How to Discriminate Between Leucine and Isoleucine by Low Energy ESI-TRAP MS^n

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In peptide sequencing experiments involving a single step tandem mass acquisition, leucine and isoleucine are indistinguishable because both are characterized by a 113 Da mass difference from the other peptide fragments in the MS^2 spectrum. In this work, we propose a new method to distinguish between these two amino acids in consecutive MS^n experiments, exploiting a gas-phase fragmentation of isoleucine that leads to a diagnostic 69 Da ion. We used this method to assess the Leu/Ile residues of several synthetic peptides. The procedure was then tested on a tryptic digest of myoglobin, assigning the correct amino acid in the majority of the peptides. This work was performed with an old and low-resolution instrument, thus demonstrating that our method is suitable for a wide number of ion trap mass spectrometers, not necessarily expensive or up-to-date. (J Am Soc Mass Spectrom 2007, 18, 57–63) © 2007 American Society for Mass Spectrometry

Proteome investigation is one of the most important topics in biomedical research. Over the last decade, liquid chromatography, coupled with electrospray mass spectrometry, has become one of the most powerful techniques for proteomic analysis. Protein sequencing by mass spectrometry is routinely achieved worldwide by collision induced fragmentation of tryptic peptides. The currently accepted nomenclature of the fragments was proposed by Roepstoff and Fohlman [1] and subsequently modified by Johnson [2, 3] and Bieman [4]. Low collision energies (a few eV) generate fragment ions carrying charges mainly over the peptidic backbone, so that *a*, *b*, and *y* ions are usually predominant in the MS/MS spectrum; *b* ions carry the charge on the amino-terminal fragment; *y* ions, on the contrary, retain the charge on the carboxy-terminal fragment. According to Bieman, b and y ions are numbered from each terminus of the backbone, respectively. At this level of analysis, leucine and isoleucine are virtually indistinguishable. Higher collision energies (keV level) can generate additional *d* and *w* fragments that enable Leu and Ile to be differentiated [5]. Unfortunately, the overwhelming majority of instruments used for routine proteomic analysis, such as triple quadrupoles and ion traps, work at low fragmentation energies and, for this reason, the information about Leu and Ile is usually lost. The Leu/Ile issue has been extensively investigated over the years: by FAB ionization [6], by electron impact [7], by formation of copper complexes [8], or by chemical derivatization [9]. In this work, we propose a new method to achieve this discrimination with a nearly obsolete, economic, and low-resolution ion trap instrument by a series of tandem mass steps, and using a relatively low sample concentration (500 fmol/μl). The idea for this work originated from a practical problem due to a mislabeling incident. Two synthetic HLA nonameric peptides (A1: VMAPRTLIL and CW3: VMAPRTLLL), identical for molecular mass and sequence and differing from each other only for a leucine instead of an isoleucine residue at position 8, were accidentally exchanged before cell culture trials. A rapid assessment of the peptide sequence was therefore requested, to avoid an expensive new synthesis of both molecules. We first performed an accurate study of the electrospray-ion trap fragmentation pattern of leucine and isoleucine as free amino acids with the purpose to find a fragment ion diagnostic for our aim. We found that the protonated molecular ion (132 Da) of Ile, unlike Leu, generates, by loss of ammonia, a high amount of 69 Da ions from its immonium fragment (86 Da). We then used this diagnostic ion to unambiguously assign the correct structure of A1 and CW3. This approach was then extended to tryptic peptides to deduce a general rule for specific Xle assessment. To this aim, we synthesized a family of 10 tryptic-like isomeric hexapeptides, consisting of a series of permutations of the same amino-acidic sequence, differing for the position of Leu and Ile in the primary structure. The correct Xle residue was characterized for every position but the last one. As a final confirmation of the validity of our method, we tested it on a myoglobin tryptic digest, obtaining satisfactory results, though with a few limitations, mostly due to instrumental restraints.

The ability to discriminate between Leu and Ile is

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important as these two amino acids are of course coded by different mRNA triplets. In a molecular cloning experiment, the correct mRNA has to be isolated to obtain the corresponding cDNA to be used, for example, to produce the recombinant species of the protein of interest [10]. For this purpose, the primers to be synthesized for polymerase chain reaction experiments should be reduced to a minimum. The primers are planned on the basis of the primary structure of the peptides of the unknown protein. These sequences are usually determined by mass spectrometry and de novo sequencing, but every Xle uncertainty in the peptide adds a 2^n multiplicity for each primer, n being the number of Xle in the structure. For this reason, a correct Xle assignment decreases the primers multiplicity, allowing significant time and costs gain.

Materials and Methods

Mass Spectrometry

All experiments were carried out on an Agilent 1100 MSD (Palo Alto, CA) ion trap mass spectrometer, equipped with an electrospray ion source. Samples were dissolved in a formic acid 0.1%/ACN 50/50 solution at a final concentration of 500 fmol/μl and analyzed by direct infusion analysis at 5 μl/min flow rate. Mass spectra were acquired in positive ion mode. Instrument parameters were set as follows:

- Dry gas (N_2): 6 L/min.
- Nebulizer (N_2): 10 L/min.
- Dry temperature: 300 °C
- HV capillary: 3500 V
- Octopole: 3.5 V
- Skimmer 1: 25 V (unless otherwise specified).
- Trap drive: 50

In the MS^n experiments, collision energies were automatically set by the acquisition software according to the precursor ion mass to charge ratio.

Peptide Synthesis

Peptides were manually synthesized using the standard 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase strategy, with minor modifications [11].

Tryptic Digestion

Horse myoglobin was dissolved in 1 mM HCl, dripped in cold acetone and centrifuged at 14,000 rpm for 10 min. The denatured protein was then separated from the supernatant and resuspended in 50 mM ammonium bicarbonate at pH 8.5. Peptide sequencing grade trypsin (Sigma Aldrich, Milano, Italy) at a final weight ratio of 1 to 100 with respect to the protein was then added to the solution. The sample was incubated at 37 °C overnight, then diluted in 50/50 water/ACN + 0.1% formic acid at a final concentration of 500 fmol/μl.

Results

Fragmentation Spectra of Leu and Ile Immonium Ions

Both Leu and Ile protonated molecular ions (132 Da) easily generate the corresponding immonium ion (86 Da) by loss of formic acid. When the immonium is further fragmented, the MS^3 spectrum in Figure 1 is recorded. Besides the 30 Da and 44 Da ions, common to both amino acids, Ile, unlike Leu, generates a 69 Da ion that is diagnostic for our aim. Leucine and isoleucine tandem mass spectra were obviously acquired at the same collision energies. This result was previously reported by Hulst and Kientz in 1996 [12], who observed this behavior of Ile when subjected to collision with inert gas, and used the abundance of this diagnostic ion to confirm the relative Leu/Ile ratio in two large peptides. The chemical nature of the 69 Da ion of Ile and the gas-phase reaction mechanism that produces it are currently under investigation, by means of quantum chemistry.

Analysis of CW3 and A1 Peptides

From the MS^2 spectrum of the doubly charged form of the HLA peptides (507.2 Da), the $y2$ ion ($^+{}_3$HN-**X**L-COOH, 245 Da) was selected and, when subjected to MS^3 fragmentation, generated the 86 Da immonium ion. The MS^4 spectrum of this last ion answered the issue of Xle assessment: only one peptide produced the 69 Da ion and was unambiguously labeled as CW3. More interestingly, another way to find this information from the MS^2 spectrum proceeded from the doubly charged $b8$ ion ($^+{}_3$HN-VMAPRTL**X**-CO$^+$). In the MS^3 spectrum of this fragment, two signals at 227 Da and 199 Da were analyzed: they matched, respectively, a $b8y2$ internal fragment ($_2$HN-L**X**-CO$^+$) and its decarbonylated form ($_2$HN-L**X**$^+$). Both ions generated the 86 Da immonium in MS^4, which allowed the identification of CW3 by the 69 Da ion produced in MS^5. This last way required an additional MS/MS step. A summarizing diagram of this analysis is reported in Figure 2a and b. The obtained result was then confirmed by the same biological trials in which HLA-E was recognized by the $\alpha\beta$ T cell receptors in presence of CW3 only, as expected for a peptide carrying an Ile residue in position 8 [13].

Xle Residue Characterization

Ten isomeric hexapeptides were specifically synthesized to achieve a complete characterization of Xle independently of its position in the peptide. The Xle residue was placed at different positions in the backbone (see Figure 3), except for the last one, which was reserved to K, to simulate the result of a tryptic digestion. The doubly charged form of each peptide (300 Da) was fragmented. In a deeper analysis of the MS^2 spectrum, often both y and b series fragments carried the analysis to a final characterization of Xle. For position 1,

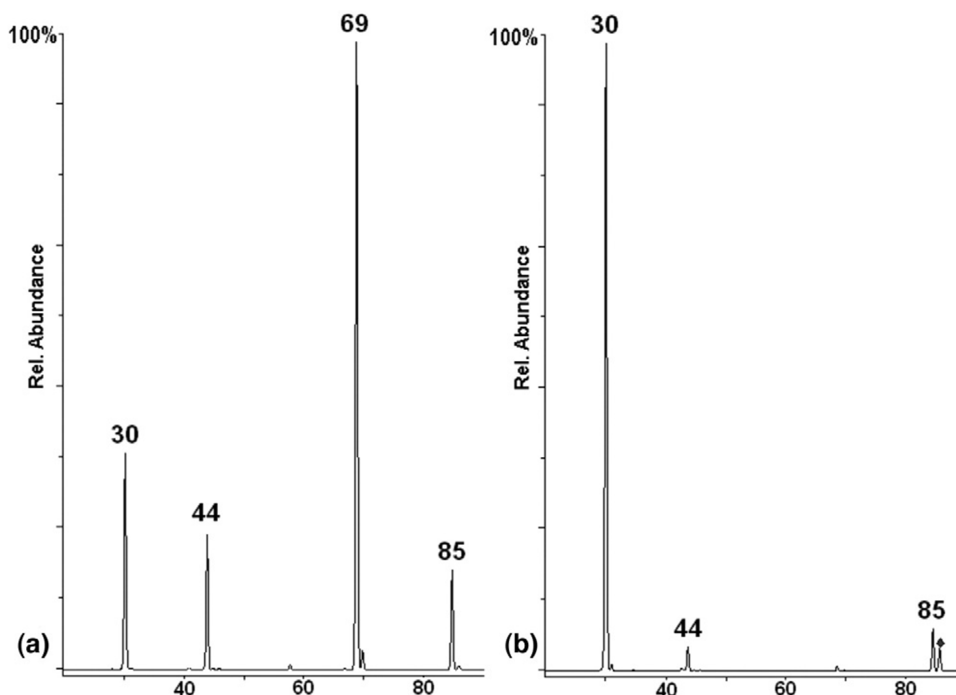

Figure 1. MS^3 spectrum of immonium ions of isoleucine (**a**) and leucine (**b**).

the result was almost obvious, since the [a1-NH_3] ion at 69 Da was clearly visible in the MS^2 spectrum of the Ile peptide only. Besides that, the b series led, through its decarbonylated a form, to the 86 Da immonium ion which, in the subsequent MS^n step, originated the 69 Da diagnostic ion for the Ile peptide only. The b series was thus used for positions 1 and 2. Getting deeper inside the peptide (positions 3 and 4), another way to generate a useful fragment ion had to be found. The y series proved to be the best choice: for Xle in position 3, the y4 fragment ion ($^+{}_3$HN-**X**AVK-COOH, 430 Da) generated the internal b4y4 ion ($_2$HN**X**A-CO^+, 185 Da) that, through the immonium form of Xle, unambiguously allowed the Xle characterization. We used the same approach for position 4: in this case, the y3 ion ($^+{}_3$HN-**X**VK-COOH, 359 Da) produced the internal b5y3 fragment ($_2$HN-**X**V-CO^+, 213 Da) and the y4 ion ($^+{}_3$HN-A**X**VK-COOH, 430 Da) produced the corresponding b4y4 ($_2$HN-A**X**-CO^+, 185 Da). Both fragmentation pathways led us to the characterization of Xle.

The Problem of Position N-1

With Xle in the second-last position immediately before K, our method did not produce reliable results. The fragmentation spectrum of the y2 ion ($^+{}_3$HN-**X**K-COOH, 260 Da) generated a very small amount of the immonium of Xle and a much higher amount of 84 Da ion (Figure 4). The latter comes from the immonium of K by loss of ammonia. The combination of weakness of 86 Da signal and the presence of a very close (by mass) 84 Da ion did not allow us to obtain a good MS^4 spectrum, since in MS^3 our instrument does not have an adequate effective resolution for isolation of 84 and 86 ions. For this position, we changed our approach: the skimmer 1 voltage was increased from 25 to 60 V, producing a considerable amount of 86 Da ion. This fragmentation is not controlled since it occurs *outside* the ion trap and it is due to collision of the protonated sample ions with the nebulizing nitrogen gas that is pumped out by the vacuum system. The resulting fragments are then moved to the trap and there analyzed and further fragmented. The MS^2 spectrum of this 86 Da produced the 69 Da ion for the Ile peptide only. This result suggests that regular ion trap fragmentation promotes the immonium form of K, while high voltage-induced unspecific decay of the protonated peptide assists the formation of immonium form of the adjacent Xle.

Analysis of Myoglobin Tryptic Peptides

The proposed method required to be tested on a real tryptic sample. For this purpose we used a 500 fmol/μl solution of horse myoglobin tryptic digest, recording the MS^n spectra of the doubly charged form of several peptides, ranging from 400 to 1800 Da. The obtained results are summarized and briefly discussed in Table 1.

Discussion

How to Get the Xle Information from a MS^2 Spectrum

Once the complete peptide sequence has been obtained, there is usually more than one way to achieve the Xle characterization, since both b and y series can often lead to the 86 Da immonium ion (see Figure 5). As shown, with

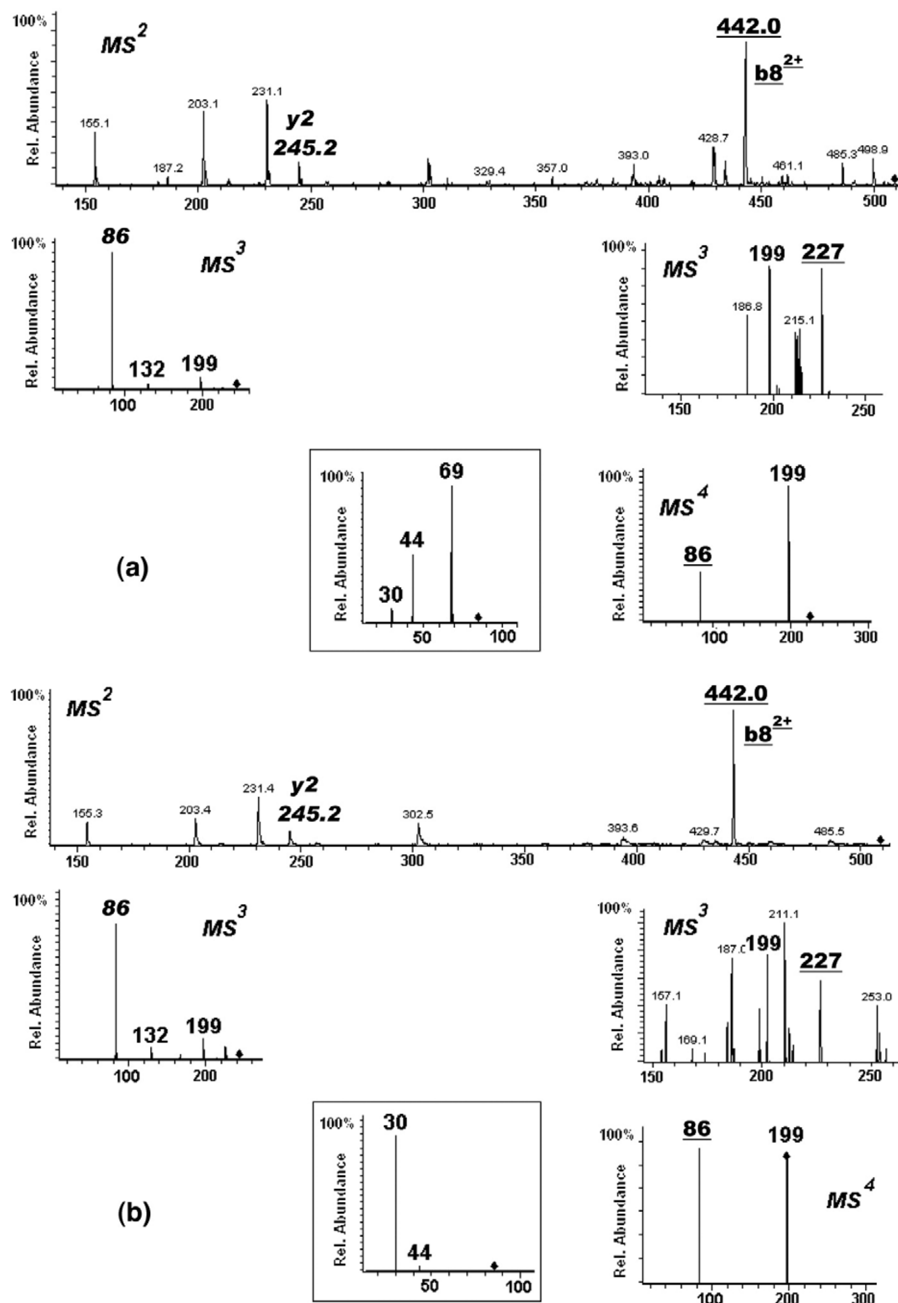

Figure 2. (**a**) Tandem mass analysis of peptide CW3: both y (italic) and b (underlined) fragment series lead to the final Ile assessment (presence of 69 Da ion). (**b**) Tandem mass analysis of peptide A1: both y (italic) and b (underlined) fragment series lead to the final Leu assessment (absence of 69 Da ion).

Xle in position n of the peptide, b_n and y_n ions carry the desired information. If these ions are weak, choosing the strongest adjacent b_{n+1} and y_{n-1} signal is sometimes a better choice, because it might generate the desired b_n and y_n ions with an additional MS/MS step. If the instrument electronics are fast enough, the immonium ion produced in MS^5 (instead of MS^4 will generate the diagnostic ions alike. As a general rule, strongest signals and lower masses are to be preferred because they usually produce a better MS^n spectrum, with a higher abundance of the fragment carrying Xle as daughter b ion. In a application-oriented approach, a few guidelines for Xle assessment can be outlined: if Xle is in the first part of the peptide, following the b series (and its decarbonylated a subseries) seems to be the best choice because it often produces the 86 Da immonium ion already in MS^3, allowing fragmentation time saving. If Xle is in the last part of a nontryptic peptide, therefore not ending by K or R, then the same result can be obtained from the y series. The inner positions of the peptide are the most difficult to characterize. In this case y ions are usually more useful than b ions because they often produce a Xle-X daughter *by* internal

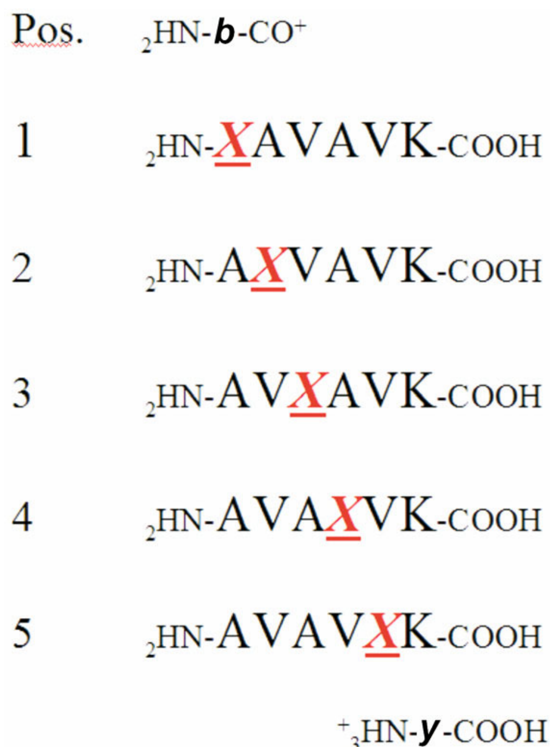

Figure 3. Synthetic hexapeptides for position-specific Xle assessment. Fragment *b* ion series begins from the *N*-terminus, *y* series from the *C*-terminus.

ion, which frequently is the key to the immonium of Xle. An example of this tandem mass behavior is represented by the y4 ion of myoglobin peptide NDIAAK (see Figure 6). Yague et al. [14] reported several examples of anomalous peptide fragmentation patterns due to rearrangement of the *b* series ions. These signals correspond to neutral losses of internal amino acids. In such a case, the effective sequence of the selected *b* ion changes, affecting the final assessment of Xle, and using the *y* series becomes a mandatory choice. However, in our experiments no direct evidence of this phenomenon was observed.

As previously described in the Results section, the second to last position of a tryptic peptide is until now not appraisable. The difference in the relative abundance of 84 and 86 Da ions in the MS^3 spectrum of the *y*5 fragment of peptides AVAVXK (Figure 4) might suggest that Ile has a little advantage respect to Leu in the formation of its own immonium ion compared with the immonium form of K. Anyway, if the researcher is confident that just one Xle is present in the peptide sequence, then the skimmer-induced fragmentation always represent a useful way to produce the 86 Da immonium ion. If more than one Xle is present but the 86 Da ion does not generate the 69 Da ion, then every Xle in the peptide is most likely a Leu.

Conclusions

It is difficult to discriminate between the intrinsic analytical limitations of this approach and the instrumental limitations of our ion trap, whose design dates back to more than ten years ago. In several cases, we were able to

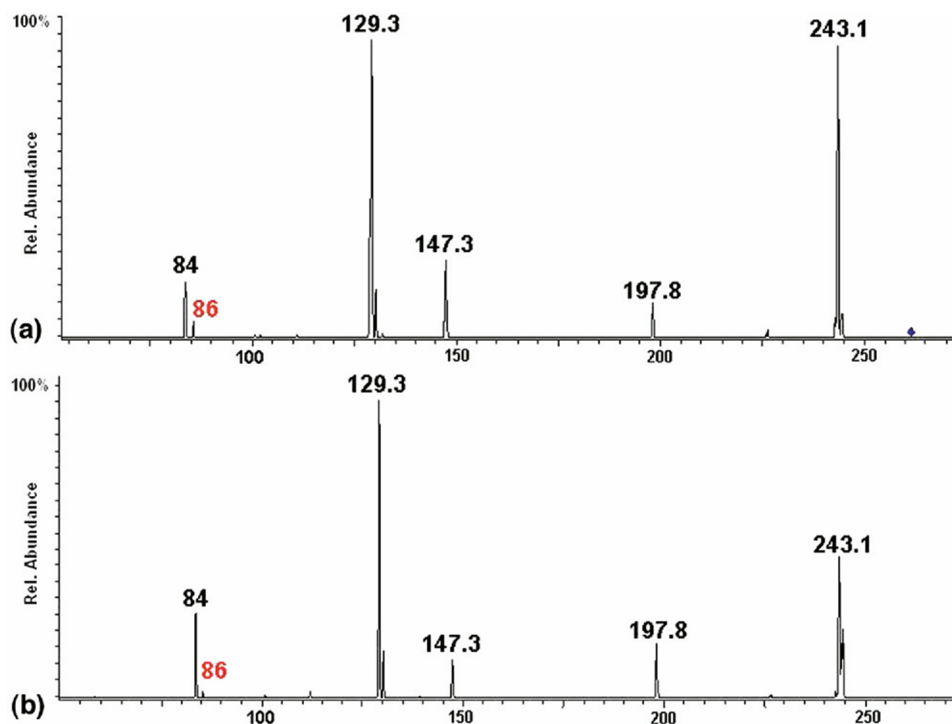

Figure 4. MS^3 spectra of the *y*5 ion of peptide AVAVIK (**a**) and peptide AVAVLK (**b**). The answer to the N-1 position issue resides perhaps in the different relative intensity of 84 and 86 Da ions.

Table 1. Myoglobin tryptic peptide analysis.

Myoglobin Peptides Underlined = correctly assigned In brackets = ambiguous or partial result	Comments
LFTGHPETLEK **MW = 1270.6**	Leu1 from a2; Leu9 from y3
ALELFR **MW = 747.4**	Leu2 from a2 and b2; Leu4 from y3
VEADIAGHGQEVLIR **MW = 1605.8**	Ile5 from b5; Leu13 from y3
NDIAAK **MW = 630.3**	Ile3 from y4
IP(I)K MW = 469.3	Ile1 from a1; Ile3 ambiguous (see text: position N-1 issue) Internal b3y3 useless probably due to proline see reference [15, 16]
Y(L)EF(I)SDA(II)HV(L)HSK **MW = 1884.0**	Analysis ended at the immonium ion (see text: instrumental limitations)
GHHEAE(L)KP(L)AQSHATK **MW = 1852.9**	Analysis ended at the immonium ion (see text: instrumental limitations)
HGTVV(L)TALGGI(L)K **MW = 1377.8**	Leu9 from y6; Ile12 from y3 Leu13 ambiguous (see text: position N-1 issue) Leu6: analysis stopped at the immonium ion (see text: instrumental limitations)
ELGFQG **MW = 649.3**	Leu2 from b2 (singly charged parent ion)

identify the dipeptide fragment carrying the Xle information, but its insufficient signal and the low capacity of the trap prevented us from reaching a further fragmentation spectrum. In our experience, we were not able to go reproducibly over a MS^5 spectrum, and frequently this was not enough because sometimes the y_n signal was not clearly visible in the MS^2 spectrum and it had to be obtained by fragmentation of a y_{n-1} or even a y_{n-2} ion. Every MS^n step dramatically reduces the ions abundance and increases acquisition times. The slow electronics of our instrument therefore represents the main instrumental limitations. We are however confident that better performing ion traps might by far increase the effectiveness of our method. In a future HPLS-MS implementation of our

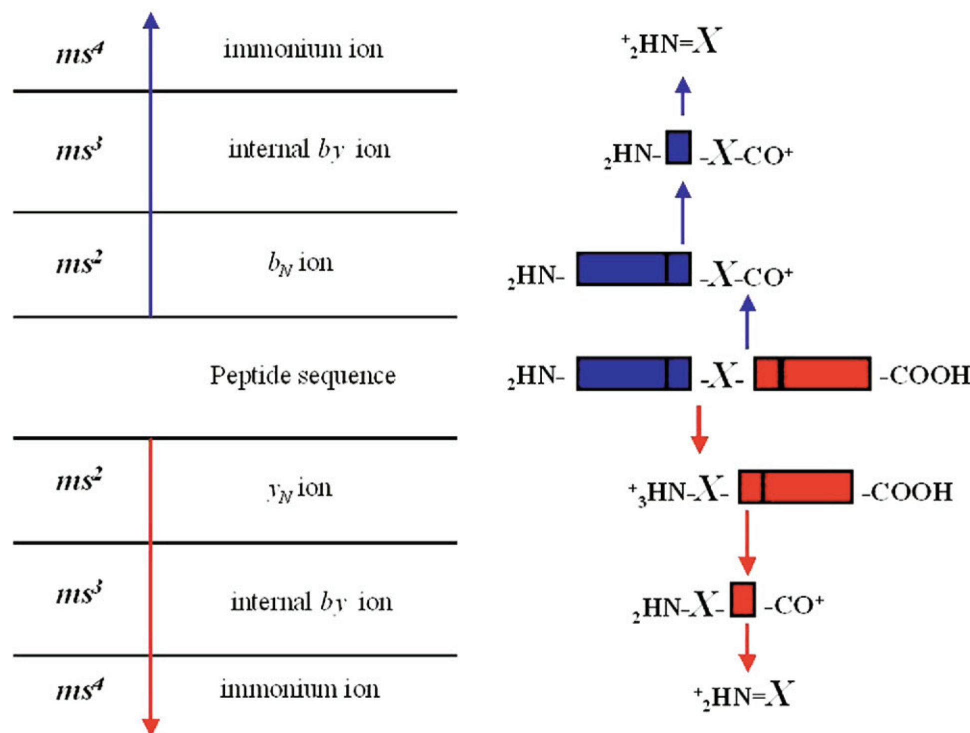

Figure 5. Summarizing diagram of the tandem mass method used to obtain the immonium ion of Xle.

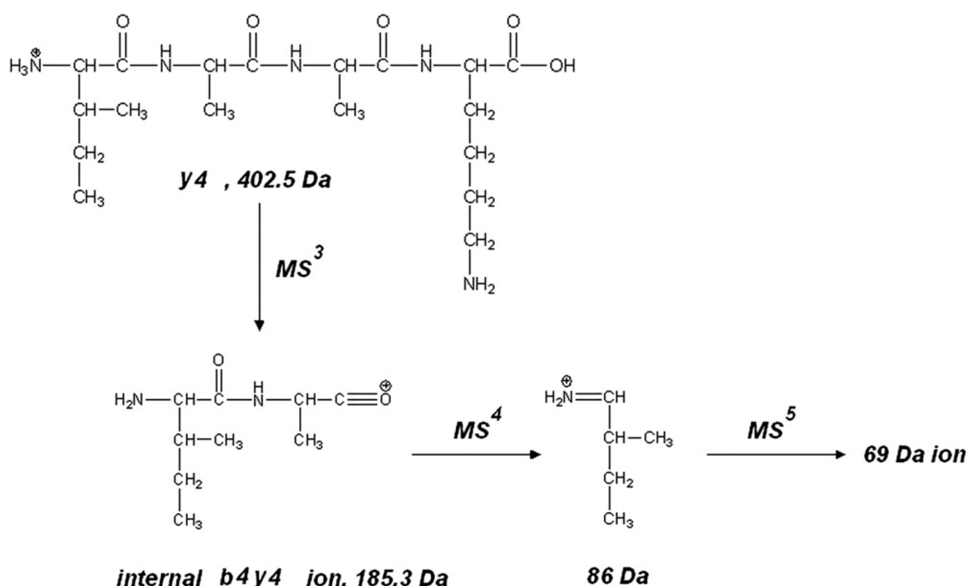

Figure 6. The y series breakdown pathway of peptide NDIAAK.

method, information dependent acquisition software, coupled with up-to-date instruments, might perhaps record the complete MS^2 spectrum, identify the critical y_n and b_n signals and proceed to their MS^3 analysis. A complete database of every possible Xle-involving dipeptide b ion could eventually lead the acquisition through the MS^4 and MS^5 steps. As for the natural intrinsic limitations, if the Xle resides in the central part of the structure, we would not recommend this approach for peptides larger than 1600 to 1800 Da, because the MS^2 fragmentation of these molecules gives rise to large b_n and y_n ions that hardly generate the desired Xle dipeptides in the subsequent MS^n steps. If, on the contrary, Xle is at the beginning of the backbone, the b series can be very often helpful, even for large peptides. Smaller peptides (500–1000 Da) are easier to analyze and the correct Xle assignment is possible in a large number of cases.

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