

# Peptide structure confirmation based on molecular weight and collision cross section obtained with trapped ion mobility separation

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## Overview

- Quality control for synthetic peptides typically involves intact mass determination. Chromatographic variables (molecular weight and retention time) alone fall short if isomeric peptides need to be safely distinguished.
- We used trapped ion mobility (TIMS) QTOF-MS data to determine collision cross sections (CCS) routinely alongside molecular weights as quality control (QC) parameters for synthetic peptides.
- Data were automatically interpreted for QC purposes using multiple quality attributes within the BioPharma Compass 2021 software.
- Several synthetic isomeric peptides, including Leu/Ile peptide isomers and  $\mu$ -conotoxin PIIIA variants with different disulfide connectivities were used to establish CCS values as reference points for future identification or synthesis quality control.

## Methods

- Peptides Two 18mer isomeric peptides from an antibody CDR (complementarity determining region) with an isobaric L vs. I exchange and five cyclic  $\mu$ -conotoxin PIIIA variants were used (Fig. 1).
- LC-TIMS-MS Peptides were purified using short reverse HPLC gradients with 15 min cycle times and analysed by TIMS-MS on a timsTOF Pro (Bruker).
- Data analysis Datasets were processed in BioPharma Compass 2021 (Bruker) and matched against previously obtained reference CCS values to distinguish isomeric peptides.

## CDR Peptides

The CDR-derived peptide isomers delivered small differences in their CCS, which were distinguishable in an automatic workflow. While the CDR-I exerted a CCS of  $477.3 \pm 0.3 \text{ \AA}^2$ , the CDR-L had a CCS of  $479.3 \pm 0.3 \text{ \AA}^2$ . Thus, given the standard deviation of the CCS determination of 0.06 %, the difference between the two forms with a 0.41 % CCS allowed for their unequivocal distinction according to their CCS in automated analysis (Fig. 2,3).

This approach may prove useful for *de novo* antibody sequencing as the unequivocal identification of isomeric peptides based on MS/MS alone remains difficult.

Sample Position: 5	Sample ID: CDR-L	Method Attribute	Narrow	Wide	Sample Result	Unit
		Base peak as expected	$\geq 80.0$	$< 60.0$	100.0	Rel. int. [%] vs base peak
		Retention Time	$< 0.30$	$\geq 0.50$	0.01	$\Delta$ Rt (min)
		Intensity Coverage	$\geq 45.0$	$\leq 35.0$	95.0	Rel. int. [%] of all target signals
		Mass Accuracy [ppm]	$< 15.0$	$\geq 20.0$	-14.5	ppm
		Collision/CrossSection (CCS)	$\geq 0.4$	$< 0.6$	0.36	$\Delta$ CCS [%]

Fig. 2 Legend to the quality attributes displayed in Figs. 3, 4. Sample CDR-L was tested with the Method to qualify CDR-I, which results in a perfect match of all attributes, except - perfectly correct - for  $\Delta$ CCS [%], thus highlighting the specificity of the analysis.

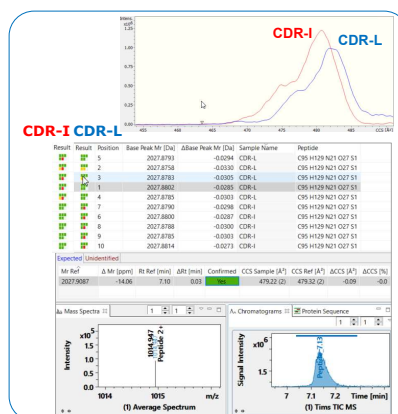


Fig. 3 Multi Attribute Analysis of 5 replicates of the peptides CDR-I and CDR-L by methods that are specific for the CCS values of either peptide. Top: mobilograms show a distinct shift between the peptides. Bottom: LC and MS signals for an analysis of CDR-L with a method specific for CDR-L. Left: same analysis with a method specific for CDR-I.



Fig. 1 Top Left: CDR-L and CDR-I are 2 CDR derived peptides that differ at position 4: Leu vs. Ile. Top Right:  $\mu$ -conotoxin PIIIA. Bottom: Disulfide connectivity of the different disulfide-bonded variants: 1: native PIIIA isomer, 5: 3-disulfide bonded isomer, 2-4: derivatives in which one disulfide bond of the native isomer was replaced by serine residues.

## Conotoxin PIIIA Isomers

Native PIIIA (1), its isomer 5 and 3 disulfide-deficient  $\mu$ -conotoxin PIIIA variants with variable disulfide connectivities (2, 3, 4) were also analysed (Fig. 1). They are indistinguishable by molar mass, MS/MS and retention time alone. TIMS established distinct mobilograms for each and enabled the identification based on the CCS values of the  $[M+5H]^{5+}$  ions.

Each dataset was tested for the presence of any of the isoforms (Fig. 4) and the 5<sup>th</sup> attribute (CCS) only matched (green) if the correct isoform was present.

The test for isomer 5 was also positive for the mixture of 1 and 5, indicating the suitability of the method for isomer detection in mixtures [1].

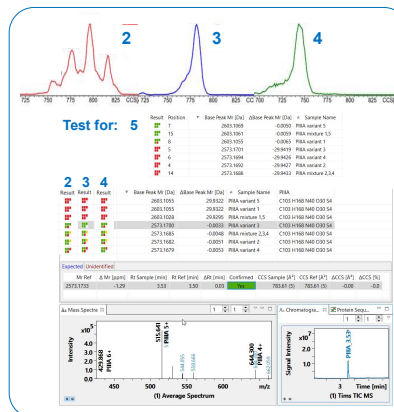


Fig. 4. Analysis of the disulfide-bonded conotoxin PIIIA variants. Top: Mobilograms are shown for isomers 2,3,4. Centre: Results of the testing for the presence of variants 2-5. Bottom: Full report of the test for variant 3 with matching Mr, Rt and CCS values (see legend in Fig. 2).

LC-TIMS-QTOF analysis permitted the reproducible determination of peptide CCS values with standard deviations smaller than 0.05% suitable to distinguish subtle isomer differences. Molecular weight, retention time and CCS were developed as acceptance criteria in the quality control of synthetic peptides which will facilitate cross-lab method transfer of peptide/substance identification.

## References

- LC-TIMS-TOF MS differentiation of 2- and 3-disulfide-bonded isomers of  $\mu$ -conotoxin PIIIA (2020) T Schmitz, S Pengelley, E Belau, D Suckau, D Imhof, submitted.

## Conclusions

- Trapped ion mobility-LC-MS permitted to distinguish peptide isomers by mass and CCS in a format suitable for automatic isomer assessment.
- The observed rel. standard deviation of CCS value was 0.06%. Isomers with CCS values differing by  $< 0.5\%$  were safely and automatically distinguished.
- Leu/Ile differentiation in CDR derived peptides was achieved, which may prove useful in mAb *de novo* sequencing studies in the future.
- The presence or absence of the isomers were automatically determined using a traffic light reporting system, which allowed to detect isomers even in mixtures based on Mr, Rt and CCS values.

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