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Streamlined Analysis of Synthetic Peptides with Non-Standard Amino Acid Chemistry

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Introduction

Synthetic peptides with unique amino acid chemistry require flexible fragment analysis software

However, many fragment analysis programs lack support for synthetic peptides with non-standard amino acids and modifications.

We demonstrate the streamlined analysis of synthetic peptides using ExDViewer, which allows customization of building blocks and modifications to create non-standard target sequences.

Using GLP-1 analogs and peptoids with unique side chain and backbone chemistry, we highlight the utility of these features.

Biomimetic peptoids are an important example of the flexibility needed for fragment analysis

Standard peptides contain the side chain R group on the backbone alpha carbon. In contrast, the side chain of peptoids is on the backbone nitrogen. (Figure 1)

For electron capture dissociation (ECD), this means the side chain mass must be included on the n-terminal ion rather than the c-terminal ion. Annotation of ECD spectra from peptoids therefore has historically required manual identification[1].

In ExDViewer v. 4.6.26, we introduce the ability to customize fragmentation rules to permit streamlined analysis of peptoid ECD data.

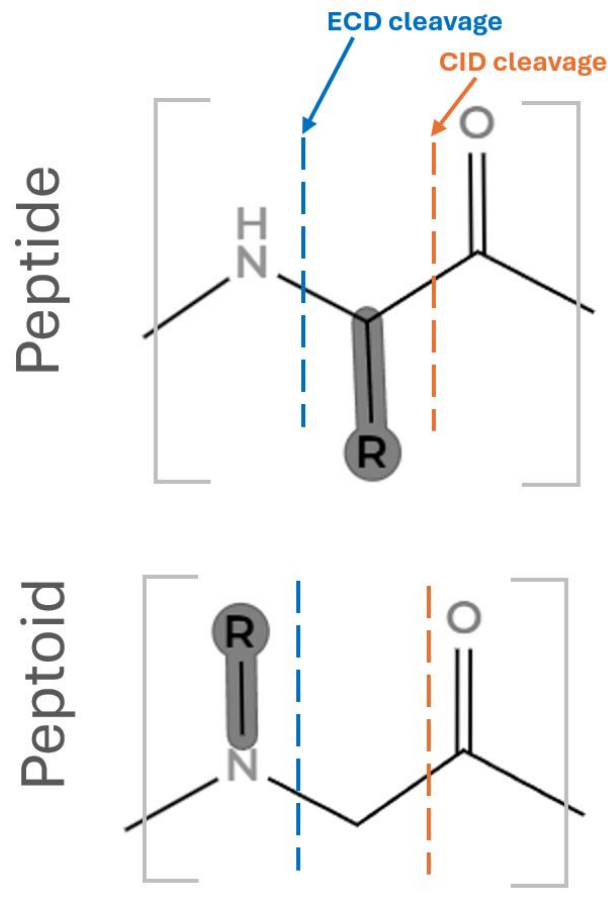


Figure 1- Comparison of peptide and peptoid side chain chemistry.

Experimental

GLP-1 samples were analyzed using the Agilent 6545XT AdvanceBio LC/Q-TOF MS with ExD cell to enable ECD.

Peptoid CID data was provided by Michael Connolly at the Molecular Foundry. Work at the Molecular Foundry was supported by the Office of Science, Office of Basic Energy Sciences, of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231. Peptoid ECD results were provided by Jianhua Ren at the University of the Pacific. (see ref. 1)

Data analysis using ExDViewer

GLP-1 analog and peptoid fragmentation was analyzed using ExDViewer V 4.6.26

This updated version is available as freeware at exdviewer.agilent.com

Streamlined analysis of synthetic peptides was enabled by the building block editor (Figure 2), modification editor (Figure 3), and customizable fragmentation rules (Figure 6). The modification editor permits configuration of neutral losses specific to each building block.

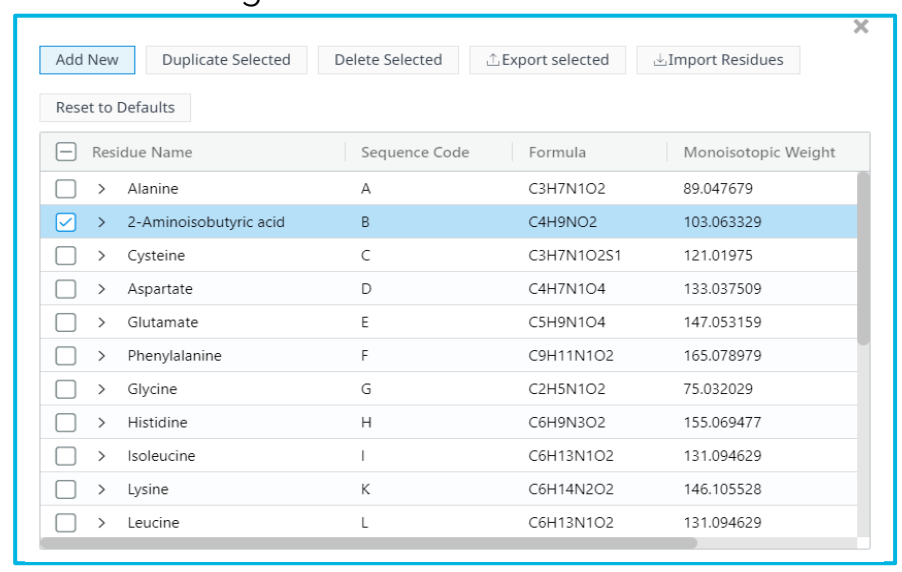


Figure 2- The building block editor user interface. The custom “B” amino acid is highlighted in blue.

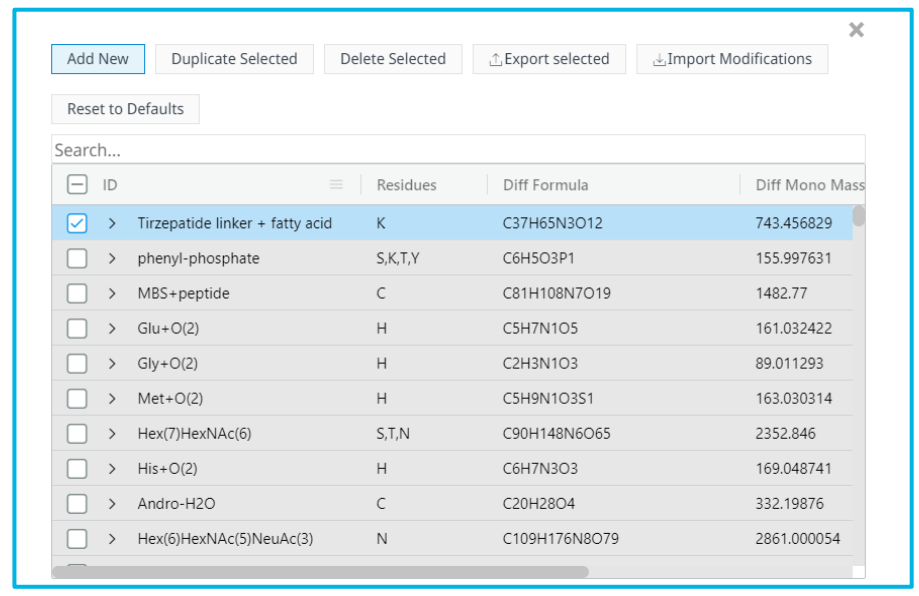


Figure 3- The modification editor user interface. The custom tirzepatide modification is highlighted.

Results and Discussion

CID Fragment analysis of N-substituted glycine polymers (peptoids)

Peptoid fragment analysis starts with creating a library of peptoid building blocks.

Peptoid sequence analysis using collision induced dissociation (CID) does not require fragmentation rules outside of the normal b/y expected patterns. CID fragment analysis was demonstrated on both purified (Figure 4) and crude peptoid samples.(Figure 5)

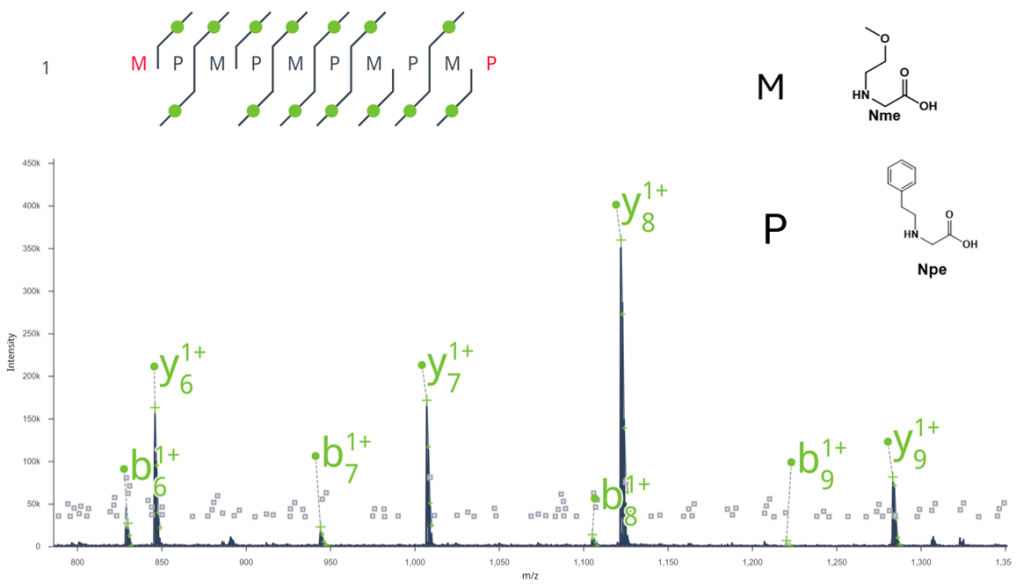


Figure 4- The CID fragmentation spectrum of a purified peptoid. The building blocks are shown in the upper right corner next to the sequence map.

Peptoid ECD analysis requires custom fragmentation rules due to non-standard backbone chemistry

Because the side chain chemistry of peptoids is on the backbone nitrogen, custom fragmentation rules are required to accommodate for ECD fragments which differ from the typical c/z-ion masses for a standard peptide. (Figure 6)

In ExDViewer, this difference is addressed by **defining custom fragmentation rules for c and z ions.**

Using ECD peptoid data from Ren lab (2014) publication, our streamlined analysis with ExDViewer aligns closely with the manually curated results. (Figure 7, 8) This is the first demonstration to our knowledge, of automated annotation of peptoid ECD data.

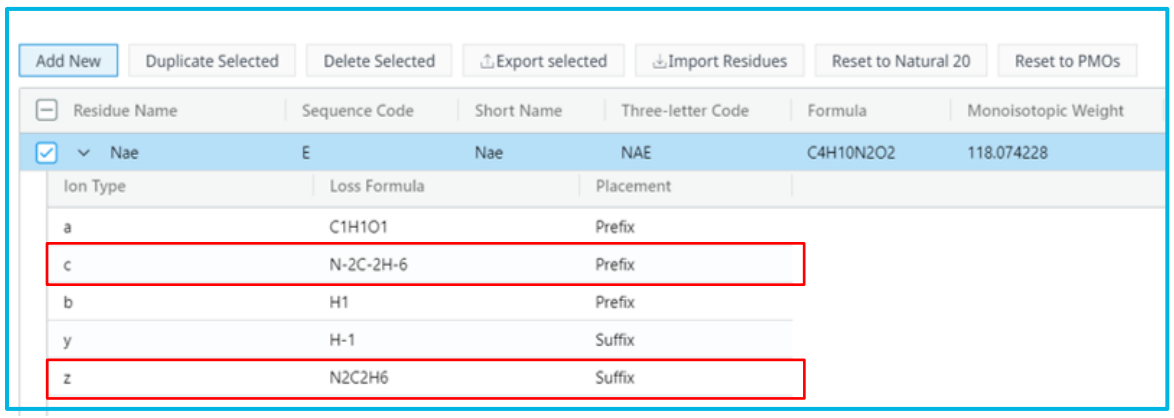


Figure 6- Custom fragmentation rules for c and z type ions. The custom rules are marked with a red box.

Nontargeted analysis of unknown peptoid samples

The caliper tool in ExDViewer's spectrum view is useful for manual de novo sequencing of unknown peptides.

Figure 5 shows how the caliper was used to identify a sequence containing sequential "S" peptoid building blocks. This method was effective for identifying the sequence of 10/11 peptoid building blocks. In this case, the sodium guided the identification of the c-terminal ion series due to loss of sodium indicator peaks.

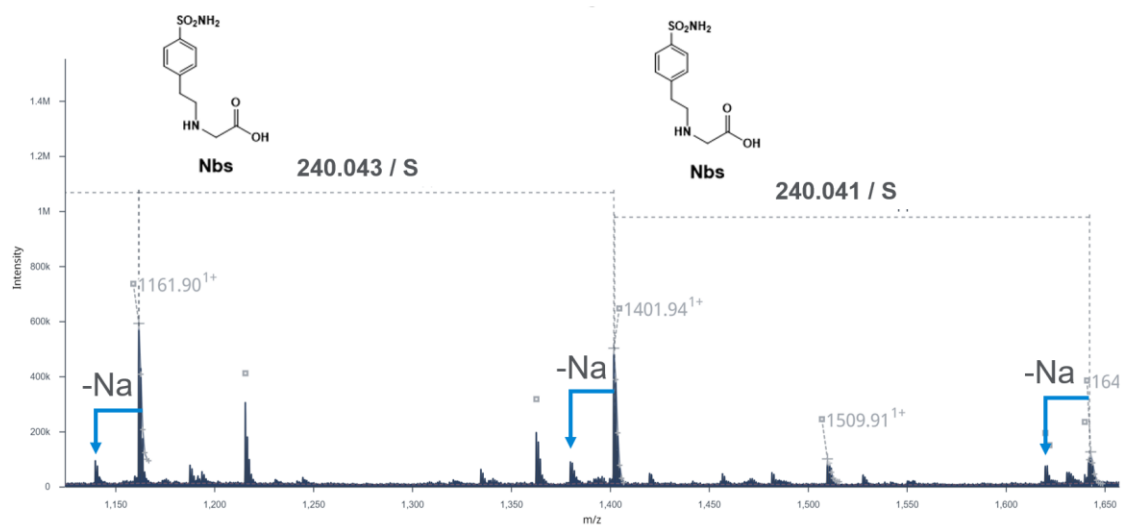


Figure 5- A CID spectrum from a crude, unknown sample. The caliper tool measures the mass between centroids and suggests the building block that matches the mass difference.

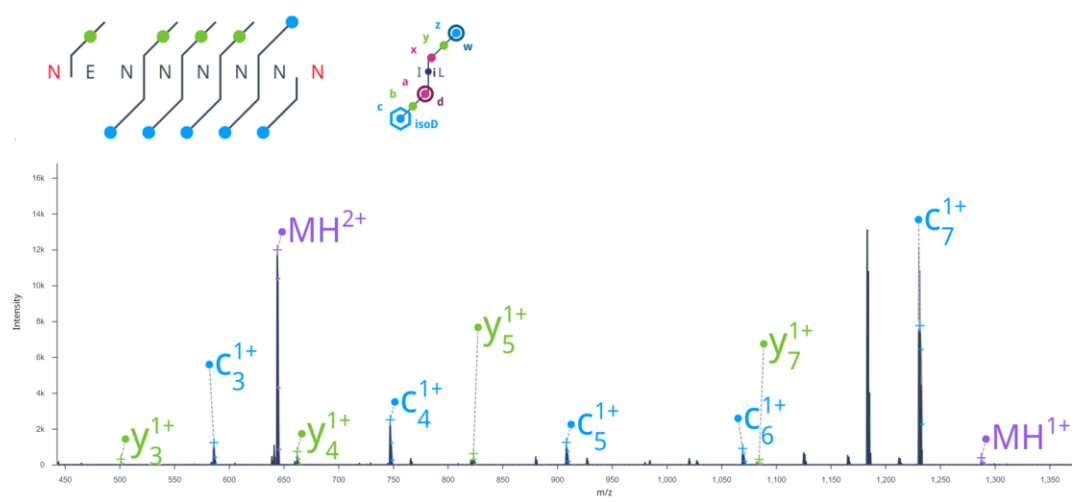


Figure 7- The ECD fragmentation spectrum of a purified peptoid.

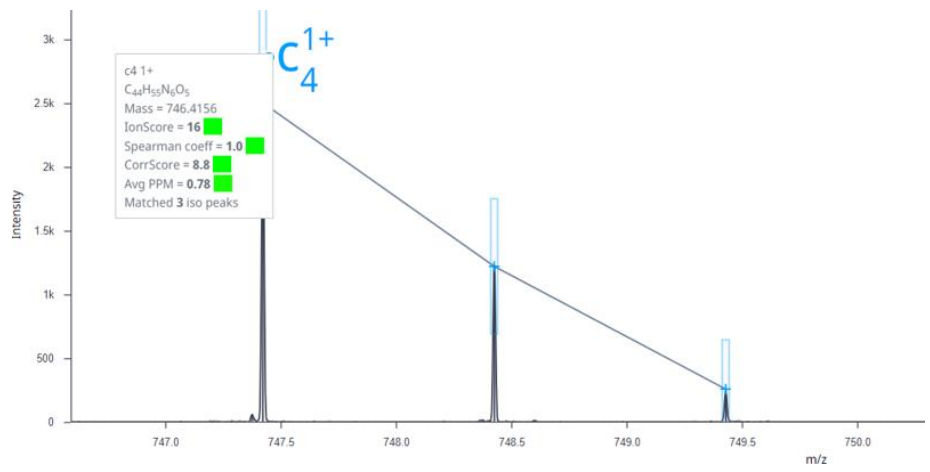


Figure 8- A zoomed in *m/z* spectrum of the Peptoid ECD fragment c_4^{1+}. Hover over the ion brings up a tool tip with information about the ion.

Results and Discussion

Fragment analysis of GLP-1 analogs with non-standard amino acid chemistry

Full sequence coverage was obtained for liraglutide, semaglutide, and tirzepatide. Excellent ion complementarity was detected, including golden pairs (b/c, y/z) and triplets (a/b/c) which improves assignment confidence. The custom amino acid, 2-aminoisobutyric acid is defined as the letter "B". (Figure 9)

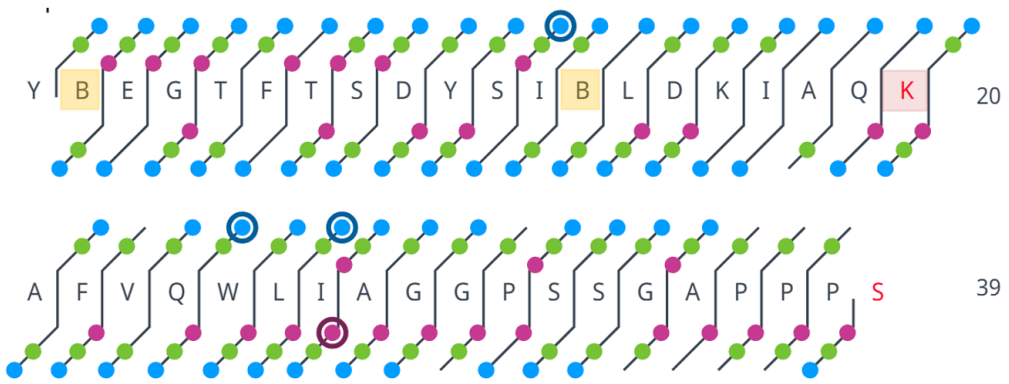


Figure 9- Sequence coverage map of tirzepatide. ECD ions are blue circles, CID ions are green, and a/x ions are pink circles.

Visualization of method suitability

Automatically generated figures of ion abundance per fragment type are useful for evaluating method suitability[2]. Figure 10 demonstrates how fragmentation changes as a function of collision energy. The amount of precursor drops while the intensity of CID fragment types increases with collision energy.

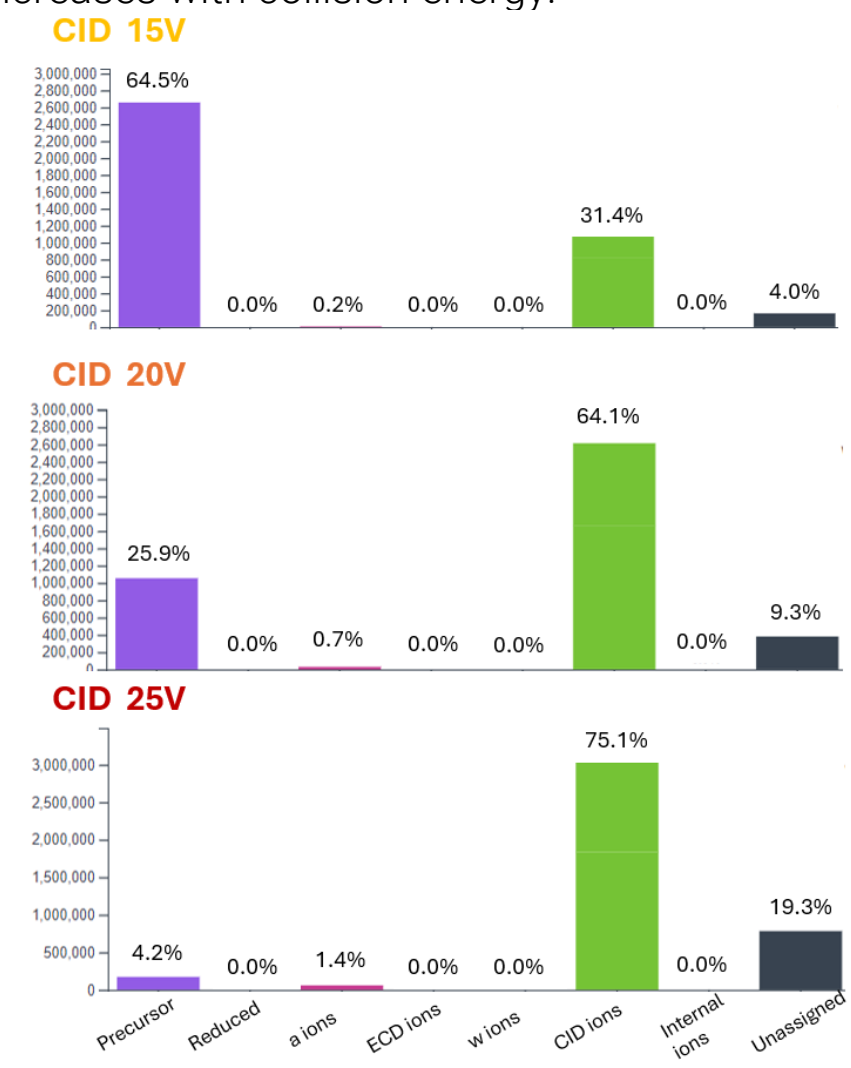


Figure 10- An ion intensities graph showing the types of ions detected as a function of collision energy for 4^+ tirzepatide.

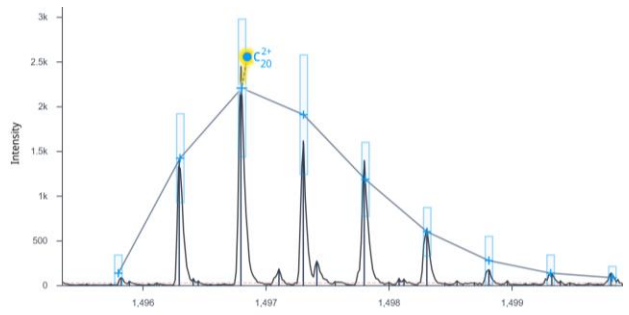
Modification localization using ECD

ECD is beneficial for the analysis of peptides with sensitive chemical groups. It is considered a 'gentle' fragmentation technique that cleaves the peptide backbone while retaining chemical modifications.

In figure 9, ECD was used to localize the fatty-acid modification, which is highlighted in red. The custom fatty acid containing modification was defined in the modification editor shown in figure 6.

Detecting consecutive ion series on both sides of the lysine modification enabled confident localization. Figure 10 highlights c_{20}^{2+}, a modification containing fragment that was used to identify the modification site.

Figure 10- The m/z spectrum of the ECD fragment (c_{20}^{2+}). Ion score = 12, where 15 is the max score.



Conclusions

Agilent ExDViewer is a versatile tool for analyzing peptide fragmentation data.

We introduce a new feature that allows custom fragmentation rules for each building block which was crucial for peptoid ECD data analysis.

Flexibility in defining building blocks and modifications enables streamlined characterization of synthetic peptides with nonstandard residues and modifications.

References

1- Bogdanov, B., Zhao, X., Robinson, D.B, and Ren, J. Electron Capture Dissociation Studies of the Fragmentation Patterns of Doubly Protonated and Mixed Protonated-Sodiated Peptoids JASMS.2014 25 (7), 1202-1216

2- Comprehensive Characterization of Multiple GLP-1 Analogs Using an Agilent 6545XT AdvanceBio LC/Q-TOF with ECD and Agilent ExDViewer software. Agilent Technologies application note, publication number 5994-7994EN, 2025.

3- Identification of Amino Acid Isomers Using Electron Capture Dissociation in the Agilent 6545XT AdvanceBio LC/Q-TOF System. Agilent Technologies application note, publication number 5994-7506EN, 2024.

Conflict of Interest Disclosure

Authors are employees of Agilent Technologies, which sells the instrumentation used in this analysis. As such, the authors disclose a potential conflict of interest, in respect to their commercial involvement.

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