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Review

Related impurities in peptide medicines

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ABSTRACT

Peptides are an increasingly important group of pharmaceuticals, positioned between classic small organic molecules and larger bio-molecules such as proteins. Currently, the peptide drug market is growing twice as fast as other drug markets, illustrating the increasing clinical as well as economical impact of this medicine group. Most peptides today are manufactured by solid-phase peptide synthesis (SPPS). This review will provide a structured overview of the most commonly observed peptide-related impurities in peptide medicines, encompassing the active pharmaceutical ingredients (API or drug substance) as well as the finished drug products. Not only is control of these peptide-related impurities and degradants critical for the already approved and clinically used peptide-drugs, these impurities also possess the capability of greatly influencing initial functionality studies during early drug discovery phases, possibly resulting in erroneous conclusions.

The first group of peptide-related impurities is SPPS-related: deletion and insertion of amino acids are related to inefficient Fmoc-deprotection and excess use of amino acid reagents, respectively. Fmoc-deprotection can cause racemization of amino acid residues and thus diastereomeric impurities. Inefficient deprotection of amino acid side chains results into peptide-protection adducts. Furthermore, unprotected side chains can react with a variety of reagents used in the synthesis. Oxidation of amino acid side chains and dimeric-to-oligomeric impurities were also observed. Unwanted peptide counter ions such as trifluoroacetate, originating from the SPPS itself or from additional purification treatments, may also be present in the final peptide product. Contamination of the desired peptide product by other unrelated peptides was also seen, pointing out the lack of appropriate GMP. The second impurity group results from typical peptide degradation mechanisms such as β-elimination, diketopiperazine, pyroglutamate and succinimide formation. These SPPS- and degradation-related impurity types can also found in the finished peptide drug products, which can additionally contain a third group of related impurities, *i.e.* the API–excipient degradation products.

Contents

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1. Introduction

Peptides can be considered as a separate drug category, situated between the classic small organic molecules and the large proteins. Given their small size, *i.e.* generally defined as up to 50 amino acid (AA) residues, peptides are able to penetrate deeper into the target tissue than proteins. Moreover, therapeutic peptides are generally less immunogenic and are more cost-efficient to consistently manufacture according to the required quality parameters than recombinant proteins and antibodies. Peptides also offer several advantages over small organic molecules, as they have a greater efficacy, selectivity and specificity [1–3]. Moreover, as peptides consist of individual amino acids, they offer enhanced possibilities for drug discovery using combinatorial peptide libraries [4,5]. These libraries can be combined with specific targets, often proteins, which are at the core of many physiological processes. The knowledge of these protein–protein interactions continues to increase, resulting in an increasing diversity of targets [6–8]. Furthermore, as traditional peptides will eventually degrade into one of the 20 endogenous amino acids, toxicity due to metabolites is greatly reduced [1–3]. However, more and more non-proteinogenic amino acids, *i.e.* amino acids not encoded in the human genetic code, are being incorporated into the sequence of peptide drug candidates [9,10]. These non-proteinogenic amino acids can be synthesized in the lab or can be found in nature. Already approximately 500 non-proteinogenic amino acids have currently been identified in nature, and are often encountered in microbial organisms and plants. Inclusion of non-proteinogenic amino acids is done to improve peptide stability, *e.g.* inclusion of D-amino acids, and to obtain (more) potent peptide drugs by altering their tissue distribution characteristics or pharmacodynamic properties [10–12]. Given the vast structural diversity of non-proteinogenic amino acids, these peptide building blocks offer great opportunities to protein/peptide engineers and medicinal chemists for the development of new peptide drugs. However, as these individual non-proteinogenic amino acids are not endogenous to humans, degradation-related toxicity issues might arise [13,14]. An overview of the current major therapeutic peptide classes with their targets and clinical use is given in Table 1.

During the last 30 years, the interest of the scientific community for peptide drugs has been continuously growing, evidenced by the continuous rise in publications focusing on various aspects of peptide research. The number of publications listing peptide as a topic term in the Web of Science database has risen from 8081 (1980–1984) to 103,426 (2010–2014) [15]. This increased scientific interest has also translated into an increased peptide drug market as evidenced by the US peptide market value, which is currently estimated at approximately $15 billion per year [16–18]. More than 85% of these sales are derived from synthetic peptides, with projected individual sales of certain blockbuster peptide drugs, *e.g.* liraglutide and leuprolide, expected to surpass $1 billion in 2015 [18]. Moreover, the peptide market value is growing much faster than for other pharmaceuticals, *i.e.* average peptide CAGR (Compound Annual Growth Rate) of 10% between 2006 and 2010. Furthermore, the success rates for bringing them to market are now approximately twice that of small organic molecule drugs [18]. In 2012, several new peptide drugs targeting a variety of disorders were approved. These included Linzess® (linaclotide) for the treatment of chronic constipation and Irritable Bowel Syndrome (IBS) with constipation in adults, Surfaxin® (lucinactant) for treatment of Infant Respiratory Distress Syndrome (IRDS), Kyprolis® (carfilzomib) a proteasome inhibitor to treat multiple myeloma, Gattex® (teduglutide) to treat adults with short bowel syndrome (SBS), Bydureon® (an extended-release version of Byetta, with the active ingredient exenatide) for patients with type 2 diabetes and Signifor® (pasireotide) for the treatment of Cushing's disease [17,19]. A global overview of currently marketed peptide drugs is given in Table 2.

Quality control of peptide active pharmaceutical ingredients (API) prior to finished drug product (FDP) manufacturing is a necessity. Currently, the pharmacopeias such as the European Pharmacopeia (Ph. Eur.). contain a number of general and peptide API specific monographs to which the quality of the peptide API material must legally adhere to. Moreover, general chapters in the Ph. Eur. – on its own not legally mandatory – become legally binding when referenced to from a general or API specific monograph [20]. A critical review regarding peptide pharmacopoeial API monographs has been performed by Vergote et al. [21]. A general lay-out for peptide drug monographs was proposed and was suggested to consist of appearance, solubility information, identification by LC-UV supplemented by other techniques like mass spectrometry (MS), related peptides by LC-UV (including higher molecular weight derivatives), residual solvents (water, acetic acid, etc.), residual reagents, inorganic impurities (such as catalysts),

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Table 1
Overview of current peptide drug classes.

Target	Indication/activity	Peptide example
26S proteasome	Multiple myeloma	Bortezomib
ACTH r	Diagnostic agent for cortisol disorder	Cosyntropin
Angiotensin II r	Anti-hypertension	Saralasin
Bacterial cellwall synthesis	Antibiotic (Gram-positive)	Vancomycin
Bradykinin B2 r	Hereditary angioedema	Icatibant
Calcitonin r	Osteoporosis	Salmon calcitonin
Cholecystokinin-B r	Diagnostic agent of gastic function	Pentagastrin
Corticotropin releasing hormone r	Diagnostic agent for cushing's or ectopic ACTH syndrome	Corticorelin
Cyclophilin protein	Immunosuppressant	Cyclosporin
Follicle-stimulating hormone r	Fertility treatment	Urofollitropin
Glucagon r	Hypoglycemia	Glucagon
Glucagon-like peptide 1 r	Diabetes mellitus type 2	Exenatide
Glucagon-like peptide 2 r	Short bowel syndrome	Teduglutide
Glycoprotein (GP) IIb/IIIa	Risk reducing of myocardial infarction	Eptifibatide
Gonadotropin-releasing hormone r	Sex hormone-responsive cancers	Buserelin
Gp41 HIV enveloppe protein	Anti-HIV	Enfuvirtide
Growth-hormone-releasing hormone r	Diagnostic agent for growth hormone deficiency	Somatorelin
Guanylate cyclase 2C	Irritable bowel syndrome	Linaclotide
Histone deacetylase	T-cell lymphone	Romidepsin
Neurokinin 1 r	Esophageal variceal bleeding	Vapreotide
N-type calcium channels	Chronic pain	Ziconotide
Nucleotide-binding oligomerization domain-containing protein 2 (NOD2)	Osteosarcoma	Mifamurtide
Oxytocin r	Postpartum bleeding	Carbetocin
Parathyroid hormone r	Osteoporosis	Teriparatide
Secretine r	Diagnostic aid for pancreatic exocrine dysfunction and gastrinoma	Secretin (human)
Somatostatin r	Neuroendocrine tumors	Somatostatin
26S proteasome	Multiple myeloma	Bortezomib
ACTH r	Diagnostic agent for cortisol disorder	Cosyntropin

r: receptor.

microbiological quality attributes and assay by LC-UV. Peptide-related substances in chemically synthesized peptide APIs are expected to adhere to thresholds of reporting (0.1%), identification (0.5%) and qualification (1.0%). The European Pharmacopeia reflect the current pharmaceutical quality level for a given marketed drug. Given their multistep manufacturing process resulting in higher impurity amounts, the general reporting, identification and qualification thresholds for peptide impurities were set higher than the corresponding thresholds for organic impurities present in the small organic molecule APIs. Moreover, it is currently perceived that impurities of peptides are of a lesser toxicological concern compared to related impurities of traditional, small molecules. Furthermore, individual impurities should primarily focus on synthesis impurities such as diastereoisomeric and deamidated peptides. The total related impurities level should not pass 5%.

Good manufacturing practices (GMPs) provide guidelines regarding the manufacturing of APIs and FDP, whether it be traditional small organic molecules or peptides [22]. The API guide covers APIs that are manufactured by chemical synthesis, extraction, cell culture/fermentation, by recovery from natural sources, or by any combination of these processes. Specific guidance for APIs manufactured by cell culture/fermentation, *i.e.* biotechnological products, and advanced therapeutic medicinal products (ATMPs) is also available. However, no specific guidance for synthetic peptides is provided. Moreover, a rationale must be designed and documented for the point at which GMP production of the API begins. For synthetic processes, this is known as the point at which "API starting materials" are entered into the process. Starting materials are defined as a raw material, an intermediate or an API that is used in the production of an API and is incorporated as a significant structural fragment into the structure of the API [23]. However, for synthetic peptides, these API starting materials are not specifically and explicitly defined. A number of principles are generally used in the consideration of the API starting material: the API starting material must (i) have sufficient propinquity from the API, *i.e.* generally at least 3 synthesis steps removed from the final API, excluding purifications, (ii) be an essential structural element of the API, (iii) be not too structurally complex, (iv) preferably be a widely commercially available material, *i.e.* not a new or unique material, (v) be a well defined, stable chemical and (vi) have a well defined impurity profile, as well as impurity limits for both related impurities as others such as residual solvents [24]. Considering this rationale, the individually Fmoc-protected amino acids can be considered as API starting materials for the solid-phase peptide synthesis (SPPS) of synthetic peptide API material. Starting material (SM) related impurities have the potential to react in the SPPS process and thus be incorporated as impurities in the final product. Such SM-related impurities can include (i) free amino acids or amino acid derivates, (ii) amino acid contaminants, *i.e.* other amino acids than the desired amino acid, (iii) incorrect enantiomers, (iv) dipeptides or oligopeptides and (v) β-alanine containing contaminants. Moreover, a (toxicity) risk assessment of the starting materials, taking into account different batches and suppliers as these might have different impurity profiles, must be performed. This requires validated analytical methods capable of detecting all possible impurities and degradants present in the starting materials. The quantitative carry-over and purging of these impurities/degradants throughout the API synthesis process and eventually toward the patient is a critical part in the control strategy and risk assessment, which can be demonstrated by means of spiking experiments. Unrelated impurities such as reagents and solvents used in the manufacturing process are unlikely to be incorporated in the final peptide product, due to purification and isolation steps within the synthesis process, and are therefore considered to be non-critical. Exception to this are the reagents and solvents used in these final purification and isolation steps. Moreover, if the final manufacturing steps include lyophilization or evaporation, any impurities can be upconcentrated in the final peptide product. Therefore, the quality attributes of the solvents and reagents used during the final manufacturing steps should be taken into account. However, applicants will also be expected to provide information regarding the origin of the Fmoc-protected

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Table 2
List of marketed peptide drugs.

Peptide name	Brand name	Indication	Administration	FDA approval[a]		EMA approval[b,c]	
				Date	No.	Date	No.
Abarelix	Plenaxis	Prostate cancer	Intramuscular	25/11/2003*	(NDA)21320	1/02/2011	BE384465
Anidulafungin	Eraxis/Ecalta	Antifungal action	Injection	17/02/2006	(NDA)21632	–	–
Anidulafungin			IV Infusion	17/02/2006	(NDA)21948	20/09/2007	EU/1/07/416/001
Atosiban	Tractocile/Antocin/ Atosiban SUN	Premature birth	Injection	–	–	20/01/2000	EU/1/99/124/001
Atosiban			IV Infusion	–	–	20/01/2000	EU/1/99/124/002
Bacitracin	Bacitracin/Baciim/ Baciguent/Baci-RX	Gram positive skin and eye infection	Injection	29/07/1948	(ANDA)060733	–	–
Bacitracin			Ophthalmic	9/11/1971	(ANDA)061212	29/03/2004	BE260811
Bentiromide	Chymex	Exocrine pancreatic insufficiency diagnostic	Oral	29/12/1983*	(NDA)18366	–	–
Bivalirudin	Angiomax/Angiox	Anticoagulant	Intravenous	15/12/2000	(NDA)20873	20/09/2004	EU/1/04/289/001
Bleomycin	Blenoxane	Cancer	Injection	31/7/1973*	(NDA)050443	11/12/1970	BE058116
Bortezomib	Velcade	Multiple myeloma	Intravenous, subcutaneous	13/05/2003	(NDA)21602	26/04/2004	EU/1/04/274/001
Buserelin			Nasal	–	–	13/06/1985	BE131826
Capreomycin	Capastat	Antibiotic	Injection	2/07/1971	(NDA)50095	–	–
Carbetocin	Duratocin/Lonactene/ Pabal	Postpartum bleeding	Injection	–	–	2/04/2007	BE293185
Carfilzomib	Kyprolis	Multiple myeloma	Intravenous	20/07/2012	(NDA)202714	3/06/2008	EU/3/08/548
Carfilzomib	Kyprolis	Multiple myeloma	IV infusion	20/07/2012	(NDA)202714	3/06/2008	EU/3/08/548
Caspofungin	Cancidas	Antifungal action	IV infusion	26/01/2001	(NDA)21227	24/10/2001	EU/1/01/196/001
Ceruletide	Tymtran	Paralytic ileus	Injection	<1/1/1982*	(NDA)18296	–	–
Cetrorelix	Cetrotide	Ovulation Induction	Injection	11/08/2000	(NDA)21197	13/04/1999	EU/1/99/100/001
Cobicistat	Stribild	HIV infection	Oral	27/08/2012	(NDA)203100	24/05/2013	EU/1/13/830/001
Colistin	Coly-mycin S	Antibiotic	Oral, ophthalmic	17/05/1962	(NDA)050356	–	–
Corticorelin	Acthrel	Diagnostic agent	Injection	23/05/1996	(NDA)20162	–	–
Corticotropin	H.P. Acthar Gel	Diagnostic agent	Injection	29/04/1952	(NDA)08372	–	–
Cosyntropin	Cortrosyn	Diagnostic agent	Injection	22/04/1970	(NDA)16750	–	–
Cyclosporin	Neoral	Immunosuppressant	Oral	14/07/1995	(NDA)50715	–	–
Cyclosporin			Oral	14/07/1995	(NDA)50716	–	–
Dactinomycin	Cosmegen	Antibiotic	Injection	10/12/1964	(NDA)50682	–	–
Daptomycin	Cubicin	Antibiotic	IV infusion	12/09/2003	(NDA)21572	19/01/2006	EU/1/05/328/001
Degarelix	Firmagon	Prostate cancer	Subcutaneous	24/09/2008	(NDA)22201	17/02/2009	EU/1/08/504/002
Depreotide	Neo Tect Kit	Diagnostic agent	Injection	3/8/1999*	(NDA)21012	–	–
Depreotide	NeoSpect	Diagnostic agent	Injection	–	–	29/11/2000	EMEA/H/C/000263
Desmopressin	DDAVP/Minirin	Diabetes insipidus	Nasal	21/02/1978	(NDA)17922	1/07/1975	BE095706
Desmopressin	DDAVP/Minirin	Diabetes insipidus	Injection	30/03/1984	(NDA)18938	8/04/1986	BE133874
Desmopressin	DDAVP/Minirin	Diabetes insipidus	Oral	6/09/1995	(NDA)19955	11/03/1997	BE181386
Edotreotide	Octreother	Neuroendocrine tumor	Injection	–	–	4/12/2008	EU/3/08/589
Enfuvirtide	Fuzeon	HIV infection	Subcutaneous	13/03/2003	(NDA)21481	27/05/2003	EU/1/03/252/001
Eptifibatide	Integrilin	Antiplatelet drug	Injection	18/05/1998	(NDA)20718	1/07/1999	EU/1/99/109/001
Exenatide	Byetta	Diabetes type 2	Subcutaneous	28/04/2005	(NDA)21773	20/11/2006	EU/1/06/362/001
Exenatide	Bydureon	Diabetes type 2	Subcutaneous	27/01/2012	(NDA)22200	17/06/2011	EU/1/11/696/001
Ganirelix	Orgalutran	Ovulation Induction	Injection	29/07/1999	(NDA)21057	17/05/2000	EU/1/00/130/001
Glucagon	Glucagon	Hypoglycemia	Injection	11/09/1998	(NDA)20928	20/04/1995	BE169145
Glutathion	BSS Plus	Inborn errors of metabolism of glutathione, cystic fibrosis	Irrigation	28/10/1981	(NDA)18469	–	–
Glutathion	Endosol extra		Irrigation	27/11/1991	(NDA)20079	–	–
Gonadorelin	Factrel	Ovulation Induction	Injection	30/09/1982*	(NDA)18123	–	–
Goserelin	Zoladex	Prostate cancer	Implantation	29/12/1989	(NDA)19726	7/09/1987	BE138731
Gramicidin	Neosporin	Bacterial infections	Ophthalmic	3/07/1968	(ANDA)60582	1/04/1962	BE049061
Histrelin	Vantas	Prostate cancer	Subcutaneous	12/10/2004	(NDA)21732	11/02/2010	BE362293

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Table 2 (*Continued*)

Peptide name	Brand name	Indication	Administration	FDA approval[a]		EMA approval[b,c]	
				Date	No.	Date	No.
Histrelin	Supprelin LA		Subcutaneous	3/05/2007	(NDA)22058	–	–
Icatibant	Firazyr	Hereditary angioedema	Subcutaneous	25/08/2011	(NDA)22150	11/07/2008	EU/1/08/461/001
Lanreotide	Somatulin depot	Acromegaly	Subcutaneous	30/08/2007	(NDA)22074	11/10/1996	BE179006
Leuprolide	Lupron	Prostate cancer	Injection	30/03/1985	(NDA)19943	–	–
Linaclotide	Linzess	Irritable Bowel Syndrome	Oral	30/08/2012	(NDA)202811	26/11/2012	EU/1/12/801/001
Liraglutide	Victoza	Diabetes type 2	Subcutaneous	25/01/2010	(NDA)22341	30/06/2009	EMEA/H/C/001026
Lixisenatide	Lyxumia	Diabetes type 2	Subcutaneous	–	–	1/02/2013	EU/1/12/811/001
Lucinactant	Surfaxin	Infant Respiratory Distress Syndrome	Intrathecal	6/03/2012	(NDA)21746	–	–
Lypressin	Diapid	Diabetes insipidus	Nasal	*	(NDA)15755	–	–
Micafungin	Mycamine	Antifungal action	Injection	16/03/2005	(NDA)21754	25/04/2008	EU/1/08/448/001
Mifamurtide	Mepact	Osteosarcoma	IV infusion	–	–	6/03/2009	EU/1/08/502/001
Nafarelin	Synarel	Endometriosis	Nasal	13/02/1990	(NDA)19886	–	–
Nesiritide	Natrecor	Heart failure	IV infusion	10/08/2001	(NDA)20920	–	–
Octreotide	Sandostatin	Acromegaly	Injection	21/10/1988	(NDA)19667	24/02/1998	BE191685
Oxytocin	Oxytocin	Labor induction	Injection	29/04/1980	(NDA)18243	1/07/1961	BE031823
Oxytocin	Pitocin	Labor induction	Injection	19/11/1980	(NDA)18261	–	–
Pasireotide	Signifor	Cushing's disease	Subcutaneous	14/12/2012	(NDA)200677	24/04/2012	EU/1/12/753/001
Pentagastrin	Peptavlon	Diagnostic agent	Injection	26/07/1974*	(NDA)17048	–	–
Pentetreotide	Octreoscan	Diagnostic agent	Injection	2/06/1994	(NDA)20314	–	–
Polymyxin B	Cortisporin	Antibiotic	Topical	26/03/1957	(NDA)50168	–	–
Polymyxin B			Topical	16/04/1963	(NDA)50218	1/07/1961	BE056585
Polymyxin B			Ophthalmic	9/12/1975	(NDA)50479	1/01/1965	0241IS0118F13/7
Pramlintide	Symlin	Diabetes type 1 and 2	Subcutaneous	16/03/2005	(NDA)21332	–	–
Protirelin	Thypinone	Diagnostic agent	Injection	5/11/1976	(NDA)17638	–	–
Romidepsin	Istodax	T-cell lymphoma	IV infusion	5/11/2009	(NDA)22393	–	–
Salmon calcitonin	Miacalcin	Osteoporosis	Injection	3/07/1986	(NDA)17808	25/01/1977	BE173031
Salmon calcitonin	Miacalcin	Osteoporosis	Nasal	17/08/1995	(NDA)20313	–	–
Saralasin	Sarenin	Hypertension	Injection	*	(NDA)18009	–	–
Secretin human	Chirhostim	Diagnostic agent	IV infusion	9/04/2004	(NDA)21256	–	–
Secretin porcine	Secroflo	Diagnostic agent	IV infusion	4/04/2002	(NDA)21209	–	–
Sermorelin	Geref	Growth hormone deficiency	Injection	28/12/1990*	(NDA)19863	–	–
Sincalide	Kinevac	Diagnostic agent	Injection	21/07/1976	(NDA)17697	–	–
Somatorelin	GHRH	Diagnostic agent	Injection	–	–	26/01/1993	BE160133
Somatostatin	Somatostatin	Oesophageal varices	Injection	–	–	20/01/1986	BE133375
Teduglutide	Gattex	Short bowel syndrome	Subcutaneous	21/12/2012	(NDA)203441	30/08/2012	EU/1/12/787/001
Teicoplanin	Targocid	Antibiotic	Injection	–	–	8/03/1989	BE147034
Telavancin	Vibativ	Antibiotic	IV infusion	11/09/2009	(NDA)22110	–	–
Teriparatide	Forteo	Osteoporosis	Subcutaneous	26/11/2002	(NDA)21318	10/06/2003	EU/1/03/247/001
Terlipressin	Glypressin	Oesophageal varices	Injection	–	–	11/04/1990	BE150717
Tesamorelin	Egriphta	HIV-associated lipodystrophy	Subcutaneous	10/11/2010	(NDA)22505	25/01/2011	EMEA-001029-PIP01-10
Thymalfasin	Zadaxin	Hepatocellular carcinoma	–	–	–	30/07/2002	EU/3/02/110
Triptorelin	Trelstar	Hormone-responsive cancers	Intramuscular	15/06/2010	(NDA)20715	14/04/1997	BE182454
Urofollitropin	Bravelle	Ovulation Induction	Subcutaneous	19/12/2002	(NDA)21484	10/09/2007	BE302687
Vancomycin	Vancocin	Antibiotic	Oral	15/04/1986	(NDA)50606	5/10/1982	BE121125
Vasopressin	Pitressin	Diabetes insipidus	Injection	*	(NDA)03402	–	–
Ziconotide	Prialt	Pain	Intrathecal	28/12/2004	(NDA)21060	21/02/2005	EU/1/04/302/001

* Discontinued.

[a] http://www.accessdata.fda.gov/scripts/cder/drugsatfda/.

[b] http://www.ema.europa.eu/ema/index.jsp?curl=pages/includes/medicines/medicines_landing_page.jsp&mid=.

[c] http://www.fagg-afmps.be/nl/items-HOME/gegevensbanken/.

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amino acids, *i.e.* (human) hair, animal, plant, fermentation, to ascertain the risk from bovine spongiform encephalopathy (BSE) and transmissible spongiform encephalopathies (TSE) [23].

Both pharmaceutical development guidance and process analytical technologies (PAT) acknowledge that the final product quality should be built in the manufacturing process, rather than tested once manufacturing is complete [25,26]. The principle of quality by design (QbD) uses the understanding of the manufacturing process to define the design spaces, in which critical process parameters are identified and controlled based upon risk assessment, thus effectively designing the final product quality into the manufacturing process [27]. PAT is an important tool for the application of QbD as it continuously monitors the manufacturing process through measurements of critical quality attributes of raw and intermittent materials, with the goal of ensuring adequate final product quality [28]. Currently, the QbD concepts are not consistently implemented in the manufacturing process of peptide APIs, thus offering the peptide manufacturers the opportunity of further optimizing their manufacturing process. In order to aid in the implementation of these QbD principles, this review will provide a structured overview of the most commonly observed related impurities in peptide medicines, encompassing the active pharmaceutical ingredients (API or drug substance) as well as the finished drug products. These impurities originate from SPPS, intrinsic peptide degradation mechanisms and peptide–excipient interaction. Moreover, the formation of these impurities will also be discussed, as well as ways to reduce their formation, thus effectively building-in the desired peptide quality. Not only is control of these peptide-related impurities critical for the manufacturing of already approved and clinically used peptide-drugs, these impurities also possess the capability of greatly influencing initial functionality studies during drug discovery phase, possibly resulting in false positive results, thus demonstrating the functional importance of peptide impurity control [29].

2. Peptide synthesis

Depending on the peptide size, *i.e.* number of amino acid residues, different peptide production techniques can be used. Recombinant DNA techniques are generally applied for the synthesis of large peptides, *e.g.* insulin, calcitonin and glucagon, whilst enzymatic synthesis is restricted to synthesis of peptides typically containing less than 10 amino acid residues, *e.g.* LVVH-7, VV-hemorphin-7 and glutathione [30]. However, chemical synthesis remains the gold standard for the production of most peptides, ranging from 5 to 50 amino acid residues. This synthesis process was originally performed in solution, but became increasingly popular after introduction of the solid-phase peptide synthesis (SPPS) mode by Merrifield, and continues to be further improved [31,32]. These chemical synthesis techniques have been thoroughly discussed in other papers, but a short summary is given here as its understanding is critical for the evaluation of possible synthesis-related impurities.

Generally, in SPPS, amino acids are linked to each other during individual coupling steps, thus constructing the desired peptide sequence. This occurs while the carboxylic end of the sequence is covalently attached to a solid support matrix. The solid support is a synthetic polymer derivatised with functional groups such as hydroxyl groups (—OH) through linkers. These groups can easily react with the carboxyl group of a N-α-protected amino acid, hereby establishing a covalent bond to the resin. The transient protection of the amino group and permanent protection of the side chain group of the incoming amino acid is necessary to avoid undesired reactions, *e.g.* the nucleophilic N-terminus of the added amino acid should not react with the carboxyl carbon of the same amino acid during the coupling reaction. The next step in the SPPS process is the removal of the transient protecting group of the resin-coupled amino acid providing a free α-NH_2 end, followed by coupling of the next N-α-protected amino acid in the presence of activators. Through these deprotections and coupling steps, accompanied with the necessary washing steps to remove reagents and byproducts, the desired peptide sequence is synthesized from C-terminal to N-terminal end. At the end of the SPPS process, a strong acid is added to cleave the bond between the C-terminal amino acid and the polymer support, thereby also dissolving the peptide in the reaction medium. This final reaction step can be combined with the removal of permanent side chain protecting groups [30,33].

The SPPS originally developed by Merrifield in 1963 used tert-butyloxycarbonyl (t-Boc) as transient (N-α-) protecting group and benzyl (Bzl) as permanent (side chain) protecting group [32]. After removal of the transient protecting group by addition of trifluoroacetic acid (TFA), the positively charged amine that is generated is neutralized with a base, *e.g.* triethylamine, before coupling to the next incoming t-Boc protected amino acid. At the end of the synthesis, the last Boc-group, the permanent (side chain) protecting groups and carboxy-terminal link with the resin are removed by acidolysis with hydrofluoric acid (HF). Both the transient and permanent protecting groups, as well as the peptide–resin bond are acid labile. So certain peptide chains will be cleaved from the resin during each synthesis step and lost in the following washing step. Moreover, due to the loss of the side chain protecting groups, undesired products will be formed as well. In a search for more suitable protecting groups, the 9-fluorenylmethoxycarbonyl group (Fmoc) was introduced by Carpino and Han in 1972 as a transient protecting group [34]. Fmoc derivates are acid-stable but sensitive to mild base, *e.g.* piperidine. The advantages of the Fmoc strategy compared to the t-Boc strategy are obvious. In this orthogonal strategy, the acid labile protecting groups on the side chains (*e.g.* tBu) and the peptide–resin link remain unaffected during each base-catalyzed deprotecting step. Depending on the amino acid residue, different permanent side-chain protecting groups can be used [30,35,36]. Tertiary butyl (tBu), trityl (Trt) and t-Boc are among the most often used permanent protection groups. Currently, Fmoc-based SPPS is the most often used technique to produce synthetic peptides and its general mechanism is given in Fig. 1 and discussed further below.

2.1. Linkage to resin

As discussed earlier, Fmoc SPPS involves assembly of a peptide chain that is anchored to a polystyrene resin through a linker, usually an ester bond. This ester is more sensitive to acids by electron-donating groups such as alkoxy, phenyl, alkoxyphenyl and halogens. The nature of the C-terminus of the target peptide determines the peptide-linker bond. For the synthesis of a peptide with a free C-terminus, a 2-chlorotrityl chloride resin can be used. This is a polystyrene resin with a trityl-based linker. The 2-chlorotrityl chloride resin reacts very efficiently with Fmoc–amino acid anions of the first added amino acid. Any unreacted chlorotrityl groups are capped by conversion to methoxy groups by reaction with methanol. The linker bond is sensitive to a weak acid, which is generally combined with nucleophiles in a cleaving solution. For synthesizing amidated C-termini, the Rink amide resin is used. This has a 2,4-dimethoxy-substituted benzhydrylamine linker affixed to the polystyrene matrix through an ether linkage. The carboxamido–methyl bonds, anchoring the peptide chains, have been rendered sensitive to TFA by the methoxy groups. The linker is presented as the Fmoc derivative, so the amino group has to be deprotected for synthesis before the first amino acid can be added [33,36].

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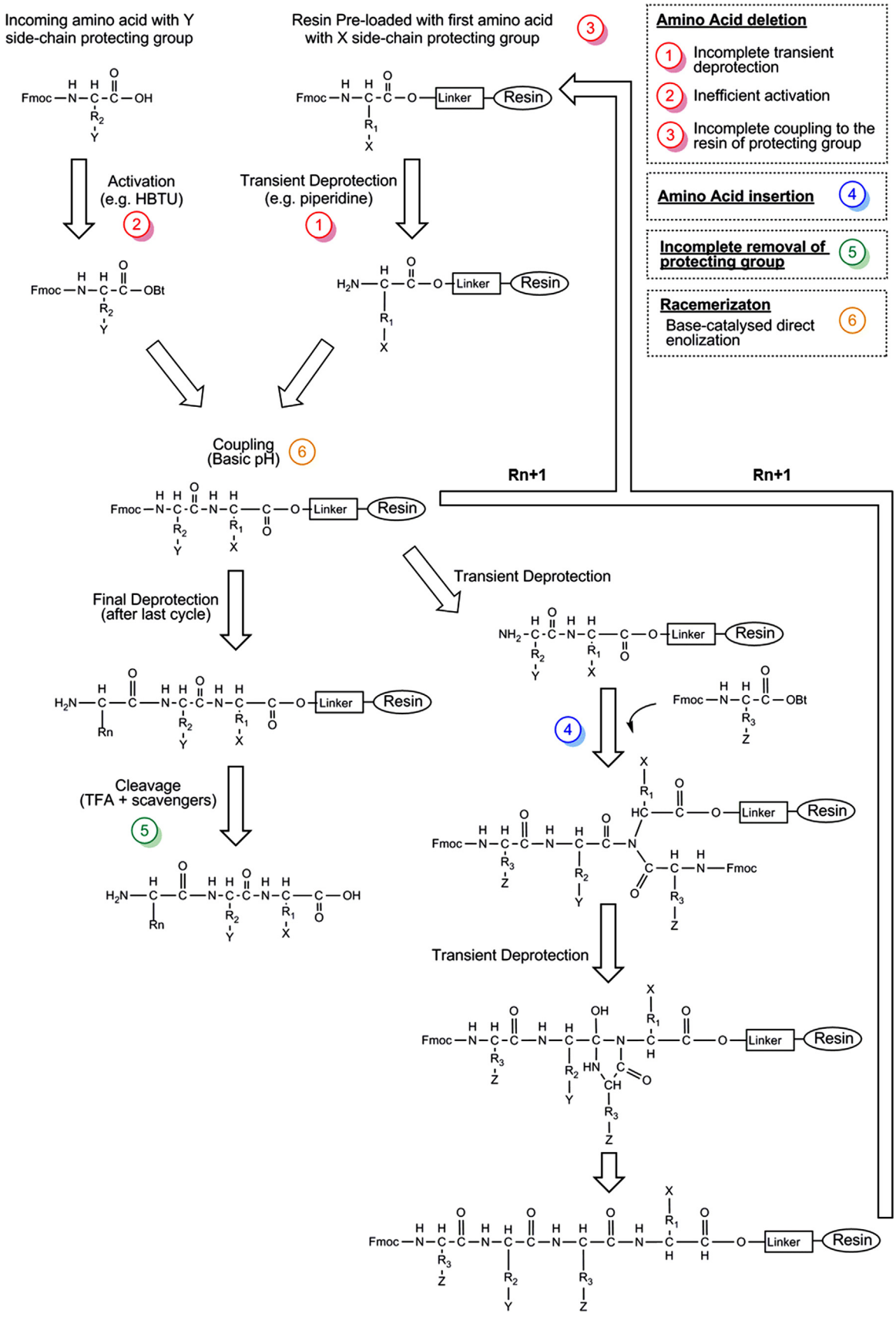

Fig. 1. Schematic overview of Fmoc solid-phase peptide synthesis (SPPS), including related impurity formation.
Adapted from N.L. Benoiton, Chemistry of Peptide Synthesis. Taylor & Francis, Boca Raton, 2006.

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2.2. Transient deprotection

The Fmoc protecting group is removed before each amino acid coupling by a base-induced β-elimination. The C-9 hydrogen atom on the Fmoc group is activated by the aromaticity of the rings. Abstraction of this proton by the piperidine is followed by a rearrangement of electrons leading to removal of the leaving group, the carboxamido anion, with generation of CO_2 and dibenzofulvene. The latter molecule has an exocyclic double bond, which reacts with the nucleophilic piperidine [33,36].

2.3. Activation and amino acid coupling

The Fmoc protected amino acid is linked with its carboxylic group to the free amino function of the last amino acid in the growing peptide chain. The nucleophilic amine attacks the electrophilic carbonyl group to form an amide bond. This reaction is a nucleophilic acyl substitution reaction as a nucleophile (the amine) replaces the substituent (the OH group) that was attached to the carbonyl group in the reactant and leaves the molecule. This reaction only occurs when activating the amino acids by transforming them into esters, anhydrides or acyl halides that easily react with an amine to form an amide plus an alcohol, carboxylate or halide, respectively. In this way, the esterification of the Fmoc protected amino acid, where the OH group is replaced with a good leaving group, also increases the electrophilicity of the carbonyl group. [33,36]. However, this activation step is associated with the formation of racemization impurities.

Uronium salts such as O-Benzotriazole-N,N,N′,N′-tetramethyluroniumhexafluorophosphate (HBTU) are commonly used to form activated esters, surpassing the carbodiimides, *e.g.* diisopropylcarbodiimide (DIC). HBTU converts the Fmoc amino acid into the active benzotriazole ester (OBt) in the presence of a base. The base generates the carboxylate of the amino acid that can attack the reagent. The bases used for binding these protons are usually tertiary amines that are very suitable because they are very weak nucleophiles that do not compete for the electrophiles. This gives the acyloxycarbenium intermediate that is converted to the benzotriazole ester while tetramethylurea is liberated, which is miscible with polar solvents. The activated ester can be easily substituted by the amine function with OBt as a leaving anion [33,36].

2.4. Resin cleavage

In this final step, the peptide is cleaved from the resin and the permanent side chain protecting groups are removed by acidolysis. The removal of the permanent protecting groups by acidolysis involves protonation, followed by spontaneous rupture of the formed cation. Trityl, tert-butyl and arylsulfonylium ions but also the resin-bound species are left as cations. By adding TFA, they are trapped. The cation-trifluoroacetate produced is an alkylating agent and has a tendency to attack nucleophilic centers on the side chains of the peptide, *e.g.* hydroxyl groups on tyrosine, serine and threonine, resulting in unwanted impurities. For this reason, one or more nucleophiles are added to the acid to trap the generated cations. These scavengers are bases that are weak enough not to be protonated by the acid, and usually include thiol compounds, *e.g.* 1,2-ethanedithiol, 2-mercaptoethanol, phenol compounds, *e.g.* anisol, p-cresol, and water. Also trialkylsilanes derivatives such as triisopropylsilane (TIS) show good efficacy in trapping tert-butyl, trityl, and arylsulfonyl carbocations [33,36].

Alternatively to conventional heating, microwave assisted SPPS introduces energy into the synthesis mixture, resulting in acceleration of the reaction. The applied electromagnetic energy is converted into thermal energy due to dielectric polarization, resulting in reduced reaction time and increased synthesis yield as well as purity [37,38].

2.5. Fermentation

Beside solid-phase synthesis, some peptides can also be derived from microorganisms by means of fermentation. These peptides are secondary metabolites produced by bacteria or fungi and generally belong to the antibiotic drug class. On an industrial scale the source microorganism is cultivated in large bioreactors containing the optimal growth-medium to ensure maximum yield. Once this process is complete, the metabolites are extracted and purified [39]. Examples of antibiotic peptides obtained by fermentation are: daptomycin, which is marketed in the USA under the trade name Cubicin®, and fusafungine, a mixture of enniatins used to treat upper respiratory infections (Locabiotal®) [40,41].

3. Analytical methods for peptide impurity/degradant profiling

3.1. Pharmacopoeial methods

Peptide API monographs are given in different pharmacopeias, often containing a suitable analytical method for the determination of related substances [20,21,30]. The majority of these methods are traditional HPLC-UV methods, using octadecylsilyl (C18) silica stationary phases with a variety of dimensions, *i.e.* difference in column length, diameter, pore size. The Ph. Eur. methods are relatively strict, as only minor changes are permitted, *e.g.* column length and internal diameter dimension may vary ±70% and ±25%, respectively. The particle size can be reduced by as much of 50%, but no increase is permitted [42]. The same variations are allowed by the United States Pharmacopeia (USP) [43]. However, in both pharmacopeias, the fundamental chemical nature of the stationary phase, *e.g.* C18, may not be modified [42,43]. Monographs may also contain an additional section on oligomerization products, where size exclusion chromatography (SEC) methods are used, *e.g.* insulin monograph [44]. Identification of the peptide and of the related impurities is based upon relative retention times and use of standard references.

The USP also provides traditional HPLC-UV methods for 'related compounds' and 'chromatographic purity'. The majority of these methods are based upon the use of octadecylsilyl (C18) silica stationary phases (L1). However, other column chemistries are also used, *e.g.* octylsilane C8 (L7) for human glucagon, irregular or spherical totally porous silica gel having a chemically bonded, strongly acidic cation-exchange coating (L9) for calcitonin and a strong cation exchange resin made of porous silica with sulfopropyl groups (L52) for aprotinin [43]. As in the Ph. Eur., identification of the related substances is based upon relative retention times. However, the identification test in USP for certain peptide APIs, *e.g.* desmopressin and gonadorelin acetate, uses mass spectrometry detection, which is currently not included in the Ph. Eur. A critical assessment of the peptide monographs, as well as suggestions for further improvements were made by Vergote et al. [21]. The major potential improvement for these peptide monographs is the inclusion of MS-based detection techniques, which is expected to be introduced in the near future, as suitable, easy-to-use and economically affordable MS equipment like the single quadrupole detector will become available for Quality Control purposes [45].

3.2. Methods used in discovery, research and development

3.2.1. UHPLC and fused-core chromatography

Ultra High Performance Liquid Chromatography (UHPLC) can be seen as an evolution of traditional HPLC. UHPLC uses smaller

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fully porous particle sizes, typically sub-2 μm in diameter, which significantly reduces the Height Equivalent to a Theoretical Plate (HETP) and results in higher efficiency and flatter Van Deemter curve. However, this decrease in particle size also results into an increase in back pressure, which was overcome with the development of newer chromatographic equipment. The major advantages of this technique are improved sensitivity and resolution, combined with significant reduction in analysis time and solvent consumption [46]. Several UHPLC peptide assays have already been reported in the literature, mostly focusing on peptide stability in different matrices [46–49]. Furthermore, stability indicating methods, focusing on peptide degradants or impurities are also reported [50–53]. Depending on the objective, *i.e.* assay or stability-indicating method, peptide concentration and matrix complexity, UHPLC is coupled to PDA or MS detection.

Alternatively, (U)HPLC chromatography with fused-core particles uses particles with a solid, inert core and a thin porous outer shell, *i.e.* superficially porous particles (SPP). With superficially porous particles, larger solutes can move rapidly in and out of the thin porous shell, resulting in reduced band broadening at higher mobile phase velocities for greater separation speeds. These SPP sizes range from 1.3 μm to 5 μm, in which the porous shell is usually around 0.5 μm thick. High efficient separations, similar to UHPLC separations with sub-2 μm fully porous particles, are obtained with 2.7 μm SPP at relatively low back pressure. Given the low back pressure, these chromatographic separations can still be performed with conventional HPLC instruments. Various fused-core chromatographic methods, coupled to PDA or MS detection, for peptide assay and peptide stability determination have already been published [54–60]. Given permitted variations in particle size dimension in the Ph. Eur. and USP, columns with 2.7 μm SPP technology are typically allowed to be used in pharmacopoeial peptide analysis, giving this SPP technology a major advantage over the sub-2 μm UHPLC technology which falls outside the permitted pharmacopoeial method adaptations, thus requiring formal variation-activities.

3.2.2. Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) uses a low viscosity and highly diffusing mobile phase, mostly CO_2 in a supercritical state, resulting in lower pressure along a packed chromatographic column, thus allowing the use of higher flow rates when compared to traditional HPLC. Moreover, SFC is seen as a green technology since it requires a smaller amount of organic solvent compared to other liquid chromatography techniques. This is especially appealing for preparative-scale chromatography since less time and energy are required to remove solvent and isolate the analyte. Traditionally, use of relatively non-polar CO_2 has limited the SFC applications to separations of non-polar and low-polar compounds. In order to expand the application range to more polar compounds such as peptides, several strategies have been employed: (i) use of relatively polar fluids, *e.g.* NH_3, SO_2, (ii) use of a polar modifier in CO_2 and (iii) addition of a highly polar or ionic component to the modifier, *e.g.* TFA or dichloroacetic acid. Furthermore, given the high volatile nature of the mobile phases, SFC technology is extremely compatible with MS detection. Tognarelli et al. reported chromatographic separation of five peptides, ranging in molecular weights from 238.2 to 1046.2, by SFC in less than 12 min, compared with 50 min using HPLC [61]. Patel et al. used SFC to separate water-soluble isomeric peptides, whereas Zheng et al. demonstrated SFC/MS separation of peptides up to 40 amino acids in length, comprising acid and basic residues [62–64]. An alternative for these aforementioned chromatographic peptide separation techniques is capillary electrophoresis (CE) [65]. Here, samples are placed in an electrolyte solution and separation is achieved under the influence of an electric field. Given their nature, peptides and proteins are ideally suited for capillary electrophoresis separation. Further information regarding CE separation of peptides can be found in the extensive review by Kašička et al. and will not be discussed in further details in this review [66].

3.2.3. Spectroscopic techniques

Circular dichroism (CD) is considered as one of the most valuable technique for examining the structure of peptides and proteins in solution. CD refers to the differential absorption of counter-clockwise (left-handed, L) and other clockwise (right-handed, R) circularly polarized light. From this, complementary structural information can be obtained from a number of spectral regions. In peptides, the chromophores of interest include the peptide bond (absorption below 240 nm) which is used to determine secondary structure, aromatic amino acid side chains (absorption in the range 260–320 nm) and disulphide bonds (weak broad absorption bands centered around 260 nm) [67]. The advantages of CD are multiple as it is a non-destructive, fast and simple technique, consuming small sample quantities. Alternatively, Fourier Transform Infrared (FT-IR) measures the absorption of energy by vibrating chemical bonds (primarily stretching and bending motions). In order to characterize the secondary structure of peptides and proteins, the different wavenumbers at which the amide peptide bonds show absorption can be used. Nine characteristic IR absorption bands for the amide bond in peptides and proteins are obtained. These are called Amide A, B and I–VII in order of decreasing frequency. The amide bands I (80% C═O stretch, near 1650 cm^{-1}), II (60% N—H bend and 40% C—N stretch, near 1550 cm^{-1}), and III (40% C—N stretch, 30% N—H bend, near 1300 cm^{-1}) are generally employed to study peptide and protein structure [68]. As both CD and IR techniques are of spectroscopic nature following Lambert–Beer's law, signals arising from low concentrated impurities will be added to the larger signal obtained from the parent peptide. Therefore, in order to fully exploit for example CD as a peptide impurity profiling technique, the use of a well characterized peptide reference standard is required or separation/isolation of the impurity from the parent peptide, which can be achieved by online coupling of CD detection to HPLC [69].

The β-amyloid peptide, comprising 42 amino acid residues, of healthy individuals and patients suffering from Hereditary Cerebral Hemorrhage with amyloidosis-Dutch-type (HCHWA-D) were compared by Fabian et al. by FT-IR and CD [70]. The Dutch-type β-amyloid peptide differs in one amino acid, *i.e.* mutation of Glu to Gln, resulting in an altered secondary structure when present in membrane mimicking solvents: the mutant Dutch-type β-amyloid peptide, containing the Gln residue, showed a significantly higher β-sheet content observed by analysis of the amide I region (1700–1600 cm^{-1}). This amide I region is extremely useful for this secondary structure analysis as different hydrogen bond patterns give rise to shifts in C═O stretching frequencies. Alternatively, the intensity of the CD signals obtained at 208 nm was used to estimate the α-helix content of both peptides and confirmed the FT-IR results. Furthermore, changes in pH were less effective in eliminating the β-sheet conformation for the mutant peptide, suggesting that its conformation is energetically favored, compared to the human-type. This β-sheet plays an important role in fibril formation, which is associated with Alzheimer's disease. Insulin fibrillation at 60 °C, induced by vortexing, was examined by Loksztejn et al. [71] using CD measurements over the course of time. The gradual transition of the native predominantly α-helical structure into aggregated β-sheets was determined by the decreasing double minima at 208 and 222 nm and the appearance of a single minimum at 216 nm. Using both FT-IR and CD, Banerjee et al. [72] found that a small nine residue peptide interferes with the fibrillation of insulin. The peptide stabilized the native-like

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secondary structure of insulin, hereby delaying the formation of the fibril nucleus.

Cell penetrating peptides (CPPs), generally described as short amphipathic or purely cationic peptides of less than 30 amino acids which possess a positive net charge, are able to penetrate biological membranes and transfer covalently or non-covalently attached bioactive cargoes into cells [73]. A conformational analysis by CD of 10 well-known CPPs (including Tat, R9, penetratin, among others) in different media, highlighted that these CPPs are random coils in water, but they become structured or partially structured in small unilamellar vesicles (SUV) prepared from negatively charged phospholipids (DOPG). Neutral (zwitterionic) phospholipids (DOPC) or a mixture of DOPC, sphingomyelin and cholesterol (40/40/20) did not induce formation of an α-helix or β-sheet. Many hydrophobic CPPs appear insensitive to sequence scrambling. This suggests that amino acid composition and their surroundings, *i.e.* membrane mimicking solvents, drive the cell-uptake of these peptides, rather than the actual primary sequence [74,75].

Alpha-helical peptides, such as enfuvirtide and C34, are derived from a 36 amino acid region of the carboxyl-terminal heptad repeat (C-HR) of gp41, an HIV-1 transmembrane envelope glycoprotein, which plays a central role in the fusion of HIV-1 with host cells. Enfuvirtide and C34 prevent the formation of a 6-helical bundle, which is comprised of a trimer of dimers formed from the amino-terminal heptad repeat (N-HR) and the carboxyl-terminal heptad repeat (C-HR) in an antiparallel orientation. Six-helix formation by physiological gp41 enables host cell and virus membranes to contact and fuse, enabling the virus entry into the cells. Therefore, inhibition of the formation of this 6-helical bundle prevents fusion of HIV-1 and targeted host cell membranes. The interaction between the fusion inhibiting peptides, *e.g.* C34 and SC34EK, and the N36 peptide, representing the N-HR of the HIV-1 gp41 surface protein, was studied by circular dichroism in function of temperature. The authors found a strong correlation between the *in vitro* anti-HIV-1 activities of these peptides and the thermostability, determined by CD measurement, of the 6-helical bundles that are formed with these peptides [76,77]. This peptide/protein temperature denaturation experiment, using the sole peptide/protein or in combination with its target receptor can be used as a fundamental quality attribute as it results in a numerical T_m value, which can be correlated with its biological function. At this time, the pharmacopoeial analysis of peptide/proteins only focuses on primary structures through peptide mapping, thus neglecting secondary and higher structures. Pharmacopoeial inclusion of denaturation experiments, *e.g.* by CD detection, will not only provide this higher structural information, but also allow for the determination of a numerical functional quality attribute, *i.e.* a T_m value, which can then be compared to a T_m specification acceptance range.

Optical rotation dispersion (ORD) measures the ability of a sample to rotate linear polarized light, in function of the wavelength [78]. This technique, closely related to CD, was very popular in the past for the conformational analysis of peptides and proteins, but is now largely surpassed by more advanced techniques, such as CD [79–81]. Vibrational circular dichroism (VCD) can be viewed as a combination of the CD and IR approaches, utilizing the bandshape variability of CD and the frequency resolution of IR. This technique can be used for both quantitative and qualitative analyses of peptides and proteins [82,83]. Alternatively, two-dimensional IR (2D-IR) can also be used, as shown by the review of Kim et al. [84].

Raman spectroscopy relies on inelastic scattering (Raman scattering) of monochromatic laser light, in the visible, near infrared or near ultraviolet range. The light interacts with molecular vibrations, phonons or other excitations in the system, resulting in the energy of the laser photons being shifted up or down. The shift in energy gives information about the vibrational modes in the system, providing similar but complementary information to IR absorption spectroscopy. The presence of specific side-chain marker bands for the cysteine sulfhydryl group (2500–2600 cm^{-1}) and methionine sulfoxide (1010 and 704 cm^{-1}, corresponding to S=O and C–S stretch) can be used as selective markers for peptide/protein oxidation [85]. Traditional Raman as well as more advanced Raman techniques such as surface-enhanced Raman (SER), resonance Raman and transient UV Raman have been used to study various conformational peptide aspects [86–89].

3.2.4. NMR

Nuclear magnetic resonance (NMR) spectroscopy measures the absorption of electromagnetic radiation of a specific frequency by an atomic nucleus that is placed in a strong magnetic field. In 2002, Wüthrich was awarded the Nobel Price in Chemistry for his involvement in the development of this technique, which can provide unique information about peptide structure, dynamics, hydration and folding in the solution state [21,90–93]. Therefore, the Ph. Eur. utilizes NMR for identity confirmation based on profile comparison *versus* a reference NMR spectrum, *e.g.* buserelin and goserelin. Furthermore, online HPLC coupling of NMR detection permits peptide impurity profiling [93]. However, due to the structural complexity of most peptides, especially those larger than 15 amino acids, ^{1}H and ^{13}C NMR spectra are full of partially overlapping signals that can only be assigned by means of two-dimensional techniques, *i.e.* 2D-COSY (2D Correlated spectroscopy) and 2D-TOCSY (2D Total Correlation Spectroscopy). In general, as NMR is a time consuming technique and the peptides are in solution, special attention must be paid to possible degradation and aggregation.

4. Impurity classes

Structurally related impurities present in peptide drug substances can have significant consequences on its functional biological function. Hemopressin (Hp), a nonamer peptide (PVNFKLLSH) derived from the α_1 chain of hemoglobin, is an antagonist of the CB_1 (cannabinoid) receptor and exhibits antinociceptive activity. Gomes et al. identified two Hp-related impurity peptides, *i.e.* VDPVNFKLLSH (VD-Hp) and RVDPVNFKLLSH (RVD-Hp) which interestingly were found to be agonists of the receptor [94]. This functionality influence of the peptide impurity is sometimes missed in the early phases of pharmaceutical R&D, especially as usually crude peptide mixtures, containing different types and levels of impurities, are used in functionality experiments, *e.g.* receptor binding or tissue organ baths. Alternatively, as mentioned earlier, physical aggregation of insulin monomers, will lead to a insulin fibrillation, which in turn will result in a lower functionality response [71,72]. An overview of the different peptide impurity classes is presented below.

4.1. Amino acid deletion

Incomplete removal of the transient protecting group of the resin-bound amino acid or insufficient activation of the incoming amino acid building block can result into an inefficient SPPS coupling step. In turn, this will result into peptide chains missing one or more desired amino acid residues. Truncated peptide impurities, missing one or more desired amino acid residues at either the N- or C-terminal end are also classified as deletion impurities and originate from overdried resin beads, causing the peptide to precipitate [95]. Furthermore, N-terminal truncated peptides can also arise from (an) inefficient coupling step(s), as discussed above, whereas C-terminal truncated peptides are the result of incomplete coupling of the first amino acid to the resin (Fig. 1). Sanz-Nebot et al. examined the impurity profiles of crude grade carbetocin, eledoisin, leuprolide, goserelin and triptorelin peptides [96–102]. A number of deletion impurities were identified and selected examples are

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Table 3
Overview of identified peptide impurities.

Peptide	MW[a]	AA deletion	AA insertion	Inc. removal PG	Ox./Red.	Racem.	Side/end chain react.
Tyr(OMe)-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 CO-CH_2-CH_2-CH_2 link between Y and C (Carbetocin)	988.2	−128.2 (−Gln)	+114.1 (+Asn)	−25.9 (Fmoc-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2)	+16.0 (Cys11 sulfoxide) +32.0 (Cys11 sulfone) +48.0 (Cys11 sulfonate) −128.2 + 16.0 (−Gln+Cys11 sulfoxide)	Yes	–
Pyr-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2 (Eledoisin)	1188.2	−97.1 (−Pro) −111.1 (−Pyr) −87.1 (−Ser) −128.2 (−Lys)	+128.2 (+Lys)	+56.0 (+tBu)	+16.0 (Met11 sulfoxide) +128.8 + 16 (+Lys+Met11 sulfoxide)	Yes	–
Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt (Leuprolide)	1209.4	−87.1 (−Ser) −156.2 (−Arg)	+186.2 (+Trp) +156.2 (+Arg) +137.1 (+His)	+56.0 (+tBu) +126.0 (+2 × tBu) +111.1 (Fmoc-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt)	+2.0 (Reduced Trp3) +16.0 (Hydroxy-Trp)	Yes	+126.0 (subst. guanidine)
Pyr-His-Trp-Ser-Tyr-D-(tBu)Ser-Leu-Arg-Pro-AzGly-NH_2 (Goserelin)	1269.4	−111.1 (−Pyr) −97.1 (−Pro) −137.1 (−His) −58.0 (−AzGly) −43.0 (Acyl-hydrazine)	+156.2 (+Arg) +163.2 (+Tyr) +163.2 (+Tyr) +97.1 (+Pro) +137.1 (+His) +113.2 (+Leu) +113.2 (+Leu) +(97.1–87.1) (+Pro−Ser)	–	–	Yes	+15.0 (Arg amination) −42 + 111.1 (Gos(Orn)+Pyr) −42 + 248.2 (Gos(Orn)+His+Pyr) −42 + 434.4 (Gos(Orn)+Trp+His+Pyr) −42 + 521.5 (Gos(Orn)+Ser+Trp+His+Pyr) −42 + 684.7 (Gos(Orn)+Tyr+Ser+Trp+His+Pyr) −42+827.8 (Gos(Orn)+Ser(tBu)+Tyr+Ser+Trp+His+Pyr)
Pyr-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2 (Triptorelin)	1311.5	−111.1 + 97.1 (−Pyr+Pro)	+137.1 (+His) +163.2 (+Tyr)	+56.0 (+tBu)	–	Yes	+15.0 (Arg amination) +1.0 (deamidation)
Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro (Tetracosactide)	2933.4	Different truncations	+381.5 (+Tyr−Ser−Met) +468.5 (+Ser−Tyr−Ser−Met) +57.0 (+Gly)	+56.0 (+tBu) +100.0 (tBoc-TCS)	+16.0 (TCS sulfoxide)	–	+42.0 (Ac-TCS)
Arg-Ile-Ile-Thr-Ser-Arg-Ile-Leu-Val-Asp-Gln-Val-Thr-Gly-Val (HuD peptide)	1670.0	−214.3 (−Ile−Thr)	–	–	–	–	–
Glu-Gln-Leu-Phe-Ser-Gln-Tyr-Gly-Arg-Ile-Ile-Thr-Ser-Arg-Ile (HuD peptide)	1811.7	−214.3 (−Ile−Thr)	–	–	–	–	–
Gly-Phe-Val-Thr-Met-Thr-Asn-Tyr-Asp-Glu-Ala-Ala-Met-Ala-Ile (HuD peptide)	1633.7	−57.0 (−Gly) −147.2 (−Phe) −99.2 (−Val) −131.2 (−Met) −101.1 (−Thr2) −114.1 (−Asn) −115.1 (−Asp) −71.1 (−Ala1) −71.1 (−Ala2) −113.2 (−Ile) −204.2 (−Gly−Phe) −246.4 (−Phe−Val) −156.2 (−Val−Gly)	–	–	–		–

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Table 3 (*Continued*)

Peptide	MW[a]	AA deletion	AA insertion	Inc. removal PG	Ox./Red.	Racem.	Side/end chain react.
Phe-Asn-Ala-Pro-Phe-Asn-Ile-Gly-Ile-Lys-Leu-Ala-Gly-Ala-Gln-Ser-Leu-Gln-His-Gly-Gln-Thr-Leu-NH_2 (bovine obestatin)	2423.3	−114.1 (−Asn6)	–	–	–	–	–
Phe-Asn-Ala-Pro-Phe-Asp-Val-Gly-Ile-Lys-Leu-Ser-Gly-Pro-Gln-Tyr-His-Gln-His-Gly-Gln-Ala-Leu-NH_2 (canine obestatin)	2522.3	–	–	–	–	–	+1.0 (deamidation)
Phe-Asn-Ala-Pro-Phe-Asp-Val-Gly-Ile-Lys-Leu-Ser-Gly-Val-Gln-Tyr-Gln-Gln-His-Ser-Gln-Ala-Leu-NH_2 (human obestatin)	2545.3	−229.2 (−Asn2−Asp6) −115.1 (−Asp6) −512.6 (−Asp−Val−Gly−Lys)	–	–	–	–	–
Phe-Asn-Ala-Pro-Phe-Asp-Val-Gly-Ile-Lys-Leu-Ser-Gly-Ala-Gln-Tyr-Gln-Gln-His-Gly-Arg-Ala-Leu-NH_2 (mouse obestatin)	2515.3	–	–	–	–	–	+1.0 (deamidation)
Phe-Asn-Ala-Pro-Phe-Asn-Ile-Gly-Ile-Lys-Leu-Ser-Gly-Ala-Gln-Ser-Leu-Gln-His-Gly-Gln-Thr-Leu-NH_2 (ovine obestatin)	2439.3	−114.1 (−Asn6)	–	–	–	–	–
Ac-Lys-Ile-Ser-Ala-Leu-Lys-Glu-Lys-Ile-Ser-Ala-Leu-Lys-Glu-Lys-Ile-Ser-Ala-Leu-Lys-Glu-NH_2 (−)	2368.0	−128.2 (−Lys) +42 − 128.2 (−Lys, Ac) −256.4 (−Lys−Lys) +42 − 256.4 (−Lys−Lys, Ac)	–	–	–	–	+42.0 (Ser3 Ac) +42.0 (Ser10/7 Ac)
Phe-Arg-Ser-Leu-Phe-Trp-Gly-Asn-His-Ser-Gln (INSL6[151–161])	1378.6	–	–	+106.0 (+Anisole/p-cresol) +56.0 (+tBu) +188.2 (Trp adduct) +243.0 (+Trityl)	–	–	–
Asn-Asn-Trp-Asn-Asn (Q19)	660.6	–	–	–	–	–	+1.0 (deamidation)
Ser-Asn-Leu-Val-Glu-Cys-Val-Phe-Ser-Leu-Phe-Lys-Lys-Cys-Asn (Q76)	1731.1	−87.1 (−Ser1)	–	–	–	–	
Asn-Asn-Trp-Gly-Asn (Q153)	603.6	−57.0 (−Gly4)	–	–	–	–	+1.0 (deamidation)
Cys-Val-Phe-Ser-Leu-Phe-Lys-Lys-Cys-Asn (Q25)	1188.5	−103.2 (−Cys1) −202.4 (−Cys1−Val2) −101.2 (−Val2+cystine) −130.2 (−Lys7+Cystine)	–	–	–	–	–
Asp-Leu-Arg-Asn-Ile-Phe-Leu-Lys-Ile-Lys-Phe-Lys-Lys-Lys (Q31)	1791.3	−115.1 (−Asp1) −156.2 (−Arg4)	–	–	–	–	–

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Table 3 (*Continued*)

Peptide	MW[a]	AA deletion	AA insertion	Inc. removal PG	Ox./Red.	Racem.	Side/end chain react.
GluSerArgLeuProLysIleLeu	2116.6	−156.2 (−Arg15) −495.6 (−Glu1−Arg4−Leu5−Pro6)	–	–	–	–	–
LeuAspPheLeuPheLeArg LysLys (Q53)	2731.2	−473.6 (−Lys22−Lys23−Cys24−Asn25)	+57.0 (Gly)	–	–	–	–
Ser-Ile-Asn-Ser-Gln-Ile-Gly-Lys-Ala-Thr-Ser-Asn-Leu-Val-Glu-Cys-Val-Phe-Ser-Leu-Phe-Lys-Lys-Cys-Asn (Q75)	2985.6	–	+106.2 (Gly10+Tyr)	–	–	–	–
Lys-Ser-Ser-Ala-Tyr-Ser-Leu-Gln-Met-Gly-Ala-Thr-Ala-Ile-Lys-Gln-Val-Lys-Lys-Leu-Phe-Lys-Lys-Trp-Gly-Trp (Q125)							
Leu-Pro-Phe-Glu-Phe (Q134)	651.7	–	–	+14.0 (methylation Glu)	–	–	–

[a] MW: average molecular weight.

listed in Table 3. Identification of this class of impurities is done by attributing the mass difference between the observed impurity and the parent peptide to a deletion of a certain amino acid residue, *i.e.* amino acid molecular mass minus H_2O.

Taichrib et al. [103] identified a number of peptide impurities present in the synthetic peptide hormone tetracosactide (SYSME-HFRWGKPVGKKRRPVKVYP, TCS). TCS (Synacthen®) is a synthetic analog of the human adrenocorticotropic hormone (ACTH) clinically used as a diagnostic agent and contains the first 24 amino acid residues of ACTH, which is composed out of 39 amino acid residues. In order to check for the presence of N- and C-terminal truncated TCS-peptide impurities, the theoretical mass of these impurities were calculated. The actual presence of truncated impurities in the TCS sample was verified by RP-C18 chromatography and capillary electrophoresis coupled with MS detection and ion extraction data treatment, using the theoretical impurity masses. The authors reported the presence of all N- and C-terminal truncated impurities down to a size of seven amino acids, *i.e.* presence of 1–23 TCS to 1–8 TCS and 2–24 TCS to 18–24 TCS.

De Beukerlaar et al. screened for the presence of T-cells directed against the onconeural HuD protein by stimulating peripheral blood cells of individuals with 15-amino acid peptides spanning the HuD protein sequence [104]. During initial screening, three different peptides with a purity of approximately 70% elicited a positive T-cell response. However, peptide batches with higher purity levels failed to reproduce these initial results. Therefore, MALDI-FT-MS was used to compare the impurity profiles of the different peptides batches. FT-MS analysis of the first two peptides, *i.e.* RIITSRILVDQVTGV and EQLFSQYGRIITSRI, revealed the presence of one major impurity type, characterized by deletion of the adjacent amino acids "IT". The impurity profile of the third 15-mer peptide, *i.e.* GFVTMTNYDEAAMAI, is more complicated as more impurities were seen. Table 3 lists 13 observed peptide-related impurities. Ten impurities were characterized by deletion of one amino acid residue, of which the majority occurred within the peptide sequence. The three other impurities were missing two amino acid residues, of which two impurities comprised adjacent amino acid deletions. Finally, the impurities characterized by deletion of the N-terminal G and GF residues can be classified as truncated impurities rather than internal deletions. No further efforts were made to pinpoint the impurity eliciting the immunological response. De Spiegeleer et al. performed a quality study evaluating obestatin peptides of different origins obtained from different peptide manufacturers [29]. Several peptide-related impurities were identified using HPLC–MS and are summarized in Table 3. Again, the majority of the impurities seen here are the result of deletion of one or more amino acid residues, but also a deletion of 5 amino acid residues was observed. Litowski et al. [105] identified several amino acid deletion impurities of a semipure 21-mer peptide, Ac-KISALKEKISALKEKISALKE-NH_2, using HPLC/Cation Exchange Chromatography (SCX), *i.e.* a lysine deletion (mass difference -128), a lysine deletion combined with a side chain acetylation, a double lysine deletion and a double lysine deletion combined with acetylation. While performing quality control on quorum sensing peptides, Verbeke et al. [53] identified a large number of deletion impurities, often combined with other impurity types, *e.g.* oxidation of cysteine residues to cystines. Selected examples of identified impurities, together with the relative mass difference toward the parent peptide, are listed in Table 3.

4.2. Amino acid insertion

During the coupling step in SPPS, an excess of Fmoc-protected amino acids is used to ensure the maximum coupling efficiency. However, this abundance can result into insertion of an additional amino acid into the desired peptide sequence, if not all reagents

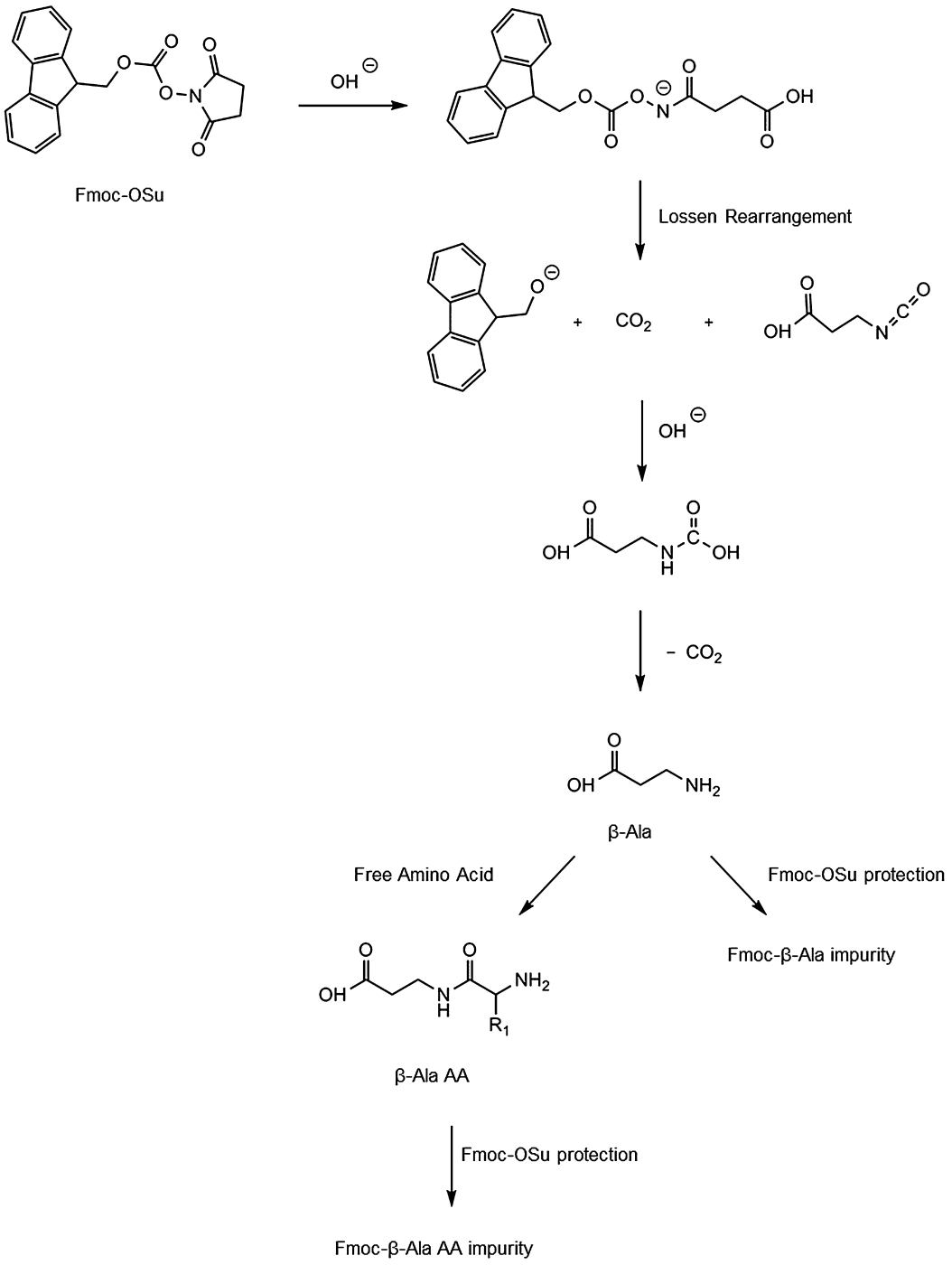

Fig. 2. Synthesis of peptide API starting materials: mechanism for the formation of Fmoc-β-Ala-OH and Fmoc-β-Ala-AA-OH impurities.

are properly washed away. Besides formation of a peptide bond with the resin-bound peptide chain, the activated incoming amino acid can also acylate an amide group of this resin-bound peptide chain (Fig. 1). During the next coupling step, transient deprotection will remove the Fmoc protecting group of this acylated amino acid, providing a free, reactive amine. This will further react with the carbonyl moiety of the peptide bond, effectively inserting the amino acid into the peptide sequence [106]. This amino acid insertion is facilitated if the peptide bond where the acylation occurs is easily accessible, *i.e.* absence of a bulky side chain in the adjacent amino acid residues. Sanz-Nebot et al. examined the impurity profiles of crude grade carbetocin, eledoisin, leuprolide, goserelin and triptorelin [96–102]. A number of insertion impurities were identified, but the location of insertion was not further clarified (Table 3). As was the case for deletion impurities, identification of these insertion impurities is done by attributing the mass difference between the observed impurity and the parent peptide to insertion of a certain amino acid residue, *i.e.* amino acid molecular mass minus H_2O. De Spiegeleer et al. [29] reported observation of an obestatin-related peptide impurity characterized by a mass increase of +137.1, corresponding to incorporation of an additional histidine residue into the desired peptide sequence. Taichrib et al. [103] also reported two tetracosactide (TCS)-related impurities characterized by a higher mass due to incorporation of additional amino acids. The authors deduced the sequences to be TCS + YMS (mass increase: 381 Da) and TCS + SYSM (mass increase: 468 Da).

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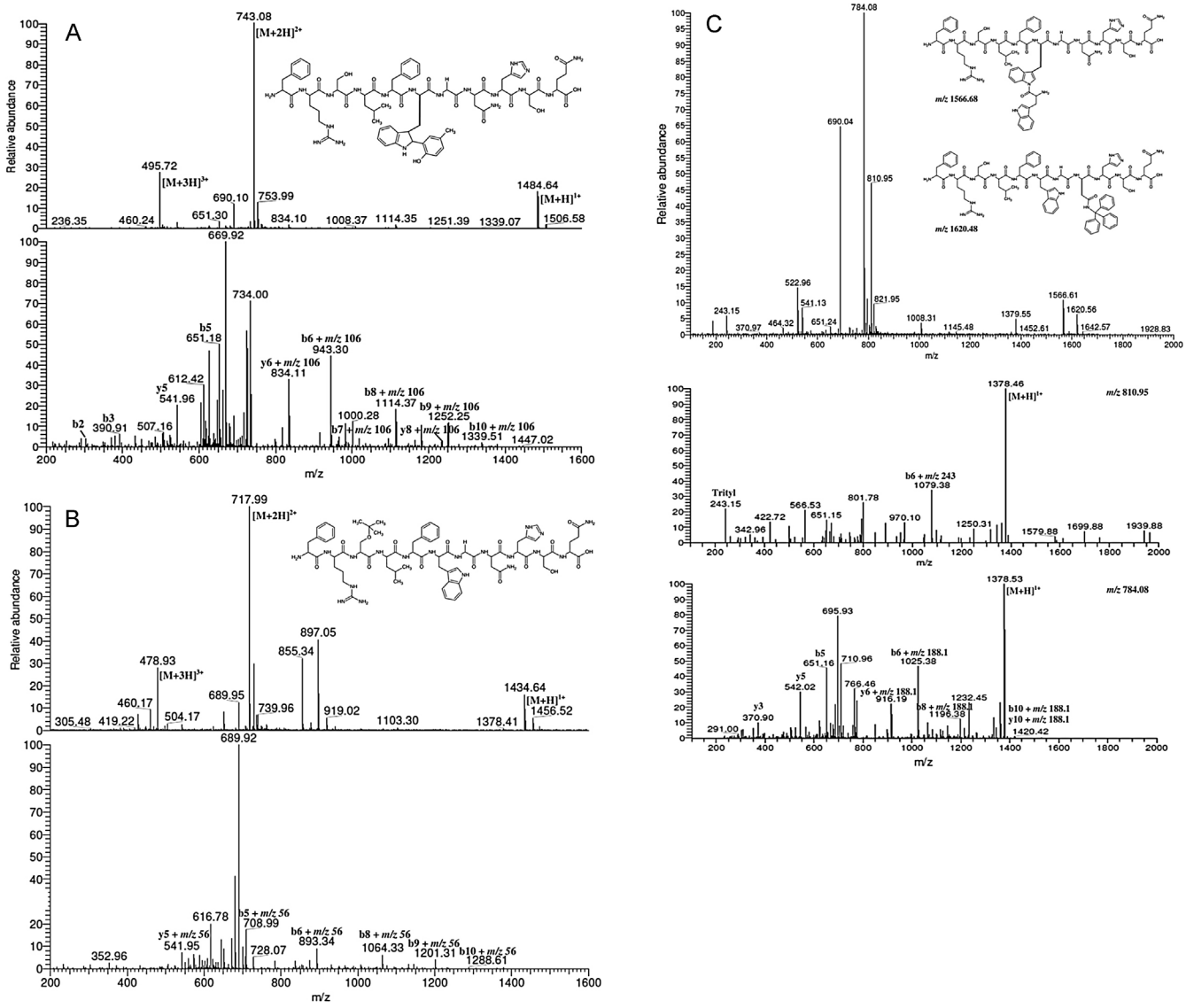

Fig. 3. (A) Typical MS and MS/MS spectra (on *m*/*z* 743.08 selected precursor ion) for the anisole/p-cresol derivative in the crude INSL6[151–161] material batch 1. (B) Typical MS and MS/MS spectra (on *m*/*z* 717.99 selected precursor ion) for the tBu derivative in crude INSL6[151–161] material batch 2. (C) Typical MS and MS/MS spectra (on *m*/*z* 784.08 and 810.95 selected precursor ions) for the major impurity peak observed in crude INSL6[151–161] material batch 3.
Adapted from Ref. [114] with permission.

A truncated TCS impurity peptide, combined with insertion of an additional Gly residue was also reported. Verbeke et al. [53] also identified a number of insertion impurities, *e.g.* insertion of Gly residue.

A different amino acid insertion mechanism was – independently from each other – observed by Obkircher et al. and Isidro-Llobet et al. Both authors observed the formation of unexpected amino acid impurities, *i.e.* Fmoc-β-Ala and Fmoc-β-Ala-AA, during the preparation of Fmoc-protected amino acids when using N-9-fluorenylmethoxycarbonyloxy succinimide (Fmoc-OSu) as a protecting agent [107,108]. The explanation for the formation of these impurities is given in Fig. 2. The β-Ala structure is formed through a Lossen rearrangement after the attack of a nucleophile, present in the reaction mixture, on one of the carbonyl moieties present in Fmoc-OSu. Finally, this results in the formation of the β-Ala amino acid residue, which subsequently can be Fmoc-protected by an additional equivalent of Fmoc-OSu, thus forming the Fmoc-β-Ala impurity. Alternatively, the free amino acid which is aimed to be Fmoc-protected, can also react with the formed β-Ala amino acid residue resulting in a dipeptide, which will then be Fmoc-protected, resulting in Fmoc-β-Ala-AA. HPLC analysis of Fmoc-OSu, showing no impurities, indicated that impurity formation occurred during preparation of the API starting materials. The same impurity type was also seen after H-tBu-Gly and H-β-cyclopropyl-Ala synthesis, and required extensive recrystallization to reduce the impurity amount to approximately 0.5%, but unfortunately dramatically reducing the desired peptide yield from 99% to 43%. In order to avoid or reduce the formation of these impurities, Fmoc-OSu was replaced with the less reactive Fmoc-2-mercaptobenzothiazole (Fmoc-2-MBT) or the Fmoc-OSu concentration reduced.

The presence of this Fmoc-β-Ala-AA impurity type in the Fmoc-protected amino acids, which are used as API starting materials in peptide synthesis, resulted in unwanted peptide impurities as was reported by Hlebowicz et al. [109]. While chemically synthesizing a non-disclosed peptide API, characterized by three Ala residues, an impurity at levels above 0.1% was observed using RP-HPLC. Using electrospray ionization-mass spectrometry, the molecular weight of this impurity was determined to be 71 Da higher than the desired

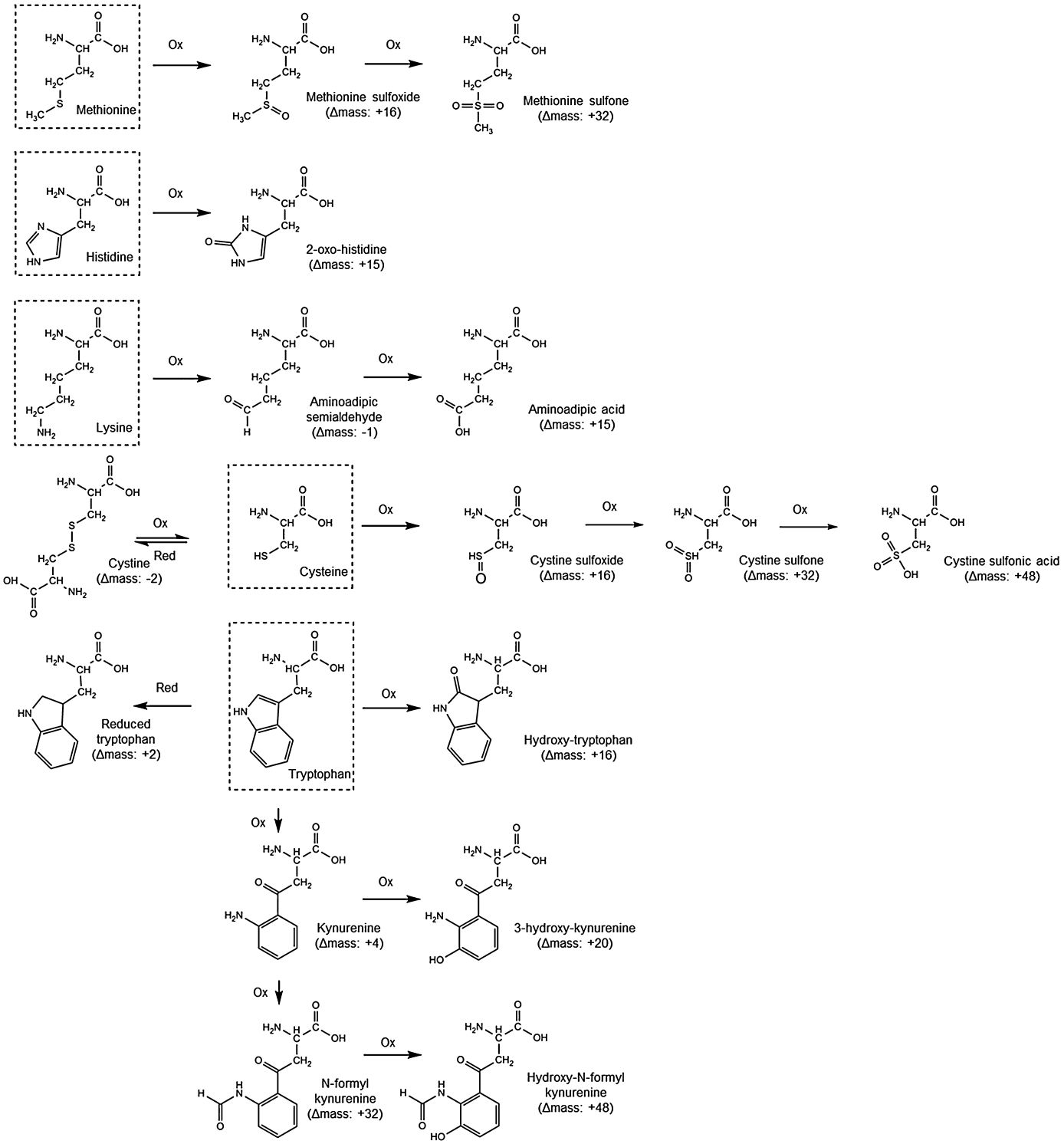

Fig. 4. Amino acid oxidation/reduction-related impurities.

peptide mass. This mass addition, which was located immediately prior to the second Ala residue by MS^2, indicated the incorporation of an additional Ala residue. Chiral amino acid analysis excluded the presence of an additional D-Ala isomer. Other suspected impurities such as incorporation of additional L-Ala, sarcosine (N-methyl-glycine) or β-Ala residues were synthesized and analyzed using the same RP-HPLC method. By retention time comparison, the nature of the impurity was identified as the inclusion of an additional β-Ala residue. Moreover, the origin of this impurity could be traced back to the Fmoc-Ala starting material, which contained approximately 0.3% of the Fmoc-β-Ala-Ala impurity. However, the provided quality certificate did not indicate this, as only the purity specification of >99% and optical purity specification of <0.5% were given. This is an example how related impurities in the API starting material, having similar structures as the desired starting material, can be carried over toward the final peptide drug. To avoid this,

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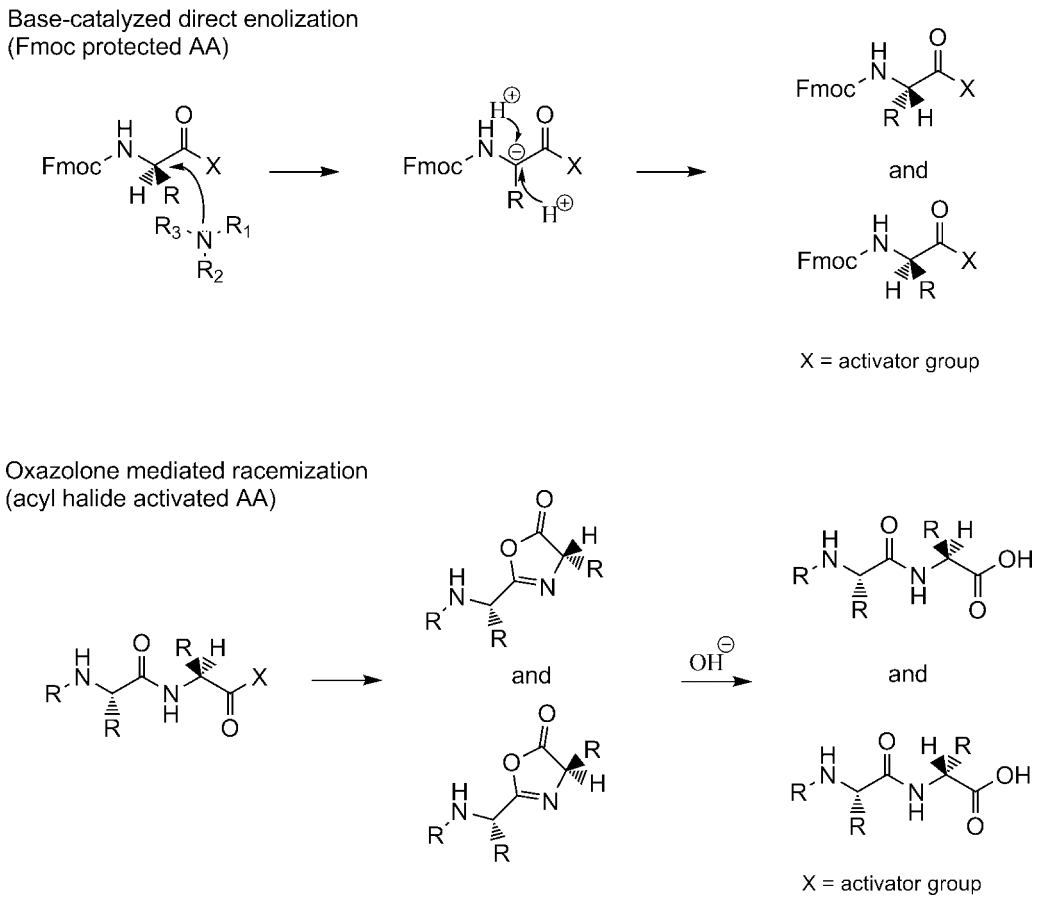

Fig. 5. Racemization during SPPS coupling step.

appropriate attention is to be paid to the impurity profiles of the peptide API starting materials, *i.e.* Fmoc-protected amino acids, requiring validated analytical methods.

4.3. Incomplete removal of protecting groups

Incomplete removal of permanent protecting groups after SPPS will result into protection groups covalently attached to peptide sequences. Covalently attached (tBu) peptide impurities, characterized by a relative mass increase of +56 Da were reported by Sanz-Nebot et al. [96–102]. The authors also reported impurities with the transient Fmoc-protecting group still attached at the amino-terminus (Table 3). The tBu adduct impurity was also seen when analyzing TCS, as well as a di-tert-butyl dicarbonate (tBoc)-TCS adduct, characterized by mass increase +100 Da [103]. Verbeke et al. [53] identified impurities, characterized by methylation of a glutamic acid residue (Table 3).

When performing tissue-organ bath experiments using isolated guinea pig longitudinal smooth muscle, Verbeken et al. [110] reported a functional activity for peptide FRSLFWGNHSQ (70% purity, *i.e.* crude grade SPPS prepared) of which the sequence is derived from the insulin-like peptide 6 (INSL6[151–161]). However, confirmatory experiments with high purity peptide material (95%) did no longer induce a functional tissue response, indicating that the parent peptide itself is not responsible for the observed activity. HPLC–ESI-MS impurity analysis of the crude peptide material revealed the presence of significant impurity levels above the applied reporting threshold of 0.5%. The major impurity peak observed in one of the crude material batches was characterized by *m*/*z* of 1484.64, which is a mass shift of +106 relative to the parent peptide (*m*/*z* 1378.6). In the MS/MS spectrum obtained from *m*/*z* 743.08, *i.e.* $[M+2H]^{2+}$, the original b_1–b_5- and y_1–y_5-fragment ions of the parent peptide were detected. On the other hand, the +106 Da mass shift, corresponding to the mass of anisole/p-cresol, was observed for b_6–b_{10} and y_6–y_{10} fragment ions, relative to the parent peptide b- and y-fragment ions (Fig. 3A). From this, it was concluded that the major impurity is an anisole/p-cresol adduct of the INSL6[151–161] peptide with the anisole/p-cresol group attached to Trp at position 6. The origin of this impurity is most likely the incomplete removal of the anisole/p-cresol protecting group. Another major impurity of a different batch was characterized by a *m*/*z* value of 1434.6, a mass shift of +56 Da compared with the parent peptide monoisotopic mass. MS/MS analysis revealed a tBu modification of the Ser residue at position 3 (Fig. 3B). Tertiary butyl was confirmed by the peptide manufacturer to be used during peptide synthesis as a protecting group for Ser residues. Yet another batch of crude peptide material contained two major impurities, characterized by monoisotopic masses of *m*/*z* 1566.68 and 1620.48, respectively. MS/MS results for *m*/*z* 1566.68 demonstrated the peptide structure to be a synthesis impurity, of the INSL6[151–161] parent peptide, with addition of tryptophanyl to the Trp residue at position 6, resulting in the observed mass increase of +188.18 Da. The mass shift of +243 of the impurity characterized by *m*/*z* 1620.56 was demonstrated by MS and MS/MS to result from incorporation of a trityl group at the Asn residue at position 8 of the parent peptide sequence (Fig. 3C).

4.4. Oxidation/reduction

Certain amino acid residues tend to experience oxidations or reductions during the SPPS procedure. Impurities caused by oxidation of cysteine and methionine residues have been found in

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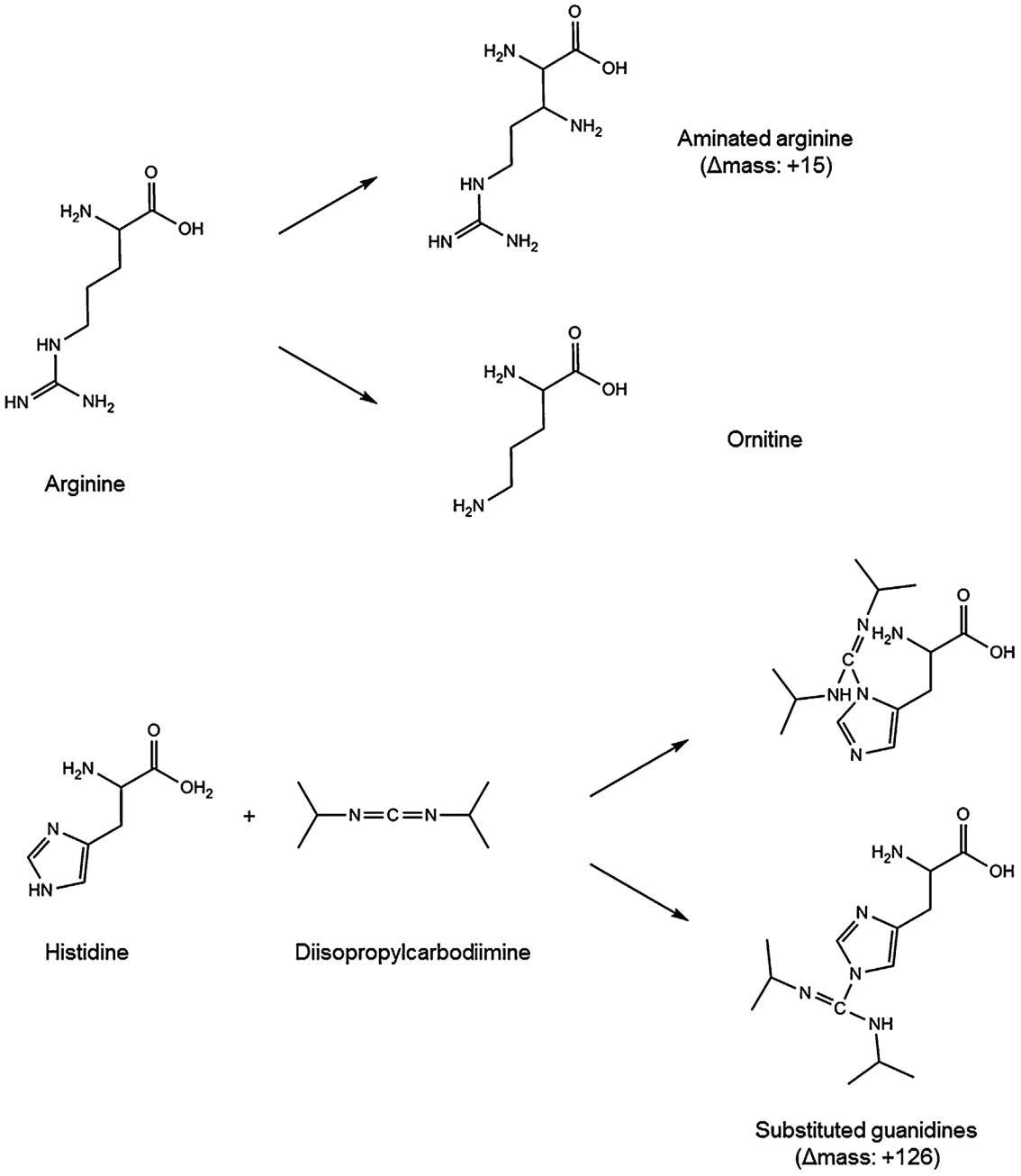

Fig. 6. Side chain reactivity.

carbetocin and eledoisin crude mixtures, respectively (Table 3). These impurities show mass differences of 16, 32 and 48 Da relative to the parent peptide, depending on the oxidation stage, *i.e.* sulfoxide, sulfone and sulfonic acid, respectively. Taichrib et al. [103] reported a sulfoxide impurity of tetracosactide, characterized by a mass increase of +16. Finally, oxidation of two cysteine residues to cystine (mass difference −2) will result into cyclization of the peptide. Verbeke et al. [53] reported this oxidation impurity type, which often also contained additional deleted amino acid residues (Table 3). Histidine and lysine are also prone to oxidation, forming 2-oxo-histidine and aminoadipic acid, respectively [111,112]. The oxidized impurities are formed by prolonged exposure to light and air during storage. On the other hand, the side chain of tryptophan is quite reactive in acidic conditions, and it can undergo oxidations resulting in hydroxy-Trp, kynurenine, 3-hydroxy-kynurenine, N-formylkynurenine and hydroxyl-N-formylkynurenine as well as undergo reduction [98,111]. A summary of the oxidized and reduced amino acid residues can be found in Fig. 4. Similar to oxidative stress caused by reactive oxygen species (ROS) in physiological processes, chemical oxidative stress in peptides and proteins caused by ROS cannot be excluded. ROS include radical species such as superoxide ($O_2^{\bullet -}$), hydroxyl ($OH^{\bullet}$), peroxyl ($RO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$) and hydroperoxyl ($HO_2^{\bullet}$), and nonradical species such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$) and peroxynitrite ($ONOO^-$) [113].

4.5. Diastereoisomerisation

The incidence of amino acid racemization has been widely investigated for many amino protected amino acid residues. Racemization mechanisms are typically classified into two main types: direct enolization and oxazolone (azalactone) formation,

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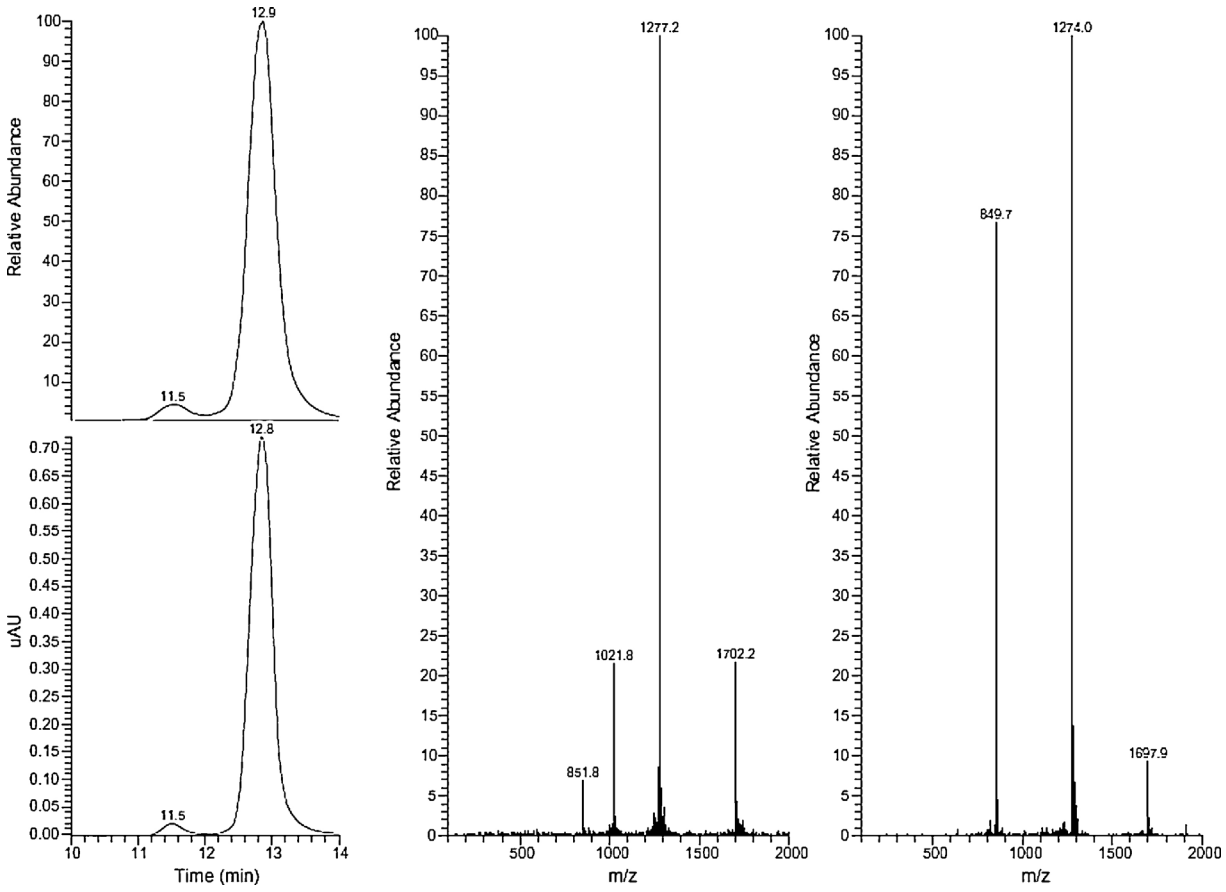

Fig. 7. Size exclusion chromatography LC–UV/MS analysis of human obestatin: (left) chromatogram showing dimeric and monomeric obestatin at 11.5 and 12.9 min, respectively (middle) mass spectra of dimeric obestatin with calculated monoisotopic molecular mass of 5104.3 Da; and (right) mass spectra of monomeric obestatin with calculated monoisotopic molecular mass of 2546.1 Da.
Adapted from Ref. [28] with permission.

which are both base-catalyzed mechanisms [114–118]. In the case of Fmoc-protected amino acid residues, direct enolization during the coupling step is the suggested mechanism. Here, a carbanion is generated due to proton abstraction catalyzed by the tertiary amine. Depending on the side chain R, this carbanion can be stabilized, *e.g.* resonance effect observed during phenylglycine racemization, facilitating the process [119]. This carbanion is then protonated from both sides of the plane, resulting in racemization (Fig. 5). Elsawy et al. reported racemization of a phenylglycine (Phg) residue during conventional and microwave-assisted Fmoc SPPS of AVPPhgY-NH_2. Optical purity of the Fmoc-Phg-OH starting material was checked, and confirmed the formation of the chiral impurity during amino acid coupling steps [120]. An alternative peptide synthesis route, using the activated carboxylic end (usually acyl halide activation) of the peptide, can also result into racemization through an oxazolone intermediate (Fig. 5). To avoid this, peptides are usually synthesized from C- to N-terminus, combined with carboxylic activation of the unbound amino acid, rather than the growing peptide chain (Fig. 1) [121].

Finder et al. observed a faster aggregation rate of recombinant amyloid β-peptide (Aβ1–42) when compared to its synthetic counterpart [122]. In order to identify the differences between recombinant and synthetic Aβ1–42, the synthetic peptide was subjected to preparative RP-HPLC isolation under conditions identical with those used for the purification of the recombinant peptide. RP-HPLC analysis of the individual amino acids showed that 1.01%, 0.14% and 0.10% of the histidine, arginine, methionine amino acid residues in the synthetic peptide was in the D-configuration, relative to the diastereoisomeric-free recombinant Aβ1–42. Histidine is indeed known for a minor, but significant tendency of racemization during peptide synthesis [123]. As three histidines, one methionine, and one arginine are contained in the Aβ1–42 sequence, this adds up to approximately 3.5% of diasteromeric Aβ1–42 impurities, containing at least one D-amino acid residue, within the synthetic Aβ1–42 peptide material. Moreover, these diastereoisomer peptide impurities resulted in altered aggregation kinetics of the synthetic Aβ1–42 peptide when compared to the all-L recombinant Aβ1–42 peptide, hereby proving that even small amount of stereoisomeric impurities, by as little as a single amino acid, can greatly influence the biological function of the parent peptide. Furthermore, conventional RP-HPLC is not always capable of separating these diastereoisomeric impurities from the all-L form, rendering detecting of these impurities difficult. Sanz-Nebot et al. also reported diastereoisomeric impurities for carbetocin, eledoisin, leuprolide, goserelin and triptorelin peptides but did not pinpoint the racemization location [96–102].

4.6. Side- and end-chain impurities

Sanz-Nebot et al. identified a number impurities arising from side- and end-chain reactivity. In the case of the crude peptide mixtures studied, the most relevant observed side-chain reactions have been aminations in the side chain of arginine, resulting in a primary amine, characterized by a mass difference of +15. Moreover, side chain reaction of the histidine residue in leuprolide with the amino acid activator DIC, lead to substituted guanidines with

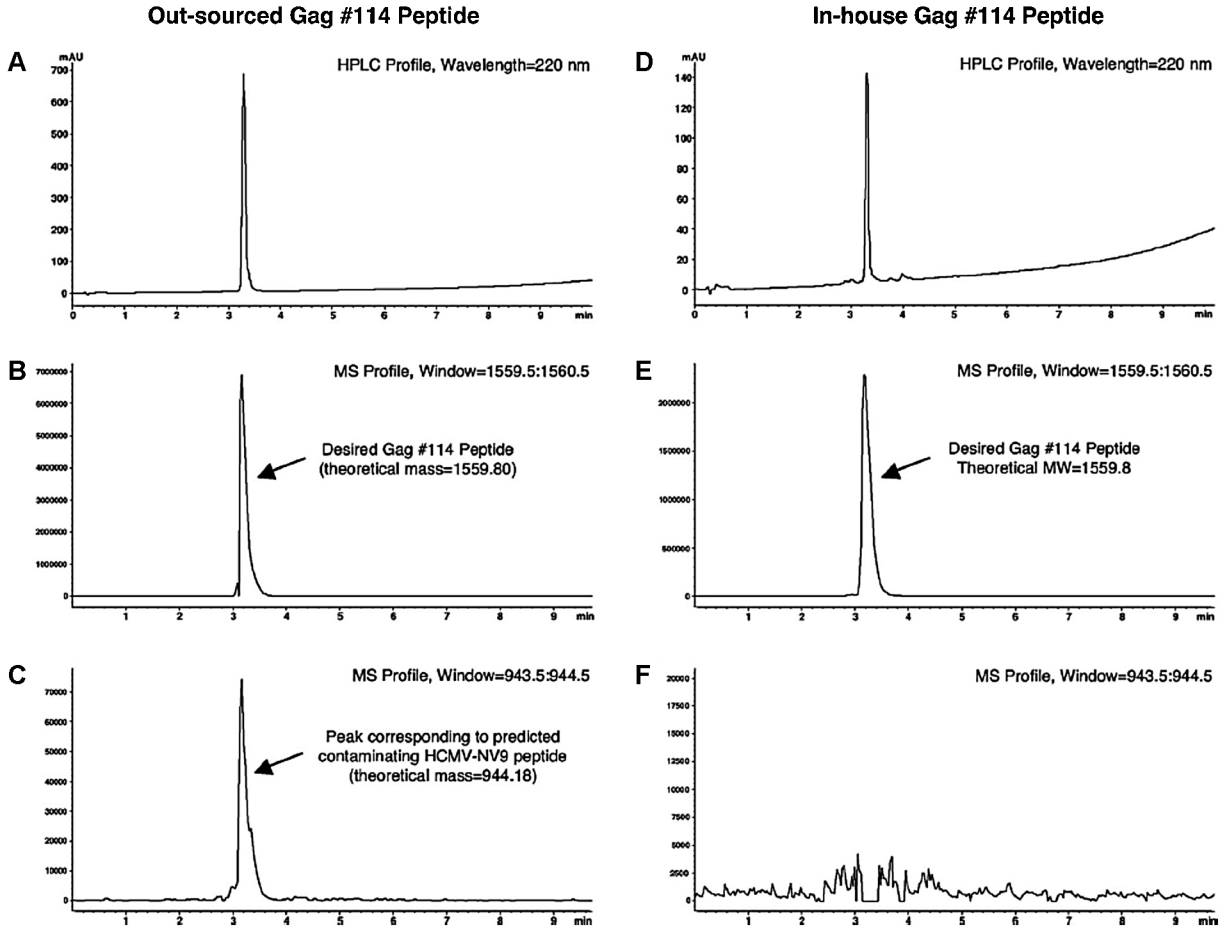

Fig. 8. LC–MS analysis of the suspect contaminated Gag 114 peptide from manufacturer A (A–C) and the in-house-resynthesized Gag 114 peptide (D–F). (A and D) UV traces of HPLC. High peptide purity is evident. The selected ion mode with mass filter adjusted to the calculated theoretical mass of the Gag 114 peptide (1559.80 $[M+H]^+$) is shown for each peptide (B and E). The desired product is clearly present in both profiles. The selected ion mode with mass filter adjusted to the calculated theoretical mass of the HCMV-HCMV-NV9 peptide (944.18 $[M+H]^+$) is shown (C and F). A signal corresponding the exact theoretical molecular weight (MW) of HCMV-NV9 is present in the outsourced Gag 114 peptide. Note the different scales on the *y* axis.
Adapted from Ref. [143] with permission.

a mass difference of 126 Da (Fig. 6). Before the SPPS of goserelin is completed by amidation, the goserelin acid precursor is cleaved from the resin, and under normal conditions amidated to form the desired goserelin API. However, this acid precursor was involved in a unwanted side-reaction with the dimethylformamide reagent used in the SPPS process, to form a dimethylamide ($-N(Me)_2$ derivative of the acid precursor (mass difference: +28). The authors also reported the modification of the arginine side chain into an ornithine (Orn) residue. This ornithine residue has an unprotected amino group, thus allowing for the coupling of additional amino acid residues. In each of the following coupling steps, the amino acid can be linked to the main peptide backbone (wanted), or to the side chain of ornithine (unwanted). Up to six goserelin-related impurities due to this ornithine side chain reactivity were reported (Table 3) [109]. The authors also observed a triptorelin deamidation product, characterized by a mass increase of 1 Da as a consequence of the $-CONH_2$ to $-COOH$ conversion. This deamidation impurity type was also reported by De Spiegeleer et al. for canine and mouse obestatin and by Lai et al. [29,124].

Litowski et al. [105] observed two single side chain acetylated peptide impurities, characterized by a mass difference of +42, in a semi-pure 21-mer peptide (sequence Ac-KISALKEKISALKEKISALKE-NH_2) by RP-HPLC and Cation Exchange Chromatography (SCX). The acetylation location was determined to be at Ser 3 and at the Ser 10 or Ser 17 residues. Similar acetylated TCS impurity was also reported by Taichrib et al. [103], but the acetylation site was not determined.

4.7. Dimers

Different aggregation or self-association mechanisms can lead to high molecular weight peptide impurities. Peptide aggregates are classified into covalent (chemical) and non-covalent (physical) interaction products, with the extent of their formation dependent on a wide variety of environmental factors. Covalent aggregates are typically formed from two or more monomers by disulfide bonds or oxidation of tyrosine to dityrosine. These covalent impurity types are thus in fact oxidation impurities (Section 4.4). Non-covalent aggregates are usually the result of weaker hydrophobic and electrostatic interactions. It is apparent that the molecular weight of a covalent dimer differ from the non-covalent type, *i.e.* two times molecular weight minus 2 (*e.g.* disulfide bond or tyrosine oxidation) *vs.* two times molecular weight, respectively. Moreover, a non-covalent aggregation impurity is usually in equilibrium with its native monomeric peptide. This equilibrium can be influenced by changes in peptide concentration or changes in solvent (*e.g.* pH), which can be used to deduce whether the aggregation is a true impurity or if it is the result of analytical sample preparation [125,126].

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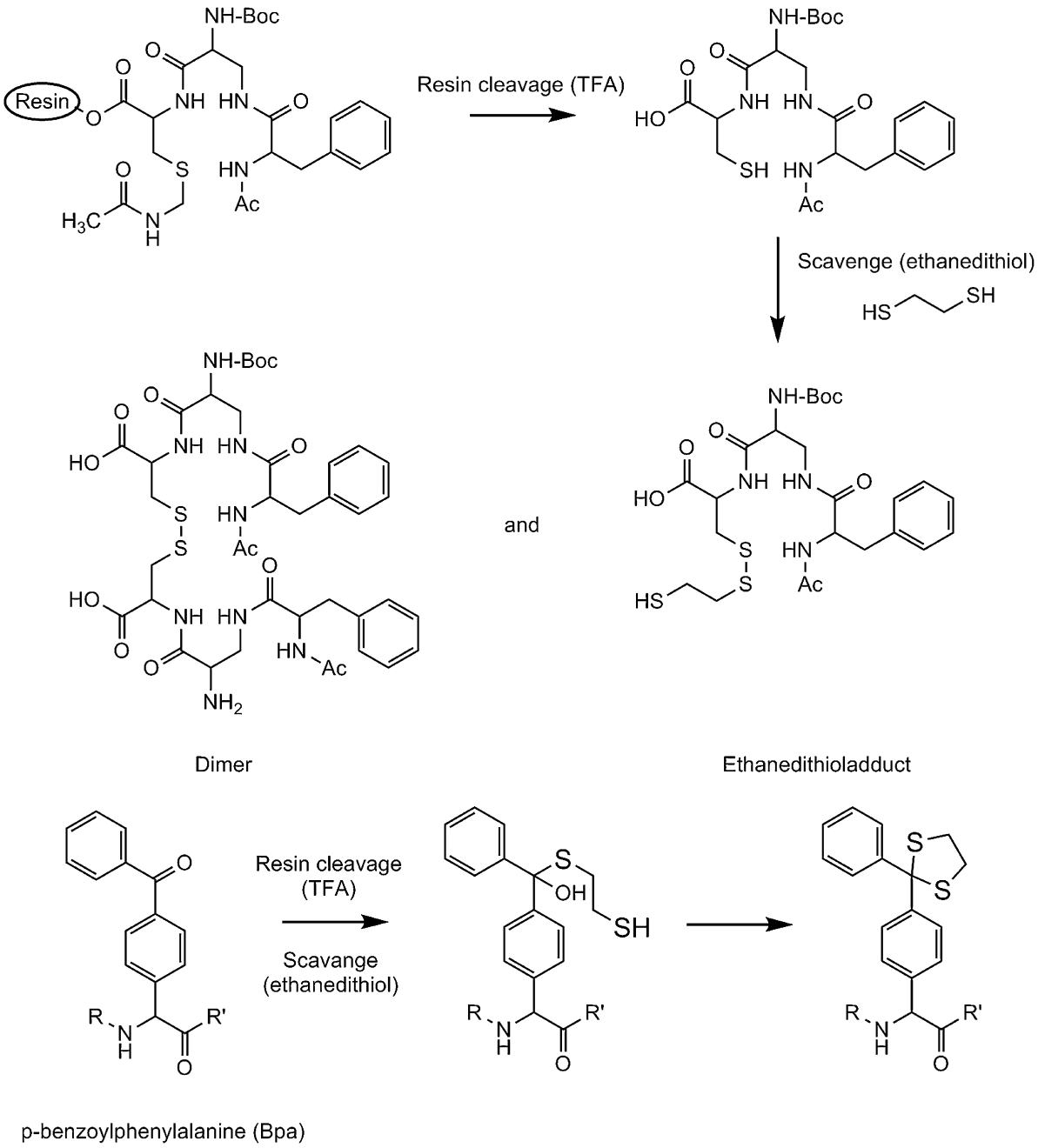

Fig. 9. Miscellaneous impurity formation.

De Spiegeleer et al. identified an impurity related to human obestatin, a 23-amino acid peptide, with molecular masses twice that of the parent peptide and with a relative retention time of approximately 1.8 relative to the parent peptide, when using a standard RP-HPLC–MS impurity profiling protocol [29]. The identity of these high-molecular-weight impurities was unequivocally proven to be obestatin dimers, by size-exclusion chromatography coupled to MS detection, as shown for human obestatin (Fig. 7).

4.8. Peptide counter ions

TFA is used in the final stage of SPPS as well as during further peptide RPLC purification steps. As a result, TFA will be present in the final peptide product. Whilst performing an infrared absorption study of the peptide dynorphin A-(1–13), Surewicz et al. [127] noticed the presence of a strong absorption band with a maximum centered at 1673 cm^{-1}. This band was completely eliminated after chromatographic purification of the dynorphin A-(1–13) peptide, suggesting that the above band originates from organic counter ions or other impurities. The authors investigated if residual TFA was responsible for this strong absorption band as TFA will interact with positively charged amino acid side chains. Infrared analysis of sodium trifluoroacetate indeed showed a single strong band at 1673 cm^{-1}, arising from the asymmetric COO— stretching vibration of the TFA ion. Other authors also reported the presence of this absorption band, which not only interferes with the peptide's amide I band, but can also distort the peptide conformation [128–136]. Andrushcenko et al. reported an optimal hydrochloric acid concentration of 2–10 mM, followed by lyophilization, to remove all TFA impurities, without modification to the peptide

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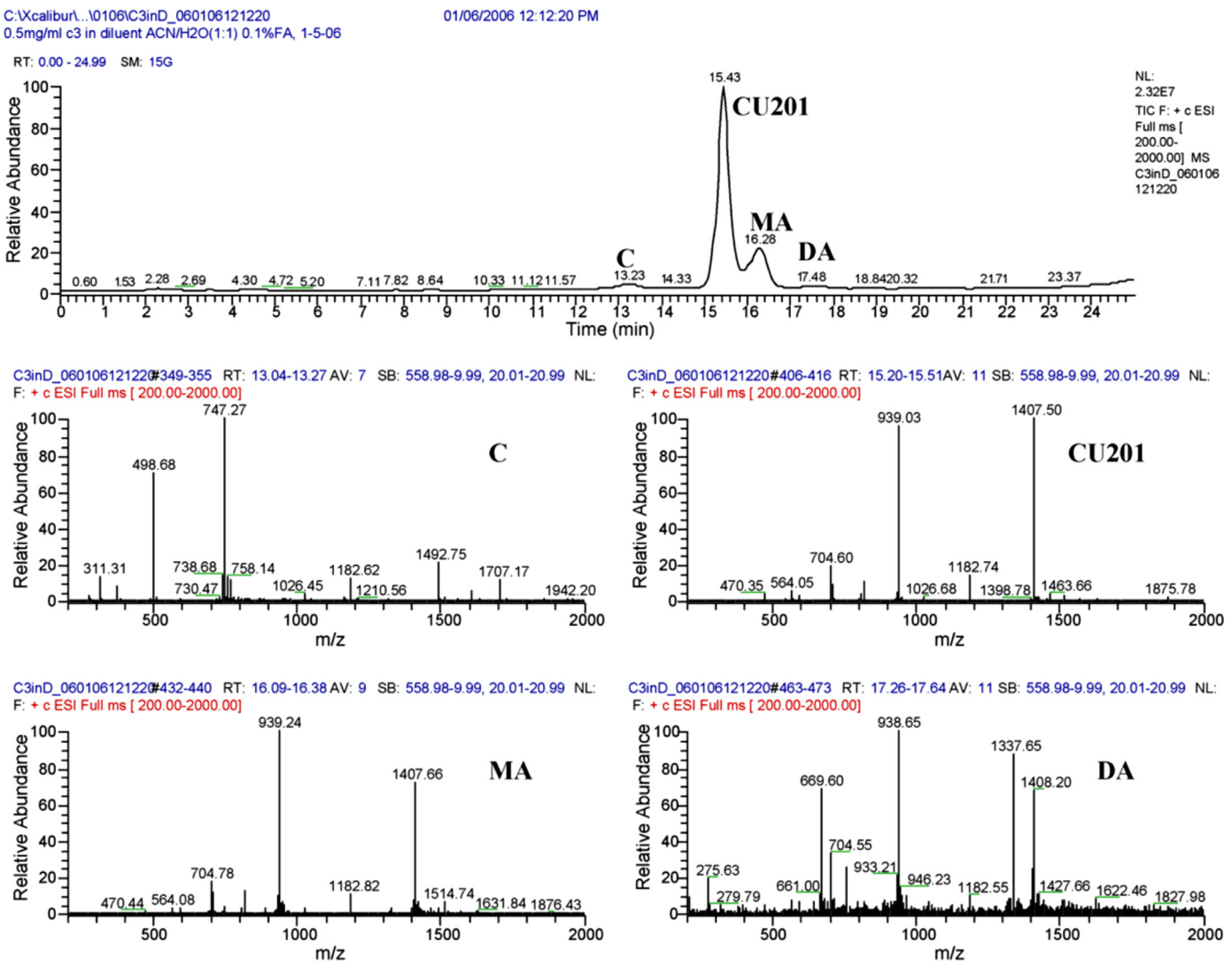

Fig. 10. TIC chromatogram (top panel) of CU201 lot –C/3, 0.5 mg/mL in ACN/H2O (1:1) with 0.1% formic acid, fresh. The mass spectra of CU201 and impurities C, MA and DA are displayed under the chromatogram.
Adapted from Ref. [150] with permission.

secondary structure [137]. Finally, De Spiegeleer et al. also reported the detection of peptide counter-ions, *e.g.* TFA, during RP-HPLC-DAD impurity profiling of obstatin peptides [29]. Although TFA salts can sometimes be tolerated during earlier drug discovery (R&D) phase, peptide APIs are generally manufactured as acetate salts, which can be achieved by an additional salt switching step [138].

4.9. Structurally unrelated contamination

Currier et al. described a contamination of individual peptides derived from the human immunodeficiency virus (HIV-1) with a commonly used peptide derived human cytomegalovirus (HCMV) [139]. The desired Gag 114 peptide (sequence: PTAPPMESLGMGEEI) was contaminated with the HCMV-NV9 peptide (sequence: NLVPMVATV) and it was proven that the contamination came directly from the manufacturer after comparison with in-house manufactured Gag 114 (Fig. 8). The desired peptide and the contaminant have a totally different sequence, illustrating that this impurity is a contamination, and not formed during the desired peptide synthesis. However, although the sequences are different, a similar chromatographic behavior is observed, rendering classic HPLC-based QC and purification of the Gag 114 peptide from the contaminant difficult. Importantly, this small amount of approximately 1% of unrelated peptide contaminant caused a false positive result for the Gag 114 peptide, demonstrating the importance of adequate peptide quality control prior to experimental use. De Graaf et al. also reported contamination of HuD peptides (each 15 amino acid residues long) with an unrelated, immunogenic peptide derived from cytomegalovirus (sequence: AIAEESDEEEAIVAY), again resulting in false positive results for the investigated peptides [140]. Brezar et al. reported false positive immonugenic stimulation due to a contaminant (sequence VYLKTNVFL) present in peptide PI_{A7-21} (sequence: CTSICSLYQLENYCN) [141]. This contaminant went undetected in several quality routine control analyses (MS detection). After a new SPPS batch of the PI_{A7-21} peptide did not reproduce the initial results, the original PI_{A7-21} peptide underwent HPLC fractionation. Each fraction was concentrated 10-fold and tested for an immunogenic response. Only one fraction provoked a response and was identified to be VYLKTNVFL using mass spectrometry. Jones et al. also described false positive results due to an HIV-1 Gag peptide contamination of 15-mer peptides derived from human endogenous retrovirus [142]. Moreover, specific guidance for the development, production and control of synthetic peptide

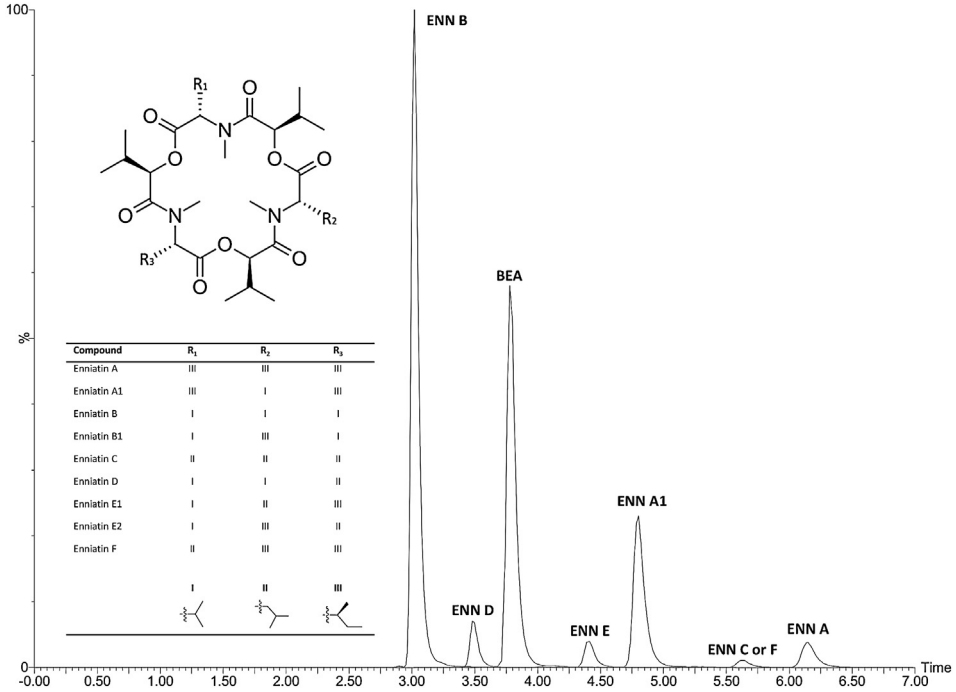

Fig. 11. Total ion chromatogram (TIC) of fusafungine (Locabiotal®).

vaccines are given by various regulatory authorities such as WHO. De Spiegeleer et al. reported delivery of an ovalbumine-derived peptide instead of the desired obestatin peptide, probably due to a complete mix-up at the peptide manufacturer. However, both peptides showed similar RP-HPLC chromatographic behavior, rendering detection of this mix-up only possible by using MS detection [29].

4.10. Miscellaneous

Singh et al. used Fmoc protected L-cysteine-acetamidomethyl (Acm) as starting material for the SPPS synthesis of the cysteine(Acm)-containing tripeptide Ac-Phe-Dap(Boc)-Cys(Acm), designed to be a bifunctional VII B metals (*e.g.* Technetium and Rhenium) chelator radiopharmaceutical [143]. However, during

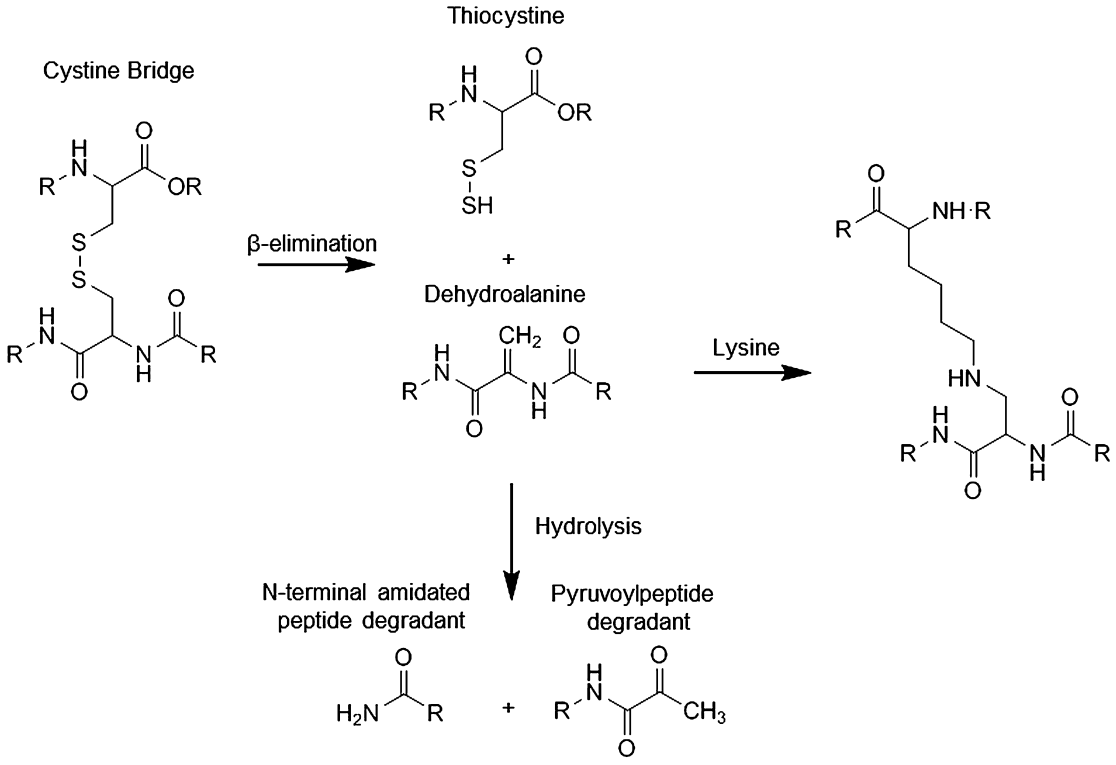

Fig. 12. Dehydroalanine formation through a β-elimination mechanism.

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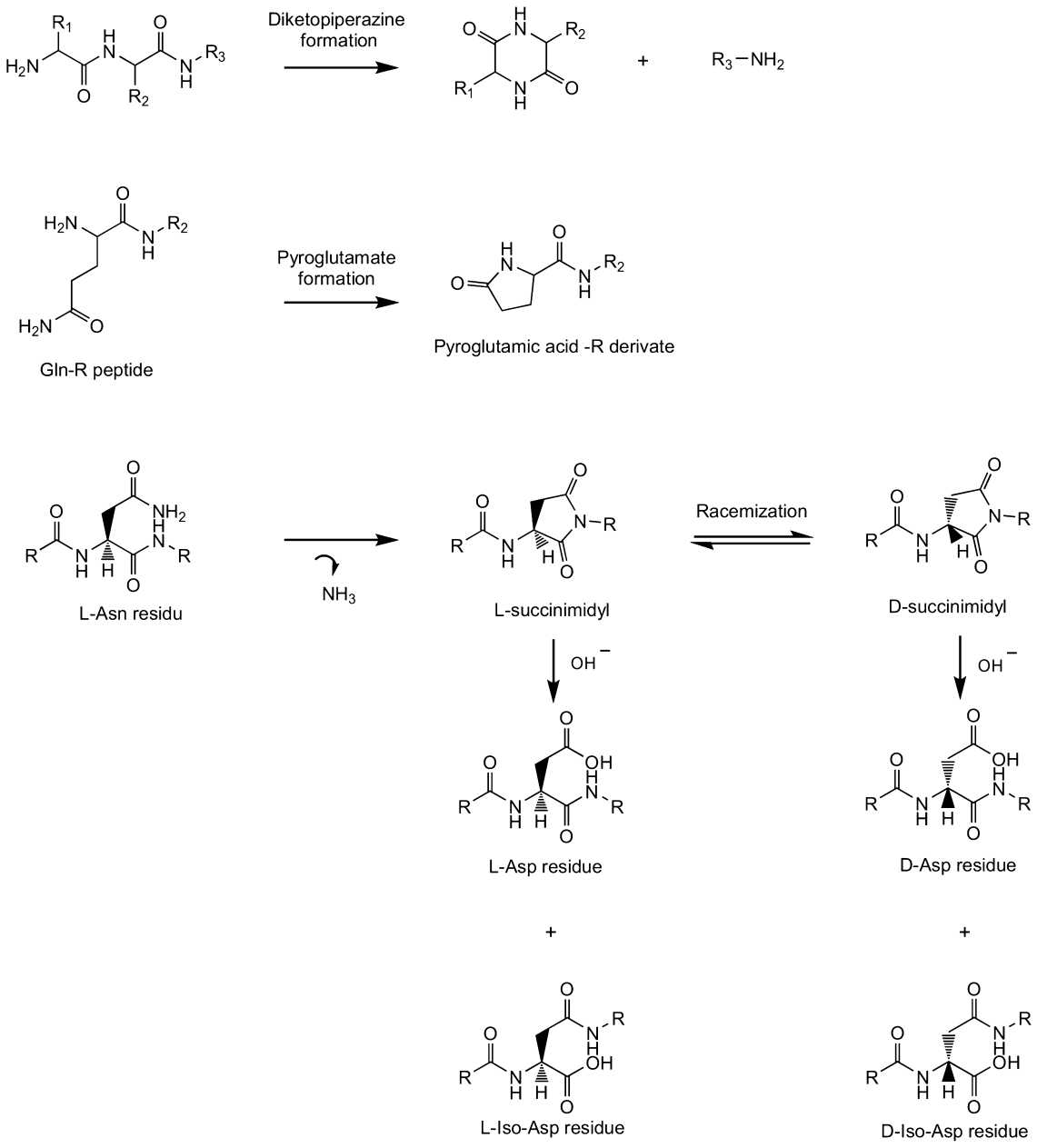

Fig. 13. Diketopiperazine (A), pyroglutamate (B) and succinimidyl (C) degradation mechanisms.

cleavage of the tripeptide from the resin using TFA, unwanted deprotection of the Cys residue was observed, thus obtaining a free thiol functional group. This reactive thiol functional group formed an ethanedithioladduct (Δmass: +92) with the ethanedithiol (EDT) scavenger as well as a disulfide dimer (Fig. 9). Identification of these impurities was performed using HPLC–ESI-MS. These impurity amounts could be reduced by using triisopropylsilane as scavenger in the absence of H_2O.

Breslav et al. performed a p-benzoylphenylalanine (Bpa) scan of a tridecapeptide, *i.e. Saccharomyces* cerevisiae α-factor, in order to define the binding site with a G-protein coupled receptor [144]. Two of these analogs, with Bpa in positions 1 and 3, respectively, were manufactured using Fmoc SPPS, and cleaved from the resin using ethanedithiol as scavenger. Besides the desired peptide, one impurity characterized by a 76 Da mass increase was observed using RP-HPLC–MS. Further sulfur and spectral analysis revealed the presence of a dithioketal group replacing the diarylketone group of Bpa (Fig. 9). Replacing the ethanedithiol with dithiothreitol as scavenger did not result in impurity formation. Sieber et al. reported similar dithioketal formation between tryptophan residues and the ethanedithiol scavenger [145].

Wang et al. [146] characterized the impurities of a dimeric antitumor peptide, CU201, with sequence SUIM-(D-Arg-Arg-Pro-Hyp-Gly-Igl-Serd-Igl-Oic-Arg)$_2$, where SUIM = suberimidyl; Hyp = trans-4-hydroxyproline; Igl = α-(2-indanyl)-glycine; Oic = octahydroindole-2-carboxylic acid. Fig. 10 shows the presence of 3 impurities in the CU201 peptide. The appearance of the mass spectrum of impurity MA is almost identical to that of CU201, with ions at *m*/*z* 1407.7, 939.2, and 704.8. Therefore, the identity of this impurity was first suspected to be a diastereoisomer of the large peptide CU201. However, careful examination of MS zoom scan data showed that the multiply charged ions for impurity MA were slightly but consistently higher than those for CU201. The average molecular masses calculated from multiple runs and multiple charge state ions of these two components are 2812.25 for CU201, and 2813.26 for impurity MA. It became apparent that

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the molecular mass of MA is approximately 1 mass unit higher than the mass of CU201 and it seemed plausible that one of the NH groups in CU201 has been replaced by an oxygen atom in impurity MA. High resolution LC–TOF/MS and NRM analysis confirmed that one of the two amidinyl = NH groups in the suberimidate residue was replaced by an oxygen atom, explaining the +1 mass difference. The DA impurity has a set of multiply charged ions that correspond to 2 mass units higher than that of CU201, whereby both amidinyl =NH groups in the suberimidate residue were replaced by oxygen atoms. Impurity C was the result of hydrolysis between Arg and suberimidate residues. Finally, Sanz-Nebot et al. reported linear impurities found in the crude carbetocin sample originating from an inefficient cyclization of the molecule [101].

5. Intrinsic peptide mixtures

Some peptide drugs consist of multiple active compounds, *i.e.* intrinsic peptide mixtures (also termed peptide complexes), which is not unusual for antibiotic drugs. An important consideration here is that each compound should have its own contributing activity, making the drug mixture acceptable for registration and marketing. If one or more components, however, have no activity or even antagonist activity, these should then be considered as impurities. Moreover, the ratio in which these different active ingredients are present in such a mixture, might vary depending on the drug product, but should at all times be consistent within a considered drug product as this links the clinical outcome with the quality of the batches.

A typical example is the antibiotic fusafungine, containing a mixture of cyclic hexadepsipeptide enniatins, which is marketed under the trade name Locabiotal® and used to treat upper respiratory infections [41]. A representative total ion chromatogram is shown in Fig. 11, indicating the presence of at least 7 different enniatin (ENN) isomers. The UHPLC–MS conditions used to obtain these data are given in Table 4. The mixture was characterized as followed: 43% ENN B, 31% ENN B1, 15% ENN A1, 4% ENN D, 3% ENN A, 3% ENN E and 1% ENN C or F. UV spectral data (not shown here) confirmed a similar pattern.

6. Degradation products

A number of peptide impurities arising from degradation, *e.g.* during storage, rather than being strictly synthesis-related, are reported here.

6.1. β-Elimination

Costantino et al. [147] proposed a hydroxide ion catalyzed β-elimination of the carbon-sulfur bond in cysteine, which would result into two new residues, *i.e.* dehydroalanine and thiocysteine. Dehydroalanine would react with lysine to form a lysinoalanine cross-link leading to insulin dimerization, whereas thiocysteine would further degrade into thiol-containing products, *e.g.* hydrosulfide ions. Indeed, the authors demonstrated a 5-fold increase of free thiol groups after incubation of insulin at 50 °C/96% R.H. Alternatively, dehydroalanine can undergo hydrolysis, yielding the N-terminal amidated peptide product and a C-terminal peptide fragment beginning with a pyruvoyl group [124]. This degradation mechanism was also reported by D'Hondt et al. [50] during short term, high temperature stressing of buserelin in its solid state. This entire degradation pathway is summarized in Fig. 12.

During microwave-assisted SPPS of C-terminal cysteine peptides, β-elimination of the C-terminal cysteine residues can also occur, forming a dehydroalanine intermediate which reacts with the deprotecting base piperidine, to form a 3-(1-piperidinyl)-alanine residue [148]. To avoid this, Ni et al. [149] introduced an Fmoc-based SPPS using an aryl-hydrazine linker for synthesis of C-terminal cysteine peptides. β-Elimination can also occur at serine and threonine residues, effectively removing the hydroxyl moiety, of which the dehydroalanine moiety can further degrade as mentioned above [150].

6.2. Diketopiperazine, pyroglutamate and Aspartimide/succinimide formation

Diketopiperazine formation can take place during SPPS and during peptide storage. It is sequence dependent and occurs if Pro or Gly residues are at position 1 or 2. The reaction involves a nucleophilic attack of the N-terminal nitrogen on the amide carbonyl between the second and third amino acid, which leads to the cleavage of the first two amino acids in the form of a diketopiperazine (Fig. 13). Good accessibility to this amide carbonyl, *i.e.* absence of a bulky side chain at position three as is the case for Gly residues, will facilitate the reaction. Different strategies to avoid this reaction during SPPS have been developed [151,152]. On the other hand, pyroglutamic acid formation is almost inevitable if Gln is the N-terminal amino acid residue. This is an analogous reaction where the N-terminal nitrogen attacks the side chain carbonyl carbon of Gln to form a pyroglutamic acid peptide analog, characterized by a mass decrease of 17, similar to deamidation [153]. This conversion also occurs when Asn is the N-terminal residue (Fig. 13) [154].

Internal Asp and Asn residues are prone to aspartimide/succinimide formation, especially when a non-bulky side chain amino acid is C-terminally adjacent to the Asp/Asn residue, reducing steric interference, *e.g.* Gly, Ala and Ser. Both the semantics "aspartimide" and "succinimide" terms are used to describe the same reaction, *i.e.* formation of a five-membered succinimide ring by nucleophilic attack of the C-terminal amide bond nitrogen on the γ-carbonyl of the Asn or Asp residue. The ring formation is favored by mildly acidic conditions and is unstable under physiological and alkaline conditions, also undergoing racemization. Subsequently, racemization and hydrolysis of the succinimide ring gives rise to a mixture of L-Asp, D-Asp, L-isoaspartate and D-isoaspartate, thus resulting in deamidation and racemization of the original Asn residue (mass difference +1) or isomerization and racemization of the original Asp residue to isoaspartate [155–159]. Moreover, succinimide impurities as such have also been identified in porcine somatotropin and during a stability study of recombinant human parathyroid hormone [160,161]. This aspartamide/succinimide formation can also occur during SPPS of Asn or Asp containing peptides. Besides the aforementioned degradation mechanisms of the succinimide ring, *i.e.* racemization and hydrolysis, a nucleophilic attack of piperidine or methanol, both used during SPPS, will result in α- or β-piperidide and α- or β-methyl ester peptides, respectively [162]. Michels et al. proposed addition of 5% formic acid to the piperidine to avoid the aspartimide formation during SPPS [163]. Presence of Asn residues can also induce peptide bond cleavage between this Asn residue and its C-terminal adjacent residue. Here, the amide nitrogen of the Asn side chain attacks the peptide bond carbonyl group, resulting in release of the carboxyl-side peptide and formation of a C-terminal succinimide on the original N-terminal peptide. Both succinimide-mediated Asn deamidation and peptide bond cleavage can occur simultaneously, with deamidation usually being the dominant reaction. Only when proline is adjacently present, which has no deprotonable peptide bond nitrogen and can thus not initiate succinimide formation, bond cleavage is the only degradation route [164,165].

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Table 4
Fusafungine (Locabiotal®) UHPLC–MS conditions.

Chromatography inlet		
Column + guard	Acquity UPLC CSH C18 130 Å 150 mm × 2.1 mm × 1.7 μm	
Flow	0.6 mL/min	
Injection volume	10 μL	
Needle wash	DMSO:isopropanol:ACN 10:10:80 (V/V/V)	Pre: 0 s Post: 6 s
Oven temperature	45 °C	
Sample temperature	25 °C	
Mobile phase	A: 0.1% FA + 0.1% isopropanol in H_2O	B: 0.1% FA + 0.1% isopropanol in ACN
Isocratic	30%	70%
Run time	7 min	
MS tuning conditions		
Capillary voltage	3.50 kV	
Cone voltage	50 V	
Source offset	60	
Source temperature	150 °C	
Desolvation gas flow	1000 L/h	
Desolvation temperature	600 °C	
Cone gas flow	150 L/h	
Collision gas flow	0.19 mL/min	
Nebulizer	7 bar	
Calibration link	Quantitative	

Compound	Parent (*m*/*z*)	Daughters (*m*/*z*)	Collision energy (V)
MS method: multiple reaction monitoring (MRM)			
ENN B	639.91	196.08 527.26	25 22
ENN D and B1	653.99	196.09 541.05	23 21
ENN E and A1	668.07	209.99 555.29	24 21
ENN C or F and A	682.47	209.93 555.01	26 23

7. Peptide–excipient interaction in FDP

The influence and interaction between excipient and APIs in FDP has already been reviewed for the classic small organic molecules and proteins. In general, four different interaction mechanisms can be distinguished: (i) Direct interaction between excipients and API, resulting in a covalent bond formation between the excipient and API molecule. Typical examples are the Maillard reaction between the side chain amino groups of lysine and arginine in proteins and reducing sugars, ester formation, transesterification, amide formation and Michael's addition. (ii) Indirect interaction, where the excipient acts as a catalyst increasing degradation rate of the API without forming a covalent bond. (iii) Excipients with ionizable functional groups can act as pH modifiers and impact the formulation pH, potentially accelerating degradation. (iv) Finally, API interactions with excipient impurities such as peroxides, aldehydes, acids and metals must also be considered [166–169]. Examples are given by D'Hondt et al. reporting a covalent bond formation between salmon calcitonin and a carbomer polymer, and Houchin et al., reporting an acylation reaction between peptides and the poly(lactide-co-glycolide) (PLGA) polymer [170,171].

8. Conclusions

Peptide impurities are not only a constant concern in the GMP manufacturing of peptide drugs during the drug development phase and clinical use, but should also be considered as possible confounders during the initial drug discovery phase. Therefore, it is crucial to verify the quality of peptide products, often manufactured using solid-phase peptide synthesis, before biological experimental use and it is highly recommended to repeat the biological experiments with a second peptide batch, obtained from another source. Commonly observed peptide-related impurities are solid-phase peptide synthesis-related: deletion and insertion of amino acids are related to inefficient Fmoc-deprotection and excess use of amino acid reagents, respectively. Fmoc-deprotection can cause racemization of amino acid residues and thus diastereomeric impurities. Inefficient deprotection of amino acid side chains results into peptide-protection adducts. Furthermore, unprotected side chains can react with a variety of reagents. Oxidation of amino acid side chains, due to prolonged exposure to air is also a possibility. Dimeric impurities were also found. Peptide counter ions, usually trifluoroacetic acid, are often present in the final peptide product, originating from the SPPS itself, or from additional purification treatments. Contamination of the desired peptide product by other unrelated peptides was also seen, pointing out the lack of appropriate GMP. Secondly, peptide impurities resulting from intrinsic peptide degradation such as β-elimination, diketopiperazine, pyroglutamate and succinimide formation are also discussed, as well as peptide-related impurities formed by peptide–excipient interaction in the finished drug product.

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References

[1] P. Vlieghe, V. Lisowksi, J. Martinez, M. Khrestchatisky, Synthetic therapeutic peptides: science and market, Drug Discov. Today 15 (2010) 40–56.
[2] R.C. Ladner, A.K. Sato, J. Gorzelany, M. de Souza, Phage display-derived peptides as therapeutic alternatives to antibodies, Drug Discov. Today 9 (2004) 525–529.
[3] D.P. McGregor, Discovering and improving novel peptide therapeutics, Curr. Opin. Pharmacol. 8 (2008) 616–619.
[4] R. Liu, A.M. Enstrom, K.S. Lam, Combinatorial peptide library methods for immunobiology research, Exp. Hematol. 31 (2003) 11–30.
[5] C. Falciani, L. Lozzi, A. Pini, L. Bracci, Bioactive peptides from libraries, Chem. Biol. 12 (2005) 417–426.
[6] T. Pawson, Protein modules and signalling networks, Nature 373 (1995) 573–580.
[7] T. Pawson, M. Raina, P. Nash, Interaction domains: from simple binding events to complex cellular behavior, FEBS Lett. 513 (2002) 2–10.
[8] T. Pawson, Assembly of cell regulatory systems through protein interaction domains, Science 300 (2003) 445–452.
[9] C.T. Walsh, R.V. O'Brien, C. Khosla, Non-proteinogenic amino acid building blocks for non-ribosomal peptide and hybrid polyketide scaffolds, Angew. Chem. Int. Ed. 52 (2013) 7098–7124.
[10] L. Gentilucci, R. De Marco, L. Cerisoli, Chemical modifications designed to improve peptide stability: incorporation of non-natural amino acids, pseudopeptide bonds, and cyclization, Curr. Pharm. Des. 16 (2010) 3185–3203.
[11] E.M. Molhoek, A. van Dijk, E.J.A. Veldhuizen, H.P. Haagsman, F.J. Bikker, Improved proteolytic stability of chicken cathelicidin-2 derived peptides by D-amino acid substitutions and cyclization, Peptides 32 (2011) 875–880.
[12] P.J. Knerr, T.J. Oman, C.V. Garcia De Gonzalo, T.J. Lupoli, S. Walker, W.A. van der Donk, Non-proteinogenic amino acids in lacticin 481 analogues results in more potent inhibition of peptidoglycan transglycosylation, ACS Chem. Biol. 7 (2012) 1791–1795.
[13] E.A. Bell, Non-protein amino acids of plants: significance in medicine, nutrition, and agriculture, J. Agric. Food Chem. 51 (2003) 2854–2865.
[14] P.B. Nunn, E.A. Bell, A.A. Watson, R.J. Nash, Toxicity of non-protein amino acids to humans and domestic animals, Nat. Prod. Commun. 5 (2010) 485–504.
[15] http://apps.webofknowledge.com (accessed 22.02.14).
[16] K. Badiani, Peptides as drugs, Int. Pharm. Ind. 4 (2012) 84–90.
[17] V. Glazer, Scaling up peptide drugs, http://www.genengnews.com (accessed 13.01.14).
[18] M. Giraud, Peptide market overview, London 23rd–24th February, 2012.
[19] D.J. Craik, D.P. Fairlie, S. Liras, D. Price, The future of peptide-based drugs, Chem. Biol. Drug Des. 81 (2013) 136–147.
[20] European Directorate for the Quality of Medicines & Healthcare, 8th ed., European Pharmacopoeia, Strassbourg, France, 2014.
[21] V. Vergote, C. Burvenich, C. Van de Wiele, B. De Spiegeleer, Quality specifications for peptide drugs: a regulatory-pharmaceutical approach, Pept. Sci. 15 (2009) 697–710.
[22] ICH Q7, Good manufacturing practice guide for active pharmaceutical ingredients, in: International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, Switzerland, 2000.
[23] I. Eggen, B. Gregg, H. Rode, A. Swietlow, M. Verlander, A. Szajek, Control strategies for synthetic therapeutic peptide APIs Part II: raw material considerations, Pharm. Tech. 38 (2014) 52–56.
[24] European Directorate for the Quality of Medicines & Healthcare, Top ten deficiencies – new applications for certificates of suitability PA/PH/CEP (12) 15, Strassbourg, France, 2012.
[25] ICH Q8R2, Pharmaceutical development, in: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, Switzerland, 2009.
[26] Food and Drug Administration, Guidance for Industry. PAT—A Framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance, US Department of Health and Human Services Food and Drug Administration Center for Biologics Evaluation and Research, Rockville, 2004.
[27] A. Tsirk, Quality by design: a peptide CMO approach, PharManufacturing, http://www.polypeptide.com/assets/002/5187.pdf
[28] ICH Q9, Quality risk management, in: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, Switzerland, 2005.
[29] B. De Spiegeleer, V. Vergote, A. Pezeshki, K. Peremans, C. Burvenich, Impurity profiling quality control testing of synthetic peptides using liquid chromatography-photodiode array-fluorescence and liquid chromatography–electrospray ionization-mass spectrometry the obestatin case, Anal. Biochem. 376 (2008) 229–234.
[30] S. Van Dorpe, M. Verbeke, E. Wynendaele, B. De Spiegeleer, Purity profiling of peptide drugs, J. Bioanal. Biomed. S6 (2011) 1–15.
[31] F. Guzman, S. Barberis, A. Illanes, Peptide synthesis: chemical or enzymatic, Electron J. Biotechnol. 10 (2007) 279–314.
[32] R.B. Merrifield, Solid phase peptide synthesis – synthesis of a tetrapeptide, J. Am. Chem. Soc. 85 (1963) 2149–2154.
[33] M. Amblard, J.-A. Fehrentz, J. Martinez, G. Subra, Methods and protocols of modern solid phase peptide synthesis, Mol. Biotechnol. 33 (2006) 236–254.
[34] L.A. Carpino, G.Y. Han, 9-Fluorenylmethoxycarbonyl amino-protecting group, J. Org. Chem. 37 (1972) 3404–3409.
[35] P. Lloyd-Williams, F. Albericio, E. Giralt, Convergent solid-phase peptide synthesis, Tetrahedon 49 (1993) 11065–11133.
[36] A. Isidro-Llobet, M. Alvarez, F. Albericio, Amino acid-protecting groups, Chem. Rev. 109 (2009) 2455–2504.
[37] V. Santagada, F. Frecentese, E. Perissutti, F. Fiorino, B. Severino, G. Caliendo, Microwave assisted synthesis: a new technology in drug discovery, Mini Rev. Med. Chem. 9 (2009) 340–358.
[38] B. Bacsa, B. Desai, G. Dibo, C.O. Kappe, Rapid solid-phase peptide synthesis using thermal and controlled microwave irradiation, J. Pept. Sci. 12 (2006) 633–638.
[39] D.T. Calam, in: J. Baddiley, N.H. Carey, J.F. Davidson, I.J. Higgens, W.G. Potter (Eds.), Process Development in Antibiotic Fermentations – Cambrdige Studies in Biotechnology, Cambridge University Press, Cambridge, 2003, p. 219.
[40] S.B. Singh, K. Young, New antibiotic structures from fermentations, Expert Opin. Ther. Pat. 20 (2010) 1359–1371.
[41] A.A. Sy-Cordero, C.J. Pearce, N.H. Oberlies, Revisiting the enniatins: a review of their isolation, biosynthesis, structure determination and biological activities, J. Antibiot. 65 (2012) 541–549.
[42] European Pharmacopoeia 8th Edition (2.2.46), Chromatographic Separation Techniques, Strasbourg, France, 2014.
[43] United States Pharmacopeia 37th edition, United States Pharmacopeial Convention, Rockvill, MD, 2014.
[44] European Pharmacopoeia 8th edition (2.2.30), Size-Exclusing Chromatograph, Strasbourg, France, 2014.
[45] B. De Spiegeleer, M. D'Hondt, Molecular weights under discussion? Pharmeuropa, Readers tribune, 2012, pp. 1–3.
[46] L. Novakova, L. Matysova, P. Solich, Advantages of application of UPLC in pharmaceutical analysis, Talanta 68 (2006) 908–918.
[47] M. D'Hondt, F. Verbeke, S. Stalmans, B. Gevaert, E. Wynendaele, B. De Spiegeleer, Derringer desirability and kinetic plot LC-column comparison approach for MS-compatible lipopeptide analysis, J. Pharm. Anal. (2013), http://dx.doi.org/10.1016/j.jpha.2013.09.001.
[48] A. Agnew, D. Calderwood, O.P. Chevalier, B. Greer, D.J. Grieve, B.D. Green, Chronic treatment with a stable obestatin analog significantly alters plasma triglyceride levels but fails to influence food intake; fluid intake; body weight; or body composition in rats, Peptides 32 (2011) 755–762.
[49] R.W. Busby, M.M. Kessler, W.P. Bartolini, A.P. Bryant, G. Hannig, C.S. Higgins, R.M. Solinga, J.V. Tobin, J.D. Wakefield, C.B. Kurtz, M.G. Currie, Pharmacologic properties, metabolism, and disposition of linaclotide, a novel therapeutic peptide approved for the treatment of irritable bowel syndrome with constipation and chronic idiopathic constipation, J. Pharmacol. Exp. Ther. 344 (2013) 196–206.
[50] M. D'Hondt, M. Fedorova, C.-Y. Peng, B. Gevaert, L. Taevernier, R. Hoffmann, B. De Spiegeleer, Dry heat forced degradation of buserelin peptide: kinetics and degradant profiling, Int. J. Pharm. 467 (2014) 48–59.
[51] S. Onoue, S. Misaka, Y. Ohmori, H. Sato, T. Mizumoto, M. Hirose, S. Iwasa, T. Yajima, S. Yamada, Physicochemical and pharmacological characterization of novel vasoactive intestinal peptide derivatives with improved stability, Eur. J. Pharm. Biopharm. 73 (2009) 95–101.
[52] A. Thomas, M. Kohler, W. Schänzer, P. Delahaut, M. Thevis, Determination of IGF-1 and IGF-2, their degradation products and synthetic analogues in urine by LC–MS/MS, Analyst 136 (2011) 1003–1012.
[53] F. Verbeke, E. Wynendaele, S. Braet, M. D'Hondt, B. De Spiegeleer, Quality evaluation of synthetic quorum sensing peptides used in R&D, J. Pharm. Anal. (2014) (submitted for publication).
[54] E.R. Badman, R.L. Beardsley, Z. Liang, S. Bansal, Accelerating high quality bioanalytical LC/MS/MS assays using fused-core column, J. Chromatogr. B 878 (2010) 2307–2313.
[55] M. D'Hondt, W. Demaré, S. Van Dorpe, E. Wynendaele, C. Burvenich, K. Peremans, B. De Spiegeleer, Dry heat stress stability evaluation of casein peptide mixture, Food Chem. 128 (2011) 114–122.
[56] J.J. Kirkland, S.A. Schuster, W.L. Johnson, B.E. Boyes, Fused-core particle technology in high-performance liquid chromatography: an overview, J. Pharm. Anal. 3 (2013) 303–312.
[57] J. Ruta, D. Guillarme, S. Rudaz, J.-L. Veuthey, Comparison of columns packed with porous sub-2 lm particles and superficially porous sub-3 lm particles for peptide analysis at ambient and high temperature, J. Sep. Sci. 33 (2010) 2465–2477.
[58] J. Ruta, D. Zurlino, C. Grivel, S. Heinisch, J.-L. Veuthey, D. Guillarme, Evaluation of columns packed with shell particles with compounds of pharmaceutical interest, J. Chromatogr. A 1228 (2012) 221–231.
[59] S.A. Schuster, B.M. Wagner, B.E. Boyes, J.J. Kirkland, Wider pore superficially porous particles for peptide separations by HPLC, J. Chromatogr. Sci. 48 (2010) 566–571.
[60] S.A. Schuster, B.E. Boyes, B.M. Wagner, J.J. Kirkland, Fast high performance liquid chromatography separations for proteomic applications using fused-core silica particles, J. Chromatogr. A, 232–241.
[61] D. Tognarelli, A. Tsukamoto, J. Caldwell, W. Caldwell, Rapid peptide separation by supercritical fluid chromatography, Bioanalysis 2 (2010) 5–7.
[62] M.A. Patel, F. Riley, M. Ashraf-Khorassani, L.T. Taylor, Supercritical fluid chromatographic resolution of water soluble isomeric carboxyl/amine terminated peptides facilitated via mobile phase water and ion pair formation, J. Chromatogr. A 1233 (2012) 85–90.
[63] J.D. Pinkston, D. Wen, K.L. Morand, D.A. Tirey, D.T. Stanton, Comparison of LC/MS and SFC/MS for screening of a large and diverse library of pharmaceutically relevant compounds, Anal. Chem. 78 (2006) 7467–7472.

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[64] J. Zheng, J.D. Pinkston, P.H. Zoutendam, L.T. Taylor, Feasibility of supercritical fluid chromatography/mass spectrometry of polypeptides with up to 40-mers, Anal. Chem. 78 (2006) 1535–1545.
[65] C. Simo, P. Lopez Soto-Yarritu, A. Cifuentes, Simulation and optimization of peptide separation by capillary electrophoresis-mass spectrometry, Electrophoresis 23 (2002) 2288–2295.
[66] V. Kašička, Capillary electrophoresis of peptides, Electrophoresis 20 (1999) 3084–3105.
[67] S.M. Kelly, T.J. Jess, N.C. Price, How to study proteins by circular dichroism, Biochim. Biophys. Acta 1751 (2005) 119–139.
[68] J.T. Pelton, L.R. McLean, Spectroscopic methods for analysis of protein secondary structure, Anal. Biochem. 277 (2000) 167–176.
[69] G. Bringmann, K. Messer, M. Wohlfarth, J. Kraus, K. Dumbuya, M. Rückert, HPLC-CD on-line in combination with HPLC-NMR and HPLC–MS/MS for the determination of the full absolute stereostructure of new metabolites in plant extracts, Anal. Chem. 71 (1999) 2678–2686.
[70] H. Fabian, G.I. Szendrei, H.H. Mantsch, L. Otvos, Comparative analysis of human and Dutch-type alzheimer β-amyloid peptides by infrared spectroscopy and circular dichroism, Biochem. Biophys. Res. Commun. 191 (1993) 232–239.
[71] A. Loksztejn, W. Dzwolak, Vortex-induced formation of insulin amyloid superstructures probed by time-lapse atomic force microscopy and circular dichroism spectroscopy, J. Mol. Biol. 395 (2010) 643–655.
[72] V. Banerjee, R.K. Kar, A. Datta, K. Parthasarathi, S. Chatterjee, K.P. Das, A. Bhunia, Use of a small peptide fragment as an inhibitor of insulin fibrillation process: a study by high and low resolution spectroscopy, PLoS ONE 8 (2013) e72318, http://dx.doi.org/10.1371/journal.pone.0072318.
[73] S. Stalmans, E. Wynendaele, N. Bracke, B. Gevaert, M. D'Hondt, K. Peremans, C. Burvenich, B. De Spiegeleer, Chemical–functional diversity in cell-penetrating peptides, PLoS ONE 8 (2013) e71752, http://dx.doi.org/10.1371/journal.pone.0071752.
[74] E. Eiríksdóttir, K. Konate, U. Langel, G. Divita, S. Deshayes, Secondary structure of cell-penetrating peptides controls membrane interaction and insertion, Biochim. Biophys. Acta 1798 (2010) 1119–1128.
[75] F. Milletti, Cell-penetrating peptides: classes, origin, and current landscape, Drug Discov. Today 17 (2012) 850–860.
[76] H. Nishikawa, S. Nakamura, E. Kodama, S. Ito, K. Kajiwara, K. Izumi, Y. Sakagami, S. Oishi, T. Ohkubo, Y. Kobayashi, A. Otaka, N. Fujii, M. Matsuoka, Electrostatically constrained α-helical peptide inhibits replication of HIV-1 resistant to enfuvirtide, Int. J. Biochem. Cell B 41 (2009) 891–899.
[77] T. Naito, K. Izumi, E. Kodama, Y. Sakagami, K. Kajiwara, H. Nishikawa, K. Watanabe, S.G. Sarafianos, S. Oishi, N. Fujii, M. Matsuoka, SC29EK, a peptide fusion inhibitor with enhanced α-helicity, inhibits replication of human immunodeficiency virus type 1 mutants resistant to enfuvirtide, Antimicrob. Agents Chemother. 53 (2009) 1013–1018.
[78] P. Polavarapu, Optical rotation: recent advances in determining the absolute configuration, Chirality 14 (2002) 768–781.
[79] B.F. Erlanger, E. Brand, Optical rotation of peptides. I: Glycine and alanine dipeptides, J. Am. Chem. Soc. 73 (1951) 3508–3510.
[80] S. Sakakibara, K. Inouye, K. Shudo, Y. Kishida, Y. Kobayashi, D.J. Prockop, Synthesis of $(Pro\text{-}Hyp\text{-}Gly)_n$ of defined molecular weights – evidence for the stabilization of collagen triple helix by hydroxyproline, Biochim. Biophys. Acta 303 (1973) 198–202.
[81] Y.H. Chen, J.T. Yang, H.M. Martinez, Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersion, Biochemistry 11 (1972) 4120–4131.
[82] T.A. Keiderling, R.A.G.D. Silva, G. Yoder, R.K. Dukor, Vibrational circular dichroism spectroscopy of selected oligopeptide conformations, Bioorg. Med. Chem. 7 (1999) 133–141.
[83] T.A. Keiderling, Protein and peptide secondary structure and conformational determination with vibrational circular dichroism, Curr. Opin. Clin. Biol. 6 (2002) 682–688.
[84] Y.S. Kim, R.M. Hochstrasser, Applications of 2D IR spectroscopy to peptides, proteins, and hydrogen-bond dynamics, J. Phys. Chem. B 113 (2009) 8231–8251.
[85] R. Tuma, Raman spectroscopy of proteins: from peptides to large assemblies, J. Raman Spectrosc. 36 (2005) 307–319.
[86] R. Schweitzer-Stenner, J.B. Soffer, S. Toal, D. Verbaro, Structural analysis of unfolded peptides by Raman spectroscopy, Methods Mol. Biol. 895 (2012) 315–346.
[87] I.K. Lednev, A.S. Karnoup, M.C. Sparrow, S.A. Asher, Transient UV Raman spectroscopy finds no crossing barrier between the peptide α-helix and fully random coil conformation, J. Am. Chem. Soc. 123 (2001) 2388–2392.
[88] S. Stewart, P.M. Fredericks, Surface-enhanced Raman spectroscopy of peptides and proteins adsorbed on an electrochemically prepared silver surface, Spectrochim. Acta A 55 (1999) 1615–1640.
[89] S.A. Oladepo, K. Xiong, Z. Hong, S.A. Asher, J. Handen, I.K. Lednev, UV resonance Raman investigation of peptide and protein structure and dynamics, Chem. Rev. 112 (2012) 2604–2628.
[90] D.S. Wishart, B.D. Sykes, Chemical-shifts as a tool for structure determination, Methods Enzymol. 239 (1994) 363–392.
[91] U. Holzgrabe, R. Deubner, C. Schollmayer, B. Waibel, Quantitative NMR spectroscopy – applications in drug analysis, J. Pharm. Biomed. Anal. 38 (2005) 806–812.
[92] U. Holzgrabe, B.W.K. Diehl, I. Wawer, NMR spectroscopy in pharmacy, J. Pharm. Biomed. Anal. 17 (1998) 557–616.
[93] J.C. Lindon, J.K. Nicholson, I.D. Wilson, Directly coupled HPLC-NMR and HPLC–NMR-MS in pharmaceutical research and development, J. Chromatogr. B 748 (2000) 233–258.
[94] I. Gomes, J.S. Grushko, U. Golebiewska, S. Hoogendoorn, A. Gupta, A.S. Heimann, E.S. Ferro, S. Scarlata, L.D. Fricker, L.A. Devi, Novel endogenous peptide agonists of cannabinoid receptors, FASEB J. 23 (2009) 3020–3029.
[95] O. Marder, F. Albericio, Industrial application of coupling reagents in peptides, Peptides June (2003) 6–11.
[96] V. Sanz-Nebot, F. Benavente, I. Toro, J. Barbosa, Separation and characterization of complex crude mixtures produced in the synthesis of therapeutic peptide hormones by liquid chromatography coupled to electrospray mass spectrometry (LC–ES-MS), Anal. Chim. Acta 521 (2004) 25–36.
[97] V. Sanz-Nebot, F. Benavente, I. Toro, J. Barbosa, Liquid chromatgraphy–mass spectrometry approach for the characterisation and purification of crude synthetic peptide hormones, Anal. Bioanal. Chem. 377 (2003) 306–315.
[98] V. Sanz-Nebot, F. Benavente, I. Toro, J. Barbosa, Liquid chromatography–mass spectrometry and capillary electrophoresis combined approach for separation and characterization of multicomponent peptide mixtures applications to crude products of leuprolide synthesis, J. Chromatogr. A 950 (2002) 99–111.
[99] V. Sanz-Nebot, F. Benavente, A. Castillo, J. Barbosa, Liquid chromatography–electrospray mass spectrometry of multicomponent peptide mixtures, characterization of a mixture from the synthesis of the hormone goserelin, J. Chromatogr. A 889 (2000) 119–133.
[100] V. Sanz-Nebot, F. Benavente, J. Barbosa, Separation and characterization of multicomponent peptide mixtures by liquid chromatography–electrospray ionization mass spectrometry, applications to crude products of the synthesis of leuprolide, J. Chromatogr. A 870 (2000) 315–334.
[101] V. Sanz-Nebot, I. Toro, A. Garcés, J. Barbosa, Separation and identification of peptide mixtures in a synthesis crude of carbetocin by liquid chromatography/electrospray ionization mass spectrometry, Rapid Commun. Mass Spectrom. 13 (1999) 2341–2347.
[102] V. Sanz-Nebot, I. Toro, A. Castillo, J. Barbosa, Investigation of synthetic peptide hormones by liquid chromatography coupled to pneumatically assisted electrospray ionization mass spectrometry: analysis of a synthesis crude of peptide triptorelin, Rapid Commun. Mass Spectrom. 15 (2001) 1031–1039.
[103] A. Taichrib, G.K.E. Scriba, C. Neusü, Identification and characterization of impurities of tetracosactide by capillary electrophoresis and liquid chromatography coupled to time-of-flight mass spectrometry, Anal. Bioanal. Chem. 401 (2011) 1365–1375.
[104] J.W. de Beukerlaar, J.W. Gratama, P.A. Sillevis Smitt, G.M. Verjans, J. Kraan, T.M. Luider, P.C. Burgers, The impact of impurities in synthetic peptides on the outcome of T-cell stimulation assays, Rapid Commun. Mass Spectrom. 21 (2007) 1282–1288.
[105] J.R. Litowski, P.D. Semchuk, C.T. Mant, R.S. Hodges, Hydrophilic interaction/cation-exchange chromatography for the purification of synthetic peptides from closely related impurities: serine side-chain acetylated peptides, J. Pept. Res. 54 (1999) 1–11.
[106] A. Isidro-Llobet, X. Just-Baringo, A. Ewenson, M. Alvarez, F. Albericio, Fmoc-2-mercaptobenzothiazole, for the introduction of the Fmoc moiety free of side-reactions, Pept. Sci. 88 (2007) 733–737.
[107] M. Obkircher, C. Stähelin, F. Dick, Formation of Fmoc-β-alanine during Fmoc-protections with Fmoc-OSu, J. Pept. Sci. 14 (2008) 763–766.
[108] A. Isidro-Llobet, X. Just-Baringo, A. Ewenson, M. Alvarez, F. Albericio, Fmoc-2-mercaptobenzothiazole for the introduction of the Fmoc moiety free of side-reactions, Pept. Sci. 88 (2007) 733–737.
[109] E. Hlebowicz, A.J. Andersen, L. Andersson, B.A. Moss, Identification of Fmoc-β-Ala-OH and Fmoc-β-Ala-amino acid-OH as new impurities in Fmoc-protected amino acid derivates, J. Pept. Res. 65 (2005) 90–97.
[110] M. Verbeken, E. Wynendaele, R.A. Lefebvre, E. Goossens, B. De Spiegeleer, The influence of peptide impurity profiles on functional tissue-organ bath response: the 11-mer peptide INSL6[151–161] case, Anal. Biochem. 421 (2012) 547–555.
[111] J.A. Ji, B. Zhang, W. Cheng, J. Wang, Methionine, tryptophan, and histidine oxidation in a model protein: PTH: mechanisms and stabilization, J. Pharm. Sci. 98 (2009) 4485–4500.
[112] K. Uchida, Histidine and lysine as targets of oxidative modification, Amino Acids 25 (2003) 249–257.
[113] I. Dalle-Donne, R. Rossi, D. Giustarini, A. Milzani, R. Colombo, Protein carbonyl groups as biomarkers of oxidative stress, Clin. Chim. Acta 329 (2003) 23–38.
[114] S.A. Palasek, Z.J. Cox, J.M. Collins, Limiting racemisation and aspartamide formation in micro-wave enhanced Fmoc solid phase peptide synthesis, J. Pept. Sci. 13 (2007) 143–148.
[115] C. Loffrendo, N.A. Assunção, J. Gerhardt, M.T.M. Miranda, Microwave assisted solid-phase peptide synthesis at 60 °C: alternative conditions with low enantiomerization, J. Pept. Sci. 15 (2009) 808–817.
[116] M.P. Souza, M.F.M. Tavares, M.T.M.M.T.M. Miranda, Racemisation in stepwise solid-phase peptide synthesis at elevated temperatures, Tetrahedron 60 (2004) 4671–4681.
[117] Y. Han, F. Albericio, G. Barany, Occurrence and minimization of cysteine racemisation during stepwise solid-phase peptide synthesis, J. Org. Chem. 62 (1997) 4307–4312.
[118] Y. Angell, J. Alsina, F. Albericio, G. Barany, Practical protocols for stepwise solid-phase synthesis of cysteine-containing peptides, J. Pept. Res. 60 (2002) 292–299.

Please cite this article in press as: M. D'Hondt, et al., Related impurities in peptide medicines, J. Pharm. Biomed. Anal. (2014), http://dx.doi.org/10.1016/j.jpba.2014.06.012

[119] T. Kaiser, G. Nicholson, H. Kohlbau, W. Voelter, Racemisation studies of Fmoc-Cys(Trt)-OH during stepwise Fmoc-solid phase peptide synthesis, Tetrahedron Lett. 37 (1996) 1187–1190.
[120] M.A. Elsawy, C. Hewage, B. Walker, Racemisation of N-Fmoc phenylglycine under mild microwave-SPPS and conventional stepwise SPPS conditions: attempts to develop strategies for overcoming this, J. Pept. Sci. 18 (2012) 302–311.
[121] C.A.G.N. Montalbetti, V. Falque, Amide bond formation and peptide coupling, Tetrahedon 61 (2005) 10827–10852.
[122] V.H. Finder, I. Vodopivec, R.M. Nitsch, R. Glockshuber, The recombinant amyloid-β peptide Aβ1–42 aggregates faster and is more neurotoxic than synthetic Aβ1–42, J. Mol. Biol. 396 (2010) 9–18.
[123] J.H. Jones, Synthesis of peptides and peptidomimetics, in: M. Goodman, A. Felix, L. Moroder, C. Toniolo (Eds.), Houben-Weyl Methods of Organic Chemistry, Thieme, Stuttgart, 2002, pp. 334–346.
[124] M.C. Lai, E.M. Topp, Solid-state chemical stability of proteins and peptides, J. Pharm. Sci. 88 (1999) 489–500.
[125] M.E.M. Cromwell, E. Hilario, F. Jacobson, Protein aggregation and bioprocessing, AAPS J. 8 (2006) E572–E579.
[126] S. Liu, L. Zhou, L. Chen, S.G. Dastidar, C. Verma, J. Li, D. Tan, R. Beuerman, Effect of structural parameters of peptides on dimer formation and highly oxidized side products in the oxidation of thiols of linear analogues of human β-defensin 3 by DMSO, J. Pept. Sci. 15 (2008) 95–106.
[127] W.K. Surewicz, H.H. Mantsch, The conformation of dynorphin A(1–13) in aqueous solution as studied by Fourier transform infrared spectroscopy, J. Mol. Struct. 214 (1989) 143–147.
[128] S.P. Brazier, B. Ramesh, P.I. Haris, D.C. Lee, S.K. Srai, Secondary structure analysis of the putative membrane-associated domains of the inward rectifier K^+ channel ROMK1, Biochem. J. 335 (1998) 375–380.
[129] M. Sharon, Z. Oren, Y. Shai, J. Anglister, 2D-NMR and ATR-FTIR study of the structure of a cell-selective diastereomer of melittin and its orientation in phospholipids, Biochemistry 38 (1999) 15305–15316.
[130] R.N. Lewis, E.J. Prenner, L.H. Kondejewski, C.R. Flach, R. Mendelsohn, R.S. Hodges, R.N. McElhaney, Fourier transform infrared spectroscopic studies of the interaction of the antimicrobial peptide gramicidin S with lipid micelles and with lipid monolayer and bilayer membranes, Biochemistry 38 (1999) 15193–15203.
[131] C. Hetru, L. Letellier, Z. Oren, J.A. Hoffmann, Y. Shai, Androctonin, a hydrophilic disulphide-bridged non-haemolytic antimicrobial peptide: a plausible mode of action, Biochem. J. 345 (2000) 653–664.
[132] W.K. Surewicz, H.H. Mantsch, D. Chapman, Determination of protein secondary structure by Fourier transform infrared spectroscopy: a critical assessment, Biochemistry 32 (1993) 389–394.
[133] P.I. Haris, D. Chapman, The conformational analysis of peptides using Fourier transform IR spectroscopy, Biopolymers 37 (1995) 251–263.
[134] M. Zhang, H. Fabian, H.H. Mantsch, H.J. Vogel, Isotope-edited Fourier transform infrared spectroscopy studies of calmodulin's interaction with its target peptides, Biochemistry 33 (1994) 10883–10888.
[135] H. Fabian, T. Yuan, H.J. Vogel, H.H. Mantsch, Comparative analysis of the amino- and carboxy-terminal domains of calmodulin by Fourier transform infrared spectroscopy, Eur. Biophys. J. 24 (1996) 195–201.
[136] R.F. Epand, N. Umezawa, E.A. Porter, S.H. Gellman, R.M. Epand, Interactions of the antimicrobial β-peptide β-17 with phospholipid vesicles differ from membrane interactions of magainins, Eur. J. Biochem. 270 (2003) 1240–1248.
[137] V.V. Andrushchenko, H.J. Vogel, E.J. Prenner, Optimization of the hydrochloric acid concentration used for trifluoroacetate removal from systhetic peptides, J. Pept. Sci. 13 (2007) 37–43.
[138] T.F. Gabriel, Simple, rapid method for converting a peptide from one salt form to another, Int. J. Pept. Protein Res. 30 (1987) 40–43.
[139] J.R. Currier, L.M. Galley, H. Wenschuh, V. Morafo, S. Ratto-Kim, C.M. Gray, L. Maboko, M. Hoelscher, M.A. Marovich, J.H. Cox, Peptide impurities in commercial synthetic peptides and their implications for vaccine trial assessment, Clin. Vaccine Immunol. 15 (2008) 267–276.
[140] M.T. de Graaf, J.W. de Beukelaar, P.C. Burgers, T.M. Luider, J. Kraan, P.A. Sillevis Smitt, J.W. Gratama, Contamination of synthetic HuD protein spanning peptide pools with a CMV-encoded peptide, Cytometry 73A (2008) 1079–1085.
[141] V. Brezar, S. Culina, T. Østerbye, F. Guillonneau, G. Chiappetta, Y. Verdier, J. Vinh, F.S. Wong, S. Buus, R. Mallone, T Cells recognizing a peptide contaminant undetectable by mass spectrometry, PLoS ONE 6 (2011) 1–9.
[142] R.B. Jones, V.M. John, D.V. Hunter, E. Martin, S. Mujib, V. Mihaljovic, P.C. Burgers, T.M. Luider, G. Gyenes, N.C. Sheppard, D. SenGupta, R. Tandon, F.-Y. Yue, E. Benko, C. Kovacs, D.F. Nixon, M.A. Ostrowski, Human endogenous retrovirus K(HML-2) Gag- and Env-specific T-cell responses are infrequently detected in HIV-1-infected subjects using standard peptide matrix-based screening, Clin. Vaccine Immunol. 19 (2012) 288–292.
[143] P.R. Singh, M. Rajopadhye, S.L. Clark, N.E. Williams, Effects of scavengers in acidolytic cleavage of Cys(Acm)-containing peptides from solid support: isolation of an ethanedithiol disulfide adduct, Tetrahedon Lett. 37 (1996) 4117–4120.
[144] M. Breslav, J. Becker, F. Naider, Dithioketal formation during synthesis of Bpa containing peptides, Tetrahedon Lett. 38 (1997) 2219–2222.
[145] P. Sieber, Modification of tryptophan residues during acidolysis of 4-methoxy-2,3,6-trimethylbenzenesulfonyl groups effects of scavengers, Tetrahedon Lett. 28 (1987) 1637–1640.
[146] J. Wang, V. Krishnamoorthi, E. Wang, C. Yang, D. Baptista, X. Wu, M. Liu, M. Gardner, P. Elkins, J. Hines, P. Liu, LC/MS characterization of impurities and degradation products of a potent antitumor peptidic dimer, CU201, J. Pharm. Biomed. 51 (2010) 824–833.
[147] H.R. Costantino, R. Langer, A.M. Klibanov, Moisture-induced aggregation of lyophilized insulin, Pharm. Res. 11 (1994) 21–29.
[148] S.L. Cohen, C. Price, J. Vlasak, β-Elimination and peptide bond hydrolysis: two distinct mechanisms of human IgG1 hinge fragmentation upon storage, J. Am. Chem. Soc. 129 (2007) 6976–6977.
[149] S. Ni, H. Zhang, W. Huang, J. Zhou, H. Qian, W. Chen, The application of an aryl hydrazine linker prevents β-elimination side products in the SPPS of C-terminal cystein peptides, J. Pept. Sci. 16 (2010) 309–313.
[150] B. Herbert, F. Hopwood, D. Oxley, J. McCarthy, M. Laver, J. Grinyer, A. Goodall, K. Williams, A. Castagna, P.G. Righetti, β-Elimination: an unexpected artifact in proteome analysis, Proteomics 3 (2003) 826–831.
[151] E. Pedroso, A. Grandas, X. de las Heras, R. Eritja, E. Giralt, Diketopiperazine formation in solid phase peptide synthesis using p-alkoxybenzyl ester resins and Fmoc-amino acids, Tetrahedron Lett. 27 (1986) 743–746.
[152] M. Gairi, P. Lloyd-Williams, F. Albericio, E. Giralt, Use of BOP1 reagent for the suppression of diketopiperazine formation in Boc/Bzl solid-phase peptide synthesis, Tetrahedron Lett. 31 (1990) 7363–7366.
[153] D.S. Rehder, T.M. Dillon, G.D. Pipes, P.V. Bondarenko, Reversed-phase liquid chromatography/mass spectrometry analysis of reduced monoclonal antibodies in pharmaceutics, J. Chromatog. A 1102 (2006) 164–175.
[154] D. Chelius, K. Jing, A. Lueras, D.S. Rehder, T.M. Dillon, A. Vizel, R.S. Rajan, T. Li, M.J. Treuheit, P.V. Bondarenko, Formation of pyroglutamic acid from N-terminal glutamic acid in immunoglobin gamma antibodies, Anal. Chem. 78 (2006) 2370–2376.
[155] T. Geiger, S. Clarke, Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides, J. Biol. Chem. 262 (1987) 785–794.
[156] R.C. Stephenson, S. Clarke, Succinimide formation from aspartyl and asparaginyl peptides as a model for the spontaneous degradation of proteins, J. Biol. Chem. 264 (1989) 6164–6170.
[157] M. Kumar, A. Chatterjee, A.P. Khedkar, M. Kusumanchi, L. Adhikary, Mass spectrometric distinction of in-source and in-solution pyroglutamate and succinimide in proteins: a case study on rhG-CSF, J. Am. Soc. Mass Spectrom. 24 (2013) 202–212.
[158] G.C. Chu, D. Chelius, G. Xiao, H.K. Khor, S. Coulibaly, P.V. Bondarenko, Accumulation of succinimide in a recombinant monoclonal antibody in mildly acidic buffers under elevated temperatures, Pharm. Res. 24 (2007) 1145–1146.
[159] B. Yan, S. Steen, D. Hambly, J. Valliere-Douglass, T.V. Bos, S. Smallwood, Z. Yates, T. Arroll, Y. Han, H. Gadgil, R.F. Latypov, A. Wallace, A. Lim, G.R. Kleemann, W. Wang, A. Balland, Succinimide formation at Asn 55 in the complementarity determining region of a recombinant monoclonal antibody IgG1 heavy chain, Pharm. Sci. 98 (2009) 3509–3521.
[160] B.N. Violand, M.R. Schlittler, E.W. Kolodziej, P.C. Toren, M.A. Cabonce, N.R. Siegel, K.L. Duffin, J.F. Zobel, C.E. Smith, J.S. Tou, Isolation and characterization of porcine somatotropin containing a succinimide residue in place of aspartate, Protein Sci. 1 (1992) 1634–1641.
[161] R. Kothari, V. Kumar, R. Jena, R. Tunga, B.S. Tunga, Modes of degradation and impurity characterization in rhPTH (1–34) during stability studies, PDA J. Pharm. Sci. Technol. 65 (2011) 348–362.
[162] P. Stathopoulos, S. Papas, S. Kostidis, V. Tsikaris, α- and β-aspartyl peptide ester formation via aspartamide ring opening, J. Pept. Sci. 11 (2005) 658–664.
[163] T. Michels, R. Dölling, U. Haberkorn, W. Mier, Acid-mediated prevention of aspartimide formation in solid phase peptide synthesis, Org. Lett. 14 (2012) 5218–5221.
[164] C.E.M. Voorter, W.A. de Haard-Hoekman, P.J.M. van den Oetelaar, H. Bloemendaal, W.W. de Jong, Spontaneous peptide bond cleavage in aging α-crystallin through a succinimide intermediate, J. Biol. Chem. 263 (1988) 19020–19023.
[165] S. Capasso, L. Mazzarella, A.J. Kirby, S. Salvadori, Succinimide-mediated pathway for peptide bond cleavage: kinetic study on Asn-Sar containing peptide, J. Pept. Sci. 40 (1996) 543–551.
[166] A.S. Narang, D. Desai, S. Badawy, Impact of excipient interactions on solid dosage form stability, Pharm. Res. 29 (2012) 2660–2683.
[167] S.R. Byrn, W. Xu, A.W. Newman, Chemical reactivity in solid-state pharmaceuticals: formulation implications, Adv. Drug Deliv. Rev. 48 (2001) 115–136.
[168] T.J. Kamerzell, R. Esfandiary, S.B. Joshi, C.R. Middaugh, D.B. Volkin, Protein–excipient interactions: mechanisms and biophysical characterization applied to protein formulation development, Adv. Drug Deliv. Rev. 63 (2011) 1118–1159.
[169] S. Ohtake, Y. Kita, T. Arakawa, Interactions of formulations excipients with proteins in solution and in the dried state, Adv. Drug Deliv. Rev. 63 (2011) 1053–1073.
[170] M. D'Hondt, S. Van Dorpe, E. Mehuys, D. Deforce, B. De Spiegeleer, Quality analysis of samon calcitonin in a polymeric bioadhesive pharmaceutical formulation: sample preparation optimization by DOE, J. Pharmaceut. Biomed. 53 (2010) 939–945.
[171] M.L. Houchin, E.M. Topp, Chemical degradation of peptides and proteins in PLGA: a review of reactions and mechanisms, J. Pharm. Sci. 97 (2008) 2395–2404.

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