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Note d'application

High-Throughput Amino Acid Analysis Using Hamilton Automated Preparation

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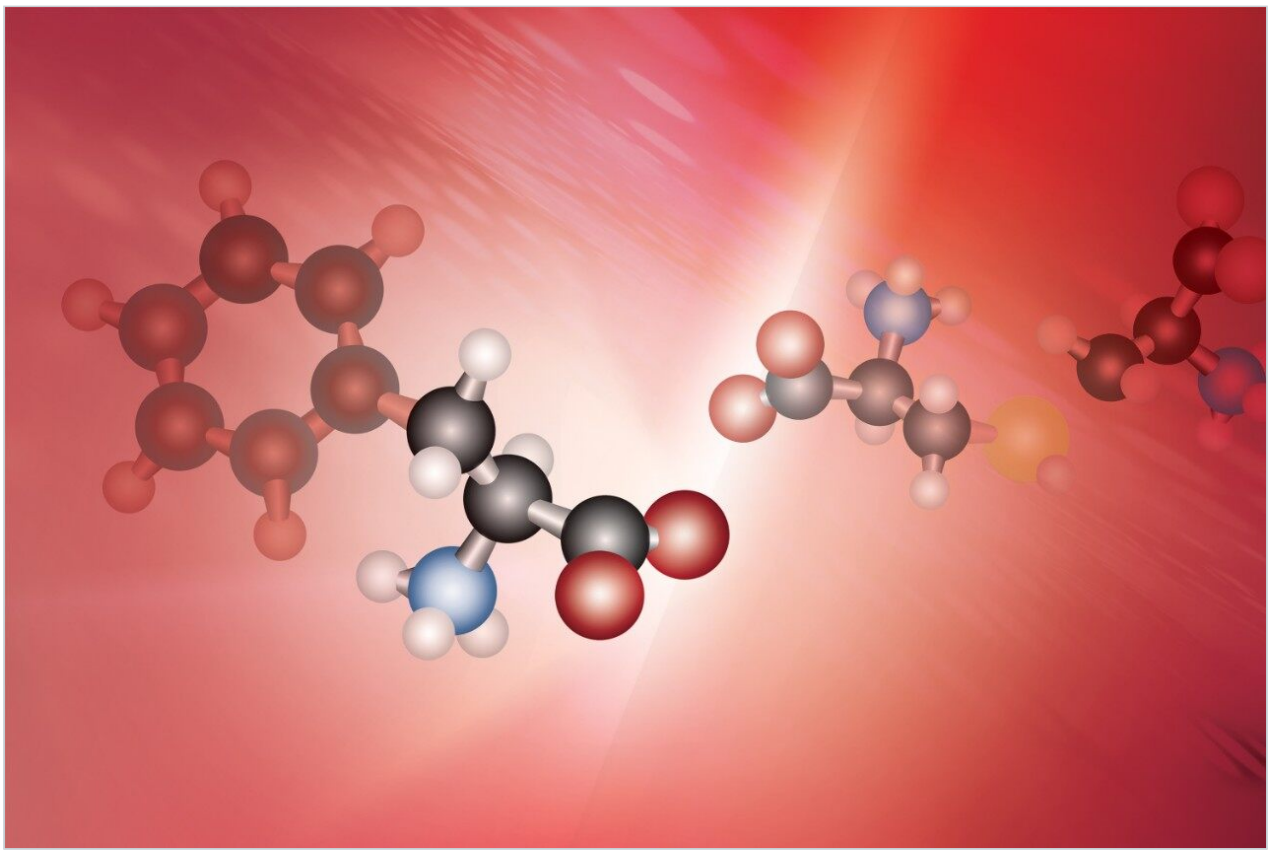

Abstract

The objective of this application note is to demonstrate the equivalency and robustness of manual preparations of AccQ●Tag-labelled amino acids to those prepared using a Hamilton Microlab (ML) STAR automation platform using the Amino Acid Cell Culture Standard Kit.

Benefits

- Sample preparation robustness and equivalency via a Hamilton automation platform
- Elimination of risks associated with human error and contamination
- Method standardization and transfer between multiple sites

Introduction

Amino acids are the primary building blocks of proteins, and the sequence in which they present is unique to each protein or peptide. They are essential for the growth and repair of cells. Determining amino acid composition is of significant relevance in fields dealing with bio-pharmaceutical preparations and protein hydrolysates. In cell culture media, the concentration of amino acids depends on the cells' metabolic and transport requirements.[1] Monitoring and optimizing the amino acid components of bioreactor media is essential for ensuring the best conditions for cell growth. This makes amino acid analysis a requirement in the pharmaceutical industry. Waters Amino Acid Cell Culture Standard Kit, which contains nine additional amino acids critical in cell cultures, was designed to complement a 17-Amino Acid Hydrolysate Standard. The addition of the cell culture amino acids is important as they include key indicator amino acids that are essential for cell growth (Table 1).

Derivatization of amino acids is a key step in amino acid analysis. The Waters UPLC Amino Acids Solution consists of a pre-column AccQ●Tag Ultra Derivatization Kit, as well as an optimal system configuration with certified column and eluents to enhance reproducibility. Automation is an ever-growing field in sample preparation. Automation platforms reduce variability compared to manual preparations and increase laboratory efficiency.[2] The AccQ●Tag Ultra Derivatization Kit provides the increased volumes of reagents required for automated preparation.

In this application note, we will demonstrate the equivalency and robustness of manual preparations of AccQ●Tag-labelled amino acids to those prepared using a Hamilton Microlab (ML) STAR automation platform with the Amino Acid Cell Culture Standard Kit.

Figure 1. Waters Amino Acid Analysis Automation Solution: (A) AccQ●Tag Ultra Eluents; (B) AccQ●Tag Ultra, 1.7 µm, 2.1 X 100 mm Column; (C) AccQ●Tag Ultra Derivatization Automation Kit; (D) Amino Acid Cell Culture Standard Kit; (E) Amino Acid Internal Standard-Norvaline; (F) Automation Scripts for Hamilton; (G) 96-Well Collection Plate; (H) Cap Mat.

Cell culture standard kit (p/n: 186009300)			
Alanine	Isoleucine	Threonine	GABA (γ-Aminobutyric acid)
Arginine	Leucine	Tyrosine	Tryptophan
Aspartic acid	Lysine	Valine	Ornithine
Cystine	Methionine	Taurine	AABA (α-Aminobutyric acid)
Glutamic acid	Phenylalanine	Hydroxy-proline	Hydroxy-lysine
Glycine	Proline	Asparagine	
Histidine	Serine	Glutamine	

Table 1. Amino Acid Composition of Cell Culture Standard Kit.

Experimental

Workflow Overview

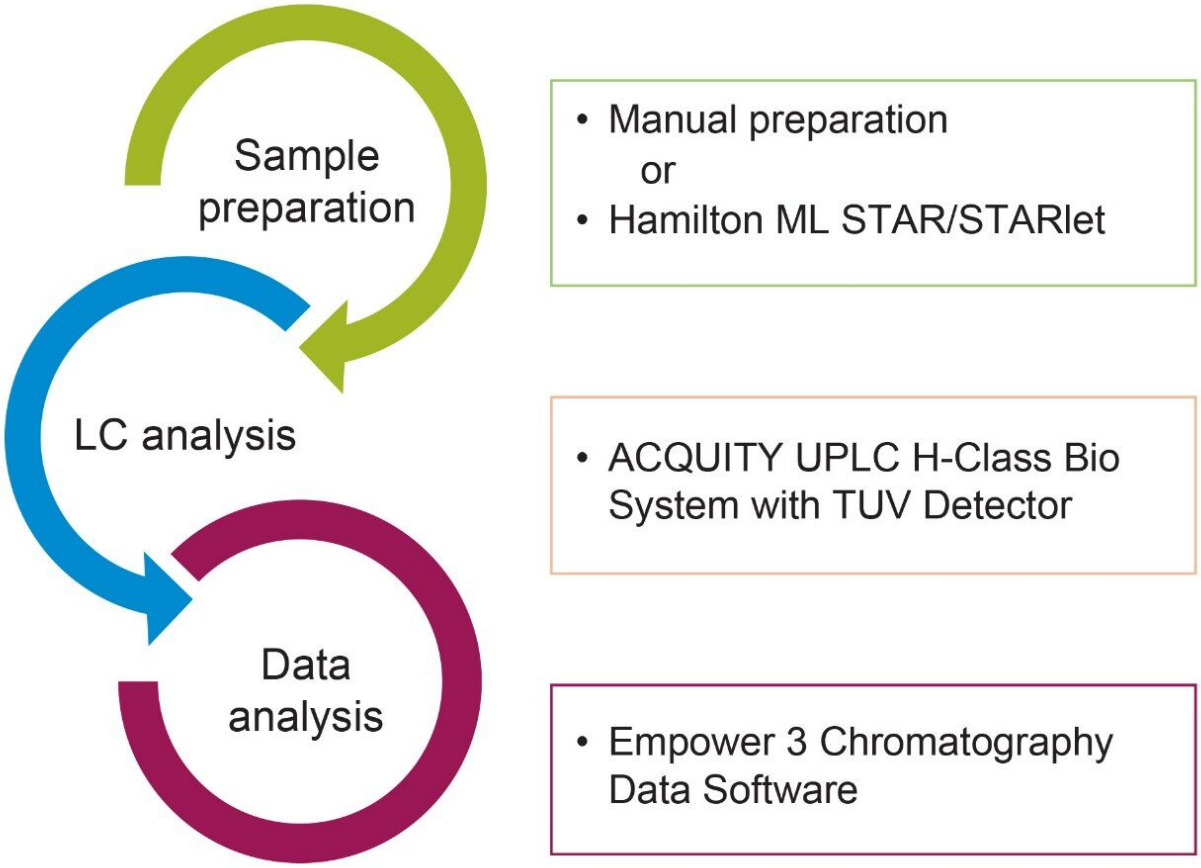

Figure 2. Example of a manual/automated sample preparation workflow.

LC Conditions

System:	ACQUITY UPLC H-Class Bio with TUV Detector
Sample temp.:	20 ˚C
Analytical column temp.:	43 ˚C
Flow rate:	700 µL/min
Injection volume:	1 µL

Column:	AccQ●Tag Ultra, 1.7 µm, 2.1 x 100 mm
UV detection:	260 nm
Mobile phase A:	100% AccQ●Tag Ultra Eluent A
Mobile phase B:	90:10 water, AccQ●Tag Ultra Eluent B
Mobile phase C:	100% HPLC-grade water
Mobile phase D:	100% AccQ●Tag Ultra Eluent B

Design Factors

A. Optional Script Features

The scripts for the Hamilton automation platform (ML STAR/ML STARlet) were created with a barcode export that provides users the functionality of building sample lists in their chromatography data system from the exported data, minimizing common mistakes made when manually inputting data. The functionality of the Hamilton scripts is enhanced by their ability to perform dilution of standards with a reference range of 500 µM to 0.5 µM (Cystine 250-0.25 µM). Additionally, cell culture samples can be diluted on deck using the available worklist import function. Selection of sample number and well starting position provides the user the advantage of running the AccQ●Tag Ultra Derivatization Automation Kit for 32, 64, or 96 samples. There is also the flexibility to include the Norvaline Internal Standard (p/n: 186009301 < https://www.waters.com/waters/partDetail.htm?partNumber=186009301>) as an optional feature when preparing samples.

B. AccQ●Tag Ultra Derivatization Automation Kit (p/n: 186009232 < https://www.waters.com/waters/partDetail.htm?partNumber=186009232>)

The AccQ●Tag Ultra Derivatization Automation Kit scales up the reagent volumes necessary for use with the automation systems due to their increased dead volume requirements. The volumes of reagents provided allow for the preparation of 96 samples and in a 3 x 32 sample format.

C. Labware

The manual preparation of amino acid samples with the AccQ●Tag Derivatization Kit is performed using Waters Total Recovery Vials. In order to make this automation compatible, these glass vials were replaced with a

96-well collection plate (p/n: 186002481 < https://www.waters.com/waters/partDetail.htm?partNumber=186002481>). Extensive feasibility testing was conducted to support the labware change, and no impact to product performance was detected.

D. Experimental Design

A minimum of three lots of eluents, columns, and AccQ●Tag Ultra Derivatization Automation Kits were evaluated during testing in order to demonstrate the assay's robustness. Details of this can be found in the section on robustness. Different analysts (n = 4) were also evaluated throughout the study to demonstrate the independence of the automated workflow from the user.

Results and Discussion

The automated preparation method using the Hamilton automation platform was assessed and compared to a pre-existing manual preparation method for robustness and equivalency. Performance characteristics were monitored across three preparations and three-replicate injections of each preparation, at three concentration levels (10 µM, 200 µM, and 400 µM) to determine the accuracy and precision (retention time, analyte peak area, and concentration) as well as the linearity of the results. The internal standard-Norvaline was used in all experiments. The use of a Norvaline internal standard best compensates for the variability generated in sample hydrolysis and amino acid analysis.

Figure 3 shows a representative chromatogram of the cell culture standard at 10 pmols on column.

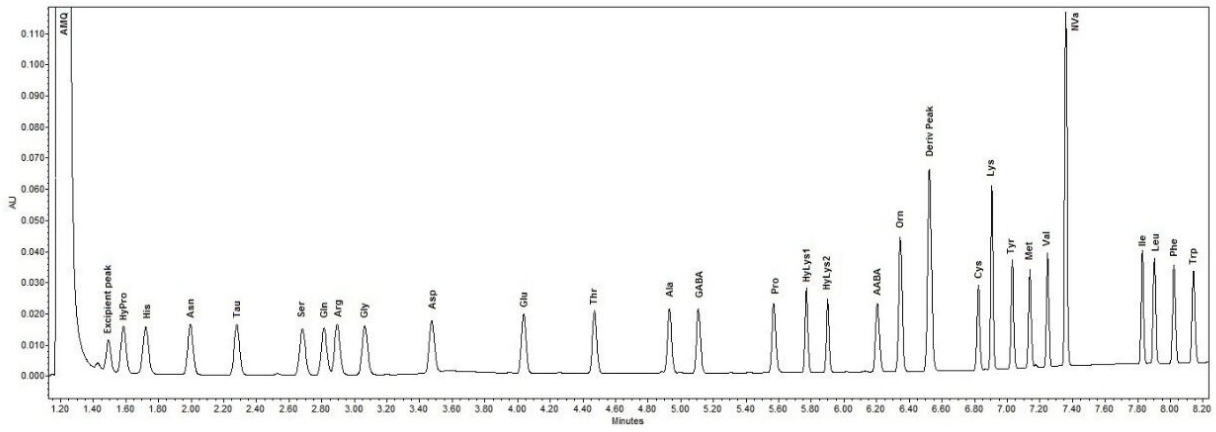

Figure 3. Separation of 10 pmols of the Cell Culture Standard spiked with 23.5 pmols of Nva on the column. Data was generated using the Empower 3 Chromatography Data System (CDS).

Precision

In liquid chromatography, retention time is the primary method of identifying chromatographic peaks. As demonstrated in Table 2, the %CV for retention time was ≤0.3%, showing good equivalence across all methods. Consistent analyte area across injections and preparations is a good indicator of the repeatability of both the sample preparation and analysis methods. This data demonstrates the excellent repeatability of the automated AccQ●Tag preparation methods and indicates a maximum %CV across all analytes of ≤2.8% for the manual preparation and ≤1.1% for the Hamilton preparation.

Amino acid	Manual % CV		Hamilton % CV	
	Retention time	Peak area	Retention time	Peak area
Hydroxy-proline	0.3	2.8	0.1	1.1
Histidine	0.3	1.9	0.2	1.1
Asparagine	0.2	1.5	0.1	1.1
Taurine	0.2	1.7	0.2	1.1
Serine	0.2	1.8	0.1	1.1
Glutamine	0.2	1.8	0.2	1.1
Arginine	0.2	2.1	0.2	1.1
Glycine	0.1	2.0	0.1	1.1
Aspartic acid	0.1	2.1	0.1	1.1
Glutamic acid	0.1	2.2	0.1	1.1
Threonine	0.0	1.8	0.0	1.0
Alanine	0.0	2.0	0.0	1.1
GABA (γ-Aminobutyric acid)	0.0	1.9	0.0	1.0
Proline	0.0	1.7	0.0	1.1
Hydroxy-lysine 1	0.0	1.8	0.0	1.1
Hydroxy-lysine 2	0.0	1.7	0.0	1.1
AABA (α-Aminobutyric acid)	0.0	1.8	0.0	1.1
Ornithine	0.0	1.8	0.0	1.1
Cystine	0.0	1.9	0.0	1.1
Lysine	0.0	1.8	0.0	1.1
Tyrosine	0.0	1.7	0.0	1.1
Methionine	0.0	1.8	0.0	1.1
Valine	0.0	1.7	0.0	1.1
Isoleucine	0.0	1.8	0.0	1.1
Leucine	0.0	1.8	0.0	1.0
Phenylalanine	0.0	1.8	0.0	1.1
Tryptophan	0.0	1.8	0.0	1.1

Table 2. Comparison of the %CVs for peak area and retention time, where N = 9, reflecting three preparations and three-replicate injections of each preparation of a 200 µM panel, across the two preparation methods, Hamilton automated preparation and manual amino acid derivatization.

Accuracy

Accuracy was assessed at concentrations of 10, 200, and 400 µM using three preparations at each concentration, and three-replicate injections of each preparation. For the 10 µM panel, the %Recovery for each amino acid prepared using the Hamilton automation method was within ±20% of the target

concentration. For the panels at 200 and 400 µM, the %Recovery for each amino acid prepared using the Hamilton automation method was within ±15% of the target concentration. This recovery data, as well as the precision data in Table 3, demonstrates the suitability of the Hamilton automated preparation platform as a considerable, time-saving alternative to manually derivatizing amino acids.

The recovery of the cell culture standard when compared to NIST SRM 2389a was also analyzed as a means of independent assessment. A 200 µM panel was prepared from the cell culture standard using the Hamilton automated preparation method, and recovery calculated using a single NIST standard (prepared at 250 µM concentration [1 in 10 dilution of SRM]), by the existing manual method but analyzed in the same UPLC analytical run. Recovery was shown to range from 91% for lysine to 104% for aspartic acid for the Hamilton automated preparation. This demonstrates the accuracy of the Amino Acid Cell Culture Standard when compared to SRM.

	R^2	
Amino acid	Manual	Hamilton
Hydroxy-proline	0.999	1.000
Histidine	0.999	1.000
Asparagine	0.999	1.000
Taurine	1.000	1.000
Serine	1.000	1.000
Glutamine	1.000	1.000
Arginine	1.000	1.000
Glycine	1.000	1.000
Aspartic acid	0.997	0.999
Glutamic acid	0.998	1.000
Threonine	1.000	1.000
Alanine	1.000	1.000
GABA (γ-Aminobutyric acid)	0.996	0.999
Proline	1.000	1.000
Hydroxy-lysine 1	1.000	1.000
Hydroxy-lysine 2	1.000	1.000
AABA (α-Aminobutyric acid)	1.000	1.000
Ornithine	1.000	1.000
Cystine	1.000	1.000
Lysine	0.999	1.000
Tyrosine	1.000	1.000
Methionine	1.000	1.000
Valine	1.000	1.000
Isoleucine	1.000	1.000
Leucine	1.000	1.000
Phenylalanine	1.000	1.000
Tryptophan	1.000	1.000

Table 3. R^2 values for line generated using the Waters Amino Acid Cell Culture Standard. All lines passed acceptance criteria of having an R^2 value > 0.995.

Linearity

Linearity was assessed using a cell culture standard prepared at seven concentration levels for each amino acid across a range of 0.5–500 µM (cystine 0.25–250 µM). All analytical runs were assessed for linearity, and all met the criteria of r^2 > 0.995 with no point deviation from the expected concentration by >15% for calibrators 2–7 (2.5-500 µM) and >20% for calibrator one (0.5 µM). The data was consistent between manual and automated preparation methods and no trends were observed.

Conclusion

The performance characteristic of precision, accuracy, and linearity were used to determine the equivalence of the Hamilton automation versus manual preparations. The results indicate good overall agreement between the two sample preparation methods for the UPLC Amino Acid Analysis Solution, however there are significant advantages to automation that must also be considered when performing a comparative analysis:

- Preparation time for the Hamilton automated instrument was significantly shorter than the manual counterpart, highlighting the efficiency of automated sample preparation versus manual preparation. The complete sample preparation time for a 96-sample run, including a standard dilution step, is <1 hour for the Hamilton automation platforms.
- The automation method developed did not require manual intervention during the run, thus allowing the analyst time to perform other laboratory tasks.
- Automation reduces the risk of human error and contamination.
- Automation removes analyst-to-analyst variation, allowing laboratories and companies to standardize analysis methods and facilitate method transfer between multiple sites.

The automated analysis of amino acids using an AccQ●Tag Ultra Derivatization Automation Kit is a promising alternative to the current manual preparation method for laboratories that suffer from time and resource constraints. The results generated show good agreement in terms of precision, accuracy, and linearity.

References

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2. Saitoh, S.; Yoshimori, T. Fully Automated Laboratory Robotic System for Automating Sample Preparation and Analysis to Reduce Cost and Time in Drug Development Process. *Journal of the Association for Laboratory Automation*, 2008, *13* (5), 265-274.

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720006953, July 2020