

# ETHcD Discrimination of Isomeric Leucine/Isoleucine Residues in Sequencing of the Intact Skin Frog Peptides with Intramolecular Disulfide Bond

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**Abstract.** Our scientific interests involve de novo sequencing of non-tryptic natural amphibian skin peptides including those with intramolecular S–S bond by means of exclusively mass spectrometry. Reliable discrimination of the isomeric leucine/ isoleucine residues during peptide sequencing by means of mass spectrometry represents a bottleneck in the workflow for complete automation of the primary structure elucidation of these compounds. MS<sup>3</sup> is capable of solving the problem. Earlier we demonstrated the advanced efficiency of ETD-HCD method to discriminate Leu/Ile in individual peptides by consecutive application of ETD to the polyprotonated peptides followed by HCD applied to the manually selected primary z-ions with the targeted isomeric residues at their N-termini and registration of the

characteristic w-ions. Later this approach was extended to deal with several (4–7) broad band mass ranges, without special isolation of the primary z-ions. The present paper demonstrates an advanced version of this method when ETHcD is applied in the whole mass range to a complex mixture of natural non-tryptic peptides without their separation and intermediate isolation of the targeted z-ions. The proposed ETHcD method showed over 81% efficiency for the large natural peptides with intact disulfide ring, while the interfering process of radical site migration is suppressed. Due to higher speed and sensitivity, the proposed ETHcD approach facilitates the analytical procedure and allows for the automation of the entire experiment and data processing. Moreover, in some cases it gives a chance to establish the nature of the residues in the intact intramolecular disulfide loops.

**Keywords:** Leucine/isoleucine differentiation, Orbitrap, ETHcD, Intact natural peptides, Radical site migration, Disulfide containing peptides, Peptide sequencing

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## Introduction

Although tandem mass spectrometry has become the most efficient and reliable tool for peptides and proteins sequencing [1, 2], discrimination of isomeric leucine/isoleucine residues remains a problem that cannot be solved automatically by the available sequence search engines. The solution to this problem would help to completely substitute the earlier widespread Edman degradation [3, 4]. An alternative possibility to discriminate the isomeric residues involves cDNA cloning. Transcriptome and genome data can unambiguously

discriminate Leu and Ile as they are encoded by different codons in mRNA; however, this approach is useful only in the case of organisms with established genome. Unfortunately it is not available for frogs used in our studies. Even when available, this information can be misleading because of protein splicing and change in amino acid sequence when converting DNA-coded protein to biologically active peptide form. The joint use of both techniques (MS sequencing and cDNA cloning) is a very powerful tool. However, creation of an independent reliable MS method to solve the mentioned problem may be considered as an important scientific task.

The identical hydrocarbon nature of the side chains of Leu and Ile prevents their differentiation by means of chemical

modifications widely used in modern MS analysis of peptides and proteins [5]. The modern approaches related to the identification of isomeric Ile/Leu residues are based on the detection of the secondary  $w$ -ions, formed by elimination of  $C_2H_5/C_3H_7$  radicals from the targeted  $z^{+}$  radical-cations with  $N$ -terminal Ile/Leu [6]. The latter arise in electron capture (transfer) dissociation (ECD/ETD) applied to the multiprotonated peptides [7, 8]. Despite of higher reactivity of odd-electron radical cations in comparison with even-electron cations, the further targeted fragmentation of the primary  $z^{+}$  ions leading to  $w$ -ions is not always easily accessible. High energy electron capture dissociation (HECD) was proposed earlier and to some extent this method resolved the problem in the case of tryptic peptides [9, 10]. Several other researchers proposed alternative approaches based on MS<sup>n</sup> techniques or registration of negative ions [11–17]. The most recent publication [16] describes quite reliable discrimination of the isomeric residues; however, it requires advanced tandem mass spectrometry experiments up to MS<sup>5</sup>. Up to now all existing techniques for Leu/Ile discrimination have certain limitations and cannot be integrated into the existing automatic sequencing algorithms.

Our scientific interest involves de novo sequencing, exclusively by mass spectrometry, of non-tryptic natural amphibian skin peptides, including those with intramolecular S–S bonds [18–21]. The first experiments on identification of isomeric Leu/Ile residues using a high performance Orbitrap analyzer (Thermo Fisher Orbitrap Fusion) in ESI-ETD-HCD (MS<sup>3</sup>) mode were conducted on six non-tryptic disulfide containing peptides from the skin secretion of the lake frog *Rana ridibunda* [22]. The peptides were preliminary separated with preparative HPLC. The targeted  $z^{+}$  ions with  $N$ -terminal Leu or Ile were manually isolated and activated further by higher energy collisional dissociation (HCD) method, while the normalized collision energy (NCE) was controlled aiming the appearance of the corresponding  $w$ -ions. The proposed approach was highly successful and allowed for the identification of isomeric residues in all 22 cases of Leu/Ile residues in the peptides' sequence. An important feature of the ETD-HCD method is its unique selectivity, as in the majority of cases only the targeted  $w$ -ions are present in the mass spectra. Further experiments [23] with 42 tryptic peptides from tryptic digests of human serum albumin and recombinant endolysin gp188 without preliminary isolation of  $z^{+}$  ions proved the efficiency of the approach with the success rate about 90%. EThcD broad-band fragmentation in this case involved all primary ETD product-ions with  $m/z$  values within a certain broad mass range, i.e., HCD was applied without manual isolation of individual  $z^{+}$ -ions.

An important interfering process involves radical site migration in the primary  $z^{+}$ -ions followed by alternative radical loss and formation of the corresponding  $u$ -ions [11, 24, 25]. The radical site migration may engage not only the neighboring residue, but go further with formation of the radical site staying up to six positions away, although the intensity of the corresponding  $u$ -ions peaks significantly drops with each

subsequent residue. Fragment ions formed by this pathway are designated as  $^1u$ ,  $^2u$ , etc., where  $w = ^0u$  [26]. So, if  $w$ -ion corresponds to leucine but  $^1u$  ion (or  $^2u$ ,  $^3u$ , etc.) is generated by neighboring isoleucine(s), both losses (29 and 43 mu) may be observed in the spectrum, making the annotation (Leu versus Ile) problematic. It is worth mentioning that the methods described in [22, 23] allow avoiding radical site migration, if the spectra are recorded at the minimal NCE sufficient to the formation of the targeted  $w$ -ions, requiring less energy in comparison with the interfering  $u$ -ions.

The present work deals with differentiation of isomeric Leu/Ile residues in non-tryptic disulphide containing peptides from the skin secretion of ranid frogs *Rana ridibunda* belonging to Slovenian and Moscow (Russia) populations [27]. HCD activation was applied to the entire array of the product ions formed due to the fragmentation of the polyprotonated peptide molecules in ETD mode, i.e., without isolation of  $z^{+}$ -ions containing  $N$ -terminal Leu/Ile residues. Therefore in this case EThcD, proposed earlier by Frese et al [28], was used. Besides peptides with the known sequence, new peptides with the primary structure not confirmed by Edman degradation or any other biochemistry-based procedures were studied. In these cases differentiation of isomeric Leu/Ile residues was done for the first time.

## Materials and Methods

### Reagents

Acetonitrile (HPLC gradient grade) was purchased from Sigma-Aldrich (St Louis, Missouri, USA), formic acid (for HPLC) was from Fluka (Buchs, Switzerland). Ultrapure water was prepared by Milli-Q water purification system (Millipore, Billerica, Massachusetts, USA).

### Peptides

Skin secretion of two specimens of the lake frog *Rana ridibunda* belonging to the Central Slovenian and Moscow region (Russia) populations were obtained by mild electrical stimulation of the dorsal skin glands [29]. The skin was moistened with deionized water and then treated with a bipolar platinum electrode connected to the laboratory electrostimulator (ESL-1). The pulse parameters were as follows: voltage, 15 V; pulse duration, 5 ms; pulse frequency, 50 Hz. The skin secretions were washed out with a small amount (up to 25 mL) of deionized water into a container with an equal volume of methanol. The latter was used to prevent the breakdown of the peptides by proteases [30]. The mixtures were then centrifuged for 15 min at 3000 rpm, the supernatants were filtered through a Millex-FH membrane (PTFE 0.45 μm; Millipore, Billerica, MA, USA), concentrated at 35 °C on a rotary evaporator to a volume of 1 mL, and lyophilized. The samples were kept in the freezer at –26 °C.

### Mass Spectrometric Analysis

Intact peptides were dissolved at 0.01 mg/mL in acetonitrile:water mixture (1:1) containing 1% formic acid. No chromatographic or any other separation was performed. Mass analyses were carried out on an electrospray ionization (ESI) Orbitrap Elite mass spectrometer (Thermo Fisher Scientific GmbH, Bremen, Germany). The instrument hardware was modified to allow ion activation by EThcD at the full mass range. Sheath gas flow rate was set to 10–25 arbitrary units, auxiliary and sweep gas flow rate was set to zero, spray voltage was held at 3.5 kV, capillary temperature 275 °C, S-Lens rf level 60%. Samples were introduced into the ion source using a syringe pump at 3 µL/min.

Mass spectra were acquired in positive ESI mode. Accurate mass measurements were carried out in the Orbitrap mass analyzer with 120,000 resolving power. For ion trap acquisitions 1e5 ions were trapped with a maximum injection time of 500 ms in MS<sup>n</sup> mode (for ETD). For Orbitrap (FT) acquisitions 1e6 ions were trapped in full MS, or 1e5 ions in tandem MS (MS<sup>n</sup>, EThcD) with a maximum injection time of 1000 ms. In ETD automatic gain control (AGC) was set at 6e5, with a maximum injection time of 100 ms. All spectra were recorded with one microscan and registered continuously during 1 min, after which they were averaged.

### Results and Discussion

In our previous study [23], we have developed a new “broad band” EThcD method for L/I discrimination that has nearly 90% efficiency for tryptic peptides. However, its disadvantage involves application of several wide “windows” to isolate primary fragment ions for future HCD. As a result, one had to work in 4–7 mass ranges consequently. In the present study, we have upgraded the proposed approach to deal with the entire mass range (standard EThcD mode). It is faster (only one

EThcD MS<sup>3</sup>-spectrum instead of 4–7 “broad band” ones). Besides, no supplemental activation to enhance ETD fragmentation is needed. A brief scheme of the MS experiment is as follows: peptide is fragmented by ETD in the ion trap. Then all ions are transmitted for analysis to orbital trap and during this transmission all ETD fragment ions are subjected to HCD without any isolation. The odd-electron z<sup>-</sup>-ions lose isopropyl radical in the case of leucine or ethyl and methyl radicals in the case of isoleucine giving corresponding w-ions. To suppress interfering processes, careful choice of normalized collision energy (NCE) for HCD is needed: it should be as low as possible but nevertheless allowing appearance of w-ions.

Differentiation of isomeric Leu/Ile residues was done for 15 intact natural peptides. Fourteen of them contained one intramolecular S–S bond (Table 1). All peptides originate from the skin secretion of the lake frog *Rana ridibunda* belonging to the Central Slovenian and Moscow (Russia) populations [27]. The sequences of half of these peptides were confirmed by Edman degradation or cDNA cloning [27] (asterisk in Table 1). Peptides 1–6 were studied earlier for the differentiation of Leu/Ile residues by ETD-HCD [22], whereas brevinin 2Ec was also studied with broad-band EThcD method [23]. Therefore, it was interesting to compare the efficiency of EThcD and ETD-HCD approaches.

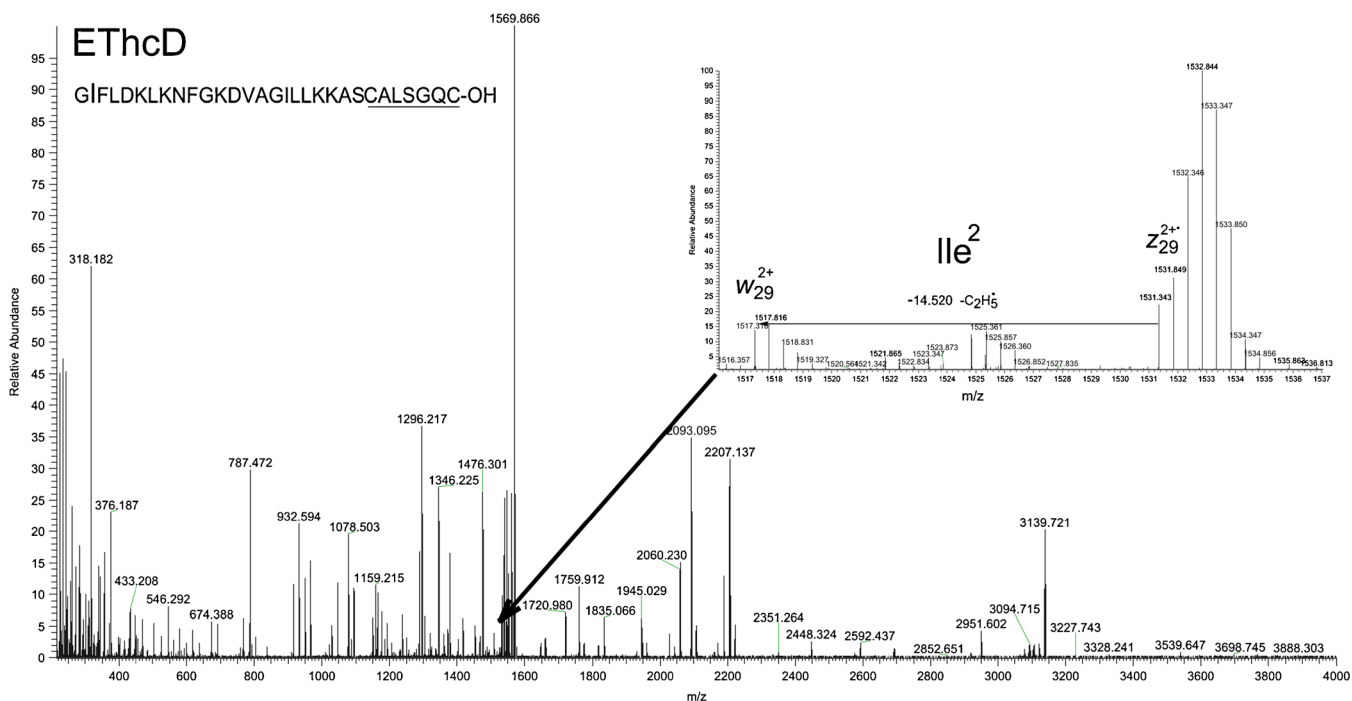
Identified residues are highlighted in bold, unidentified are represented as X. Sixty-one out of seventy-five (81.3%) isomeric Leu/Ile residues in the sequences of 15 peptides including “Rana box” (Table 1) were identified by EThcD approach. The result is even better if we exclude “Rana box” usually requiring derivatization (60 out of 66 – 90.9%). Since EThcD involves application of HCD to the entire array of the primary ions, the resulting spectra appear to be much more complex than in ETD-HCD mode [22]. Nevertheless the spectral complexity does not interfere with the correct discrimination of the isomeric residues (Figure 1). One should note that differentiation was carried out with rather large non-tryptic peptides (MW

**Table 1.** Results of the Discrimination of Leu/Ile in 15 Non-Tryptic Peptides with Disulfide Bonds

No.	Peptide name	MW	Sequences	Efficiency %
1	Brevinin 1E*	2674.5216	FL <b>PLLAGLAANFLPKIFCKITRKC</b> -OH	100 (7/7)
2	Brevinin 1Ra*	2636.2481	VIPFVASVAEMMQHVYCAASRRC-OH	100 (1/1)
3	Brevinin 2Ec *	3516.9157	GIL <b>LDKLN</b> FN <b>FAKTAGKGV</b> L <b>QSL</b> LN <b>TASCK</b> LSGQC-OH	87,5 (7/8)
4	Ranaturerin 2R*	1939.0321	AVNIPFKVKFRCKAAFC-OH	100 (1/1)
5	Ranaturerin 2Ra*	1891.0321	AAK <b>IIL</b> NP <b>KFRCKAAFC</b> -OH	100 (3/3)
6	Esculentin 2R	3823.0889	GILSIVKGVA <b>KL</b> AG <b>KT</b> FAKEGGK <b>F</b> GLE <b>FL</b> ACKV <b>T</b> NQ <b>C</b> -OH	100 (6/6)
7	Brevinin 1R*	2663.5056	FFPAIFRLVAKVVP <b>S</b> H <b>CS</b> SV <b>T</b> KK <b>C</b> -OH	100 (4/4)
8	Brevinin 2Rb	2918.5388	GXMSTLKGAATNAAV <b>T</b> X <b>LN</b> KL <b>QCK</b> X <b>T</b> GT <b>C</b> -OH	50,0 (3/6)
9	[Asp <sup>3</sup> ,Lys <sup>6</sup> ,Phe <sup>13</sup> ]* 3-14-bombesin	1385.7297	NLGKQWAVGHFM-NH <sub>2</sub>	100 (1/1)
10	Brevinin 2Re	2946.5352	GXMNTLKGAATNAAV <b>T</b> L <b>X</b> DKL <b>QCK</b> X <b>T</b> GT <b>C</b> -OH	50,0 (3/6)
11	Brevinin 2Rf	3549.9165	GLFLNALKN <b>FAKTAGKGV</b> L <b>QSV</b> LN <b>TASCK</b> X <b>SK</b> Q <b>C</b> -OH	83,3 (5/6)
12	Brevinin 2Rg	3087.6574	GIL <b>X</b> DKL <b>N</b> FN <b>FAK</b> GV <b>AQ</b> S <b>LL</b> N <b>K</b> A <b>S</b> C <b>A</b> X <b>S</b> G <b>Q</b> C-OH	71,4 (5/7)
13	Brevinin 2Rj	3134.6985	GIFL <b>DKL</b> KN <b>F</b> NG <b>KD</b> VAGIL <b>L</b> KK <b>A</b> S <b>C</b> A <b>X</b> S <b>G</b> Q <b>C</b> -OH	85,7 (6/7)
14	Brevinin 2Rh	3376.7704	GIMDTLKNLAK <b>TAGK</b> GAL <b>Q</b> S <b>LL</b> K <b>M</b> A <b>S</b> C <b>K</b> X <b>S</b> G <b>Q</b> C-OH	85,7 (6/7)
15	Brevinin 2Rk	3504.8620	GLWKT <b>X</b> K <b>D</b> SA <b>K</b> SA <b>V</b> T <b>N</b> VA <b>V</b> TM <b>L</b> DKL <b>R</b> CK <b>X</b> T <b>G</b> GC-OH	60,0 (3/5)

\*- The structures are confirmed by Edman degradation [27]

Underlined sequence means that the highlighted residues constitute S–S ring  
The residues established by the proposed MS approach are marked with bold



**Figure 1.** Full  $m/z$  range EThcD spectrum of brevinin 2Rj. The insert demonstrates discrimination of  $Ile^2$  by  $C_2H_5^\cdot$  loss from  $z_{29}^{2+}$  ion as an example of successful application of the method to the rather complex EThcD spectra

in the range of 2.5–3.5 kDa). A number of targeted  $z$ -ions with  $N$ -terminal Leu/Ile residues have masses around 2000 Da, representing certain challenges to achieve the goal [16, 23]. This is due to the fact that the kinetic shift increases when fragmenting species become more complex, since the excessive internal energy is distributed among many bonds [31, 32]. At the same time the increase of NCE often leads to the activation of multiple fragmentation routes, competing formation of the targeted  $w$ -ions [22, 23]. In the present research, the spectra with HCD NCE energy 20–30 were most informative (excluding peptides with Leu and Ile residues in neighboring positions). Usually,  $z$ -ions with  $m/z$  below 1500–1600 Da were singly charged and over 1600 Da, doubly charged. Actually doubly charged  $z$ -ions represented the major group in the discrimination experiments. Figure 1 represents an example of application of doubly charged  $z$ -ion for the identification of  $Ile^2$  in brevinin 2Rj.

It is fortunate that peptides belonging to the brevinin 2 and esculentin 2 families usually contain a fair number of basic Lys residues, thus each forming  $MH_n^{n+}$  ions ( $n \gg 2$ ) in ESI mode. That feature allows overcoming the problem of ions of high  $m/z$  values mentioned in [16, 23]. Differentiation of the isomeric residues was carried out detecting  $C_2H_5^\cdot/C_3H_7^\cdot$  radical losses in EThcD spectra of singly-, doubly-, and triply charged  $z^{n+}$  ions. Thus, Figure 2 represents HCD spectrum of  $z_{33}^{3+}$  ion (NCE 30) of brevinin 2Rf. The targeted pair of the corresponding  $z_{33}$  and  $w_{33}$  ions allowed reliable identification of  $Leu^2$  ( $z_{33}^{3+}$  1159,967  $\rightarrow$   $w_{33}^{3+}$  1145,617). Similarly, the loss of isopropyl radical from  $z_{22}^{3+}$  ion of brevinin 2Rj allowed establishing  $Leu^4$  ( $z_{22}^{3+}$  934,843  $\rightarrow$   $w_{22}^{3+}$  920,4914), whereas the loss of ethyl radical from  $z_{32}^{3+}$  ion of brevinin 2Rh and

from  $z_{33}^{3+}$  ion of brevinin 2Ec helped to identify isoleucine residues in the second position of their sequences.

Nevertheless, the applied EThcD approach demonstrated excellent results in the cases of singly charged  $z^+$ -ions as well. Thus, Figure 3 represents the identification of  $Leu^7$  on the basis of fragmentation of heavy (2433,251 Da) singly charged  $z_{24}^+$  ion of brevinin 2Rg. Therefore, the doubts expressed in [16] concerning applicability of the approached for the heavy  $z$ -ions are not always justified.

It is also important to note that the results of Leu/Ile discrimination by EThcD totally match earlier ETD-HCD results [22] obtained with the individual peptides and preliminary selection of the targeted  $z$ -ions. Despite of the extremely rich mass spectra, differentiation was possible even in the case of the heaviest brevinin 2Ec and esculentin 2R (MW 3516.9157 and 3823.0889 Da, respectively). Therefore, the proposed approach may be used in the future in automatic peptide sequence search engines.

### Radical Site Migration

The problem of radical site migration was mentioned in the introduction and was discussed earlier in detail by many [11, 16, 22–26, 33]. It was shown that its realization highly depends on the initial internal energy of  $z$ -ions and on the NCE applied at the HCD step. It was shown [22] that the NCE should be slightly higher than a threshold to trigger radical site initiated fragmentation and should be specific for every single peptide (peptides 1–6 in Table 1). Identification of the isomeric residues in the peptides studied earlier [22] by ETD-HCD and broad-band EThcD [23] and having both isomeric residues in

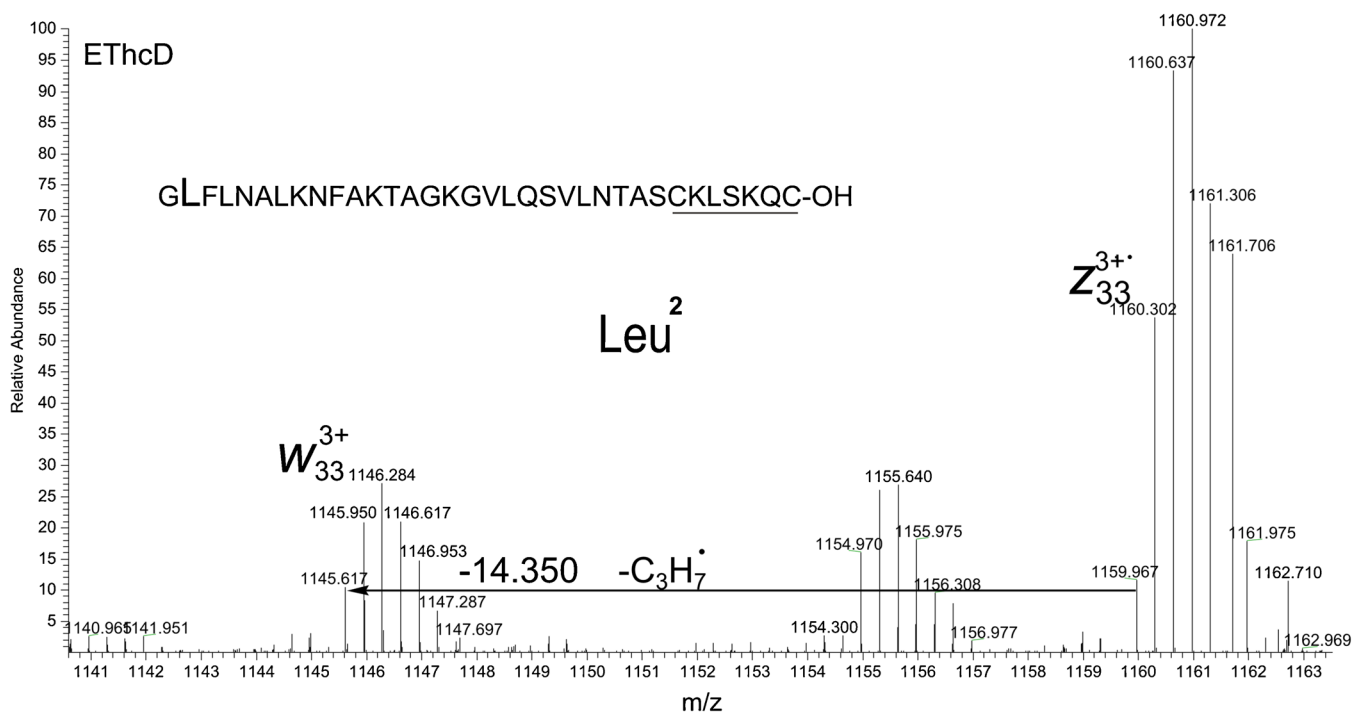


Figure 2. Discrimination of Leu<sup>2</sup> by the loss of C<sub>3</sub>H<sub>7</sub><sup>·</sup> from z<sub>33</sub><sup>3+</sup> ion of brevinin 2Rf

neighboring positions (brevinin 2Ec (Ile<sup>2</sup> Leu<sup>3</sup> Leu<sup>4</sup>), ranaturin 2Ra (Ile<sup>4</sup> Ile<sup>5</sup> Leu<sup>6</sup>), and esculentin 2R (Ile<sup>2</sup> Leu<sup>3</sup>) with EThcD approach proves that statement, while the current experiments confirmed the earlier [22, 23] conclusions.

Besides the three peptides mentioned above, brevinins 2Rg and 2Rj with Leu/Ile residues in adjacent positions were tested as well to assess the efficiency of the method. Both these peptides are minor components of the skin secretion of Slovenian population of *R. ridibunda*. They have the same structure of a disulfide induced ring and contain 4

or 5 Lys residues in their sequence. However, protonation in conditions of the experiment was not exhaustive: only charge states MH<sub>3</sub><sup>3+</sup> (brevinin 2Rg) and MH<sub>4</sub><sup>4+</sup> (brevinin 2Rj) were realized. Since fragmentation efficiency in ETD/ECD modes directly depends on the charge state of the precursor, we could not detect the full array of z-series ions, including the targeted z<sub>27</sub> ion of brevinin 2Rg G-ILXDKLKNFAKGVAQSLLNKASCALSGQC-OH. Without this it was impossible to identify the corresponding isomeric residue.

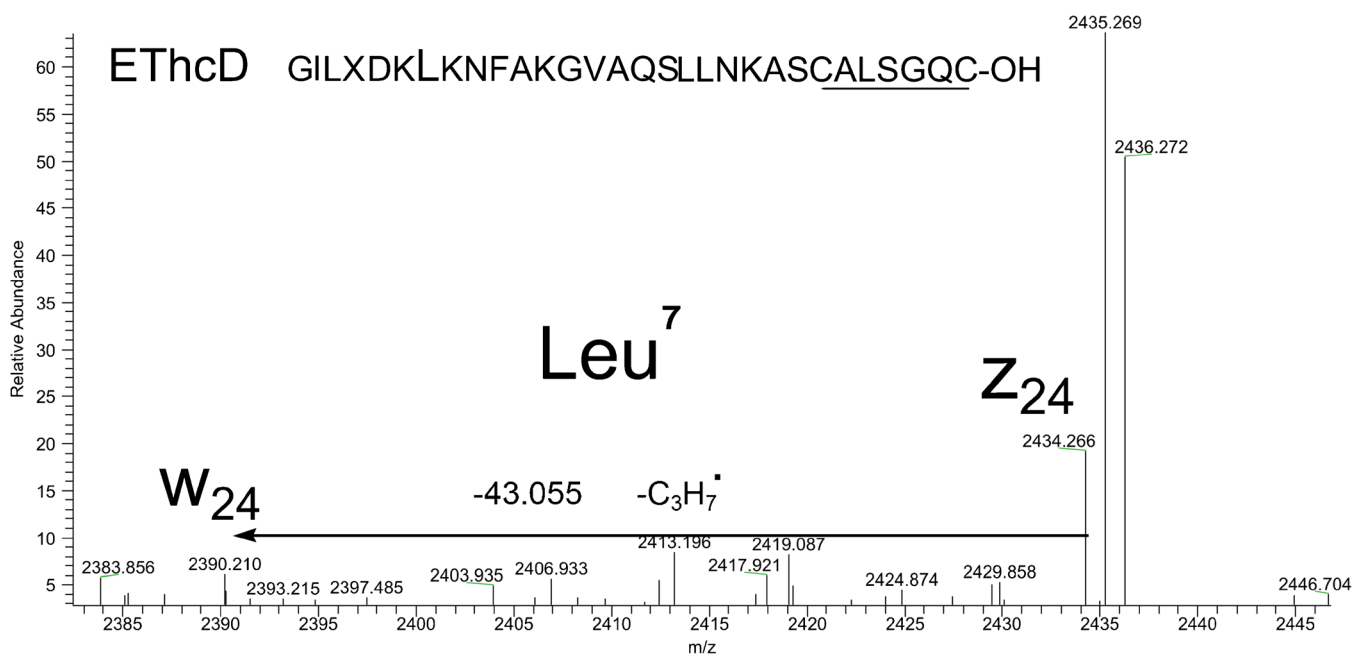


Figure 3. Discrimination of Leu<sup>7</sup> by C<sub>3</sub>H<sub>7</sub><sup>·</sup> loss from z<sub>24</sub><sup>3+</sup> ion of brevinin 2Rg

The cleavage of the polypeptides chains in ECD/ETD involves radical site reaction initiation [34, 35]. Usually amino groups of the lysine residues trigger the process. Certain peculiarity of the peptides belonging to brevinin 2 family engages the presence of numerous (6–8) lysine residues uniformly spread along the backbone. This feature leads to the formation of multiprotonated peptide molecules and correspondingly to the informative ECD/ETD spectra. The higher the charge state of the precursor ion, the better the sequence that may be obtained [36]. The efficiency of L/I discrimination is low (Table 1) for some brevinins 2 (i.e., 2Rb and 2Re) due to low content of lysine residues in their sequence. The latter issue is relevant for some other brevinins 2 (i.e., 2Rg) as well.

Experiments with  $\text{MH}_3^{3+}$  ion of brevinin 2Rg demonstrated also complete suppression of radical site migration. Scanning of NCE from 0 to 50 at the beginning brought about the increase of the targeted  $w$ -ions intensities and then to their decrease due to competitive pathways of fragmentation. Optimal spectra were recorded at NCE 20. Figure 4 illustrates the loss of  $\text{C}_2\text{H}_5\cdot$  (1493.7971) and the absence of the alternative loss of  $\text{C}_3\text{H}_7\cdot$  from the  $z_{29}^{2+}$  ion (1508.3166) of brevinin 2Rg, proving the presence of  $\text{Ile}^2$  in the sequence of that peptid

At NCE 0 the secondary fragmentation of the side chain, requiring some excessive energy, is not observed. At the same time, an increase of NCE from 20 to 50 completely stops formation of  $w_{29}^{2+}$  ions due to competitive fragmentation pathways. It should be noted that alternative loss of isopropyl radical was not observed at any NCE.

Radical site migration was also completely suppressed during HCD fragmentation of the  $z_{28}^{2+}$  ion of brevinin 2Rg (Figure 5). The intensities of the  $w_{28}^{2+}$  and  $z_{28}^{2+}$  ions peaks were quite high, while alternative loss of ethyl radical ( $u$ -ion

of  $m/z$  1437.2551,  $2^+$ ) was not observed, proving  $\text{Leu}^3$  in the sequence of brevinin 2Rg. An increase of NCE from 20 to 50 significantly decreased the competitiveness of the  $w_{28}^{2+}$  ion formation; however, even at NCE 40 both  $w_{28}^{2+}$  and  $z_{28}^{2+}$  ions peaks were still present in the spectrum. It is important that at any NCE there was no loss of ethyl radical from the  $z_{28}^{2+}$  ion. Therefore, two isomeric residues ( $\text{Ile}^2\text{Leu}^3$ ) were reliably determined in the trio of neighboring Leu/Ile residues in the sequence of brevinin 2Rg.

All three isomeric neighboring residues were successfully identified in brevinin 2Rj - GFLDKLKNFGKDVAG-**ILLKASCALSGQC**-OH. Figure 6 illustrates the loss of ethyl radical from the singly charged  $z_{14}^{+}$  ion of brevinin 2Rj.

Despite its low abundance  $m/z$  value of  $w_{14}^{+}$  ion was measured with high precision 0.0005 Da (0,3 ppm). The alternative loss of  $\text{C}_3\text{H}_7\cdot$  radical from  $z_{14}^{+}$  ion of brevinin 2Rj was not observed, and even NCE increase to 50 did not help to make it appear. Therefore, we conclude that there is likely an  $\text{Ile}^{17}$  in the sequence of brevinin 2Rj. The neighboring 18 position of the sequence occupies Leu as the corresponding  $z_{13}^{+}$  ion eliminated exclusively  $\text{C}_3\text{H}_7\cdot$  radical (Figure 7).

The accuracy of mass measurements of ions  $z_{13}^{+}$  and  $w_{13}^{+}$  was 0.0018 Da (1.3 ppm) and 0.0024 Da (1.9 ppm) correspondingly. The absence of the alternative loss of  $\text{C}_2\text{H}_5\cdot$  confirmed  $\text{Leu}^{18}$  in brevinin 2Rj sequence.

Finally the third isomeric residue in the Leu/Ile triada of brevinin 2Rj was established by the loss of  $\text{C}_3\text{H}_7\cdot$  from the  $z_{12}^{+}$  ion at NCE 30 (Figure 8). An increase of NCE from 20 to 50 did not change the character of fragmentation of the side chains, demonstrating the absence of the radical site migration. The following sequence of the doubtful residues in brevinin 2Rj was obtained:  $\text{Ile}^{17}\text{Leu}^{18}\text{Leu}^{19}$ .

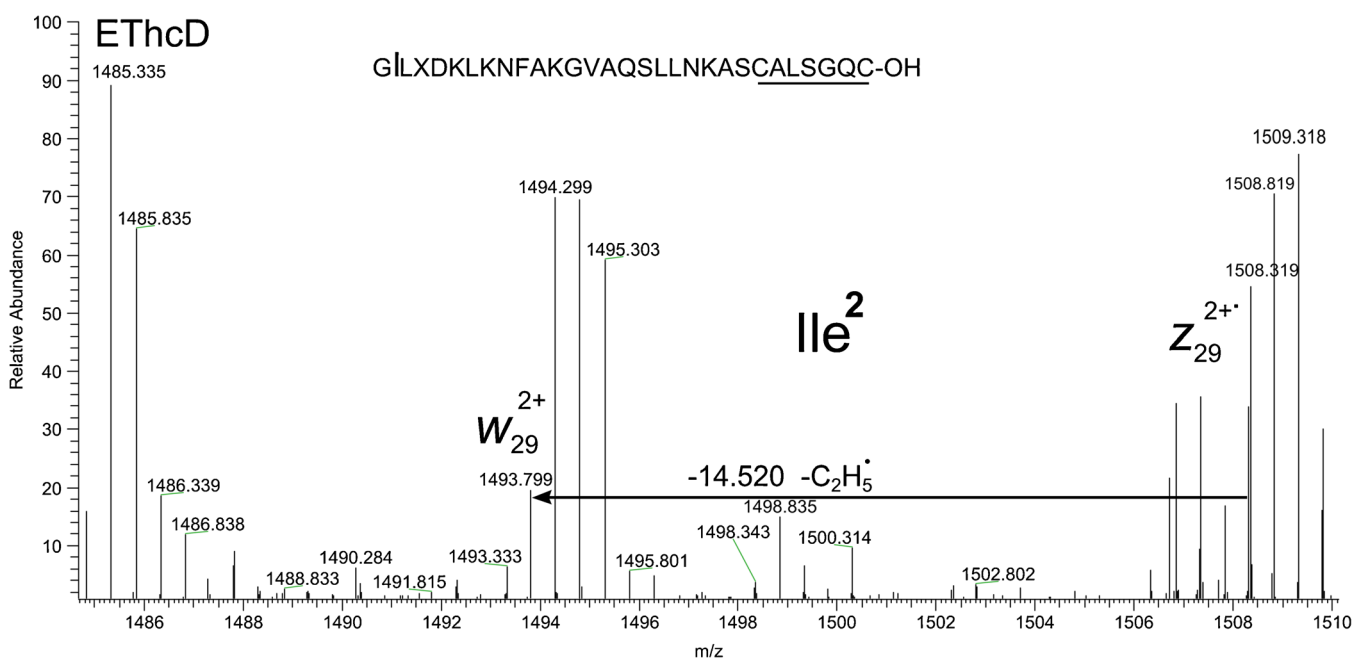


Figure 4. Identification of  $\text{Ile}^2$  by  $\text{C}_2\text{H}_5\cdot$  loss from  $z_{29}^{2+}$  of brevinin 2Rg, NCE 20

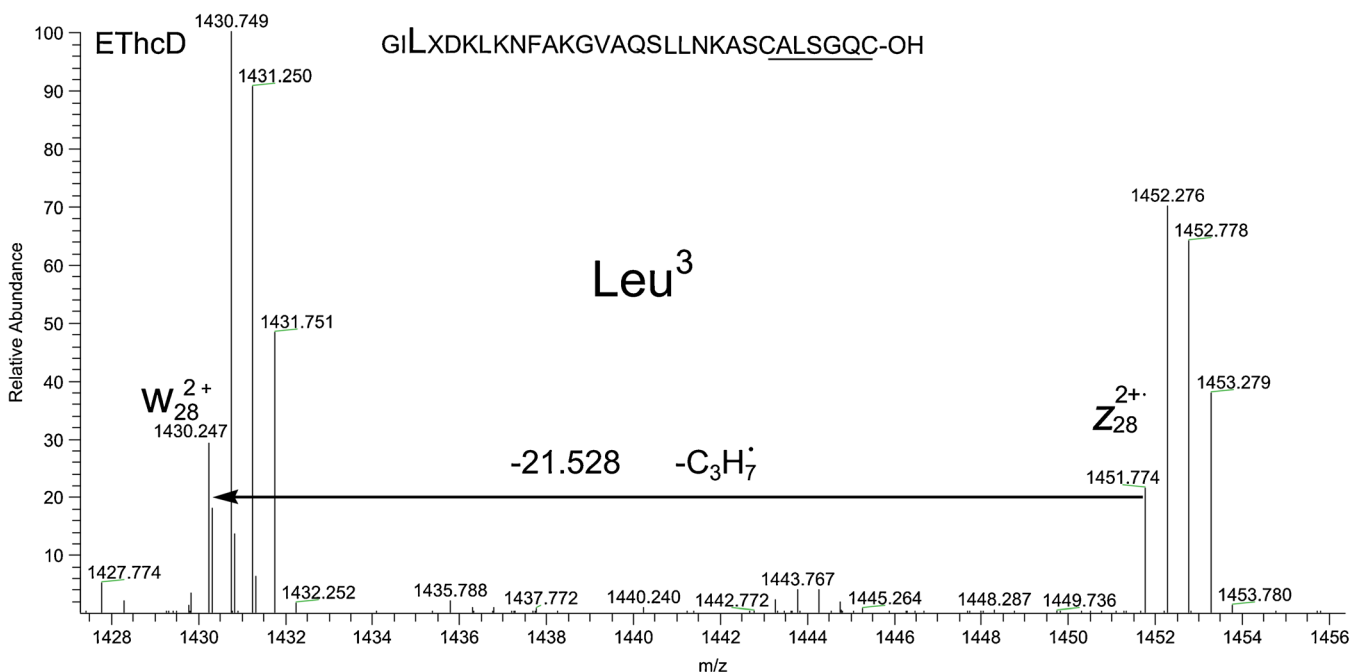


Figure 5. Identification of Leu<sup>3</sup> by the loss of C<sub>3</sub>H<sub>7</sub><sup>•</sup> from Z<sub>28</sub><sup>2+</sup> ion of brevinin 2Rg, NCE 20

### Disulfide Bonds Cleavage in EThcD Process

The structure of the C-terminal disulfide ring “Rana box” presents a marker for the belonging of a peptide to the certain peptide family. Thus brevinins 1 and esculentins 1 usually contain two basic residues in the loop (lysines, sometimes arginines), brevinins 2, ranaruerins 2, and esculentins 2 are characterized by the presence of one lysine inside the “Rana box” [37, 38]. However, this rule has many exceptions. Since electron capture/transfer is known to trigger the cleavage of intra- and intermolecular S–S bonds [39], we expected we

would be able to get information about the isomeric residues working with unmodified peptides. Therefore, the recorded spectra were thoroughly studied to discriminate Leu/Ile residues inside the S–S terminal loop.

Unfortunately the results did not match our expectations. We observed the ring opening only for three peptides with the disulphide bridge. The opening of the ring did not happen in any peptide with a single Lys in the “Rana box”. These results correlate with the accepted mechanism of S–S and N–C $\alpha$  bond cleavage in peptides in conditions of ECD/ETD [39–41]. The

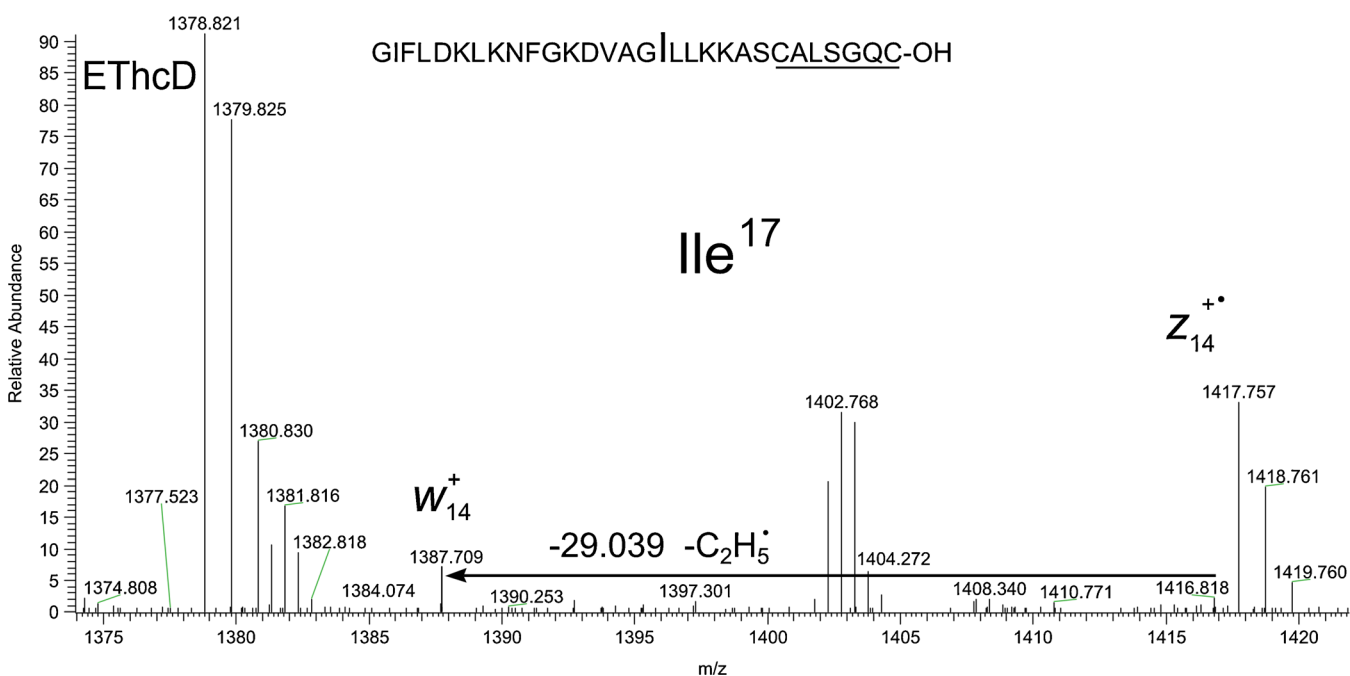


Figure 6. Identification of Ile<sup>17</sup> by the loss of C<sub>2</sub>H<sub>5</sub><sup>•</sup> from Z<sub>14</sub><sup>+</sup> ion of brevinin 2Rj, NCE 20

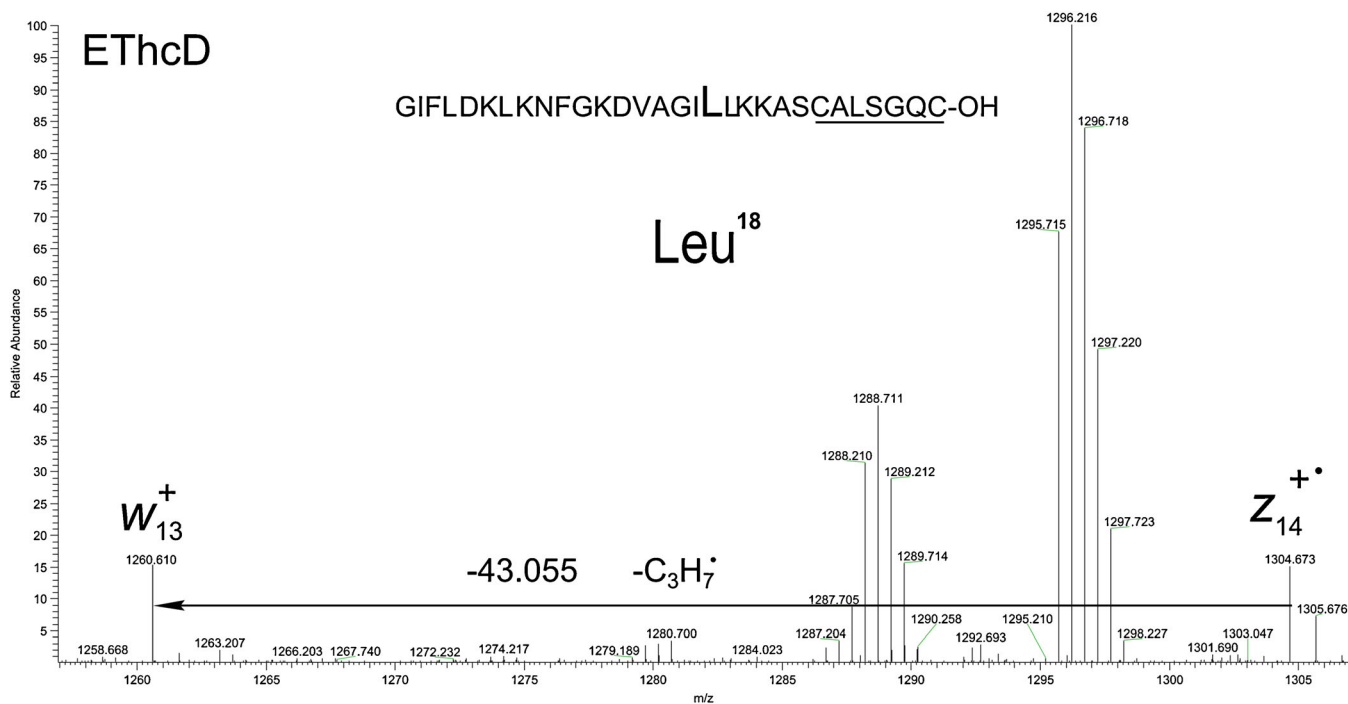


Figure 7. Identification of Leu<sup>18</sup> by the loss of C<sub>3</sub>H<sub>7</sub><sup>•</sup> from z<sub>13</sub><sup>+•</sup> ion of brevinin 2Rj

dominant portion of electrons go into a Rydberg orbital located on one of the peptides positive sites while a small portion can attach directly into SS  $\sigma^*$  or amide  $\pi^*$  orbital. The direct attachment becomes possible if those orbitals are situated in the vicinity of the positive charge localization, playing a role of Coulomb stabilizer (ca. 1 eV for SS and 2.5 eV for amide orbital) [42]. The presence of two stabilizing sites at a distance less than 41 Å from the S–S bond (i.e., side chains of Lys)

favors electron capture by an S–S bond. Electron may attach to the S–S bond directly even in the case of a single positive charge site; however, it should be located not further than 20 Å from this S–S bond. For the peptides studied, two Lys residues inside the “Rana box” in brevinin 1E, brevinin 1R, and brevinin 2Rf seems to facilitate the electron attachment directly to the S–S bond, leading to its cleavage and formation of the corresponding z-ions. However, a single Lys residue in the

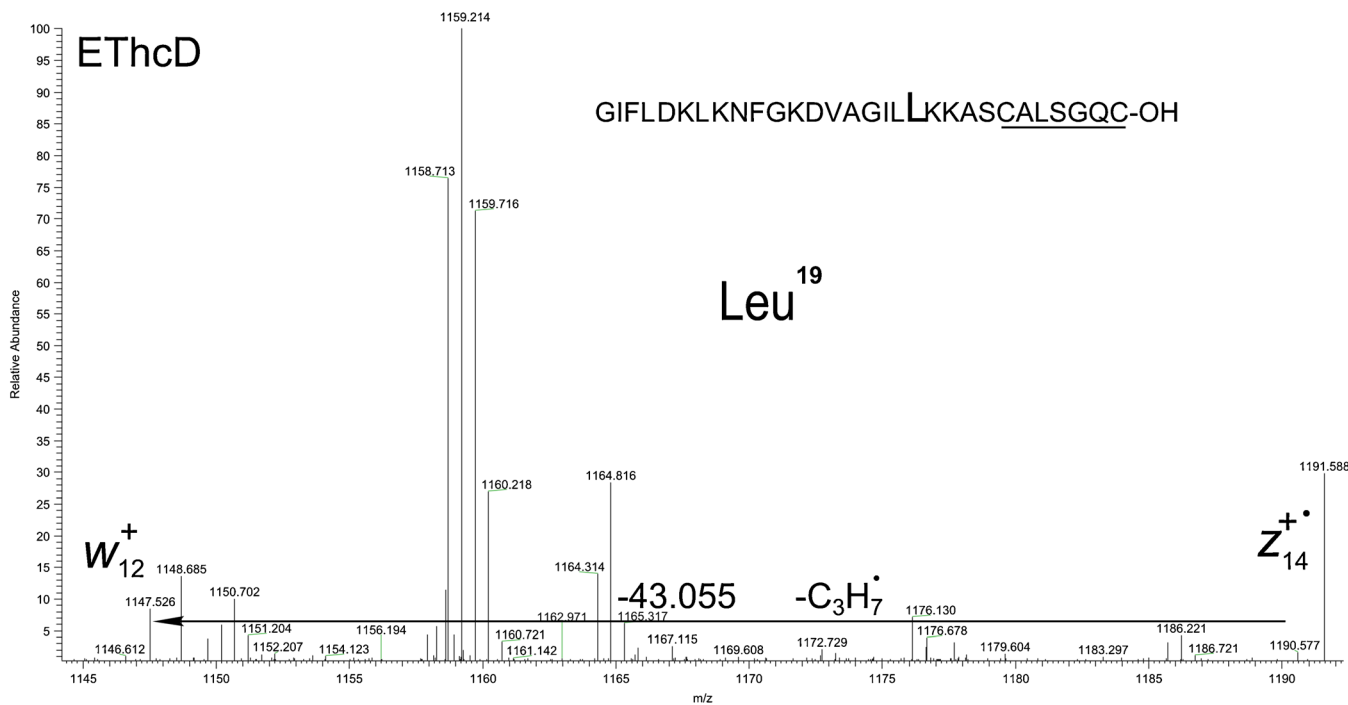


Figure 8. Discrimination of Leu<sup>19</sup> by HCD loss of C<sub>3</sub>H<sub>7</sub><sup>•</sup> from z<sub>12</sub><sup>+•</sup> of brevinin 2Rj



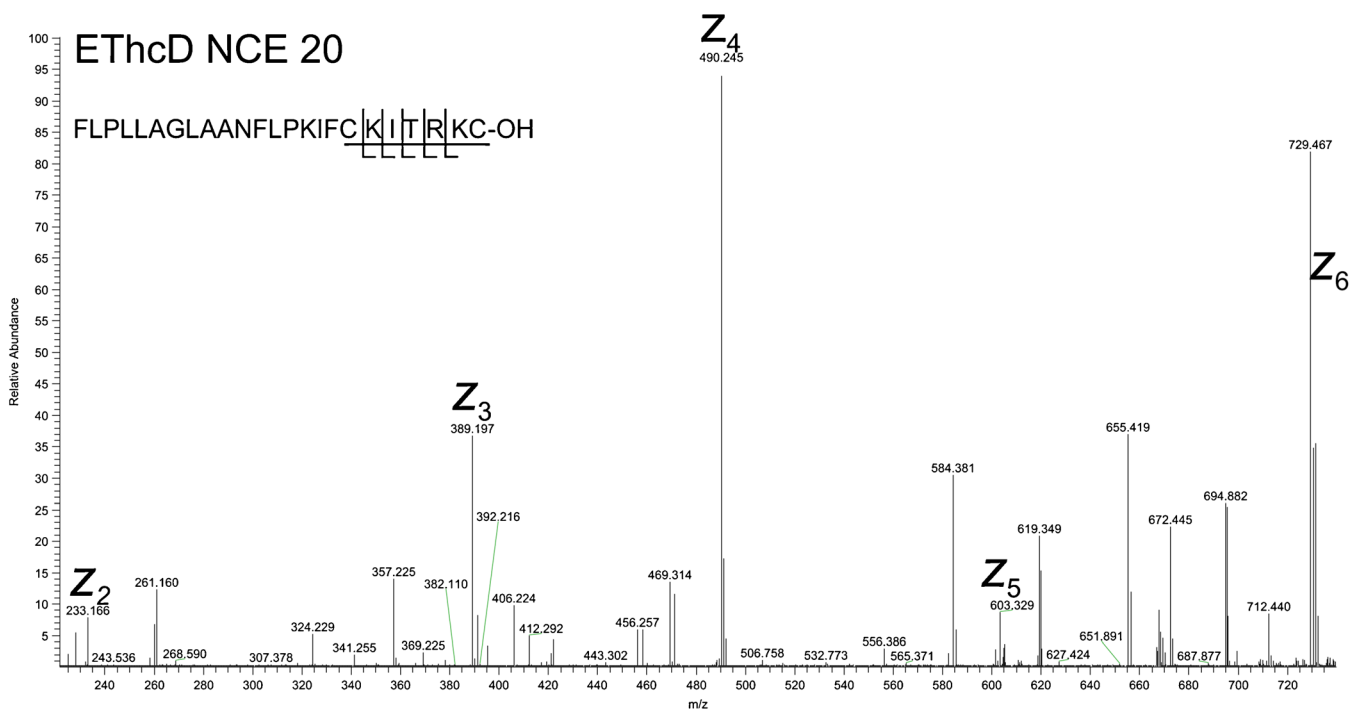


Figure 9. EThcD spectrum of  $MH_4^{4+}$  ion of intact brevinin 1E (low  $m/z$  range, NCE 20)

vicinity to the interior Cys residue in other disulfide containing peptides (listed in Table 1) apparently does not lead to the cleavage of the S–S bond. It is also worth mentioning that two arg residues inside the “Rana box” in brevinin 1Ra do not promote to cleave S–S bond either (*CAASRRC*). One explanation might be the too strong proton affinity of arginine and, therefore, the suppressed migration of the hydrogen from its original position to the S–S bond.

The analysis of EThcD spectra of the peptides listed in Table 1 showed that the cleavage of S–S bond took place during fragmentation of three peptides with two Lys in S–S terminal cycle: brevinin 1E, brevinin 1R, and brevinin 2Rf. Figure 9 illustrates fragmentation inside the cycle of the intact brevinin 1E.

There are five ions of  $z$ -series:  $z_2, z_3, z_4, z_5, z_6$ , formed due to four cleavages inside the S–S ring (Figure 9). Brevinin 1E

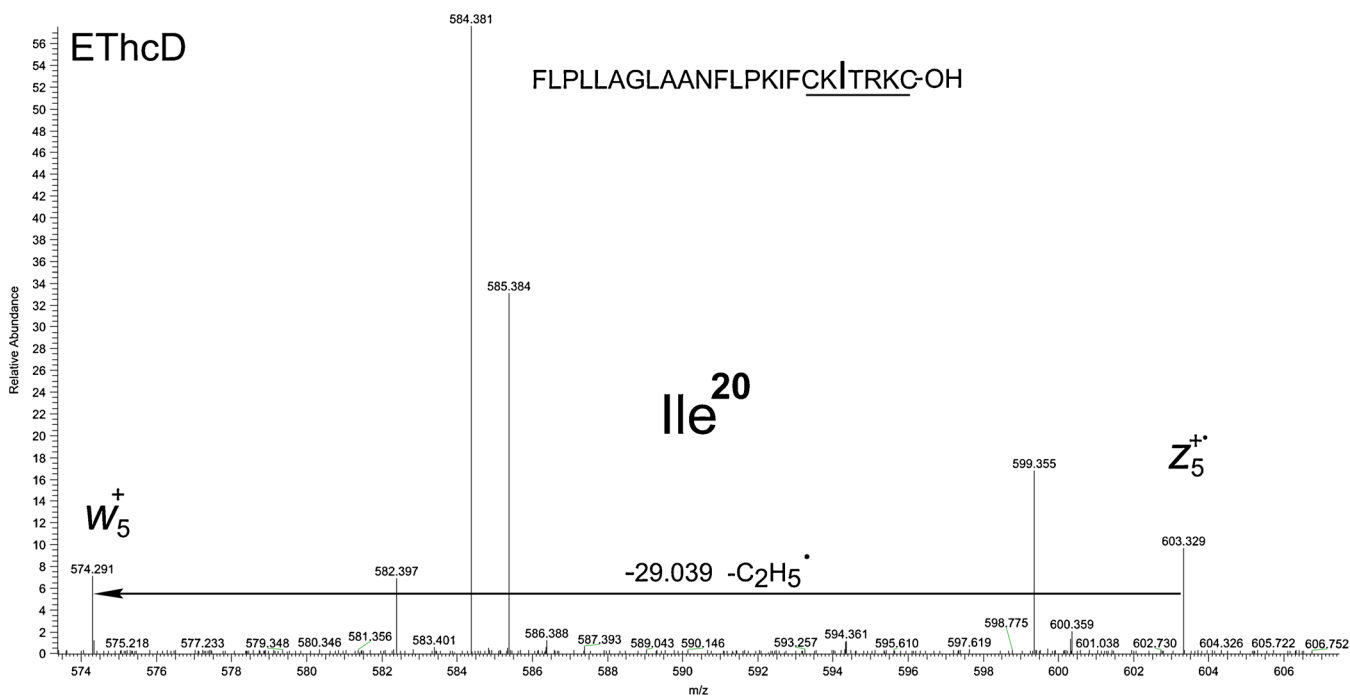


Figure 10. Identification of  $Ile^{20}$  by the loss of  $C_2H_5^\cdot$  from  $z_5^{+}$  ion of brevinin 1E

contains three Lys and one Arg in its sequence, meaning that the number of potential protonation sites equals 5. However, only one charge state ( $MH_4^{4+}$ ) was observed in the applied ESI conditions. The cleavage of the S–S bond became visible at NCE 20. At NCE 40 fragmentation of the side chain of Ile<sup>20</sup> of intact brevinin 1E was recorded (Figure 10).

Similar ring cleavage took place in EThcD conditions in  $MH_4^{4+}$  ion of brevinin 1R at NCE 20 (no Leu/Ile in “Rana box”). The spectrum contained  $z_2 - z_6$  ions due to fragmentation of the C-terminal part of the intact peptide.

Brevinin 2Rf contains Leu/Ile residue inside the “Rana box” [27]. ETD spectrum of its  $MH_6^{6+}$  ion demonstrated formation of  $z_4$  and  $z_5$  ions, while spectrum of  $MH_5^{5+}$  ion, only  $z_5$  ion. However, discrimination of Leu/Ile failed as there was no targeted  $w_5$  ion peak in the EThcD spectrum. An increase of NCE to 50 did not add to resolving the problem.

The results obtained demonstrate that for the majority of natural peptides with the disulphide ring at the C-terminus, some sort of preliminary transformation for the sequence inside the ring is required [18, 21, 27]. However it may be avoided if at least two lysine residues are present inside the ring.

## Conclusions

1. EThcD allows successful discrimination of 61 out of 75 (81.3%) isomeric Leu/Ile residues in the full sequences of 14 non-tryptic disulfide bridge containing peptides. The result is even better (60 out of 66-90.9%) if we exclude “Rana box” usually requiring derivatization. Notable complexity of EThcD spectra due to the HCD fragmentation of the entire array of primary ETD product ions does not interfere with a reliable Leu/Ile discrimination. In addition, EThcD significantly decreases time, facilitates the analytical procedure, and allows for the entire experiment and data processing to be automated.
2. Radical site migration does not represent any problem in the proposed EThcD conditions, even in the case of adjacent Leu/Ile residues, while the most efficient HCD fragmentation requires NCE in the range 5–20.
3. EThcD results obtained from unseparated mixtures of *R. ridibunda* skin secretion peptides were similar to ETD-HCD method with preliminary isolation of  $z$ -ions of purified peptides. A limitation may be due to the syringe pump introduction of the sample, as minor components may be lost due to ion suppression. Application of LC/MS should help to resolve this problem.
4. The best results are obtained when applying the method to the multiprotonated peptides in their highest charge states. Besides better ETD efficiency, the highest precursor charge allows obtaining a full array of the targeted  $z$ -ions at different charges. Their following HCD fragmentation provides complementary spectra for the reliable discrimination of the isomeric residues.

5. S–S bond cleavage in ETD mode followed by the formation of the targeted  $z$ -ions depends on the sequence inside the “Rana box” C-terminal disulfide formed intramolecular ring. The presence of basic residues inside the loop favors formation of the targeted ions.

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