International Journal of Mass Spectrometry 280 (2009) 213–217

Contents lists available at ScienceDirect

International Journal of Mass Spectrometry

journal homepage: www.elsevier.com/locate/ijms

Magnesium interference and different efficiencies of diastereoisomeric cluster formation in phenylalanine enantiomeric discrimination by the kinetic method

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ARTICLE INFO

Article history:
Received 20 July 2008
Received in revised form
12 September 2008
Accepted 19 September 2008
Available online 30 September 2008

Keywords:
Enantiomeric discrimination
Kinetic method
Diastereoisomeric cluster
Mass spectrometry
Magnesium interference

ABSTRACT

The kinetic method has been applied for determination of D-Phe/L-Phe enantiomeric ratio. Discrimination of enantiomers was inferred from product ion mass spectra of trimeric cluster ions containing the analyte (L,D-Phe), Cu^{2+} as a central metal and L-Trp as a chiral reference ligand. Unsatisfactory quantitative results achieved on an ion trap were rationalized by high-resolution mass spectrometry. The formation of Mg^{2+}-containing cluster isobaric to trimeric cluster $[Cu(L\text{-}Trp)_2Phe]^+$ was observed. Interference like this was identified as a possible reason for deterioration of quantitative low-resolution mass spectrometric analyses of real-world samples based on the kinetic method. Cation-exchanger was used for easy removal of magnesium from a sample and improvement of quantitation.

Chiral dependence of formation of the Cu^{2+}-containing trimeric cluster was also observed. Heterochiral diastereoisomeric ions were created less effectively.

1. Introduction

Chirality represents a fascinating phenomenon with a significant influence on living systems. In this context, the development of fast and robust tools for chiral analysis is one of the most important analytical tasks today. Multiple methods are based on chromatographic or electrophoretic separations [1,2] but mass spectrometry can be applied too [3]. Chiral discrimination by Cooks' kinetic method is one of most reliable mass spectrometric approaches [4–10]. Target diastereoisomeric clusters with a general formula $[M(ref)_2(A)\text{-}H]^+$ (M—stands for a central metal, ref—is an optically pure reference ligand, A—an enantiomer of an analyte) are transferred into the gas phase by electrospray ionization. Fragmentation of this target cluster leads to a competitive release of a reference or analyte ligand. The intensity ratio (R) of product ions depends on enantiomeric ratio (A_L:A_D) in a sample:

$$R = \frac{[M(ref)_2\text{-}H]^+}{[M(ref)A\text{-}H]^+} \quad (1)$$

Chiral selectivity is expressed by R_{chiral}:

$$R_{chiral} = \frac{R_L}{R_D} \quad (2)$$

R_L and R_D are obtained by a measurement and dissociation of diastereomeric metal-mediated clusters containing pure L- or D-enantiomer, respectively. An R_{chiral} value closer to one indicates lower chiral selectivity.

Cooks' kinetic method has been successfully applied in the analysis of various isomers using direct infusion of samples to mass spectrometers [5,7,11–17]. It has also been used in combination with flow injection and chromatographic formats [8].

The formation of the diastereoisomeric cluster ion represents the first step in the chiral analysis by the kinetic method [4–6]. In general, quantitative results can be hampered by chemical interferences. In this paper we report on the formation of Mg^{2+} containing cluster, which deteriorates quantitative results in chiral analysis of phenylalanine.

2. Experimental

2.1. Chemicals

Tryptophan (Trp), phenylalanine (Phe), $CuCl_2$ (all analytical grade) and HPLC grade water were obtained from Sigma–Aldrich

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doi:10.1016/j.ijms.2008.09.009

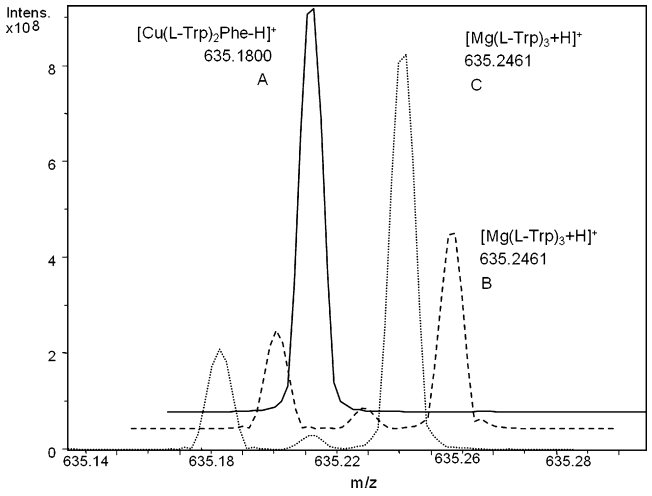

Fig. 1. Detail of FTICR spectra of the target copper cluster: (A) no addition of Mg^{2+}, exit capillary voltage 300 V; (B) 2.5×10^{-4} mol/L Mg^{2+}, exit capillary voltage 300 V; (C) 2.5×10^{-4} mol/L Mg^{2+}, exit capillary voltage 320 V. Internal calibration using ions [L-Trp+H]$^+$, [(L-Trp)$_2$+H]$^+$ and [Cu(L-Trp)$_2$Phe-H]$^+$ was performed.

(Prague, Czech Republic) and used as received. Isotopically labeled L-tryptophan (U-$^{13}C_{11}$, 98%, U-$^{15}N_2$, 98%), L-phenylalanine (Ring-$^{13}C_6$, 99%) and D,L-phenylalanine (Ring-D_5, 98%) were purchased from Cambridge Isotope Laboratories (Andover, USA). HPLC grade methanol was supplied by Merck (Prague, Czech Republic). $Mg(NO_3)_2$ (analytical grade) was received from Lachema (Brno, Czech Republic). Strong cation-exchanger Dowex 50WX8 50–100 mesh size in H^+-form (Dow Water Solutions, Midland, USA) was used for elimination of magnesium in some experiments. After 20 min equilibration with the exchanger the supernatant was sampled and immediately measured.

2.2. Instrumentation

The electrospray sources of the quadrupole ion trap LCQ Classic (Thermo Electron, San Jose, USA), Fourier transform ion cyclotron resonance APEX-Q (9.4 T, Bruker Daltonics, Billerica, USA) and quadrupole time-of-flight mass spectrometer Premier (Waters, Manchester, UK) were tuned to gain satisfactory intensities of trimeric cluster ions using direct infusion of aqueous:methanol (1:1, v:v) solution mixtures of phenylalanine (1.0×10^{-4} mol/L), Cu^{2+} (2.5×10^{-5} mol/L) and L-tryptophan as a chiral reference ligand (2.5×10^{-4} mol/L). Selected LCQ parameters were as follows: spray voltage +5.6 kV, sheath gas 60 arb. units, heated capillary temperature 175 °C. Q-Tof Premier spray voltage was set to +2.4 kV, source temperature to 100 °C, desolvation gas temperature and flow-rate to 100 °C and 250 L/h, respectively. In FTICR the spray voltage was adjusted to −3.5 kV (the voltage of opposite polarity with regard to polarity of analyzed ions was applied to a shield of the capillary entrance, spray capillary was grounded), nebulizer gas flow-rate to 60.0 L/h, drying gas temperature and flow-rate to 200 °C and 180.0 L/h, respectively. Working solutions were directly infused by a linear pump into the ion sources at a 1–5 μL/min flow-rate.

2.3. Results and discussion

The kinetic method has been successfully applied to the isomeric analysis of different standard model samples [5,7,8,11–17]. Interestingly, it has not found a wide use in an analysis of real samples yet. Potential difficulties in quantitative analysis may arise from matrix suppression effects or the occurrence of isobaric species interfering with the signal of the analyte of interest. The latter case can increase the signal intensity at the given nominal mass and thus can have adverse effects on quantitative results.

In this work, the cluster [Cu(L-Trp)$_2$Phe-H]$^+$ at nominal mass 635 u was isolated in an ion trap and fragmented during the chiral analysis of phenylalanine by the kinetic method. L-Tryptophan was used as the reference ligand and copper(II) as the central metal ion. The ratio of enantiomers was calculated from intensities of product ions at m/z 470 and 431, respectively. Interestingly, high-resolution experiments performed with the same chiral system on the FTICR instrument revealed the presence of two peaks at nominal mass 635 u. The first ion at m/z 635.1808 corresponded to [Cu(L-Trp)$_2$Phe-H]$^+$ (calculated m/z 635.1800, error 1.3 ppm). The second ion was identified as a cluster of magnesium and tryptophan (Fig. 1). It was recorded at m/z 635.2461 (calculated m/z 635.2463, error −0.3 ppm).

The cluster with Mg^{2+} did not appear in a pure standard solution (Fig. 1A) but can rise to significant intensity in real samples (see Fig. 1B and C). The reported presence of Mg^{2+} negatively influenced quantitative results. Absolute intensity of the target copper cluster decreased due to competition of both metals (Cu^{2+} and Mg^{2+}) for the ligands (compare Fig. 1A vs. B). It is noteworthy, that the addition of Mg^{2+} into the system supports the formation not only of [Mg(L-Trp)$_3$-H]$^+$ but also [Mg(Trp)$_2$Phe-H]$^+$ and [Mg(Phe)$_2$-H]$^+$ ions. Further, both clusters at m/z 635 fragmented to product ions with the same nominal mass 431 u (data not shown), assigned as [Cu(L-Trp)Phe-H]$^+$ (calculated m/z 431.0901) and [Mg(L-Trp)$_2$-H]$^+$ (calculated m/z 431.1564). These product ions were not resolved in the ion trap and the ion intensity at m/z 431 used in the calculation of the enantiomeric ratio was skewed due to the presence of [Mg(L-Trp)$_2$-H]$^+$ species. Interestingly, the change of entrance capillary voltage of the FTICR instrument from 300 to 320 V increased absolute intensity of the Mg^{2+}-containing cluster while absolute intensity of the copper cluster remained unchanged (Fig. 1B and C). Intensity ratio of [Mg(L-Trp)$_3$-H]$^+$/[Cu(L-Trp)$_2$Phe-H]$^+$ changes from 2.0 to 4.0.

The formation of the Mg^{2+}-containing cluster in the ion trap instrument was verified using labeled tryptophan (Trp*, molecular weight 217). The corresponding clusters [Mg(L-Trp*)$_3$-H]$^+$ and [Cu(L-Trp*)$_2$Phe-H]$^+$ were observed at m/z 674 and 661, respectively. The absolute intensities of these clusters are temperature dependent (Table 1).

Table 1
Influence of the heated capillary temperature on the abundances of the copper and magnesium clusters [ion trap, Cu^{2+} (2.5×10^{-5} mol/L), Mg^{2+} (2.5×10^{-4} mol/L), triplicates, *indicates labeled compound].

Temperature (°C)	Absolute intensity ($\times 10^3$ counts)		Ratio[a]
	[Mg(L-Trp*)$_3$-H]$^+$ m/z 674	[Cu(L-Trp*)$_2$Phe-H]$^+$ m/z 667	Ion 674/ion 667
100	0.2	1.0	0.2 (13.8)
150	94.2	180.3	0.5 (3.0)
200	264.0	219.3	1.2 (5.4)
225	142.5	34.1	4.2 (10.0)

[a] Relative standard deviation (%) is given in a bracket.

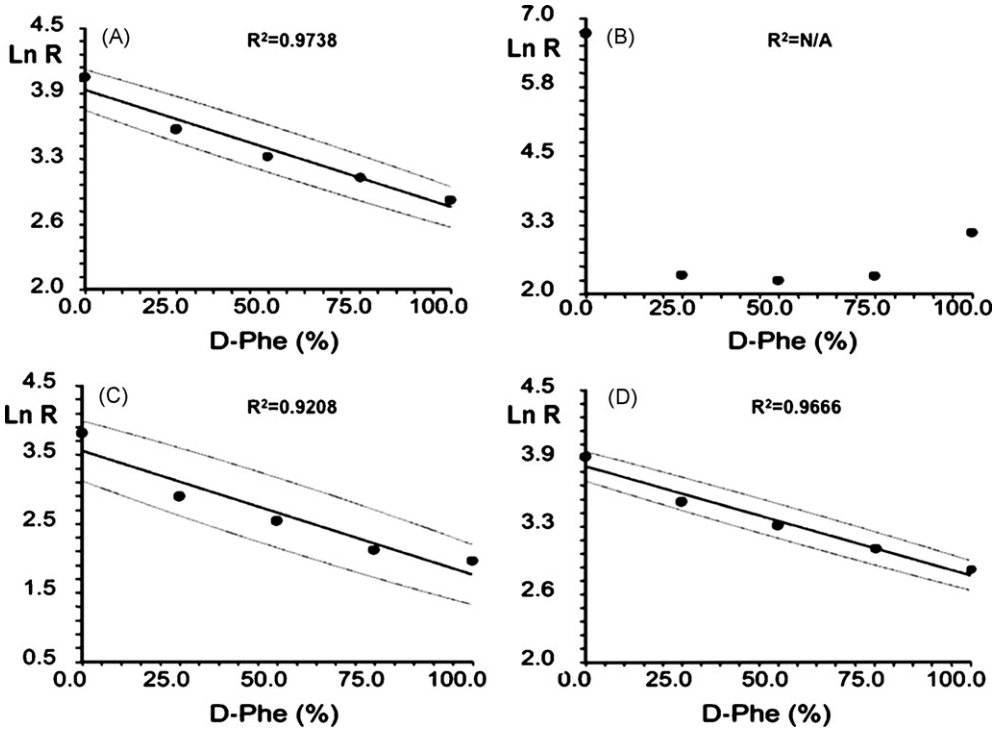

Fig. 2. Calibration curves indicating the relationship between product ion ratio (R) and D-Phe content. (A) No Mg^{2+} added, (B) with Mg^{2+} (1.5×10^{-3} mol/L), (C) with Mg^{2+} (1.5×10^{-3} mol/L), L-Trp substituted by L-Trp*, (D) Mg^{2+} (1.5×10^{-3} mol/L) added, removed by a cation-exchanger, finally Cu^{2+} and L-Trp mixed with a sample. All points repeated five times, *indicates labeled compound.

The intensity ratio of $[Mg(L\text{-}Trp^*)_3\text{-}H]^+/[Cu(L\text{-}Trp^*)_2Phe\text{-}H]^+$ changes from 0.2 at 100 °C to 1.2 at 200 °C. Further increase of heated capillary temperature to 225 °C leads to a drop of the intensities of both clusters due to their decomposition. Simultaneously, the ratio of their intensities is shifted even more in favor of the magnesium cluster. Analogous experiments with labeled tryptophan performed on Q-TOF instrument demonstrated the same trend. The cluster ratio was 1.6 at 100 °C and 2.1 at 120 °C (temperature of the ion source and of desolvation gas). The absolute intensities of both clusters increased from 100 to 120 °C but again more rapidly for the magnesium cluster. A decrease in the absolute intensities of both clusters was observed at 150 °C. Their respective ratio was 3.3 in the latter case.

The increase of energy content supplied either by voltage offset (FTICR, Fig. 1) or by temperature gain (ion trap and Q-TOF) led to higher concentration of magnesium-related cluster ions in the mixed ion population. The tuning of an API part of any low-resolution instrument (ion trap and quadrupole) likely influences the absolute intensity of both the target diastereoisomeric cluster and an interfering ion at the given nominal mass. This can result in a higher contribution of interference to signal and thus, deterioration of quantitative results.

The deleterious effect of magnesium on calibration in our particular system is evident from Fig. 2. While the satisfactory calibration was obtained in absence of magnesium, its presence (1.5 mmol/L) made calibration fully impossible. The use of a high-resolution instrument can solve this problem since product ions $[Cu(L\text{-}Trp)Phe\text{-}H]^+$ and $[Mg(L\text{-}Trp)_2\text{-}H]^+$ at m/z 431 can be resolved. For low-resolution instruments, two approaches were tested. The use of labeled tryptophan separated the target diastereoisomeric cluster ($[Cu(L\text{-}Trp^*)_2Phe\text{-}H]^+$, m/z 661) from the Mg^{2+}-containing cluster ($[Mg(L\text{-}Trp^*)_3\text{-}H]^+$, m/z 674). It improved the calibration dramatically but magnesium was not removed from analyzed solutions and was still available in the system (at concentration 1.5 mmol/L) to compete with copper for ligands. Removal of magnesium by a cation-exchanger should be more useful, as demonstrated by the calibration curves and analyses shown in Fig. 2 and Table 2, respectively. It is noteworthy, that R_{chiral} values need not point to the magnesium interference (no addition: $R_{chiral} = 3.5$; 1.5 mmol/L: $R_{chiral} = 3.4$).

Lower contents of magnesium (0.25 and 0.50 mmol/L) do not obstruct the construction of the calibration curves. However, higher errors of quantitation were observed (Table 2). The elimination of magnesium is crucial to analysis even at its lower concentrations.

Table 2
Calibration equations and results of analyses of model samples (ion trap, each measurement repeated five times).

Mg^{2+} (mmol/L)	Elimination of Mg^{2+}	[a]Calibration line $\ln R = A^*(\%\ D\text{-Phe}) + B$			Sample (% D-Phe)[b]	
		A	B	coef. of determination	10	90
Not added	None	−0.0100 (±0.0007)	3.80 (±0.04)	0.9738	10.8 (0.8)	94.3 (4.3)
0.25	None	−0.0112 (±0.0046)	3.91 (±0.26)	0.9580	14.7 (4.7)	108.6 (18.6)
0.50	None	−0.0126 (±0.0036)	3.91 (±0.22)	0.9759	19.3 (9.3)	101.8 (11.8)
1.50	Labeled Trp*	−0.0180 (±0.0023)	3.61 (±0.14)	0.9208	8.0 (−2.0)	81.6 (−8.4)
1.50	Cation-exchanger	−0.0151 (±0.0012)	3.58 (±0.07)	0.9666	10.3 (0.3)	82.2 (−7.8)

[a] Confidence interval.
[b] Differences from actual values are given in brackets.

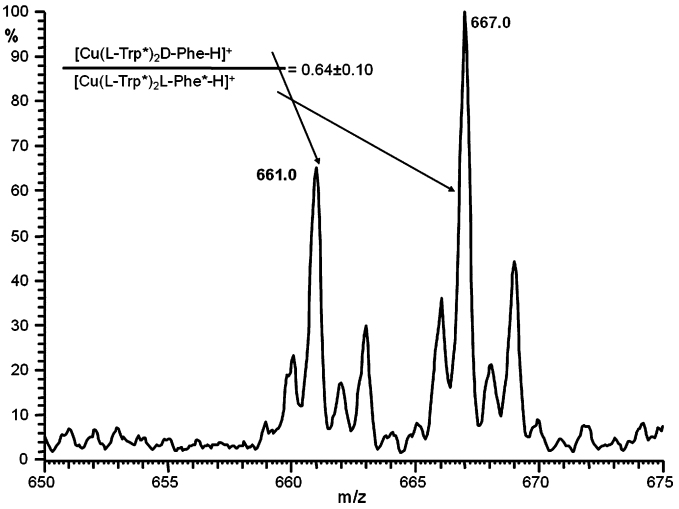

Fig. 3. Unequal formation of diastereoisomeric clusters (*indicates labeled compound). Analyzed mixture of D-Phe and L-Phe* (1:1), ion trap, triplicates, measured ratio and confidence interval are shown.

Further, calibration in the absence of magnesium was used for both samples (10% and 90% D-Phe). Evaluation of the results returned values of 74.3 and 156.9%, respectively, in Mg^{2+}-containing samples (1.5 mmol/L). While the value 156.9% is clearly wrong, the value 74.3% could be erroneously accepted as a reasonable enantiomeric excess in an unknown sample. Lower concentrations of magnesium induced errors too, e.g., 110.7% D-Phe was determined for 90% sample at 0.25 mmol/L magnesium.

The Mg^{2+}-containing product ion increases the observed signal at *m*/*z* 431. Since D-Phe showed the same effect (higher intensity of [Cu(L-Trp)Phe]$^+$ in comparison to L-Phe), magnesium mimicked its presence in samples. However, competition among the metals occurred during the cluster formation and the stability of both metal clusters differed which further complicated the quantitation of enantiomers.

Quantitative results can also be influenced by different efficiencies of diastereomeric cluster formation. As consequence, the measured ratio can differ from ratio of enatiomers in a solution [8]. Labeled compounds were used to distinguish diastereoisomers. Ion trap spectra showed significantly lower intensity of diastereoisomeric clusters containing D-Phe (Fig. 3). An isotope effect could be a possible explanation for this observation. However, similar experiments performed on FTICR using only labeled L-Phe* supported unequal formation of diastereoisomeric complexes (Fig. 4). The diastereoisomeric ratio differed from one and reversed with substitution of L-Trp by D-Trp. The observed ratios did not exactly reflect the expected cross-chiral relationship, which indicates the potential contribution of isotope effects.

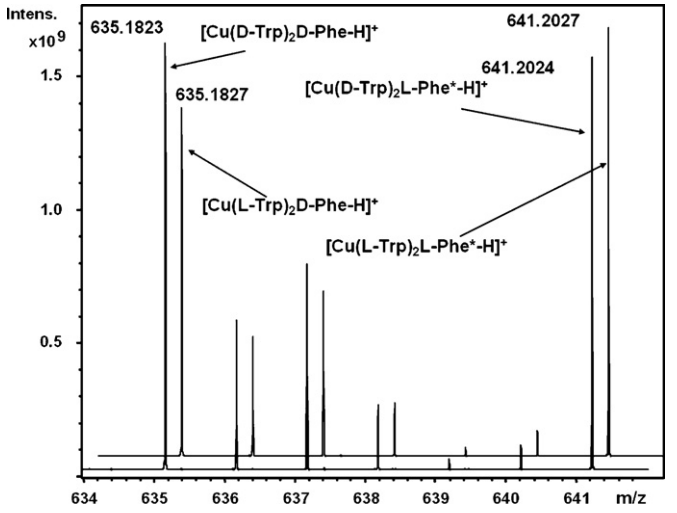

Fig. 4. FTICR spectra demonstrating unequal formation of diastereoisomeric clusters (D-Phe/L-Phe* = 1:1; *indicates labeled compound).

Further, sample solutions containing labeled phenylalanine racemate, unlabeled L- or D-Phe, respectively, with constant ratio Phe*/Phe= 1 and L-Trp were prepared and measured using the ion trap instrument. The copper cluster containing Phe* appeared at *m*/*z* 640 and the cluster with unlabeled Phe was observed at *m*/*z* 635. Their intensity ratio could differ from one due to isotope effect. If no chiral discrimination in formation of the diastereoisomeric clusters occurs, the ratio should be constant independent of substitution of unlabeled L-Phe by unlabeled D-Phe. Experimental average intensity ratio (five measurements) was 0.93 and 0.57 for sample with unlabeled L-Phe and D-Phe, respectively. Since the prepared samples differed only in unlabeled enantiomer, the difference of measured ratios cannot be explained by isotope effect. This indicates chiral dependence of the cluster formation. Heterochiral diastereoisomeric clusters (D-Phe/L-Trp or L-Phe/D-Trp) were created less effectively.

2.4. Conclusions

The formation of diastereoisomeric clusters can strongly influence quantitation of enantiomers of phenyalalanine by the kinetic method. The presence of magnesium in the sample deteriorated or even made impossible determination due to creation of interfering ions, providing isobars to both initial trimeric copper cluster and the resultant dimeric product ion. Removal of magnesium using cation-exchanger gave satisfactory results, better than elimination of interference by labeled tryptophan. Generally, all matrix constituents that suppress the signal of or are isobaric to the ions of interest are unwanted. The presence of isobaric interferences should, if possible, be checked by a high-resolution instrument.

Further, chiral discrimination occurred in the process of cluster formation. Heterochiral diastereoisomeric ions were created less effectively in comparison to homochiral ones. As a consequence, the ratio of enantiomers bound in diastereoisomeric clusters in the gas phase differs from their ratio in a solution, but it is still possible to compensate the effect by the calibration [8].

Although the chiral analysis was investigated, analogous phenomena can play role in analyses of other isomers using the kinetic method.

Acknowledgements

This paper is dedicated to the 75th birthday of Professor Zdenek Herman. We would like to express thanks for his support of our research work.

Financial support of the Ministry of Education, Youth and Sports of the Czech Republic (MSM6198959216 and LC7017), the Grant agency of Czech Republic (203/07/0765) and the European Commission (MKTD-CT-2004-014407) are gratefully acknowledged.

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