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Instrument Considerations for Reliable Amino Acid Analysis Using AccQ•Tag™ Ultra C_{18} 2.5 µm Column

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Abstract

Amino acid analysis is performed regularly for a wide range of samples including hydrolyzed proteins, cell culture, as well as and food and feeds samples. However, traditional HPLC methods for these analyses can have long run times and lower sensitivity. To address these challenges, Waters created an ACQUITY™ UPLC™ AAA Solution which includes a pre-column derivatization kit, sub-2 µm columns and pre-packaged mobile phases suitable for UPLC analyses. By scaling and adapting these reliable and reproducible methods from sub-2 µm particles to HPLC particle sizes (i.e., 2.5 µm), the AccQ•Tag™ Ultra solution chemistry can be used at lower pressures with conditions compatible for a wider range of systems. This study will demonstrate the considerations for adaptation of this pre-column derivatization method from UPLC to UHPLC/HPLC systems. After adaptation and optimization of the separation method, performance will be demonstrated, including linearity, repeatability, reproducibility, limit of quantitation, and limit of detection.

Benefits

- Scaling of proven AccQ•Tag UPLC methods for analysis on HPLC systems, including Arc HPLC and

ACQUITY Arc™ Chromatographic Systems

- AccQ•Tag Ultra C_{18} 2.5 µm Column performance is maintained using AccQ•Tag Ultra C_{18} 2.5 µm VanGuard™ Cartridges that can be effectively used to extend analytical column lifetime reducing cost per analyses
- Using the AccQ•Tag Ultra C_{18} 2.5 µm Column enables a shorter run time compared to legacy HPLC methods resulting in higher throughput and lower solvent consumption

Introduction

Analysis of amino acids (AAA) can be very challenging due to low or a lack of UV absorbance and the wide range of chemical properties of amino acids. Given these challenges, pre-column derivatization using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) followed by a reversed-phase separation has been found to be a highly reproducible technique for quantitative analysis of amino acids using a UHPLC/UPLC systems. However, higher pressure systems are not always available. While there are traditional HPLC methods that use older legacy columns, these may have lower sensitivity, longer runtimes and require the use of complicated mobile phases. Therefore, an HPLC method that uses a stable derivatization technique as well as modern, reproducible columns can provide a highly reproducible method for amino acid analysis at lower pressures.

Experimental

Sample Description

All calibration standards were prepared from Waters Amino Acid Standard (p/n: WAT088122 < https://www.waters.com/nextgen/global/shop/standards--reagents/wat088122-amino-acid-standard-accq-tag-pico-tag-accq-tag-ultra.html>) using norvaline (p/n: 186009301 < https://www.waters.com/nextgen/global/shop/standards--reagents/186009301-amino-acid-internal-standard-norvaline.html>) as the internal standard and 0.1 N HCl as the diluent.[1] The internal standard stock was prepared at 2500 µM in 0.1 N HCl. The final concentration of the calibrants were 1, 5, 10, 20, 50, 100, 200, and 500

µM for all amino acids (except cysteine which was present in ½ the concentration) and 250 µM for norvaline (internal standard). The precision sample was prepared at 500 µM (250 µM cysteine). Norvaline was kept constant at 250 µM.

LC Conditions

LC systems:	Arc HPLC System with 30 cm CHC with passive preheater ACQUITY Arc System with CH-A Column heater
Detection:	Arc HPLC-2489 TUV Detector with 10 mm HPLC Analytical Flow Cell (p/n: 176248901) ACQUITY Arc-2489 TUV Detector with low dispersion 10 mm UHPLC flow cell (p/n: 176017007)
Wavelength:	260 nm
Sampling rate:	10 Hz
Vials:	LCGC Certified Clear Glass 12 x 32 mm screw neck vial, total recovery with cap and PTFE/Silicone septum (not pre-slit) (p/n: 186000384C)
Column(s):	AccQ•Tag Ultra C_{18}, 2.5 µm 4.6 x 150 mm (p/n: 186010407)
Column temperature:	43 °C
Sample temperature:	20 °C

Injection volume:	3 µL (Arc HPLC) and 2µL (ACQUITY Arc)
Flow rate:	1.5 mL/min
Mobile phase A:	AccQ•Tag Eluent A (p/n: 186003838)
Mobile phase B:	90:10 (v/v) Water:AccQ•Tag Ultra Eluent B
Mobile phase C:	Milli-Q Water
Mobile phase D:	AccQ•Tag Eluent B (p/n: 186003839)
Sample manager wash	95:5 (v/v) Water:Acetonitrile
Sample manager purge	95:5 (v/v) Water:Acetonitrile

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	%C	%D	Curve
Initial	1.500	10.0	0.0	90.0	0.0	Initial
0.36	1.500	10.0	0.0	90.0	0.0	11
19.67	1.500	9.0	80.0	11.0	0.0	7
25.07	1.500	8.0	16.0	60.0	16.0	7
25.74	1.500	8.0	16.0	58.0	18.0	6
27.05	1.500	7.8	0.0	70.9	21.3	6
28.20	1.500	4.0	0.0	36.3	59.7	6
30.08	1.500	4.0	0.0	36.3	59.7	6
30.38	1.500	10.0	0.0	90.0	0.0	6
35.48	1.500	10.0	0.0	90.0	0.0	6

Data Management

Chromatography data system:	Empower™ 3, FR 3.6.1

Results and Discussion

The separation of a wide range of amino acids is challenging as slight changes in temperature, gradient delivery, and other conditions can impact retentivity and selectivity. Given these challenges, differences across liquid chromatography (LC) systems can make direct scaling more challenging and require optimization of the method on a system-by-system basis. For these studies, the method conditions for the separation of amino acids from protein hydrolysate were adapted from the separation on a 2.1 x 100 mm[1] to a 4.6 x 150 mm column. Pre-column derivatization of amino acids was performed using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC),[2] followed by separation of the derivatives with reversed-phase liquid chromatography.

Instrument Considerations

For initial separation scouting, the method obtained from the ACQUITY UPLC H-Class AccQ•Tag Ultra Solution was scaled and tested on the ACQUITY Arc System. Adjustment of the method was required to achieve adequate resolution and chromatographic performance. To assess the final method conditions, both the ACQUITY Arc and Arc HPLC Systems were selected and included to control for variability across systems. Both systems consist of quaternary pumps, flow through sample managers, column heaters with passive preheaters and TUV (tunable wavelength) detectors.

The two systems were selected to understand and control for differences in extra-column volume or bandspread. The major differences across the two systems include tubing internal diameter (ID) from the needle to the column, post column tubing and the flow cell volume. The ACQUITY Arc uses lower dispersion tubing (0.005" ID), a passive preheater and a low dispersion 10 mm flow cell, with extra-column volume measurements of approximately 25–35 µL (4 σ).[3] In contrast, the Arc HPLC System uses wider bore tubing (0.010" ID), a passive preheater and a standard 10 mm flow cell with stainless steel inlet tubing, resulting in extra-column volumes of approximately 45–75 µL (4 σ).[3] The extra-column dispersion or bandspread for each system will be impacted by different column heater options, needle dimensions, tubing dimensions, detectors, and flow cells.

For each system, differences in extra-column dispersion can result in lower resolution of critical pairs and/or

peak distortion of early eluting peaks from strong solvent effects. For example, larger internal diameter (ID) post-column tubing can lead to loss of resolution for critical pairs, while lower ID pre-column tubing can result in peak distortion of early eluting peaks due to strong solvent effects. To assess suitability for a wide range of LC systems, the final method was tested for minimum chromatographic performance, reproducibility, precision, linearity and limit of detection, and quantitation on both LC systems.

Method Optimization of HPLC Amino Acid Separation

While the original method was scaled from the existing UPLC solution, method optimization was required due to the sensitivity of the analysis. Optimization included adjustment of column temperature and injection volume. Injection volumes were assessed because in previous studies slight changes in the system were found to impact to peak shape for histidine.[4] The peak distortion seen in the referenced work was caused by inadequate mixing of the sample with the mobile phase resulting in fronting from strong solvent effects. While several adjustments can be made to minimize the asymmetrical peak shape, adjusting the injection volume has been found to be effective.

For the two systems evaluated, different injection volumes were tested to assess the optimum injection volume for symmetrical peak shape of histidine. The Arc HPLC System, was found to produce significant peak distortion for injections greater than 4 µL. In addition, no difference in limit of detection (LOD) or limit of quantitation (LOQ) was observed for 4 µL injection as compared to 3 µL, thus the lower injection volume was selected to provide good results (Figure 1). The ACQUITY Arc System produced significantly greater peak distortion for histidine above 2 µL, thus for this system a 2 µl injection volume was determined to be acceptable (Figure 2).

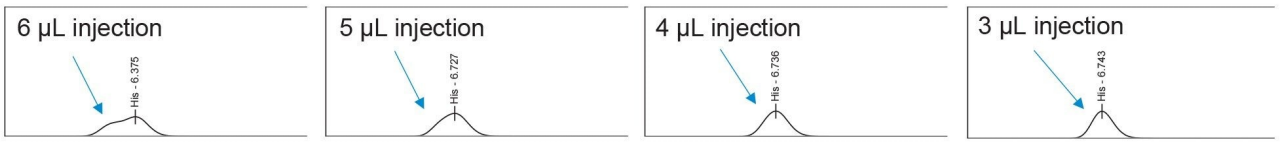

Figure 1. Injection volume determination of the Protein Hydrolysate AA Standard (500 µM) chromatogram on the Arc HPLC.

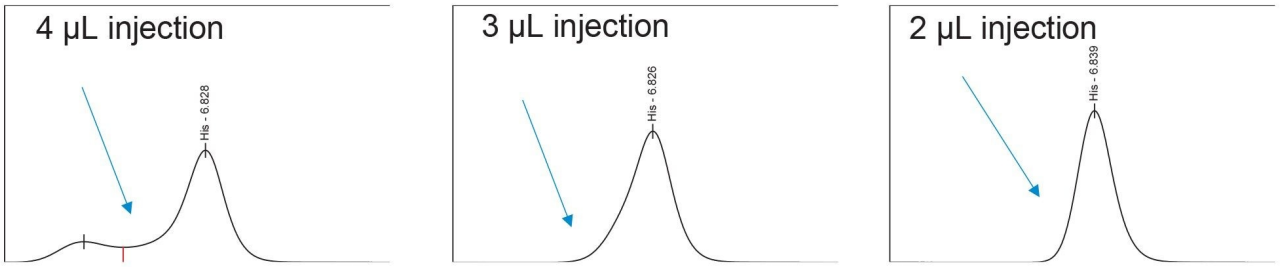

Figure 2. Injection volume determination of the Protein Hydrolysate AA Standard (500 µM) chromatogram on the ACQUITY™ Arc.

After optimization of injection volumes for each system, chromatographic performance was compared (Table 1). Both systems were found to have acceptable USP resolution (>2.0) and peak shape for the hydrolysate amino acids (Figures 3 and 4).

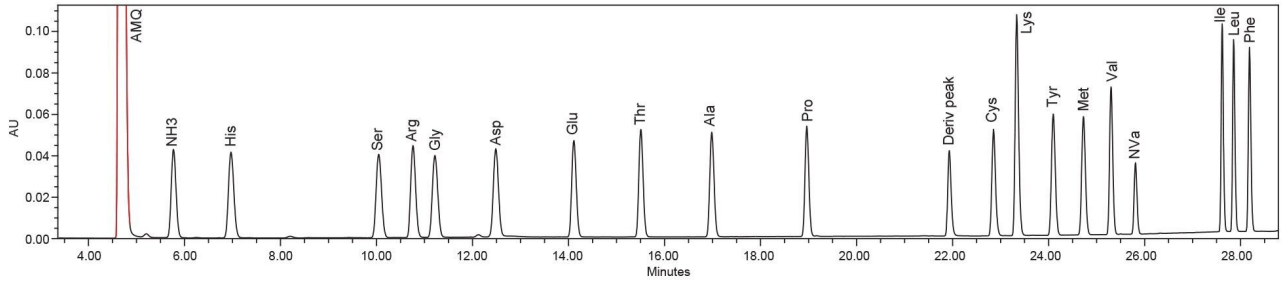

Figure 3. Protein Hydrolysate AA Standard (500 µM) chromatogram using an AccQ•Tag Ultra C_{18} 2.5 µm Column on an Arc HPLC System.

ACQUITY Arc retention time and USP resolution		
Compound	Retention time	USP resolution
His	6.97	6.96
Ser	10.15	17.42
Arg	10.83	3.59
Gly	11.33	2.93
Asp	12.67	7.46
Glu	14.36	10.08
Thr	15.77	9.11
Ala	17.29	9.99
Pro	19.34	14.24
Cys	23.62	8.92
Lys	23.96	3.18
Tyr	24.61	6.12
Met	25.10	4.31
Val	25.57	4.60
Ile	27.62	20.22
Leu	27.85	2.99
Phe	28.18	4.10

Arc HPLC retention time and USP resolution		
Compound	Retention time	USP resolution
His	6.67	6.19
Ser	9.75	15.65
Arg	10.39	3.38
Gly	10.89	2.70
Asp	12.17	6.62
Glu	13.78	9.18
Thr	15.16	8.57
Ala	16.63	9.42
Pro	18.59	13.31
Cys	22.3	6.46
Lys	22.76	3.53
Tyr	23.62	5.89
Met	24.29	4.41
Val	24.93	4.61
Ile	27.42	19.63
Leu	27.66	2.86
Phe	27.99	3.69

Table 1. Hydrolysate Standard (500 µM) retention time and UPS resolution results on an AccQ•Tag Ultra C_{18} 2.5 µm Column on both the ACQUITY Arc HPLC and the Arc HPLC Systems.

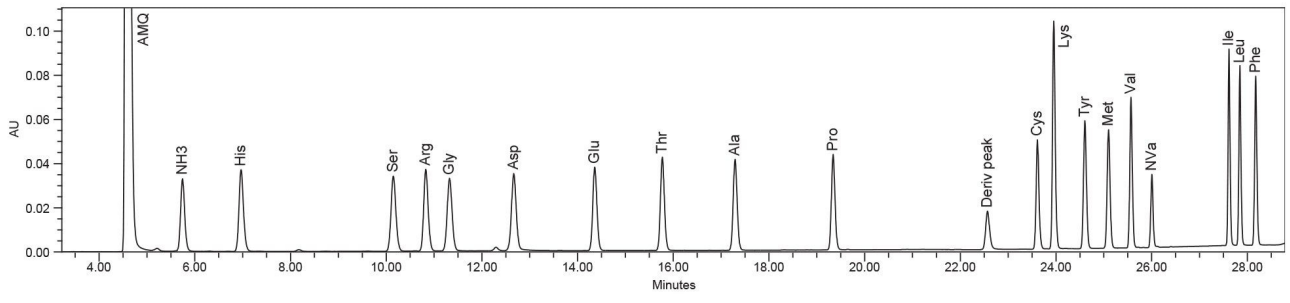

Figure 4. Protein Hydrolysate AA Standard (500 µM) using an AccQ•Tag Ultra C_{18} 2.5 µm Column on an ACQUITY Arc System.

Verification of Amino Acid Analysis Using AccQ•Tag Ultra C_{18} 2.5 µm Column on Arc HPLC

and ACQUITY Arc Systems

After method adjustments were made, each system was tested using the protein AA hydrolysate standard to ensure adequate repeatability for both retention time and peak area. While retention time repeatability is a measure of the ability of the pump to reproducibly deliver the gradient, area repeatability reflects the precision of the injector. Both systems were tested using replicate injections of the same sample at the pre-identified injection volumes discussed above. For both systems, the retention time repeatability showed standard deviations of less than 0.005 minutes or 0.3 seconds, demonstrating the high reproducibility of the quaternary pump performance. Given the similarities in each pump the comparable results were expected.

The area precision for each system also produced comparable results, despite difference in injection volume. The Arc HPLC System, tested at 3 µL injection volume, produced area RSDs from 0.2% to 0.6% for all the hydrolysate amino acids. For the ACQUITY Arc System, which was tested at 2 µL the area RSDs were 0.1% to 0.3% for the hydrolysates. Both systems demonstrated good area repeatability. Results for both retention time and peak area reproducibility are shown in Tables 2 and 3.

AccQ•Tag Ultra™ C_{18}, 2.5 µm 4.6 × 150 mm Column RT repeatability 50 pmol (n=6)				AccQ•Tag Ultra™ C_{18}, 2.5 µm 4.6 × 150 mm Column area repeatability 50 pmol (n=6)			
Compound	RT	STDEV	%RSD	Compound	Area	STDEV	%RSD
His	6.591	0.005	0.1	His	297275	497	0.2
Ser	9.803	0.005	0.1	Ser	292694	649	0.2
Arg	10.438	0.005	0	Arg	296522	449	0.2
Gly	10.974	0.004	0	Gly	288133	595	0.2
Asp	12.364	0.003	0	Asp	288095	1819	0.6
Glu	14.036	0.003	0	Glu	279484	1131	0.4
Thr	15.444	0.003	0	Thr	297706	653	0.2
Ala	16.959	0.003	0	Ala	297846	1380	0.5
Pro	18.971	0.003	0	Pro	272782	846	0.3
Cys	23.219	0.004	0	Cys	253242	189	0.1
Lys	23.596	0.004	0	Lys	483176	2528	0.5
Tyr	24.324	0.004	0	Tyr	303705	498	0.2
Met	24.854	0.004	0	Met	291717	99	0
Val	25.379	0.004	0	Val	299546	996	0.3
Ile	27.537	0.004	0	Ile	303121	984	0.3
Leu	27.764	0.004	0	Leu	297853	986	0.3
Phe	28.075	0.004	0	Phe	302390	513	0.2

Table 2. Protein Hydrolysate AA Standard (500 µM) retention time and area repeatability results using a AccQ•Tag Ultra C_{18} 2.5 µm Column on an Arc HPLC System.

AccQ•Tag Ultra™ C_{18}, 2.5 µm 4.6 × 150 mm Column RT repeatability 50 pmol (n=6)				AccQ•Tag Ultra™ C_{18}, 2.5 µm 4.6 × 150 mm Column area repeatability 50 pmol (n=6)			
Compound	RT	STDEV	%RSD	Compound	Area	STDEV	%RSD
His	6.743	0.002	0.03	His	295476	481	0.2
Ser	9.951	0.001	0.01	Ser	286932	532	0.2
Arg	10.593	0.001	0.01	Arg	287057	479	0.2
Gly	11.119	0.001	0.01	Gly	277464	488	0.2
Asp	12.484	0.001	0.01	Asp	272035	377	0.1
Glu	14.185	0.001	0.01	Glu	268891	557	0.2
Thr	15.601	0.001	0.01	Thr	292470	580	0.2
Ala	17.118	0.002	0.01	Ala	288072	493	0.2
Pro	19.177	0.002	0.01	Pro	266489	469	0.2
Cys	23.464	0.002	0.01	Cys	254701	483	0.2
Lys	23.8	0.002	0.01	Lys	478242	586	0.1
Tyr	24.466	0.002	0.01	Tyr	302908	554	0.2
Met	24.962	0.002	0.01	Met	279338	800	0.3
Val	25.449	0.002	0.01	Val	295199	602	0.2
Ile	27.537	0.001	0	Ile	299435	593	0.2
Leu	27.76	0.001	0	Leu	294579	418	0.1
Phe	28.085	0.001	0	Phe	298659	613	0.2

Table 3. Protein Hydrolysate AA Standard (500 µM) retention time and area repeatability results using a AccQ•Tag Ultra C_{18} 2.5 µm Column on an ACQUITY Arc System.

Intra-Day and Inter-Day Precision for Retention Time and Area RSD

In addition to repeatability, intra-day and inter-day precision are essential for analysis and quantitation of amino acids. Both the ACQUITY Arc and Arc HPLC, were tested over the course of three days with six samples tested per day. An internal standard was used to normalize for differences in sample preparation. The analysis evaluated the intra-day precision for six injections and the aggregate precision across all three days for six injections.

The results on both systems demonstrated high intra-day retention time precision and area precision. All retention times were within 0.35% RSD over the course of three days, with a precision that is well within the expected retention time range for each system. The peak area %RSD were within 0.5% within one day and 0.6% across all three days. The results demonstrate the reproducibility of the analysis on both the ACQUITY Arc and Arc HPLC Systems.

RT %RSD								
ACQUITY Arc					Arc HPLC			
Compound	Day 1	Day 2	Day 3	Interday	Day 1	Day 2	Day 3	Interday
His	0.00	0.10	0.00	0.03	0.10	0.80	0.10	0.33
Ser	0.00	0.00	0.00	0.00	0.10	0.30	0.00	0.13
Arg	0.00	0.00	0.00	0.00	0.00	0.30	0.00	0.10
Gly	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.07
Asp	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.07
Glu	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.03
Thr	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.03
Ala	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.03
Pro	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.03
Cys	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.07
Lys	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.07
Tyr	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.07
Met	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.03
Val	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.03
Ile	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Leu	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Phe	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Area %RSD								
ACQUITY Arc					Arc HPLC			
Compound	Day 1	Day 2	Day 3	Interday	Day 1	Day 2	Day 3	Interday
His	0.20	0.20	0.30	0.23	0.20	0.20	0.20	0.20
Ser	0.20	0.20	0.30	0.23	0.20	0.30	0.40	0.30
Arg	0.20	0.20	0.30	0.23	0.10	0.20	0.20	0.17
Gly	0.20	0.20	0.30	0.23	0.20	0.30	0.30	0.27
Asp	0.10	0.10	0.40	0.20	0.50	0.40	0.70	0.53
Glu	0.20	0.10	0.30	0.20	0.40	0.30	0.60	0.43
Thr	0.20	0.10	0.30	0.20	0.20	0.30	0.30	0.27
Ala	0.20	0.10	0.30	0.20	0.40	0.40	0.70	0.50
Pro	0.20	0.10	0.30	0.20	0.30	0.30	0.50	0.37
Cys	0.20	0.20	0.30	0.23	0.10	0.30	0.10	0.17
Lys	0.10	0.10	0.30	0.17	0.60	0.40	0.80	0.60
Tyr	0.20	0.10	0.30	0.20	0.20	0.30	0.20	0.23
Met	0.30	0.20	0.30	0.27	0.00	0.20	0.20	0.13
Val	0.20	0.10	0.30	0.20	0.30	0.30	0.50	0.37
Ile	0.20	0.20	0.30	0.23	0.30	0.30	0.50	0.37
Leu	0.10	0.20	0.30	0.20	0.30	0.40	0.50	0.40
Phe	0.20	0.10	0.30	0.20	0.20	0.30	0.20	0.23

Table 4. Inter-day retention time and area reproducibility using a AccQ•Tag Ultra C_{18} 2.5 µm Column on Arc HPLC and ACQUITY Arc Systems.

Linearity and Limit of Detection/Limit of Quantitation

To ensure accurate quantitation of all the amino acids, linearity as well as LOD and LOQ were evaluated for both the ACQUITY Arc System (2 µL injection) and the Arc HPLC System (3 µL injection). Linearity was determined by injecting eight concentration levels of hydrolysate standard ranging from 1 µM to 500 µM. The calibration curves for both instruments resulted in acceptable R^2 of > 0.995 for all amino acids. Given the distribution of standards across the dynamic range, weighting of 1/x was used for the calibration curve. LOQ, as defined by signal-to-noise equal to or greater than 10:1, was 1 µM for both systems. All values above the LOQ exhibited deviations of < 5% and deviations of <25% at the LOQ. Despite the differences in injection volume, the LOD was determined to be 0.5 µM based on a signal to noise ratio of 3:1 for both instruments. Although the ACQUITY Arc used a lower injection volume as compared to the Arc HPLC System, the LOQ and LOD were maintained due to the lower extra-column dispersion or bandspread of the ACQUITY Arc System, which resulted in narrower peaks as compared to the Arc HPLC System.

ACQUITY Arc										Arc HPLC									
% Deviation @ concentration level (µM)										% Deviation @ concentration level (µM)									
Compound	R^2	1	5	10	20	50	100	200	500	Compound	R^2	1	5	10	20	50	100	200	500
His	0.9999	10.11	-4.19	-2.26	-2.21	-1.21	-0.41	-0.32	0.48	His	0.9995	24.95	-8.47	-7.48	-6.17	-2.55	-0.65	-0.75	1.12
Ser	0.9999	-5.97	5.31	2.00	-0.92	-0.40	0.34	-0.47	0.12	Ser	0.9999	7.65	-0.82	-2.92	-1.76	-2.18	-0.01	-0.52	0.55
Arg	0.9999	-0.53	2.18	3.21	-2.43	-1.44	-0.99	-0.60	0.59	Arg	0.9998	0.83	7.43	-3.11	-1.85	-2.98	-0.43	-0.57	0.67
Gly	0.9998	15.12	-6.65	-3.34	-2.66	-1.86	-0.76	-0.65	0.81	Gly	0.9999	-3.31	4.21	-0.96	0.86	-0.46	0.36	-1.05	0.34
Asp	0.9979	-0.10	-24.81	3.94	24.25	-4.44	0.99	1.21	-1.04	Asp	0.9997	4.84	-3.1	-4.67	-0.87	0.58	3.17	1	-0.94
Glu	0.9998	-2.62	-1.21	1.45	-0.29	0.70	1.70	1.15	-0.87	Glu	0.9999	-4.11	2.23	-1.08	0.87	0.63	1.65	0.38	-0.57
Thr	0.9999	-0.15	0.14	-0.12	0.66	-0.36	-0.10	-0.16	0.09	Thr	0.9998	-6.3	10.76	-0.94	-1.89	-1.55	0.15	-0.6	0.36
Ala	0.9999	-5.41	1.48	2.76	0.84	-0.13	0.67	0.01	-0.22	Ala	0.9999	-6.06	4.1	0.23	1.24	-0.22	1.1	-0.18	-0.21
Pro	0.9999	-3.13	1.77	2.19	-0.06	-0.68	0.22	-0.47	0.16	Pro	0.9999	4.16	-0.75	-0.97	-1.45	-1.27	0.41	-0.42	0.29
Cys	0.9999	9.21	-4.01	-0.98	-1.89	-1.80	-0.62	-0.57	0.65	Cys	0.9999	5.7	0.05	-1.99	-1.05	-2.23	-0.36	-0.8	0.69
Lys	0.9999	4.37	-0.64	-0.41	-1.29	-1.76	-0.61	0.00	0.36	Lys	0.9999	1.55	-0.19	-1.78	-0.25	-0.26	0.98	0.14	-0.18
Tyr	0.9999	-0.13	-0.09	1.42	-0.51	-0.34	-0.10	-0.49	0.24	Tyr	0.9999	1.35	-0.48	1.29	-0.31	-1.38	-0.08	-0.91	0.51
Met	0.9999	7.74	-3.12	-0.33	-1.86	-1.78	-0.68	-0.63	0.66	Met	0.9998	-0.87	8.15	-2.06	-1.6	-2.69	-0.66	-1.17	0.89
Val	0.9999	0.33	-1.78	1.97	0.02	-0.30	0.06	-0.49	0.19	Val	0.9999	5.4	-1.42	-2.13	-0.94	-1.32	0.54	-0.41	0.27
Ile	0.9999	2.58	-3.05	1.74	-0.63	-0.78	0.39	-0.44	0.19	Ile	0.9999	6.97	-0.68	-1.8	-3.22	-1.87	0.7	0.5	-0.41
Leu	0.9999	-1.83	0.08	2.77	-0.17	-0.78	0.26	-0.50	0.18	Leu	0.9999	8.02	-2.67	-2.89	-1.59	-1.23	0.51	-0.49	0.35
Phe	0.9999	-1.96	-0.86	2.87	-0.30	0.34	0.39	-0.54	0.07	Phe	0.9999	4.53	-0.36	-1.76	-0.68	-1.52	0.01	-0.72	0.5

Table 5. Protein Hydrolysate AA Standard (1–500 µM) R^2 and % deviation at concentration level linearity results using a AccQ•Tag Ultra C_{18} 2.5 µm Column on ACQUITY Arc and Arc HPLC Systems.

Impact of AccQ•Tag Ultra™ C_{18} 2.5 µm VanGuard Cartridge on Chromatographic Performance

While analysis of amino acids is challenging, complex sample matrices of live samples can add an additional level of complexity. To preserve the life of the AccQ•Tag Ultra C_{18} 2.5 µm analytical use of the AccQ•Tag Ultra C_{18} 2.5 µm VanGuard Cartridge is recommended. However, it is essential to ensure the chromatographic separation is not adversely impacted by the use of a guard device.

To assess the impact of a guard device, the AccQ•Tag Ultra C_{18}, 2.5µm 4.6 x 150 mm Column was tested with and without a VanGuard Cartridge. The VanGuard Cartridge is designed to protect the column while preserving chromatographic performance. The testing was conducted on the ACQUITY Arc System to probe for the impact on resolution and peak tailing. The results show that there was no significant loss of resolution with the VanGuard Cartridge and for some critical pairs there was an improvement in resolution (Leu/Ile). Furthermore, all USP resolution values were baseline resolved (>2.0) ensuring reproducible quantitation. Additional analysis revealed that USP tailing and peak width values were all comparable, indicating no adverse effects from the addition of the guard Cartridge (Figure 5). Numerical data is shown in Table 6.

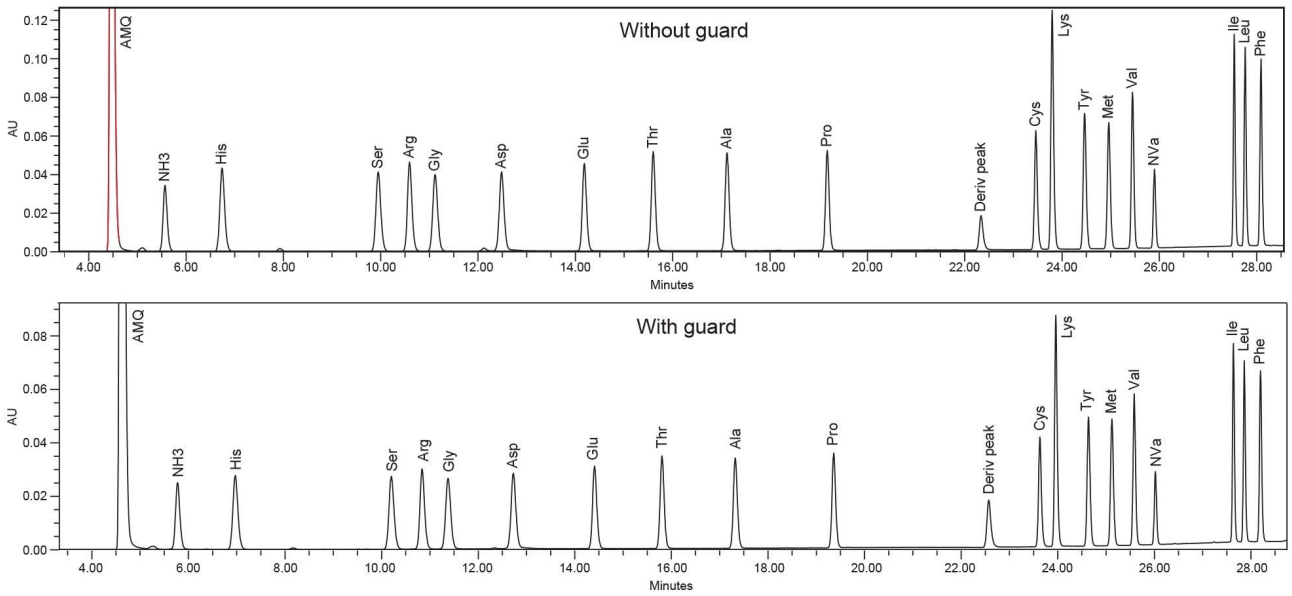

Figure 5. Analysis of Protein Hydrolysate AA Standard on the AccQ•Tag Ultra C_{18}, 2.5 µm 4.6 x 150 mm Column with (top) and without AccQ•Tag Ultra C_{18}, 2.5 µm VanGuard Cartridge 3.9 x 5 mm (bottom) installed on a ACQUITY Arc with 2489 Detector.

Without guard				With guard			% Difference with guard without guard
Compound	USP resolution	USP tailing	Width at 4.4%	USP resolution	USP railing	Width at 4.4%	USP resolution
His	6.96	1.11	0.24	6.70	1.10	0.25	3.8%
Ser	17.42	1.11	0.24	16.77	1.11	0.25	3.7%
Arg	3.59	1.14	0.22	3.45	1.13	0.23	3.9%
Gly	2.93	1.09	0.24	2.91	1.08	0.25	0.6%
Asp	7.46	1.07	0.23	7.18	1.08	0.24	3.8%
Glu	10.08	1.10	0.20	9.75	1.11	0.21	3.4%
Thr	9.11	1.10	0.20	8.78	1.10	0.2	3.6%
Ala	9.99	1.09	0.19	9.77	1.09	0.2	2.2%
Pro	14.24	1.10	0.18	13.87	1.10	0.18	2.6%
Cys	8.92	1.15	0.15	8.23	1.15	0.15	7.7%
Lys	3.18	1.18	0.14	3.19	1.17	0.14	-0.2%
Tyr	6.12	1.11	0.15	6.40	1.11	0.15	-4.5%
Met	4.31	1.09	0.15	4.32	1.09	0.15	-0.1%
Val	4.60	1.09	0.13	4.43	1.11	0.13	3.6%
Ile	20.22	1.12	0.10	20.07	1.12	0.1	0.8%
Leu	2.99	1.13	0.10	3.01	1.13	0.1	-0.7%
Phe	4.10	1.13	0.11	4.17	1.13	0.11	-1.6%

Table 6. Analysis of Protein Hydrolysate AA Standard on the AccQ•Tag Ultra C_{18}, 2.5 µm 4.6 x 150 mm Column for USP Resolution, USP Tailing and Width @4.4% with and without AccQ•Tag Ultra C_{18}, 2.5 µm VanGuard Cartridge 3.9 x 5 mm.

AccQ•Tag Ultra™ C_{18}, 2.5 µm Column- to-Column and Batch-to-Batch Reproducibility

In addition to evaluating the performance of the ACQUITY Arc and Arc HPLC systems, column-to-column and batch-to-batch variability was assessed. For column-to-column reproducibility two columns of the same lot were tested on the ACQUITY Arc System using the same mobile phases. Each column was tested with six injections, and the average values were reported (Table 7). While there was some variation in retention time, all values were within expected retention time windows and unequivocally identifiable between the two columns. Retention time standard deviations were all within 0.29 min or 30 seconds for both columns as well indicative of good chromatographic performance.

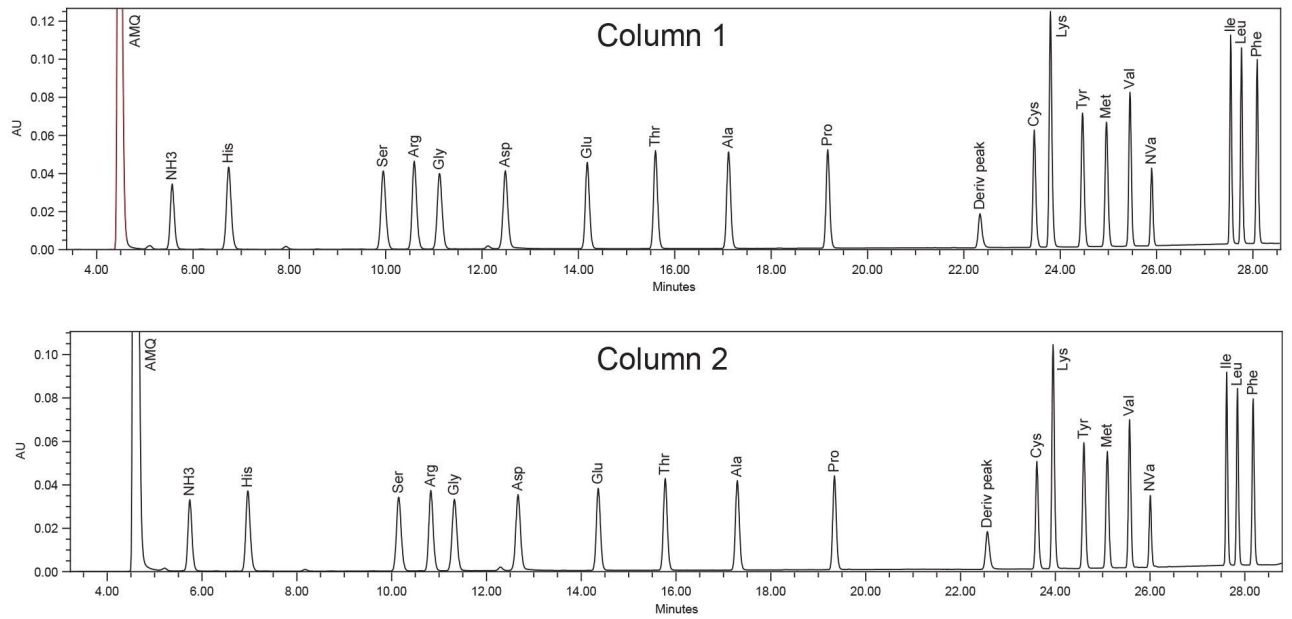

Figure 6. Analysis of Protein Hydrolysate AA Standard on the AccQ•Tag Ultra C_{18}, 2.5 µm 4.6 x 150 mm Column intra-batch reproducibility on ACQUITY Arc System.

AccQ•Tag Ultra™ C_{18}, 2.5 µm 4.6 × 150 mm Column batch to batch retention time reproducibility				
Compound	**Packing 1**	**Packing 2**	**Average**	**STDEV**
His	7.37	6.97	7.17	0.29
Ser	10.47	10.15	10.31	0.22
Arg	11.25	10.83	11.04	0.30
Gly	11.65	11.33	11.49	0.23
Asp	12.93	12.67	12.80	0.19
Glu	14.61	14.36	14.48	0.17
Thr	16.03	15.77	15.90	0.18
Ala	17.55	17.29	17.42	0.18
Pro	19.60	19.34	19.47	0.18
Cys	23.78	23.62	23.70	0.11
Lys	24.16	23.96	24.06	0.15
Tyr	24.76	24.61	24.68	0.11
Met	25.27	25.10	25.19	0.12
Val	25.73	25.57	25.65	0.12
Ile	27.76	27.62	27.69	0.10
Leu	28.00	27.85	27.92	0.11
Phe	28.34	28.18	28.26	0.11

Table 7. Analysis of Protein Hydrolysate AA Standard on the AccQ•Tag Ultra C_{18}, 2.5 µm 4.6 x 150 mm Column Batch-to-Batch retention time reproducibility on the ACQUITY Arc.

While column-to-column variability is essential, testing across three different batches of packing material is recommended to ensure robustness of the method. For this testing, three AccQ•Tag Ultra C_{18}, 2.5 µm 4.6 x 150 mm columns were tested on an Arc HPLC System with each column containing a different batch of packing material. As with the column-to-column testing the same batch of mobile phase and samples were used to minimize potential variability. The results show good reproducibility with no significant changes in resolution across the three batches tested. All the amino acids were within expected retention time values, with similar separation between the peaks. These results demonstrated the robustness of the method across different lots of packing material (Figure 7 and Table 8).

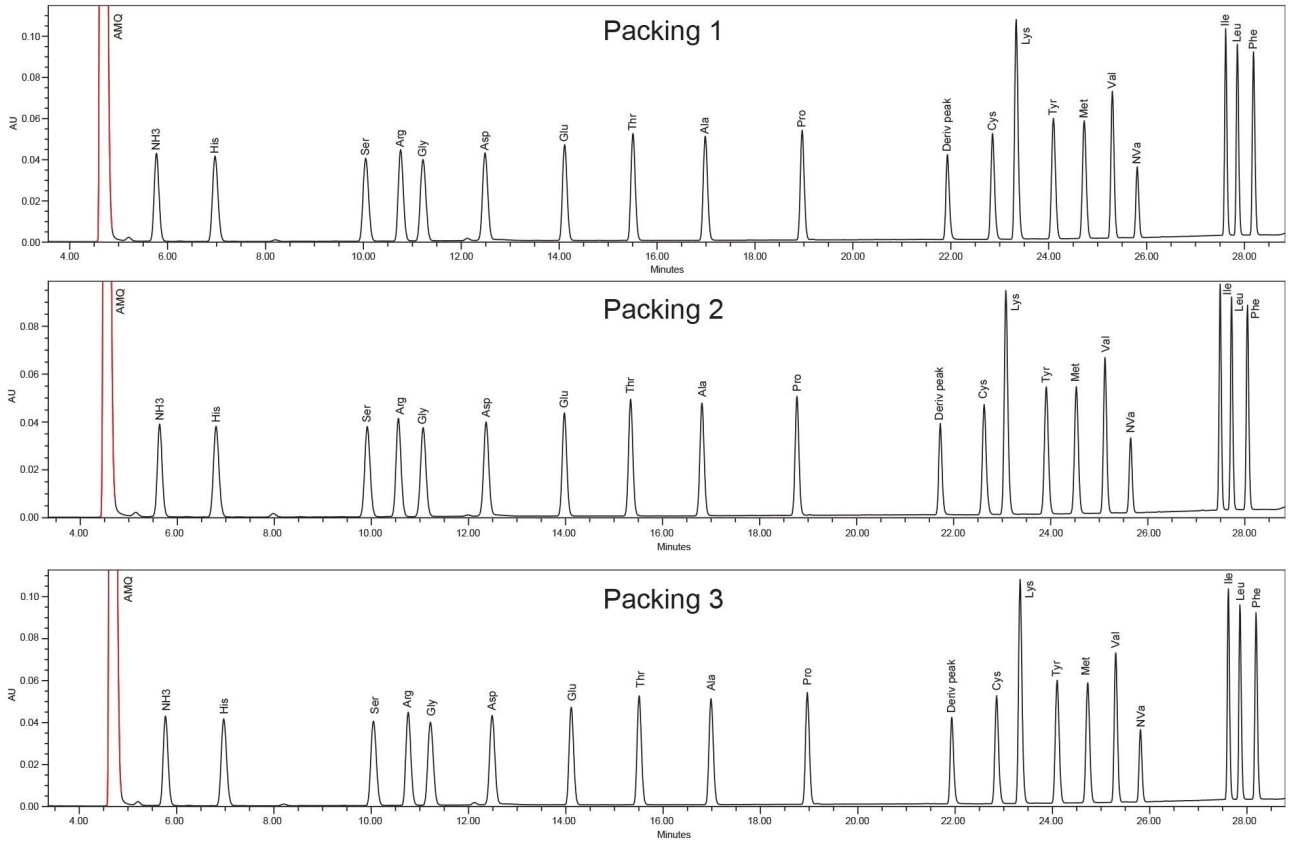

Figure 7. Analysis of Protein Hydrolysate standard on the AccQ•Tag Ultra C_{18}, 2.5 µm 4.6 x 150 mm Column batch-to-batch reproducibility.

AccQ•Tag Ultra™ C_{18}, 2.5 µm 4.6 × 150 mm Column batch to batch retention time reproducibility						
Compound	**Packing 1**	**Packing 2**	**Packing 3**	**Average**	**STDEV**	**%RSD**
His	6.59	6.97	6.67	6.75	0.20	2.98
Ser	9.80	10.05	9.75	9.87	0.16	1.64
Arg	10.44	10.76	10.39	10.53	0.20	1.94
Gly	10.97	11.22	10.89	11.03	0.17	1.54
Asp	12.36	12.49	12.17	12.34	0.16	1.31
Glu	14.04	14.11	13.78	13.98	0.17	1.24
Thr	15.44	15.51	15.16	15.37	0.19	1.21
Ala	16.96	16.98	16.63	16.86	0.20	1.18
Pro	18.97	18.96	18.59	18.84	0.22	1.17
Cys	23.22	22.86	22.30	22.79	0.46	2.04
Lys	23.60	24.10	22.76	23.49	0.67	2.87
Tyr	24.32	24.73	23.62	24.22	0.56	2.32
Met	24.85	25.30	24.29	24.81	0.51	2.04
Val	25.38	25.81	24.93	25.37	0.44	1.74
Ile	27.54	27.62	27.42	27.53	0.10	0.35
Leu	27.76	27.86	27.66	27.76	0.10	0.35
Phe	28.08	28.19	27.99	28.08	0.10	0.35
					Average	1.54

Table 8. Analysis of Protein Hydrolysate standard on the AccQ•Tag Ultra C_{18}, 2.5 µm 4.6 x 150 mm Column batch-to-batch retention time reproducibility on the Arc HPLC.

Conclusion

Separation of amino acids can be challenging, with system characteristics impacting method performance. While UHPLC/HPLC systems are generally adequate for many analyses, given the requirements of the separation for amino acids, it is important to assess the impact of each system's extra-column volume or bandspread on the separation of amino acids. Differences in extra-column dispersion can result in either lower resolution from larger bore post-column tubing or peak distortion due to lower bore tubing pre-column.

In this work, a robust, precise, and efficient method for amino acid analysis was successfully developed and optimized for use on the ACQUITY Arc and Arc HPLC Systems using the AccQ•Tag Ultra C_{18}, 2.5 µm, 4.6 x 150 mm Column. Injection volumes were optimized for each system to ensure the required sensitivity was achieved while minimizing any strong solvent effect.

To assess suitability for a wide range of LC systems, the final method was tested for minimum chromatographic performance, reproducibility, precision, linearity, and limits of detection and quantitation. The results demonstrate the ability to perform the separation of hydrolysate amino acids on the Arc HPLC and ACQUITY Arc Systems with the using the AccQ•Tag Ultra C_{18}, 2.5 µm, 4.6 x 150 mm Column.

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