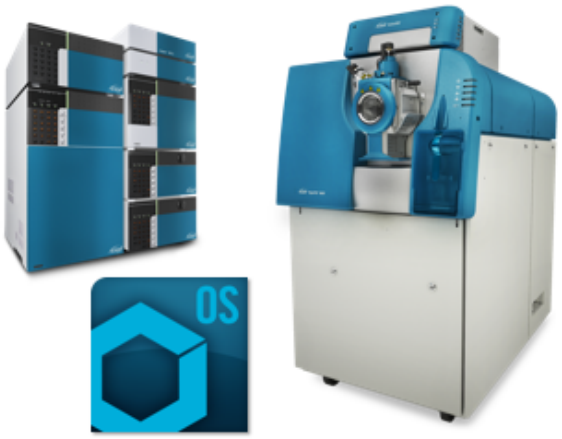
Quantitative Amino Acid Analysis in Cell Culture Media Using SWATH® Acquisition

Featuring data independent acquisition on the SCIEX TripleTOF® 6600 LC-MS/MS System

Zuzana Demianova[1], Jeff Layne[2], Brain Rivera[2], Elsa Gorre[3], Andrew Mahan[3] and Lei Xiong[4]

[1] SCIEX, Brea, CA, USA; [2] Phenomenex, Torrance, CA, USA; [3] Janssen Research & Development, Spring House, PA, USA; [4] SCIEX, Redwood Shore, CA, USA

Amino acids are important nutrients in cell culture media that support cell growth and impact critical quality attributes of biotherapeutics production. In order to optimize the process yield, drug efficiency, safety and quality consistency, the levels of amino acids in cell media are critical to monitor across the entire therapeutic manufacturing process. Although there is an urgent need to develop reliable analytical workflow for amino acid quantification, amino acid analysis using LC-MS is challenging due to multiple reasons: 1) some compounds are very polar and hard to retain on a reserve phase HPLC column; 2) isomers are usually coeluted and can hardly be differentiated from each other; 3) HILIC (hydrophilic interaction liquid chromatography) based separation allows better retention, but is often struggled with poor reproducibility. To overcome these challenges, SCIEX has developed a reliable quantification workflow for amino acid analysis in cell media, by coupling the HILIC separation with the SWATH Acquisition. The Phenomenex bioZen® glycan column provides highly reproducible HILIC based retention and resolution of analytes including isomers, while SWATH Acquisition on the TripleTOF 6600 System allows solid quantification and confirmation of amino acids as well as unknown compound identification in cell media.



ExionLC™ System coupled to TripleTOF® 6600 System and SCIEX OS 1.5 for data processing

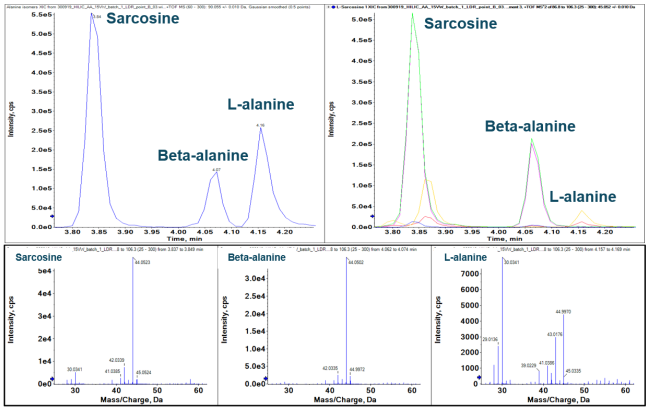


Figure 1. Basic separation of alanine isomers (L-alanine, Beta-alanine, sarcosine) by using HILIC separation. Bottom panel lists the SWATH MS/MS spectra for the three isomers that are used for compound confirmation.

Key Features of Quantitative Amino Acid Analysis Using SWATH Acquisition

SWATH Acquisition on the TripleTOF 6600 System offers:

- Wide analyte coverage by quantifying and confirming every ESI MS detectable compound in the medium
- Superior quantification quality based on high resolution MS/MS spectra, compared to single MS spectra
- Ease of method development by using generic method parameters with minimum method optimization requirements
- Variable Q1 window acquisition by optimizing window width based on *m/z* density of precursors, which allows for greater selectivity in chromatographic periods of high precursor ion density
- Phenomenex bioZen glycan column provides excellent HILIC based retention and resolution of target analytes with high reproducibility
- All-in-one powerful software solution with SCIEX OS offers versatile qualitative and quantitative workflows

Methods

Sample preparation: Three groups of samples, amino acid standard solutions, CD CHO media (Gibco) and cell culture component master mix, were prepared serving different experimental purposes. The amino acids standard stock mix (Sigma-Aldrich) was serial diluted with LC mobile phase buffers to prepare amino acid standard solutions. Undiluted and 300x diluted CD CHO media were used to prepare matrix samples. The cell culture component master mix was prepared by mixing standards of 111 compounds with final concentration ranges from 6.67 to 20 µg/mL.[1]

Chromatography: Samples were subjected to LC-MS analysis in triplicate by using a TripleTOF 6600 system coupled with an ExionLC system. Analytes were separated using a Phenomenex bioZen glycan column (100 mm x 2.1 mm ID, particle size 2.6 µm) with an 8 min gradient (12 min total run time) at a flow rate of 500 µL/min. Mobile phase A was 10 mM ammonium formate in acetonitrile, and mobile phase B was 10 mM ammonium formate in water. Column oven temperature was 40°C and the injection volume varied from 1 to 5 µL.

Mass spectrometry: The data independent workflow using SWATH Acquisition was applied in this project. The SWATH data were generated with variable window SWATH Acquisition using 15 variable windows, with a total cycle time of 0.67 sec. The window widths were determined based on the MS ion intensity distribution using the SWATH Variable Window Calculator.[2] The method details are listed in Table 1.

Table 1. Summary of mass spectrometry parameters.

Parameter	Value	Parameter	Value
MS mass range	*60-300 m/z*	*MS/MS mass range*	25-300 *m/z*
MS accumulation time	*100 ms*	*MS/MS accumulation time*	*35* ms
Curtain gas:	*35 psi*	*Source temperature:*	400 *°C*
Ion source gas 1:	*40 psi*	*Ion source gas 2:*	40 *psi*
Declustering potential	*40 V*	*Collision energy*	*7 V*
Polarity:	+	*Ion spray voltage:*	5500

Data processing: The data were analyzed using SCIEX OS 1.5 Software. The MRM transition library of amino acids only or all cell culture components[1] was incorporated into SCIEX OS to generate the quantification method. Peak integration was performed with the AutoPeak algorithm. For compound confirmation and unknown compound screening, the acquired SWATH MS/MS spectra were matched against MS/MS spectra in the built-in high resolution spectral library. This built-in spectral library contains entries from the SCIEX all-in-one library and NIST library.

HILIC Method Development and Reproducibility Evaluation

Some amino acids are polar and hydrophilic, have weak retention on reverse phase HPLC columns in general. Therefore, the HILIC chromatography is implemented here to provide better retention and isomer separation. Table 2 summarizes the amino acid analytes monitored in this method and their retention times. The retention times are widely distributed over the LC gradient, suggesting the good resolution and strong retaining power of the HILIC column. Moreover, this method also provides the baseline separation of multiple pairs of isomers. A few examples are shown: 1) leucine and iso-leucine (Figure 2), 2) sarcosine, beta-alanine and L-alanine (Figure 1).

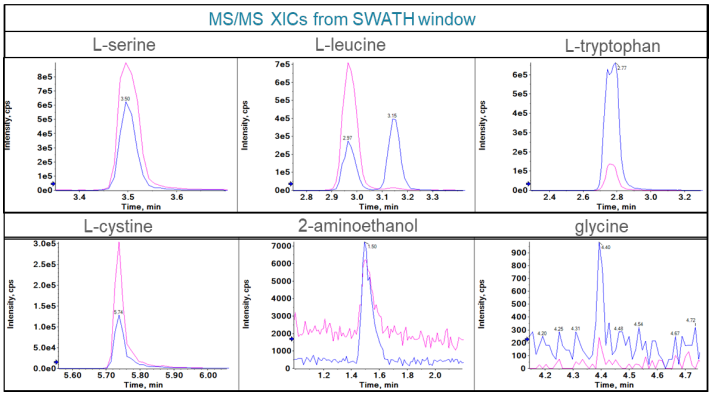


Figure 2. XICs of representative compounds from Gibco's CD CHO medium. Selected analytes abundances cover four orders of magnitudes. In the L-Leucine XIC (top middle window), the two peaks are Leucine (earlier) and iso-leucine (later), baseline separated.

Maintaining retention time reproducibility is crucial in quantitative LC-MS analysis, but it could be challenging while using the HILIC methodology. Comparing with reverse phase method, the retention time in HILIC method is more sensitive to any minor changes related with column solid phase chemistry, mobile phase preparation, column equilibration and sample condition. Therefore, an extensive evaluation of retention time reproducibility is executed here to ensure the method robustness. The reproducibility test covers inter-day, intra-day, column lot-lot and multiple injection volumes. As shown in Figure 3, the RTs of all analytes remain consistent among the entire test, suggesting a solid HILIC chromatography for amino acid quantification.

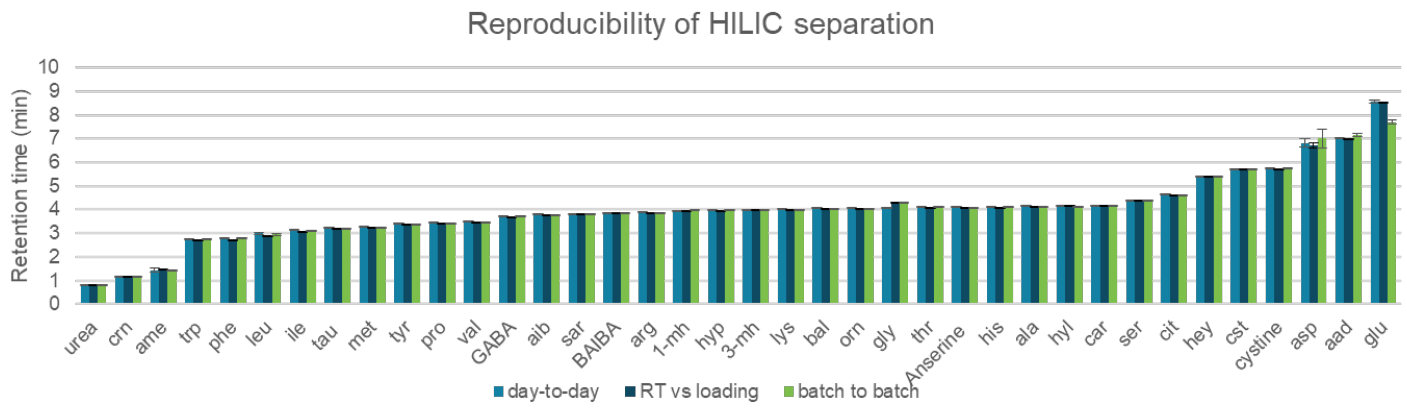


Figure 3. Retention time reproducibility evaluation of HILIC separation for 38 amino acids. Batch-to-batch evaluation includes: 3 batches and 15 injections per batch; Day-to-day evaluation includes: 4 days and 5 injections per day; RT-vs.-loading evaluation includes: 1, 2, 3, 4, 5 uL injection volumes, 3 injections per volume.

SWATH Acquisition workflow

In this project, SWATH Acquisition is utilized to develop a comprehensive workflow for quantification and confirmation of amino acids, as well as unknown compound identification in cell culture media. SWATH Acquisition (Figure 4), also known as data independent acquisition (DIA), demonstrates significant advantages in analyte coverage, quantification capability and ease of method development.[1] SWATH Acquisition offers variable window acquisition strategy[2, 3] by allowing the user to vary the size of the Q1 isolation window based on the density of analyte precursor masses. Herein an optimal method with 15 variable isolation windows (Figure 5) is built to allow mass range coverage and high quantification performance.

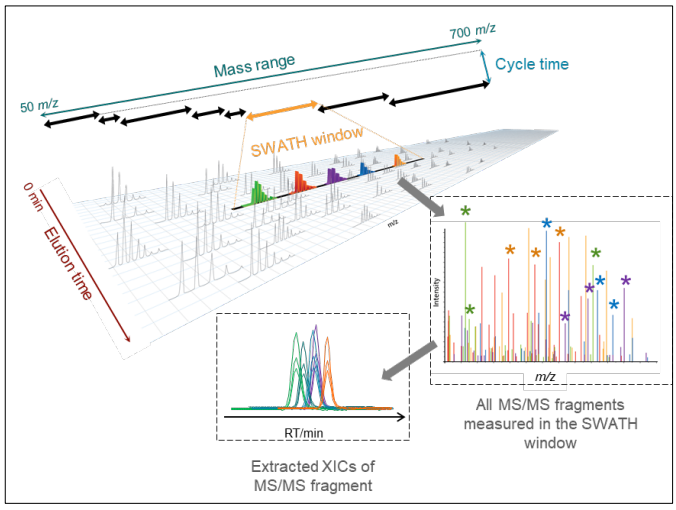


Figure 4. The general workflow of SWATH® Acquisition.

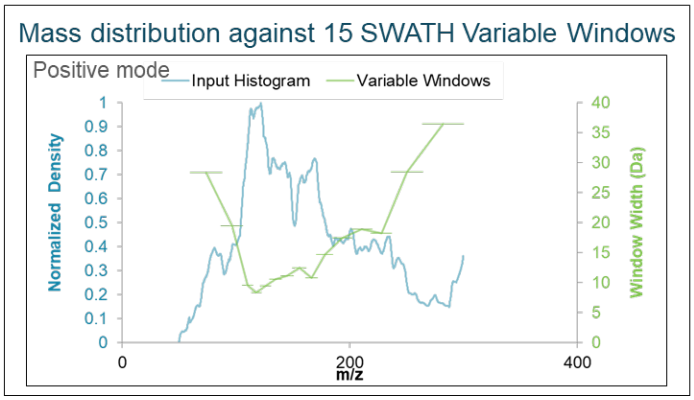
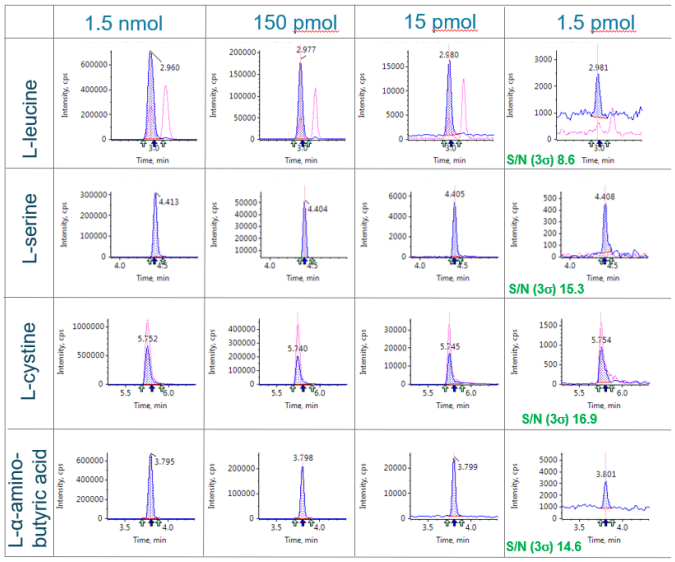


Figure 5. Graphic representation of windows size variation. The size of the Q1 isolation window (green line) is determined by the density of component precursor masses (blue line).

In the targeted (quantification and confirmation) workflow, an MRM transition library of amino acids is incorporated into SCIEX OS to generate the quantification method. The abundant fragment ions of the target analyte are automatically extracted by software for peak integration. Figure 6 shows the quantification of representative amino acids with concentrations ranging from 1.5 pmol to 1.5 nmol on column, with high S/Ns and good linearity demonstrated. Another example (Figure 2) is shown as the quantification of multiple components in the Gibco's CD CHO cell medium, in which the analyte abundances cover over four orders of magnitudes. The MS/MS level confirmation is executed in parallel with quantification to prove the identity of the XIC peaks, especially among isomers (Figure 1, Figure 7).



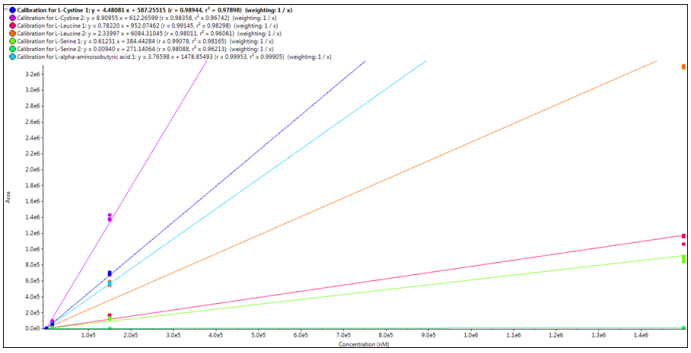


Figure 6. Quantitation of representative amino acids, L-cystine, L-leucine, L-serine, L-α-aminoisobutyric acid. Top: XICs with concentrations ranging from 1.5 pmol to 1.5 nmol on column. Bottom: calibration curves.

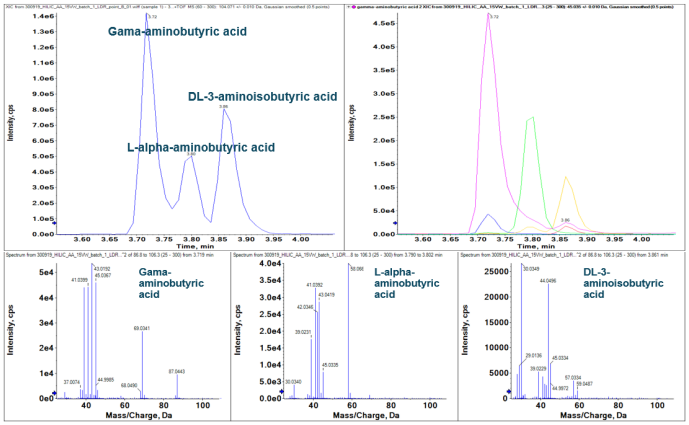


Figure 7. MS/MS confirmation of aminobutyric acid isomers. Top panel shows the XICs of the TOF MS and MS/MS of the three isomers. Bottom panel lists the SWATH MS/MS spectra of gama-, DL-3- and L-alpha-aminobutyric acid.

In the untargeted compound screening workflow, both MS and MS/MS spectra are processed by SCIEX OS-Q for compound identification. Figure 8 shows an example of niacinamide identification in the medium sample. The TOF MS spectrum is linked with predicted compound formula by FormulaFinder and searched against ChemSpider for structure matching and fragment ion prediction. The MS/MS spectrum is searched against MS/MS spectral library for fragment ion matching. This workflow significantly reduces false discovery rate by providing MS/MS level confirmation in addition to the retention time and precursor mass information.

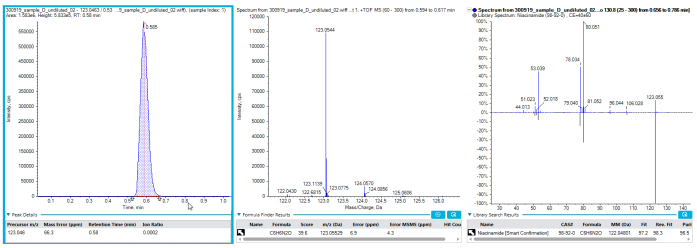


Figure 8. Untargeted compound screening using SCIEX OS-Q software. Niacinamide is identified as an addition to amino acids in cell culture media. The TOF MS spectrum ([M+H]+; 123.05 m/z) was matched to compound formula (C6H6N2O). The MS/MS spectrum is searched against MS/MS spectral library, showing a good matching (Fit 97.2, Purity 96.5%) between measured MS/MS spectrum (blue MS/MS spectrum) and library spectrum (grey MS/MS spectrum).

Conclusions

A powerful data independent workflow using SWATH Acquisition coupled with HILIC chromatography is presented for quantitative amino acid analysis in cell media. It offers 1) powerful retention and isomer separation of polar amino acids by using the highly reproducible HILIC chromatography; 2) the high resolution MS/MS based quantification and confirmation of amino acids, as well as unknow compounds screening in cell media; 3) the all-in-one data processing software solution to cover both targeted and untargeted workflows. This workflow demonstrates not only superior quantification performance for amino acids in cell media, but also outstanding capabilities on MS/MS level confirmation and unknown compound identification.

References

1. Quantitative and Qualitative Analysis of Cell Culture Medium Using SWATH® Acquisition, SCIEX Technical Note RUO-MKT-02-10242-A.

2. SWATH® Acquisition Variable Window Calculator - Excel tool. Download from http://sciex.com/support/software-downloads.

3. Improved Data Quality Using Variable Q1 Window Widths in SWATH® Acquisition, SCIEX Technical Note RUO-MKT-02-2879-B.

Table 2. List of amino acids monitored in this workflow and their retention times with HILIC chromatography.

Amino acid compound	RT (min)
Urea	0.81
L-Creatinine	1.17
2-Aminoethanol	1.44
L-Tryptophan	2.74
L-Phenylalanine	2.78
L-Leucine	2.99
L-Isoleucine	3.12
Taurine	3.22
L-Methionine	3.27
L-Tyrosine	3.39
L-Proline	3.46
L-Valine	3.48
gamma-aminobutyric acid	3.71
L-alpha-aminoisobutyric acid	3.78
L-Sarcosine	3.82
DL-3 aminoisobutyric acid	3.85
L-Arginine	3.87
1-Methyl-L-histidine	3.96
Hydroxy-L-proline	3.98
3-Methyl-L-histidine	4.00
L-Lysine	4.01
beta-Alanine	4.05
L-Ornithine	4.06
Glycine	4.07
L-Threonine	4.10
Anserine	4.10
L-Histidine	4.11
L-Alanine	4.14
Hydroxylysine	4.17
L-Carnosine	4.18
L-Serine	4.40
L-Citrulline	4.64
L-Homocystine	5.40
L-Cystathionine	5.70
L-Cystine	5.75
L-Aspartic acid	6.81
2-Aminoadipic acid	7.01
L-Glutamic acid	8.55

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
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