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Separation of Low Levels of Isoleucine from Leucine Using the ACQUITY UPLC H-Class Amino Acid System

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APPLICATION BENEFITS

Resolution of leucine and isoleucine at levels as low as 0.05% Ile/Leu using the UPLC® Amino Acid Analysis Solution.

WATERS SOLUTIONS

UPLC Amino Acid Analysis Solution

ACQUITY UPLC® H-Class System

AccQ•Tag™ Ultra Chemistry

Empower® 3 Software

KEY WORDS

Amino acid analysis, leucine, isoleucine, European Pharmacopoeia, USP

INTRODUCTION

The European Pharmacacopoeia (Ph. Eur.) defines requirements for the qualitative and quantitative composition of amino acids and mixtures of amino acids. The requirements for allowed impurities are also defined. Manufacturers of amino acids are legally bound to prove that their amino acids meet these specifications before they can distribute their products in Europe.

Leucine (Leu) is a branched-chain α-amino acid and is produced by the fermentation process. During this process, isoleucine can be produced as a by-product. The European Pharmacopoeia states that leucine and isoleucine should have a resolution of 1.5 to levels as low as 0.05%. This application note is intended to demonstrate that the Waters ACQUITY UPLC H-Class Amino Acid System can be used to suitably resolve isoleucine from leucine at these low levels.

The Waters ACQUITY UPLC H-Class Amino Acid System combines UPLC separation technology with AccQ•Tag Ultra derivatization chemistry, providing improved resolution and sensitivity, leading to improved sample characterization, all achieved within a shorter analysis time than conventional methodologies.

The amino acids are derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) under largely aqueous conditions. The derivatives are then separated utilizing the ACQUITY UPLC H-Class System, enabling analysts to achieve accurate, precise, and robust amino acid analysis utilizing a reversed-phase separation, and quantification with either UV or fluorescence detection.

EXPERIMENTAL

LC conditions

System:	ACQUITY UPLC H-Class
Detector:	ACQUITY UPLC TUV at 260 nm
Column:	AccQ•Tag Ultra C_{18}, 2.1 x 100 mm, 1.7 µm
Sample temp.:	20 °C
Column temp.:	43 °C
Injection vol.:	0.8 µL
Flow rate:	0.7 mL/min
Mobile phase A:	AccQ•Tag Eluent A
Mobile phase B:	90/10 Water/Eluent B
Mobile phase C:	Water
Mobile phase D:	AccQ•Tag Eluent B

Gradient:

Time	%A	%B	%C	%D	Curve
0.00	10.0	0.0	90.0	0.0	N/A
0.29	9.9	0.0	90.1	0.0	11
5.49	9.0	80.0	11.0	0.0	7
7.10	8.0	15.6	57.9	18.5	6
7.30	8.0	15.6	57.9	18.5	6
7.69	7.8	0.0	70.9	21.3	6
7.99	4.0	0.0	36.3	59.7	6
8.59	4.0	0.0	36.3	59.7	6
8.68	10.0	0.0	90.0	0.0	6
10.20	10.0	0.0	90.0	0.0	6

Data Management

Empower 3 Software, SR2

Standards, reagents, separation column, and turnkey methodologies within Empower Software projects, are sold as a system solution.

Sample preparation

To a leucine solution, different amounts of isoleucine were spiked to prepare isoleucine/leucine mixtures at 0.0, 0.05, 0.1, and 0.2%. A calibration standard and samples were prepared by transferring 70 µL Borate buffer and 10 µL of the standard/sample to a Waters total recovery vial, vortexing to mix. The derivatization reagent was dissolved in 1 mL of acetonitrile and then 20 µL of the solution was transferred to each vial. Each vial was capped, vortexed, and then heated to 55 °C for 10 minutes prior to analysis.

RESULTS AND DISCUSSION

A calibration standard of 17 amino acids was prepared. The standard consisted of a single point calibration curve with each standard at a concentration of 50 pmoles/µL (except cystine at 25 pmoles/µL). As can be seen from the figure below, isoleucine and leucine were resolved at around 7.8 minutes.

The 0.0% Ile/Leu (unspiked) was analyzed and the leucine sample was found to be free of interferences at the retention time of isoleucine. The spiked Ile/Leu samples at 0% (Black line) 0.05% (Blue line), 0.1% (Brown line), and 0.2% (Green line) were analyzed and the peaks were found to have a USP resolution of 2.0 for the 0.05%, 0.1%, and 0.2% levels.

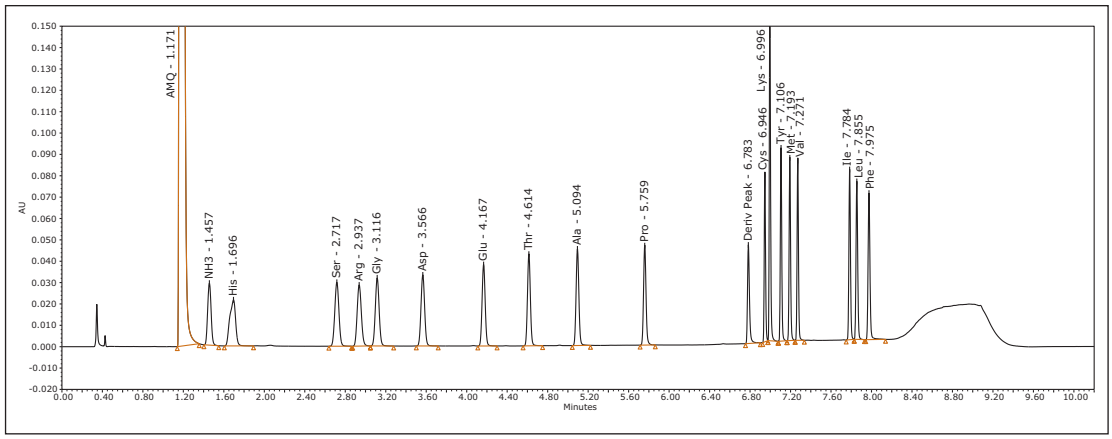

Figure 1. Standard chromatogram.

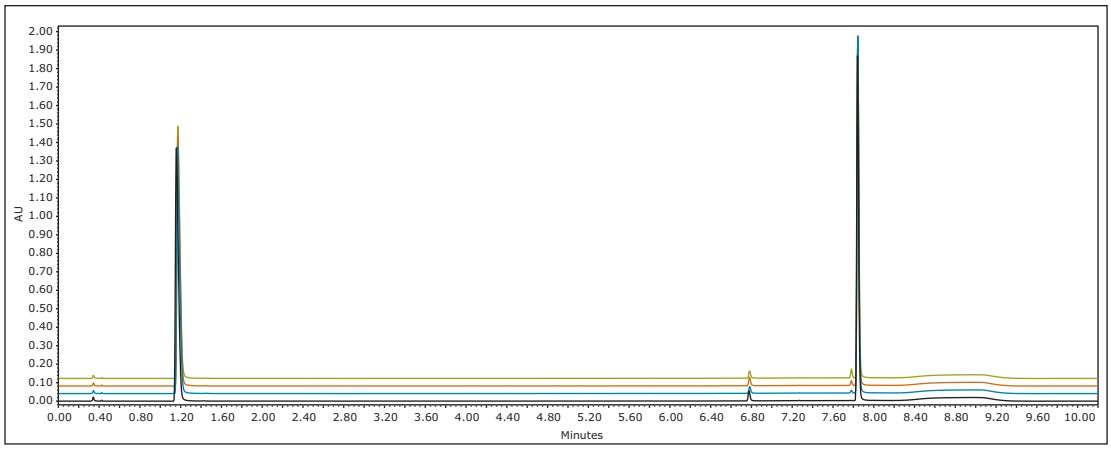

Figure 2. 0%, 0.05%, 0.1%, and 0.2% Ile/Leu chromatogram overlay.

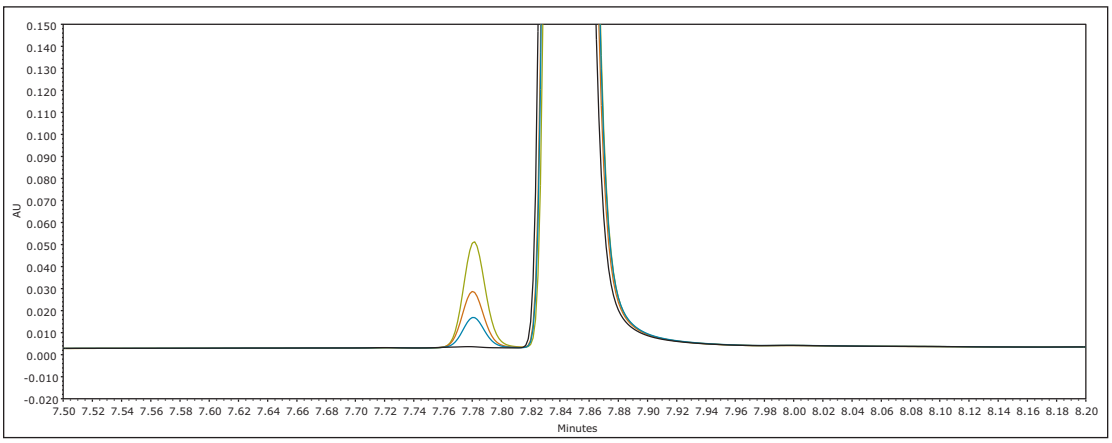

Figure 3. 0%, 0.05%, 0.1%, and 0.2% Ile/Leu chromatogram overlay (zoomed).

The Ile area was found to be linear when compared to the %Ile/Leu, with an R^2 of 0.9999.

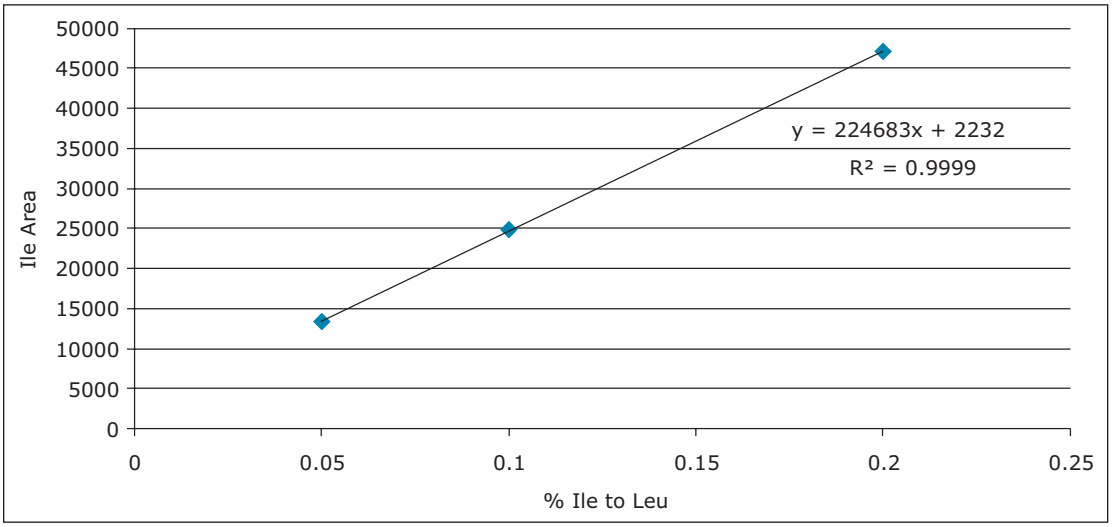

Figure 4. Linearity of Ile area vs. %Ile/Leu.

CONCLUSIONS

The Waters ACQUITY UPLC H-Class System provided chromatographic separation of all 17 amino acids in a commercially available amino acid mix within a very short run time. Baseline resolution of isoleucine and leucine was confirmed at levels as low as 0.05% Ile/Leu, meeting the regulatory requirements for these components.

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