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## **Introducing protein deamidation: landmark discoveries, societal outreach, and tentative priming workflow to address deamidation**

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### **ABSTRACT**

Our current knowledge on protein deamidation results from a journey that started almost 100 years ago, when a handful of researchers first described the non-enzymatic “desamidation” of glutamine, and the effect of different anions on the catalytic rate of the reaction. Since then, the field has tremendously expanded and now finds outreach in very diverse areas. In light of all the recent articles published in these areas, it seemed timely to propose an integrated review on the subject, including a short historical overview of the landmark discoveries in the field, highlighting the current global positioning of protein deamidation in biology and non-biology fields, and concluding with a workflow for those asking if a protein can deamidate, and identify the residues involved. This review is essentially intended to provide newcomers in the field with an overview of how deamidation has penetrated our society and what tools are currently at hand to identify and quantify protein deamidation.

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## 25 INTRODUCTION

### 1- General description of deamidation

Deamidation is a peculiar post-translational modification (PTM) because it has the specificity to be either enzymatically catalyzed (in bacteria or viruses as reviewed in [1]) or chemically catalyzed in any proteinaceous sample, even outside cells. In the latter case, deamidation falls in the category of  
30 spontaneous and irreversible PTMs, just like protein oxidation or carbonylation for example.

Deamidation occurs in peptides and proteins, and removes the amino group on the lateral chain of the neutral polar residues asparagine (Asn, N) and glutamine (Gln, Q). It is a very frequently observed modification in proteins. Indeed, with an occurrence of 4.3% for Asn and 4.2% for Gln in eukaryotic proteins (source: the Biological Magnetic Resonance Data Bank), and if an average length of 400  
35 residues is assumed in the ~10,000 different proteins estimated in a typical eukaryotic cell, one may calculate ~400,000 potential deamidation sites [1] vs ~640,000 for phosphorylation [2]. Of note, not all Asn or Gln residues are equally eligible to deamidation, as essentially two criteria must be fulfilled: (1) Internal criteria relate to the structural environment of the Asn or Gln residues: the probability to deamidate is higher when they are located in a poorly structured environment and when the n+1  
40 residue is small. (2) External criteria relate to the physical and chemical properties of the solution containing the peptide/protein: deamidation is accelerated by high temperature, low or high pH; also the catalytic rate depends on the ionic strength and the type of buffer ions [3].

Asn and Gln residues may lose the amino group on their lateral chain by two mechanisms (see paragraph 2): either through direct hydrolysis (acid- or base-catalyzed), or through the formation of a  
45 cyclic intermediate (Figure 1) which is a five-membered ring called succinimide for Asn or a six-membered ring called glutarimide for Gln. Cyclic imide formation is the limiting step of the reaction. Once formed, this intermediate is unstable and hydrolysis rapidly opens the ring at two potential sites, thus producing  $\alpha$ -linked L-Asp or  $\beta$ -linked L-IsoAsp (or L-Glu and L-IsoGlu). Of note, the cyclic imide formation is thermodynamically far less favorable for Gln than for Asn, accounting for the fact that Gln  
50 deamidation is slower, and thus less frequently observed than that of Asn : deamidation rates measured in pentapeptides (pH = 7.4, 37.0°C, 150 mM Tris-HCl) range between 500 – 17,900 days for Gln residues, while those for Asn range between 1 – 500 days [4]. For each deamidation reaction, one -NH<sub>2</sub> group is replaced by an -OH group, thus increasing the mass of the deamidated product by

+0.984 Da, and conferring an additional negative charge at physiological pH. These differences are so small that detecting deamidation might prove technically challenging, as will be explained below.

From the biological standpoint, deamidation essentially intersects with the concept of protein stability. Converting Asn and Gln into Asp/IsoAsp and Glu/IsoGlu not only alters the composition, but also the structure of proteins compared to their time of synthesis. This is frequently referred to as “protein degradation” in the literature. Of note, deamidated proteins containing isomerized Asp or Glu are potentially even more “degraded” because their structure is more altered: the rearrangement leading to  $\beta$ -linked (IsoAsp) or  $\gamma$ -linked (IsoGlu) residues lengthens the backbone of the protein by one or two carbon atoms respectively ( $C\beta$  alone or  $C\beta + C\gamma$  are incorporated in the peptide chain) (Figure 1A). The generic term of “protein degradation” thus encompasses the possible consequences entailed by deamidation in biology : the half-life of the protein may be shortened due to increased susceptibility to proteolysis (e.g. crystallins [5], triosephosphate isomerase [6] or the adrenocorticotrophic hormone - ACTH), the subcellular localization may be modified, protein-protein interactions may be lost, activity may be impaired (e.g. cytochrome c [7]), the supramolecular arrangement of subunits might be challenged (e.g. triosephosphate isomerase [8,9]), or deamidation products may tend to aggregate (e.g. crystallins [10]). All in all, deamidation is mostly considered to impart a loss of biological function, although gain of functions are also reported [11–13]. Importantly, neosynthesis is the only way to retrieve the native protein because there is no repair mechanism to restore Asn or Gln from Asp/IsoAsp or Glu/IsoGlu residues. An immediate implication is that deamidation essentially applies to proteins with a slow turnover (*e.i.* long-lived proteins), and since deamidation unrelentingly proceeds with time, deamidated proteins resolutely build up with age. Phrasing deamidation as a “time-related degradation of proteins” led to “the molecular clock” concept first proposed in the 1970’s by Robinson [14]. This concept enunciates that deamidation rates are encoded in the structure of proteins, and that evolution has conserved these naturally unstable residues to serve as molecular timer of biological events. This is how Robinson first proposed that controlled deamidation of glutaminy and asparaginy residues is one of the mechanisms implemented at the molecular scale to orchestrate organisms development and aging [15].

## 2- Landmark discoveries in protein deamidation

85 This section is not intended to provide an exhaustive review of about 100 years of research on protein deamidation, but its aim is to give newcomers in the field an overview of important discoveries (Figure 2), and to redirect them toward excellent reviews already available on specific points.

It is important to stress that most of the information regarding protein deamidation was initially  
90 brought by the use of short model peptides. They proved a remarkably powerful tool to run systematic comparisons on sequence permutations; fewer studies were carried out on whole proteins, and even today, case by case analysis of deamidation of specific proteins remains of high interest to confront the knowledge gained with peptide studies. Nevertheless, protein deamidation may essentially be broken down into 4 main points that have been addressed throughout the last century.

95 1- What are the parameters controlling the catalytic rate of deamidation (with respect to the internal and external criteria previously listed)?

2- What is (are) the reaction mechanism(s)?

3- The serendipitous intersection of deamidation with the methylating enzyme PIMT.

4- The development of tools to predict, detect and quantify peptide/protein deamidation.

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### 2-a) Parameters modifying deamidation rates

The first experiments carried out on asparagine and glutamine deamidation are recounted by Robinson & Rudd in their 1974 article [16]. They indicate that the first works began around 1935, where the degradation rates of glutamine alone or included as the carboxy-terminal residue in  
105 dipeptides or tripeptides were studied, and appeared very early on to be temperature, pH, and ionic strength dependent. Between 1970 and the turn of the century, the Robinson lab have dedicated a significant part of their work to the systematic characterization of Asn and Gln deamidation rates (mainly on peptides) (Figure 2). Owing to the decisive input provided by their comprehensive studies [3], it is certainly fair to acknowledge their contribution to the foundation of the field, even if many  
110 other contributions were also evidently essential to reach the current understanding of deamidation.

Between 1970 and 1990, how internal and external factors determine the deamidation rates of Asn and Gln in peptides was addressed. Internal factors include the primary sequence and the local structure surrounding the deamidable residue, which may be worded also as “structural flexibility” or

“solvent accessibility”. The external factors describe the physico-chemical traits of the medium (pH, temperature, ion buffer, ionic strength – noted *I*). It is however difficult to break down individual sections for each point, as internal and external parameters have often been modified along in many studies.

Regarding the effect of external factors, in 1971, Mc Kerrow & Robinson measured the deamidation rates of Asn in 2 peptides exposed to different pH adjusted either with borate, carbonate, phosphate or Tris [17]. In the 1990’s, several other studies were carried out : in 1991 Tyler-Cross & Schirch used pentapeptides VSNXV exposed to different ions (LiCl, NaCl) and different pH (adjusted with phosphate, NH<sub>4</sub>OH, glycine or triethylamine) [18]. Capasso et al. also provided several reports to describe the dependence of Asn deamidation on salts, buffers and organic solvents [19,20]. In some studies, the sequence of peptides were random [17,21,22], while in others, they recapitulated sequences found in known proteins like cytochrome c [14], histone IV [23], rabbit muscle aldolase [24] or ACTH [25]. At the same time, the question was also addressed in whole proteins in a few studies [6,26,27]. All in all, these reports highlighted that deamidation rates are the lowest between pH=4 to 6, but significantly accelerate outside of this range [3,16,25]; deamidation rate is also higher at *I*= 1.4 than at *I*=0.2 [3,16], and at 37°C compared to 4°C; finally, phosphate and NH<sub>4</sub>OH are often better catalysts than other ions for deamidation.

Regarding the control of deamidation by internal factors, in 1973, the deamidation half-times of Asn or Gln were measured in 42 pentapeptides while keeping the external parameters constant (phosphate pH=7.4, 37°C, *I*=0.2) [21]. This study was followed in 1974 by another one collating data from different projects in the Robinson lab and presenting the deamidation rates of Asn and Gln in 64 peptides, measured in phosphate pH=7.4, 37°C, 0.15<*I*<0.2. All these data provided solid ground to show that deamidation was favored when the n+1 residue was small, but the study was brought to completion much later, with the use of mass spectrometry to determine the deamidation half-times for 306 synthetic GXNYG asparaginylyl peptides at pH=7.4, 37.0°C, 0.15 M Tris-HCl buffer [28]; deamidation “hot spots” or “canonical motifs” could thus be identified in sequences, where NG > NS > NN > NT > NH. But it was also clear that deamidation rates were controlled by higher order structure than just the primary sequence. The Clarke lab established that a key parameter was the distance between the nucleophilic NH group of the n+1 residue and the C $\gamma$  of the Asn side chain (called the C $\gamma$ -N distance) [29]. This parameter was related to the angle for rotation around the C $\alpha$ -C bond ( $\psi$  angle)

and angle for rotation around the C $\alpha$ -C $\beta$  bond ( $\chi_1$  angle). The ideal peptide geometry for succinimide  
145 formation was proposed to be obtained when C $\gamma$ -N=1,89 Å with  $\psi=-120^\circ$  and  $\chi_1=120^\circ$ , a configuration  
rarely allowed in proteins because of the constraints imparted by higher order structures.

## 2-b) Reaction mechanism(s) of Asn and Gln deamidation

150 The reaction mechanism of Asn and Gln deamidation has long been assumed to proceed through  
direct hydrolysis of the lateral -NH $_2$  group [30] thus exclusively producing Asp and Glu. But  
chromatography analyses played a critical role in revealing that the reaction actually generated  
intermediates (cyclic imides) and additional terminal products (IsoAsp and IsoGlu). This finding led to  
unraveling the mechanism in the mid 1980's. Figure 1C recapitulates the pathways for Asn  
155 deamidation at acidic pH (left part) or at physiological pH and temperature (right part), and the  
ensuing Asp isomerization and racemization reactions. Gln deamidation proceeds through the same  
scheme, although with slower kinetics [31]. The existence of the intramolecular rearrangement  
leading to the cyclic succinimide intermediate was hypothesized as of 1970 [32] (Figure 2), but its  
experimental proof was only brought in 1987, with reverse phase chromatography applied to  
160 deamidated ACTH-related peptides to separate all the products [29]. The possibility to open the ring  
on either side of the nitrogen atom justified how L-Asp and L-IsoAsp were formed, with a favored  
production of IsoAsp as shown by the 1:3 ratio consistently found [19,29,33]. L-conformed but also D-  
conformed products are generated (Figure 1C), and this racemization essentially occurs on the cyclic  
intermediate, converting L-succinimide into D-succinimide; hydrolysis of the latter produces D-Asp and  
165 D-IsoAsp [29].

The pathway depicted in Figure 1C highlights how Asn deamidation, and Asp isomerization and  
racemization are interconnected. Deamidation, isomerization and racemization are three prevalent  
protein degradation mechanisms at physiological pH and temperature, but it is important to note that  
they have slow kinetics. Also, while deamidation is irreversible, isomerization and racemization may  
170 proceed toward equilibrium. The dynamics of these reactions were compared using synthetic peptides  
and computer-assisted kinetic modeling [29,34]. Over 6 days of reaction in 0,1M phosphate (pH=7.4)  
at 37°C, deamidation of Asn-containing peptides was calculated to produce L-IsoAsp (70,2%) and L-Asp  
(19,1%) (consistent with experimental data) and low amounts of D-Asp (1,2%) and D-IsoAsp (3,7%). By

175 comparison, Asp-containing peptides in the same conditions were calculated to produce only 6,6% of degradation products. These data were obtained with model peptides, and proteins are expected to display even slower kinetics due to superior structural constraints. The time scale of these degradation reactions remains none the less relevant to modify long-lived proteins, and specific examples like glucagon or calmodulin show that Asp degrade in these proteins faster than Asn [29,34].

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### **2-c) Protein deamidation intersects with research on PIMT**

The fact that aging mammalian proteins accumulate abnormal (“non natural”) residues has been known since the 1960’s, with D-aspartyl residues found in old eye lens, and L-isoaspartyl residues documented in urine for example. But it was unclear whether the latter  $\beta$ -linked residues could be 185 incorporated during protein synthesis, or were degradation products of hydrolytic enzymes [35] or deamidation [16].

The Aswad lab and the Clarke lab are the two groups who provided experimental evidence that isoaspartyl residues are produced by deamidation [36,37], and who elucidated the mechanisms of racemization of L-aspartyl into D-aspartyl residues [29,34]. They further demonstrated that D-Asp 190 [38,39] and L-IsoAsp [36,37] are substrates of PIMT, an enzyme characterized in 1965 for its methylating activity [40]. Why a subset of proteins was “methyl-marked” by PIMT remained elusive until Aswad and Clarke identified that PIMT accepts damaged proteins as substrates, and therefore acts as a repair enzyme. As indicated in Figure 1B, L-isoaspartyl/D-aspartyl methyl transferase (PIMT: EC.2.1.1.77), also known as protein carboxymethyl transferase (PCMT), transfers the methyl group 195 from S-adenosyl-L-methionine (SAM) to the  $\alpha$ -carbonyl group of IsoAsp or the  $\beta$ -carbonyl group of D-Asp. The methylester converts into the succinimidyl intermediate, the hydrolysis of which generates again L-Asp or L-IsoAsp. Each cycle thus repairs a fraction of L-IsoAsp (and D-Asp).

A comprehensive review on PIMT proteins was published in 2006 by O’Connor [41], which collates 200 historical, phylogenic, biochemical and physiological data on this family of proteins distributed throughout all phylogenetic kingdoms and domains. The functional importance of PIMT is discussed in all the organisms where it was studied (bacteria, plants, Drosophila, Caenorhabditis elegans and mammals) as well as functional analysis in transgenic models.

While the loss of function of PIMT has moderate phenotype in bacteria, studies in mammals highlighted that neurons are the cell type where PIMT loss has the most severe effects. More recently, PIMT defect has been correlated to several diseases. Behavioral disorders as well as abnormal synaptic transmission have confirmed its impact in neuronal disorders [42], but other studies also suggest links with diabetes [43], aging [44], and PIMT has also been proposed as a novel predictor for poor prognosis in cancer [45,46].

Discussing PIMT-related repair hypothesis with respect to aging echoes with the “molecular clock” hypothesis, and further emphasizes that repair enzymes and protein deamidation are actually two sides of a same coin: proteins are spontaneously damaged by deamidation, racemization and isomerization, and these damages are essentially visible on proteins with a slow turnover. The fact that they are only slowly replaced by newly synthesized proteins drives the unrelenting build up of damaged proteoforms. Their loss of functionality is a main contributor to the slow decline of normal cellular functions that accompanies aging. PIMT is one of the few factors alleviating this functional decay, but the effects are mitigated because the repair is only partial in the case of deamidation, and because PIMT expression declines with age. Thus the age-dependent increase in damaged proteins results from the slow accumulation of modified forms, added to an inefficient repair mechanism. The intersection of research on deamidation and PIMT essentially allowed to investigate aging at the molecular level, and pointed out that processes driving age-related accumulation of damaged proteins are promising therapeutic intervention points.

#### **2-d) Towards high-resolution tools to predict, detect and quantify peptide/protein deamidation**

Figure 1C explicitly shows that 7 proteoforms coexist (in variable amounts) in any sample containing one mono-deamidable protein. For a polydeamidable protein,  $7^n$  proteoforms will coexist, and the number further increases if complex protein samples are considered rather than purified proteins.

Detecting deamidation implies that one can discriminate the products from the precursor on physical/chemical criteria. The charge heterogeneity between the carboxylic acid products and the precursor can essentially be exploited by techniques like chromatography (anionic or cationic exchange), IsoElectroFocusing (IEF) which is particularly suited to detect sequential deamidation in proteins harboring more than one deamidable site, and Capillary Zone Electrophoresis (CZE), which

235 produces a high-resolution separation of deamidated products. All these techniques have previously  
been reviewed in [47] and [48]. IEF [49] and CZE have recently been used to estimate deamidation  
rates of antibodies [50] or characterize the deamidation products of insulin [51] for example. It should  
240 be stressed that charge heterogeneity can of course result from other modifications, and the above  
techniques need companion studies to first ascertain the occurrence of deamidation. Another  
limitation is that isomerized and racemized proteoforms are overlooked. Indeed physical/chemical  
criteria are lacking to discriminate each carboxylic product because they have the same mass, same  
charge and similar hydrophobicity.

To detect Iso-Asp and Iso-Glu, techniques initially developed by chemists to detect  $\beta$ -linked peptides  
can be implemented . (i) Two-dimensional chromatography and ninhydrin staining can distinguish  $\beta$ -  
aspartyl peptides from their  $\alpha$ -isomers as only the former develops a blue color [35]. (ii) Unlike  $\alpha$ -  
amino acids,  $\beta$ -amino acids cannot react with phenyl isothiocyanate to form the phenylthiocarbamoyl  
245 derivative during the Edman degradation procedure, thus isoAsp and IsoGlu in peptides can be  
indirectly deduced by failure of the Edman sequencing procedure [52]. (iii) Changes in the secondary  
structures of  $\beta$ -linked peptides compared to  $\alpha$ -linked peptides are instrumental for reverse phase  
HPLC separation, and the sequential elution of deamidation products is isoaspartyl first, followed by  
aspartyl, and then the aminosuccinyl form of the peptide [29]. (iv) Native gel electrophoresis allowed  
250 to highlight the retarded mobility of deamidated calmodulin compared to the native, due to an  
increase in the Stokes radius probably caused by IsoAsp-driven unfolding [53]. (v) Specific  
polyacrylamide gel compositions in denaturing conditions have also recently been reported to assist  
the separation of native and deamidated species [54].

In the case of Asn deamidation, enzymatic detection of IsoAsp is also allowed by PIMT-based assays,  
255 which were developed around the 1990's: a radioactive assay with [ $^3\text{H}$ ]methyl-labeled SAM can be  
used, and [ $^3\text{H}$ ]methanol release is detected [55]; SAH may be detected in non-isotopic assays using  
HPLC [56,57]. Several PIMT-based enzymatic tests have been developed and coupled to UV-vis [58],  
fluorescence [59,60] or bioluminescent detection methods [61,62]. These assays allow a bulk  
detection of IsoAsp residues in samples ranging from purified proteins to more complex protein  
260 extracts. Some have been designed to carry out high throughput assays [59,61].

The main limitation of all the above techniques is that they do not allow to specify the site(s) of deamidation, and that the analysis of complex samples is only possible if an antibody detects the protein of interest after electrophoresis/western blot for example.

The development of mass spectrometry (MS) at the turn of the century has undoubtedly provided the most powerful tool for high throughput, sensitive and accurate analysis of deamidation. Using analytic techniques to separate the different species contained in a sample prior to MS analysis has now become a gold standard to establish deamidation and locate the residue(s). MS analysis was initially only deployed on simple samples (peptides or protein digests) to detect the increase between monoisotopic mass of Asn (114,0429) and Asp/IsoAsp (115,0269) which generates the typical 0,984-Da difference. The first report describing the use of MS for the detection of deamidation was by Teshima et al. [63]: a charge variant of their protein of interest was isolated by high-performance cation-exchange chromatography and submitted to tryptic mapping analysis by fast atom bombardment mass spectrometry (FAB MS); the mass shift accompanying Asn→Asp was detected on one of the peptides, and Asp-N endopeptidase was further used to confirm the site of deamidation. Over the last 20 years, major improvements have been brought on MS instrumentation to refine the ionization methods (Fast Atom/ion Bombardment, ElectroSpray Ionization, Matrix-Assisted Laser Desorption/Ionization), to make mass analyzers (quadrupole, iontraps, Time Of Flight, orbitrap, Fourier transform mass spectrometers) faster, more accurate with better resolution, and to make detectors more sensitive. The introduction of diverse ion activation procedures, where selected ions are further collided and fragmented in MS/MS, has finally enabled structural determination of molecules : sites of deamidation were specified in peptide sequences, and in 2005, discrimination of Asp from IsoAsp was achieved when Electron Capture Dissociation was used as fragmentation method to generate IsoAsp specific reporter ions [64,65].

No other technique, to this date, has outperformed mass spectrometry, and it comes in so many flavors that one can tailor its use to virtually any sample, from pure peptides/protein digests (bottom up approaches) to complex protein mixtures (middle down and bottom down approaches).

Recent reviews may be found in this Special Issue [66–68], which highlight the latest advances in MS at multi-level scales since 2015, in protein samples of varied complexity. All three reviews bring a special focus on how to minimize deamidation during samples preparation, a major challenge to limit loss of information and avoid false positive. Jin et al. provide numerous examples of MS technologies

used on large polypeptides (middle down) and complex protein samples (top down), and discuss their respective differences and limitations [67]; Ying et al. review chromatographic separation methods prior to MS analysis depending on the complexity of the sample [68]. Sze et al. present new analytical technologies to tackle the separation of the highly similar deamidated proteoforms, and discuss recent improvement in MS instrumentation and data processing for deamidome analysis [66].

Looking how far deamidation has come with the help of MS, there is no question that middle down and top down MS analyses hold high promises to understand the role of deamidation in biology and harness protein degradation in pharmaceuticals; to meet this goal, effective separation of intact proteins, without size restriction, with high resolution and high throughput are the next challenges MS will need to face.

One of the latest items in the deamidation tool box is computer-assisted methods to predict the probability with which a given Asn or Gln residue might be deamidated in a specific protein. The Robinson lab should here again be credited for this endeavor. The easier detection/quantification of deamidation granted by MS analyses gave them the impetus for a large scale quantification of deamidation rates of a library of 306 peptides. Based on this large amount of measures, the Robinson lab elaborated a the computerized prediction method. Initially fed with rates and sequences from the library of 306 synthetic peptides [28], the procedure was refined by 3D-structures available of deamidable proteins [69]; its performance was then tested on a subset of human proteins [70], and lastly validated on more than 13,000 proteins [71], thus leading to the first prediction algorithm. Since the first algorithm proposed by Robinson, several other computational tools have been published [72–76], including in this Special Issue [77]. The parameters that are generally computed include local structure conformation, structure flexibility and solvent accessibility: some algorithms require that the crystal structure of the protein is known [69,72,75,76], while others only rely on primary sequences [74,77]. Some of them may be applicable to any protein and have indeed investigated deamidation probabilities at the scale of whole proteomes [77] while others are essentially focused on a specific category of proteins, namely antibodies [75,76]. One can foresee that their use will be routinely implemented for the purpose of antibodies development in biotechnology and pharmaceutical industries. But the advance of such computational tools will also undoubtedly help widen even more the outreach of deamidation: not only do they have the potential to become the first intention

approach for newcomers in the field (Figure 4), but they may also provide a invaluable input in the understanding of deamidation from the evolution standpoint.

325 **3- Global positioning : deamidation spreads across diverse areas**

Nowadays the use of protein deamidation has spread beyond the mere biological interest of determining the functional consequences in cells (Figure 3). It has been claimed by areas distant from biology, that have in common the dating of samples or the concern to maintain proteins in their original state: it routinely serves as a quality control assay in biotechnology, and owing to the fact that  
330 deamidation occurs in any proteinaceous sample even outside of cells, and that the reaction may take years to centuries to occur, deamidation now serves in ancient art, archaeology and forensic.

The development of high-resolution mass spectrometry techniques over the last 20 years has had a decisive impact on shifting the focus on deamidation and turning it into a tool for other studies. Accurate quantification in trace-amounts of samples and identification of site-specific reactions has  
335 helped expanding the use of deamidation outside biology.

**3-a) Deamidation is a molecular marker of historical objects and ancient artwork.**

All the historical objects that have been passed on to us constitute our cultural heritage. They are the link to our past and provide a wonderful means to analyze how our ancestors lived and how these  
340 objects were taken care of through time. Many historical objects and artworks are composed of proteinaceous samples. Historical objects encompass religious and royal collections containing textiles made of wool or silk, and leather handcraft; ancient artworks include paint, binding media and protective coatings used in paintings. These organic materials naturally decay. Analyzing how these historical specimens stood the test of time is not only helpful to reconstruct their preservation history,  
345 but also mandatory to protect them from further degradation, as well as to design the appropriate restoration procedure.

MS being a minimally invasive technology, it is well suited to describe the deamidation state of trace amounts of precious but complex samples. Protein deamidation has been analyzed in historical objects in several studies, allowing to specify more precisely what deamidation may say, and what it  
350 may not say of an object.

While studying frescoes from the Camposanto Monumentale (13<sup>th</sup> century) in Pisa, Leo et al. [78] had the initial goal to identify molecular markers that could be used as aging/deterioration signatures potentially applicable to other ancient artworks. They focused on the organic material used in the binders of the painting, and designed, as a reference sample, a modern replica containing the same animal glues as in the original painting binder. LC MS/MS analysis of the chemical modifications undergone at specific sites by the original samples were compared to the reference sample. Deamidation could be detected in virtually all the different proteins identified, both in control and original samples, therefore confirming that limiting artifactual deamidation during sample preparation is a crucial parameter in these kinds of analyses. Nevertheless, they identified selected peptides from casein that were poly or mono-deamidated in the original artwork, while their respective counterparts in the replica were mono- or non deamidated. This work suggests that comparative deamidation analysis of known casein peptides in original artworks vs proper reference can be used for the diagnostic of the stability of the materials and conservation of ancient artworks. It must be stressed that this approach is only valid if the history and composition of the artwork is know well enough to design a proper replica, where modern proteins are assembled in a medium that mimics as closely as possible all the different processes that have been applied to the original artwork, sometimes during sequential interventions.

In another study, Mackie et al. [79] recently applied paleoproteomic to the Italian wall painting “A Group of Four Poor Clares” (14<sup>th</sup> century), with the goal to better characterize the artistic technique employed and elucidate the function of different proteinaceous layers found in the artwork. Nano LC MS/MS was used to identify the protein composition of the layers, and characterize protein modifications/damage to reconstruct the successive interventions applied to this painting. Deamidation analysis could unfortunately not be very informative because the extent of Asn and Gln deamidation in each layer was different, and unlike the study by Leo et al., no common peptide could be found in the layers to allow direct comparison. Furthermore, the preparation of some layers had probably involved boiling of the proteinaceous samples, which is known to tamper with natural deamidation rates. This work therefore exemplifies some limits inherent to deamidation analyses.

Another analysis which involves ancient textiles, was carried out by Araki and Moini [80]. They provided out a proof-of-concept study on well-dated ancient textiles stored in museums; their work perfectly illustrates the prerequisites for deamidation to be used to date samples: a similar

environmental history is needed to minimize the influence of external factors on natural deamidation rates, and samples of the same species must be considered to minimize variations of deamidation rates due to internal factors (sequence/structure). Granting the above, well-dated textiles of different ages could be used to establish a calibration curve for the dating of an unknown sample.

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### **3-b) Deamidation in archaeology.**

Archaeology essentially aims for two goals: (1) establish the absolute dating of any biological sample for phylogenetic classifications [81–83], and (2) use remains and manufactured objects to reconstruct human history and establish a time-line for cultural transitions/acquisitions [84,85]. Archaeology therefore needs to authenticate the nature and origin of samples (to discriminate contamination from original proteins). To this end, extraction protocols have been refined to limit artifactual deamidation and to maximize the recovery rate from total proteins [86–88]. In spite of such improvements, deamidation studies have provided inconsistent success to meet these goals.

Deamidation rates of collagen from bone fossils have been analyzed in many studies. Collagens are very abundant proteins in bodies, and notably in bones (85-90%) where they largely exceed non collagenous proteins (0-15%). In addition, these organic samples are naturally confined in the mineral inorganic phase (mainly calcium phosphate) that composes 50-70% of bones, thus accounting for a remarkable preservation in prehistoric samples, even temperate. Owing to the highly ordered arrangement in triple helix of Type I collagen, deamidation rate is also kept considerably slow.

Different MS-derived tools have been developed to tailor the measurement of deamidation to archaeology specific needs. A method called ZooMS (for Zoology Mass Spectrometry), developed by M. Collins and M. Buckley [89], is widely used for the taxonomic identification of bones: the MALDI-ToF-MS finger printing analysis of collagen type I provides spectrometric patterns that are compared and matched to a reference database. Variations in amino acid sequences from different taxa allow robust, fast and cost-effective species identification. The levels of deamidation in archaeological collagen can be further measured from the same ZooMS analyses. The usefulness of deamidation for absolute dating of bone samples was unfortunately disproved by most studies : Orlando et al. compared the proportion of deamidated sites in collagen samples from a Middle Pleistocene horse bone (see suppl data in [81]) to Late Pleistocene mammoth samples previously analyzed by Cappellini et al. [82]: Dent mammoth (-11 000 years) and La Sena mammoth (-19 000 years) preserved in

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temperate environments show ~80% deamidation, while the Siberian mammoth (-43 000 years) and the Middle Pleistocene horse (-780 000 to -560 000 years) preserved in permafrost show ~53% deamidation, in spite of their age difference. This proves that thermal variations over time at the burial site tamper with deamidation rates, and preclude their use to reflect chronological age.

415 In some cases, the expected deamidation rates were corrected by a metric called “thermal age” [90] to account for the environmental history of fossils in different places (temperature, hydration, burial depth...); better correlation of deamidation rates with chronological age was achieved [90,91].

The Collins lab further designed a new software called DeamiDATE 1.0 [92] which relies on site specific deamidation rather than bulk deamidation to compare samples. In DeamiDATE the relative deamidation of samples determined by MS/MS analysis is weighted by the half-time specific to each X-N-Y or X-Q-Y combination as calculated by Robinson and Robinson under controlled pH and temperature conditions (37 °C, pH 7.4) [4]. DeamiDATE allowed authenticating samples in complex protein mixtures; applied to a case study from Neolithic dental calculus, efficient sorting of contaminants and authentic ancient proteins was achieved. However, very recently Brown et al. [83] 425 also used DeamiDATE to assay the potential of bone collagen deamidation as an age-predictor using both MALDI-TOF MS and LC-MS/MS methods on well-dated samples from different stratigraphic layers of the East Chamber of Denisova Cave. High levels of variation in samples of the same or similar age were found, which even calls into question the use of deamidation as a relative age dating method.

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In conclusion, the current consensus is that Gln deamidation in archaeology may be used more as an indicator of the preservation quality of the samples than for absolute dating [93]. It may indeed serve as a comparative method to assess the chronological relationship between samples from different deposits and to identify outliers. Current endeavor is to establish whole proteomes in given 435 archaeological samples [82] to widen the array of deamidable proteins to be studied and mitigate the inherent variability of deamidation that results from specific sequences and 3D structures. Other remains than bones also seem to be better suited for the use of deamidation in sample dating, as was recently shown with ancient dentine [94].

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### **3-c) Deamidation in forensic analyses.**

Forensic avidly looks for specific biomarkers that may assist the estimation of the time elapsed since death (also called Post-Mortem Interval, PMI), (see [95] for detailed state of the art). High resolution proteomic analyses of soft tissues have been repeatedly used to describe time-dependent degradation, but they are excessively susceptible to cumulative alterations over the time, caused by  
445 multiple external factors, and they are therefore not ideally suited for advanced decomposition stages. Recent works indicate that bone proteomics gives promising results because these proteins are more stable against extrinsic factors, and may be extracted and analyzed whatever the decomposition state of the remains.

Procopio et al. used pigs bones as model systems and demonstrated that as PMI increases, post  
450 mortem proteins in bones decay, which mirrors the proteome simplification also observed in soft tissues. But most interestingly, they established a positive correlation between biglycan deamidation and PMI. This small leucine-rich proteoglycan is involved in osteoblast differentiation and matrix mineralization, assisting new bone formation and reparation [92]. This long-lived protein was also recovered from archaeological specimens, opening the possibility that biglycan might become a  
455 forensic tool to estimate PMI over long time spans.

In the work by Mizukami et al., deamidation was identified as a potential means to discern specific post mortem circumstances, with certain proteins being deamidated only in aquatic environment and not in terrestrial ones, and other proteins being deamidated in certain types of waters but not in others [96].

460 But deamidation was shown to be of interest for other forensic applications than PMI estimation. Notably, Oonk et al. performed differential studies of fresh and aged fingermarks up to 16 days [94]. After establishing a fingermark proteome (essentially material shed from skin and sweat), they assessed its dynamic changes at different stages of aging, including protein damage and modification. Increasing protein modification levels were detected between 0 and 12 days, and keratin deamidation  
465 was found. The authors stress however that the analysis of fingermark proteome to establish a time-line reconstruction in crime investigations is still in its infancy and will require more work, notably including climatic variations and using diverse substrates for fingermarks.

### **3-d) Deamidation in industrial pharmaceuticals.**

470 The production of protein drug products (hormones, growth factors, immuno-modulators, antibodies,  
etc...) by pharmaceutical industries is a constantly expanding field due increasing needs worldwide,  
that are fortunately met by the advance of large scale engineering methods. Rigorous technical  
specifications are enacted to ensure safety and bio-activity of protein therapeutics. Here again,  
protein stability is sought: from the chemical standpoint, the amino-acid sequence, structural folding  
475 and post-translational modifications that characterize the original and functional protein must be  
preserved. From the physical standpoint, unfolding, dissociation, denaturation, aggregation, and  
precipitation must be prevented. Hence, pharmaceutical industries have adapted specific formulations  
of solutions to warrant the preservation of therapeutic proteins in their active state, and they have  
included analytical characterization steps to ensure product integrity and manufacturing consistency  
480 [95,96]. Large peptides/proteins are more likely than small molecules to harbor complex three-  
dimensional structure and include many functional groups; as a consequence, the former are more  
susceptible to spontaneous modifications (including conformational and chemical changes) [100].  
Today, machine learning models have been refined to the point that algorithms now allow to compute  
complex datasets and assist protein design and development with unprecedented accuracy : as a  
485 result, spontaneous degradation may be averted in therapeutic proteins [101].

Deamidation, isomerization and racemization have clearly been identified as spontaneous processes  
potentially challenging the biological activity of proteins because they proceed at physiological pH  
with rates relevant to the time-scale of protein production [29].

The specific case of monoclonal antibodies (mAbs) will be reviewed here as an example of  
490 deamidation-related issues in the stability for pharmaceutical proteins [102]. This example is  
exquisitely suited to point how the knowledge earned from all the early works before 2000 has now  
been implemented to predict hot-spots of deamidation and avert protein instability.

Antibodies are composed of 2 heavy chains (HC) and 2 light chains (LC) linked by disulfide bridges. The  
specificity of an antibody is mediated by 2 antigen-binding fragments (Fab), for which one HC and one  
495 LC each contribute a constant domain (noted CH and CL respectively) and a variable domain (noted VH  
and VL respectively). VH and VL each contain 3 complementary determining regions (CDRs) that are  
responsible for the specificity toward the antigen. Asn deamidation and/or Asp isomerization in CDRs  
has long been known to reduce antigen binding and limit biological efficacy of therapeutic antibodies  
[103,104]. Structural data have recently provided molecular grounding for how Asn→ IsoAsp disrupts

500 mAb potency [105]. Improving the engineering of mAbs requires to detect as early as possible in the production pipeline the molecules combining biological activity and chemical stability. To meet this goal, the Holy Grail would of course be to assist the very design of the molecule. Over the last 20 years, several studies have run the systematic analysis of Asn deamidation in mAbs for which the patent had expired and the sequence was published. Experimental deamidation rates (spontaneous and accelerated) were established [76,106]; when matched to the primary sequence, the correlation with deamidation rates established on pentapeptides by Robinson and Robinson sometimes confirmed the rates NG>NS>NT but not systematically, and sometimes no deamidation was monitored on these motives. Conversely, low-probability motives like NT, NF, NY [106] or NW and NI [76] sequences actually deamidated in whole molecules. A recent structural analysis ran a comprehensive correlation of antibodies 3-D structures with deamidation rates, and proposed a decision tree implementing structural parameters like backbone flexibility (corresponding to the  $\psi$  and  $\chi_1$  angles), the distance between the NH group and the C $\gamma$  of the Asn side-chain, and solvent accessibility (to account for the critical role of water in the reaction). As a result, Asn deamidation in antibodies can be predicted more accurately than based on sequence alone [76]. This work might serve in the future to refine prediction algorithms, not only for antibody also for other protein therapeutics.

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One last important information regarding protein drug products stability is that, even if buffer optimal manufacturing formulation and storage conditions are secured to warrant the best handling before injection, deamidation continues to occur in vivo (at least for recombinant mAbs). Fortunately, methods are reported to measure this parameter in vivo, which opens the possibility to explore further the bioactivity of the engineered protein once in the patient's system [107].

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#### **4- Workflow to investigate deamidation**

As a conclusion to this review, a possible workflow can be proposed to researchers entering the field and wondering if their protein of interest (POI) is deamidated, and if so, how to proceed from the most simple to the most refined method to determine the residues involved (Figure 4).

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The first tool to determine the probability that a given protein deamidates is the in silico approach, carried out through a bio-informatic analysis with one (or ideally several) of the algorithms available. It is however important to stress that even prediction utilizing the most refined data (experimental

530 crystal structure) might be flawed. Structure determination might identify intrinsically disordered regions in a protein, which will be credited with the highest deamidation probability; however these regions frequently acquire an induced fold upon interaction with a binding partner. The lack of structure in a purified protein might thus not hold true in a cellular environment. This is why experimental proof of deamidation should always be sought, even after algorithms have provided a  
535 deamidation score.

A further step in the analysis could be to submit the POI to conditions prone to accelerated deamidation. A range of temperatures, pH, ionic strengths and buffer ions should be assayed (preferably with NH<sub>4</sub>OH or phosphate). The efficiency of the treatment will then be checked: the IsoAsp content could be assayed with one of the PIMT-derived enzymatic assays. The limitations are  
540 (1) that the POI should be available as a purified protein, (2) that IsoAsp detection is mostly a qualitative assay, especially if several deamidation sites coexist in the POI, and (3) that this method only reveals Asn deamidation (IsoGlu is not a substrate of PIMT), and might also reveal Asp isomerization. Because Asn anyway deamidate much faster than Gln, assaying IsoAsp content is nonetheless helpful because these tests are easy to set up and do not require access to any specific  
545 tool or equipment.

An alternative very straightforward method, that is even applicable to complex protein samples, is the electrophoretic separation and immunodetection of the POI. Denaturing polyacrylamide gels with specific formulations have been described in this Special Issue [54] to highlight various deamidation states of Bcl-xL, Calmodulin and small GTPases; native gels have also been used to separate  
550 deamidated and native Calmodulin on the basis of the changes in the molecular radius of the IsoAsp containing form [53]. Of note, these techniques may not be suited to all proteins because the migrations shifts might be too small or the size of the protein might not be in the best range, so only a case by case analysis can tell their applicability. Lastly, IEF and CZE are also useful to show with high resolution the shifts of charge during (poly)-deamidation, but the separation performed on a pH  
555 gradient gel requires a specific type of material.

As already indicated before, diverse chromatography techniques can be used to separate the species contained in a complex sample, and they are ideally coupled to mass spectrometry analysis to further specify the extent and the sites of deamidation.

Mass spectrometry allows to detect deamidation based on the typical +0,984 Da mass shift. However, discrimination of Asp and IsoAsp by simple MS is tricky because they have an identical chemical composition, identical mass, and similar physico-chemical properties. MS/MS is thus necessary, and allows the separation of specific fragment ions. For example, electron-capture dissociation (ECD) or electron-transfer dissociation (ETD) can be employed to generate an IsoAsp-specific diagnostic ion. Regarding the preparation of samples, top-down and bottom-up approaches may be implemented in MS/MS analysis. In bottom-up approaches, a complex protein sample is first subjected to enzymatic digestion to generate peptides that are more easily solubilized and separated than whole proteins. But the main drawback is that artefactual post-translational modifications (including deamidation) can be introduced due to the pH conditions used for enzymatic digestions. In top-down approaches, intact proteins are directly analyzed, and the whole procedure therefore spares lengthy sample preparation steps. These approaches have been reviewed and confronted in this Special Issue [67].

## 5- Conclusion

Our knowledge of protein deamidation has made significant progress in a little less than 100 years. Chemists brought the first insights, with an initial descriptive approach to fathom how it occurs and what influences its catalysis at the molecular level. It very rapidly spread to biology when it was investigated at the cellular level : the endeavor started then, to integrate its functions in physiology and pathology. In parallel, it was also harnessed as a tool which now finds applications outside of basic research in biology.

As in many other fields of investigation, it is remarkable how different basic research areas have collided at some point to advance our knowledge, and how technical breakthrough have allowed a significant acceleration. Facilitating the detection of deamidation then fostered its spreading to areas so diverse as history/arts, biotechnology and forensic. Given the different stakes in each of these areas, one can foresee a ramping utilization of deamidation. But because deamidation is potentially used in such ubiquitous ways, it makes it all the more unsettling that this topic currently receives so little attention from basic research and academia (most students never hear about deamidation, while other PTMs are extensively taught). Detecting this PTM still remains less straightforward than other PTMs : to date, only one PIMT-based detection kit is commercially available to allow the detection of IsoAsp; if the proof of concept was made in 2000 that specific antibodies could discriminate between

590 native and IsoAsp-deamidated A $\beta$  peptide in Alzheimer's disease, biotechnology industries are yet to invest in the development of a wide array of antibodies to IsoAspartyl proteins. The fact that protein deamidation relates more and more to pathology (neurodegeneration, aging, diabetes and cancer...) will perhaps be an incentive powerful enough for industries to commit in the development of these tools. There is no question that such tools would be powerful kick-starters for researchers to import deamidation in their scope of interest.

595 **FIGURE LEGENDS:**

**FIGURE 1: Deamidation of asparagine residues and partial repair mechanism**

A- When the flexibility of the polypeptide chain is permissive, the nitrogen atom of the n+1 residue operates a nucleophilic attack (green arrow) on the gamma-carbonyl group of the L-asparagine. A metastable succinimide (cyclic imide) intermediate is formed, which hydrolyzes in two possible ways (dashed green line) and produces a mixture of L-aspartyl and L-isoaspartyl residues (in IsoAsp, the peptide bond between Asp and the n+1 residue is made with the side chain beta-carboxyl of aspartate instead of the conventional alpha-carboxyl). The succinimide is also prone to racemisation, generating D-succinimidyl, D-aspartyl and D-isoaspartyl residues. Of note, D-isoAsp are no substrates for PIMT. B - The alpha-carboxyl group generated during L-isoAsp or D-Asp formation is methylated by PIMT to form a methyl ester; S-adenosyl-L-methionine (SAM) is the methyl donor, which is converted into S-adenosyl-homocysteine (SAH). Spontaneous ester hydrolysis leads to reformation of the cyclic imide intermediate in A, which may again hydrolyze to a mixture of Asp and isoAsp.

C- Connection between degradation pathways involving Asn and Asp residues. L-Asn in peptides or proteins irreversibly deamidates either through direct hydrolysis in acidic solutions, or through the formation of L-succinimide at more alkaline pH. Racemization (purple box) essentially occurs on L-succinimide to produce the D- enantiomer. Reactions involving Asp residues are reversible. L-Asp in peptides or proteins are prone to isomerization (orange box) into L-IsoAsp. D-Asp residues sometimes found in damaged proteins are formed after hydrolysis of D-succinimide. The dynamics of these reactions is discussed in the text and in references [33] and [35]. L-IsoAsp and D-Asp labeled in blue are substrates of the repair enzyme PIMT.

**FIGURE 2: Time-line of the landmark discoveries in protein deamidation**

Discoveries pertaining to the catalytic parameters that influence deamidation are in blue. Discoveries pertaining to the reaction mechanisms at play during deamidation are in orange. Technical milestones are indicated in pink. PIMT related discoveries are indicated in green. The time-line emphasizes that the mechanisms and the products of deamidation were known at the end of the 70's. The time-line also stresses that the advent of mass spectrometry revolutionized the measurement of deamidation. No other technique has outperformed the sensitivity and accuracy granted by MS to allow quantifying

and specifying the deamidation sites. The discoveries post-dating 2005 are described in the rest of the  
625 manuscript.

**FIGURE 3: Societal outreach of protein deamidation**

Current fields in which the use of deamidation has penetrated. For each area, examples are given of  
what may be achieved though the measurement of Asn or Gln deamidation.

630

**FIGURE 4: Tentative workflow to investigate if and where a protein deamidates.**

See text for detailed description of the proposed workflow.

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640 **Author Contributions**

M.P. wrote the manuscript and generated the figures. All authors reviewed and edited the manuscript.

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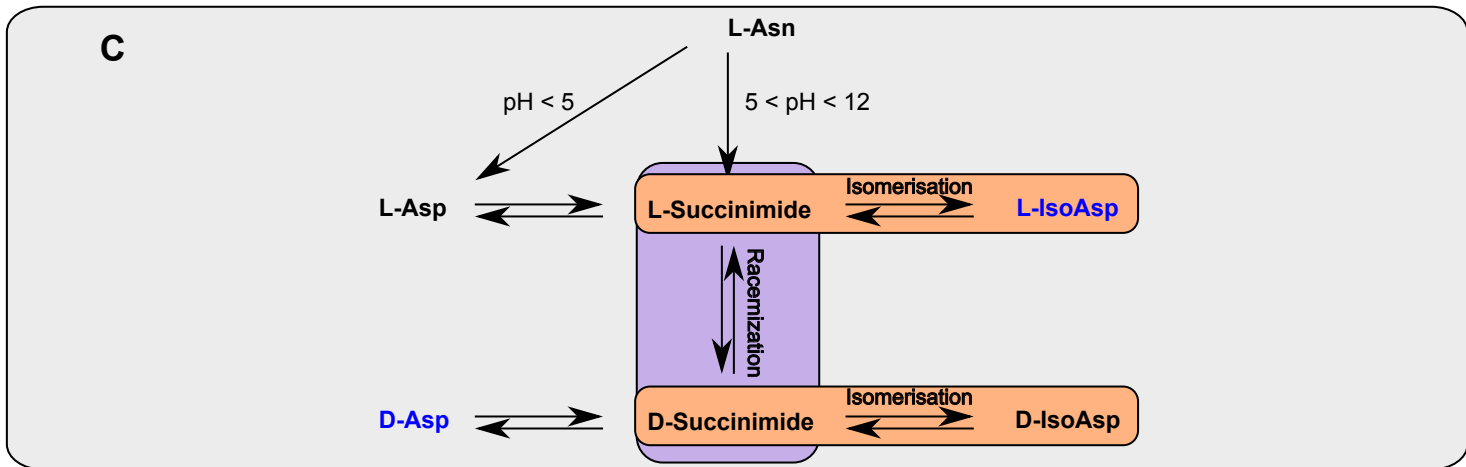
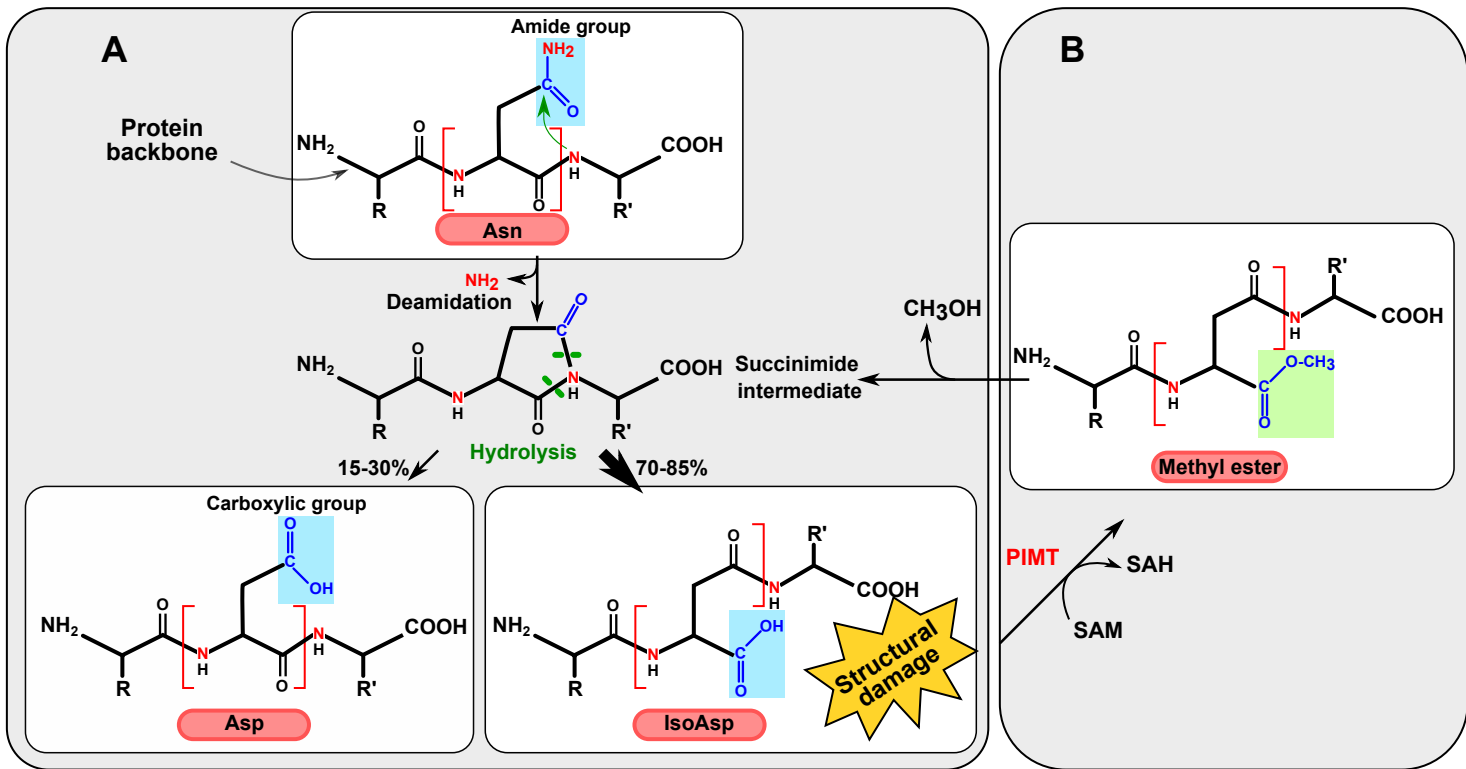
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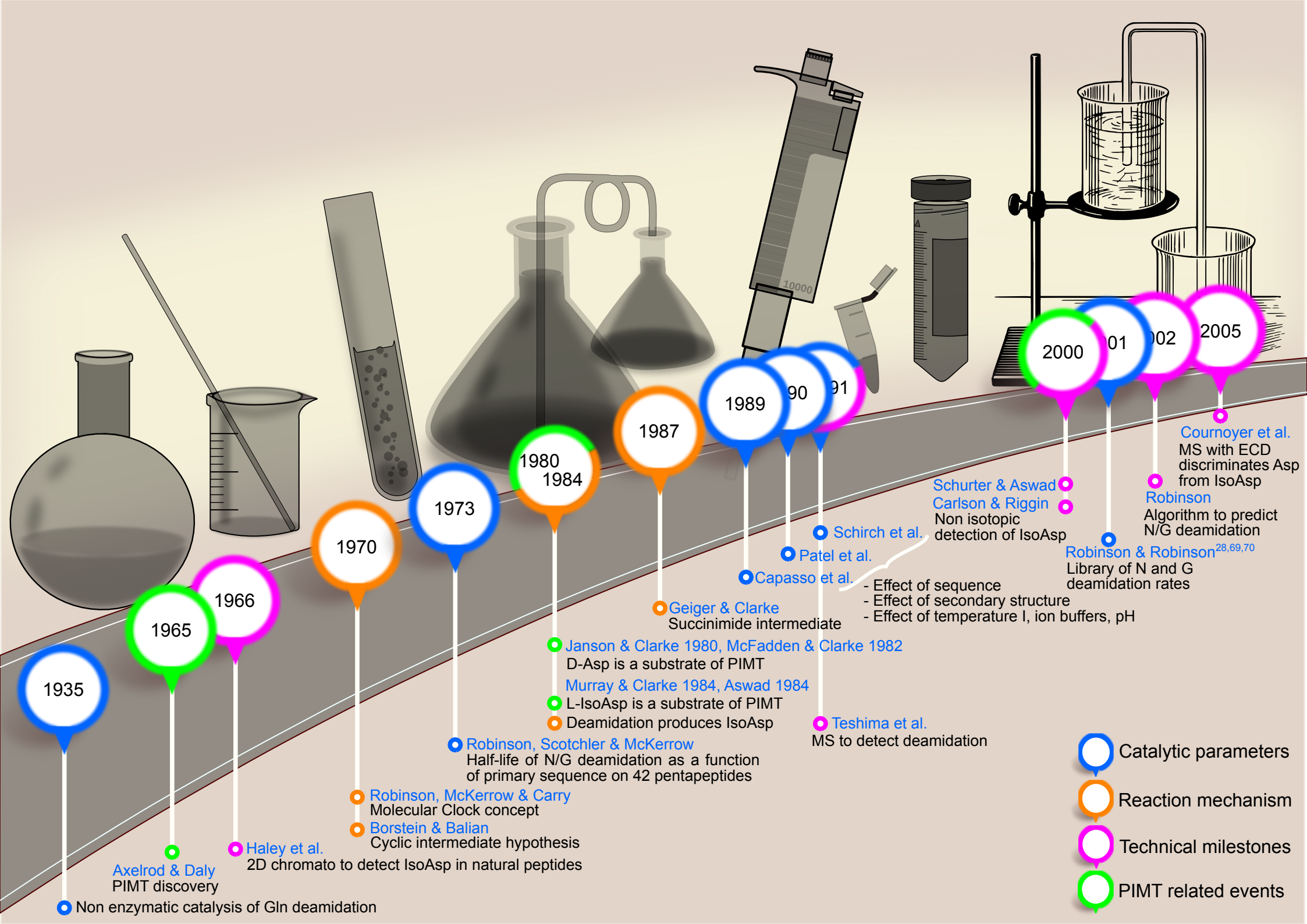
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1935

Non enzymatic catalysis of Gln deamidation

1965

Axelrod & Daly  
PIMT discovery

1966

Haley et al.  
2D chromato to detect IsoAsp in natural peptides

1970

Robinson, McKerrow & Carry  
Molecular Clock concept  
Borstein & Balian  
Cyclic intermediate hypothesis

1973

Robinson, Scotchler & McKerrow  
Half-life of N/G deamidation as a function of primary sequence on 42 pentapeptides

1980  
1984

Janson & Clarke 1980, McFadden & Clarke 1982  
D-Asp is a substrate of PIMT  
Murray & Clarke 1984, Aswad 1984  
L-IsoAsp is a substrate of PIMT  
Deamidation produces IsoAsp

1987

Geiger & Clarke  
Succinimide intermediate

1989

Capasso et al.

Patel et al.

Schirch et al.

- Effect of sequence
- Effect of secondary structure
- Effect of temperature I, ion buffers, pH

Patel et al.

Schurter & Aswad  
Carlson & Riggin  
Non isotopic detection of IsoAsp

2000

Robinson & Robinson<sup>28,69,70</sup>  
Library of N and G deamidation rates

2001

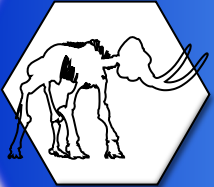
Robinson  
Algorithm to predict N/G deamidation

2002

Cournoyer et al.  
MS with ECD discriminates Asp from IsoAsp


2005

- Catalytic parameters
- Reaction mechanism
- Technical milestones
- PIMT related events




### Archaeology

comparison of preservation conditions



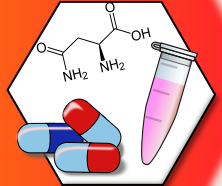
### Art

- textile dating
- painting dating
- profiling of restoration methods



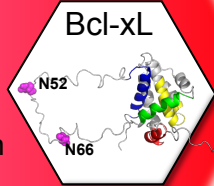
### Forensics

- estimate post-mortem interval
- discern post-mortem circumstances
- fingerprints dating



### Industry

- formulation of preservation media
- sequence adaptation for improved stability



### Biology

**Bcl-xL**

- function modification
- aging
- sequence evolution

