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Mass spectrometric analysis of asparagine deamidation and aspartate isomerization in polypeptides

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Abstract

One of the most frequent modifications in proteins and peptides is the deamidation of asparagine, a spontaneous non-enzymatic reaction leading to a mixture of ***L,D-succinimidyl, L,D-aspartyl, and L,D-isoaspartyl forms, with L-isoaspartyl dominating. Spontaneous isomerization of L-Asp yields the same products. In vivo, these unusual forms of aspartate are repaired by the PIMT enzyme, with the balance between isomerization and repair affecting the organism physiology. Mass spectrometric analysis of this balance involves isomer separation, iso-Asp/Asp quantification and iso-Asp site identification. This review highlights the issues associated with these steps and discusses the prospects of high-throughput iso-Asp analysis.

Introduction

Proteins and peptides are susceptible, both *in vivo* as well *in vitro*, to a variety of chemical modifications that can affect their structure and biological functions. One of the most frequent modifications is the deamidation of asparagine, a spontaneous non-enzymatic reaction [1]. Usually this reaction is associated with protein degradation [2–5], but recently deamidation has been linked to triggering apoptosis in cancer cells [6–9] as well as other regulatory functions [10–13]. Under physiological conditions, deamidation proceeds through formation of a five-membered succinimide ring intermediate by the nucleophilic attack of the nitrogen atom in the following (C-terminal to Asn) peptide bond on the carbonyl group of the Asn side chain (Figure 1). A similar nucleophilic attack on the Asp side chain leads to dehydration of aspartic acid. The L-succinimidyl intermediate then undergoes a relatively rapid hydrolysis at either the α- or β-carbonyl group to generate L-isoaspartate (L-iso-Asp) and normal L-aspartate (L-Asp) in a ratio of approximately 3 : 1 [1, 14]. The formation of L-succinimide can also be accompanied by enhanced racemization at the α-carbon to generate a mixture of D-succinimidyl, D-aspartyl, and D-isoaspartyl forms. Among the products of non-enzymatic reaction, L-iso-Asp is typically the predominant form.

While *in vivo* deamidation is an irreversible process, isomerization is not. The free α-carbonyl group of L-iso-Asp can be methylated by the protein L-isoaspartyl *O*-methyltransferase (PIMT) enzyme with S-adenosylmethionine (SAM) as the methyl donor that is transformed to S-adenosylhomocysteine (SAH). Enzymatic methylation followed by spontaneous ester hydrolysis leads to reformation of the L-succinimide intermediate, which is again hydrolyzed to a mixture of L-Asp and L-iso-Asp. The overall result of this “repair” process is that a certain fraction (15%–25%) of the original L-iso-Asp population is converted back to L-Asp. D-Asp residues undergo similar enzymatic repair.

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In the cell, polypeptides with unusual forms of Asp (L-iso-Asp, D-Asp and D-iso-Asp) can be either inactive or directly damaging, and the balance between these forms and normal Asp forms is very important. In homeostasis, there is equilibrium between the spontaneous deamidation and isomerization on the one hand, and PIMT repair action on the other hand. Persistent misbalance in this equilibrium, e.g. due to PIMT inactivation, can lead to a number of pathologies [13, 15–19]. Thus there is an analytical need to measure the levels of L-iso-Asp and other unusual Asp isomers in biological samples relative to normal L-Asp isomers.

Antibodies specific against iso-Asp and D-Asp appeared only recently [20–23] and still haven't been commercialized. Global analysis of iso-Asp residues content in the protein sample is usually performed by the ISOQUANT detection kit (Promega, Madison, WI) as follows. The protein of interest or protein mixture is incubated with PIMT and S-adenosyl-L-methionine. The formation of the methylation byproduct of the repair process, S-adenosyl homocysteine, is then detected by reversed phase HPLC and its quantity estimated by comparing the chromatographic peak area with a standard curve [24–26].

ISOQUANT and similar global approaches require at least picomolar quantities of sample and cannot identify proteins where this process occurs, not to mention the position of deamidation/isomerization in the protein. Therefore, there is a growing interest in the use of mass spectrometry, which is a much more sensitive technique (femtomoles to attomoles) that produces site- and molecule-specific information.

The task of isomer analysis by mass spectrometry can be divided into isomer separation, iso-Asp/Asp quantification and iso-Asp site identification. The general procedure is shown in Figure 2. Crude protein mixture is a usual starting point of the analysis, which can be followed by separation on a SDS-PAGE or 2D gel. The band or spot density in the stained gel serves as a semi-quantitative measure of the relative abundances of different isoforms. Deamidation reduces the protein pI value (pKα of Asp side chain is 3.86 while pKα of Asn side chain is not well known; being close to that of acetomide it is probably slightly negative) and therefore the deamidated form shifts the protein position along the pH gradient on a 2D gel, but in practice this method of deamidation detection is rarely used. Electrophoresis-separated proteins, or starting materials can then undergo additional separation by reverse-phase HPLC, with UV absorption representing the quantification aspect. The separated isoforms (alternatively, unseparated protein mixture) are then subjected to enzymatic digestion, typically with trypsin. The tryptic mixture is either directly analyzed with MALDI TOF mass spectrometry, or additionally separated by reverse-phase HPLC and ionized by on-line electrospray ionization (ESI). In the "top-down" approach, mixtures of protein isoforms are directly ionized by ESI and undergo tandem mass spectrometry (MS/MS), which is used for sequence identification and localization of modification position.

Mass spectrometric identification of deamidated peptides is relatively straightforward, as deamidation adds to the mass of intact molecule +0.984 Da (the mass difference between –OH and $–NH_2$ groups). Since deamidation is a modification stable in the gas phase, CAD MS/MS spectra can reveal the position of deamidation even in the presence of several potential deamidation sites.

Unlike Asn deamidation, Asp isomerization analysis presents a significant challenge for mass spectrometry, as isomerization is the subtlest posttranslational modification (PTM) of amino acids in polypeptides [27]. Isomerization doesn't change the elemental composition by definition and thus it is "silent" in the mass spectrometric sense. Fortunately, structural changes induced by isomerization usually change the retention time of the peptide in

reversed-phase liquid chromatography, which thus provides isomer separation. The task of mass spectrometry is then reduced to differentiation between different isomers, which still remains a significant challenge.

In this review we discuss the use of mass spectrometry for detection and relative quantification of deamidation and Asp isomerization, especially in high-throughput proteomics analysis.

MS analysis of Asn deamidation

In general, the half-times of asparaginyl deamidation and aspartyl isomerization under physiological conditions (pH 7.4, 37 °C) vary between about 1 and 1000 days [28]. L-Asn residues form L-succinimides about 10 times more rapidly than comparable L-Asp residues [29]. A similar series of reactions occur in glutamine and glutamate residues, generating L- and D-iso-glutamate and glutamate residues, but these reactions are much slower than those of asparagines and aspartic acid residues [30, 31].

In short peptides, formation of cyclic intermediates is controlled by primary sequence, while in proteins secondary, tertiary, and quaternary structures have an additional effect. Thus the deamidation rate of each protein amide is uniquely determined. On average, the deamidation rate is affected ≈60% by primary structure and ≈40% by higher order structures in those proteins in which it has been carefully characterized [32] and about 50% primary and 50% secondary and tertiary in the 17,935 proteins for which three-dimensional structures are available (Table 1).

Deamidation introduces a negative charge into a protein, which can change protein function or make it more (or less) susceptible to degradation. In most instances in which asparagine deamidation has been identified *in vivo*, it is involved in pathology. However, since the change of even a single neighboring amino acid can have a marked effect on the rate of deamidation of an asparagine, the underlying rate of deamidation of any asparagine is genetically programmable. Thus asparagines are believed to serve as molecular timers that regulate protein function and stability [28, 32, 33].

Deamidation can be recognized by B and T lymphocytes and can potentially make “self”-proteins appear foreign to the immune system. Using the tetanus toxin C fragment (TTCF) protein as a test case, Moss et al. [34] showed that spontaneous deamidation of asparagine residues interferes with processing by the enzyme asparagine endopeptidase (AEP) and contributes to diminished antigen presentation. It was shown that deamidation can inhibit AEP action both directly, when asparagine residues targeted by AEP are modified, and indirectly, when adjacent Asn residues are deamidated. Thus it was concluded that deamidation of long-lived self-proteins may contribute to the onset or exacerbation of autoimmune disease. The locations of AEP deamidation sites have been determined as follows. First, the presence of iso-Asp residues in the isolated TTCF protein was established using the ISOQUANT kit. For MS identification of the deamidation position in the deamidated sample TTCF37 compared to the control TTCF4 (Figure 3), the protein was digested with trypsin and tryptic peptides were separated by reversed phase HPLC. HPLC fractions were analyzed individually by MALDI MS. Peptides 1214–1223 (DGNAFNNLDR) and 1179–1191 (YTPNNEIDSFVK) showed positive molecular mass shifts in their isotopic distributions compared to the control (insets in Figure 3). These peptides were sequenced by tandem MS (TOF/TOF) to determine the sites of deamidation, Asn residues 1183 and 1219.

In some cases, such as in therapies involving monoclonal antibodies (mAbs), the modification position is less important than the abundance of any modified isoform relative

to unmodified molecule. Like other proteins, therapeutic mAbs undergo various reactions affecting their structural integrity and stability. Among the degradation reactions, deamidation and isoaspartate formation are major sources of concern. Zhang and Czupryn reported the detection and quantification of iso-Asp in a recombinant mAb [3]. The intact molecule and its charge isoforms were resolved by cation exchange HPLC (Figure 4). With the ISOQUANT assay, the mAb was found to contain an average 0.2 mol of iso-Asp per mole of protein, with the most acidic isoforms containing 0.7 mol and without iso-Asp in the most basic isoform. The authors concluded that the majority of iso-Asp in the mAb is formed as a result of asparagine deamidation in the process of protein storage, but did not identify the positions of modification.

This latter task was addressed by Chelius et al. who identified and characterized deamidation sites in the conserved region of a recombinant monoclonal human IgG antibody artificially aged by storage in solution at pH 7.5 and 37 °C [31]. The molecule was first digested by trypsin, and peptides as well as their isoforms, including the succinimide, isoaspartic, and aspartic acid isoforms, were separated by reversed-phase HPLC (Figure 5). Each of the isoforms was unambiguously identified using tandem mass spectrometry coupled with electrospray ionization (Figure 6). It should be noted that the employed MS/MS based on collision-activated dissociation could only distinguish succinimide, Asn and isoforms of Asp, but could not differentiate between Asp isoforms (see below). The Asp isoforms were assigned based on the known order of peptide elution at given HPLC conditions (iso-Asp, L-Asp, D-Asp and succinimide). Deamidation at the identified sites was found slow for the intact, folded antibody, and accelerated for partially unfolded molecules at degradation conditions. Deamidation was also enhanced after reduction, alkylation, and tryptic digestion, i.e. typical sample preparation procedures used in proteomics, which sent a warning signal for straightforward attempts to quantify deamidation at proteome level using standard approaches.

Recently, the same group suggested using a short, modified digestion protocol that does not induce measureable deamidation *in vivo* [35]. Alternatively, *in vitro* and *in vivo* occurring deamidation processes can be distinguished by using $H_2{}^{18}O$ instead of ordinary water for protein digestion and sample storage [36]. Deamidation that occur during protein digestion and storage will then give peptides 2 Da heavier than those undergoing *in vivo* deamidation.

Protein digestion and associated *in vitro* deamidation can be avoided altogether if “top-down” approach is applied, involving tandem mass spectrometry of individual protein molecules having the same or similar mass. Because of technical and other limitations, top-down approach works best for molecules below 20–25 kDa. Zabrouskov et al. applied this approach to reduced bovine ribonuclease A (13 689 Da) [37]. Despite extensive studies on this molecule, only Asn67 deamidation has been positive identified by previous research. Top-down tandem MS on a high-resolution mass spectrometer showed that Asn67 deamidation is followed by conversion of Asn71 and Asn94. When these residues are more than half deamidated, Asn34 reacts, after which deamidation of Gln74 is initiated. Up to the date, the top-down approach remains relatively low-throughput and is limited in terms of protein molecular mass and sample amount.

MS analysis of Asp isomerization

Iso-Asp forms most easily in sequences where the side chain of the C-flanking amino acid is relatively small and hydrophilic, and is less likely to be formed where bulky or hydrophobic residues are in this position. The most favorable for Asp-isomerization C-flanking amino acids are Gly, Ser, and His [38].

To analyze the isoaspartate content in proteins in PIMT knock-out (KO) mice that exhibit brain enlargement and fatal epileptic seizures, Zhu et al. [39] undertook a global analysis of endogenous substrates for PIMT in mouse brain. Extracts from PIMT-KO mice were subjected to two-dimensional gel electrophoresis and blotted onto membranes. Isoaspartyl proteins were radiolabeled on-blot using [methyl-^{3}H]S-adenosyl-L-methionine and recombinant PIMT (a variant of the ISOQUANT assay). Fluorography of the blot revealed 30–35 ^{3}H-labeled proteins, 22 of which were identified by MALDI MS peptide fingerprinting (Figure 8). The following five proteins, all of which are rich in neurons, accumulated exceptional levels of isoaspartate: collapsin response mediator protein 2 (CRMP2/ULIP2/DRP-2), dynamin 1, synapsin I, synapsin II, and tubulin. But even in this study, the sites of the Asp isomerization remained unidentified.

Identification of the iso-Asp sites by mass spectrometry requires MS/MS method specific to iso-Asp. Lehmann et al. [40] have noticed that replacement of L-Asp by L-iso-Asp resulted in the shifts in b/y intensity ratio of complementary b and y ions generated by cleavages N- and C-terminal to the iso-Asp, with the Asp immonium ion abundance at m/z 88 also decreased. However, these authors conceded that the b/y ion intensity ratio and the immonium ion intensity vary considerably depending on the peptide sequence and instrumental settings. Thus the abundance changes are difficult to use in practice for detection of iso-Asp presence.

As a more reliable criterion, Cournoyer et al. have found iso-Asp specific fragments in electron capture dissociation (ECD [41]) of synthetic peptides [42]. Besides the conventional in ECD N-$C_{\underline{\alpha}}$ bond cleavage leading to complementary c and z fragments, these authors detected a diagnostic cleavage giving $c_n\bullet$ + 58.0054 ($C_2H_2O_2$) and z_{l-n} − 56.9976 (C_2HO_2), where *n* is the position of the isoaspartyl residue and *l* is the peptide length. These diagnostic fragments are usually less abundant than the adjacent conventional c_n and z_{l-n} species. The same fragments are observed with electron transfer dissociation (ETD [43]), the technique that is similar to ECD but that employs radical anions instead of free electrons. Andreazza et al. have recently reported that collisional dissociation of negative peptide ions does not provide Asp/iso-Asp differentiation [44]. In our laboratory, we have found that higher-energy collisional dissociation (HCD [45]) of both positive as well as negative ions also fails in this task. This means that ECD/ETD are the only MS/MS technologies applied to electrospray-produced ions that provide reliable diagnostic ions for iso-Asp detection. It should be noted that MALDI TOF/TOF that utilizes higher collision energies than HCD has not been evaluated in this study.

However, in high-throughput proteomics experiment the presence of a peak at a certain mass is not specific enough for reliable iso-Asp identification because of the noise present in MS/MS spectra as well as the probability of accidental match by unrelated ionic species. Our recent study of 32 proteomics datasets acquired using ECD provided 466 iso-Asp candidates, of which only 219 could be validated using at least one more additional criterion [46]. Based on the presence of adjacent c/z fragments - 160 candidates were validated, the chromatographic peak shape (two or more peaks corresponding to isomeric components) - 113 candidates, accurate mass of the diagnostic fragment (<5 ppm) - 113 cases, presence of the same diagnostic peak in more than one ECD mass spectrum of the same peptide (or its modified version) - 89 cases, presence of the isotopic distribution in the iso-Asp fragment signal - 43 cases, absence of specific for Asp loss (−60.02 Da) from the reduced molecular species – 25 cases, CO_2 loss from the adjacent c/z fragments (“soft” criterion) - 25 cases, presence of complementary iso-Asp-specific fragment - 5 candidates.

As an example, top panel in Figure 9 shows the extracted ion chromatogram for isomerized peptide L(D->isoD)LAGR from the full proteome of human cell culture [46]. According to

CAD MS/MS data, the two chromatographic peaks are due to identical sequences LDLAGR. In the ECD spectrum of the first-eluting minor component (bottom panel, upper spectrum), an iso-Asp specific fragment z_5 - 57 was found. In the spectrum of the Asp-variant (lower spectrum), the loss of ($\bullet C_3H_7 + C_2H_4O_2$) from the reduced species (m/z 542.305) was attributed to a combined loss from the adjacent Leu and Asp residues. Such a combined loss is similar to the combined radical + molecule loss, e.g. the specific for Leu ($\bullet C_3H_7 + NH_3$) loss. Since $C_2H_4O_2$ originates from the full Asp side chain, the combined ($\bullet C_3H_7 + C_2H_4O_2$) loss cannot occur in the case of iso-Asp. Consistent with that, the iso-Asp ECD spectrum does not contain this loss (the peak at m/z 541.309 is not related). The ratio of the areas of chromatographic peaks on the upper panel of Figure 9 provides the occupancy of the isomerized site, e.g. the relative degree of Asp isomerization.

The above approach to iso-Asp analysis provides high throughput in respect to peptide candidate detection, but requires time-consuming validation of the detected iso-Asp candidates. It is however possible to formalize the validation criteria and automate the whole iso-Asp analysis. Another limitation of the current approach is the relatively low speed and sensitivity of ECD/ETD when high-resolution mass analyzers are used. We are currently investigating the analytical utility of ETD MS/MS with detection in high-sensitivity, high-speed linear ion trap analyzer combined with high-resolution HCD MS/MS of the same peptide. This combined approach lacks in the accurate mass specificity of the iso-Asp specific ETD fragments, but on the other hand it provides significantly enhanced throughput, which is advantageous for a large-scale iso-Asp detection experiment.

Summary and Outlook

The important role isomerized residues start to play in today's biological research requires high-sensitivity, high-specificity methods of their detection and quantification in complex protein mixtures. Mass spectrometric challenges include artifact-free sample preparation, efficient on-line separation of isoforms and specific MS/MS identification of isomerized residues. Not surprisingly, Asn deamidation and Asp isomerization, due to their ubiquitous nature, drew most attention of the research community. Taking into account recent developments, one can postulate that the goal of analyzing the cell "isomerome" in a high throughput manner, with identification of the modified sites and determination of their relative occupancies, is fully realistic. We project this goal to be reached within a few years. Among the problems remaining to be solved is the reliable automatic analysis of the shape, position and composition of the peaks in extracted ion chromatograms combined with the detailed analysis of MS/MS spectra, especially those produced by ECD/ETD.

Acknowledgments

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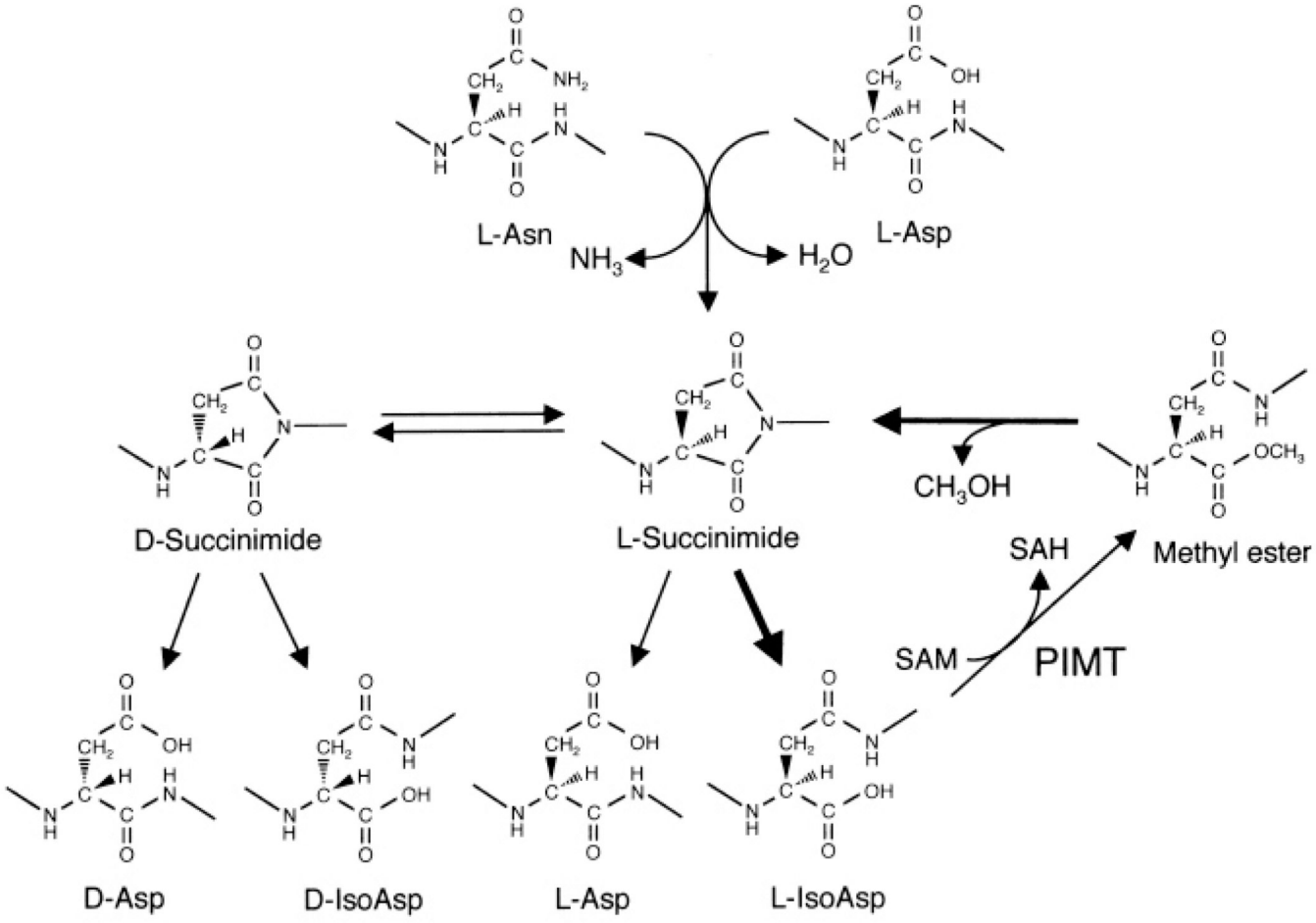

Figure 1.
Spontaneous deamidation of aspargine (L-Asn), isomerization of aspartic acid (L-Asp) residues and their repair by the PIMT enzyme using S-adenosylmethionine (SAM) as the methyl donor. L-Succinimide reversely racemizes to D-succinimide, which leads to a variety of isomerized products. The nonenzymatic reactions denoted by the thick arrows are faster than those denoted by the thin arrows. SAH represents S-adenosylhomocysteine.

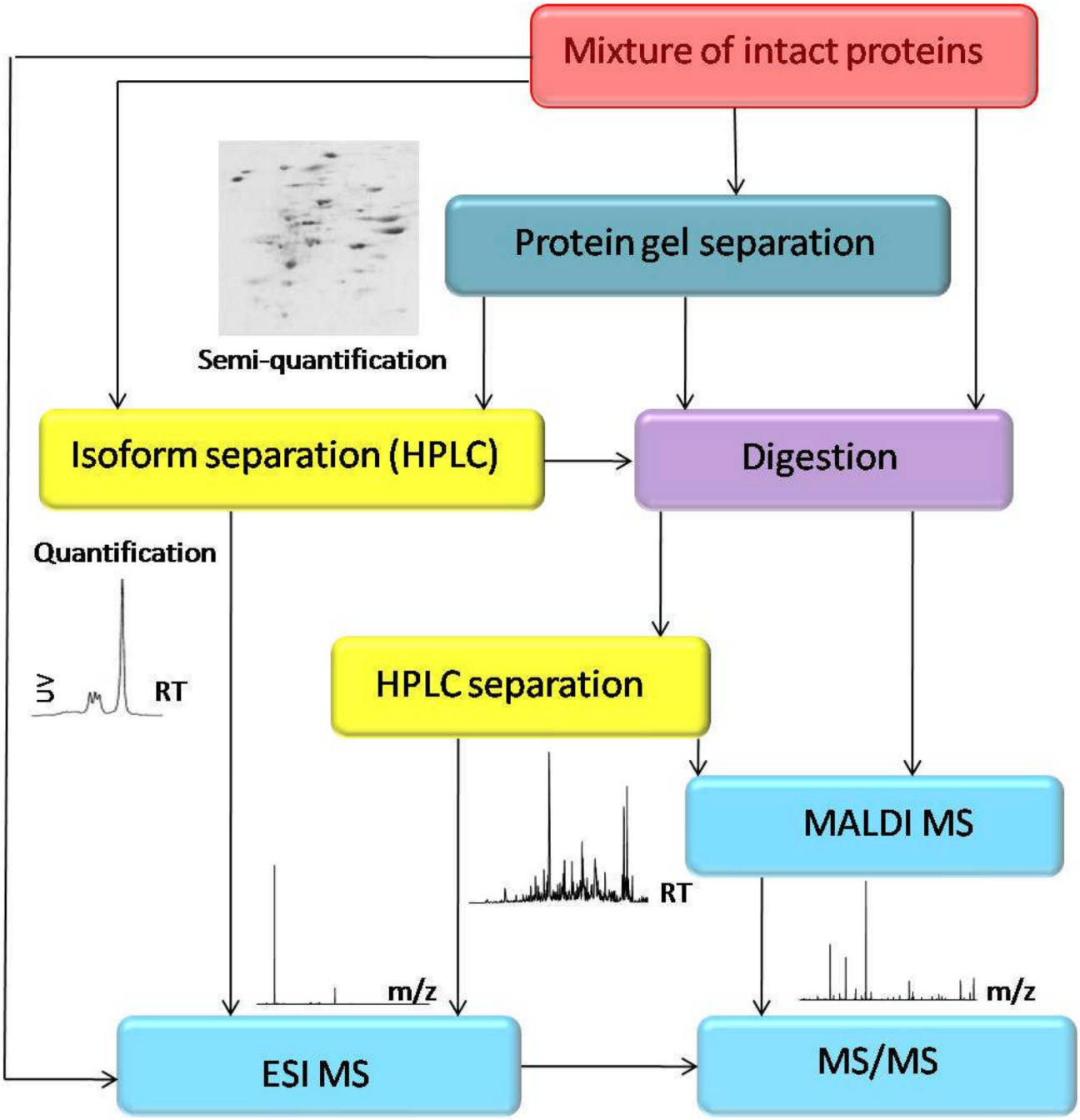

Figure 2.
Generic workflow of mass spectrometric identification and quantification of isomerized residues.

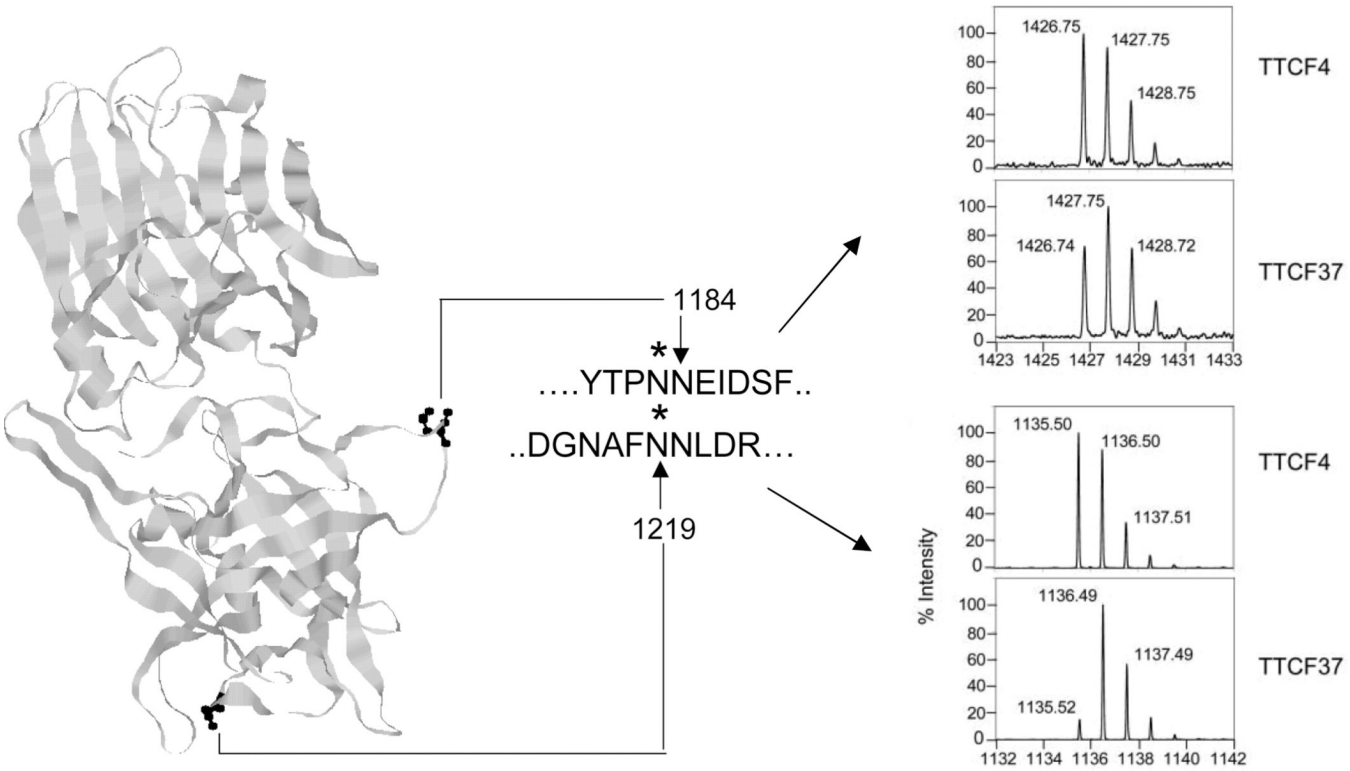

Figure 3.

Identification of deamidated Asn residues in the tetanus toxin C fragment (TTCF) protein. Deamidation position in the sample TTCF37 compared to the control TTCF4 was performed by MALDI MS upon protein digestion with trypsin and tryptic peptides separation by reversed phase HPLC. The peptides 1214–1223 (DGNAFNNLDR) and 1179–1191 (YTPNNEIDSFVK) that showed positive molecular mass shift compared to the control were sequenced by tandem MS analysis to determine the sites of deamidation, Asn residues 1219 and 1183 [34].

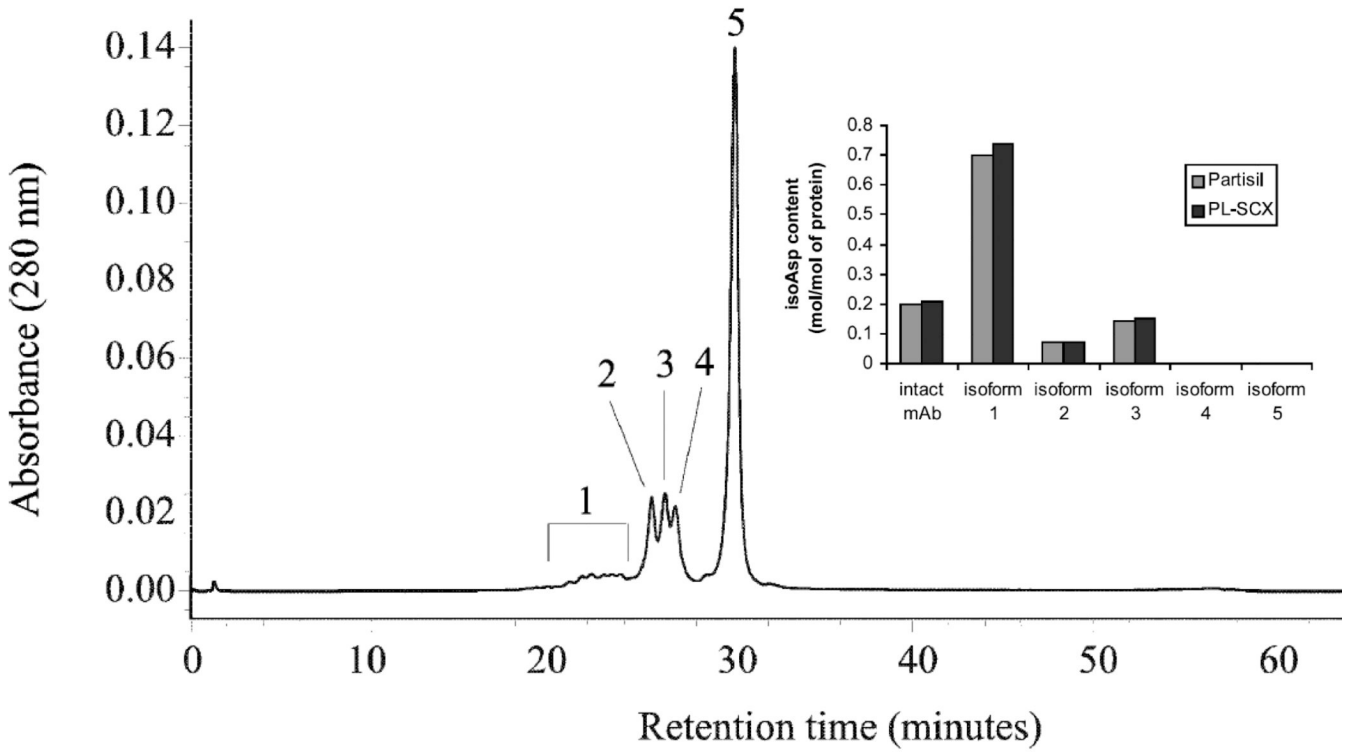

Figure 4.
CEX-HPLC profile of the mAb. Inset – iso-Asp content of the unfractionated mAb and its charge isoforms [3].

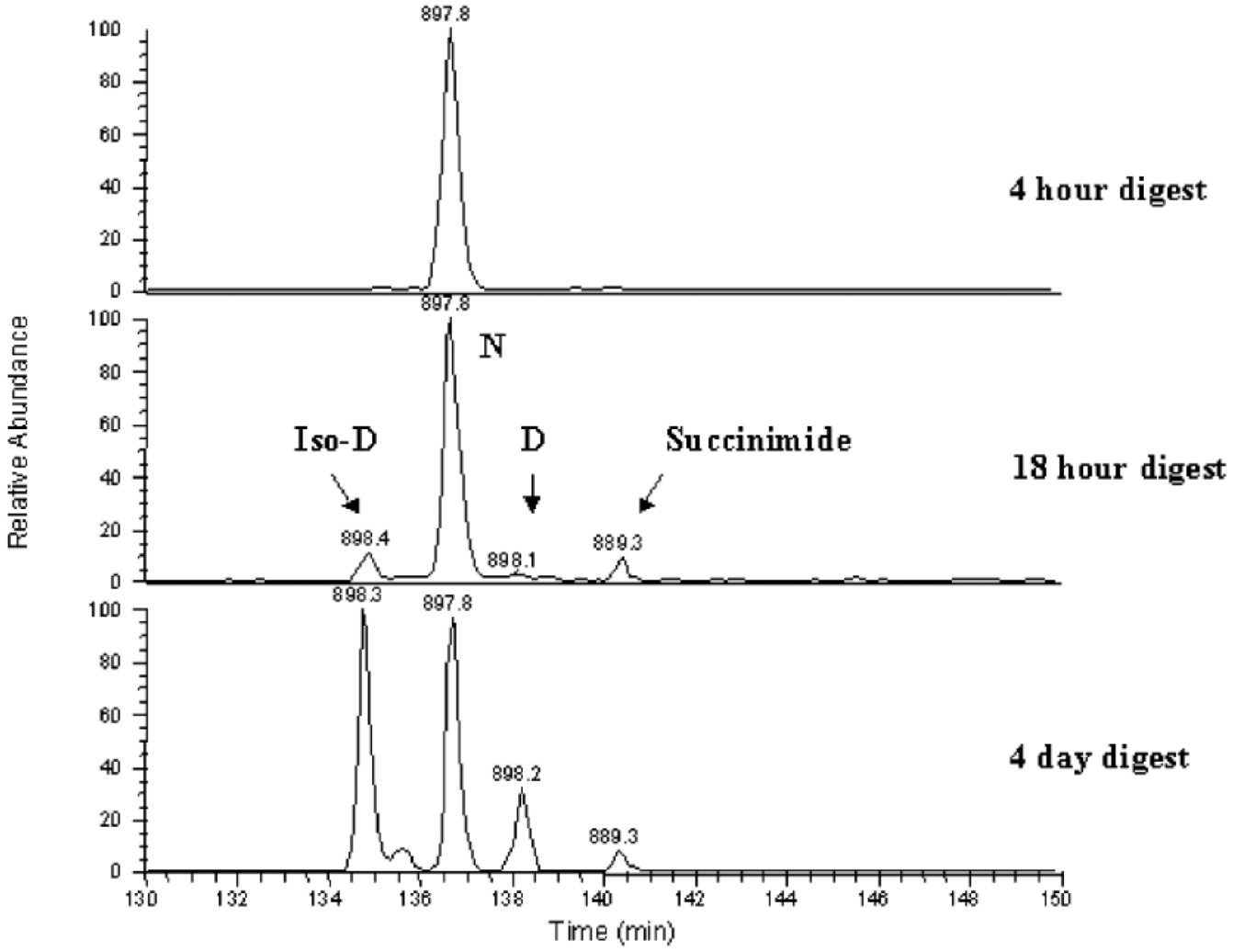

Figure 5.
Base peak ion chromatograms of $(M + 2H)^{2+}$ precursor ions of the four different isoforms of the peptide VVSVLTVVHQDWLNGK incubated at pH 7.5 and 37 °C in 100 mM Tris buffer for different periods of time: normal (N), succinimide (Su), iso-Asp (iso-D) and L-Asp (D) [31].

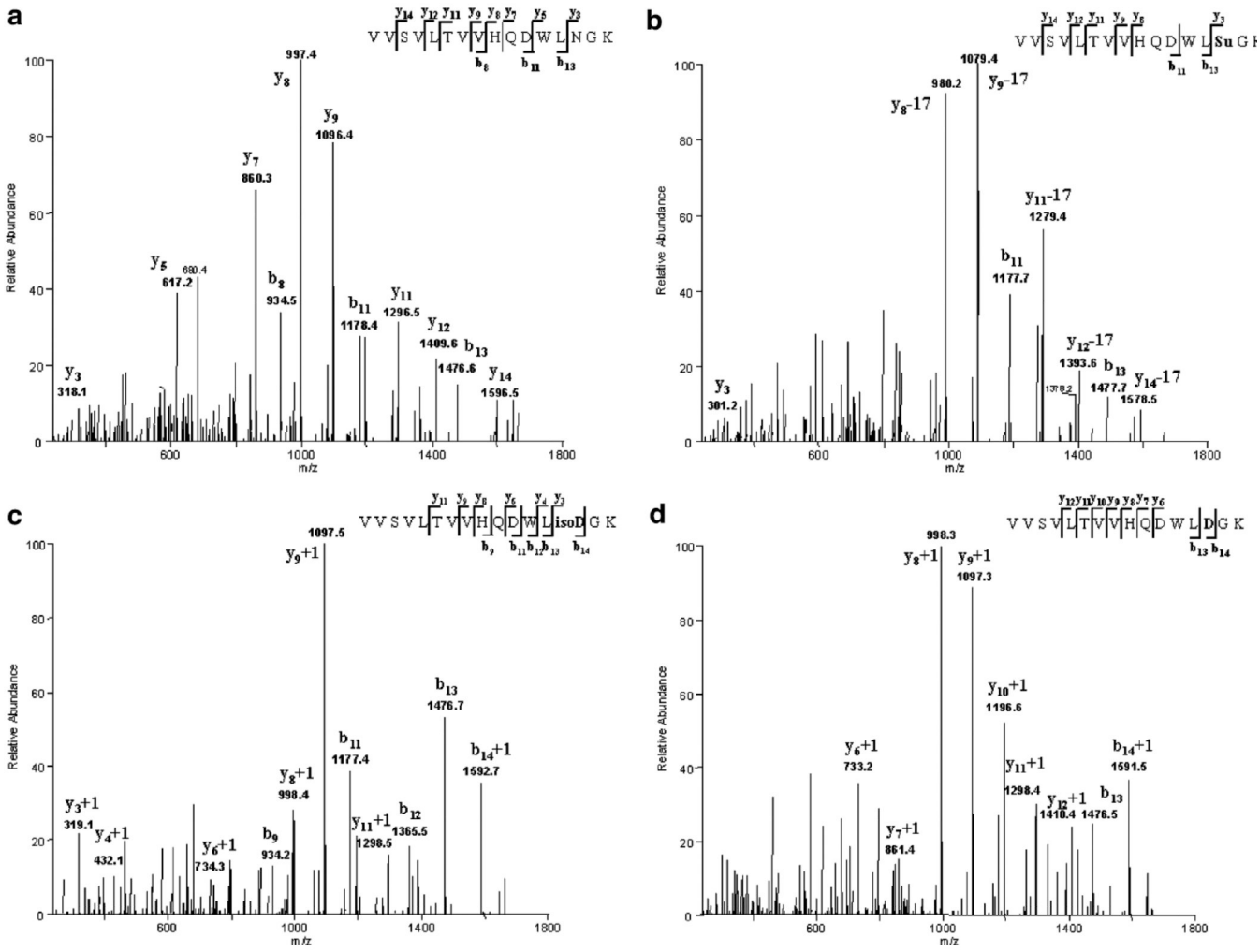

Figure 6.

Tandem mass spectra derived by collision-induced dissociation (CID) of the $(M + 2H)^{2+}$ precursor ions of the peptide VVSVLTVVHQDWLNGK (a) and its three isoforms: succinimide (b), iso-Asp (c), and Asp (d) [31]. The two last isoforms were assigns based on the HPLC retention times (Figure 5), as the CID MS/MS spectra of these molecules are very similar.

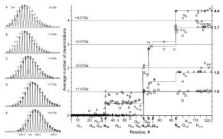

Figure 7.
ESI spectra of 13+ molecular ions of RNase: (A) untreated and (B–E) after deamidation that caused a mass increase of 1.0, 1.8, 3.7, and 4.4 Da, respectively. (A) Best fits of the theoretical abundance distribution corresponding to the protein deamidated at n and n + 1 sites, respectively. (B–E) Best fits of the indicated fractional number of deamidations [37].

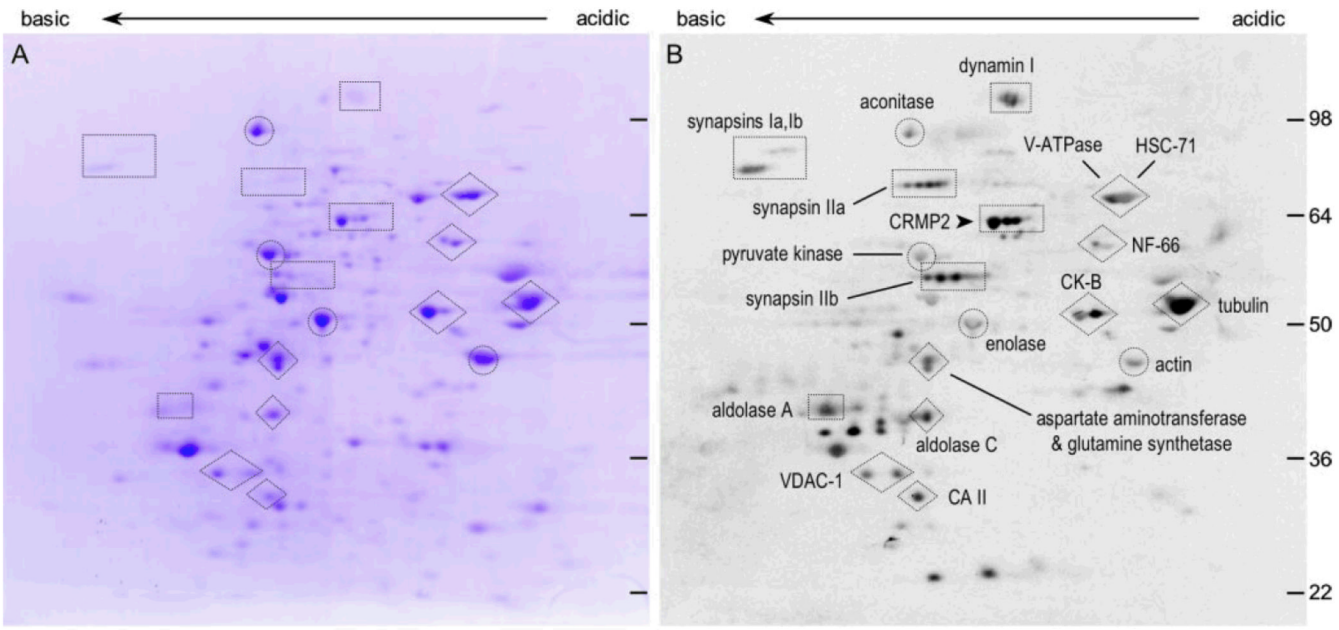

Figure 8.

A comparison of protein staining and 3H-methylation patterns indicates a wide range of isoaspartate content in proteins from the PIMT knock-out mouse. A - Coomassie Blue stain of a PVDF membrane after ^{3}H-methylation and fluorography. B - ^{3}H fluorogram of the same membrane prior to staining. Protein spots that were subsequently identified by peptide-mass fingerprinting are marked. Rectangles mark proteins that appear to have a relatively high level of isoaspartate per unit protein; diamonds and circles mark proteins with intermediate and low levels of isoaspartate, respectively [39].

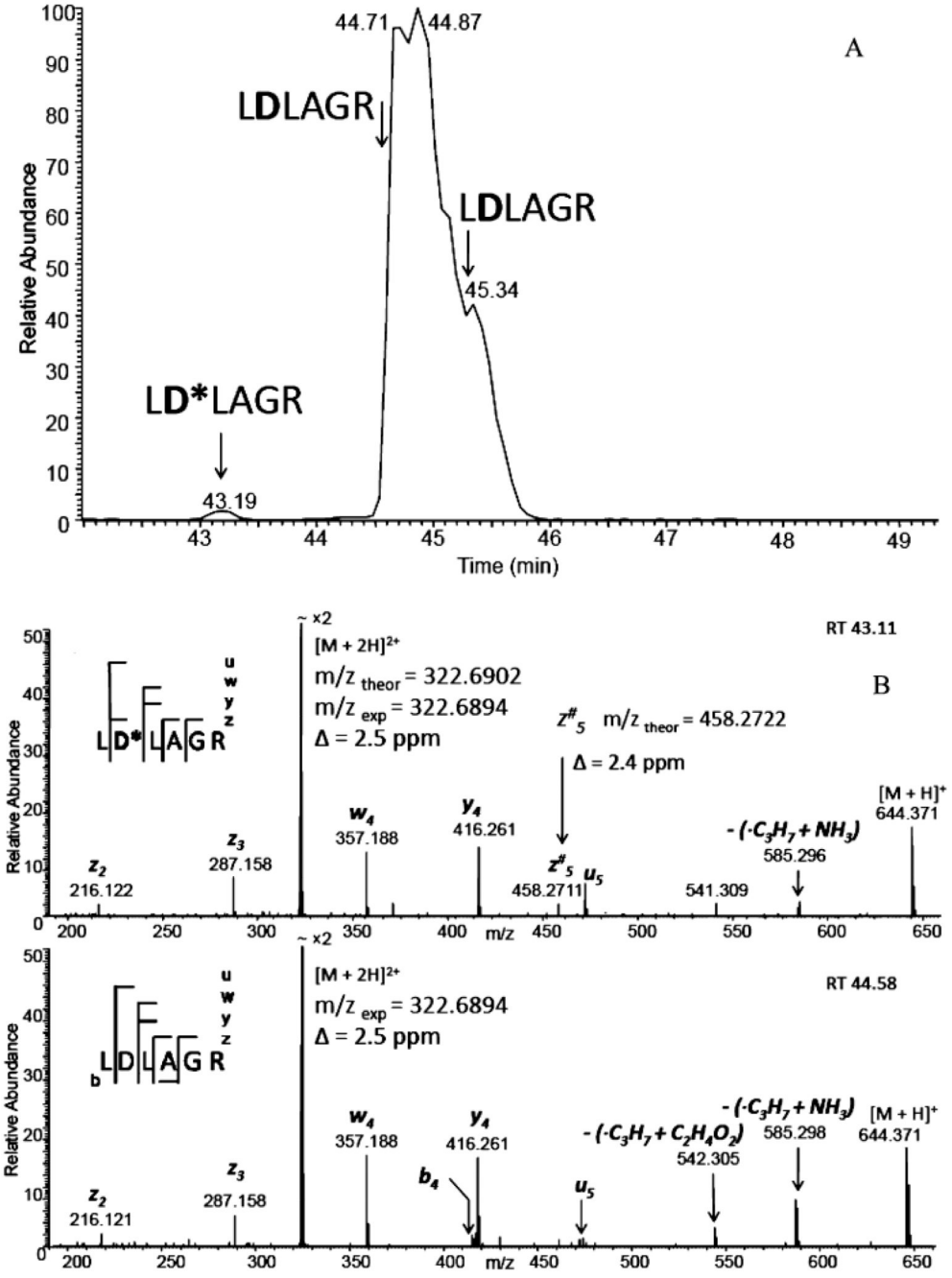

Figure 9.

Top panel: extracted ion chromatogram for isomerized peptide L(D->isoD)LAGR. Bottom panel: ECD MS/MS spectra for LisoDLAGR (upper), and spectra for LDLAGR (lower). *D indicates the presence of iso-Asp [46].

Table 1

Deamidation percentages in 0.15 M Tris–HCl, pH 7.4, 37 °C, for 17,935 peptides and proteins [32].

	Days	%
Deamidated by ≥one-half of an amide	1	0.3
	5	3.7
	10	7.5
Deamidated by ≥one-tenth of an amide	1	4.7
	5	26.6
	10	42.8
Deamidated by ≥one-half of an amide at 2× rate	1	1.6
	5	7.5
	10	17.2
Deamidated by ≥one-tenth of an amide at 2× rate	1	10.5
	5	42.8
	10	57.3