APPLICATION NOTE

Chiral analysis of short peptides and peptide fragments with Vaast column

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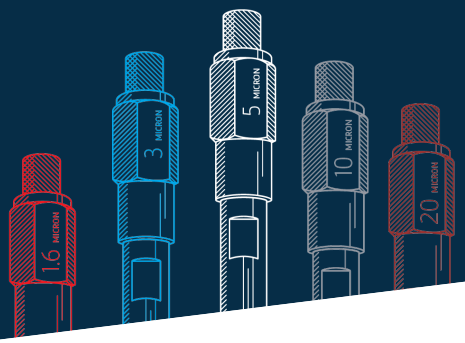

DAICEL Chiral Technologies

INTRODUCTION

Peptides are short chains orderly arranged of amino acids that can be linear, branched or cyclic, typically consisting of fewer than 50 amino acids, linked together by peptide bonds. They occur naturally in the body and play essential roles in cell signaling, hormonal regulation, immune response and metabolism. Well known biological examples include insulin, oxytocin or endorphins. Consequently, the growing importance of peptide-based therapeutics, such as GLP-1 analogues (Glucagon like peptide 1) for diabetes treatment as well as peptide fragments used to enhance biological transport or drug delivery, is unsurprising. Peptides thus play an increasingly pivotal role in modern medicine and biotechnology, offering innovative solutions for a range of health, food, and cosmetic applications[1].

With the widespread introduction of peptide based molecules into therapeutic pathways, control of their purity has become a critical health and safety issue. However, several analytical and manufacturing challenges remain. Peptides are mostly synthetized using solid-phase peptide synthesis (SPPS), a widely used technique that is easily automated. In this approach, Fmoc-protected amino acids are sequentially assembled, on a resin support to form the peptide chain[2]. During peptide synthesis, impurity formation can arise mainly from two sources: (i) impurities present in the starting amino acid building blocks and (ii) degradation or chemical transformation occurring during peptide assembly.

Quality control of starting amino acid derivatives typically focuses on chemical and enantiomeric purity, often using chiral stationary phases. Nevertheless, the simultaneous assessment of both the derivative's purity and the presence of free amino acids within the same starting material is still uncommon. A methodology addressing this limitation has recently been developed by our team and is described in separate communication[3].

In addition, certain amino acids are prone to epimerization once incorporated into a peptide chain. This chemical transformation, which converts a stereogenic center into its epimer, can significantly affect the overall conformation of the

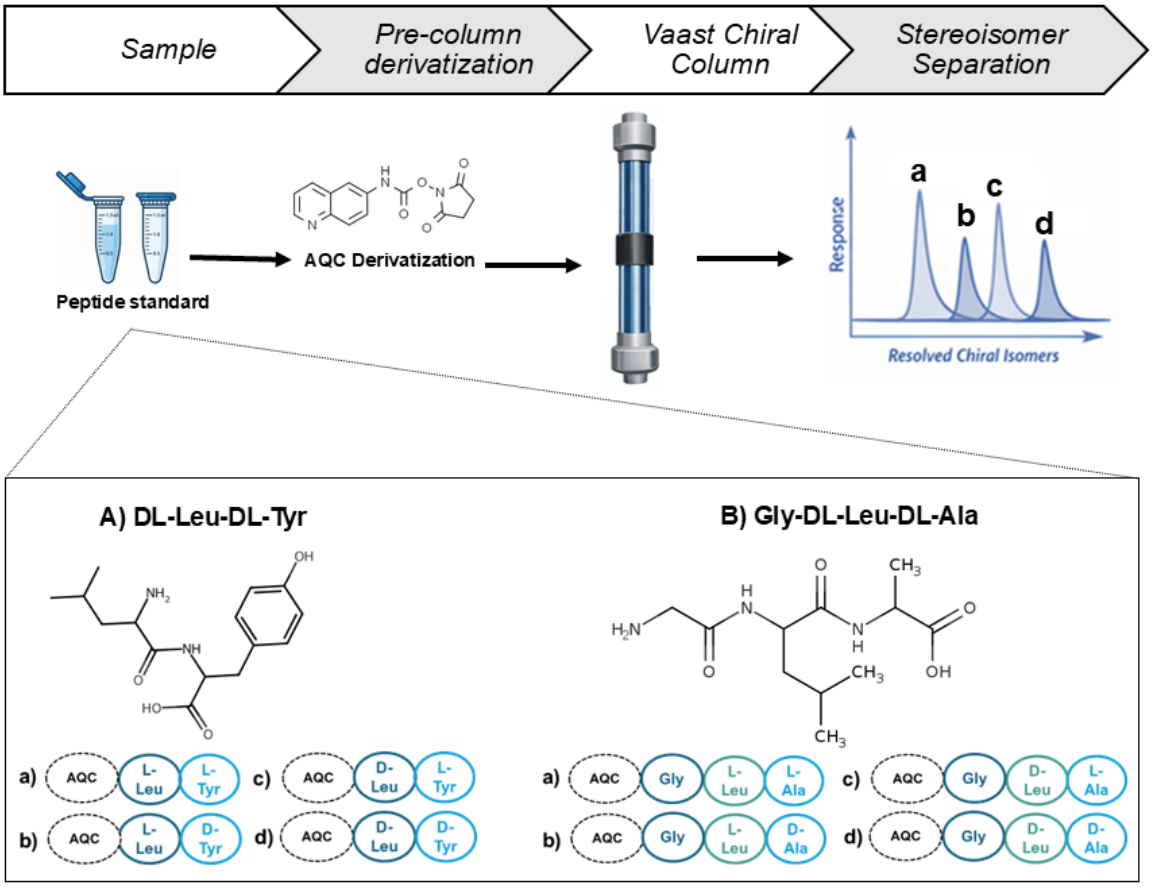

Figure 1. Representative scheme of the pre-column AQC derivatization protocol and the chromatographic separation of the resulting derivatized peptide isomers on the Vaast column.

molecule and may ultimately alter its biological activity. Epimerized products exhibit highly similar physicochemical properties, making their purification particularly challenging[4]. Furthermore, peptides containing multiple stereogenic centers often form complex stereoisomeric mixtures that are difficult to resolve using conventional reversed-phase liquid chromatography. These limitations underscore the need for alternative separation strategies capable of addressing the stereochemical complexity inherent to peptide based systems.

Due to the high analytical complexity associated with long peptides and their related impurities, several analytical strategies have been developed that rely on the partial digestion of peptides into smaller fragments or even into their individual amino acid building blocks[5,6]. However, the short peptides generated by this approach frequently retain multiple stereogenic centers, hereby increasing the analytical complexity of peptide characterization. Therefore, is critical that digestion conditions strictly preserve the stereochemical integrity of all resulting species, as any racemization would directly compromise the reliability of the analysis[7].

Short peptides themselves have also attracted increasing interest in recent years. In particular, dipeptides and tripeptides have emerged as a promising class of functional excipients in parenteral protein formulations. Their modular architecture enables precise modulation of key physicochemical properties such as polarity, charge distribution, and hydrogen-bonding capacity, offering formulation flexibility beyond that of individual amino acids[8]. In addition, short peptides can also serve as transport moieties, further expanding their functional relevance[9].

Within this context, the analytical characterization of short peptides has become a critical component not only for the control of these excipients themselves, but also for ensuring the quality of larger peptide and protein-based molecules. Effective stereochemical control of short peptides or peptide fragments can be achieved through direct chiral analysis using appropriate chiral stationary phases (CSPs), selected according to the structural features of the peptide fragment. Zwitterionic and crown ether–based selectors have proven effective for the separation of various small peptides and digested peptide fragments under chromatographic conditions[6].

Nevertheless, the inherently limited number of functional groups available for interaction with the chiral phase, combined with the relatively weak UV absorbance of short peptides, presents significant challenges for achieving sufficient resolution and sensitive detection. Against this backdrop, a pre column derivatization strategy was envisaged as an effective means to enhance chiral recognition, improve detection sensitivity and expand impurity profiling capabilities. Pre column derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) is widely employed for the analysis of amino acids due to its high reaction efficiency and the introduction of a strong chromophore and fluorophore, enabling sensitive detection by LC-UV and LC-MS[10].The recent introduction of the DAICEL Vaast ion exchange column has enabled the simultaneous chiral resolution of 21 natural amino acids derivatized with AQC and the chromatographic conditions can be extended to many other amino acid types[11,12].

While AQC-derivatization strategy is well established for amino acid profiling, its application to chiral separation of structurally complex peptides remains limited. Nevertheless, this approach represents a promising strategy to address the previously described analytical challenges.

In this study, we demonstrate the capability of the Vaast column to resolve stereochemical complexity in short peptide systems containing multiple chiral centers (Figure 1).

EXPERIMENTAL

Isocratic conditions for separation of AQC-DL-Leu-DL-Tyr	
Mobile phase	10 mM formic acid + 10 mM ammonium formate in methanol/acetonitrile/water (49/49/2 – v/v/v)

Gradient conditions for separation of AQC-Gly-DL-Leu-DL-Ala			
Mobile phase	Mobile phase A: 10 mM formic acid + 10 mM ammonium formate in methanol/water (93/7 – v/v) Mobile phase B: 10mM formic acid+ 10 mM ammonium formate in acetonitrile/water (93/7 – v/v)		
Gradient program	**Time (min)**	**Solvent A (%)**	**Solvent B (%**
	0.0	100	0
	4.0	100	0
	30.0	0	100
	34.0	0	100
	34.1	100	0
	50.0	100	0

General chromatographic conditions	
Column	Vaast (100 mm x 2.1 mm i.d, 1.7 µm)
Flow rate	0.2 ml/min
Detection	UV at 254 nm
Temperature	25°C
Sample	1 mM in prepared Derivatization Solution (following derivatization protocol[3])
Injection volume	2 µl

DISCUSSION

To evaluate the applicability of the Vaast ion-exchange column for the analysis of short peptides, two peptide systems were investigated. The selected models consisted of a dipeptide and a tripeptide. Despite their different length, both systems contain two stereogenic centers overall, resulting in four stereoisomers expected for each sample, as glycine is achiral and present in the tripeptide.

It is important to mention that the stereoisomers of these two peptides were previously separated without derivatization using the zwitterionic chiral stationary phase, CHIRALPAK ZWIX(+)[13] with an Evaporative Light Scattering Detection (ELSD) detection, due to the limited UV-absorbance. In this work, the AQC-derivatization was required to resolve the four stereoisomers on the Vaast column.

At the beginning, the stereoselective performance of the Vaast stationary phase was evaluated using the dipeptide AQC-DL-Leu-DL-Tyr under optimized isocratic conditions with a mixture of methanol/acetonitrile and a low water percentage (49/49/2). We noticed an enabled efficient separation of stereoisomeric species (Figure 2A). Four peaks were detected at 9.1, 9.8, 10.9, and 18.2 minutes. Despite the close structural similarity of the stereoisomers, the stationary phase exhibited sufficient selectivity to resolve the peptide variants within a relatively short analysis time.

To further assess the versatility of the method, a second, more complex peptide system was examined. For the tripeptide AQC-Gly-DL-Leu-DL-Ala, after method optimization steps, a baseline stereochemical discrimination

between these stereoisomers was achieved under gradient conditions (Figure 2B and experimental section). Four well-resolved chromatographic peaks were observed at retention times of 4.1, 4.8, 5.7, and 8.1 minutes, corresponding to the four distinct stereoisomeric peptide species as depicted in Fig. 1.

Overall, the observed stereoselective separations demonstrate the ability of the Vaast ion-exchange stationary phase to recognize subtle differences in peptide stereochemistry. Furthermore, AQC-derivatization not only enhanced chromatographic resolution, but also facilitated straightforward UV detection at 254 nm.

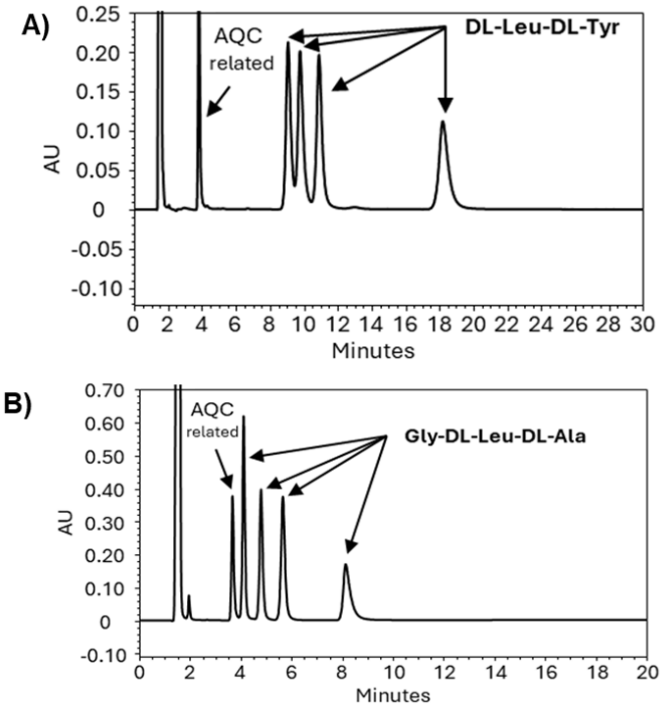

Figure 2. UHPLC-UV chromatograms at 254 nm of AQC-derivatized short peptide enantiomers obtained on a Vaast column. (A) AQC-DL-Leu-DL-Tyr under isocratic conditions. (B) AQC-Gly-DL-Leu-DL-Ala under gradient conditions. Chromatographic conditions as described in Experimental section. The elution order was not assigned because only stereoisomeric mixtures were available.

CONCLUSIONS

This study demonstrates that the Vaast ion-exchange column, when combined with pre column AQC derivatization, provides an effective and practical solution for the stereochemical analysis of short peptides or peptide fragments. Baseline separation of all expected stereoisomers was achieved for both di- and tripeptide model systems, underscoring the column strong chiral recognition capability even for closely related peptide isomers. These findings position the approach as a compelling alternative to conventional chromatographic methods, which often struggle with the inherent stereochemical complexity of peptides.

Efficient resolution of stereoisomeric species of short peptide systems was achieved, representing the first reported application of this methodology to AQC-derivatized peptides beyond single amino acid analysis. Moreover, AQC-derivatization proved essential for this approach, enhancing chiral selectivity while simultaneously enhancing detection sensitivity through the introduction of a strong UV and fluorescence chromophore. This dual improvement in selectivity and sensitivity enables the direct extension of established amino acid analytical workflows to more complex peptide systems of significant interest for biopharmaceutical research and industry. The robustness and flexibility of the method indeed make it well suited for routine impurity profiling, epimer monitoring and quality control of peptide-based excipients and therapeutic products across multiple stages of peptide manufacturing, including starting material characterization, in-process monitoring, and final product analysis. It is therefore positioned as a valuable addition to the analytical toolbox for peptide-based drug development.

Future work will focus on expanding this methodology to a wider range of peptide sequences and evaluating its performance or more complex peptide entities, further strengthening its potential as a routine analytical solution in peptide development and manufacturing. In addition, the separation of unprotected peptides will be explored, as partial resolution may also be achievable for these species, despite the column being less specifically optimized for such molecules.

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