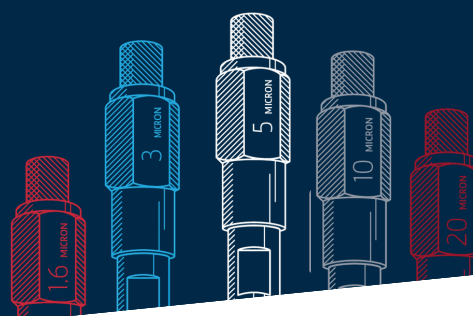


Vaast: Resolving Chiral Isobaric Amino Acids

Antoine Falatas, Imane El Barmaki, Nicolas Hausser, Pilar Franco



INTRODUCTION

Isobaric amino acids (compounds with identical nominal masses but distinct structures) pose a significant analytical challenge in biochemical and clinical research. Among these, threonine, homoserine, leucine, isoleucine and their stereoisomeric forms (allo- and their enantiomers) are particularly important due to their roles in metabolism, protein synthesis and disease biomarkers. Accurate identification and quantification of these species are essential for understanding metabolic pathways and diagnosing disorders such as maple syrup urine disease, where allo-isoleucine serves as a critical marker^{1,2}.

Threonine (Thr) is an essential proteinogenic amino acid (AA) that plays a key role in protein structure and metabolic regulation. **Homoserine** (Hse), although non-proteinogenic, serves as an important intermediate in the biosynthesis of methionine and **isoleucine** (Ile) in microorganisms and plants. **Leucine** (Leu) and **isoleucine** are branched-chain essential AAs critical for muscle metabolism and energy balance. Their diastereomeric counterparts, **allo-threonine** (allo-Thr) and **allo-isoleucine** (allo-Ile), occur only in trace amounts but have clinical significance, particularly in metabolic disorders. Regarding stereochemistry, the L-forms of these AAs

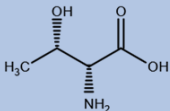
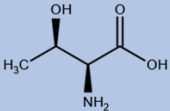
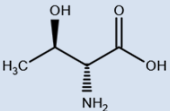
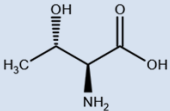
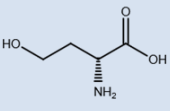
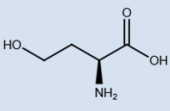
dominate in proteins, while D-forms are found in bacterial cell walls and specialized peptides, highlighting their diverse biological roles.

These two series of AAs share **identical molecular formulas and exact masses** (see Table 1):

- Leu/Ile/Allo-Ile: $C_6H_{13}NO_2$ (m/z 131.094 Da monoisotopic mass)
- Thr/Allo-Thr/Hse: $C_4H_9NO_3$ (m/z 119.058 Da monoisotopic mass)

Accurate analysis of these AAs in biological samples faces several obstacles. **Isobaric interference is a major issue**, as Leu and Ile, as well as Thr and Hse, produce overlapping mass spectrometric signals. Enantiomeric resolution adds complexity because L- and D-forms require chiral separation for precise quantification. Detecting diastereomers such as allo-Ile and allo-Thr is equally challenging since they occur only at trace levels but hold clinical significance. Furthermore, the complexity of biological matrices often leads to ion suppression and co-elution, hindering detection. To overcome these challenges, advanced analytical strategies—such as chiral liquid chromatography, ion mobility spectrometry and high-resolution tandem mass spectrometry—are essential for reliable identification and quantification^{3,4}.

Table 1. Comparative Table: Isobaric AAs and their properties

Amino Acid	Formula	Chemical Structures		Proteinogenic	Clinical Relevance
Threonine	$C_4H_9NO_3$	 D-Threonine (2R,3S)	 L-Threonine (2S,3R)	Yes	Essential AA
Allo-Threonine	$C_4H_9NO_3$	 D-Allo-Threonine (2R,3R)	 L-Allo-Threonine (2S,3S)	Rare	Trace component in metabolism
Homoserine	$C_4H_9NO_3$	 D-Homoserine (2R)	 L-Homoserine (2S)	No	Intermediate in methionine synthesis

Leucine	$C_6H_{13}NO_2$	D-Leucine (2R)	L-Leucine (2S)	Yes	Branched-chain AA
Isoleucine	$C_6H_{13}NO_2$	D-Isoleucine (2R,3R)	L-Isoleucine (2S,3S)	Yes	Branched-chain AA
Allo-Isoleucine	$C_6H_{13}NO_2$	D-Allo-Isoleucine (2R,3S)	L-Allo-Isoleucine (2S,3R)	Rare	Biomarker for maple syrup urine disease

The recent introduction of the DAICEL Vaast ion-exchange column has enabled simultaneous chiral resolution of 21 natural amino acids derivatized with AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate). Under the initial conditions developed, enantiomeric pairs such as threonine (Thr) and leucine (Leu) were successfully resolved^{5,6}.

Importantly, these same chromatographic conditions also separate the enantiomers of homoserine (Hse), allo-threonine (allo-Thr), isoleucine (Ile) and allo-isoleucine (allo-Ile) (see Figure 1 and Table 2). In all pairs, the D-enantiomer elutes first. This is a favorable feature, as the D-enantiomers are most often biologically present as minor peaks.

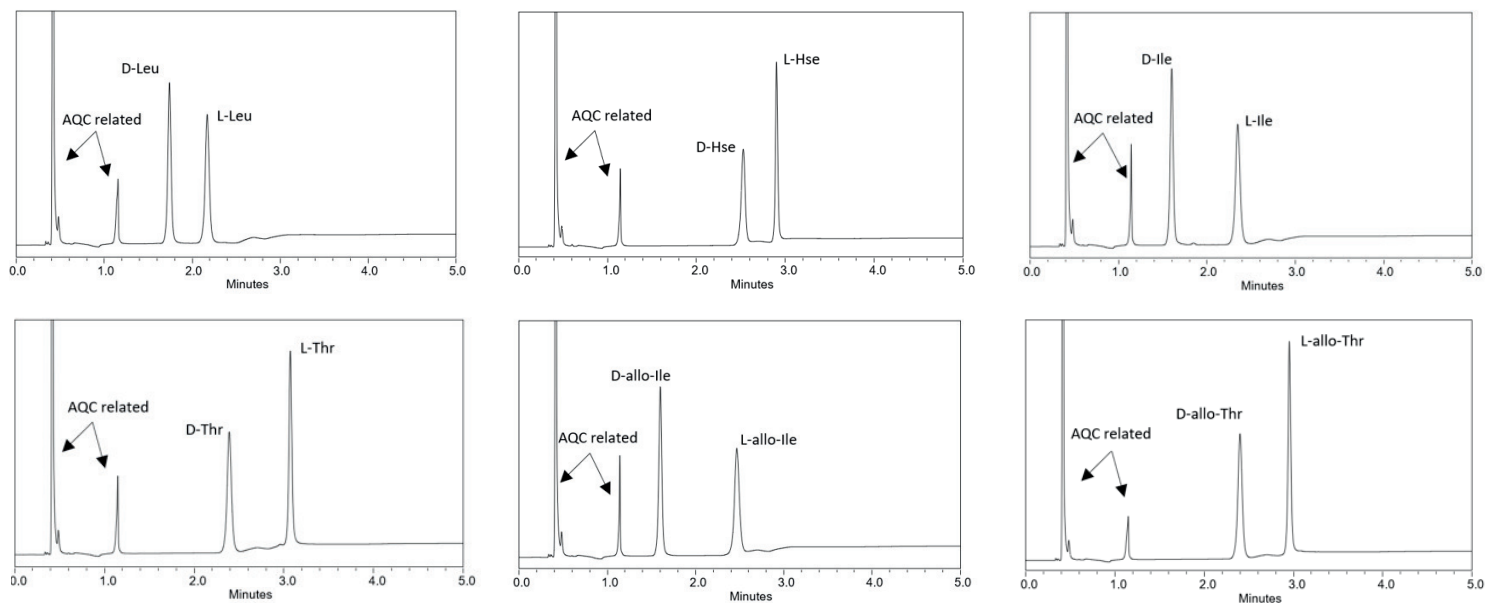


Figure 1: Individual UV chromatograms at 254 nm HPLC conditions of the 6 AQC-derivatized AAs on the Vaast column (1.7 μ m, 100 x 2.1 mm). Gradient conditions as described in Experimental Section.

Table 2. Retention time of the 6–AQC derivatized AAs on the Vaast column (gradient conditions)

AQC-AA*	RT1 (D) in min	RT2 (L) in min	α
Isoleucine (Ile)	1.60	2.35	2.7
Allo-Isoleucine (allo-Ile)	1.60	2.47	2.9
Leucine (Leu)	1.74	2.17	1.7
Threonine (Thr)	2.39	3.07	1.6
Allo-Threonine (allo-Thr)	2.40	2.95	1.4
Homoserine (Hse)	2.53	2.90	1.3

(*): If detected by LC-MS; exact (calculated) m/z value of the protonated species would be 3022 for the Leu series and 2901 Thr series as AQC-derivatives.

Furthermore, in the absence of allo-forms, the method allows the simultaneous determination of enantiomeric excesses for the corresponding isobaric pairs (see Figure 2).

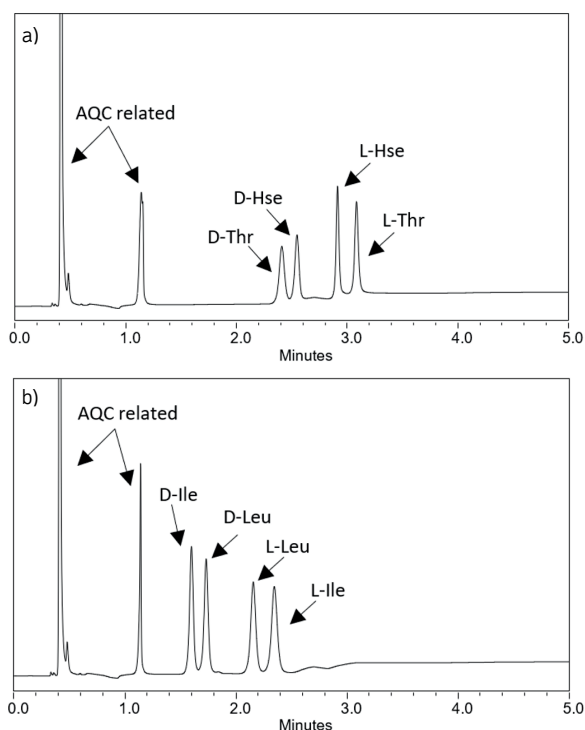


Figure 2. UV chromatograms at 254 nm under HPLC conditions of the two series of isobaric enantiomers AQC-derivatized AAs on the Vaast column (1.7 μ m, 100 x 2.1 mm). Simultaneous injection: a) Thr and Hse enantiomers; b) Leu and Ile enantiomers. Gradient conditions as described in Experimental Section.

If allo-amino acids are present together with other isobaric isomers, the mobile phase must be adjusted as shown in Figure 3 (retention times in Table 3). Under isocratic modified conditions, all six isobaric compounds are baseline resolved for the Thr series in just 13 minutes by the Vaast column (see Figure 3a). In the case of the Leu series, five peaks are baseline resolved in 7 minutes, as D-Ile and D-Allo-Ile coelute (see Figure 3b). None of the conditions tested could resolve those two isobaric isomers, often quantified as the sum of the two species³⁷.

Table 3. Retention time of the two series of isobaric enantiomers of AQC-derivatized AAs on Vaast column (isocratic conditions – see Figure 3)

AQC-AA	RT1 (D) in min	RT2 (L) in min
Isoleucine (Ile)	3.34	6.03
Allo-Isoleucine (allo-Ile)	3.34	6.48
Leucine (Leu)	3.79	5.52
Threonine (Thr)	5.22	12.13
Homoserine (Hse)	5.61	9.19
Allo-Threonine (allo-Thr)	5.96	11.24

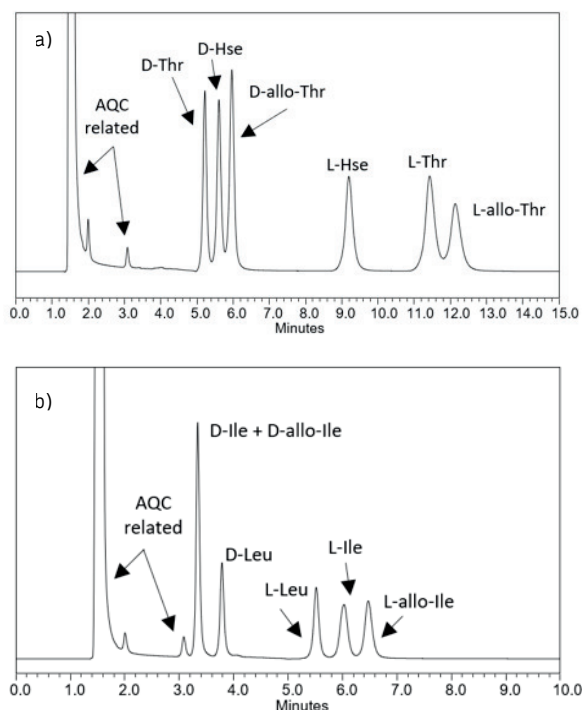


Figure 3. UV chromatograms at 254 nm under HPLC conditions of the two complete series of isobaric enantiomers AQC-derivatized AAs on the Vaast column (1.7 μ m, 100 x 2.1 mm). Simultaneous injections: a) Thr and Hse, plus allo-Thr enantiomers; b) Leu and Ile, plus allo-Ile enantiomers. Isocratic conditions : 10 mM Formic Acid + 10 mM Ammonium Formate in MeOH (100%) at FR = 0.21 ml/min. The column temperature was maintained at 50°C.

Experimental Section: gradient conditions:

Chromatographic conditions for Separation of Isobaric AAs			
Column	Vaast (100 mm x 2.1 mm i.d, 1.7 µm)		
Mobile phase	A: 10 mM Formic Acid + 10 mM Ammonium Formate in Acetonitrile/Water (93/7; v/v) B: 50 mM Formic Acid + 50 mM Ammonium Formate in Methanol/Acetonitrile (75/25; v/v)		
Gradient program	Time (min)	Solvent A (%)	Solvent B (%)
	0	90	10
	2.1	75	25
	2.5	0	100
	5.8	0	100
	6.0	90	10
9.0	90	10	
Flow rate	0.8 ml/min		
Detection	UV at 254 nm		
Temperature	50°C		
Injection volume	1 µl		

Screening and optimization were performed on a Waters Acquity I-Class UPLC + PDA + QDa Performance MS. The MS detector was set with the following parameters:

- Mode: ESI positive (ESI+)
- Cone voltage: 15 V
- Sampling rate: 15 points/sec
- Capillary voltage: 1.0 kV
- Probe temperature: 600 °C
- Gain: 1

All mobile phases and reagents were prepared according to the Derivatization Protocol and Mobile Phase Preparation Protocol for Vaast column.

CONCLUSIONS

Resolving isobaric and enantiomeric AAs is no longer a challenge thanks to the DAICEL Vaast column.

This innovative ionic-exchange technology delivers simultaneous chiral resolution of 21 natural amino acids as AQC-derivatives, including critical isobaric pairs such as **threonine/homoserine and leucine/isoleucine**.

With proven **capability to separate allo-forms** and determine enantiomeric excess under a single set of conditions, Vaast sets a new benchmark for precision and efficiency in AA analysis.

Complete separation of the six threonine isobaric forms was achieved in **under 13 minutes** using an LC-MS-ready workflow, which also supports **UV- and fluorescence-based enantiomeric determination**. The leucine isobaric series was resolved into five distinct peaks.

Whether for metabolic research, clinical diagnostics or quality control, Vaast empowers laboratories to achieve robust, reproducible results with confidence and speed.

REFERENCES

1. Strauss KA, Puffenberger EG, Carson VJ. Maple Syrup Urine Disease. 2006 Jan 30 [Updated 2020 Apr 23]. In M. P. Adam, H. H. Ardinger, R. A. Pagon, et al. (Eds.), *GeneReviews*® [Internet]. University of Washington, Seattle. Retrieved from <https://www.ncbi.nlm.nih.gov/books/NBK1319/>
2. Ozaki, H., Takahashi, Y., & Saito, K. (2023). Separation and identification of isoleucine enantiomers and diastereomers using an original chiral resolution labeling reagent. *Chemical and Pharmaceutical Bulletin*, 71(5), 439-446. <https://doi.org/10.1248/cpb.c23-00439>
3. Konya, Y., Taniguchi, M., Fukusaki, E. (2017). Novel high-throughput and widely-targeted liquid chromatography–time-of-flight mass spectrometry method for D-amino acids in foods. *Journal of Bioscience and Bioengineering*, 123(1), 126–133. <https://doi.org/10.1016/j.jbiosc.2016.07.009>
4. Jaag, S., Valadbeigi, Y., Causon, T., Gross, H., Lämmerhofer, M. (2024). Three-minute enantioselective amino acid analysis by ultra-high-performance LC drift tube ion mobility-MS using a chiral core-shell tandem column approach. *Analytical Chemistry*, 96(2), 1234–1242. <https://doi.org/10.1021/acs.analchem.3c05426>
5. Vaast: An Innovative, Single Column Solution for the Chiral and Achiral Separation of 21 natural Amino Acids. Antoine Falatas, Nicolas Hausser and Pilar Franco, 2025, *Daicel Chiral Technologies Application Note*. https://chiraltech.com/wp-content/uploads/2025/12/Daicel-Chiral-Tech_App-Note_Vaast-Vaast-A-Single-Column-Solution-for-the-Chiral-and-Achiral-Separation-of-21-Natural-Amino-Acids.pdf
6. Falatas, A., Hausser, N., Kinderstuth, L., Schaeffer, M., Franco, P. (2025). Method for the simultaneous enantioselective analysis of 21 natural amino acids and its application to analytics of fluorenylmethoxycarbonyl-derivatives under liquid chromatography conditions, 100286. <https://doi.org/10.1016/j.jcoa.2025.100286>
7. Karongo, R., Li, F., Fiessinger, F., Horak, J., Lämmerhofer, M. (2025). Automated derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate for the enantioselective amino acid analysis of neurotensin synthesized by liquid-phase peptide synthesis. *J. Biomed. Anal.* 263, 116916. <https://doi.org/10.1016/j.jbpa.2025.116916>