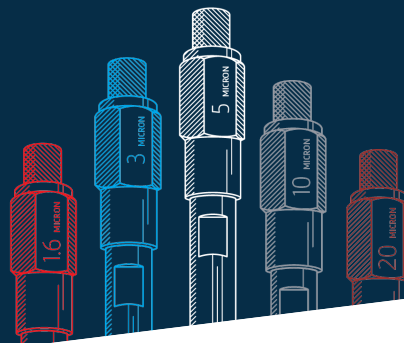


Expanding Peptide Analysis Chiral and Achiral Separation of Native Peptide Variants on the Daicel Vaast Column

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INTRODUCTION

The **Daicel Vaast ion-exchange column** exploits its unique ionic separation mechanism to resolve short peptides, including variants with subtle stereochemical and minimal structural differences. This advanced capability addresses a critical need in modern biopharma, where peptide therapeutics are rapidly expanding due to their high specificity, potent bioactivity and favorable safety profiles.

Regulatory agencies emphasize the characterization of product- and process-related impurities as a critical element of quality assessment.^{1,2} However, peptide characterization remains analytically challenging, due to their inherent structural complexity, susceptibility to degradation and the fact that peptides are particularly prone to generate structurally related impurities during synthesis, purification and storage.^{3,4} Consequently, no single analytical method can typically resolve all peptide variants.⁵

A methodology combining the Daicel Vaast column with chemical derivatization was recently developed to improve impurity

identification capabilities while enabling enhanced UV or fluorescence detection of peptide impurities present at very low levels. This approach proved highly effective.⁶

Building upon these findings, the present work extends the application of **Vaast column** and demonstrates its complementary capability for the **direct chromatographic separation** of native short peptides and peptide fragments, without requiring derivatization (Figure 1). Direct analysis preserves the native molecular composition, which is especially valuable in peptidomics and biomarker discovery.^{7,8} It is also compatible with complementary LC-MS strategies such as peptide mapping, degradation profiling and structural confirmation.⁹

The results highlight the potential of the Vaast column as a rapid and versatile complementary tool for impurity profiling, process development and product quality assessment in comprehensive peptide analysis.

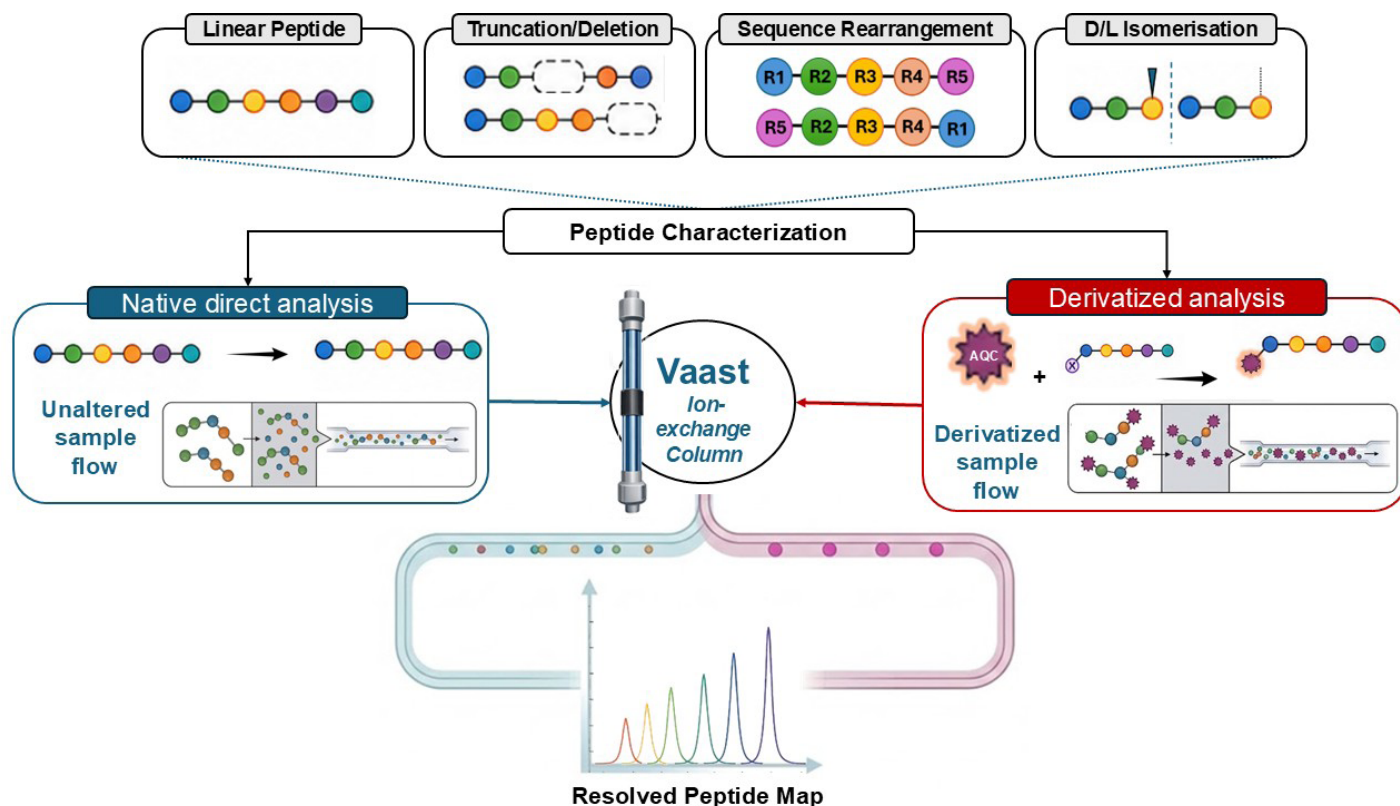
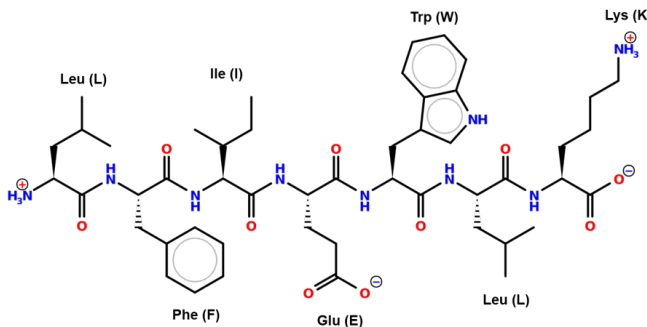


Figure 1. Schematic representation of the analytical workflow for profiling and characterization of peptide modifications via native direct and derivatized analysis using Vaast.

DISCUSSION

The investigation was conducted using a model peptide composed of seven L amino acids (L AAs) and several sequence variants (see Table 1). Variants containing D amino acid substitutions, as well as positional and deletion modifications, were included to assess the specific discrimination capabilities of the method.

In a first step, the stereoselective performance of the Vaast stationary phase was evaluated using the native seven L AA model peptide and three variants, each incorporating a single D-amino acid substitution at either the N-terminus, a central position or the C-terminus.

Evaluated model peptide variants and their comparative properties			
Peptide*	MW (g/mol)	Isoelectric point (pI)	Hydrophobicity (Eisenberg scale)
LFIEWLK	948.2	6.94	3.26
			
Stereoisomeric variants			
IFIEWLK	948.2	6.94	3.26
LFIEWLK	948.2	6.94	3.26
LFIEWLk	948.2	6.94	3.26
Positional variant			
KFIEWLL	948.2	6.94	3.26
Deletion variant			
LFIEWL(-K)	820.0	4.00	4.76
(-L)FIEWLK	835.0	6.61	2.20

One-letter codes are used to describe the AA sequence; the lowercase letters denote the D-forms; capital letters are used for the L-forms of the peptide. Variant's changes from the parent peptide are in red.

Successful chromatographic discrimination for all four stereoisomeric variants was achieved, without the need of pre-derivatization, despite only a single stereocenter differing from the parent peptide. Optimal separation was obtained under isocratic conditions using 100% methanol; the salts required for the ionic recognition mechanism (25 mM formic acid and 25 mM ammonium formate) (Figure 2).

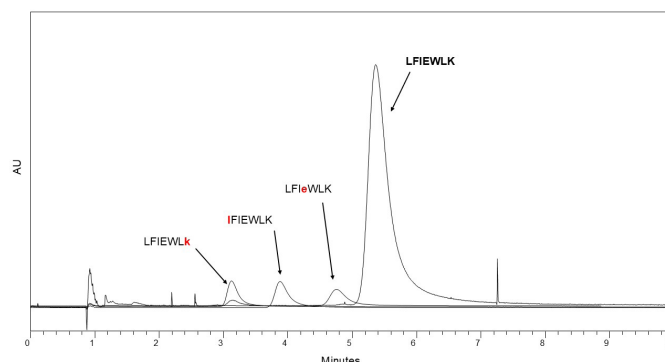


Figure 2. Overlaid UV chromatograms at 270 nm under UHPLC conditions of the four native peptide stereochemical variants (7-AAs) on the Vaast column using the conditions described in Experimental Section. Adapted scale to differentiate parent peptide from variants.

The observed elution order was LFIEWLk < IFIEWLK < LFIEWLK < LFIEWLK (parent peptide) indicating that peptide variants containing D-amino acids in their sequence consistently eluted earlier than the parent peptide under the conditions employed in this study. The enhanced chiral recognition capability of the Vaast column enabled discrimination between the stereoisomeric peptide variants, resulting in distinct chromatographic behaviors and retention times. These results demonstrate the column's ability to resolve subtle stereochemical differences within peptide sequences. The peptide containing a D-Lysine variant (LFIEWLk) consistently exhibited the shortest retention time of 3.1 min because lysine is located at the C-terminus and possesses a long, positively charged side chain. The D-Leucine variant (IFIEWLK) produced an intermediate chromatographic effect, showing a retention time of 3.9 min. Leucine primarily contributes to the hydrophobic character of the peptide. Changing stereochemistry does not modify global hydrophobicity but may alter local side-chain orientation and peptide conformation, producing a significant reduction in retention in comparison to the native peptide sequence being eluted at 5.4 min. The D-Glutamic acid variant (LFIEWLK), eluted at 4.7 min, exhibited the highest retention among the stereoisomeric variants. Indeed, glutamic acid is centrally located within the peptide and participates in hydrogen-bonding and local electrostatic interactions.

On the second part of the investigation, positional and deletion variants were analyzed. Under the same chromatographic conditions, complete resolution was also achieved for peptide variants exhibiting sequence rearrangements (Figure 3). The rearranged peptide KFIEWLL exhibited a longer retention time (7.7 min) than the native peptide LFIEWLK. Although both peptides contain the same amino acid composition, the altered sequence changes the spatial distribution of hydrophobic and charged residues, which may modify their interactions with the stationary phase and result in increased retention.

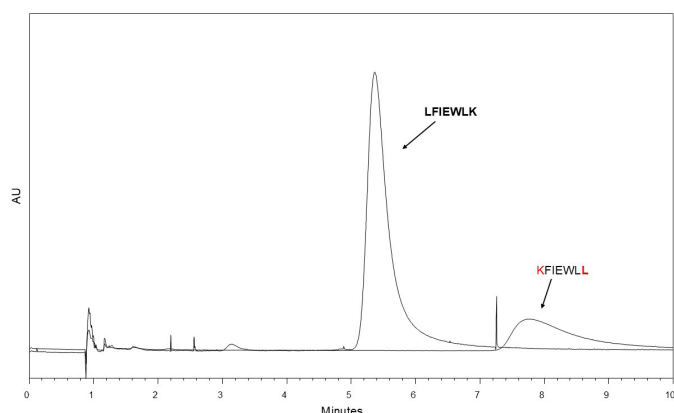


Figure 3. Overlaid UV chromatograms at 270 nm under UHPLC conditions of the native peptide with only one amino acid positional variant in the N-terminus/C-terminus (7-AAs) on the Vaast column using the conditions described in Experimental Section. Adapted scale to differentiate parent peptide from variants.

Distinct chromatographic behavior was also observed for peptide truncation variants (Figure 4). Deletion of the C-terminal lysine residue (LFIEWL(-K)) resulted in a markedly shorter retention time (2.7 min), whereas deletion of the N-terminal leucine residue ((-L)FIEWLK) produced a considerably longer retention time (6.0 min). These results indicate that chromatographic retention depends not only on the loss of an amino acid residue, but also on its position within the peptide sequence. Removal of the positively charged lysine substantially alters the overall charge distribution and interaction profile of the peptide, while deletion of the N-terminal leucine primarily affects the hydrophobic character and local sequence environment, leading to distinct retention behaviors.

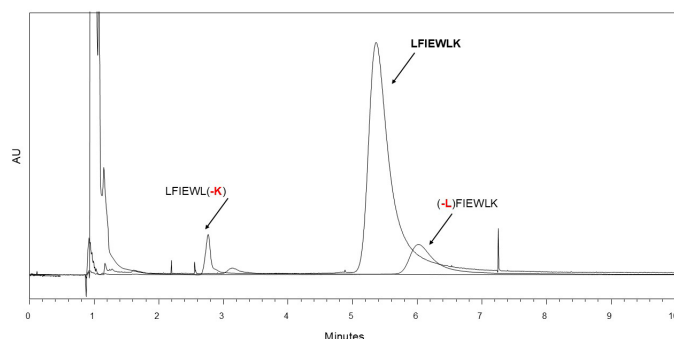


Figure 4. Overlaid UV chromatograms at 270 nm under UHPLC conditions of the native peptide (7-AAs) and its deletion variants (6-AAs) on the Vaast column using the conditions described in Experimental Section. Adapted scale to differentiate parent peptide from variants.

Overall, these results demonstrate the capability of the Vaast column to discriminate structurally related peptide variants, stereochemical inversion, sequence rearrangements and truncation products. Such variants often possess nearly identical molecular masses and highly similar physicochemical properties (Table 1), making their chromatographic discrimination particularly challenging using conventional analytical methods. These observations further highlight the complementary chromatographic selectivity provided by the Vaast column for the analysis of closely related peptide variants (see full overlay in Figure 5).

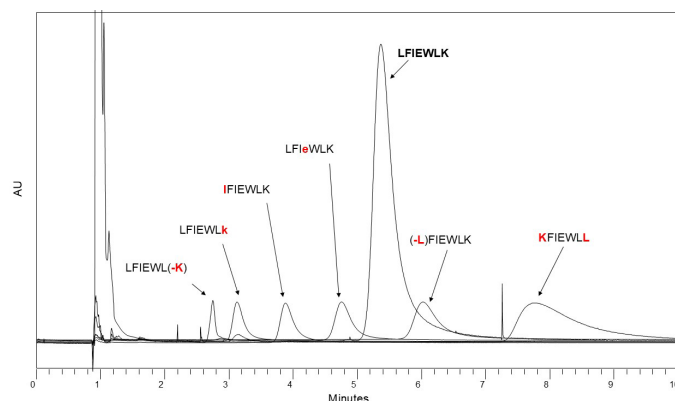


Figure 5. Overlaid UV chromatograms at 270 nm under UHPLC conditions of the native structurally related peptide variants on the Vaast column as described in Experimental Section. Adapted scale to differentiate parent peptide from variants.

EXPERIMENTAL SECTION

Chromatographic conditions for peptide separation

Column	Vaast (100 mm x 2.1 mm i.d. 1.7 µm)
Mobile phase	25 mM formic acid + 25 mM ammonium formate in methanol 100%
Mode	Isocratic
Flow rate	0.3 mL/min
Detection	UV at 270 nm
Temperature	25°C
Sample	100 µg/mL in appropriate diluent
Injection volume	3 µL

Diluent: All variants were dissolved in a water/acetonitrile mixture (1:1, v/v) containing 0.1% formic acid, except for the Lys-deletion variant (LFIEWL), which was dissolved in an acetic acid/water mixture (9:1, v/v).

CONCLUSION

This study demonstrates that the Daicel Vaast column provides a powerful and differentiated approach for the direct analysis of native peptide variants. Excellent discrimination was achieved for stereochemical, positional and truncation-related modifications of a model peptide, even when the variants exhibited highly similar molecular characteristics.

By enabling the separation of closely related peptide species without derivatization, Vaast delivers an additional dimension of selectivity that complements established analytical techniques that are currently used in peptide research, development and manufacturing. This analytical capability is increasingly valuable as peptide therapeutics continue to grow in importance and complexity.

The peptide examples presented here represent only a first step in exploring the full analytical potential of technology. Future investigations will evaluate a wider range of peptide lengths, linear, and cyclic sequences and variant types to further expand application opportunities and deepen understanding of Vaast selectivity mechanisms.

These results reinforce the value of Vaast Column as an innovative solution for next-generation peptide characterization challenges

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