Diagnostic 7-Mer Model Peptide for Rapid Evaluation of Cysteine Racemization in Solid-Phase Peptide Synthesis

Saundra Mason, Wesley Grenier, Dario Slavazza, Hanson Chang
CSBio Instrumentation, Mountain View, CA, 94043

https://doi.org/10.17952/38EPS.2026.P002

Introduction

- High-temperature synthesis reduces reaction time and can improve crude purity and yield.
- Elevated temperatures increase the risk of racemization in sensitive residues like Cysteine (Cys).
- Racemization is difficult to detect as L and D forms may co-elute.
- Epimerization can change peptide 3-D structure, affecting biological activity and safety.
- Regulatory bodies expect impurities to be appropriately characterized and controlled.
- We developed a diagnostic 7-mer (KGP**X**GEG) that can be used to screen synthesis conditions that cause racemization. This sequence can be used to identify Cysteine racemization on a standard RPHPLC C18 column without pre-column derivatization.

Method

Syntheses were carried out on a CSBio II utilizing conduction heating with preheated solvents.



Coupling and deprotection times are specified in the figure legends. Synthesis methodology and analytical methods are summarized below.

Parameter	Specification/Condition
Model Sequence	KGP**X**GEG-NH_2 (X = L-Cys or D-Cys)
Resin	Rink-Amide Resin
Coupling Conditions	DIC/Oxyma: 90 °C, 110 °C HBTU/DIEA: 30 °C, 45 °C, 60 °C, 90 °C
HPLC	Stationary Phase: C18 Buffer A: 0.1 % TFA:H_2O Buffer B: 0.1 % TFA:ACN Phenomenex Luna C18 (250 x 4.6 mm) 214 nm Flow rate: 1 mL/min 0 to 30 % B in 20 min Column temperature: 25 °C
MS	ITMS ESI+ (Identity Confirmation Only)
Chiral AA Analysis of DIC/Oxyma 90 °C L-Cys and D-Cys Peptides by GC-MS	C.A.T. Labs method A.0.3 Enantiomeric Purity

Table 1. Synthesis parameters and analytical methods.

DIC/Oxyma Synthesis

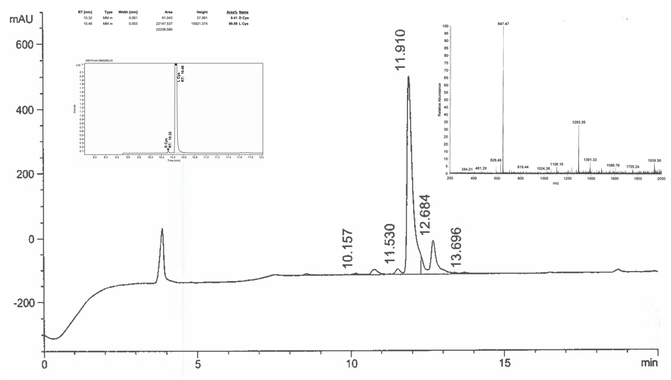


Figure 1. HPLC of peptide synthesized with **L-Cys in sequence position X using DIC/Oxyma at 90 °C**, 1 min deprotection and 5 min coupling. L-Cys variant elutes at ~11. 9 min. MS shows m/z 647. Enantiomeric purity 99.59% L-Cys (C.A.T. Lab Method A.03).

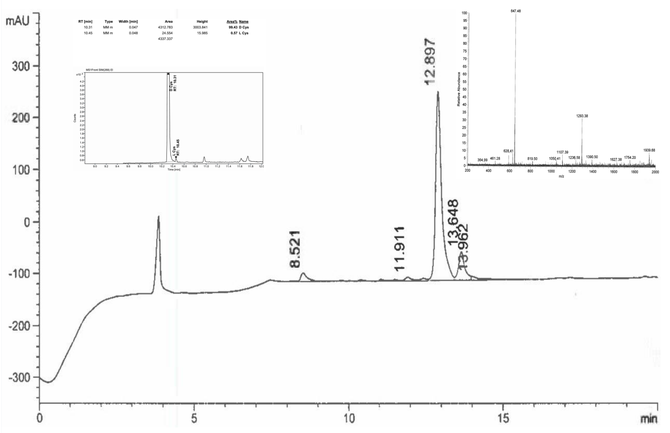


Figure 2. HPLC of peptide synthesized with **D-Cys in position X using DIC/Oxyma at 90 °C**, 1 min deprotection and 5 min coupling. D-Cys variant elutes at ~12.9 min. MS shows m/z 647. Enantiomeric purity 99.43% D-Cys (C.A.T. Lab Method A.03).

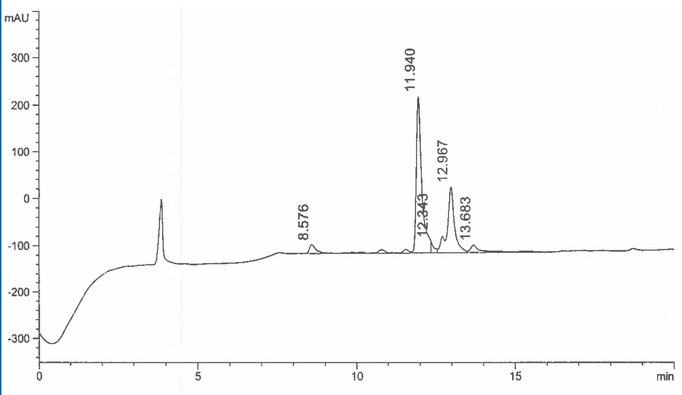


Figure 3. Co-injection HPLC of L-Cys and D-Cys enantiomerically pure peptides synthesized with DIC/Oxyma at 90 °C, 1 min deprotection and 5 min coupling. The L-Cys and D-Cys variants elute at ~11.9 min and ~12.9 min, respectively.

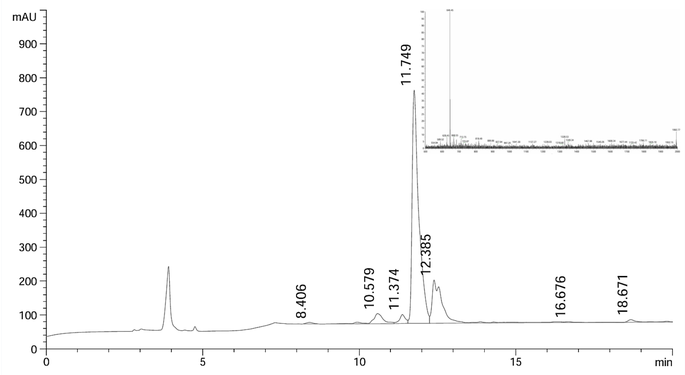


Figure 4. HPLC of peptide synthesized with **L-Cys in position X using DIC/Oxyma at 110 °C**, 1 min deprotection and 5 min coupling. MS shows m/z 646.

HBTU/DIEA Synthesis

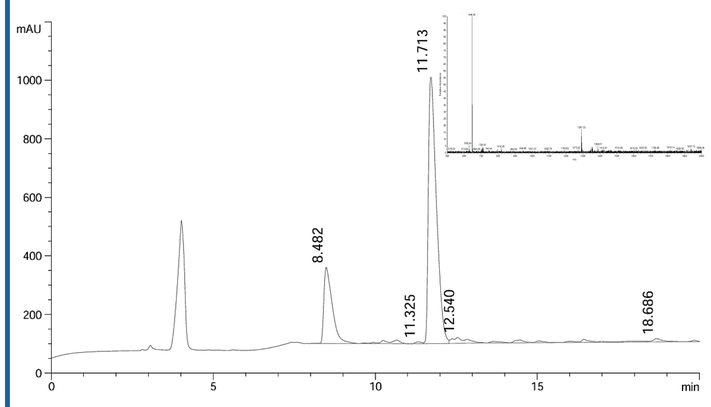


Figure 5. HPLC of peptide synthesized with **L-Cys in position X using HBTU/DIEA at 30 °C**, 2 x 5 min deprotection and 60 min coupling. MS shows m/z 646.

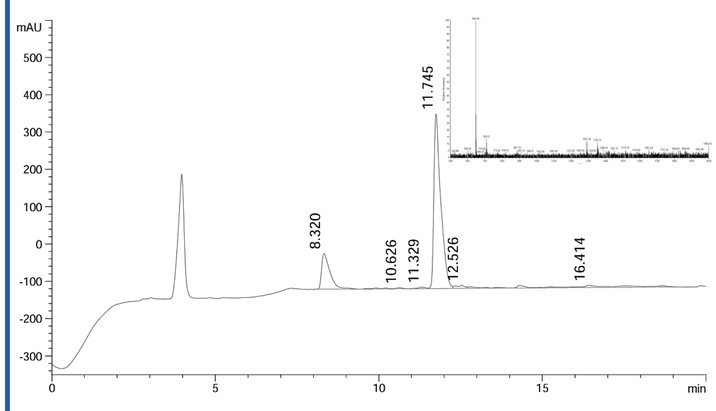


Figure 6. HPLC of peptide synthesized with **L-Cys in position X using HBTU/DIEA at 45 °C**, 2 x 5 min deprotection and 30 min coupling. MS shows m/z 646.

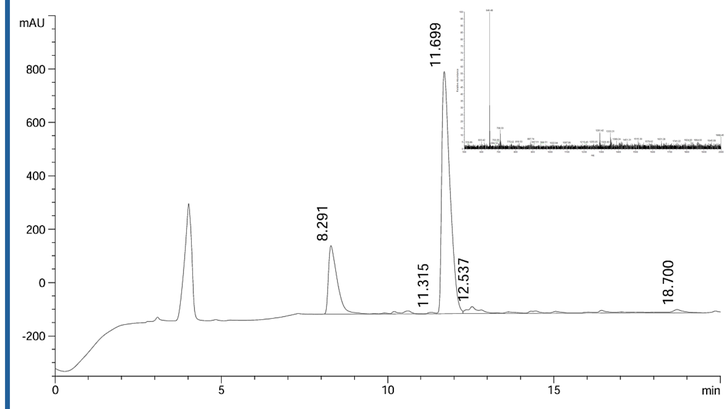


Figure 7. HPLC of peptide synthesized with **L-Cys in position X using HBTU/DIEA at 60 °C**, 2 x 5 min deprotection and 5 min coupling. MS shows m/z 646.

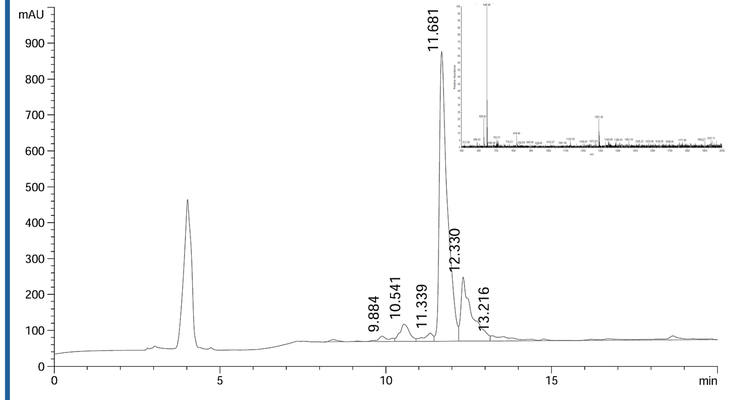


Figure 8. HPLC of peptide synthesized with **L-Cys in position X using HBTU/DIEA at 90 °C**, 1 min deprotection and 5 min coupling. MS shows m/z 646.

Process Workflow Impact and Conclusions

- We designed a sequence that resolves Cysteine racemization on standard RPHPLC. This method is accessible, with no pre-column derivatization or chiral column required.
- DIC/Oxyma coupling chemistry maintains stereochemical integrity of Cysteine at 90 °C using conduction heating as confirmed by chiral amino acid analysis using GC-MS.
- Bypassing the recommended high temperature for each coupling chemistry causes racemization; tight temperature control, like that conferred by conduction heating, is critical for maintaining stereochemistry at chiral centers.
- Easy screening tool for identifying conditions that minimize racemization in a target peptide sequence. However, racemization may be sequence-dependent and temperature is the critical synthesis condition to control. Uniform heating may be difficult to control with other heating methods when changing reaction volume, resin loading, and peptide mass.

References

[1] Duengo, S., Muhajir, M.I., Hidayat, A.T., Musa, W.J.A., Maharani, R. (2023) Epimerisation in Peptide Synthesis. Molecules, 28, 8017. https://doi.org/10.3390/ molecules28248017

[2] Huang, Y.; Pan, L.; Zhao, L.; Mant, C.T., Hodges, R.S.; Chen, Y. (2014). Structured Guided RP-HPLC Chromatography of Diastereomeric Alpha-Helical Peptide Analogs Substituted with Single Amino Acid Stereoisomers, Biomedical Chromatography, 28(4), 511-517. doi:10.1002/bmc.3061

CSBio INSTRUMENTATION