APPLICATION NOTE

Vaast®: Simultaneous determination of protected and unprotected impurities in Fmoc-L-amino acid samples

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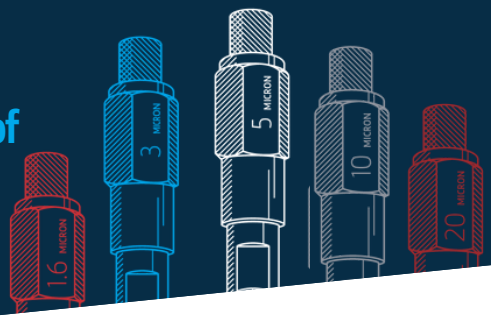

DAICEL Chiral Technologies

INTRODUCTION

Enantiomeric purity of fluorenylmethoxycarbonyl-protected or Fmoc-L-amino acids, is controlled on a routine basis to determine the content of the Fmoc-D-counterpart, which can be present as impurity. This is a standard practice in peptide production, for example.

However, the current analytical methods do not allow the simultaneous determination of potential unprotected L- and D-amino acids (AAs) which can be also present as impurities in the same sample. Free AAs require different recognition mechanisms (and thus, different columns/methods) and detection. The presence of such unprotected AA impurities can be the source of multiple by-products during the peptide production and should be avoided.

Coinciding with the recent commercial introduction of the Vaast® ion-exchanger column from Daicel, we revisited this unmet analytical need to determine whether a viable solution could be achieved. Although the Vaast® column was not originally designed for the simultaneous resolution of Fmoc-protected amino acid (Fmoc-AA) enantiomers, its ionic characteristics suggested potential for effective interaction with these compounds.

Based on this hypothesis, method development was initiated using the chromatographic conditions previously described[1]. This approach is of particular interest for facilitating the implementation of robust quality control (QC) procedures for raw materials in peptide synthesis, offering a streamlined and efficient alternative to conventional workflows.

In a separate application note[1] and journal publication[2], the use of the Vaast ion-exchanger column for the simultaneous chiral resolution of 21 natural amino acids derivatized with AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) was described. Achieving baseline separation of AQC-AA enantiomers in a single run prompted us to explore whether a similarly straightforward approach could be applied to the impurity analysis of Fmoc-protected AAs.

All Fmoc-AA enantiomers commercially available (homoserine missing) were resolved in the same conditions described in the previous Application, with only two exceptions: Fmoc-Aspartic acid and Fmoc-Proline. Those results were exceeding initial expectations, because this means that one can envisage to overcome the unmet analytical challenge by simple pre-derivatization treatment of the Fmoc-L-samples with the AQC-reagent and then proceed to the simultaneous analysis of Fmoc- and AQC-derivatives, as they are resolved in the same conditions and differ in their mass (see Figure 1). An abstract of retention times, elution orders and conditions for all Fmoc-samples tested are summarized in Table 1.

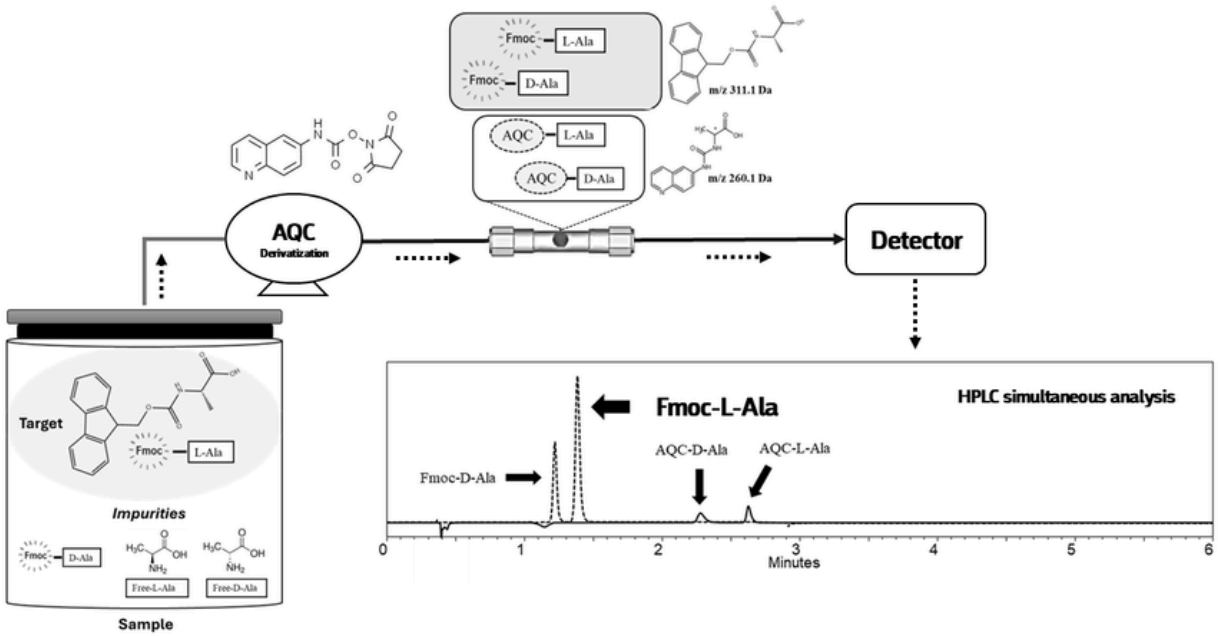

Figure 1: Figure 1: UV chromatograms at 254 nm (intensity vs LC retention time) under HPLC conditions of the 21 AQC-derivatized AAs on the Vaast column (1.7 µm, 100 x 2.1 mm). Gradient conditions as described in Experimental Section.

Subsequently, we came back to the case of Fmoc-Asp that could not be resolved using the gradient previously described. However, it was possible to develop alternative conditions with the same column in isocratic conditions with mobile phase B only. In contrast, no resolution was achieved in the case of Fmoc-Pro on that column, despite the multiple attempts. Further investigations were made in multiple columns and the simultaneous baseline resolution for the 4 enantiomeric forms on CHIRALPAK® QD-AX in less than 8 min. It is interesting to highlight that the mobile phase chosen is the same used for the resolution of Asp derivatives in the other column, reducing the number of experimental parameters.

Moreover, it is important to mention that CHIRALPAK QD-AX shows inversion of the elution order observed in the Vaast columns for AQC-Pro derivative. Therefore, in the present case, Fmoc L-Pro and AQC-D-Pro elute before their corresponding enantiomeric counterparts (see chromatograms in the figure below and results in Table 1).

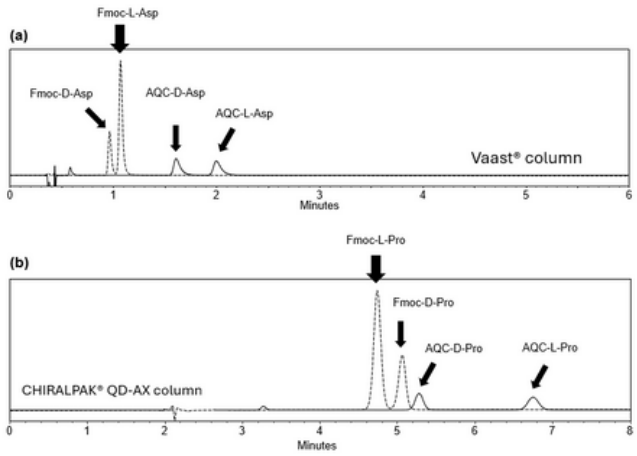

Figure 2: Overlaid chromatograms showing the baseline resolution of both Fmoc- and AQC-AA enantiomers in the same chromatographic conditions for aspartic acid and proline (see conditions in Table 1).

	Fmoc-AAs		AQC-AAs		
Amino Acids	**RT1 (min)**	**RT2 (min)**	**RT1 (min)**	**RT2 (min)**	**Analytical conditions**
Alanine	1.22 (D)	1.36 (L)	2.28 (D)	2.63 (L)	A
Arginine	2.81 (D)	3.21 (L)	3.61 (D)	4.93 (L)	A
Asparagine	1.67 (D)	2.00 (L)	2.91 (D)	3.56 (L)	A
Aspartic Acid	0.97 (D)	1.07 (L)	1.61 (D)	2.00 (L)	B
Cysteine	1.46 (D)	1.72 (L)	2.70 (D)	3.03 (L)	A
Glutamic Acid	2.76 (D)	2.94 (L)	3.32 (D)	3.55 (L)	A
Glutamine	1.54 (D)	1.81 (L)	2.78 (D)	3.04 (L)	A
Glycine	1.51		2.85		A
Histidine	2.23 (D)	2.70 (L)	3.24 (D)	4.08 (L)	A
Isoleucine	1.09 (D)	1.29 (L)	1.62 (D)	2.34 (L)	A
Leucine	1.06 (D)	1.21 (L)	1.76 (D)	2.17 (L)	A
Lysine	2.67 (D)	2.85 (L)	3.21 (D)	3.55 (L)	A
Methionine	1.25 (D)	1.46 (L)	2.22 (D)	2.66 (L)	A
Phenylalanine	1.35 (D)	1.57 (L)	2.13 (D)	2.68 (L)	A
Proline	4.74 (L)	5.06 (D)	5.27 (D)	6.75 (L)	C
Serine	1.47 (D)	1.81 (L)	2.73 (D)	3.29 (L)	A
Threonine	1.31 (D)	1.68 (L)	2.45 (D)	3.10 (L)	A
Tryptophan	1.63 (D)	2.01 (L)	2.62 (D)	3.62 (L)	A
Tyrosine	1.69 (D)	1.97 (L)	2.66 (D)	3.05 (L)	A
Valine	1.12 (D)	1.35 (L)	1.71 (D)	2.49 (L)	A

Table 1: Retention times of Fmoc-AA and AQC-AA derivatives analyzed on Vaast column under conditions detailed above.

(A) Gradient conditions : Mobile phase A – 10 mM formic acid + 10 mM ammonium formate in Acetonitrile/water (93/7; v/v) and mobile phase B - 50 mM formic acid + 50 mM ammonium formate in Methanol/Acetonitrile (75/25; v/v) (Gradient details can be found in experimental section), flow rate: 0.8 ml/min, temperature: 50°C; Vaast column.

(B) Isocratic conditions : 50 mM formic acid + 50 mM ammonium formate in Methanol/Acetonitrile (75/25; v/v), flow rate: 0.8 ml/min, temperature: 50°C; Vaast column.

(C) Isocratic conditions : 50 mM formic acid + 50 mM ammonium formate in Methanol/Acetonitrile (75/25; v/v), flow rate: 0.8 ml/min, temperature: 50°C; CHIRALPAK®.

CONCLUSIONS

The systematic control the enantiomeric purity of Fmoc-L-AAs with simultaneous determination of potential unprotected L- and D-AA impurities becomes easier with the Vaast column. The analytical conditions described here have been specifically achieved on a single chiral ion-exchanger column, except for Fmoc-Pro. Such a simplified workflow opens new perspectives in the control of AA impurities.

The developed methods were tested with AA standards and their derivatives, delivering exceptional resolutions of a broad range of natural AA enantiomers under consistent conditions. This versatility opens new horizons for chiral quality assurance and impurity profiling.

Complete separations were achieved in under 10 min, with an LC-MS-ready workflow that also supports UV- and fluorescence-based enantiomeric determination.

REFERENCES

1. 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.
2. 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. *Journal of Chromatography Open*, 100286. https://doi.org/10.1016/j.jcoa.2025.100286

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