0 dissociation (Q0D). Q0D was operated in 'Simple mode'. Both systems were optimized for maximum efficiency and operated in ESI mode with polarity switching. Optimized source parameters for the SCIEX 7500+ system are found in

Table 2; previously optimized source parameters were used for the QTRAP 6500+ system.[1] The same LC system was used and moved between the instruments to eliminate variability and allow direct MS instrument performance comparison. Data was acquired using the Scheduled MRM algorithm with at least two selective MRM transitions per analyte. Dwell times were optimized to ensure reliable integration, quantitation and confirmation of the peak for each target analyte with >15 points across each peak at the LLOQ (Figure 4).

Table 2: Source and gas parameters for the analysis of steroid compounds using the SCIEX 7500+ system

Parameter	Value
Polarity	Positive and Negative
Ion source gas 1	30 psi
Ion source gas 2	70 psi
Curtain gas	40 psi
Source temperature	500 °C
Ion spray voltage	2000 V and -2250 V
CAD gas	9

Data processing: Data collection and analysis were performed in SCIEX OS software, version 3.4.5. Peaks were automatically integrated using the MQ4 algorithm, noise determined with the peak-to-peak algorithm and a weighting of 1/x was used for quantitation. The relative mean error (RME) for accuracy and relative standard deviation (%RSD) for precision were calculated. The LLOQ and the upper limit of quantitation (ULOQ) were defined as the lowest and highest concentration that could be quantified within ± 15% accuracy and precision at ULOQ, ± 15% of accuracy and ± 20% precision at LLOQ.

Technology improvements on the SCIEX 7500+ system

The SCIEX 7500 and 7500+ systems have several front-end technology improvements as compared to the previous versions of high-end triple-quadrupole mass-spectrometry systems such as the QTRAP 6500+ system. These include a redesign of the previous IonDrive Turbo V source, with the introduction of the OptiFlow Pro ion source, and the new D Jet+ assembly, incorporating a dodecapole geometry to the existing ion path. As part of this assembly, a voltage differential can now be applied to ions entering the vacuum region, and in Simple mode, this is applied between the Q-Jet ion guide and 1Q0 lens to achieve declustering, rather than the declustering potential (DP) value used on previous systems. This QOD parameter is tuneable and can be useful for removal of interferences and decreasing baseline noise. The MRM transitions for each steroid were taken from the previously optimized method on a QTRAP 6500+ system, but were reoptimized, including the QOD value for individual MRMs. Further improvements on the SCIEX 7500+ system to the front-end include the incorporation of Mass Guard technology to reduce the risk of instrument contamination,[2,3] and an increase in scanning speed on the SCIEX 7500+ system, allowing for scan speeds as low as 1.2 ms.[4]

The QOD parameter, operated in Simple mode, was used to reduce the overall noise level. While both the signal and noise may be impacted, typically the noise is reduced to a greater extent. As shown in Figure 2, this noise reduction resulted in a significant S/N increase.

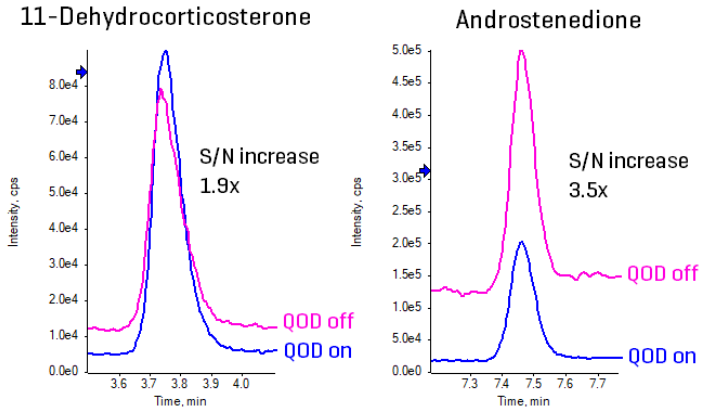


Figure 2. Improved signal to noise (S/N) on the SCIEX 7500+ system when using optimized QOD values. A decrease in baseline noise was observed when using optimized QOD values for many MRMs, resulting in an increase in the S/N, even when overall signal was decreased. QOD off is shown in pink, QOD on, with optimized value, is shown in blue.

Sensitivity improvements on SCIEX 7500+ system

The SCIEX 7500+ system demonstrated high levels of sensitivity, allowing for the low-level quantitation of 17 steroids at sub-pg levels, reaching as low as 2.5 fg on column. The LLOQ for the quantifier MRM transition for each steroid is shown in Figure 3.

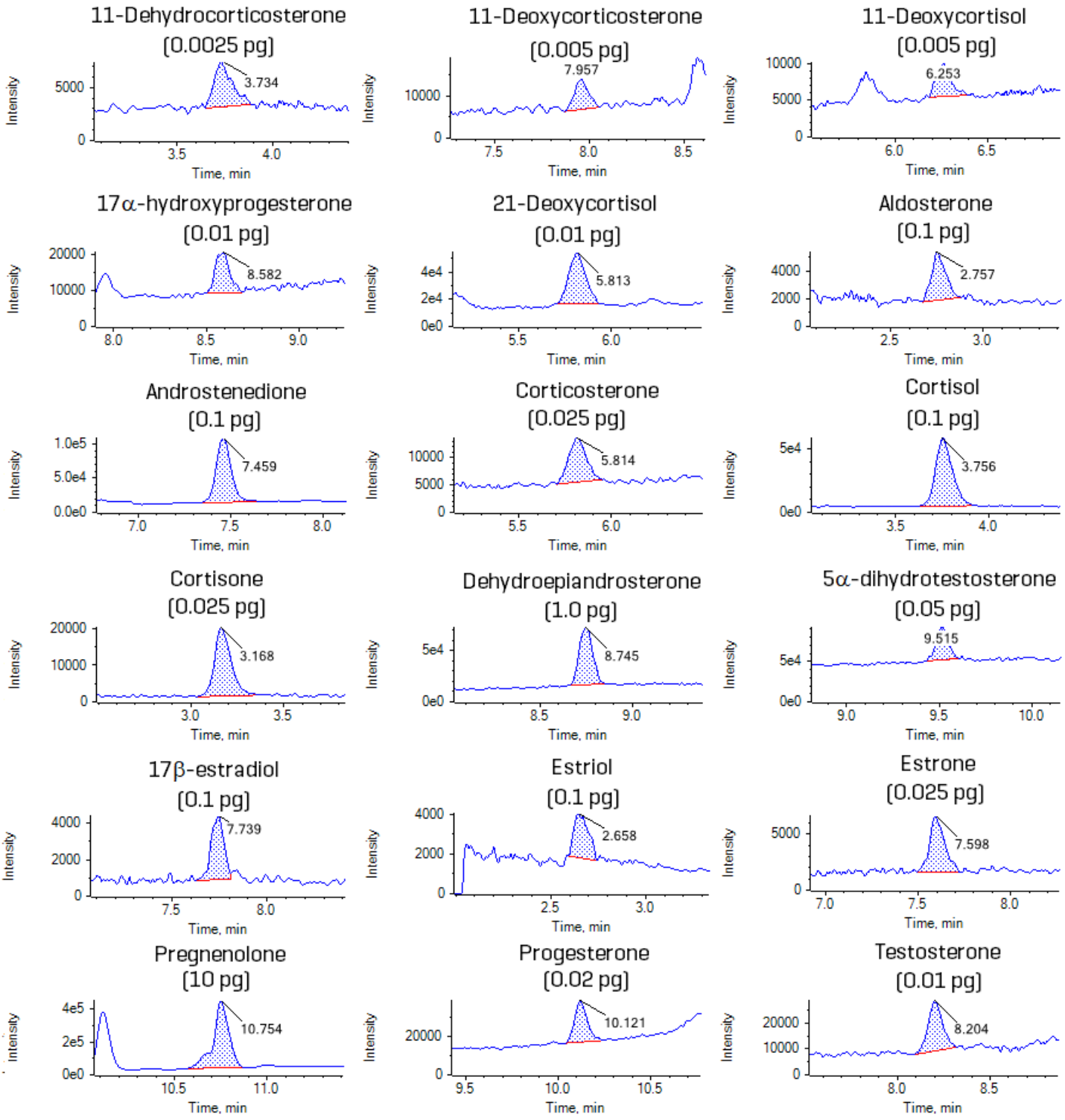


Figure 3: Representative XICs for the quantifier MRM transition at the LLOQ for 18 steroids on the SCIEX 7500+ system. LLOQ peaks shown with S/N > 3, accuracy of 85-115% and %RSD < 13% for three replicates (Table 3). All values are reported as pg-on-column for a 10 µL injection.

Linearity (1/x weighting, $r^2 > 0.99$) was achieved for the calibration curves of all steroids, with at least three orders of magnitude achieved for more than half the steroids tested, and at least two for all others (except pregnenolone). Average accuracy and precision were assessed for 3 replicates and was within acceptable validation requirements with average accuracy of 85–115% and precision of <15% at all concentrations. Full data for both MRM transitions for each steroid is shown in Table 3, including quantitative performance data at the LLOQ.

Table 3: Quantitative performance for steroid analysis on the SCIEX 7500+ system. Reproducibility and accuracy results were determined from the calibration curve across 3 replicates at each concentration. Statistical results were summarized using the Analytics module in SCIEX OS. The quantifier ion (1) is listed first, followed by the qualifier ion (2) for all steroids tested.

Compound	Calibration range (pg on column)	Linear correlation	LLOQ (pg on column)	Accuracy at LLOQ (%, n=1)	LLOQ peak area %RSD (%, n=3)
11-Dehydrocorticosterone 1	0.0025-0.5	0.9992	0.0025	86.3	2.4
11-Dehydrocorticosterone 2	0.0025-0.5	0.9993	0.0025	85.5	8.1
11-Deoxycorticosterone 1	0.005-25	0.9987	0.005	88.8	3.9
11-Deoxycorticosterone 2	0.005-25	0.9994	0.005	94.1	7.1
11-Deoxycortisol 1	0.005-10	0.9981	0.005	91.3	5.9
11-Deoxycortisol 2	0.005-10	0.9990	0.005	95.9	4.9
17α–hydroxy progesterone 1	0.01-10	0.9998	0.01	104	6.7
17α-hydroxy progesterone 2	0.01-10	0.9998	0.01	100	3.7
21-Deoxycortisol 1	0.01-5	0.9998	0.01	97.7	5.5
21-Deoxycortisol 2	0.01-5	0.9997	0.01	102	8.2
Aldosterone 1	0.1-50	0.9997	0.1	102	12
Aldosterone 2	0.1-50	0.9996	0.1	103	9.7
Androstenedione 1	0.1-20	0.9997	0.1	96.3	3.5
Androstenedione 2	0.1-20	0.9998	0.1	99.7	5.3
Corticosterone 1	0.025-50	0.9956	0.025	110	2.5
Corticosterone 2	0.025-50	0.9961	0.025	112	7.5
Cortisol 1	0.1-50	0.9979	0.1	94.9	1.3
Cortisol 2	0.1-50	0.9997	0.1	100	2.8
Cortisone 1	0.025-25	0.9998	0.025	88.3	0.98
Cortisone 2	0.025-25	0.9996	0.025	96.3	2.3
Dehydroepiandrosterone 1	1-250	0.9988	1	90.4	2.6
Dehydroepiandrosterone 2	1-250	0.9968	1	85.2	3.6
Dihydrotestosterone 1	0.05-10	0.9986	0.05	95.3	7.6
Dihydrotestosterone 2	0.05-10	0.9992	0.05	112	8.9
17β-estradiol 1	0.1-50	0.9995	0.1	109	6.9
17β-estradiol 2	0.1-50	0.9996	0.1	107	7.4
Estriol 1	0.1-50	0.9995	0.1	109	3.4
Estriol 2	0.1-50	0.9996	0.1	110	13
Estrone 1	0.025-50	0.9997	0.025	110	11
Estrone 2	0.025-50	0.9996	0.025	115	8.9
Pregnenolone 1	10-100	0.9963	10	109	2.0
Pregnenolone 2	10-100	0.9957	10	104	0.64
Progesterone 1	0.02-20	0.9985	0.02	95.5	11
Progesterone 2	0.02-20	0.9985	0.02	97.4	7.0
Testosterone 1	0.01-20	0.9993	0.01	86.8	5.6
Testosterone 2	0.01-20	0.9994	0.01	93.5	5.0

The same calibration standards were analyzed on a QTRAP 6500+ system as a direct comparison with the SCIEX 7500+ system. Figure 1 shows the clear increase in sensitivity seen on the SCIEX 7500+ system for 5 steroids, in terms of raw peak area and S/N.

The sensitivity differences between the two systems were investigated further by normalizing the distribution of the peak areas and S/N (n=3) for each of the two MRM transitions monitored for each steroid, at the LLOQ concentration determined on the QTRAP 6500+ system. Across all transitions, the average area gain was 14-fold and the average increase in S/N was 4.7-fold (Figure 5).[6]

In addition to the increase in sensitivity, the reproducibility of the method was assessed by comparing the %RSD at the same concentrations for 3 replicate injections. As shown in Figure 6, excellent precision was seen for the SCIEX 7500+ system with an average %RSD of 2.1% compared with 5.9% for the QTRAP 6500+ system (Figure 6). It was not possible to compare the precision (%RSD) results of the 7500+ LLOQ values shown in Table 3 with the QTRAP 6500+ system as more than 50% of the steroids analysed for one or both transitions at these concentrations were not detectable (S/N < 3) on the QTRAP 6500+ system (see Figure 4).

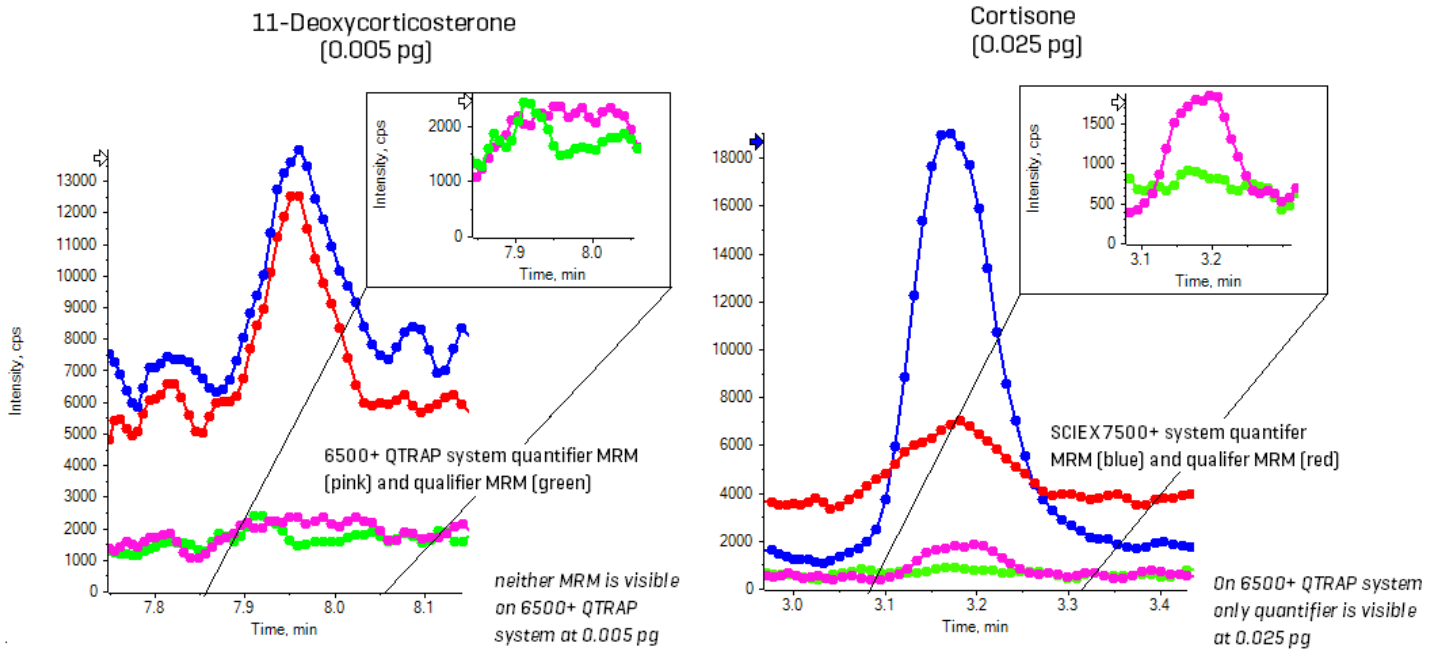
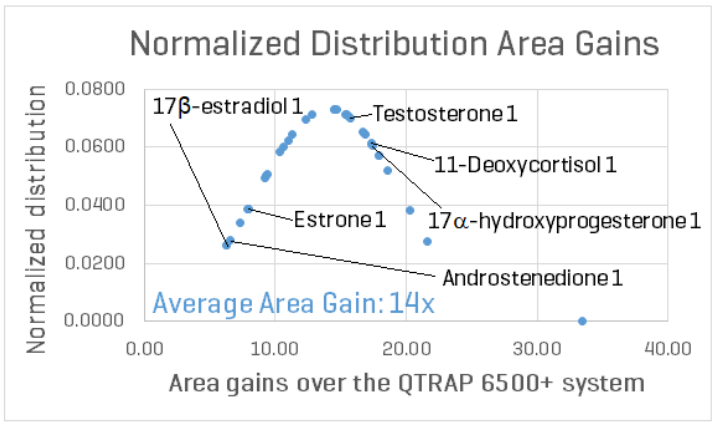


Figure 4: Sensitivity improvements at the LLOQ on the SCIEX 7500+ system. A comparison of the LLOQ on the SCIEX 7500+ system (quantifier ion in blue, qualifier ion in red) with an injection of the same sample on the QTRAP 6500+ system (quantifier ion in pink, qualifier ion in green). 10 steroids were not detectable for either transition on the QTRAP 6500+ system (example 11-deoxycorticosterone shown here), and a further 4 were only detectable for the quantifier ion and so ion ratios could not be confirmed for a positive identification (example cortisone shown here). All transitions were required to have at least 15 points across the peak to ensure reliable integration.

These results demonstrate that greater sensitivity can be achieved on the SCIEX 7500+ system, in both raw signal gains, S/N values and improved precision at low levels with clear improvements in %RSD at a higher concentration, and %RSD values that meet most validation requirements at the reduced LLOQ values achieved. This implies the possibility of achieving comparable LLOQs for steroid concentrations commonly found in biological samples with smaller sample volumes, giving greater scope to the number of sample types and species of which this analysis can be applied. An additional benefit is the greater instrument robustness over time with the reduced injection volume. Reduced sample loading is also likely to minimize matrix interferences giving reduced likelihood of ion suppression or difficulties in accurate peak integration. Alternatively, the SCIEX 7500+ system's enhanced sensitivity, accuracy, and precision at low concentrations enable simplified sample preparation methods that may result in sample dilution. The increased performance of the SCIEX 7500+ system ensures that even with diluted samples, the quantitation remains reliable and accurate, making it a valuable tool for high-throughput laboratories aiming to streamline their processes without compromising data quality.



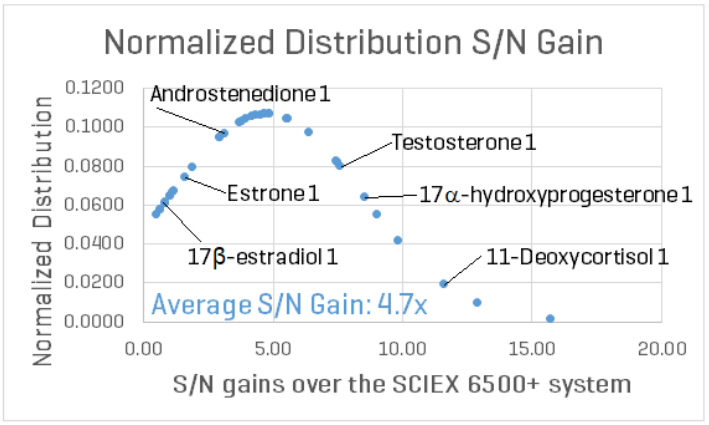


Figure 5: Peak area and S/N gains for a panel of 18 steroids on the SCIEX 7500+ system. Averaged (n=3) peak areas at the LLOQ for the 6500+ QTRAP system for the 36 MRM transitions monitored in this assay were normalized and the gains observed for the SCIEX 7500+ system over the QTRAP 6500+ system were plotted. Average peak area gains were 14-fold (range of 6.2-33-fold for quantifier MRMs). Average S/N gains were 4.7-fold (range of 0.83-13-fold) for quantifier MRMs. Selected quantifier ions are labelled.

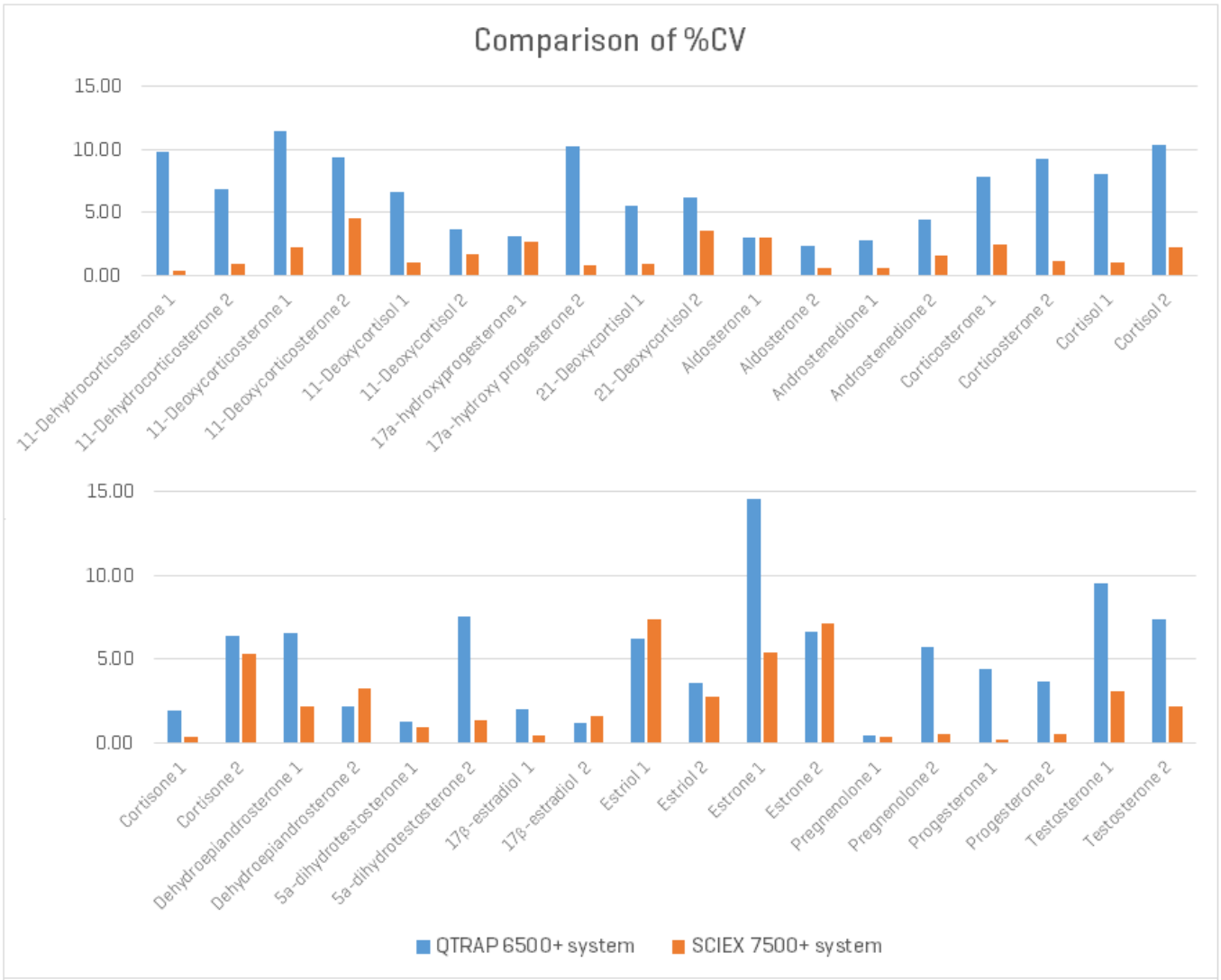


Figure 6: Increased sensitivity on the SCIEX 7500+ system gives significantly improved precision (N=3). Superior precision was seen on the SCIEX 7500+ system with an average %RSD of 2.1%. When compared to the %RSD on the QTRAP 6500+ system with an average of 5.9% (at the LLOQ for the 6500+ QTRAP system), this represented a nearly 3-fold improvement.

Conclusions

- High sensitivity was achieved with most steroids having an LLOQ less than 1 pg-on-column, reaching as low as 2.5 fg for 11-dehydrocorticosterone
- Good linearity was achieved for all steroids, spanning 2-3 orders of magnitude for all steroids except pregnenolone
- The method demonstrated accurate (85-115%) and precise (%RSD <13%) quantitative performance at all concentrations
- Sensitivity and precision were improved for the SCIEX 7500+ system with raw signal gains of 14-fold and S/N gains of 4.7-fold, as well as an almost 3-fold improvement in %RSD over the QTRAP 6500+ system
- The overall improvements in sensitivity, accuracy and precision of the SCIEX 7500+ system may allow for simplification of sample preparation and overall workflows, a useful tool for high-throughput laboratories

References

1. Denham, S.G.; Simpson, J.P.; Diez, F.; Lee, P.; Kyle, C.; Morgan, R.; Homer, N.Z.M. A practical approach to supported liquid extraction and measurement of 18 steroids in plasma and serum by targeted liquid chromatography tandem mass spectrometry. *MethodsX* **2024,** *12*, 102728. DOI: 10.1016/j.mex.2024.102728
2. Discover unparalleled sensitivity and extended instrument uptime on the highly robust SCIEX 7500+ system. *SCIEX technical note,* **2024,** *MKT-32166-A*. https://sciex.com/tech-notes/food-beverage/food-and-beverage/Discover-unparalleled-sensitivity-and-extended-instrument-uptime-on-the-highly-robust-SCIEX-7500--system
3. Redefine bioanalysis with enhanced robustness on the SCIEX 7500+ system. *SCIEX technical note,* **2024**, *MKT-31350-A*. https://sciex.com/tech-notes/pharma/bioanalysis-pk/redefine-bioanalysis-with-enhanced-robustness-on-the-sciex-7500-plus-system
4. Fast scanning quantitative lipidomics analysis using the SCIEX 7500+ system. *SCIEX technical note,* **2024**, *MKT-32067-A*. https://sciex.com/tech-notes/life-science-research/lipidomics/fast-scanning-quantitative-lipidomics-analysis-using-the-sciex-7
5. Leveraging sensitivity improvements for low-level detection of drugs, metabolites, and endogenous hormones in complex biological matrices, *SCIEX technical note,* **2022**, *RUO-MKT-02-14409-A*. https://sciex.com/tech-notes/forensic/toxicology/leveraging-sensitivity-improvements-for-low-level-detection-of-d

Headquarters
250 Forest Street, Marlborough,
MA 01752 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please all the division headquarters or refer to our website at
sciex.com/offices