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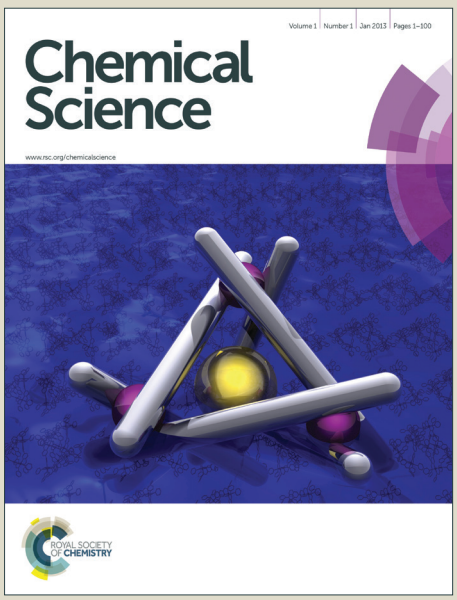

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EDGE ARTICLE

Enantiomers differentiation of aromatic amino acids by traveling wave ion mobility – mass spectrometry

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The present work describes the first differentiation of enantiomers using the coupling of traveling wave ion mobility and mass spectrometry (TWIM-MS). This study was carried out on amino acids, the building blocks of proteins, which are with nucleotides, polysaccharides or lipids, the main constituents of all living organisms. Herein, enantiomers of aromatic amino acids (AA) such as phenylalanine, tryptophan and tyrosine are differentiated by TWIM-MS through their cationisation with copper II and multimers formation with D-Proline (Pro) as a chiral reference compound. This methodology can be considered as an alternative approach to conventional methods for the enantiomers separation. Moreover, quantification of enantiomers can be performed easily and quickly using TWIM-MS analysis of the ionic complex $[(^{D}Pro)_2+^{D/L}AA+Cu^{II}–H]^+$.

Introduction

The importance of chirality is recognized due to the behavior of enantiomers in biological systems. Owing to the chiral environment within a living organism, the biological activities of each enantiomeric form of molecules can differ dramatically.[1] Therefore, chiral recognition of biological molecules, such as carbohydrates, peptides and amino acids has attracted increasing interest for many years.[2] The basis of chiral separation is to form interactions between enantiomers and a chiral selector in order to obtain diastereomers.[3] Chiral separation techniques such as high performance liquid chromatography, gas chromatography and capillary electrophoresis are the most commonly used techniques to distinguished enantiomers.[4] However, most of them require expensive chiral columns and/or complicated sample pretreatment.

In an effort to eliminate the slow and tedious steps of enantiomers separation by chromatography, several mass spectrometric methods have been developed.[5] The majority of chiral recognition by mass spectrometry experiments are based on (i) the measurement of the relative abundance of non-covalent diastereomeric adducts involving a enantiopure reference compound and the enantiomers of the molecule of interest,[6] (ii) the determination of the relative kinetic stability of diastereomeric adducts by collision-induced dissociation (CID) experiments,[7] (iii) the measurement of the rates of gas-phase ion/molecule reactions, often exchange reactions between diastereomeric adducts typically generated from a chiral ligand and a chiral host like cyclodextrin.[8] These approaches are most often based on relatively complex data analysis of fragmentation patterns or involve specifically modified instruments.

The coupling of ion mobility and mass spectrometry (IM-MS) can be considered as a new alternative method for stereochemistry study. Ion mobility is a post-ionization separation method based on the drift of ions in a gas-filled cell under the influence of an electrical field. This technology allows for measuring the time taken by each ion to travel the ion mobility cell. This time on the millisecond range is called drift time and depends on the ion mobility of each ion. Ion mobility varies according to mass (m), charge (z) and collision-cross section (CCS: Ω) of the ions. CCS is related to the conformation and size of ions in the gas phase. Coupled to mass spectrometry, ion mobility adds an extra dimension to the separation. Ions with different CCS can be separated thanks to IM-MS even if they have the same m/z ratio. Traveling wave ion mobility (TWIM) is a type of IM based on low-voltage waves pushing the ions across a gas-filled ion guide.[9] TWIM cell was incorporated recently in commercially available hybrid quadrupole/time-of-flight mass spectrometers.[10] TWIM-MS was successfully applied to biomolecules, synthetic polymer analysis[11] and also to isomer differentiation.[12] This coupling was also used for diastereoisomer differentiation. In fact, works carried out by Revesz *et al.* showed the identification and interconversion of diastereomeric Tröger bases by TWIM-MS.[13] The difference between these two isomers is the position of the stereogenic nitrogen centers in bridgehead position,

which gives them a very different configuration (ΔCCS=6 Å²). A few works on differentiation of diastereomers having almost identical CCS values have been also performed. Campuzano *et al.* showed a small drift time difference between two diastereomers of betamethasone and dexamethasone analyzed separately (drift time difference 0.07 ms, ΔCCS=1 Å²).[14] Our recent work presented the TWIM-MS separation of a mixture of diastereomers (M) which presented very close CCS (ΔCCS<1 Å²), thanks to the cationization with lithium and the formation of multimers $[2Y+M+Li]^+$ with a chiral compound Y.[15] We also reported the use of transition metals, particularly copper II, which allowed an increase of the diastereomer differentiation.[16] Regarding the enantiomer separation by IM-MS, very few examples were described. The most widespread study have been carried out with FAIMS (High-field asymmetric waveform ion mobility spectrometry), particularly the combination of FAIMS and MS/MS analyses for the analysis of terbutaline enantiomers[17] or structural identification of contaminants in drinking water.[18] One example of analysis of amino acids enantiomers has been also carried out with FAIMS.[19] Another example of chiral separation has been described with a drift tube ion mobility spectrometry constructed at Washington State University. Dwivedi *et al.* demonstrated that gas-phase separation of the two enantiomers (S) and (R) of atenolol was possible when the drift gas in ion mobility cell is modified with a chiral vapor.[20] For that, nitrogen was used as drift gas and (S)-(+)-2-butanol as the chiral drift gas modifier. The chiral modifier was infused by a syringe pump into the nitrogen drift gas line using a T-junction before the drift gas inlet into the IMS. However, these analyses, realized with a non-commercial ion mobility spectrometer, required some instrumental modifications.

Herein, we report the first TWIM-MS enantiomer differentiation with an unmodified commercial instrument. This approach was applied to the enantiomer separation of aromatic amino acids (phenylalanine, tyrosine and tryptophan), which are the building blocks of proteins. Amino acids are together with nucleotides, polysaccharides or lipids, the main constituents of living organisms. In order to perform these differentiations, $[(X)_2+^{D/L}AA+Cu^{II}-H]^+$ ions (with $^{D/L}AA$ is amino acids enantiomers and X is a chiral reference compound) are formed and analyzed on a commercial hybrid ion-mobility-mass spectrometer. Copper II has been chosen for its coordination properties and its use in differentiation of amino acids enantiomers by high voltage capillary zone electrophoresis[21] or by mass spectrometry in the Cook's kinetic method.[22] We began our study with the phenylalanine enantiomers. For the choice of the suitable chiral reference compound, many amino acids were tested. The best differentiation for the phenylalanine enantiomers was obtained with proline. This chiral reference compound was then used for the differentiation of other aromatic amino acids.

Results and discussion

TWIM-MS analysis of D and L Phenylalanine

As expected, drift times obtained for each enantiomer of phenylalanine were identical. A chiral reference compound (Y) was therefore added to the solution in order to form diastereomeric complexes, which could enable the differentiation of these molecules. Moreover, a cationisation with copper II was carried out in order to form multimers. For the choice of the suitable chiral reference compound, eleven amino acids were tested. Worth of note is that for each reference compound, the following ratio $^{D/L}Phe/Y/Cu^{II}$ was optimized to obtain heterodimers and heterotrimers. Resulting mass spectra are quite dense due to the presence of several hetero/homodimers and hetero/homotrimers. For instance, the mass spectrum obtained for the analysis of phenylalanine and proline as chiral reference compound is presented in Figure 1. The drift time obtained for the heterodimer $[Y+^{D/L}Phe+Cu^{II}-H]^+$ and the heterotrimer $[(Y)_2+^{D/L}Phe+Cu^{II}-H]^+$ were extracted. Table 1 presents the drift time difference and the experimental collision cross section difference obtained for these species between D and L proline with AA: arginine, tryptophan, glutamic acid, threonine, histidine, proline or tyrosine. (CCS_{exp} is given for each specie in Table S1 supplementary information). Worth of note is that no adduct was formed with alanine, glutamine, cysteine or lysine. These values correspond to the average value obtained from 5 replicates after a Gaussian fit of each peak (coefficient of variation (CV) < 5%).

Gaussian fit of the IMS data was required in order to improve the precision of the drift time measurements. In fact, the ion mobility spectrum obtained from our instrument present a relatively low number of data points per peak (between seven and eleven points per peak depending on the drift time of each peak and the peak width).

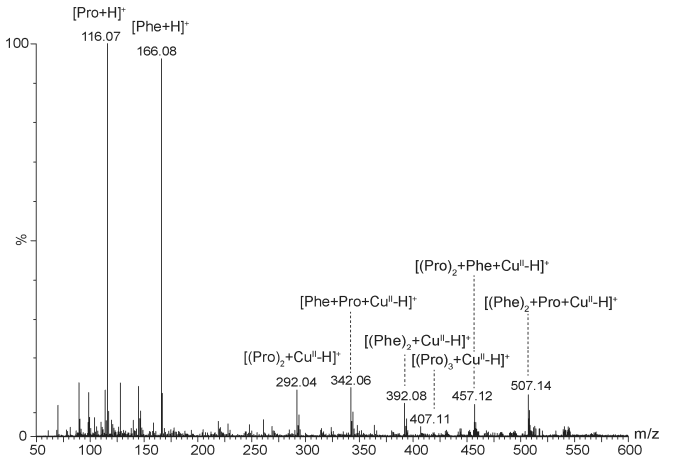


Figure 1. Mass spectrum obtained for a mixture of phenylalanine (Phe), proline (Pro) and $CuCl_2$.

Table 1. TWIM-MS analysis results obtained for $[Y+^{D/L}Phe+Cu^{II}–H]^+$ and $[2Y+^{D/L}Phe+Cu^{II}–H]^+$ ions.

Y Compound	*m/z*	Δt_d (ms)[a]	ΔCCS_{exp}(Å²)[b]
Arginine			
$[^{D}Arg+^{D/L}Phe+Cu–H]^+$	401.12	0.04±0.01	1.4
$[(^{D}Arg)_2+^{D/L}Phe+Cu^{II}–H]^+$	575.23	0.06±0.01	1.9
Tryptophan			
$[^{D}Trp+^{D/L}Phe+Cu^{II}–H]^+$	431.10	0.02±0.01	0.6
$[(^{D}Trp)_2+^{D/L}Phe+Cu^{II}–H]^+$	635.19	0.04±0.01	1.2
Glutamic acid			
$[^{D}Glu+^{D/L}Phe+Cu^{II}–H]^+$	374.06	0.01±0.01	0.3
$[(^{D}Glu)_2+^{D/L}Phe+Cu^{II}–H]^+$	521.12	0.05±0.01	1.6
Threonine			
$[^{D}Thr+^{D/L}Phe+Cu^{II}–H]^+$	346.07	0.02±0.01	0.7
$[(^{D}Thr)_2+^{D/L}Phe+Cu^{II}–H]^+$	465.13	0.04±0.01	1.3
Histidine			
$[^{D}His+^{D/L}Phe+Cu^{II}–H]^+$	382.08	0.02±0.01	0.7
$[(^{D}His)_2+^{D/L}Phe+Cu^{II}–H]^+$	537.15	0.04±0.01	1.3
Proline			
$[^{D}Pro+^{D/L}Phe+Cu^{II}–H]^+$	342.07	0.04±0.01	1.4
$[(^{D}Pro)_2+^{D/L}Phe+Cu^{II}–H]^+$	457.14	**0.11±0.01**	**3.8**
Tyrosine			
$[^{D}Tyr+^{D/L}Phe+Cu^{II}–H]^+$	408.08	0.01±0.01	0.3
$[(^{D}Tyr)_2+^{D/L}Phe+Cu^{II}–H]^+$	589.16	0.06±0.01	1.8

[a] Δt_d is the experimental drift-time difference between D and L phenylalanine. [b] ΔCCS_{exp} is the experimental collision cross section difference between D and L phenylalanine.

The ion mobility spectrum sampling rate is indeed related to the time of flight (TOF) acquisition speed. As a consequence, under standard conditions, variation of the drift time is in the order of 0.03 ms from the raw data whereas it is below 0.01 ms after the Gaussian fit. A description of the data analysis is given in Figure S1 supplementary information.

Thanks to these analyses, several conclusions appeared. First, for all amino acids chosen as chiral reference compound, the greatest drift time difference between the L and D phenylalanine was obtained with the $[(AA)_2+^{D/L}Phe+Cu^{II}–H]^+$ ions. Then, the use of proline as chiral reference compound allowed for obtaining a significant increase of drift time difference between these two enantiomers (Δt_d between D and L phenylalanine for the $[(^{D}Pro)_2+^{D/L}Phe+Cu^{II}–H]^+$ ion was in the order of 0.11 ms). Although the increase of the drift time difference was significant, it was not sufficient to obtain the separation of the two enantiomers. Nevertheless, mixtures with various proportions of D and L phenylalanine were analyzed by TWIM-MS. For each solution, drift time for $[(^{D}Pro)_2+^{D/L}Phe+Cu^{II}–H]^+$ ion (*m/z* 457.14) was determined. Figure 2 presents the results obtained for these mixtures from 0% to 100% of L compound. These results were obtained with

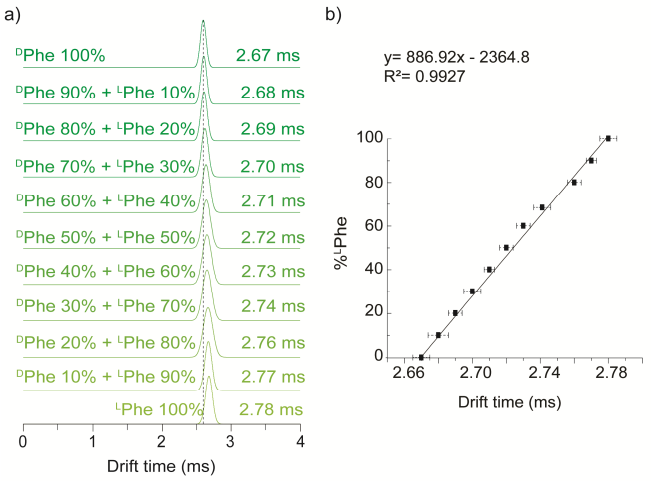


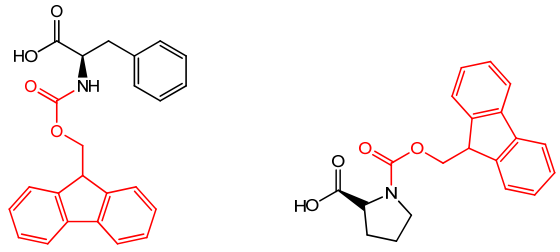
Figure 2. Correlation between D/L Phenylalanine ratio and measured drift-time for $[(^{D}Pro)_2+^{D/L}Phe+Cu^{II}–H]^+$ ion. a) Drift time plot obtained for each proportion of D and L enantiomers of Phenylalanine. b) Calibration curve.

a reproducibility study on 10 analyses. These analyses showed a good correlation between D/L ratio and the mixture drift time. Data were sufficiently accurate and reproducible to obtain a linear calibration curve with R^2=0.9927.

Therefore, the proportion of each enantiomer in a mixture solution could be easily determined by the drift time measurement of $[(^{D}Pro)_2+^{D/L}Phe+Cu^{II}–H]^+$ ion.

Recent work on separation of steroids by ion mobility mass spectrometry has shown that the derivatization of the alcohol function by *p*-toluenesulfonyl isocyanate improved their separation in the ion mobility cell.[23] In fact, this derivatization allowed for increasing the difference of ions shape.

In order to improve the enantiomer differentiation of phenylalanine, substituents on the asymmetric carbon were modified with larger chemical groups. For that, several analyses were carried out with phenylalanine, proline and modified molecule with an amine protecting group: Fluorenylmethyloxycarbonyl (Fmoc) (Figure 3). Results obtained for $[(^{D}Pro)_2$ + $(^{D/L}Fmoc\text{-}Phe)+Cu^{II}–H]^+$, $[(^{D}Fmoc\text{-}Pro)_2+(^{D/L}Phe)+Cu^{II}–H]^+$ and $[(^{D}Fmoc\text{-}Pro)_2$ + $(^{D/L}Fmoc\text{-}Phe)+Cu^{II}–H]^+$ are presented in Figures 4b, 4c and 4d respectively.



DFmoc-Phe DFmoc-Pro

Figure 3. Structures of amino acids derivatives with Fmoc as amine protecting group.

It can be observed that the analysis of Fmoc-Phenylalanine with proline (Figure 4b), shows an increase of the drift time difference between the two Fmoc-Phe enantiomers (Δt_d: 0.16 ms for Fmoc-Phe compared to 0.11 ms for Phe (Figure 4a)). However, in those conditions, drift times were shifted to higher values and the width of peaks was significantly increased, due to the diffusion of ions in the TWIM cell. The peak to peak resolution (R_{p-p}) was calculated for each ion using the following equation:

$$R_{p-p}=\frac{2(t_{d_2}-t_{d_1})}{W_{b1}+W_{b2}}$$

in which t_{d2} and t_{d1} are the drift time obtained respectively for the second and the first peaks and W_{b2} and W_{b1} are the width at the base of each peak. Resolving power R_p (related to ion mobility peak) and the separation factor α (related to ion mobility peak separation) have been therefore calculated using the following equations:

$$R_p=\frac{t_d}{W_{1/2}} \qquad \alpha=\frac{t_{d2}}{t_{d1}}$$

with $W_{1/2}$, the peak width at half of maximum intensity. The peak to peak resolution was the same between the Phe and the Fmoc-Phe (R_{p-p}: 0.44). Therefore, in this case, the derivatization of asymmetric carbon did not allow for an increase of the enantiomer differentiation.

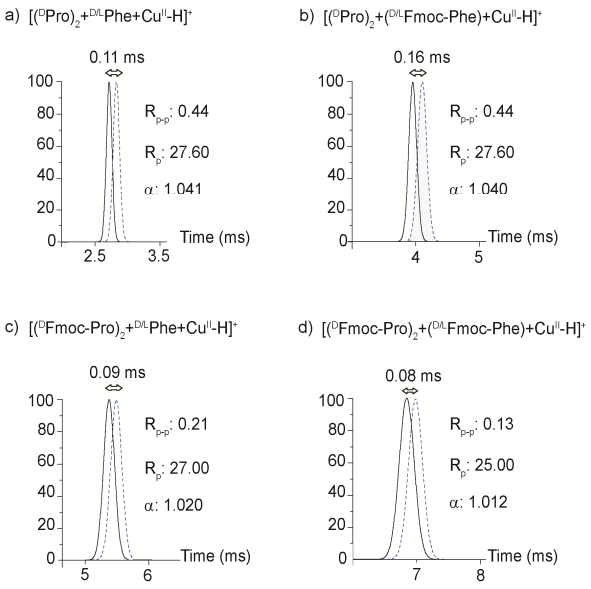


Figure 4. Overlay of ion mobility spectra for $[(Y)_2+^{D/L}M+Cu^{II}–H]^+$. a) Y: DPro, M: D/LPhe; b) Y: DPro, M: D/LFmoc-Phe ; c) Y: D Fmoc-Pro, M: D/LPhe; d) Y: D Fmoc-Pro, M: D/L Fmoc-Phe. In solid line: ion mobility spectra for DM; in dotted line: ion mobility spectra for LM compound.

TWIM-MS analysis of D and L amino acids with D Proline

In order to know if proline enabled the differentiation of other amino acid enantiomers, other TWIM-MS analyses were carried out. Nine amino acids were therefore tested that are representative of the different types (with basic, acid, aromatic and aliphatic side chains). Table 2 presents the results obtained for $[(^{D}Pro)_2+^{D/L}AA+Cu^{II}–H]^+$ ions with AA as arginine, tryptophan, glutamic acid, threonine, glutamine, tyrosine and lysine. (CCS_{exp} is given for each specie in Table S2 supplementary information). Worth of note is that no adduct was formed with histidine and cysteine. The drift time difference values were obtained from 5 replicates after a Gaussian fit of each peak (coefficient of variation (CV) < 5%). The formation of multimers with D-proline allowed for increasing the drift time difference between enantiomers for all the investigated amino acids.

In fact a slight drift time difference was obtained in the case of glutamic acid and threonine (Δt_d: 0.03 ms). This difference was slightly higher with glutamine and lysine (Δt_d: 0.04 ms) and the best differentiation was obtained for aromatic amino acids: tyrosine and tryptophan (Δt_d: 0.14 and 0.18 ms respectively). An overlay of ion mobility spectra obtained for each case is presented on Figure 5. Control experiments carried out with L-Proline ($[(^{L}Pro)_2+^{D/L}Tyr+Cu^{II}–H]^+$ and $[(^{L}Pro)_2+^{D/L}Trp+Cu^{II}–H]^+$) yielded identical separation (Figure S2 supplementary information). It was shown in a previous work that the use of a polarizable drift gas could improve the separation in ion mobility experiments.[24]

Table 2. TWIM-MS analysis results obtained for $[(^{D}Pro)_2+^{D/L}AA+Cu^{II}–H]^+$ ions

Y Compound	*m/z*	Δt_d (ms)[a]	ΔCCS_{exp}(Å²)[b]
Arginine $[(^{D}Pro)_2+^{D/L}Arg+Cu^{II}–H]^+$	466.17	0.04±0.01	1.3
Tryptophan $[(^{D}Pro)_2+^{D/L}Trp+Cu^{II}–H]^+$	496.15	**0.18±0.01**	**6.2**
Glutamic acid $[(^{D}Pro)_2+^{D/L}Glu+Cu^{II}–H]^+$	439.11	0.03±0.01	1.0
Threonine $[(^{D}Pro)_2+^{D/L}Thr+Cu^{II}–H]^+$	411.12	0.03±0.01	1.0
Glutamine $[(^{D}Pro)_2+^{D/L}Gln+Cu^{II}–H]^+$	438.12	0.04±0.01	1.3
Lysine $[(^{D}Pro)_2+^{D/L}Lys+Cu^{II}–H]^+$	438.16	0.04±0.01	1.3
Tyrosine $[(^{D}Pro)_2+^{D/L}Tyr+Cu^{II}–H]^+$	473.13	**0.14±0.01**	**4.8**

[a] Δt_d is the experimental drift-time difference between D and L amino acids. [b] ΔCCS_{exp} is the experimental collision cross section difference between D and L amino acids.

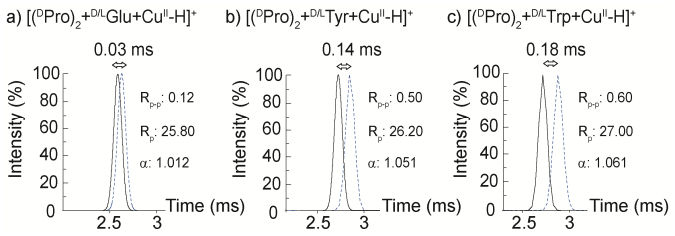


Figure 5. Overlay of ion mobility spectra for $[(^{D}Pro)_2+^{D/L}M+Cu^{II}–H]^+$ a) M: Glutamic acid; b) M: Tyrosine; c) M: Tryptophan. In solid line: ion mobility spectra for ^{D}M; in dotted line: ion mobility spectra for ^{L}M compound.

However, for these particular systems no significant improvement was observed with the use of CO_2 compared to N_2 (Figure S3 supplementary information).

For tyrosine and tryptophan, the drift time difference seemed to be sufficiently high to obtain a separation of the enantiomers in the mixture. Analyses of D and L tryptophan mixtures were therefore carried out by TWIM-MS. For each solution, the drift time for the ion $[(^{D}Pro)_2+^{D/L}Trp+Cu^{II}–H]^+$ (*m/z* 496.15) was extracted. These results were obtained with a reproducibility study on 10 analyses. Drift time plots obtained for each solution are presented Figure 6a. It clearly shows that the enantiomers of tryptophan are separated in the mixture.

Note that the ionization efficiency of the two enantiomers was different. Indeed, the ^{L}Trp yielded $[(^{D}Pro)_2+^{D/L}Trp+Cu^{II}–H]^+$ ions in a lower abundance than the ^{D}Trp explaining why the areas of the peaks corresponding to the ^{L}Trp were smaller than those obtained for the ^{D}Trp. However, it was possible to link proportion of each enantiomer to the area of peak as shown Figure 6b. Similar results were obtained for the differentiation of enantiomers of tyrosine (Figure S4 supplementary information).

Preliminary quantum chemical calculations were carried out to cast the light on the structures of such complexes. They revealed that many coordination schemes are possible, and that intramolecular bonds between the ligands play a crucial role. Furthermore, using state-of-the-art computational techniques, experimental CCS differences can be accurately reproduced, as described in the supplementary material (Figure S5 supplementary information).

Analyzes have been carried out with other transition metals such as Ni^{II}, Mn^{II} and Co^{II} (Figure S6 supplementary information). However, drift time difference is greatest with Cu^{II}, which is an agreement with previous tandem mass spectrometry experiments.[22]

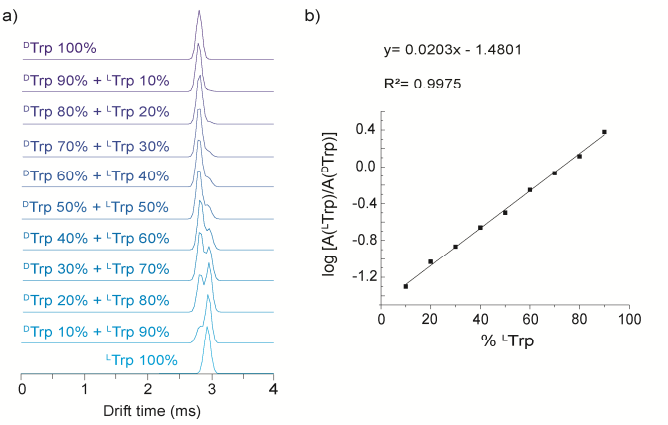


Figure 6. Analysis of D and L enantiomers of tryptophan in mixture. a) Drift time plot obtained for each proportion of D and L enantiomers of tryptophan for [(DPro)2+D/LTrp+CuII–H]+ ion. b) Curve obtained when log[A(LTrp)/A(DTrp)] is reported in function of proportion of ^{L}Trp. (A ($^{L/D}Trp$) is the area of the pic corresponding to $^{L/D}Trp$)

Conclusions

In summary, this work reported the first TWIM-MS enantiomeric differentiation of aromatic amino acids based on the formation of trimeric Cu(II)-bound complexes. Using copper II and proline as chiral reference compound, enantiomers of phenylalanine, tryptophan and tyrosine can be readily differentiated. After optimization of the Pro/AA/Cu^{II} ratio, the implementation of this type of analysis was quite straightforward to simply adding proline and copper II to the enantiomers sample (AA). Moreover, this implement does not require an instrument modification. Ion such as $[(^{D}Pro)_2+^{D/L}AA+Cu^{II}–H]^+$ can then be analyzed on the millisecond time scale by TWIM-MS. The total analysis time does not exceed five minutes. Even if the IMS resolution is not sufficient to obtain resolved peaks, the very high accuracy and reproducibility of the determined drift time, allowed for obtaining information about the proportion of each enantiomer thanks to a calibration curve. This work demonstrates that TWIM-MS is a powerful approach for the enantiomer separation and quantification.

Experimental section

Chemicals

All amino acids (D and L forms) were purchased from Serva. Copper chloride ($CuCl_2$), Fmoc-Pro and Fmoc-Phe were purchased from Sigma. These amino acids and copper were dissolved separately in water to a concentration of 1 mg mL^{-1}.

For ion mobility studies of $[(X)_2+^{D/L}Phe+Cu–H]^+$, each stock solution was diluted with copper II in methanol to obtain a final solution of 20 µM of the $^{D/L}Phe$ and a $^{D/L}Phe$/ X/ Cu^{II} ratio as follow: X= Arginine or Proline: 1/ 2/ 2 ratio; X= Tyrosine or Threonine: 1/ 2/ 4 ratio; X= Tryptophan 1/ 4/ 4 ratio; X= Glutamic acid: 1/ 4/ 2 ratio and X= Histidine: 1/ 2/ 6 ratio.

For ion mobility studies of Fmoc derivatives, each stock solution was diluted in methanol to obtain a final solution of 20 µM of each species.
For ion mobility studies of $[(^{D}Pro)_2+^{D/L}AA+Cu–H]^+$, each stock solution was diluted with copper II in methanol to obtain a final solution of 20 µM of the ^{D}Pro and a $^{D/L}AA$ / ^{D}Pro / Cu^{II} ratio as follow: AA=Arginine, Tryptophan, Tyrosine: 1/ 1/ 1 ratio and AA= Glutamic acid, Threonine, Glutamine: 4/ 1/ 1 ratio.

ESI – TWIM – MS

TWIM-MS analyses were carried out on a Synapt G2 HDMS (Waters, Manchester, United Kingdom). This instrument is a hybrid quadrupole/time-of-flight mass spectrometer, which features a traveling wave ion mobility (TWIM). The instrument and the T-Wave device have been described in detail previously.[9-10] Experiments were performed by direct infusion of samples at 10 µL min^{-1} flow rate into the ESI source of the instrument, with capillary and sampling cone voltages fixed between 2.8-3.2 kV and 20-30 V respectively under a nitrogen flow of 500 L h^{-1} heated at 250 °C. For all experiments, IM-MS spectra were acquired in a positive mode over the *m/z* 50-600 range. Helium cell gas flow was set at 180 mL min^{-1}. IMS gas flow (N_2) was set at 70 mL min^{-1} of 2.96 mbar for IMS cell pressure, traveling wave height and velocity in the IMS cell were set, respectively, at 40 V and 600 m s^{-1}. CO_2 was also used as drift gas in following conditions (Figure S2 supplementary information): IMS gas flow was set at 60 mL min^{-1} of 2.00 mbar for IMS cell pressure, traveling wave height and velocity in the IMS cell were set, respectively, at 40 V and 500 m s^{-1}. Data acquisition and mass spectra processing were carried out using the MassLynx v4.1 software (Waters, Manchester, United Kingdom).
For ion mobility spectra a Gaussian fit of each peak was carried out with Origin® software v9.1 (OriginLab). All masses and *m/z* reported in the manuscript correspond to monoisotopic values.
IMS cell calibration was carried out with polyalanines at the concentration of 10 ng $µL^{-1}$ as described by Smith *et al.*[25] Polyalanines CCS values used for calibration come from Pr. Clemmer CCS database.[26]

Acknowledgements

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