



Crown ethers as shift reagents in peptide epimer differentiation –conclusions from examination of ac-(H)FRW-NH₂ peptide sequences

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Abstract

Crown ethers with different ring sizes and substituents (18-crown-6, dibenzo-18-crown-6, dicyclohexano-18-crown-6, a chiral tetracarboxylic acid-18-crown-6 ether, dibenzo-21-crown-7, and dibenzo-30-crown-10) were evaluated as shift reagents to differentiate epimeric model peptides (tri- and tetrapeptides) using ion mobility mass spectrometry (IM-MS). The stable associates of peptide epimers with crown ethers were detected and examined using traveling-wave ion mobility time-of-flight mass spectrometer (Synapt G2-S HDMS) equipped with an electrospray ion source. The overall decrease of the epimer separation upon crown ether complexation was observed. The increase of the effectiveness of the microsolvation of a basic moiety - guanidine or ammonium group in the peptide had no or little effect on the epimer discrimination. Any increase of the epimer separation, which referred to the specific association mode between crown substituents and a given peptide sequence, was drastically reduced for the longer peptide sequence (tetrapeptide). The obtained results suggest that the application of the crown ethers as shift reagents in ion mobility mass spectrometry is limited to the formation of complexes differing in stoichiometry rather than it refers to a specific coordination mode between a crown ether and a peptide molecule.

Keywords Peptide epimers · Ion mobility mass spectrometry (IM-MS) · Non-covalent complexes · Crown ether · TWIMS

Introduction

Peptides belong to the most biologically relevant group of molecules. Although it has long been assumed that in higher animals proteins and peptides are built exclusively from L-amino acids, the analysis of D-amino acid-containing peptides as the source of naturally and unnaturally occurring modifications in living organisms is becoming an increasingly important issue. [1, 2] Similarly to other types of isomers, although differing in chemical, physical, and biological properties, they remain undistinguishable using direct mass spectrometry measurements, due to the identical ratio of the mass of ion to its charge (m/z). Therefore, additional mass spectrometry-based methodology, which takes advantage of the differences in

physicochemical gas-phase properties of isomeric ions, is required to allow for isomer discrimination. [3] Distinguished fragmentation pathways, intensities of ionic fragments, or decomposition energy of isomeric ions or their non-covalent complexes with a selector molecule are commonly used strategies towards isomer analysis. [4–9] Indirect approaches involve other techniques used in tandem with mass spectrometry (*e. g.*, chromatographic or capillary electrophoretic techniques). [10, 11] Among mass spectrometry-based approaches, mass spectrometry combined with ion-mobility (IM-MS) allows for additional separation of ions according to their mobilities (K) through an inert gas in a drift tube with an applied electric field. [12]

The ability of IM-MS to separate isomeric ions is determined by the difference of ion mobilities as well as the resolving power of ion mobility drift tube. Due to the limited resolving power of ion mobility drift tubes and at the same time the small difference in mobilities of isomeric ions, the supplementary methodology of the addition of a shift reagent is used. The shift reagents are the additives that form associates with ions to enable their mobility shifts to different regions of the mobility spectrum. They may introduce the conformational diversity among isomeric ions and make operative different coordination

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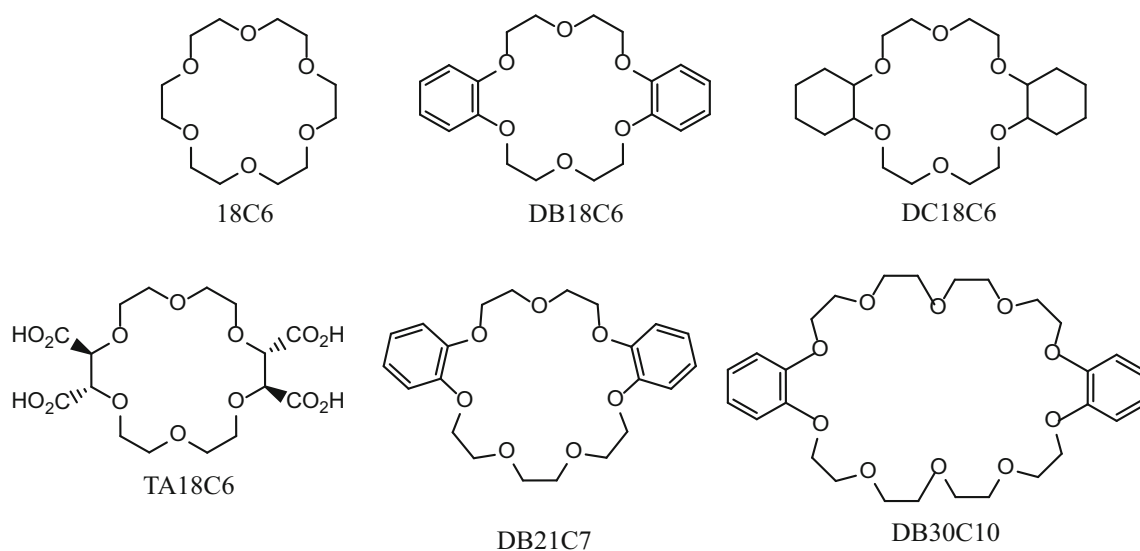
schemes for a given isomeric system. These additives may also induce significant differences of electrostatic charge distributions and dipole moments between isomeric ionic species, and hence improve the ion mobility separation. [13]

This general approach of using shift reagents was followed to induce separation of enantiomers of the amino acids tryptophan and phenylalanine in differential ion mobility spectrometry with *N*-*tert*-butoxycarbonyl-*O*-benzyl-L-serine as the shift reagent. [14] The formation of trimeric Cu(II)-bound complexes with aromatic amino acid enantiomers allowed for their partial separation using TWIMS. [15] Recently, dimerization, oligomerization and metalation (K^+) was shown to improve the mobility separation of selected epimeric peptides of dermorphin and achatin-I. [16] The employment of a new technique of trapped ion mobility (TIMS) [17] available in custom-built instrument [18, 19] turned out to be a very promising in the separation of D-amino-acid-containing peptides. [20] This technique has shown substantially high ion mobility resolving power R up to 400, in comparison to the earlier mentioned TWIM spectrometry, which offers a modest resolving power ($R \approx 25$ –60) in ion separation. The site-specific strategy combining the reversed-phase liquid chromatography separation assisted with fragmentation by collision-induced dissociation (CID), and followed by IMS analysis turned out to be a very useful approach in determining the exact position of D-amino acid in selected epimeric peptides. [21] The recent advance in improving mobility resolving power was reported for cyclic ion mobility device (about 750), [22] implemented in a commercially available Waters SYNAPT G2-Si instrument. In such a device, the resolving abilities are related to the number of cycles which ions are passing through the cyclic tube. Hence, a drop of ion transmission while increasing the number of subsequent cycles may be a major disadvantage in this approach.

In this manuscript, the effects of the complexation with crown ethers (Scheme 1) on the ion mobility shift of model epimeric peptides is discussed. The crown ethers with various ring sizes and substituents are an important group of complexing reagents used in ion recognition and molecular scaffold. [23, 24] The ability of crown ethers to bind positively charged side chain of lysine and arginine [25–28] was used in structure determination and surface characterization of peptides and proteins using ESI-MS. [29–32] For instance, the 18-crown-6 (18C6) effectively solvated the side chain of a lysine residue in the protein, providing the information about their exterior exposition. The selectivity of dibenzo-30-crown-10 (DB30C10) and 18-crown-6 (18C6) association was used to determine the presence of lysine or arginine in a peptide sequence. [30]

A series of crown ethers was previously examined as shift reagents for peptide analysis by ion mobility spectrometry. [33, 34] The isobaric or having similar masses or cross section ions formed complexes of different stoichiometry with ethers, depending on the peptide sequence. This complexation affected the mobility shift of ions. The improvement of ion separation was mainly associated with the formation of complexes differing in stoichiometry rather than with a specific coordination mode between a crown ether and a peptide. For example, the ion mobility shifts of the dipeptides isobars: RA, KV, LN upon complexation with crown ethers were mainly due to the different complexation stoichiometry, which in turn was closely related to the selectivity and specificity of the formation of the complexes between basic amino acid side chain or NH_2 -terminus with crown ethers depending on the crown size.

In this manuscript the effect of microsolvation of the amino acid basic side-chain on the ion mobility shift of epimeric peptides is described. The microsolvation of the protonated basic side chain of a peptide by a crown ether may drastically change the peptide conformation and may lead to the shift of



Scheme 1 Crown ethers examined as ion-mobility shift reagents

the peptide mobility. In the gas phase the peptide conformation is associated with the enhancing role of electrostatic interactions, as a result of which intramolecular interactions between charged protonated side chains and backbone carbonyl groups become important. This collapse of charged site chains onto the backbone along with the conformational change was observed for protein ions upon their transfer from solution to the gas phase, investigated by ion mobility mass spectrometry. [35] The conformational change was induced by the formation of new intramolecular interactions between charged, protonated basic sites and backbone carbonyls, which would then no longer be involved in the structure-stabilizing system of hydrogen bonds. It was shown that the microsolvation of protonated sites of protein by 18-crown-6 effectively inhibits this process. In a smaller molecular systems, the conformational change associated with the microsolvation by the crown ether is also observed. [36] The IM-MS studies of the alanine-based peptides with different locations of lysine residue have shown that the position of the basic-lysine amino acid in a peptide chain and associated with this the ability to form intramolecular interactions between amino group of lysine side chain and C-terminal carbonyl group may tune the peptide conformation between globular to helical forms. The propensity of peptide to helix formation upon microsolvation by 18-crown-6 ether was observed as a result of combination of lysine isolation effect and the C-terminal capping effect of 18C6.

The above mentioned examples clearly show that the microsolvation of the basic sites in peptides have a relevant role in their conformations in the gas phase. These studies were the inspiration for further detailed consideration on the conformational changes induced by the microsolvation by the crown ethers. While microsolvation has been shown to drastically alter the conformation of proteins and larger peptides, its effects are virtually unknown on smaller peptide systems. In this manuscript tri- and tetrapeptide models are examined. Previous studies have shown small enhancement of ion mobility separation of two isobaric dipeptide ions upon the same binding stoichiometry with the crown ($[RA + 3(18C6) + 2H]^{2+}$ and $[KV + 3(18C6) + 2H]^{2+}$). [33] On the other hand, a decrease of separation was observed upon increasing the crown ether size. In the case of tri- and tetrapeptides this effect should have a little impact, as the sizes of tri- and tetrapeptides should be larger than for the crown ether even in the case of the largest DB30C10 analyzed in this study. The collision cross section (based in nitrogen drift gas) of the simple alanine containing tripeptide and tetrapeptide are as follows: 151 \AA^2 , 166 \AA^2 , [37] while the collision cross section of the largest crown ether used in this study, DB30C10 is reported to be 155 \AA^2 . [33]

In our model peptide system: Ac-(H)FRW-NH₂, arginine (R) is implemented in the tri- and tetrapeptide as the basic amino acid. The efficacy of microsolvation of the arginine guanidinium group obtained with the use of crown ethers of various sizes, is examined in terms of enhancing separation of

epimeric peptides. In tetrapeptide the presence of the additional basic amino acid, histidine (H) may enable the formation of higher aggregates and may allow the higher charge state of ions to be analyzed. The remaining amino acids in the analyzed sequence belong to the aromatic amino acids, phenylalanine (F) and tryptophane (W) and have been chosen to enable evaluation of the effect associated with the interactions between peptide aromatic side chains and substituent groups incorporated in a crown ring on the mobility separation of the model epimers. All possible epimeric structures with respect to the L-amino acid containing Ac-(H)FRW-NH₂ were analyzed as they represent different local environment of the peptide basic site, and its different access to the backbone carbonyl groups, which in turn is crucial for the formation of the gas-phase stable peptide conformations. In addition, the substitution of arginine with lysine residue (Ac-HFKW-NH₂) sheds a light on the origin of the observed effects depending on the nature of the basic site.

Experimental

Materials

Peptides were custom synthesized and delivered by TriMen Chemicals. Crown ethers were obtained from Sigma Aldrich Chemical Co.

IM-MS experiments and data processing

IM-MS measurements were performed on a commercially available from Waters quadrupole traveling-wave ion mobility time-of-flight mass spectrometer (Synapt G2-S HDMS). A mixture of a tri- or tetrapeptide: Ac-(H)F(d₅)RW-NH₂ (containing all L-amino acids) and its respective epimer (^DH-, ^DF-, ^DR-, and ^DW-epimers), and a crown ether (previously dissolved in acetonitrile) in methanol: water (3:1) was infused through a standard electrospray ion source into the instrument at a concentration of ca. 1 μM of the peptide (peptide: crown ether 1: 2). The samples were infused at a flow rate of 10 μL/min and were analyzed in positive ion mode with a capillary voltage at 1.8 kV and source temperature at 80 °C. The nitrogen was used as a drift gas in ion mobility measurements. The detailed IM-MS experimental parameters and detailed drift time data are given in the Supporting Information. The IM peaks were fitted with Gaussian distributions using SigmaPlot 12.0.

Results and discussion

Addition of any crown ether presented in Scheme 1 to the solution containing a model tri- or tetrapeptide with a general

sequence Ac-(H)FRW-NH₂ led to the formation of non-covalently bound complex, which was detected as a protonated ion in the mass spectrum. The exemplified Q1 mass spectrum recorded for the solution of the model peptide Ac-FRW-NH₂ and DB18C6 is shown in Fig. 1. Although the specificity was observed in the association of DB30C10 and 18C6 to the guanidinium group and the ammonium group, respectively, and therefore it was suggested that it may be used to determine the presence of arginine or lysine moiety in a peptide sequence, [30] in the non-competitive binding conditions, both ethers along with these shown in Scheme 1 form stable associates with the model peptides containing the arginine residue. These associates were stable under the conditions of increased pressure that prevail in the ion mobility drift tube, and their influence on the epimer separation was further evaluated.

In the first series of experiments, the effect of the ring size of the crown ether on the epimer separation in the model peptide was examined. The epimeric peptides in which each of the L-amino acid in the peptide Ac-FRW-NH₂ was sequentially substituted by the corresponding D-counterpart, i.e., Ac-^DFRW-NH₂ Ac-F^DRW-NH₂, and Ac-FR^DW-NH₂ were studied using IM-MS in pairs. Each pair contained L-peptide and one of its epimer, to keep the mobility spectra more clearly and straightforwardly to interpret. Deuterated L-peptide (F-d₅, i.e., hydrogen atoms in the phenyl ring of phenylalanine were substituted by deuterium atoms) was used to induce the mass specified differentiation while keeping the value of ion mobility indistinguishable from the L-peptide, which was

Fig. 2 IM spectra of selected and extracted ions corresponding to the (a) epimers of Ac-FRW-NH₂ and their associates with (b) DB18C6, (c) DB21C7, and (d) DB30C10

helpful in the analysis of unseparated or hardly separated IM peaks.

Figure 2 shows the results of the ion mobility separation of selected and extracted ions corresponding to the protonated epimeric peptides (Fig. 2a) and their associates with the crown ethers of increasing ring sizes: DB18C6 (Fig. 2b), DB21C7 (Fig. 2c), and DB30C10 (Fig. 2d).

The separation efficacy was evaluated based on the IM-MS peak resolution determined in terms of the peak-to-peak resolution (R_{p-p}), calculated according to the equation:

$$R_{p-p} = \frac{2(t_{d1} - t_{d2})}{\omega_{b1} + \omega_{b2}} \quad (1)$$

where t_{d1} and t_{d2} are the drift times of two subsequent peaks corresponding to the epimers and ω_{b1} and ω_{b2} are their baseline peak width. The peak-to-peak resolution reflects the effective separation of ions taking into account their drift time difference and their peak width, which, in turn, is characteristic for a given ion and in a simplified approach, refers to the diffusion process through ion mobility cell. [38]

The IM separation efficacy of the protonated ions corresponding to the bare epimers depends on the peptide site-specific stereochemistry. All the D-epimers exhibit a slight

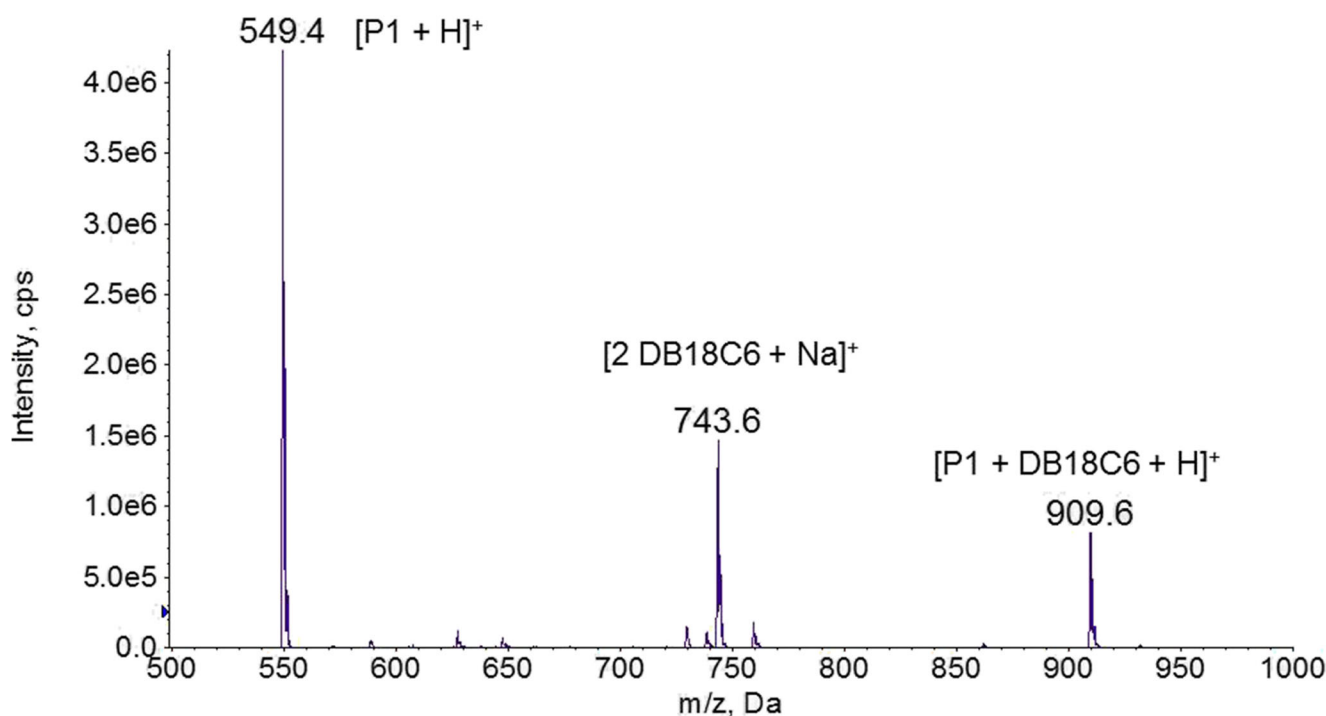
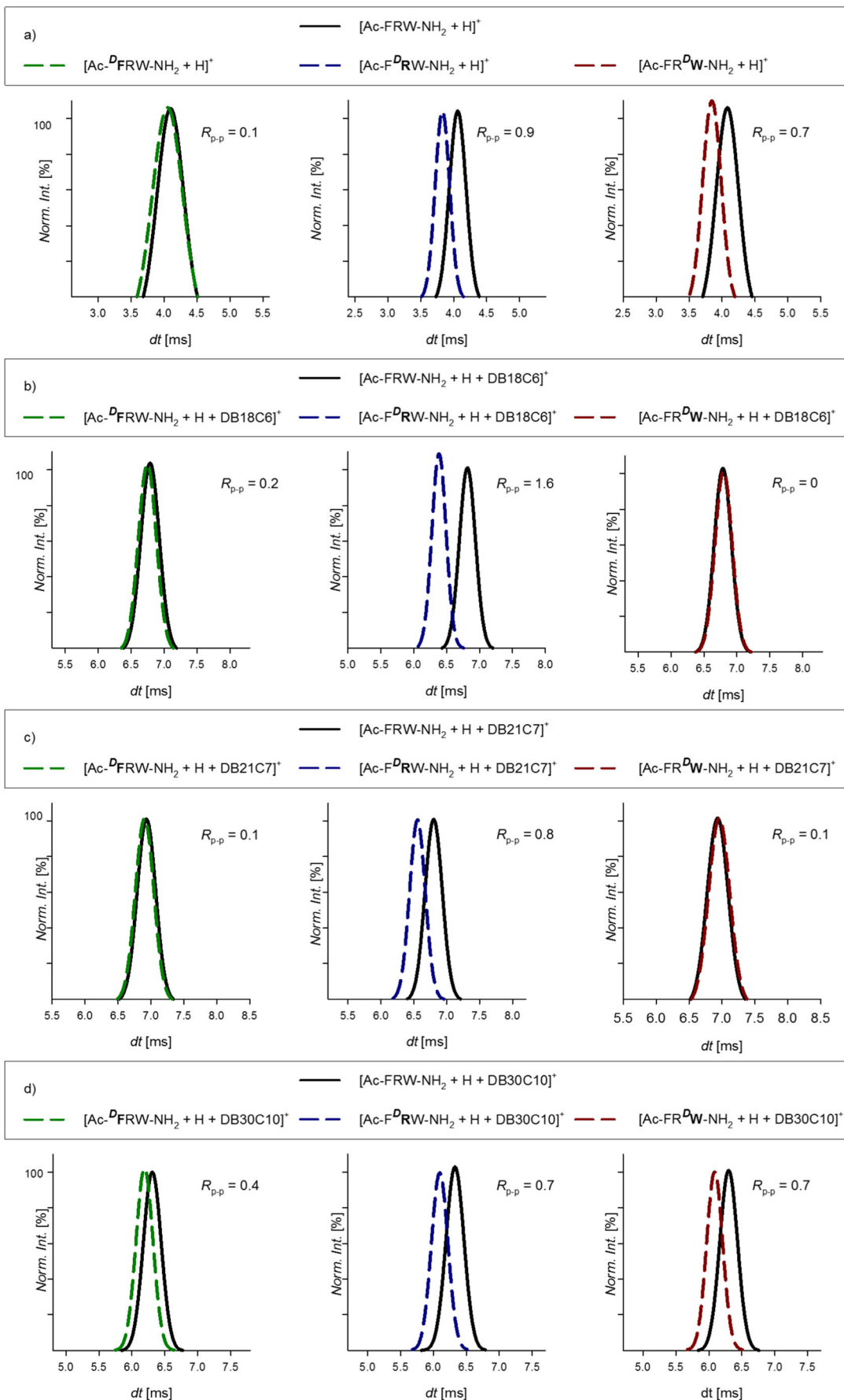


Fig. 1 Q1 mass spectrum recorded for a mixture of the peptide Ac-FRW-NH₂ and DB18C6



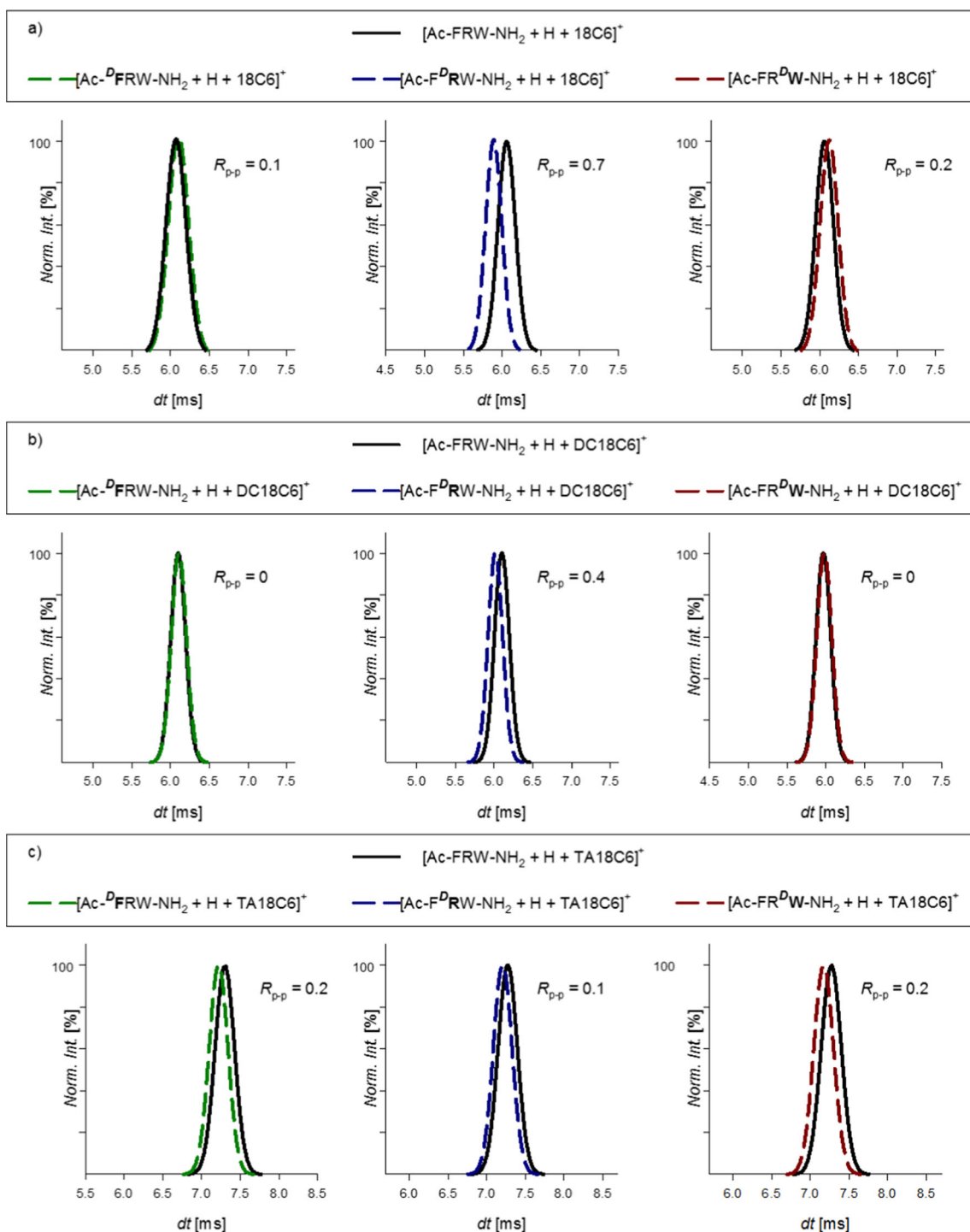


Fig. 3 IM spectra of selected ions and extracted ions corresponding to the epimeric peptides of Ac-FRW-NH₂ and their associates with (a) 18C6, (b) DC18C6, and (c) TA18C6

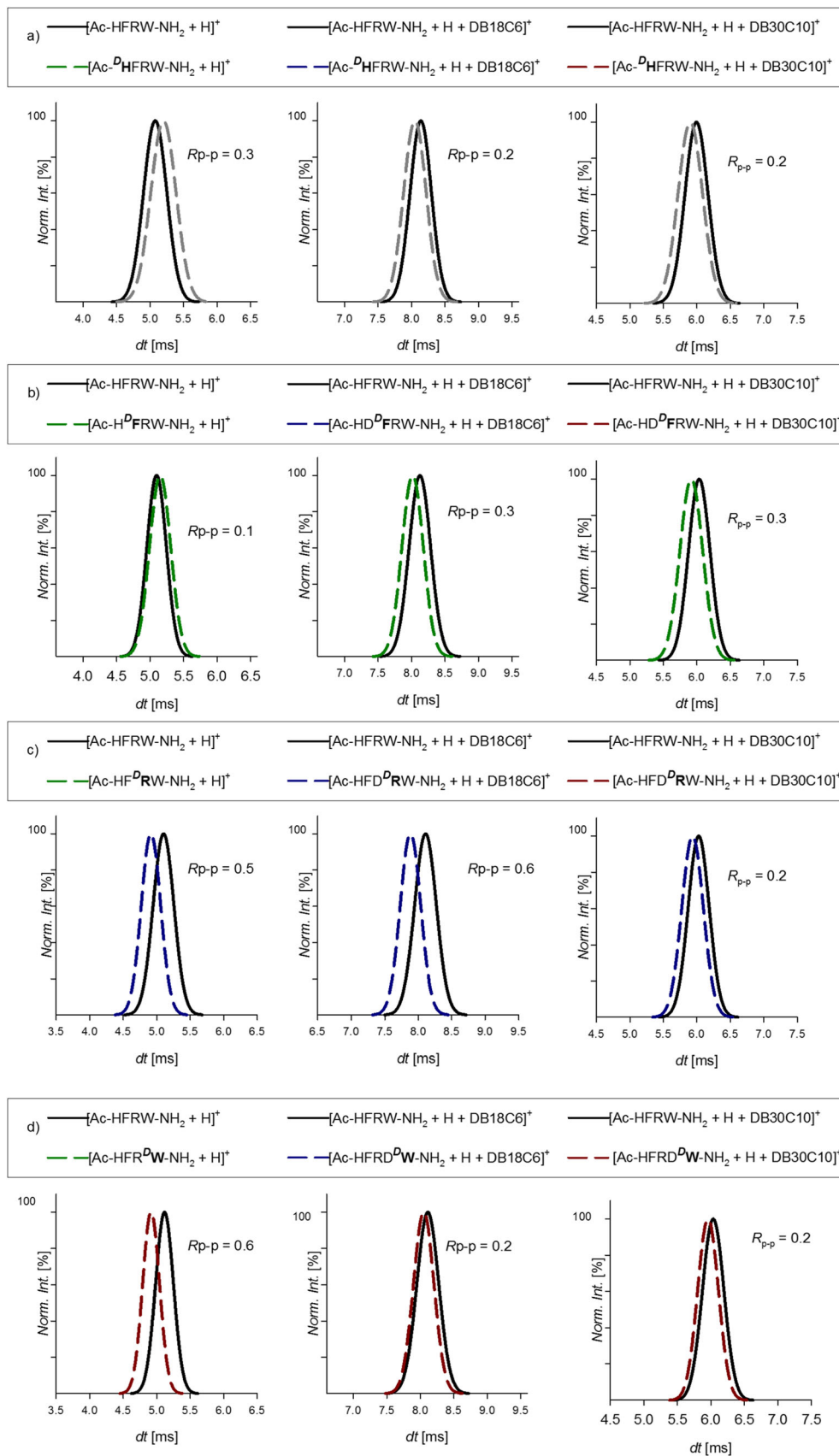


Fig. 4 IM spectra of selected and extracted ions corresponding to the peptide Ac-HFRW-NH₂ and its a) D H-, b) D F-, c) D R-, and d) D W-epimers, and their associates with DC18C6 and DB30C10

shift to lower drift times. This change in the drift time is negligible in the case of ^DF -epimer ($\text{Ac-}^D\text{FRW-NH}_2$, $R_{p-p} = 0.1$), while a similar shift is observed for ^DR - and ^DW -epimers.

The association of the first series of crown ethers with different ring sizes: DB18C6, DB21C7, and DB30C10 to the examined tripeptide and its epimers (Fig. 2) evokes site-specific epimer separation as is observed for the associates with DB18C6 and DB21C7 (Fig. 2b-c). The significant ^DR -specific separation ($R_{p-p} = 1.6$) is observed for the associate with DB18C6. The increase of the size of the crown ether ring from DB18C6 to DB21C7 and DB30C10 erased this specific separation. Moreover, the complexation with the largest crown ether – DB30C10, that assures the effective solvation of the arginine guanidinium group, [30] only slightly improves the separation in the case of ^DF -epimer.

The influence of the type of the substituents in the ether's crown on the efficacy and specificity of the separation of the examined peptide epimers was further evaluated by the analysis of the shift of the drift times under the complexation with 18C6, dicyclohexano-18-crown-6 (DC18C6) and a chiral tetracarboxylic acid-18-crown-6 ether (TA18C6).

The addition of the simplest 18C6 reduced the separation efficacy between all epimeric pairs (Fig. 3a) giving evidence that the sole solvation of the arginine's guanidinium charge site

by the simple crown in the absence of any additional functionality in the crown structure is not sufficient to induce or enhance separation. In comparison to the benzannulated 18C6, the dicyclohexano substitution does not improve the separation of epimers giving evidence of the significant contribution of the interactions between the aromatic groups of ether and the peptide molecule on their complex conformation (Fig. 3b). The analyzed epimers contain amino acids with aromatic side chains: benzyl group of phenylalanine and indole group of tryptophan. Thus, the significant increase of discrimination between the peptide and its ^DR -epimer observed for the complex with DB18C6 may be the result of the specific interactions between aromatic groups within the complex.

The chiral tartaric acid-derived ether is an approved chiral selector for the separation of the enantiomers of chiral primary amines including amino acids. [39, 40] It has been utilized as an effective chiral selector for the separation of chiral amine-containing pharmaceutical compounds in the chiral capillary electrophoresis method, [41, 42] as well as in the enantiodiscrimination of amino acid by NMR spectroscopy. [43] The TA18C6 possesses four carboxylic groups, which may interact with the peptide backbone via hydrogen bonds. These additional interactions may induce a specific conformational change of peptide-TA18C6 associates, depending on the type of the amino acid stereochemical inversion in the peptide

Fig. 5 IM spectra of selected and extracted ions corresponding to the $[\text{Ac-HFRW-NH}_2 + 2\text{H} + 2\text{DB18C6}]^{2+}$ of epimers

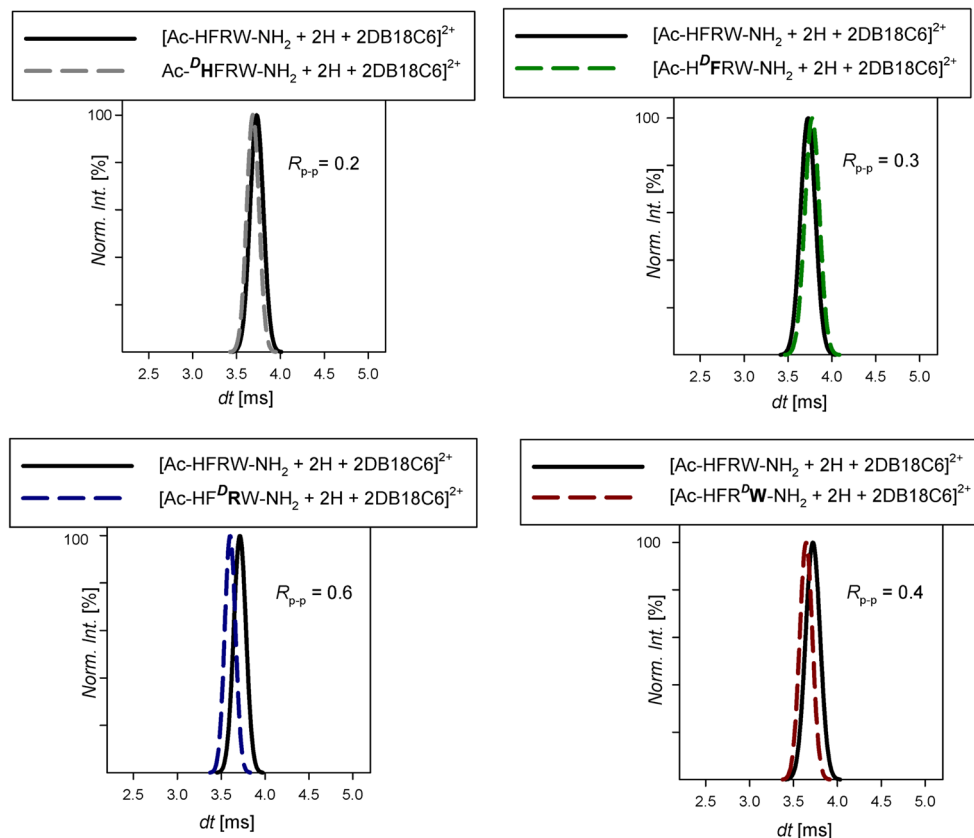


Table 1 R_{p-p} of doubly protonated epimeric ions, 1:1, and 1:2 complexes with DB18C6 and DB30C10 (in parentheses)

EPIMER	R_{p-p}		
	[Peptide + 2H] ²⁺	[Peptide + Crown Ether + 2H] ²⁺	[Peptide + 2xCrown Ether + 2H] ²⁺
Ac- ^D HFRW-NH ₂	0.1	0.3 (0.1)	0.2 (0.1)
Ac-H ^D FRW-NH ₂	0.3	0.3 (0.3)	0.3 (0.5)
Ac-HF ^D RW-NH ₂	0.5	0.1 (0.1)	0.6 (0.1)
Ac-HFR ^D W-NH ₂	0.4	0.4 (0.0)	0.4 (0.1)

sequence and, in a consequence, effect their separation. However, the results obtained for associates with TA18C6 show unequivocally that the complexation with TA18C6 decreases the R_{p-p} of epimeric peptides (Fig. 3c).

To shed more light on the relationship between peptide size and the ion mobility shift upon crown ether complexation, an additional model tetrapeptide Ac-HFRW-NH₂ and its epimers were examined (Fig. 4).

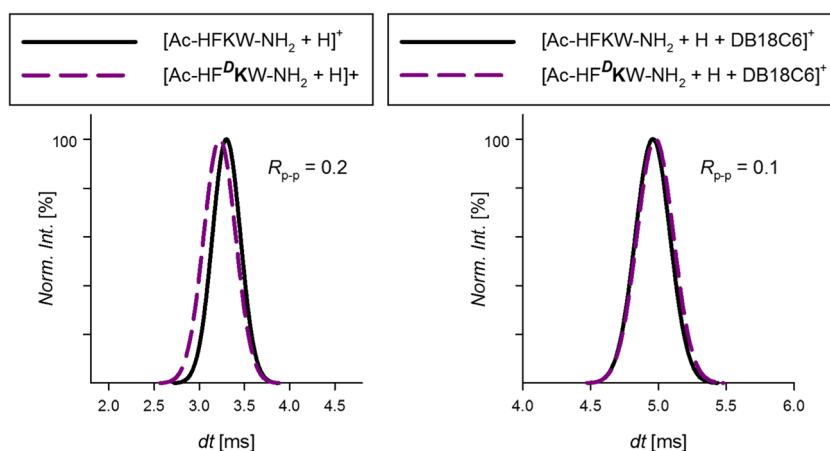
The association of DB18C6 has no or little effect on the enhancement of epimer separation in the studied peptide sequence, while the use of DB30C10 drastically decreases the separation. The site-specific separation of ^DR-epimer observed for Ac-FRW-NH₂ peptide has hardly been observed as the R_{p-p} increases only by 0.1 upon DB18C6 complexation and for DB30C6 associates the opposite process, the unification of the mobilities of these epimers is observed. These results indicate the overall peptide-size dependence of the epimer discrimination upon complexation with a crown ether.

The presence of histidine moiety as a second basic amino acid in the analyzed tetrapeptide epimers enables the association of the additional molecule of crown ether to the peptide and formation of doubly charged ion [Ac-HFKW-NH₂ + 2H + 2DB18C6]²⁺. The association of more than one crown ether molecule has not been observed for the tripeptide model. The IM spectra of [Ac-HFKW-NH₂ + 2H + 2DB18C6]²⁺

epimers (Fig. 5) show no increase of epimer separation upon association of additional crown ether molecule to the peptide. The R_{p-p} values of the analyzed epimers are less or similar to the values of bare or singly complexed peptides.

Full analysis of doubly charged ions present in the spectra recorded for tetrapeptides are summarized in Table 1. Doubly protonated bare epimer ions show overall similar or lower separation efficacy in comparison to singly charged counterparts with one exception, an increase the separation of ^DF-epimer. The small changes in R_{p-p} are seen for doubly charged ionic adducts of both DB18C6 and DB30C10 with respect to the bare epimers. A positive effect on separation is seen for 2:1 adduct formation of ^DF-epimer with DB30C10. This increase of the shift of ^DF-epimer may be associated with the steric factors, since the F amino acid residue is located in between two basic amino acids in the peptide sequence.

The site-specific recognition of ^DR-epimer observed for tri- and tetrapeptide upon complexation with DB18C6 (Figs. 2b and 4c, respectively) was additionally examined in terms of the nature of the amino acid basic residue. To this end, the discrimination of epimeric structures upon crown ether complexation was evaluated for a peptide, in which the arginine residue was substituted with the lysine moiety, i.e., Ac-HFKW-NH₂, and its corresponding ^DK-epimer. The

Fig. 6 IM spectra of selected and extracted ions corresponding to the a) Ac-HFKW-NH₂ and its epimer Ac-HF^DKW-NH₂, and b) their complexes with DB18C6

comparison of the separation of the bare protonated epimers and their complexes with DB18C6 is shown in Fig. 6.

The low separation efficacy of the protonated epimers ($R_{p,p} = 0.2$) is further reduced to $R_{p,p} = 0.1$ as a result of the complexation with DB18C6. This example confirms the previous observation that the enhancement of the solvation of the basic residue by the crown ether decreases the epimer separation. In the case of lysine, the DB18C6 enables the effective solvation of the ammonium group. Although the identical chemical environment around the basic amino acid moiety i.e., the presence of W- and K- residues, the effect of DB18C6 complexation is quite different. This supports the previous conclusions that the effective microsolvation of the basic residue diminishes the epimer separation in IM-MS.

The above reported studies on microsolvation of the basic sites in tri- and tetrapeptides by crown ethers suggest that the microsolvation itself has small effect on introduction the conformational diversity between epimers, given as an increase of their separation efficacy in IM-MS. This also shows that conformational differences, observed for the longer peptide sequences and associated with significant change in conformation of peptide upon microsolvation by crown ethers are not noticeable for shorter peptide sequences. Any significant increase of separation in the studied examples may be attributed to some steric effects, which in turn dependent on the peptide sequence. The results presented in this work also suggest that the presence of the strong electrostatic interactions between basic sites and acidic sites, by formation of intramolecular salt-bridge interactions in peptide may be more sensitive to the microsolvation by the crown ethers. This issue needs to be additionally explored and will be the subject of the separate study.

Conclusions

In the non-competitive complexation conditions, the crown ethers with different ring sizes and substituents form associates with the model peptides containing the arginine residue. These complexes are stable under increased pressure of the ion mobility drift tube. The overall effect associated with the crown ether complexation on the ion mobility shift of the epimeric peptides results from the combined effectiveness of solvation of the basic amino acid residue, the interactions between the crown ether substituents and the peptide residues, and the peptide length. In general, the effective microsolvation of the basic amino acid reduced the epimer separation, although some steric interactions may be still relevant and may slightly change this trend. Additionally, the increase of peptide sequence from tri- to tetrapeptide drastically decrease any positive effect associated with the crown ether complexation on the epimer separation process. The studies on the crown ether microsolvation of the basic site of the peptide also imply the relevant role of the basic amino acid residue in the conformation

features of the peptide, especially those with the short amino acid sequence. Hence, the solvation of the basic moiety rather reduces than increases peptide epimer separation.

Availability of data and material Not applicable.

Author's contributions Not applicable.

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Compliance with ethical standards

Conflict of interest There are no conflicts to declare.

Code availability Not applicable.

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