Sensitivity improvement for the detection of steroid hormones and endocannabinoids in keratinized matrices

Using the SCIEX 7500 system, powered by SCIEX OS software

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Steroid hormones and endocannabinoids (ECs) are endogenous substances that play a key role in the regulation of the human stress response. The concentrations of these regulators greatly fluctuate throughout the day, making it challenging to correlate their relationship to the range of stress-related conditions experienced by humans. Analysis of these substances is analytically challenging due to their low levels in biological matrices and chemical similarity to one another. As a result, sensitive analytical techniques are required to provide accurate quantification of these stress markers in biological matrices.

Detection of these endogenous markers can be performed in many biological matrices including urine, blood, saliva and keratinized matrices, such as hair and nails. Among these matrices, hair and nails are becoming extremely valuable for assessing the long-term and retrospective determination of these endogenous stress markers, which are known to steadily accumulate in the keratinized matrix over time. Other benefits include the non-invasive nature of sample collection, minimal storage requirements and long-term stability with low risk of sample degradation over time.

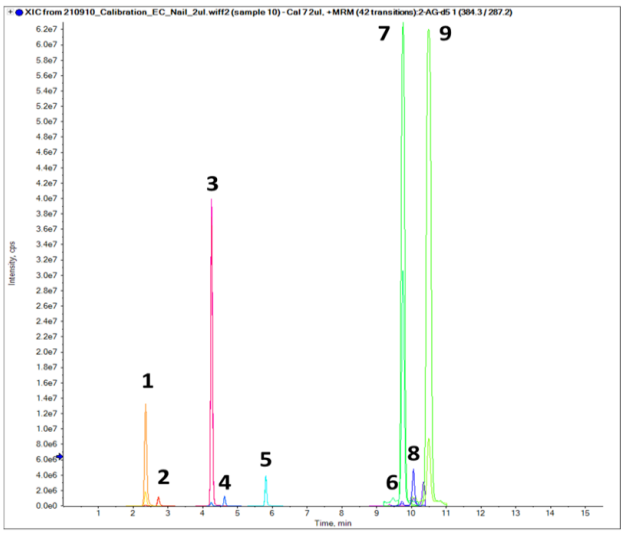

Figure 1. Chromatographic profile of the 5 steroid hormones and 4 endocannabinoids targeted in this study. Extracted ion chromatogram (XIC) resulting from the baseline separation of the 9 compounds in the panel. The numbered peaks are assigned as follows: 1) cortisone $^{13}C_3$, 2) cortisol $^{13}C_3$, 3) androstenedione $^{13}C_3$, 4) testosterone $^{13}C_3$, 5) progesterone $^{13}C_3$, 6) AEA D_4, 7) PEA D_4, 8) 2-AG D_5 and 9) N-OEA D_4. The total LC runtime was 15.5 min.

One of the challenges associated with the detection and accurate quantification of endogenous compounds in biological matrices is that the matrices themselves already contain varying levels of the endogenous analytes of interest. Therefore, no blank, analyte-free matrices are available that can be used for calibration and validation. An approach commonly used to overcome this challenge is the use of surrogate analytes, such as $^{13}C_3$-labeled or deuterated analogs that enable calibration in the biological matrix of interest. This approach was successfully used previously for the accurate quantification of endogenous steroids and endocannabinoids in keratinized matrices[1].

Here, an analytical workflow that combines the use of a supported liquid extraction (SLE)-based sample preparation and the SCIEX 7500 system for sub-pg/mg calibration of 5 steroid hormones and 4 ECs is described. Calibration results from the SCIEX 7500 system were compared with results from the QTRAP 6500+ system, an established platform that is routinely used for low-level quantification of endogenous species. Significant gains in peak area and signal-to-noise ratio (S/N) were observed on the SCIEX 7500 system, demonstrating the benefits of this system for sensitive quantification of low-level stress markers in keratinized matrices.

Advantages of the SCIEX 7500 system for high-sensitivity stress marker analysis in keratinized matrices

- Analyte extraction followed by a SLE-based sample preparation in combination with a robust detection method using the scheduled MRM algorithm in SCIEX OS software enabled calibration in the sub-pg/mg range using surrogate endogenous steroid hormones and ECs in keratinized matrices
- Sensitivity gains observed on the SCIEX 7500 system improved quantification of low-level stress markers in keratinized matrices

Experimental details

Target analytes: The following surrogate analytes were used for quantification: cortisone $^{13}C_3$, cortisol $^{13}C_3$, androstenedione $^{13}C_3$, testosterone $^{13}C_3$, progesterone $^{13}C_3$, AEA D_4, PEA D_4, 2-AG D_5 and N-OEA D_4. The internal standard included cortisone D_7, progesterone D_9 and AEA D_{11}.

Calibrator preparation: Seven levels of calibrators were prepared by spiking the surrogate analyte mixture and internal standard in hair and nail samples across various concentrations. The concentrations tested included: 5 to 200 pg/mg for 2-AG D_5; 0.1 to 10 pg/mg for AEA D_4 and testosterone $^{13}C3$; 1 to 500 pg/mg for androstenedione $^{13}C3$, cortisone $^{13}C3$ and progesterone $^{13}C3$; 500 to 10,000 pg/mg for N-OEA D_4 and PEA D_4 and 0.5 to 50 pg/mg for cortisol $^{13}C3$.

Hair and nail sample washing: Hair and nail samples were first washed for 3 minutes with deionized water, followed by a 2-minute wash with acetone. The tubes containing the hair and nail samples were thoroughly shaken by hand during the washing steps and the washing solutions were decanted and discarded after each washing step. The washed hair and nail samples were dried overnight at room temperate.

Hair and nail sample preparation: Nail clippings (20 mg) were weighed into an Eppendorf tube and 3 milling balls (stainless steel, 5 mm diameter) were added. Nail clippings were pulverized for 10 minutes at 30 Hz. Hair segments were manually cut into snippets and 20 mg of hair was weighed into an Eppendorf tube. Then, 1 mL of methanol and 50 uL of the internal standard were added to each tube containing either the hair or pulverized nail samples. The tubes were briefly shaken and placed in a sonicated bath (35 kHz, 600 W) for 4 hours (hair) or 1 hour (nail) at 55°C for extraction. The tubes were centrifuged at 9000 g for 5 minutes and the methanolic extracts were transferred to a column rack for SLE.

SLE procedure: A supported liquid extraction (SLE) was performed using an automated Biotage Extrahera system (Biotage, Sweden). Sample extracts were automatically loaded onto Isolute SLE+ columns and allowed to absorb for 5 minutes. Analytes were then eluted 2 times with 2.5 mL ethyl acetate with a wait time of 5 minutes between the 2 elution steps. The extracts were dried in a Turbovap solvent evaporator system (Biotage, Sweden) and resuspended in 60 µL of methanol and 140 µL of a reconstitution solution consisting of 0.2mM ammonium formate in 97:3, water/methanol.

Liquid chromatography: HPLC separation was achieved using a Phenomenex Kinetex XB-C_{18} column (50 x 2.10 mm, 2.6 µm, 00B-4496-AN). The column was held at 40°C on a Prominence UFLC system on the QTRAP 6500+ system or on a Nexera 40 Series UHPLC system on the SCIEX 7500 system. Mobile phases A and B consisted of 0.2 mM ammonium formate in 97:3, water/methanol and 3:97, water/methanol, respectively. The LC flow rate was 0.4 mL/min and the total runtime was 20 min. The injection volume was 2 µL.

Mass spectrometry: A SCIEX 7500 system equipped with an OptiFlow Pro ion source with an electrospray ionization (ESI) analytical probe and E Lens probe was used. For comparison purposes, a QTRAP 6500+ system equipped with an IonDrive Turbo V ion source was also used. Both instruments were optimized for maximum sensitivity and operated in positive ESI mode. Source parameters and compound-dependent parameters for all compounds and their corresponding internal standards were also optimized on each system, including the Q0D dissociation on the SCIEX 7500 system.

Data acquisition and processing: Data were acquired using SCIEX OS software on the SCIEX 7500 system. For the QTRAP 6500+ system, data were acquired using Analyst software, version 1.7. All data were processed using SCIEX OS software, in which detection and integration of the peaks from the background were accomplished within the viewing window using the MQ4 algorithm. The peak-to-peak algorithm was used for S/N calculations in the Analytics module of SCIEX OS software.

Analytical methodology for robust and accurate quantification of stress markers

For the initial method development, a diluted, 10 ng/mL neat standard mixture containing the 9 target analytes was used. Figure 1 shows the chromatographic profile of the 5 steroid hormones and 4 endocannabinoids targeted in this study. The combination of an appropriate gradient, adequate mobile phase compositions and ideal column choice (Phenomenex Kinetex XB-C_{18}) enabled baseline separation of the 9 analytes in a 15-minute total runtime.

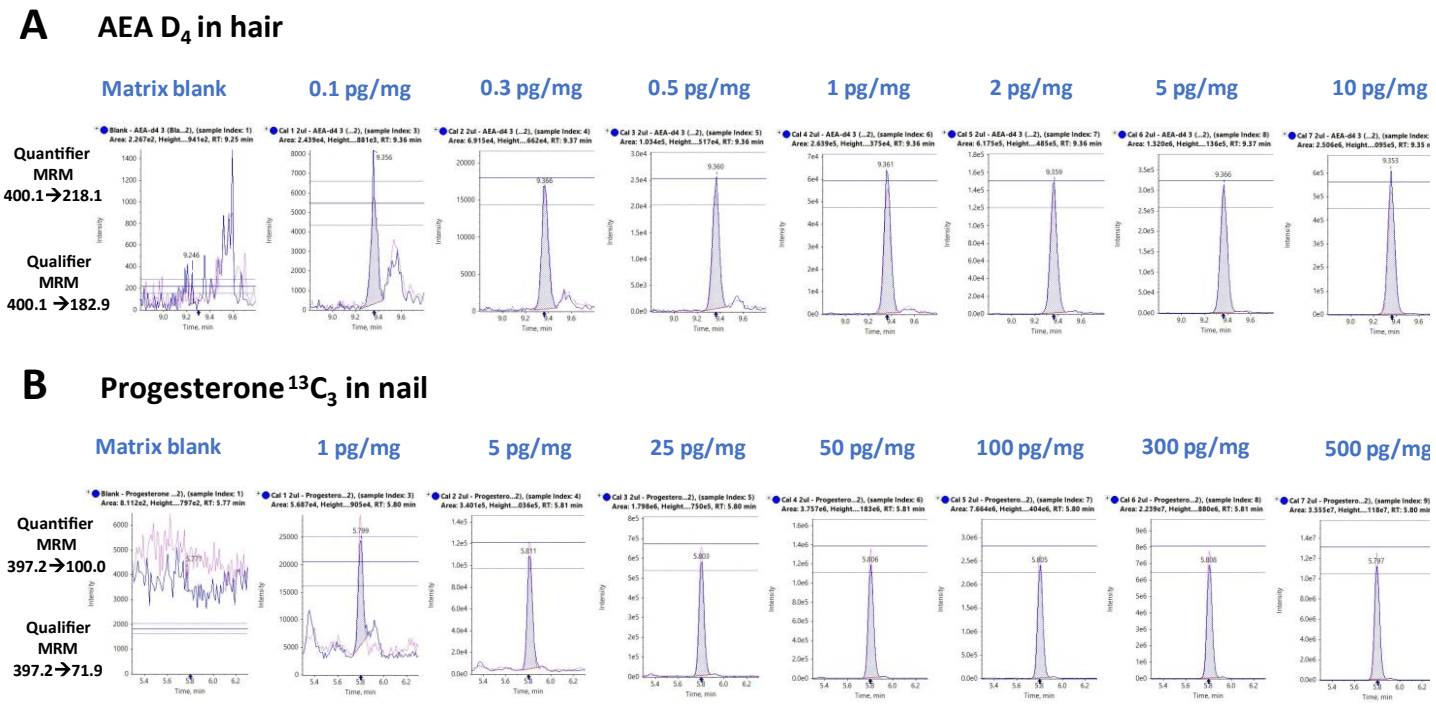

Figure 2. Representative XICs acquired on the SCIEX 7500 system for an endocannabinoid and steroid hormone extracted from hair or nail samples. XICs for A) the deuterated EC analog, AEA D_4, extracted from hair samples at concentrations ranging from 0.1 to 10 pg/mg and B) the $^{13}C_3$ labeled steroid hormone, progesterone $^{13}C_3$, extracted from nail samples at concentrations ranging from 1 to 500 pg/mg. Both the quantifier and qualifier traces are shown. Ion ratios were monitored across the dataset, indicated by the tolerance lines.

Authentic human hair and nail samples spiked with 5 $^{13}C_3$-labeled steroid hormones and 4 deuterated ECs were prepared at concentrations ranging from 0.1 to 10,000 pg/mg, extracted using the SLE procedure and injected to the Nexera 40 Series UHPLC system on the SCIEX 7500 system to build a data processing method. The series of calibrator solutions was injected to evaluate the quantification performance of the system and its ability to accurately measure low levels of endogenous biomarkers with high levels of precision and accuracy.

Figure 2 shows representative extracted ion chromatograms (XICs) acquired on the SCIEX 7500 system for 2 MRM transitions monitored for AEA D_4 extracted from hair samples (Figure 2A) and progesterone $^{13}C_3$ extracted from nail samples (Figure 2B). The series of XICs displayed includes overlays of both the quantifier and qualifier ions for a blank injection (left) and for concentrations ranging from 0.1 to 10 pg/mg for AEA D_4 and from 1 to 500 pg/mg for progesterone $^{13}C_3$. Also displayed in Figure 2 is the tolerance set for these analyses, indicated by the ion ratio line overlay that helps visualize the confidence levels. The ion ratio difference was <20% for the quantifier and qualifier ions of each of the targeted analytes across the calibration range. Overall, the developed method enabled robust and accurate quantification of endogenous steroids and endocannabinoids in keratinized matrices.

Calibration curves were generated using the quantifier MRM transition for each of the analytes targeted in this method. Figure 3 shows the resulting regression lines plotted across the calibrator levels for androstenedione $^{13}C_3$, cortisone $^{13}C_3$ and progesterone $^{13}C_3$ from to 500 pg/mg (Figure 3A), 2-AG D_5 from 5 to 200 pg/mg (Figure 3B), N-OEA D_4 and PEA D_4 from 500 to 10,000 pg/mg (Figure 3C) and AEA D_4 and testosterone $^{13}C_3$ from 0.1 to 10 pg/mg (Figure 3D) extracted from hair samples. The resulting calibration curves showed excellent linearity across the calibration ranges with R^2 values greater than 0.99 for all the steroid hormones and endocannabinoids targeted in this study. Excellent linearity was also observed for the analytes extracted from nail samples (data not shown).

Leveraging sensitivity improvement for low-level detection of endogenous steroids and endocannabinoids in hair and nail samples

The use of sensitive mass spectrometry instrumentation is critical to accurately quantify low levels of these endogenous species in complex biological matrices. In this study, the sensitivity of the SCIEX 7500 system was compared to that of a previous generation instrument, the QTRAP 6500+ system using both the peak area and signal-to-noise ratio (S/N). Figure 4 shows the XIC series, respective peak area and S/N values for

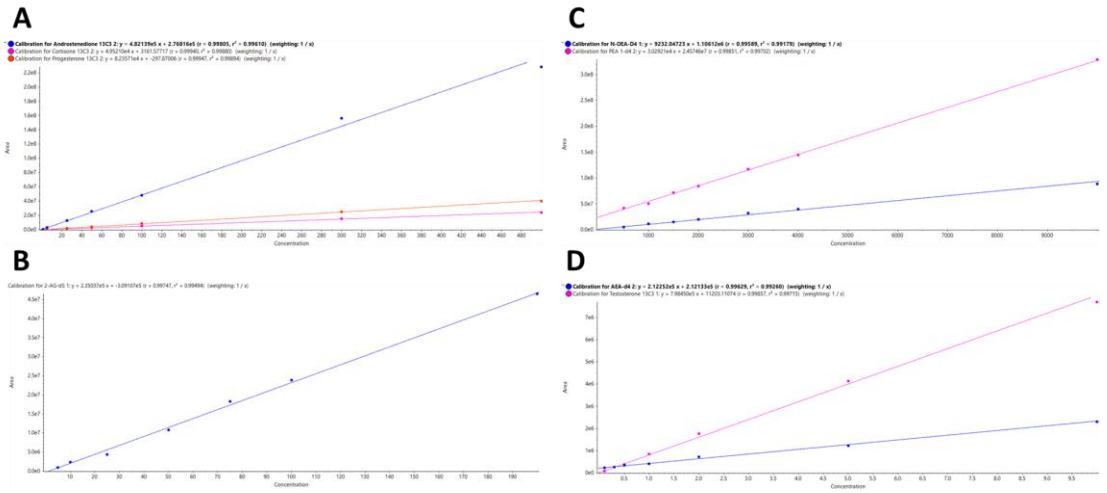

Figure 3. Excellent linearity for 8 steroids and endocannabinoids targeted in this panel. Calibration curves were generated using the quantifier MRM transition for A) androstenedione $^{13}C3$, cortisone $^{13}C3$ and progesterone $^{13}C3$ from 1 to 500 pg/mg, B) 2-AG D_5 from 5 to 200 pg/mg, C) N-OEA D_4 and PEA D_4 from 500 to 10,000 pg/mg and D) AEA D_4 and testosterone $^{13}C3$ from 0.1 to 10 pg/mg extracted from hair samples and acquired on the SCIEX 7500 system. The assay showed excellent linearity with R^2 values greater than 0.99 for all the analytes.

androstenedione $^{13}C_3$ in hair samples acquired on the SCIEX 7500 system (top) and QTRAP 6500+ system (bottom) for 7 concentration levels, ranging from 1 to 500 pg/mg. The average peak area and S/N gains across the 7 concentration levels for androstenedione $^{13}C_3$ were 9.9 ±1.2 and 4.4 ±2.2, respectively. Similar observations were made for the other 7 analytes included in the panel, with peak area gains ranging from 9.9x for androstenedione $^{13}C_3$ to 71.2x for PEA D_4 and S/N gains ranging from 0.6x for progesterone $^{13}C_3$ to 13.8x for PEA D_4 in hair samples. Overall, the SCIEX 7500 system provided a significant increase in both peak areas and S/N ratios across all the analytes extracted from hair samples, with average peak area gains of 30.0x and average S/N gains of 5.6x across all the analytes extracted from hair samples.

Figure 5 shows the XIC series for 2-AG D_5 in nail samples acquired on the SCIEX 7500 system (top) and QTRAP 6500+ system (bottom) for 7 concentration levels ranging from 5 to 200 pg/mg. Similarly, the average peak area and S/N gains across the 7 concentration levels for 2-AG D_5 were 35.5 ±4.8 and 4.5 ±4.0, respectively. As observed with analytes from hair samples, significant gains in the peak area and S/N were observed for the analytes extracted from nail samples. Peak area gains ranged from 3.7x for progesterone $^{13}C_3$ to 426x for PEA D_4 and S/N gains ranged from 0.7x for progesterone $^{13}C_3$ to 19.0x for PEA D_4 in nail samples. Overall, the peak area gains and S/N gains averaged 15.4x and 5.6x, respectively, across all the analytes extracted from nail samples. Table 1 summarizes the peak area gains and S/N gains observed for the 9 analytes targeted in this study in the 2 keratinized matrices investigated.

Conclusions

- The technology improvements of the SCIEX 7500 system were leveraged to improve the sensitivity for accurate detection of endogenous markers in keratinized matrices
- The ion ratio difference was <20% for the quantifier and qualifier ions of the targeted analytes, showing the quantitative robustness of the developed workflow
- The overall performance of the system resulted in excellent linearity with R^2 values >0.99 for all the steroids and endocannabinoids targeted in this study
- The impact of these sensitivity gains was investigated by comparing the signals observed on the SCIEX 7500 system and a previous generation instrument, the QTRAP 6500+ system
- Improvements were observed in both peak area and S/N gains for the 9 analytes targeted in this study in the 2 keratinized matrices investigated
- Average peak area gains were attained that ranged from 9.85x to 71.21x and 3.69x to 42.55x for compounds extracted from hair and nails, respectively
- Average S/N ratio gains were achieved that ranged from 0.62x to 13.82x and 0.68x to 19.04x for compounds extracted from hair and nails, respectively
- The use of the SCIEX 7500 system demonstrated the ability to routinely and robustly detect ultra-low levels of analytes extracted from challenging biological matrices
- The presented workflow provided the sensitivity levels required for the long-term retrospective measurement of endogenous biomarkers in keratinized matrix

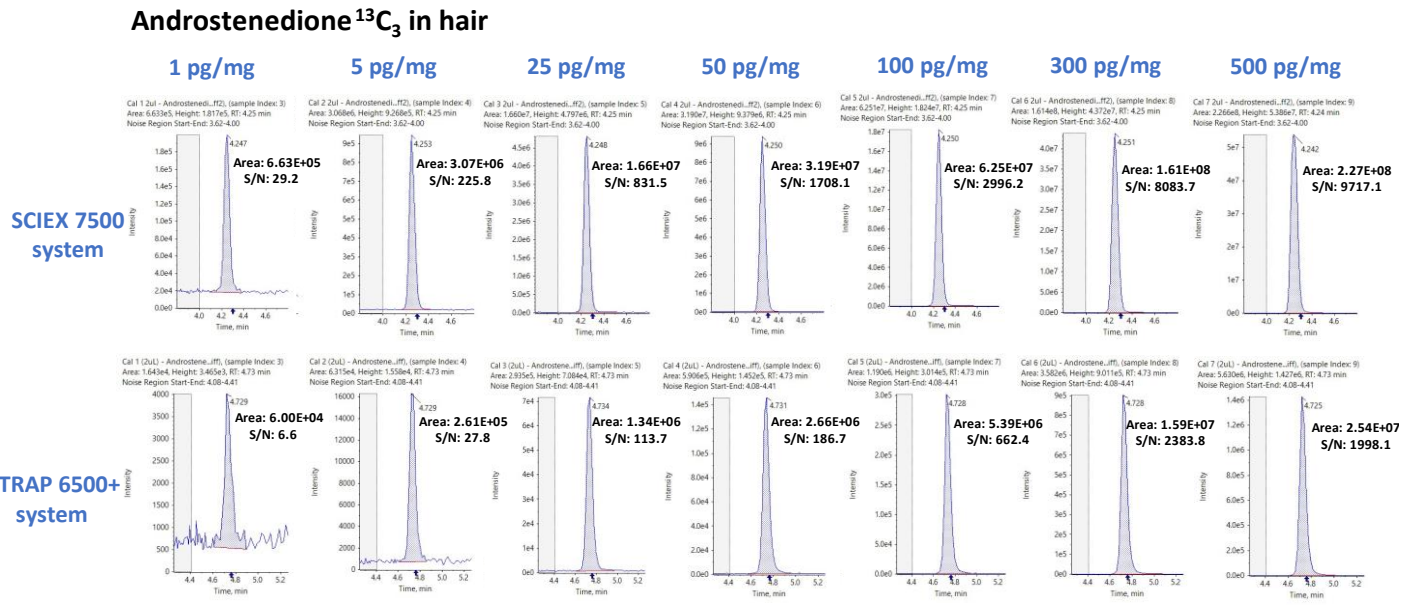

Figure 4. Sensitivity improvement for the detection of steroid hormones extracted from hair samples. XIC comparisons between the SCIEX 7500 system (top) and the QTRAP 6500+ system (bottom) for the 7 levels androstenedione $^{13}C_3$ calibrators extracted from hair samples at concentrations ranging from 1 to 500 pg/mg. The SCIEX 7500 system showed significant improvements in both peak area and S/N gains over the QTRAP 6500+ system.

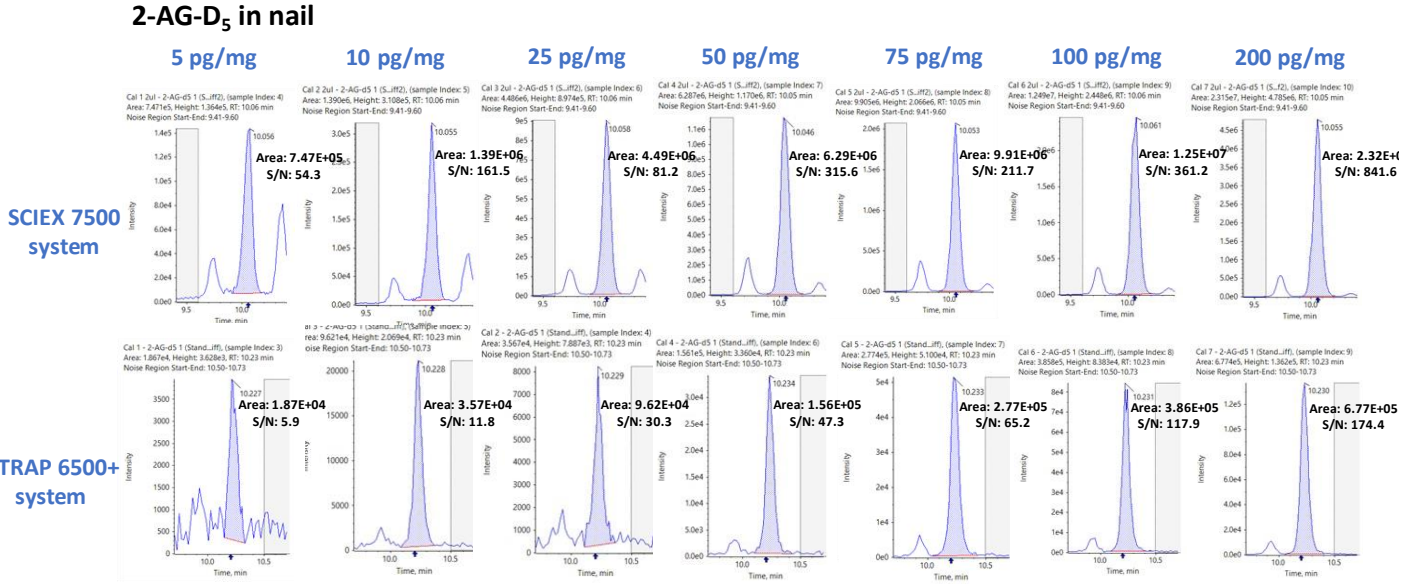

Figure 5. Increased sensitivity for the detection of endocannabinoids extracted from nail samples. XIC comparisons between the SCIEX 7500 system (top) and the QTRAP 6500+ system (bottom) for the 7 levels of 2-AG D_5 calibrator extracted from nail samples at concentrations ranging from 5 to 200 pg/mg. The SCIEX 7500 system showed significant improvements in both peak area and S/N gains over the QTRAP 6500+ system.

References

1. Voegel CD, Baumgartner MR, Kraemer T, Wüst S, Binx TM (2021) "Simultaneous quantification of steroid hormones and endocannabinoids (ECs) in human hair using an automated supported liquid extraction (SLE) and LC-MS/MS - Insights into EC baseline values and correlation to steroid concentrations", *Talanta*, **222**: 121499.

Table 1. Mean peak area and S/N ratios of the 7 levels of calibrators for each of the 9 analytes spiked in hair and nail samples. Seven levels of calibrators were prepared and injected to the SCIEX 7500 system and QTRAP 6500+ system. The ratios of the peak areas and S/N were determined at each calibrator level and averaged for each of the 5 steroid hormones and 4 endocannabinoids for each of the 2 keratinized matrices analyzed (hair and nail).

Compound	Hair matrix		Nail matrix	
	Mean peak area ratio	Mean S/N ratio	Mean peak area ratio	Mean S/N ratio
2-AG D_5	*63.0 ± 11.0*	*11.7 ± 4.2*	*35.5 ± 4.8*	*4.5 ± 4.0*
AEA D_4	*57.6 ± 10.2*	*1.2 ± 0.5*	*21.4 ± 121.0*	*3.9 ± 7.4*
Androstenedione $^{13}C3$	*9.9 ± 1.2*	*4.4 ± 2.2*	*6.5 ± 0.3*	*1.1 ± 0.2*
Cortisol $^{13}C3$	*15.2 ± 0.5*	*3.4 ± 1.0*	*6.7 ± 0.7*	*1.8 ± 0.4*
Cortisone $^{13}C3$	*13.9 ± 0.9*	*3.5 ± 1.4*	*6.1 ± 0.5*	*2.7 ± 0.8*
N-OEA D_4	*13.7 ± 5.6*	*9.2 ± 60.9*	*8.1 ± 2.2*	*14.4 ± 65.5*
PEA D_4	*71.2 ± 21.0*	*13.8 ± 11.8*	*42.6 ± 9.0*	*19.0 ± 12.2*
Progesterone $^{13}C3$	*10.8 ± 1.8*	*0.6 ± 0.1*	*3.7 ± 0.6*	*0.7 ± 0.3*
Testosterone $^{13}C3$	*14.4 ± 1.7*	*2.6 ± 1.0*	*8.2 ± 1.1*	*2.0 ± 0.3*

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