APPLICATION NOTE

Liquid Chromatography

Authors:

Chi Man Ng

Wilhad M. Reuter

PerkinElmer, Inc.
Shelton, CT

# The Analysis of Underivatized Amino Acids by HPLC with MS Detection

## Introduction

The analysis of amino acids plays an important role in a wide range of applications, including those in food, beverage and biomedical industries. A common method for amino acid analysis is liquid chromatography with pre- or post-column derivatization to improve sensitivity and/or increase retention of the analytes. The most common derivatization methods have used ninhydrin, o-phthalaldehyde (OPA), fluorenylmethyloxycarbonyl (FMOC) and AccQ-Tag™ Waters®, Milford, MA as derivatizing agents. Although these methods are well understood and have been historically well received, they are often labor intensive and time-consuming.

The ability to analyze underivatized amino acids provides the advantages of increased convenience, simplicity and repeatability, while still providing the desired sensitivity and separation speed. The elimination of the derivatization step reduces the possibility of side reactions and reagent interference or the possibility of altering the sample through contamination/degradation.[1]

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This application note focuses on the HPLC analysis of underivatized amino acids using MS detection. Method conditions and performance data, including linearity and repeatability, are presented.

## Experimental

### Hardware/Software

For all chromatographic separations, a PerkinElmer Altus™ HPLC System was used, including the Altus A-10 solvent and sample module, integrated vacuum degasser/column oven and an Altus SQ MS detector. All instrument control, analysis and data processing was performed using the Waters® Empower® 3 Chromatography Data System (CDS) platform.

### Method Parameters

The HPLC method parameters are shown in Table 1 and the MS method parameters are shown in Table 2.

*Table 1.* LC Method Parameters.

| | |
|---|---|
| Column: | Intrada™ Amino Acid, 5 µm 100 x 3-mm column (Imtakt, Portland, OR) |
| Mobile Phase: | Solvent A: Acetonitrile/tetrahydrofuran (THF)/ 25-mM ammonium formate/formic acid; 9 / 75 / 16 / 0.3 (v/v/v/v)<br>Solvent B: Acetonitrile/ 100-mM ammonium formate; 20 / 80 (v/v)<br>Solvent Program: |
| Analysis Time: | 25 min. |
| Flow Rate: | 0.400 mL/min. |
| Pressure: | 2100 psi |
| Oven Temp.: | 35 °C |
| Detection: | Altus SQ MS |
| Injection Volume: | 5 µL |
| Sampling (Data) Rate: | 2 pts./sec |

| | Time (min.) | Flow Rate (mL/min.) | %A | %B | Curve |
|---|---|---|---|---|---|
| 1 | Initial | 0.400 | 100.0 | 0.0 | Initial |
| 2 | 5.00 | 0.400 | 100.0 | 0.0 | 6 |
| 3 | 12.00 | 0.400 | 83.0 | 17.0 | 6 |
| 4 | 19.00 | 0.400 | 0.0 | 100.0 | 6 |
| 5 | 25.00 | 0.400 | 0.0 | 100.0 | 11 |
| 6 | 25.10 | 0.400 | 100.0 | 0.0 | 6 |

*Table 2.* MS Method Parameters.

| | |
|---|---|
| Ionization Mode: | Electrospray (+) |
| Capillary Voltage: | 3.50 kV |
| Source Temp.: | 150 °C |
| Cone Voltage: | 35 V |
| Desolvation Temp.: | 400 °C |
| Desolvation Gas: | 700 L/hr |
| Cone Gas Flow: | 25 L/hr |

### Solvents, Standards and Samples

All solvents and diluents used were HPLC grade and filtered via 0.45-µm filters. The diluent used was 0.1N HCl.

The Pierce 17-Amino Acid Standard H Mix (protein hydrolysate) was obtained from Thermo Scientific™ (Rockford, Illinois). This included: alanine (Ala), arginine (Arg), aspartic acid (Asp), cysteine (Cys), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tyrosine (Tyr), and valine (Val). The concentration for each amino acid was 2.50 µmol/mL in 0.1N HCl, except for cysteine, which was present at 1.25 µmol/mL. The standard was diluted with diluent to 1.0 µmol/mL for the working standard 1 (WS1) and to 0.1 µmol/mL for the working standard 2 (WS2). WS1 was used for the calibration of alanine and cysteine, as these two amino acids have lower sensitivity. The lower level standards were prepared from each of the two working standards via serial dilution with diluent.

Prior to injection, all calibrants and samples were filtered through 0.45-µm filters to remove any small particles.

## Results and Discussion

Figure 1 shows the overlay of the selected ion recordings (SIRs) for all 17 amino acids, using the optimized conditions described above. The analysis time was under 25 minutes. Table 3 shows the retention times (RT) and SIR windows/values used for each amino acid analyzed.

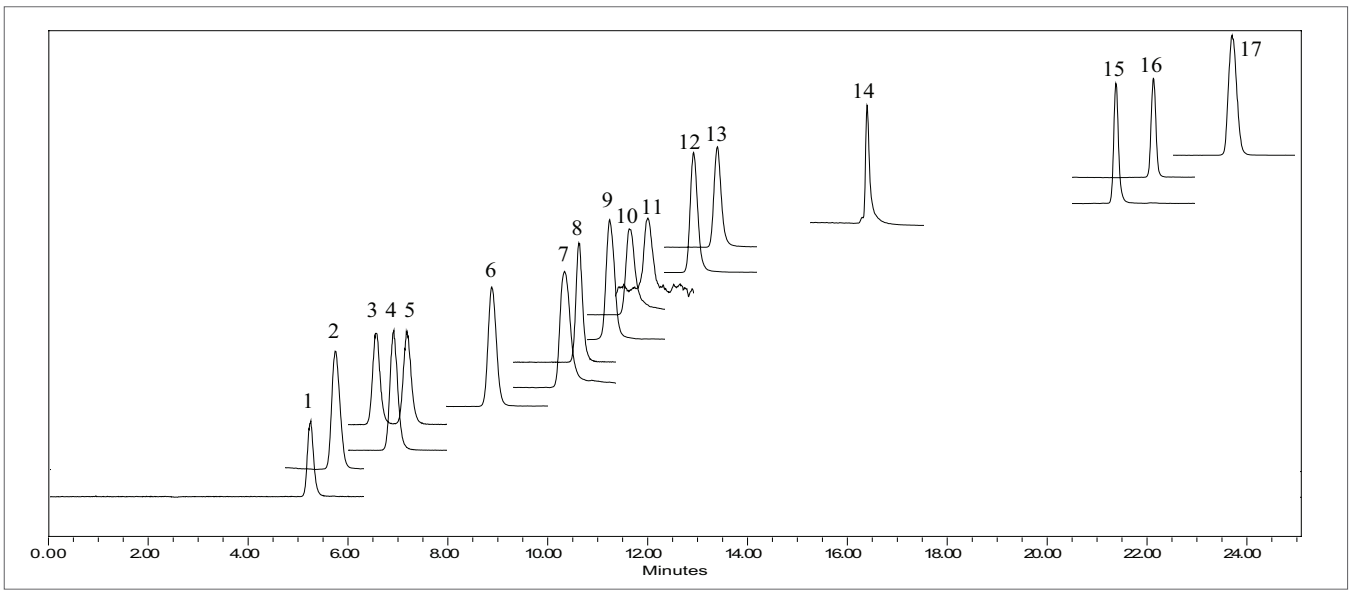


*Figure 1.* Overlay of SIRs for all 17 analytes: 1-Phe; 2-Tyr; 3-Leu; 4-Met; 5-Ile; 6-Val; 7-Glu; 8-Pro; 9-Thr; 10-Asp; 11-Ala; 12-Ser; 13-Gly; 14-Cys; 15-His; 16-Lys; 17-Arg.

Figure 2 shows the overlay of 10 replicate injections of the 0.1-µmol/mL histidine in WS2, demonstrating exceptional reproducibility. The RT %RSD was 0.016 %.

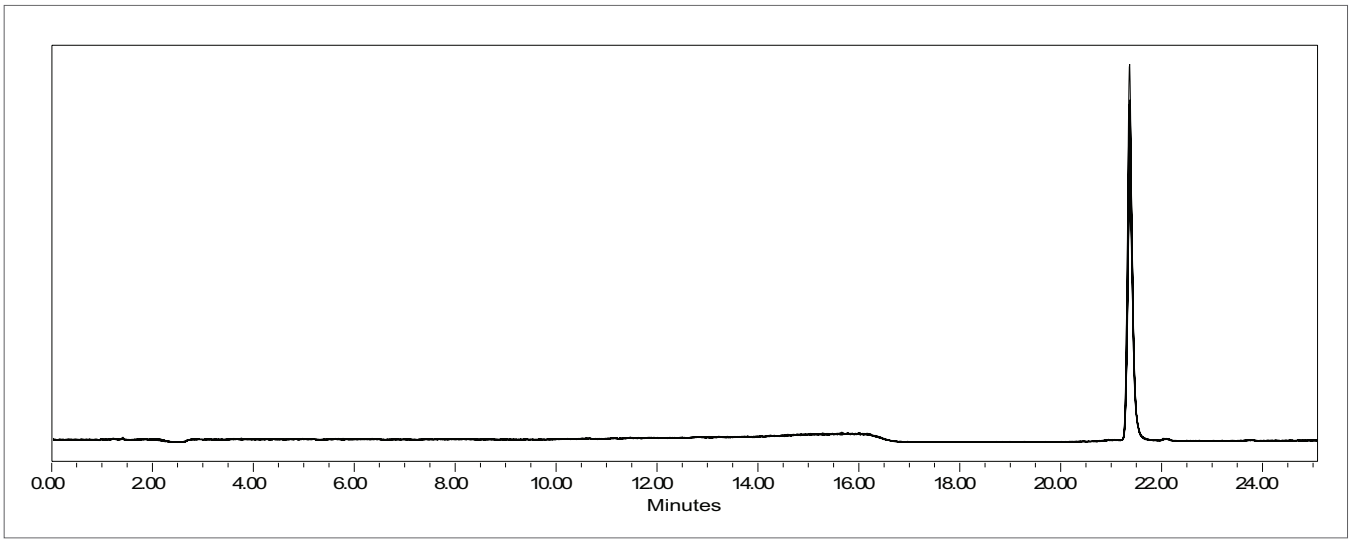


*Figure 2.* Overlay of 10 replicates of the 0.1-µmol/mL histidine standard.

*Table 3.* RTs and SIR windows/values for the 17 analytes.

| RT (min.) | Amino Acid | SIR – RT windows | SIR - m/z |
|---|---|---|---|
| 5.18 | Phe | 0.00-6.40 | 166.0 |
| 5.80 | Tyr | 4.60-6.50 | 182.0 |
| 6.57 | Leu | 6.00-8.00 | 132.0 |
| 6.90 | Met | 6.00-8.00 | 150.0 |
| 7.19 | Ile | 6.00-8.00 | 132.0 |
| 8.87 | Val | 8.00-10.00 | 118.2 |
| 10.40 | Glu | 9.30-11.50 | 148.0 |
| 10.70 | Pro | 9.30-11.50 | 116.0 |
| 11.22 | Thr | 10.40-12.20 | 120.1 |
| 11.68 | Asp | 10.60-12.90 | 134.0 |
| 11.97 | Ala | 11.40-12.90 | 90.0 |
| 12.90 | Ser | 12.30-14.20 | 106.1 |
| 13.40 | Gly | 12.30-14.20 | 76.0 |
| 16.40 | Cys | 15.30-17.50 | 241.0 |
| 21.40 | His | 20.50-22.50 | 156.0 |
| 22.20 | Lys | 20.50-22.50 | 147.0 |
| 23.80 | Arg | 22.50-25.0 | 175.1 |

A 5-level calibration set was used, covering a concentration range of 0.006 to 0.10 µmol/mL, except for alanine and cysteine, each covering a concentration range of 0.06 to 1.00 µmol/mL. Figure 3 shows the representative calibration results for aspartic acid, glycine, histidine and methionone. All amino acids followed a quadratic (2$^{nd}$ order) fit. The $R^2$ coefficients for the 17 amino acids are shown in Table 4, all values exceeding 0.999 (n = 3 at each level).

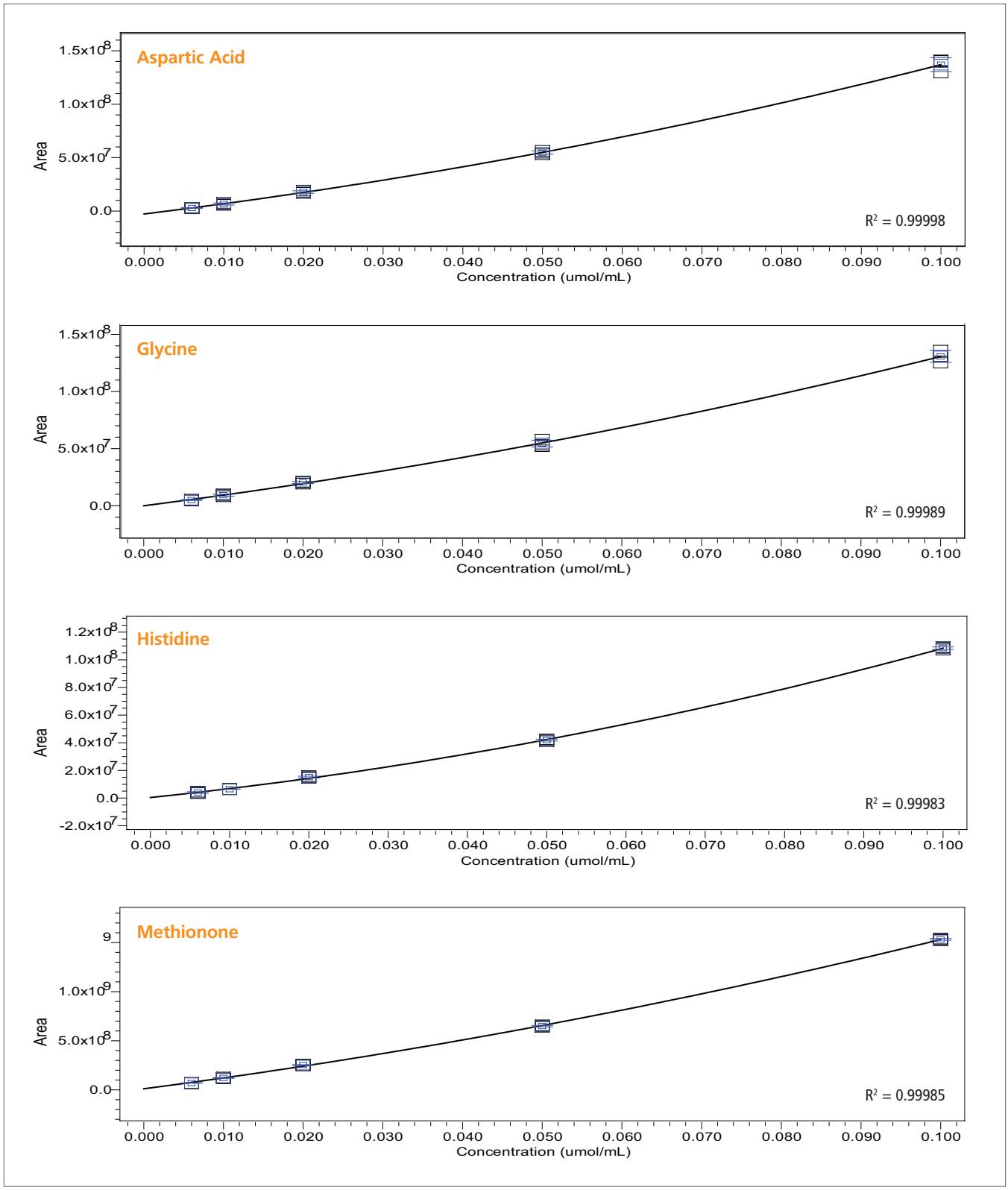


*Figure 3.* Results of 5-level calibration sets for aspartic acid, glycine, histidine and methionone; n=3.

As listed in Table 4, LOD (limit of detection) and LOQ (limit of quantitation) levels were established for each analyte, based upon a signal-to-noise (s/n) of > 3/1 for the LOD and >10/1 for the LOQ. The considerably higher values for alanine are due to its lower ionization efficiency.

*Table 4.* Curve fit and LOD/LOQ results.

| Amino Acid | $R^2$ Values | LOD (nmol/mL) | LOQ (nmol/mL) |
|---|---|---|---|
| Ala | 0.99967 | 35.07 | 116.9 |
| Arg | 0.99908 | 0.360 | 1.200 |
| Asp | 0.99998 | 1.104 | 3.680 |
| Cys | 0.99982 | 1.439 | 4.798 |
| Glu | 0.99987 | 0.129 | 0.430 |
| Gly | 0.99989 | 0.631 | 2.104 |
| His | 0.99983 | 0.428 | 1.426 |
| Ile | 0.99998 | 0.157 | 0.524 |
| Leu | 0.99996 | 0.155 | 0.516 |
| Lys | 0.99915 | 0.779 | 2.596 |
| Met | 0.99985 | 0.147 | 0.490 |
| Phe | 0.99995 | 0.198 | 0.659 |
| Pro | 0.99997 | 0.121 | 0.404 |
| Ser | 0.99982 | 0.246 | 0.820 |
| Thr | 0.99983 | 0.549 | 1.830 |
| Tyr | 0.99989 | 0.261 | 0.870 |
| Val | 0.99983 | 0.199 | 0.662 |

## Conclusion

This work demonstrated a simple, rapid and direct HPLC method for amino acids. The detection of amino acids was achieved without pre- or post-column derivatization while providing high sensitivity and selectivity. The results also exhibited a very good curve fit and retention time repeatability over the tested concentration range.

Apart from the higher limit for alanine (due to its lower ionization efficiency), the LODs for all other analyzed amino acids ranged from 0.12 to 1.44 nmol/mL. Also, other than for alanine, all LOD/LOQ values were over five-fold lower than historical values obtained for OPA-derivatized amino acids.[2]

## References


1. P. Agrafiotou, S. Sotiropoulos, A. Pappa. J. Sep. Sci., 2009, 32, 949-954.
2. M. P. Bartolomeo and F. Maisano. J. Biomol. Tech., 2006, 17, 131-137.



**PerkinElmer, Inc.**
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
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