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Case study

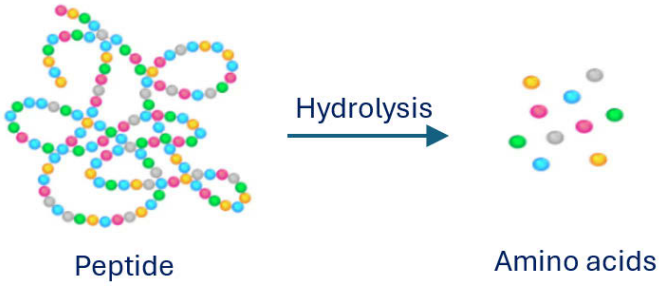
# Determination of Peptide Chiral Purity Using Marfey's Reagent and Accurate Mass Liquid Chromatography Mass Spectrometry

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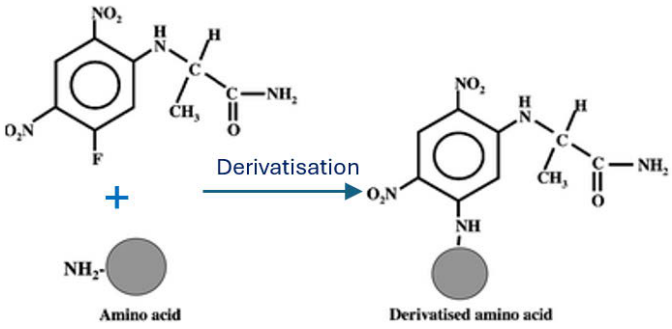
# Determination of Peptide Chiral Purity Using Marfey's Reagent and Accurate Mass Liquid Chromatography Mass Spectrometry


## Abstract

Accurate determination of chiral purity in synthetic peptides is essential for evaluating the quality and safety of peptide-based pharmaceutical products. D-isomeric forms may arise during synthesis or storage due to impurities in amino acid starting materials or racemisation processes. This study presents a rapid and precise LC-ESI-MS (liquid chromatography–electrospray ionisation–mass spectrometry) method for quantifying the enantiomeric composition of amino acids within peptides. Peptides are hydrolysed using deuterated hydrochloric acid, followed by derivatisation with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA), commonly known as Marfey's reagent, to form diastereomers. These D- and L-amino acid derivatives are then separated in a single run using conventional reverse phase high performance liquid chromatography (RP-HPLC). Deuterated hydrolysis mitigates racemisation artifacts by incorporating a deuterium atom at the α-carbon of racemised amino acids, allowing for their exclusion from MS detection. The developed protocol demonstrated excellent selectivity and reproducibility in distinguishing D-amino acids from their L counterparts. Furthermore, its successful application to complex biological samples at Almac highlights its potential use in proteomics and peptidomics.


### 1. Acid Hydrolysis




### 2. Derivatisation




### 3. Separation and Detection by LC-MS

## Introduction

L-amino acids are the predominant structural units of naturally occurring proteins, making the assessment of chiral purity in synthetic peptide-based medicinal products essential for ensuring patient safety and therapeutic effectiveness. Synthetic peptides may contain unwanted D-isomer impurities, which can originate from several sources e.g. residual D-isomers in protected L-amino acid starting materials, racemisation during activation steps prior to coupling in solid-phase peptide synthesis (SPPS), during subsequent processing steps such as hybrid coupling methods or long-term storage.

To minimise the formation of D-isomer impurities, control strategies should include the specification of starting materials, the use of reagents that suppress racemisation during activation, parametric controls to avoid conditions favourable to racemisation, and appropriate storage protocols. Effective analytical methods are crucial to support these controls by enabling accurate and precise quantification of D-isomer impurities.

Although high-performance liquid chromatography (HPLC) is widely used to separate and quantify chemical impurities, detecting diastereomers generated through amino acid racemisation or epimerisation remains challenging. This is due to the subtle structural differences and identical masses between isomers. Chiral stationary phases have demonstrated limited success in separating D-isomer impurities within intact peptides, and reversed-phase chromatographic resolution is both difficult to predict and often unsuccessful.

In the current study, the chiral purity of amino acids within a peptide is assessed by first subjecting the peptide to acid hydrolysis using deuterated HCl, which breaks it down into its constituent amino acids. These amino acids are then derivatised and separated using hyphenated chromatographic techniques[1,2]. Acid hydrolysis can however induce racemisation, converting L-amino acids to D-amino acids and vice versa therefore during this process, the hydrogen on the α - carbon may be replaced with deuterium, as illustrated in Figure 1, resulting in a mass increase of +1 Da. Such racemised amino acids can be excluded using MS detection from further analysis[3].

To detect only the non-racemised amino acids, the derivatisation is carried out using Marfey's reagent as shown in Figure 2. The resulting derivatives are analysed by liquid chromatography–mass spectrometry (LC–MS) on an achiral column, enabling selective identification of amino acids that retain their original chirality. This approach allows for accurate determination of the initial chiral purity of each amino acid in a peptide using standard achiral reversed-phase HPLC columns.

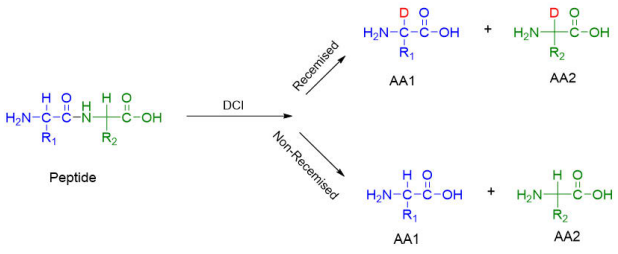



**Figure 1: Amino Acid Racemisation during Peptide Hydrolysis using DCl**

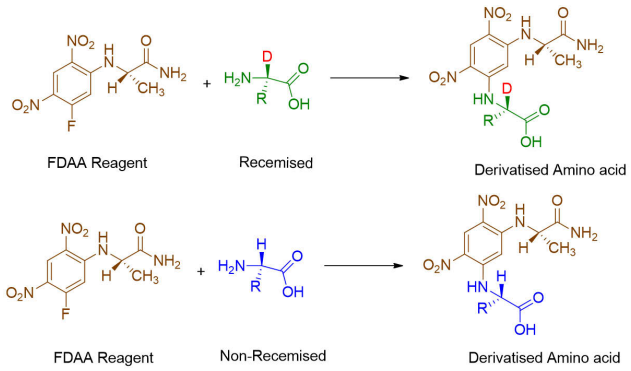



**Figure 2: Reaction of FDAA with Amino Acid**

## Method

Standard and Sample Preparation L- and D- amino acid mixed standards were prepared at a concentration of 50µg/mL and samples were hydrolysed in using 6N DCl. After hydrolysis the standards and samples were freeze dried using liquid nitrogen and derivatised using Marfey's reagent. The derivatised amino acids were separated by RP-HPLC and analysed by high resolution LC-ESI-MS. Mass Spectrometry detection using single ion monitoring mode (SIM) for each individual FDAA-amino acid derivative was employed.

## Results and Discussion

Derivatisation of amino acids with FDAA reagent produces diastereomers with high sensitivity and specificity. All amino acids were robustly derivatised with FDAA resulting in 2,4-dinitrophenyl-5-L-alanineamide (DNPA) derivative amino acids. Chromatographic peaks were extracted using specific m/z values for each derivatised amino acid. Figure 3 shows typical separation of all D- and L- derivatised amino acid derivatives by RP-HPLC-MS and the retention times of L- and D- amino acids are listed in Table 1. In this MS-platform multiple deprotonated molecular ions [M-H]- were monitored for each expected m/z value corresponding to a non-racemised DNPA-amino acid present in the peptide prior to hydrolysis.

Glutamine and asparagine were deaminated during hydrolysis to form glutamic acid and aspartic acid. Therefore, glutamine and asparagine were identified as the corresponding acid derivative. Accurate mass determination provided extra selectivity that allowed additional confidence in results. Enantiomeric purity is calculated via the following equation for each amino acid.

$$\text{Individual D Isomer (\%)} = \frac{T_{\text{Area of D}}}{T_{\text{Area of D}} + T_{\text{Area of L}}} \times 100$$

Where:

$T_{\text{Area of D}}$ = Area of Individual D – Amino Acid in hydrolysis sample

$T_{\text{Area of L}}$ = Area of Individual L – Amino Acid in hydrolysis sample

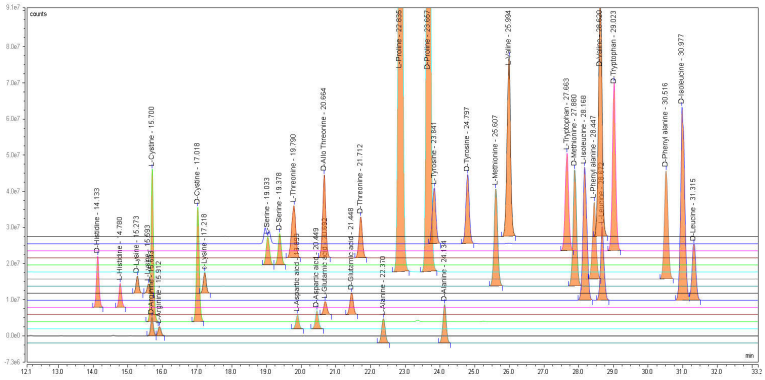


**Figure 3: SIM Channels of D-Amino Acids and L-Amino Acids**

| Amino Acid | L-AA RT (minutes) | D-AA RT (minutes) |
|---|---|---|
| Alanine (Ala, A) | 22.370 | 24.134 |
| Arginine (Arg, R) | 15.912 | 15.693 |
| Aspartic acid (Asp, D) | 19.890 | 20.449 |
| Asparagine (Asn, N) | | |
| Cystine (Cys, C) | 15.700 | 17.018 |
| Glutamic acid (Glu, E) | 20.692 | 21.448 |
| Glutamine (Gln, Q) | | |
| Histidine (His, H) | 14.780 | 14.133 |
| Isoleucine (Ile, I) | 28.168 | 30.977 |
| Leucine (Leu, L) | 28.672 | 31.315 |
| Lysine (Lys, K) | 15.593 | 15.273 |
| Methionine (Met, M) | 25.607 | 27.880 |
| Phenylalanine (Phe, F) | 28.447 | 30.516 |
| Proline (Pro, P) | 22.835 | 23.657 |
| Serine (Ser, S) | 19.033 | 19.378 |
| Threonine (Thr, T) | 19.790 | 21.712 |
| Tryptophan (Trp, W) | 27.663 | 29.025 |
| Tyrosine (Tyr, Y) | 23.841 | 24.797 |
| Valine (Val, V) | 25.994 | 28.620 |

**Table 1: Retention Time of Amino Acids**

## Conclusion

This study presents a robust chiral HPLC-ESI-MS method for the accurate determination of enantiomeric purity in synthetic peptides. By integrating Accurate Mass LC-MS with FDAA derivatisation, the approach enables highly sensitive and selective quantification of D-amino acid residues, even at low levels ranging from 0.10% to 1.0%. The use of deuterated hydrolysis minimises racemisation artifacts, ensuring reliable assessment of original stereochemistry. Overall, this analytical strategy offers a powerful tool for the detailed characterisation of peptide chirality, thereby supporting the development and quality control of bioactive synthetic peptides intended for pharmaceutical applications.

## References


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