Combination of Capillary Electrophoresis and Ion mobility coupled to Mass Spectrometry and Theoretical Calculations for cysteine connectivity identification in peptides bearing two intramolecular disulfide bonds

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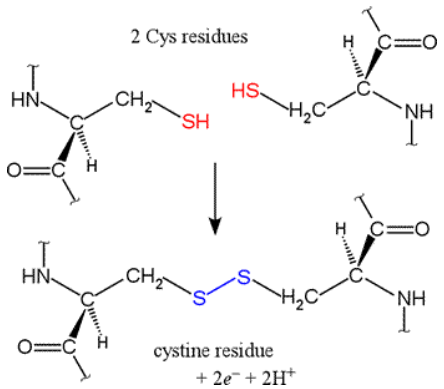
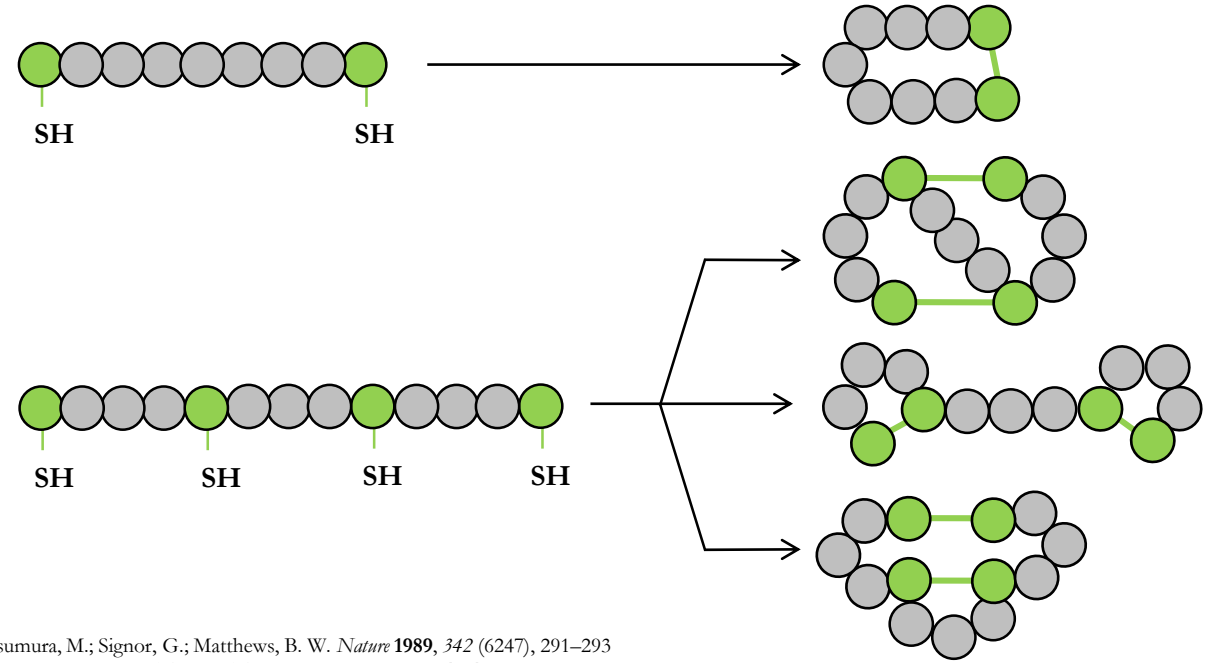
38th International Symposium on the Purification of Proteins, Peptides and Polynucleotides (ISPPP), 4-7th November 2018, Berlin

Context of the study : disulfide connectivity assignment



- Major post translational modification playing crucial roles in peptide stabilization and protein structures
- In some cases, the native disulfide pattern is essential for biological activities(1) or to preserve the biological activity(2)
- Misfolded variants can lead to reduced biological activity(2) and are generally degraded or recycled by enzymes to the native form(3)

The presence of multiple disulfide bonds leads to various disulfide isomers/variants :



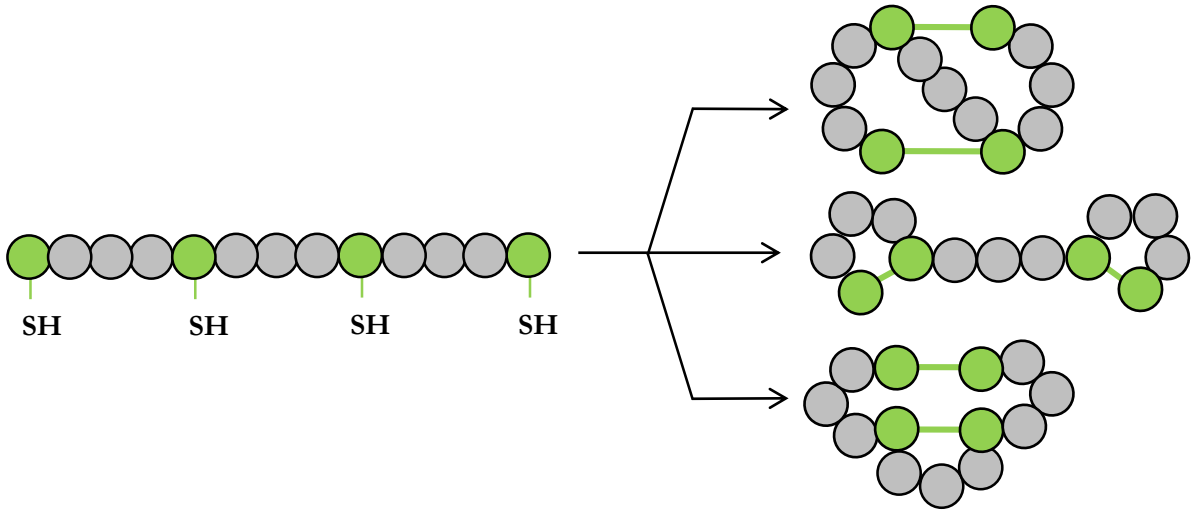
Peptide isomers

Same amino acid sequence but different disulfide connectivities

Potential variable biological activities

(1) Matsumura, M.; Signor, G.; Matthews, B. W. *Nature* **1989**, *342* (6247), 291–293
(2) Wu, Y.; Wu, X.; Yu, J.; Zhu, X.; Zhangsun, D.; Luo, S. *Molecules* **2014**, *19* (1), 966–979
(3) Trivedi, M. V.; Laurence, J. S.; Siahaan, T. J. *Curr. Protein Pept. Sci.* **2009**, *10* (6)

Characterization methods for S-S bonds connectivities in peptides and proteins: State-of-the-art

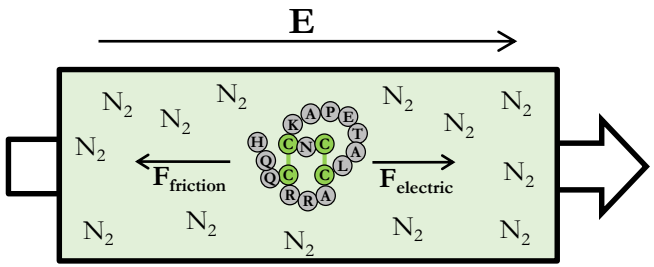


Method	Main Advantage	Main Drawback
X-Ray Crystallography	High structural resolution	Need for a crystal/fold
Nuclear Magnetic Resonance	High structural resolution	Large amount of sample needed
Bioinformatics	Only sequence is required	Not experimentally confirmed
Mass Spectrometry	Large number of approaches available (MALDI-ISD, ETD, CID, IM-MS, LC-MS/MS,…)	Complex spectral information

The use of Ion Mobility Spectrometry (IMS) for disulfide connectivity identification

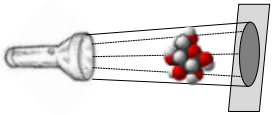
Ion Mobility :

Separation **in the gas phase** according to both charge (**q**) and collision cross section (**Ω**)



Courtesy of Bowers group Mobility Cell

Mason-Schamp



Projection approximation

$$K = \frac{3q}{16N}\cdot\left(\frac{2\pi}{kT}\right)^{\frac{1}{2}}\cdot\left(\frac{m+M}{mM}\right)^{\frac{1}{2}}\cdot\left(\frac{1}{\Omega}\right)$$

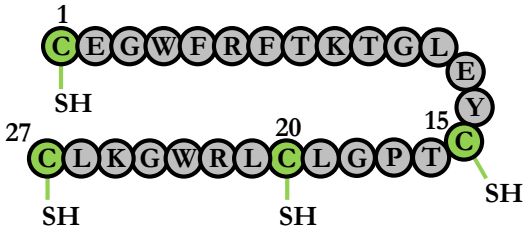
$$= \text{constant}.\left(\frac{m+M}{mM}\right)^{\frac{1}{2}}\cdot\frac{q}{\Omega}$$

K: mobility in gas phase ($m^2.V^{-1}.s^{-1}$)
q: charge of the ion (C)
T: temperature (K)
M: mass of ion (Da)
k: Boltzmann's constant ($1.38065.10^{-23}$ $J.K^{-1}$)
N: density number of buffer gas
m: mass of buffer gas (Da)

Ω: Collision Cross Section (m^2, $Å^2$) is accessible through a calibration

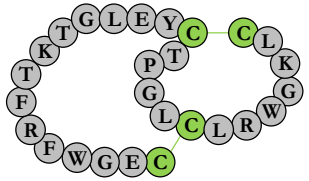
Model peptide :

27-residue synthetic peptide containing 4 cysteines with 3 possible intramolecular disulfide pairings (**conceptual rendering**) :



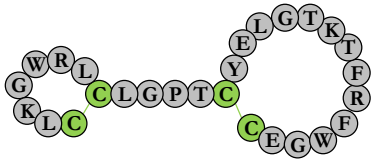
1) ModGlo

(Cys1-Cys20 / Cys15-Cys27)
C_1-C_3 / C_2-C_4



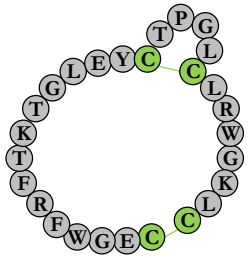
2) ModBea

(Cys1-Cys15 / Cys20-Cys27)
C_1-C_2 / C_3-C_4



3) ModRib

(Cys1-Cys27 / Cys15-Cys20)
C_1-C_4 / C_2-C_3



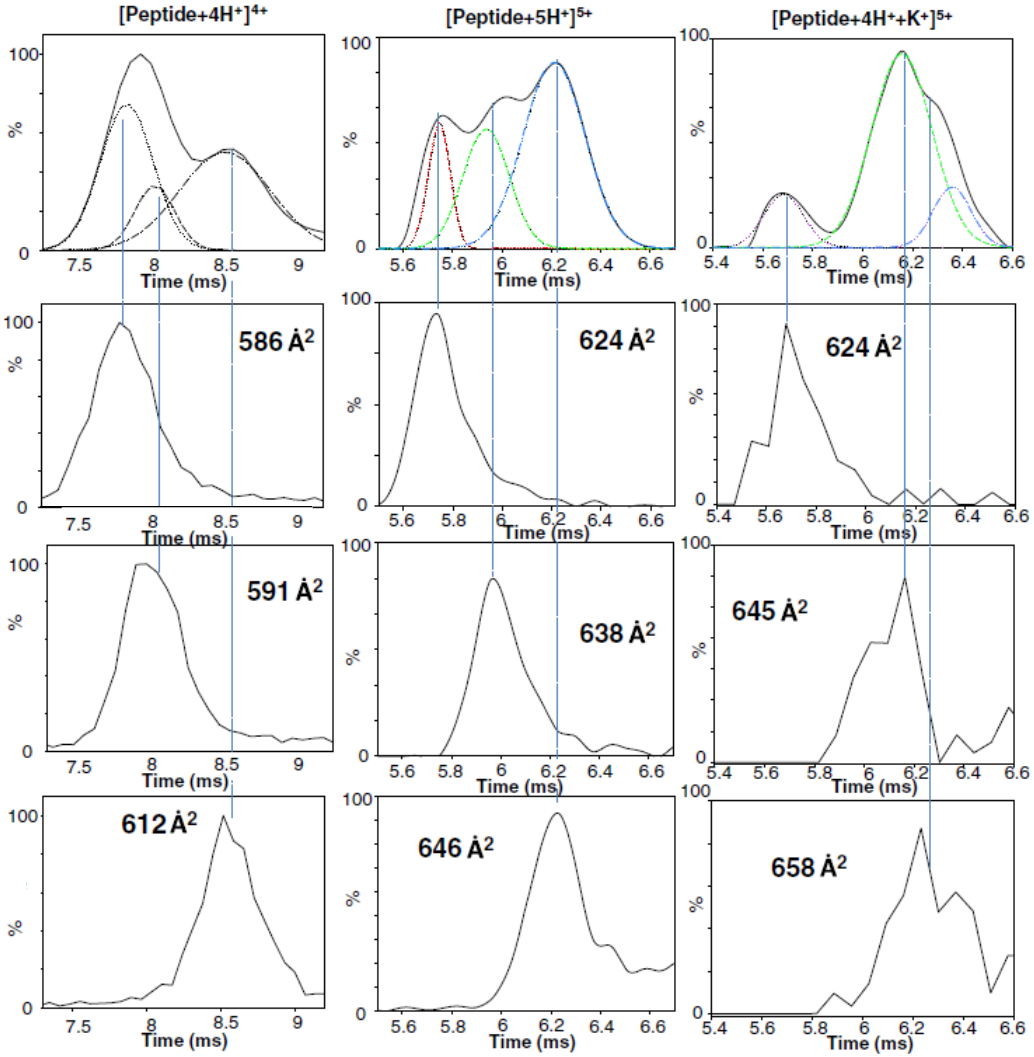
Published IM-MS method(1) on a synthetic model peptide

Mix of 3 model peptides isomers at equal concentrations

ModGlo
(Cys1-Cys20 / Cys15-Cys27)
C_1-C_3 / C_2-C_4

ModRib
(Cys1-Cys27 / Cys15-Cys20)
C_1-C_4 / C_2-C_3

ModBea
(Cys1-Cys15 / Cys20-Cys27)
C_1-C_2 / C_3-C_4

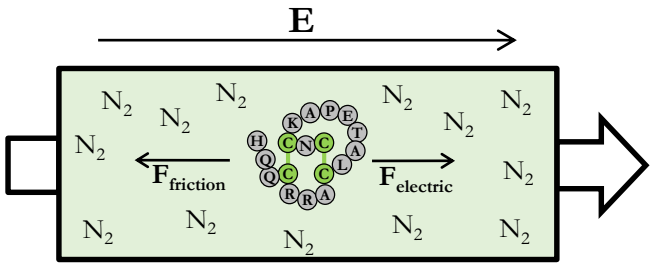


(1) Massonnet, P.; Haler, J. R. N.; Upert, G.; Degueldre, M.; Morsa, D.; Smargiasso, N.; Mourier, G.; Gilles, N.; Quinton, L.; De Pauw, E. **2016**, *27* (10), 1637–1646

Ion Mobility Spectrometry and Capillary Electrophoresis : mobility-based separation techniques

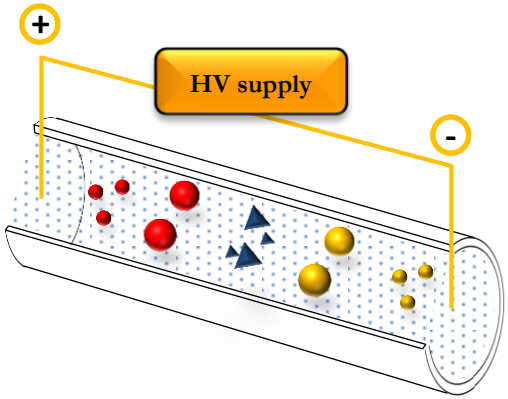
Ion Mobility :
Separation **in the gas phase** according to both charge (**q**) and collision cross section (**Ω**)

Capillary Electrophoresis :
Separation **in solution** according to both charge (**q**) (pH dependent) and hydrodynamic radius ($\mathbf{R_h}$)



Courtesy of Bowers group

Mobility Cell



$$F_{electric} = F_{friction} \rightarrow v_{stat} = \textbf{mobility constant}.\ E$$

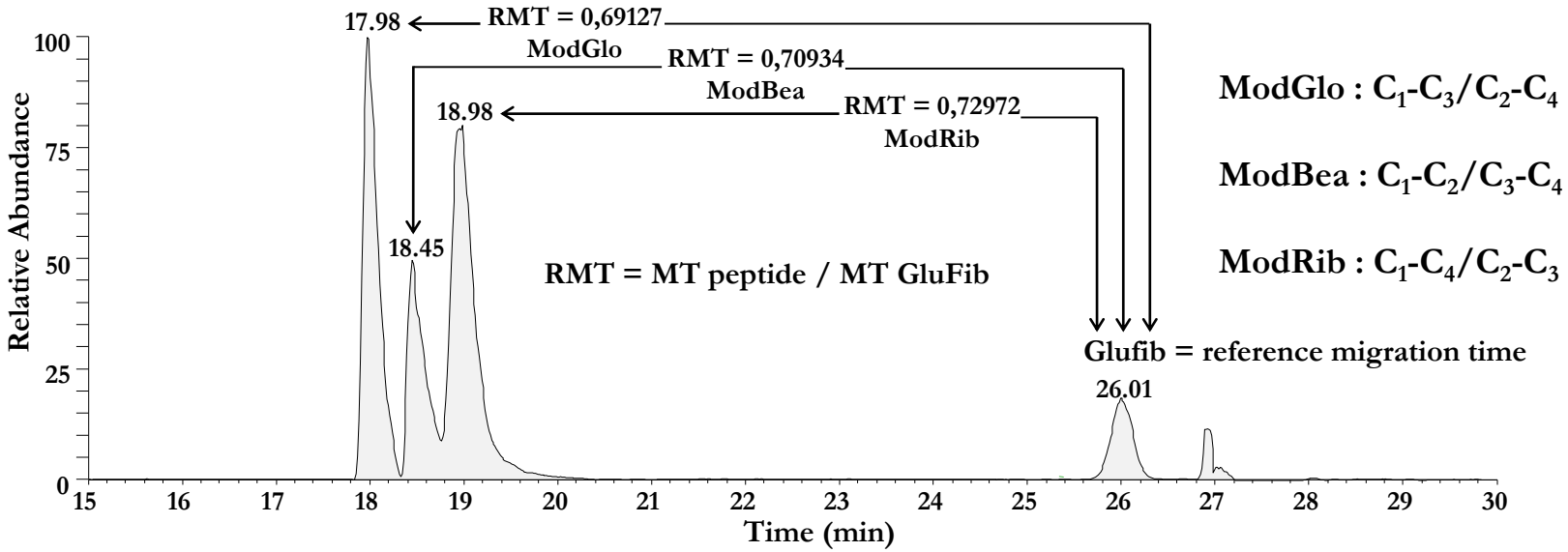
$$K = \frac{3q}{16N}.\left(\frac{2\pi}{kT}\right)^{\frac{1}{2}}.\left(\frac{m+M}{mM}\right)^{\frac{1}{2}}.\left(\frac{1}{\Omega}\right)$$

$$= \text{constant}.\left(\frac{m+M}{mM}\right)^{\frac{1}{2}}.\frac{q}{\Omega}$$

$$\mu_e = \frac{q}{6\pi\eta R_h} = \frac{1}{6\pi\eta}.\frac{q}{R_h}$$

CZE method development on a synthetic model peptide

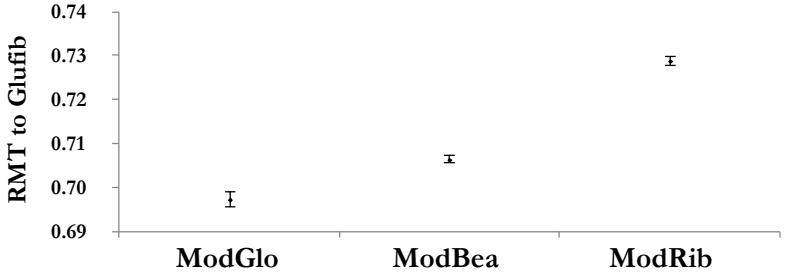
BGE = 80mM formic acid in 20% isopropanol 30µm x 150µm x 90cm BFS @+30kV



Determination of RMT (separate disulfide isomers)

Peptide	RMT (n=6)	σ (n=6)	%σ (n=6)
ModGlo	0,69741	0,00158	0,23%
ModBea	0,70650	0,00088	0,12%
ModRib	0,72882	0,00110	0,15%

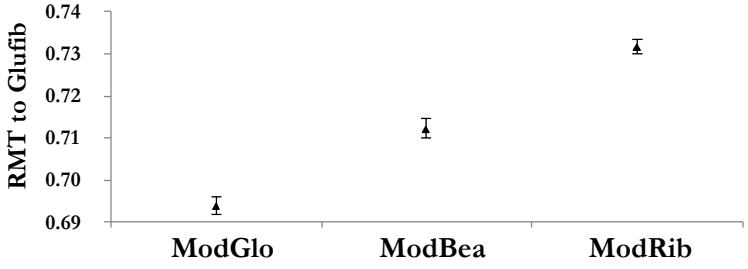
RMT = relative migration time σ = standard deviation
n = number of replicates



Determination of RMT (mix of the disulfide isomers)

Peptide	RMT (n=6)	σ (n=6)	%σ (n=6)
ModGlo	0,69388	0,00217	0,31%
ModBea	0,71225	0,00228	0,32%
ModRib	0,73169	0,00185	0,25%

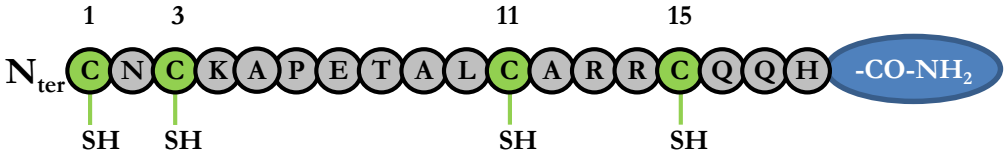
RMT = relative migration time σ = standard deviation
n = number of replicates



Expanding the method to biologically relevant peptides : apamins

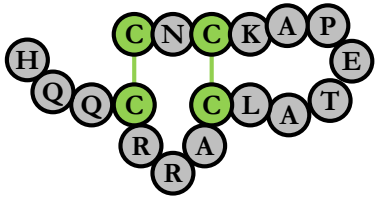
1st biologically relevant peptide : **Apamin**

Naturally occurring 18-residue peptide contained in the venom of bees



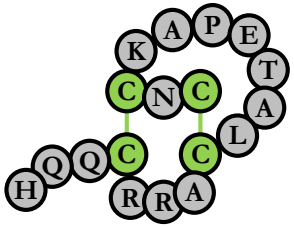
With 3 possible intramolecular disulfide pairings (**conceptual rendering**) :

ApaRib



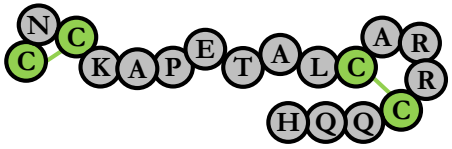
(Cys1 – Cys15 / Cys3 – Cys11)
C_1-C_4/C_2-C_3
Purely synthetic

Apamin



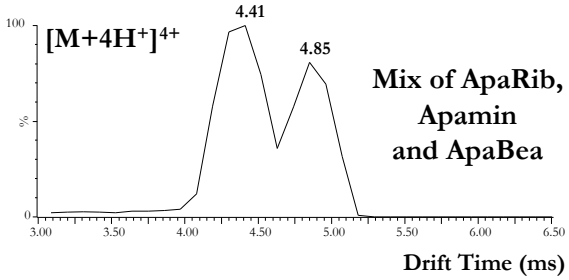
(Cys1 – Cys11 / Cys3 – Cys15)
C_1-C_3/C_2-C_4
Naturally occuring Apamin

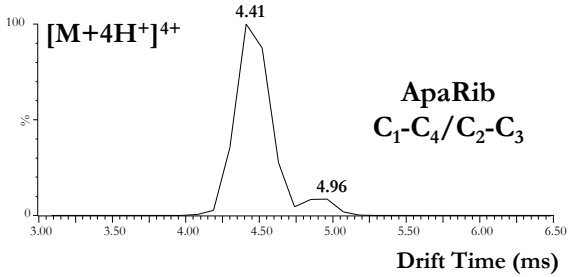
ApaBea

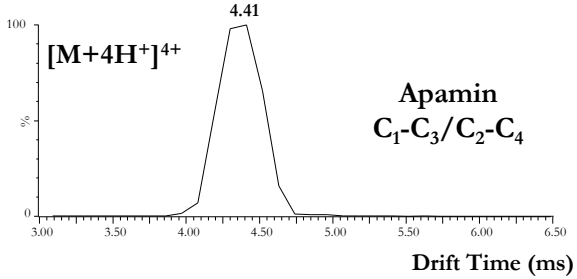


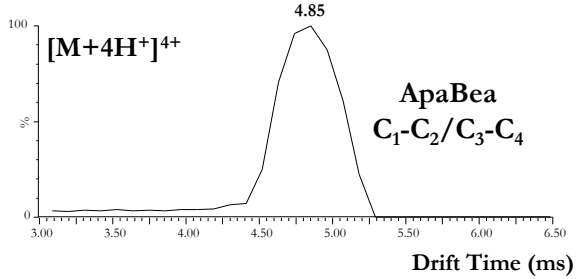
(Cys1 – Cys3 / Cys11 – Cys15)
C_1-C_2/C_3-C_4
Purely synthetic

IM-MS/MS results of the apamins

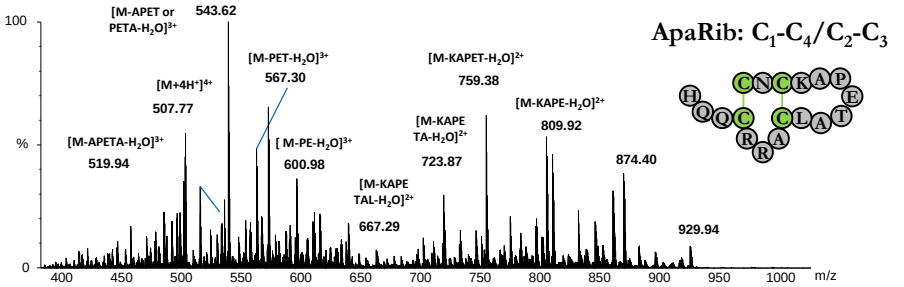


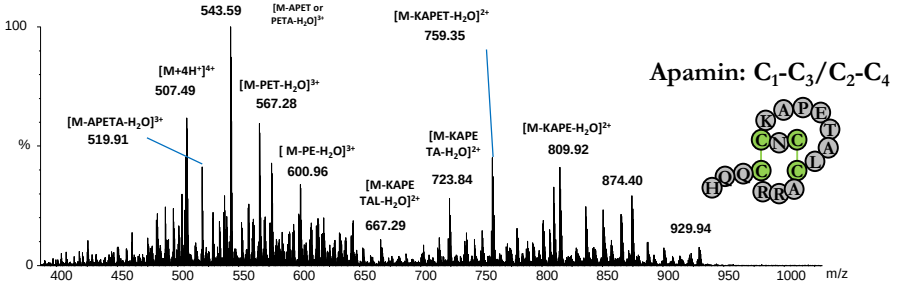


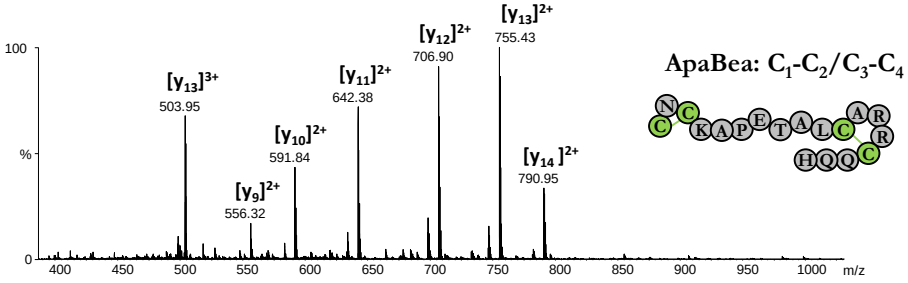




MS/MS spectra



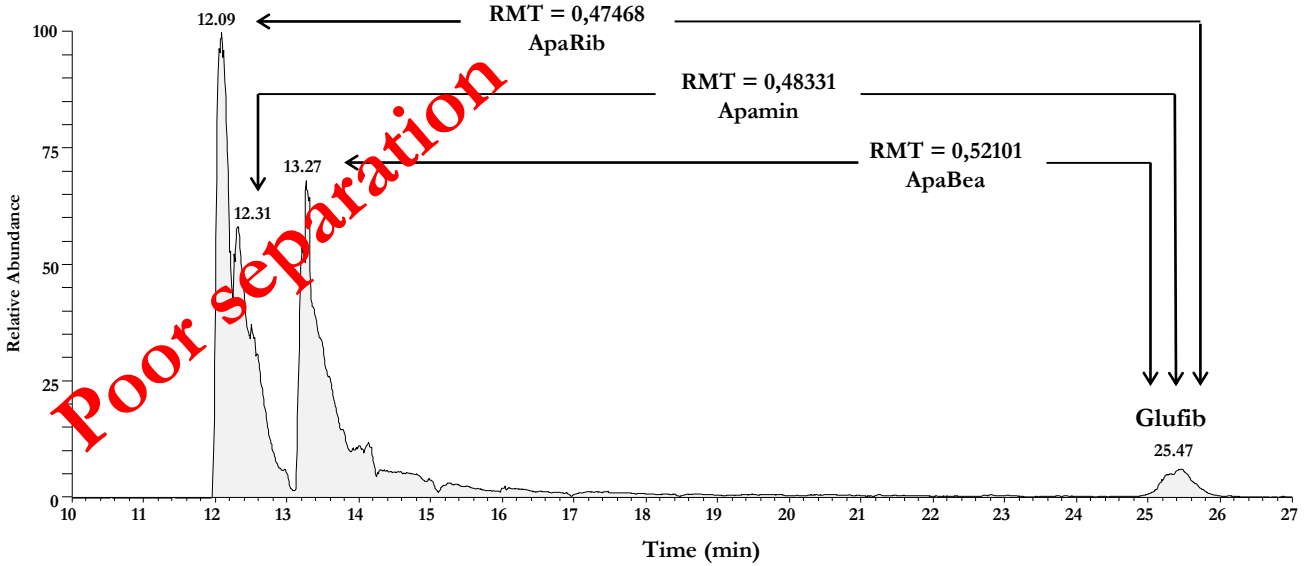




CZE-MS results of the apamins in an acidic buffer

BGE = 100mM formic acid

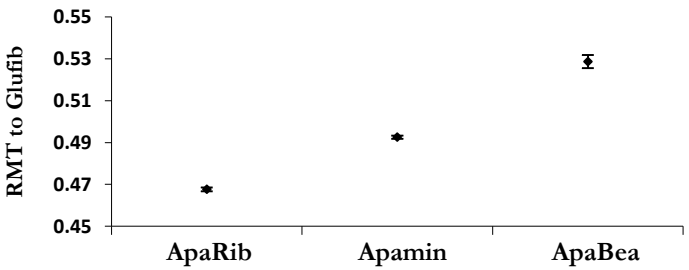
30µm x 150µm x 90cm BFS @+30kV



Determination of RMT (separate disulfide isomers)

Peptide	RMT (n=6)	σ (n=6)	%σ (n=6)
ApaRib	0,46764	0,00086	0,18%
Apamin	0,49250	0,00075	0,15%
ApaBea	0,52866	0,00312	0,59%

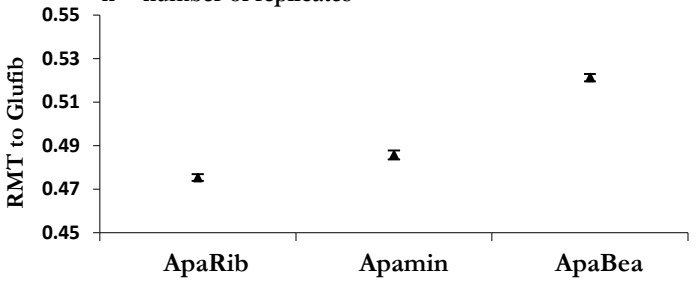
RMT = relative migration time σ = standard deviation
n = number of replicates



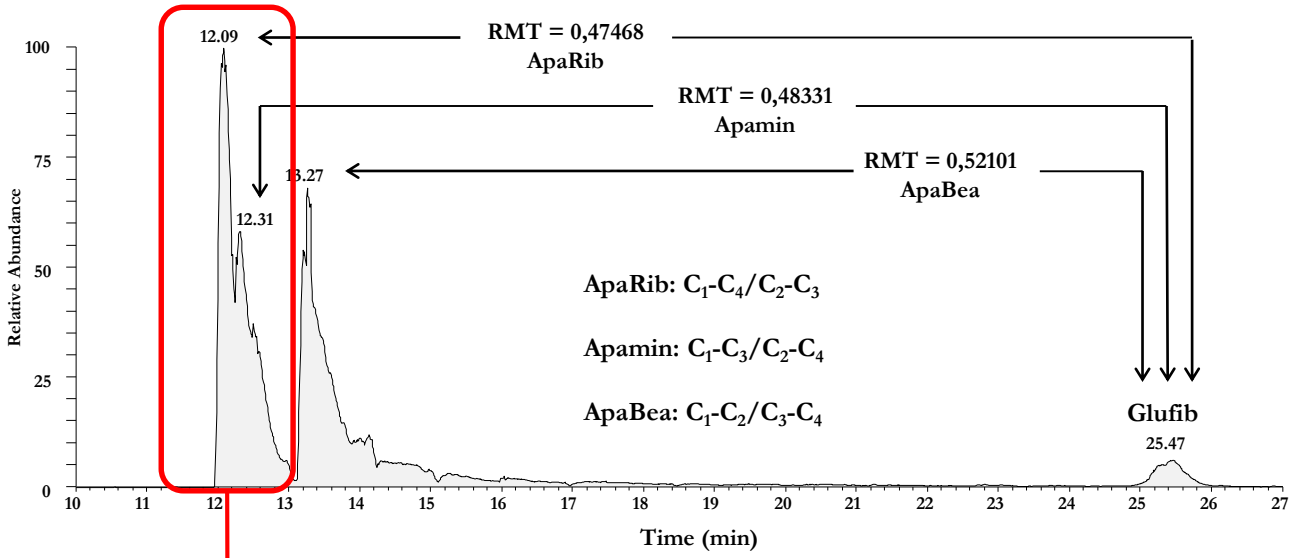
Determination of RMT (mix of the disulfide isomers)

Peptide	RMT (n=6)	σ (n=6)	%σ (n=6)
ApaRib	0,47529	0,00154	0,32%
Apamin	0,48571	0,00211	0,43%
ApaBea	0,52124	0,00169	0,32%

RMT = relative migration time σ = standard deviation
n = number of replicates

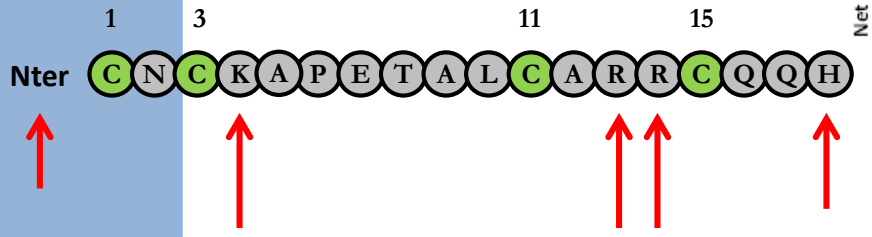


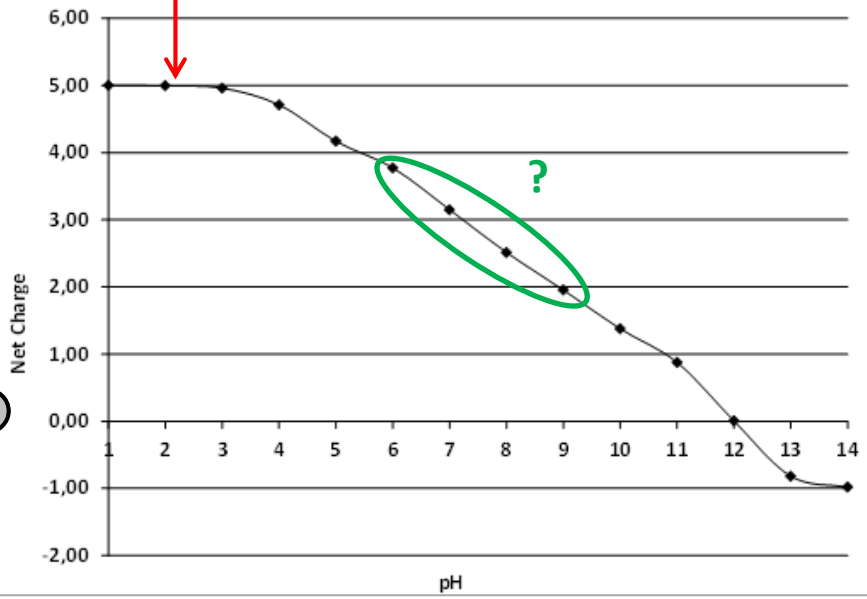
Could pH optimization of the buffer improve the CZE-MS separation of the apamins ?



Insufficient separation

Diagram of theoretical average charge distribution (in-solution) of apamins according to Henderson-Hasselbalch

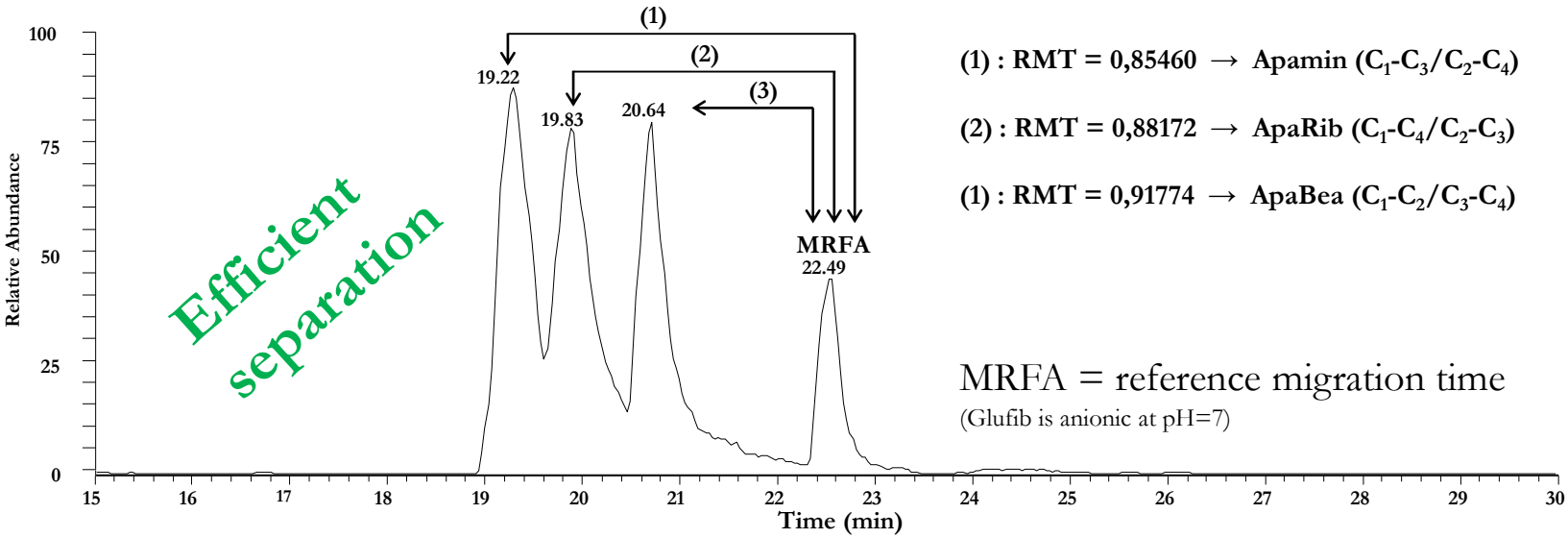




CZE-MS results of the apamins in a neutral buffer

BGE = NH_4Ac 50mM pH 7

30µm x 150µm x 90cm BFS @+20kV



(1) : RMT = 0,85460 → Apamin (C_1-C_3/C_2-C_4)

(2) : RMT = 0,88172 → ApaRib (C_1-C_4/C_2-C_3)

(1) : RMT = 0,91774 → ApaBea (C_1-C_2/C_3-C_4)

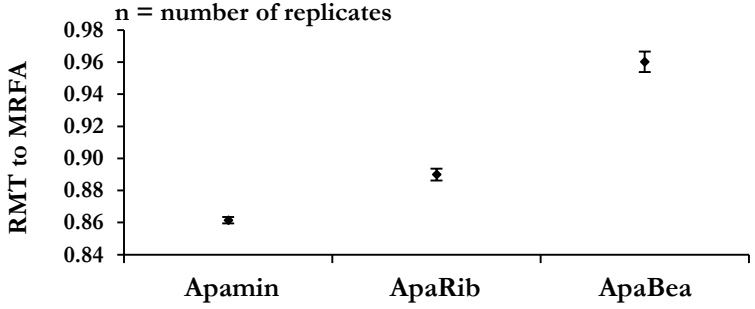
MRFA = reference migration time

(Glufib is anionic at pH=7)

Determination of RMT (separate disulfide isomers)

Peptide	RMT (n=6)	σ (n=6)	%σ (n=6)
Apamin	0,86142	0,00199	0,23%
ApaRib	0,88990	0,00376	0,42%
ApaBea	0,96019	0,00644	0,67%

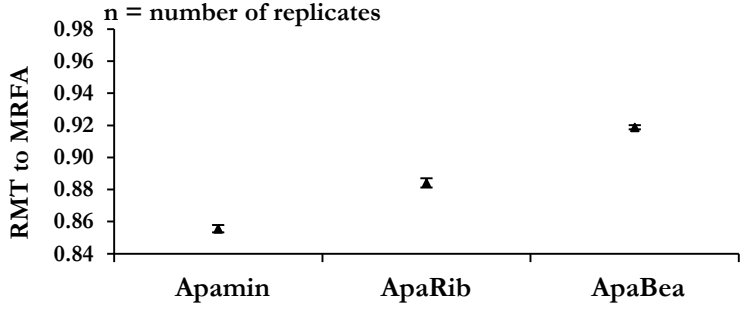
RMT = relative migration time σ = standard deviation
n = number of replicates



Determination of RMT (mix of the disulfide isomers)

Peptide	RMT (n=6)	σ (n=6)	%σ (n=6)
Apamin	0,85550	0,00230	0,27%
ApaRib	0,88404	0,00292	0,33%
ApaBea	0,91875	0,00133	0,15%

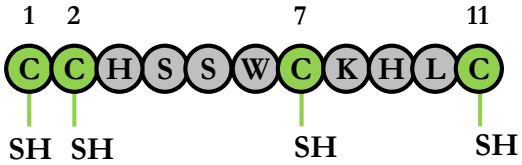
RMT = relative migration time σ = standard deviation
n = number of replicates



Expanding the method to biologically relevant peptides : conotoxins

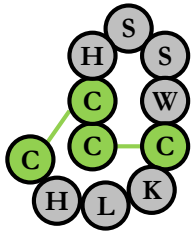
2nd biologically relevant peptide : **α and χ conotoxins**

Naturally occurring 11-residue peptide contained in the venom of marine cone snails



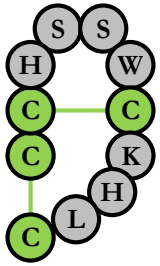
With 2 possible intramolecular disulfide pairings (**conceptual rendering)** :

Conotoxin α



(Cys1 – Cys7 / Cys2 – Cys11)
C_1-C_3 / C_2-C_4
Purely synthetic

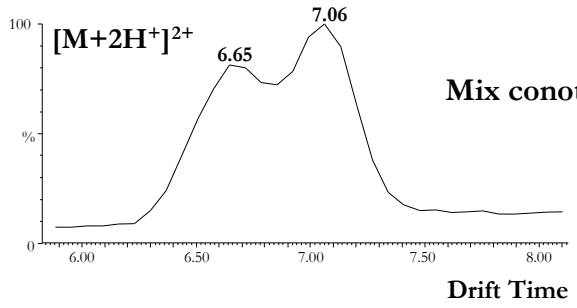
Conotoxin χ



(Cys1 – Cys11 / Cys2 – Cys7)
C_1-C_4 / C_2-C_3
Naturally occuring

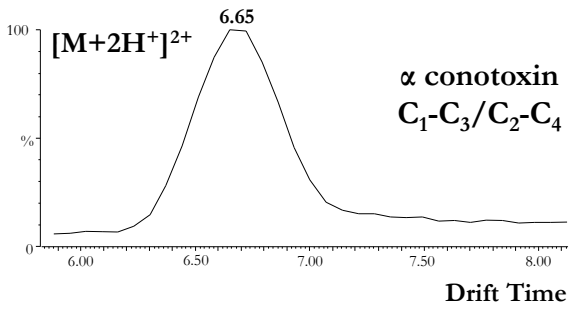
IM-MS/MS results of α and χ conotoxins

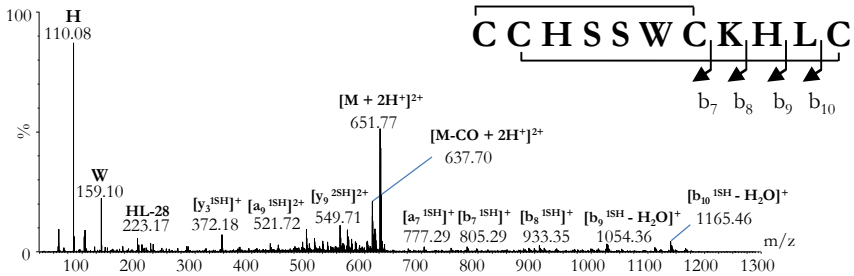
(A)



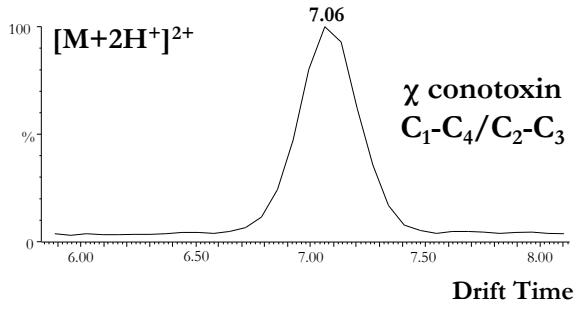
MS/MS spectra

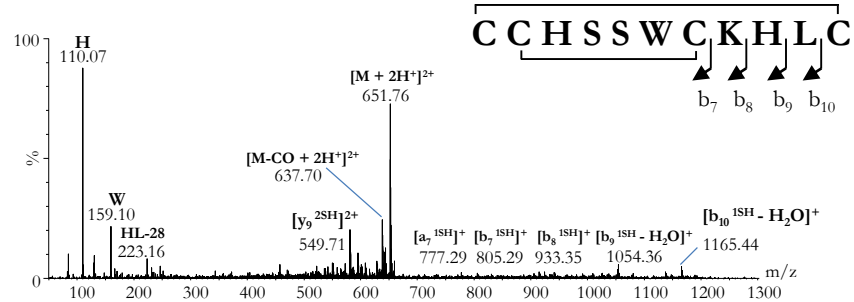
(B)





(C)





CZE-MS results of the conotoxins in an acidic buffer

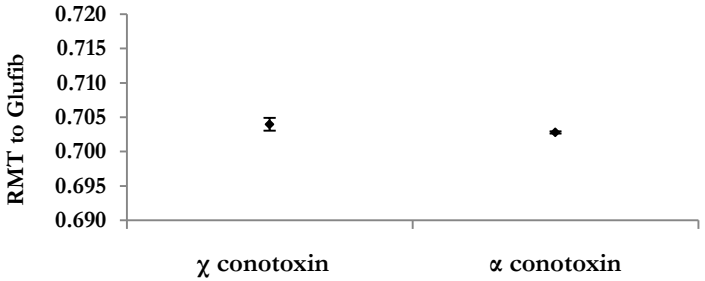
BGE = 100mM formic acid

30µm x 150µm x 90cm BFS @+30kV

(A) **Determination of RMT (separate disulfide isomers)**

Peptide	RMT (n=6)	σ (n=6)	%σ (n=6)
χ conotoxin	0,70396	0,00094	0,13%
α conotoxin	0,70281	0,00016	0,02%

RMT = relative migration time σ = standard deviation
n = number of replicates



(B) **Electropherogram of the disulfide isomers mix**

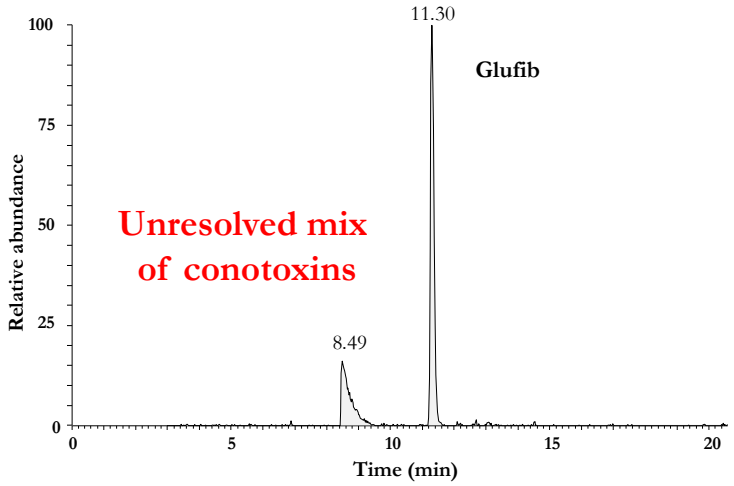
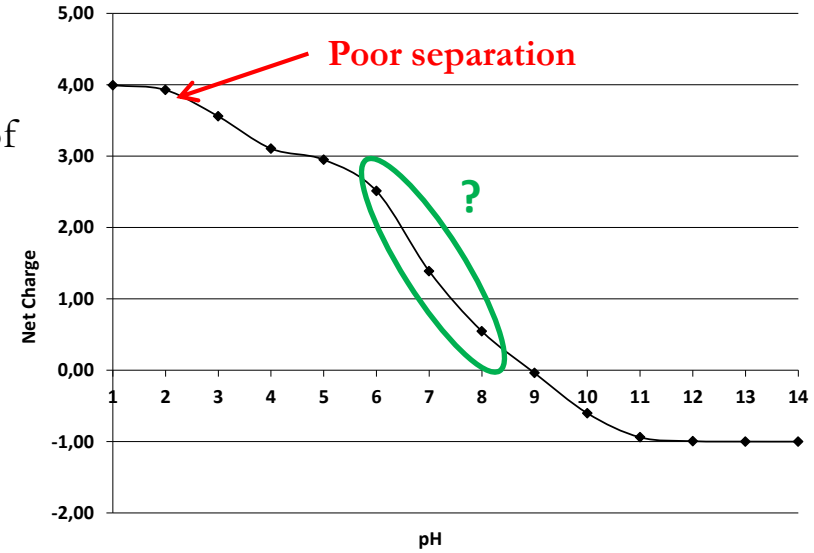


Diagram of theoretical average charge distribution (in-solution) of conotoxins according to Henderson-Hasselbalch



CZE-MS results of the conotoxins in a neutral buffer

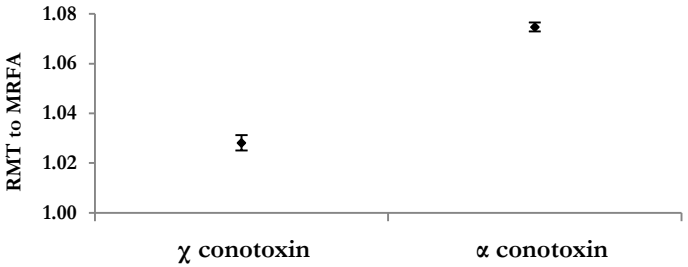
BGE = NH_4Ac 25mM pH 7

30µm x 150µm x 90cm BFS @+30kV

(A) **Determination of RMT (separate disulfide isomers)**

Peptide	RMT (n=6)	σ (n=6)	%σ (n=6)
χ conotoxin	1,02815	0,00305	0,30%
α conotoxin	1,07469	0,00181	0,17%

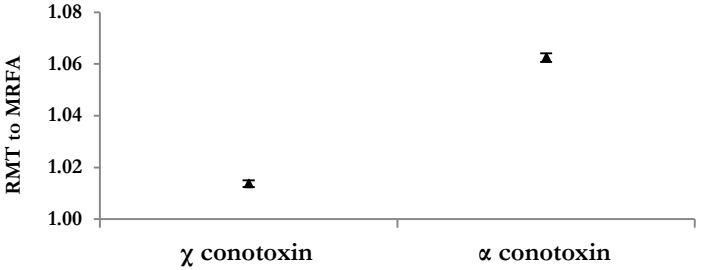
RMT = relative migration time **σ = standard deviation**
n = number of replicates



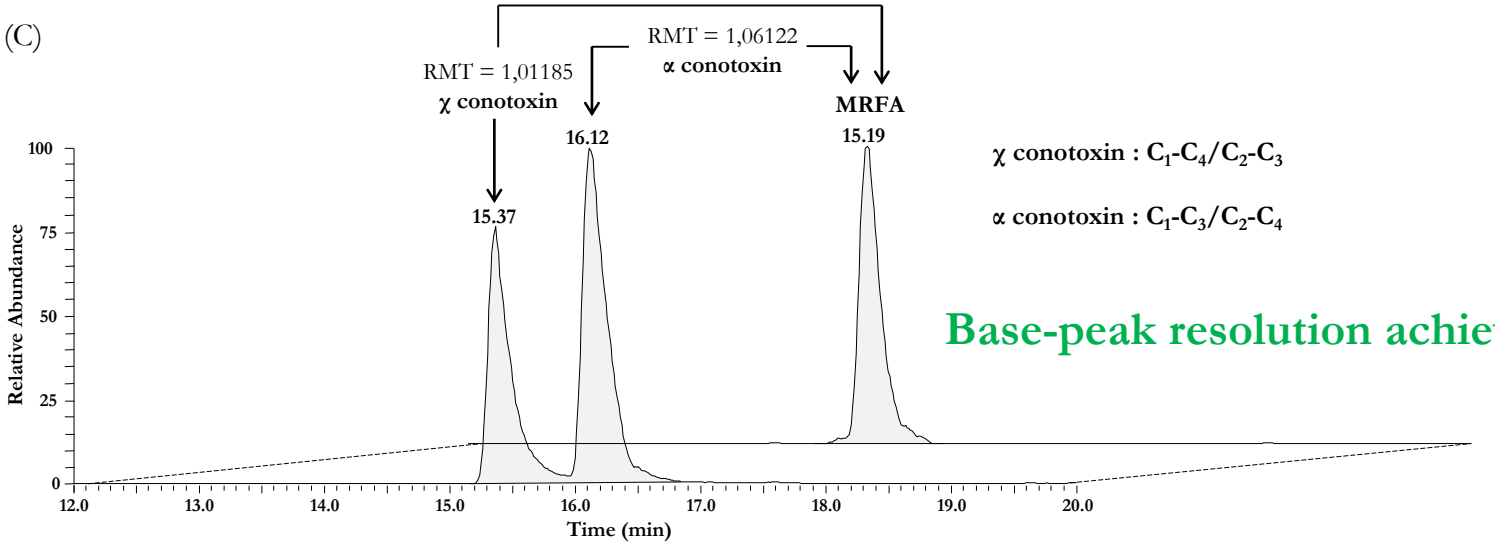
(B) **Determination of RMT (mix of the disulfide isomers)**

Peptide	RMT (n=6)	σ (n=6)	%σ (n=6)
χ conotoxin	1,01371	0,00128	0,13%
α conotoxin	1,06252	0,00166	0,16%

RMT = relative migration time **σ = standard deviation**
n = number of replicates



(C)



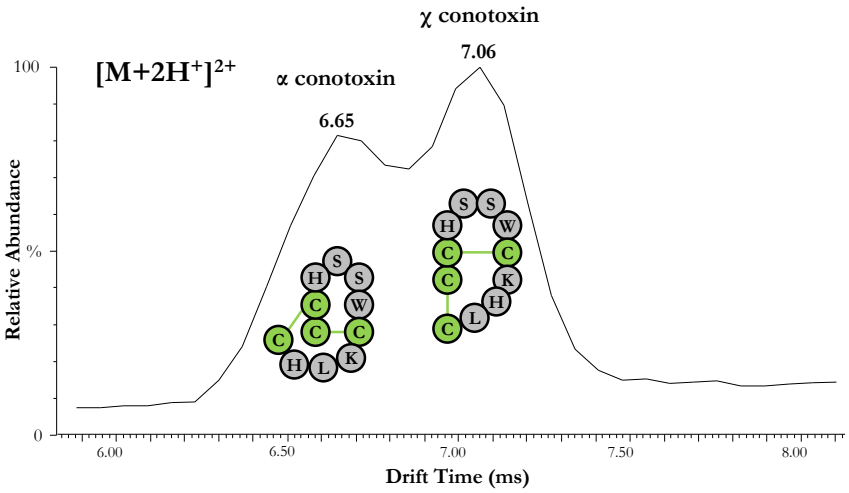
χ conotoxin : C_1-C_4/C_2-C_3

α conotoxin : C_1-C_3/C_2-C_4

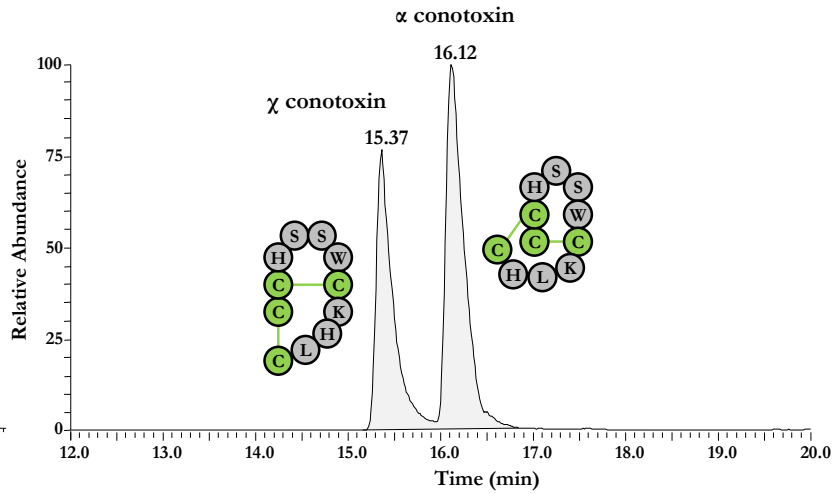
Base-peak resolution achieved

Comparison between the migrations in the gas phase (IMS) and the solution (CZE)

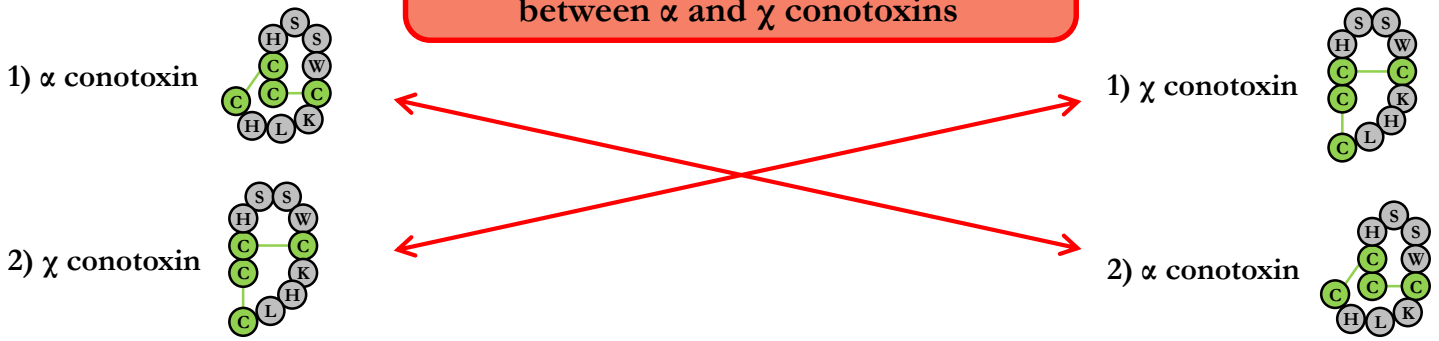
IM-MS



CZE-MS

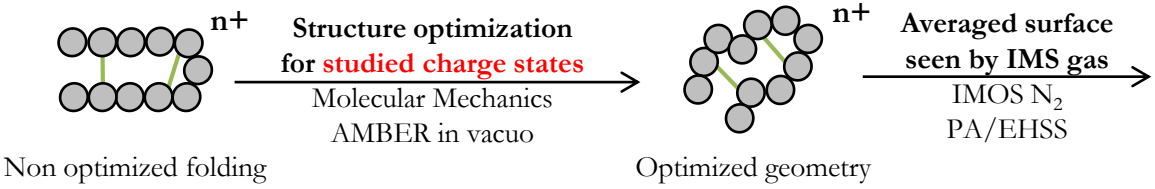


IM-MS and CZE-MS results highlight differential migration behaviors between α and χ conotoxins

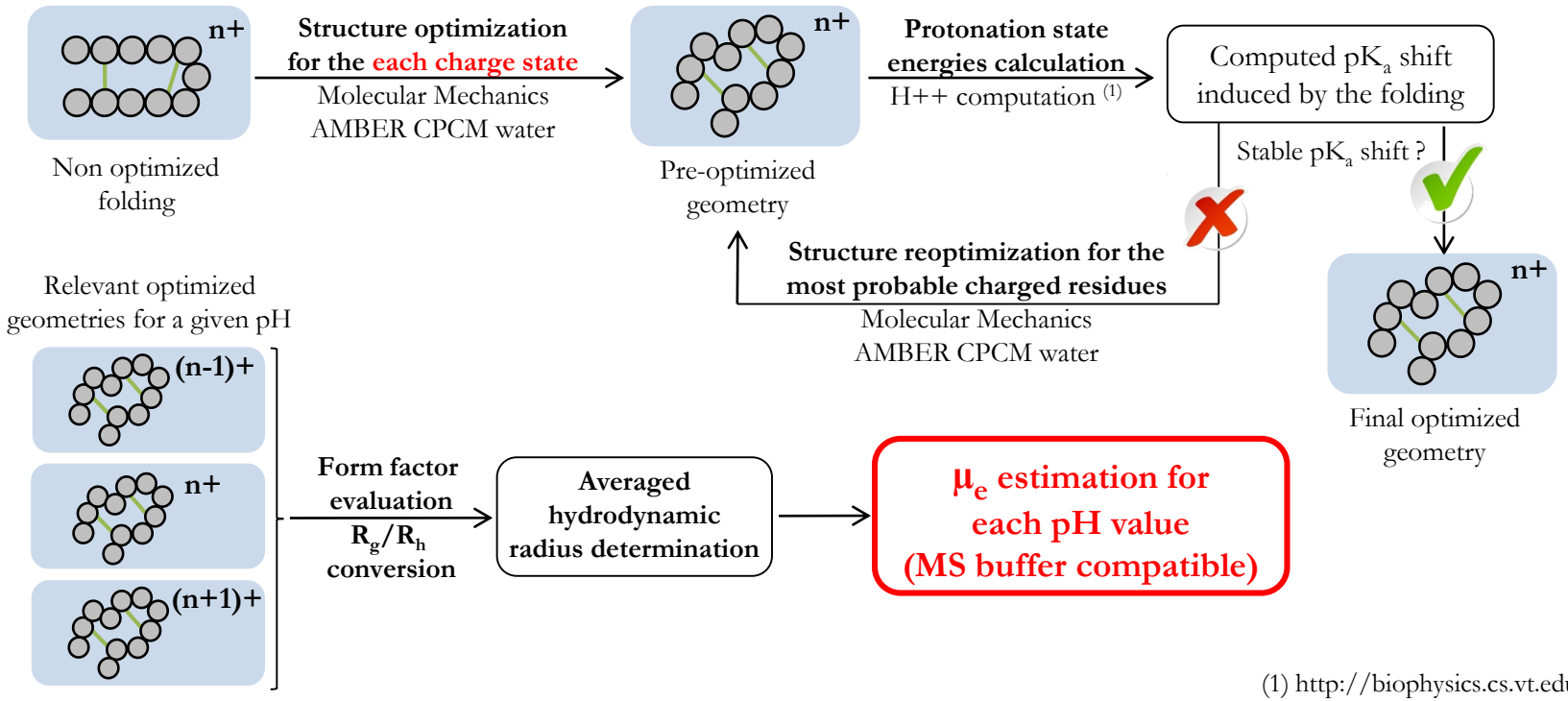


Theoretical calculations for structure elucidation

IMS: CCS estimation from structure optimization (in vacuo)



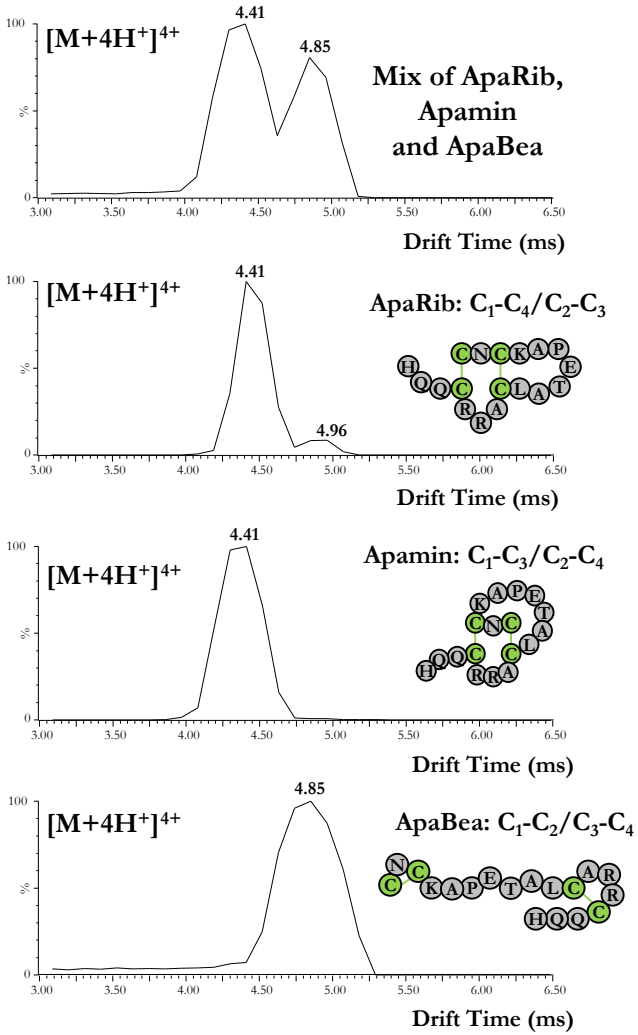
CZE: μ_e estimation from structure and charge state optimization (in water)



(1) http://biophysics.cs.vt.edu/

Theoretical calculations for structure elucidation

IMS: CCS estimation from structure optimization (in vacuo)



Disulfide isomer	Calculated CCS (EHSS)
ApaRib	377Å
	7% CCS difference < IMS resolution
Apamin	405Å
	17% CCS difference > IMS resolution
ApaBea	474Å

Conclusions

- IMS (drift time), CZE (relative migration time) and fragmentation patterns (MS/MS spectra) provide complementary set of data for a complete disulfide connectivity characterization in all studied cases
- CZE-MS : improvement of the separation by pH optimization → taking advantage of the influence of the folding on the pI value of the peptide
- Theoretical calculations allow to get an insight into structural differences of the disulfide isomers in both gas phase and solution
  - Optimized structures : explanation of the observed switch in the migration order between the gas phase and the solution
  - IM-MS : Structural effects at fixed charge state provide the CCS difference in the gas phase
  - CZE-MS : Averaged charge states due to buffer pH affect both the peptide structure and charge, leading to differential electrophoretic mobilities

Acknowledgment