Analysis of Underivatized Essential Amino Acids by HILIC Separation using the HALO® Penta-hydroxy based Column and Single Quadrupole Mass Detection

GOAL

- To develop a sensitive and reproducible non-derivatization method for the analysis of 20 standard amino acids
- Determine LOD and LOQ for each amino acid
- Evaluate column and method robustness by analyzing 50 injections of a cell lysate sample

APPLICATION BENEFITS

- Simple sample preparation without derivatization procedure
- Method is reproducible and sensitive enough to identify the 20 standard amino acids
- Mass detection facilitates the identification of amino acids not fully resolved by liquid chromatography

INTRODUCTION

Amino acid analysis (AAA) is an important technique for determining the quantity of amino acids in a sample. In the biopharmaceutical industry, amino acid analysis has become an important tool in quantifying amino acid content in both feed and spent media during upstream fermentation, enabling efficient monitoring of the cell-culture process.

Despite its prevalence, underivatized analysis of amino acids by liquid chromatography still remains a challenge. The lack of chromophore or fluorophore present on the majority of amino acids renders detection by UV difficult. As a result, chemical derivatization, often through pre- or post-column reaction with o-phthalaldehyde (OPA) or fluorenylmethyloxycarbonyl (FMOC) is required to enable detection, chromatographic separation, and quantification of the amino acids. However, chemical derivatization often requires long chromatographic run times limiting sample throughput, a lack of analyte specificity, and the inability to distinguish between chemical isotopes. Additionally, these derivatization reagents are considered toxic, exhibit limited stability, yield byproduct interference, and at times demonstrate limited reactivity toward secondary amino acids[5]. Therefore, methods that enable for underivatized amino acids in a fast, robust, and reproducible manner are highly desirable.

Hydrophilic interaction chromatography (HILIC) presents a solution to avoid the challenges highlighted above and enable underivatized amino acid analysis. In HILIC chromatography, the separation of an analyte is based on its partitioning between a high organic containing mobile phase and a water enriched layer adsorbed onto the hydrophilic stationary phase. The more hydrophilic the analyte, the more the partitioning equilibrium shifts toward the analyte being retained in the immobilized water layer. Therefore, longer retention times for an analyte typically correlates with increased hydrophilicity of the solutes[3,4]. By utilizing volatile organic solvents and incorporating low concentrations of volatile salts in both the initial high concentration acetonitrile mobile phase and the aqueous elution buffer, the separation method can be effectively integrated with mass spectrometry instruments. This integration enables the accurate and sensitive quantification of compounds in complex samples.

This work describes the use of the HALO® penta-hydroxy based column (Advanced Materials Technology) for the HILIC separation of underivatized amino acids. The column is a bonded silica phase which contains a highly polar ligand with five hydroxyl groups tethered via novel proprietary linkage chemistry to Fused-Core® silica particles. The Fused-Core® particles provide higher efficiencies with improved resolution and lower detection limits, lower back pressure, as well as excellent reproducibility and lot-to-lot robustness. To demonstrate the quantitative application, the HILIC separation method is coupled to a single quadrupole (QDa) mass spectrometer for mass detection analysis.

The main objective of this work is to evaluate the HALO® penta-hydroxy based column for HILIC separation coupled to the QDa Mass Spectrometer for mass identification of common amino acids. In this work, a sensitive and reproducible non-derivatization method for the quantification of 20 essential amino acids was developed.

MATERIALS

- Although Pierce™ Amino Acid Standard H (Thermo Scientific™, PN: 20088)
- Formic acid, LC-MS grade, CAS No. 64-18-6 (Thermo Scientific™, PN: 28905)
- Hydrochloric acid 36.5 – 38.0%, CAS No. 7647-01-0 (JT Baker, PN: 9535-00)
- L-Cysteine 97.0%, CAS No. 52-90-4 (Sigma, PN: 168149-25G)
- L-Tryptophan ≥98.0%, CAS No. 73-22-3 (Sigma, PN: T0254-5G)
- L-Asparagine ≥98.0%, CAS No. 70-47-3 (Sigma, PN: A0884-25G)
- L-Glutamine 99.0-101.0%, CAS No. 56-85-9 (Sigma, PN: G8540-25G)
- L-Isoleucine, 99%, CAS No. 73-32-5 (Acros Organics, PN: 166170050)
- Ammonium formate crystals ≥99.0%, CAS No. 540-69-2 (Sigma, PN: 70221)
- Acetonitrile, LC-MS grade, CAS No. 75-05-8 (Fisher Scientific™, PN: AA47138K7)
- Milli-Q filtered water (in-house, Pelican Expression Technology®, Ligand Pharmaceuticals Inc.)
- *Pseudomonas fluorescens* cell lysate (Pelican Expression Technology®, Ligand Pharmaceuticals Inc.)

INSTRUMENTATION

Waters ACQUITY UPLC H-Class PLUS System which includes:

- Sample Manager FTN
- Binary Solvent Manager
- Column compartment
- TUV Optical Detector
- Single Quadrupole Mass Detector

MOBILE PHASE PREPARATION

Mobile phase A consisted of 0.15% formic acid in acetonitrile and 10 mM ammonium formate buffer (85:15 v/v). Mobile phase B consisted of 0.15% formic acid in 10 mM ammonium formate buffer at pH 3.0. Ammonium formate stock solution was prepared at 1M concentration. For mobile phase A, 10 mL of ammonium formate stock solution and 1.5 mL of formic acid were added into 100 mL of water, followed by 850 mL of acetonitrile, and QS to 1L final volume with water. The prepared mobile phase A was placed on top of a stir plate to slowly mix with low heat to ensure constant miscibility. For mobile phase B, 10 mL of ammonium formate stock solution and 1.5 mL of formic acid were added into water, followed by QS to 1L final volume with water. The final buffer concentration in each mobile phase was 10 mM ammonium formate.

SAMPLE PREPARATION

The amino acid mixture was prepared using the Pierce™ Amino Acid Standard H containing a mixture of seventeen amino acids (Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val, and Cystine) and four additional amino acids (Asn, Cys, Gln, Trp) from Sigma-Aldrich to make up the 20 standard amino acids at a uniform 1 mM concentration in solution. Further preparation of the standard solution down to 0.1 µM concentration was done by serial dilution to determine the LOD and LOQ of each amino acid.

The concentration of each amino acid in Standard H was 2.5 mM in 0.1N HCl – with the exception of Cystine at 1.25 mM . The four additional amino acids were dissolved in 0.1N HCl and prepared to 10 mM initial concentration. The combined Standard H and four additional amino acids standard solution was prepared to a final concentration of 1 mM (refer to Table 1) – with the exception of Cystine which became 0.5 mM final concentration.

For assay robustness determination, the cell culture media was carefully aspirated and aliquoted for analysis following collection and centrifugation of a fermentation sample. The remaining pellet was reconstituted with 1x PBS solution and glass microbeads were added, followed by vortexing for 30 seconds to lyse the cells. The pellet sample was centrifuged to separate cell debris from the solution, and finally the cell pellet supernatant was aliquoted for analysis.

Solution	Initial Conc. (mM)	Final Conc. (mM)	Initial Sample Volume (µL)	Combined Total Volume (µL)
PIERCE™ AMINO ACID STANDARD H	2.5	1	160	400
L-CYSTEINE	10	1	10	
L-GLUTAMINE	10	1	10	
L-ASPARAGINE	10	1	10	
L-TRYPTOPHAN	10	1	10	

Table 1: Preparation of standard solution of 20 essential amino acids. Consisting of Standard H and four additional amino acids to 1 mM final concentration using 0.1 N HCl as diluent.

CHROMATOGRAPHIC CONDITIONS

Column: Advanced Materials Technology HALO® 90 Å Glycan, 2.1 x 100 mm, 2.7 µm
Part Number: 92812-605
Mobile Phase A: 0.15% formic acid, 10 mM ammonium formate in 85% acetonitrile
Mobile Phase B: 0.15% formic acid, 10 mM ammonium formate in water, pH 3.0
Flow Rate: 0.4 mL/min
Column Temperature: 30 °C
Sample Manager Temperature: 5 ± 3 °C
Injection Volume: 1 µL
Column Pressure Limit: 9990 psi or 689 bar

Gradient:

Time (min)	% MP-B
0.0	0.0
10.0	5.0
15.0	37.5
15.5	95.0
20.0	95.0
20.5	0.0
25.0	0.0

Mass Detector Settings

Ionization Mode: ESI
Polarity: Positive
Full Scan: *m/z* 50 – 1000
Selected Ion Recording: SIR masses are listed in Table 1
Cone Voltage: 15 V
Gas Flow Pressure: 70 – 100 psi

Table 2: List of 20 essential amino acids for analysis of the method. Selected Ion Recording (SIR) mass [M+H$^+$] for positive ion mode.

Amino Acid	Acronym	Monoisotopic mass[ref 2] [M]	SIR mass[ref 6] [M+H$^+$]
Alanine	Ala, A	89.1	90
Arginine	Arg, R	174.2	175
Asparagine	Asn, N	132.1	133
Aspartic acid	Asp, D	133.1	134
Cysteine	Cys, C	121.2	122
Cystine [ref 7]	n/a	240.3 [ref 7]	241
Glutamic acid	Glu, E	147.1	148
Glutamine	Gln, Q	146.2	147
Glycine	Gly, G	75.1	76
Histidine	His, H	155.2	156
Isoleucine	Ile, I	131.2	132
Leucine	Leu, L	131.2	132
Lysine	Lys, K	146.2	147
Methionine	Met, M	149.2	150
Phenylalanine	Phe, F	165.2	166
Proline	Pro, P	115.1	116
Serine	Ser, S	105.1	106
Threonine	Thr, T	119.1	120
Tryptophan	Trp, W	204.2	205
Tyrosine	Tyr, Y	181.2	182
Valine	Val, V	117.1	118

RESULTS

❖ Optimization of mobile phase salt composition, pH, and method flow rate

The buffer salt content in the mobile phase is a crucial parameter in HILIC methods as it influences peak shape, analyte retention, and secondary interactions. Volatile salts such as ammonium acetate or ammonium formate, are considered suitable for mass detection. Three concentrations of ammonium formate buffer at 5, 10 and 20 mM were tested. Figure 1 shows a selection of 9 amino acids – Ala, Pro, Leu, Ile, Gln, Glu, Arg, Tyr and Trp – that were analyzed for the effects of mobile phase salt composition at pH 3.0. Differences in the buffer composition affected the retention time, (RT), signal intensity, peak resolution, and baseline noise observed; however, there were no observed differences in selectivity, with the order of elution being consistent across the three salt concentrations tested. The lowest composition of 5 mM ammonium formate showed earlier RT for the selected 9 amino acids, and lower signal intensity with less noise compared to the higher buffer concentrations. Increasing the concentration of ammonium formate to 10 mM resulted in a later elution of the amino acids. The highest composition of 20 mM ammonium formate showed similar retention times to the 10 mM concentration but was observed to have lower signal intensity when analyzed the mass spectrometer. The separation of amino acid isomers, Leu and Ile, was selected as the critical measure for peak resolution. The elution order for the isomers is Leu first followed by Ile, represented in Figure 1. Optimum resolution of the two amino acids was observed at 10 and 20 mM concentration of ammonium formate buffer where both compositions showed almost-to-baseline peak resolution. However, given that the 20 mM buffer concentration showed an elevated noise signal, 10 mM was set as the final ammonium formate concentration for the mobile phase.

Two additional mobile phase pH were tested at pH 2.8 and 3.5, to assess the effect on peak shape and selectivity depicted in Figure 2. No differences in selectivity were observed, but some differences were observed in peak shape and signal response. For example, Gln and Lys showed increased signal response at pH 2.8, indicating better ionization for mass spectrometry detection at lower pH conditions.

The mobile phase flow rate of 0.4 mL/min and ammonium formate buffer pH 3.0 were used as the initial conditions. Testing higher flow rates at 0.6 mL/min resulted in earlier retention time overall compared to 0.4 mL/min flow rate as expected (Fig. 3). However, this early RT was also coupled with reduced peak signal for Tyr and Trp when analyzed by mass spectrometry. Additionally, a loss in peak signal and resolution was observed for the amino acid isomers, Leu and Ile. Therefore, the initial flow rate at 0.4 mL/min was maintained as the preferred operating condition.

Figure 4 depicts representative chromatograms displaying signal response and retention times of 20 standard amino acids following method optimization completion.

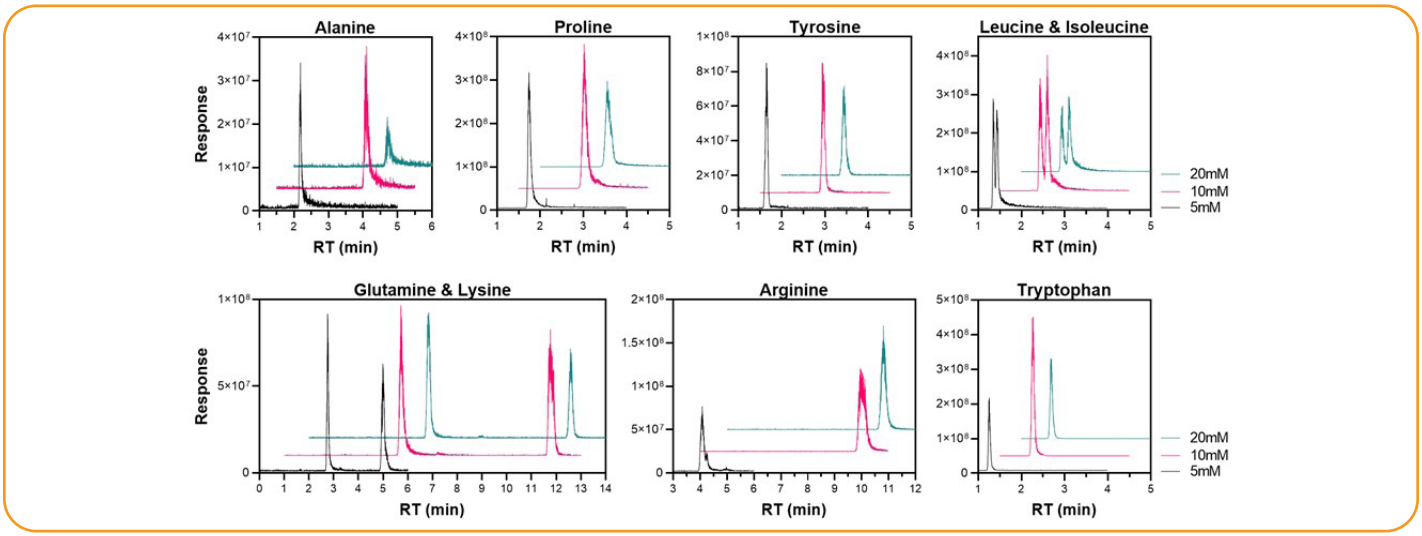

Figure 1. Optimization of ammonium formate buffer concentration at pH 3.0, showing the peak shape and retention of 9 selected amino acids with RT (x-axis) offset. 5 mM buffer concentration shows earlier RT and higher noise compared to higher concentrations. At 10 mM and 20 mM concentrations, greater resolution was achieved for Leu & Ile isomers, but ultimately overall better signal response and reduced noise was achieved with 10 mM buffer concentration.

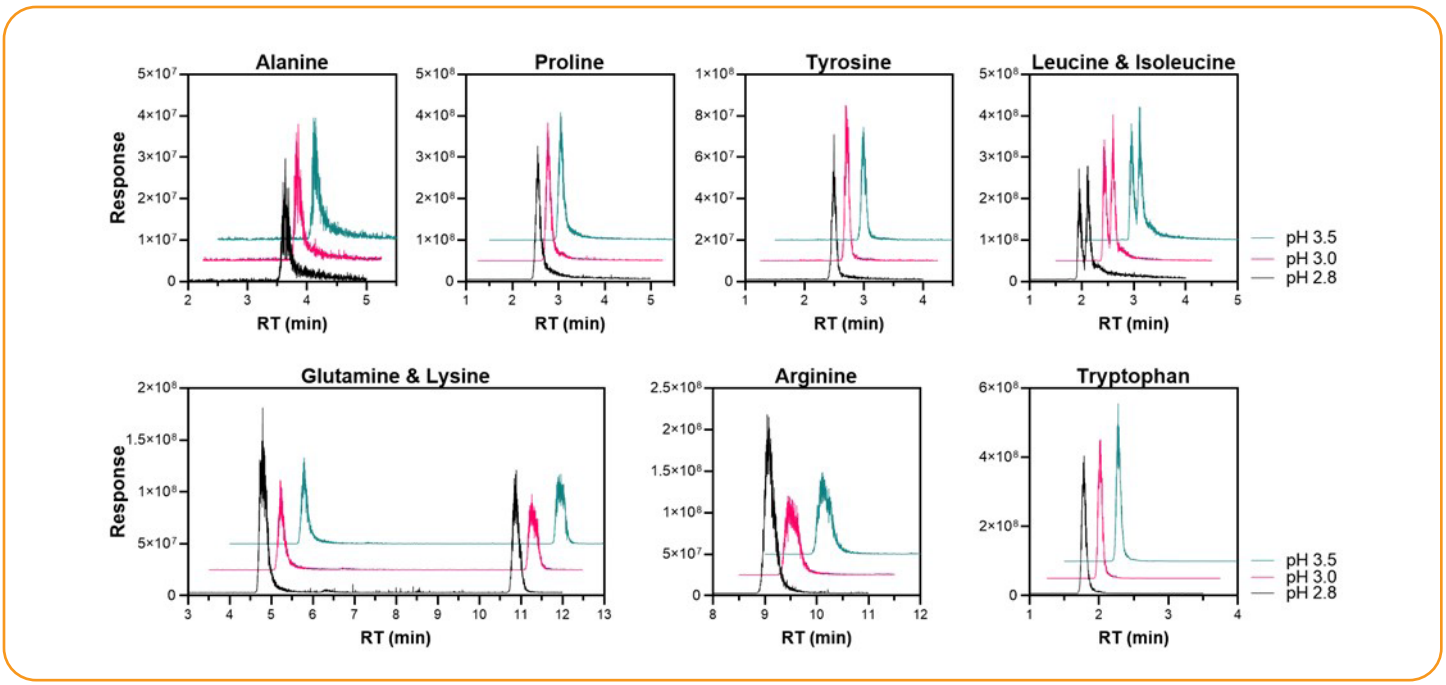

Figure 2. pH screening of 10 mM ammonium formate buffer at pH 2.8 and 3.5 compared to pH 3.0 with RT (x-axis) offset. No differences in the selectivity were observed, but some differences were observed in peak shape and signal response for the amino acids, therefore pH 3.0 was selected.

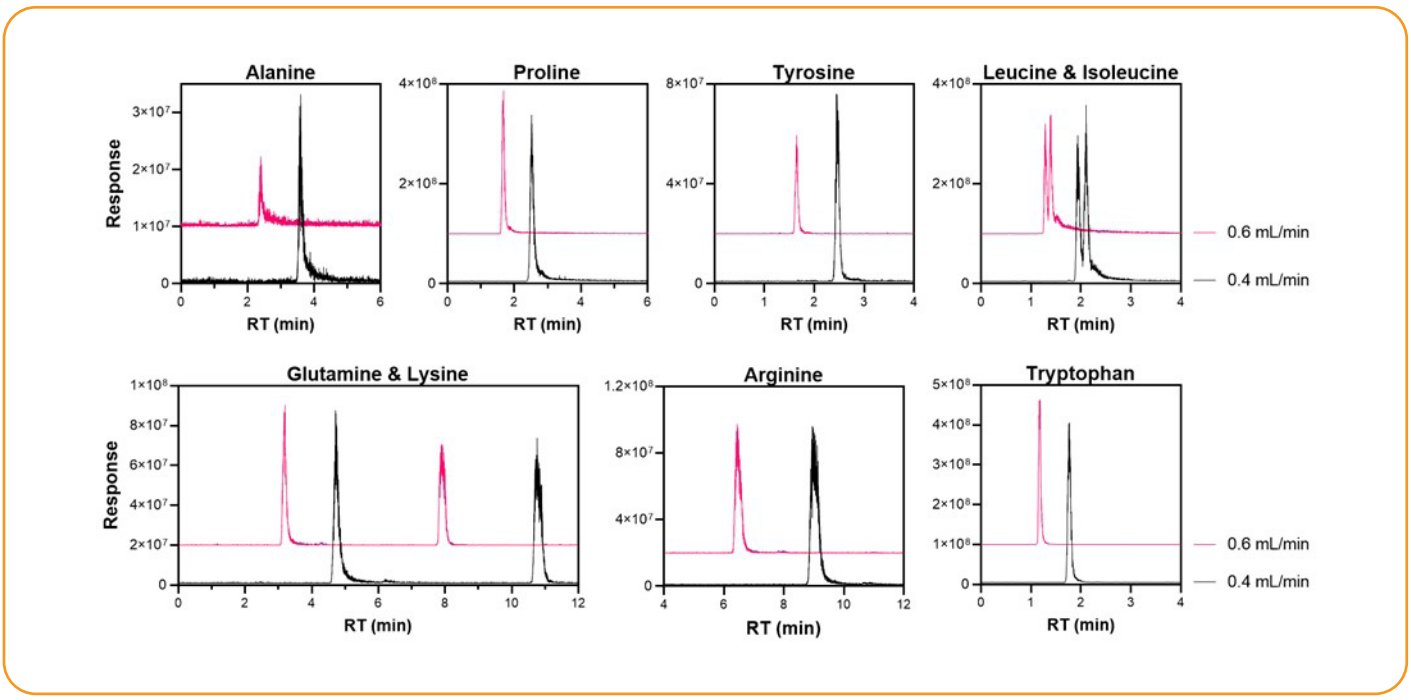

Figure 3. Flow rate optimization of 10 mM ammonium formate buffer at pH 3.0, showing the differences in peak signal, profile, and RT for 9 selected amino acids (without x-axis offset). Faster flow rate produced earlier RT as expected but lower signal overall, therefore the original 0.4 mL/min flow rate was selected.

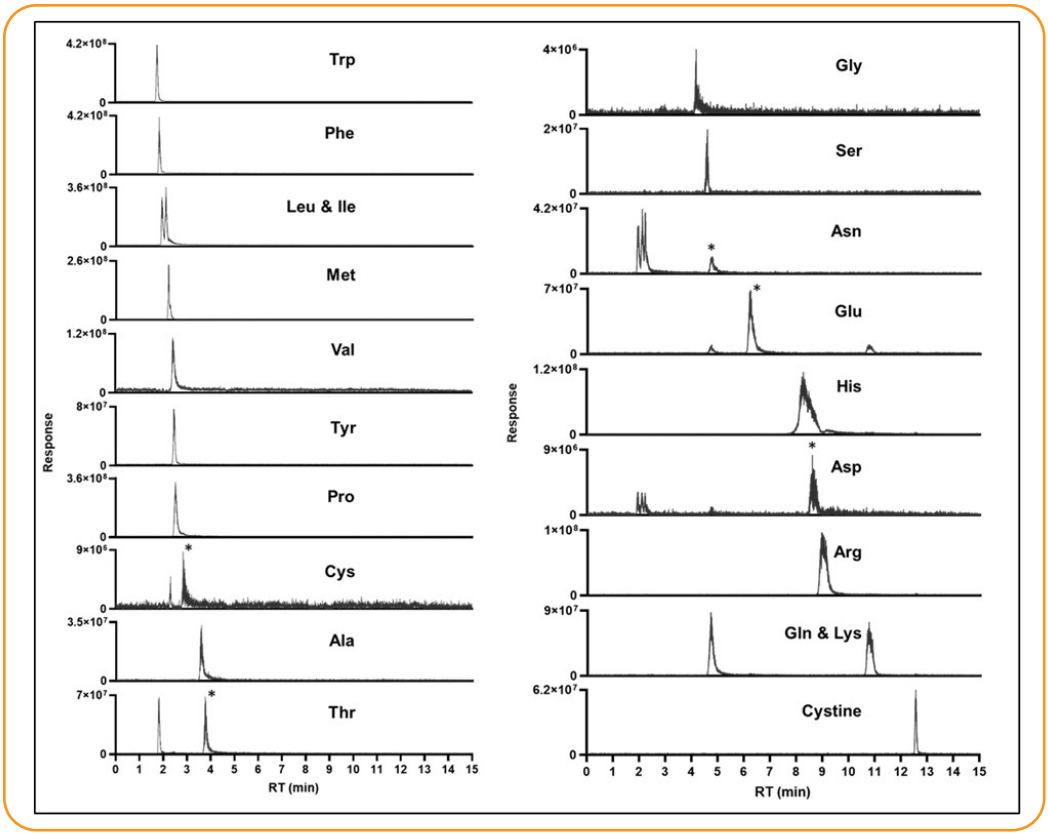

Figure 4. Representative chromatograms of 21 standard amino acids. Amino acid isomers (e.g., Leu, Ile), as well as amino acids: Gln and Lys, are detected under the same mass channels (m/z 132.1 and 147.1, respectively). The elution order for the isomers is Leu first, followed by Ile. The target amino acid is specified with an asterisk (*) symbol in chromatograms showing multiple peaks (e.g., Cys, Thr, Asn, Glu, Asp).

❖ Determination of Limit of Detection and Limit of Quantitation

The solution of Standard-H mixture with four additional amino acids was used starting from 1 mM concentration. The solution was prepared by serial dilution until the S/N ratios of each analyte is measured greater-than or equal-to 10 and 3, for LOQ and LOD, respectively. The LOQ for the amino acids ranged from 0.1 to 80 µM, and the LOD ranged from <0.1 to 40 µM. Table 3 contains the LOQ and LOD values for all amino acids tested.

Table 3. List of observed LOD and LOQ concentrations for the amino acids.

Amino Acid	LOD[1] (µM)	LOD (mg/mL)	LOQ[2] (µM)	LOQ (mg/mL)
Alanine	6.0	0.54	40.0	3.56
Arginine	0.2	0.04	0.5	0.09
Asparagine	4.0	0.53	40.0	5.28
Aspartic acid	10.0	1.33	40.0	5.32
Cysteine[3]	6.0	0.73	60.0	7.27
Cystine	2.0	0.48	0.6	1.44
Glutamic acid	2.5	0.37	10.0	1.47
Glutamine	1.0	0.15	6.0	0.88
Glycine	40.0	3.00	80.0	6.01
Histidine	0.1	0.02	0.4	0.06
Isoleucine	0.1	0.01	0.4	0.05
Leucine	0.2	0.03	0.6	0.08
Lysine	2.0	0.29	6.0	0.88
Methionine	0.6	0.09	4.0	0.60

Amino Acid	LOD[1] (µM)	LOD (mg/mL)	LOQ[2] (µM)	LOQ (mg/mL)
Phenylalanine	<0.1[4]	<0.02	0.2	0.03
Proline	0.1	0.01	0.4	0.05
Serine	20.0	2.10	80.0	8.41
Threonine	0.6	0.07	2.0	0.24
Tryptophan	<0.1[4]	<0.02	0.1	0.02
Tyrosine	0.2	0.04	0.6	0.11

[1] The LOD was estimated by analyzing the peak signal-to-noise (S/N) ratio of greater-than or equal-to (≥) 3. This was determined using MassLynx software for the mass spectrometry, by first applying the smoothing algorithm to the Total Ion Chromatogram (TIC), then applying the signal-to-noise function to the smoothed chromatogram.

[2] The LOQ was estimated by analyzing the peak signal-to-noise (S/N) ratio of greater-than or equal-to (≥)10. This was determined using MassLynx software for the mass spectrometry, by first applying the smoothing algorithm to the Total Ion Chromatogram (TIC), then applying the signal-to-noise function to the smoothed chromatogram.

[3] For the purposes of LOD & LOQ determination, cysteine was analyzed individually from commercially available standard without combining with the standard mixture at 1mM final concentration.

[4] Observed S/N ratio value was (greater-than) > 3 at the lowest concentration (0.1 µM) tested.

❖ Repeatability and Robustness

Repeatability was analyzed by measuring the retention time and peak area for ten selected amino acids over 10 consecutive injections of Standard H plus four additional amino acids solution. For data analysis a smoothing algorithm was applied using the TargetLynx software sample processing for the mass spectrometry instrument. The mean average as well as subsequent standard deviation and relative standard deviation (RSD) were calculated for peak area and RT of each selected amino acid, shown in Table 4. The overall RSD for peak area showed great results as it was calculated to be less than 10%. In addition, the overall RSD for the RT was excellent as the value reached no greater than 0.5%, indicating exceptional accuracy and precision of amino acid separation using the HALO® Penta-hydroxy based column.

Table 4. Repeatability of retention time (RT) and peak area for 10 selected amino acids.

Amino Acid	Average RT (min)	RT %RSD	Average Peak Area	Peak Area %RSD
Asparagine	4.84	0.14	1.14E+06	0.58
Glutamine	4.84	0.17	7.92E+06	1.95
Lysine	10.98	0.06	7.25E+06	2.40
Histidine	8.41	0.21	1.07E+07	2.30
Arginine	9.18	0.10	1.05E+07	2.79
Cysteine	2.92	0.49	9.01E+05	9.08
Glycine	4.30	0.26	4.32E+05	4.60
Phenylalanine	1.85	0.36	3.52E+07	4.14
Tryptophan	1.80	0.27	4.65E+07	2.94
Isoleucine	2.15	0.31	3.49E+07	1.43

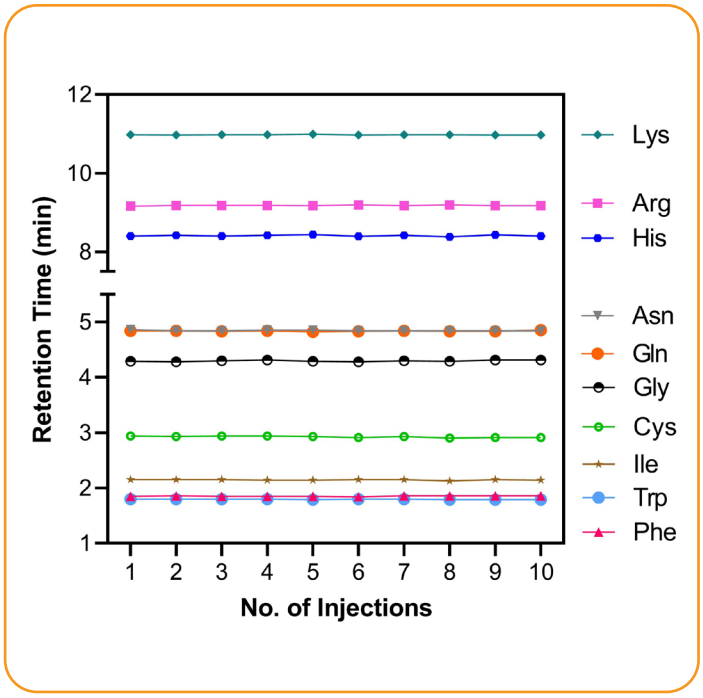

Figure 5. Plot of retention time repeatability for 10 selected amino acids show <0.5% RSD, indicating consistent peak RT throughout 10 injections.

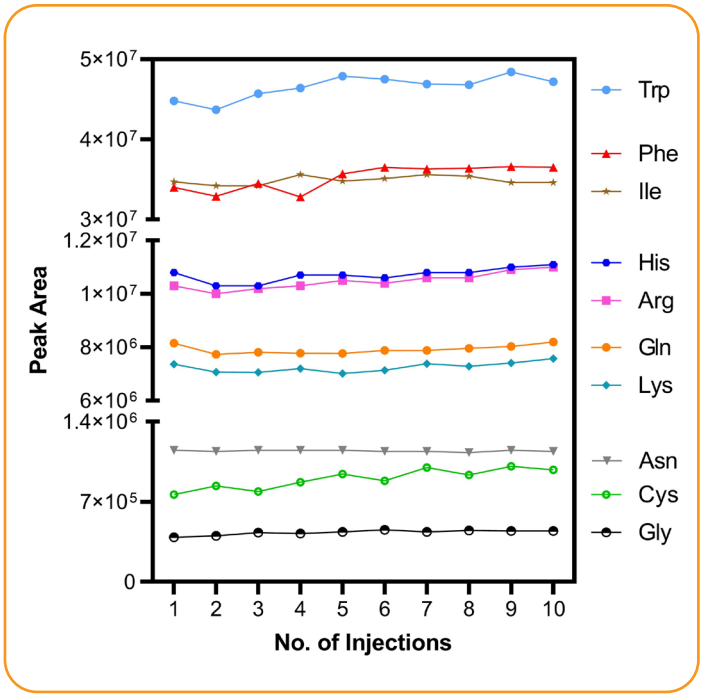

Figure 6. Plot of peak area repeatability for 10 selected amino acids show <10% RSD, indicating consistent peak area was observed throughout 10 injections.

Column robustness was demonstrated using both spent cell culture media and cell-lysate, depicted in Figure 7. The spent cell culture media sample and cell-lysate sample were each injected 50 times. Phenylalanine was selected as the target amino acid for analysis due to its strong signal. The analytical column demonstrated strong robustness showing <0.5% RSD for retention time and <9.0% RSD for peak area of signal response after 50 injections for each supernatant and cell pellet samples. To note, these results were obtained from a single column that was used for the entirety of the method development, thereby further demonstrating the strong performance of the column.

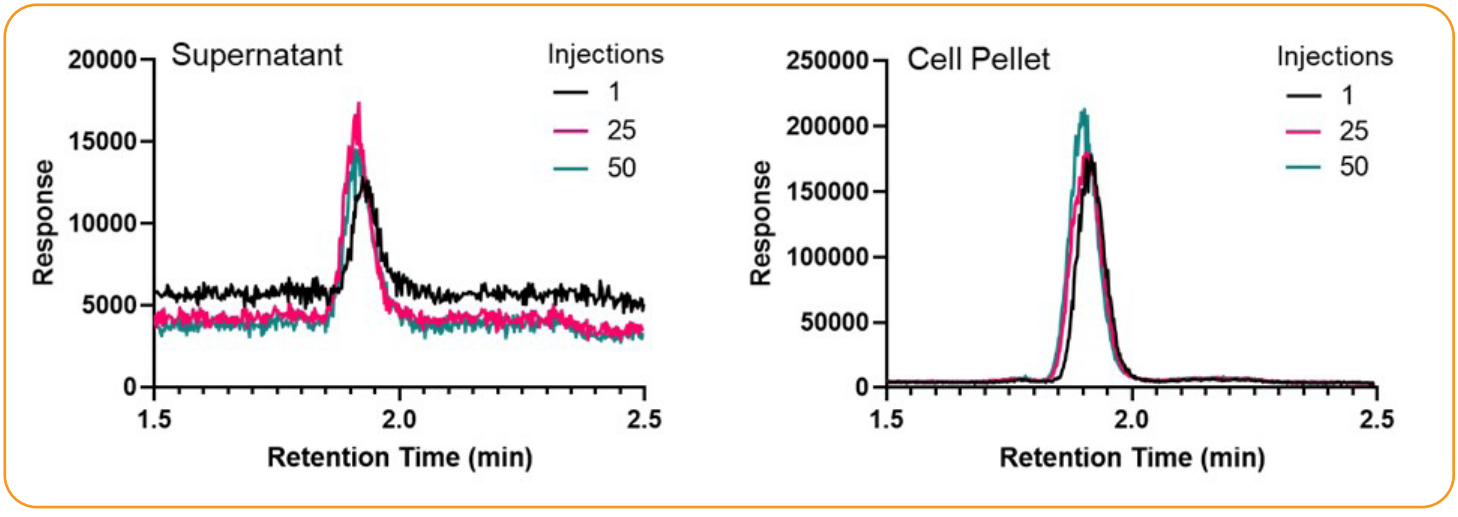

Figure 7. Robustness results monitoring Phe at RT 1.91 min, and the 1st, 25th, and 50th injection of cell lysate supernatant and resuspended cell pellet. The results show consistent peak retention time (<0.5% RSD) and peak area observed (<10% RSD) for 50 injections of each sample.

CONCLUSION

An underivatized amino acid analysis method was developed using the HALO® penta-hydroxy based column for HILIC separation coupled to the QDa Mass Spectrometer for mass identification. The method demonstrated excellent repeatability and sensitivity to detect LOD and LOQ for each amino acid at concentrations <1 mM. This HILIC column was highly effective in separating polar compounds and demonstrated outstanding robustness when used with lysate samples. The HALO® Penta-hydroxy based column serves as an ideal choice for HILIC-based separations, ensuring consistent results and an impressively long lifetime.

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ABOUT PELICAN EXPRESSION TECHNOLOGY

The Pelican Expression Technology® (PET) platform is a validated and versatile *Pseudomonas fluorescens*-based expression system for the development of protein-based therapeutics from concept-to-market. With more than 20 years of experience and five marketed products, the platform provides a robust and highly scalable means for protein engineering, accelerated CMC development, and the production of a broad range of complex and difficult-to-express proteins. Through a combination of state-of-the-art HTP screening and analytics, along with fermentation and purification development capabilities, the PET platform enables gene-to-protein production in as little as four weeks, and final production strain selection in less than 10 weeks.

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