

# Rapid Detection of Growth Hormone-Releasing Peptides in Dried Blood Spots: a Proof of Concept Workflow for Anti-Doping Analysis

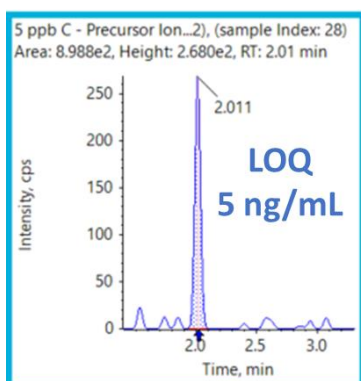
## Using Independent Data Acquisition (IDA) on the SCIEX X500R QTOF System

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Growth hormone-releasing peptides (GHRPs) are synthetic peptides that stimulate the release of growth hormones from the hypothalamus and pituitary gland. These peptides have received considerable attention in sports drug testing and are listed on the World Anti-Doping Agency (WADA) Prohibited List due to their ability to induce secretion of endogenous growth hormone (GH).

Among GHRPs, ipamorelin is a new potent and selective pentapeptide that belongs to the class of growth hormone secretagogues. This particular peptide has been shown to display high growth hormone potency and efficacy in vitro and in vivo. Due to their high efficacy as novel performance-enhancing substances, detection of GHRPs in biological matrix has become a focus in anti-doping analysis.

Analysis of GHRPs for doping controls is typically performed in matrices such as urine, plasma or whole blood. While these conventional matrices provide a reliable means of detecting enhancing substances that might be present in an athlete's body, dried blood spots (DBS) are becoming extremely valuable in testing the presence of these prohibited substances. Benefits of using DBS for anti-doping analysis include but are not limited to (1) small sample volume required, (2) absence of storage requirement, and (3) long term stability and minimal risk of sample adulteration and degradation overtime. However, there are two major analytical challenges associated with detecting enhancing substances in DBS: (1) low concentration of these compounds and (2) high abundance of matrix interference associated with the use of DBS cards.



**Figure 1: Highly Sensitive Detection of Ipamorelin in Dried Blood Spots (DBS).** Example XIC of the observed precursor ion of ipamorelin at m/z 356.7002 Da detected at the LOQ (5 ng/mL).



In this technical note, a comprehensive workflow is presented combining the use of the SCIEX X500R QTOF System with a simple extraction procedure for specific and sensitive detection of ipamorelin in dried blood spots.

### Key Features of IDA Acquisition Method for GHRPs Detection in Dried Blood Spots

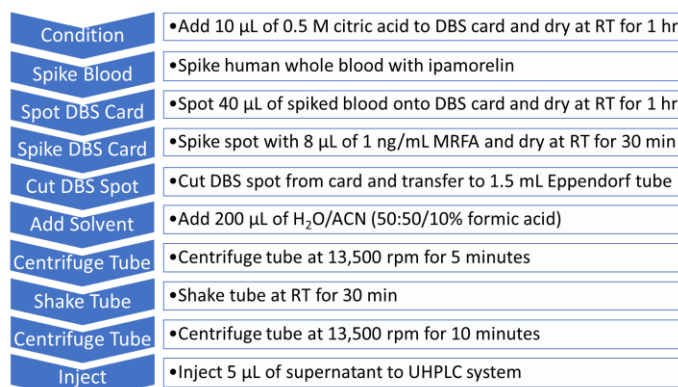
- 10-step extraction protocol provided an easily implemented sample preparation procedure for selective and sensitive detection of ipamorelin in dried blood spots (DBS)
- Independent Data Acquisition (IDA) method generated comprehensive and high-quality TOF MS and TOF MS/MS spectra, enabling reliable and reproducible fragmentation for accurate peptide characterization
- Sensitivity of the method was evaluated with an LOD of 2.5 ng/mL and an LOQ of 5 ng/mL for ipamorelin extracted from DBS while maintaining accuracy and precision across the calibration range (5 to 100 ng/mL)
- Assay showed excellent linearity with an  $R^2$  value  $>0.99$  for ipamorelin
- Method can be extended to include a larger panel of GHRPs listed on the World Anti-Doping Agency (WADA) Prohibited List

## Experimental Details

### DBS Cards Conditioning, Sample Preparation and

**Extraction Procedures:** Protein saver cards (also known as DBS cards) were purchased from Whatman (Piscataway, NJ). Prior to their use, DBS cards were conditioned using 10  $\mu$ L of 0.5 M citric acid and dried at room temperature for 1 hour. Human whole blood aliquots were spiked with various concentrations of ipamorelin acquired from Millipore Sigma (Saint Louis, MO). 40  $\mu$ L of spiked human whole blood were spotted onto the DBS card and dried at room temperature for 1 hour. Each spot was spiked with 8  $\mu$ L of a 1 ng/mL solution of MRFA (tetrapeptide used as internal standard) and dried at room temperature for 30 minutes. The circular spots were carefully cut and transferred into a 1.5 mL Eppendorf tube. 200  $\mu$ L of a 50:50 water: acetonitrile solution with 10% formic acid was added to each tube and centrifuged at 13,500 rpm for 5 minutes. Each tube was then shaken at room temperature for 30 minutes prior to being centrifuged again at 13,500 rpm for 10 minutes. 5  $\mu$ L of the supernatant was injected into the UHPLC system. A summary of the DBS cards conditioning and sample preparation procedures is shown in Figure 2.

**Calibrator Preparation:** A 1  $\mu$ g/mL stock standard ipamorelin solution was prepared by diluting with methanol. A series of 7 calibrators were prepared in blood spiking ipamorelin at a final concentration of 100, 50, 25, 10, 5, 2, and 1 ng/mL. Three of each calibrator solutions were spiked on the DBS cards and injected over three consecutive days for validation purposes and to evaluate the quantitative and qualitative attributes of the method such as linearity, LOD, LOQ, accuracy and precision. Validation was performed using the calibrator solutions from 5 to 100 ng/mL due to the lack of selectivity below the LOQ (5 ng/mL).



**Figure 2. DBS Cards Conditioning, Sample Preparation and Extraction Workflow.** A 10-step sample preparation and extraction protocol was used for selectively extracting ipamorelin and MRFA (IS) from DBS cards for analysis with the SCIEX X500R QTOF System.

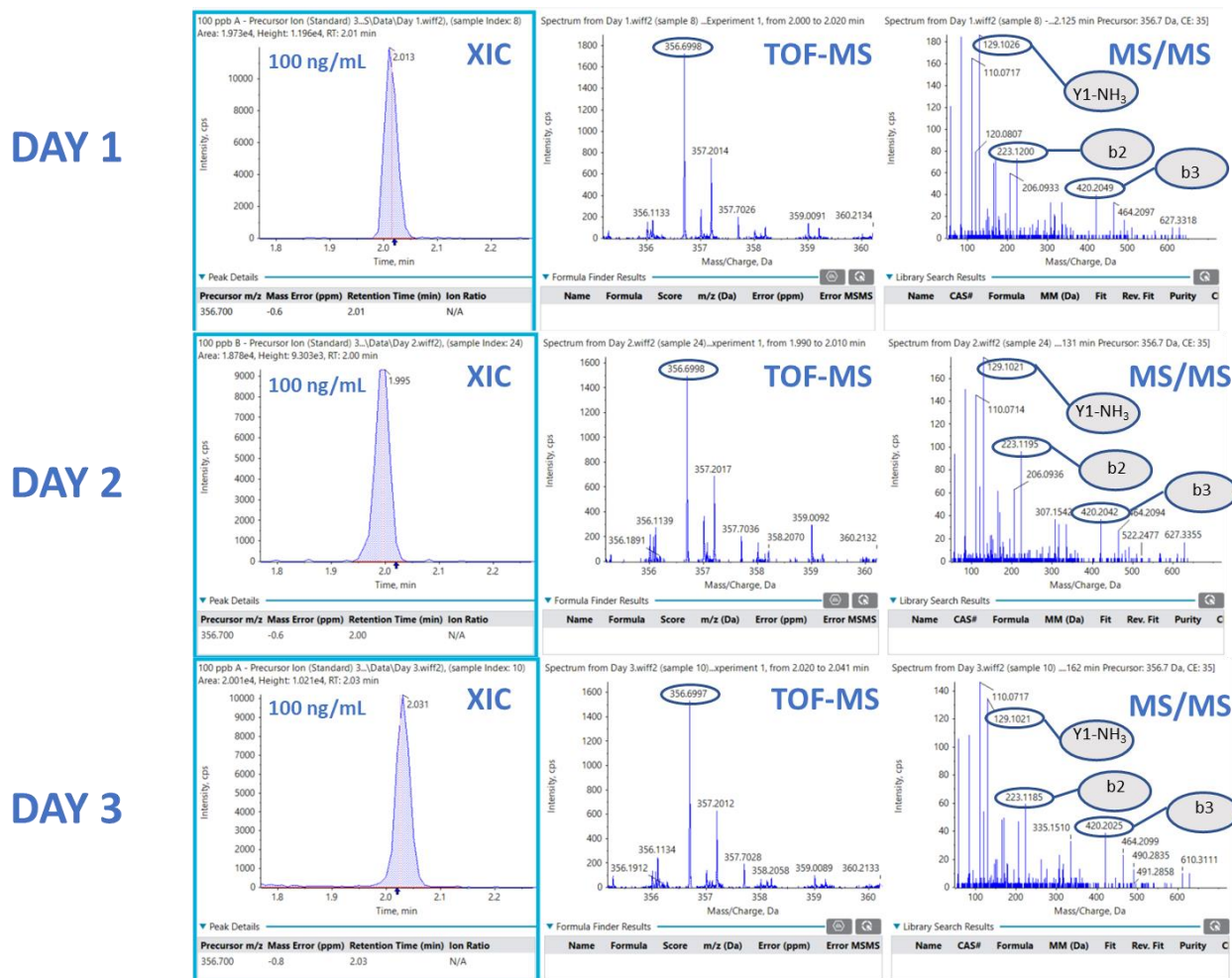
**Liquid Chromatography:** UHPLC separation was performed on a Phenomenex C18 column (100 x 2.1 mm, 1.7  $\mu$ m, 00D-4475-AN) at 40°C on a SCIEX ExionLC™ AC System. Mobile phases used consisted of water, acetonitrile, and modifiers. The LC flow rate was 0.5 mL/min and the total run time was 7 min. The injection volume was 5  $\mu$ L.

**Mass Spectrometry:** MS and MS/MS data were collected for each sample using Independent Data Acquisition (IDA) on the SCIEX X500R QTOF System in positive ESI mode. Data acquisition was TOF MS scan (230-800 Da) followed by a TOF MS/MS experiment to monitor the most abundant fragment ions in the mass range from 50 to 720. Da. Data was acquired using SCIEX OS Software 1.5. Each extracted sample was injected in triplicate over the course of three consecutive days.

**Data Analysis:** Data processing was performed using SCIEX OS Software 1.5.

**Table 1: Ipamorelin's Mass Spectral Fingerprint.** Combined TOF MS and TOF MS/MS results from the detection of Ipamorelin's precursor Ion [M+2H]<sup>2+</sup> at 356.7000 Da and its three main fragment ions at 129.1022, 223.1189, and 420.2030 Da.

| Analyte    | Elemental Composition   | Charge State ESI     | Theoretical Precursor Ion (m/z) | Theoretical Fragment Ion (m/z) | Conc (ng/mL) | Observed Precursor Ion (m/z) | Mass Error (ppm) | Observed Fragments (m/z)         | Fragment Mass Error (ppm) |
|------------|---|----------------------|---------------------------------|--------------------------------|--------------|------------------------------|------------------|----------------------------------|---------------------------|
| Ipamorelin | C <sub>38</sub> H <sub>49</sub> N <sub>9</sub> O <sub>5</sub> | [M+2H] <sup>2+</sup> | 356.7000                        | 129.1022                       | 5            | 356.7002                     | 0.56             | 129.1016<br>223.1189<br>420.2038 | -4.64<br>0<br>1.90        |
|            |   |                      |                                 | 223.1189                       | 25           | 356.7003                     | 0.84             | 129.1024<br>223.1194<br>420.2031 | 1.55<br>2.24<br>0.23      |
|            |   |                      |                                 | 420.2030                       | 100          | 356.6998                     | -0.56            | 129.1021<br>223.1195<br>420.2042 | -0.77<br>2.69<br>2.86     |



**Figure 3: Independent Data Acquisition (IDA) Leads to Reproducible Characterization of Ipamorelin's Precursor Ion and Peptide Fragments.** Selected Extracted Ion Chromatograms (XICs) and TOF MS spectra showing detection of ipamorelin's precursor ion  $[M+2H]^{2+}$  at 356.7000 Da at 2.01 min as well as MS/MS spectra showing reproducible detection of ipamorelin's three main fragment ions at 129.1022, 223.1189, and 420.2030 Da over the course of three consecutive days. The XICs, TOF MS and TOF MS/MS spectra show a high level of reproducibility over the course of three consecutive days.

## Developing a Reproducible Workflow for Accurate Mass Detection of Ipamorelin and its Main Fragment Ions

Human whole blood samples were spiked with ipamorelin at various concentrations ranging from 5 to 100 ng/mL. 40  $\mu$ L of these spiked human whole blood samples were spotted onto pre-conditioned DBS cards, dried at room temperature for 1 hour, extracted using the aforementioned procedure and injected in triplicate over the course of three consecutive days to build a data processing method.

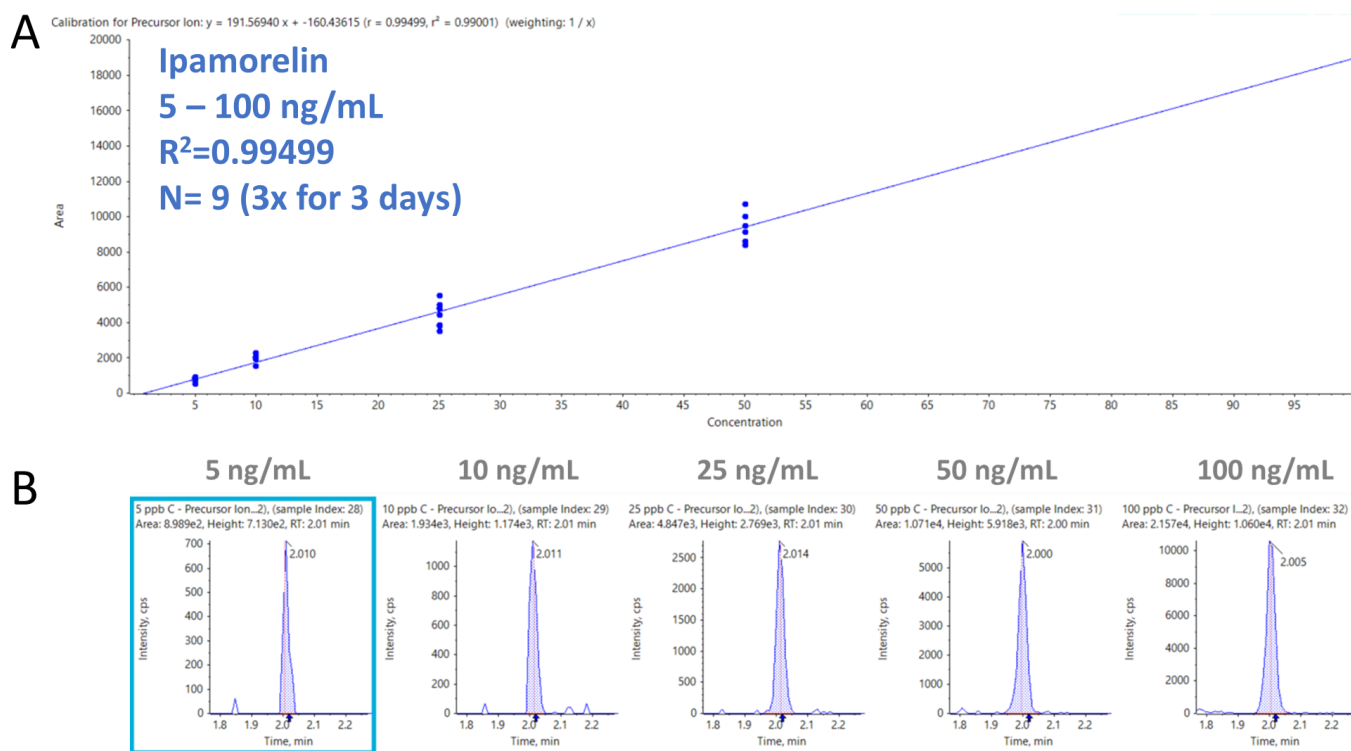
Figure 3 shows the extracted ion chromatograms (XICs) and the TOF MS spectra for the precursor ion of ipamorelin  $[M+2H]^{2+}$  at 356.7000 Da as well as the MS/MS spectra of the peptide fragments collected over three consecutive days at a concentration of 100 ng/mL. The three main fragment ions extracted from the MS/MS spectra were observed at 129.1022, 223.1189, and 420.2030 Da and assigned to the Y1-NH3 (129.1022 Da), b2 (223.1189 Da) and b3 (420.2030 Da) peptide fragments of ipamorelin, respectively. As seen in Figure 3, the data collected over the course of three consecutive days showed a high level of reproducibility across the three concentrations. In particular, the three peptide fragments of ipamorelin observed in the MS/MS spectra were consistent over the course of the validation study.

**Table 2: Validation Study.** Average (n=9, 3x for 3 days) results from the validation study showing inter-day and intra-day precision (%CV) and accuracy (bias%) as well as the LOD and LOQ for the detection of Ipamorelin's precursor ion  $[M+2H]^{2+}$  at 356.7000 Da.

| Analyte    | Linearity $R^2$ | Concentration (ng/mL) | Intra-Day Accuracy (%) | Inter-Day Accuracy (%) | Intra-Day Precision (CV%) | Inter-Day Precision (CV%) | LOD (ng/mL) | LOQ (ng/mL) |
|------------|-----------------|-----------------------|------------------------|------------------------|---------------------------|---------------------------|-------------|-------------|
| Ipamorelin | 0.99499         | 5                     | 15.2                   | 7.2                    | 32.7                      | 18.5                      | 2.5         | 5           |
|            |                 | 25                    | -19.4                  | -19.3                  | 10.6                      | 23.5                      |             |             |
|            |                 | 100                   | -12.8                  | 5.9                    | 15.7                      | 20.1                      |             |             |

Using an analytical workflow that consistently delivers reproducible and accurate results for every injection of every batch is essential to attaining reliable results. In this experiment, a series of three replicate injections were run over the course of three consecutive days to evaluate the reproducibility and robustness of the method. Table 1 summarizes the results obtained from the detection of ipamorelin extracted from dried blood spots spiked at three concentration levels (5, 25, and 100 ng/mL). The mass error for the precursor ion of ipamorelin  $[M+2H]^{2+}$  at 356.7000 Da as well as the fragment mass error for theoretical peptide fragments at 129.1022, 223.1189, and 420.2030 Da are shown. The assay showed great reproducibility over the course of three consecutive days and across the three concentrations, proving the robustness of the overall workflow.

The robustness of the workflow was further investigated by analyzing spiked human whole blood samples at concentrations ranging from 5 to 100 ng/mL and extracted from dried blood spots. Detection and integration of the peaks from the background were achieved within the viewing window using the MQ4 algorithm. This ease of use enabled streamlined analysis of replicate injections average (n=9, 3x for 3 days) which were used to verify the following validation parameters: linearity, inter- and intra-day accuracy and precision, as well as the LOD and LOQ. Table 2 shows the average results from the validation study for ipamorelin spiked in human whole blood on DBS cards at three concentrations (5, 25 and 100 ng/mL).



**Figure 4: High Sensitivity and Linearity for Ipamorelin Extracted From Dried Blood Spots.** A) calibration curve resulting from the calibration series of ipamorelin from 5 to 100 ng/mL. B) XIC traces and peak integration for ipamorelin for the calibrator series. The calibration curves and XIC traces demonstrate excellent linearity and sensitivity.

Inter-day and intra-day precision (expressed as percent variation coefficient, CV%) and accuracy (expressed as bias%) were found to be below 25% and 20%, respectively, for the calibrators at 5, 25 and 100 ng/mL concentration. The only exception was the intra-day precision percentage value for the concentration corresponding to the LOQ (5 ng/mL). The percent variation coefficient was found to be 32.7% at 5 ng/mL. Moreover, limits of quantitation (LOQ) and detection (LOD) in matrix were found to be 5 and 2.5 ng/mL, respectively. These results show that in addition to being robust, the presented workflow allows excellent sensitivity of detection of ipamorelin extracted from dried blood spots.

## Robust Workflow Leads to Highly Sensitive Detection of Ipamorelin in DBS

Achieving high sensitivity and linearity of calibration is essential to validate the robustness of any newly-developed assay. The sensitivity of the workflow was investigated by injecting a series of samples extracted from dried blood spots spiked with ipamorelin at five concentration levels (5, 10, 25, 50 and 100 ng/mL). These samples were injected in triplicate over the course of three consecutive days. Calibration curves were generated to evaluate the sensitivity and linearity of the workflow. Figure 4 shows the calibration curve (Figure 4A) and the extracted ion chromatogram (XIC) traces for the precursor ion of ipamorelin  $[M+2H]^{2+}$  at 356.7000 Da for the five calibration levels (Figure 4B). The results show a high level of consistency and precision across the calibration series. In addition, excellent linearity across the calibration range was observed with an  $R^2$  value of 0.99499.

## Conclusions

A novel and comprehensive workflow for the detection of growth hormone-releasing peptides from dried blood spots was successfully developed using the SCIEX X500R QTOF System. The combination of a rapid sample extraction procedure and a highly selective MS/MS acquisition method enabled sensitive detection of ipamorelin in dried blood spots with ng/mL detection limits.

- A rapid sample preparation procedure was developed for efficient extraction of ipamorelin from dried blood spots
- Data Dependent Acquisition (IDA) generated reproducible and comprehensive quality TOF MS and TOF MS/MS spectra of the most abundant precursor/candidate ions, enabling reproducible peptide fragmentation
- Data generated using IDA method allowed reliable characterization and quantitation of ipamorelin's precursor ion and peptide fragments
- Workflow showed excellent sensitivity, with an LOD of 2.5 ng/mL and LOQ of 5 ng/mL for ipamorelin extracted from DBS while maintaining accuracy and precision
- Assay showed excellent linearity with an  $R^2$  value of 0.99499 for ipamorelin across the calibrator series ranging from 5 to 100 ng/mL
- Developed workflow can be modified to include larger panel of analytes, including additional GHRPs listed on the World Anti-Doping Agency (WADA) Prohibited List