

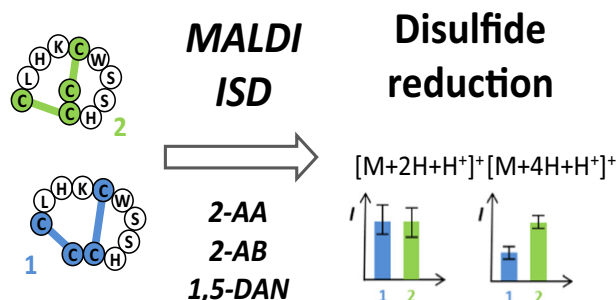
RESEARCH ARTICLE

Disulfide Connectivity Analysis of Peptides Bearing Two Intramolecular Disulfide Bonds Using MALDI In-Source Decay

Philippe Massonnet,¹ Jean R. N. Haler,¹ Gregory Upert,² Nicolas Smargiasso,¹ Gilles Mourier,² Nicolas Gilles,² Loïc Quinton,¹ Edwin De Pauw¹

¹Mass Spectrometry Laboratory, MolSys Research Unit, University of Liège, Quartier Agora, Allée du six Aout 11, B-4000, Liege, Belgium

²Commissariat à l'Énergie Atomique, DRF/SIMOPRO, 91191, Gif sur Yvette, France



Abstract. Disulfide connectivity in peptides bearing at least two intramolecular disulfide bonds is highly important for the structure and the biological activity of the peptides. In that context, analytical strategies allowing a characterization of the cysteine pairing are of prime interest for chemists, biochemists, and biologists. For that purpose, this study evaluates the potential of MALDI in-source decay (ISD) for characterizing cysteine pairs through the systematic analysis of identical pep-

ptides bearing two disulfide bonds, but not the same cysteine connectivity. Three different matrices have been tested in positive and/or in negative mode (1,5-DAN, 2-AB and 2-AA). As MALDI-ISD is known to partially reduce disulfide bonds, the data analysis of this study rests firstly on the deconvolution of the isotope pattern of the parent ions. Moreover, data analysis is also based on the formed fragment ions and their signal intensities. Results from MS/MS-experiments (MALDI-ISD-MS/MS) constitute the last reference for data interpretation. Owing to the combined use of different ISD-promoting matrices, cysteine connectivity identification could be performed on the considered peptides.

Keywords: Mass spectrometry, Peptide, Disulfide bonds, MALDI, ISD, Isomers

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Introduction

Among all post-translational modifications, disulfide bonds stand out due to their formation mechanism based on the oxidation of two cysteine residues creating S-S bonds. This modification is for example found in animal venoms [1], cyclotides [2, 3], or again in antimicrobial peptides [4, 5]. Disulfide bonds provide structural constraints to peptide and protein backbones [6–10]. The presence of such bonds can also

be mandatory in order to stabilize secondary structures [11–14] such as α -helices and β -sheets, or to maintain a specific biological activity [2, 15]. For instance, it has been shown that the biological activity of peptides bearing two disulfide bonds can be affected by the cysteine connectivity (C_1 - C_2 / C_3 - C_4 , C_1 - C_3 , C_2 / C_4 , C_1 - C_4 / C_2 - C_3) [16]. Indeed, when having multiple intramolecular disulfide bonds, the number of possible cysteine connectivities rapidly increases with the number of disulfide bonds (relation described by combinatorics) and the biological relevance of the cysteines becomes even more important [17].

In this context, characterization techniques allowing having access to cysteine connectivity are important. For this purpose, NMR-based strategies have been developed [18, 19]. The downsides of NMR are, however, the need for large amounts of sample and, most of the time, the requirement of ^{13}C and/or

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Correspondence to: Philippe Massonnet; e-mail: pmassonnet@uliege.be

Table 1. List of the peptides used in this study

Name	Sequence	Cysteine connectivity
α -Conotoxin	C ₁ C ₂ HSSWC ₃ KHLC ₄	C ₁ -C ₃ /C ₂ -C ₄
χ -Conotoxin	C ₁ C ₂ HSSWC ₃ KHLC ₄	C ₁ -C ₄ /C ₂ -C ₃
ModBea	C ₁ EGWFRFTKTGLEYC ₂ TPGLC ₃ LRWGKLC ₄ *	C ₁ -C ₂ /C ₃ -C ₄
ModGlo	C ₁ EGWFRFTKTGLEYC ₂ TPGLC ₃ LRWGKLC ₄ *	C ₁ -C ₃ /C ₂ -C ₄
ModRib	C ₁ EGWFRFTKTGLEYC ₂ TPGLC ₃ LRWGKLC ₄ *	C ₁ -C ₄ /C ₂ -C ₃

*Peptides with a C-terminus amidation

¹⁵N-labeled analogues. Techniques involving tryptic digestion and liquid chromatography separations have also been developed [20, 21]. Nevertheless, these techniques are time consuming and not always efficient when there are not enough cleavage sites between the cysteines.

More recently, techniques involving gas-phase reactions have been developed for the characterization of peptides bearing intramolecular disulfide bond(s) [22–25]. The presence of disulfide bonds was for example probed by collision-induced

dissociation (CID) [22] and the disulfide connectivities were determined using electron transfer dissociation (ETD) [24, 25]. MALDI in-source decay (ISD) reactivity of disulfide bonds has also been investigated on various peptides [26–32]. These studies pointed out the ability of 1,5-diaminonaphthalene (1,5-DAN) matrix to reduce disulfide bonds (addition of two radical hydrogens on the sulfur atoms) and the possibility to determine the number of S-S bonds in a given peptide (from 0 to 3). For these studies, peptides containing one disulfide bond were

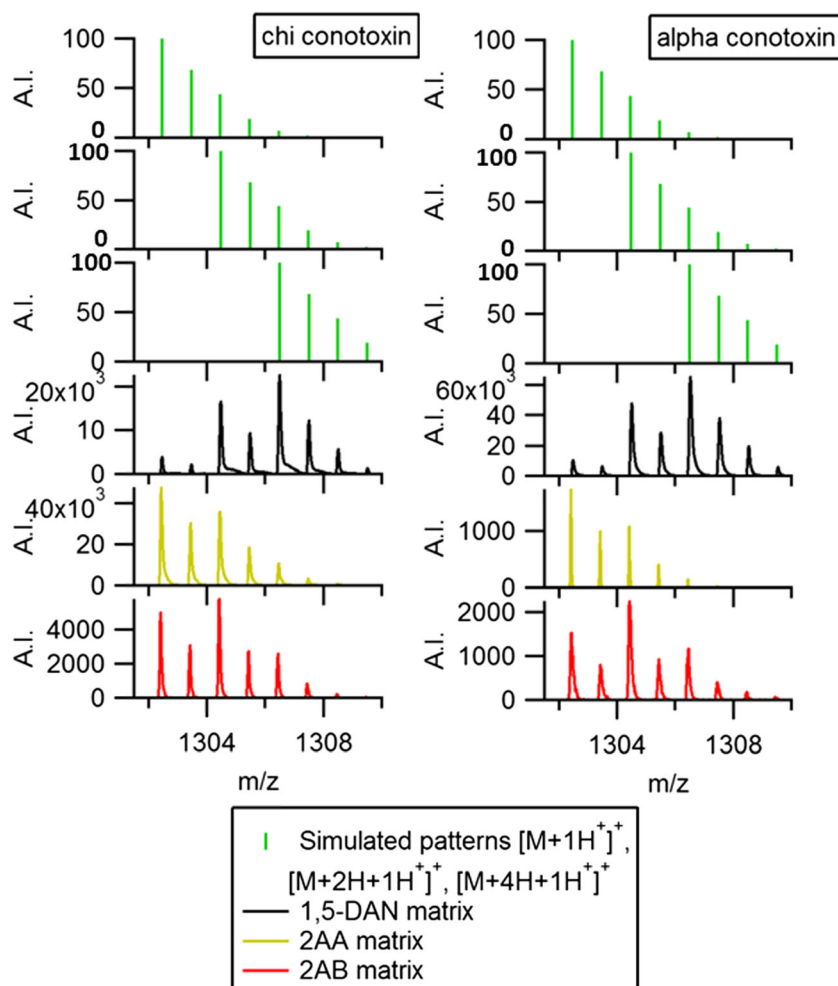


Figure 1. On the left, 1+ charge state series of χ -conotoxin using different matrices. On the right, 1+ charge state series of α -conotoxin. The first row (green spectra) represents the simulated isotope pattern without disulfide bond reduction ($[M+1H]^+$). The second and the third rows represent respectively the simulated isotope pattern with 1 disulfide bond reduction ($[M+2H+1H]^+$) and 2 disulfide bond reductions ($[M+4H+1H]^+$). The fourth, fifth, and last rows of spectra represent the isotope patterns of the (reduced) parent ions using respectively the 1,5-DAN (black), 2-AA (yellow), and 2-AB (red) matrices

mostly investigated as proof of concept. Fukuyama and co-workers studied a peptide with two disulfide bonds using MALDI-ISD followed by CID activation [26]. They concluded that using theoretical and experimental mass differences of fragment ions having reduced or intact disulfide bonds could enable the prediction of the disulfide bond connectivity. However, these hypotheses have not yet been systematically or experimentally surveyed on multiple peptides with identical sequences but differing disulfide bond connectivities.

In this work, we investigate the effects of the cysteine connectivity on disulfide bond reactivity undergoing MALDI-ISD. The reduction and fragmentation capabilities of three ISD matrices are examined: 1,5-diaminonaphthalene (1,5-DAN), 2-aminobenzamide (2-AB), and 2-aminobenzoic acid (2-AA). For this purpose, two peptide sequences bearing two disulfide bonds were chosen.

Materials and Methods

Chemicals and Peptides

2-Aminobenzamide (2-AB), 2-aminobenzoic acid (2-AA), 1,5-diaminonaphthalene (1,5-DAN), and formic acid (FA) were purchased from Sigma-Aldrich (Saint Louis, MO, USA), while acetonitrile (ACN) was purchased from Biosolve (Dieuze, France). 1,5-DAN has been chosen because of its well known high efficiency to reduce disulfide bonds while 2-AA and 2-AB matrices are known to have a lower efficiency to reduce disulfide bonds [28]. The sequences and the connectivities of the peptides used in this study are summarized in Table 1. They were chemically synthesized as previously described [24, 33].

Mass Spectrometry Analysis

All MALDI mass spectrometry experiments were performed on a rapifleX TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a smartbeam™ 3D laser in positive and/or in negative mode. The analyzer was operated in reflectron mode and all spots were prepared using the dried droplet method: 1 μ L of peptides at 10 μ M in a H₂O/ACN/FA 49.9/50/0.1 solvent mixture was first placed on the target plate and allowed to dry at room temperature followed by the deposition of 1 μ L of matrix solution. The 2-AA and 2-AB matrices were used at a concentration of 20 mg/mL in a H₂O/ACN/FA 49.9/50/0.1 (v/v/v) while 1,5-DAN was prepared at saturation in the same solvent mixture. Due to the instability of 1,5-DAN in ACN [34], 1,5-DAN solutions were prepared just before mass spectrometry analysis. For the 2-AA and 2-AB matrices, fresh solutions were also used. Each spectrum is a sum of 5000 laser shots (5 times 1000 shots at 1000 Hz) at a laser intensity of 40%. A laser intensity of 50% has been used for the spots containing 2-AA and 2-AB matrices. The detector gain was set to 2.45 kV in the positive mode and to 2.0 kV in negative mode. The measurements of each peptide with each matrix (one peptide/matrix combination per spot) were performed 5 times (5 different days) with fresh solutions and matrices on

each spot in order to verify the reproducibility of the measurements.

MS/MS (CID) Experiments

For the CID experiments, the MS/MS mode of the rapifleX has been used and the laser intensity was set to 65%. The laser boost was set to 60% and all presented spectra are constituted by a sum of 10,000 shots (10 times 1000 shots at 1000 Hz). The collision chamber was filled with Argon.

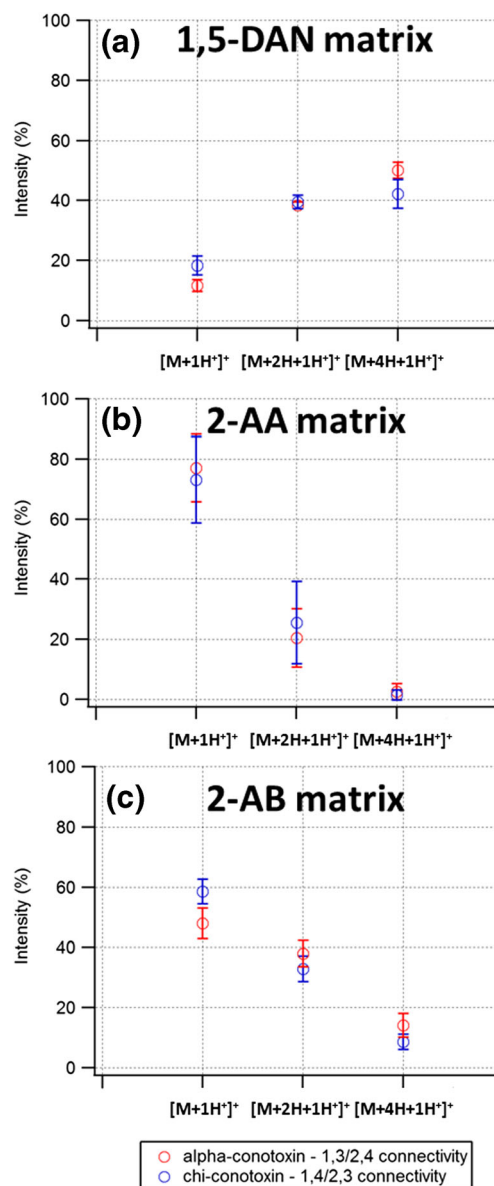


Figure 2. Calculated normalized intensities (see Eq. 1) after isotope pattern deconvolution of the parent ion $[M+1H]^+$, of the partially reduced parent ion $[M+2H+1H]^+$ and the totally reduced species $[M+4H+1H]^+$ of the conotoxin-based peptides using (a) the 1,5-DAN matrix; (b) the 2-AA matrix, and (c) 2-AB matrix. The error bars originate from the standard deviation of 5 independent measurements (5 different days)

Data Analysis

All presented data were extracted using the FlexAnalysis 3.4 (Bruker Daltonics) software while isotope pattern deconvolutions were performed using Microsoft Excel 2010. All figures were generated using Igor Pro 6.37 and FlexAnalysis 3.4.

Results and Discussion

Peptides Inspired from Conotoxins

First, the α - and χ -conotoxins are investigated (see Table 1). The comparison of the experimental isotope patterns of the parent ions with the simulated patterns for non-reduced species demonstrates that all the matrices (1,5-DAN, 2-AA, 2-AB) have the ability to reduce disulfide bonds (Figure 1). Disulfide bond reduction modifies the isotope patterns by superimposing signatures of the non-reduced species, of the species that have undergone the reduction of only one disulfide bond (additional 2 mass units) and the ones that are totally reduced (additional 4 mass units). When further analyzing the results, it appears that the 1,5-DAN matrix reduces more efficiently the disulfide bonds than the 2-AA and 2-AB matrices, as already demonstrated in literature [28, 35]. Moreover, when focusing on the fragment ions of both conotoxins using the three matrices (see

Fig. SI 1), only few fragments are observed (losses of H_2S and CH_2S_2 as already reported elsewhere [28, 36]). Based on these results, we have chosen to focus our attention only on the parent ions for the determination of the disulfide bond reduction capabilities of the matrices.

Figure 2 depicts the normalized intensities obtained after isotope pattern deconvolutions of non-reduced ($[M + 1H]^+$, see Eq. 1), partially ($[M + 2H + 1H]^+$) and totally reduced ($[M + 4H + 1H]^+$) species of the two peptides using the three different matrices. Equation 1 illustrates the normalized intensity calculation on the non-reduced parent ion $[M + 1H]^+$. To verify the reproducibility of the data, the MALDI-ISD measurement of each peptide connectivity with each matrix was performed 5 times, summing 5000 laser shots for each replicate measurement.

$$Intensity_{[M+1H]^+}(\%) = \frac{\sum I_{isotope\ pattern}[M + 1H]^+}{\sum I_{isotope\ pattern}([M + 1H]^+, [M + 2H + 1H]^+, [M + 4H + 1H]^+)} * 100 \quad (1)$$

Figure 2 reveals that the 2-AA matrix yields similar normalized intensities for both conotoxins and all parent ion species (non-reduced, partially reduced, and totally reduced). 2-AA does hence not allow to unambiguously distinguish between the two disulfide connectivities. This behavior is confirmed by

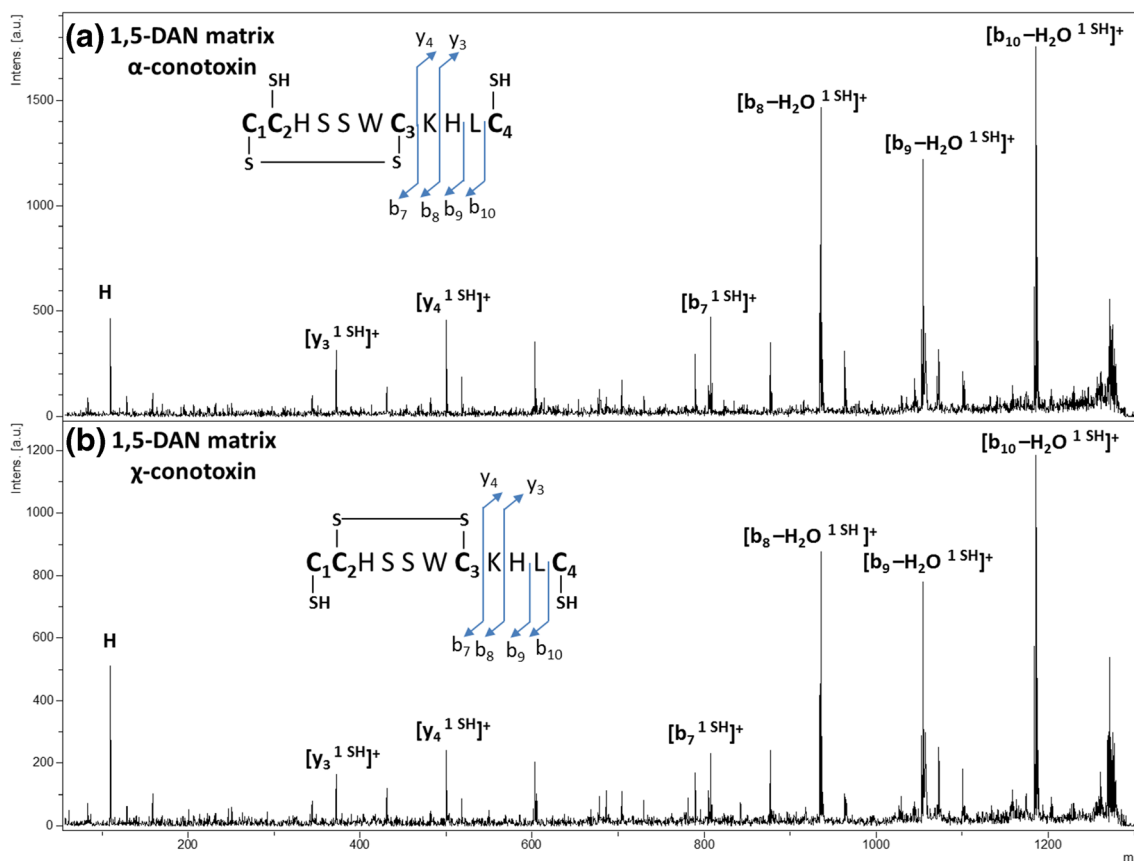


Figure 3. MS/MS (high energy CID) spectra after isolation of the partially reduced species $[M + 2H + 1H]^+$ of (a) α -conotoxin (C_1 , C_3/C_2 , C_4) and (b) χ -conotoxin (C_1 , C_4/C_2 , C_3) using 1,5-DAN matrix. The fragment ion peaks are annotated with their identities (1 SH designates the presence of one 1 SH in the selected fragment) and a peptide sequence scheme with all the observed fragments for the different disulfide isomers is represented in each spectrum. The spectrum was recorded with a laser boost of 60%

the p values obtained after t test (see Figure S12). Indeed, the obtained p values for the 2-AA matrix are always higher than 0.05 (significant value). However, the connectivities of the two conotoxin isomers are differentiated using the 1,5-DAN and 2-AB matrices. For both matrices, the α -conotoxin (C_1 - C_3 / C_2 - C_4 connectivity) exhibits an increased reactivity towards the ISD process compared to the χ -conotoxin peptide (C_1 - C_4 / C_2 - C_3 connectivity). This is demonstrated by the smaller normalized intensity of the non-reduced α -conotoxin parent ion $[M + 1H^+]^+$. Again, these discussions are supported by the p values obtained after t tests (see Figure S13 and Figure S14). In both cases (2-AB and 1,5 DAN matrices), the p values are lower than 0.05. By looking at the totally reduced ion species $[M + 4H + 1H^+]^+$ obtained using 1,5-DAN and 2-AB, the two conotoxins are also clearly distinguishable (see Figure S13 and Figure S14; p values < 0.05). These results could be related to an increased accessibility of the disulfide bonds of α -conotoxin (C_1 - C_3 / C_2 - C_4 connectivity) for the radical hydrogens produced by the ISD-inducing matrix.

The same measurements were also performed in negative mode using the 1,5-DAN matrix. However, no significant differences are obtained between both connectivity isomers (see Figure S15 and Figure S16 for results and p values). This behavior could be explained by a low ionization efficiency

resulting in low intensities in the spectra, thus making the signal extraction difficult.

In positive mode, in order to further analyze the partially reduced species, MS/MS (high energy CID) experiments were performed (see Figure 3). Unfortunately, resulting spectra were the same for both conotoxins, avoiding then any distinction between the two species. Indeed, as seen in Figure 3, only fragments between C_3 and C_4 are obtained in both cases (opening by ISD of the C_2 - C_4 disulfide bond for the α -conotoxin and of the C_1 - C_4 disulfide bond for the χ -conotoxin).

Model Peptides

Next, we analyzed three model peptides containing 2 disulfide bridges using the same approach as for the conotoxin-inspired peptides. Figure 4 reveals that all three matrices reduce the disulfide bonds of the mod-based peptides. From Figure 4, the 1,5-DAN matrix seems to reduce more efficiently the disulfide bonds than the two other matrices.

In positive mode, MS/MS (high energy CID) experiments on the non-reduced and partially reduced species were performed (see Figure S17 and Figure S18). Unfortunately, resulting spectra were the same for all isomers (see y_{11} , y_{12} , y_{14} , and c_{14} ions). This could be due to the high energy CID that opens disulfide bonds in a similar way for all isomers.

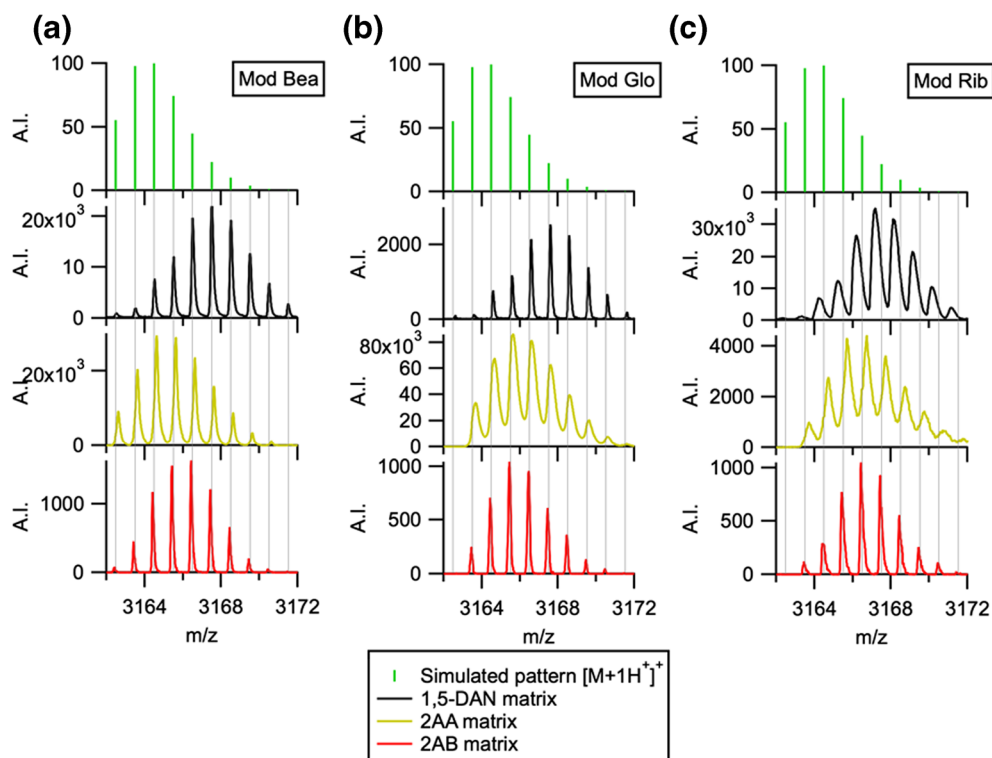


Figure 4. (a) 1+ charge state series of the ModBea peptide (C_1 , C_2 / C_3 , C_4 connectivity) using different matrices (1,5-DAN in black, 2-AA in yellow, and 2-AB in red) compared with the simulated isotope pattern without reduction ($[M + 1H^+]^+$, green spectrum); (b) 1+ charge state series of the ModGlo peptide (C_1 , C_3 / C_2 , C_4 connectivity) using different matrices (1,5-DAN in black, 2-AA in yellow, and 2-AB in red) compared with the simulated isotope pattern without reduction ($[M + 1H^+]^+$, green spectrum); (c) 1+ charge state series of the ModRib peptide (C_1 , C_4 / C_2 , C_3 connectivity) using different matrices (1,5-DAN in black, 2-AA in yellow, and 2-AB in red) compared with the simulated isotope pattern without reduction ($[M + 1H^+]^+$, green spectrum)

When focusing on the ISD fragments using 1,5-DAN, Figure S19 shows small differences between the three isomers. For example, fragment ion peak at m/z 999.4 is only found for the ModBea isomer (C_1 , C_2/C_3 , C_4 connectivity) while the peak at $m/z = 2781.7$ (*GWFRFTKTGLEICTPGLCLRWGKL-H₂O-NH₃*) is only found for the ModGlo isomer (C_1 , C_3/C_2 , C_4 connectivity). The ModRib isomer does not exhibit any specific fragment ion. However, these fragments cannot be explained by a specific disulfide opening for any of the connectivity isomers. Unfortunately, the 2-AA and 2-AB matrices did not yield any fragment ions (data not shown).

Even if, in the case of the mod-based peptides using the 1,5-DAN matrix, connectivity-specific fragment ions could be found, this might not be the case for other disulfide bond-containing peptides or when using other matrices (e.g., 2-AA and 2-AB). We therefore still go further into the ISD data exploitation by calculating the normalized intensities of the non-reduced ($[M + 1H^+]^+$), partially disulfide-reduced ($[M + 2H + 1H^+]^+$) and totally disulfide-reduced ($[M + 4H + 1H^+]^+$) parent ion species (cf. Eq. 1). In addition, for the 1,5-DAN matrix, given that the mod-based peptides yielded fragment ions after the ISD reaction, the normalized intensity of all fragment ions (sum of all fragments) can be calculated as well for each of the three isomers using the three different matrices. Equation 2 was then used to calculate the normalized intensities after isotope pattern deconvolutions; Eq. 2 illustrates the calculation of the normalized intensity of the non-reduced parent ion. The fragment ions were considered as sum of both $[M + 2H + 1H^+]^+$ and $[M + 4H + 1H^+]^+$ ion contributions given that the “model-peptide” fragments do most of the time not allow for the identification of its partially reduced or totally reduced nature (see Figure S19, *c*-type fragments).

$$Intensity_{[M+1H^+]^+}(\%) = \frac{\sum I_{isotope\ pattern}([M + 1H^+]^+)}{\sum I_{isotope\ pattern}([M + 1H^+]^+, [M + 2H + 1H^+]^+, [M + 4H + 1H^+]^+, Fragments)} * 100 \quad (2)$$

In order to verify the reproducibility of the measurements, the spectra of each peptide isomer (three matrices) were recorded 5 times (5 different days). Figure 5 depicts the normalized intensities for all considered species.

By calculating the p values of the results obtained with the 2-AA and 2-AB matrices (see Figure S110 and Figure S111), it appears that no significant distinction between the isomers can be obtained (p values > 0.05).

Concerning the 1,5-DAN matrix, the $[M + 1H^+]^+$ and the $[M + 2H + 1H^+]^+$ species do not allow distinguishing between the isomers (see Figure S112). However, the $[M + 4H + 1H^+]^+$ species allow to distinguish the ModBea from the ModRib isomer (p value = 0.0298) and the ModBea from the ModGlo isomer (p value = 0.0034). Moreover, when focusing on the fragments obtained with ISD, ModBea and ModGlo can also be distinguished (p value = 0.0186). The fact that the fragment ion intensities enable the discrimination between isomers could be of great importance for disulfide bond connectivity identifications. Indeed, in the absence of connectivity-specific

fragments, a qualitative look at the fragmentation spectra would not have allowed to distinguish the isomers. If, additionally, the parent ion species do not provide discriminative normalized intensities, fragment ion intensities, if present, could bring help.

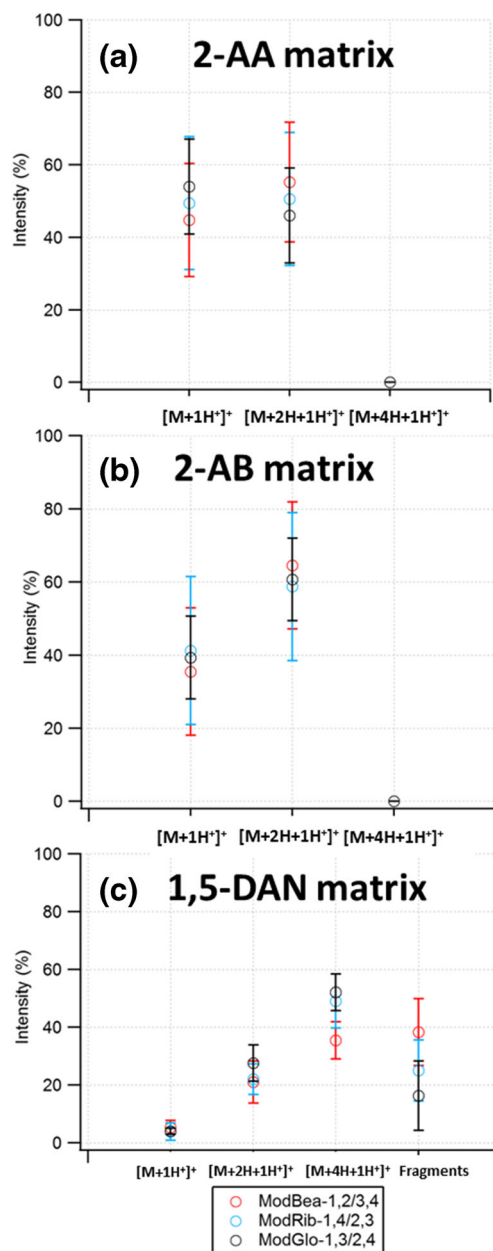


Figure 5. Calculated normalized intensities after isotope pattern deconvolution of the non-reduced parent ion $[M + 1H^+]^+$, of the partially reduced $[M + 2H + 1H^+]^+$ and the totally reduced $[M + 4H + 1H^+]^+$ parent ion species of mod-based peptides using (a) the 2-AA matrix (see Eq. 1), (b) the 2-AB matrix (see Eq. 1), and (c) the 1,5-DAN matrix. For the 1,5-DAN matrix, the normalized intensity of the fragment ions was calculated as well (Eq. 2). The error bars originate from the standard deviation of 5 independent measurements (5 different days)

Conclusions

The aim of this study was to investigate the ability of MALDI-ISD to experimentally predict the disulfide connectivity of peptides bearing two intramolecular disulfide bonds. Different data analysis methods were developed in order to cover different potential scenarios of peptide fragmentation and disulfide reduction signals yielded by MALDI-ISD.

For this purpose, MALDI-ISD experiments were performed (5 replicates each on different days) on two series of peptides bearing the same sequence but differing cysteine connectivities using 3 different ISD matrices (2-AA, 2-AB, and 1,5-DAN). Generally, the 1,5-DAN matrix resulted in the highest disulfide bond reduction yield.

First, we interpreted the fragment ions yielded by the ISD process. Only few fragment ions after the 1,5-DAN ISD reduction were formed for the conotoxin-based peptides. They were non-specific to the connectivities (loss of H_2S and CH_2S_2). For the Mod-based peptides, the 1,5-DAN matrix generated fragment ions, where some were found to be connectivity-specific (fragment ion peak at m/z 999.4 for the C_1 , C_2/C_3 , C_4 isomer and m/z = 2781.7 for the C_1 , C_3/C_2 , C_4 isomer).

Then, given that one cannot expect to form specific fragment ions for all peptides through the ISD process, as seen for the conotoxin peptides, we tried further fragmenting the partially reduced parent ions (opening of one of the two disulfide bonds) using MS/MS (high energy CID). Unfortunately, MS/MS (high energy CID) did not allow distinguishing between the different disulfide connectivities.

After interpreting the ISD and MS/MS (high energy CID) fragment ions, the disulfide-reduced parent ions were analyzed. Using isotope pattern deconvolutions, normalized intensities were calculated for the intact non-reduced parent ion, for the partially disulfide-reduced parent ion and for the totally reduced parent ion. Significant differences, taking into account the standard deviations from the replicate measurements (owing to t tests), could thus be found in isomer-ISD reactivities. The conotoxin peptides were distinguishable using the 1,5-DAN and the 2-AB matrices, in positive ionization mode. Regarding the Mod-based peptides, using the 1,5-DAN in conjunction with the 2-AB matrices allowed distinguishing disulfide connectivity isomers.

Finally, the calculation of the normalized intensities for the Mod-based peptides was expanded to their fragment ions formed using 1,5-DAN. This last result showed a distinction between ModBea and ModGlo peptides.

The obtained results pave the way for a quick and simple method for cysteine connectivity identification of peptides bearing two intramolecular disulfide bonds, without consuming much sample. However, the presented method necessitates the use of isomer standards of the chosen sequence to compare the normalized intensity plots with attributed connectivities. The positive MALDI-ISD analysis of such compounds using the 1,5-DAN matrix could also be useful for peptide sequencing without prior chemical disulfide reduction.

In terms of prospects, further ISD matrices having different reduction efficiencies could be tested and different peptides could be sampled to extend the presented data interpretation methodologies to other peptide sequences. The next step in the disulfide bond characterization could be the coupling of MALDI-ISD with ion mobility. Indeed, such analyses of the disulfide-reduced species and of the obtained fragment ions could then give a better understanding of their structures.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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